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Bio-Effectors for Improved Growth, Nutrient Acquisition and Disease Resistance of Crops

Case Studies to the Development of Sustainable Cropping Systems Emphasizing Soil-Plant-Microbial Interactions

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“The world is on the brink of a new agriculture, one that involves the marriage of plant biology and agroecology under the umbrella of biotechnology and germplasm improvement.”

(C. P. Vance, 2001)

“… here I miss the importance of simple agricultural management issues!”

(pers. com. Volker Römheld, 2007)

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LIST OF ABBREVIATIONS

%	Percent
®	Registered Trade Mark
°C	Degree Celsius
µg	microgram (10^{-6} g)
µl	Microliter (10^{-6} liter)
µmol	Micromole (10^{-6} mol)
$\cdot O_2^-$	Superoxide radical
16S	16 Svedberg (non-SI unit for sedimentation rate)
$^1O_2$	Singlet oxygen
ACC	1-aminocyclopropane-1-carboxylate
ADP	Adenosine diphosphate
AG	Aktiengesellschaft [English: joint-stock company]
AHLs	N-acyl-homoserine lactones
Al	Aluminum
Al_2O_3	Aluminium oxide
Al^{3+}	Aluminium ion
$AlPO_4$	Aluminum phosphate
$AlPO_4 \cdot 2H_2O$	Aluminum phosphate dihydrate (variscite)
AM	Arbuscular mycorrhiza
AMF inoc.	Arbuscular mycorrhizal fungi inoculum
AM-fungi	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
ANOVAs	Analyses of variance
Apr	April

ATP	Adenosine triphosphate
Aug	August
aut.	autoclaved
avr genes	Avirulence genes
BBA	Biologische Bundesanstalt für Land- und Forstwirtschaft [English: Federal Biological Research Centre for Agriculture and Forestry]
B	Boron
B.	*Bacillus*
B.V.	Besloten vennootschap met beperkte aansprakelijkheid [English: private company with limited liability]
Bio-super	Biological superphosphate
BMELV	Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz [English: Federal Ministry of Food, Agriculture and Consumer Protection]
BVL	Bundesamt für Verbraucherschutz und Lebensmittelsicherheit [English: Federal Office of Consumer Protection and Food Safety]
Bt	*Bacillus thuringiensis*
C	Carbon
$C_3H_7O_6PNa_2$	Glycerophosphate disodium salt
$C_6H_6Na_{12}O_{24}P_6$	Na-phytate
Ca	Calcium
$Ca(H_2PO_4)_2$	Calcium dihydrogen phosphate
$Ca(NO_3)_2$	Calcium nitrate
Ca^{2+}	Calcium ion
$Ca_3(PO_4)_2$	Calcium phosphate
CAB	Commonwealth Agricultural Bureaux

$CaCl_2$	Calcium chloride
$CaCO_3$	Calcium carbonate
CAGR	compound annual growth rate
CAGR	Compound annual growth rate
CAL	Extractant consisting of Ca lactate + Ca acetate + acetic acid
$CaSO_4$	Calcium sulfate
CAT	Extractant consisting of $CaCl_2$ and DTPA
Cd^{2+}	Cadmium ion
cfu	Colony forming units
Cl^-	Chloride ion
C-loess	Loess material taken from the calcareous C-horizon (subsoil) of a Luvisol
cm	Centimeter (10^{-2} m)
CO_2	Carbon dioxide
C_{org}	Organic carbon
cp.	compare
Cu	Cuprum [English: copper]
Cu^+	Cuprous ion
$CuSO_4$	Copper sulfate
Cu-Zn SOD	Copper-zinc superoxide dismutase
DAPG	Diacetylphloroglucinol
Dec	December
DGGE	Denaturant gradient gel electrophoresis
DLR	Dienstleistungszentrum Ländlicher Raum [English: Service center for the rural area]
DNA	Deoxyribonucleic acid
DRB	Deleterious rhizobacteria

DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen [English: German Collection of Microorganisms and Cell Cultures]
DTPA	Diethylene-triamine-pentaacetic acid
DüMV	Düngemittelverordnung [English: Fertilizer Ordinance]
DüngG	Düngegesetz [English: Fertilization Law]
EBIC	European Biostimulant Industry Council
e.f.s.t.	Excluded from statistical tests
e.g.	for example [Latin: *exempli gratia*]
EC	European Community
EDTA	Ethylenediaminetetraacetate
EEC	European Economic Community
EPA	Environmental Protection Agency
et al.	and others [Latin: *et alii*]
ET	Ethylene
EU	European Union
f. sp.	special form [Latin: *forma specialis*]; informal taxonomic grouping applied to parasites with adaptation to a specific host
f. sp.	Forma specialis
FAO	Food and Agriculture Organization of the United Nations
Fe	Iron
Fe^{2+}	Ferro ion
Fe^{3+}	Ferric ion
Feb	February
$FeCl_2$	Iron chloride
$FePO_4$	Iron phosphate
$FePO_4 \cdot 2H_2O$	Iron phosphate dihydrate (strengite)
Fig.	Figure

FORL	*Fusarium oxysporum f. sp. radices-lycopersici*
g	Gram
G.	*Gaeumannomyces*
GbR	Gesellschaft bürgerlichen Rechts [English: civil law association]
Gga	*Gaeumannomyces graminis* var. *avenae*
Ggg	*Gaeumannomyces graminis* var. *graminis*
Ggt	*Gaeumannomyces graminis* var. *tritici*
GmbH & Co. KG	Gesellschaft mit beschränkter Haftung & Compagnie Kommanditgesellschaft [English: Limited partnership with a limited liability company as general partner]
GmbH	Gesellschaft mit beschränkter Haftung [English: limited liability company]
GMOs	Genetically modified organisms
h	Hour
H	Hydrogen
H^+	Hydrogen ion
H_2O	dihydrogen monoxide (water)
H_2O_2	Hydrogen peroxide
$H_2PO_4^-$	di- hydrogen phosphate ion
H_2SO_4	Sulfuric acid
H_3BO_3	Boric acid
ha	Hectare (1 ha = 10^4 m^2)
HCl	Hydrochloric acid
HCN	Hydrogen cyanide
HF	Hydrofluoric acid
HNO_3	Nitric acid
HPO_4^{2-}	mono-hydrogen phosphate ion
HR	Hypersensitive cell death response

i.e.	that is [Latin: *id est*]
IAA	indole-3-acetic acid
ICP-OES	Inductively coupled plasma optical emission spectrometry
Inc.	Incorporated company (i.e. joint stock company)
ISR	Induced systemic resistance
JA	Jasmonic acid
Jacq.	Jacquin
Jan	January
JKI	Julius Kühn-Institut - Bundesforschungsinstitut für Kulturpflanzen [English: Federal Research Centre for Cultivated Plants]
Jul	July
Jun	June
K	Kalium [English: potassium]
K^+	Potassium ion
K_2SO_4	Potassium sulfate
KCl	Potassium chloride
kg	Kilogram (10^3 g)
KH_2PO_4	Potassium dihydrogen phosphate
KMnO4	Potassium permanganate
KOH	Potassium hydroxide
l	Liter
L.	Linné
LMWCs	Low molecular weight compounds
log	Logarithm
LRR domains	Leucine rich repeat domains
Ltd.	Limited Company
m	Meter

M	Molar concentration (1 M = 1 mol liter^{-1})
MAMPs	Microbe-associated molecular patterns
Mar	March
Merr.	Merrill
Mg	Magnesium
mg	Milligram (10^{-3} g)
Mg^{2+}	Magnesium ion
$MgSO_4$	Magnesium sulfate
MHB	Mycorrhiza helper bacteria
Mill.	Miller
min	Minute
ml	milliliter (10^{-3} liter)
mm	Millimeter (10^{-3} m)
mM	Millimolar (10^{-3} molar)
Mn	Manganese
Mn^{2+}	Manganous ion
$MnCl_2$	Manganese chloride
MnO_2	Manganese dioxide
MnOOH	Manganese oxyhydroxide
$MnSO_4$	Manganese sulfate
MnSOD	Manganese superoxide dismutase
Mn_{total}	Total manganese
mol	Mole
N	Nitrogen
n	Number of replicates
N_2	Dinitrogen
Na	Natrium [English: Sodium]

Na_2MoO_4	Sodium molybdate
Na_3PO_4	Sodium phosphate
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
NAS	National Academy of Sciences
NB domains	Nucleotide binding domains
n.d.	Not determinable
$(NH_4)_2SO_4$	Ammonium sulfate
$(NH_4)_6Mo_7O_{24}$	Ammonium heptamolybdate
NH_4^+	Ammonium
NH_4F	Ammonium fluoride
NH_4OAc	Ammonium acetate
NH_4VO_3	Ammonium metavanadate
Ni	Nickel
nm	Nanometer (10^{-9} m)
n.m.	Not measured
No.	With the number [Latin: *numero*]
NO_3^-	Nitrate
No-P	Without phosphate fertilizer addition
Nov	November
NPC	Novobiocin, Penicillin G, and Cycloheximide
NPR	nodulation promoting rhizobacteria
NPR1	Non-expressor of pathogenesis-related genes1
N_{total}	Total nitrogen
O	Oxygen
O_2	Dioxygene

Oct	October
OECD	Organization for Economic Cooperation and Development
OH^-	hydroxyl ion
$OH^·$	hydroxyl radical
p	probability of the statistical test value when the null hypothesis is true
P	Phosphorus
P.	*Pseudomonas*
p.a.	pro analysis
PAMPs	Pathogen-associated molecular patterns
PAMPs	Pathogen-associated molecular patterns
Pb	Plumbum [English: lead]
PCA	Phenazine-1-carboxylic acid
PCR	Polymerase chain reaction
Pers. com.	Personal communication
PflSchG	Pflanzenschutzgesetz [English: Plant Protection Law]
PGPMs	Plant growth-promoting microorganisms
PGPR	Plant growth-promoting rhizobacteria
pH	Decimal logarithm of the reciprocal of the hydrogen ion activity in a solution
Phyl.	Phylazonit (Corax-Bioner, Budapest, Hungary)
P_i	Inorganic phosphate
PIPs	Plant-incorporated protectants
PLFA	Phospholipid fatty acid
PLFAs	Phospholipid fatty acids
PMIRL	Percentage of mycorrhizal infected root length
PNP	Para(p)-nitrophenol
PO_3^{3-}	Phosphite ion

PO_4^{3-}	Phosphate ion
P_{org}	Organic phosphate
PR	Pathogenesis-related
PSM	Phosphate solubilising microorganisms
PTFE	Polytetrafluoroethylene
puriss.	Purissimum
Putt.	Putterlick
PVC	Polyvinyl chloride
P_{wsi}	Water soluble inorganic phosphate
R genes	Resistance genes
r	Correlation coefficient
RA56	*Pseudomonas fluorescens* strain RA56 (Phytobacter Production AG, Berlin, Germany)
$R\text{-}COO^-$	Carboxylate anion (R are organic groups)
RNA	Ribonucleic acid
Rock-P	Phosphate rock
rRNA	Ribosomal ribonucleic acid
RuBisCO	Ribulose-1,5-bisphosphate carboxylase
S	Sulfur
SA	Salicylic acid
Sacc.	Saccardo
SAR	Systemic acquired resistance
SE	European society or company [Latin: *Societas Europaea*]
SEM	Standard error of the mean
Sep	September
Si	Silicon
$Si(OH)_4$	Orthosilicic acid
SiO_2	Silicon dioxide

Si_xO_y	Silicates
SO_4^{2-}	Sulphate ion
SOD	superoxide dismutase
sp.	Species (singular) [Latin: *species*]
SP11	Vitalin SP11 (Vitalin Pflanzengesundheit GmbH, Ober-Ramstadt, Germany)
SPAD units	Unites based on differences in optical density at two wavelengths as measured with a chlorophyll meter (Minolta SPAD-502)
spp.	Species (plural) [Latin: *species pluralis*]
ssp.	Subspecies
Superphos	Super phosphate
t	Tonne ($1\ t = 10^6\ g$)
T.	*Trichoderma*
T50	Vitalin T50 (Vitalin Pflanzengesundheit GmbH, Ober-Ramstadt, Germany)
Tab.	Table
TAD	Take-all decline
TEA	Triethanolamine
Ti	Titanium
TiO_2	Titanium dioxide
TM	Unregistered Trade Mark
T-RFLP	Terminal restriction fragment length polymorphism
U.S.	United States
UK	United Kingdom of Great Britain and Northern Ireland
USA	United States of America
UV	Ultraviolet
v.	von
var.	Variety [Latin: *varietas*]

w/w	Weight per weight
WHC	Water holding capacity
w-o-w	Wheat-oat-wheat
w-o-w-w	Wheat-oat-wheat-wheat
w-w-o-w	Wheat-wheat-oat-wheat
w-w-w	Wheat-wheat-wheat
w-w-w-o	Wheat-wheat-wheat-oat
w-w-w-w	Wheat-wheat-wheat-wheat
Zn	Zinc
Zn^{2+}	Zinc ion
$ZnSO_4$	Zinc sulfate

A GENERAL PART

1 General background and objectives

1.1 Aims and challenges in modern agricultural production

A global challenge for agriculture is to ensure safe nutrition for the growing world population. To meet additional food demand by enhanced productivity necessitates innovative cropping technologies for a more efficient management of the limited natural resources, including fertile land, non-renewable reserves of mineral nutrients, energy and water. There is a common understanding that the establishment of sustainable agro-ecosystems for the environmentally friendly and profitable production of healthy food plays a key role for the sound conjunction of socio-economic growth and ecological integrity on local and global scales (McCalla, 2001; Roy et al., 2006; Turkson, 2014).

Issues of agricultural sustainability

The innovations of the 20th century, spread over the world as technologies of the "Green Revolution", were mainly based on progress in agricultural chemistry combined with genetically improved crop varieties. But beside exceeding achievements in productivity the widespread use of chemical fertilizers and chemo-synthetic pesticides turned out to be accompanied by massive problems related to the environment and public health, such as contaminations of food, pollutions of air and water bodies, degradation of soil fertility and loss of biodiversity, calling long-term sustainability into question (Matson et al., 1997; Swaminathan, 2006). At the same time, the closure of nutrient cycles and biological processes retaining soil fertility have tended to be neglected since the former dependence on sustainable utilization of internal resources has been overridden by the use of industrial inputs in agricultural production, becoming increasingly reliant upon the support of agro-chemistry (Welbaum et al., 2004).

Nevertheless, to alleviate persistent human undernourishment, mostly in Africa and South Asia, and to keep pace with current population projections, further raises in agricultural productivity will be required in the years ahead at an accelerated environmental impact (Rosegrant et al., 2001; Tilman et al., 2002). Data from the FAO (Food and Agriculture Organization of the United Nations) indicate, however, that annual yield growth rates for the major cereal crops, as paddy rice, wheat and maize, have been slowing down globally in recent years, even in many of the world's most fertile areas, despite high agrochemical input intensity (Gruhn et al., 2000; Heisey, 2002; Wiebe, 2003; Swaminathan, 2004). This slowdown, caused by a combination of factors, is notably associated with little or missing progress in increasing the genetic yield potential and with the degradation of agro-ecosystems (Cassmann et al., 2003). The latter includes the loss of soil fertility and increased plant health problems, due to the exploitative consequences of production intensification, lacking the perspective of a holistic natural resource base management (Conway and Toenniessen, 1999; Wiebe, 2003). With intensive management in some of the world's most productive regions farm yields reach about 80 % of existing yield ceilings (Cassman, 1999), but it has been estimated that on average 65 % to 87 % of the potential yield of major crops is not realized due to biotic and in particular abiotic stresses (Boyer, 1982; Bray et al., 2000). These findings suggest that not only plants need better adaptations to the environment in which they are grown, but also crop and soil management practices must be improved to achieve high yields under various stresses. Even though prevailing agricultural systems have the potential to feed today's world population, concerns are growing because they threaten previous productivity gains and further growth by deterioration of the prerequisites that make agriculture possible (Wiebe, 2003; Gliessman, 2007).

Scientific approaches towards sustainable agriculture

Regarding the throwbacks of the past, recent scientific approaches focus on a better understanding of biological processes supporting soil fertility, healthy plant growth and resource efficiency (Uphoff et al., 2006). This includes the complex interaction between plants and microorganisms in their environment, either catalyzing or hampering the working of the whole system (Artursson et al., 2006; Watt et al., 2006; Nadeem et al.,

2013), as well as the formation of plant own adaptations and defense reactions under various abiotic and biotic stresses (Römheld and Neumann, 2006). An overall aim is to develop integrated management strategies that make optimal use of the biological potential in agriculture in a way that, at the same time, leads to a profitable and sustained food production at high quality and yield levels while maintaining the natural resource base (Altieri and Rosset, 1995; Roy et al., 2006).

A key functional domain in this regard is the **"*rhizosphere*"**, first mentioned by the phytopathologist Lorenz Hiltner as the soil compartment influenced by the root (Hiltner, 1904; cited in: Hartmann et al., 2007) and further described as the narrow zone in the vicinity of living roots, manifested by their release of materials and determined by an interacting trinity of the soil, plant and associated organisms (Curl and Truelove, 1986; Lynch, 1990; Lavelle, 2002). Already a century ago, Hiltner envisioned the application of rhizosphere management practices comprising the use of bacterial inoculants for improved soil fertility and sustained agricultural production, based on his observation that both the mineral nutrition of plants and their resistance towards pathogenesis are dependent on the composition of the root microflora (Sen, 2005; Hartmann et al., 2007).

Nowadays, the application of biotechnological measures as alternatives or at least partial substitutes to chemo-synthetic pesticides and chemical fertilizers is regarded as an attractive but controversially discussed policy which may help to realize integrated and resource efficient cropping concepts in practice (Persley, 2000). Beside genetic engineering for enhanced plant breeding (Swaminathan, 2000; FAO, 2004) there is an increasing attention for biotechnology applications based on living organisms or active natural compounds, which are as "bio-stimulants" and "bio-fertilizers" designed to improve plant growth and nutrient acquisition, or as "bio-pesticides" and "bio-control agents" to protect the healthiness of crops (Dent, 2000; Banerjee et al., 2006; Martinez Viera and Dibut Alvarez, 2006; Selvamukilan et al., 2006). Such commercially manufactured or otherwise produced bio-preparations, also more generally referred to as "bio-agents" and by various other names, may contain a wide range of micro- and macro-organisms, microbial metabolites, plant and algae extracts, rock powders and other naturally occurring inorganic and organic materials (Hall and Sullivan, 2001; Wheeler,

2002; Montesinos, 2003; Hamer, 2004). However, the definitions of these terms are not obvious and vary depending on the view and interest of the definer, as discussed explicitly in Chapter 2.

On the one, there is a lack of clarity because single microbial strains and abiotic compounds may comprise multifunctional properties to affect growth, nutrient acquisition and healthiness of plants at the same time. Moreover, individual preparations often contain several active components in combined formulations together with supportive carrier materials, which are likely to affect the outcome interactively. If so, it becomes evident that the terms "bio-stimulant", "bio-fertilizer" and "bio-pesticide/bio-control agent" can be used appropriately with respect to the intended effect in case of specific applications, but will often be too constrict regarding the diverse nature of microorganisms and other ingredients (see Kloepper, 1993).

Likewise, material criteria are of limited usefulness for the definition of bio-preparations. More than on their physicochemical properties, their assumed effects are expected to be based on the activity of biological mechanisms. These include growth promotion by microbial-produced phytohormones, diazotrophic dinitrogen (N_2) fixation, microbial solubilization of soil minerals for enhanced availability of plant nutrients (e.g. phosphorus (P), iron (Fe)) and the suppression of pathogens by antibiosis, predation, parasitism and antagonistic competition or induction of systemic resistance in plants (Dent, 2000; Banerjee et al., 2006). Application quantities are typically but not necessarily small. A further characteristic is that direct substrate or nutritional effects are negligible, whereas the beneficial interference with biological processes, particularly those involved with direct and indirect soil-plant-microbe interactions, seems to be essential for the functioning of bio-preparations.

Introduction of the term "bio-effector"

Taking into account the considerations outlined above, the generic term "bio-effector", invented by Römheld and Neumann (2006), is defined as follows to emphasize the essential attributes of living (or at least viable) organisms and natural compounds as active ingredients in bio-preparations, which are used as supplements for crop production:

"***Bio-effectors***" are viable organisms or active natural compounds whose direct or indirect effects on plant performance are based on the functional implementation or activation of biological mechanisms, in particular those interfering with soil-plant-microbe interactions. In contrast to conventional fertilizers and pesticides, the effectiveness of bio-effectors is essentially not based on the substantial direct input of mineral plant nutrients, neither in inorganic nor organic forms, nor of a-priori toxic compounds.

"*Bio-preparations (bio-agents)*", as ready formulated products, applied with the purpose of stimulated plant growth (bio-stimulants), improved plant nutrient acquisition (bio-fertilizers), to protect plants from pathogens and pests (bio-pesticides/bio-control agents) or generally to advance cropping efficiency, can contain one or more bio-effectors as active agents along with other additive materials (Fig. 1.1).

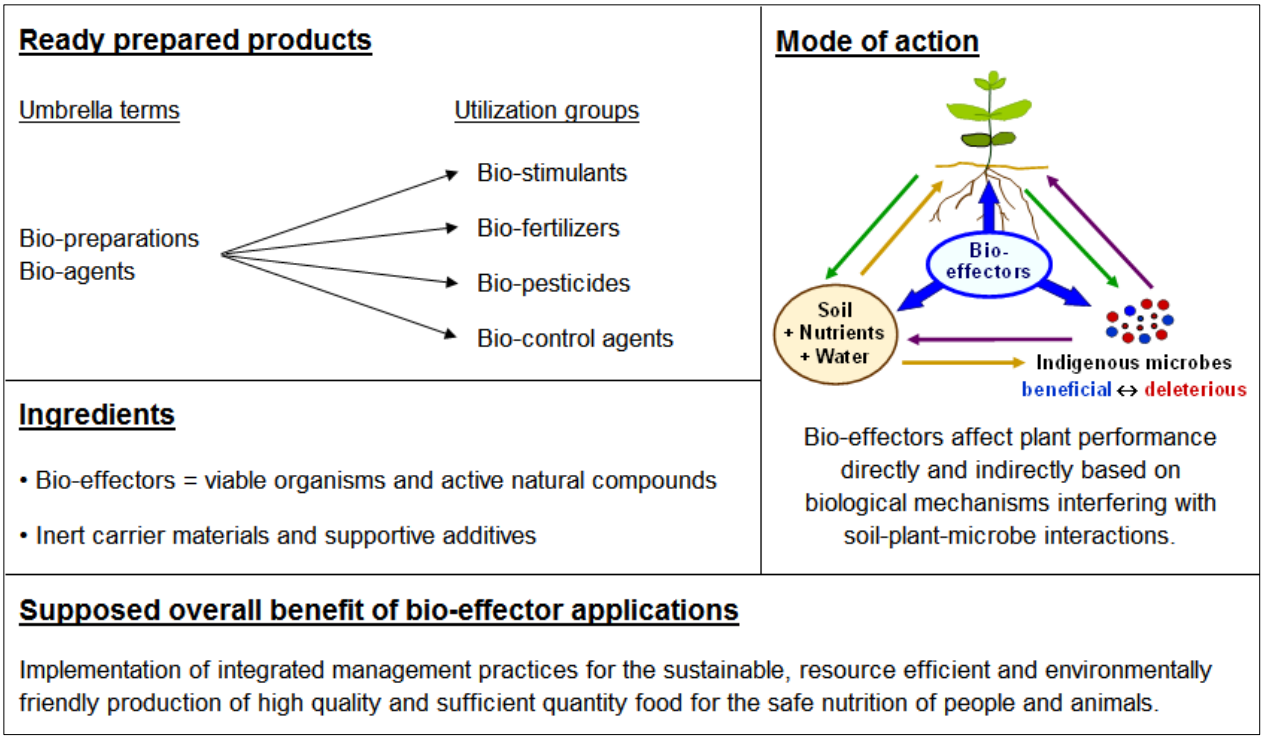

Fig. 1.1: A concept of bio-effectors as active ingredients of various groups of bio-preparations and their integrated action in soil-plant-microbe relationships

Present use of bio-effectors in agricultural practice

The successful application of bio-effectors might be worthwhile particularly in organic farming systems and developing countries where agrochemicals are legally and economically restricted or simply not available. But they may also alleviate negative

impacts on the environment by diminished or more efficient use of agrochemicals, when appropriately integrated with conventional high input farming technologies (Schmitt and Seddon, 2005; Gentili and Jumpponen, 2006).

Indeed, there is advanced knowledge on the potential mechanisms of selected microorganisms and specific natural compounds which are often contained in bio-preparations and there is objective evidence for their beneficial properties derived from experiments under controlled conditions. In recent reviews different aspects involved with phytohormonal stimulation of root growth (Kolbe, 2006), microbial mobilization of sparingly available nutrient sources such as recalcitrant soil phosphates (Richardson, 2001; Rodriguez et al., 2006), associative N_2-fixation (Bashan et al., 2004), and also pathogen control via improved resistance or antagonistic suppression (Harman et al., 2004a; Kloepper et al., 2004; Ma, 2004; Weller, 2007) have been discussed in detail. But, this knowledge is insufficiently exploited in agricultural practice and reliable data from field experiments are often missing for practical advice or application. The various individual aspects of single measures have not been integrated into a whole system approach and the complexity of multi factorial interactions in real cropping situations is only poorly understood. Consequently, unknown factors often retard the successful application of innovative products under variable environmental conditions (Alabouvette et al., 2006; Martinez Viera and Dibut Alvarez, 2006). Nevertheless, during last years, the number of commercially available bio-agents increased rapidly on the worldwide market, in developing as well as in industrialized countries, while sufficient effectiveness is usually not strictly required for their legal approval, as, for instance, according to the German Plant Protection Law – PflSchG-new, 2012, §§ 45,1-6 (Selvamukilan et al., 2006; Whipps and Gerhardson, 2007).

Novel research needs on bio-effectors for agricultural practice

Biological methods are commonly seen as environmentally friendly and preferable to agrochemicals in public opinion. Nevertheless, among farmers, being exposed to the risk of crop failure, an atmosphere of distrust towards bio-preparations exists (Martinez Viera and Dibut Alvarez, 2006). Only a few products like bio-fertilizers based on rhizobia inoculants to establish symbiotic nitrogen fixation in leguminous plants (Catroux et al.,

2001), biological insecticides based on *Bacillus thuringiensis* Berliner (1915; cited in: Milner, 1994) ssp. (Cannon, 1993; Thakore, 2006), or the use of pheromones for the control of insect pests by mating disruption (Jones, 1998b; Harari, 2007) have been established well in agricultural practice. To meet farmers' constraints bio-preparations must be easily accessible, predictably effective, easy to handle and reliable in operation, consistent in quality, and finally cost-efficient (Herrera-Estrella and Chet, 2004; Selvamukilan et al., 2006). The biological potential for developing such improvements in crop management strategies clearly exists. To reach these criteria, the bio-effectors in question have to be characterized by screening and efficacy testing in greenhouse and laboratory studies but also in field trials, exposed to the complexity of diverse environmental conditions (Saleh-Lakha and Glick, 2007; Whipps and Gerhardson, 2007). In this process, a better understanding needs to be worked out how purposeful changes in the physiological response of plants in interaction with soils and microorganisms can be induced by certain measures with respect to the intricacies of cropping practice. This is of particular concern when crops are grown under unfavorable environmental conditions with constraints from poor soils, water deficits, nutrient deficiencies, pathogens and other stresses (Neumann and Römheld, 2002; Drinkwater and Snapp, 2007). The latter are increasingly important issues in a world of declining resources and expanding demands, all the more taking into account the possible impact of global warming on local climates and agriculture (Desanker et al., 2001; Feddema and Freire, 2001; Vance, 2001; Yohe et al., 2007).

The present study assumed that the integration of thus achieved innovative knowledge on biological processes and mechanisms within overall strategies of agro-ecosystem management is a perquisite to achieve better plant growth, improved nutrient acquisition and enhanced resistance to biotic and abiotic stress conditions with the application of bio-effectors. Farmers, scientists, and producers of bio-preparations need to be prepared to make optimal benefit and to avoid possible disadvantages that could arise in the future from such methods (Martinez Viera and Dibut Alvarez, 2006). According to Römheld and Neumann (2006), it is anticipated that, facing tightening resource constraints and growing environmental concerns, the potential merits of bio-effectors will become more demanding than ever to sustain food security in the 21st century. It will be a great

challenge to support farmers worldwide, particularly those on less favorable land with adverse soil factors and weather conditions, in the optimization of soil fertility to achieve ever-increasing productivity with less input of precarious agrochemicals.

1.2 General objectives and scientific approaches of the present study

This study aimed to improve the state of empirical and conceptual understanding concerning the utilization of biological methods supporting improved soil fertility and healthy crop growth in agricultural practice (Part A). Focus was set on the application of commercially available bio-preparations based on bio-effectors including microbial inoculants and active natural compounds following the conceptual principles of (1) plant growth stimulation, (2) bio-fertilization and (3) bio-control via direct and indirect mechanisms (see Fig. 1.1).

The work was organized in successive operations with specific objectives:

1. To structure existing knowledge and definitions dealing with various bio-preparations in view of specific agricultural application areas based on literature reviews;
2. The development of fast screening methods for the preliminary assessment of bio-effectors relying on the expression of specific mechanisms to determine their potential uses;
3. To investigate the operation of functional mechanisms of selected bio-effectors in target oriented soil, plant and pathogen systems under controlled conditions;
4. To characterize the effectiveness of promising bio-effectors under field conditions to collect reliable data for practical implementation;
5. To develop further research perspectives facilitating the design of advanced bio-preparations and adequate application techniques.

Bio-preparations are often supposed to invigorate plants more generally rather than being exclusively targeted on specific purposes and may not be categorized within strictly defined groups according to their various organic, inorganic and microbial components. To structure the work in an application-oriented way, that meets farmers' constraints, the

investigations were conducted in concrete case studies regarding prevailing problems of current agricultural practices (Part B – C). Based on the results obtained, principle prerequisites for the effectiveness and successful application of specific bio-preparations were derived in order to evaluate their prospective economical and ecological benefit. This knowledge should make a contribution towards the development of resource efficient crop management strategies for safe food production within more self-sufficient agro-ecosystems. Since the investigated approaches were particularly concerned on general biological mechanisms, the represented case studies can serve as exemplary systems to provide new relevance for the comprehension of similar problems.

Experimental studies were performed to address the following major aspects:

Part B: Plant growth stimulation and bio-fertilization with emphasis on improved phosphorus acquisition efficiency

Soil fertility depends on physical, chemical and biological processes providing nutrients for crop growth. Knowledge of these processes is critical to maintain soil quality and productivity (Havlin 2005; Roy et al. 2006,43). Plant roots respond dynamically to their specific soil environment and play an active role in the spatial or chemical acquisition of nutrients and water (Marschner 1998; Neumann and Römheld 2002). Furthermore, plants nourish mutual interactions with a wide variety of soil microorganisms, which may assist or hamper the functions of the root (Hinsinger et al. 2005; Gregory 2006). Phytohormones and other signaling compounds are involved with the intra- and interspecific communication between roots, shoots and microorganisms (Dakora 2003; Kulaeva and Prokoptseva 2004; Bouwmeester 2007; Pierson and Pierson 2007). Phytostimulation and bio-fertilization are approaches to manage this complex network of interactions for optimized microbial activity, adequate nutrient availability, improved root and shoot growth, and finally enhanced crop yield. The effectiveness to achieve these results was tested with selected bio-effectors described in Chapter 4. This part of the study was directed particularly towards the improvement of phosphorus acquisition efficiency in crop plants from sparingly available phosphate pools through microbial associations.

Part C: Biological control with emphasis on soil borne pathogens

Soil health as biological component of soil quality is vital to both healthy crop growth in agronomic production systems and the ecological functionality of soils (Doran, 2002; Harris and Romig, 2005). Healthy soils represent living buffer systems that provide adequate supply of mineral nutrients to growing plants and limit the establishment of pathogens (Primavesi, 2006; Robertson and Grandy, 2006). Crop rotations are used to manage soil fertility and health by improving the use efficiency and availability of nutrients and avoiding the build-up of disease problems, thereby lowering the need for fertilizers and pesticides (Karlen et al., 1994; Finck, 1998). On the contrary, soil sickness due to monocultures and poorly designed crop rotations has been referred to the accumulation of toxins, unbalanced mineral nutrition, and soil degradation including the loss of functional biodiversity, particularly concerning the microorganisms (Hoestra, 1994; Politycka, 2005; Donn et al., 2015). The increased incidence of soil borne pathogens in replant-diseased soils is seen as a sign of biological imbalance within the soil ecosystem where the natural antagonists are impaired and pathogens become prevalent (Kloepper, 1993; Pankhurst and Lynch, 2005). Contrariwise, agricultural strategies to optimize the supply of mineral nutrients and/or to promote beneficial soil microorganisms are regarded as promising approaches to enhance the resistance of crop plants and maintain the microbial balance of pathogen infested soils, thus mimicking the effect of crop rotations (Buchenauer, 1998; Römheld and Neumann, 2006; Janvier et al., 2007). This may be achieved by enhancement of specific soil indigenous microbial communities through an adapted rhizosphere management, by addition of isolated microbial strains as seed or soil inoculants, and/or by the application of distinct mineral nutrients (Pankhurst and Lynch, 2005; Römheld and Neumann, 2006). The present study investigated the feasibility of such approach in the host-pathogen system of wheat and *Gaeumannomyces graminis* (Sacc.) v. Arx & Olivier, the fungus causing take-all root rot disease.

2 Review on definitions and properties of various bio-agents

The terms “bio-preparation” and “bio-agent” are used for products based on a wide variety of living organisms and active natural compounds applied for improved crop performance and yield. According to their supposed beneficial effects, bio-preparations may become classified as “bio-fertilizers”, or “bio-pesticides” and “bio-control agents” following the paradigm of the two types of supplements for agriculture, namely fertilizers and pesticides (Banerjee et al., 2006). A third group may be referred to as “bio-stimulants” accounting for those, which enhance plant performance by various growth stimulatory mechanisms (Chen et al., 2002; Saleh-Lakha and Glick, 2007). It can be generally assumed that the prefix “bio” suggests the presence of something living or the implementation of a biological mode of action [from Greek: βίος, bios, ‘life’ and λόγος, logos, ‘reason, speech, study, word’]. Beyond this, the scientific literature and commercial producers have filled these expressions with manifold but inconsistent interpretations. In April 2015 the international bibliographic database CAB Abstracts (CAB International, formerly Commonwealth Agricultural Bureaux, Wallingford, Oxfordshire, UK) indicated more than 1.300, 2.100, 8.300, and 4.000 citations related to bio-agents, bio-stimulants, bio-fertilizers and bio-pesticides respectively, when searching for these terms in different variations of spelling. Searching the world-wide-web with the search engine Google™ resulted in 45.000 hits for the item “bioagent”, 106.000 for “biostimulant”, 347.000 for “biofertilizer”, and 302.000 for “biopesticide”. These numbers have been increasing rapidly during the recent years. Modern dictionaries, however, do not contain definitions for anyone of these novel word combinations (see Vessey, 2003). To avoid misunderstanding it is therefore necessary to determine precisely their meaning before they are used as technical terms in scientific essays, as well as by companies and retailers. This is particularly important when complex problems are discussed interdisciplinary among scientists, but also when the same vocabulary is used in non-scientific branches referring to science. It can be presumed that proper definitions should be reasonable and clearly distinguish between that which is included and that which is excluded to set out unmistakably the character of the things defined.

2.1 Definitions and properties of bio-pesticides and bio-control agents

The terminology has been most elaborated in the case of "***biological pesticides (or bio-pesticides)***" which are subject to governmental pesticide regulations, covering risk and efficacy assessments in numerous countries. During a survey on data requirements for the registration of biological pesticides conducted by the Organization for Economic Cooperation and Development (OECD, Paris, France) 16 OECD member countries, the European Community (EC) and Hungary were asked to list categories of products they identified as bio-pesticides (OECD, 1996). The results showed vast conformity to identify microorganisms as bio-pesticides and the majority of countries also listed macro-organisms. Several countries mentioned that a pesticide must be a living organism to be "biological", but only three countries listed transgenic plants. Furthermore biologically derived chemicals (biochemicals) including, semiochemicals (signaling compounds like pheromones), growth regulators as well as plant and animal extracts were designated as bio-pesticides (see also Hamer, 2004). An overview on the spectrum of "bio-pesticide" definitions used in different countries is given in Chapter 2.1.1 of this work.

More scientifically coined, than according to administrative purposes, is the terminology of "***biological control (bio-control)***", with "***bio-control agents***" considered as the media used to achieve biological control. The roots of biological control go back to ancient times when growers started to account for the beneficial work of predaceous insects or to practice crop rotation (Doutt, 1964; Cook and Baker, 1983). Already Julius Kühn (1825-1910), regarded by Wilhelm and Tietz (1978) as a founder of scientific plant pathology, noted that the natural enemies of animal parasites represent a helpful element of pest control in crops. In this regard, Kühn emphasized the suppression of parasitic sugar beet nematodes by an antagonistic fungus, which he named *Traychium auxiliarium* (Kühn, 1877; Hallmann, 2002). In an article about fungal diseases, Carl von Tubeuf (1862-1941) introduced the expression "Biologische Bekämpfung" [English: biological combat] to microbial plant pathology, which at that time was already in common use regarding the method to control insect pests by their natural enemies (von Tubeuf, 1914). However, von Tubeuf's attempt to control *Cronartium ribicolum* causing blister rust of Weymouth Pine (*Pinus strobus* L.) by the hyperparasitic fungus *Tuberculina maxima* did not fulfill his

expectations. Subsequent scholars largely neglected the biological control of microbial pathogens, which remained an undeveloped subject for a long time, in favor of that of insects, mites and weeds, achieving highly successful practical results in that field (DeBach, 1964; Baker and Cook, 1974; Maloy and Lang, 2003). A changing scope and perspective under which scientific definitions of "bio-control" have been proposed in the literature by different authors is reviewed in Chapter 2.1.2.

2.1.1 Definitions of "bio-pesticides" according to governmental registration authorities

As "bio-pesticides" a broad spectrum of crop-protection agents derived from natural sources such as of micro- and macro-organisms, biogenic materials and their synthetic analogues, certain minerals as well as genes used to transform crops to express resistance have been labeled (Copping and Menn, 2000; Sudakin, 2003; Joshi, 2006). The target of bio-pesticide applications encompasses all kinds of pest problems (insects, fungal, bacterial and viral diseases, weeds, nematodes and molluscs etc.) in agriculture (Rodgers, 1993). Therefore, bio-pesticides can be grouped in "bio-insecticides", "bio-fungicides" "bio-herbicides", and so forth (Menn and Hall, 1999). Referring to their active ingredients among others microbial and viral bio-pesticides as well as phytochemical bio-pesticides (botanicals) based on plant extracts have been described (Kurstak and Tijssen, 1982; Isman, 2001; Koul and Dhaliwal, 2002).

Even though representing little more than 5 % of the total crop protection market, the worldwide demand for different types of bio-pesticides has been growing steadily in recent years (Menn and Hall, 1999; Thakore, 2006; Olson, 2015; MarketsandMarkets, 2016). Driving forces behind this development are the growing sensitivity to environmental and health risks, the rise of organic food sales, and the pressure of consumers, environmental activists, food traders and governments to produce food commodities free from pesticide residues but also free from pest damage (Wanjama, 2004; Wilson and Otsuki, 2004; Thakore, 2006). Further, the interest in bio-pesticides has been gaining attention since pests have been increasingly acquiring resistance to conventional pesticides (Butt et al., 1999). North America (40 %), Europe (20 %) and the

Oceanic Countries (20 %) have been reported to consume about 90 % of the global bio-pesticide production (Thakore, 2006; Aneja et al., 2016). Yet, global and regional markets for bio-pecticides have been growing rapidly in recents years (Pesticide Action Network 2003; Olson, 2015). The European bio-pesticide market is predicted to be among the most fast growing, more than doubling from about $834 million in 2015 to approximately $2100 million within the next years until 2020 (Olson, 2015; Micromarketmonitor, 2016). This would be still less than 10 % of the total European market for crop protection pesticides, which amounted to $19.6 billion in 2015 and is expected to reach $24.9 billion in the year 2020 (Market Data Forecast, 2016a). Nevertheless, the subject of bio-pesticides is receiving growing attention not only in Asian (Grzywacz, 2004; Keswani et al., 2016), African (Cherry, 2004; Giller et al. 2013) and Latin American countries (Rosset and Moore, 1997; Olson, 2015). Moreover, also the global players of the chemical industry that are dominating the markets for chemical pesticides appear to recognize bio-pesticides as a lucrative option for investments (Borriss, 2015; Market Data Forecast, 2016b; Agra-Europe, 2017). To facilitate and control the market authorization and use of bio-pesticides, governments have been drawing up regulation and registration guidelines paying particular attention to the active ingredients.

Classification of bio-pesticides in the United States of America (USA)

In the USA, bio-pesticides must be approved by the Environmental Protection Agency (EPA; Washington, D.C.) before marketing and use in practice. According to their definition bio-pesticides fall into three major classes:

1) "***microbial pesticides*** *consist of a microorganism (e.g., a bacterium, fungus, virus or protozoan) as the active ingredient*";
2) "***plant-incorporated protectants (PIPs)*** *are pesticidal substances that plants produce from genetic material that has been added to the plant*"; and
3) "***biochemical pesticides*** *are naturally occurring substances that control pests by non-toxic mechanisms*" (U.S. EPA 2017a;b).

In spite of this classification, the effectiveness of different types of bio-pesticides might be based on the same intrinsic compounds. For example, the pesticidal activity of

microbial pesticides containing strains of the soil-borne bacterium *Bacillus thuringiensis* (*Bt*) spp. is based on the formation of crystal proteins that are toxic against specific insect-pests after ingestion by the larvae (Crickmore, 2006). In transgenic *Bt*-crops the expressed *Bt*-proteins and the encoding genes, but not the plants themselves, are regulated as PIPs. And, even though biochemical pesticides are supposed not to be directly toxic, the crystal *Bt*-toxins are listed as active ingredients of bio-pesticides as well (U.S. EPA 2017c).

Pesticide regulation in the European Union and Germany

The European Union (EU) aims to uniform the rules concerning the authorization of plant protection products. The main item of regulatory legislation concerning plant protection products that applies to all EU member countries is the European Commission Regulation (EC) No 1107/2009. In Germany the evaluation, market authorization and use of plant protection products are regulated by the Federal Office of Consumer Protection and Food Safety (BVL, Braunschweig, Germany) based on the German Plant Protection Law (PflSchG-new, 2012). The European Union (EU) and the German legislation do not classify plant protection products concerning the nature of their ingredients and the term "bio-pesticide" is not officially applied there. Microorganisms and active natural compounds with "*general or specific action against harmful organisms or plants*" are rather put on a par with chemical pesticides as "active substances" respectively "Wirkstoffe" (Article 2,2 of Regulation (EC) No 1107/2009; § 41,1 PflSchG-new). Basically, this implies that for the approval of microorganisms as bio-control agents the same requirements concerning sufficient effectiveness and safety for human and animal health or the environment must be fulfilled as for the approval of chemical pesticides (Article 4; 7; and 8 of Regulation (EC) No 1107/2009). In this context, "micro-organisms" have been defined as cellular or non-cellular microbiological entities capable of replication or transferring genetic material. This applies, but is not limited, to bacteria, fungi, protozoa, viruses and viroids (Article 3,15 of Regulation (EC) No 1107/2009).

With respect to the experience that microorganisms differ from chemicals in safety relevant issues, the former European Council Directive 91/414/EEC for the market authorization of plant protection products had been amended by the European Commission Directive 2001/36/EC to introduce more precision concerning the

requirements for their registration (Hamer, 2004). Yet, when the new European Commission Regulation (EC) No 1107/2009 entered into force not only the Directive 91/414/EEC, but also the Directive 2001/36/EC to differentiate requirements for the legal approval of microorganisms or chemical pesticides was repealed. In consequence of the increasingly strict regulatory policy for the registration of plant protection produtcs in the European Union, the numbers of "active substances" on the market shrinked from about 1000 to 250 in recent years (Chapman, 2014). Nevertheless, taking account to the possibilities of cultural and biological control in plant protection is emphasized as a basic principle of "good plant protection practice" according to the EU legislaton (Article 3,18 of Regulation (EC) No 1107/2009). It is furthermore mentioned in Article 77 of Regulation (EC) No 1107/2009 that the Comission may adopt or amend guidance documents concerning micro-organisms and other biological products.

The German Plant Protection Law (PflSchG-new, 2012) distinguishes between "***Pflanzenschutzmittel***" [English: plant protection agents] and "***Pflanzenstärkungsmittel***" [English: plant strengthening agents]. "Pflanzenschutzmittel", recognized as pesticides in the stricter sense, are substances "*protecting plants or plant products against harmful organisms or preventing the action of harmful organisms*" (Article 2,1a of Regulation (EC) No 1107/2009). They also include herbicides and plant growth regulators (Article 2,1b-e of Regulation (EC) No 1107/2009; § 1-2 PflSchG-new, 2012). As "Pflanzen-stärkungsmittel", only products registered in a list of the BVL are permitted to be marketed (§ 45,3 PflSchG-new). Those are defined as substances and mixtures inclusive microorganisms which are intended (a) exclusively to maintain the healthiness of plants in general terms, but without being "Pflanzenschutzmittel", and (b) to protect plants from non-parasitic impairment (§ 2,10 PflSchG-new). This implies that "Pflanzenstärkungsmittel" in contrast to "Pflanzenschutzmittel" are supposed to have neither direct toxic nor any other effects protecting plants against harmful organisms. Coherently, several preparations containing strains of *Bacillus thuringiensis* ssp. or certain members of the *Bacillus subtilis* Cohn (1872) group (Priest et al., 1987) have been listed for years as "Pflanzenschutzmittel" regarding their direct insecticidal respectively fungicidal effect (BMELV, 2007a; BVL, 2017a). Other microbial products and active natural compounds, which had been listed as "Pflanzenstärkungsmittel" before the

European Commission Regulation (EC) No 1107/2009 was transposed into national law with the novel Plant Protection Law (PflSchG-new, 2012) of Germany, have not been further approved under this more stringent regulatory regime. The repeal of the former Plant Protection Law (PflSchG-old, 1998), which still allowed that "Pflanzenstärkungsmittel" improve the resistance of plants against harmful organisms, through the novel Plant Protection Law (PflSchG-new, 2012), thus, was associated with a decrease in the number of listed "Pflanzenstärkungsmittel" from about 380 in the year 2007 (BMELV, 2007b) to 244 in the year 2017 (BVL, 2017b).

Basically, three groups of active ingredients of "Pflanzenstärkungsmittel" can be distinguished (PflSchG-new, 2012, FiBL, 2016):

1) **Microorganisms**: Microbial preparations that had been registered in the list of "Pflanzenstärkungsmittel" (BMELV, 2007b) before the novel Plant Protection Law (PflSchG-new, 2012) entered legal force contained among other things strains of *Bacillus* spp., *Pseudomonas* spp., and *Trichoderma* spp. (JKI, 2008). Yet, in those genera mechanisms of direct antibiosis have been assumed to play a key role for the suppression of pathogens as well (Fravel, 1988; Raaijmakers, Vlami and de Souza, 2002; Viterbo et al., 2002; Chen et al., 2006). However, the restriction of the definition that "Pflanzenstärkungsmittel" must have no protective effect against harmful organisms, virtually led to the situation that products containing viable microorganisms as active ingredients are not anymore indexed in the current list of "Pflanzenstärkungsmittel" (BMELV, 2007b; BVL, 2017b).

2) **Abiotic compounds**: Beside microorganisms, a wide variety of inorganic (e.g. silicates (Si_xO_y), chalk ($CaCO_3$), alumina (Al_2O_3), baking soda ($NaHCO_3$)) and organic (e.g. humic acids, extracts from algae, plants and animals) substances of, in most cases, natural origin, have been described as active components of "Pflanzenstärkungsmittel" (BMELV, 2007b; BVL, 2017b). Nevertheless, also such compounds can have direct or indirect effects against pathogens and pests. Silicates (Bélanger et al., 2003) and baking soda (Horst et al., 1992) as well as extracts from field horsetail (*Equisetum arvense* L.; Bélanger et al., 1995) or garlic (*Allium sativum* L.; Singh et al., 1995), for instance, are known for their effectiveness to protect plants against powdery mildews, induced by

pathogenic fungi in the order Erysiphales (Gwynne-Vaughan, 1922), and other microbial diseases. Despite legal definitions, even in the case of abiotic "Pflanzenstärkungsmittel", it seems to be difficult to attribute their possible modes of action exclusively to general plant strengthening functions, which protect plants only from non-parasitic impairments.

3) **"Information" on carrier materials**: Regarding their material characteristics, homeopathic and "energized" products are not comprehensively to classify, because their proposed efficacy has been associated with the transmission of "information" contained in indifferent carrier materials like water or Epsom salt ($MgSO_4 \cdot 7H_2O$) (Hahnemann, 1921,§ 269; Vithoulkas, 1980; Maddox, Randi and Steward, 1988; Kunz, 1995; BioAktiv, 2010). The importance of homeopathics must not be underestimated, as they accounted for more than 30 % of all listed "Pflanzenstärkungsmittel" (JKI, 2009) before the novel Plant Protection Law (PflSchG-new, 2012) came into effect. In the current list of "Pflanzenstärkungsmittel" (BVL, 2017b) 9 from 244 products (less than 4 %) can be identified as homeopathics. In contrast to former lists (e.g. BMELV, 2007b), however, only the product designations, but not the ingredients and application purposes are now described (BVL, 2017b). Thus, it is more difficult for consultants and users to find suitable products and to compare them with other products of similar or same ingredients.

A point of practical relevance is that, in contrast to "Pflanzenschutzmittel" which need to proof sufficient effectiveness before approval (European Commission Regulation (EC) No 1107/2009; § 33 PflSchG-new), for "Pflanzenstärkungsmittel" it only must be assured that an intended and appropriate application causes no harmful effect on the health of humans and animals, the groundwater, and the ecosystem (§ 45 PflSchG-new). Consequently, also no official recommendations concerning the purposeful application of "Pflanzenstärkungsmittel" are made by the BVL or other authorities. Impartial information is usually rare and users of "Pflanzenstärkungsmittel" often have to rely on the manufacturers' instructions only. As a remedy to improve the market transparency, an internet database facilitating the access on available information collected from the literature and diverse sources had been established on a webpage of the former Federal Biological Research Centre for Agriculture and Forestry (Biologische Bundesanstalt für Land- und Forstwirtschaft, BBA i.e. now Julius Kühn-Institut, JKI; Braunschweig,

Germany) under the domain "http://pflanzenstaerkungsmittel.bba.de" (Marx, Kühne and Jahn, 2005; JKI, 2008). A drawback was still that most of the available data did not origin from scientifically rigorous tests and efficiency standards were not guaranteed. However, in consequence of the restrictions imposed by the novel Plant Protection Law (PflSchG-new, 2012) on the registration of "Pflanzenstärkungsmittel" the internet database has been detained for the time being.

This decline in public information together with the far-reaching loss of "Pflanzenstärkungsmittel" with effectiveness against pathogens and pests, due to the increasingly stringent regulatory regime, especially hits organic farming and the cultivation of minore crops with fairly limited market potential compared to the costly registration of specific "Pflanzenschutzmittel" (Chapman, 2014). In organic farming, the use of "Pflanzenschutzmittel" is restricted to a few active substances besides miroorganisms, as defined in Article 16 of the European Council Regulation (EC) No 834/2007 and lists in Annex II of the European Commission Regulation (EC) No 889/2008. "Pflanzenstärkungsmittel", by contrast, are exempt from being listed there and, thus, generally allowed to be used in organic farming according to the regulations of the European Commission (European Commission Regulation (EC) No 889/2008; FiBL, 2016). Before the novel Plant Protection Law (PflSchG-new, 2012) entered legal force, "Pflanzenstärkungsmittel" were attractive to desperate farmers as unprohibited alternative to "Pflanzenschutzmittel", as they could help to protect crop plants under the restrictions of organic production systems. Many active natural compunds and microbial agents, such as viruses, bacteria and fungi, which could be used as bio-control agents against specific pathogen problems requiring targeted remedial actions, are not available furthermore as commercial products, unless they have been registered as "Pflanzenschutzmittel" (European Commission Regulation (EC) No 1107/2009).

Conclusion on prevailing definitions of "bio-pesticides"

Bio-pesticides are gaining increasing momentum for the control of pests and diseases in agriculture. This development disposed governments to draw up guidelines for the regulation and market authorization of these new products. Thereby, even though in most cases remarkably different, the conception of bio-pesticides has been logically developed

from the paradigm of chemical pesticides, which are toxic materials usually not found in nature, but applied occasionally as the need arises. This might have been the reason why Cook (1993) esteemed the term "bio-pesticide" as an unfortunate label, thus referring to Garrett (1965) that biological control needs whole system approaches, which eventually involve a complete knowledge of the pathogen and host ecology. It can be seen as indicative in that connection, that the newer editions of the book previously published as "The BioPesticide Manual" (Copping, 1998) has been launched with a new title as "Manual of Biocontrol Agents" (Copping, 2004, 2011) to be more inclusive of the agents it describes.

2.1.2 Scientific conceptions and definitions of biological control

Bio-control based on antagonistic organisms

The first broadly recocnised scientific definition of the term "*biological control*" was given by Harry Scott Smith (1919; cited in: Wilson and Huffaker, 1976; Garcia et al., 1988) as the use of natural or introduced enemies to control insect pests. Traditionally, three main approaches of bio-control have been differentiated: (1) importation and establishment of non-native natural enemy populations; (2) augmentation of native natural enemies through laboratory-reared culture and periodic release; and (3) conservation of resident natural enemies, e.g. by use of selective pesticides that spare beneficial organisms, to allow them to realize their potential to control undesirable organisms (Gnanamanickam et al., 2002). This classical categorization of biological control originates from entomology where it has been developed with emphasis on "*the action of parasites, predators, or pathogens in maintaining another organism's population density*", in particular insect pests and weeds, "*at lower average than would occur in their absence*" (DeBach, 1964). Nevertheless, also for the control of microbial pathogens the application of antagonistic microorganisms as inoculants or the management of resident populations are the basic methods applied to make maximum use of biocontrol agents (Baker and Cook, 1974; Cook, 1993). In this regard, three principle mechanisms of antagonism between microorganisms have been described: (1) antibiosis, which means production of toxic metabolites by one organism inhibitory or destructive to another; (2)

competition for nutrients and space; and (3) hyperparasitism and predation, where the antagonist directly feeds on the pathogen (Park, 1960; Tronsmo, 1993; Agrios, 2005). Because all living organisms are subject to predation, parasitism or competition from other organisms, antagonistic interactions between living organisms at virtually all trophic levels might be explored for their bio-control potential (Rogers, 1993; Lingappa and Hegde, 2001). So, for example, hyper-parasitic fungus-fungus, fungus-insect and fungus-weed relationships have been described (Te Beest et al., 1992; Chet, 1987; Butt and Copping, 2000; Shah and Pell, 2003).

Importance of population dynamics for the bio-control of microbial pathogens

Understanding the ecology and dynamics of beneficial and deleterious populations has been recocnised as key prerequisite to develop effective control strategies in microbial plant pathology, likewise as in entomology, too. The importance of population dynamics for the suppression of microbial diseases becomes obvious in particular, when the delicate ecological balance of a system is disturbed, which can among others be the case when broad-spectrum pesticides are applied (Evans, 1999). To that effect, the outbreak of epidemic root diseases after reintroduction of pathogens has been accounted to the co-destruction of the beneficial microflora in fumigated soils (Hiltner, 1904; Ebbels, 1969).

An intensively studied example is the phenomenon of take-all decline (TAD), which has been attributed to the buildup of antagonistic microbial communities in soils becoming suppressive to the fungal pathogen (*Gaeumannomyces graminis* (Sacc.) von Arx & Olivier var. *tritici* Walker (Ggt)) with continuous wheat cropping (Cook and Rovira, 1976). Changes in the population structure of fluorescent pseudomonads, which are a group of bacteria commonly found in the rhizosphere of wheat (Elsherif and Grossmann, 1990; Mittal and Johri, 2007), have been shown to play a major role in this naturally occurring form of biological control (Andrade, 1994; Cook, 2003). The specific disease suppression that operates in TAD soils has been largely attributed to the production of antibiotics, e.g. phenazine-l-carboxylate and 2,4-diacetylphloroglucinol (2,4-DAPG), by certain strains of *Pseudomonas* species (Thomashow and Weller, 1988; Raaijmakers and Weller, 1998). Recently, it has been demonstrated that the cell-density-dependent control of gene expression (quorum-sensing) in biocontrol strains of *Pseudomonas* spp. is

employed in regulating root colonization and production of the respective antibiotics (Wei and Zhang, 2006; Maddula et al., 2008; Zhang et al., 2008). It has been further suggested by Fajardo and Martínez (2008) that antibiotics, beside their function as competitive inhibitors, may have an important role as signaling molecules in quorum sensing as they modulate gene transcription in bacterial cells at sub-inhibitory concentrations (Goh et al., 2002; Yim et al., 2007). Vice versa, Molina et al. (2003) demonstrated the degradation of pathogen own quorum sensing molecules (N-acyl-homoserine lactones, AHLs) by a transgenic *Pseudomonas* sp. carrying a gene from *Bacillus* sp. that encodes an appropriate enzyme (lactonase) as a possible biocontrol mechanism against potato soft rot caused by *Erwinia carotovora* and crown gall of tomato caused by *Agrobacterium tumefaciens*.

As such findings make clear, the prevalence and severity of microbial diseases is partly controlled by a dynamic balance between interacting microbial populations, competing for growth limiting resources within the ecological niches that they occupy. The ecological niche, in this regard, describes not only the environmental factors of the habitat but also all the pro- and re-active adaptations which an organism uses to survive and reproduce (Hutchinson, 1957; Pianka, 2000; Lomolino et al., 2006). Consistently, Campbell (1989,2) concluded, that similar effects, which are classically studied on the level of higher plant and animal ecology, also apply to microbial populations in both natural and agro-ecosystems. In this sense, Shurtleff and Averre (1997) encompassed the control of virtually all kind of undesirable organisms (e.g. rodents, insects, mites, nematodes, bacteria, fungi, weeds, etc.) through counterbalance of microorganisms and other components of the environment under the broad umbrella of biological control. Agrios (2005), in his widely used standard textbook of plant pathology, simply described biological control as the "*total or partial inhibition or destruction of pathogen populations by other organisms*" in the widest sense.

Host resistance as a mechanism of bio-control

Plant pathologists initially adopted from entomologists the concept of population dynamics in biological control, but also stressed on major differences between biological control of microbial pathogens and macro-organisms like insects and weeds. With regard to the control strategies of microbial antagonists, Baker and Cook (1974) argued that the

principle of population ecology, that repressive environmental factors intensify as the population increases and relax as it declines, might only partially explain the suppression of plant pathogens. According to the opinion of these authors, biological control is the control of one organism by another (Beirne, 1967; cited in: Cook, 1989). Yet, this refers not only to the decrease of the inoculum (population) density of a pathogen due to direct antagonisms (DeBach, 1964), but also includes the restriction or prevention of disease producing activity, as due to various types of crop resistance, without regard to the size of the pest population (Cook and Baker, 1983).

In general, improved resistance of plants can be either achieved by conventional breeding and gene technology. In this case, the plant itself becomes the agent of biological control. Beyond this, the expression of host-plant resistance can be stimulated by other organisms (Cook and Baker, 1983). The mechanisms involved in such indirect bio-control activity include the improved acquisition of nutritional elements with specific roles in host resistance (e.g. Si, Mn, Cu, Zn; Graham and Webb, 1991; Dordas, 2008), compensation for pathogen damage as due to morphological changes of the root system or generally enhanced growth, and, more specifically, forms of induced resistance, predisposing the plant to parry future attacks (Azcón-Aguilar and Barea, 1997). On basis of the elicitors (signaling compounds) and regulatory pathways by which the plant's intrinsic defense mechanisms are activated two forms of induced resistance have been differentiated: (1) systemic acquired resistance (SAR), which is induced by necrotizing pathogens and associated with salicylic acid (SA) as a possible signaling molecule activating the expression of pathogenesis-related (*PR*) genes in non-infected plant parts; and (2) induced systemic resistance (ISR), which is activated upon root colonization by certain non-pathogenic rhizobacteria (especially *Pseudomonas* spp.) independent from SA and *PR* gene expression but dependent on jasmonic acid (JA) and ethylene as signaling molecules (Kloepper et al., 1992; Pieterse et al., 1996; reviewed in: van Loon, 1997; Sticher et al., 1997; Mauch-Mani and Métraux, 1998; Pieterse and van Loon, 2004). Investigations with resistance-defective plant mutants have shown that the protein NPR1, which is encoded by the "non-expressor of pathogenesis-related genes1" (*NPR1*) gene, seems to play a central regulator role in both, SA and JA mediated, signaling pathways (Cao et al., 1994; Pieterse et al., 1998). In contrast to SAR, downstream of NPR1 in the cascade of defense

responses, ISR is commonly not associated with the accumulation of pathogenesis-related (PR) proteins, while different defensive compounds involved with ISR remain to be identified (Pieterse et al., 1998; Vallad and Goodman, 2004; van Loon and Bakker, 2006).

As these illustrations reveal, biological control can be seen as a continuum that ranges from direct antagonistic suppression over indirect agency through induced resistance by associated pathogenic and non-pathogenic organisms until the pre-resistant plant acting alone. This perception led Cook and Baker (1983) to define biological control as "*the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man*". The organisms included hereby as interacting components of biological control systems are the pathogen itself, the host plant manipulated towards enhanced resistance and antagonists that directly or indirectly impair the activities of the pathogen.

Bio-control based on biological processes and products

Today biological control of plant pathogens and pests emerges as a broad concept, whose scope has been expanded over decades with respect to an increasing understanding of the mechanisms of bio-control, involving the genetic basis of pathogenesis, antagonism and resistance. In particular the advent of molecular technologies including the transformation of microorganisms and plants to express gens for the production of novel compounds has led the United States National Academy of Sciences (NAS) to propose a redefinitions of the term "biological control" as "*the use of natural or modified organisms, genes or gene products to reduce the effects of undesirable organisms (pests) and to favor desirable organisms such as crops, trees, animals and beneficial insects and microorganisms*" (NAS, 1987,57). It was further outlined in the same report, that in addition to natural antagonists as the classic agents, the host as well as the pest against itself can be used as components (agents) in three major strategies of biological control: (1) regulation of the pest population; (2) systems of protection that exclude infection or deter pest attack; and (3) self-defense of the host due to enhanced resistance. According to this broad definition cultural practices (e.g. crop rotation or tillage to maximize the effect of indigenous agents), genetic manipulation resulting in host resistance or incapable pest genotypes to replace capable ones, as well as the application of biochemical compounds (e.g.

pheromones) are considered as approaches to achieve biological control (NAS, 1987,59). As an example that demonstrates all three strategies of biological control, the NAS report cited *B. thuringiensis* and its toxins (see Chapter 2.1.1). Thus, the bacillus used as a natural enemy regulating the pest population would fit with strategy 1, the toxin gene expressed in transgenic rhizosphere bacteria colonizing the host roots as a living barrier against pest attack corresponds to strategy 2, and production of the toxic protein as a self-defense system by transgenic crops, finally, is consistent with strategy 3 (NAS, 1987,59).

The NAS-definition, which broadened the scope of "biological control" beyond the use of living organisms, provoked considerable controversy between proponents and those who advocated a restriction of the meaning to the use of natural enemies in contrast to other biologically based methods (Gabriel and Cook, 1990; Garcia et al., 1988; U.S. Congress, 1995; Pal and McSpadden Gardener, 2006). In this regard, Garcia et al. (1988) criticized that the *B. thuringiensis*-toxin would protect the plant from pest attack in the same manner as would any chemical pesticide do, thus they considered its use merely as a "biotechnique". Similarly the release of sterile males or the use of pheromones to control insect pest, plant breeding and cultural control methods have been described as "biology-based forms of pest control", "biotechnical control", and "non-chemical control", all accounted to be different from classical biological control by divers authors (Van Driesche and Bellows, 1996; Simmonds et al., 1976; Wilson and Huffaker, 1976). In contrast, Gabriel and Cook (1990) simply separated the manifold methods of pest and disease control in chemical, physical and biological, proposing that biological control should include all of its aspects. Cultural methods, as for example crop rotations, these authors regarded as one means to achieve biological control insofar as the suppression of pathogens and pests is facilitated for biological reasons. Accordingly, Cook (1993) argued that gene products such as endotoxins of *Bacillus thuringiensis* delivered by living microorganism or within the transgenic host plant, becoming thus resistant against insect pests would be biological control. But, the *B. thuringiensis*-toxin extracted from the organisms and applied directly as a biochemical compound would fit more logically with the concept of chemical control.

However, as emphasized by Hardy (1993) the transitions between biological, chemical, and physical/cultural approaches can be smooth like in the case of synthetic analogs of naturally occurring toxins or mixed cropping systems for the control of weeds by allelopathy and competition (cp. Chou et al., 1987; Vandermeer, 1989). Correspondingly, more recent conceptions of biological control have tended to reconsider the agency of living organisms tantamount with the use of biological mechanisms, processes and products (Campbell, 1989; Wilson, 1997). By this means, Wilson (1997) proposed to define biological control as: "*The control of a plant disease with a natural biological process or the product of a natural biological process*." In order to facilitate the multisided search for effective alternatives to synthetic pesticides, this would include biological chemicals regardless whether they are delivered by or extracted from living organisms as well as any form of host resistance (Wilson and El Ghaouth, 1993).

Bio-control based on the implementation of knowledge on biotic systems

In spite of all things considered so far, the agency of man himself in biological control has been either explicitly excluded or neglected in prevalent definitions of biological control (cp. DeBach, 1964; Cook and Baker, 1983; NAS, 1987; Wilson, 1997). So, if human's activity or management is not implicitly implied, the question arises who the controller is. In this connection, Barbosa and Braxton (1993) made clear that all the processes, which determine the survival and abundance of populations unaided by human intervention, are natural controls. *Biological control, in contrast, is based on human's understanding of living organisms which is implemented for the purposeful management of natural controls or the direct limitation of pests (undesirable organisms) and their negative impact.* This conception comes closest to the common meaning of "biology" as the study of living organisms and their vital processes (Biology, 2008), because the awareness and intension of humans is regarded as the motive force behind biological control.

As one can control only the known and understood, the successful realization of biological control eventually necessitates a complete understanding of the biology and epidemiology of a disease and of the ecology of the host plant (Garrett, 1965). Thus, the best knowledge on each of the three categories of control methods (biological, chemical, physical) should be used and combined into integrated management systems to realize

plant-health care in the most effective, economical, safest and sustainable way (Cook, 1989; Hardy, 1993). Yet, to encompass the complex interactions between crop, pathogens and antagonists, that occur in biological control systems and become apparent upon thorough investigation, requires a broad perspective that goes beyond the narrow limits of traditional bio-control definitions focusing on the direct action of antagonists versus parasitic organisms or the derivation of the products used (Wilson, 1997).

Conclusion on conceptions and definitions of biological control

With respect to the changing scope and perspective under which definitions have been proposed in the literature by different authors, it is concluded here that biological control refers to the purposeful implementation of effective strategies based of the knowledge how to manage biotic processes for improved plant health-care respectively to limit the undesirable effect of pests (weeds, insects, nematodes, pathogens etc.) in the widest sense. To realize this aim, biological control favors the use of living organisms and natural compounds (bio-effectors) as well as physical/cultural methods instead of chemo-synthetic pesticides.

2.2 Definitions and properties of bio-stimulants and bio-fertilizers

According to a definition of the International Fertilizer Industry Association (IFA), "bio-fertilizers" are products based on microbial inoculants (e.g. *Rhizobium* species) or organic growth stimulants, which affect the soil biotically, chemically or physically (IFA, 2005). This is in close agreement with the German Fertilization Law (DüngG, 2009, § 2,6) that describes "Bodenhilfsstoffe" (English: soil auxiliary supplies) as *substances without substantial nutrient content as well as microorganisms, which affect the soil biotically, chemically or physically to improve the growth conditions for crop plants or to promote the biological nitrogen fixation.* The range of "Bodenhilfstoffe" may comprise microbial inoculants, soil conditioners, soil stabilizers, rock powders and even substances with significant content of mineral nutrients when they are used in restricted quantities (DüMV, 2012). Notwithstanding, the term "bio-fertilizer" is widely used with a broad meaning regardless of the mineral nutrient content of its active ingredient itself, as the purpose of their application is not only to ensure the adequate supply of mineral nutrients

to the plant, but also to maintain or improve the fertility of soils by biological modes of action (DüngG, 2009, § 1,1-2; du Jardin, 2015).

2.2.1 Principle modes of action of different bio-stimulants and bio-fertilizers

In the recent scientific literature and political debate there has been incited to separate "bio-stimulants" (also referred to as "bio-enhancers", "phytostimulants") from "bio-fertilizers" to indicate preparations which enhance crop growth and yields due to immediate stimulatory effects on plants themselves. The intension is to distinguish "bio-stimulants" from agents that improve the fertility of soils, directly enhance the plant availability of mineral nutrient, and especially from those which have direct effects against pathogenic organisms (Bloemberg and Lugtenberg, 2001; Ohyama, 2006; Saleh-Lakha and Glick, 2007; du Jardin, 2015; EBIC, 2016). Likewise the term "Pflanzenhilfstoffe" [English: plant helping agents] has been defined in the German Fertilization Law (DüngG, 2009, § 2,7) as *substances without substantial nutrient content that are supposed to affect plants biologically or chemically in oder to achieve benefits in plant production or application technology, insofar as they are not "Pflanzenstärkungsgmittel" according to the German Plant Protection Law (see Chapter 2.1.1).* Nevertheless, while detailed definitions vary, the terms "bio-stimulant" ("phyto-stimulator") and "bio-fertilizer" haven been used interchangeably for the same kind of materials as for example in the case of phytohormone rich algae extracts or certain root associated bacteria (Crouch and van Staden 1994; Zodape, 2001; Tilak and Singh, 2002; El Zemrany, 2006). In any case, it can be stated from a practical perspective that an adequate uptake of essential nutrients by the plant is an indispensable prerequisite for substantial growth gains. Concerning plant nutrient acquisition, however, two groups of active compounds can be distinguished which differ principally by their mode of action:

I. Preparations providing pre-contained mineral nutrients

The one group of compounds identified as "bio-fertilizers" is likely to increase the supply of nutrients to the plant most notably by mineral elements already present in them. Those include for instance animal manures (Abdel Magid et al., 1995), earthworm composted

organic wastes (Mba, 1997; Benítez et al., 2000) and green manures from nitrogen fixing legumes (Becker et al., 1988; Kalidurai and Kannaiyan, 1991).

II. Preparations enhancing the plant accessibility of mineral nutrients

The second group, comprising various microorganisms (Subba Rao, 2002; Hafeez et al., 2006) and substances with plant growth regulative activity (e.g. phytohormone rich seaweed extracts; Zodape, 2001), unlike fertilizers in the common sense merely carries the ability to improve plant nutrient acquisition from other sources. In principle, this can happen either directly via increased delivery of nutrients to the plant but also indirectly via stimulation of plant own nutrient acquisition strategies, comprising aspects of chemical and spatial nutrient availability in both cases (Marschner, 1989; Jungk, 2002).

Direct mechanisms facilitating plant nutrient acquisition

In this regard, biological nitrogen fixation by diazotrophic bacteria and solubilization of nutrients from recalcitrant soil pools (e.g. by phosphate solubilising microorganisms (PSM)) are mechanisms which directly improve the chemical availability of nutrients to the host plant (Graham and Vance, 2000; Khan et al., 2007). An example for directly improved spatial nutrient availability is arbuscular mycorrhiza (AM), the widespread rhizosphere association between terrestrial plants, including many crop species, and obligate symbiotic fungi (Smith and Read, 1997). Arbuscular mycorrhizal fungi (AM-fungi) spread their hyphae from the root surface into a larger soil volume than do roots alone (see 3.2.1). Thus, they can amplify the surface area to absorb phosphorus and other nutrients with low mobility in soils, whereas their ability to chemically mobilize insoluble or strongly adsorbed soil phosphates is quite limited, as it has been shown in earlier and recent studies (Mosse, 1986; Marschner and Dell, 1994; Bagyaraj, 2002; Antunes et al., 2007).

Indirect mechanisms facilitating plant nutrient acquisition

Indirect effects on the nutrient acquisition of plants are associated with corresponding alterations in root morphology and physiology. Enlargement of the root surface by increasing the number of lateral roots and root hairs and thereby also of their spatial

uptake capacity are typical plant own responses to specific nutrient limitations (more precisely N, P, sulfur (S) and iron (Fe)) which can be mimicked due to microbial in situ production as well as external supply of ready prepared phytohormones (Forde and Lorenzo, 2001; Dobbelaere et al. 2003; López-Bucio et al., 2003). Because roots hairs and apical root zones are preferred sites for the release of exudates with nutrient mobilizing properties (e.g. carboxylates, phytosiderophores), also plant own chemical mechanisms for nutrient acquisition can be addressed by these means (Marschner et al., 1987; Hoffland et al., 1989; Jones, 1998a; Michael, 2001). Furthermore, microbial activity in the rhizosphere may stimulate root exudation by a number of mechanisms (e.g. degradation of root exudates, release of microbial metabolites, generally improved root activity), but also counteract chemical nutrient acquisition of plants due to continuous consumption of root released organic solvents as a carbon source (Barber and Lynch, 1977; Meharg and Killham, 1995; Watanabe and Wada, 1989). Nevertheless, plants have developed ways to prevent microbial degradation of root exudates (e.g. temporal and spatial variation in root exudation, release of phenolics with antibiotic activities). The net result of their interaction with microorganisms depends on the plant genotype and is influenced by environmental factors such as temperature, light intensity and various chemical and physical soil properties (Takagi et al., 1984; von Wirén et al., 1993; Dinkelacker et al., 1995; Neumann, 2007).

2.2.2 Critical appraisal of current “bio-stimulant” and “bio-fertilizer” definitions

Accentuating the above described qualitatively different principles how the growth and mineral nutrient status of plants can be improved by application of appropriate substances, and microorganisms, controversial attempts to define the terms “bio-stimulant” and “bio-fertilizer” can be found in the literature.

Bio-fertilizer versus organic fertilizer

In this context Vessey (2003) suggested to distinguish “bio-fertilizers” from any kind of organic fertilizers and not to use the term interchangeably with “green manure”, “manure”, “intercrop”, and other terms already used for manifold organic materials which immediately or after their decay deliver nutrients already containing in them. In contrast

to this argumentation, which restricts "bio-fertilizers" to the second group described above, water-fern of the genus *Azolla* Lamarck (1783; cited in: Bergman et al., 2007) is commonly referred to as a "bio-fertilizer" which has been used for centuries to increase the fertility of rice fields (Shi and Hall, 1988; Peters and Meeks, 1989; Vaishampayan et al., 2001). *Azolla*, living in symbiosis with the N_2-fixing cyanobacterium *Anabaena azollae* Strasburger (1873; cited in: Bergman et al., 2007), can add more than 60 kg N per hectare and season when applied by incorporation into the soil as a green manure before planting or grown as an intercrop among the target crop. But in any case, the fixed nitrogen is released in the main only when *Azolla* decomposes, as it is the case with any other green manure (Ito and Watanabe, 1985; Lem and Glick, 1985; Ventura and Watanabe, 1993).

Replacement of nutrients versus improved availability

Okon and Labandera-Gonzalez (1994) argued that the name "bio-fertilizer" is not appropriate if the application of a microbial inoculum only improves the utilization of fertilizers, respectively of nutrients present in the soil, but does not replace them like ordinary fertilizers do. This understanding, based on the comprehension that fertilization is identical with adding nutrients to the system, conclusively confines microbial "bio-fertilizers" to actively N_2-fixing microorganism. Contrary to this position, Vessey (2003) defined "bio-fertilizer" as: *"a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant"*. Hence, independent whether soil nutrients are replaced or just made more available by any of the direct and indirect mechanisms described in Chapter 2.2.1, microbial preparations would be considered as "bio-fertilizers" as long as they enhance the nutrient status of plants. This conception of "biofertilizers" includes most of the preparations which other authors described as "bio-stimulants", as far as their immediate growth promoting activity has been chiefly attributed to pre-contained or microbially produced phytohormones such as auxins, cytokinins and gibberellins (Beckett and van Staden, 1989; Dobbelaere et al., 1999; Paczynski and Dobrzanski, 2004), which are known to be involved with the induction of lateral roots, root elongation and root hair

formation (Ridge and Katsumi, 2002; Aloni et al., 2006). Coherently, in many studies improved nutrient uptake and water status of plants due to root interception of a greater soil volume has been assumed to be the main factor enhancing crop yields in response to the application of "bio-stimulators" (Lin et al., 1983; Finnie and van Staden, 1985; Steenhoudt and Vanderleyden, 2000).

Plant nutrient status versus overall soil fertility

As mentioned by Roy et al. (2006,130), the word "bio-fertilizer" strictly speaking is a misnomer when it is used under the following premises: (1) the suffix "fertilizer" is understood in the common sense, equivalent to mineral fertilizer, dung or manure, which are substances pre-containing essential mineral elements for plant growth, but (2) "bio-fertilizer" is used to denote microbial inoculants which are supposed to improve the nutrient status of the host plant through on-going processes while living in association with the plant, instead of providing nutrients present in them in advance (cp. Okon and Labandera-Gonzalez, 1994; Vessey, 2003). Interpreted with respect to the conventional meaning of "fertilizer", the term "bio-fertilizer" would fit most accurately with legumes or *Azolla* water fern used as green manure, which provide biologically fixed nitrogen and other nutrients previously bio-accumulated in their biomass. Yet, soil fertility [from Latin: fertilis, 'bearing in abundance, fruitful, productive'] as a whole comprises a complexity of chemical, physical and biological properties beyond the pure nutrient effect. The use of "bio-fertilizers" may have several further advantages like increasing the content of organic matter in soils or improving the physical soil structure, which can promote plant growth without necessarily affecting the nutrient status of the soil (Singh and Singh, 1987; Wagner, 1997). As an example, bacteria of the genus *Rhizobium* Frank (1889; cited in: Young et al., 2001) and related genera, well known for their ability to form nodules and fix nitrogen in legume host roots, may serve. For centuries legume cropping has been used as a principal biological method to improve and maintain soil fertility in many agricultural systems. Long time before the bacteria responsible for nitrogen fixation in legume nodules were identified (Beijerinck, 1888; Hellriegel and Willfarth, 1888; cited in: Mikola, 1986) and became available as soil inoculants (Nobbe and Hiltner, 1893; cited in: Hartmann et al., 2007), the ancient Greek writer Theophrastus (370-285 B.C.)

reported: "*... the bean best reinvigorates the soil. Beans are not a burdensome crop to the ground: they even seem to manure it, because the plant is of loose growth and rots easily ...*" (Fred et al., 1932; cited in: van Kessel and Hartley, 2000). This historical quotation has been proven to be true not only due to the ability of beans and other legumes to fix nitrogen, but also with respect to their further advantages in crop rotations. Such non-N effects can account for more than 90 % of the rotational benefit of legume crops and are attributable to the breaking of pathogen and pest cycles, soil structure improvement or the efficient use of nutrients from recalcitrant pools and deep soil layers (Hoshikawa, 1991; Stevenson and van Kessel, 1996; Lupwayi et al., 2005). Among the most widespread limitations to N_2-fixation by legumes, beside adverse climate factors are nutritional problems, mainly deficiencies of N, P and molybdenum (Mo), on marginal soils (Gibson, 1976; Hafner et al., 1992; Bagayoko et al., 2000a; O'Hara, 2001; Uliassi and Ruess, 2002). In soils poor of available nitrogen, legume seedlings may not be able to establish an effective N_2-fixing symbiosis without supply of additional nitrogen fertilizer as a starter dose (Schomberg and Weaver, 1990). Thus, although selected strains of rhizobia applied as microbial inoculants to legume crops belong to the most prominent group of microorganisms described as "bio-fertilizer" (Brahmaprakash and Hegde, 2002; Cocking, 2003; Zahran, 2006), it is rather the whole process of growing legumes than the application of rhizobia inoculants per se which can make a soil more fertile. Rhizobial nitrogen fixation is a dynamic process, performed together with the legume host plant and determined by various environmental conditions (Schultze and Kondorosi, 1998; Perret et al., 2000; Vance and Lamb, 2001). The performance of the whole soil-plant-bacteria system is of importance in order that rhizobia inoculants can become an efficient source of fixed nitrogen for the plant (Döbereiner, 1977; Slattery et al., 2001).

Living microorganism versus the management of biological processes

As argued by Vessey (2003) in accordance with Subba Rao (1981; cited in: Banerjee et al., 2006), "bio-fertilizers" must contain living (or at least viable) microorganisms, in particular when the term "bio-fertilizer" is interpreted as a contraction of the term "biological fertilizer". However, as biology is the study of living things, but also of their vital processes with all the physicochemical aspects of life (Biology, 2008), it is

reasonable as well that any material applied might be referred to as a "bio-fertilizer", as long as its effectiveness is achieved based on the knowledge how to engineer biotic processes which casually improve plant productivity or soil fertility. This is of considerable relevance because almost every rhizosphere process involves a response cascade of interdependent soil-plant-microbe reactions and very little is known about the sum of factors that lead to reliable applications of microbial preparations (Kloepper et al., 1989; Lugtenberg et al., 2001; Pinton, 2004). An illustrative example for this can be derived from the requirement of Mo for symbiotic nitrogen fixation in legume root nodules because of its specific role in their nitrogenase enzyme system (Dilworth and Loneragan, 1991; O'Hara, 2001). Experiments with diverse leguminous species have shown that inoculation with rhizobia did not improve the nitrogen nutrition of the plants on molybdenum deficient soils even though a higher number of nodules might be formed under these conditions. In contrast, small amounts of molybdenum applied with the seeds (19 ppm) or on the leafs (40 g ha^{-1}) improved the effectiveness of inoculated as well as soil indigenous rhizobia in terms of nitrogen fixation rates and crop yields (Mulder, 1954; Gurley and Giddens, 1969; Vieira et al., 1998a,b,c; Hafner et al., 1992; Kaiser et al., 2005). These results demonstrated that, under certain soil conditions, rather the biological effect of an abiotic compound, namely Mo, was necessary to replace N fertilizer by biological N_2-fixation than the application of living microorganisms. Therefore, with respect to the complexity of biological systems, sufficient knowledge of the biological mechanisms involved and of the prerequisites for their appropriate functioning might be considered as the most critical factor for the successful implementation of biological approaches to improve the fertility of soils beyond all material conceptions (Kloepper et al., 1989; Brimecombe et al., 2007).

2.3 Multi-functional and multi-partite soil-plant-microbe interactions

As presented above bio-effectors are used for phytostimulation, bio-fertilization and bio-control. But, from a functional view, clear boundaries between these categories do not exist. In fact, the response of plants on treatment with miscellaneous bio-effectors results largely from complex and multi-functional interactions between the associated crop, introduced organisms and others already present in the habitat. Each of these interactions

is affected by countless environmental variables such as climate and soil factors (Kloepper et al., 1989; Requena et al., 1997; Bloemberg and Lugtenberg, 2001; Whipps, 2001, 2004; Khan et al., 2014). Among the most versatile known microbial bio-effectors, are plant growth-promoting rhizobacteria (PGPR), a heterogeneous group of non-infective microorganisms colonizing the root system and improving plant performance by various mechanisms (Schroth and Hancock, 1981; Kloepper, 1993). In that field two main research directions have been followed (reviewed in Bashan and Holguin, 1998). One of them is the direct growth-promoting effect of free living diazotrophs like *Azospirillum* spp. (Tarrand et al., 1978) and *Azotobacter* spp. (Beijerinck, 1901) which has been attributed to several mechanisms including biological N_2-fixation and, even more important, the production of growth stimulatory substances such as phytohormones and vitamins (Döbereiner and Day, 1976; Bashan and Levanony, 1990; Steenhoudt and Vanderleyden, 2000; Dobbelaere et al., 2003). Another research area is the suppression of soil borne pathogens by use of biocontrol PGPR, for example antagonistic strains of *Pseudomonas* spp. and *Bacillus* spp. as the best-known genera, to enhance plant growth indirectly by the suppression of pathogens (Kloepper and Schroth, 1978; Kloepper et al., 2004; Weller, 2007). Further, in recent essays instead of PGPR also the abbreviation PGPM for plant growth-promoting microorganism has been used to include fungal root colonizers such as *Trichoderma* spp. and arbuscular mycorrhizal fungi under one roof for beneficial rhizosphere associations (Avis et al., 2008; Gravel et al., 2007). Single microbial species and even strains from all these PGPM-groups have been shown to possess a number of beneficial effects which may result in enhanced plant growth, nutrient acquisition and healthiness at the same time (Yao et al., 2006; Avis et al., 2008). An overview on exemplary literature reports accounting for the multiplicity of plant growth-promoting properties of selected groups of rhizosphere microorganisms based on bilateral relations with their host or the direct suppression of pathogens is given in Table 2.1 and the subsequent Chapters 2.3.1 to 2.3.4. Beyond these one-on-one interactions with pathogens or their host, certain strains of PGPMs also interact with other beneficial microorganisms and may thus improve plant growth synergistically in tri- or multi-partite interactions (Banerjee et al., 2006; Saxena et al., 2006), which is briefly elucidated in Chapter 2.3.5.

Table 2.1: Potential properties of selected types of PGPMs reported in the literature

Type of PGPMs	Bio-stimulation and Bio-fertilization	Bio-control
Bacillus spp.	• Phytohormone-like actions (Idris et al., 2004) • Phosphate solubilization (Hariprasad and Niranjana, 2008) • Biological N_2-fixation (Rennie et al., 1983)	• Competitive colonization of plant surfaces (Bais et al., 2004) • Production of antibiotics (Asaka and Shoda, 1996) • Induction of plant's own defenses (Kloepper et al., 2004)
Pseudomonas spp.	• Modulation of phytohormonal balances (Glick et al., 1997) • Phosphate solubilization (Trivedi and Sa, 2008) • Biological N_2-fixation (Lalucat et al., 2006) • Siderophore-mediated iron mobilization (Bar-Ness et al., 1991)	• Competition for space and nutrients (Kloepper et al., 1980b) • Production of antibiotics (Hamdan et al., 1991) • Induction of plant's own defenses (Bakker et al., 2007)
Trichoderma spp.	• Modulation of phytohormonal balances (Gravel et al., 2007) • Phosphate solubilization (Anusuya and Jayarajan, 1998) • Solubilization of micronutrients (e.g. Mn; Altomare et al., 1999) • Enhancement of the plant's nitrogen use efficiency (Harman, 2000) • Degradation and buffering of toxins (Neumann and Laing, 2006)	• Competition for resources (Hjeljord and Tronsmo, 1998) • Production of antibiotics (Schirmböck et al., 1994) • Inhibition of pathogenesis-related enzymes (Zimand et al., 1996) • Hyperparasitism (Mycoparasitism; Chet, 1987) • Induction of plant's own defenses (De Meyer et al., 1998)
Arbuscular mycorrhizal fungi	• Spatial acquisition of mineral nutrients (Marschner and Dell, 1994) • Improvement of the drought resistance of plants (Augé, 2001) • Alleviation of salt and heavy metal stresses (Quilambo, 2003) • Stabilization of soil structure (Miller and Jastrow, 1992)	• General raise of plant vigor (Azcón-Aguilar and Barea, 1996) • Induction of plant's own defenses (Cordier et al., 1998) • Induction of changes in the composition of soil microbial populations (Secilia and Bagyaraj, 1987) • Interference with pathogenesis-related signaling pathways (Bouwmeester et al., 2007)

2.3.1 Multi-functionality of beneficial *Bacillus* species

Taxonomy and description of Bacillus species

Bacillus species (Cohn, 1872) are characterized as facultative or obligate aerobic, gram-positive rods, ubiquitous in nature and primarily found in soils (Harwood, 1989; Madigan et al., 2003). Their ability to form a dormant, highly resistant cell type, named endospore, to survive periods of environmental stresses and starvation, makes them appealing candidates to be formulated as long-term viable bio-preparations (Weller, 1988; Dirks, 2004). Many *Bacillus* spp. are known for the production of extracellular enzymes to perform a wide range of chemical transformations, antibiotics as well as probiotic compounds (Harwood, 1989; Hong et al., 2008). Among them, *Bacillus subtilis* is not only one of the most intensively studied prokaryotes, but also widely used in traditional and industrial fermentation processes as well as in agriculture (Fritze, 2004). Closely related *Bacillus* species are not easy to distinguish on the phenotypic level. Thus, increasing numbers of additional species have been identified by genotypic analysis, such as *Bacillus amyloliquefaciens,* which has been described as a strain of *B. subtilis* in prior studies (Priest et al., 1987; Krebs et al., 1998; Idriss et al., 2002; Fritze, 2004; Borriss et al., 2011). *B. subtilis*-like strains, however, have been found to improve plant health and production by a variety of mechanisms upon root and shoot colonization (Rytter et al., 1989; Sharga, 1997; Krebs et al., 1998; McSpadden Gardener, 2004; Blom et al., 2012). Although this is not a character of taxonomic use in this genus, most *Bacillus* species including *B. amyloliquefaciens* and other members of the *B. subtilis* group (Priest et al., 1987) are motile by means of peritrichous flagella (Claus and Berkeley, 1986). Chemotaxis in *B. subtilis* has been reviewed by (Ordal and Nettleton, 1985).

Direct growth promotion and bio-fertilization effects of Bacillus species

Major activities of direct plant growth-promotion and bio-fertilization performed by strains of the *B. subtilis* group include phytohormone (e.g. auxin and cytokinin)-like actions (Idris et al., 2004; Arkhipova et al., 2005) as well as the solubilization of inorganic and organic phosphates increasing the availability and uptake of phosphorus in the rhizosphere of host plants (Idriss et al., 2002; Yao et al., 2006; Hariprasad and Niranjana,

2008). While diazotrophy does not seem to appear in type strains of the *B. subtilis* group (Achouak, 1999), Rennie et al. (1983) showed that up to 32 % of N in wheat plants was derived from the atmosphere following inoculation with *Bacillus polymyxa*, which has been identified as a N_2-fixing species by some strains (Hino and Wilson, 1958).

Bio-control effects of Bacillus species

Bio-control of *B. subtilis* is mediated by direct antagonism, promotion of host nutrition and stimulation of plant own defenses (McSpadden Gardener, 2004). Reported mechanisms of direct pathogen suppression involve the production of antifungal and antibacterial metabolites such as peptide antibiotics, the secretion of lytic enzymes (e.g. chitinases which can degrade the cell wall of fungal pathogens), and the competitive colonization of plant surfaces (Asaka and Shoda, 1996; Bais et al., 2004; Nalisha et al., 2006; Trotel-Aziz et al., 2008). The literature on the potential of *B. subtilis* and other *Bacillus* spp. as inducers of host resistance against a broad range of microbial, viral and other parasites was recently reviewed by Kloepper et al. (2004). A main conclusion drawn from this paper is that *Bacillus* species elicit systemic resistance and growth-promotion by multiple mechanisms. Studies on the specific signal transduction pathways suggest that, dependent on the strain, the host plant and the pathogen, *Bacillus* spp. are not only able to induce jasmonic acid- and ethylene-dependent ISR (e.g. against late blight in tomato caused by *Phytophthora infestans* (Mont.) de Bary); Yan et al., 2002). As it is the case with SAR, specific *Bacillus* strains also have been shown to elicit salicylic acid-dependent defense responses in plants, leading to the accumulation of PR proteins, in some cases (e.g. against *Pseudomonas syringae* pathovar *tabaci* in tobacco; Park and Kloepper, 2000). Investigations by Ryu and associates demonstrated that volatile organic compounds (i.e. 2,3-butanediol) produced by a *Bacillus subtilis* strain induced resistance (Ryu et al., 2004) and also promoted plant growth (Ryu et al., 2003) in *Arabidopsis thaliana*. But, the elicited ethylene-dependent signaling pathway for systemic resistance against a soft root-causing fungus (*Erwinia carotovora*) appeared to be different from the cytokinin-dependent pathway for growth promotion. In a recent review publication, Borriss (2015) argued based on results obtained with subtypes of the *Bacillus amyloliquefaciencs* group that the effectiveness of these bacteria as bio-control agents is

critically due to the induction of systemic resistance in plants, whereas direct effects from the production of diverse metabolites with antimicrobial activity could be of minor importance for the suppression of root associated pathogens.

2.3.2 Multi-functionality of beneficial *Pseudomonas* species

Taxonomy and description of Pseudomonas species

The genus ***Pseudomonas*** (Migula, 1894; cited in: Palleroni, 2008) belongs to the larger ubiquitous group of pseudomonads, which are able to use a broad spectrum of simple organic materials, such as many root exudate compounds, for their respiratory metabolism (Lugtenberg et al., 1999; Madigan et al., 2003). Beside *Bacillus* spp., pseudomonads are among the most abundant bacterial populations inhabiting the rhizosphere and phyllosphere of diverse crops (Lambert, 1990; Duineveld et al., 2001; Yang et al., 2001; Behrendt et al., 2003; Mittal and Johri, 2007). Current phenotypic characterization of *Pseudomonas* spp. as Gram-negative, aerobic, non-sporulating rods that are mobile by polar flagella does not allow a clear distinction from other groups of Gram-negative bacteria (Palleroni, 1984; Palleroni, 2008). Recent phylogenetic revisions of the genus *Pseudomonas* based on 16S rRNA gene sequence analysis revealed the close relationship to the nitrogen fixing genus *Azotobacter* (Anzai et al., 2000; Young and Park, 2007). Other strains previously described as *Pseudomonas* have been reclassified in different genera such as *Burkholderia* and *Ralstonia*, which are well-known for their human-, animal- and plant-pathogenic members (Anzai et al., 2000; Genin and Boucher, 2004; Compant et al., 2008; Pellegrino, 2008). However, when characterized by phenotypic methods the currently recognized *Pseudomonas* species can be distinguished in the two groups of those which, especially when grown under iron limited conditions, form pigments that fluoresce under ultraviolet (UV) light of around 260 nm wavelength and others which do not do not fluoresce (Garibaldi, 1967; Palleroni, 2008).

Fluorescent pseudomonads, in particular rhizosphere competent strains of *Pseudomonas fluorescens* and *P. putida*, have been recognized for their plant growth-promoting effects in the presence and absence of pathogens (Burr et al., 1978; Suslow et al., 1978; Suslow and Schroth, 1982b; García de Salamone et al., 2001; Gravel et al., 2007). Other strains of

fluorescent *Pseudomonas* spp. were identified as deleterious rhizobacteria (DRB), which as minor pathogens are detrimental to overall plant vigor without causing obvious disease symptoms but may enhance infection by pathogenic fungi (Suslow and Schroth, 1982a). Similarly *P. aeruginosa*, a human pathogen commonly found in soils, attacks root and foliar tissues of weakened plants on occasion (Elrod and Braun, 1942; Schroth et al, 1981; Walker et al., 2004), whereas pathovars of *P. syringae* are known as aggressive major pathogens, causing blights, leaf spots and galls in a wide range of host plants (Singh, 1989; Bender et al., 1999). Beneficial *Pseudomonas* spp. have been attracting much attention to be commercialized as bio-agents because they can be propagated in fermenter culture and subsequently be reintroduced into the rhizosphere by seed bacterization (Savithiry and Gnanamanickam, 1987; Weller, 1988; Dekkers et al., 1998; Ashnaei et al., 2008). A difficulty in the preparation of pseudomonads inoculants of practical value is their inability to form dormant spores, which complicates the development of preservable products (Weller, 2007). Nevertheless, the ascendance of research on PGPR concentrates on fluorescent pseudomonads and their many traits that can be exploited for improved crop production (Chin-A-Woeng, 2003; Kloepper et al., 2004; Mercado-Blanco and Bakker, 2007).

Direct growth promotion and bio-fertilization effects of Pseudomonas species

Direct growth promotion by strains of *Pseudomonas* spp. can be ascribed to the synthesis of auxins, especially indolacetic acid (IAA), stimulating the development of the host plant root system when present in adequate concentrations (Benizri 1998; Patten and Glick, 2002). García de Salamone et al. (2001), characterized the production of three cytokinins by a strain of *P. fluorescens* and proposed a meaningful role of rhizobacterially produced cytokinins to enhance yield and quality of crops (García de Salamone et al., 2006). Another possible mechanism reported for *P. putida*, is the degradation of 1-aminocyclopropane-1-carboxylate (ACC) through its ACC deaminase activity. Because ACC is the immediate precursor of ethylene, this action may prevent the synthesis of plant growth inhibiting concentrations of this phytohormone in roots (Glick et al., 1997; Penrose et al., 2001).

Prospects for using *Pseudomonas* spp. to improve the availability of nutrients for plants have been linked to their ability to mobilize various forms of precipitated phosphates (Richardson, 2001). A major compound used by *Pseudomonas* spp. and other Gram-negative bacteria to solve inorganic phosphates seems to be gluconic acid produced from extracellular glucose (Illmer and Shinner, 1992; Buch et al., 2008; Trivedi and Sa, 2008). Phosphohydrolases are largely involved with the mineralization of organically bound phosphates in soils (Bünemann and Condron, 2007). The microbial excretion of such enzymes (i.e. phosphatases), which has been shown for *P. fluorescens* and *P. putida* (Gügi et al., 1991; Richardson and Hadobas, 1997), may play an important role for the P-acquisition of plants (Richardson et al., 2001). Correspondingly, increased growth and P-uptake after inoculation with phosphate-solubilizing *Pseudomonas* spp. has been observed in pot and field experiments (Sharma and Prasad, 2003; Egamberdiyeva, 2007). Nitrogen-fixation was generally not considered as a trait of *Pseudomonas* spp. until the property was detected in some *P. stutzeri* related strains (Lalucat et al., 2006; Palleroni, 2008). Furthermore, first results obtained by Mirza et al. (2006) indicate that nitrogen-fixing *Pseudomonas* strains are worthwhile to be investigated as growth-promoting inoculants for rice. The fluorescent pigments released by respective *Pseudomonas* spp. in response to nutritional iron deficiency are named pyoverdines (Greek: pyo, 'pus' and verdine, 'green'), or synonymously pseudobactins (Elliott, 1958; Visca et al., 2007). A primary role of these low molecular weight compounds (LMWCs) is to act as ferric ion (Fe^{3+}) chelating ligands, called siderophores (Greek: sidero, 'iron' and phore, 'carrier'), to make iron available to the microbial cell as part of an iron uptake system that also includes the formation of membrane bound receptor systems (Neilands 1981; Neilands, 1995; Leach and Lewis, 2006). Both, pyoverdines and corresponding membrane-receptors are typically very specific and a given strain may only incorporate its own ferri-pyoverdine complexes (Hohnadel and Meyer, 1988; Meyer, 2000). Nevertheless, Bar-Ness et al. (1991) have shown that siderophores produced by a strain of *P. putida* can serve as an iron source for diverse crop species. But, the mechanisms by which plants may acquire iron from ferri-pyoverdines remain to be further elucidated with respect to their ferro-ion (Fe^{2+}) specific respectively phytosiderophore mediated uptake systems (Marschner and Römheld, 1994; von Wirén et al., 1995; Curie, 2001; Lemanceau et al., 2007; Vansuyt et al., 2007).

Bio-control effects of Pseudomonas species

Kloepper et al. (1980a,b) presented evidence that the suppression of fungal and bacterial soil borne diseases (e.g. take-all in wheat, *Fusarium* wilt in flax, potato soft rot caused by *Erwinia carotovora*) by fluorescent *Pseudomonas* spp. is partially caused by the production of pyoverdines due to their high affinity for ferric iron, making it unavailable to pathogenic microorganisms which lack uptake specificities for ferri-pyoverdine complexes. This hypothesis has not only been further supported by subsequent research (Misaghi et al., 1982; Buyer and Leong, 1986; Höfte, 1991; Ambrosi et al., 2002), but also controversial results have been reported. In such way, Ahl et al. (1986) reasoned that siderophores produced by a strain of *P. fluorescens*, rather than depleting iron, seem to increase its concentration to highly toxic concentrations. Because, as shown in studies with *Thielaviopsis basicola*, iron-free siderophores did not show inhibitory effects on the growth of this fungal pathogen, whereas siderophores complexed with Fe^{3+} were more toxic than non-chelated iron. Further, investigations with mutants of *P. fluorescens*, either defective in the production of pyoverdine or antibiotics (e.g. phenazine-1-carboxylic acid (PCA); 2,4-DAPG), indicated the overriding importance of the antibiotics for the suppression of take-all in wheat and other soil borne diseases (Hamdan et al., 1991; Fenton et al., 1992; Keel et al., 1992; Schnider-Keel et al., 2000). Besides, there are a number of reports on the involvement of hydrogen cyanide (HCN) and diverse other antibiotics (e.g. pyocyanine, pyoluteorin, pyrrolnitrin, viscosinamide) in pathogen control by *Pseudomonas* spp. (Howell and Stipanovic, 1978; Hassan and Fridovich, 1980; Howell and Stipanovic, 1980; Voisard et al., 1989; Thrane et al., 2000). However, siderophores and antibiotics seem to play more complex roles than previously suspected, as both have been recognized as elicitors of systemic resistance in several plant-pathogen relations (Bakker et al., 2007). Rhizosphere colonizing *Pseudomonas* spp. strains have been shown to induce defense responses against pathogen attack, such as the accumulation of plant own antibiotics (phytoalexins, e.g. fungitoxic phenolic compounds), in above ground plant parts (van Peer et al., 1991; Wei et al., 1991; Alström, 1995) or spatially separated parts of the root systems (Zhou and Paulitz, 1994; Leeman et al., 1995). For systemic resistance induced by PGPR-strains of *Pseudomonas* spp. the jasmonate/ethylene-inducible defense pathway of ISR seems to be predominant, whereas the activation of SA-

dependent SAR by avirulent *P. syringae* strains is accompanied by the synthesis of PR proteins (Hoffland et al., 1995; van Wees et al., 2000; Ran et al., 2005a). Like SAR, ISR induced by rhizosphere colonizing *Pseudomonas* spp. can be effective against pathogenic strains of *P. syringae* and multiple fungal pathogens (Uknes et al., 1992; Hoffland et al., 1996; Pieterse et al, 1996; Bakker et al., 2007). The involvement of bacterial siderophores (i.e. pseudobactin) in ISR has been clearly demonstrated for derivates of a *P. putida* strain in the suppression of bacterial wilt in eucalyptus caused by *Ralstonia solanacearum* (Ran et al., 2005a) and *Botrytis cinerea* in tomato (Meziane et al., 2005). In these host-pathogen systems, siderophore-postive genotypes as well as the purified siderophore induced resistance, whereas the respective siderophore-negative mutants did not. However, defectiveness in siderophore production did not compromise resistance induction against *B. cinerea* in bean and *P. syringae* in tomato. Similarly in comparative studies with *P. fluorescens* all, the parental strain, the siderophore mutant, as well as the purified siderophore, were effective in suppression of *R. solanacearum* in eucalyptus. These observations indicate that the importance of siderophores as determinants for the inductions of resistance depends on the *Pseudomonas* strain, the host plant and the pathogen involved. In addition, beside siderophores, also other factors may trigger the immune response of plants, which becomes apparent when knockout strains still show effectiveness in pathogen control. To that effect, the antibiotic 2,4-DAPG (Iavicoli et al., 2003; Weller et al, 2004), cell surface compounds (i.e. flagellar proteins, lipopolysaccharides; van Peer and Schippers, 1992; Meziane et al., 2005), N-acyl-L-homoserine lactone (Schuhegger et al., 2006), and more determinants of *Pseudomonas* spp. have been discovered as potential elicitors of ISR (Mercado-Blanco and Bakker, 2007; Van Wees et al., 2008), which could be extensively discussed in more details.

Indeed, it is concluded here that *Pseudomonas* not only produce manifold bioactive metabolites with plant growth promoting and biocontrol properties. Single compounds have been shown to perform multiple functions and the beneficial effect of individual *Pseudomonas* spp. most likely results from the combined effects of more than one mode of action (Avis et al., 2008). Recent reviews and the literature cited therein document that this largely applies down to the level of single strains of *Pseudomonas fluorescens* and *P.*

putida (Ownley, 2002; Martins dos Santos et al., 2004; Loper et al., 2007; Subashri et al., 2013).

2.3.3 Multi-functionality of *Trichoderma* species

Taxonomy and description of Trichoderma fungi

The anamorphic (asexual or imperfect) fungal genus ***Trichoderma***, introduced by Persoon (1794; cited in: Druzhinina and Kubicek, 2005), is classified in the hyphomycetes of the imperfect fungi (*Deutromoycetes*). Most *Trichoderma* spp. can be readily assigned to the genus by some general characteristics that include rapid growth and abundant formation of green or less frequently white conidia on repetitively branched, but otherwise highly variable, conidiophores (Samuels, 1996; Gams and Bissett, 1998; Samuels, 2006). Yet, close similarities hinder the clear morphological identification of *Trichoderma* species and over the years an increasing number of new species has been described (Samuels, 2006). Rifai (1969; cited in: Samuels, 2006) differentiated nine "species aggregates", such as for instance *Trichoderma viride* (Persoon, 1794; Fries, 1829; cited in: Gams and Bissett, 1998) and *T. harzianum* Rifai (1969). Later it has been shown by use of molecular taxonomic methods that these fungal taxa comprise two or more genetically different species or biotypes (Lieckfeldt et al., 1999; Kullnig et al., 2000; Kullnig-Gradinger et al., 2002). Another important species, *Gliocladium virens*, had been excluded from the genus *Trichoderma* in early works because of its morphological similarities with fungi of the genus *Gliocladium*, such as conidia held in drops of watery liquid (Rifai, 1969; cited in: Samuels, 2006). Nevertheless, later studies revealed that the genus *Gliocladium* comprises several phylogenetically distinct groups, while *G. virens*, henceforth considered identical with *Trichoderma virens* (Miller et al., 1957; von Arx, 1987; cited in: Gams and Bissett, 1998), proved to be a species of *Trichoderma* and not *Gliocladium* (Rehner and Samuels, 1994; Lieckfeldt et al, 1998; Samuels, 2006). Associated sexual states, or teleomorphs, of *Trichodema* spp. have been found among the *Hypocrea* and closely related genera in the order *Hypocreales* of the *Ascomycetes* (Gams and Bissett, 1998). Chaverri et al. (2001), for instance, introduced the new species *Hypocrea virens* as teleomorph of *T. virens* and

T. harzianum was connected to *Hypocrea lixii* Patouillard (1891) by Chaverri and Samuels (2002), based on morphological and molecular analyses.

Trichoderma fungi are cosmopolitan in widely varying habitats and among the most prevalent saprophytes in soils of all climatic zones. This may be attributable to their minimal nutritional requirements, very versatile metabolic capabilities and aggressively competitive nature (Gams and Bissett, 1998; Klein and Eveleigh 1998). The ability of some *Trichoderma* spp. to produce a broad range of enzymes and to suppress other fungi has led to their commercial exploitation for industrial purposes and bio-control applications in crop production (Samuels, 1996; Azin et al., 2007). But, they also cause epidemic infections in mushroom cultivation and have been identified as opportunistic human pathogens (Samuel, 1996; Samuels et al., 2002). The interactions of *Trichoderma* spp. with plants have been widely shown to result in beneficial effects on growth and development (Harman, 2006), although there are several reports that certain isolates caused plant diseases under conductive environmental conditions (Hjeljord and Tronsmo, 1998). It has been shown by Yedidia et al. (1999) in axenic cultures, that root inoculation of cucumber (*Cucumis sativus* L.) seedlings with a biocontrol strain of *Trichoderma harzianum* initiated a series of morphological and biochemical changes in the plant, which were partly considered as initial defense responses. In consequence, the fungal colonization was mainly restricted to the root epidermis and outer cortex, but ingress into the vascular stele was apparently prevented by the formation of callose barriers by the plant. During the subsequent course of the interaction, however, certain typical plant defense responses (i.e. increased chitinase and peroxidase activity in root and shoot tissues) were systemically suppressed after their transient appearance. Enhanced plant growth furthermore indicated that the interaction with the *Trichoderma* strain resulted in the establishment of a beneficial rather than a parasitic association. Therefore, although *Trichoderma* species virtually have an inherent ability to attack plants, they usually seem to function as opportunistic, avirulent symbionts (Harman et al., 2004a). Different strains may thereby vary considerably in their ability to colonize the rhizosphere (Ahmad and Baker, 1987). It has been amply documented by Harman (2000) that selected rhizosphere competent *Trichoderma* strains proved to competitively establish and persist on developing roots for several months or the life span of annual crops after application to

soil or seed under diverse field conditions. Some strains of *Trichoderma* also persist on leaf surfaces (Elad and Kirshner, 1992; Lo et al., 1998), and recently several *Trichoderma* species living as endophytes in stem tissues of certain plant species have been isolated (Evans et al., 2003; Samuels et al., 2006; Zhang et al., 2007).

As they are highly interactive inhabitants of root and shoot environments, particular strains of *T. harzianum* and *T. virens* (*G. virens*) have been studied for the versatile advantages, which they may provide in crop production (Hjeljord and Tronsmo, 1998; Harman et al., 2004a; Druzhinina and Kubicek, 2005). Primary activities for which *Trichoderma* fungi are used are those of their broad based antagonism against root and shoot pathogens. But, they also have been shown to stimulate plant growth in the absence of pathogens and to confer tolerance against many adverse environmental conditions such as nutritional stresses, drought and water logging (Harman et al., 2004a; Neumann and Laing, 2006). Due to the multiple beneficial effects exerted on the colonized plant, increases in crop productivity up to 300 % have been reported from greenhouse and field experiments with diverse plant species, which indicates that the host specificity of beneficial *Trichoderma* spp. is not distinct (Vinale et al. 2004; cited in: Vinale et al., 2008). However, extensive trials suggest at least for maize a genotype specificity of the plant growth promoting effect, as far as the same strain of *T. harzianum* increased the growth of some maize breeds while it adversely affected others (Harman, 2006). This in turn would offer the opportunity to select combinations of host plants and *Trichoderma* strains with maximum compatibility, also taking into consideration the influence of the many uncontrollable variables that operate under field conditions (Harman et al., 2004c; Harman, 2006). So far, *Trichoderma* species are among the most thoroughly investigated fungal bio-effectors and commercially marketed as bio-stimulants, bio-fertilizers and bio-pesticides (Morales-Payan and Stall, 2004; Vinale et al., 2008).

Direct growth promotion and bio-fertilization effects of Trichoderma species

Apart from their inhibitory effects on potential pathogens, the production of growth regulating compounds and improved mineral nutrition through enhanced solubilization and/or uptake of mineral nutrients (e.g. N, P, Fe, Mn, Zn, Cu) are known as the main mechanisms that account for the plant growth promoting effect of *Trichoderma* fungi

(Harman, 2000; Yedidia et al., 2001; Avis et al., 2008). Studies performed under axenic growth conditions demonstrated that certain *Trichoderma* strains exert direct stimulation of plant development. Among the reported effects are: improved seedling emergence, increased root length, stimulated formation of lateral roots and root hairs, enhanced shoot elongation and leaf area, and greater dry weight production (Lindsey and Baker, 1967; Windham et al., 1986; Yedidia et al., 2001; Neumann and Laing, 2006; Contreras-Cornejo, 2009). Few reports have elucidated the signaling mechanisms by which *Trichoderma* species affect plant growth (Vinale et al., 2008). Evidence indicates a role of phytohormonal signals in mediating the developmental alterations induced by *Trichoderma* inoculation in plants. The production of auxin-, gibberellin-, and cytokinin-like compounds by isolates of *Trichoderma* has been detected by means of bioassays or analytical tools (Kampert and Strzelczyk, 1975; Kampert et al., 1975; Tsavkelova et al., 2006). In a screening of 27 different seed borne fungi of maize (*Zea mays* L.) that were incubated in liquid culture medium for 16 days, Reddy and Reddy (1987) found that *T. viride* produced maximum amounts of auxin (IAA). However, the effects of auxins and other growth regulators on plant development are dependent on their concentration. While low concentrations may be stimulatory, high concentrations can inhibit plant growth (Frankenberger and Arshad, 1995; Khalid et al., 2006). For example, the synthesis of high amounts of IAA by an overproducing mutant strain of *Pseudomonas putida* has been shown to retard the root growth of canola (*Brassica campestris*) seedlings rather than to increase it (Xie et al., 1996). This finding was probably due to the stimulatory effect of IAA on the synthesis of ACC, the precursor of the growth inhibiting hormone ethylene (Kende, 1993). The beneficial influence of certain *Trichoderma* strains on plants is therefore likely to result from a fine tuned balance between growth inhibitory and promoting effects, as suggested by Ousley et al. (1993). Consistent with this assumption, Gravel et al. (2007) reported that *Trichoderma atroviride* (Karsten, 1892; cited in: Gams and Bissett, 1998), which has been often confused with *T. harzianum* in the literature (Gams and Meyer, 1998; Dodd et al., 2003), is not only able to produce, but also to degrade IAA in-vitro. Further, *T. atroviride* may lower the concentration of ethylene within the plant because of its ACC deaminase activity (Gravel et al., 2007). Thus, a combined regulative effect of *T. atroviride* on phytohormone activities could have been

the cause for an optimized development of the root system and enhanced fruit yield of tomato plants observed in the same study.

Through the stimulation of root growth, *Trichoderma* species are able to improve plants' tolerance against nutrient deficient and toxic soil conditions (Neumann and Laing, 2006). Yedidia et al. (2001) characterized the effect of a *T. harzianum* strain on the root morphology and nutrient uptake of cucumber (*Cucumis sativus* L.) plants that were grown in hydroponic or soil culture. Under both cultivation systems inoculated plants showed significant increases in the number of root tips, total root length and biomass production as well as enhanced concentrations of P and other nutritional elements in roots and shoots. As phosphate was readily available in the hydroponic solution it was concluded that increased uptake rates rather than P-solubilization were the main factor that led to the higher P-status of *Trichoderma* treated plants. However, the relative increases in growth and tissue concentrations of P in response to *T. harzianum* appeared to be more distinct in soil than in hydroponic grown plants. The ability of several *Trichoderma* species to solubilize calcium phosphates has been demonstrated by use of in-vitro tests (Anusuya and Jayarajan, 1998). Furthermore, Altomare et al. (1999) characterized the importance of different mechanisms for the chemical mobilization of diverse sparingly soluble minerals by a strain of *T. harzianum*. In an in-vitro assay, the utilization of rock phosphate by the fungus could only be indirectly detected by an increase of soluble calcium, while the corresponding phosphate ions were obviously sequestered in the fungal mycelium and therefore immediately removed from the culture medium. Because acidification of the medium and production of organic acids did not seem to play a major role, it was assumed by the authors in accordance with Halvorson et al. (1990) that a continuous disturbance of the equilibrium between unsolved and solved P due to uptake by the fungus led to the subsequent delivery of P from the solid form by dissociation under slightly acidic or alkaline conditions. By contrast, Benítez et al. (2004; citing previous work by Gómez-Alarcón and de la Torre, 1994) alluded that most strains of *Trichoderma* release organic acid anions (e.g. carboxylates such as gluconate, citrate, fumarate) into their surroundings, which may contribute to the solubilization of sparingly available Ca-phosphates in calcareous soils and Fe/Al-phosphates in acidic soils by mechanisms of ligand exchange, dissolution, and occupation of P sorption sites, but also mobilize Mn, Zn and Cu by

complexation of the respective metal cations (Altomare et al., 1999; Neumann and Römheld, 2002; Neumann and Römheld, 2007). A concomitant extrusion of H^+ ions as the counter ion could further contribute to the mobilization of acid-soluble mineral nutrients, such as P, Fe, Mn, Zn, Si, and B, in calcareous soils (Neumann and Römheld, 2002). In addition, Altomare et al. (1999) found that the same strain of *T. harzianum* produced Fe^{3+}-chelating compounds and reduced Fe(III) and Cu(II) oxides to soluble, plant available Fe^{2+} and Cu^+ ions. Unknown reductive metabolites were also suggested to account for the dissolution of MnO_2 to Mn^{2+}, whereas the solubilization of metallic zinc was probably performed by its oxidation to Zn^{2+}, which are the forms usable by plants.

The effect of *Trichoderma* spp. on plant growth and uptake of mineral elements also makes them interesting agents to enhance the efficiency of plants used for the restoration of problem sites (Harman, 2006). Thus, it has been shown that inoculation with *Trichoderma* strains increased the biomass production and accumulation of toxic metals, such as Cd and Ni, in above ground parts of willow (*Salix fragilis*) and *Brassica* species grown on contaminated soils for remediation purposes (Adams et al., 2007; Cao et al., 2008; Wang et al., 2009). Moreover, *Trichoderma* fungi are resistant or tolerant to high levels of a range of toxic compounds such as anthropogenic pollutants, pesticides and antimicrobial metabolites produced by plants and other microorganisms (Harman et al., 1996; Harman et al., 2004a). This robustness has been associated with the expression of active efflux systems that prevent the intracellular accumulation of toxic compounds and/or with the catabolic degradation of some of these materials (Ezzi and Lynch, 2002; Lanzuise et al., 2002; Harman et al., 2004b). Beyond the development of remediation strategies for serious pollutants such as cyanide, the ability of plant associated *Trichoderma* spp. to withstand diverse toxicities also makes them competitive antagonists for biocontrol applications under adverse environmental conditions (Benítez et al., 2004; Harman et al., 2004b; Ruocco et al., 2009).

Bio-control effects of Trichoderma species

The bio-control efficacy of *Trichoderma* isolates has been attributed primarily to direct antagonistic interactions, including competition for resources and space, hyperparasitism and antibiosis. But also, mechanisms of indirect effect to pathogens such as systemic or

localized induced resistance, enhanced nutrition and growth of the host plants, as well as changes in the composition of microbial communities on roots have been recently described for *Trichoderma* species (Harman, 2000; Harman, 2006).

Reported instances suggest that the suppression of single pathogens by *Trichoderma* fungi is likely to result from the simultaneous operation of more than one mechanism (Jeger et al., 2009). Thus, control of the grey mould fungus *Botrytis cinerea*, an important pre- and post-harvest pathogen of fruits and vegetables, has been supposed to involve competitive colonization of floral debris, necrotic tissues, and wound sites in the case of application on respective shoot parts (Dubos, 1987; O'Neill et al., 1996; Hjeljord and Tronsmo, 1998). *Trichoderma* fungi may further destroy the mycelium, sclerotia and conidiophores of *B. cinerea* in a hyperparasitic way, thereby inhibiting dissemination of the pathogen (Dubos, 1987; El-Naggar et al., 2008). The parasitism on other fungi (mycoparasitism) by *Trichoderma* species is a phenomenon that involves several consecutive steps, starting with chemotrophic growth towards the target fungus, followed by lectin-mediated host recognition and attachment to its hyphae, and finally attack and lysis of the prey cells (Barak et al., 1985; Chet, 1987). Experimental evidence suggests that hydrolytic enzymes (chitinases, ß-1,3-glucanases, proteases) exhibiting fungal cell wall degrading activity and peptide antibiotics released by *Trichoderma* isolates may act synergistically in this process (Schirmböck et al., 1994; Lorito et al., 1996; Zaldivar et al., 2001; Vinale et al., 2008). At the same time, *Trichoderma* fungi are able to protect their own cell walls from enzymatic breakdown, which is likely due to a distinct cell wall protein that is co-induced with chitinolytic enzymes and acts as a local inhibitor of chitinase activity (Lora et al., 1994; Lorito, 1998). Because different enzymes released by *Trichoderma* fungi may work synergistically at concentrations below those where the single enzymes are effective, the specific inhibition of a key activity such as chitinolysis on the cell walls should also break the synergistic impact of the other enzymes (Lorito et al., 1993; Lorito, 1998). This selective mechanism has been proposed to enable the hyphae of *Trichoderma* fungi to perforate the cell walls of prey fungi without suffering damage to their own cell walls (Elad et al., 1983; Lorito, 1998). Inoculation of bean (*Phaseolus vulgaris* L.) leaves with a strain of *T. harzianum* also has been reported to lower the activity of pectolytic enzymes

produced by *B. cinerea*, which play an important role in fungal pathogenesis as they disrupt the structure of plant cell walls (Zimand et al., 1996).

The bio-control effect of induced resistance in diverse crop plants against *B. cinerea* has been demonstrated by suppression of grew mould disease after application of a *T. harzianum* strain to roots and leafs spatially separated from the site of pathogen inoculation (De Meyer et al., 1998). Studies with mutants of *Arabidopsis thaliana* L., impaired either in salicylic acid (SA) or ethylene/jasmonic acid (ethylene/JA) dependent signaling pathways, indicated that the type of *Trichoderma*-mediated systemic resistance against *B. cinerea* resembles that of ethylene/JA regulated ISR caused by rhizobacteria (Korolev et al., 2008). Nevertheless, in another investigation root-inoculation with a strain of *T. harzianum* induced enhanced activity of PR proteins (i.e. chitinase and peroxidase) in leaves of cucumber (*Cucumis sativus* L.) seedlings (Yedidia et al., 1999), which is indicative of SA-dependent SAR responses (van Loon and Strien, 1999; Harman, 2000). As elicitors of *Trichoderma*-induced resistance proteins with enzymatic activity and peptides (Lotan and Fluhr, 1990; Hanson and Howell, 2004), avirulence-like gene products similar to those found in avirulent pathogens (Woo et al., 2006), and degradation products of low molecular weight released from fungal or plant cell walls through the action of *Trichoderma* enzymes have been recognized (Harman et al., 2004a). Beside the induction of defense related reactions in the plant, some of these low-molecular weight compounds (LMWCs) released from the cell wall of fungal pathogens are supposed to stimulate the expression of mycoparasitism-related genes in *Trichoderma* species (Woo et al., 2006; Vinale et al., 2008).

Trichoderma species not only exert different suppressive mechanisms against a single disease, these fungi also function as versatile agents for the biocontrol of a wide range of important air- and soil-borne fungal pathogens in diverse crops (Papavizas, 1985; Chet, 1987). Furthermore, phytopathogenic nematodes (Spiegel and Chet, 1998), bacteria (Yedidia et al., 2003; Harman et al., 2004a), and even viral diseases (Lo et al., 2000; Jakubíková et al., 2006) are reportedly suppressed by the direct or indirect action of certain *Trichoderma* species. Elad (2000) attributed the control of powdery (*Sphaerotheca fusca*) and downy mildew (*Pseudoperonospora cubensis*) in cucumber (*Cucumis sativus*

L.) by an isolate of *T. harzianum* (T39) to the combined effect of several mechanisms. The same isolate was commercialized for the control of *Botrytis*-incited diseases and also suppressed the severity of downy mildew (*Plasmopara viticola*) in grape vines (*Vitis vinifera*; Perazzolli et al., 2008). Higher propagule numbers of individual *T. harzianum* species in soils from organic farming systems have been shown to be related with suppressiveness to *Sclerotium rolfsii* Sacc., a fungal pathogen of many agronomic crops and causative agent of Southern blight of tomato (*Lycopersicon esculentum* Mill.; Liu et al., 2008). But also for the control of *Rhizoctonia solani* (Elad et al., 1981; Howell et al., 2000), *Fusarium* spp. (Sivan and Chet, 1986; Sivan et al., 1987), *Pythium ultimum* (Lynch et al., 1991), and more species of root infecting fungal parasites the application of diverse *Trichoderma* inoculants has received considerable attention (Scala et al., 2007). Many reports have shown a suppressive effect of *Trichoderma* isolates on the take-all fungus *Gaeumannomyces graminis* var. *tritici*, which was in the main attributed to antibiosis and mycoparasitism (Dunlop et al., 1989; Ghisalberti et al., 1990; Küçük and Kivanç, 2004; Vinale et al., 2006; Zafari et al., 2008). Another antagonistic mechanism of particular relevance for the suppression of take-all disease might be the solubilization of Mn by *Trichoderma* fungi, by reason that the Mn-oxidizing (immobilizing) capacity of *G. graminis* var. *tritici* (*Ggt*) isolates has been identified as an important virulence factor (Pedler et al., 1996; Rengel, 1997a). Considering the physiological key role of Mn in the synthesis of defense-related compounds, such as phenols and lignin, in plants, it can be further assumed that *Trichoderma* colonization may contribute to the development of resistance against a broad spectrum root and foliar pathogens due to an enhanced Mn-status of the host (Graham and Webb, 1991; Altomare et al., 1999; Dordas, 2008). In the action of *Trichoderma* species, other components of the soil microflora may play a role, too. Thus, Simon and Sivasithamparam (1988a,b) compared the composition of microbial communities in soils suppressive or conductive to the take-all pathogen (*Ggt*). In the suppressive soil, they found more *Trichoderma* spp., which were in the main identified as strains of *Trichoderma koningii*, and more bacterial isolates that were antagonistic to *Ggt*. Furthermore, metabolites produced in vitro by bacteria isolated from the suppressive soil were less inhibitory to the growth of *T. koningii*. At the same time, the bacterial community of the suppressive soil was characterized by a lower percentage of isolates

that showed growth inhibition in response to metabolites from *T. koningii*. These results suggest the involvement of *Trichoderma* spp. in mutual selection processes that result in the build-up of an adapted antagonistic microflora in take-all suppressive soils.

In conclusion of the above considerations it is apparent that the mechanisms by which *Trichoderma* spp. accomplish beneficial influences on plants are complex and multifaceted. What is observed as *Trichoderma*-mediated plant growth or health effect may be the cumulative result of multiple interactions with environmental factors and the native soil microflora. Because single *Trichoderma* strains have a number of different capabilities, it is very likely that under conditions were a certain mechanism employed proves ineffective other mechanisms come into play and compensate (Harman, 2000; Howell, 2003).

2.3.4 Multi-functionality of arbuscular mycorrhizal fungi

Mycorrhiza (Greek: μυκης, mykes, 'fungus' and ριζα, rhiza, 'root') is the symbiotic association between soil-borne fungi and plants forming a morphological unit with root-like functions (Frank, 1885a). The fungus provides the plant with mineral nutrients and water from the soil versus the delivery of photosynthates (Frank, 1885b). Plant species that support mycorrhizal colonization, even though their dependency on mycorrhiza for nutrient acquisition is not distinct, may still benefit in other ways (Newsham et al., 1995; Tawaraya, 2003; Finlay, 2005). Such secondary functions of mycorrhizal fungi include the assistance for plants to stave off harmful organisms, their role in soil conservation, and the cooperation with other beneficial organisms (Duchesne, 1994; Cardoso and Kuyper, 2006). Mycorrhizal fungi are thought to play a key role in the rhizosphere, which they extend to the mycorrhizosphere, as they mediate the transfer of carbon from roots to the soil as a source of energy for microbial life as well as the uptake of mineral nutrients and water by the plant, thus serving as a broker between plants and the soil that they inhabit (Römheld and Neumann, 2006; Timonen and Marschner, 2006).

Description of arbuscular mycorrhizal fungi

Depending on the fungal associates and the characteristic structures they form, different types of mycorrhiza have been distinguished (Read, 2002). Among them, the arbuscular

mycorrhiza (AM) represents the most relevant group for agriculture (Smith and Read, 1997). Arbuscular mycorrhizal fungi (AM-fungi) have been classified in the order *Glomales* of the phylum *Zygomycota* (Morton and Benny, 1990) until the *Glomales* were raised to the rank of a separate phylum *Glomeromycota* Walker and Schuessler (Schüßler et al., 2001), which probably derived from an ancestor common with the *Ascomycota* and *Basidiomycota* (Redecker and Raab, 2006). AM-infections arise from asexual spores, residual mycelium or existing hyphal systems in the soil (Carling and Brown, 1982). Spores, as well as non-septate hyphae, appear to be multinucleate (Cooke et al., 1987).

On the basis of spore wall characteristics currently only about 200 morphological species of the *Glomeromycota* have been described (Redecker and Raab, 2006). But, the range of plant hosts that form root symbioses with AM-fungi seems to be extremely wide and ubiquitous in terrestrial ecosystems, though only a relatively small fraction of plants and fungi has been examined so far (Smith and Read, 1997; Bever et al., 2001). Among the most intensively investigated AM-fungi are strains of the species *Glomus mosseae* (Nicolson and Gerdemann, 1968) and *Glomus intraradices* (Schenck and Smith, 1982), probably because they are genuine host generalists (Koide and Mosse, 2004; Parniske, 2008). Recent studies, however, indicate a greater diversity of AM-fungal species than previously recognized (Scheublin et al., 2004; Santos et al., 2006). Fossil and molecular evidence that arbuscular mycorrhiza was instrumental to ancient plants invading the land more than 400 million years ago, underlines the close relationship between AM-fungi and plants (Simon et al., 1993; Remy et al., 1994; Taylor et al., 1995). Hosted by almost all temperate and tropical crops AM-fungi could be the outstanding resource for nutrient acquisition and healthy plant growth in agriculture (Winter, 1951; Gerdemann, 1968; Norman et al., 1995). Even among the *Brassicaceae* (e.g. *Brassica napus* L.), *Chenopodiaceae* (e.g. *Beta vulgaris* L.) and in the genus *Lupinus* of the *Fabaceae*, often thought to be non-mycorrhizal, AM establishment was inconsistently found (Trinick, 1977; Harley and Harley, 1987; Smith et al., 2003).

During root internal colonization the hyphae of AM-fungi establish an intimate contact with cortical cells. Characteristic is the formation of highly branched haustoria, called “arbuscules” from the Latin word for little tree, inside plant cells, thought to facilitate the

exchange of signals, mineral nutrients and carbohydrates (Parniske, 2008). It has been estimated that AM-fungi, representing a considerable sink for photosynthates, receive about 10 % of the carbon transported to the root (Fitter, 1991). The arbuscules are enveloped by a plant-derived periarbuscular membrane that excludes the fungus from the plant cytoplasm and harbors different transport systems (Bücking and Shachar-Hill, 2005; Chalot et al., 2006; Uehlein et al., 2007). In ectomycorrhizas, by contrast, the fungus builds complex intercellular hyphal systems, the Hartig net, but without or little intracellular penetration. Typical ectomycorrhizal symbioses are formed by certain basidio- and ascomycetes, colonizing the roots of certain tree species growing on humus rich soils of boreal and temperate forest ecosystems (Smith et al., 2003). Especially in the later stages of the symbiosis some, but not all AM-fungi, also form small bladders or "vesicles" within the root tissue that seem to function as fungal storage organs as they contain lipids (Carling and Brown, 1982; van Aarle and Olsson, 2003). Phosphate deposited in vesicles might be used by the plant during P-deficient growth phases (Srivastava et al., 1996). The formation of AM leads to changes in root morphology and physiology of the whole plant that are likely to be controlled by specific expression patterns of host genes (Wulf et al., 2003). Examples are altered tissue concentrations of growth regulating compounds such as auxins, cytokinins and gibberellins (Hause et al., 2007), enhanced photosynthesis rates (Smith and Gianinazzi-Pearson, 1988), decreased ratios of root to shoot biomass allocation (Taylor et al., 2008), and increased root branching (Berta et al., 1995).

By their external mycelium AM-fungi explore a great soil volume and tremendously increase the absorptive surface area of the mycorrhized root, in a way that can be more carbon cost efficient than the investment in root biomass (Fitter, 1991): (i) the extraradical hyphae can extend several centimeters from the root surface and thus beyond the nutrient depleted zone that develops around roots as a consequence of their own uptake processes (Mosse, 1986; Li et al., 1991); (ii) hyphal length densities may exceed 100 m per cubic centimetre of soil (Miller et al., 1995); and (iii) the low diameter of AM-hyphae (1.2 to 18.0 µm; Dodd et al., 2000) enables them to enter soil pores smaller than those accessible to root hairs (5 to 17 µm in diameter; 80 to 1,500 µm in length; Dittmer, 1949). In addition, external mycorrhizal mycelia can link together the roots of different hosts by

establishing symbioses with diverse plant species growing in the same area, and by means of anastomosis, the fusion process that connects hyphal branches of the same or different origin (Newman et al., 1994; Giovannetti et al., 2001; Southworth et al., 2005). It is discussed controversially whether the resulting networks of interconnected hyphae may facilitate the direct exchange of photosynthetates or mineral nutrients such as nitrogen and phosphorus from one plant to another (He et al., 2003).

Bio-fertilization effects of arbuscular mycorrhiza

Due to its generally low concentration and low diffusion rate in the soil solution, in particular the uptake of phosphate is largely determined by the size of the absorptive surface area of the root (Newman and Andrews, 1973). Therefore, the ability of AM to increase P supply to the plant via hyphal translocation has been regarded as their most important function from an agronomic perspective (Mosse, 1986). In several studies the effect of mycorrhiza to improve the P status and growth of diverse crops was most pronounced on soils with moderate phosphorus availability, whereas excessive P fertilization not only lowered mycorrhizal efficiency, but also decreased the mycorrhization of the root (Stribley et al., 1980; Bolan et al., 1984; Amijee et al., 1989; Hayman, 1982; Branzanti et al., 1992). The degree to which plants depend on AM is also largely determined by the specificity of their own facilities for nutrient acquisition. Plants species or genotypes with roots that are finely branched and form many root hairs tend to be less responsive to mycorrhizal colonization than those with comparatively course root systems (Baylis, 1970; Hetrick, 1991; Wilson and Hartnett, 1998; Tawaraya, 2003). Menge et al. (1978) compared mycorrhizal with non-mycorrhizal citrus seedlings growing at varying P-fertilization levels. From the results of this experiment, Menge (1985) later estimated that, depending on the citrus genotype, the activity of mycorrhizal fungi could save the application of more than 100 to 500 kg P per hectare.

Similar to phosphorus, mycorrhiza also contributes considerably to the plant acquisition of ammonium (NH_4^+) and other nutrients, such as zinc (Zn), copper (Cu), iron (Fe), and potassium (K), that move through the soil mainly by diffusion (Marschner and Dell, 1994; Caris et al., 1998; Rengel and Marschner, 2005). Nitrate (NO_3^-) and sulphate (SO_4^{2-}) are usually not assumed to be uptake limited by their chemical mobility and may move faster

through the soil than through hyphae (Srivastava et al., 1996). But, under conditions where mass flow and diffusion to the root are inhibited, as for example under drought-stress, hyphal translocation can also be an important factor in increasing nitrate and sulfur uptake by the plant (Rhodes and Gerdemann, 1978a; Tobar et al., 1994). Under field conditions, high nitrate, even more than phosphate, fertilization levels are closely correlated with suppressed AM-establishment (Azcón et al., 1982; Hayman, 1982). Furthermore, the higher drought and salinity tolerance of mycorrhizal plants has been attributed to improved water relations and increased acquisition of nutritional elements (Ruiz-Lozano et al., 1996; Augé, 2001; Neumann and George, 2004). In addition, AM may selectively inhibit the sodium (Na) uptake of plants under salt stress (Al-Karaki, 2000; Colla et al., 2007). Some studies indicate that specific species or ecotypes of AM fungi are particularly adapted to dry or saline environments and that the selection of effective strains could offer a great potential to advance crop production under such stress conditions (Ruiz-Lozarno et al., 1995; Hildebrandt et al., 2001; Landwehr et al., 2002). Also for calcium (Ca) and magnesium (Mg), the latter in the case of an ectomycorrhizal fungus, a certain uptake and translocation capacity of the external mycelium has been shown (Rhodes and Gerdemann, 1978b; Jentschke et al., 2000). In particular on acid soils, where these base cations are depleted, AM-fungi were observed to improve the plant uptake of Ca and Mg (Clark and Zeto, 1996; Alloush and Clark, 2001).

Hyphal translocation may be inhibited when plants are exposed to harmful elements. In soils containing high levels of heavy metals like manganese (Mn), (Zn) and cadmium (Cd), AM-fungi colonizing the root can decrease the uptake of these elements into plant cells, thus alleviating toxicity to some degree (Gildon and Tinker, 1983; Heggo et al., 1990; Quilambo, 2003). It has been assumed that the high sorption capacity of the AM-mycelium plays a role in plant protection against excessive heavy metal uptake, but it is not clear which mechanisms regulate the selective retention of only undesired elements (Li et al., 1991; Joner et al., 2000a; Lee and George, 2005). In experiments with different hosts, however, AM-fungi maintained their conductibility for heavy metal uptake, when plants were exposed to high Zn, Cu, nickel (Ni) and lead (Pb) supply, thus enforcing adverse effects (Killham and Firestone, 1983; Symeonidis, 1990). More specific is the mitigation of Mn-toxicity by AM-fungi, which has been explained by changes in

microbial populations of the rhizosphere with a possible impact on the balance between Mn-reducing (mobilizing) and Mn-oxidizing (immobilizing) bacteria (Kothari et al., 1991; Posta et al., 1994). Beside a generally inhibitory effect of AM on Mn-uptake, results from Bethlenfalvay and Franson (1989) showed a positive correlation between increasing AM-fungal colonization rates and Mn concentrations in roots and shoots of soybean (*Glycine max* (L.) Merr.). These findings suggest the co-existence of such AM-mediated mechanisms that protect the plant from toxic Mn in the soil and others that improve acquisition when the Mn-availability is low. Further, AM-fungi may also enable their host to tolerate higher Mn-concentrations in the plant tissue. This assumption is supported by studies that have shown the prevention of Mn-toxicity in leaves by silicon (Si) supply (Horst and Marschner, 1978a; Rogalla and Römheld, 2002; Dragišić Maksimović et al., 2012) and the possible contribution of AM to the uptake of Si in some plant species (Yost and Fox, 1982). Similarly, on acid soils a lower uptake but also a higher tissue tolerance of mycorrhizal plants against toxic aluminum (Al) species has been reported (Rufyikiri et al., 2000; Lux and Cumming, 2001). To what extent the use of AM-fungi could be helpful for the clean up (e.g. by phytoremediation) and re-cultivation of contaminated soils is a matter of current debate that requires further investigation (Joner and Leyval, 2003; Regvar et al., 2003; Hildebrandt et al., 2007).

In contrast to ectomycorrhizal fungi, there is only little evidence for AM-fungi that they contribute actively to the chemical mobilization of nutrients in soils (Marschner and Dell, 1994; Northup et al., 1995; Hodge, 2001). It is questionable to what extent pH changes induced by AM-hyphae observed in root organ cultures that were established on agar media amended with pH indicator (Bago et al., 1996), or the hyphal production of extracellular phosphatases demonstrated under axenic conditions (Tarafdar and Marschner, 1994; Koide and Kabir, 2000), may lead to an increased solubilization respectively mineralization of sparingly available P sources for uptake by field grown plants. In comparison to other soil microbes and autolysis, the phosphatase activity of AM-fungi is relatively small (Joner et al., 2000b). Although recent reports have shown an enhanced acquisition of nitrogen from patches of organic matter in soil via AM-fungal hyphae, this was probably due to an enhanced mineralization of these materials by other microorganisms in the hyphal environment (Hodge et al., 2001). Whether AM-fungi are

able to actively decompose complex organic compounds or to directly capture organic N sources remains controversial (Hodge, 2001). Several plant species that are adapted to growth on soils with extremely low phosphate solubility have developed own strategies for enhanced P-acquisition. The *Proteaceae* that inhabit nutrient-impoverished landscapes in Western Australia, for instance, as well as some members of the *Cyperaceae* (sedges) are typically non-mycorrhizal and form dense clusters of lateral roots respectively root hairs for intense chemical mining of confined soil patches (Dinkelaker et al., 1995; Neumann and Martinoia, 2002; Shane and Lambers, 2005; Shane et al., 2006).

Bio-control effects of arbuscular mycorrhiza

Many records attest the suppression of soil-borne root diseases caused by fungal pathogens including species of *Fusarium* (Caron et al., 1985; Akköprü and Demir, 2005), *Phytophthora* (Norman et al., 1996), *Pythium* (Larsen et al., 2003), *Rhizoctonia* (Kasiamdari et al., 2002) and *Verticillium* (Karagiannidis et al., 2002). Also the deleterious effect of root parasitic nematodes like species that form root-cysts (*Heterodera* sp.; Francl and Dropkin, 1985; Todd et al., 2001), root-knots (*Meloidogyne* sp.; Li et al., 2006), or root-lesions (*Pratylenchus* sp.; Talavera et al., 2001) was often found to be less severe on mycorrhizal plants. But, AM-fungi do not always provide consistent pathogen control. Most notable is the enhanced susceptibility to viral diseases, which has been attributed to the enhanced development and physiological activity of mycorrhizal plants (Dehne, 1982). A higher infestation by certain shoot and foliage pathogens, such as powdery mildew and rust fungi, has been observed in some cases, too (Schönbeck and Dehne, 1979; Gernns et al., 2001; Whipps, 2004).

Biological control conferred by AM-fungi could be seen as the outcome of the simultaneous operation of several mechanisms that contribute to the protection of plants with varying success (Pozo and Azcón-Aguilar, 2007). Dense cocoon like hyphal structures, encapsulating the root like the “mantle” of the ectomycorrhizas, are not formed by AM-fungi. Their protective function for the root is therefore not accomplished by constructing a mechanical barrier (Duchesne, 1994). Also direct forms of antagonism through antibiosis or mycoparasitism have not been shown for AM-fungi (Harrier and Watson, 2004). The plant health promoting effect of AM is rather achieved indirectly by

physiological changes of the host or due to the induction of microbial changes in the rhizosphere (Linderman, 1994).

Nutrition-mediated effects of arbuscular mycorrhiza on plant health

An improved nutritional status of mycorrhizal plants may increase their resistance, but also make them more attractive for pathogens (Dehne, 1982). A higher uptake of P and possibly other mineral nutrients via AM was related with a lower incidence of take-all disease in wheat (Graham and Menge, 1982) and of a root rot disease caused by *Phytophthora parasitica* Dast. in *Citrus* sp. (Davis and Menge, 1980). Contrarily, Davis et al. (1979) described a more severe occurrence of Verticillium wilt (*Verticillium dahliae* Kleb.) due to the improved P-nutrition of mycorrhized cotton (*Gossypium hirsutum* L.). In all three studies, the same AM-fungal species (*Glomus fasciculatus* (Thaxter) Gerd. and Trappe) was used as inoculum. The role of mineral nutrients in resistance varies depending on the pathogen involved and nutritional effects can not solely explain the benefit of AM for plant health (Huber and Graham, 1999; Harrier and Watson, 2004).

Arbuscular mycorrhiza induced resistance

Many protective processes activated within the plant by AM-fungi follow the principle of induced resistance, but the elucidation of the underlying mechanisms has only begun (Pozo and Azcón-Aquilar, 2007). Split-root experiments, where mycorrhizal and non-mycorrhizal parts of the root system were physically separated, confirmed localized and systemic AM-effects (Cordier et al., 1998; Pozo et al., 2002; Zhu and Yao, 2004; Khaosaad et al., 2007). It has been shown in this context that AM formation triggers the accumulation of several plant defense related compounds either before or in response to pathogen attack. Enhanced synthesis of phenolics and lignification of root and stem tissues for instance can provide a mechanical and chemical barrier against pathogen invasion (Daft and Okusanya, 1973; Dehne and Schönbeck, 1979). Critical for the understanding of such processes could be that there are some similarities in plant response to AM-fungi with those found in plant-pathogen associations (Güimil et al., 2005). SA-dependent signaling pathways elicited in root cells as a first reaction to AM-fungal invasion seem to be modified in order to achieve a compatible interaction (Blilou et al.,

2000b; García-Garrido and Ocampo, 2002). Coincidently, local and only weak defense responses have been observed in host plants during early stages of AM-colonization, which were then subsequently repressed (Kapulnik et al. 1996; Liu et al., 2003). Accordingly, transient enhancements of enzyme activities (e.g. peroxidases, catalases, chitinases) that are generally involved in plant defense have been observed in AM-inoculated roots (Lambais and Mehdy, 1993; Blilou et al., 2000a). Later, during arbuscule formation and mutualistic interaction, jasmonate-dependent signaling pathways are likely to be associated with the functioning of the fully established symbiosis. To that effect, Hause et al. (2002) have shown that colonization of barley (*Hordeum vulgare* L.) roots by *Glomus intraradices* led to elevated levels of endogenous JA and JA-isoleucine. These findings were accompanied by the specific expression of genes, involved in JA-biosynthesis and responsiveness, within root cells that contained arbuscules. Furthermore, in and around arbuscule-containing cells the local expression of genes (mRNA accumulation), encoding an acidic endochitinase, a β-1,3-endoglucanase and other defense related enzymes that are probably involved in the regulation of AM development, has been detected (Lambais and Mehdy, 1995; Blee and Anderson, 1996; Azcón-Aguilar and Barea, 1996). In summary, it has been assumed by Pozo and Azcón-Aquilar (2007) that AM-colonization results in the suppression of SA-dependent responses, which is partially compensated by the priming of JA-dependent defense mechanisms, allowing the plant to respond faster in the case of pathogen attack. This hypothesis is in line with results of Cordier et al. (1998) and Pozo et al. (2002), who demonstrated that AM-induced resistance against *Phytophthora parasitica* arose from localized and systemic defense responses in tomato (*Lycopersicon esculentum* Mill.) roots. Local resistance mechanisms comprised the induction of enzymes (chitinase; chitosanase; β-1,3-glucanase) that can hydrolyse pathogen cell walls and the formation of host cell wall reinforcements (callose deposits) by arbuscule containing cells in reaction to intercellular growth of *P. parasitica*. The pathogen thus was never found in cells that were already colonized by arbuscules. Systemic effects were characterized by the formation of callose-rich encasements around hyphae of *P. parasitica* that were penetrating root cells and by cell wall thickening with pectins and PR proteins in response to intercellular pathogen growth. Interestingly, in this research only a strain of *G. mosseae* but not of *G. intraradices* conferred significant

protection against *P. parasitica* (Pozo et al., 2002). Furthermore, the observations of different mechanisms that were made in studies with other host plants, pathogens and AM-fungi indicate the specificity of mycorrhizal interactions (Zhu and Yao, 2004; Khaosaad et al., 2007).

Arbuscular mycorrhiza-induced changes in soil microbial populations

Enhanced mineral nutrition, altered carbon allocation patterns and higher metabolic activity reported for mycorrhizal plants may lead to quantitative and qualitative changes in materials released by the root (Jones et al., 2004; Neumann, 2007). Because loss of organic materials from plant roots provides energy for growth and activity of microbial populations in soils, this in turn can be decisive for the balance between beneficial and deleterious microorganisms in the rhizosphere (Hiltner, 1904; Linderman, 1988). Colonization by AM-fungi has been shown to result in a decreased release of sugars and total amount of amino acids by the root, but increased exudation of phenolics, gibberellins and specific amino acids that were not found in root exudates of non-mycorrhizal plants (Graham et al., 1981; Mada and Bagyaraj, 1993). Corresponding influences of AM-establishment on the composition of microbial communities in the rhizosphere, stimulating some microbial groups while decreasing others, have been reported congruently (Paulitz and Linderman, 1989; Kothari et al. 1991; Marschner et al., 2001). More seldom in the literature is evidence that AM-induced shifts in microbial populations of the rhizosphere resulted in effective pathogen suppression. Secilia and Bagyaraj (1987) studied the effect of AM on bacteria and actinomycetes associated with pot cultures of guinea grass (*Panicum maximum* Jacq.). They found that different AM-fungi selectively stimulated different actinomycetes that showed specific antagonistic activity towards diverse pathogens. Waschkies et al. (1994) observed lower numbers of probably deleterious pseudomonads on the root surface of grapevine (*Vitis* sp.) cuttings after inoculation of replant diseased soil with *Glomus mosseae*.

Further bio-control mechanisms of arbuscular mycorrhiza

In addition, also the following modes of action have been discussed with respect to plant health effects of AM-fungi in several reviews: (i) compensation of root damage by the

functions of the external AM-mycelium; (ii) competition with pathogens for nutrients, infection sites or photosynthates; and (iii) phytohormonal induced changes in the root system resulting in altered root architecture and longevity (Linderman, 1994; Azcón-Aguilar and Barea, 1996; Harrier and Watson, 2004; Demir and Akkopru, 2007). However, these aspects can be recognized as side effects of the main AM-functions already described above in this chapter, also (Schönbeck et al., 1994). A recently studied topic is the influence of AM on the release of secondary plant compounds that are important signals in several symbiotic and pathogenic interactions (Steinkellner et al., 2007). Strigolactones for example are signaling compounds that mediate host location in AM-fungi and parasitic plants of the genera *Striga* and *Orobanche* (Akiyama et al., 2005; López-Ráez et al., 2008). Flavonoids act as signals for rhizobia and AM-fungi, but also for fungal pathogens including species of *Phytophthora* and *Fusarium* (Chabot et al., 1992; Morris and Ward, 1992; Ruan et al., 1995). Plants exuding lower amounts or altered patterns of these chemo-attractants upon mycorrhizal colonization could therefore be less attractive to respective parasites (Bouwmeester et al., 2007; Carlsen et al., 2008).

The effect of arbuscular mycorrhiza on soil structure

A third important role of AM-fungi is their contribution to the process of creating and maintaining a favorable soil structure (Miller and Jastrow, 1992; Cardoso and Kuyper, 2006). The arrangement of solid soil parts in aggregates and the pore spaces between them have a major influence on physical, chemical and biological processes that determine soil fertility (Marshall et al., 1996; Gobat et al. 2004). Conditions of low density and adequate porosity in aggregated soils allow water and air movement, support root growth, enhance the accessibility of nutrients, provide shelter to soil organisms, and confer stability against soil erosion (Bronick and Lal, 2005). AM-fungi affect soil structure via their influence on plants or by effects that are mediated through the fungal mycelium itself (Rillig and Mummey, 2006). The water-stability of aggregates in many soils largely depends on organic matter as a biochemical cementing agent (Tisdall and Oades, 1982). AM-fungi directly move carbon resources from the plant into the soil through hyphal turnover and exudation (Staddon et al., 2003; Tawaraya et al. 2006), which in cooperation with other soil organisms contributes to the organic stabilization of

soils (Miller and Jastrow, 2000). Growing through the soil, external hyphae enmesh soil particles and hold them together in larger units, what makes a mechanical contribution to the stability of soil aggregates (Rillig et al., 2002). In addition, it was discovered that AM-hyphae produce large amounts of a hydrophobic glycoprotein, called "Glomalin", which may serve a highly persistent binding agent to soil aggregates (Wright and Upadhyaya, 1996; Cardoso and Kuyper, 2006). The improvement of soil aggregate stability has been recognized as a major factor determining the response of plants to AM-fungi under field conditions (Hamel et al., 1997).

Prospects for the application of mycorrhizal technologies in agriculture

The manifold potential benefits of arbuscular mycorrhiza on plant performance suggest the possibility of remarkable applications in agriculture (Menge, 1983). There are some considerations, which can be taken into account in this connection. Because AM-fungi are ubiquitous soil inhabitants, it can be assumed that their unique plant growth-promoting abilities are already being utilized by most crop plants on healthy soils. Advantages from measures that aim at the promotion of mycorrhization by the use of AM-inoculants can therefore be expected in situations where the natural populations of AM-fungi are destroyed or diminished, such as in fumigated soils and disturbed ecosystems (Gentili and Jumpponen, 2006). But, also when the abundance of autochthonous AM-propagules is high, adverse environmental factors may suppress the establishment or functioning of the symbiosis (Waschkies et al., 1994). In such cases target-oriented management practices to remediate the individual blockages, which probably require a profound understanding of the particular cause and effect relationships, could be more demanded than the supply of additional AM-inoculum. It is noteworthy that the greatest benefit to the host was often achieved with soil indigenous and not introduced isolates of AM-fungi, which indicates the importance of ecotypical adaptation to the local environment (Requena et al., 1997; Cumming and Ning, 2003; Davies et al., 2005). Baertschi et al. (1981) reported that an unidentified mixed population of naturally occurring AM-fungi was considerably more effective in suppressing pathogen development than a pure culture of *G. mosseae*. As a general rule, it can be recognized in several studies that AM symbioses need to be well established before they confer their beneficial effects (Graham and Menge, 1982; Azcón-

Aguilar and Barea, 1996; Cordier et al., 1998; Khaosaad et al., 2007). According to Feldmann and Boyle (1998) only a high degree of arbuscular mycorrhizal root colonization correlated with enhanced resistance of *Begonia* leaves against powdery mildew, whereas a low degree of root colonization was associated with enhanced susceptibility to the respective pathogen (*Oidium begoniae* Putt.). Furthermore, distinct specificities in host response to different strains of AM-fungi have been described, which demonstrate the need for selection of desirable host-fungus combinations in case AM-inoculants should be applied (Bethlenfalvay, 1989). The same AM-fungal strain can cause opposite growth effects in different plant species. Diverse responses also occur when the same plant species is colonized by different AM-fungi (Feldmann et al., 1996). The outcome of the AM-symbiosis largely depends on the genotypes of both partners and is further affected by environmental influences (Feldmann, 1997). Reliable prediction of AM-effects is therefore only possible when the combination of plant and fungus has been tested under a set of similar conditions in advance (Feldmann and Boyle, 1997; Dehne, 1982).

Nevertheless, in recent years growing emphasis has been put on the selection of effective and adapted isolates of AM-fungi to address specific problems in agriculture by replacing or reinforcing the native mycorrhiza (Gianinazzi and Vosátka, 2004). A major constraint to the commercial production of AM-inoculants is still seen in the obligate biotrophy of AM-fungi, which are usually propagated in costly cultures with living host plants (Hildebrandt et al., 2006; Raja, 2006; Barbosa da Silva et al., 2007). Such basic problems of inoculum production seem to be overcome by a recently discovered root-colonizing basidiomycete *Piriformospora indica* (Verma, 1998), which mimics the capabilities of a typical arbuscular mycorrhizal fungus and performs similar multi-beneficial tasks (Varma et al., 1999; Singh et al., 2000). *P. indica* has been first isolated from Indian desert plants and can be easily grown on simple substrates where it forms durable vegetative spores (Verma, 1998). The host range of this endophytic fungus seems to be even broader than that of AMF as it also colonizes the roots of typical non-mycorrhizal plants (Kumari et al., 2003; Pham et al., 2004). Root colonization by *P. indica* has been shown to result in tremendous growth enhancement of important crop plants (Varma et al., 1999) and to provide protection against biotic and abiotic stresses (Singh et al., 2000; Waller et al.,

2005; Deshmukh and Kogel, 2007). Disease resistance is conferred not only to the roots but also to the shoots (Waller et al., 2008). However, on the cellular level the interaction of *P. indica* with the host plant is entirely different from the mutualism of AMF with living root cells. Although *P. indica* colonizes large areas of the root without provoking visual tissue necrotization, Deshmukh et al. (2006) discovered that this fungus requires dead host cells for successful proliferation and seems to interfere with the cell death program of plants. *P. indica* is related to the phytopathogenic *Rhizoctonia solani* group (Verma et al., 1998), and the outcome of its interaction with plants could revert from mutualistic to antagonistic. Depending on the cultural conditions, its behavior can alter drastically and the fungus starts to grow parasitically on roots and aerial plant parts (Kaldorf et al., 2005). It also needs critical appraisal that there is little or no information concerning the interaction of *P. indica* with AM-fungi (Pham et al., 2004). Regarding the similar niche that these fungi occupy it might be speculated that their relationship is rather competitive than commensalistic. In vitro tests have shown that *P. indica* can completely block the growth of some mould fungi (Pham et al., 2004), but other research indicated a synergistic interaction with *Trichoderma viride* and *Pseudomonas fluorescens* in plant growth promotion (Margode et al., 2003).

As a conclusive remark, it can be stated that there are no simple shortcuts to better harness the beneficial effects of mycorrhizal fungi for agricultural purposes. The changes brought about by mycorrhizal colonization of roots amplify the functional space of the rhizosphere in terms of a mycorrhizosphere, in which many processes take place (Lindermann, 1988; Johansson et al., 2004). Favorable influences of AM-fungi on crops arise from highly complex interactions with the plant, the soil and other organisms, whereby several mechanisms can be operative at the same time (Timonen and Marschner, 2005). Successful application of mycorrhizal technologies in agriculture, therefore require a thorough understanding of the mycorrhizal ecology (Whipps, 2001). Extensive screening programs may enable the selection of effective AM-fungi with respect to specific host and environmental factors, but it is unlikely that a limited number of mycorrhizal strains contained in commercial inoculants will confer equal tolerance to all the stresses, which plants encounter under field conditions (Abbott et al., 1992). And, even when the most effective fungal partners for one host can be found, those must not necessarily be

beneficial for other crops that follow in a rotation. Assumed that effective AM-fungi already occur naturally in the soil, it seems to be more promising under most circumstances to adjust the cropping practices in a way that optimally utilizes the infection potential of the indigenous mycorrhizal populations (Abbott and Robson, 1982).

2.3.5 Multi-partite interactions, synergistic effects and feedback loops

In many laboratory and greenhouse investigations the interaction of a single inoculant organism with a single pathogen or host plant has been concerned while other organisms that exist in the phytosphere have often been ignored. However, multiple plant-microbe-microbe interactions are common under natural and field conditions and resulting effects can prove very diverse (Leggett et al., 2001). Recent attempts not only aim at a better understanding of the mechanisms involved in such complex operations, but also to enhance the effectiveness of microbial agents by combined application, in particular if they exhibit complementary modes of action (Requena et al., 1997; Whipps, 2001; Saxena et al., 2006).

Multi-partite interactions

Some of the best known instances are nodulation promoting rhizobacteria (NPR) which support rhizobia to establish a nitrogen fixing symbiosis with legume roots (Polonenko et al., 1987), and mycorrhiza helper bacteria (MHB) assisting not only the formation (i.e. mycorrhization promoting bacteria) but also the functioning (i.e. mycorrhiza helping bacteria) of the plant fungal-symbiosis (Meyer and Linderman, 1986a; Garbaye, 1994; Frey-Klett et al., 2007). NPR and MHB have been indentified from different groups of microorganisms including species of *Azospirillum*, *Azotobacter*, *Pseudomonas*, and *Bacillus* in both cases (Bagyaraj and Menge, 1978; Burns et al., 1981; Subba Rao et al., 1985b; Meyer and Linderman, 1986a; Halverson and Handelsman, 1991; von Alten et al., 1993; Burdman et al., 1997; Villacieros et al., 2003). While some MHB show a certain degree of fungal-specificity (Duponnois et al., 1993; Bending, 2007), diverse *Pseudomonas* and *Bacillus* spp. interact beneficially with different ecto- and arbuscular mycorrhizal fungi (Duponnois, 2006; Frey-Klett et al., 2007). By contrast, strains of *P. fluorescens* and *P. putida* suppressed the growth of the mycorrhiza-like fungus *P. indica*.

It is, however, not known if the same *Pseudomonas* strains acted as helper-bacteria to real AM-fungi (Pham et al., 2004). Because bacteria are easier and faster to propagate in commercial quantities than arbuscular mycorrhizal fungi, helper bacteria provide additional perspectives to improve the performance of fungal inoculants with low extra cost (Frey-Klett et al., 2007) or to make enhanced profit of the indigenous, well adapted mycorrhizal populations already present in soils (see Chapter 2.3.1).

The mechanisms most commonly implicated with the pre-symbiotic action of helper bacteria are phytohormonal stimulation of root growth resulting in more infection sites for the plant-microbe symbioses, but also the enhanced production of plant derived signaling compounds (i.e. flavonoids) that act as chemo-attractants and regulators of genes involved with the initiation of rhizobial and mycorrhizal symbioses (Azcón et al., 1978; Mayo et al., 1986; Gryndler, 2000; Hirsch and Kapulnik, 1998; Parmar and Dadarwal, 1999). On the functional level, the synergistic cooperation between MHB and already established mycorrhizae includes aspects of improved plant development, nutrient acquisition and biological control of pathogens (Barea et al., 2005; Frey-Klett et al., 2007).

Synergistic effects

Toro et al. (1997) found that dual inoculation of onion (*Allium cepa* L.) with the AM fungus *Glomus intraradices* and *B. subtilis*, acting as MHB, not only promoted the AM establishment, but also increased biomass and N and P accumulation in plant tissues when compared to single applications. The effect on nutrient uptake, observed in this study, was attributed to the exploitation of an extended soil volume for P and N uptake by mycorrhizal hyphae and to the solubilization of otherwise less available phosphate sources by *B. subtilis* in the mycorrhizosphere, thus completing the function of the external mycelium. Similar results of other authors approved the beneficial cooperation between different phosphate solubilizing bacteria (e.g. *Enterobacter agglomerans*, *Bacillus circulans*, *Pseudomonas* sp.) and mycorrhizal fungi in experiments with diverse plant species (Kim et al., 1998a; Singh and Kapoor, 1998; Widada et al., 2007). The compatibility of mycorrhizal fungi with biocontrol strains of *Bacillus*, *Pseudomonas* and *Trichoderma*, even those antagonistic to fungal pathogens, has been proven in respective studies (Paulitz and Linderman, 1989; Schelkle and Peterson, 1996; Barea et al., 1998;

Mar Vásquez et al., 2000; Haggag et al. 2001). In fact, there is strong evidence that changes in the composition of resident microbial soil populations triggered by mycorrhiza formation facilitate those components that can be antagonistic to root pathogens (Secilia and Bagyaraj, 1987; Frey-Klett et al., 2005; Barea et al., 2005).

Mycorrhizal fungi also interact synergistically with symbiotic and free-living nitrogen fixing bacteria (Barea et al., 2002a). As shown by using nitrogen isotope tracer techniques, N_2-fixation rates in legumes nodulated by *Rhizobium* sp. were higher in mycorrhizal than in non-mycorrhizal plants, particularly in soils with limited N and P availability (Kucey and Paul, 1982; Barea et al., 1987; Toro et al., 1998). Taking into account the importance of P supply for biological N_2-fixation, such effect has been partially explained by an improved P acquisition via mycorrhiza (Smith and Draft, 1977; Fitter and Garbaye, 1994). Moreover, the AM effect on N_2-fixation by rhizobia could be further improved by co-inoculation with phosphate-solubilizing rhizobacteria, as demonstrated in pot and field experiments (Barea et al., 2002b). Similarly, the growth of non-legume crops like maize (*Zea mays* L.; Barea et al., 1983), barley (*Hordeum vulgare* L.; Subba Rao et al., 1985a) and wheat (*Triticum aestivum* L.; Singh et al., 1990) was increased by the combined application of *Azospirillum* bacteria and AM-fungi, though the effect on nitrogen fixation was not pronounced in these studies.

Feedback loops

The interactions between plants, rhizosphere inhabitants and the soil involve feedback loops driven by the transfer of organic carbon as energy source from the root to associated organisms. Plant growth can be amplified if the investment of assimilates into the rhizosphere results in improved nutrient acquisition and in feedback enhanced photosynthetic activity itself (DeAngelis et al., 1986; Gobran et al., 1998). To that effect, protozoa and nematodes grazing on bacterial populations in the rhizosphere are of importance to unlock and increase the plant availability of nutrients, in particular N, otherwise sequestered in bacteria (Clarholm 1985; Bonkowski et al., 2000; Bonkowski, 2004). Where nitrogen and phosphorus are limiting, the positive back coupling between P mobilizing and N_2-fixing microorganisms may overcome many of the limitations on low-fertile soils and thus provide a useful basis for the functionality of low-input systems

(Barea et al., 2005). For example, if increased P-uptake via mycorrhizal fungi benefits N_2-fixing bacterial symbionts, the improved N status of the plant in turn may promote root growth and mycorrhizal development (Fitter and Garbaye, 1994; Puppi et al., 1994). AM-fungi act as integral mediators in plant-soil systems because they transport mineral nutrients to the plant and carbon compounds to the soil and its living organisms. Healthier and more vigorous mycorrhizal plants represent an upgraded carbon source for enhanced microbial activity and soil aggregation which again may result in sustained soil fertility (Bethlenfalvay and Linderman, 1992).

2.4 Synopsis on definitions and activities of various bio-preparations

The previous sections have reviewed definitions and properties of bio-stimulants, bio-fertilizers and bio-pesticides with respect to regulatory and applied aspects. It became clear that there are distinct discrepancies between the definitions of different authors, saying which materials and objects they included respectively excluded from being considered as active bio-agents. As summarized in a simplified form in Table 2.2, much of this controversy can be ascribed to dissenting interpretations of the prefix “bio”. Translation of “bio” as “living” consequentially restricts the range of possible active agents to living organisms. When in addition materials derived from living organisms and genes are regarded as “bio”, then every kind of method or application that relies on biotic processes can be included in the bio-concept. The most comprehensive approach certainly is that one which aims to integrate the most advanced knowledge on living organisms and their vital processes into the realization of optimized crop management systems. This broad bio-logical (including meanings that can be translated as “bio-rational and reasonable”; Perelman, 1979; Chapter 9.3) perspective is inclusive of all aspects regarding the complex nature of plant interactions with their environment.

Table 2.2: Interpretations of the prefix "bio" and corresponding conceptions leading to the derivation of diverse definitions what is regarded as active agents of bio-preparations

Interpretation	Basic concept	Included active agents	Examples (not exclusive)
Bio = living	Activity of living organisms	· Living organisms	· AM-fungi, PGPR
Bio = biotic	Mechanisms, processes and products related to living organisms	· Living organisms · Genes and gen-products · Materials derived from organisms	· AM-fungi, PGPR · Resistance genes · Extracts from plants, algae, or microbes
Bio = biological	Rational and reasonable use of knowledge on living organisms and their vital processes	· Living organisms · Genes and gen-products · Biotic and abiotic materials · Human beings as initiators	· AM-fungi, PGPR · Resistance genes · Extracts from organisms, resistance-enhancing minerals · Farmers, companies, scientists

The interdependency of microbial processes in the rhizosphere has been emphasized by the examples of *Pseudomonas* spp., *Bacillus* spp., *Trichoderma* spp. and AM-fungi. Indeed these organisms exert multiple actions that can result in beneficial but also in adverse effects on plant performance. Therefore, particularly in the case of microorganisms, which are always multi-players, but also for other natural compounds that affect plants in sophisticated ways, a categorization into **bio-stimulants**, **bio-fertilizers** or **bio-pesticides** may hamper a holistic view of plant ecology and agricultural problem-solving. With respect to the state of knowledge, it appears to be more appropriate and scientifically correct to use the term "**bio-effector**" as defined in Chapter 1.1, when the active agent and not the purpose of a specific application is meant.

From an applied perspective, the understanding of the modes of action of certain organisms or materials is surely more interesting than definitions, which are finally meaningless for the effectiveness per se. But, during the developmental phase of a new biological method, prevailing definitions constitute mini-theories, which eventually become paradigms that encapsulate ideas and direct the thinking of investigators (Wilson, 1997). In scientific research works, such patterns may influence the questions asked, the hypotheses formulated, the experiments conducted, the results obtained and finally the

conclusions drawn. This, in consequence, will limit the search for effective biological methods to the a priori expected framework (Wilson and Ghaouth, 1993). Therefore, instead of being restricted by present definitions, those might rather be regarded as preliminary "working-definitions", that are open for revision as new insights become available and the understanding of biological systems increases (Cook, 1993; Wilson, 1997).

Knowledge of the multiple ecological interactions taking place in agricultural systems has been recognized as the key to predict the conditions under which successful, reproducible results can be achieved with biological methods (Whipps, 2001; Welbaum et al., 2004). But, an often occurring problem is that with increasing information on complex systems initially some questions can be answered, but simultaneously other knowledge-gaps arise that were previously not recognized. This experience may lead to frustration or to the conclusion that, even if all the conditions that are effectively operating to control the course of events could be understood, it might still be necessary to find ways that allow the application or management of an tremendous number of site-, plant-, and pathogen-specific entities, before advanced practices can be implemented (Garrett, 1965; Cook, 1993). Yet, despite all progress made, science is still far from having an all-encompassing theory of everything. Nevertheless, for proceeding investigations on biological systems, it might be helpful to structure the phenomenon of complexity as well as the corresponding research work. Regarding the ecology of cropping systems, three conceptual levels can be exposed for that purpose: (1) single factor components including the soil, plant and microorganisms, (2) dual or multi-partite interactions between single entities that may antagonize each other or cooperate synergistically for a final outcome, and (3) feedback loops which may amplify the effect of small initial events by self-reinforcing circuits (Willy, 2003; Dhurjati and Mahadevan, 2008). Probably the understanding of such key functional concepts may offer access to the control and management of agricultural ecosystems without getting bogged down in details. In natural ecosystems, sustained plant growth occurs because of the balance that has developed between associated deleterious and beneficial organisms (Hairston et al., 1960). In frequently disturbed habitats, such as agricultural fields, considerable inputs and management efforts are necessary to compensate for the effect of disturbance and to shift the balance of functionally distinct

populations towards those, which support plant health and productivity (Linderman, 1992; Avis et al., 2008). Innovative *bio-logical* approaches that make better use of the synergistic interplay between cultivated plants and related organisms in their site-specific environments are a promising tool to optimize the production of crops as yields can be increased while the dependency on agrochemicals is decreased. But, the great variation between experimental results and their reproducibility in practice indicates that more studies are needed to explore the multi-functionality of single bio-effectors and their multi-factorial interactions (Vestberg et al., 2004; Barea et al. 2005; Römheld and Neumann, 2006).

B CASE STUDIES ON PLANT GROWTH STIMULATION AND BIO-FERTILIZATION WITH EMPHASIS ON IMPROVED PHOSPHORUS ACQUISITION IN CROPS

3 Introduction to the issue of phosphate in agriculture

3.1 The importance and problems of phosphorus in agriculture

Phosphorus: a key mineral nutrient determining the productivity of crops

Without anthropogenic inputs, nitrogen (N) and phosphorus (P) are the most common growth limiting mineral nutrients in natural as well as agricultural ecosystems and essential to all known forms of life (Vitousek and Howarth, 1991; Cowling et al., 2002; Hart et al., 2004). Abundant as dinitrogen (N_2) gas in the atmosphere not the element nitrogen is scarce. The availability of N is limited by the ability to perform the highly energy demanding conversion of nonreactive N_2 into biologically active nitrogen compounds which can be assimilated by living organisms (Galloway et al., 2008). Phosphorus, by contrast, is predominately found as compounds of the phosphate ion (PO_4^{3-}) in both living organisms and global cycles (Goldwhite, 1981). While some plant species in mutualistic association with diazotrophic bacteria have developed sophisticated methods to acquire N from the atmosphere through biological N_2-fixation, the primary source of P for plants and microorganisms are phosphates originating from minerals of the earth's crust (Richardson, 2001; Condron and Tiessen, 2005). Unlike nitrate (NO_3^-) and sulphate (SO_4^{2-}), phosphate is not reduced in plants but phosphorus remains in its highest oxidation state as P^V (Marschner, 1995). In many terrestrial and aquatic environments the productivity of N_2-fixing organisms and hence the net primary production of the whole system is ultimately limited by the amount of available phosphate (Smith, 1984; Smith, 1992; Kitayama et al., 2000; Vitousek et al., 2002).

The pivotal role of phosphate in life processes results from its ability to provide ester linkages in organic molecules that are of fundamental biological importance, such as nucleic acids (DNA, RNA) carrying the genetic information, energy transferring nucleotides (ADP, ATP), and phospholipids that are constituents of cellular membranes (Goldwhite, 1981; Marschner, 1995). The reversible addition of phosphate groups to organic molecules (phosphorylation / dephosphorylation) within the primary cellular metabolism is further an important regulatory mechanism controlling the activity of cellular proteins and part of intricate signaling pathways (Graves and Krebs, 1999; Raghothama, 2005). Due to its basic biochemical functions, phosphate is required by plants in relatively large amounts (about 3 to 5 g P kg^{-1} plant dry matter) for photosynthesis, respiration, and other processes critically involved in growth and reproduction (Marschner, 1995; Bundy et al., 2005).

Trends and issues related to P-fertilizer consumption

The industrial production of nitrogen fertilizers, predominantly through the energy-intensive Haber-Bosch process (Galloway et al., 2002; Smil, 2004) and the exploitation of phosphate deposits for use in agriculture (Stewart et al., 2005), have greatly advanced crop productivity in the past, and continuous delivery maintains high yield levels. However, limited natural resources, especially of energy and phosphate reserves, but also adverse environmental impacts as well as increasing costs of high fertilizer input rates, necessitate alternative strategies for an efficient and resource-saving management of nitrogen and phosphorus in agricultural production (Vance, 2001). As shown in Fig. 3.1 on a global scale the consumption of N- and P-based fertilizers in agriculture has been increasing more than eight- respectively three-fold during the last 50 years. But, the global production of grains as the major source of food has grown only by the factor 2.5 within the same time frame. This indicates a decrease in the use efficiency of both fertilizer types, which is particularly distinct regarding the widening discrepancy between nitrogen input and corresponding yields. On average, the production of one metric ton of grain appears to require an uptake of about 4 kg phosphorus by the plant, with rice (1.8 – 4.8 kg P t^{-1} grain; Dobermann, 1996) having a similar yield specific phosphate demand than maize (2.8 – 3.3 kg P t^{-1} grain; Tang et al., 2008) or wheat (4.0 – 4.4 kg P t^{-1} grain;

Tang et al., 2008). Nitrogen is required at amounts of 15 – 20 kg by rice (Mae, 1997; Swain et al., 2006), 20 – 25 kg by maize (Presterl et al., 2002) and 20 – 30 kg by wheat (Sharma, 1992) to produce 1 t of grain. Assuming a moderately high yield of 4 to 5 t per hectare, the P uptake by the plant would be around 18 kg P ha^{-1} and the N uptake about 100 kg N ha^{-1} (Goswami et al., 1990). However, low use efficiencies of applied nutrients during a single season (< 10 – 30 % for P, < 30 – 50 % for N) necessitate proportionately higher fertilizer input rates in agricultural production (Ouyang et al., 1999; Raun and Johnson, 1999; Fageria et al., 2008).

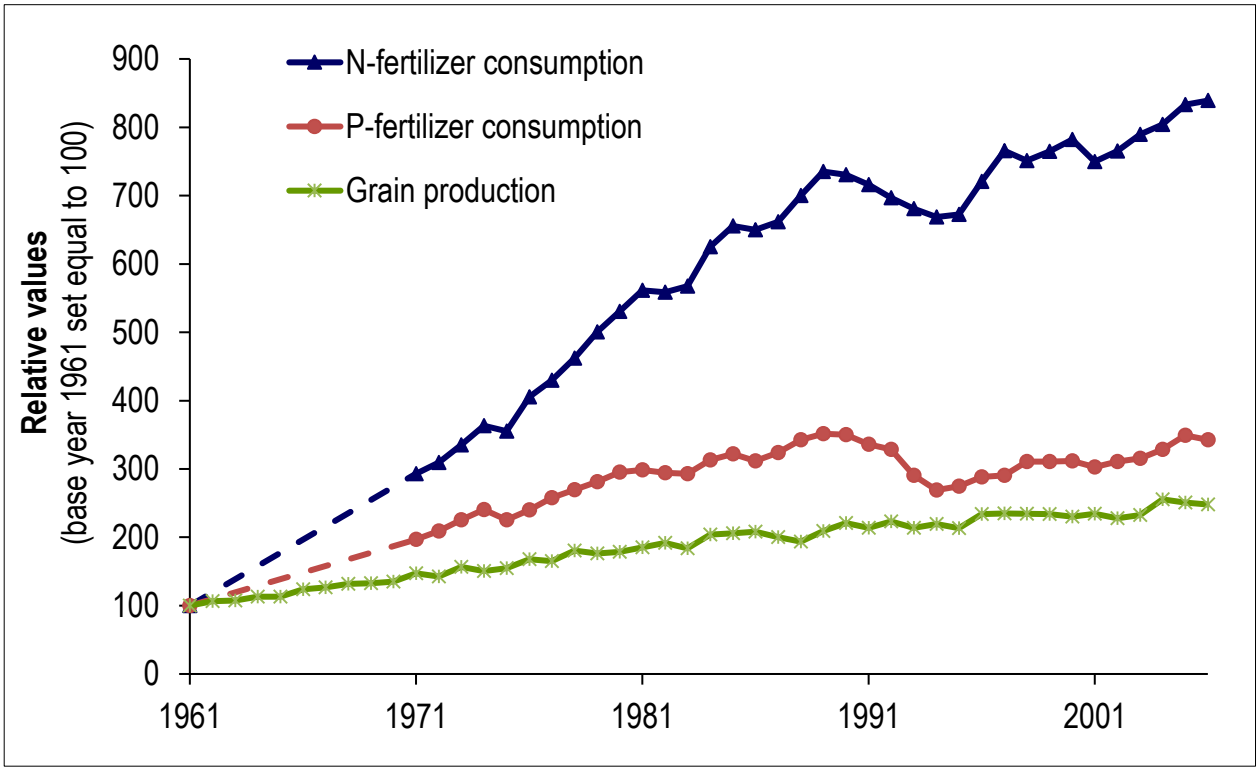

Fig. 3.1: Trends of worldwide consumption of nitrogen (N) and phosphorus (P) fertilizers and grain production of the years 1961-2006. Total weight values of each year were calculated relative to the base year 1961 set equal to 100. Dashed lines indicate missing values. Data sources: IFA (2006a,b); USDA (2006; cited in: Earth Policy Institute, 2006).

The main raw material used in the production of phosphate fertilizers are rock phosphates mined from sedimentary and igneous deposits to an amount of currently 150 million metric tons per year to meet the annual consumption of worldwide 17 million metric tons phosphorus (Stewart et al., 2005; Bickert, 2008). Rock phosphates are minerals rich in sparingly soluble calcium phosphates that need to be chemically processes to produce

water soluble P-fertilizers (Leikam and Achorn, 2005). Finely ground rock phosphates can also be used directly as a slow-release fertilizer in agricultural production, but have a low effectiveness due their low solubility in many soils, beside some strongly acidic soils (Barea et al., 2002b). Depending on the assumed development of future patterns of consumption and economic conditions for their exploitation, estimates of the duration of global phosphate reserves range from less than 50 to more than 500 years. Nevertheless, it is widely acknowledged that the quality of remaining phosphate stocks is decreasing whereas the fertilizer production costs are increasing (Stewart et al., 2005; Cordell et al., 2009). Major criteria for the quality of rock phosphate are the concentration of phosphorus in it, but also the load of undesired contaminants such as toxic cadmium, given that the processing of lower grade raw material causes significantly higher expenses (Bickert, 2008; Cordell, 2008). Another reason for the tremendous increase of the price of rock phosphate in the recent past is seen in the growing demand of fertilizers for the production of food and fuel crops together with a limited hauling capacity of existing phosphate mines (Bickert, 2008; Cordell and White, 2008).

Beside economic considerations, environmental issues necessitate an optimized management of P-fertilizer inputs in agricultural systems, too, so as to minimize any loss of phosphate from soil to water bodies regarding its role in eutrophication. Phosphate discharged into surface water is known to accelerate the growth of algae and hydrophytes, which may lead to oxygen shortages due to their subsequent decomposition. For this reason, phosphate entry can cause severe damages of aquatic ecosystems (Sharpley and Rekolainen, 1997; Tiessen, 2008). According to Tiessen (2008), approximately 10 % of the export of phosphorus from land occurs by leaching and ground water transport, while 90 % of the losses result from surface runoff and erosion, which can be explained by the relative immobility of phosphates in most soils (Sims et al., 1998). In contrast to water and other major elements such as carbon, nitrogen and sulfur in global cycles, phosphorus has no gaseous phase that enters the atmosphere but rather becomes incorporated in ocean sediments over geological periods of time (Begon et al., 2006).

Sustainable phosphorus management also is an integral component of agricultural low-input systems to ensure adequate P availability for crop production. In particular, many

highly weathered, acidic soils of the tropics are notoriously deficient in plant available phosphorus (Buerkert et al., 2001; Singh and Lal, 2005). Mineral P-fertilizers are not only financially or logistically unavailable to many farmers in tropical regions, but also often show limited effectiveness due to the strong sorption of phosphate in these soils (Bationo and Buerkert, 2001; Turner et al., 2006). However, even on favorable soils of the temperate regions insufficient external inputs of organic or mineral P-fertilizers combined with negative nutrient balances at the farm or field level may lead to the re-occurrence of P-deficiency situations in modern cropping systems due to the continuous depletion of previously supplied P-fertilizer surplus (Fig. 3.2; Condron, 2004).

Fig. 3.2: Visual symptoms of phosphorus deficiency (i.e. stunted growth and poor tillering, darkish blue-green or purple color of older leaves, yellowing of leaves that senesce prematurely, and rigid erect appearance of the plants; Bergmann, 1992) in spring barley (Hordeum vulgare L. var. Braemar). The plants were grown on a field with sandy loam soil near Worms, Rhineland-Palatinate, Germany after several years without application of phosphate fertilizers. The picture was taken on 07th May 2009.

3.2 Prospects to improve the efficiency of phosphorus acquisition in crops by the use of bio-effectors

Phosphorus in soils and its availability to plants

Though most soils contain abundant amounts of phosphorus, with total concentrations ranging from 200 to 2000 mg P kg^{-1} dry soil and equivalent total contents amounting from 400 to 4000 kg P ha^{-1} in the top 20 cm soil layer, the bulk of this nutrient is present in forms not readily available to plants (Jarvis and Oenema, 2000; Mengel and Kirkby, 2001; Tiessen, 2008). Phosphorus is taken up by plants almost exclusively from the soil solution as inorganic phosphate ions, with the di- ($H_2PO_4^-$) and mono-hydrogen phosphate (HPO_4^{2-}) anions being the major forms for plant uptake (Richardson, 2001; Raghothama, 2005). In alkaline soils the divalent anion HPO_4^{2-} is predominant, whereas high proton (H^+) concentrations shift the equilibrium to the monovalent anion $H_2PO_4^-$ in acidic soil, according to the equation $HPO_4^{2-} + H^+ \leftrightarrows H_2PO_4^-$ (Mengel and Kirkby, 2001; Brady and Weil, 2008). Even in the case of fertile arable land the concentration of phosphate in the soil solution is typically only in the range between 1 to 10 µmol l^{-1} (Mengel and Kirkby, 2001; Richardson, 2001). These values equate to 0.03 to 0.3 mg P l^{-1} or less than approximately 20 to 200 g P ha^{-1} in the top 20 cm soil layer, assuming a water content of 200 ml kg^{-1} soil and a soil density of 1.3 g cm^{-3} (Loomis and Connor, 1992; Schachtschabel et al., 1992; Or and Wraith, 1999; Chan, 2006). To meet the total phosphorus requirement of high-yielding crops, which can amount to more than 40 kg per hectare and season, the quantity of phosphorus directly present in the soil solution is much too low (Mengel and Kirkby, 2001). Mass flow driven by transpiration would supply on the average only 1 % of phosphate used by the plant (Barber, 1980) and due to their strong affinity to the solid soil phase phosphate ions may diffuse less than a half mm in ten days (Jungk, 2001). Thus, the phosphate depletion zone is constricted to a distance of few mm (ca. 1 – 4 mm) from the root surface and only about 20 % of the top soil volume contributes phosphate to the plant in one season (Jungk and Claasen, 1986), as the roots of annual crops have a volume that is usually less than 1 % of the soil volume (Barber, 1963). Plants therefore depend on the continuous replenishment of the soil solution with phosphate from solid pools existing in the soil (Marschner, 1995; Kirkby and Römheld,

2006). This phosphorus, comprising native soil phosphates as well as phosphate that has accumulated as a consequence of previous fertilizer applications, is retained in organic (P_{org}) and inorganic (P_i) phosphate forms of varying accessibility to plant roots or microorganisms (Bieleski, 1973). Due to the continuous immobilization of phosphates in soils by adsorption and precipitation processes usually only 10-30 % of the phosphorus supplied as water-soluble P-fertilizers is utilized by the crop in the year of application (Bolland and Gilkes, 1998).

Organic phosphate in soils

Organic phosphate compounds, originating from plant remains or synthesized by soil organisms, typically account for between 30 and 65 % of the total phosphorus existing in soils (Condron et al., 2005). Included in this fraction are to a small part relatively easily decomposable phospholipids and nucleic acids, whereas a large proportion is comprised by more resistant inositol phosphates and uncharacterized high molecular weight substances (Richardson, 2001; Brady and Weil, 2008). In particular derivates of inositol hexaphosphate (phytic acid), which is an important storage form of phosphorus in plant tissues, can account for 10 to 50 % of the total organic phosphorus content in soils (Richardson, 2001; Grotz and Guerinot, 2002). However, once released into soil from decomposing organic matter, plants are largely unable to access phosphate from inositol phosphates as they become strongly adsorbed to the soil matrix or rapidly stabilized in binding forms that are highly recalcitrant to mineralization, such as sparingly soluble phytate salts formed with iron and aluminum in acidic soils or with calcium in alkaline soils (Tisdale et al., 1990). Another important storage pool of phosphate in soils is the microbial biomass, which can hold a share of the total amount of organically bound phosphate exceeding 25 % in arable, 50 % in grassland, and 80 % in forest soils, as reported by Khan and Joergensen (2012).

Inorganic phosphate in soils

The concentration of inorganic phosphate in the soil solution is largely determined by sorption-desorption reactions on the surface of soil constituents, and by the pH dependent solubility of defined phosphate minerals in soils (Tisdale et al., 1990; Brady and Weil,

2008). In neutral to alkaline calcareous soils the dominant form of inorganic phosphate are various Ca-phosphates, which become more soluble as the pH and the calcium concentration of the soil solution decreases. Under alkaline conditions $H_2PO_4^-$ anions in the soil solution rapidly react with Ca^{2+} cations to form a sequence of Ca-phosphates decreasing in solubility with aging time, and ending up in the formation of least soluble apatites (Mengel and Kirkby, 2001; Brady and Weil, 2008). In very acidic soils with pH-values below 4, by contrast, the iron and aluminum phosphates strengite ($FePO_4 \cdot 2H_2O$) and variscite ($AlPO_4 \cdot 2H_2O$) are stable and become more soluble as the pH increases (Larsen, 1967; cited in: Mengel, 1991). The concentration of inorganic phosphorus in the soil solution of most acidic to neutral soils is largely governed by the adsorption of phosphate to the surfaces of iron or aluminum (Fe/Al) hydrous oxides, clay minerals, or humic materials by exchange with other anions such as the hydroxyl ions (OH^-). Because this type of adsorption is reversible it is obvious that the solubility of phosphate in acidic soils can be increased by raising the soil pH or adding other anions such as carboxylate ($R\text{-}COO^-$) or silicate ions in particular (Mengel and Kirkby, 2001; Brady and Weil, 2008). Like Ca-phosphates, also, phosphate adsorbed on the surface of iron and aluminum oxides may undergo sequential reactions, which decrease the solubility of the aging phosphate compounds to extremely low levels. This can either happen by the establishment of chemical bonds to adjacent Fe or Al atoms or due to the additional precipitation of Fe/Al hydrous oxides, which occlude previously adsorbed phosphate as an overlying layer (Brady and Weil, 2008). Reversely, conditions leading to the reduction of ferric iron (Fe^{III}), as it is contained in Fe hydrous oxides and Fe-phosphates, to soluble Fe^{2+} can result in the concomitant release of phosphate (Welp et al., 1983; Reddy and DeLaune, 2008).

The concept of labile and non-labile phosphate factions in soils

With respect to the phosphorus acquisition by plants, the capacity of solid-phase phosphorus in soils can be roughly divided in a labile and a non-labile fraction, although the transitions between these fractions are smooth (Larsen, 1964; Olsen and Khasawneh, 1980). The labile phosphate fraction in soils, amounting to between 150 and 500 kg P ha^{-1} in the top 20 cm soil layer, is in a rapid equilibrium with the soil solution that buffers the

phosphate concentration by desorption and adsorption processes and can hence be regarded as potentially plant available (Mengel and Kirkby, 2001). Major labile constituents of soil phosphate are easily soluble Ca-phosphates, exchangeably adsorbed phosphate ions, easily mineralizable organic phosphate compounds, and phosphate that is occluded by reducible Fe-oxides (Clarholm, 1993; Mengel and Kirkby, 2001). The non-labile phosphate fraction, including strongly adsorbed and tightly bound organic and mineral phosphates, can account for more than 90 % of the total soil phosphorus, amounting from about 400 to 4000 kg P ha^{-1} in the top 20 cm soil layer (Mengel and Kirkby, 2001; Jarvis and Oenema, 2000; Bünemann and Condron, 2007). Even some highly weathered tropical soils, where severe phosphate deficiency is a major constraint on agricultural productivity, have been reported to contain large amounts of phosphorus in recalcitrant forms (Nziguheba and Bünemann, 2005; Turner et al., 2006; Oberson et al., 2006). By complex reactions in the soil, a significant proportion of phosphate from fertilizers can become incorporated in the non-labile fraction, which limits the use efficiency of P fertilizer applications. Nevertheless, the non-labile pool also is a source of slow phosphate release, in particular when more soluble phosphate forms in the soil are becoming depleted. But, as the rate of phosphate mobilization from the non-labile fraction is generally far too low to meet the demand of rapidly absorbing roots, it can be regarded as virtually inaccessible to plants (Olsen and Khasawneh, 1980; Tisdale, 1990). In view of the increasing need to develop more P-efficient agricultural systems, it is therefore an important question how phosphate from non-labile pools can be made more accessible to crops (Jakobsen et al., 2005).

Biological mechanisms and strategies to improve the acquisition of phosphate by plants

As most phosphate in soils is strongly adsorbed or bound to the soil matrix, the concentration of phosphate in the soil solution is generally very low. Thus, only a relatively small amount of phosphate is directly available at the root surface or supplied with the flow of water (mass flow) to the root for uptake by the plant (Barber, 1980). Furthermore, the mobility of phosphate ions is impaired by their high interactivity with the soil matrix and hence low diffusion coefficient in most soils (Cullimore, 1966; Jungk, 2001; Lambers et al., 1998). Active uptake mechanisms enable plants to accumulate

phosphate ions more rapidly than the movement of phosphate in soils even against a step concentration gradient, which can result in the formation of a zone depleted in the concentration of phosphate ions in the soil solution around the roots (Hase et al., 2004; Raghothama, 2005). Physical-chemical soil factors, such as moisture, temperature, pH and redox potential, determine the buffering of phosphate concentrations in the soil solution (Barber, 1980; Pierzynski et al., 2005). In most soils, however, abiotic soil processes alone do not replenish phosphate to the depleted soil solution around roots at rates that are adequate for optimal plant growth (Richardson, 2001). Therefore, plants have developed a range of mechanisms either to economize the internal use of scarcely absorbed phosphate or to improve the acquisition of sparingly available external phosphate sources (Föhse et al., 1988). The latter include the induction of chemical changes in the rhizosphere and/or adapted root growth characteristics such as the formation of root hairs and arbuscular mycorrhizas to enhance the spatial acquisition of phosphorus (Jungk, 2002; Kirkby and Römheld, 2006). Although the combination of low internal requirement and high phosphate acquisition efficiency appears to be a desirable goal in crop breeding programs in order to achieve maximum yields under phosphate-limited conditions, none of seven crop species studied by Föhse et al. (1988) showed a simultaneous pursuit of both adaptation strategies. Rather species with the lowest internal phosphate requirement, i.e. onion and bean, were the least efficient in phosphate acquisition. Furthermore, species efficient in the acquisition of phosphate, such as wheat and rape, were not only able to accumulate higher P concentrations in their shoot biomass but also were less suppressed in their shoot growth in treatments with a low phosphate supply in the soil solution. Very different findings, however, have been made for wild plants, such as many species of the *Proteaceae*. These plants, which are adapted to extremely phosphorus impoverished soils, combine efficient chemical mobilization of recalcitrant phosphate forms by a timely and spatially concentrated release of organic chelators from their specialized cluster roots with a high internal phosphorus use efficiency (Neumann and Martinoia, 2002; Neumann, 2010). Although the sophisticated specialization of these plant species is coupled with slow growth rates, as they take up and store phosphorus mainly during the rainy winter season to be used for shoot growth

during spring and summer (Jeschke and Pate, 1995), this example shows that individual plants can express versatile modes of adaptation for phosphorus efficiency.

Biological factors affecting the spatial availability of phosphate to plants

Plant adaptations towards an improved uptake of sparingly available phosphate include morphological changes such as enhanced root growth, formation of lateral roots and root hair development to increase the surface area of roots for phosphate absorption (Drew, 1975; Schmidt and Linke, 2007). In addition, the association of many major crop plants with arbuscular mycorrhizal (AM) fungi, growing their hyphae several centimeters beyond the phosphate-depleted zone surrounding the roots, allows the absorption of phosphate from considerably larger soil volumes (Tinker, 1984; Li et al., 1991). The extra phosphate taken up via arbuscular mycorrhizas, however, comes in the main from pools of similar chemical availability than accessible to non-mycorrhizal roots. There is little evidence that AM-fungi contribute directly to the chemical mobilization of recalcitrant soil phosphates in a range that would be significant for plant growth (Mosse, 1986; Hodge et al., 2001; Richardson, 2001). Furthermore, Schweiger et al. (1995) have shown that the importance of AM-fungi in phosphate uptake of different pasture species was inversely related to the root hair length of these host plants, as root hairs and external AM-hyphae may provide alternative ways of bypassing the phosphate depletion zone around the root. The kinetic characterization of the phosphate uptake activity of plant cells indicated the presence of two different transporter systems to take up phosphate at lower respectively higher concentrations. The low-affinity transporters are expressed constitutively in plants whereas the expression of high-affinity transporters in root tissues is regulated by the availability of phosphate (Barber, 1972; Furihata et al., 1992; Raghothama et al., 2005). In this regard, molecular studies confirmed that genes encoding high-affinity phosphate transporters are preferentially expressed in roots under phosphate deficient conditions to accelerate the uptake of phosphate from the soil solution (Leggewie et al., 1997; Liu et al., 1998a; Hase et al., 2004). Also arbuscular mycorrhizal fungi have been shown to express transporters for the uptake of phosphate in external hyphae, whereas the expression of root own transporters for the direct uptake of phosphate from the environment seems to be suppressed upon mycorrhizal infection (Harrison and van Buuren, 1995; Liu et al.,

1998b). However, strategies for increasing the acquisition of phosphate by intensive spatial exploitation of the soil or enhanced influx rates due to the function of high-affinity transporters are likely to be successful in situations where reasonable phosphate concentrations can be maintained in the soil solution (Jungk, 2001). As the maintenance of such concentrations is often the ultimate constraint affecting the spatial availability of phosphate in deficient soils, emphasis is on strategies to improve the solubility and movement of readily available phosphate ions to the root surface, respectively mycorrhizal sites of phosphate uptake (Jungk, 2001; Smith, 2002).

Biological factors affecting the chemical availability of phosphate to plants

To increase the concentration of phosphate in the soil solution that is available for immediate uptake, plants, but also soil microorganisms, may use five major chemical forms of phosphate mobilization (Whitelaw, 2000; Richardson, 2001; Neumann and Römheld, 2007; Marschner, 2008):

1) The exudation of organic acid anions (i.e. carboxylates) can be effective to mobilize phosphate adsorbed to Fe/Al-oxides through ligand exchange reactions (Jones, 1998a).

2) Carboxylates may also increase the solubility of Ca-, Fe- and Al-phosphates by chelating the respective metal ions (i.e. Ca^{2+}, Fe^{3+}, andAl^{3+}), thus releasing phosphate anions into the soil solution (Ryan et al., 2001). Similarly, the chelation of iron by siderophores or phenolic compounds can result in the solubilization of phosphate, too (Römheld, 1987; Jayachandran et al., 1989; Dakora and Phillips, 2002).

3) The release of protons (H^+), lowering the soil pH, may contribute to the solubilization of precipitated Ca-phosphates in neutral and alkaline soils (Hedley et al., 1982).

4) Soil acidification, the exudation of carboxylates or phenolics, and the consumption of oxygen by plant roots or microorganisms may further enhance the reduction of Fe^{III} and thus the solubility of iron-bound phosphate (Dinkelaker, 1995; Jones, 1998a; Neumann and Römheld, 2002).

5) To liberate phosphate from organic materials by hydrolysis the production and secretion of extracellular enzymes (i.e. phosphatases) is of particular importance, as it

is generally assumed that organic phosphate compounds need to be mineralized in order to become available for uptake by plants (Tarafdar and Claassen, 1988; Raghothama, 2005).

By these various mechanisms, the activity of plant roots and associated microorganisms can shift the equilibrium from non-labile and labile phosphates fractions in soils towards a higher concentration of phosphate ions in the soil solution of the rhizosphere as schematically summarized in Fig. 3.2.

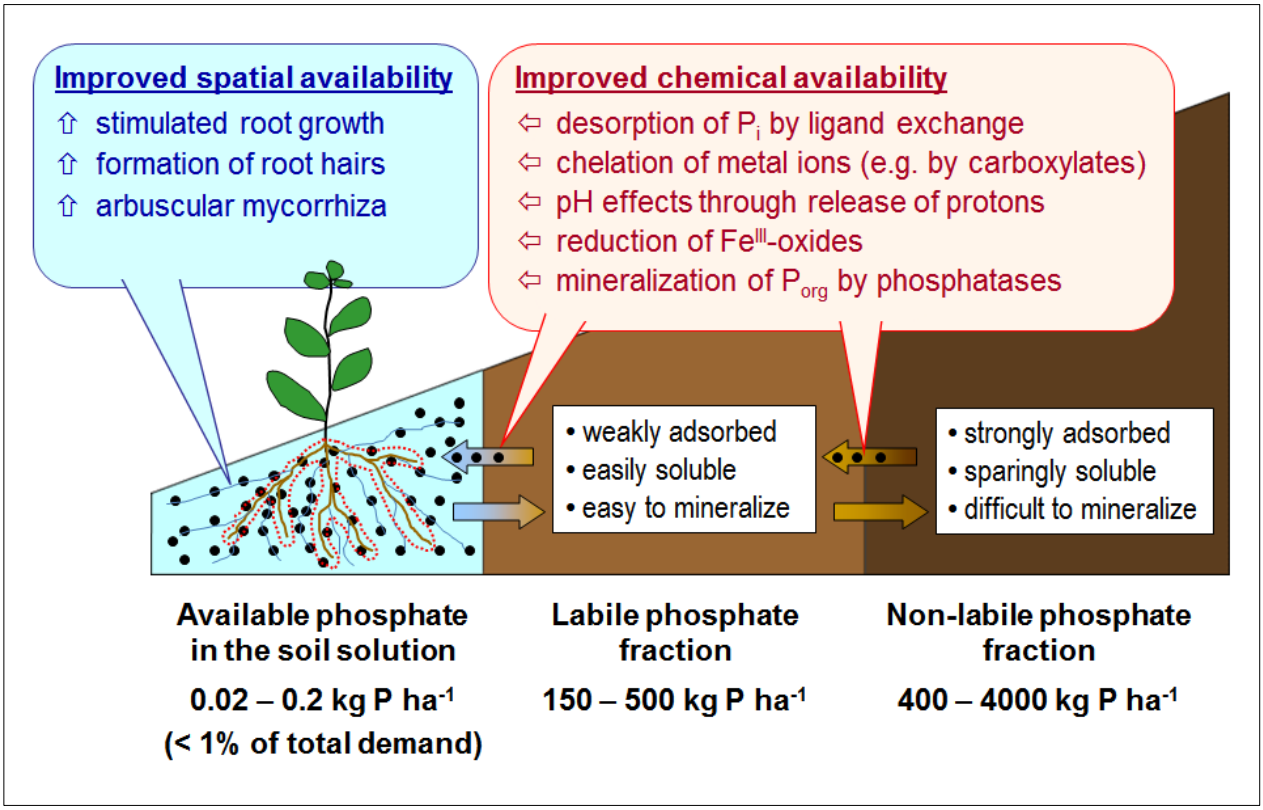

Fig. 3.3: Schematic illustration of phosphate (•) pools in soils and principle mechanisms to increase the plant availability of phosphate. Phosphate is generally taken up from the relatively small pool of soluble inorganic phosphate ions in the immediate surroundings of roots. Enhanced root growth, formation of root hairs, or mycorrhizas can increase the absorptive surface (blue color). However, the main factor affecting the spatial availability of phosphate is often the phosphate concentration in the soil solution controlled by the buffering capacity of the soil around the roots or mycorrhizal sites of uptake. Therefore, plants and in particular soil microorganisms have developed a range of chemical mechanisms (red color) to shift the equilibrium (⇆) from the much larger pools of non-labile and labile phosphates in the rhizosphere soils towards a higher concentration of phosphate ions in the soil solution of the rhizosphere (adapted from: Mengel and Kirkby, 2001; Richardson, 2001).

Although the relative importance of plants or microorganisms in enhancing the chemical availability of soil phosphate in the rhizosphere might be difficult to determine, it is generally accepted that the amount and composition of root exudates as carbon and energy source selectively affect the activity and composition of associated microbial populations in a host plant specific way (Richardson, 2001; Hartmann et al., 2009; Marschner, 2008). Reversely, microorganisms can influence the conditions in the rhizosphere by enhancing the release of root exudates (Meharg and Killham, 1995) or producing phytohormonal compounds that modify the growth patterns of roots (Wittenmayer and Merbach, 2005). Different plant species and even varieties of the same species can vary widely in their ability to grow on soils with low phosphate concentration in the soil solution (Ray and van Diest, 1979; Föhse et al., 1988; Jemo et al., 2006; Corrales et al., 2007). But, for the nutrition of most crops, the direct effect of root exudates on the chemical mobilization of soil phosphates appears to be of limited significance.

Model experiments with different soils and a range of organic compounds have demonstrated the phosphate mobilizing effect of organic acids. Thereby tricarboxylic acids (e.g. citric) proved to be more effective than dicarboxylic acids (e.g. malic, oxalic), whereas monocarboxylic (e.g. acetic, formic, lactic) acids were weaker still, due to the decreasing stability of their complexes formed with metal ions (i.e. Fe^{3+}, Al^{3+}, Ca^{2+}) in this order (Amann and Amberger, 1988; Bolan et al., 1994; Jones, 1998a; Ryan, 2001). Yet, even though enhanced release of carboxylates from roots is a common response to P deficiency in many plant species, particularly dicotyledonous ones, it is questionable whether this can be generally regarded as an effective mechanism for chemical phosphate acquisition in soils (Neumann and Römheld, 1999; Gerke et al., 2000; Neumann and Römheld, 2007). In dependence from the pH-value and a complexity of other soil properties significant phosphate desorption typically requires carboxylate concentrations of at least10 µmol g^{-1} soil for citrate and even more for less efficient carboxylates (Jones, 1998a; Gerke et al., 2000). While such high concentrations (> 40 µmol g^{-1} soil) have been reported to be reached in the rhizosphere of highly specialized root clusters formed by white lupine (*Lupinus albus* L.; Dinkelaker et al., 1989; Gerke et al., 1994) and members of the *Proteaceae,* for most other plant species carboxylate concentrations found in their

rhizospheres are far too low to effectuate significant phosphate mobilization (Dinkelaker, 1995; Jones et al., 1998a). Carboxylates exuded from roots are further susceptible to rapid decomposition by microorganisms proliferating in the rhizosphere (Matsumoto et al., 1979). Therefore, the localized and temporally controlled release of carboxylates in apical root zones, where the density of microbial populations is still lower than in older root parts, has been discussed as an adaptive strategy of plants to limit the microbial degradation of carboxylates (Hoffland et al., 1992; Schönwitz and Ziegler, 1986; Römheld, 1991; Zhang et al., 1997; Neumann and Römheld, 2002). In cluster roots of white lupines, additionally, a drastic decrease in pH and the release of antagonistic compounds (e.g. phenolics, fungal cell wall-degrading enzymes) has been attributed to the suppressed development of microorganisms, and thus enhanced phosphate mobilizing effectiveness of carboxylates (Kania et al., 2003; Weisskopf et al., 2006; Marschner, 2008).

Plants also encounter severe restrictions to enhance the availability of organically bound phosphate in soils. The low solubility of root-secretory phosphatases (i.e. acid phosphatase; Tarafdar and Claassen, 1988) in soils, which are in the main bound to cell walls of the root (Ridge and Rovira, 1971; Barrett et al., 1998) or inactivated by adsorption to soil mineral and organic components (Kandeler, 1990; Rao et al., 2000), largely restricts their mineralizing activity to those organic soil phosphates that are in contact with or mobile enough to reach the root surface (Adams and Pate, 1992; Firsching and Claassen, 1996). Yet, in particular inositol phosphates, which account for the dominant form of organic soil phosphates, are strongly adsorbed or precipitated as sparingly soluble phytates and hence very immobile in soils, too (Richardson et al., 2007). A further constraint is the low specific activity of extracellular root phosphatases (i.e. phytases) to hydrolyze phytates, as it has been found in several plant species (Hübel and Beck, 1996; Hayes et al., 2000; Phillippy, 2002; Konietzny and Greiner, 2004). A major function for the phosphate deficiency-induced release of phosphatases by roots, therefore, seems to lie in the hydrolysis of organic phosphate compounds that are permanently lost into the rhizosphere from sloughed-off and damaged root cells in order to permit the rapid retrieval of phosphate in competition with rhizosphere microorganisms (Lefebvre, 1990).

Apart from the limited ability of plants to chemically mobilize phosphate in soils, rhizosphere microorganisms are considered to play an important role in the transformation of phosphate from recalcitrant pools into forms available to plants (Gerretsen, 1948; Whitelaw, 2000; Marschner, 2008). The capacity to mobilize phosphate retained in soils by solubilization of inorganic and/or mineralization of organic phosphate forms appears to be widespread among soil and rhizosphere microorganisms. Most predominant phosphate-mobilizing bacteria belong to the genera *Bacillus*, *Pseudomonas*, and *Rhizobium* (Kucey et al., 1989; Rodríguez and Fraga, 1999), and phosphate-mobilizing fungi were often found within the genera *Penicillium*, *Aspergillus*, and *Trichoderma* (Kucey et al., 1989; Anusuya and Jayarajan, 1998; Whitelaw, 2000; Tarafdar et al., 2003).

Many studies have demonstrated that a high proportion (up to 40 %) of the microflora isolated from diverse soils or rhizosphere samples and tested on the basis of laboratory screening assays is capable of solubilizing sparingly soluble phosphates (Kucey et al., 1989). While there is little evidence for any specific promotion of phosphate-solubilizing microorganisms in the rhizosphere (Kucey et al., 1989; Richardson, 2001), the vitamin producing activity of phosphate-solubilizing bacteria isolated from the rhizospheres of different plant species has been observed to be higher than of those isolated from control soil when determined by use of agar plate tests (Baya et al., 1981). Such rhizosphere effect probably also applies to the microbial production and release of carboxylates improving the solubility of inorganic phosphates in soils, which has been suggested to be an important mechanism of plant growth promotion by root associated microorganisms (Richardson, 2001). Diverse strains of *Bacillus* spp. and *Pseudomonas* spp., for instance, have been shown to produce organic acids (e.g. citric, gluconic), to solubilize Ca-phosphate, and to improve the growth and phosphate acquisition of plants grown under greenhouse conditions (Zhong and Huang, 2004; Hariprasad and Niranjana, 2008; Trivedi and Sa, 2008). Several in vitro studies have further attributed the solubilization of Ca-phosphates to decreases in pH of the culture media during microbial growth, as reviewed by Whitelaw (2000). This effect, however, might be strongly influenced by the nutrient composition and buffering capacity of the culture media. Thus, ammonium (NH_4^+) instead of nitrate (NO_3^-) nutrition, is known to favor the release of protons (H^+) in both microorganisms as well as plants and may hence improve the solubilization of Ca-

phosphates as an unspecific side effect (Whitelaw, 2000; Neumann and Römheld, 2002; Rahmatullah et al., 2006). Likewise, many soil microorganisms will be inevitably assessed as powerful phosphate solubilizers when cultivated on nutrient rich media, because these conditions favor high growth rates, which are often accompanied with proton release. In less nutrient rich environments, such as in soils or the rhizosphere, however, microbial growth rates are usually much lower (Marschner, 2008). In addition, the strong buffering capacity of calcareous soils is likely to counteract strong pH effects on the solubility of Ca-phosphates (Neumann and Römheld, 2002).

In fact, microorganisms are considered to be the major source of phosphatase activity in soils and of an enhanced phosphatase activity in the rhizosphere (Tarafdar and Jungk, 1987; Rodríguez and Fraga, 1999; Badalucco and Nannipieri, 2007). In a screening of diverse soil bacteria, it has been shown by Richardson and Hadobas (1997) that in particular fluorescent *Pseudomonas* strains exhibited marked extracellular phytase activity. Subsequent tests conducted under laboratory conditions further proved that a specific isolate of *Pseudomonas* sp., selected for its pronounced phytase activity, was able to improve the P nutrition of pasture plants supplied with inositol phosphate (Richardson et al., 2001). Similar results have been reported by Idris et al. (2002), who found that culture filtrates of a strain of *Bacillus amyloliquefaciens* (FZB45), able to degrade extracellular phytate, improved the growth of maize seedlings under phosphate limitation in the presence of phytate. As compared to other fungi, tested by Tarafdar et al. (2003), a strain of *Trichoderma harzianum* was found to be most efficient mobilizer of organic phosphates.

Management of microbial bio-effectors for improved phosphate mobilization

The ability of microorganisms to mobilize otherwise unavailable phosphate in soils and enhance its available to plants makes them interesting from the view of agriculture because of the possibility to manage them for optimized production. This includes the perspective to maintain productive yields with less or cheaper P-fertilizer use in low input cropping systems as well as to avoid phosphate losses by a more efficient phosphate management in high input cropping systems (Tinker, 1980; Jakobsen et al., 2005). These objectives may be achieved by two principle strategies: (1) the management of microbial

populations existing in the soil to optimize their phosphate mobilizing activity; and/or (2) the application of selected microbial inoculants (Richardson, 2001). Besides, there are still many uncertainties regarding the successful implementation of such approaches in agricultural practice. Rhizosphere microorganisms may not only increase, but also decrease the availability of phosphate to plants by immobilization of phosphate in their microbial biomass, decomposition of root exudates, or pathogenic suppression of root growth (Marschner, 2008). The proportion of phosphate contained in the microbial biomass has been estimated to account for 1-10 % of the total soil P (Hedley and Stewart, 1982; Brookes et al., 1984, Richardson, 2001). Higher proportions of microbial phosphate are likely to be found in the rhizosphere compared to the bulk soil (Marschner, 2008). Although appropriate microorganisms are capable to dissolve sparingly soluble phosphates in pure culture, it is questionable whether they will mobilize significant surplus to support plants beyond their own requirements, regarding the competitive conditions prevailing in the rhizosphere of soil grown plants (Tinker, 1984; Richardson, 2001). The critical analysis of the biochemical processes involved in phosphate solubilization rather suggests that plants are more likely to release the required amounts of materials (e.g. carboxylates) directly than associated microorganisms could do, as those depend on root exudates as their main carbon source (Tinker, 1984).

However, microorganisms may not be as ineffective as the above considerations suggest. Differentiated population dynamics in response to the spatially and temporally controlled release of root exudates may result in the wave-like formation of “hot spots” along the growing root axis, where the localized effect on phosphate mobilization is quite high (Römheld, 1991). Thus, initially enhanced microbial growth rates in response to the concentrated release of root exudates as organic carbon source in young apical root zones, may lead to the rapid accumulation of phosphate in the microbial biomass. During these processes, the microbial transformation of non-phosphate-mobilizing root exudates, such as sugars, into P-mobilizing compounds may increase the availability of soil phosphates to the microorganisms predominantly (Schilling et al., 1998; cited in: Horst, 2001; Marschner, 2008). However, as root growth continues and the exudation by maturing root parts decreases, carbon starvation may lead to the net release of plant available phosphate from the perishing microbial biomass (Richardson, 2001; Marschner, 2008). In addition,

components of the soil fauna, such as protozoa and nematodes, grazing on the root-associated microflora, can have an important influence on the turnover of microbial phosphate (Cole et al., 1978; Turner, 2006).

While numerous microbial isolates have been characterized for their high phosphate solubilizing activity in laboratory screening assays, less research has investigated the specific interaction with potential host plants, and only few reports describe the application in field trials (Kucey et al., 1989; Whitelaw, 2000; Leggett, 2001). An exception to this is made by studies performed in the former Soviet Union on the use of bacterial bio-fertilizers. Thus, as reviewed by Brown (1974), in the late 1950's about 10 million hectares were treated among others with a preparation named "phosphobacterin" based on *Bacillus megaterium*, in particular the strain "*phosphaticum*", to supply phosphate to various crops. Benefitting approximately 50 to 70 % of the field crops, reported yield increases ranged from 10 to 20 %. Regrettably, it is not comprehensible whether the observed plant growth promoting effect was caused by phosphate mobilization or other mechanisms, such as enhanced root growth, suppression of pathogens or nitrogen fixation. Later studies conducted in other countries under different conditions could not consistently reproduce the former results and come to diverse conclusions regarding the possible modes of action (Smith et al., 1961; Brown, 1974; Kabesh et al., 1975). Recent studies, nevertheless, have reemphasized the phosphate solubilizing ability of *Bacillus megaterium* strains as one of their multiple plant promoting properties (Çakmakçı et al., 2007; Elkoka et al., 2008; Raju and Uma, 2008; Trivedi and Pandey, 2008). Inconsistency of performance is basically not surprising considering the complexity of interacting processes that govern phosphate acquisition by plant roots growing in soil. In this system, microbial effects on phosphate availability are only one link of the chain and determined by many variables such as physical/chemical soil factors (e.g. temperature, water content, pH, buffer capacity, redox potential), the activity of concomitant soil biota, and characteristics of the host plant (Kloepper, 1989; Horst et al., 2001; Kovar and Claassen, 2005). Insufficient knowledge to predict how the phosphate mobilizing activity of microorganisms is influenced by environmental factors and how to establish them as a significant component of the soil and rhizosphere

microflora appears to be a major constraint to their effective utilization (Richardson, 2001).

Objectives of Part B of this study

A major objective, according to the above discussion, is the coherent characterization of commercially available bio-fertilizers for improved phosphate acquisition by plants as basis for the sound evaluation of their potential effectiveness as well as of their suitability to be used effectively in agricultural cropping practice.

For this purpose selected preparations based on microbial bio-effectors as well as abiotic compounds supposed to stimulate the activity soil microorganisms have been tested in screening assays, greenhouse experiments and under field conditions at two sites in Germany, as presented in the following three chapters.

4 Screening the potential effectiveness of bio-fertilizers for improved phosphorus acquisition by plants

4.1 Background and objectives

The application of bio-fertilizers containing microbial inoculants to solubilize sparingly available phosphates in soils has been shown to result in higher phosphate uptake and tremendous yield increases (up to 40 %; Brown, 1974) in various crop plants under greenhouse and field conditions (Gerretsen, 1948; Şahin et al., 2004; Rudresh et al., 2005b; Zarei et al., 2006; Richa et al., 2007; Wani et al., 2007; El-Azouni, 2008; Linu et al., 2009). However, the knowledge of the biological mechanisms and environmental factors actually involved is rather vague (Richardson, 2001). While few critical reports have also described the absence or even detrimental responses to inoculation with phosphate solubilizing microorganism (Smith et al., 1961; Kucey, 1989; Whitelaw, 2000; Valverde, 2006), inconsistency of performance under field conditions is still a major obstacle for the application of commercial bio-fertilizers in agricultural practice. It is a matter of speculation how many greenhouse and field tests have been done, because the "negative" results have often not been reported for this reason (Leggett et al., 2001). Nevertheless, the use and commercialization of specific microbial inoculants as bio-fertilizers has received increasing attention in recent years (Thakuria et al., 2004; Martinez Viera and Dibut Alvarez, 2006; Selvamukilan et al., 2006). In addition, many commercial preparations also contain diverse non-living compounds such as humic acids or algae extracts with expected potential to stimulate plant growth, thus further complicating the traceability of cause and effect (NIIR, 2004). Hence, there is a need to develop adequate methods to investigate the principle effectiveness of bio-fertilizers and conditions for their successful use, to test the quality of such products, and to optimize their application.

The reliability of bio-fertilizer applications can finally only be ascertained in field experiments to study the cause-effect relationships under praxis conditions. However, field and even greenhouse experiments are generally speaking too labor intensive, costly

and time consuming in order to assess the influence of a large number of microbial isolates and environmental variables. For these reasons, the development of simple but significant screening tests is required in order to gain useful information for the more effective arrangement of expensive experiments prior to their setup. Suitable methods to evaluate the potential effectiveness of bio-fertilizers for improved phosphate nutrition are likely to emphasize on the expression of mechanisms that are obvious to affect the phosphate acquisition of plants under certain conditions. Important modes of action, that have been generally discussed in this regard, are (1) mobilizing sparingly available phosphates in soils, (2) increasing the active surface area for uptake of readily available phosphate from the soil solution, and (3) the promotion of soil borne microorganisms exerting these abilities. In addition to these mechanisms, an overall improved plant vigor, as due to the enhanced availability of different mineral nutrients, the effect of plant growth promoting compounds, or the suppression of deleterious organisms, is likely to support a higher phosphate acquisition by the plant (Richardson, 2001; Vessey, 2003; Marschner, 2008).

In the present study, commercially available bio-fertilizers were characterized by the use of literature based screening methods that were adapted further to test their potential effectiveness under defined conditions. In detail, the capability of microbial isolates, contained in the bio-fertilizers, to solubilize sparingly soluble phosphates was estimated by in vitro tests. The direct effect of bio-fertilizers on root growth and morphology was studied by means of filter paper based germination and seedling growth tests. In addition, the direct effect on plant vigor and shoot development was observed in hydroponic culture tests. The main objective of this work was to create a basis for the rational selection of promising bio-fertilizer candidates for following greenhouse and field experiments.

4.2 Material and Methods

4.2.1 Bio-fertilizer products used in this study

Seven commercial bio-fertilizers based on diverse bacterial strains (*Bacillus* spp., *Pseudomonas* spp.; *Azotobacter chroococcum; Streptomyces* spp.), one fungal strain

Table 4.1: Manufacturers' information on the commercial bio-fertilizers used in the experiments

Product name and manufacturer	**Active compounds**	**Supposed effects on plants**
RhizoVital®42 TB, Abitep GmbH, Berlin, Germany	▪ *Bacillus amyloliquefaciens* strain FZB42; 10^9 cfu g^{-1}	▪ Growth stimulation and enhanced vitality ▪ Improved resistance to biotic and abiotic stresses ▪ Bio-fertilizing effects ▪ Suppression of diseases
Vitalin SP11, Vitalin Pflanzengesundheit GmbH, Ober-Ramstadt, Germany	▪ *Bacillus subtilis*; $8*10^7$ cfu g^{-1} ▪ *Streptomyces* spp.; $1*10^7$ cfu g^{-1} ▪ *Pseudomonas* sp.; $1*10^7$ cfu g^{-1} ▪ Humic acids ▪ Extract from the brown alga (*Ascophyllum nodosum*)	▪ Growth stimulation and enhanced vitality ▪ Improved resistance to biotic and abiotic stresses ▪ Microbial N_2-fixation and phosphate solubilization ▪ Stimulation of beneficial soil microorganisms
Proradix®WG, Sourcon Padena, Tübingen, Germany	▪ *Pseudomonas* sp. *Proradix* strain DSMZ 13134; $5*10^{10}$ cfu g^{-1}	▪ Growth stimulation and enhanced vitality ▪ Improved resistance to biotic and abiotic stresses ▪ Increased availability of mineral nutrients ▪ Competitive suppression of pathogens
Phylazonit MC, Corax-Bioner, Budapest, Hungary	▪ *Bacillus megatherium* varietas *phosphaticum* ▪ *Azotobacter chroococcum*	▪ Growth stimulation ▪ Microbial N_2-fixation ▪ Suppression of pathogens
Phylazonit CE, Corax-Bioner, Budapest, Hungary	▪ *Bacillus megatherium* varietas *phosphaticum* ▪ *Azotobacter chroococcum* ▪ *Bacillus* sp. ▪ *Pseudomonas putida*	▪ Growth stimulation ▪ Microbial N_2-fixation ▪ Suppression of pathogens ▪ Degradation of cellulose ▪ ACC deaminase activity
Vitalin T50, Vitalin Pflanzengesundheit GmbH, Ober-Ramstadt, Germany	▪ *Trichoderma harzianum*; 10^8 spores g^{-1}	▪ Growth stimulation and enhanced vitality ▪ Improved resistance to biotic and abiotic stresses ▪ Suppression of soil borne fungal pathogens
KELPAK, Kelp Products, Ltd., Simon's Town, South Africa	▪ Phytohormones extracted from the brown alga *Ecklonia maxima* - Auxins (11 mg l^{-1}) - Cytokinins (0.031 mg l-1)	▪ Enhanced root development ▪ Improved nutrient utilization ▪ Enhanced resistance to biotic and abiotic stresses

(*Trichoderma harzianum*), and/or non-living compounds (phytohormones, humic acids, etc.) as active ingredients were tested in the following screening assays. More detailed descriptions and supposed effects of these products, according to the manufacturers' specifications, are given in Table 4.1. Unfortunately, for most of these products, aside

from the supposed active compounds, the exact composition of other ingredients, such as carrier materials, is considered as a trade secret and therefore not actually disclosed on the product label.

One of the microbial bio-fertilizers (Vitalin SP11, Vitalin Pflanzengesundheit GmbH, Ober-Ramstadt, Germany) also contained humic acids and an extract from the brown alga *Ascophyllum nodosum* as supportive additives. These organic compounds are supposed to affect plant growth directly and to support the establishment of the microbial inoculants, which are strains of *Bacillus subtilis*, *Streptomyces* spp. and *Pseudomonas* sp., in the rhizosphere. While *Streptomyces* species are rarely contained in commercial bio-fertilizer preparations, they have been reported in the literature to potentially cause a similarly broad range of effects on plant growth like certain strains of *Bacillus* and *Pseudomonas* species (see Chapter 2.3). Belonging to the order *Actinomycetales* within the phylum *Actinobacteria* (*Actinomycetes*), species of *Streptomyces* were originally classified as fungi due to their ability to form highly differentiated mycelium-like filaments (Waksman and Henrici, 1943), but are in fact gram-positive bacteria (Anderson and Wellington, 2001; Ventura et al., 2007). In contrast to the related genus *Frankia* (Brunchorst, 1886; cited in: Becking, 1970), comprising obligate symbiotic bacteria with N_2-fixing capacity living in root nodules of certain non-leguminous plants, most species of *Streptomyces* are saprophytic bacteria that are ubiquitous in soils where they play an important role in the decomposition of organic materials (Goodfellow and Williams, 1983; Ventura et al., 2007). During their life cycle *Streptomyces* spp. form vegetative mycelia in their growth substrate and reproductive aerial mycelia that at maturity develop into chains of spores. The spores are non-motile resting cells, which can be dispersed passively and allow the organism to withstand periods of nutrient depletion, desiccation or extreme temperatures (Williams et al., 1989; Dworkin, 1992; Ensign, 1992). The interaction between plants and diverse species of *Streptomyces* can be deleterious or beneficial for the plants. While some streptomycetes are plant pathogens, such as *Streptomyces scabies* causing common scab in potato tubers (Locci, 1994), others may contribute to the chemical mobilization of phosphorus and/or other sparingly available nutrients (Banerjee et al., 2006; Kannaiyan and Kumar, 2006), or have been recognized as free living nitrogen-fixing bacteria (Ribbe et al., 1997). In agriculture, however, commercial strains of *Streptomyces* are mainly used

to protect plants against soil and air-borne fungal pathogens (e.g. *Rhizoctonia solani*, *Botrytis cinerea*) and insect pests, as they are able to produce a wide variety of antibiotics and/or hydrolytic enzymes (Valois et al., 1996; Wan et al., 2008).

Another product, KELPAK (Kelp Products, Ltd., Simon's Town, South Africa), solely based on phytohormone rich extract from the brown alga *Ecklonia maxima*, was chosen to serve as a kind of positive control to assess the direct effect of phytohormonal compounds on plant growth. Many brown algae (*Phaeophyceae*) are large seaweeds called "kelps" and widely distributed inhabitants of seacoasts around the world (Steinberg, 1989; Khan et al., 2009).

4.2.2 In vitro tests to assess the phosphate solubilizing ability of microbial inoculants

Preparation of the culture media

The capability of microorganisms to solubilize sparingly soluble phosphates is routinely screened by agar plate assays. In such tests, the formation of a halo/clear zone around the microbial colony growing on a solid medium clouded by precipitated phosphates gives a qualitative indication of the phosphate solubilizing ability (Katznelson et al., 1962; Mehta and Nautiyal, 2001). In addition, the calculation of a solubilization index from the ratio of the diameter of the clear zone to the colony diameter can provide a half-quantitative measure of the solubilizing activity (Edi-Premono et al, 1996; cited in: El-Azouni, 2008). In order to detect the influence of the nutritional properties of the growth medium on the phosphate solubilizing activity of microorganisms, in this study a nutrient-poor and a nutrient-rich basis medium were used in combination with different sparingly soluble phosphate forms. As a nutrient-poor basis medium (Table 4.2) served glucose-asparagine agar according to Muromcev (1958; cited in: Deubel, 1996). The nutrient-rich basis medium (Table 4.3) was prepared in modification to Bunt and Rovira (1955) as described by Fankem et al. (2006). In this medium, bromocresol green was optionally used as pH indicator, which at pH values above 6.0 is green but changes its color into yellow within the transition pH range of 3.8 to 5.5, so that a respective acidification of the medium could be recognized visually in addition.

Table 4.2: Nutrient-poor basis medium in adaptation to Muromcev (1958)

To be autoclaved at 121 °C for 20 min:	
K_2SO_4, puriss., p.a. (Riedel de Haën, No. 31270)	0.20 g
$MgSO_4 \cdot 7H_20$, puriss., p.a. (Fluka, No. 63140)	0.40 g
Agar agar (Oxoid, No. LP 0028 purified agar)	20.00 g
Deionized water, sufficient amount to adjust the final volume of the medium to	1000.0 ml
pH adjustment to 6.9 with 1 M NaOH or 1 M HCl	
To be added separately by sterile filtration to the medium cooled to 60 °C after autoclaving:	
D (+) Glucose monohydrate (Merck, No. 9658675), solved in 110.0 ml deionized water	10.00 g
L-Asparagine monohydrate (Fluka, No. 11160), solved in 50.0 ml deionized water	1.00 g

Table 4.3: Nutrient-rich basis medium in adaptation to Fankem et al. (2006)

To be autoclaved at 121 °C for 20 min:	
NaCl, puriss., p.a. (Fluka, No. 71380)	3.00 g
Yeast extract, (Bacto™, No. 212720)	3.00 g
Proteose Peptone No. 3 (Bacto™, No. 211693)	5.00 g
Agar agar (Oxoid, No. LP 0028 purified agar)	20.00 g
Deionized water (DW), sufficient amount to adjust the final volume of the medium to	1000.0 ml
Bromocresol Green (Fluka, No. 17470) 0.5 % in 70 % ethanol, optionally as pH indicator	5.0 ml
pH adjustment to 6.9 with 1 M NaOH or 1 M HCl for Ca- and rock phosphate assays	
pH adjustment to 7.5 with 1 M NaOH or 1 M HCl for Fe- and Al-phosphate assays	

To prepare a homogenous clouding of the agar media by calcium phosphate (**Ca-P**), 2.20 g $CaCl_2 \cdot 2H_2O$ (Merck, No. 2818) and 3.80 g $Na_3PO_4 \cdot 12H_2O$ (Merck, No. 6578) were solved separately in each 20 ml deionized water. After autoclaving at 121 °C for 20 min these components were added to the basal media cooled to 60 °C under permanent shaking in order to form a precipitate of sparingly soluble tricalcium phosphate, according to the equation $3CaCl_2 + 2Na_3PO_4 = Ca_3(PO_4)_2 + 6NaCl$. Similar approaches have been described in the literature (de Freitas et al., 1997; Deubel, 1996). The ability of microbial inoculants to solubilize sparingly soluble rock phosphate (**Rock-P**) was assessed by

adding 2.00 g of such a fine ground (90 % ≤ 0.063 mm) product, containing 13 % P (DC Naturphosphat, Thomasdünger, Untergruppenbach, Germany), to the basal agar media before autoclaving. In order to estimate capacity of microbial cultures to solubilize iron (**Fe-P**) or aluminum phosphate (**Al-P**), only the nutrient-rich basis medium was used by adding either 4.2 g $AlPO_4 \cdot 3H_2O$ (Merck, No. 101098) or 6.7 g $FePO_4 \cdot 4H_2O$ (Sigma-Aldrich, No. 436038), as modified from Fankem et al. (2006).

Because agarose is commonly supposed to have several advantages over agar agar, such as greater purity, higher gel strength, and lower possible charge (Armisén, 1991), it was also tried to use agarose (Roth, No. 2267) as solidifying agent in the media. However, this was not possible in combination with the Ca-P treatment, as the medium did not solidify in this case.

Plating of the microbial inoculants

The following steps were performed under sterile conditions. First, the test media were poured into Petri dishes (94 mm in diameter, 16 mm in height) up to an approximate height of 3 to 4 mm (respectively 45 ml per dish). Before plating on the surface of the solidified media, suitable test portions of the bio-fertilizers were suspended in 1 % proteose peptone solution at ratios according to the manufacturers' instructions. Dilution series were prepared to obtain appropriate inoculum densities of 10 to 50 colony-forming units per plate when spreading an aliquot of 50 µl diluted suspension. This procedure, however, was modified in the case of Vitalin SP11 and Vitalin T50, because earlier attempts had shown that the respective microorganisms did not grow when inoculated in this way. Therefore, 5.0 g of Vitalin SP11 were incubated in 300 ml Erlenmeyer flasks containing 100 ml liquid medium, suitable for the propagation of *Bacillus and Pseudomonas* strains (Table 4.4; DSMZ, 2007a), for 12 hours at 25 °C on a rotary shaker (120 rotations min^{-1}). Although *Streptomyces* spp. can grow on a wide variety of media, they are frequently outgrown by other genera that are more competitive (Dart, 1996). Sterile needles were used to transfer adhering Vitalin T50, which has a powdery formulation, at five equally distributed punctures into the test media. Upon inoculation, the Petri dishes were sealed with laboratory film, to avoid medium dehydration, and incubated at 25 °C in darkness.

Table 4.4: Growth medium suitable for the propagation of Bacillus and Pseudomonas strains according to DSMZ (2007a)

To be autoclaved at 121 °C for 20 min:	
Peptone	5.0 g
Meat extract	3.0 g
$MnSO_4 \cdot H_2O$, recommended for sporulation of *Bacillus* strains	10.0 mg
Deionized water	1000.0 ml
pH adjustment to 7.0	

Evaluation of the in vitro tests

During incubation, the culture plates were regularly monitored with regard to the number, size and visible activities of forming colonies. After a growth period of 14 days, when most of the microbial inoculants had achieved a considerable developmental stage, the experiments were terminated in order to perform destructive measurements. At that time, each four Petri dishes with comparable colony densities, containing the same bio-fertilizer and growth medium, were evaluated as replicates for statistical analysis. First, the number of colonies per agar plate, visible to the naked eye, was recorded. To differentiate between phosphate solubilizing and phosphate non-solubilizing colonies, the formation of a clear zone surrounding the colony was taken as indicator. The diameters of each five colonies and clear zones, if present, were determined with a measuring rod or, in the case of small colonies, by means of a binocular microscope with digital camera and image analysis software (AxioVision, Carl Zeiss AG, Jena, Germany). Changes of the pH-values inside the colonies and clear zones in comparison to the bulk agar were determined with an antimony microelectrode. For this purpose, the electric potential between the microelectrode and a reference electrode was measured with a digital pH/volt-meter. To convert the values of electrical potential differences so obtained into pH values, seven standard buffer solutions ranging from pH 3.0 to 8.0 were used for calibration (Schaller and Fischer, 1981).

4.2.3 Germination tests to determine the direct effect of bio-fertilizers on early plant development and root morphology

Cucumber (*Cucumis sativa* L. var. Vorgebirgstrauben, Württembergische Samenzentrale A. E. Gehrke GmbH, Göppingen, Germany) and spring wheat (*Triticum aestivum* L. var. Paragon, RAGT Seeds Ltd., Cambridge, UK) were chosen as indicator plants to characterize the direct effect of commercial bio-fertilizers, described in Table 4.1, on early plant development and root morphology in filter paper based germination tests.

Preparation of seeds and bio-fertilizer treatments

To avoid contamination with pathogenic or other seed borne microorganisms, cucumber seeds were surface disinfected for 10 min, and wheat seeds for 15 min, in a defined volume (20 ml per 100 seeds) of 3 % hydrogen peroxide. Subsequently the tenfold volume of sterile 10mM calcium sulfate ($CaSO_4$) was added to let the seeds soak for 5 hours at room temperature. In previous trials, also applications of 30 % hydrogen peroxide for 3 min, followed by 70 % ethanol for 2 min, or 1.1 % sodium hypochlorite solution in 5 % ethanol for 10 min had been tested for seed disinfection, while only 2.5 mM $CaSO_4$ had been added to the seeds for soaking. These applications, however, damaged in particular the cumber seeds and diminished the germination rates of both plant species, for which reason they were not applied in further germination tests.

The bio-fertilizers were used as fresh and autoclaved preparations. For treatment with the fresh products, each 3.0 g l^{-1} RhizoVital®, Phylazonit MC, Phylazonit CE, or 0.06 g l^{-1} Proradix® were suspended in sterile 2.5 mM $CaSO_4$ solution. These concentrations of the were charged three times higher than suggested by the manufacturers instruction, because in a previous trial, with the recommended application rates, no clear effects on plant growth could be observed. As control treatments only sterile 2.5 mM $CaSO_4$ solution (Blank), autoclaved (121 °C, 20 min) bio-fertilizer suspensions of respective compositions, and 1 % Kelpak in sterile 2.5 mM $CaSO_4$ were prepared. According to the product label, a 1 % dilution of Kelpak should contain adequate concentrations of cytokinins (0.31 µg l^{-1}) and auxins (110 µg l^{-1}) to optimize root development by phytohormonal effects.

Setup of the germination test

Squares of filter paper (Type 710; Machery-Nagel, Düren, Germany) with an edge length of 58 cm were folded horizontally into stripes of 58 cm length and 20 cm breadth. After soaking the so prepared filter papers with each 60 ml of the above described bio-fertilizer suspensions or control treatments, the surface sterilized seeds were sown. On each filter paper, 15 cucumber or 20 wheat seeds were arranged at regular intervals in line with the long axis of the folded paper, leaving 2.5 cm from its upper edge. Finally, the filter papers stripes were closed by folding them three times vertically and placed in upright position into germination boxes. Thereby, the experiment was arranged following a randomized block design with four replicates. To prevent diffusion and intermixture of the bio-fertilizers the packages of filter paper were separated by plastic bags, taking care to ensure the air exchange (Fig. 4.1). For incubation, the germination boxes were kept in a climate cabinet at 25 °C in the dark. Light was supplied for 12 hours per day after the first shoot tips had appeared at the upper edges of the filter papers.

Fig. 4.1: Germination boxes with cucumber and wheat seedlings seven days after sowing in packages of filter paper.

Evaluation of the germination test

The rate of seedling emergence was recorded daily during the incubation period by counting the number of shoot tips that had appeared on the upper edge of the germination packages. Seven days after sowing, the seedlings had reached an adequate developmental stage to record morphological characteristics of roots and shoots. At that time, maximum lengths of roots and shoots, and the numbers of lateral roots per plant were scored in up to five plants per filter paper. However, in some replicates of cucumber less seeds had germinated. Subsequently, roots and shoots were separated and dried at 65 °C until weight constancy. To determine total root to shoot ratios, dry weights were measured with an analytical balance.

4.2.4 Hydroponic tests to study the direct effect of bio-fertilizers on plant vigor and shoot development

Pre-culture of the test plants

As test plants, seedlings of cucumber (var. Vorgebirgstrauben) and wheat (var. Paragon) were used (see Chapter 4.2.3). To obtain seedlings, seeds were pre-germinated within folded filter paper sheets as described in Chapter 4.2.3, but without bio-fertilizer treatment. As measure of disinfection, cucumber seeds were treated with a 1.1 % solution of sodium hypochlorite in 5 % ethanol for 10 min, whereas wheat (var. Paragon) seeds were immersed in 30 % hydrogen peroxide for 4 min, followed by 70 % ethanol for 2 min. Both kinds of seeds were then rinsed three times and subsequently soaked for two hours in 2.5 mM $CaSO_4$, before sowing. After seven days of pre-culture, uniformly growing seedlings of suitable size were selected for transplanting into the hydroponic system.

Setup of the hydroponic test

Each four cucumber or six wheat seedlings were inserted in round plastic pots (Haugolit-Plastik, Ammerbuch, Germany) filled with 2.5 l standard nutrient solution of the following composition (Mori and Nishizawa, 1987; Yousfi et al., 2007): 2 mM $Ca(NO_3)_2 \cdot 4\ H_2O$, 0.1 mM KH_2PO_4, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.5 mM $MgSO_4$, 20µM Fe-

EDTA ($C_{10}H_{12}FeN_2NaO_3$), 0.5 µM $MnSO_4 \cdot H_2O$, 0.5 µM $ZnSO_4 \cdot 7\ H_2O$, 0.2 µM $CuSO_4 \cdot 5\ H_2O$, and 0.01 µM $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$. Boron, a nutrient required in specifically higher amounts by dicotyledonous than by graminaceous plants (Marschner, 1995), was added as boric acid (H_3BO_3) at adjusted concentrations of 10 µM or 1 µM to pots with cucumber or wheat seedlings, respectively. In addition, a scoopula tip of $CaSO_4 \cdot H_2O$ (about 1.0 g) was supplied per pot to prevent calcium deficiency in young plant tissues of high growth rates and low transpiration intensity (Bangerth, 1979).

The bio-fertilizer treatments consisted of four pots per plant species as replicates and were applied at concentrations of 1.0 g l^{-1} RhizoVital®, 0.02 g l^{-1} Proradix®, 1.0 g l^{-1} Phylazonit MC, 1.0 g l^{-1} Phylazonit CE, or 10 ml l^{-1} Kelpak. For detailed descriptions of the bio-fertilizers see Table 4.1. Untreated control pots were kept without bio-fertilizers. The experiment was conducted in a greenhouse at the Institute of Plant Nutrition, Universität Hohenheim, Stuttgart, Germany, where the pots were positioned on tables in a randomized complete block formation (Fig. 4.2). After six days, the nutrient solutions and bio-fertilizer treatments were renewed.

Fig. 4.2: Hydroponic system with cucumber and wheat plants 18 days after sowing.

As the natural light intensity varied over the experimental growth phase from the 13th June until the 26/27th June 2008, additional light was supplied between 06:00 a.m. and 06:00 p.m. by use of high pressure sodium lamps (SON-T AGRO, 400 watt, Philips, Amsterdam, The Netherlands), during periods of low natural light intensity. The air temperature was maintained by an automatic ventilation system, opening the windows of the greenhouse when the temperature rose above 20 °C. Shading screens were automatically closed with a delay of 10 min whenever the natural illuminance exceeded 60.000 lux. Cucumber plants were cultivated for 13 days and wheat plants for 14 days in the hydroponic system before they were harvested for analytical measurements.

Evaluation of the hydroponic test

On the sixth day after transplanting, it was observed that exceptionally high light irradiation appeared to cause stress symptoms in cucumber plants of some treatments but not in others. At that time, the first true leaf of the cucumber plants underwent expansion and was most severely affected, as it could be later recorded on the basis of necrotic spots in the leaf blade. To quantify the degree of the damage, a visual rating scheme for estimating the percentage of damaged leaf area was used (Moll et al., 2000).

At harvest of the experiment, the effect of different bio-fertilizer treatments on root and shoot characteristics of the seedlings was evaluated by measuring the length of root hairs, maximum and total root length, the elongation of wheat leaves, the length and width of cucumber leaves, as well as the biomass production per plant. Measurements of length were taken on five root hairs of five representative root segments for a total of 25 root hairs per pot, using a binocular microscope with digital camera and image analysis software (AxioVision, Carl Zeiss AG, Jena, Germany). Digital images of individual root systems were obtained with a flatbed scanner and analyzed with the WinRHIZO Pro software (Regent Instruments Inc., Quebec, Canada) for automatic root analysis, to measure the total root length of one cucumber respectively two wheat plants per pot. The specific root length to weight ratio was thereafter calculated by dividing the total root length by the dry weight of the particular root. Further morphological variables were determined throughout all plants of the experiment. In wheat plants, which were uniformly in the three- to four-leaf stage, shoot height was recorded as the leaf length

from the top of the hydroponic pot to the tip of the tallest leaf. The length and width of cucumber leaves was measured to estimate the leaf area of each seedling according the following equation derived from Blum and Dalton (1998): Leaf area = -1.457 + 0.00769 (L * W), where leaf area is calculated in cm^2 and length (L) and width (W) are values in mm. Biomasses of roots and shoots were weighed after drying at 65 °C until weight constancy.

Furthermore, mineral nutrient analyses were performed on shoots of the cucumber plants and the commercial bio-fertilizer materials. The purpose was to check whether an improved nutritional status due to the supply of nutritional elements contained in the biofertilizers could be responsible for growth stimulation or an enhanced light stress tolerance of the plants. Before elemental analysis, dried plant samples were ground to finest powder in an agate disc swing mill. To be digested in a wet ashing procedure in modification to Fridlund et al. (1994), subsamples of plant material (150 mg) or appropriate portions of the bio-fertilizer materials (between 0.075 and 1.5 g in three replicates) were placed in polytetrafluoroethylene (PTFE) crucibles. After adding the digestion mixture, consisting of 3 ml HNO_3 (65 %) and 2 ml H_2O_2 (30 %), to the samples, the crucibles were firmly closed and placed into a high performance microwave unit (MLS 1200 MEGA, Microwave Laboratory Systems GmbH, Leutkirch, Germany) for digestion. The samples were cooled to room temperature, before the PTFE crucibles were opened again. Immediately after rinsing the samples solutions with 15 ml supra-pure water (ELGA LabWater, Celle, Germany) into 25 ml volumetric flaks of plastic, 1 ml concentrated hydrofluoric acid (HF) was added to completely digest the samples and inhibit the precipitation of silicon. Glassware was not used to avoid contamination of the samples with silicon. The samples were allowed to react with the HF over night (12-14 h), before 2.5 ml boric acid were added to neutralize remaining HF, because HF represents a potential health hazard and could corrode glass pieces on analytical instruments. At last, the samples were brought up to a final volume of 25 ml with supra-pure water. The analysis of elemental (P, K, Mg, Ca, Fe, Mn, Zn, Cu, Si) concentrations in the sample solutions was done through use of inductively coupled plasma optical emission spectrometry (ICP-OES) at the Landesanstalt für Landwirtschaftliche Chemie, Universität Hohenheim, Stuttgart, Germany.

4.2.5 Statistical analyses of the screening tests

Statistical calculations were done using the computer program SigmaStat Version 2.03 (Systat Software Inc., Erkrath, Germany). Data are presented as means with their standard errors. If the respective data sets failed the normality or equal variance test, the diameters of microbial colonies grown on agar media and mineral nutrient concentrations in plant tissues were square root transformed. Analyses of variance (ANOVAs; significance level $p \leq 0.05$), followed by Tukey's or Dunnett's multiple comparison test, were performed to compare results between different treatments, as described in figure and table footnotes. Tukey's test compares all pairs of treatment groups, whereas the Dunnett's test compares one control group to all other treatments for significant difference between means. As a measure of the strength of the association between two variables (e.g. leaf area and biomass production of plants), the Pearson's product-moment correlation coefficient (Pearson's r) was calculated. Positive correlation coefficients indicate that pairs of variables increase together, whereas negative values indicate an inverse relationship. For pairs with p values below 0.05 there is a significant relationship between the two variables.

4.3 Results

4.3.1 Results of the *in vitro* tests to assess the phosphate solubilizing ability of microbial inoculants

Microbial isolates contained in six commercial bio-fertilizers, that were tested on a nutrient-poor and a nutrient-rich basis agar medium supplied with different sparingly soluble phosphate forms (Ca-P, Rock-P, Fe-P, Al-P), showed clear distinctions in their growth rates and phosphate solubilizing capacity (Fig. 4.3, 4.5 - 4.7). A general observation was that the performance of the microbial inoculants was stronger affected by the composition of the basis media than by the form of sparingly soluble phosphate added. The growth of the bacterial inoculants was broadly suppressed on the nutrient-poor medium, while larger colonies were formed on the nutrient rich medium (Fig. 4.3a-d).

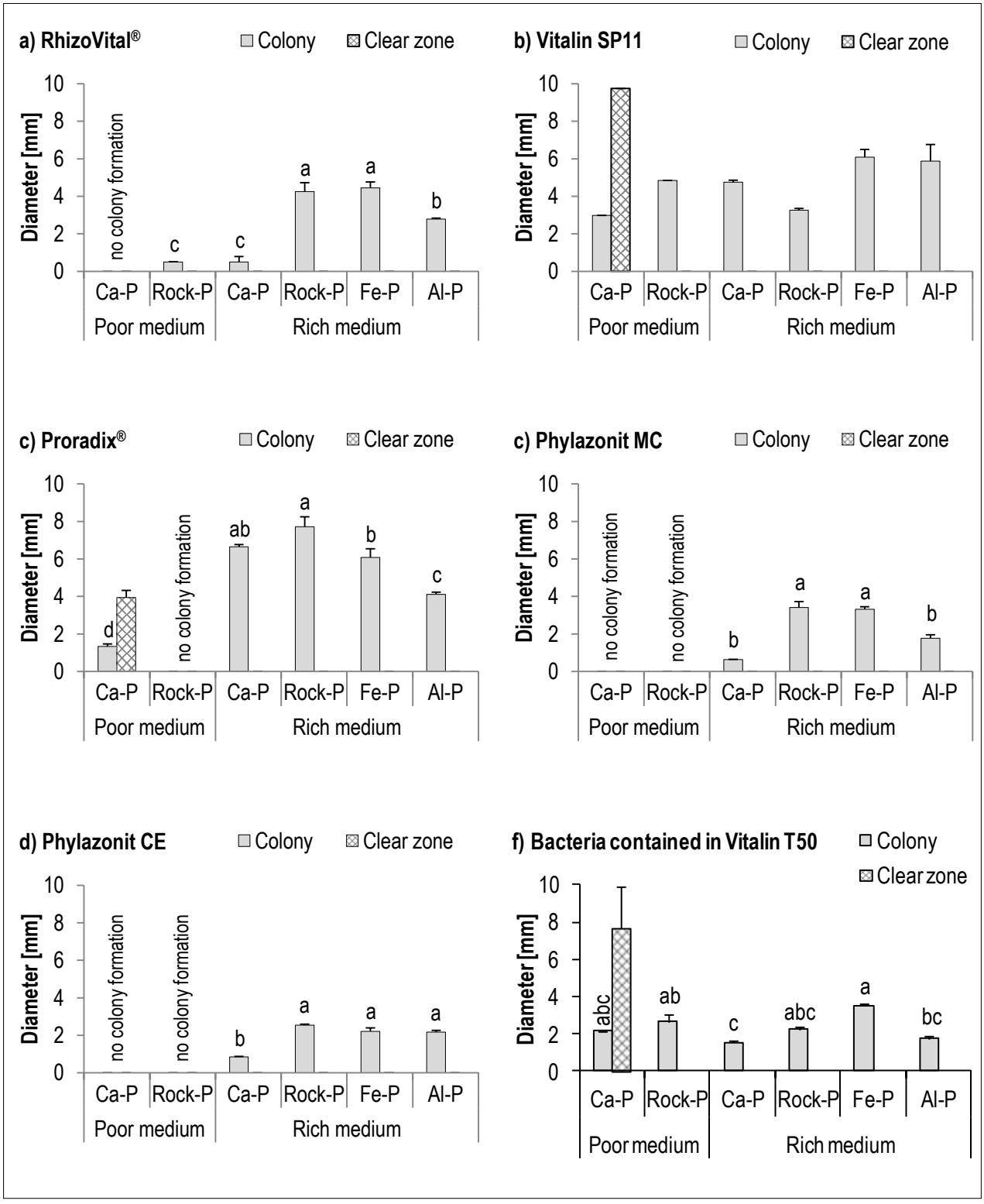

Fig. 4.3: Diameters of colonies and surrounding clear zones as indicators for the growth performance and phosphate solubilizing activity of diverse microbial inoculants (a-f) cultivated for 14 days on a poor or rich basal medium supplied with different sparingly soluble phosphate forms (Ca-P, Rock-P, Fe-P, or Al-P) in Petri dishes.

Columns represent mean values ± SEM (n = 4). Values not sharing common letters are significantly different [Tukey's test, $p \leq 0.05$], whereas those without or sharing letters are not significantly different.

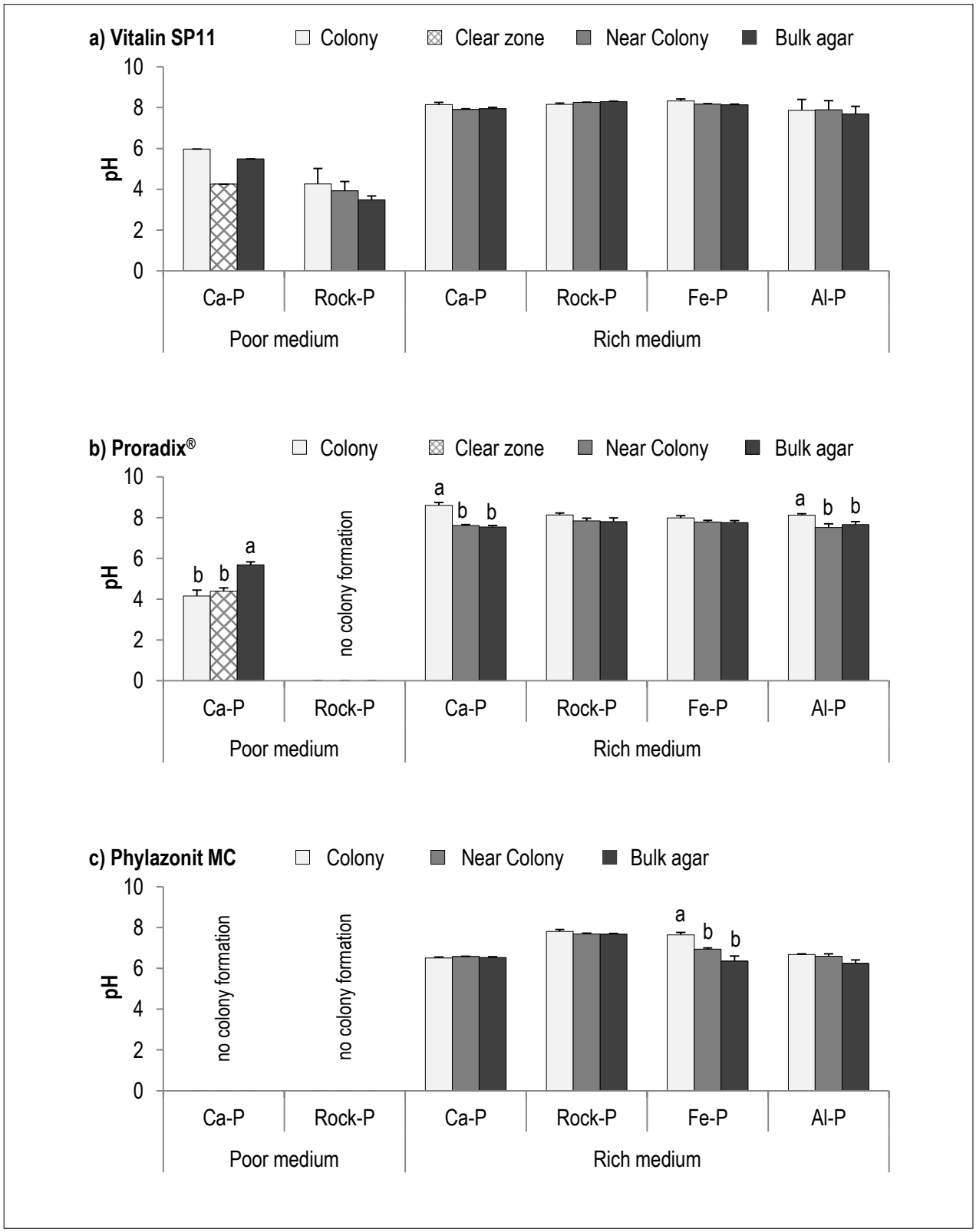

Fig. 4.4: pH values in colonies and their near (3 mm) surrounding (clear zone or near colony) formed by diverse microbial inoculants (a-f) compared to the pH-values far from colonies (bulk agar) after 14 days of cultivation on a poor or rich basal medium supplied with different phosphate forms (Ca-P, Rock-P, Fe-P, or Al-P) in Petri dishes.

Columns represent mean values ± SEM (n = 4). Values of the same treatment group not sharing common letters are significantly different [Tukey's test, $p \leq 0.05$], those without or sharing letters not.

Clear zones of phosphate solubilization, however, were only formed on the nutrient-poor but not on the rich medium supplied with Ca-P, after inoculation with Vitalin SP11 (Fig. 4.3b; 4,5a), Proradix® (Fig. 4.3c; 4.6a), and Vitalin T50 (4.3f; 4.7a).

Much varied growth responses were shown between bacterial species from RhizoVital® and Vitalin SP11. The strain of *Bacillus amyloliquefaciens* contained in RhizoVital® was obviously not able to utilize Ca-P regarding its absent or low growth on both media containing this phosphate form (Fig. 4.3a). Bacterial organisms contained in Vitalin SP11, by contrast, formed remarkable colonies and wide clear zones on the nutrient-poor medium with Ca-P (Fig 4.3b). This effect was further accompanied by a considerable pH decrease in the clear zones compared to the bulk agar (Fig. 4.4a). But also on the nutrient-poor medium with Rock-P as well as on the rich medium with Ca-P, where no clear zones or pH-effects were detectable, the bacteria contained in Vitalin SP11 did not show such growth depressions as the strain of *B. amyloliquefaciens* did (Fig. 4.3a,b; Fig. 4.4a).

More similarities were found comparing the growth performance of the bacterial organisms derived from Vitalin SP11 (Fig. 4.3b; Fig. 4.5a,b) to that of the pseudomonads contained in Proradix® (Fig. 4.3c; Fig. 4.6a,b).

Inoculation of the nutrient-poor medium with Proradix®, containing a strain of fluorescent *Pseudomonas* spp., resulted in the formation of relatively small colonies (< 1.6 mm) in the treatment with Ca-P and no colony growth on the same medium with Rock-P. Interestingly, only the small colonies formed on the poor medium induced clear zones of phosphate solubilization and significant pH decreases in their surroundings, whereas the significantly larger colonies formed on the nutrient-rich medium did not show such effect (Fig. 4.3c; Fig. 4.4b; 4.6a,b). It was further observed that the large colonies growing on the nutrient-rich medium only produced yellow-green pigments (pyoverdines), which fluoresced under ultraviolet (UV) light (366 nm), when treated with Ca-P or Al-P, but not when supplied with Rock-P or Fe-P (representative pictures are shown in Fig. 4.4a-d). This indicates, that the pseudomonads contained in Proradix® were able to mobilize iron, and therefore probably also phosphate, contained in the rock respectively iron phosphate, as the production of pyoverdines to function as siderophores is a typical reaction of *Pseudomonas* spp. to low iron availability (Visca et al., 2007).

The microorganisms contained in Phylazonit MC and Phylazonit CE did not appear to grow on the nutrient-poor medium at all, but only on the rich medium where they formed comparatively small colonies (Fig. 4.3d,e). Both inoculants showed significantly lower growth performance in response to the Ca-P treatment as compared to Rock-P or Fe-P application. For the bacterial isolates of Phylazonit MC, also Al-P revealed to be a less suitable substrate. Nevertheless, each Phylazonit preparation consisted of a mixture of different bacterial species, which varied remarkably in their specific properties. Thus, it was recognized by observation under the binocular microscope that beside from the colonies that were visible to the naked eye, many transparent colonies in the order of magnitude of less than 100 µm had formed on the nutrient-rich medium. Furthermore, the comparison of different inoculum densities showed a predominance of fast growing species on agar plates containing many easily visible colonies, whereas on agar plates with few colonies different species, that were obviously less competitive, were able to develop noticeable colonies. Examples are shown in Fig. 4.5c and d. An important finding in this regard was that a certain species only induced strong pH decreases on the rich medium with Al-P when grown under conditions of low inoculum density (Fig. 4.5d). The species that were predominant on densely colonized agar plates, by contrast, did not show such effect, as documented in Fig. 4.4c).

The highest index for Ca-P solubilization on the poor medium, expressing the size of clear zones in relation to that of microbial colonies, could be estimated for Vitalin T50 (Fig. 4.3f). Yet, the exact determination of this value seemed to be questionable. Agar plates containing Ca-P or Rock-P were completely covered by the *Trichoderma*-fungus within few days after inoculation with Vitalin T50. The fungal mycelium itself, however, did not show any visible phosphate solubilizing activity. The clear zones found after inoculation with Vitalin T50, were rather attributable to the activity of bacterial organisms forming distinct colonies on the same agar plates (Fig. 4.7a). The provenience of these bacteria is not known, although they emerged consistently. According to the manufactures specifications, Vitalin T50 should only contain spores of *Trichoderma harzianum* as microbial ingredients. Nevertheless, an intrusion of microbial contaminants during the experimental procedure can be reasonably excluded, because the same observation had

been made in independent earlier trials. Moreover, no such colonies were found in Petri dishes inoculated with different bio-fertilizers as well as in non-inoculated controls.

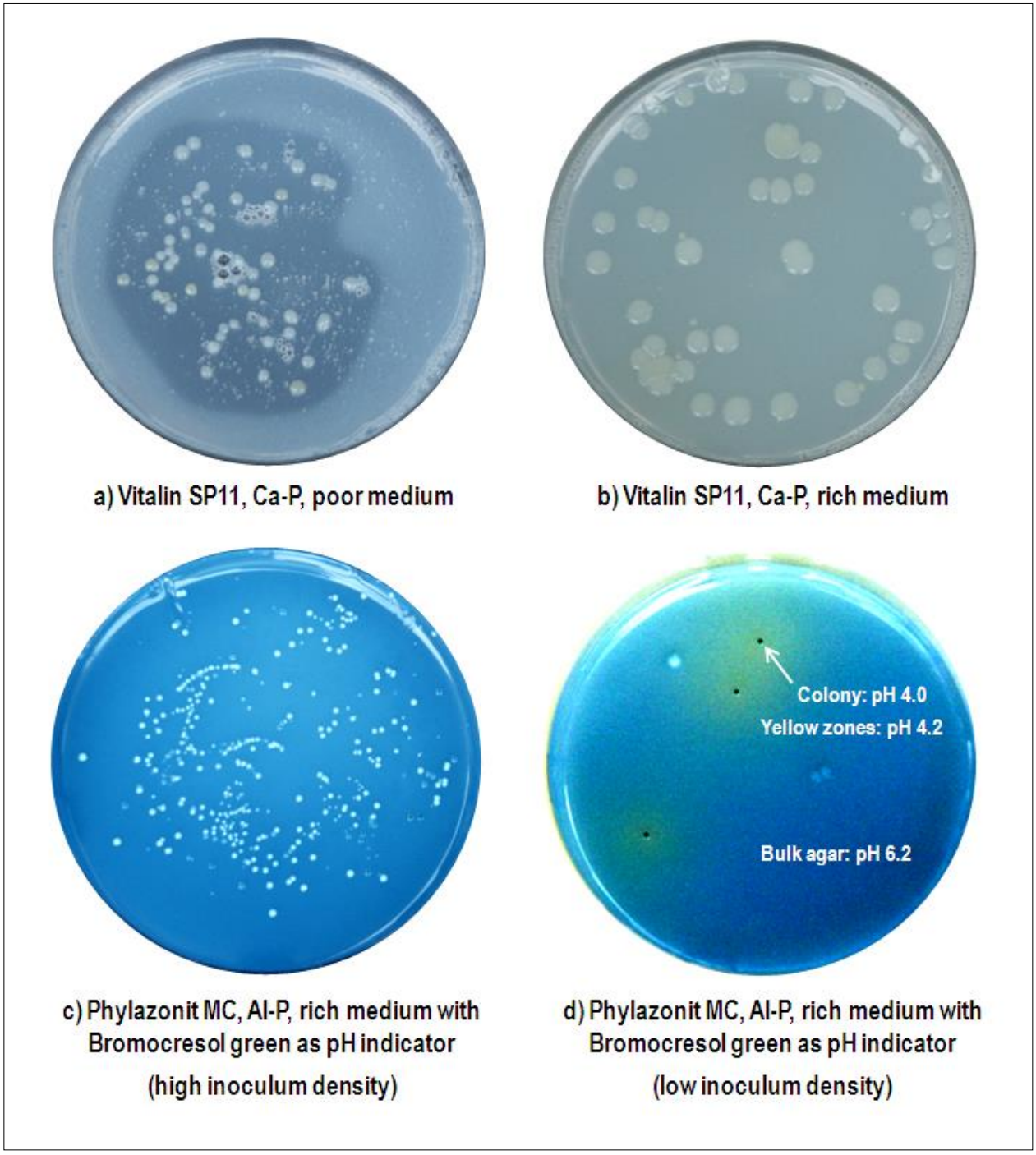

Fig. 4.5: Colonies formed by microbial inoculants cultivated for 14 days on a poor or rich basal medium supplied with different sparingly soluble phosphate forms (Ca-P, Al-P) in Petri dishes. Bacterial organisms contained in Vitalin SP11 formed clear zones on the poor (a) but not on the rich-medium with Ca-P (b). A bacterial species of Phylazonit MC cultivated on the rich-medium with Al-P induced strong pH decreases under conditions of low (d) but not of high (c) inoculum density.

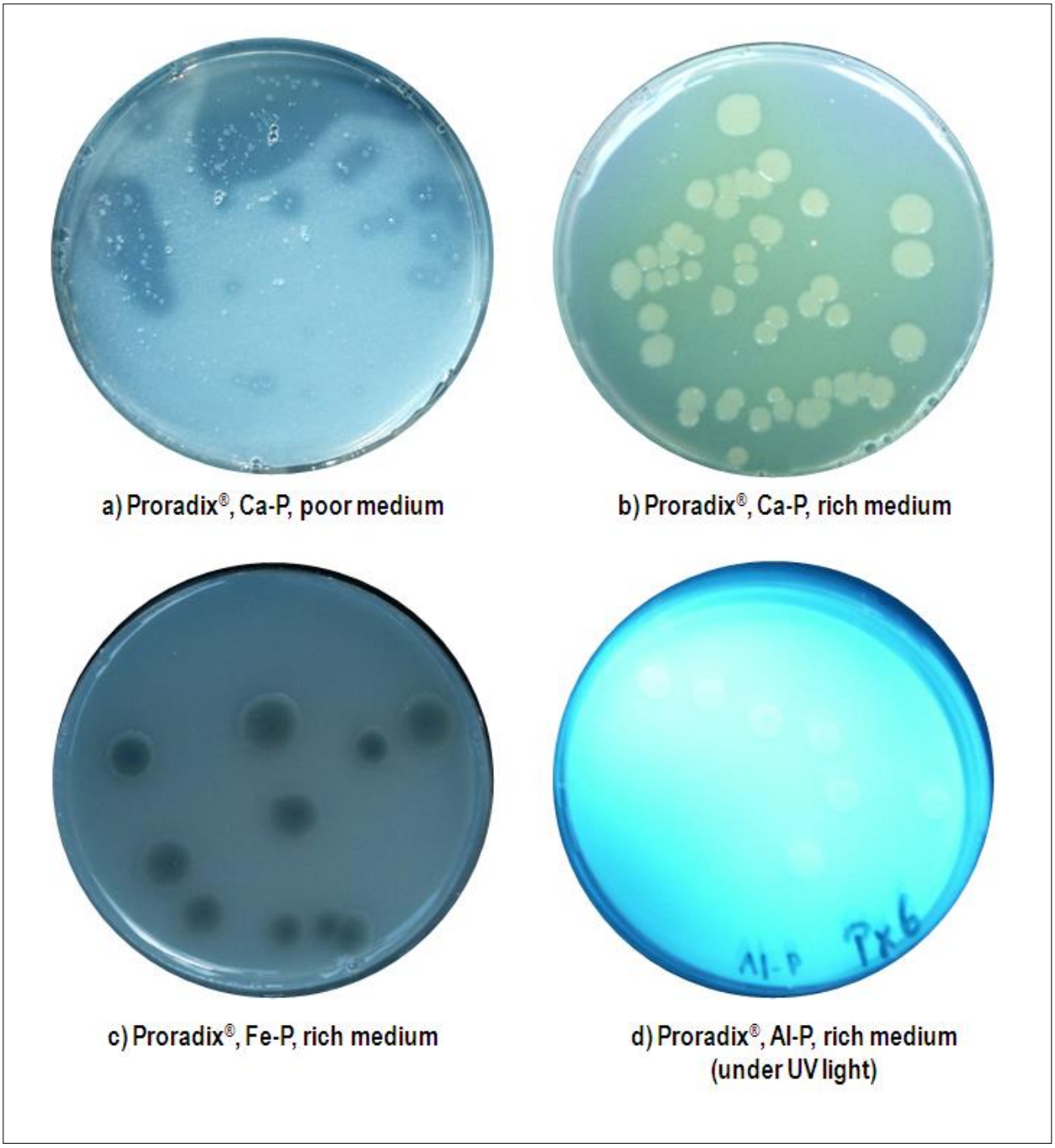

Fig. 4.6: Colonies formed by microbial inoculants cultivated for 14 days on a poor or rich basal medium supplied with different sparingly soluble phosphate forms (Ca-P, Fe-P, Al-P) in Petri dishes. The Pseudomonas strain of Proradix® formed clear zones on the poor (a) but not on the rich-medium with Ca-P where it released abundant amounts of yellow-green pyoverdines into the medium (b). Pyoverdines were also produced on rich medium with Al-P, as visualized by fluorescence under UV light (d), but not when the Pseudomonas strain was supplied with Fe-P (c), which indicates a mobilization of iron contained in the iron phosphate.

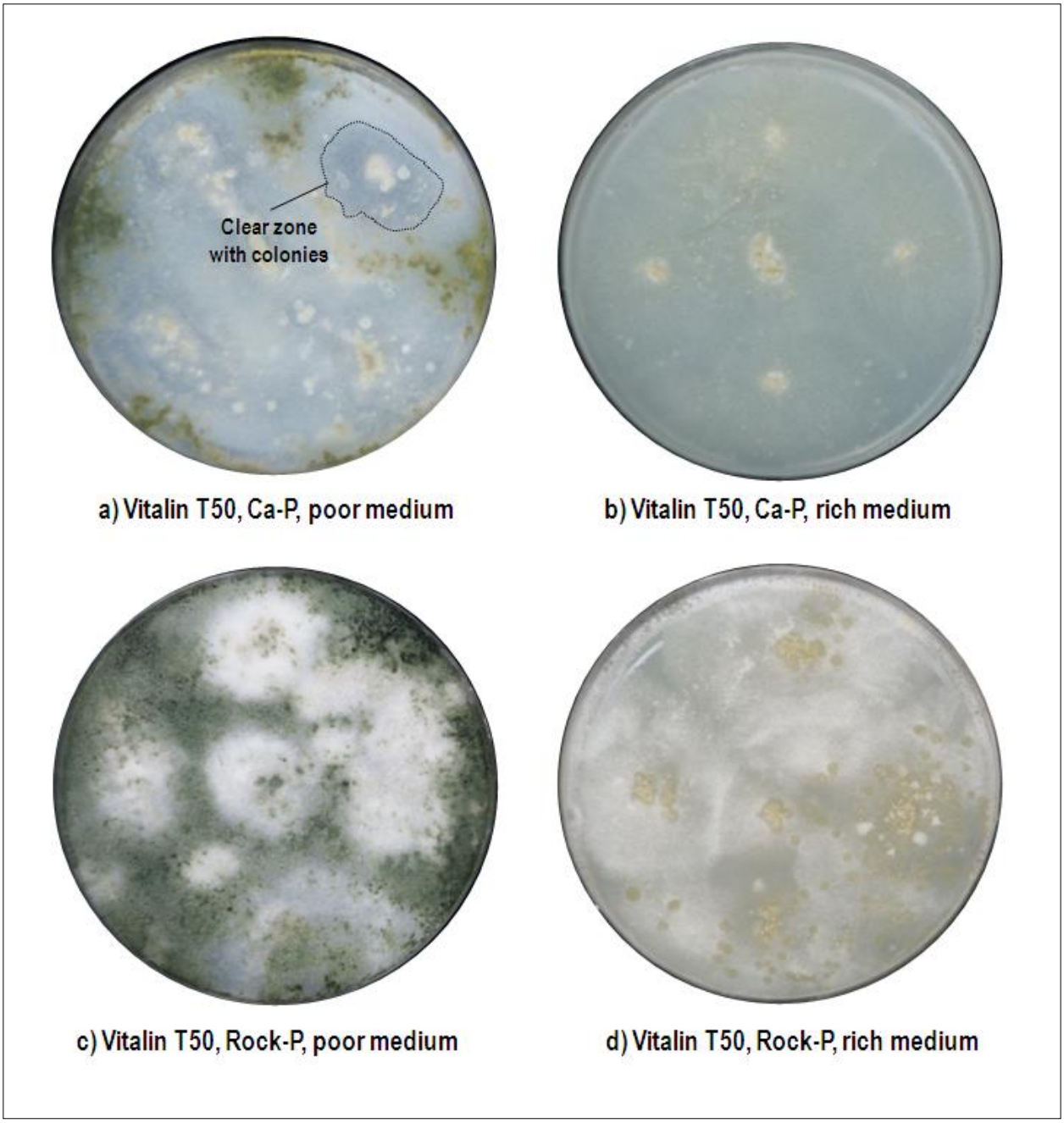

Fig. 4.7: Growth of Trichoderma harzianum and bacterial organisms 14 days after inoculation of Vitalin T50 on a poor or rich basal medium supplied with different sparingly soluble phosphate forms (Ca-P, Rock-P) in Petri dishes. The agar plates were completely covered by the Trichoderma strain (a-d). Green conidia were formed on the poor medium (a,c), in particular when supplied with rock phosphate (c), but not on the rich medium (b,d). The formation of clear zones of phosphate solubilization on the poor medium with Ca-P was rather attributable to the activity of bacterial colonies than to the influence of the fungal mycelium (a).

Contrary to other microorganisms tested in the present screening assays, the growth of the *T. harzianum* strain was visibly enhanced on the nutrient-poor compared to the rich medium. Abundant amounts of white mycelium and green conidia were in particular produced when Vitalin T50 was inoculated on the poor medium supplemented with rock phosphate, which indicated the preferential utilization of this phosphate form by the *Trichoderma* fungus (Fig. 4.7a-d). Iron and aluminum phosphate, by contrast, seemed to be less suitable substrates for the *Trichoderma* strain, as its mycelial growth was restricted to small patches around the inoculation sites (pictures and data not shown). The average diameter of these patches formed on the nutrient-poor medium with Al-P (13 mm) was significantly (Student's t-test for unpaired samples, $p \leq 0.001$) lower than on the same medium with Fe-P (23 mm).

4.3.2 Results of the germination tests to determine the direct effect of bio-fertilizers on early plant development and root morphology

Seeds of cucumber and wheat, germinated in filter papers soaked with diverse commercial bio-fertilizers, showed few differences between treatments. The statistical evaluation of the rates of seedling emergency (Fig. 4.8) did not indicate distinct effects of the bio-fertilizer applications, rather fresh nor autoclaved, when compared to the untreated control (Blank).

However, there were some significant differences noted by pair wise comparisons between certain bio-fertilizer treatments. Thus, five days after sowing cucumber seedlings treated with Phylazonit CE had emerged at a significantly higher rate than seedlings treated with Phylazonit MC (Fig. 4.8a). The fastest development of wheat seeds was induced by Kelpak, when compared to the treatment with the lowest of rate of seedling emergency, autoclaved RhizoVital®, on the third day after sowing (Fig. 4.8b). No significant effects on the rates of seedling emergency were found on other days of the incubation phase (Tukey's test, $p > 0.05$; data not shown). At the end of the germination test, seven days after sowing, more than 80 % of the wheat seeds had emerged on the top of the filter paper packages throughout all treatments. In cucumber, the germination rates were generally lower and only about 34 % of the seeds had emerged at the end of the test.

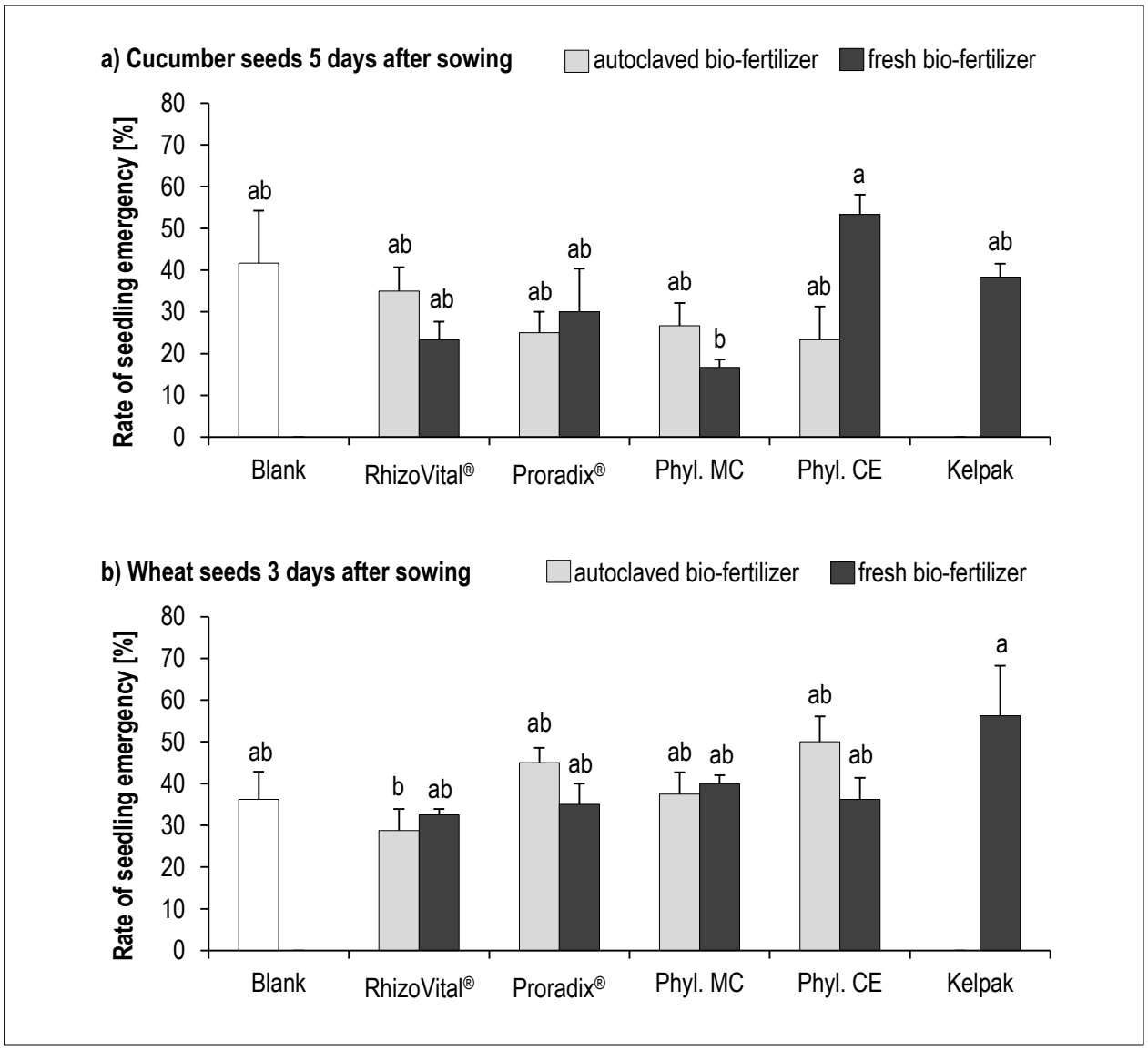

Fig. 4.8: Germination rate of cucumber (a) and wheat (b) seedlings sown on filter paper soaked with different fresh or autoclaved bio-fertilizer preparations (Phyl. = Phylazonit as MC or CE product).

Columns represent mean values ± SEM (n = 4). Values not sharing common letters are significantly different [Tukey's test, $p \leq 0.05$].

Although, certain bio-fertilizers, such as Phylazonit CE and Kelpak in particular, seemed to induce strong morphological alterations in some cases, the variation between individual plants was very high, as can be seen from the pictures shown in Fig. 4.9.

The comparison of average values by statistical methods showed no significant differences between bio-fertilizer treated seeds and the untreated Blank group with respect to the number of lateral or crown roots per plant (Fig. 4.10), the maximum shoot and root length (Fig. 4.11), and the partitioning of biomass between shoots and roots (Fig. 4.12.).

Although, bio-fertilizer treated wheat seedlings generally seemed to form a higher number of crown roots when compared to the untreated control (Blank), this appearance should not be over interpreted because the same trend was observable in response to the application of the autoclaved bio-fertilizer preparations (Fig. 4.10b).

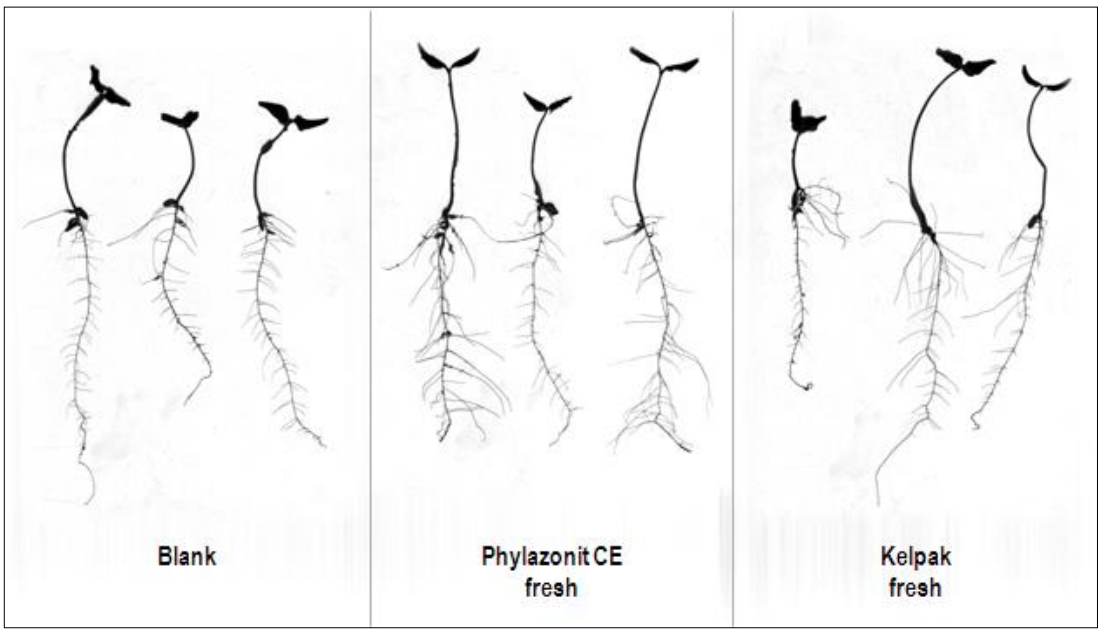

Fig. 4.9: Selected cucumber seedling germinated on filter paper that was soaked with Phylazonit CE or Kelpak appeared to have formed particularly long lateral roots. But, there was a huge variation between single plants.

A lower number of lateral roots in cucumber plants (Fig 4.10a) after use of the autoclaved Proradix® preparation, however, was probably caused by the formation of growth inhibitory compounds during the autoclaving process. In addition, the shoot elongation of wheat seedlings that had been treated with the autoclaved Proradix® preparation was less pronounced compared to other treatments (Fig. 4.11b).

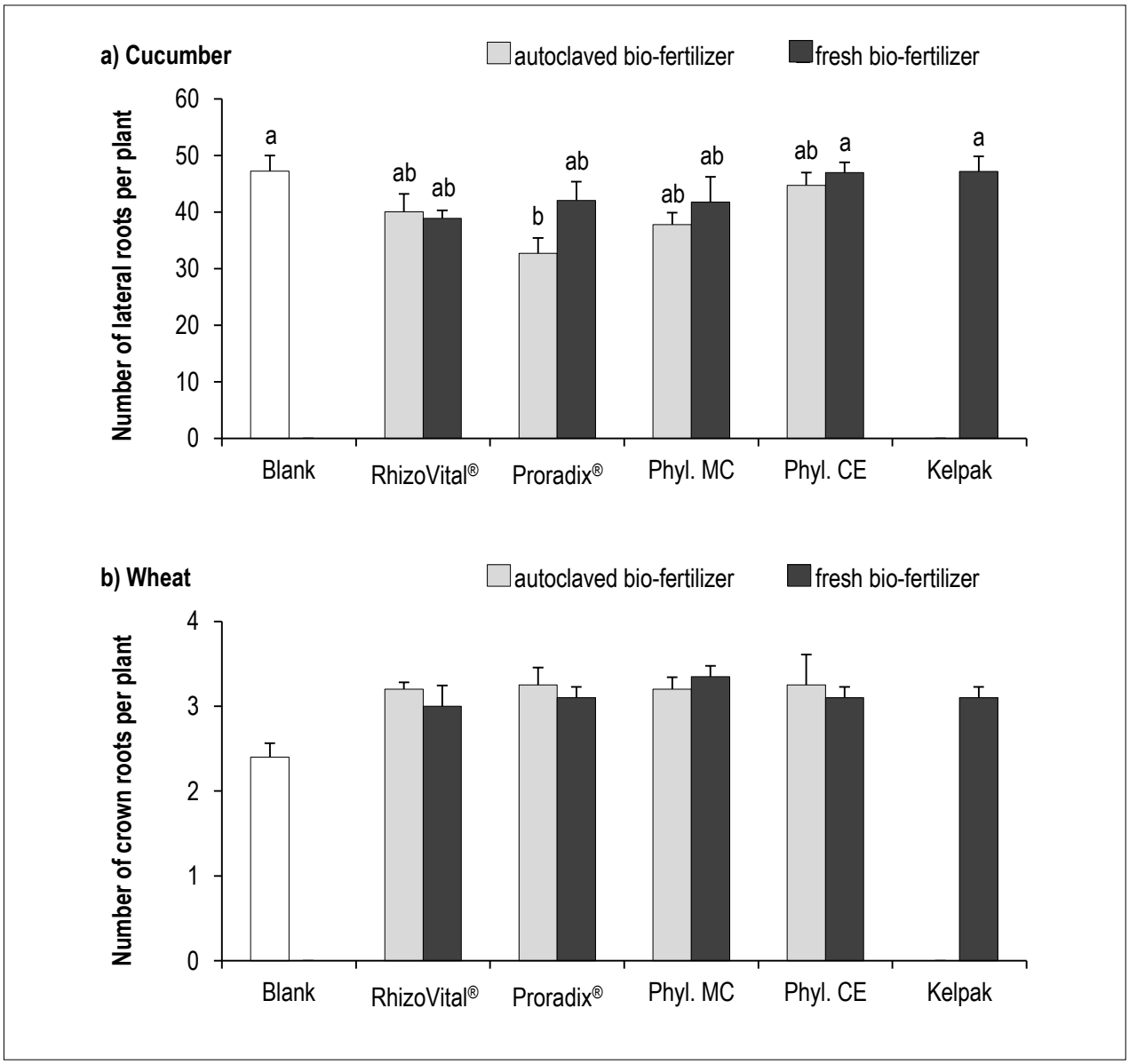

Fig. 4.10: Number of lateral roots, respectively crown roots, formed by cucumber (a) or wheat seedlings (b) seven days after sowing on filter paper soaked with different fresh or autoclaved bio-fertilizer preparations (Phyl. = Phylazonit as MC or CE product).

Columns represent mean values ± SEM (n = 4). Values not sharing common letters are significantly different [Tukey's test, $p \leq 0.05$], those without or sharing letters are not significantly different.

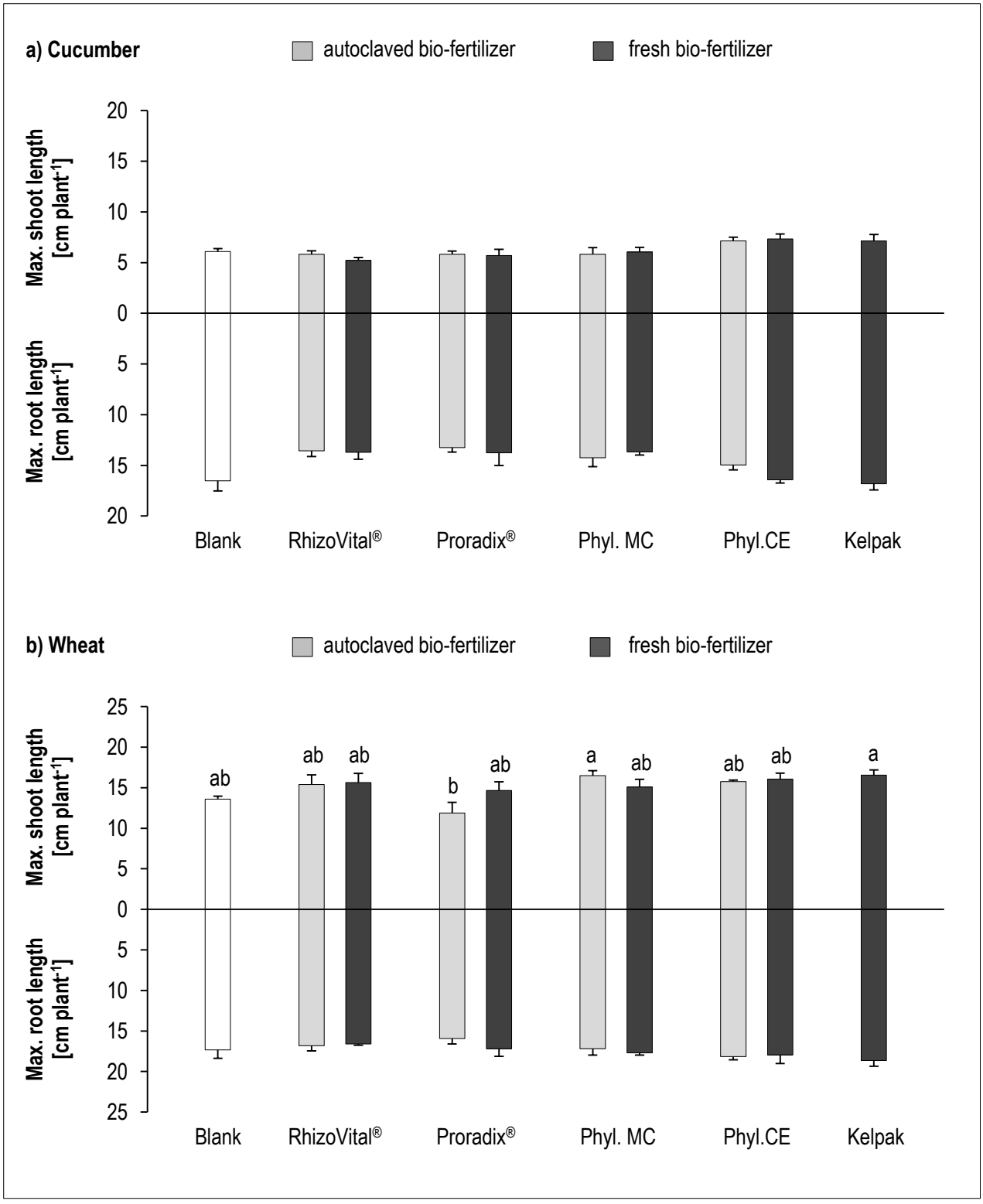

Fig. 4.11: Maximum root and shoot length of cucumber (a) and wheat (b) seedlings seven days after sowing on filter paper soaked with different fresh or autoclaved bio-fertilizer preparations (Phyl. = Phylazonit as MC or CE product).

Columns represent mean values ± SEM (n = 4). Values on the same side of the vertical axis not sharing common letters are significantly different [Tukey's test, $p \leq 0.05$], whereas those without or sharing letters are not significantly different.

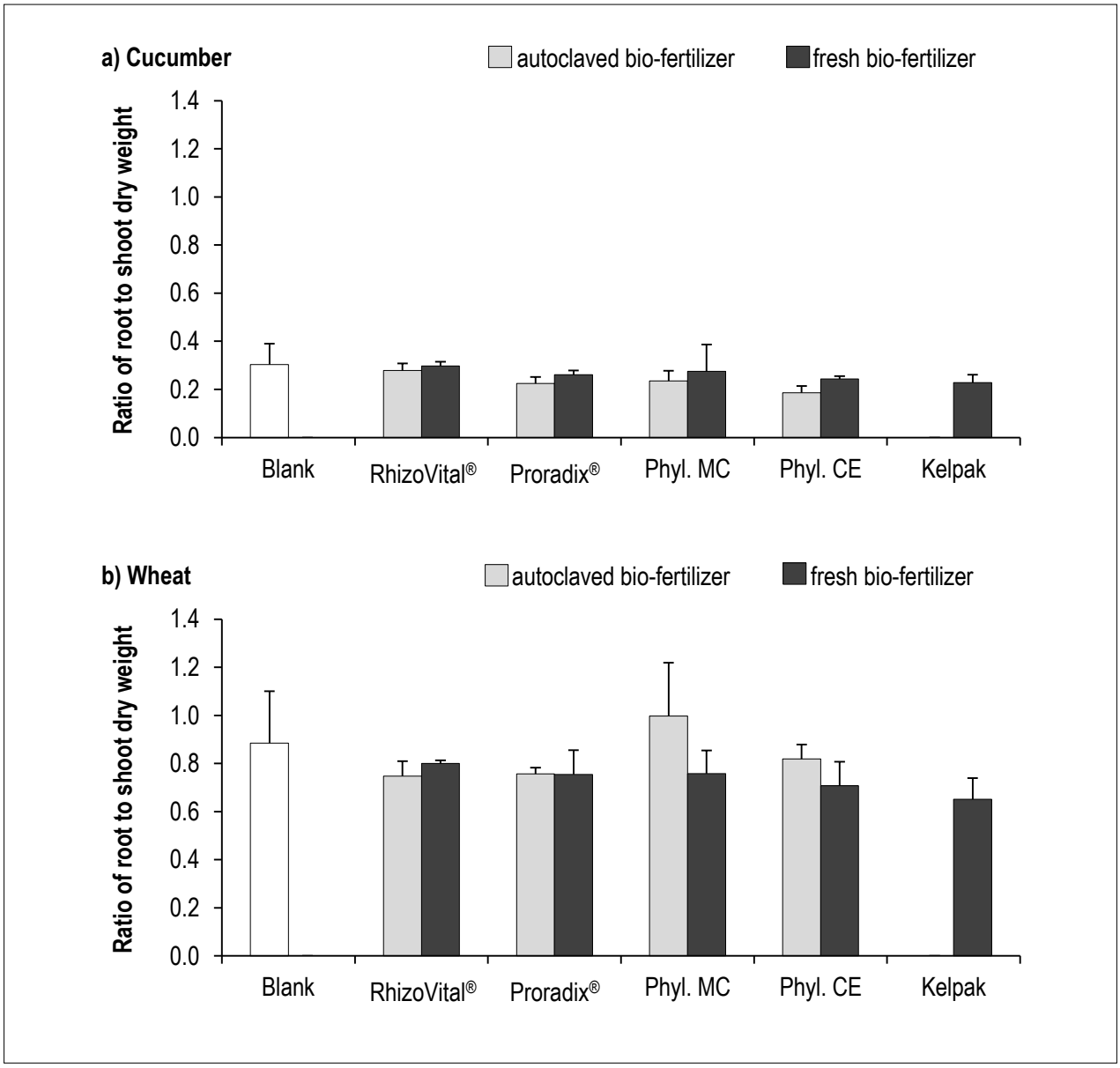

Fig. 4.12: Ratio of root to shoot dry weight (root to shoot ratio) of cucumber (a) and wheat (b) seedlings seven day after sowing on filter paper soaked with different fresh or autoclaved bio-fertilizer preparations (Phyl. = Phylazonit as MC or CE product).

Columns represent mean values ± SEM (n = 4). Values are not significantly different [Tukey's test, $p > 0.05$].

In both, wheat and cucumber seedlings, the highest root to shoot ratios were found in the untreated control (Blank), whereas the application of fresh bio-fertilizers was consistently accompanied by a lower partitioning of the total biomass to the roots (Fig. 4.12). Even though this trend was not significant, too (Tukey's test, $p \leq 0.05$), it can be stated that none of the tested bio-fertilizer products showed clear stimulatory effects on the root

growth of cucumber and wheat seedlings in the present filter paper based germination test.

4.3.3 Results of the hydroponic tests to study the direct effect of bio-fertilizers on plant vigor and shoot development

Seedlings of cucumber and wheat were cultivated in hydroponic systems supplemented with different commercial bio-fertilizers. The growth response, morphological characteristics of roots and shoots, and indices for the susceptibility of the seedlings towards abiotic stress factors (i.e. high light intensity) were assessed 13 to 14 days after transplanting of seven days old seedlings to the hydroponic system. In contrast to the results of the germination test, described in the previous Chapter 4.3.2, the seedlings of both plant species showed clear responses to some of the bio-fertilizer treatments. As shown in Fig. 4.13, the application of the phytohormone based product, Kelpak, had a strong effect of increasing the length of root hairs in cucumber by about 75 % compared to other bio-fertilizer treatments and the untreated control (Blank).

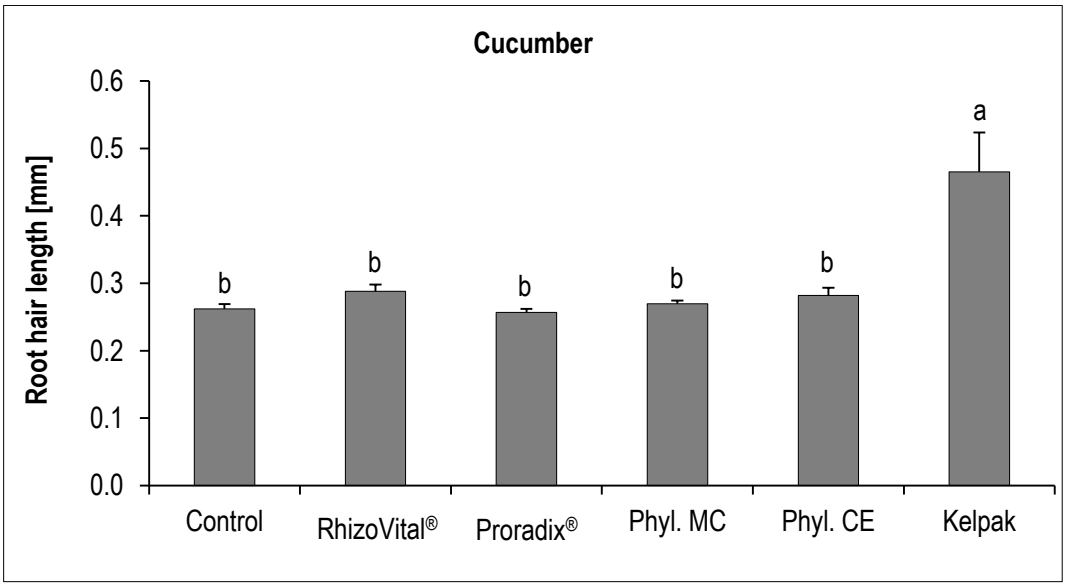

Fig. 4.13: Root hair length of cucumber seedlings 13 days after transplanting into the hydroponic system supplied with different commercial bio-fertilizer preparations (Phyl. = Phylazonit as MC or CE product).

Columns represent mean values ± SEM (n = 4). Values not sharing common letters are significantly different [Tukey's test, $p \leq 0.05$].

The root hairs of untreated wheat plants (0.46 mm) were generally longer than those of untreated cucumber plants (0.26 mm) and the use of Kelpak did not induce an additional increase here. By contrast, the inoculation with Phylazonit CE decreased the root hair length of wheat significantly. An effect that was by trend also observable in response to the Phylazonit MC treatment (Fig. 4.14).

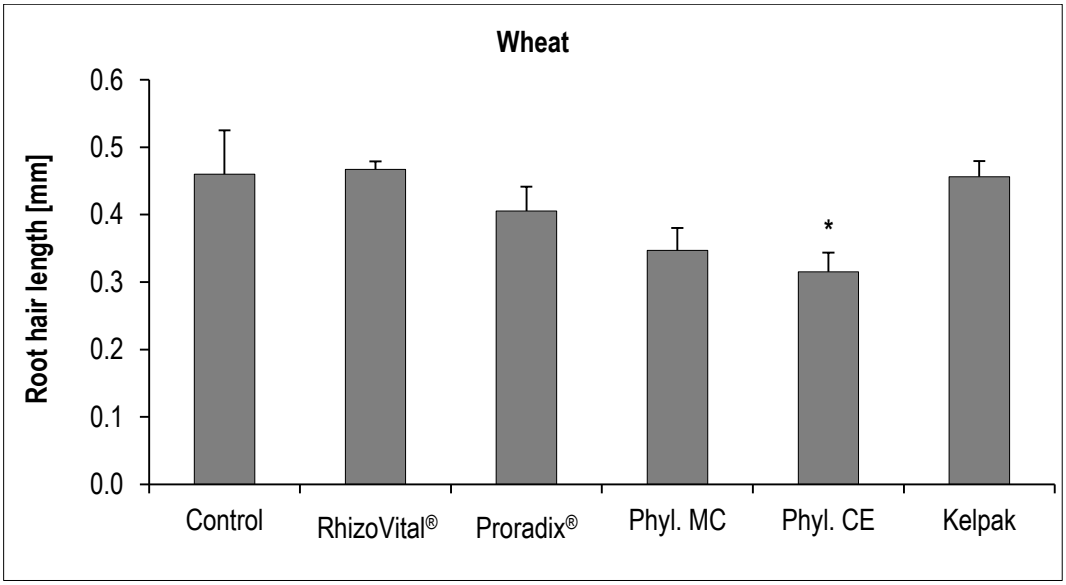

Fig. 4.14: Root hair length of wheat seedlings 14 days after transplanting into the hydroponic system supplied with different commercial bio-fertilizer preparations (Phyl. = Phylazonit as MC or CE product).

Columns represent mean values ± SEM (n = 4). Values with asterisks are significantly different from the untreated control [Dunnett's test, $^{*}p \leq 0.05$]. No significant differences were indicated by Tukey's test ($p > 0.05$).

Maximum root lengths, measured in wheat and cucumber plants, were highest after treatment with RhizoVital® and lowest after treatment with Proradix®. Although trends were similar in both plants, the difference between these two treatments was only significant in wheat as shown in the lower side of Fig. 4.15, while the respective insignificant data obtained from cucumber plants are not presented here. The maximum root length of Proradix® treated wheat plants was furthermore significantly lower than that of the untreated control plants. In addition, the upper side of Fig. 4.15 compares the influence of different bio-fertilizer treatments on the shoot height of wheat. As with roots, the tallest shoots were obtained by treatment with RhizoVital®, but the lowest shoot height was recorded in plants supplemented with Kelpak.

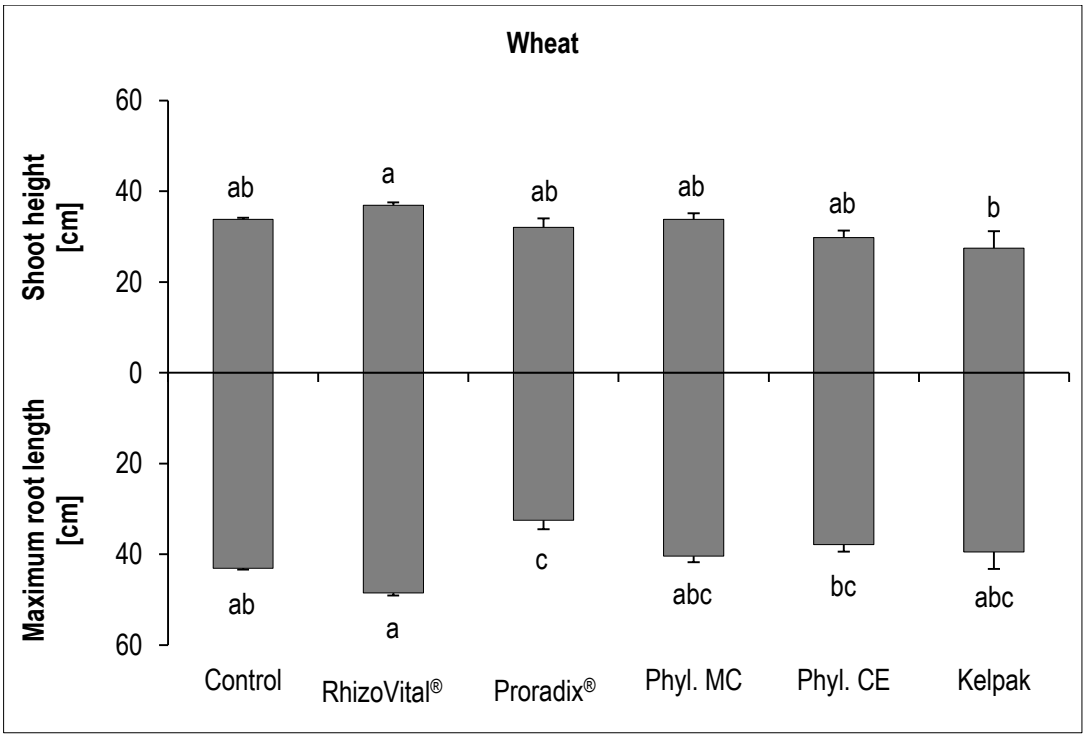

Fig. 4.15: Maximum root length and shoot height of wheat seedlings 14 days after transplanting into the hydroponic system supplied with different commercial bio-fertilizer preparations (Phyl. = Phylazonit as MC or CE product).

Columns represent mean values ± SEM (n = 4). Values on the same side of the vertical axis not sharing common letters are significantly different [Tukey's test, $p \leq 0.05$].

Cucumber plants showed differences between treatments in the specific root length to weight ratio. A significantly higher root length per unit of dry matter investment was found in response to the application of Phylazonit CE when compared to RhizoVital® but not to other bio-fertilizer treatments and the untreated control (Fig. 4.16). The specific length of wheat roots (about 200 m g^{-1} dry matter) was generally lower than that of cucumber roots (290 to 400 m g^{-1} dry matter), but was not significantly affected by the bio-fertilizer treatments (data not shown).

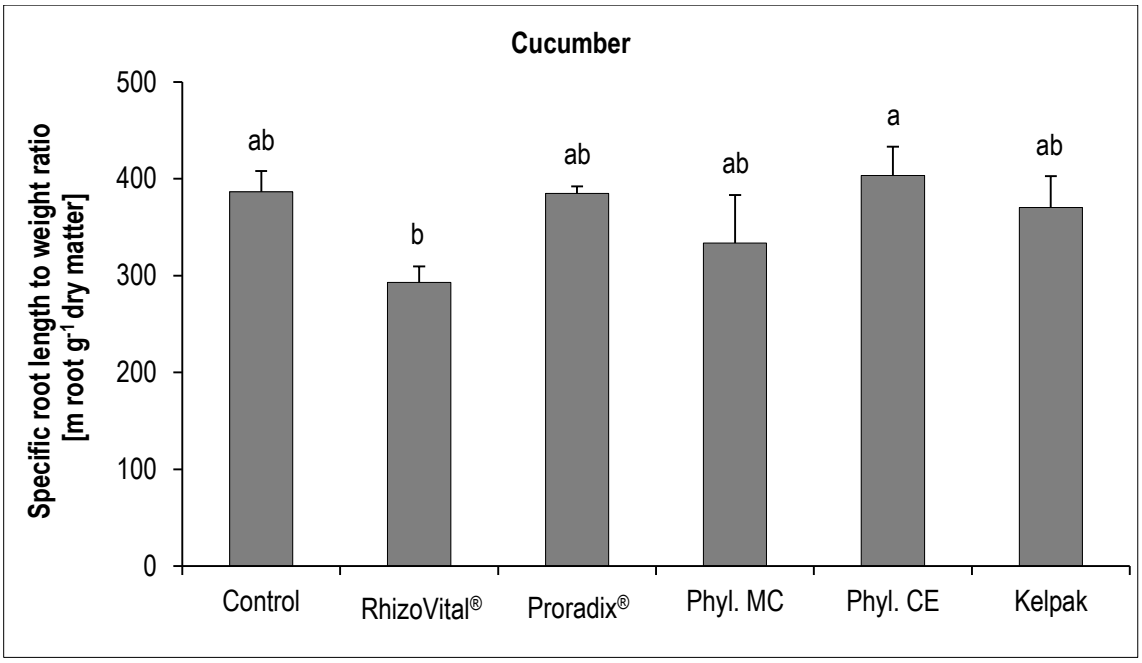

Fig. 4.16: Specific root length to dry weight ratio of cucumber seedlings 14 days after transplanting into the hydroponic system supplied with different commercial bio-fertilizer preparations (Phyl. = Phylazonit as MC or CE product).

Columns represent mean values ± SEM (n = 4). Values not sharing common letters are significantly different [Tukey's test, $p \leq 0.05$].

Most pronounced effects were seen in the leaves of cucumber plants. Thus, plants treated with RhizoVital® were able to develop first true leaves that had significantly larger blade areas (up to factor 4.4) than those of plants treated with Proradix®, Phylazonit CE, or without bio-fertilizers (control) as documented in Fig. 4.17. The second largest first true leaves were formed by plants supplied with Kelpak, followed by Phylazonit MC. While no such clear differences were observed in younger leaves (2nd and 3rd true leaf) at the time of harvest, the area of the first true leaf was closely correlated with the degree of damage caused by an exceptionally high light irradiation on the sixth day after transplanting (Pearson's correlation coefficient $r = -0.84$, $p \leq 0.001$). Pictures of the respective leaf blades together with estimates of the percentage of damaged leaf area are presented in Fig. 4.18. Reciprocally to the ranking order by the size of the leaf area, the significantly lowest percentage of damaged leaf area was scored in the treatments of RhizoVital® and Kelpak followed by Phylazonit MC, whereas the severest damage rate was found in plants supplied with Proradix®, followed by the untreated control and Phylazonit CE.

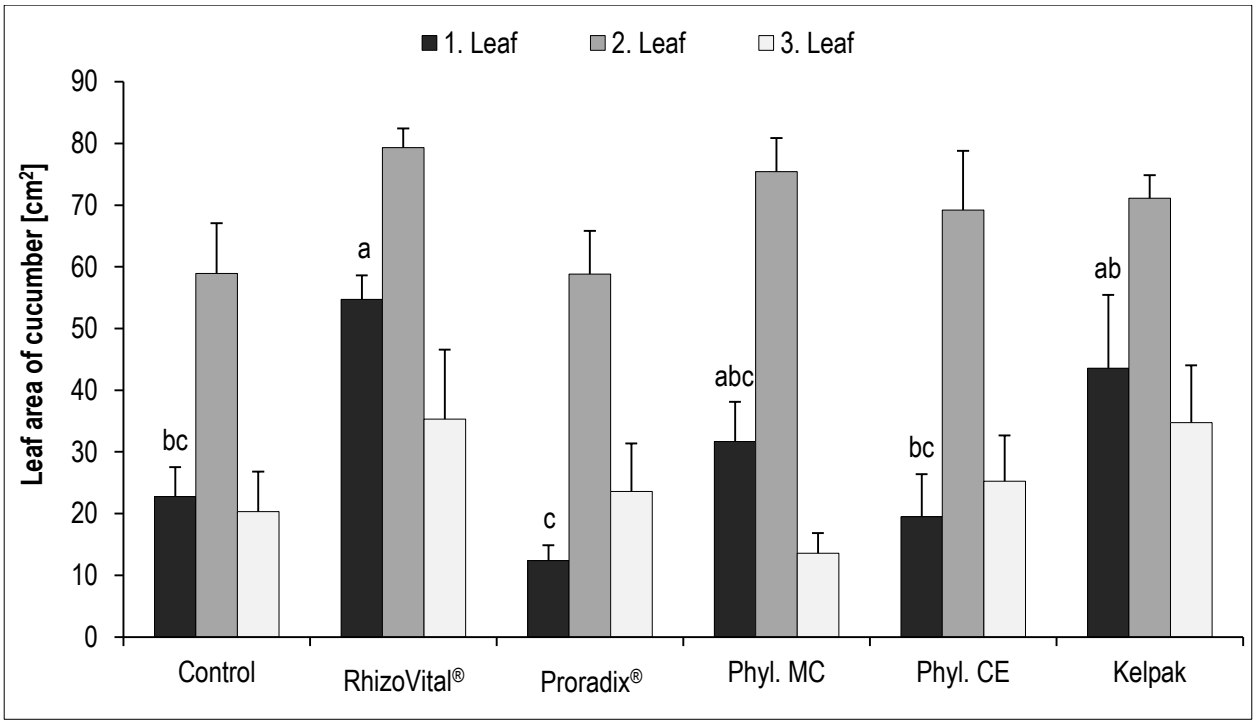

Fig. 4.17: Area of leaves of different insertion level (1st, 2nd and 3rd true leaf) formed by cucumber seedlings 13 days after transplanting into the hydroponic system supplied with different commercial bio-fertilizer preparations (Phyl. = Phylazonit as MC or CE product).

Columns represent mean values ± SEM (n = 4). Values of the 1st leaf not sharing common letters are significantly different. No significant differences within the 2nd or 3rd leaf [Tukey's test, $p \leq 0.05$].

The analysis of elemental concentrations in the shoots of cucumber and in the commercial bio-fertilizer materials furthermore revealed that the resistance to light stress was interrelated with the nutritional status of the cucumber plants. In particular, higher concentrations of Mg (Pearson's $r = -0.61$, $p \leq 0.01$) or Si (Pearson's $r = -0.51$, $p \leq 0.05$) in the shoot tissue were related with a lower degree of leaf damage. As shown in Table 4.5, the highest concentrations of Mg were found in plants treated with Kelpak, followed by RhizoVital® and Phylazonit CE, whereas only plants supplied with RhizoVital® had notably increased Si concentrations. Significantly lower Si concentration than in untreated control plants were measured in plants that had been supplied with Proradix®, Phylazonit MC and Phylazonit CE. The cucumber plants of different bio-fertilizer treatments further differed significantly in their concentrations of P, Mn, and Zn, but not of K, Ca, Fe and Cu in the shoot tissue (Table 4.5; Table 4.6). While dilution effects, due to an increased biomass production of plants that were supplied with RhizoVital® or Kelpak (Fig. 4.19a),

are attributable to the decreased concentrations of P (Pearson's r = -0.92, $p \leq 0.001$) and Zn (Pearson's r = -0.89, $p \leq 0.001$) in the shoot tissue, no significant correlations with the variation of Mn concentrations were found. According to literature data, however, the analyzed concentrations of mineral nutrients in shoot tissues of the test plants were in ranges regarded as sufficient for adequate growth of cucumber plants, aside from Mg which was found in low (2.2 – 5.9 g kg^{-1}) but not yet deficient (< 2.2 g kg^{-1}) concentrations (Bergmann and Neubert, 1976; Bergmann, 1992).

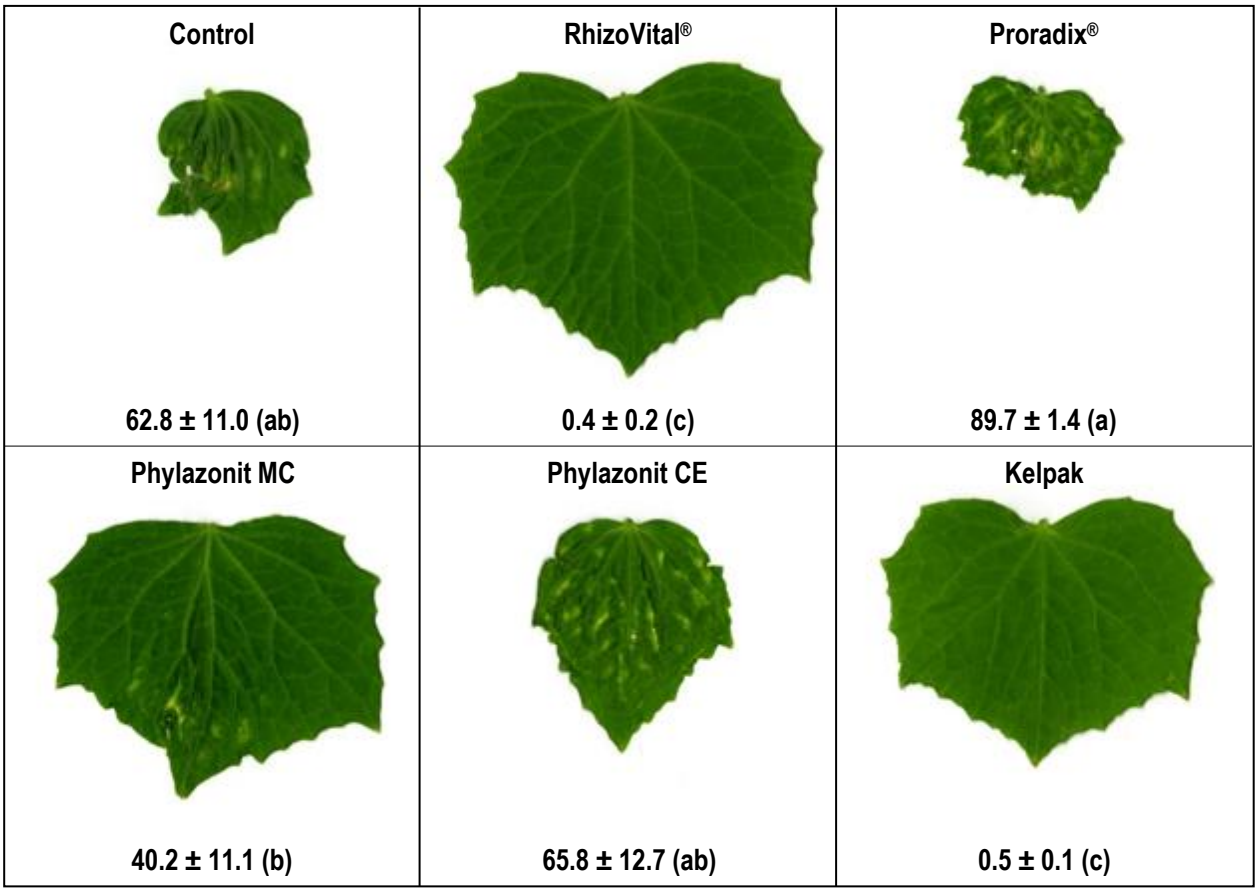

Fig. 4.18: First true leaf of cucumber seedlings treated with Proradix®, Phylazonit MC, Phylazonit CE, or without bio-fertilizer supply (control) showed deformations and necrotic spots in the leaf blade in response to high light irradiation on the sixth day after transplanting into the hydroponic system, whereas nearly no such damage was observed in cucumber seedlings treated with RhizoVital® or Kelpak. Ratings of the percentage of the damaged leaf area in different treatments that were taken on the 13th day after transplanting are given below the corresponding pictures.

Values represent mean values ± SEM (n = 4). Values not sharing common letters are significantly different [Tukey's test, $p \leq 0.05$].

Table 4.5: Concentrations of nutritional macroelements in the shoot dry matter of cucumber seedlings 13 days after transplanting into the hydroponic system supplied with different commercial bio-fertilizer preparations. Ranges indicated as sufficient for adequate growth are given below (Bergmann and Neubert, 1976; Bergmann, 1992).

Values represent mean values ± SEM (n = 4). Values in columns not sharing common letters are significantly different [Tukey's test, $p \leq 0.05$], whereas those not followed by letters or followed by sharing letter are not significantly different.

	P	K	Mg	Ca	Si
	[g kg^{-1} dry matter]				[mg kg^{-1} dry matter]
Control	8.1 ± 0.4 (ab)	60.8 ± 1.2	3.8 ± 0.2 (ab)	44.7 ± 0.8	330 ± 1 (b)
RhizoVital®	5.4 ± 0.3 (c)	63.0 ± 0.9	4.2 ± 0.1 (ab)	44.5 ± 1.3	416 ± 35 (a)
Proradix®	9.3 ± 0.3 (a)	61.5 ± 2.1	3.8 ± 0.2 (ab)	48.0 ± 2.1	218 ± 12 (c)
Phylazonit MC	7.8 ± 0.6 (ab)	61.5 ± 2.7	3.8 ± 0.1 (b)	47.8 ± 1.9	225 ± 12 (c)
Phylazonit CE	8.4 ± 0.2 (ab)	62.8 ± 0.8	4.1 ± 0.2 (ab)	48.3 ± 1.4	247 ± 7 (c)
Kelpak	7.4 ± 0.4 (b)	63.9 ± 1.8	4.4 ± 0.2 (a)	45.3 ± 1.7	283 ± 4 (bc)
Sufficiency ranges	3.0 – 6.0	31.5 – 33.2	6.0 – 13.0	57.0 – 110.0	

Table 4.6: Concentrations of nutritional microelements in the shoot dry matter of cucumber seedlings 13 days after transplanting into the hydroponic system supplied with different commercial bio-fertilizer preparations. Ranges indicated as sufficient for adequate growth are given below (Bergmann and Neubert, 1976; Bergmann, 1992).

Values represent mean values ± SEM (n = 4). Values in columns not sharing common letters are significantly different [Tukey's test, $p \leq 0.05$], whereas those followed by letters or followed by sharing letter are not significantly different.

	Fe	Mn	Zn	Cu
	[mg kg^{-1} dry matter]			
Control	253 ± 77	77.1 ± 11.9 (a)	70.3 ± 2.4 (a)	12.6 ± 1.7
RhizoVital®	180 ± 8	62.8 ± 2.3 (ab)	48.7 ± 2.3 (b)	9.7 ± 0.1
Proradix®	124 ± 8	68.0 ± 6.1 (ab)	72.1 ± 2.3 (a)	10.1 ± 0.4
Phylazonit MC	143 ± 9	52.6 ± 7.1 (b)	68.5 ± 3.7 (a)	9.8 ± 0.0
Phylazonit CE	144 ± 18	49.1 ± 4.6 (b)	69.9 ± 0.8 (a)	10.6 ± 0.4
Kelpak	130 ± 8	46.8 ± 3.8 (b)	46.8 ± 2.8 (b)	10.0 ± 0.5
Sufficiency ranges	150 – 250	40 – 120	40 – 80	10 -18

As seen from the concentrations of nutritional elements added to the nutrient solution by supply of the different bio-fertilizer materials shown in Table 4.7., in particular RhizoVital® and Kelpak have to be considered as potential sources of minerals to the plant.

Table 4.7: Concentrations of nutritional macro- and microelements in the standard nutrient solution used in the hydroponic system and respective amounts of these elements added to the nutrient solution by the supply of different commercial bio-fertilizer materials in the present experiment.

	P	K	Mg	Ca	Si	Fe	Mn	Zn	Cu
	[mg l^{-1} nutrient solution]					[µg l^{-1} nutrient solution]			
RhizoVital®	1.50	2.63	118.28	7.89	187.67	13177.5	241.4	26.7	3.9
Proradix®	0.24	0.19	0.13	0.28	0.13	<10.2	n.d.	n.d.	n.d.
Phylazonit MC	0.04	0.58	0.07	0.76	0.01	29.4	1.0	n.d.	n.d.
Phylazonit CE	0.04	0.56	0.07	0.66	0.01	27.3	1.0	n.d.	n.d.
Kelpak	1.07	57.57	2.05	1.46	0.02	<19.4	n.d.	n.d.	2.0
Nutrient solution	3.10	63.84	12.15	180.32	0.00	1116.8	27.5	32.7	12.7

n.d. = not determinable

RhizoVital® revealed to be foremost rich in Si, Mg, Fe, and Mn, but also Zn, P and Cu were detected in concentrations appropriate to those already contained in the standard nutrient solution. Nevertheless, the corresponding increases of the Si (by 25 %) and Mg (by 10 %) concentrations in the shoot tissue of cucumber plants in response to the treatment with RhizoVital® were comparatively small (Table 4.5). While the amount of Si supplied with RhizoVital® would have been sufficient on a calculatory basis to increase the concentration up to more than 400 g Si kg^{-1} in the plant dry matter, only about 0.4 g Si kg^{-1} dry matter were detected in the shoots of cucumber.

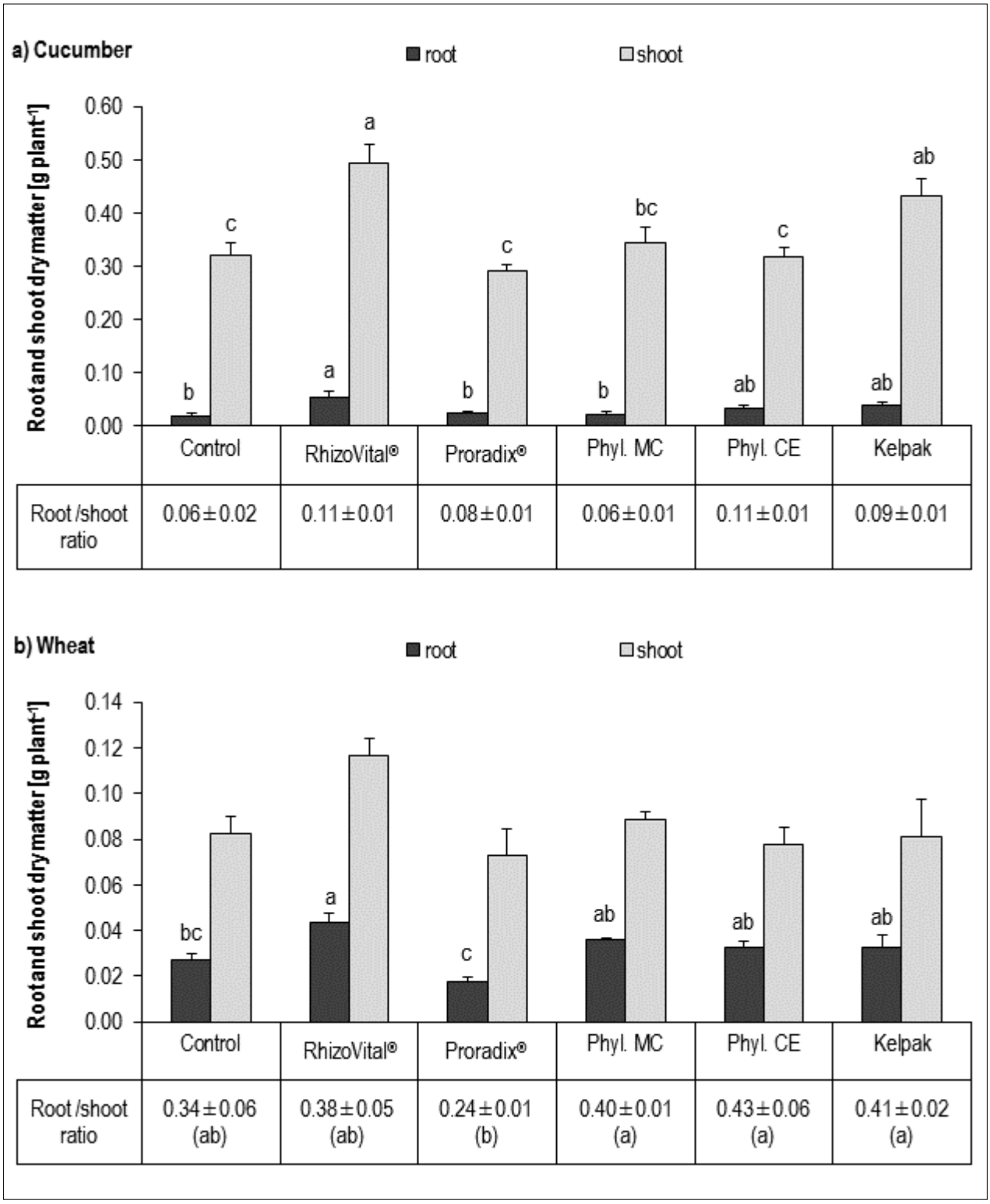

Fig. 4.19: Root and shoot dry matter of cucumber (a) and wheat (b) seedlings 13 respectively 14 days after transplanting into the hydroponic system supplied with different commercial bio-fertilizer preparations (Phyl. = Phylazonit as MC or CE product). Calculated ratios of root to shoot dry weights are given below the graph.

Columns and root/shoot ratios represent mean values ± SEM (n = 4). Values in columns or root/shoot ratios not sharing common letters are significantly different [Tukey's test, $p \leq 0.05$]. Values without or sharing letters are not significantly different.

The amount of Mg supplied by RhizoVital® was ten times higher than that already contained in the standard nutrient solution, but the total biomass production was only increased by factor 1.6 compared to the untreated control. An overview on the responses to of cucumber and wheat to the different bio-fertilizer treatments in terms of biomass production is provided in Fig. 4.19a and b. For other elements such as Fe and Mn, which were found to be abundantly contained in RhizoVital® too (Table 4.7), even lower concentrations were detected in the shoots of so treated cucumber plants (Table 4.6). Kelpak was found to be primarily rich in K and to contain P and Mg in considerable concentrations (Table 4.7), but in cucumber shoots, only the concentration of Mg was significantly increased in response to the application of Kelpak (Table 4.5).

In cucumber and wheat, the largest shoot and root dry weights were achieved with RhizoVital®, whereas Kelpak enhanced the shoot growth of cucumber only (Fig 4.19a,b). A greater biomass production by cucumber plants could be partially attributed to a lower light stress susceptibility, which allowed the formation of larger healthy leaves (Fig. 4.17) and hence increased photosynthetic gains as a secondary effect. To that effect, there was a significant correlation between the area of the first true leaf and the total biomass production per plant (Pearson's $r = 0.68$, $p \leq 0.01$). In wheat, where no symptoms of leaf damage were found, the significantly enhanced production of root biomass appeared to be a more direct response to the application of RhizoVital®. However, neither in cucumber nor wheat a significant shift toward increasing values of root to shoot ratio was found, which indicates that the tested bio-fertilizers did not induce a specifically enhanced partitioning of carbohydrates to the roots.

4.4 Discussion

4.4.1 Compilation of main findings

The results obtained from the presented screening assays to test the potential effectiveness of commercial bio-fertilizers for improved phosphate acquisition by plants support three main statements:

1) Bacterial organisms contained in Vitalin T50, Vitalin SP11 and Proradix® showed a strong capacity to solubilize sparingly available tricalcium phosphate when cultivated on a nutrient-poor agar medium.

2) Aside from Kelpak, based on phytohormone rich algae extracts, none of the bacterial bio-fertilizers included in the present germination and hydroponic tests (i.e. RhizoVital®, Proradix®, Phylazonit MC, Phylazonit CE) showed a clear stimulatory effect on the morphological development of cucumber and wheat roots.

3) An improved resistance towards light stress and an increased biomass production of cucumber seedlings in the hydroponic test were interrelated with an enhanced Si or Mg status in the shoot tissue in response to the application of RhizoVital® or Kelpak respectively. Both of these bio-fertilizer materials have been shown to contain remarkable amounts of the respective and other nutritional elements.

4.4.2 The significance of phosphate solubilization activity under *in vitro* conditions

Many soil microorganisms have been reported to show a high capacity for solubilizing mineral phosphates *in vitro* (Sperber, 1957; Banik and Dey, 1982; Whitelaw, 2000; Akintokun et al., 2007). In an attempt to investigate the mechanisms by which bacterial and fungal isolates dissolve sparingly soluble calcium phosphate, Sperber (1957; 1958a,b) detected the production of organic acids from fermentable carbohydrates (i.e. glucose, maltose, glycerol) which was accompanied by decreases in pH of the culture media. Because high microbial growth rates are often associated with proton release, many soil microorganisms are likely to show calcium phosphate solubilizing ability when grown on

nutrient-rich media (Marschner, 2008). In several studies, however, the degree of solubilization was not proportional to the change in pH (Sperber, 1958a; Deubel and Merbach, 2005), which indicated that proton release was not the only mechanism involved in the mobilization of calcium phosphates. Furthermore, Illmer and Schinner (1992), who tested the influence of varying concentrations of carbon sources and mineral nutrients in the growth medium on the activity of microbial isolates, found that phosphate solubilization was higher when the medium was low in carbon (4 g sugar L^{-l}). According to their explanation, this low optimum of carbon supply is reasonable as suboptimal conditions for growth and biomass production may be optimal for the production of phosphate mobilizing metabolites (Illmer and Schinner, 1992). This assumption coincides with observations made in the present study, where clear zones of calcium phosphate solubilization were only formed by the relatively small colonies of the Vitalin SP11 and Proradix® inoculants grown on the nutrient-poor medium but not by their larger colonies on the nutrient-rich medium. Such a pattern in the expression of phosphate solubilizing ability could be of particular benefit in soils, where microbial activity is generally limited by the availability of carbon. As root exudation may provide minimum threshold levels of organic carbon to fuel the process of phosphate solubilization, it appears to be possible, that suitable micro-site conditions for intensive microbial phosphate mobilization exist in the rhizosphere (Leggett et al., 2001). Accordingly, Sperber (1957; 1958a) found consistently higher proportions of phosphate solubilizing microorganisms in rhizosphere isolates than in those from nearby soil, which indicated a selective influence of root exudates on the composition of microbial populations. The nutrient poor medium used in the present study contained 1 % glucose and 0.1 % asparagine as carbon and nitrogen sources, which corresponds well with the ratio of sugars to amino acids in root exudates (Gransee and Wittenmayer, 2000; Deubel et al., 2000). While neutral sugars were commonly found to represent the largest part of organic rhizodeposites (Gransee and Wittenmayer, 2000), these compounds are hardly able to directly influence the solubility of phosphate in soils (Deubel et al., 2000). The transformation of such ineffective precursor materials into effective carboxylic acids is regarded as an important means by which root associated microorganisms can mobilize recalcitrant calcium, but also iron and aluminum phosphates in the rhizosphere (Banik and Dey, 1982; Whitelaw, 2000; Deubel

et al., 2000). In that regard, the production of gluconate, lactate, citrate and a broad spectrum of other carboxylic anions by diverse members of the fungal and bacterial rhizosphere community has been discussed in topical reviews (Kucey et al., 1989; Whitelaw, 2000; Deubel and Merbach, 2005). Furthermore, Goldstein and Krishnaraj (2007) proposed the direct extracellular oxidation of glucose to gluconic acid and 2-ketogluconic acid as a phosphate deficiency inducible mechanism for mineral phosphate solubilization in gram-negative bacteria (Bagyaraj et al., 2000), which may provide the biochemical basis for the development of mutualistic plant-bacteria relationships in phosphate-limited soil ecosystems. To that effect, Deubel et al. (2000) reported that plants, in response to phosphate deficiency, not only release a relatively higher proportion of carboxylates (Schilling et al., 1998), which may directly increase the solubility of inorganic phosphates in soils, but also show qualitative modifications within the sugar fraction of their root deposits. As suggested by the results of concomitant in vitro experiments, such alterations in the sugar composition can be suitable to facilitate the phosphates solubilizing activity of specific rhizobacteria (Deubel et al., 2000).

However, even though sophisticated laboratory assays may indicate the potential effectiveness of an organism, they cannot predict its functionality with plants under field conditions (Bashan et al., 2013). It rather must be considered that the results are often misleading and methodically influenced by certain test parameters. For example, if despite of phosphate solubilization the production of carboxylates leads to the formation of precipitates, such as of calcium citrate or oxalate, preventing the development of clear zones, many effective microorganisms may not be detected as phosphate solubilizers on agar plates clouded by calcium phosphates (Deubel and Merbach, 2005; Fankem et al., 2006). It has been frequently reported that microbial isolates, which did not produce any visible zones of phosphate solubilization an agar plates, exhibited a high efficiency for solubilizing inorganic phosphates in liquid culture to quantify the amount of dissolved phosphate in the supernatant (Louw and Webley, 1959; Ahmad and Jha, 1968; Gupta et al., 1994; Whitelaw, 2000). Contrariwise, some isolates forming large clearance zones on agar only showed low phosphate solubilization in liquid culture (Ahmad and Jha, 1968), which might be attributed to the infeasibility of these growth conditions for the respective microorganisms to express their phosphate solubilizing ability.

A major prerequisite to grow and function in the rhizosphere is the root-colonizing ability of a microbial inoculant (Weller, 1988). The traits involved in this "rhizosphere competence" (Ahmad and Baker, 1987) of microorganisms are not fully understood. Yet, in particular motility, high growth rates, the utilization of complex carbohydrates, the production of certain metabolites (e.g. antimicrobial compounds, amino acids, vitamins), and rapid adaptation to changing conditions have been attributed to the ability for competitive root colonization (Weller, 1988; Lugtenberg and Dekkers, 1999; de Weert and Bloemberg, 2007). In this respect, it might be concluded that even the most sensitive in vitro tests can only be regarded as preliminary screens, as they cannot reflect the full range of conditions prevailing in soils and the rhizosphere.

4.4.3 Discussion of the germination and hydroponic tests

The release of root growth regulating compounds by rhizosphere microorganisms comprising species of *Azotobacter*, *Bacillus*, *Pseudomonas*, and *Trichoderma* can indirectly enhance the phosphate acquisition of plants (Vessey, 2003; Tsavkelova et al., 2006). Increases in root length, formation of lateral roots and root hair elongation, resulting in a greater absorptive surface area of the root system, have been related to the production of phytohormones such as auxins, cytokinins and gibberellins, by microbial inoculants (Höflich et al., 1994; Frankenberger and Arshad, 1995; Khalid et al., 2006; Ortíz-Castro et al., 2008). In the present germination and hydroponic assays, however, the investigated commercial bio-fertilizers containing diverse microbial strains showed few specific stimulatory effects on root growth and morphology. Only Kelpak, the product based on phytohormone rich algae extracts, clearly increased the root hair length of cucumber seedlings in the hydroponic system. Vitalin SP11 and Vitalin T50 have not been included in this work, because it has been shown earlier by Akter et al. (2007) that both products enhanced the germination rate, the leaf area as well as the biomass production in roots and shoots of cucumber seedlings in analogous screening assays. In addition, the application of Vitalin T50 induced a specific increase in the maximum elongation of cucumber roots. In accordance with these findings, Yedidia et al. (2001) reported increases in the number of root tips, cumulative root length, and root surface area in response to the application of a *Trichoderma harzianum* strain to cucumber seedlings

grown under axenic hydroponic conditions and in soil culture, suggesting a common role of the fungus in improving the plant root system. Different results, have been obtained in hydroponic experiments performed by Beata Szabo et al. (2005, personal communication of unpublished data) under conditions slightly modified from those described by Akter et al. (2007), where the treatment with Vitalin T50 or another *Trichoderma* based bio-preparation (BioHealth®-Granulate, Humintech GmbH, Düsseldorf, Germany), appeared to inhibit the shoot development of cucumber seedlings (Fig. 4.20). A possible reason for these diverging observations could be that variations in test conditions, such as pretreatments during the germination phase, light intensity within the growth chambers, or application rates of the microbial inoculants influenced the results to a large extent. Thus, Akter et al. (2007) had already pretreated the seedlings by germinating them in filter papers soaked with 2.5 mM $CaSO_4$ containing 1 g l^{-1} (personal communication Zafrin Akter) of the different commercial bio-fertilizers before cultivating them in hydroponic systems supplied with about 1 g L^{-1} of the respective preparations. Szabo et al. (2005, unpublished data), by contrast, only used concentrations of 1 g l^{-1} of the *Trichoderma* based products in the hydroponic system, but probably germinated the cucumber seeds without bio-fertilizer pre-treatment, as it was done in the present work, too.

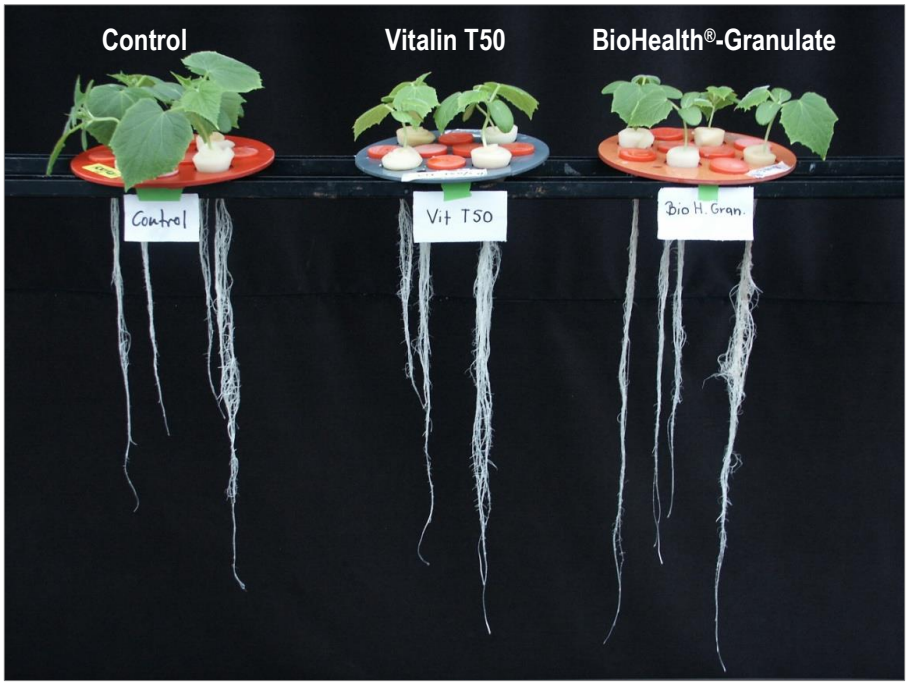

Fig. 4.20: Root and shoot growth of cucumber seedling in response to application of Vitalin T50 or BioHealth®-Granulate after about 14 days of cultivation in hydroponic systems.

Inconsistent growth promotion by *Trichoderma* isolates has also been reported by Rabeendran et al. (2000), who related the contradicting results obtained from two trials with cabbage (*Brassica oleracea* L.) to differences in cultivation conditions, in particular a varying light intensity. Even though water and nutrients were never limiting in the those investigations, *Trichoderma* inoculation only increased root and shoot dry weights in the first trial with lower light intensity, where the plants were in general less vigorously growing, but not in the second trial with higher light intensity. Therefore, the same authors (Rabeendran et al., 2000) in accordance with Harman (2000) postulated that enhanced growth by the application *Trichoderma* isolates is hardly achieved when plants are already grown under near-optimal conditions, whereas the expression of growth promotion is likely to be observed under suboptimal cultivation conditions. This assumption has also been met by results of Björkman et al. (1998) who found a growth promoting effect of *Trichoderma* in sweet corn (*Zea mays* L.) that had been weakened by various abiotic stress treatments, but not in vigorous plants. The increased biomass production of cucumber seedlings in response to the application of RhizoVital® or Kelpak, which has been observed in the present hydroponic assays, could be attributed to an increased resistance against high light intensity stress.

Possible mechanisms increasing the light stress resistance of cucumber plants

Photo-oxidative pressure due to intensive solar radiation inducing the accumulation of reactive oxygen species, such as hydrogen peroxide (H_2O_2), the superoxide radical ($\cdot O_2^-$), the hydroxyl radical ($OH^\cdot$), and singlet oxygen ($^1O_2$) in shoot tissues, is a common stress factor for plants (Foyer et al., 1994; Cakmak, 2005; Grill, 2005). Healthy plants, therefore, are equipped with effective defense systems comprising of antioxidant enzymes (e.g. superoxide dismutase (SOD), catalase, peroxidases) and molecular antioxidants (e.g. ascorbate, glutathione, α-tocopherol, carotenoid pigments, phenolic and floavonoid compounds) to scavenge these radicals (Foyer et al., 1994; Tewari et al., 2006). Reactive oxygen species are also formed during regular cell processes such as photosynthesis and respiration (Smirnoff et al., 1993; Kiyoshi et al., 1999; Apel and Hirt, 2004), but severe light stress conditions and/or additional discomfort, such as mineral nutrient deficiencies, can disturb the intricate balance between formation and scavenging of reactive oxygen

species in plant tissues (Cakmak, 2000; Tewari et al., 2006). An increase in the level of free radicals and a decrease in detoxification mechanisms can lead to the damage of vital cell constituents such as chlorophyll, nucleic acids, membrane lipids and proteins, and result in symptoms of leaf bleaching and tissue necrosis (Cakmak, 2000). Furthermore, it has been recognized that reactive oxygen species are involved in the initiation of signaling responses, enzyme activation, gene expression, and programmed cell death as typical defense reactions in plants (Neill et al., 2002; Mahalingam and Fedoroff, 2003).

A particular role in the light stress resistance of plants is played by magnesium, which is an indispensable element for chlorophyll synthesis and photosynthetic activity (Cakmak and Marschner, 1992; Kiyoshi et al., 1999). In magnesium deficient plants leaf symptoms of chlorosis and necrosis, and corresponding growth impairments have been reported to become more severe under high light irradiation (Cakmak and Marschner, 1992). This enhanced photosensitivity is attributable to several factors inhibiting the photosynthetic CO_2 fixation, insofar as the excess energy absorbed by chlorophyll pigments may alternatively lead to the activation of oxygen to reactive species (Huner et al., 1998; Foyer et al., 2002; Cakmak et al., 2005). In particular, the activity of ribulose-1,5-bisphosphate carboxylase (RuBisCO), the key enzyme in photosynthetic carbon assimilation catalyzing the incorporation of CO_2 into ribulose-1,5-bisphosphate, is highly dependent on magnesium in the chloroplasts (Pierce, 1986; Sugiyama, 1968; cited in: Marschner, 1995). Also an inhibited sucrose export from leaves due to an impaired phloem loading and transport in magnesium deficient plants may impair CO_2 fixation and hence lead to the formation of reactive oxygen species (Cakmak and Marschner, 1992; Cakmak et al., 1994; Hermans et al., 2005). The accumulation of reactive oxygen species, in turn, may not only inhibit the activity of photosynthetic enzymes (Kaiser, 1976), but also induce the closure of stomata (Desikan et al., 2004), which may additionally enhance the formation of reactive oxygen species by limiting the rate of CO_2 uptake (Tewari et al., 2006). The consequent photooxidation of the thylakoid membranes in chloroplasts, rather than an inhibited synthesis of chlorophyll, is regarded as a major contributing factor to the development of interveinal chlorosis in magnesium deficient plants (Cakmak and Marschner, 1992; Cakmak and Kirkby, 2008).

With respect to the above referenced considerations, the improved light stress resistance of cucumber seedlings that had been treated with Kelpak in the present hydroponic test can be reasonably explained by an enhanced concentration of magnesium in the shoot tissue achieved by supply with the nutrient rich algae extract. RhizoVital®, which had a similar protective effect, contains about 98 % of weight talcum powder as a carrier material, according to the product data sheet. Talc is a mineral composed of hydrated magnesium silicate with the chemical formula $Mg_3Si_4O_{10}(OH)_2$ (Schober and Canalini, 2003). Even though talc is sparingly soluble in water (Janssen, 2004), an enhanced magnesium status and a significantly increased silicon concentration was found in the shoot tissue of cucumber seedlings after application of RhizoVital® in the present hydroponic test. In addition to magnesium, also silicon can improve the light stress resistance of plants. In that regard, Li et al. (2004) recently reported that the silicon supplementation of rice (*Oryza sativa* L.) grown in solution culture retarded the occurrence of brown spots and strips of damage symptoms caused by intensive irradiation of ultraviolet-B light in untreated control plants. As a possible mechanism behind the elevated resistance of silicon-treated plants, Li et al. (2004) suggested that silicon induced an increase of phenolic compounds in the leaf epidermis that functioned as absorbents for ultraviolet light. Other studies have shown that silicon application can increase the activity of antioxidant enzymes in plants (Zhu et al., 2004; Gong et al., 2008). Beside its role in the generation of reactive oxygen species, ultraviolet light causes direct damage of DNA, proteins, lipids and membranes in plant cells (Foyer et al., 1994; Jansen et al., 1998; Hollósy, 2002). Compared to silicon concentrations in the shoot tissues of soil grown and silicon-fertilized cucumber, reported to range in the order of 2 to 20 g Si kg^{-1} dry matter (Miyake and Takahashi, 1983), the investigated hydroponic cultured plants of the present work only had a low silicon status of 0.2 to 0.3 g Si kg^{-1} dry matter. Slightly but significantly higher values of about 0.4 g Si kg^{-1} dry matter were found in RhizoVital® treated cucumber seedlings, indicating a low plant availability of the abundant amount of silicon supplied with the talc powder contained as carrier material in this product. Nevertheless, while silicon is generally not considered an essential element for plants and therefore routinely left out from standard formulations of culture solutions (Epstein, 1999), the presented results suggest that already a small surplus of silicon supply can have

considerable beneficial effects on the stress resistance of silicon deprived cucumber plants.

4.4.4 Conclusions

A general conclusion might be that the microbial bio-fertilizers tested in the present study revealed little effectiveness to directly improve the plant growth or morphological development of roots in a way that could affect the spatial capacity for nutrient uptake at the root surface. With respect to controversial findings, reported by different authors as discussed above, it appears, that even under controlled laboratory conditions microbial inoculants may require a narrow range of conductive environmental settings to reveal their potential effectiveness. Under field conditions, however, microbial inoculants should be able to function while being exposed to a broad range of varying factors. The strong phosphate solubilizing activity of bacterial organisms contained in Vitalin T50, Vitalin SP11 and Proradix®, which has been demonstrated on a nutrient poor agar medium, makes these bio-fertilizers the most promising preparations for further studies investigating the chemical mobilization of phosphates in soil and rhizosphere environments.

5 Testing bio-fertilizers for improved phosphate acquisition by crop plants under greenhouse conditions

5.1 Background and objectives

Rhizosphere competence or the successful colonization of roots of plants grown in soil is a major requirement in the effective application of microbial inoculants for beneficial purposes such as bio-fertilization and bio-control (Lugtenberg and Dekkers, 1999; Lugtenberg et al., 2001). This also applies to microorganisms involved in phosphate solubilization, mineralization and/or improved spatial acquisition of readily available phosphate from the soil solution by the plant (Leggett et al., 2001). In the rhizosphere, carbon sources are available to microbes to sustain their own metabolism as well as to produce phosphate solubilizing and plant growth regulatory compounds (Richardson, 2001; Marschner, 2008). The host plant may effectively utilize phosphate that has been mobilized by microorganisms in the rhizosphere. In the bulk soil, by contrast, liberated phosphate ions are likely to be removed from the solution phase again by microbial uptake (Hoberg et al., 2005; Marschner, 2008) and by rapid precipitation or adsorption reactions with the soil matrix (Goldstein, 1986; Gyaneshwar et al., 2002; Delvasto et al., 2006). Although large numbers of microorganisms with the ability to solubilize poorly soluble phosphates under laboratory conditions have been isolated from diverse soils, results obtained after inoculation of soil grown plants with respective strains have been highly variable (Banik and Dey, 1982; Kucey et al., 1989; Whitelaw, 2000). To that effect, it has been suggested that the laboratory conditions employed to detect phosphate solubilizing microorganisms do not adequately reflect soil conditions and that microorganisms capable of mobilizing considerable amounts of phosphate in the rhizosphere are less abundant in soils as indicated by earlier studies (Gyaneshwar et al., 2002; Marschner, 2008). Relying on microbial characters that allow competition for nutrients and space in the rhizosphere environment, rhizosphere competence is affected by various environmental factors including water potential, pH, temperature, soil type, interaction with other microorganisms and the plant genotype (Weller, 1988). However, the influence of soil properties, seasonal determinants and plant characteristics on the

phosphate solubilizing activity of microbial populations has rarely been investigated and the ability to establish specific strains as a significant component of complex rhizosphere communities remains poorly defined (Richardson, 2001; Gyaneshwar et al., 2002). In this regard, new results can be expected from pot experiments to characterize the functionality of microbial inoculants with soil-grown plants under controlled conditions. The expected results, however, will need to be validated in further steps under field conditions.

For microbial inoculants contained in the commercial bio-fertilizer preparations Vitalin SP11 (mixture of *Bacillus subtilis*, *Streptomyces* spp., *Pseudomonas* sp.), Vitalin T50 (*Trichoderma harzianum* together with unidentified bacterial organisms), and Proradix® (*Pseudomonas* sp. *Proradix*, strain DSMZ 13134) a strong activity to solubilize tricalcium phosphate has been observed by use of adapted in vitro tests, as described in the previous Chapter 4. The aim of the following greenhouse experiments was to ascertain the effectiveness of Vitalin SP11 in enhancing the phosphate acquisition of plants that were grown on different soil types containing specific sparingly available phosphate forms. In addition, rock phosphate, organic phosphate compounds, or water soluble inorganic phosphate were supplied in respective treatments in order to test the influence of the microbial bio-fertilizers on the utilization of these different forms of chemical P fertilizers. Beside analyses of the phosphate status of the experimental plants, data of root and shoot growth characteristics were collected as indices of performance. The subsequent discussion in Chapter 5.3 addresses own results as well as findings obtained in complementary studies with Vitalin SP11, Vitalin T50 and Proradix® that were performed in cooperation with other researchers (Hong et al., 2002, unpublished; Singh et al., 2002, unpublished; Win et al., 2002, unpublished; Yusran, 2009) under similar conditions.

5.2 Materials and Methods

5.2.1 Origins and properties of the experimental soil materials

Soil materials low in plant available phosphate but rich in certain sparingly available mineral or organic phosphate forms, which had been collected from three different origins, were used as experimental growth substrates in the pot experiments:

1) Ca-phosphate predominated loess material taken from the calcareous C-horizon (subsoil) of a Luvisol at Wippenhausen near Freising, Bavaria, Germany.

2) Sandy material derived from the top horizon of an acidic Arenosol at Niger, West Africa. Typical precipitation products in acidic soils are Fe- and Al-phosphates (Delgado and Torrent, 2000).

3) Humic soil material with 2.8 % organic carbon (C_{org}) originating from the top horizon of a Cambisol at Ravensburg-Bavendorf, Baden-Württemberg, Germany. A major fraction of the total phosphorus in this soil was organic in nature.

The soil units were classified according to the classification system of the Food and Agriculture Organization of the United Nations (FAO, 2003). In the following, for simplification, also the experimental growth substrates prepared from material derived from these soils will be referred to as “C-loess”, “Arenosol” and “Cambisol”, respectively. Detailed data on selected physical and chemical properties of these soil materials are listed in Table 5.1 and 5.2. The presented values were obtained from Dinkelaker (1990), Bagayoko et al., (2000b), Li (2001), the Landesanstalt für Landwirtschaftliche Chemie (710) at Universität Hohenheim, Stuttgart, Germany, and from own analyses.

Table 5.1: Physical properties of the experimental soil substrates

Soil unit	Soil type	C_{org}	N_{total}	Soil texture			WHC	Bulk density
		[%]	[%]	Clay [%]	Silt [%]	Sand [%]	[% w/w]	[g cm^3]
"C-loess"	loam	0.10	0.02	18 – 25	40 – 50	25 – 45	30	1.36
Arenosol	loamy sand	0.14	n.m.	10 – 18	5 – 40	45 – 90	21	1.61
Cambisol	silt loam	2.80	n.m.	18 – 30	50 – 70	0 – 30	39	1.06

C_{org} = organic carbon; N_{total} = total nitrogen; WHC = water holding capacity; n.m. = not measured

Table 5.2: Chemical properties of the experimental soil substrates

Soil unit	pH	Mineral nutrient concentrations [mg kg^{-1} dry soil]							
		P_i				P_{org}		K	Mg
	($CaCl_2$)	(CAL)	(Bray 1)	($NaHCO_3$)	(H_2SO_4)	($NaHCO_3$)	(H_2SO_4)	(extractant)	(extractant)
"C-loess"	7.6	4.5	2.0	0.6	332.0	n.m.	n.m.	17 (CAL)	150 ($CaCl_2$)
Arenosol	4.5	3.0	5.6	n.m.	n.m.	n.m.	n.m.	31 (NH_4OAc)	27 (NH_4OAc)
Cambisol	6.3	6.9	8.0	5.8	122.6	8.0	95.6	241 (CAL)	165 ($CaCl_2$)

Notations in round brackets refer to the respective extraction method; n.m. = not measured

For estimating the concentration of "plant available" phosphate in soils, a number of chemical extractants are routinely used in standard methods. However, while the solubility of phosphates in soils is largely determined by the pH of the soil, in soil extracts it depends on the pH of the extractant. Therefore, no single extraction solution can be suitable for all soils (Schüller, 1969). Methods using pH neutral extractants such as water (H_2O), calcium chloride ($CaCl_2$) or ammonium acetate (NH_4OAc) are likely to reflect the fraction of adsorbed phosphates and exchangeable cations such as K^+ and Mg^{2+} (Schachtschabel, 1954; Thomas, 1982). Alkaline extractants such as 0.5 M $NaHCO_3$ (Olsen et al., 1954; cited in: Mengel and Kirkby, 2001), by contrast, may correlate with Fe- and Al-phosphates, whereas acidic extractants such as CAL (Ca lactate + Ca acetate + acetic acid; Schüller, 1969) or 1 M H_2SO_4 (Bowman and Cole, 1978) will primarily extract Ca-phosphates (Schüller, 1969). Likewise, dilute acids can extract some non-exchangeable K^+ from the interlayer spaces of soil clay minerals that is potentially plant available (Mengel and Kirkby, 2001). Another extractant, consisting of 0.03 M NH_4F and

0.025 M HCl (Bray 1), that can desorb adsorbed phosphate and dissolve available forms of Ca phosphates, has been proposed by Bray and Kurtz (1945). Soil extraction with 0.5 M H_2SO_4 can yield about 90 % of the total inorganic phosphate in calcareous loess soils (Schachtschabel and Beyme, 1980; Schachtschabel et al., 1992).

In Germany and some other European countries, the CAL-method is commonly used to estimate the plant availability of P and K in soils as a basis for side specific fertilizer recommendations and applications (Schachtschabel et al., 1992). As suggested by the Association of German Agricultural Investigation and Research Centers (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, VDLUFA), soils with CAL-extractable phosphate concentrations below 22 mg P kg^{-1} soil dry matter can be regarded as having a very low level of plant available phosphate (Kerschberger et al., 1997). The experimental soil materials used in the present study, having CAL-extractable phosphate concentrations of less than 10 mg P kg^{-1} soil dry matter in common, would thus require strongly enhanced rates of fertilizer applications to achieve optimum crop yields and qualities (Kerschberger et al., 1997).

According to the methods described by Bowman and Cole (1978), also the availability of organic phosphate (P_{org}) fractions in soils can be characterized with chemical extractants by calculating the increase of inorganic phosphate (P_i) in the respective extract after oxidative digestion with perchloric acid. The labile fraction of organic phosphate in soils can thus be extracted with 0.5 M $NaHCO_3$, whereas some moderately labile forms are represented by the difference between 0.5 M $NaHCO_3$ and 1 M H_2SO_4 extractable organic phosphates. More resistant organic compounds, containing phosphate that is scarcely available to plants, can be grouped together as phytates, fulvic acids and humic acids. Yet, in soils with low levels of available phosphate, microorganisms can slowly mobilize phosphate also from highly resistant organic and inorganic sources (He and Zhu, 1998; Li, 2001; Marschner, 2008). For the soil material derived from the Cambisol, which was used in the present study, about 60 % of the 0.5 M $NaHCO_3$ extractable phosphate and 45 % of the 1 M H_2SO_4 extractable phosphate was attributable to organic compounds (Li, 2001).

5.2.2 Setup of the pot experiments

First, the soil materials were sieved through a 5 mm mesh to remove larger particles and thoroughly mixed to obtain homogenous growth substrates. The actual water content, the maximum water holding capacity, and the bulk density of the sieved soil substrates were determined gravimetrically. For that purpose, subsamples of the soil substrates, which had been filled in metal cylinders of defined volume, were subsequently saturated with water, drained of the excess water by gravity for 48 hours, and dried at 105 °C until weight constancy. Based on the weight of the water held in the sample in relation to the sample dry weigh, the water holding capacity (WHC) was calculated in percent (Öhlinger, 1996).

Soil treatment with the bio-fertilizer and chemical fertilizers

As appropriate to the particular treatments and described in the next sections, the bio-fertilizer preparation together with the chemical fertilizers was dispersed in an adequate amount of deionized water (circa 35 % water holding capacity) and sprayed on the soil substrates under continuous mixing to ensure an even distribution. Subsequently the soil substrates were sieved a second time (2 mm mesh size) and homogenized by thorough mechanical mixing.

Application of the bio-fertilizer product

For soil amendment with a bio-fertilizer intended to improve the phosphate nutrition of plants, the commercial product Vitalin SP11 was applied at amounts of 0.21, 0.29 and 0.27 g kg^{-1} dry soil matter of the C-loess, Cambisol and Arenosol substrates, respectively. According to the instructions of the producer, these rates were equivalent to concentrations of 0.1 % (w/w) in the soil solution of the C-loess and Cambisol substrates at 70 % WHC. The sandy Arenosol substrate, by contrast, was treated with a higher bio-fertilizer concentration in the soil solution (0.2 % w/w at 70 % WHC) to allow for its low water retention. Control treatments were not inoculated with the bio-fertilizer. A detailed description of the ingredients and proposed effects of Vitalin SP11 is given in Table 4.1.

Basal and phosphate fertilization

For basal fertilization 100 mg N (7.1 ml 0.5 M $Ca(NO_3)_2$), 150 mg K (3.8 ml 0.5 M K_2SO_4), and 50 mg Mg (4.2 ml 0.5 M $MgSO_4$) kg^{-1} dry soil matter were added in common to the different types of soil substrates. To increase the availability of iron, the calcareous C-loess material was additionally supplied with 20 µ-mole FeEDTA kg^{-1} dry matter.

The soil materials were furthermore treated or not (No-P) with different inorganic and organic phosphate forms:

1) Water soluble inorganic phosphate (P_{wsi}) was added as a 0.05 M solution of calcium dihydrogen phosphate ($Ca(H_2PO_4)_2$) at a moderate dose of 80 mg P kg^{-1} dry soil matter to all kinds of substrates.

2) As a type of sparingly soluble Ca-phosphate, finely ground apatitic phosphate rock (Rock-P) called "Hyperphos" (8-13 % P) was applied to the C-loess material only at the amount of 1.5 g Hyperphos (ca. 200 mg P) kg^{-1} dry soil matter.

3) Organically bound phosphate (P_{org}) was fertilized to the C-loess material only at rates of each 100 mg P kg^{-1} soil dry soil matter in the form of Na-phytate ($C_6H_6Na_{12}O_{24}P_6$; Sigma, No. P-1916) and phosphoglycerate ($C_3H_7O_6PNa_2$; Sigma, No. G-6014) to obtain a total supply of 200 mg P kg^{-1} dry soil matter.

In the following, these treatments without or with different phosphate fertilizers are referred to as "No-P", "P_{wsi}", "Rock-P", and "P_{org}", respectively.

Experimental design, plants, their sowing and cultivation

The pot experiment was conducted with wheat (*Triticum aestivum* L., var. Triso; Deutsche Saatveredelung – DSV, Lippstadt, Germany). Black plastic pots (11 x 11 cm wide, 12 cm high; Lamprecht-Verpackungen, Göttingen, Germany) were separately filled with the readily prepared growth substrates at amounts according to 1000 g dry weight of the contained soil materials. To stop soil substrate being lost, fine mesh gauze was used for covering the drainage holes on the bottom of the pots. For each combination of soil type, phosphate fertilizer and bio-fertilizer treatment, four pot replicates were prepared. Nine wheat seeds per pot were sown in seeding holes of 1.5 – 2.0 cm depth on 08th May

2002. The seeding holes were then filled up with fine quartz sand (grain size 0.6 – 1.2 mm; Dorsilit®, Gebrüder Dorfner, Hirschau, Germany) to optimize germination conditions. Finally, the surface of the growth substrates was covered by a 1 cm layer of coarse quartz sand (grain size 1.6 – 2.5 mm; Dorsilit®), which avoided pore sealing and crust formation upon watering. Immediately after sowing and throughout the experiment as needed by the plants, the pots were watered with deionized water at 70 % water holding capacity of the soil substrates. The plants were cultivated under greenhouse conditions with natural light at Universität Hohenheim, Stuttgart, Germany. Passive ventilation and shading devices counteracted overheating of the greenhouse during bright sunshine hours. A complete randomized experimental design was used for arrangement of the pots, which were regularly rotated to minimize locational effects. After 14 days from sowing, the germinated wheat seedlings were thinned out to five plants per pot, which were grown until experimental harvest at the developmental stage between inflorescence emergency and beginning of anthesis (stages 50 – 60 on Zadoks' scale; Zadoks et al., 1974) on 19th June 2002.

5.2.3 Experimental harvest and evaluation of the pot experiments

Collection of root and shoot samples

After six weeks of growth, plants from each pot were harvested and combined as one sample. Shoots were cut at soil surface level and the numbers of emerged tillers per plant were counted. The roots were carefully washed free of soil substrate by using an adjustable water sprayer (Gardena, Ulm, Germany) and a sieve frame (50 x 50 cm) with 2 – 3 mm mesh size to avoid the loss of small root pieces. For dry weight determinations, root and shoot samples were dried at 60 °C until weight constancy.

Phosphate analysis in shoot tissues

For analysis of phosphate concentrations in the shoot tissue, dried samples were powdered, using agate disc swing mills (Fritsch, Idar-Oberstein, Germany). Subsamples of about 250 mg were weighed into porcelain crucibles and dry ashed in a muffle furnace at 500 °C for 4 hours. After cooling and oxidation with some drops of 3 % H_2O_2, the

samples were muffled again for 1 hour, then two times evaporated with 2.5 ml 1:3 diluted HNO_3 (65 %) to precipitate SiO_2. The digested samples were then dissolved in 2.5 ml 1:3 diluted HCl (37 %), rinsed with hot water into calibrated flasks and boiled for 2 min to convert meta- and pyrophosphates to orthophosphate. Cooled to room temperature, the samples were made up to volume (25 ml) before being blue-band filtered. Phosphate concentrations in the filtered samples were determined using a colorimetric assay according to Gericke and Kurmies (1952a,b). For that purpose, a color reagent was prepared in a 1:1:1 volume ratio of (1) 1:3 diluted HNO_3 (65 %), (2) 0.25 % ammonium metavanadate (NH_4VO_3) solution in 5.2 % HNO_3, and (3) 5.0 % ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$) solution. To form a yellow dye, sample aliquots of 1 ml were mixed with 1.5 ml of the molybdate-vanadate reagent and 2.5 ml of 1:30 diluted HCl (37 %) to a total volume of 5.0 ml. After 20 hours of color development and stabilization, the light absorption at 436 nm wavelength was measured with a spectral photometer (U-3300, Hitachi, Tokyo, Japan) and compared to that of standard solutions with known phosphate concentrations.

Statistical analyses

Statistical calculations were performed separately for each soil type with the software program SigmaStat Version 2.03 (Systat Software Inc., Erkrath, Germany). Two-way ANOVAs followed by Tukey's test were used to assess significant differences within and interactions between the groups of chemical P fertilizer and bio-fertilizer treatments. If necessary, numeric values of dry matter production and phosphate content in shoot tissues were transformed to their natural logarithm to meet the requirements of equal variance and normal distribution.

5.3 Results

5.3.1 Phenological development of the wheat plants

During the experimental growth phase under late season conditions of long photoperiod (about 15 h day^{-1}) and warm temperatures (13.4 – 18.8 °C mean monthly temperatures), wheat plants grown on soil substrates with different sparingly available phosphate forms showed a rapid phenological development (Gries et al., 1956; Jame et al., 1998). After a short duration of the vegetative growth phase, the wheat plants started to set ears just six weeks after sowing. In general only one elongated tiller per plant was formed. An exception was noted for the treatment of water soluble inorganic phosphate (P_{wsi}) to the Cambisol, which led to the formation of, on average, 3.2 stem tillers per plant. The bio-fertilizer treatment, however, had no clear effect on tiller development by the plants (data not shown in graphs).

Also at harvest of the pot experiment, wheat plants showed little significant response to the application of Vitalin SP11 in terms of biomass production. Strong growth increases, by contrast, were observed following treatment with easily available phosphate forms, as reflected by the dry weight of root and shoots in Fig. 5.1 and 5.2.

5.3.2 Plant growth promoting effectiveness of the chemical phosphate fertilizers

The effect of chemical P fertilization was most pronounced in wheat plants that were grown on the C-loess or on the Arenosol, where the application of water soluble inorganic phosphate (P_{wsi}) enhanced the root biomass production by a factor of 4.4 or 4.0 (Fig. 5.1) and the shoot biomass production by a factor of 4.5 or 5.3 (Fig. 5.2), respectively. The performance of plant growth on these soil substrates without phosphate supply (No-P), however, was especially weak and characterized by symptoms of severe phosphate deficiency.

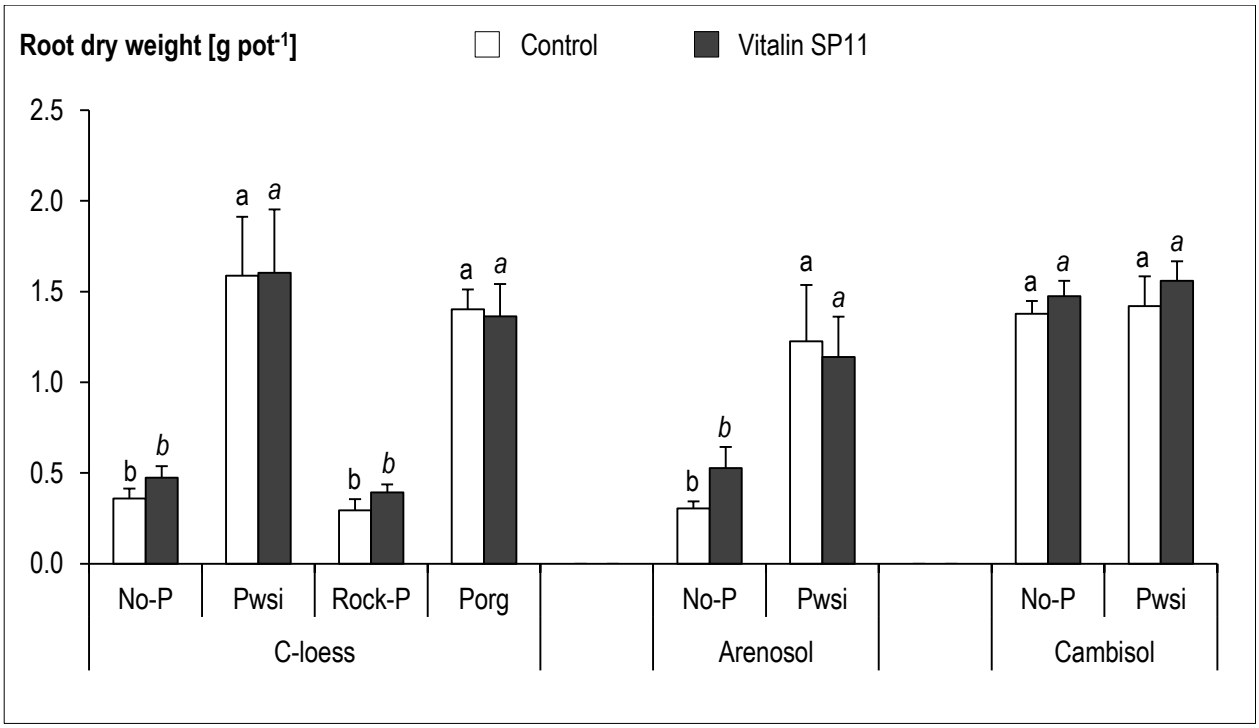

Fig. 5.1: Root dry weight of wheat (var. Triso) 42 days after sowing in the pot experiment with different soil substrates (C-loess, Arenosol, Cambisol) and phosphate fertilizer treatments (No-P, P_{wsi}, Rock-P, P_{org}). Soils substrates were treated or not (Control) with a commercial bio-fertilizer (Vitalin SP11).

Columns represent mean values ± SEM (n = 4). Data were tested separately for each soil type in two-way ANOVAs followed by Tukey's test comparing phosphate fertilizer and bio-fertilizer treatments. Significant differences between phosphate fertilizer treatments within the same bio-fertilizer treatment group are indicated by values not sharing common letters ($p \leq 0.05$), which are printed in non-cursive or cursive characters for values of the Control or SP11 treated group, respectively. There were no significant bio-fertilizer effects and no significant interactions between phosphate fertilizer and bio-fertilizer treatments.

On the humic Cambisol, rich in organically bound phosphate, the availability of phosphate was apparently less growth limiting for the plants, as the No-P plants were less growth-retarded and the P_{wsi} treatment only increased the shoot dry matter by a factor of 1.5 (Fig. 5.2), but not the root dry matter (Fig. 5.1). Correspondingly, the ratios of root to shoot dry weights were lowered in response to the chemical fertilizer application as depicted in Fig. 5.4. A high utilization of phosphate from organic sources was furthermore indicated by the growth response of wheat plants that were grown on the C-loess and fertilized with organic phosphate compounds (P_{org}), as this treatment had a similar growth promoting effectiveness than the P_{wsi} application (Fig. 5.1 and 5.2). Rock phosphate was obviously not an accessible source of phosphorus for wheat on the neutral

to alkaline C-loess, as its application had no significant effect on root and shoot biomass production (Fig. 5.1 and 5.2).

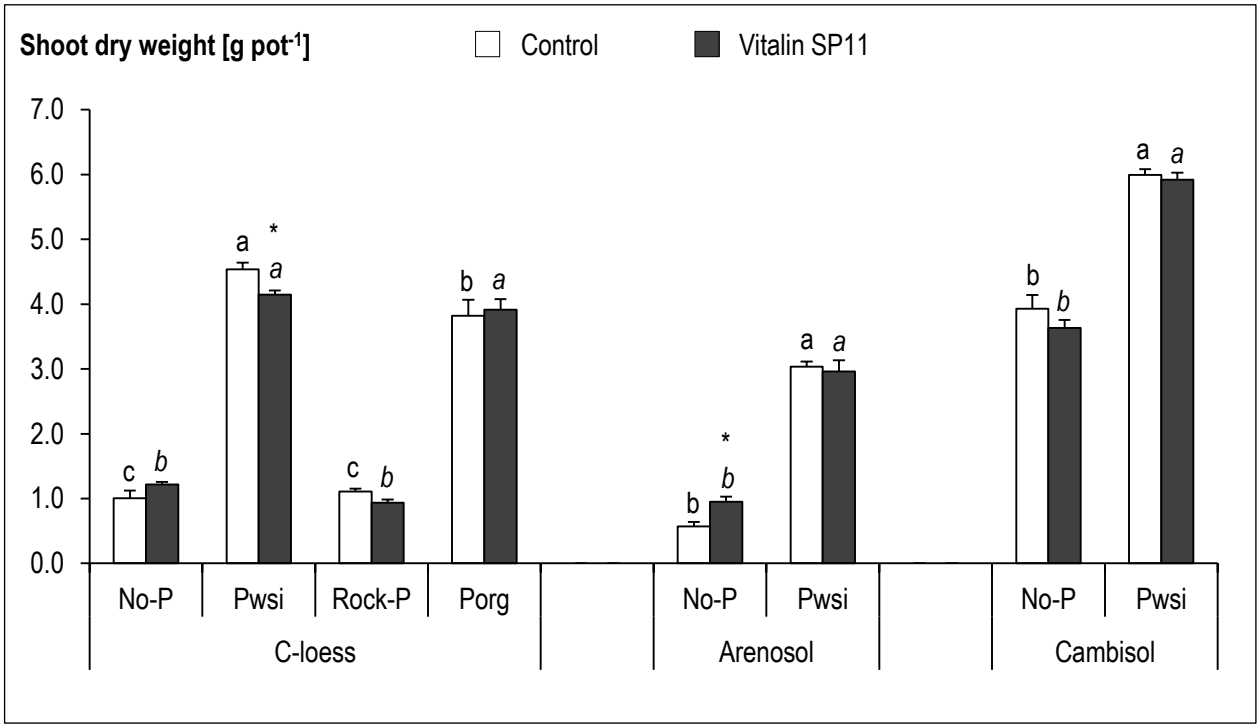

Fig. 5.2: Shoot dry weight of wheat (var. Triso) plants 42 days after sowing in the pot experiment with different soil substrates (C-loess, Arenosol, Cambisol) and phosphate fertilizer treatments (No-P, P_{wsi}, Rock-P, P_{org}). Soil substrates were treated or not (Control) with a commercial bio-fertilizer (Vitalin SP11).

Columns represent mean values ± SEM (n = 4). Data were tested separately for each soil type in two-way ANOVAs followed by Tukey's test comparing phosphate fertilizer and bio-fertilizer treatments. Significant differences between phosphate fertilizer treatments within the same bio-fertilizer treatment group are indicated by values not sharing common letters ($p \leq 0.05$), which are printed in non-cursive or cursive characters for values of the Control or SP11 treated group, respectively. Effects of the Vitalin SP11 against the control treatment are indicated separately by asterisks (* $p \leq 0.05$) for each phosphate fertilizer group. There were no significant interactions between phosphate fertilizer and bio-fertilizer treatments.

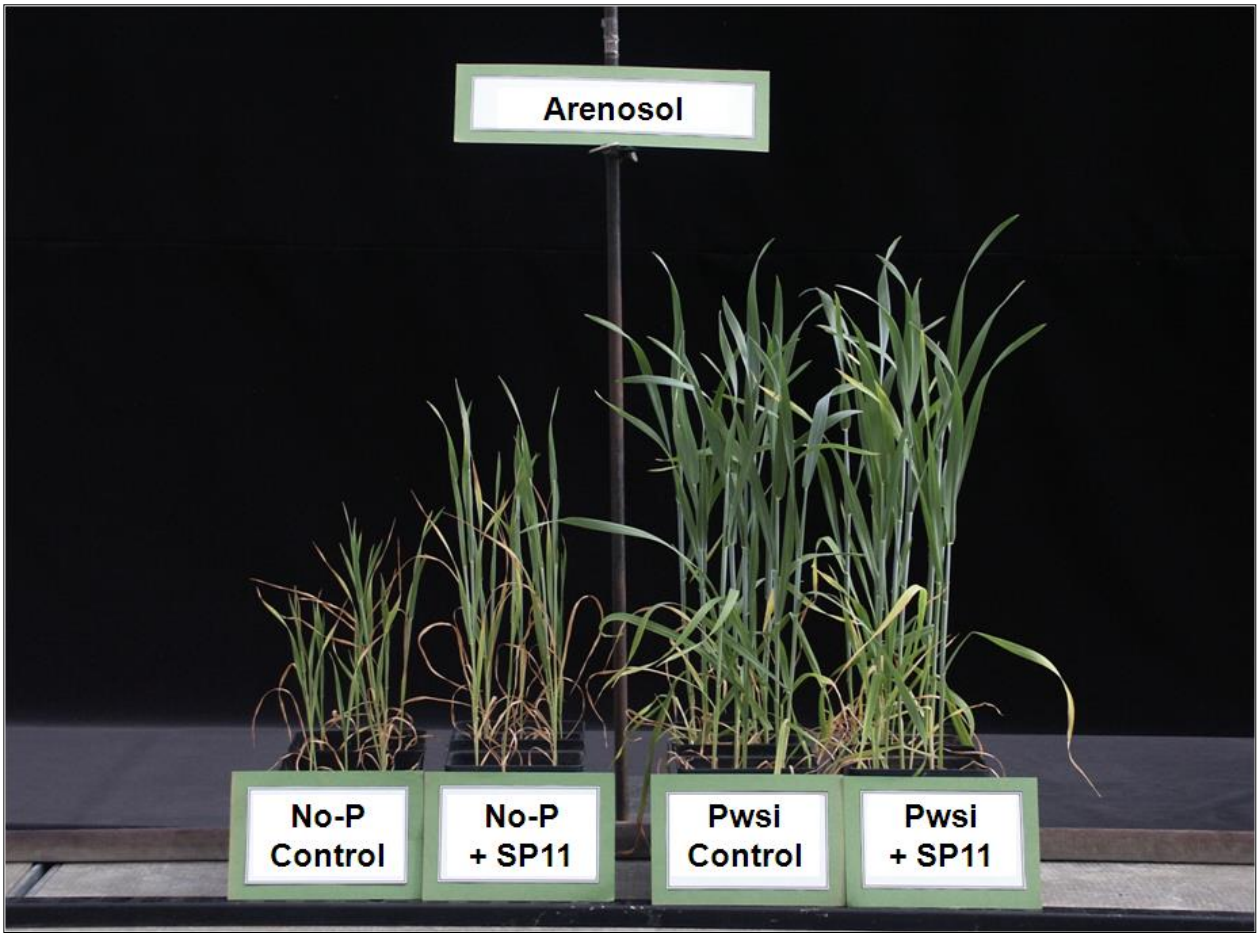

Fig. 5.3: Shoot growth of wheat (var. Triso) plants on soil material of an acidic Arenosol used as substrate in response to the treatment with Vitalin SP11 (+ SP11) and/or water soluble inorganic phosphate (P_{wsi}) compared to the untreated reference plants (Control, No-P) 42 days after sowing in the pot experiment.

5.3.3 Plant growth promoting effectiveness of the bio-fertilizer

A significant growth stimulatory effect of the bio-fertilizer (Vitalin SP11) could only be detected in terms of an enhanced shoot biomass formation (+ 66 %) on the Arenosol without phosphate fertilization (No-P) as shown in the graph (Fig. 5.2). The picture shown in Fig. 5.3 provides a comparative visualization of this effect by means of the shoot developmental status.

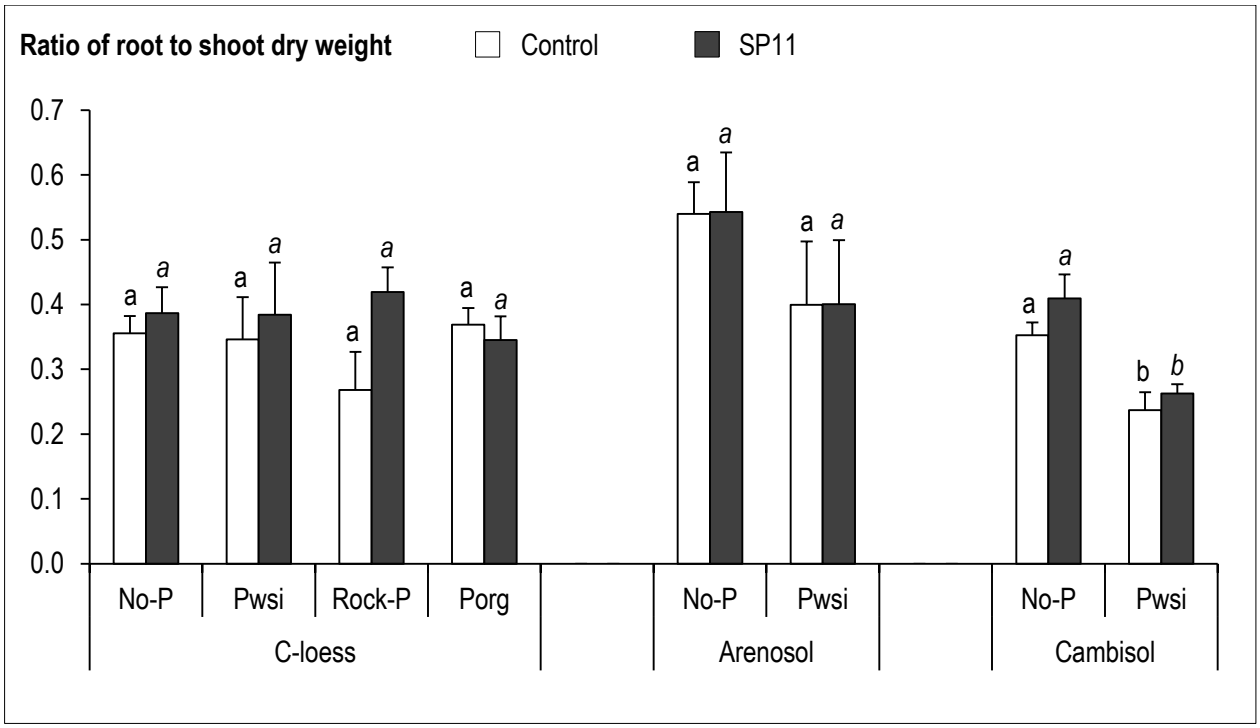

Fig. 5.4: Root to shoot ratio (root dry weight divided by the shoot dry weight) of wheat (var. Triso) plants 42 days after sowing in the pot experiment with different soil substrates (C-loess, Arenosol, Cambisol) and phosphate fertilizer treatments (No-P, P_{wsi}, Rock-P, P_{org}). Soil substrates were treated or not (Control) with a commercial bio-fertilizer (Vitalin SP11).

Columns represent mean values ± SEM (n = 4). Data were tested separately for each soil type in two-way ANOVAs followed by Tukey's test comparing phosphate fertilizer and bio-fertilizer treatments. Significant differences between phosphate fertilizer treatments within the same bio-fertilizer treatment group are indicated by values not sharing common letters ($p \leq 0.05$), which are printed in non-cursive or cursive characters for values of the Control or SP11 treated group, respectively. There were no significant bio-fertilizer effects and no significant interactions between phosphate fertilizer and bio-fertilizer treatments.

However, when water soluble inorganic phosphate (P_{wsi}) was supplied to the same soil substrate, the additional treatment with Vitalin SP11 did not induce further differences in growth. These results give evidence that the growth promoting effect of Vitalin SP11 observed after inoculation to the Arenosol resulted from improved phosphate availability to the plant. At the same time, Vitalin SP11 did not significantly alter the root to shoot ratio of the wheat plants (Fig. 5.4).

The assumption that treatment with Vitalin SP11 improved the phosphate availability to the plant is further supported by the results of the analysis of phosphate concentrations in

shoot tissues (Fig. 5.5) and the thereof calculated total phosphate contents in the shoot biomass produced per pot (Fig. 5.6).

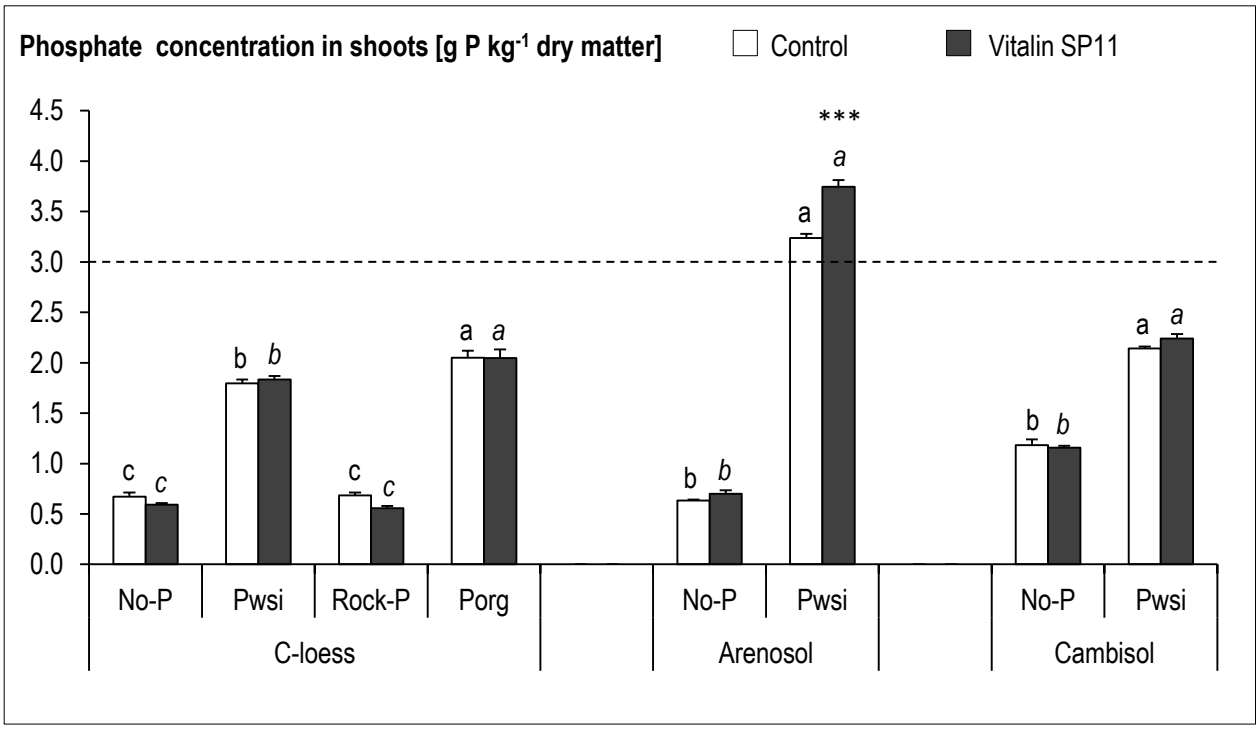

Fig. 5.5: Phosphate concentration in the shoot tissue of wheat (var. Triso) plants treated or not (Control) with a commercial bio-fertilizer (Vitalin SP11) after 42 days in the pot experiment with different soil substrates (C-loess, Arenosol, Cambisol) and phosphate fertilizer treatments (No-P, P_{wsi}, Rock-P, P_{org}). The dashed line (----) indicates the minimum P concentration in the above ground biomass of wheat that is sufficient for adequate growth as reported by Bergmann (1992).

Columns represent mean values ± SEM (n = 4). Data were tested separately for each soil type in two-way ANOVAs followed by Tukey's test comparing phosphate fertilizer and bio-fertilizer treatments. Significant differences between phosphate fertilizer treatments within the same bio-fertilizer treatment group are indicated by values not sharing common letters ($p \leq 0.05$), which are printed in non-cursive or cursive characters for values of the Control or SP11 treated group, respectively. Effects of the Vitalin SP11 against the control treatment are indicated separately by asterisks (*** $p \leq 0.001$) for each phosphate fertilizer group. There was a significant interaction between phosphate fertilizer and bio-fertilizer treatments of plants that had been grown on the Arenosol ($p \leq 0.001$) but not on the C-loess or Cambisol substrate.

Notwithstanding, the phosphate concentration in the shoot tissue was only slightly enhanced (+ 11 %) and still in a deficient range after the phosphate deficient Arenosol was treated with Vitalin SP11 (Fig. 5.5), the increase of the total shoot content of phosphate (+ 83 %) was highly significant under these conditions (Fig. 5.6).

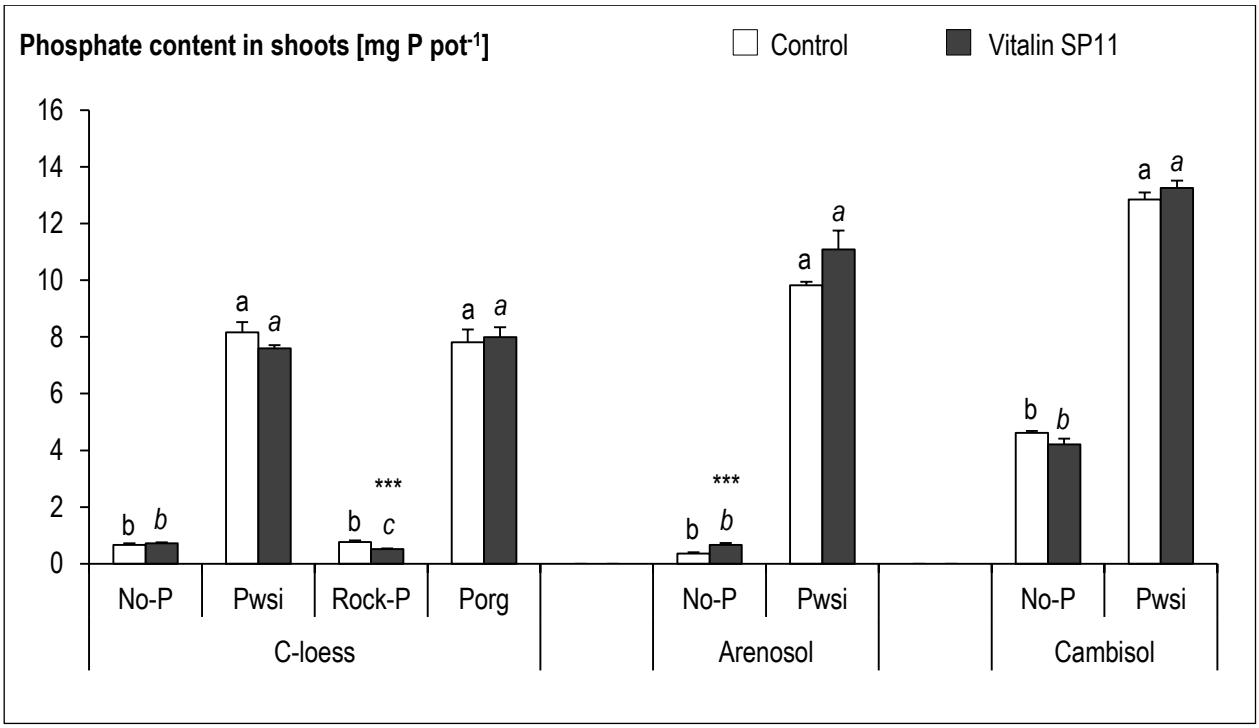

Fig. 5.6: Total phosphate content in the shoot tissue of wheat (var. Triso) plants treated or not (Control) with a commercial bio-fertilizer (Vitalin SP11) after 42 days in the pot experiment with different soil substrates (C-loess, Arenosol, Cambisol) and phosphate fertilizer treatments (No-P, P_{wsi}, Rock-P, P_{org}).

Columns represent mean values ± SEM (n = 4). Data were tested separately for each soil type in two-way ANOVAs followed by Tukey's test comparing phosphate fertilizer and bio-fertilizer treatments. Significant differences between phosphate fertilizer treatments within the same bio-fertilizer treatment group are indicated by values not sharing common letters ($p \leq 0.05$), which are printed in non-cursive or cursive characters for values of the Control or SP11 treated group, respectively. Effects of the Vitalin SP11 against the control treatment are indicated separately by asterisks (*** $p \leq 0.001$) for each phosphate fertilizer group. There was a significant interaction between phosphate fertilizer and bio-fertilizer treatments of plants that had been grown on the Arenosol or C-loess ($p \leq 0.05$) but not on the Cambisol substrate.

Inoculation with Vitalin SP11 furthermore induced a highly significant increase in the shoot concentration of phosphate when combined with the application of P_{wsi} to the Arenosol (Fig. 5.5). In the latter case, however, also the non-inoculated control plants achieved a sufficient phosphate status with only the supply of P_{wsi} and the additionally enhanced phosphate status of the Vitalin SP11 treated plants was not accompanied by further increases in root or shoot biomass production (see Fig. 5.1 and Fig. 5.2). This finding gives an indication that the improved phosphate status of wheat after inoculation with Vitalin SP11 was due to chemically enhanced phosphate availability in the acidic Arenosol. There was no clear evidence for an improved spatial phosphate acquisition by

the plant as a possible consequence of root growth stimulatory properties of Vitalin SP11, as its application to the Arenosol did not alter the root to shoot ratio of wheat (Fig. 5.4).

For the calcareous C-loess or the humic Cambisol, inoculation with Vitalin SP11 did not influence the root and shoot biomass production or the phosphate uptake of wheat to a remarkable extent. Although on these soil substrates a tendency towards increased biomass allocation to the roots at the cost of the shoots in response to the bio-fertilizer application can be suspected, particularly in case of the No-P and Rock-P treatments (Fig. 5.1, 5.2, and 5.4), there was no respective increase in phosphate acquisition by the plant (Fig. 5.5 and 5.6). In fact, the combined treatment of the C-loess with Vitalin SP11 and Rock-P was followed up by a decrease in the concentration and total content of phosphate in shoots. With respect to the results obtained in related investigations (Singh et al., 2002, unpublished), which are discussed in the following, it is furthermore noteworthy that Vitalin SP11 slightly but significantly decreased the shoot dry weight of wheat (- 9 %; Fig. 5.2) when combined with the application of P_{wsi} to the C-loess substrate.

5.4 Discussion

5.4.1 Possible roles of bacterial inoculants, humic acids and algae extracts contributing to the solubilization of inorganic phosphates

The above results demonstrated that the application of Vitalin SP11, a commercial bio-fertilizer containing a mixture of *Bacillus subtilis*, *Streptomyces* spp., and *Pseudomonas* sp. strains along with humic acids and extracts from brown algae, improved the growth and phosphate acquisition of wheat grown on an acidic soil substrate (Arenosol). However, no such improvements in plant performance were observed on a calcareous (C-loess) or a humic (Cambisol) soil substrate. The possible mechanisms by which Vitalin SP11 promoted plant growth on the acidic substrate may have been associated with the activity of different components, comprising bacterial inoculants, humic acids, and algae extracts. As there was no clear evidence for a specific stimulation of root growth, the improved growth and phosphate status of the wheat plants in response to the treatment with Vitalin SP11 most likely resulted from processes of chemical phosphate mobilization in the acidic soil substrate. In numerous studies, phosphate release from sparingly soluble

inorganic sources by diverse soil microorganisms (e.g. *Pseudomonas* or *Bacillus* spp.) has been attributed mainly to the production of carboxylates (Kucey et al., 1989; Richardson, 2001; Vyas and Gulati, 2009), which may function as anion exchangers displacing phosphate ions from the soil matrix and as chelating ligands for metal ions such as Al^{3+}, Fe^{3+} and Ca^{2+} (Buerkert et al., 2000; Neumann and Römheld, 2007; see Chapter 3.2). Recent studies furthermore indicated that phosphate solubilization by strains of *Streptomyces* spp. could be accomplished by the release of Ca^{2+}, Fe^{3+}, Al^{3+} and other metal ions chelating siderophores instead of organic acid anions (Dimkpa et al., 2008; Hamdali et al., 2008). Humic acids are a relatively stable product of organic matter decomposition containing an abundance of carboxylic and phenolic functional groups in their complex and heterogeneous molecules (Burdon, 2001; von Lützow et al., 2006; Baigorri et al., 2009). When applied to soils, humic acids can improve the structural stability of the soil matrix, provide pH buffering, form complexes with metal ions, and may thus increase the availability of phosphate to plants (Wang et al., 1995; Mackowiak et al., 2001). The growth promoting effect of humic acids could further include microbial stimulation through provision of carbon substrates and enhanced uptake of mineral nutrients (Valdrighi et al., 1996; Bünemann et al., 2006). Also for algae extracts, beside of their direct plant growth stimulatory properties (Beckett and van Staden, 1989; Kolbe, 2006), metal chelating and thus potentially phosphate mobilizing abilities have been reported (Crouch et al., 1990). Irrespective of these possible nutrient mobilizing mechanisms of action, the use of Vitalin SP11 would not appear to directly introduce considerable amounts of mineral nutrients as it was applied at rates below 0.3 g kg^{-1} soil material. Analytical data furthermore indicated a low concentration of nutritional elements such as N (9.1 mg g^{-1}) and P (0.2 mg g^{-1}) in the dry matter of Vitalin SP11 (Akter, 2007; Stecher and Ochott, unpublished).

5.4.2 Relative importance of bacterial inoculants contained in Vitalin SP11

Because in soils, minerals, organic constituents, and microorganisms are constantly interacting with each other, also the individual components contained in Vitalin SP11 may not be regarded as separate entities, but rather their mixture can be expected to be more efficient due to their combined activity (Huang et al., 2005). By testing a mixture of

several compounds with similar properties (see previous section) it remains, however, difficult to say which of them were active or not and if one or more of them caused the phenomena observed.

For that reason another experiment, analogous to that described above in Chapter 5.2, was conducted by Singh et al. (2002, unpublished) in which autoclaved Vitalin SP11 was used additionally to the non-autoclaved (fresh) bio-fertilizer material to separate the effect of the living microorganisms from that of the non-living components. Both the treatments of fresh and autoclaved Vitalin SP11 were, however, only applied to the C-loess substrate. To the Arenosol and Cambisol substrate, like in the above experiment, only fresh Vitalin SP11 was applied. The chemical fertilization was performed as specified in Chapter 5.2.2, with the exception that as organic phosphate form only Na-phytate was applied at the rate of 200 mg P kg^{-1} dry matter to the C-loess material. Furthermore, the Cambisol material was mixed with 10 % weight/weight fine quartz sand before fertilizer application for improvement of the soil structure. The experiment was conducted with spring wheat (*Triticum aestivum* L., var. Star; KWS Lochow, Bergen, Germany), which was sown on the 20th March 2002 and harvested on the 15th May 2002.

Some results of the work from Singh et al. (2002, unpublished), are shown in Fig 5.7, 5.8., 5.9, and 5.10. These findings, however, differ widely from those reported for the above experiment (Chapter 5.3) and do not indicate any plant growth enhancing properties of Vitalin SP11, which could be attributed unequivocally to the activity of the bacterial inoculants contained in this bio-fertilizer product.

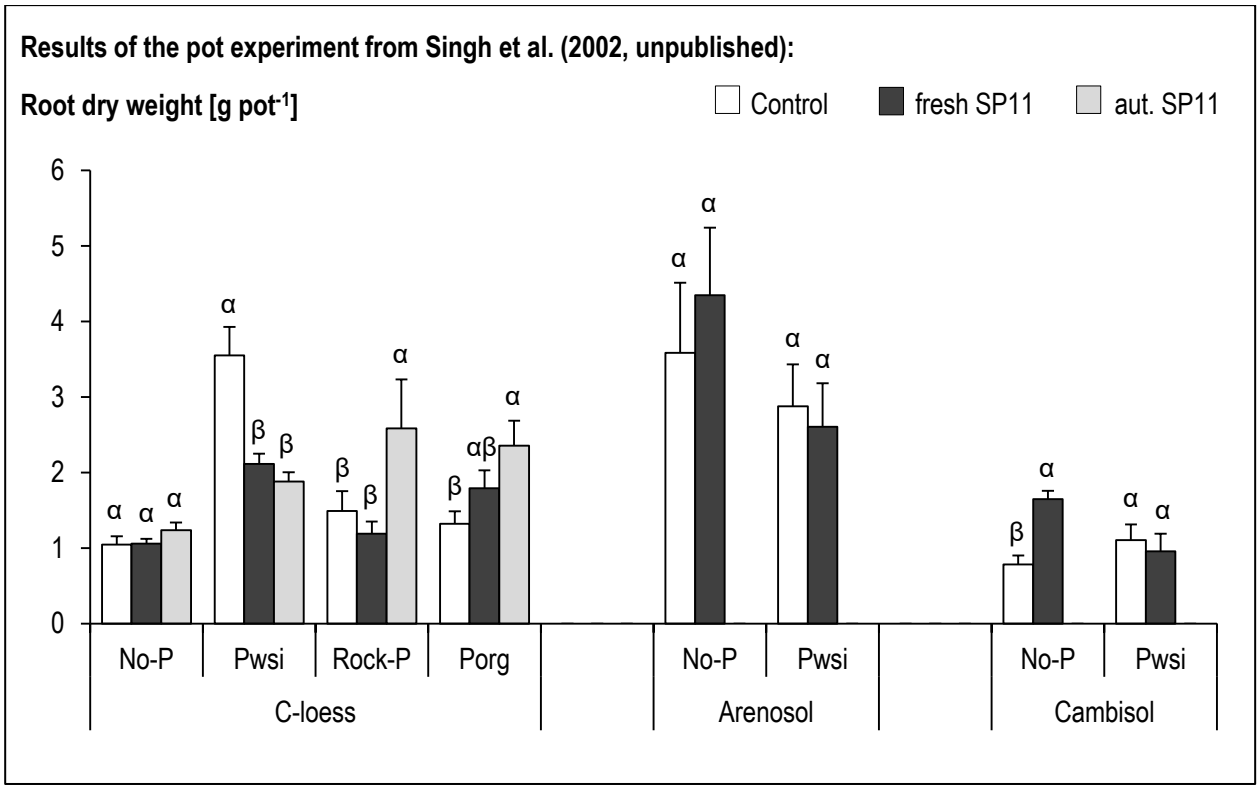

Fig. 5.7: Root dry weight of wheat (var. Star) plants treated or not (Control) with a commercial bio-fertilizer (Vitalin SP11) in non-autoclaved (fresh SP11) and autoclaved (aut. SP11) form after 57 days in the pot experiment from Singh et al. (2002, unpublished) with different soil substrates (C-loess, Arenosol, Cambisol) and phosphate fertilizer treatments (No-P, P_{wsi}, Rock-P, P_{org}).

Columns represent mean values ± SEM (n = 4). Data were tested separately for each soil type in two-way ANOVAs followed by Tukey's test comparing phosphate fertilizer and bio-fertilizer treatments. Significant ($p \leq 0.05$) differences between bio-fertilizer treatments within the same group of phosphate fertilizer treatments are indicated by values not sharing Greek letters. Although there were significant ($p \leq 0.05$) differences between phosphate fertilizer treatments within the same group of bio-fertilizer treatments to the C-loess and Cambisol, these are not indicated in the graph to avoid overcrowding of the figure. There were significant interactions between phosphate fertilizer and bio-fertilizer treatments to the C-loess ($p \leq 0.001$) and Cambisol ($p \leq 0.05$). No significant effects or interactions were observed between phosphate fertilizer and bio-fertilizer treatments to the Arenosol.

Plant growth promoting effects of Vitalin SP11 in combination with organic phosphates

In contrast to the above results (Chapter 5.3), in the experiment of Singh et al. (2002, unpublished) the application of fresh Vitalin SP11 did not clearly affect the growth and phosphate uptake of wheat on the Arenosol, but this time it significantly increased the root and shoot biomass production on the humic Cambisol when no phosphate fertilizer (No-P) was added (Fig. 5.8 and 5.10).

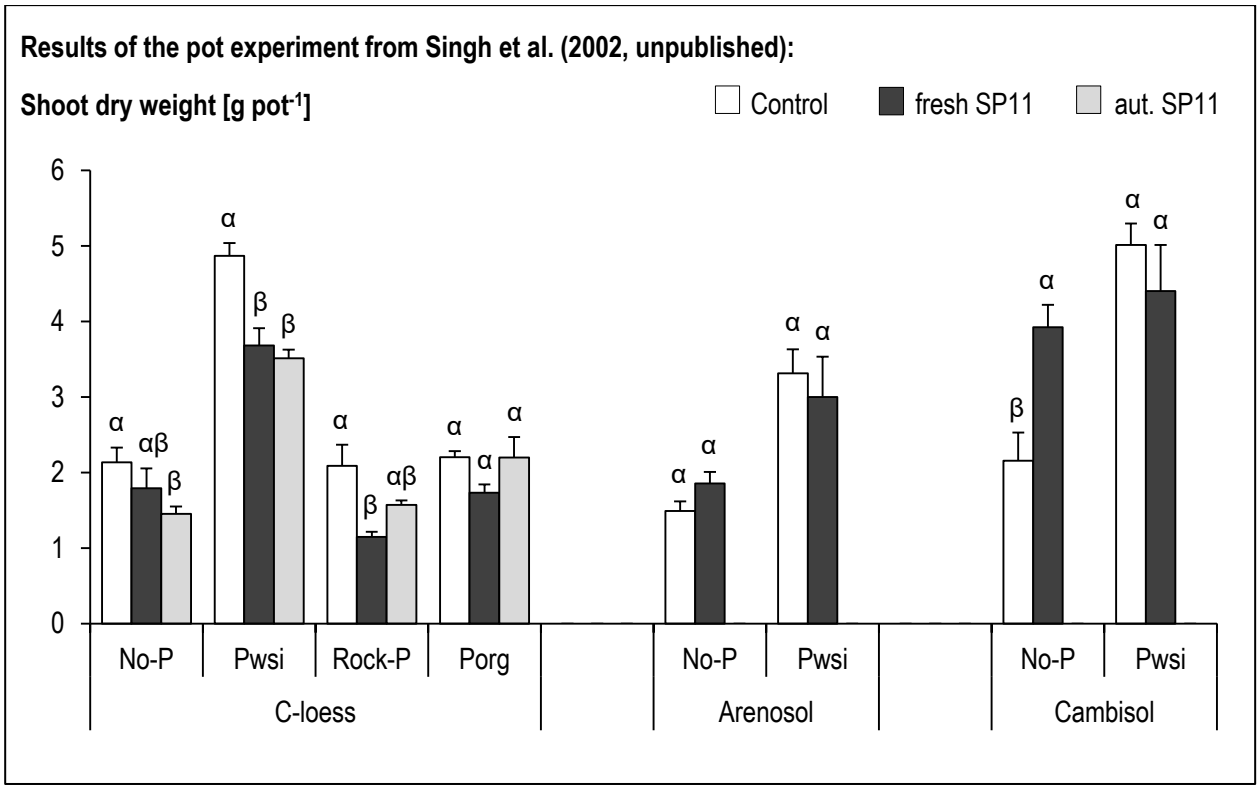

Fig. 5.8: Shoot dry weight of wheat (var. Star) plants treated or not (Control) with a commercial bio-fertilizer (Vitalin SP11) in non-autoclaved (fresh SP11) and autoclaved (aut. SP11) form after 57 days in the pot experiment from Singh et al. (2002, unpublished) with different soil substrates (C-loess, Arenosol, Cambisol) and phosphate fertilizer treatments (No-P, P_{wsi}, Rock-P, P_{org}).

Columns represent mean values ± SEM (n = 4). Data were tested separately for each soil type in two-way ANOVAs followed by Tukey's test comparing phosphate fertilizer and bio-fertilizer treatments. Significant ($p \leq 0.05$) differences between bio-fertilizer treatments within the same group of phosphate fertilizer treatments are indicated by values not sharing Greek letters. Although there were significant ($p \leq 0.05$) differences between phosphate fertilizer treatments within the same group of bio-fertilizer treatments to the C-loess, Cambisol and Arenosol, these are not indicated in the graph to avoid overcrowding of the figure. There were significant interactions between phosphate fertilizer and bio-fertilizer treatments to the C-loess ($p \leq 0.05$) and Cambisol ($p \leq 0.05$). No significant interactions were observed between phosphate fertilizer and bio-fertilizer treatments to the Arenosol.

Because the respective increase in shoot dry matter formation (+ 82 %; Fig. 5.8) was not accompanied by a comparably enhanced phosphate uptake (+ 45 %; Fig. 5.10), thus leading to a significant decrease of the phosphate concentration in the shoot tissue (- 20 %, Fig. 5.9), mechanisms different from phosphate mobilization might have contributed to these findings. The role of the bacterial inoculants in this effect, however, remains unclear as the treatment with autoclaved Vitalin SP11 was only tested on the C-loess but not on the Arenosol and Cambisol substrates.

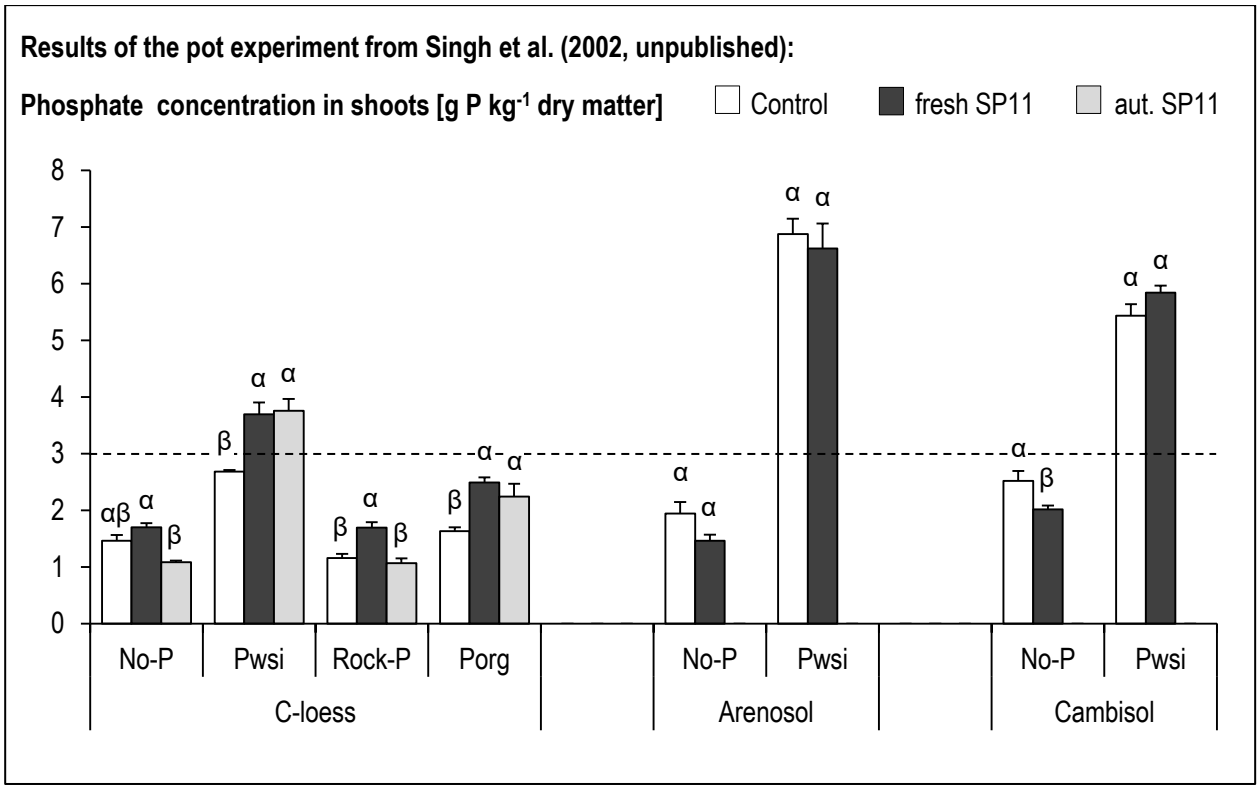

Fig. 5.9: Phosphate concentration in the shoot tissue of wheat (var. Star) plants treated or not (Control) with a commercial bio-fertilizer (Vitalin SP11) in non-autoclaved (fresh SP11) and autoclaved (aut. SP11) form after 57 days in the pot experiment from Singh et al. (2002, unpublished) with different soil substrates (C-loess, Arenosol, Cambisol) and phosphate fertilizer treatments (No-P, P_{wsi}, Rock-P, P_{org}). The dashed line (----) indicates the minimum P concentration in the above ground biomass of wheat that is sufficient for adequate growth as reported by Bergmann (1992).

Columns represent mean values ± SEM (n = 4). Data were tested separately for each soil type in two-way ANOVAs followed by Tukey's test comparing phosphate fertilizer and bio-fertilizer treatments. Significant ($p \leq 0.05$) differences between bio-fertilizer treatments within the same group of phosphate fertilizer treatments are indicated by values not sharing Greek letters. Although there were significant ($p \leq 0.05$) differences between phosphate fertilizer treatments within the same group of bio-fertilizer treatments to the C-loess, Cambisol and Arenosol, these are not indicated in the graph to avoid overcrowding the figure. There were significant interactions between phosphate fertilizer and bio-fertilizer treatments to the C-loess ($p \leq 0.001$) and Cambisol ($p \leq 0.05$). No significant interactions were observed between phosphate fertilizer and bio-fertilizer treatments to the Arenosol.

Quantitative analyses of the acid phosphatase activity on the root surface of wheat revealed generally higher activities in the No-P compared to the P_{wsi} treatments of the Cambisol substrate, which was particularly pronounced in combination with Vitalin SP11 (Fig. 5.11). The increase in these enzyme activities in response to the application of Vitalin SP11 to the substrate without chemical phosphate fertilization (No-P) was,

however, not significant when compared to the respective control without bio-fertilizer (Fig. 5.11).

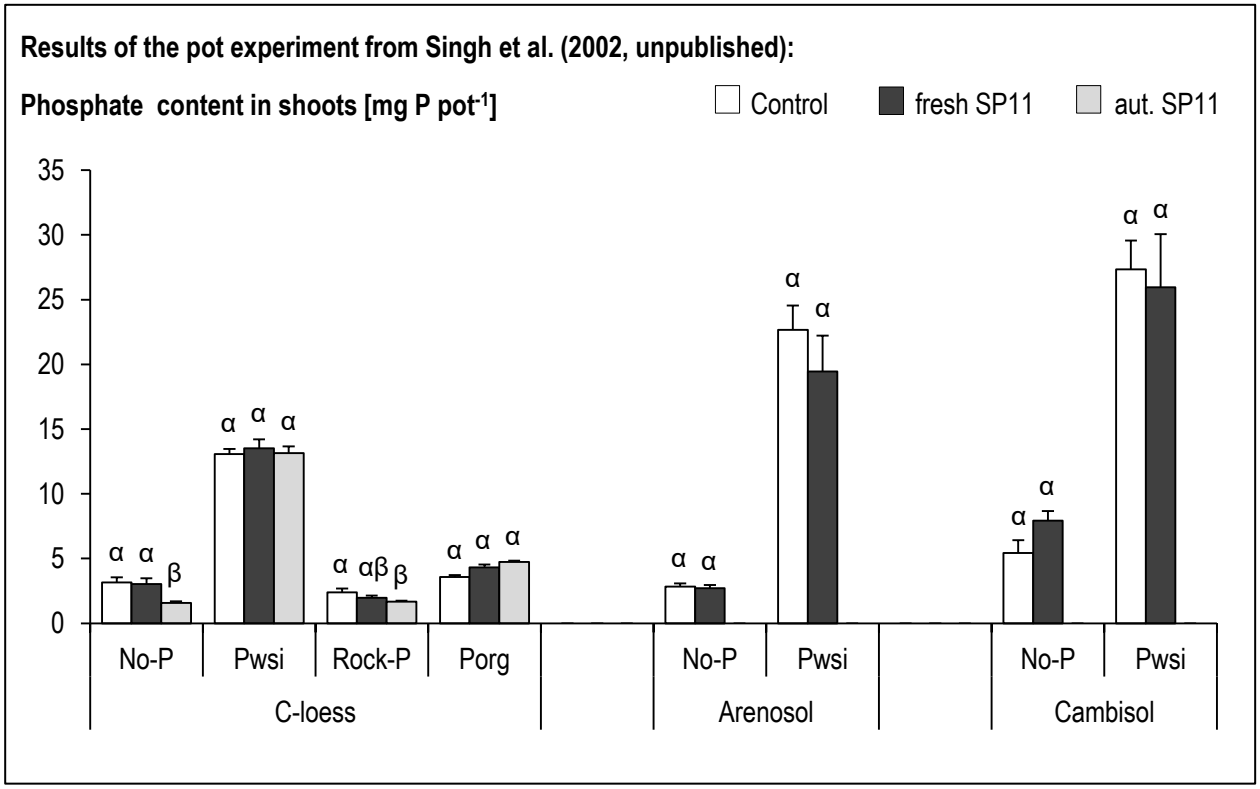

Fig. 5.10: Total phosphate content in the shoot tissue of wheat (var. Star) plants treated or not (Control) with a commercial bio-fertilizer (Vitalin SP11) in non-autoclaved (fresh SP11) and autoclaved (aut. SP11) form after 57 days in the pot experiment from Singh et al. (2002, unpublished) with different soil substrates (C-loess, Arenosol, Cambisol) and phosphate fertilizer treatments (No-P, P_{wsi}, Rock-P, P_{org}).

Columns represent mean values ± SEM (n = 4). Data were tested separately for each soil type in two-way ANOVAs followed by Tukey's test comparing phosphate fertilizer and bio-fertilizer treatments. Significant ($p \leq 0.05$) differences between bio-fertilizer treatments within the same group of phosphate fertilizer treatments are indicated by values not sharing Greek letters. Although there were significant ($p \leq 0.05$) differences between phosphate fertilizer treatments within the same group of bio-fertilizer treatments to the C-loess, Cambisol and Arenosol, these are not indicated in the graph to avoid overcrowding of the figure. There were significant interactions between phosphate fertilizer and bio-fertilizer treatments to the C-loess ($p \leq 0.001$). No significant interactions were observed between phosphate fertilizer and bio-fertilizer treatments to the Arenosol and Cambisol.

In contrast to plant roots, microorganisms may not only produce and release acid phosphatases but also alkaline phosphatases (Tarafdar and Claassen, 1988; Tarafdar et al., 2002). Analyses of the alkaline phosphatase activity in the rhizosphere, therefore, could have been more closely related to the presence of root associated microbial populations,

but were not performed by Singh et al. (2002, unpublished), probably due to the rather acidic pH value (6.3) of the Cambisol.

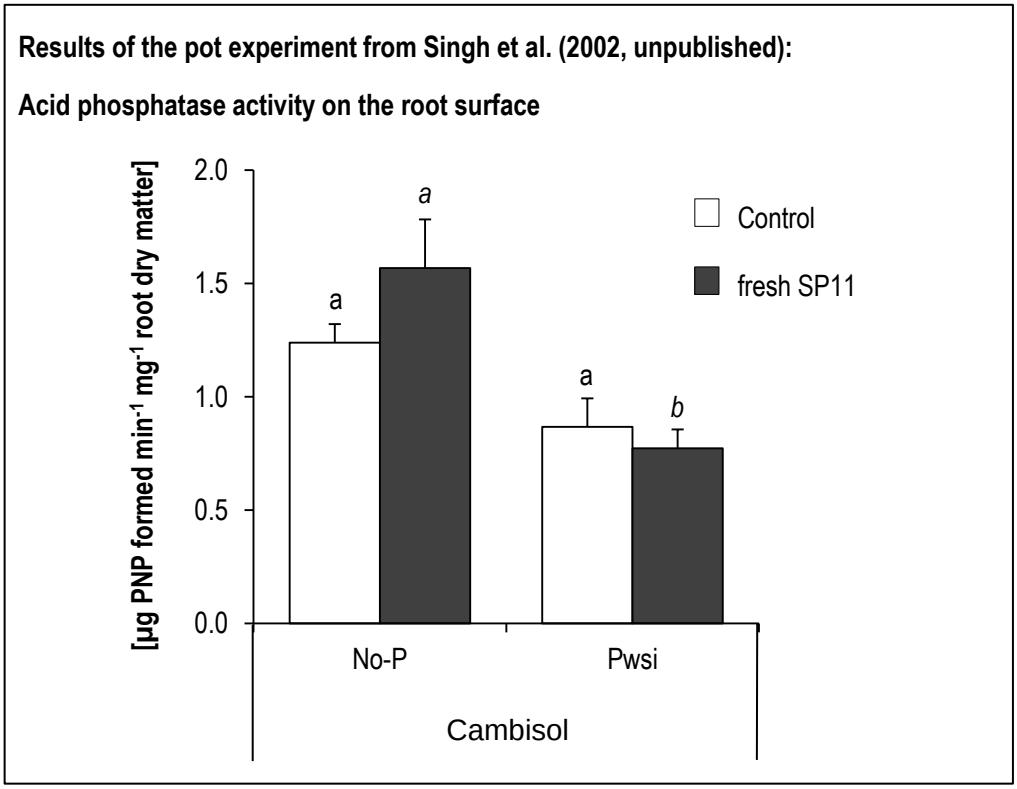

Fig. 5.11: Acid phosphatase activity on the root surface of wheat (var. Star) 57 days after sowing in the pot experiment from Singh et al. (2002, unpublished). The plants were grown on a humic Cambisol substrate without (Control) or with application of non-autoclaved material of the commercial bio-fertilizer Vitalin SP11 (fresh SP11) under two different levels of phosphate fertilization (No-P, P_{wsi}). Samples were analyzed in a spectrophotometric assay according to the method of Tabatabai and Bremner (1969), modified by Alvey et al. (2001). The phosphatase activity is expressed in units of p-nitrophenol (PNP) formed by enzymatic hydrolysis of the chromogenic substrate p-nitrophenyl phosphate after incubation of fresh root pieces in reaction tubes.

Columns represent mean values ± SEM (n = 4). Data were tested in a two-way ANOVA followed by Tukey's test comparing phosphate fertilizer and bio-fertilizer treatments. Significant differences between phosphate fertilizer treatments within the same bio-fertilizer treatment group are indicated by values not sharing common Latin letters ($p \leq 0.05$), which are printed in non-cursive or cursive characters for data sets of the Control or SP11 treated group, respectively. There were no significant bio-fertilizer effects and no significant interactions between phosphate fertilizer and bio-fertilizer treatments.

Adverse growth effects of Vitalin SP11

The most pronounced effect upon treatment of the C-loess substrate with fresh Vitalin SP11 in the experiment of Singh et al. (2002, unpublished) was a decreased root (- 40 %) and shoot (- 24 %) biomass production observed within the treatment group that had received water soluble inorganic phosphate (P_{wsi}) (Fig. 5.7 and 5.8). Though far less strongly expressed, a somewhat similar finding can be seen in the beforehand described results of the present study, where only the shoot (- 9 %; Fig. 5.2) but not the root growth (Fig. 5.1) was suppressed within the same treatment variants. The work of Singh et al. (2002, unpublished) furthermore revealed that autoclaving of the bio-fertilizer did not significantly alter the magnitude of its adverse effect on root (Fig. 5.7) and shoot (Fig. 5.8) growth when combined with the treatment of P_{wsi} to the C-loess substrate. A possible explanation for this phenomenon could, however, still be seen in the activity of soil biota, because also the application of dead organic matter, contained in the fresh and even more in the autoclaved bio-fertilizer material might stimulate the activity of autochthonous or accidentally introduced microorganisms, as for instance through the irrigation water. Depending on the composition of organic materials, their incorporation into soils as a carbon source may not only increase the growth and activity of microbial populations but also lead to the competitive immobilization of otherwise plant available nutrients, such as particularly nitrogen, in the microbial biomass (Kuzyakov et al., 2000). Accordingly, microbial decomposers are thought to require at least 1.5 to 1.7 % N, corresponding to a C/N ratio of ca. 25 or 30, in organic matter to meet their nitrogen demand without added fertilizer or mineral N sources from the soil (Schmalfuß und Kolbe, 1959; Parr and Papendick, 1978; cited in: Kennedy et al., 2004). Organic inputs containing less nitrogen can thus be expected to decrease rather than to enhance the plant availability of nitrogen during the growing season following incorporation (Williams, 1968). In soil biota, nitrogen is generally enriched compared to other pools of soil organic matter, with bacteria being reported to have higher nitrogen concentrations in their microbial biomass (C/N ratio = 3 – 6) than fungi (C/N ratio = 5 – 15) (Harris, 1997; Wallander et al., 2003). The production of microbial biomass therefore is likely to cause a demand for nitrogen whereas their decay or consumption by protozoa may lead to the release of NH_4^+ and NO_3^- after nitrification (Clarholm, 1985; Mengel and Kirkby, 2001; Whitmore, 2007;

Krome et al., 2010). Because the complexes formed between NH_4^+ and humic acids are reported to release nitrogen slowly into soil, humic acids contained in Vitalin SP11 could have contributed to the immobilization of nitrogen, too (Fataftah, 2001). The relevance of this argumentation may appear limited with respect to the low amount of Vitalin SP11 (0.02 % w/w) added to the C-loess substrate with its low organic matter content (0.10 %). Nevertheless, also if the application of autoclaved or fresh Vitalin SP11 was sufficient only to initially stimulate microbial populations, which after enhanced root colonization consumed root exudates as their main carbon source, these microorganisms would have required other sources of nitrogen to sustain their growth, as root exudates characteristically have high C/N ratios (Priha et al., 1999; Ekelund et al., 2009).

Similarly to the above considerations on nitrogen, the phosphate uptake of plants may not be improved if the quantity of phosphate released by the activity of phosphate solubilizing microorganisms does not exceed their own demand (Laheurte and Berthelin, 1988; Hoberg et al., 2005). Soil microorganisms can also compete with plants for available phosphate (Schachtman et al., 1998; Jakobsen et al., 2005). The simple interpretation that a relationship existed between nutrient immobilization in the microbial biomass and suppressed plant growth is however contradicted by the higher phosphate concentration (Fig. 5.9) and unaffected total phosphate content (Fig. 5.10) in the shoot dry matter after application of fresh or autoclaved Vitalin SP11 to the C-loess substrate with P_{wsi}. It remains a matter of speculation whether microbial populations only competed with the wheat plants for nitrogen and possibly other nutrients, but covered their demand for phosphorus from different sources than plant available pools (Hoberg et al., 2005).

With the C-loess substrate the experiment from Singh et al. (2002, unpublished) gave more conflicting results. A major observation in the treatment variants with Na-phytate (P_{org}), rock phosphate (Rock-P) and by tendency also in the variant without phosphate supply (No-P) was that only the autoclaved but not, or to no significant extent, the fresh Vitalin SP11 material enhanced the root biomass production of wheat (Fig. 5.7). In the No-P and Rock-P treatments of the C-loess substrate, providing only sparingly available Ca-phosphates from the soil and rock phosphate fertilizer, this root growth enhancement was not accompanied by an improved phosphate status of the plants (Fig. 5.10) which

were rather suppressed in their shoot development (Fig. 5.8). The combination of fresh or autoclaved Vitalin SP11 with Na-Phytate (P_{org}) by contrast appeared to increase the utilization of this organic phosphate form at least to some extent, taking into account the higher concentration and tendentiously increased total phosphate content in the shoot dry matter (Fig. 5.10). The generally lower or less distinct growth and phosphate uptake enhancing effect of Na-phytate (P_{org} treatments in Fig. 5.7 and 5.8) compared to the combined application of Na-phytate with phosphoglycerate (P_{org} treatments in Fig. 5.5 and 5.6) can be explained by the low stability of phosphoglycerate in soils. While phosphoglycerate can be easily mineralized through the activity of phosphatases produced by plant roots and microorganisms, the hydrolysis of Na-phytate might have been prevented by the rapid formation of insoluble Ca-phytates in the calcareous C-loess substrate (Tarafdar and Claassen, 1988; Dou and Steffens, 1993; Tang et al., 2006).

5.4.3 Further discrepancies in reproducibility of results

Conflicting results were furthermore obtained by Hong et al. (2002, unpublished) who tested Vitalin SP11 and the *Trichoderma harzianum* product Vitalin T50 in a pot experiment with wheat (*Triticum aestivum* L.) that was run from the 18th September 2001 until the 01th November 2001 under greenhouse conditions at the Institute of Plant Nutrition, Universität Hohenheim, Stuttgart, Germany. In this experiment, only the C-loess substrate with adequate basal fertilization (N, K, Mg, Fe) was used and treated without phosphate fertilizer (No-P), with rock phosphate (Rock-P; 200 mg P kg^{-1} soil substrate), or with water-soluble inorganic phosphate (P_{wsi}; 80 mg P kg^{-1} soil substrate).

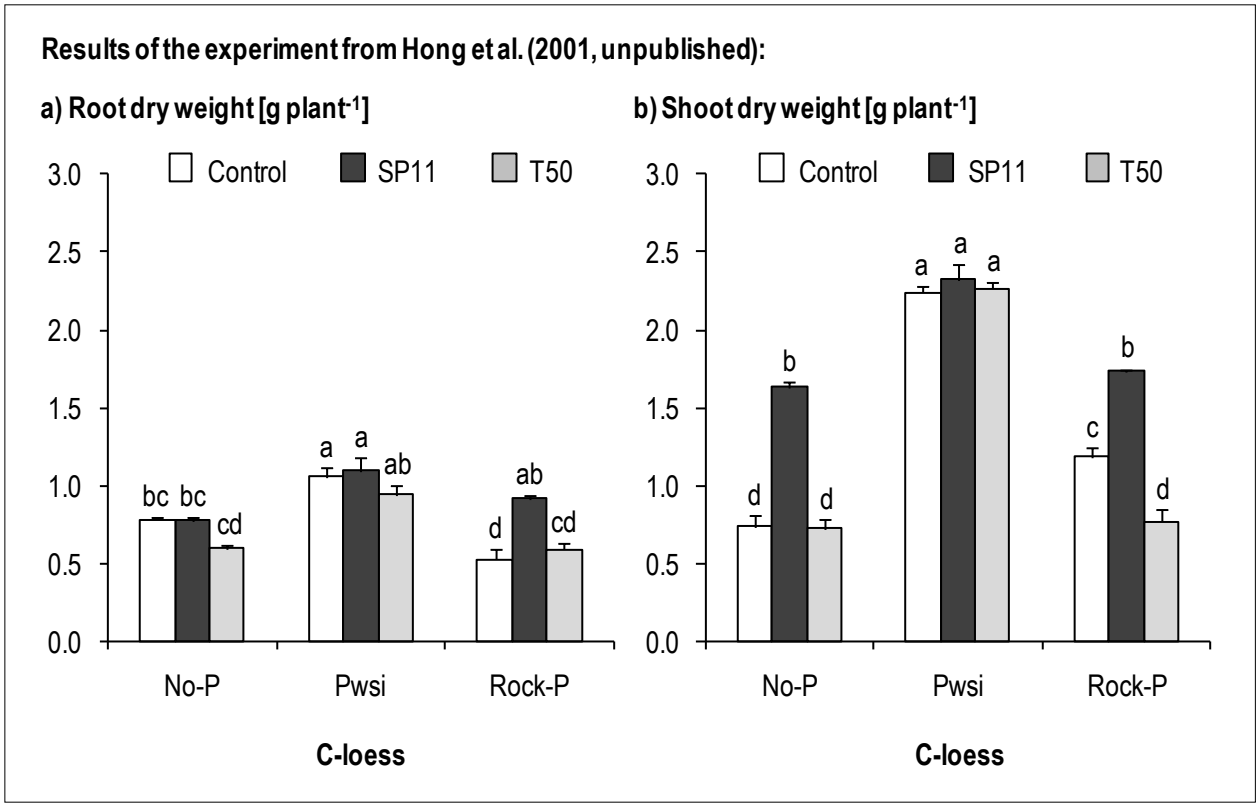

Fig. 5.12: Root (a) and shoot (b) dry weight of wheat (Triticum aestivum L.) plants treated or not (Control) with the bio-fertilizers Vitalin SP11 (SP11) or Vitalin T50 (T50) after 44 days of cultivation on the C-loess substrate with different phosphate fertilizer treatments (No-P, Rock-P, Pwsi) in the pot experiment from Hong et al. (2002, unpublished).

Columns represent mean values ± SEM (n = 4). Data were tested by one-way ANOVA followed by Tukey's test. Values not sharing letters indicate significant ($p \leq 0.05$) differences between combined bio-fertilizer and phosphate fertilizer treatments.

Contradictory to the beforehand described results, in the study from Hong et al. (2002, unpublished) Vitalin SP11 showed a plant growth promoting effect when applied to the C-loess substrate without phosphate fertilization (No-P), where it strongly increased the shoot (Fig. 5.12a) but not the root (Fig. 5.12b) growth of wheat. The combination with rock phosphate (Rock-P), however, did not show an enhanced effectiveness compared the application of Vitalin SP11 alone and the application of water-soluble inorganic phosphate (P_{wsi}) nullified the growth promoting effect of Vitalin SP11. These findings, together with respective increases of the phosphate concentration in the shoot tissue (Fig. 5. 13) indicate that the growth promotion by Vitalin SP11 was mainly due to an improved acquisition of phosphate from sparingly available pools contained originally in the C-loess material. The phosphate status of the wheat plants grown with rock phosphate

(Rock-P) was not significantly altered compared to that of the plants without phosphate fertilization (No-P), in both the Vitalin SP11 treated and untreated control variants. The application of Vitalin T50, however, did not induce changes in the growth and phosphate acquisition of the wheat plants in the same experiment.

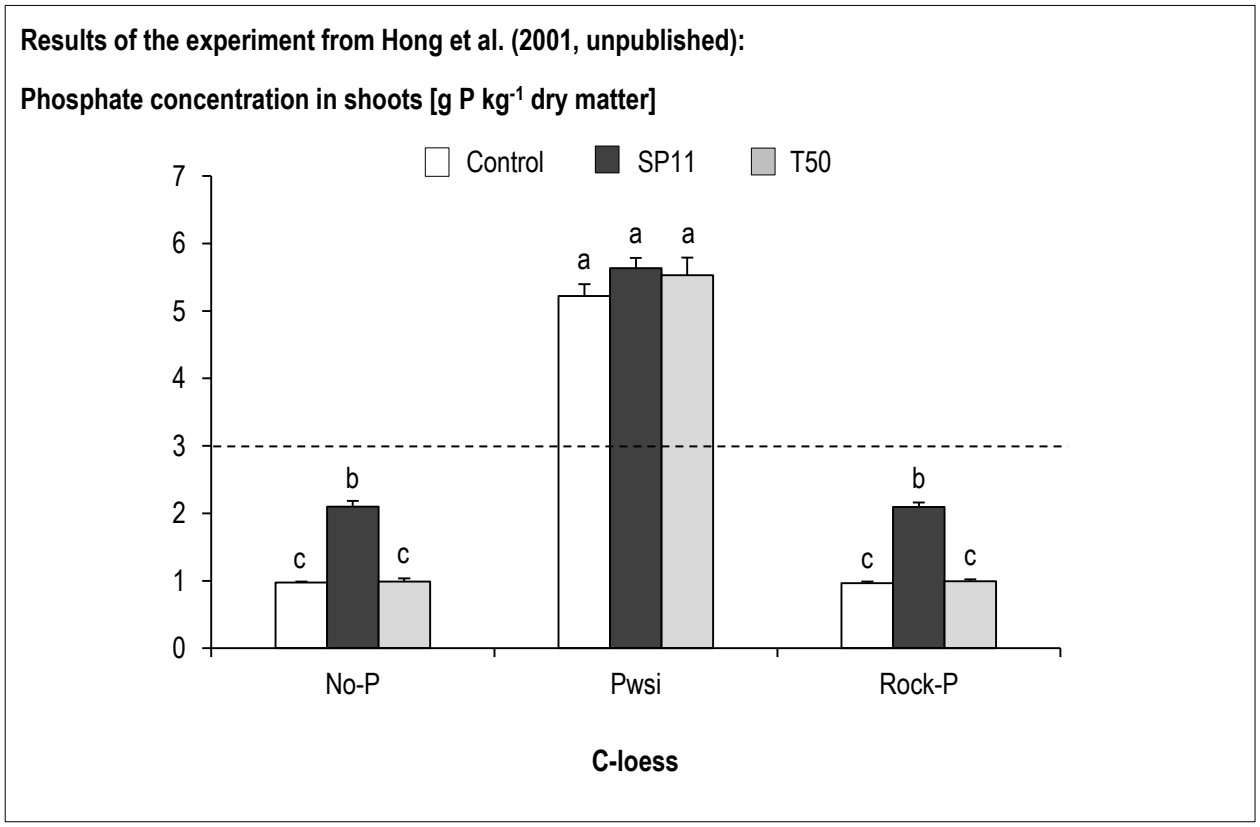

Fig. 5.13: Phosphate concentration in the shoot tissue of wheat (Triticum aestivum L.) plants treated or not (Control) with the bio-fertilizers Vitalin SP11 (SP11) or Vitalin T50 (T50) after 44 days of cultivation on the C-loess substrate with different phosphate fertilizer treatments (No-P, Rock-P, Pwsi) in the pot experiment from Hong et al. (2002, unpublished). The dashed line (----) indicates the minimum P concentration in the above ground biomass of wheat that is sufficient for adequate growth as reported by Bergmann (1992).

Columns represent mean values ± SEM (n = 4). Data were tested by one-way ANOVA followed by Tukey's test. Values not sharing letters indicate significant ($p \leq 0.05$) differences between combined bio-fertilizer and phosphate fertilizer treatments.

Fig. 5.14: Shoot development of Andrographis paniculata Nees after 13 weeks in the pot experiment from Win et al. (2002, unpublished). Plants were cultivated on the C-loess substrate supplied with sparingly available organic phosphates (Porg) in combination without or with Vitalin SP11 (+ SP11; left picture). A further treatment was the application of only water soluble inorganic phosphate at a rate of 80 mg P kg^{-1} soil dry matter (80Pwsi; right picture).

That Vitalin SP11 can induce strong growth effects under certain, but hardly comprehensible, conditions has also been shown by Win et al. (2002, unpublished) in pot experiments conducted with the traditional medicinal plant *Andrographis paniculata* Nees (Fig. 5.14), which is widely cultivated in southern Asia (Jarukamjorn and Nemoto, 2008). In this investigations, the C-loess substrate with basal fertilization as described above in Chapter 5.2.2 was treated without phosphate supply (Control), with water soluble inorganic phosphate (P_{wsi}) at low rates of 20 or moderate rates of 80 mg P kg^{-1} dry soil matter, or with organic phosphates (P_{org}; each 100 mg P kg^{-1} dry soil matter in the form of Na-phytate and phosphoglycerate). The tropical plants were maintained under greenhouse conditions from the 30th August 2002 until the 21st November 2002 at the Institute of Plant Nutrition, Universität Hohenheim, Stuttgart, Germany.

As represented in Fig. 5.14 the application of Vitalin SP11 in combination with organic phosphate forms (P_{org}) induced tremendous growth enhancements, while plants supplied

only with P_{org} remained strongly retarded in their development throughout the experimental growth phase of 13 weeks. This effect was also highly significant in terms of root and shoot dry matter production by the plants ($p \leq 0.001$, Student's t-test for unpaired samples, numeric values of shoot dry matter production were transformed to their natural logarithms before analysis, data not shown in graphs).

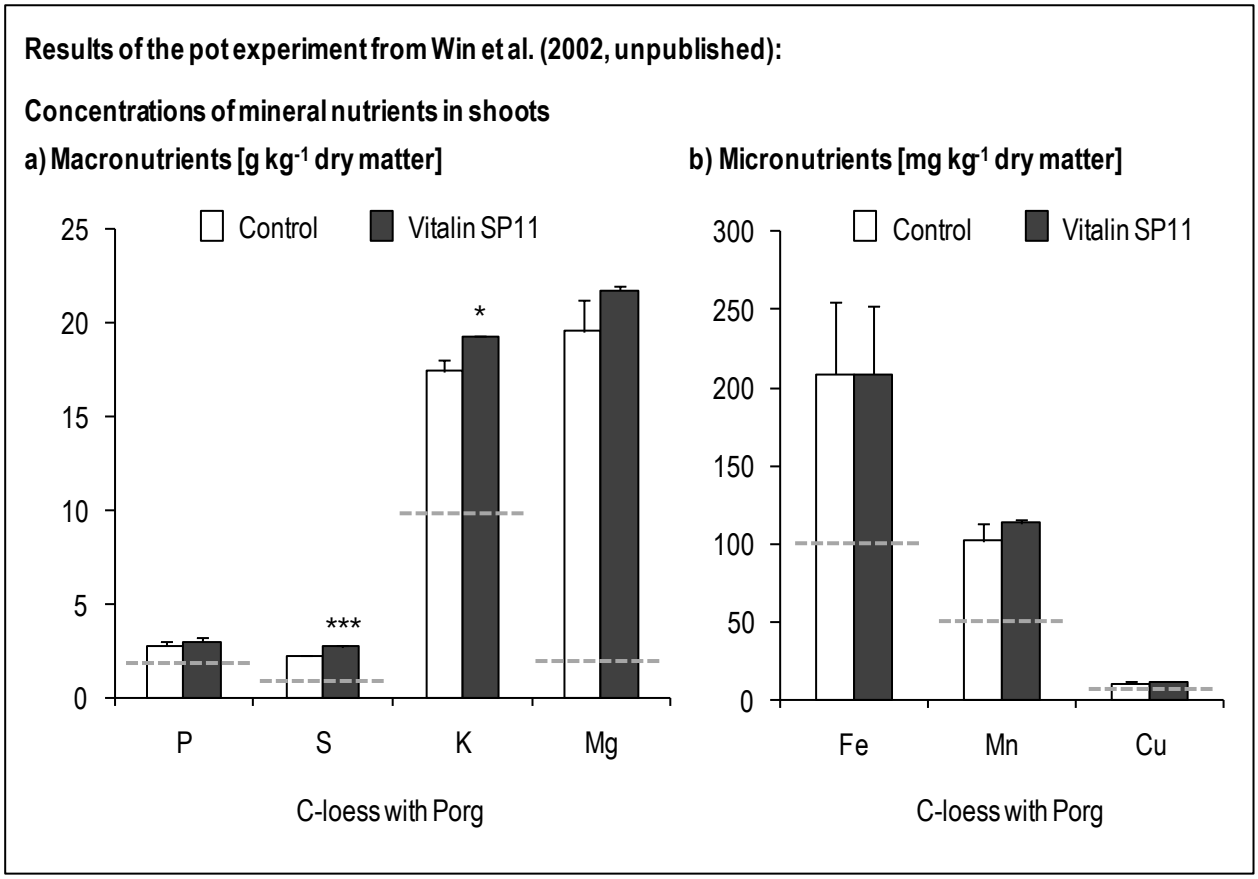

Fig. 5.15: Concentrations of (a) macro- (P, S, K, Mg) and (b) micronutrients (Fe, Mn, Cu) in the shoot dry matter of Andrographis paniculata Nees after 13 weeks in the pot experiment from Win et al. (2002, unpublished). Plants were cultivated on the C-loess substrate supplied with sparingly available organic phosphates (P_{org}) in combination without (Control) or with Vitalin SP11. The dashed lines (----) indicate average concentrations of mineral nutrients in the shoot dry matter of plants that are sufficient for adequate growth as reported by Marschner (1995).

Columns represent mean values ± SEM (n = 4). Data were tested using Student's t-test for unpaired samples, comparing Vitalin SP11 treated plants versus control plants. If necessary, numeric values of nutrient concentrations in shoot tissues were transformed to their natural logarithm to meet the requirements of equal variance and normal distribution of residuals. Significant differences are indicated by asterisks (*$p \leq 0.05$, ***$p \leq 0.001$).

Analyses of mineral nutrients in shoot tissues, however, indicated that the growth promotion of *A. paniculata* by Vitalin SP11 was probably not due to an enhanced nutritional status (Fig. 5.15a,b). Although the application of Vitalin SP11 resulted in a significant increase in the shoot concentrations of sulfur (S) and potassium (K), there was no clear effect on the supply of phosphate, the nutrient which was not applied in directly plant available forms (Fig. 5.15a). Furthermore, neither the Vitalin SP11 treated nor the respective control plants showed deficiencies in P, S, K or other mineral nutrients such as Mg, Fe, Mn and Cu under these conditions (Fig. 5.15a,b). And also, the weak development of plants, which were fertilized with water soluble inorganic phosphate at a sufficient rate of 80 mg P kg^{-1} soil dry matter ($80P_{wsi}$), suggested different reasons than nutrient deficiencies for their growth suppression (Fig. 5.14).

Indeed, as shown in Fig. 5.16a, the application of Vitalin SP11 increased the activity of acid phosphatase activity on the root surface. However, because incubation of root samples did not show alkaline phosphatase activities, root associated microorganisms, as potential producers of both acid and alkaline phosphatases (Tarafdar and Claassen, 1988; Tarafdar et al., 2002) did not seem to make a direct contribution to the production of these enzymes in the rhizosphere. Rather, it can be assumed that the enhanced acid phosphatase activities associated with the roots of Vitalin SP11 treated plants were produced by the plants themselves as due to their generally enhanced vitality. Besides, the application of Vitalin SP11 did not significantly affect the activities of acid and alkaline phosphatases in the bulk soil (Fig. 5.16b).

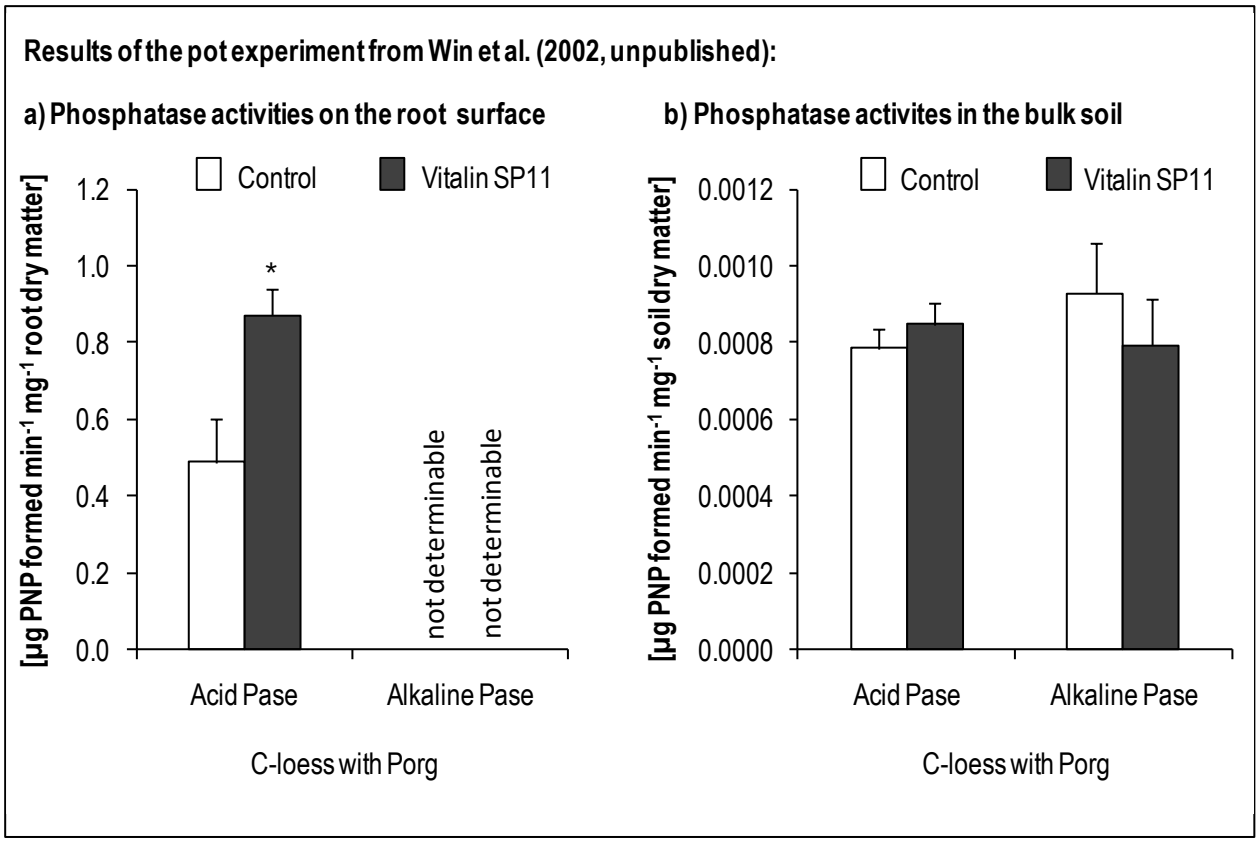

Fig. 5.16: Activities of acid and alkaline phosphatases on the root surface of Andrographis paniculata Nees (a) and in the bulk soil (b) after 13 weeks in the pot experiment from Win et al. (2002, unpublished). Plants were cultivated on the C-loess substrate supplied with sparingly available organic phosphates (P_{org}) in combination without (Control) or with Vitalin SP11. Samples were analyzed in a spectrophotometric assay according to the method of Tabatabai and Bremner (1969), modified by Alvey et al. (2001; see also: Eivazi and Tabatabai, 1977; Tabatabai, 1982). The phosphatase activities are expressed in units of p-nitrophenol (PNP) formed by enzymatic hydrolysis of the chromogenic substrate p-nitrophenyl phosphate after incubation of fresh root pieces respectively bulk soil samples in reaction tubes.

Columns represent mean values ± SEM (n = 4). Data were tested using Student's t-test for unpaired samples, comparing Vitalin SP11 treated plants versus control plants. Significant differences are indicated by asterisks (*$p \leq 0.05$).

An even weaker growth than in the phosphate-fertilized treatments was, however, observed on the C-loess substrate without phosphate supply (No-P). Here, Vitalin SP11 stimulated the formation of fine and lateral roots (Fig. 5.17a), but was not suitable to enhance the root and shoot biomass production of *Andrographis paniculata* (Student's t-test for unpaired samples, data not shown). In combination with low supply rates of 20 mg P_{wsi} kg^{-1} soil dry matter, by contrast Vitalin SP11 increased the formation root and shoot biomass significantly (Fig. 5.17b). These findings indicate that the root growth

stimulatory effect of Vitalin SP11 was only beneficial for the plant development when a certain level of plant available phosphate was readily supplied to the soil, whereas the solubilization of Ca-phosphates from the C-loess material did not appear to play a major role.

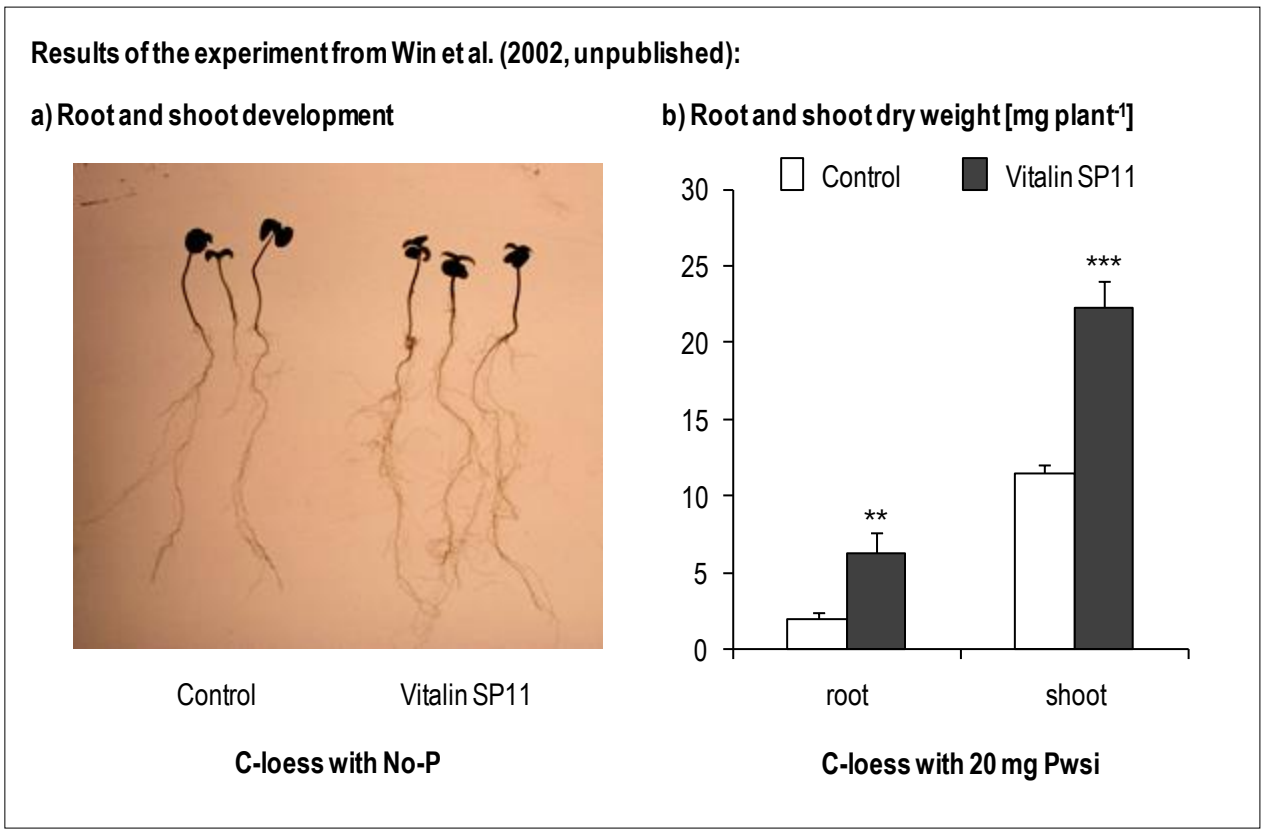

Fig. 5.17: Andrographis paniculata Nees cultivated for 13 weeks in the pot experiment from Win et al. (2002, unpublished). The plants were treated without (Control) or with the bio-fertilizer Vitalin SP11. (a) Root and shoot development on the C-loess substrate without phosphate fertilizer supply (No-P). (b) Root and shoot dry weight production on the C-loess substrate supplied with water soluble inorganic phosphate at a low rate of 20 mg P kg^{-1} (20 mg P_{wsi}) soil dry matter.

Columns represent mean values ± SEM (n = 4). Data were tested using Student's t-test for unpaired samples, comparing Vitalin SP11 treated plants versus control plants. Numeric values of root dry weights were transformed to their natural logarithm to meet the requirements of equal variance and normal distribution of residuals. Significant differences are indicated by asterisks (**$p \leq 0.01$, ***$p \leq 0.001$).

The induction of alterations in root morphology allowing the plant to explore a greater soil volume for the acquisition of mineral nutrients is regarded as an important effect of plant growth promoting microorganisms including strains of *Pseudomonas* spp., *Bacillus* spp. and *Streptomyces* spp. (Vessey, 2003; Hasegawa et al., 2008; Richardson et al.,

2009). Such beneficial stimulation of root development has been mainly attributed to the microbial production of phytohormones such as auxins, cytokinins, and gibberellins (Aldesuquy et al., 1998; Khalid et al., 2004a,b; Wittenmayer and Merbach, 2005; Ortíz−Castro, 2008). In addition, the modification of plant hormone levels, as by lowering of plant ethylene levels through deamination of 1-aminocyclopropane-1-carboxlate (ACC), the immediate precursor of ethylene in higher plants, has been reported as a major mechanism utilized by rhizobacteria to facilitate plant development (Penrose and Glick, 2003; Blaha et al., 2006; Naveed et al., 2008; see also Chapter 2). Furthermore, phytohormones from algae extracts (Featonby-Smith and van Staden, 1983) as well as phytohormone-like activities of humic compounds (Muscolo et al., 2007) contained in Vitalin SP11 could have been responsible for the stimulated root growth of *Andrographis paniculata*. However, enhanced root branching and production of fine roots are common morphogenetic responses in plants to phosphate starvation and other abiotic stresses (Marschner, 1998; Neumann and Römheld, 2002; Potters et al., 2007). The application of Vitalin SP11, therefore, might have encouraged these plant own strategies by tightening the stress situation due to competition between inoculated microorganisms and roots for a severely limited phosphate supply.

5.4.4 The possible role of environmental factors in the variability of results

The presented research has shown that the effects of Vitalin SP11 varied largely among different experiments and were transient. Inoculation with Vitalin SP11 increased plant growth and phosphate acquisition in some cases, but it was also found that it had no or even adverse effects. The producer (Vitalin Pflanzengesundheit GmbH, Ober-Ramstadt, Germany), however, confirmed that the composition and quality did not vary between the different charges of this bio-fertilizer preparation. Therefore, uncontrolled factors such as varying temperature and light conditions in the greenhouse with limited climate control, but probably also the plant genotype, appeared to have a stronger influence on the effectiveness of Vitalin SP11 than the investigated soil or fertilizer treatments. Contemporary climate data from a nearby meteorological station, including monthly sunshine duration and average air temperature as illustrated in Fig. 5.18, reflect the variability in environmental conditions experienced by the plants during the growth

periods of different experiments. Intensive solar irradiation caused already at moderate outside temperatures high temperatures peaks in the greenhouse and thus adverse conditions for the vegetative growth of wheat (Porter and Gawith, 1999). Accelerated early development without tillering and premature shift to the generative growth phase probably intensified the deficiency situation due to the high rates of phosphate uptake required by wheat plants during early stages of development (Whitfield and Smika, 1971; Römer and Schilling, 1986). Moreover, retarded root growth due to high soil temperatures (> 20 °C) and low photosynthetic capacity of the weakened plants could have inhibited the activity of phosphate solubilizing microorganisms in the rhizosphere (Přikryl and Vančura, 1980; Porter and Gawith, 1999).

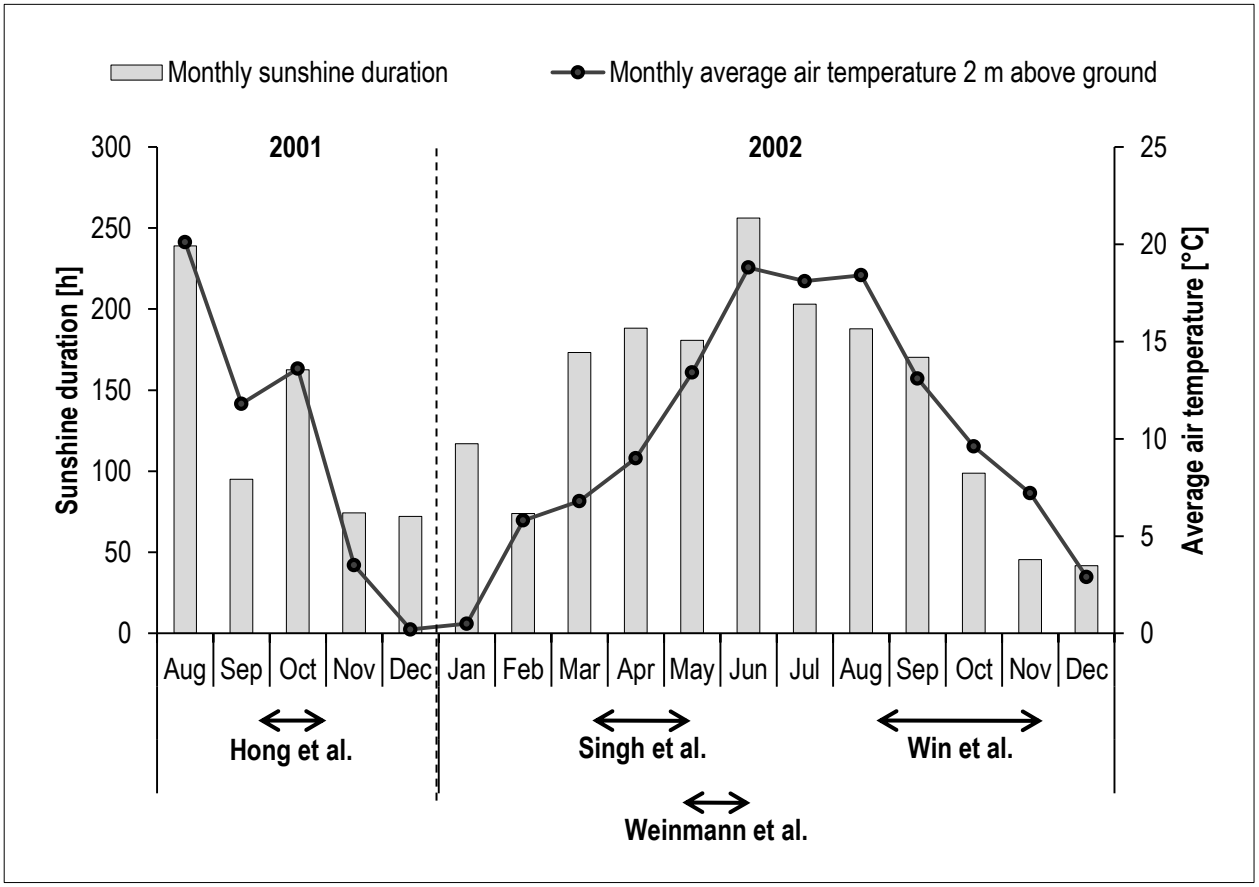

Fig. 5.18: Monthly sunshine durations and average temperatures 2 m above ground during the experimental growth phases (↔) of the experiments from Hong et al. (2001, unpublished), Singh et al. (2002, unpublished), Win et al. (2002, unpublished) and of the own investigations presented in Chapter 5.2 and 5.3 (Weinmann et al.). Data were derived from the meteorological station of the Insitut für Physik und Meteorologie at Universität Hohenheim, Stuttgart, Germany.

Outlook on the interaction of bacterial bio-fertilizers with mycorrhizal fungi

Besides achieving a better understanding of how factors of the abiotic environment affect the effectiveness of microbial inoculants, interactions with other microorganisms such as those of soil indigenous populations are of considerable interest. In this regard, arbuscular mycorrhizal fungi (AM-fungi) which are important for the mineral nutrition of most surveyed plant species (Wang and Qiu, 2006) could be particularly worthwhile for further investigation. These fungi are well known to explore a great soil volume by their fine mycelium to take up mineral nutrients such as phosphate from the soil solution for transfer to the symbiotic root in exchange for carbohydrates (Smith and Read, 1997; Ferrol et al., 2000). However, due to their limited capacity to mobilize sparingly available phosphates, arbuscular mycorrhizas alone may not appreciably improve the phosphate acquisition of plants grown on soils with extremely low plant available phosphate concentrations in the soil solution (Mosse, 1986; Bolan, 1991; Richardson, 2001; Richardson et al., 2009). Under such conditions, mycorrhization will rather suppresses plant growth as the mycorrhizal fungi represent a strong sink for photosynthates independent whether the plant may benefit or not (Douds et al., 1988; Marschner, 1995).

Table 5.3: Results from Elke Neumann (unpublished) demonstrating the influence of arbuscular mycorrhiza (AM) on the biomass production, mycorrhizal root colonization and phosphate status of potato (Solanum tuberosum L.) plants grown on soil substrates extremely low in plant available (CAL extractable) phosphate. The substrates, containing diverse sparingly soluble phosphate forms such as Ca-phosphates (C-loess), Fe- and Al-phosphates Arenosol, or organically bound phosphates (Cambisol), were inoculated with arbuscular mycorrhizal fungi (+ AMF inoc.) or not (No AMF inoc.). The study was conducted as a pot experiment under greenhouse conditions at the Institute of Plant Nutrition, Universität Hohenheim, Stuttgart, Germany.

Soil unit (CAL extractable P [mg kg^{-1} dry matter])	Dry matter [g $plant^{-1}$]		AM infected root length [%]	Phosphate concentration in shoots [g P kg^{-1} dry matter]	
	No AMF inoc.	+ AMF inoc.	+ AMF inoc.	No AMF inoc.	+ AMF inoc.
C-loess (4.5)	4.30	3.85	42.0	1.5	1.8
Arenosol (3.0)	3.50	3.15	14.0	1.3	1.6
Cambisol (6.9)	4.85	4.13	8.8	1.4	1.7

For the phosphate deficient soil materials which were also used in the present study, this issue has been conclusively demonstrated by Elke Neumann (unpublished) in pot experiments with potato (*Solanum tuberosum* L.) plants that were colonized or not by AM-fungi (Table 5.3).

In recent years, however, the possibility of beneficial interactions between arbuscular mycorrhizal fungi and phosphate solubilizing or mineralizing microorganisms has been attracting increasing attention (Azcon et al., 1976; Kucey, 1987; Tarafdar and Marschner, 1995; Toro et al., 1997; Kim et al., 1998a; Toro et al., 1998; Frey-Klett et al., 2007; Richardson et al., 2009). Consistently, research from Yusran (2009) demonstrated that dual treatment with AM-fungi and an inoculant of phosphate solubilizing bacteria, i.e. Proradix® (see Chapter 4.3), improved the growth and phosphate acquisition of tomato (*Lycopersicon esculentum* Mill.) plants to a greater extent than separate inoculation with either AM-fungi or the bacterial preparation alone did (Table 5.4). Thereby, the bacterial inoculant promoted the establishment of the mycorrhizal symbiosis. Measured in terms of mycorrhizal infected root length, this effect was particularly pronounced when the application rate of the mycorrhizal inoculum was low (i.e. 2.3 times higher infection rate at the level of 0.5 % AMF inoc.; Table 5.4). In subsequent experiments, culture assays with selective growth media furthermore indicated that inoculation with Proradix® increased absolute numbers of fluorescent pseudomonads and decreased the population densities of certain fungal pathogens (i.e. *Fusarium* and *Phytopthora* spp.) in the rhizosphere of tomato plants, suggesting competitive colonization by the respective strain of *Pseudomonas* sp. *Proradix*® (Yusran, 2009). Insufficient rhizosphere colonization, in contrast, is regarded as a major reason for the often observed ineffectiveness of microbial inoculants under field conditions (Lugtenberg et al., 2001; Compant et al., 2010).

Table 5.4: Results from Yusran (2009) showing the interactive effect of a bacterial bio-fertilizer preparation and arbuscular mycorrhizal fungi on the root and shoot biomass production, mycorrhizal infection, and phosphate acquisition of tomato (Lycopersicon esculentum Mill. var. Hellfrucht Hillmar) six weeks after sowing. The plants were treated without (Control) or with Proradix® (Pseudomonas sp. Proradix, strain DSMZ 13134; Sourcon Padena, Tübingen, Germany) and additionally supplied with increasing amounts of arbuscular mycorrhizal fungi inoculum (No, 0.5, 2.5, and 5.0 % AMF inoc.; Glomus intraradices strain 510; Myko plant, Mykotek Biotechnik GbR, Hannover, Germany). As growth substrate, a C-loess/sand mixture with adequate basal fertilization (N, K, Mg, Fe) and a moderate phosphate supply of 50 mg P kg^{-1} C-loess dry matter was used. The study was conducted as a pot experiment with controlled soil temperature (27 °C) under greenhouse conditions at the Institute of Plant Nutrition, Universität Hohenheim, Stuttgart, Germany.

Values represent mean values ± SEM (n = 5). Data were tested in two-way ANOVAs followed by Tukey's test. Significant effects of the Proradix® versus the control treatment are indicated by asterisks (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001) for each level of inoculation with arbuscular mycorrhizal fungi. Values in columns not followed by sharing letters indicate significant differences between different levels of inoculation with arbuscular mycorrhizal fungi within the control or Proradix® treated group respectively [Tukey's test, p ≤ 0.05]. Significant p values (p ≤ 0.05; two-way ANOVA) indicative of interactive effects (Intreactions) between the two factors of variability "bacterial bio-fertilizer treatment (Control, Proradix®)" and "inoculation with arbuscular mycorrhizal fungi" are given below columns.

	Root dry weight [g $plant^{-1}$]		**Shoot dry weight [g $plant^{-1}$]**	
	Control	Proradix®	Control	Proradix®
No AMF inoc.	0.31 ± 0.02 (b)	0.51 ± 0.06 (b**)	2.55 ± 0.13 (c)	4.02 ± 0.12 (c***)
0.5 % AMF inoc.	0.61 ± 0.03 (a)	0.68 ± 0.03 (b)	3.63 ± 0.07 (b)	4.30 ± 0.23 (bc*)
2.5 % AMF inoc.	0.71 ± 0.02 (a)	0.91 ± 0.04 (a**)	4.52 ± 0.29 (a)	5.23 ± 0.27 (a*)
5.0 % AMF inoc.	0.78 ± 0.04 (a)	1.01 ± 0.10 (a**)	4.50 ± 0.24 (a)	4.97 ± 0.08 (ab)
Interactions	no significant interaction		no significant interaction	

	Mycorrhiza infected root length [%]		**Phosphate concentration in shoots [g P kg^{-1} dry matter]**	
	Control	Proradix®	Control	Proradix®
No AMF inoc.	0.5 ± 0.1 (d)	0.6 ± 0.1 (d)	1.87 ± 0.04 (b)	2.01 ± 0.03 (b*)
0.5 % AMF inoc.	5.8 ± 0.0 (c)	13.4 ± 0.6 (c***)	2.12 ± 0.05 (a)	2.24 ± 0.07 (a)
2.5 % AMF inoc.	78.9 ± 0.9 (b)	89.7 ± 0.5 (b***)	2.22 ± 0.05 (a)	2.40 ± 0.04 (a*)
5.0 % AMF inoc.	92.7 ± 0.5 (a)	97.8 ± 0.2 (a***)	2.30 ± 0.04 (a)	2.30 ± 0.06 (a)
Interactions	p ≤ 0.001		no significant interaction	

5.4.5 Conclusions

The pot experiments with different types of soil substrates and forms of phosphate fertilizers revealed that beneficial effects were possible in connection with sparingly available Ca-phosphates, Fe- and Al-phosphates, as well as organic phosphates. In some cases, improved plant growth was associated with enhanced phosphate acquisition, which was attributable to mechanisms of increased spatial and chemical nutrient availability to the plant roots. The variability and low reproducibility of the observed effects, however, revealed that general conclusions drawn from the results of isolated experiments could be very misleading. Rather, it became evident that based on the diversity of plant genotypes and environmental influences, a wide range of potential factors needs to be considered for determining a set of conditions required by introduced rhizosphere microorganisms to establish and interact with the plant root for a targeted effect. For example, climatic or environmental simulation chambers could help to limit the number of unpredictable factors to well designed factorial analyses while more nearly approximating to the complexity of natural ecosystem conditions (Hartmann et al., 2009). Such approach would allow to investigate the performance of plants, associated microorganisms and their interactive rhizosphere processes under extreme conditions in order to isolate critical components for the successful application of bio-fertilizers in the field.

Nevertheless, the presented results already demonstrated clearly that the most significant effects of Vitalin SP11 on plants were achieved under conditions of severely to moderately low phosphate availability but not with sufficiently fertilized soils. It is still uncertain whether the magnitude of such effects can be large enough to allow economically feasible production on poor soils or with lower fertilizer inputs in high yielding agriculture. Although, in most of the above reported cases the overall performance of plants showing enhanced growth after treatment with Vitalin SP11 was still far below optimum levels, the example with the tropical herb *Andrographis paniculata* showed that under certain circumstances tremendous gains in productivity were possible. These findings, together with the observed synergy of bacterial inoculants and arbuscular mycorrhizal fungi, indicate an important role of highly complex interactions between plants, microorganisms and their physico-chemical environment for

the expression of versatile bio-fertilizer effects, beyond direct mechanisms of phosphate solubilization or phytohormonal growth stimulation.

Thus, besides investigating the mechanisms that are directly involved in phosphate mobilization in the rhizosphere of crop plants, future research could focus on two major aspects. One is directed towards gaining advanced understanding on how and under which conditions beneficial microorganisms efficiently colonize the plant environment and express their targeted traits in situ (Compant et al., 2010). The second issue is the application and revision of by such means gained knowledge in multi-year and multi-site field trials with a range of crops taking into account the full range of conditions prevailing in agricultural practice (Leggett et al., 2001). Such approaches may not only lead to a more successful and reliable use of bio-fertilizer inoculants, but also include the management soil indigenous populations as the majority of the microflora in the rhizosphere is naturally recruited from the soil environment (Jakobsen et al., 2005; Turner et al., 2006; Hartmann et al., 2009).

6 Bio-fertilizer applications for improved phosphate availability in organic farming

6.1 Background and objectives

Phosphate balances in organic agriculture

Organic farming systems rely on the activity of healthy soil life and on closed nutrient flows for maintaining soil fertility in agricultural production (Francé, 1921; Steiner, 1924; Rusch, 1968; Lampkin, 2002). Therefore, the use of easily soluble mineral fertilizers and synthetic pesticides is strictly limited or prohibited, whereas high levels of biodiversity, the use of natural compounds, and the sustainable management of natural resources are emphasized for an environmentally friendly and ecologically safe food production (e.g. IFOAM, 2006; European Council Regulation (EC) No 834/2007). Accordingly, also the use of water-soluble phosphate fertilizers is not permitted in organic agriculture, while certain naturally occurring phosphate minerals (e.g. rock phosphates) or basic slag, a phosphate bearing by-product of the steel industries, can be applied as sparingly soluble mineral P-fertilizers, besides a range of organic wastes and manures (e.g. IFOAM, 2006; European Commission Regulation (EC) No 889/2008).

Organic farms, however, are typically characterized as low external input agro-ecosystems, following the concept that the farm as a whole is an autarkic organism with self-sustaining interactions between its components and nearly closed material flows (Steiner, 1924). Among other inputs, all fodder used in organic husbandry should come from the farm itself or be produced within the region, according to the rules of organic farming (IFOAM, 2006). The recycling and efficient management of organic matter to minimize the loss of mineral nutrients and the furtherance of an active healthy soil life, therefore, are the primary means to sustain adequate nutrient availability in organic crop production (IFOAM, 2006; Haas et al., 2007). Exports in form of livestock products and crops from the farm to the markets, by contrast, can significantly disturb the delicate whole-farm balance of nutrient flows (Gysi and Schwaninger, 1998; Goulding et al., 2008). Furthermore, the content of available phosphate in soils tends to decrease with

time after application, due to its continuous conversion into sparingly soluble forms (Bezama and Aomine, 1977; Pierzynski et al. 2005). Although it may take decades until critical levels of available phosphate are reached in well-buffered soils, the decrease of available soil phosphate can become an important yield-limiting factor and thus a key question in organic agriculture, even on farms with import surpluses for phosphate (Oehl et al. 2002; Oberson and Frossard, 2005). For example, John et al. (1990; cited in: Oberson and Frossard, 2005) observed symptoms of phosphate deficiency in crops grown on a farm that had been organically managed for 37 years. The positive phosphate balance of this farm was achieved mainly by the input of rock phosphate and other rock powder to compensate for the output of phosphate. In general, however, the phosphate input of organic farms is much lower than in conventional or integrated agriculture, due to the restrictions imposed by organic production standards. A number of input/output studies have shown negative farm gate budgets for phosphate in organic farming systems with and without animal husbandry (Wieser et al., 1996; Lindenthal, 2000; Öborn et al., 2005).

Moreover, also in conventional farming systems where the input of easily soluble mineral phosphate fertilizers, such as superphosphates, is allowed according to sound farming practices (e.g. LfL, 2012), increasing fertilizer costs have been conducive to an insufficient replacement of phosphorus removals in recent years (Kerschberger und Preusker, 2014; Seibert und Wiesler, 2015).

Current strategies to improve the availability of phosphate in organic agriculture

To address the obstacle of phosphate depletion and low availability, farmers, and organic farmers in particular, need to employ a range of measures for improving the acquisition of phosphate from sparingly available soil reserves and fertilizer sources, besides to prevent avoidable losses from the farm internal cycles. Management strategies that are routinely adopted in this regard include the cultivation of efficient crops and varieties, adjusted crop rotations, mixed cropping, and cultural practices that help to improve the structure and biological activity of soils (Lindenthal, 2000; Oberson and Frossard, 2005; Kirkby and Römheld, 2006). Furthermore, composting with organic materials can improve the availability of phosphorus from rock phosphates (Döring, 1956; Bangar et al., 1985), whereas directly applied rock phosphates as well as basic slag are generally considered as

poor phosphate sources for field crops, particularly in neutral or alkaline soils (Mishra et al., 1981; Davies, 1984; Bekele and Höfner, 1993).

Microbial bio-fertilizer preparations

Regarding the central role of root-induced and microbial processes in the transformation of fertilizer and accumulated soil phosphates, besides indirect management through cultural practices also the direct application of root growth stimulating and phosphate mobilizing bio-fertilizers appears as an attractive proposition to improve the phosphate availability to crop plants (Jakobsen et al., 2005; Richardson et al., 2009). As previously shown in laboratory and greenhouse experiments with commercial bio-fertilizers such as Vitalin SP11 and Vitalin T50 (Vitalin SP11, Vitalin Pflanzengesundheit GmbH, Ober-Ramstadt, Germany), beneficial effects on plant growth and phosphate uptake are most likely to be expected with soils that are low in plant available phosphate concentrations (see Chapter 4 and 5). As described in Chapter 4, Table 4.1, Vitalin SP11 contains bacterial inoculants (i.e. strains of *Bacillus subtilis*, *Streptomyces* spp., and *Pseudomonas* sp.) together with humic acids and algae extracts, and the active ingredient of Vitalin T50 are spores of the fungal species *Trichoderma harzianum*.

Some articles in the literature provide evidence how appropriate application strategies and an adapted crop management can enhance the effectiveness of microbial bio-fertilizers. Mixtures of different microbial species, for example, proved advantageous when compared to the application of single strains (Kundu and Gaur, 1984). Accordingly, the combination with *Trichoderma* fungi can increase the effectiveness of *Bacillus* spp. and *Pseudomonas* spp. based bacterial inoculants, which has been attributed to cumulative effects, such as enhanced supply of mineral nutrients to the crop, production of growth promoting compounds, and the suppression of soil borne pathogens by these organisms (Duffy et al., 1996; Rudresh et al., 2005a). In addition, the supply of organic manure can enhance the proliferation of phosphate solubilizing bacteria in the rhizosphere of crop plants by ameliorating the chemical and physical soil environment, as reported by Kundu and Gaur (1980). So far, however, no previous investigations are known which tested the effectiveness of the microbial bio-fertilizer preparations Vitalin SP11 and Vitalin T50 under field conditions.

Biological superphosphate

Another biological strategy relies on the microbially mediated oxidation of elemental sulfur (S) to sulfuric acid (H_2SO_4), which in turn can decrease the pH and enhance the plant availability of mineral nutrients, especially phosphate, in neutral and alkaline soils (Lipman et al., 1916; Rajan, 1981; Lindemann et al., 1991; Germida and Janzen, 1993; Müller et al., 2007). Like with the production of regular superphosphate fertilizer, in which finely ground rock phosphate is dissolved with sulfuric acid (Leikam and Achorn, 2005), several experiments have indicated that the sulfuric acid produced by sulfur oxidizing microorganisms can increase the release of phosphorus from rock phosphate. Correspondingly, the admixture of sulfur to enhance the dissolution of rock phosphate by microbial means has been announced as a biological superphosphate or "bio-super" (Swaby, 1975; cited in: Swaby and Fedel, 1977). Many different microorganisms oxidize elemental S and reduced sulfate compounds as a source of energy, among which members of the genus *Thiobacillus* (Beijerinck, 1904; cited in: Kelly and Wood, 2000) are regarded as an important group in agricultural soils (Germida and Janzen, 1993; Robertson and Kuenen, 2006; Besharati, 2007). Cultures of *Thiobacillus* spp., added to the soil or to mixtures of sulfur and rock phosphate, can be useful to ensure the effectiveness of bio-super applications if the soil does not already contain sufficient populations of sulfur oxidizers (Rajan, 1981; Rajan, 1982).

Biological dynamic preparations

A very distinct form of organic farming is the method of biological dynamic (biodynamic) agriculture, which was inspired by a series of eight lectures concerning the "spiritual foundations for the prosperity of agriculture", which was given by the philosopher Rudolf Steiner in 1924 (Steiner, 1924; Kirchmann et al., 2008). A central emphasize in Steiner's philosophy as well as in today's practice of biodynamic farming is directed towards the transfer of "terrestrial" and "cosmic forces" into soil and crops, not only to improve the agricultural production, but also to sustain the physical and spiritual live of human beings on earth. For that purpose, Steiner instructed farmers to manufacture eight preparations by using certain minerals, plant materials and animal remains, which are to be applied on the field (e.g. 5 g cow horn-quartz per hectare) or to composting farm yard manure (e.g. 1 to 2

cm³ plant material per 10 cm³ manure) at very low concentrations (Steiner, 1924; von Wistinghausen et al., 2005 and 2007; Kirchmann et al., 2008). As a treatment, which stimulates manure to relate in the right way to phosphorus, Steiner (1924) suggested in particular the admixture of diluted press sap from the flowers of *Valeriana officinalis*. The instructions Steiner gave were not based on natural sciences but rather derived from inspirations gained during mental exercises, and the existence of the "forces" he proposed has not been confirmed empirically so far (Steiner, 1924; Raupp, 1999; Carpenter-Boggs et al., 2000a; Zaller and Köpke, 2004; Jayasree and George, 2006; Kirchmann et al., 2008; Scheper et al., 2009; Turinek et al., 2009).

Objectives of the present study

In the present work, Vitalin SP11 and Vitalin T50 were applied under the conditions of organic farming practice in multi year and site experiments, intended to study their influence on the growth, phosphate acquisition and yield of various field crops of the local crop rotations. Both products were tested in single and combined applications in order to detect possible synergistic actions. Furthermore, the influence of farmyard manure on the effectiveness of these microbial bio-fertilizers was investigated.

In addition to the studies on the microbial bio-fertilizer preparations, different approaches to improve the phosphate nutrition of crops in organic farming systems were tested for their effectiveness under field conditions, too. At one experimental site, treatments with "bio-super" phosphate according to the method described by Rajan (1981) were performed in cooperation with Schulz (2009). Another innovative product, "BioAktiv" (BioAktiv-Pulver Produktions- und Vertriebs GmbH, Würchwitz, Germany), has been proposed to improve the growth and mineral nutrition of plants by the action of "activated" oxygen. Referring to the manufactures information, BioAktiv contains only water-soluble magnesium sulfate heptahydrate (Epsom salt, $MgSO_4 \cdot 7\ H_2O$) which is "activated" by the influence of unknown "cosmic forces" during the production process, thus sharing some similarity with the biodynamic preparations described by Steiner (1924). The "activated" product is further supposed to exert its effect by propagating beneficial soil bacteria, which indirectly stimulates the root growth of crops and may lead to yield increases of up to 20 %.

6.2 Material and Methods

6.2.1 Sites of the field experiments

Geographic locations and farm management systems

The present investigations were conducted at two organic farms in Baden-Württemberg, Germany.

The first experimental site (Longitude: 48°44'28" N; Latitude: 9°11'54" E) was located at the research farm for organic farming Kleinhohenheim of the Universität Hohenheim, in Stuttgart, which has been managed according to organic farming principles since 1994. The area, which lies approximately 435 m above sea level, receives on average about 700 mm of precipitation per year and has a mean annual temperature of 8.8 °C. To maintain soil fertility, an eight-year crop rotation including two years of clover and grass mixed stands followed by winter wheat (*Triticum aestivum* L.; 3rd year), oat (*Avena sativa* L.; 4th year), fava bean (*Vicia faba* L.; 5th year), spelt wheat (*Triticum spelta* L.; 6th year), hoeing row crops such as potato or maize (*Solanum tuberosum* L. or *Zea mays* L.; 7th year), and triticale (*Triticum aestivum* L. x *Secale cereale* L.) with undersown clover (8th year) is used. Furthermore, cover crops are cultivated after the main crops of the 3rd until 6th year for improved soil quality, such as by improving soil structure, capturing nutrients, and suppression of weeds. Manure is obtained from organic sheep husbandry and applied to the fields with arable crops (Friedel and Gabel, 2001; Sarrantonio, 2007; Funk and Zikeli, 2010). During the years of investigations, however, the experimental plots were excluded from the customary fertilizer management and were also not treated with biodynamic preparations.

The second experimental site (Longitude: 48°16'07"N, Latitude: 8°45'24" E) was placed in a field of the farm Schönberghof near Rosenfeld-Isingen, which has been managed organically since 1968 and lies at about 620 m above sea level in the hilly country between Black Forest and Swabian Alb. Annual mean temperatures of 6.5 to 6.9 °C and rates of rainfall of 700 to 1,000 mm per year characterize the local climate. In the regular scheme of the crop rotation mixed stands of clover and grass (1st and 2nd year), wheat followed by *Fabaceae* spp. as cover crop (3rd year), oat followed by mustard (*Sinapis*

spp.) as cover crop (4th year), fava bean (5th year), spelt wheat (6th year), and rye (*Secale cerale* L., 7th year) are comprised. In addition, also emmer (*Triticum dicoccum* Schrank), an ancient wheat type, is cultivated on some fields. Manure to fertilize the field crops is in the main derived from the farm's own mother cow husbandry, whereby the animals are kept on pastures during summer and in a stable during winter. A site-specific problem was further seen in low temperatures early in the spring season which can inhibit the activity of roots and microorganisms and thereby the mobilization of phosphate in the soil (Dibb et al., 1990).

Soil characteristics and phosphate availability

At the experimental site in Kleinhohenheim the soil type consisted of a loamy clay with a slightly acidic pH value of 6.2 ($CaCl_2$). The estimation of the plant available phosphate concentration in this soil by use of the CAL extraction method according to Schüller (1969) revealed a concentration of 4.6 mg P 100 g^{-1} dry soil. This is in a low but still adequate range (> 4.5 mg P 100 g^{-1} dry soil) for crop production where no tremendous yield increases are to be expected from the addition of phosphate fertilizers (Kerschberger et al., 1997; Kerschberger, 2012).

The soil of the experimental site at the Schönberghof was rich in clay and silt with near neutral pH value of 6.5 ($CaCl_2$) and thus able hold large amounts of water and mineral nutrients. However, due to negative field balances for phosphate, the soil has been depleted over the past decades of organic production. According to the farmer's estimation, the annual net-export of phosphate averaged to a continuous loss of 5 kg P from arable crop fields and of 15 kg P from grassland per hectare. Therefore, symptoms of phosphate deficiency, such as decreased quantities and qualities of yields in crop production as well as nutritional disturbances in animal husbandry, became increasingly noticeable at the farm level in recent years. Analyses of the CAL-extractable phosphate, furthermore, indicated very low concentrations of less than 2.0 mg P 100 g^{-1} dry soil for samples taken from most of the land lots of the farm including the place of the present investigation. Total concentrations of phosphorus and organic carbon in the soil of this experiment, however, amounted to 1500 mg P kg^{-1} dry soil and 4.5 % C respectively. The use of phosphate fertilizers, but also adequate measures to enhance the availability of

recalcitrant soil phosphates, could therefore strongly increase the productivity of crop plants at the Schönberghof.

6.2.2 Crops, treatments and experimental set-up

The experiment at Kleinhohenheim was conducted in the years from 2002 until 2004 with oat (*A. sativa*), fava bean (*V. faba* var. Divine), and spelt wheat (*T. spelta*) respectively, according to the rotational crop sequence. Because the initially selected area of the experiment (initial area) revealed to be located in a very heterogeneous part of the field in 2002, it was moved to a more homogenous section of the same field (replacement area) and with slightly modified treatments and design in the following years.

In the two years of investigations at the Schönberghof, 2003 and 2004, wheat (*T. aestivum*) respectively emmer (*T. dicoccum*) were grown at the site of the studies. In both years, the experiment was replicated at two separate areas of the same field, whereby only one of them was fertilized in 2003 with liquid manure derived from the farm's own animal husbandry at the Schönberghof. For technical reasons, the manure treatments were not randomized, as a tank spreader equipped with a 12 m broad system of drag hoses was used to distribute the liquid manure. For farm internal reasons, however, in 2004 no liquid manure was supplied at all, so that only the residual effect of the previous year's manure treatment could be evaluated for that year.

Table 6.1 provides an overview of the various bio-fertilizer treatments per sites and years. Details regarding the application of the different preparations are further described in the following sections, whereas "control" plots were not subjected to any of these measures.

Application of the microbial bio-fertilizers

Vitalin SP11 and Vitalin T50 were applied two times per growing season, once at the beginning of the vegetation period (beginning of April – mid of May) and again about three to six weeks later (mid of May – beginning of June), which was at the end of the tillering stage of the cereal crops. Detailed information regarding the manufacturer, ingredients and supposed effects for each product have already been provided in Chapter 4, Table 4.1.

In the first year of the studies, 2002, the microbial bio-fertilizers were distributed with a customary garden sprayer on the field plots. By this method, however, the amount of water employed to disperse and spread the powdery products was very limited. In the following years, by contrast, watering cans with spray roses (polyethylene cans, Gebrüder Lippert GmbH, Alzenau, Germany) were used to apply the bio-fertilizers together with about 14 liters of water per plot (24 or 30 m^2), which allowed an improved soil intrusion. For that purpose, stock suspensions containing about 3 g per liter of Vitalin SP11 and/or Vitalin T50 were prepared with 2.5 mM $CaSO_4$ in deionized water at the laboratory. Tap water, which is usually chlorinated, was not used to avoid any inhibition of the microbial inoculants. Before application to the field plots, appropriate aliquots of the stock suspensions were further diluted with locally available unchlorinated water, such as from fresh ground or surface water bodies, to fill the watering cans. The preparations were applied at rates according to the instructions of manufacturers. Exact doses for each experiment are given above in Table 6.1.

Table 6.1: Overview of the different treatments performed in the present field experiments at Kleinhohenheim and Schönberghof from 2002 until 2004

Experimental site	Years (crops)	Acronym	Application rates
Kleinhohenheim	2002	Control	untreated control
(initial area)	(oat)	SP11	2 times 2 kg Vitalin SP11 ha^{-1}
		SP11+T50	2 times 2 kg Vitalin SP11 plus 1 kg Vitalin T50 ha^{-1}
		Superphos	35 kg P ha^{-1} as super phosphate (ca. 54 kg S ha^{-1} as sulfate)
		Rock-P+TiO_2	35 kg P ha^{-1} as rock phosphate with TiO_2
		Bio-super	35 kg P ha^{-1} as bio-super phosphate (1070 kg S ha^{-1})
Kleinhohenheim	2003	Control	untreated control
(replacement area)	(fava bean)	T50	2 times 2 kg Vitalin T50 ha^{-1}
		T50+SP11	2 times 2 kg Vitalin T50 plus 2 kg Vitalin SP11 ha^{-1}
		Superphos	40 kg P ha^{-1} as super phosphate (ca. 61 kg S ha^{-1} as sulfate)
		Rock-P	40 kg P ha^{-1} as rock phosphate
		Sulfur	30 kg S ha^{-1} as elemental S (Sferosol®, 87 % S)
		Gypsum	30 kg S ha^{-1} as natural gypsum (15 % S)
		Bio-super	40 kg P ha^{-1} as bio-super phosphate (1223 kg S ha^{-1})
Kleinhohenheim	2004	Control	untreated control
(replacement area)	(spelt wheat)	T50	2 times 2 kg Vitalin T50 ha^{-1}
		T50+SP11	2 times 2 kg Vitalin T50 plus 2 kg Vitalin SP11 ha^{-1}
		Superphos	25 kg P ha^{-1} as super phosphate (ca. 38 kg S ha^{-1} as sulfate)
		Rock-P	25 kg P ha^{-1} as rock phosphate
		Sulfur	25 kg S ha^{-1} as elemental S (Sferosol®, 87 % S)
		Gypsum	25 kg S ha^{-1} as natural gypsum (15 % S)
		Bio-super	25 kg P ha^{-1} as bio-super phosphate (765 kg S ha^{-1})
Schönberghof	2003	Control	untreated control
(with or without manure)	(wheat)	SP11	2 times 2 kg Vitalin SP11 ha^{-1}
		SP11+T50	2 times 2 kg Vitalin SP11 plus 2 kg Vitalin T50 ha^{-1}
		Epsom salt	2 kg $MgSO_4$•7 H_2O ha^{-1} (0.2 kg Mg and 0.3 kg S ha^{-1} as sulfate)
		BioAktiv	2 kg “activated” $MgSO_4$•7 H_2O ha^{-1}
Schönberghof	2004	Control	untreated control
(no manure application)	(emmer)	SP11	2 times 2 kg Vitalin SP11 ha^{-1}
		SP11+T50	2 times 2 kg Vitalin SP11 plus 2 kg Vitalin T50 ha^{-1}
		Epsom salt	2 times 2 kg $MgSO_4$•7 H_2O ha^{-1}
		BioAktiv	2 times 2 kg “activated” $MgSO_4$•7 H_2O ha^{-1}

Application of biological super superphosphate

The approach to improve the agronomic effectiveness of rock phosphate by microbial sulfur oxidation was only investigated at the experimental site Kleinhohenheim. For treatment with biological superphosphate ("Bio-super"), a product obtained from the BASF (Baden Aniline and Soda Factory, Ludwigshafen, Germany) was applied, which contained rock phosphate (Morocco apatite, 13.08 % P; 0.15 mm mesh) and elemental sulfur in the ratio of 4:1 by weight. The mixture of rock phosphate and sulfur was shaped in the form of small granules by using titanium dioxide (TiO_2) as a binding additive, in order to facilitate handling and to ensure proper contact between sulfur and rock phosphate when applied to the soil (Rajan, 1983; Müller et al., 2007). Thiobacilli were not added to the granules as populations of sulfur oxidizing microorganisms can be assumed to be ubiquitous in agricultural soils (Germida and Janzen, 1993). In further treatments only rock phosphate ("Rock-P"), rock phosphate with titanium oxide ("Rock-P+TiO_2"), elemental sulfur ("Sulfur"; Sferosol®, Esseco, Trecate, Italy, 87 % S), natural gypsum ("Gypsum"; 15 % S), or regular super phosphate ("Superphos"; 7.85 % P; ca. 12 % S) were applied separately for comparison. As recommended by the Landesanstalt für Landwirtschaftliche Chemie, Universität Hohenheim, Stuttgart, Germany and indicated in Table 6.1, the annual application rates were adjusted to the nutrient demand of the respective crop and incorporated into the soil before sowing.

Application of the "activated" Epsom salt

BioAktiv, the product consisting of "activated" Epsom salt, was only tested at the Schönberghof and distributed with watering cans at rates of 2 kg $MgSO_4$•7 H_2O per hectare as described in the previous section for the microbial bio-fertilizers, too. In the same manner, also "non-activated" raw material of the BioAktiv production, namely normal Epsom salt, was applied in order to separate the proposed specific effect of the "activated" product from the regular influence that magnesium or sulfate as mineral nutrients can have on the crops. According to the producers instruction, in 2003 only one application at the beginning of the vegetation period (17th May 2003) was performed. However, because there were no growth responses observed during that year, in 2004 the

treatments were supplied two times, first at the beginning of the vegetation period (19th May 2004), and second at the end of the tillering stage (09th June 2004).

Experimental design

At both sites, Kleinhohenheim and Schönberghof, the field experiments were arranged in randomized block designs in order to compensate for previously observed fertility gradients on the sloping fields (EPPO, 2006).

In 2002 at Kleinhohenheim, four replicate blocks were each divided into five main plots of 10 m in length and 8 m in width, which were treated with bio-super, Rock-P+TiO_2, Superphos or not (Control). One main plot per block was further divided in two subplots (5 m x 8 m) that were inoculated with SP11 or SP11+T50. The first year of the study, however, showed that the initially selected area of the experiment (initial area) was located in a part of the field appearing to be very heterogeneous in soil properties and fertility. For the following years of 2003 and 2004, therefore, the experiment was re-established at a more homogenous field section (replacement area) but with seven instead of five main plots (10.0 x 8.0 m) in each of four replicate blocks. On one of the additional main plots per block, the experiment was extended by the treatments of Sulfur and Gypsum, which were supplied to subplots of 5.0 x 8.0 m in size. Furthermore, the treatment of Rock-P+TiO_2 was replaced by Rock-P without TiO_2, for reasons outlined in the discussion section below. Two main plots per block, however, were divided into each three subplots of 3.3 m x 8 m in size that were either treated with T50, T50+SP11 or remained untreated as an internal control within each of the respective main plots. This part of the experiment with microbial bio-fertilizers could therefore be evaluated as a separate sub-experiment.

At the site of Schönberghof, five replicates for each treatment were carried out in the experiments with or without manure application. Plots of 5 m x 6 m in size were treated with SP11, SP11+T50, Epsom salt, BioAktiv or not (Control).

6.2.3 Evaluation of the field experiments

Not all types of the analyses described in the following sections were performed in all sites and years of investigations, as it was recognized that remarkable results could not be expected in most cases.

Experimental harvest and collection of yield data

Total above ground biomass of the cereal crops was harvested by cutting plants to a height of about 5 cm above the soil surface from patches of 60 cm in diameter. In this way, three subsamples were taken for each replicate. At Kleinhohenheim, also a plot combine of 1.60 m cutting width was available to harvest grain from the central 12.8 m^2 of each treatment plot, thus minimizing edge effects. Yields of straw, spikes and grain of the cereal crops were determined on air-dry weight basis. If necessary, plant samples were dried in drying cabinets with air ventilation at 35 °C. The hulled grains (groats) of oat and spelt wheat, which have bracts adherent tightly to the caryopsis even though after threshing, were not dehusked before analyses. In fava beans, leaf samples for mineral analysis were taken during flowering (26^{th} May 2003; 11 weeks after sowing) by picking the youngest fully expanded leaf from 16 plants per treatment plot and dried at 60 °C. At ripening at the end of July 2003, fava beans were harvested by collecting the pods of 10 plants per treatment plot at ripening. Besides numbers per plant of pods and seeds, dry weights (30 °C) were recorded to calculate yields of fava beans per hectare, which had an average crop density of 32 plants m^{-2}.

Mineral nutrient analyses in plant tissues

Before mineral nutrient analyses, dried plant materials were first shredded with a cutting mill (Brabender, Duisburg, Germany), and then ground to fine powder with mills agate disc swing mills (Fritsch, Idar-Oberstein, Germany). Bean seeds had to be freeze-dried (Piatkowski - Forschungsgeräte, München, Germany) to make them more suitable for grinding. Subsamples of ground plant material were dry ashed (500 °C) and acid digested (HNO_3 65 %) according to the procedure described previously in Chapter 5.2.3. Phosphate concentrations in the digests were estimated photometrically (U-3300, Hitachi, Tokyo, Japan) with ammonium-vanadate-molybdate as the color reagent (Gericke and

Kurmies, 1952b). With particular respect to the possible activities of the microbial bio-fertilizers to improve the plant availability of other nutritional elements than P, also Mn and Zn concentrations in the digested plant tissues were determined by atomic absorption spectroscopy (ATI Unicam Solaar 939, Thermo Electron, Waltham, USA). To eliminate interferences in the air-acetylene flame of the atomic absorption spectrometer, a buffer solution containing cesium chloride and lanthanum chloride (Merck, No. 116755) was added before measuring Mn concentrations in the sample solutions (Schinkel, 1984).

Statistical analyses

The statistical software program SigmaStat Version 2.03 (Systat Software Inc., Erkrath, Germany) was used to analyze all data sets. Data from each experimental unit, location and year were tested separately. The effects of the treatments and the blocks on the measured variables were distinguished by two-way analysis of variance (ANOVA). When ANOVA results indicated significant treatment effects, the analyses were followed by Tukey's test for multiple pairwise comparisons of means of the different treatment types. Treatments that were not appropriately randomized were excluded from statistical tests, as indicated below the respective graphs.

6.3 Results

In the present field experiments, treatments with microbial bio-fertilizers, biological superphosphate, or “activated” Epsom salt resulted in few considerable changes of the investigated crop characteristics.

6.3.1 Yields and mineral nutrition of crops grown at Kleinhohenheim

No data are shown for the experiment that was conducted with oat at Kleinhohenheim in the year 2002, as no significant responses to the experimental treatments were recorded in terms of biomass yields and phosphate concentrations in straw and grain samples. Likewise, the harvested seed yields and mineral nutrient concentrations (i.e. P, Mn, and Zn) in shoot tissues of fava beans grown at Kleinhohenheim in 2003 were not obviously affected by the different treatments. Exemplarily, data of the unchanged phosphate concentrations in the leaf tissue of fava beans are shown in Fig. 6.1., which were consistently in a sufficient range throughout all treatments as well as in the untreated controls.

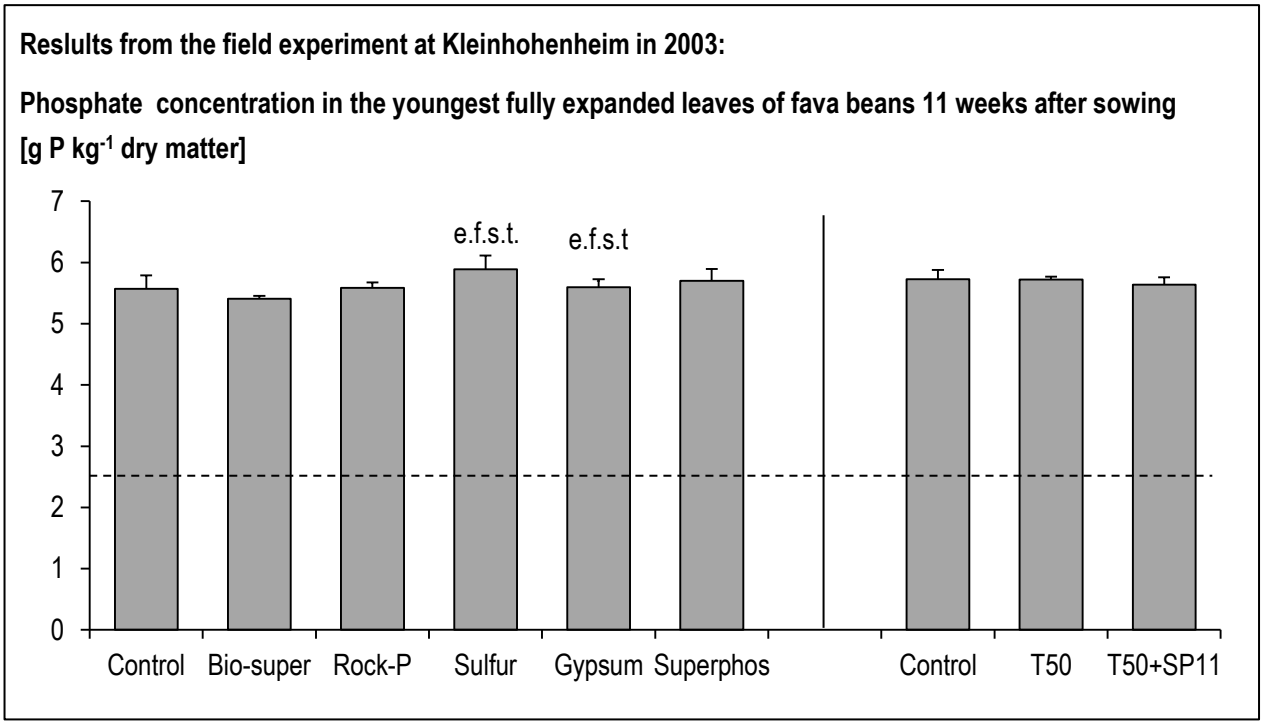

Fig. 6.1: Phosphate concentrations in the youngest fully expanded leaves of fava beans at flowering stage 11 weeks after sowing in the field experiment at Kleinhohenheim in 2003. Plants had been treated or not (Control) with biological superphosphate (Bio-super), rock phosphate (Rock-P), elemental sulfur (Sulfur), natural gypsum (Gypsum), superphosphate (Superphos), or microbial bio-fertilizers (T50, T50+SP11). The dashed line (----) indicates the minimum P concentration in the uppermost fully developed leaves at onset of blossom that is sufficient for adequate growth of fava beans as reported by Bergmann (1992).

Columns represent mean values ± SEM (n = 4). Data were tested separately for the part with bio-super phosphate (left side of the graph) and for the part with microbial bio-fertilizers (right side of the graph) of the experiment in two-way ANOVAs with treatment (including Control) and block as factors. Treatments of "Sulfur" and "Gypsum" were not strictly randomized and therefore excluded from statistical tests (e.f.s.t.). There were no significant treatment effects detected.

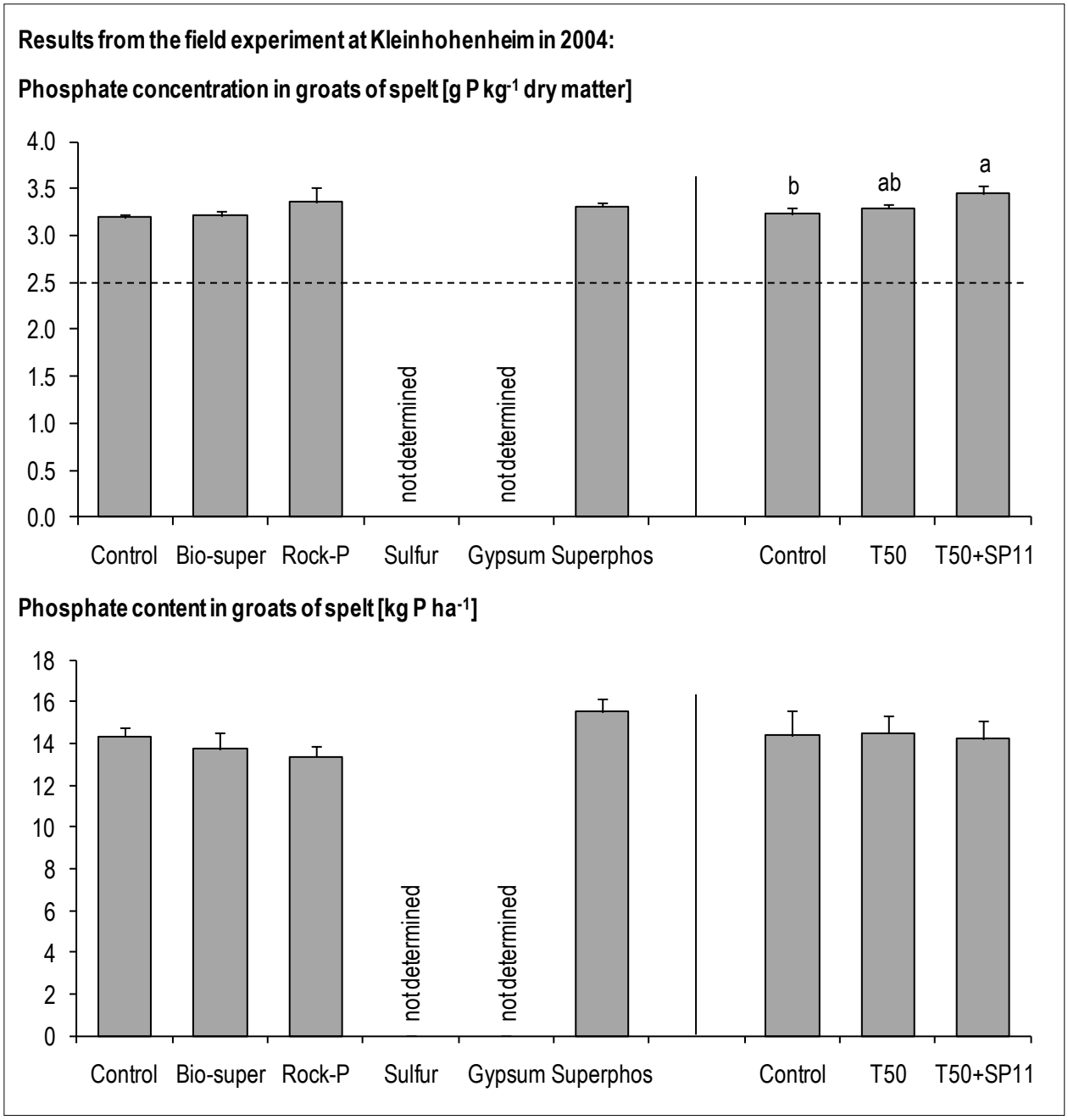

Fig. 6.2: Phosphate concentration (a) and total phosphate contents per hectare (b) in groats of spelt wheat harvested at full ripening stage from the field experiment at Kleinhohenheim in 2004. Plants had been treated or not (Control) with biological superphosphate (Bio-super), rock phosphate (Rock-P), superphosphate (Superphos), or microbial bio-fertilizers (T50, T50+SP11). Samples from "Sulfur" and "Gypsum" treated plots were not analyzed. The dashed line (----) indicates the minimum P concentration in the above ground biomass of diverse cereal species that is sufficient for adequate plant growth as reported by Bergmann (1992).

Columns represent mean values ± SEM (n = 4). Data were tested separately for the part with bio-super phosphate (left side of the graph) and for the part with microbial bio-fertilizers (right side of the graph) of the experiment in two-way ANOVAs with treatment (including Control) and block as factors. In cases of significant treatment effects, the analyses were followed by Tukey's test. Mean values without superscripts or sharing common letters are not significantly different ($p > 0.05$).

Some significant changes were observed with spelt wheat at Kleinhohenheim in 2004. These findings may not meet the expectations of agricultural producers, but are noteworthy for the interpretation and agronomic validation of the findings derived. As illustrated in Fig. 6.2a, the combined application of Vitalin T50 and Vitalin SP11 (T50+SP11) significantly increased the phosphate concentration in the groats of spelt wheat by about 7 %. This effect, however, did not appear to be accompanied by absolute increases in phosphate uptake (Fig. 6.2b), but was rather attributable to a decreased biomass production per unit area (4.1 t groats ha^{-1}) when compared with the untreated control (4.3 t groats ha^{-1}; Fig. 6.3).

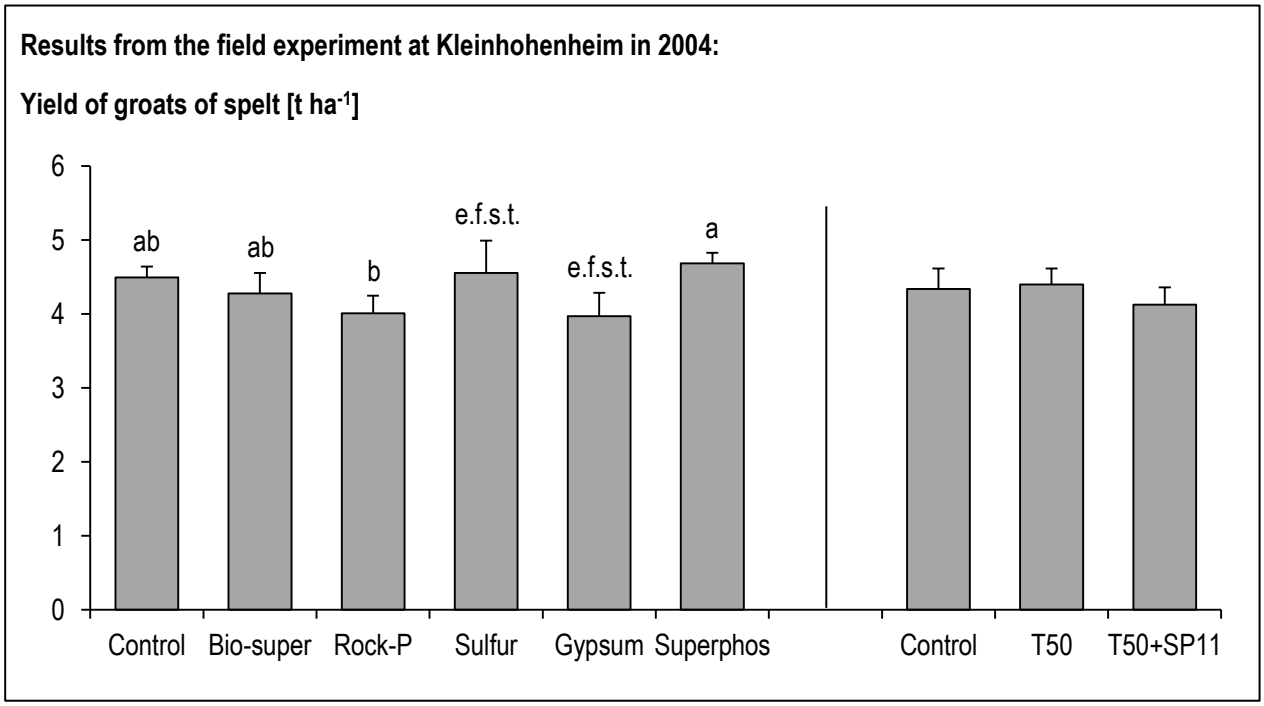

Fig. 6.3: Yield of groats of spelt wheat harvested at full ripening stage from the field experiment at Kleinhohenheim in 2004. Plants had been treated or not (Control) with biological superphosphate (Bio-super), rock phosphate (Rock-P), elemental sulfur (Sulfur), natural gypsum (Gypsum), superphosphate (Superphos), or microbial bio-fertilizers (T50, T50+SP11).

Columns represent mean values ± SEM (n = 4). Data were tested separately for the part with bio-super phosphate (left side of the graph) and for the part with microbial bio-fertilizers (right side of the graph) of the experiment in two-way ANOVAs with treatment (including Control) and block as factors. In cases of significant treatment effects, the analyses were followed by Tukey's test. Mean values not sharing common letters are significantly different ($p \leq 0.05$). Treatments of "Sulfur" and "Gypsum" were not strictly randomized and therefore excluded from statistical tests (e.f.s.t.). There were no significant treatment effects detected for the experimental part with microbial bio-fertilizers.

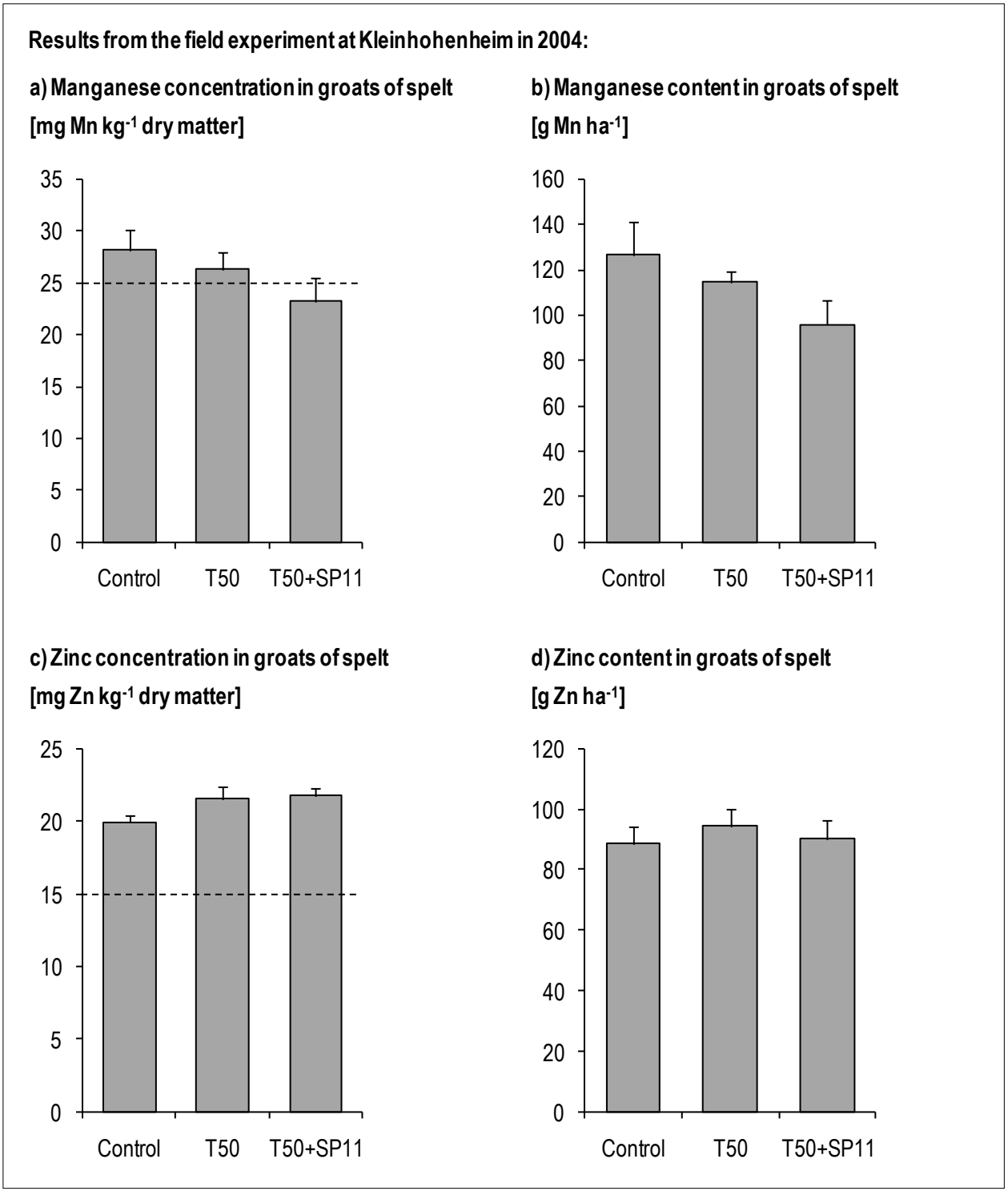

Fig. 6.4: Concentrations and total contents per hectare of manganese (a, b) and zinc (c, d) in groats of spelt wheat harvested at full ripening stage from the field experiment at Kleinhohenheim in 2004. Plants had been treated or not (Control) with microbial bio-fertilizers (T50, T50+SP11). The dashed lines (----) indicate minimum concentrations of the respective element in the above ground biomass of diverse cereal species that are sufficient for adequate plant growth as reported by Bergmann (1992).

Columns represent mean values ± SEM (n = 4). Data were tested separately in two-way ANOVAs with treatment (including Control) and block as factors. There were no significant treatment effects detected.

In the experimental part with biological super phosphate (left side of Fig. 6.3), the largest yield of spelt wheat (4.7 t groats ha^{-1}) was achieved in response the application of regular superphosphate. Thereby, superphosphate was significantly more yield effective than rock phosphate (4.0 kg groats ha^{-1}), but not when compared to the respective untreated control (4.5 kg groats ha^{-1}). Correspondingly, the treatment with superphosphate also resulted in the largest total phosphate content in the harvested groats of spelt wheat (15.5 kg P ha^{-1}), whereas the lowest phosphate removal per hectare was achieved by the application of rock phosphate (13.4 kg P ha^{-1}, Fig. 6.2b). There was, however, no clear difference found between rock phosphate and biological superphosphate application regarding their influence on the phosphate concentration in the groats of spelt wheat (Fig. 6.2 – 6.3).

Furthermore, as shown in Fig. 6.4a, the treatments with microbial bio-fertilizer preparations decreased the manganese concentration in the groats of spelt wheat tendentiously ($p = 0.054$), which was particularly pronounced in response to the combined application of Vitalin T50 and Vitalin SP11. The same trend was observed for the total manganese content in the groats of spelt wheat (Fig. 6.4b), thus indicating an inhibited mobilization in the rhizosphere, uptake by the plant and/or translocation to the grains of manganese. By contrast, no such adverse effect was observed for the concentrations and total contents of zinc in the groats of spelt wheat (Fig. 6.4c and d). Not represented in the graphs are the manganese and zinc levels in the groats of spelt wheat from the experimental part with biological superphosphate, as these values were not clearly affected by the respective treatments.

6.3.2 Yields of crops grown at Schönberghof

Exemplarily for the experimental site at Schönberghof, in Fig. 6.5 the weights of ears obtained with wheat in 2003 are represented, which indicate a certain yield response to the supply of liquid manure, but no clear reactions to the applications of microbial bio-fertilizers or Epsom salt ($MgSO_4$•7 H_2O, activated or not) treatments. In the same year, similarly poor results were found in terms of straw yield or numbers of ears per unit area that were formed by the wheat crop (data not shown). Because these findings indicated that similarly non-significant results were to be expected, the grains were not separated

from the ears to measure grains yields. Also in the following year 2004 when emmer was planted, there were no appreciable treatment effects observed, such as measured in terms of total aboveground biomass production per hectare (data not shown).

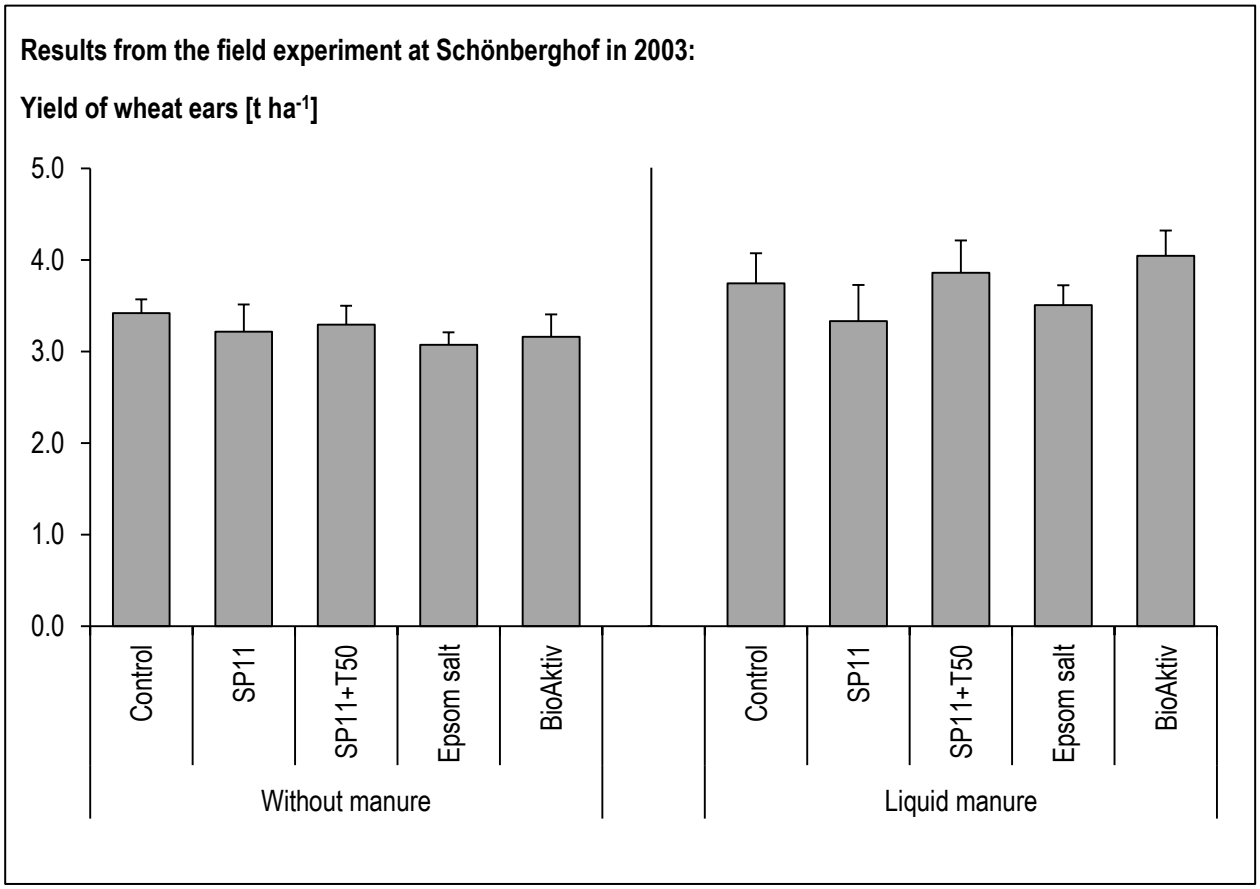

Fig. 6.5: Yield of wheat ears harvested at full ripening stage from the field experiments with or without supply of liquid manure at Schönberghof in 2003. Plants had been treated or not (Control) with microbial bio-fertilizers (T50, T50+SP11), "non-activated" Epsom salt, or "activated" Epsom salt (BioAktiv).

Columns represent mean values ± SEM (n = 5). Data were tested separately for the experiment without (left side of the graph) and with supply of liquid manure (right side of the graph) in two-way ANOVAs with treatment (including Control) and block as factors. There were no significant treatment effects detected.

6.4 Discussion

Previous Chapters 4 and 5 provided results from in vitro screenings, hydroponic culture tests, and greenhouse experiments, which revealed a considerable potential of microbial bio-fertilizers to solubilize sparingly available phosphate forms, to stimulate the root growth, and to enhance the phosphate acquisition of plants. In contrast to these findings, the present field experiments showed that applications of microbial bio-fertilizers, but also biological superphosphate, or "activated" Epsom salt were not effective to improve the phosphate acquisition and growth of crop plants grown in organic farming systems. The reasons accounting for these inconsistencies are probably manifold, as discussed in the following sections.

6.4.1 Factors involved with the failure of microbial bio-fertilizers under field conditions

As outlined in Chapter 2, introduced microbial inoculants, associated crops and the autochthonous soil microflora are known to interact in complex ways while exerting effects on the performance of plants. It is, however, poorly understood how the influences of multiple environmental factors such as the soil type, nutrient availability, moisture and temperature are determining the outcome of these mutual interdependencies (Kloepper et al., 1989; Yarzábal, 2010; Musarrat and Khan, 2014). Other important factors, such as the origin and properties of the microbial isolates used as inoculants, the application method, the plant genotype, and the crop management are not inherent to the system of unpredictable environmental influences, but are more suitable to be controlled by producers of bio-fertilizer preparations respectively farmers.

Soil type

Result from Gyaneshwar et al. (1998) indicated that a high buffering capacity of alkaline soil completely inhibited the phosphate solubilizing ability of bacterial species (i.e. *Bacillus coagulans, Citrobacter koseri*), which had been previously isolated for their ability to dissolve calcium phosphates when grown on an un-buffered medium. Strains of *Enterobacter asburiae*, by contrast, were able to release phosphate from an alkaline soil and to dissolve calcium phosphate on a buffered medium (pH 8.0) (Gyaneshwar et al.,

1999), but failed to release phosphate in an acidic soil, which was attributed to the high sorption capacity of iron and aluminum oxides (Srivastava et al., 2007). Further evidence for the soil specificity of phosphate solubilizing microorganisms has been reported by Toro (2007), whose results showed that Al- and Fe-phosphate solubilizing organisms were predominant in the rhizosphere of native savanna plants when Al- and Fe-phosphates were important phosphate fractions in soil. Similar coincidence was found in calcareous soils, where Ca-P solubilizing organisms and Ca-P fractions were predominant, suggesting a well-adapted composition of the indigenous microflora in soils of natural ecosystems.

Pre-existing fertility status of the soil

The plant growth promoting effect of microbial inoculants can also vary considerably depending on the fertility level of the soil as shown in potted soil and field experiments (Chabot et al., 1996; Çakmakçi et al., 2006). In this regard, the pot experiments with different soils and phosphate fertilizer levels presented in Chapter 5 indicated that plant growth promoting effects of phosphate solubilizing microorganisms are more likely in less fertile soils. Accordingly, results from Deubel et al. (2007) suggested that phosphate starvation stress in plants affects quantitative and qualitative changes in the composition of root exudates, which selectively enhance the phosphate mobilizing effectiveness of specific rhizobacteria, as demonstrated under in vitro conditions. Furthermore, isotopic (^{32}P) dilution approaches have been employed, demonstrating that the improved plant use of sparingly available phosphate sources in soils, that were inoculated with phosphate solubilizing bacteria (i.e. *Enterobacter* sp.; Barea et al., 2002b) or fungi (i.e. *Penicillium bilaji*; Asea et al., 1988), is a major mechanism contributing to corresponding yield increases. In soils with relatively high levels of plant available phosphate, by contrast, the application of phosphate solubilizing microorganisms would therefore have little or no effect on plant performance. This argumentation seems to be particularly plausible for the studies performed at Kleinhohenheim, as the plants did not even show pronounced responses to the application of regular superphosphate, there. At this site, the CAL extractable phosphate concentration of 4.6 mg P 100 g^{-1} dry soil was still in a sufficient range and about ten times higher than in the calcareous substrate that was used in the

greenhouse trials described in Chapter 5, where Vitalin SP11 improved the shoot growth and P uptake of wheat (Hong et al., 2001, unpublished).

At the experimental site Schönberghof, by contrast, CAL-P values were lower than 2.0 mg P 100 g^{-1} dry soil, and hence regarded as severely limiting for plant growth, according to standard recommendations (Kerschberger et al., 1997). Nevertheless, the crops still appeared to be adequately supplied with phosphate, which was probably derived from organic pools in the soil. This assumption has been recently challenged by Redel (2010), who measured high levels of organic phosphorus in labile (11.5 mg P 100 g^{-1} soil) and moderately labile (47.9 mg P 100 g^{-1} soil) fractions for soil samples collected at the Schönberghof, which were accompanied by a high acid phosphatase activity in the soil. For the phosphorus fractionation into labile and moderately labile pools, Redel (2010) extracted the soil samples successively with 0.5 M $NaHCO_3$ and 0.1 M NaOH, thereby following a modified "Hedley procedure" (Hedley et al., 1982; Tiessen and Moir, 1993; Hedley et al., 1994) described by Redel et al. (2007 and 2008). Furthermore, it is well known that the CAL-method may underestimate the phosphate availability to plants with a high acquisition capability for phosphate and other mineral nutrients (Gahoonia et al., 1992; Römheld and Neumann, 2006). This applies in particular to fava bean (Nuruzzaman et al., 2005), but also spelt wheat (Zhang et al., 2002; Mielke and Rodemann, 2007), as well as modern cereal cultivars which have been bred especially for organic farming purposes (Wolfe et al., 2008).

Nevertheless, regarding the possible multi-functionality of *Bacillus, Pseudomonas, Streptomyces* and *Trichoderma* species described in Chapter 2 and 4 and proposed by the producers of commercial inoculants (see Table 4.1), it is appropriate to question why the microbial strains contained in Vitalin T50 and Vitalin SP11 could not improve plant growth by mechanisms other than the alleviation of phosphate deficiency. As emphasized by Whitelaw et al. (1997), for instance, a phosphate-solubilizing isolate of *Penicillium radicum*, that increased phosphate uptake and yield in greenhouse experiments, was found to improve plant growth under field conditions independently of whether the phosphate level of the soil was low or not. Moreover, field trials with strains of *Rhizobium*, *Serratia*, *Pseudomonas*, and *Rhizopus* species have indicated that the phosphate solubilizing effect

of these microorganisms was of importance for plant growth promotion in moderately and very fertile soils. In a poorly fertile soil with low phosphate availability, by contrast, only the crop yield but not the phosphate uptake by the plants was increased in response to the microbial treatments (Chabot et al., 1996).

Climate factors

Environmental variability, such as fluctuations in soil temperature (Pandey et al., 2002; Das et al., 2003) and moisture (Vyas et al., 2009; Sandhya et al., 2010), also has been shown to severely affect the activity of phosphate-solubilizing microorganisms. Microbial isolates which retain their plant growth promoting properties under stress conditions, such as cold (Mishra and Goel, 1999; Das et al., 2003; Katiyar and Goel, 2003; Trivedi and Pandey, 2007) or drought tolerant (Vyas et al., 2009; Sandhya et al., 2010) *Pseudomonas* strains, could therefore be of particular benefit to crops grown under low temperature respectively dry conditions.

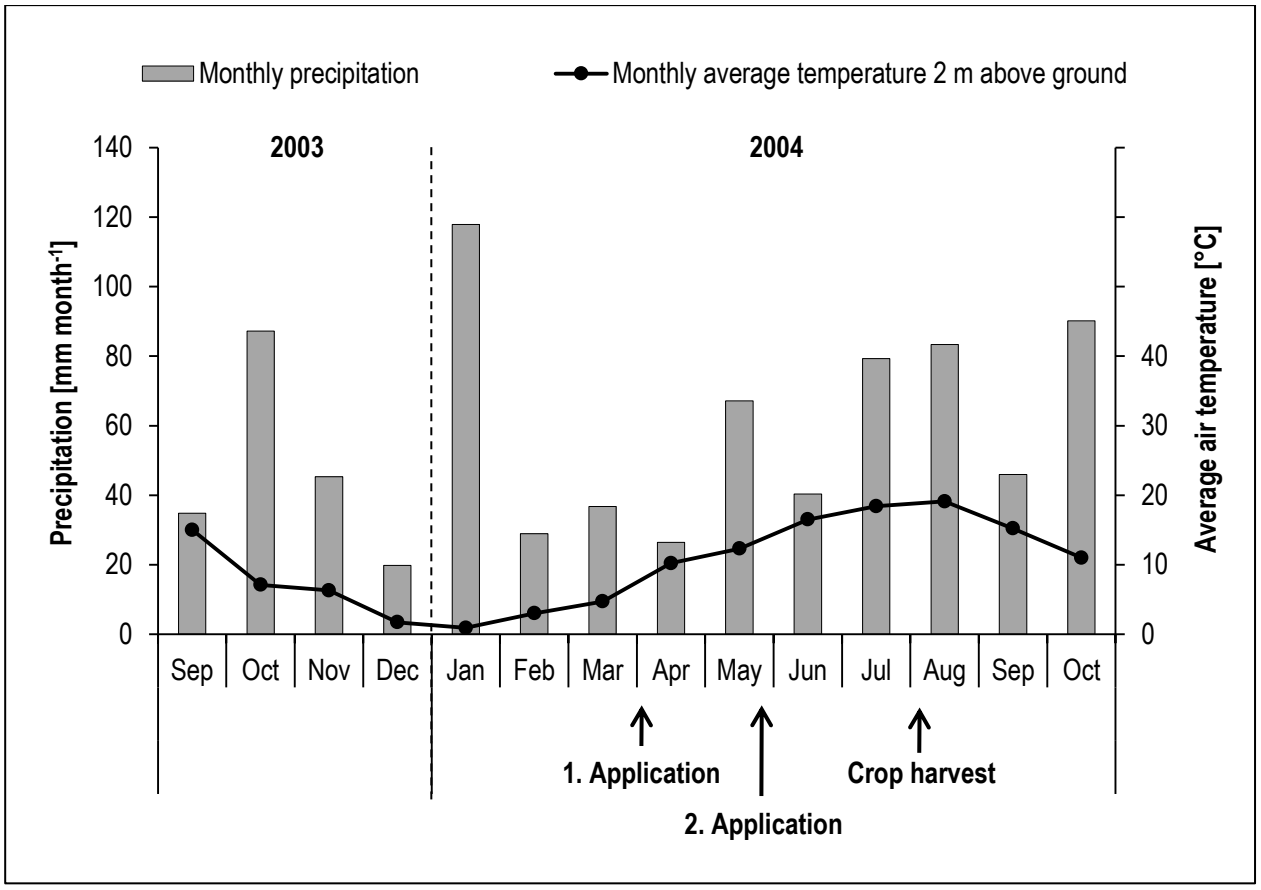

Fig. 6.6: Monthly precipitations and monthly average temperatures 2 m above ground during the field experiment with spelt wheat at Kleinhohenheim. Arrows indicate the first (1.) and second (2.) application date of the bio-fertilizers Vitalin T50 and Vitalin SP11 as well as the date of harvest at ripening of the spelt crops. Data were derived from the meteorological station of the Insitut für Physik und Meteorologie at Universität Hohenheim, Stuttgart, Germany.

Contrariwise, favorable conditions for the activity of microbial inoculants during the early developing stages of crops may result in enhanced uptake of mineral nutrients by the plant. If adverse conditions follow later on, then plants may have allocated more of their resources into shoot growth, just when root growth would be required to overcome stresses such as drought and low nutrient availability in soils. This probably also was the case for spelt wheat grown at the experimental site Kleinhohenheim in the year 2004, when conditions of adequate soil moisture and temperature in spring were followed by heat and low precipitation during the phase of grain development in June, as indicated by the climate data in Fig. 6.6. According to Walter and Lieth (1967), conditions are regarded as arid when the value of the monthly precipitation in mm is less than than twice the value of the monthly average temperature in °C. In addition, a high phosphate status

of the plant due to the microbial mobilization of soil phosphate, which in the case of wheat plants is mainly taken up in their early stages of growth (Römer and Schilling, 1986), might lead to an inhibited manganese acquisition in later growth stages. While plants respond to P, N, S, and Fe starvation with adaptations in root growth (McDonald et al., 1996; Neumann and Römheld, 2002), and to P, Fe and Zn deficiencies with enhanced root exudation (Marschner et al., 1987; Neumann and Römheld, 2007), there is few evidence for such specific reaction to shortages in manganese supply (Gherardi and Rengel, 2004). The role of microorganisms in the rhizosphere, however, is particularly pronounced for the availability of manganese to plants (Marschner, 1988; Marschner et al., 2003). Furthermore, the remobilization and translocation of manganese from older plant parts to developing wheat grains is quite limited, in contrast to the efficient redistribution of phosphate and zinc in the plant (Batten et al. 1986; Pearson and Rengel, 1994).

Resident soil microflora

Many reports attributed the failure of microbial inoculants to improve plant growth to their inability to compete successfully with indigenous antagonists, which are probably better adapted to local conditions (van Veen et al., 1997; Yarzábal, 2010). This may explain the frequently observed rapid decline of the population sizes of exogenous microorganisms upon introduction into soils with intact microbial communities (Katznelson, 1940; Bowen and Rovira, 1976; van Elsas, 1986; Joe and Sivakumaar, 2009). Beyond counterproductive relationships of antagonism, however, the possibility of mutualistic interactions between inoculated and native microorganisms has been attracting considerable attention in more recent publications (Bashan and Holguin, 1997; Barea et al., 2005; Saxena et al., 2006; Castro-Sowinski et al., 2007; Tarkka et al., 2008; Nezarat and Gholami, 2009). In this connection, it is increasingly perceived that individual microorganisms exert their influence as integral, functional components of biotic communities, through producing, sensing and responding to intra- and inter-specific signals that are mediated by chemical messengers, rather than by acting as isolated entities (Straight and Kolter, 2009). On the molecular level, microbial signaling circuits, monitoring environmental and intracellular parameters and effecting behavioral responses

in microorganisms, have been demonstrated to share many similarities with those of multi-cellular organisms (Galperin, 2004; Cashin et al., 2006; Straight and Kolter, 2009). Microbial inoculants produced from local isolates, such as they were found to be more effective than exogenous strains in several studies (Sattar et al., 1995; Barea et al., 2002a; Salantur et al., 2006), may therefore not only be better adapted to their abiotic soil environment, but also functionally better integrated into the prevalent signaling networks of the resident microflora. In this regard, the perspective of Rudolf Steiner, who described the farm as a living organism (Steiner, 1924), appears to be of particular relevance in developing a better understanding of soil microbial ecology.

Specificities between plants and rhizosphere microorganisms

Some investigations revealed that certain microbial strains promoted growth only in certain crop species or even cultivars, thus indicating a high degree of specificity in plant-microbe interactions (Halverson and Stacey, 1986; Bashan and Holguin, 1997; Lucy et al., 2004). For example, as reported by Kloepper et al. (1980), only one out of four isolates from celery roots of *Pseudomona*s spp. was found to increase the growth of potato, whereas all four of them had been shown to increase the growth of radish in previous experiments. Similarly, Chanway and Nelson (1990) observed that strains of the genus *Bacillus* isolated from the rhizosphere of a certain wheat variety increased seedling emergence, shoot weight and tiller number of the same but not of another wheat variety under field conditions.

Application method

Choice of application method can have a strong influence on the effectiveness of microbial treatments in agriculture. Because the activity of phosphate solubilizing microorganisms is more likely to exert a beneficial effect on plant growth in the rhizosphere than in the bulk soil, microbial inoculants should be introduced in way that enables them to reach and colonize the plant root (Leggett et al., 2001). Seed inoculation or placed application to the root zone may therefore appreciably enhance the effectiveness of introduced microorganisms, supposed that their movement is more or less retarded by the soil matrix (Brown, 1974; Fallik and Okon, 1996; Leggett et al., 2001). In addition,

the release of exudates from germinating seeds and developing seedlings may stimulate the initial multiplication and establishment of seed inoculated microorganisms in the rhizosphere, and so provide a competitive advantage over the resident soil micro-flora (Kloepper et al., 1989; Thompson et al., 1990). In contrast to these considerations, however, results from Boelens et al. (1994) have shown that seed inoculation resulted in significantly lower colonization rates of roots than homogeneous admixture of *Pseudomonas* strains to the soil substrate. These differences, however, may also depend on the amount of inoculum applied.

With respect to successful root colonization by introduced rhizobacteria, the importance of passive transport and active chemotactic movement towards exudates from plant roots have been discussed as a matter of controversy (Weller, 1988; Boelens et al., 1994; Lugtenberg and Dekkers, 1999; Lugentberg et al., 2001). It has been shown by Carvalhais (2010), in this regard, that unknown compounds in the root exudates of phosphate deficient maize plants induced the enhanced expression of genes in a strain of *Bacillus amyloliquefaciens*, which are involved in motility and chemotaxis. Interestingly, no such response was observed with root exudates from nitrogen, potassium or iron deficient maize plants. Further evidence for the selective recruitment of specific root colonizers from the pool of microorganisms present in the soil under the interactive influence of plant and soil factors shaping the microbial community in the rhizosphere has been provided recently (Berendsen et al., 2012; Bakker et al., 2013; Hunter et al., 2014; Patel et al., 2015). Bashan (1986a) reported that upon inoculation strains of *Azospirillum brasilense* and *Pseudomonas fluorescens* migrated with increasing speed of up to 3.5 cm per day towards the roots of wheat seedlings. Fastest migration rates were obtained in light and moist soil substrates, whereas low levels of humidity and heavy soil substrates impaired the movement of these bacterial organisms. Distances of more than 17 cm have been demonstrated to be migrated through by rhizobia in soil (Frazier and Fred, 1922; cited in: Bashan, 1986a and Kluepfel, 1993). Further evidence for the important role of motility in rhizosphere competence has been provided by several studies, which have consistently shown severe impairment in root colonization by non-motile mutants of *Pseudomonas* spp. (De Weger et al., 1987; Simons et al., 1996; Dekkers et al., 1998; Chin-A-Woeng et al., 2000). In some other studies, however, non-motile mutants of

Pseudomonas spp. colonized roots and promoted plant growth as well as their motile counterparts, thus indicating the action of different distribution mechanisms (Howie et al., 1987; Sher et al., 1988; Boelens et al., 1994). It should be noted in this connection, that the role of flagella in root colonization remains unclear from these studies. In some investigations, mutants impaired in motility due to the lack of flagella were used. Howie et al. (1987) and Boelens et al. (1994), by contrast, explicitly used non-motile mutants still producing flagella that were paralyzed by different mechanisms. Flagella, however, are not only involved with motility but also with adherence to surfaces and the development of interacting bacterial communities know as biofilms, which can be of significance relative to the retained colonization capacities of non-motile, but still flagellated bacterial mutants (Boelens et al., 1994; O'Toole and Kolter, 1998; Bodenmiller et al., 2004).

Other investigations with non-motile strains provided evidence for the carriage of bacteria through the soil by adherence to advancing root tips (Howie et al., 1987). Results from Bahme and Schroth (1987), by contrast, indicated that growing root tips are not efficient in transporting colonizing bacteria, which was attributed to physical removal, adsorption to the soil matrix, competition from resident microorganisms, and slow growth rates of the inoculum strain. Similarly, Parke et al. (1986) found that a strain of *Pseudomonas fluorescens* could not keep pace with growing wheat roots and was no longer detectable on the root tip after four days, if there was no water allowed to drain through the soil profile as transporting agent. Coherently, diverse authors emphasized the importance of water flow through macro-pores, wormholes and channels formed by plant roots to explain the rapid movement of microorganisms over significant distances in soils (Parke, 1986; Abu-Ashour et al., 1994; Boelens et al., 1994; Abu-Ashour et al., 1998).

Dose of inoculum

Another point of controversy is the amount of inoculum applied to the plant or soil, which is appropriate to achieve the desired effect. The concentrations of microbial numbers in the bio-fertilizer preparations used in the present field experiments are noted in Table 4.1. With application rates of 2 kg ha^{-1} of Vitalin SP11 or Vitalin T50, as suggested by the manufacturer, only doses of about 2 x 10^7 cfu or spores m^{-2} of the bacterial respectively

fungal inoculants have been applied onto the soil. These amounts equate to inoculum doses of less than 2 x 10^2 microbial entities cm^{-3} in top 10 cm of the soil, whereas non-inoculated bulk soil typically contains 10^5 to 10^9 cfu of culturable organisms g^{-1} dry weight (Leggett et al., 2001; Janssen et al., 2002). Several studies indicated that above certain threshold levels, which are the minimum required for effectiveness, increasing the dose of inoculum generally fortified the beneficial effect on plants (Lucy et al., 2004; Yarzábal, 2010). Accordingly, results from Raaijmakers et al. (1995) have shown that for strains of two *Pseudomonas* species an inoculum dose of at least 10^5 cfu g^{-1} soil was required for significant suppression of *Fusarium* wilt in radish. Bull et al. (1991) observed decreasing severity of disease symptoms on wheat roots produced by the take-all pathogen *Gaeumannomyces graminis* var. *tritici* with increasing application rates of a suppressive *Pseudomonas* strain ranging from about 3 x 10^1 to 3 x 10^8 cfu peer seed. In both studies, the population sizes of the antagonistic pseudomonads strains detected on the roots were directly related to the initial doses of bacteria applied (Bull et al., 1991; Raaijmakers et al., 1995). Comparable relationships between the dose of inoculum and the level of effectiveness also have been described for other genera of rhizosphere microorganisms. A strain of *Bacillus subtilis* provided adequate control against fungal diseases when it was applied to roots at concentrations above 3 x 10^5 cfu g^{-1} root fresh weight (Berger et al., 1996). Increasing application rates of a *Trichoderma harzianum* isolate, ranging from 4.3 x 10^6 to 1.0 x 10^7 spores g^{-1} soil, were associated with decreasing disease incidence of seedling damping-off caused by *Rhizoctonia solani* in several crop species (Hadar et al., 1979).

In other studies, opposite dose-effect responses were found, indicating that the adequate inoculum rate of a certain microbial strain may vary, depending on the targeted purpose. Frey-Klett et al. (1999) observed that within the range from 10^6 to 10^9 cfu m^{-2} applied onto the soil surface, which equates to an amount of less than 10^1 to 10^4 cfu cm^{-3} of soil, the lowest doses of a *Pseudomonas* strain were the most efficient ones in provoking a mycorrhiza helper effect. Likewise, the plant growth promoting effect of an *Azospirillum* strain increased in spite of a rapid decrease in root colonization density in maize (Jacoud et al., 1998), and phosphate-solubilizing bacteria did not necessarily need to colonize roots in high numbers to improve the yield and phosphate acquisition of wheat plants

(Harris et al., 2006). Other authors observed detrimental effects on root growth in response to treatments with large doses of otherwise beneficial rhizobacteria such as 10^8 cfu g^{-1} growth substrate (Kapulnik et al., 1985; Bashan, 1986b).

6.4.2 Possible reasons for the missing effectiveness of biological superphosphate

The ineffectiveness of biological superphosphate might be simply explained by the fact that also the supply of conventional superphosphate did not significantly improve the phosphate status and yield of crop plants, thus indicating that the availability of phosphate in the soil was not a major growth-limiting factor. Nevertheless, several other factors might have played a role in hampering the occurrence of beneficial effects from the application of biological superphosphate, too.

Physical and chemical characteristics of the rock phosphate

When compared to many minerals and mineral compounds of the soil matrix, certain rock phosphates can be regarded as relatively easily soluble materials (Müller, 1968). The solubility of the apatitic phosphate fraction in rock phosphates strongly depends on the grain size of their crystalline structure. The low solubility of the Russian Kola apatite, for instance, has been attributed to its coarse-grained texture. Reactive phosphate rocks, such as the Tunisian Gafsa phosphate, in contrast, are characterized by a very fine (cryptocrystalline) granularity with high inner surface area, which facilitates their solubility. In addition, the comparatively high solubility of Gafsa phosphate has been attributed to its content of phosphate that is included in sorbed and organic forms (Müller, 1968; Scheel, 1968; Faithfull, 2002). Results from Welp et al. (1983), who investigated the influence of soil reaction (pH in soil suspension), redox potential, and organic matter indicate that the phosphate concentration in the soil solution is mainly governed by adsorption/desorption processes, rather than by the dissolution or precipitation of defined phosphate compounds. Up to 70 % of the total phosphorus in the soil solution may be attributable to soluble organic phosphate compounds (Welp et al., 1983).

Rock phosphate to sulfur ratio

Results of an accompanying pot experiment conducted by Martini (2001) indicated that the composition of the bio-super phosphate used could have been suboptimal. In that investigation, soil material taken from Kleinhohenheim was diluted by mixing it with 40 % by volume of soil material derived from a phosphate depleted Cambisol (see Chapter 5.2.1) and 20 % by volume of quartz sand, in order to obtain a growth substrate decreased in its concentration of available phosphate. Nevertheless, applications of bio-super could only increase the concentration and total uptake of phosphate in the shoot tissue of spring barley (*Hordeum vulgare* L.), when either the ratio of rock phosphate to elemental sulfur was enhanced from 4:1 to 1:1, or when the soil substrate was additionally inoculated with a strain of *Thiobacillus thioparus*. Although, the role of the *Thiobacillus* inoculum remained unclear due to the possible introduction in to the soil substrate of phosphate with the bacterial culture medium, these findings clearly indicated that the indigenous populations of sulfur oxidizing microorganisms in the soil substrate were only effective to dissolve admixed rock phosphate when sulfur was applied at the higher rate (Martini, 2001). While the industrial production of superphosphate requires sulfuric acid at a respective rock phosphate to sulfur ratio of about 5:1, only (Raijan, 1983; Leikam and Achorn, 2005), the adequate proportion of sulfur used in bio-super preparations appears to be dependent on the initial pH and the buffer capacity of the soil, besides of the reactivity of the rock phosphate. Kittams and Attoe (1965) used different soil substrates to study the application of bio-super with rock phosphate to sulfur ratios ranging from 1:1 to 20:1. Their result showed that, when mixed with a silt loam substrate having an initial pH of 6.6, only treatments with high sulfur ratios (1:1 to 2:1) were similarly effective like superphosphate as a phosphate fertilizer for ryegrass. No such efficient phosphate recoveries occurred, however, when bio-super was added to a more alkaline silt loam (pH 7.9) or to a calcareous substrate (pH 7.5). Rajan (1981), by contrast, prepared bio-super with a rock phosphate to sulfur ratio of 5:1 and found it as effective as superphosphate in a limed soil substrate of pH 6.2, given that a reactive type of rock phosphate with a suitable mineral composition, such as carbonate and fluorapatite instead of iron-aluminum phosphates, was used.

Direct influence of rock phosphate and sulfur on the phosphate availability in soils

For comparison, in the present study, rock phosphate and sulfur were also directly applied. In soils less acidic than pH 5.5 – 6.0 the plant availability of apatitic rock phosphates is generally very low (Khasawneh and Doll, 1978; Rajan et al., 1996; Reddy, 2002). Therefore, no significant plant response could be expected from the direct application of such material to a soil with a pH of 6.2, as it was found in the field experiment conducted at Kleinhohenheim. The application and subsequent oxidation of elemental sulfur, by contrast, can be particularly beneficial in alkaline soils to decrease the pH, increase the plant availability of phosphate and micronutrients (i.e. Fe, Mn, Zn), and supply sulfate to plants (Burns, 1967; cited in: Lindemann, 1991; Soliman et al., 1992). Accordingly, Stamford et al. (2002) reported that the supply of sulfur at a level equivalent to 600 kg ha^{-1} lowered the pH of an alluvial sodic saline soil substrate, which was irrigated with saline water, from the initial value of pH 7.9 to pH 3.5. Such strong pH effect might not be observed in calcareous soils with high buffering capacity in the pH range between 7.5 and 8.5 (Marschner, 1995), where the H_2SO_4 formed by sulfur oxidation might be neutralized by $CaCO_3$, thus counteracting any significant mobilization of acid-soluble phosphates and micronutrients in the soil (DeLuca et al., 1989; Lindemann et al., 1991). However, values of soil pH between 6 and 7, such as they were given at the experimental site Kleinhohenheim, generally represent a range for optimal availability of phosphate and other mineral nutrients to plants, which do not require further pH amendment (Haynes, 1982; Finck, 1991; Mengel and Kirkby, 2001).

Direct influence of titanium dioxide on plants

Treatments with rock phosphate plus titanium oxide (Rock-P+TiO_2) were only performed in 2002, but later replaced by Rock-P treatments without TiO_2, because first results did not provide evidence for side effects produced by the admixture of TiO_2 as binding agent in bio-super preparations. TiO_2 is generally regarded as a chemically inert compound, which is widely used as a white pigment in products such as paints, plastics, papers, and food colorants, although recent research indicated the induction of adverse health effects in mice supplemented with TiO_2 in nano-scale form (Trouiller et al., 2009). In contrast to the present results, Lopez-Moreno et al. (1996) reported that the application of titanium as

Ti(IV)-ascorbate complex enhanced the biomass production of pepper crops (*Capsicum annuum* L.), which was particularly pronounced under low phosphate supply. As it is soluble in oxalic acid, also TiO_2 may become available to plants (Dumon and Ernst, 1988). The exact mechanisms by which titanium can stimulate the biomass production of plants are not known, but have been related to the activation of enzymes, enhanced chlorophyll content and photosynthetic activity, and interactions with the uptake and function of mineral nutrients (Dumon and Ernst, 1988; Carvajal and Alcaraz, 1998; Tlustoš et al., 2005). However, because titanium is ubiquitous in soils and among the most common elements in earth's crust, the possible effects of the amount of TiO_2 contained in the bio-super preparation were regarded as negligible.

Direct influence of sulfate on plants

Gypsum, which represents an already oxidized and Ca-bound form of sulfur, cannot be expected to mobilize significant amounts of phosphate in slightly acidic to alkaline soils, but may rather facilitate the adsorption of phosphate ions on reactive surfaces as well as the precipitation of calcium phosphates (Waksman and Joffe, 1922; Kordlaghari and Rowell, 2006). Nevertheless, gypsum may still show effects of sulfate fertilization, such as including improved yield and disease resistance of crops (Davis et al., 1974; Tiwari et al., 1986; Hell, 1997; Riley et al., 2000; Rausch and Wachter, 2005). S-containing metabolites of plants, which are involved directly or indirectly with the defense of plants against microbial pathogens, include amino acids (e.g. cystein; Bloem et al., 2004), sulfur-rich proteins such as thionins (Florack and Stiekema, 1994) and defensins (Broekaert, 1995), glucosinolates (Ludwig-Müller, 1997; Tierens et al., 2001), crucifer phytoalexins (Pedras, 2006), the garlic antiseptic alliin (Ankri and Mirelman, 1999), and glutathione (Gullner and Kőmíves, 2006). Furthermore, it has been observed in diverse plant species that they produce elemental sulfur as a phytoalexin-like component of active defense against xylem-invading fungal and bacterial pathogens (Cooper et al., 1996; Resende et al., 1996; Williams and Cooper, 2003; Cooper and Williams, 2004). Some reported examples in which sulfur fertilization increased the pathogen resistance of crops are *Alternaria* spp. in rape (Sadowski et al., 2000), *Rhizoctonia cerealis* in wheat (Wang

et al., 2003; cited in: Bloem et al., 2007a), *Phytophthora brassicae* in rape (Dubuis et al., 2005), and *Rhizoctonia solani* in potatoes (Klikocka et al., 2005).

Indeed, due to the drastic decrease of sulfur emissions from the combustion of fossil fuels, sulfur deficiency developed into a widespread nutrient disorder of crops in Western Europe after the introduction of strict controls on industrial emissions in the 1970's (Riley, 2000; Bloem et al., 2004). In consequence, not only adverse effects on the yield and quality of agricultural crops were observed, but also an increasing susceptibility of plants to pathogens, which could be countervailed by the application of sulfur fertilizers (Dubuis et al., 2005; Haneklaus et al., 2007; Bloem et al., 2007b). Disease problems, however, and in particular those which are produced by soil borne pathogens, are often the result of inadequate cropping systems and poor soil conditions. Organic farming along with balanced crop rotations, by contrast, can be an effective management option to counteract the proliferation of pathogen populations in soils (Abawi and Widmer, 2000; Peters et al., 2003; Cunfer et al., 2006; Janusauskaite and Ciuberkis, 2010). Therefore, the prevalence of healthy soil conditions, apart from the non-appearance of sulfur deficiency as a nutritional disorder, could have been anticipating the occurrence of beneficial effects on plant growth in response to sulfur treatments in the present field study.

Application form of phosphate fertilizer

Finally, the mode of application can have a strong influence on the use efficiency of phosphate fertilizers. Field experiments with durum wheat conducted by the International Center for Agricultural Research in the Dry Areas (ICARDA, 1985; Matar and Brown, 1989a) on phosphorus deficient calcareous soils (< 0.4 mg $NaHCO_3$-extractable P 100 g^{-1} soil in the plow layer) at three locations of Syria showed that for equal rates of phosphorus application the placement of triple superphosphate as band with the seed at sowing was more effective than broadcast with soil incorporation before sowing. Banding of the phosphorus fertilizer generally improved the growth and phosphorus uptake of plants early in the season and in most cases resulted in higher dry matter yields of straw and grain compared to broadcasting (Matar and Brown, 1989a). Investigations on root growth and phosphorus dynamics, furthermore, indicated that the advantageous effect of fertilizer banding on crop performance and phosphorus use efficiency was mainly due to

the localized proliferation of root growth in the soil section with the fertilizer band, increasing the probability of root contact with fertilizer granules, rather than a decreased immobilization of fertilizer phosphorus in the soil (Matar and Brown, 1989b).

6.4.3 Considerations on the scientific rigidness of using unknown "cosmic forces"

The "cosmic forces" tapped to "activate" Epsom salt during the production process of Bioaktiv, as proposed by the manufacturer, are unknown to natural science. Even though, this is not a proof of their non-existence, there is also no clear evidence for beneficial effects, which could be unambiguously attributed to the impact of these "cosmic forces". In the present study, neither the application of "non-activated" Epsom salt, nor that of BioAktiv showed significant influences on the measured crop characteristics. Other investigations, demonstrating the effectiveness of BioAktiv in enhancing the growth and quality of crop plants, compared the "activated" product with untreated controls only, but not with applications of the "non-activated" raw material (BioAktiv, 2010). The beneficial effects which Epsom salt as a mineral fertilizer can have on the yield and quality of crop plants are well documented (Bolton, 1973; Mondy et al., 1987; Siegfried and Jüstrich, 2006), although it is typically applied at considerably larger doses (ca. 20 to 50 kg ha^{-1}) than those recommended for BioAktiv (2 kg ha^{-1}). Nevertheless, a reasonable discussion about the utilization of "comic forces" in agricultural production would require a clear evidence of the extra benefits derived from such efforts. Similar difficulties have been described with isolating the influence of biodynamic preparations in organic farming systems (Kirchmann, 1994; Raupp, 1999). Some reports attributed significant effects of the biodynamic preparations, such as on compost quality and earthworm communities, to empirically detectable reasons such as the propagation of microorganisms (Carpenter-Boggs et al., 2000b; Zaller and Köpke, 2004; Scheper, 2009). Other results indicated that the use of biodynamic preparations did not affect the yield or nutritional value of crops, beyond the effects of organic management without them (Zaller and Köpke, 2004; Mäder et al., 2007). Indeed, such materialistic research is not directly concerned with Rudolf Steiner's intension to enrich crops with "terrestrial and comic forces", which he regarded as major quality criteria for agricultural products as he thought them to sustain not only

the physical, but also the spiritual live of humans (Steiner, 1924; Kirchmann, 1994). From a critical perspective, one may question whether his attempt aims to control unknown physical forces by the application of purely mentally gained insights, or probably to control non-physical forces by human-made means. In particular, for the latter case, such effort seems to contradict rational expectations, as it would strive for the technical implementation of some kind of hidden knowledge, exceeding cognitive skills. Rudolf Steiner, however, was convinced that spiritually gained insights are true themselves, and do therefore not need to be confirmed by empirical methods (Steiner, 1924).

6.4.4 Conclusions

Microbial bio-fertilizers and biological superphosphate can improve the availability of phosphate to plants, as recorded in the literature and partly approved by investigations under controlled conditions described in Chapters 4 and 5. The contrasting results obtained from the present field experiments, however, showed no responses in plant performance and yield, which would suggest convincing advantages from the application of such products. This confirms the conclusion of other authors that the complexity of interacting factors hampers the effective implementation of biological strategies under praxis conditions (Kloepper et al., 1989; Leggett et al., 2001; Lucy et al., 2004). Unfavorable soil factors and environmental variability can have a huge impact on the activity of microbial populations associated to plant roots, resulting in hardly predictable difficulties to obtain an intended result (Bashan, 1998). If biological approaches, such as the use of bio-fertilizers or bio-super phosphate, are supposed to be successfully adopted in crop production, these intricacies have to be carefully taken into account. Continued work in this area could either, lead to the development of versatile products that function with crops in diverse environments, or yield advanced knowledge regarding the application of products with a narrow functional range under specific soil and climate conditions. However, controversy regarding the effectiveness of different application strategies and the unforeseeable variability of interacting environmental factors suggest that conclusive results are considerably more difficult to achieve by the latter option. Apart from that, it is obvious that very specific applications will provide only a small market for commercial products.

Another explanation, of particular relevance to the observed ineffectiveness of microbial bio-fertilizers, emerges to be that organically managed soils with diversified crop rotations favor the establishment of beneficial microbial populations out of the indigenous flora, providing suitable conditions for healthy root growth. The introduction of external strains may therefore provide little additional benefit, as plants together with associated soil microorganisms may already express to a nearly optimal extend the diverse biological mechanisms involved with the mobilization of mineral nutrients and the suppression of pathogens (Römheld and Neumann, 2006; Toro, 2007; Yarzábal, 2010). Further investigations will show whether results that are more significant can be achieved in soils with less balanced microbial activity and enhanced disease pressure from soil borne pathogens.

Little consistent evidence is available for the effectiveness of biodynamic preparations and BioAktiv, a commercial product supposed to improve plant growth by means of unknown “cosmic forces”. The discussion of these matters, however, revealed aspects concerning the derivation of these methods, which expand beyond the scope of empiric research. The technical implementation of such approaches in agricultural management, therefore does not appear to be based on rational logic, but rather seems to represent the expression of intuitive insights and convictions, whose understanding and testing is not entirely possible by the methods of natural sciences.

A major conclusion of the present work, however, is that field experiments are an indispensable tool to evaluate the functionality of biological approaches for improved nutrient acquisition and growth of plants under praxis conditions, but also very laborious, time-consuming and financially expensive.

C CASE STUDIES ON BIO-CONTROL OF SOIL BORNE PATHOGENS WITH EMPHASIS ON TAKE-ALL DISEASE

7 Introduction to the role of soil microorganisms and mineral nutrition in healthy crop growth

"Healthy soils" are often described as living buffer systems against abiotic and biotic disturbances that sustainably support biological productivity and fulfill important ecosystem functions (Doran, 2002; Harris and Romig, 2005). In particular high levels of biological diversity and activity have been associated with the long-term capacity of natural and agricultural soils to provide adequate supply of mineral nutrients to growing plants and to limit the establishment of pathogens (Brussaard et al., 2004; van Bruggen et al., 2006). The mechanisms by which healthy soils suppress the development of plant diseases include microbial antagonism as well as induced resistance and the role of mineral nutrition in plant defense (Huber and Graham, 1999; Sullivan, 2004). "Soil sickness", on the contrary, has been attributed to complex interactions between biotic and abiotic factors such as the accumulation of toxic materials, unbalanced supply of mineral nutrients, and soil degradation including the loss of functional biodiversity in particular concerning the microbial soil community (Hoestra, 1994; Politycka, 2005). According to this concept, the increased incidence of soil-borne pathogens in diseased soils is regarded as a sign of biological imbalance within the soil ecosystem where the natural antagonists are impaired and pathogens become prevalent (Kloepper, 1993; Alabouvette et al., 2004; Pankhurst and Lynch, 2005). Soil-borne diseases impose serious restrictions on production efficiency and are among the most difficult problems to control in intensive agriculture and horticulture (Shipton, 1977; Schippers et al., 1985; Hoestra, 1988). Genetic resistance to root diseases of plants is rare and chemical pesticides show limited utility to solve the problem persistently (Weller et al., 2002). A major constraint is to target only the niches where soil-borne pathogens are found, without treating the whole

soil profile or destroying the beneficial microflora, too (Lucas, 2006). Furthermore, broad spectrum chemicals like methyl bromide, which has been widely used for soil fumigation, have been banned for human health and ecological reasons (Yates et al., 1997; Carter et al., 2005). Therefore, the development of alternative control measures against soil-borne diseases represents an important issue for the sustainability of agriculture.

Traditionally, crop rotations have been performed to avoid the build-up of disease problems and improve the availability of mineral nutrients in soils, thereby sustaining soil health and lowering the need for pesticides and fertilizers (Karlen et al., 1994; Finck, 1998; Zhao et al., 2011). Yet, crop rations are generally not effective to control pathogens that are dispersed by wind or other vectors from outside the area (Atkinson et al., 2004; Sullivan; 2004), although in such cases mineral nutrients and beneficial microorganisms can still make an important contribution to improved disease control (Andrews, 1992). In addition, many soil-borne pathogens (e.g. subspecies of *Fusarium oxysporum*) can persist in soils for long periods of time without a host, thus seriously limiting the agronomic effectiveness and flexibility of crop rotations (Sullivan, 2004; King et al., 2008). Economic reasons, nevertheless, are urging farmers to adjust their production to the market demand and to restrict crop rotations to a few intensive cash crops, leading to narrow crop rotations or even monocultures (Shipton, 1979; Mielke, 1998; Kocjan-Ačko, and Šantavec, 2010). Although, in some cases the benefit of crop rotation may financially compensate for a reduction in the cultivation frequency of the most profitable crop, the complex interplay of plants with abiotic and biotic soil components bearing the "crop rotation effect" has been rarely understood and is difficult to reproduce by alternative means (Lampkin, 2002). As an alternative approach to control soil-borne pathogens, cropping strategies to specifically promote beneficial soil microorganisms and/or to optimize the supply of mineral nutrients have been considered to maintain the microbial balance of pathogen infested soils and to enhance the resistance of crop plants (Buchenauer, 1998; Römheld and Neumann, 2006; Janvier et al., 2007). This may be achieved through a purposeful rhizosphere management such as comprising the utilization of effective microbial inoculants, the encouragement of soil indigenous microbial populations, and/or by the application of distinct mineral nutrients (Harrier and Watson, 2004; Pankhurst and Lynch, 2005; Römheld and Neumann, 2006). However, current

knowledge on the influence of microbiota and abiotic soil factors, including mineral nutrients, needs to be extended and well-integrated to maintain or restore soil quality for healthy plant growth by improved agricultural practices (Alabouvette et al., 2004).

7.1 The importance of microbial diversity for biological soil fertility and plant health

Soil microbial diversity is a measure of the heterogeneity within the community of soil inhabiting microorganisms. This comprises aspects of structural diversity, regarding the presence and population densities of different species within the soil environment, and functional diversity, referring to the different processes carried out by the interacting groups within the soil microbial community (Brussaard et al., 2004; Nelson and Spaner, 2010). A balanced diversity of microbial populations in soils can play an important role in conferring resistance and resilience to various stresses that may affect plants cultivated in agro-ecosystems (Brussaard et al., 2007; Fig. 7.1). One important function of diversified and abundant populations of microorganisms in soils is to maintain soil fertility by biological processes that ameliorate the physical and chemical state of soil and rhizosphere environments, thus affecting the availability of mineral nutrients to plants (Uphoff et al., 2006; Abbott and Murphy, 2007). This includes the root-like functions of mycorrhizal symbioses, the nitrogen-fixing activity of diazotrophic bacteria, the turnover of organic matter, mechanisms involved with the dissolution and immobilization of soil minerals, and the formation and stabilization of soil aggregates (Beare et al., 1997; Nelson and Spaner, 2010). In addition, a healthy soil provides not only a favorable physico-chemical substrate for root development and plant nutrient acquisition, but hosts an intact biotic community that enables healthy plant growth by suppressing pathogens and improving the resistance of plants (Alabouvette et al., 2004; Janvier et al., 2007).

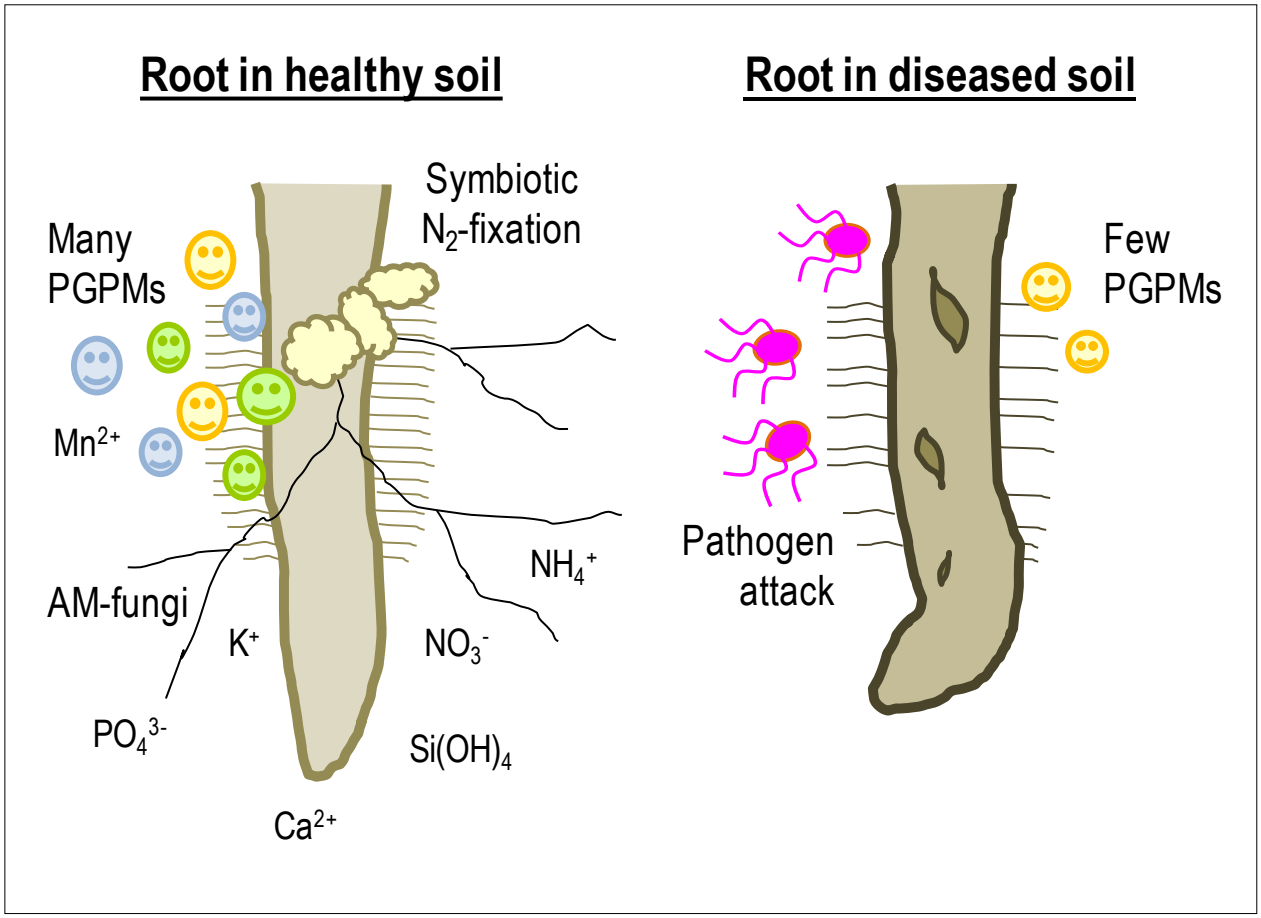

Fig. 7.1: Plant roots growing in healthy soils typically support large and diverse populations of microorganisms in the rhizosphere. Plant growth-promoting microorganisms (PGPMs) and the establishment of effective symbioses with arbuscular mycorrhizal fungi (AM-fungi) and N_2-fixing rhizobia contribute to the acquisition of water and mineral nutrients and to the suppression of phytopathogenic organisms. In diseased soils, a disturbed balance among functional microbial groups may favor the development of deleterious populations resulting in the buildup of diseases (adapted from: Gunawardena et al., 2006).

Diseased soils, in contrast, are characterized by disturbance of the balance among functional microbial groups, favoring the development of deleterious populations and resulting in the buildup of soil-borne diseases (Nitta, 1991; Alabouvette et al., 2004). These conditions may also affect the nutritional status of plants adversely to such extend that the symptoms of pathogenic diseases frequently include mineral deficiencies or toxicities (Huber and Wilhelm, 1988). Conversely, the adequate availability of mineral nutrients in soils is not only important for optimal plant growth and development, but also the disease resistance and general tolerance of plants to various stresses largely depends on their nutritional status (Huber and Graham, 1999). It should be possible, therefore, to

maintain or restore healthy plant growth through an adapted crop management that optimizes the conditions in the soil environment by integrated approaches, including an optimized availability of mineral nutrients and a balanced diversity of microorganisms in the rhizosphere (Huber and Graham, 1999; Alabouvette et al., 2004; Morgan et al., 2005; Römheld and Neumann, 2006). However, the complexity and specificity of the manifold interactions between plants, soil biota and abiotic soil factors contributing to the suppression of soil-borne diseases are only incompletely understood (Beare et al., 1995; Postma et al., 2008). In the following sections this issue is elaborated by reviewing the current knowledge on the range of microbial diversity in soils and the prevailing difficulties to characterize soil microbial communities consistently (Chapter 7.1.1 and 7.1.2). Furthermore, specificities of interactions between plants, microorganisms in their soil environment, and mineral elements that contribute to soil fertility and healthy plant growth for sustainable crop production are emphasized (Chapter 7.2 and 7.3).

7.1.1 The range of microbial diversity in soils

Assumptions on the diversity of microbial life in soils are in the range of $2*10^4$ to $4*10^4$ bacterial species, as assessed by use of cultivation independent genomic methods (Torsvik et al., 1990; Tiedje, 1995; Brussaard et al., 1997). Little information is available on the numbers of coexisting fungal (van Elsas et al., 2000; Kirk et al., 2004), protozoan (Esteban et al., 2006), and nematodal (Bongers and Bongers, 1998) species in soils. It is likely, however, that less than 5 to 25 % of the globally existing species in these groups have been described, which impedes accurate estimations (Hawksworth, 1991; Coleman and Whitman, 2005). Yet, as analytical improvements by Gans et al. (2005) have recently revealed, even the diversity of bacterial species in soils could have been underestimated by two orders of magnitude in previous studies and may actually account to more than $8*10^6$ different species, with rare taxa accounting for 99.9 % of the diversity. Similar careful consideration is necessary when interpreting total numbers of different microbial groups, which have been estimated to be as high as 10^8-10^9 bacterial, 10^5-10^6 fungal, 10^3-10^5 protozoan, and 10^1-10^2 nematodal organisms per gram of soil (Metting, 1993). In particular, estimates of fungal numbers in soil are complicated by their indeterminate mycelial growth habit, while fungal biomass (500-5000 kg ha^{-1}) is generally found to be

greater than bacterial biomass (300-3000 kg ha^{-1}) in agricultural soils (Metting, 1993; de Vries et al., 2006; Finlay, 2007; Joergensen and Wichern, 2008). Protozoa (5-200 kg ha^{-1}) and nematodes (1-100 kg ha^{-1}) revealed to contribute only a small share to the total microbial biomass in soils (Vickerman, 1992; Metting, 1993), but have the capacity to significantly influence the structure of bacterial communities in soils (Rønn et al., 2002; Blanc, 2006; Clarholm et al., 2007). In their function as bacterial grazers, also the role of protozoa and bacterial-feeding nematodes in re-mineralizing and thus increasing the availability of mineral nutrients to plants is out of proportion to their biomass (Vickerman, 1992; Bonkowski, 2004; Djigal et al., 2004).

7.1.2 Approaches and limitations to study microbial communities in soil

Regarding the enormous diversity and abundance of microorganisms in soils and the associated difficulties to study soil microbial communities at a high level of resolution, recent approaches have been focusing on the characterization of functional groups rather than the investigation of single species (Pankhurst, 1997; Emmerling et al., 2002; Nybroe et al., 2007; Torsvik and Øvreås, 2007). This comprises the measurement of microbial biomasses, respiration, nitrogen mineralization, and the analysis of enzyme activities involved in the cycling of carbon and mineral nutrients such as N, P, or S (Kandeler et al., 1996). In further approaches, the analysis of nucleic acid sequences, such as genes encoding enzymes for important ecosystem processes like nitrification (Kowalchuk et al., 2000), denitrification (Priemé et al., 2002; Cantera and Stein, 2007), or nitrogen fixation (Widmer et al., 1999), can be accomplished. Likewise, the detection of specific components of microbial cell membranes, such as phospholipid fatty acids (PLFAs), can be deployed to facilitate the separation of large functional groups within the soil microbial community (Frostegård and Bååth, 1996; Kandeler et al., 1998). However, as recently reviewed, all these methods have their biases and limitations and only the combination of molecular/genetic and physiological characterization with improved isolation and cultivation techniques may reveal a holistic view on the role of microbial diversity in soil ecosystem functioning (Muyzer and Smalla, 1998; Kandeler, 2007; Thies, 2007, 2008). The results of enzymatic assays have to be interpreted with caution, as they do not distinguish between the activity of intracellular, extracellular and those enzymes that are

stabilized by adsorption to the soil matrix, and thus they do not accurately reflect microbial activity (Nannipieri et al., 2003; Kandeler, 2007). Direct microscopic counts of soil bacteria are typically 100 to 1000 times higher than counts obtained by culturing methods, but many prokaryotic species cannot be distinguished by visual analyses alone (Skinner et al., 1952; Torsvik and Øvreås, 2007). Molecular techniques have a huge potential to provide analytical access to the more than 99 % of soil microorganisms that cannot be cultivated with current growth media (Amann et al., 1995; Felske, 1999; Zengler et al., 2002). Yet, due to biases in the extraction and amplification of nucleic acids from soil (Oros-Sichler et al., 2007) as well as technical constraints related with the in-situ detection of genomic elements in soil (Schmid et al., 2007), the currently available molecular methods provide only fragmentary information on the actual composition of soil microbial communities, too (Thies, 2007). Fingerprinting methods for nucleic acids such as denaturant gradient gel electrophoresis (DGGE; Muyzer and Smalla, 1998) or terminal restriction fragment length polymorphism (T-RFLP; Liu et al., 1997) may not resolve more than about 100 operational units that, moreover, do not necessarily reflect different species or taxonomic groups. Such levels of taxonomic resolution, however, are still far from estimates of the actual microbial diversity in soils (Thies, 2007). Likewise, the quantities of extracted PLFAs are not directly related to microbial biomass in soil. Rather, error-prone conversion factors from mass of PLFA to mass of microbes are in use to estimate microbial biomasses based on PLFA concentrations. In addition, the taxonomic resolution obtainable from PLFA analyses is inherently limited because lipids are not as diverse as nucleic acid sequences (Frostegård and Bååth, 1996; Green and Scow, 2000; Petsch et al., 2001). A more general challenge to soil ecology lies in the up-scaling of results obtained at the micro level of soil samples, which are typically small, to larger units such as field plots, because patterns of spatial variability are largely unknown (Kandeler, 2007; Joschko et al., 2008). Hot spots, or localities of exceptionally high concentrations of microbial life, typically show a heterogeneous distribution within the soil matrix and may account for only a small proportion of the total soil volume, while representing zones of most intensive biological activity in soils (Beare et al., 1995; Nannipieri et al., 2003).

7.2 The relevance of specificity in rhizospheric plant-microbe interactions

7.2.1 The role of reciprocity in the development of microbial rhizosphere communities

In future, the development of advanced molecular and physiological tools together with improved isolation and cultivation techniques may enable the characterization of soil microbial communities as a whole (Muyzer and Smalla, 1998; Alabouvette et al., 2004). Until recently, however, the importance of single species in the numerous and complex interactions between organisms in soils and their contribution to ecosystem functioning and diversity remain poorly understood (Anderson and Cairney, 2004; Kirk et al., 2004; Rajendhran and Gunasekaran, 2008; Donn et al., 2015). Natural ecosystems and organic farming systems are generally thought of as being more resilient against the proliferation of soil-borne pathogens due to a higher biodiversity than found in conventionally managed soils. Nevertheless, the suppression of distinct diseases may be largely due to specific relationships between pathogens and antagonists (van Bruggen et al., 2006). Accordingly, Postma et al. (2008) have shown that the suppressiveness of soils from organic farms against three pathogens (*Rhizoctonia*, *Streptomyces*, *Verticillium* sp.) in distinct crops was related to the presence of specific bacterial antagonists besides of general soil characteristics such as pH, bacterial diversity, fungal activity or bacterial biomass. Although, there is evidence that some species can replace each other in certain functions, no species and even no member of the same species are identical and therefore will not play exactly the same role in every case (Beare et al., 1995; Muyzer and Smalla, 1998; Emmerling et al., 2002). This genetic variability within and between species allows microbial communities a rapid adaptation to environmental changes if there are sufficient numbers of suitable genotypes present in the soil. In contrast, highly specific functions that are performed by a limited number of community members with narrow niche requirements are particularly sensitive to disturbances (Giller et al., 1997). An extreme instance for the latter case are symbiotic *Rhizobium* bacteria, where the absence of a single species can result in the total loss of symbiotic nitrogen fixation for a specific legume host (Giller et al., 1997). Furthermore, plant specific patterns of rhizodeposition, in particular the amount and composition of root exudates, exert selective effects in

attracting and promoting distinct microbial populations (Marschner et al., 2004; Dennis et al., 2010). To that effect, rather few species out of the diverse microbial community in soils can be found as predominant colonizers and major actors in the rhizosphere (Yang et al., 2001; Hartmann et al., 2009). Different plant species growing in the same soil appear to be characterized by distinct patterns of the microbial community structure in their rhizospheres (Ibekwe and Kennedy, 1998; Marschner et al., 2001). Conversely, a given plant species may attract and host similar microbial communities when grown in different soils (Grayston et al., 1998; Miethling et al., 2000; Marschner et al., 2004). In addition, also microbial activity can modify the conditions in the rhizosphere by affecting root exudation (Meharg and Killham, 1991), producing growth factors that influence root growth (Müller et al., 1989), altering physico-chemical soil properties as to increase the availability of nutrients (Marschner and Rengel, 2007), or due to interactive processes within the complex community of soil organisms (Whipps, 2001). A central role in plant-microbial interactions is played by mycorrhizal fungi, as they mediate the allocation of carbon, which they obtain from their photosynthetic host plant, through their root-external mycelia to the soil (Finlay and Rosling, 2005; Finlay, 2008). It has been shown, in this regard, that mycorrhization induces quantitative and qualitative alterations in root exudation (Jones et al., 2004; Lendzemo et al., 2009), which can results in respective changes of the microbial community composition in the rhizosphere (Ames et al., 1984; Marschner and Baumann, 2003).

From this brief recapitulation, of what has already been discussed in further details (Chapters 2 and 3), it is evident that microbial communities in soil and rhizosphere ecosystems are participating in a complexity of interactive and dynamic processes that are likely to govern the instantaneous population sizes and activities of single species (Loper et al., 1985; Kent and Triplett, 2002). Thereby, different plants develop characteristic microbial colonization patterns in their rhizospheres, which may also vary during plant growth stages (Wieland et al., 2001). Approaches to investigate the specificities of such interactions, therefore, appear to be more relevant for understanding the processes that regulate soil fertility and healthy plant growth, than those aspiring to structure the richness of biological diversity into relatively few functional groups (Beare et al., 1995; Brussaard et al., 2004).

7.2.2 Induction or suppression of soil-borne plant diseases by plant-specific rhizosphere communities

The above sections indicated that, from an agronomic perspective, specific interactions between plants and certain microorganisms could be of greater interest than structural or functional profiles of the complete microbial diversity in soils. This applies not only to those interactions that influence plant nutrient uptake and growth, but also the resistance of plants against pathogenesis depends on the specific functions and activities exerted by distinct species out of the existing microbial soil community. The microbiological status of a given soil, therefore, is of particular relevance in relation to its plant specific response potential, which may result in beneficial, neutral, or deleterious health outcomes (Dommergues, 1978). The literature provides numerous examples of specific interrelationships between crop plants and resident microbial populations in soils that are involved in pathogenesis or disease suppression (Weller et al., 2002). Probably one of the first detailed descriptions of such phenomena was made by Hiltner (1904), who explained the growing incidence and subsequent decline of replant disease in pea (*Pisum sativum* L.) crops by the selective effect of specific root deposits, which after repeated cropping induced the successive adaptation of pathogenic respectively antagonistic populations of root colonizing soil bacteria.

In the more recent past, qualitative changes in the community structure of rhizobacterial species in response to crop management practices have been receiving renewed attention with respect to the receptivity of soils to soil-borne diseases of diverse crop plants (Schippers et al., 1987; Alabouvette et al., 2004). There is considerable evidence that cropping sequence can affect the balance of deleterious (DRB) (Suslow and Schroth, 1982a) and plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1980; Kloepper and Schroth, 1981). As thought to be minor pathogens, deleterious rhizobacteria not necessarily parasitize the plant tissue, but they may affect plant growth adversely by inducing detrimental alterations in water and mineral availability, phytohormone levels, root development, and weakening the resistance of plants towards major pathogenic diseases (Salt, 1979; Schippers et al., 1985; Schippers et al., 1987). The converse mechanisms by which plant growth-promoting rhizobacteria exert their benefits also

include the suppression of minor (Suslow and Schroth, 1982a) and major pathogens (Kloepper et al., 1980a;b).

Deleterious rhizosphere microorganisms and replant diseases

Prevalent rhizosphere colonization by deleterious pseudomonads seems to be critically involved in the formation of specific replant diseases in apple orchards (Čatská, 1982), citrus groves (Gardner et al., 1984), and grapevine nurseries (Waschkies et al., 1994). Furthermore, there is evidence that the roots of sugar beets (Suslow and Schroth, 1982a), wheat (Elliott and Lynch, 1984; Fredrickson and Elliott, 1985), and potato (Bakker and Schippers, 1987) can be colonized by diverse bacteria that are inhibitory to plant growth, which has been largely attributed to the bacterial production of toxins. For citrus cultivars, the host specificity of root colonizing pseudomonads has been demonstrated by Gardner et al. (1984), whereby the effect of distinct bacterial strains was found to alternate between stimulation and inhibition of plant growth depending on the given citrus variety. Besides hampering root and shoot growth, biotic factors associated with the replant disease of grapevine appeared to decrease the formation of arbuscular mycorrhiza (AM) in grapevine rootstocks, whereas maize grown in the same soil neither showed disease symptoms nor an inhibited mycorrhization, thus indicating the specificity of the plant-microbial interactions (Waschkies et al., 1994). While mitigating the severity of the disease, the application of additional mycorrhiza inoculum not only increased the mycorrhization of grapevine roots, but also appeared to decrease the predominance of deleterious pseudomonads on the rhizoplane (Waschkies et al., 1994). Similar effects of improved plant growth, root health and mycorrhization could, however, also be achieved with the application of a biocontrol agent based on a PGPR strain of *Pseudomonas fluorescens* "RA 56" (Phytobacter® GmbH, Wallmow, Germany), which might be explained by direct antagonism against the deleterious pseudomonads as well as by mycorrhization helper effects (Fig. 7.2; Weinmann, 2000; Kaließ, 2008).

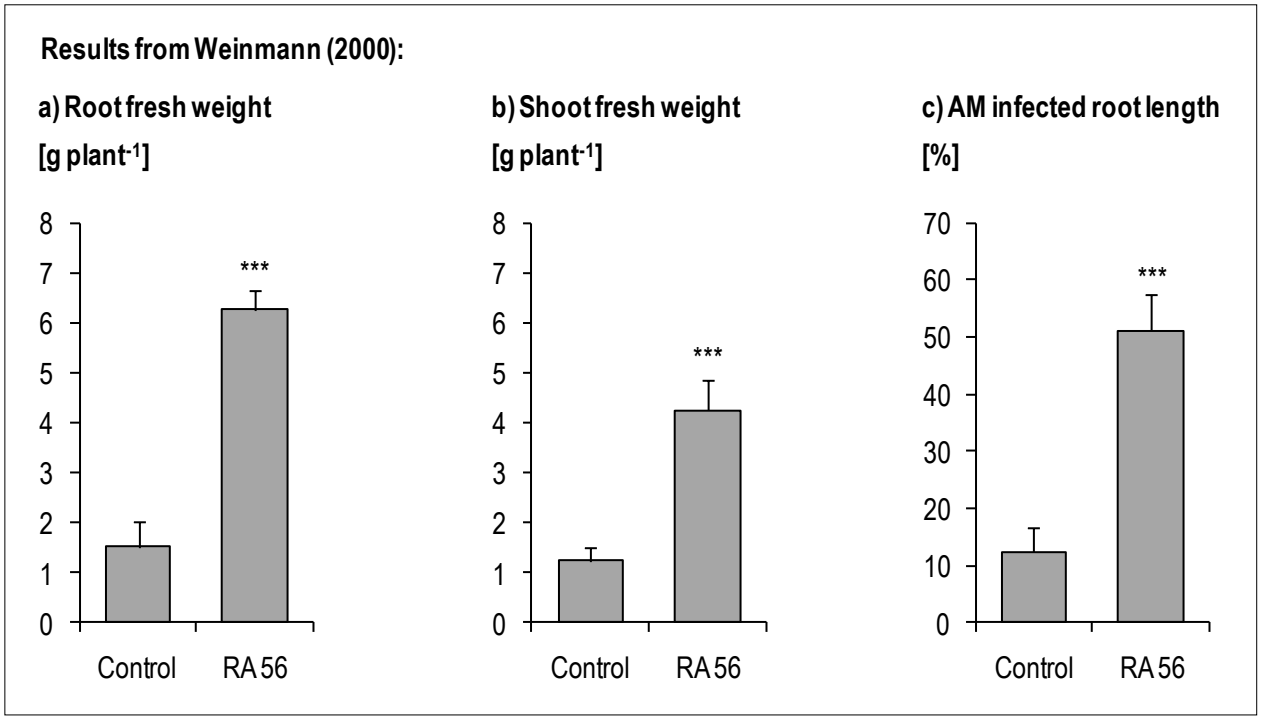

Fig. 7.2: Root (a) and shoot (b) biomass production and arbuscular mycorrhiza (AM) infected root length (c) of two-bud green cuttings of grapevine rootstock clone Teleki-Kober 5 BB (Vitis berlandieri Planch. x Vitis riparia Michx.) grown on a replant disease affected soil inoculated with a bio-control strain of Pseudomonas fluorescens "RA 56" (Phytobacter® GmbH, Wallmow, Germany) or not (control). Plants were cultivated in a pot experiment under controlled temperature and light conditions in a climate chamber at the Dienstleistungszentrum Ländlicher Raum (DLR) Rheinpfalz, Neustadt-Mußbach, Germany from 31st May until 31st August 1999.

Columns represent mean values ± SEM (n = 6). Data were tested using Student's t-test for unpaired samples, comparing RA 56 treated plants versus control plants. Significant differences are indicated by asterisks (***$p \leq 0.001$). Although locational effects should have been largely excluded due to the controlled conditions in the climate chamber, results should be interpreted with care because pots were not installed consistently in a randomized design for practical reasons.

Plant growth-promoting microorganisms and disease suppressive soils

Plant growth-promoting strains of *Pseudomonas* spp. have been found to predominate in soils that are naturally suppressive to take-all root rot disease of wheat caused by the soil-borne fungus *Gaeumannomyces graminis* (Sacc.) v. Arx. & Olivier var. *tritici* Walker (*Ggt*) (Cook and Rovira, 1976; Smiley, 1979; Weller and Cook, 1983; Sarniguet et al., 1992a) or *Fusarium* wilt pathogens of diverse crops (Scher and Baker, 1982; Lemanceau and Alabouvette, 1991). The suppressiveness of soils against take-all can develop as a consequence of continuous cropping of wheat or barley over a number of years after one or more severe outbreaks of the disease (Hornby, 1983; Andrade et al., 1994; Mazzolla,

2002; Weller et al., 2002). This decrease in disease incidence, also known as take-all decline (TAD), has been shown to correlate with multiple changes in community composition of bacteria in the rhizosphere, beyond the sole case of antagonistic pseudomonads (Sanguin et al., 2009). Furthermore, in different soil types and under varying environmental conditions, different taxonomic groups of microbial antagonists and diverse mechanisms of suppression may be responsible for take-all decline that occurs worldwide in agro-ecosystems (Andrade et al., 1994; Weller et al., 2002). Among the bacterial and fungal genera that have been frequently reported for their contribution to the suppressiveness of soils against take-all, but also *Fusarium* diseases, are *Pseudomonas* (Leeman et al., 1996; Barnett et al., 1998), *Bacillus* (Kim et al., 1997; Cavaglieri et al., 2005), and *Trichoderma* fungi (Dunlop et al., 1989; Simon, 1989; Sivan and Chet, 1989a). Both, the enhanced population of the take-all fungus as well as the continuous presence of wheat roots as specific carbon source, could select for increased populations of antagonistic microorganisms emerging in take-all decline soils (Alabouvette et al., 2004). As reported by Gerlagh (1968), for instance, the repeated addition of *Ggt* inoculum induced suppressiveness in soils irrespective of whether wheat had been grown or not, which indicated that presence of the pathogen alone was sufficient to the buildup of antagonistic populations. In contrast, the observation that grass weeds hosting the take-all fungus disrupt or delay the development of take-all decline in wheat, suggests that the microflora associated with take-all decline in wheat is specific to the rhizosphere of wheat plants (Hornby, 1998; Alabouvette et al., 2004; Chng et al., 2005). Although, soil inoculation with AM-fungi, has been suggested to protect crop plants against soil-borne fungal diseases, such as *Fusarium* wilt of cucumber plants, little is known about the possible interactions between DRB, PGPR, AM-fungi and pathogenic fungi in the rhizosphere (Torres-Barragán et al., 1996; Hao et al., 2005; Hu et al., 2010). Graham and Menge (1982) reported a suppressive effect of AM against take-all disease in wheat, which appeared to be a consequence of increased phosphate status and decreased root exudation by the plants. Because hyphae of *Gaeumannomyces* appear to respond chemotropically towards wheat roots and their exudates, mycorrhization-induced changes in the quality or quantity of such compound releases might also alter the infection process of the take-all fungus (Pope and Jackson, 1973). Recently, Khaosaad et al. (2007)

demonstrated that a high degree of mycorrhizal root colonization preceding pathogen attack could strengthen the systemic resistance of barley (*Hordeum vulgare* L.) roots against *Ggt*. In this connection, it is furthermore worthwhile to re-emphasize that the antagonistic effect of certain *Pseudomonas* strains against take-all and *Fusarium* pathogens has been largely related to their capacity to produce antibiotics, such as phenazine-1-carboxylic acid (PCA) (Gurusiddaiah et al., 1986; Brisbane et al., 1987; Upadhyay and Srivastava, 2010) and 2,4-diacetylphloroglucinol (DAPG) (Keel et al., 1992; Harrison et al., 1993; Duffy and Défago, 1997; Raaijmakers and Weller, 1998; Schnider-Keel et al., 2000). As recently shown by Siasou et al. (2009), selected strains of *Pseudomonas fluorescens* revealed to produce the antibiotic DAPG only when they were supplied with soluble carbon extracted from soil in which wheat plants had been grown in the presence of *Ggt* or AM-fungi, but not in the absence of these fungi. Moreover, the bacterial production of DAPG increased by two orders of magnitude in response to extracts from soil containing both the pathogenic and mycorrhizal type of fungal inoculants (Siasou et al., 2009). These results, however, were obtained with bacteria grown on artificial media and the influence of AM-fungi on the populations of pseudomonads in the rhizosphere, which can be selectively affected by root exudates and mycorrhizal root colonization (Sands and Rovira, 1971; Meyer and Linderman, 1986b), has not been investigated in this study on take-all disease. Further, Bergsma-Vlami et al. (2005) demonstrated soil and plant species-specific influences on the population dynamics, genotypic diversity, and DAPG-producing activity of rhizosphere colonizing *Pseudomonas* species. When grown in a take-all suppressive soil, the rhizospheres of wheat and potato supported higher amounts of DAPG production per colony forming unit of indigenous *Pseudomonas*, carrying a gene indicative of the ability to produce DAPG, than the rhizospheres of sugar beet. In a take-all conductive soil, this pattern of DAPG production did not change for potato and sugar beet, whereas no DAPG production was detectable in the wheat rhizosphere. In the rhizosphere of lily, however, DAPG production was not detectable for both soils. These findings indicate that in take-all conductive soils the inoculum potential of antagonistic *Pseudomonas* spp. not necessarily needs to be low. Rather, the influence of unknown but wheat specific factors appears to

inhibit the establishment of DAPG producing populations in the rhizosphere of wheat when grown in take-all conductive soils.

7.3 The importance of genotypic and nutritional factors for the disease resistance of plants

7.3.1 Preformed and inducible mechanisms of parasite defense in plants

Plants have developed a versatile arsenal of preformed and inducible mechanisms of resistance against pathogenic organisms (Ingham, 1973). In general, this comprises physical barriers and metabolic defenses of the plant that counteract the mechanical and biochemical activities exerted by diverse parasites during initial and advanced stages of pathogenesis on host tissues (Marschner, 1995; Agrios, 2005). Preformed physical or chemical barriers include wax layers, rigid cell walls, and preexisting antimicrobial metabolites (i.e. phytoanticipins), which may initially stop or impede the establishment of infection structures (VanEtten et al., 1994; Dixon, 2001; Heath, 2000; Nürnberger et al., 2004). Pathogens that manage to overcome barriers that are constitutively present on plant surfaces, however, might be recognized at the plasma membrane of plant cells, which is likely to activate additional resistance mechanisms (Nürnberger and Lipka, 2005). Such inducible defense responses comprise the synthesis and accumulation of reactive oxygen species, the expression of pathogenesis related genes, the de novo synthesis of antimicrobial metabolites (i.e. phytoalexins), the localized reinforcement of plant cell walls, and the hypersensitive cell death response (HR) (Heath, 2000; Lam et al., 2000., Thordal-Christensen, 2003; Mysore and Ryu, 2004). In recent reviews, furthermore, the system of inducible resistance in plants has been referred to comprise a continuum of layered defense responses following pathogen attack (da Cunha et al., 2006; Jones and Dangl, 2006; de Wit, 2007; Boller and He, 2009). The primary line of inducible resistance in plants is thought to be activated by the recognition of microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) through pattern recognition receptors (PRRs) that mediate primary defense responses in plants (de Wit, 2007; Pieterse et al., 2009). These basal resistance reactions include the production of reactive oxygen species and ethylene, localized cell wall reinforcements such as depositions of callose, lignin and

silicon, and the accumulation of defense related proteins including chitinases, glucanases and proteases, which may inhibit colonization by potential pathogens (van Loon et al., 2006; Ade and Innes, 2007; Adams-Phillips et al., 2010). MAMPs or PAMPs are synonymously called general microbe or pathogen-derived elicitors, as they constitute highly conserved determinants, such as bacterial flagellin (Gómez-Gómez and Boller, 2002; Jones et al., 2007), that are characteristic of whole classes of microbial organisms (Montesano et al., 2003; Nürnberger and Lipka, 2005; Suzuki et al, 2007). In addition, breakdown products of the plant cell wall, which are probably released by hydrolytic activities from attacking pathogens, can function as plant-derived elicitors of defense responses in plants (Nürnberger, 1999; Vorwerk et al., 2004). During evolution, however, individual races or strains of given pathogen species, have acquired additional virulence factors, also called "effectors", enabling them either to evade or to suppress reactive defense mechanisms of certain host plants (Abramovitch and Martin, 2004; Alfano and Collmer, 2004; Chang et al., 2004). Conversely, the selective pressure exerted by virulent pathogens has resulted in the co-evolution of specific resistance factors that enable distinct plant genotypes to recognize specific virulence factors, thus allowing for the expression of pathogen strain- or race-specific forms of resistance (de Wit, 1992; Abramovitch and Martin, 2004; Chang et al., 2004; Espinosa and Alfano, 2004; Jones and Takemoto, 2004). The ongoing arms race of virulence and resistance thus results in a continuous succession of co-evolutionary specialization in both parasite and plant genotypes (Jones and Dangl, 2006; Anderson et al., 2010). Genetically, the more specific secondary forms of inducible resistance in plants are thought to be determined by complementary pairs of plant resistance (*R*) genes and pathogen-encoded virulence genes, which, due to the recognition of their gene products by the plant, function as avirulence (*avr*) genes (Flor, 1971; Gabriel and Rolfe, 1990; Newton and Andrivon, 1995; van de Weg, 1997; Dixon et al., 2000). Most *R* genes encode NB-LRR proteins, named after their characteristic nucleotide binding (NB) and leucine rich repeat (LRR) domains, which recognize corresponding virulence factors (e.g. avirulence proteins encoded by *avr*-genes) either directly or by detecting virulence activity-induced perturbations of host targets (Jones and Dangl, 2006). Although, little is known about the intracellular signaling events involved in these processes, the recognition of virulence factors initiates strong local

defense reactions that often culminate in a hypersensitive cell death response blocking further growth of the pathogen (Jones and Dangl, 2006). In addition to inducible local defenses, plants possess inducible systemic responses that establish an enhanced defensive capacity in tissues distant from the site of primary attack, protecting the plant against subsequent infestation of parasites (Pieterse and Van Loon, 2004). The regulation of local and systemic defense responses in plants is generally associated with signaling pathways that involve salicylic acid (SA) and the combination of jasmonic acid (JA) and ethylene (ET), besides the interference of other phytohormones (Anderson et al., 2004; Pieterse and Van Loon, 2004; Bostock, 2005; Jones and Dangl, 2006). For instance, the SA-dependent pathway of systemic acquired resistance (SAR) is induced by necrotizing pathogens, but most effective against biotrophic pathogens. The JA- and ET-dependent pathway of induced systemic resistance (ISR), in contrast, which is activated upon root colonization by certain non-pathogenic rhizobacteria and fungi (see Chapter 2.3), is conferring resistance mainly towards necrotrophic pathogens (Ton et al., 2002; Hammond-Kosack and Parker, 2003; Pieterse and Dicke, 2007). However, there is increasing evidence for overlaps and interactions between different defense pathways (see Chapter 2.1.2), including both up and down regulatory mechanisms, which may allow plants to specifically modulate their defense responses according to the nature of invading pathogens (Glazebrook et al., 2003; Rojo et al., 2003; Anderson et al., 2004; Pieterse and Van Loon, 2004; Bostock, 2005; Ahn et al., 2005; Beckers and Spoel, 2006).

7.3.2 The influence of mineral nutrients on the disease resistance of plants

Although, the susceptibility or resistance of plants depends, directly or indirectly, on the genetic material of the host and of the parasite, mineral nutrients play particularly important roles in plant-parasite interactions, besides other environmental factors (Staskawicz et al., 1995; Staskawicz, 2001; Agrios, 2005). Thus, not only the growth and development of plants and related parasites, but also the successful expression of plant defense and parasite virulence genes depends on their respective status of mineral nutrition (Marschner, 1995; Huber and Graham, 1999; Walters and Bingham, 2007). For instance, enhanced levels of silicon and manganese in foliar tissues, due to improved acquisition through the root, have been associated with increased resistance against

powdery mildews in wheat (Colquhoun (1940); cited in: Wilhelm et al., 1985; Leusch und Buchenauer, 1988b) and grape wine plants (Fig. 7.3).

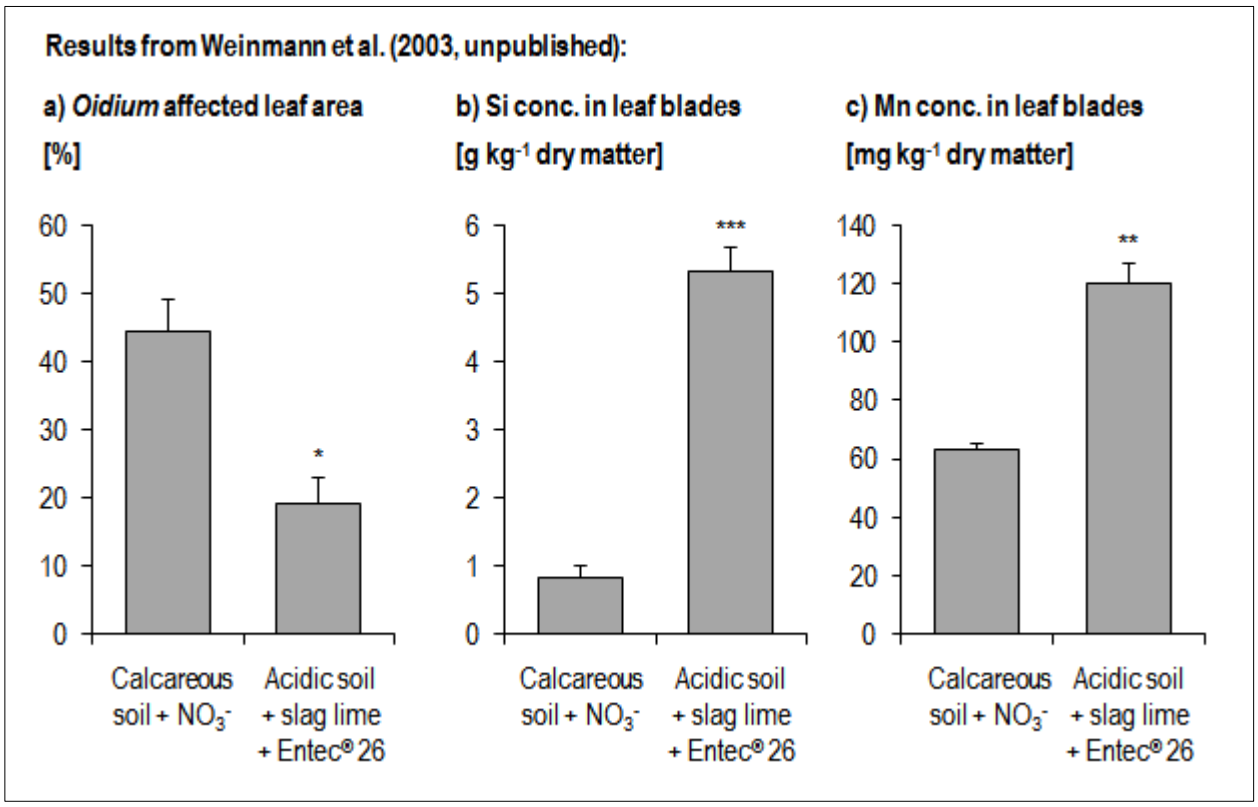

Fig. 7.3: Percentage of leaf area affected by symptoms of powdery mildew (Oidium tuckeri Berk. [teleomorph Uncinula necator (Schwein.) Burrill]) and concentrations (conc.) of silicon (Si) and manganese (Mn) in the blades of young fully developed leaves of the grape vine (Vitis vinifera L.) variety Bacchus (Geilweilerhof 33-29-133). The plants, two-bud wooden cuttings, were grown on a calcareous loamy substrate (pH 7.6) fertilized with nitrate-N (NO_3^- supplied as $Ca(NO_3)_2$) and iron (supplied as FeEDTA) or on an acidic sandy substrate (pH 4.6) fertilized with slag lime and ammonium nitrate plus ammonium sulfate with nitrification inhibitor supplied as Entec® 26(+13S) (K+S Nitrogen GmbH, Mannheim, Germany). Both substrates were supplied with basal fertilization of P, K, and Mg. The experiment was conducted as a pot experiment with randomized block design in a greenhouse at Universität Hohenheim, Stuttgart, Germany from 12th May until 18th July 2003.

Columns represent mean values ± SEM (n = 8 for disease ratings, n = 4 for mineral nutrient analyses). Data were tested in two-way ANOVAs with substrate treatment and block as factors. In cases of significant treatment effects, the analyses were followed by Tukey's test. Significant differences between treatments are indicated by asterisks (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

In addition, the availability of mineral nutrients affects the ability of plants to escape from or tolerate parasitism by enhanced growth rates or morphological changes, but may also

induce alterations in the external environment of plants thus influencing the activity and survival of parasites (Huber and Wilhem, 1988; Marschner, 1995).

As shown in extensive literature surveys (Huber and Graham, 1999; Dordas, 2008), all the essential minerals for plant nutrition can affect the severity of parasitic diseases. There are, however, no general trends. Depending on the plant genotype, the environmental conditions, as well as its amount and chemical form, a particular mineral nutrient may suppress the establishment of some parasites but increase the devastating incidence of others. A balanced nutrient supply that is optimal for plant growth, therefore, should provide optimal conditions for resistance to a broad spectrum of parasitic diseases, too. In contrast, deficiencies or excessive supplies of mineral nutrients often predispose plants to invasion by parasites (Marschner, 1995; Huber and Graham, 1999). Nevertheless, due to their specific functions in distinct plant-parasite interactions, the supply of certain mineral elements that is necessary for an effective expression of resistance mechanisms by the plant may exceed their basic needs for an optimal growth (Huber and Graham, 1999). On the contrary, some pathogens are known to increase the disease susceptibility of plants by immobilizing mineral nutrients in the rhizosphere or in infected host tissues, while others cause disturbances in mineral nutrition by interfering with the nutrient uptake, translocation or use efficiency of plants (Timonin, 1946; Huber, 1978).

Functions of distinct nutritional elements in pathogen defense

The most intensively studied mineral nutrients with respect to their influence on the disease resistance of plants are nitrogen, potassium, calcium, and, among the micronutrients, manganese (Huber and Graham, 1999), but also for other mineral nutrients specific roles in pathogen defense have been proposed (Graham and Webb, 1991; Walters and Bingham, 2007; Dordas, 2008). Furthermore, silicon is considered as a particularly beneficial element for the healthy growth of plants (Huber and Graham, 1999).

Nitrogen

The effect of nitrogen on plant diseases may depend on the form, amount, and timing of nitrogen supply, as well as on the genotypes of hosts and pathogens (Huber and Graham,

1999; Hoffland et al., 2000). Both, the ammonium (NH_4^+) and the nitrate (NO_3^-) form of nitrogen are taken up and assimilated by plants, but they frequently have opposite effects on the severity of pathogenic diseases (Huber and Graham, 1999). As shown in a literature overview by Huber and Watson (1974), many pathogenic diseases that are suppressed by ammonium nutrition of the host plant are exacerbated by nitrate nutrition, and vice versa. This differential influence of the form of nitrogen nutrition on disease severity has been mainly explained by its respective effect on the pH in the rhizosphere, as for many diseases the effect of ammonium or nitrate nutrition is consistent with that of low or high soil pH, respectively (Huber and Wilhelm, 1988; Huber and Graham, 1999). *Fusarium* wilt of eggplants, *Verticillium* wilt of vegetables, blast (*Pyricularia grisea*) of rice, take-all disease of cereals, and *Streptomyces* scab of potatoes for instance are less severe in acidic soil and ammonium nourished plants (Huber and Graham, 1999). In contrast, the severity of *Fusarium* wilt of cucumber, tomato, and pepper, but also *Rhizoctonia solani* induced diseases of potato, bean (*Phaseolus vulgaris*), and pea (*Pisum stativum*) are decreased by nitrate and alkaline soil conditions (Huber and Graham, 1999). As shown by Smiley and Cook (1973) in diverse soils, with uptake of ammonium the pH in the rhizosphere of wheat plants decreased by more than one pH unit, whereas it increased with uptake of nitrate. In addition to the acidification of the rhizosphere, an ammonium based plant nutrition may intensify the formation and elongation of lateral roots (Marschner et al., 1986), which may improve the availability of water and mineral nutrients due to an enhanced spatial and chemical acquisition by the plant. It has been shown in this regard that ammonium supply increased the nutritional status of phosphorus (Jungk et al., 1993), silicon (Leusch and Buchenauer, 1988a) and of the micronutrients iron, manganese, zinc and copper (Thomson et al., 1993) in plants, particularly when they were grown on neutral to alkaline soils. Furthermore, ammonium supply has been reported by Huber and Wilhelm (1988) to provide a reductive environment and decrease the population of Mn-oxidizing microorganisms in the rhizosphere of wheat, thereby increasing the availability of manganese to the plant. The opposite effect is induced by nitrate, which provides a readily available source of oxygen for microbial oxidations and increases the population of Mn-oxidizing microorganisms.

Besides its influence on rhizosphere processes, the level of nitrogen nutrition also directly affects the physiological basis of plant resistance. In plants, nitrogen stimulates vigorous growth, delays maturity, and is necessary for the synthesis of nucleic acids, amino acids, proteins, phytohormones, and defense related secondary metabolites such as alkaloids and phenolic compounds (Huber, 1980; Herrmann, 1995a; Daniel et al., 1999; Mengel and Kirkby, 2001). Regarding diseases in the phyllosphere, the effect of nitrogen supply has been generally referred to differ depending on the trophic lifestyle of the pathogen (Marschner, 1995). While diseases induced by obligate biotrophic parasites such as powdery mildews (*Erysiphales*), rusts (*Pucciniales*), and downy mildews (*Peronosporaceae*) increase in severity when there is a high nitrogen supply, the opposite response occurs for facultative parasites such as *Alternaria* and *Fusarium* species as well as many pathogenic bacteria (Marschner, 1995; Riethmüller et al., 2002; Mandal et al., 2008). These differences in response of the two types of parasites have been related to their specific nutritional requirements. Obligate parasites require assimilates supplied by living cells, whereas facultative parasites are semisaprophytes that prefer senescing tissues or produce toxins to kill the host tissue before using it as a growth substrate (Dubery et al., 1994; Marschner, 1995). Therefore, nitrogen supply and other factors that support the metabolic activity and delay the senescence of host plants may also enhance their resistance or tolerance to facultative parasites (Marschner, 1995). Additional factors such as the plant species and cultural conditions, however, may modify this general pattern of nitrogen influence on the disease susceptibility of plants. Hoffland et al. (1999), for instance, observed increasing resistance of tomato plants to the grey mold fungus *Botrytis cinerea* with increasing nitrogen status of the plants, which were grown under controlled climate conditions. In contrast, the common field observation that high nitrogen supply increases bunch rot of wine grapes has been attributed to nitrogen induced increases in canopy density providing micro-climatic conditions that are more conducive to the development of the same pathogen (Mundy and Beresford, 2007). In the case of obligate parasites, increased susceptibility of plants following high nitrogen supply has been associated with various biochemical and histological alterations in host tissues. During vegetative growth, nitrogen stimulates the formation of young plant tissues that are typically less advanced in the development of preformed barriers such as cell wall

lignifications or silicon depositions and therefore more susceptible to invading pathogens (Marschner, 1995). Furthermore, it has been proposed that due to plant internal competition between metabolic pathways that require carbon skeletons for nitrogen or carbohydrate assimilation, the nitrogen level also affects the balance of biosynthetic processes in plants (Bryant et al., 1983; Marschner, 1995; Jones and Hartley, 1999). High nitrogen availability in plant tissues generally increases the proportion of low-molecular-weight organic nitrogen compounds such as amino acids (Solomon et al., 2003), whereas it decreases the concentration of phenolic compounds, including important defense related metabolites such as lignin and flavonoids with antimicrobial properties (Sander and Heitefuss, 1998; Leser and Treutter, 2005), thus providing favorable conditions for pathogen development. Although the amino acids phenylalanine and tyrosine, produced in the shikimate pathway, are precursors in the synthesis of phenolics (Herrmann, 1995b), high nitrogen supply has been reported to decrease the activity of key enzymes of lignin and flavonoid synthesis (Matsuyama and Dimond, 1973; Treutter, 2005). Alternatively, however, the synthesis of proteins, alkaloids and other nitrogen-based defense compounds in plants may increase with increasing nitrogen availability and thus compensate for a decreased production of phenolics (Mattson, 1980; Lundberg P. and Åström, 1990; Sander and Heitefuss, 1998). Exceptions rather than rules have been reported in many cases and there is increasing evidence that general assumptions do not accurately predict the influence of nitrogen nutrition on the specific interactions between plants and parasites (Hoffland et al., 1999; Hoffland et al., 2000; Hamilton et al., 2001). Yet, even more complex than in the phyllosphere the situation appears to be for soil-borne pathogens. Because plant roots and their rhizospheres are colonized by a wide variety of beneficial and pathogenic microorganisms in soils, the effect of nitrogen, along with other nutrients, is likely to result not only from its influence on the host plant and its pathogens, but also from interactions with other soil organisms and the abiotic soil environment (Huber and Watson, 1974). As an example, the suppressive effect of ammonium supply on take-all disease of wheat in soils with high pH has not only been related to rhizosphere acidification (Smiley and Cook, 1973; Brennan, 1992), but also to qualitative changes within the rhizosphere populations of fluorescent pseudomonads in favor of pathogen-antagonistic strains (Smiley, 1978b; Sarniguet et al., 1992a,b). Furthermore, manganese

reduction and increased uptake of manganese by the host plant seem to be important mechanisms by which such antagonistic rhizobacteria suppress the take-all pathogen (Marschner et al., 1991; Ownley et al., 2003).

Phosphorus

Foliar sprays of dissolved phosphate salts controlled fungal diseases such as anthracnose (*Colletotrichum lagenarium*) in cucumber (Gottstein and Kuć, 1989), rust (*Uromyces viciae-fabae*) in fava bean (Walters and Murray, 1992) and powdery mildews in diverse crops plants (Reuveni and Reuveni, 1995; Reuveni et al., 1996 and 1998a,b; Mitchell and Walters, 2004). These findings have been attributed to the induction of systemic resistance by release of elicitor-active compounds from plant cell walls (Gottstein and Kuć, 1989; Walters and Muarry, 1992), or the initiation of localized cell death (Orober et al., 2002), which suggest certain perturbations of plant integrity, rather than a nutritional effect of the phosphate salts. Likewise, foliar sprays of boric acid (H_3BO_3), copper sulfate ($CuSO_4$), manganese chloride ($MnCl_2$), or potassium permanganate ($KMnO_4$) induced systemic resistance in cucumber plants against powdery mildew (*Sphaerotheca fuliginea*), thus indicating non-specificity of the mechanisms involved (Reuveni et al., 1997; Reuveni and Reuveni, 1998). However, Reuveni et al. (2000) have shown that also root-applied phosphate enhanced the resistance against *Sphaerotheca fuliginea* in hydroponically grown cucumber plants, probably by different and more nutrition-based mechanisms.

Phosphorus in the form of phosphite (PO_3^{3-}) is widely marketed as a P-fertilizer, bio-stimulant, or as a pesticide for the control of oomycetes, in particular *Phytophthora* species and other members of the *Peronosporales* (Brunings et al., 2005; Thao and Yamakawa, 2009). The effectiveness of phosphite, however, seems to rely mainly on direct toxic or at least the metabolism of pathogens inhibiting modes of actions that need to be further investigated (Dercks and Creasy, 1989; Smillie et al., 1989; Grant et al., 1990; Guest and Bompeix, 1990; Jackson et al., 2000). There is no scientific evidence that plants can use phosphite as a nutritional source of phosphorus, unless it is oxidized to phosphate by microbial activity, which is a slow process in soils (McDonald et al., 2001; Thao and Yamakawa, 2009). The treatment with phosphite rather accelerates the severity of phosphorus deficiency in plants by disrupting the coordinated expression of plant

responses to phosphate starvation, as shown in a number of studies (Seymour et al., 1994; Carswell et al., 1996; Förster et al., 1998; Varadarajan et al., 2002; Lambers et al., 2006, Ratjen and Gerendás, 2009). Phosphite, therefore, is regarded as particularly toxic to plants deprived of phosphate and may not improve the growth of healthy plants (Thao and Yamakawa, 2009).

Potassium

Potassium is not a constituent of organic compounds in plants, but it has important regulatory functions in plant metabolism. Thus, it is necessary for photosynthesis, phloem loading and transport of photosynthates, protein synthesis, control of cation-anion balance, osmotic functions (e.g. opening and closure of stomata, cell extension, plant movements), and the activation of numerous enzymes (Huber, 1980; Marschner, 1995). In the cytoplasm, the potassium ion (K^+) is the most abundant cation and very mobile by means of K^+-specific membrane-bound transport proteins (Lea, 2001; Britto and Kronzucker, 2008). Potassium concentrations in the apoplasm, in contrast, are generally low except for specialized cells or tissues such as stomatal guard cells, where the apoplastic K^+ concentration may rise transiently (Outlaw, 1983; Marschner, 1995). Under potassium deficiency, in particular an inhibited synthesis of high-molecular-weight compounds such as proteins, starch and cellulose together with a depressed translocation of photosynthates from source to sink organs, typically leads to the accumulation of low-molecular-weight organic compounds in photosynthetically active leaves (Cakmak et al., 1994, Marschner, 1995). Therefore, potassium deficient plants tend to be more susceptible to pathogen infection than adequately supplied plants, whereas a surplus in potassium supply does not further improve the organic constitution and resistance of plants (Marschner, 1995). As reviewed by Amtmann et al. (2008), however, the metabolic profiles and disease susceptibility of potassium deficient plants show a high degree of variability, thus questioning a close relationship between these characteristics. Nevertheless, examples of successful disease suppression through optimized potassium nutrition include *Fusarium* wilt of flax (Dastur and Bhatt, 1964), spot-type net blotch (*Pyrenophora teres* f. *maculate*) of barley (Brennan and Jayasena, 2007), and *Fusarium graminearum* stalk rot of maize (Li et al., 2010). In other cases, including take-all disease

(Trolldenier, 1985) and leaf rust (*Puccinia triticina*) of wheat (Sweeny et al., 2000), as well as powdery mildew (*Blumeria graminis*) of barley (Brennan and Jayasena, 2007), potassium chloride (KCl) revealed to be more effective in disease suppression than potassium sulfate (K_2SO_4). To explain this effect, it has been suggested that the chloride ion (Cl^-) supplied with KCl can compete with nitrate (NO_3^-) absorption by the plant, suppress nitrification, and thus affect the pH and availability of other mineral nutrients, particularly Mn, in the rhizosphere (Huber, 1980; Dordas, 2008).

Calcium

In contrast to potassium, calcium is to a high proportion located in the apoplast and not effectively transported in the symplast, as the calcium concentrations in the cytosol and phloem sap are maintained at very low levels by active membrane transport (Marschner and Richter, 1974; Clarkson, 1984; Mengel and Kirkby, 2001). High concentrations of calcium ions (Ca^{2+}) in the cytosol are toxic, as due to the low solubility product of calcium and phosphate ions (Sanders et al., 1999; White and Broadley, 2003), and implicated in programmed cell death processes such as the hypersensitive response to pathogen attack (Levine et al., 1996). A major function of calcium is the stabilization of plant tissues and cell walls by establishing bridges between pectin polymers of the middle lamella and cell wall (Jarvis, 1982; Fry, 2004). Likewise, cell membranes are stabilized by calcium as it bridges phosphate and carboxyl groups of membrane constituents such as phospholipids and proteins (Seimiya and Ohki, 1973; Legge et al., 1982; Pedersen et al., 2006). Calcium deficiency, on the contrary, increases the permeability of membranes and subsequently leads to an enhanced efflux of low-molecular-weight solutes from the cytoplasm into the apoplast of calcium-deficient tissues (van Goor, 1968; Shay and Hale, 1973; Bangerth, 1979). Many parasitic fungi, such as *Fusarium oxysporum* spp. (Blais et al., 1992); *Botrytis cinerea* (Johnston et al., 1994), and *Gaeumannmyces graminis* var. *tritici* (Martyniuk, 1988), but also bacteria (e.g. *Erwinia* spp.) invade the apoplast by releasing pectolytic enzymes, such as polygalacturonases, which cleave structural polymers in the cell wall and middle lamella (Collmer and Keen, 1986). Plant tissues high in calcium are less susceptible to maceration by certain pectolytic enzymes (e.g. endo-polygalacturonase) that are inhibited by calcium (Bateman and Millar, 1966) and can thus

be more resistant to infection by certain pathogens (e.g. *Botrytis cinerea*; Krauß, 1971). Other pectolytic enzymes, however, are stimulated by calcium (e.g. exo-polygalacturonate trans-eliminase) or have even been shown to be calcium dependent (Bateman and Millar, 1966). Accordingly, for different pathogens (e.g. certain strains of *Erwinia* spp.) only a weak correlation between the calcium content and disease resistance of plant tissues has been reported (Pagel and Heitefuss, 1989). Furthermore, some pathogens (e.g. species of *Sclerotium*) produce oxalic acid as chelating agent that can bind calcium ions and thus enhance the activity of calcium-inhibited pectolytic enzymes (Bateman and Beer, 1965; Stone and Armentrout, 1985).

Another important role of calcium, based on its low concentrations in the cytosol and high concentrations in adjacent compartments, such as the apoplast, vacuoles, endoplasmic reticulum, and chloroplasts, is to function as a second messenger in plants and other eukaryotic organisms (Marschner, 1995; White and Broadley, 2003; Harper and Harmon, 2005). In this regard, the perception of extracellular signals (i.e. first messengers; Robison et al., 1968), such as those indicative for pathogen attack or other environmental challenges, at the cell surface can activate calcium channels in the membranes of cell compartments and thus induce transient changes in cytosolic Ca^{2+} levels (Drøbak and Ferguson, 1985; White and Broadley, 2003). These calcium signals are sensed by intracellular secondary receptors (i.e. Ca^{2+} binding proteins), which subsequently modulate the expression of various plant responses (Cheung, 1980; Roberts and Harmon, 1992; McLaughlin and Wimmer, 1999). As reviewed by White and Broadley (2003), specific cellular responses have been attributed to specific signatures of calcium signals, which may vary in frequency and amplitude of the perturbations in cytosolic Ca^{2+} concentrations. In addition, different stimuli induce contrasting perturbations in cytosolic Ca^{2+} concentrations by mobilizing calcium from different cellular stores and/or by activating Ca^{2+} cannels in restricted subcellular locations (Sanders et al., 2002). Osmotic stress, for example, has been shown to induce a biphasic pattern of calcium entry into the cytoplasm involving the sequential influx of Ca^{2+} from the extracellular milieu and the release of Ca^{2+} from intracellular stores such as the vacuole (Knight et al, 1997; Cessna et al., 1998; Pauly et al., 2001). In contrast, the induction of defense reactions against pathogenic bacteria (*Pseudomonas syringae*) in tobacco (Atkinson et al., 1990), fungi

(*Cladospovium fulvum*) in tomato (Gelli et al., 1997), or oomycetes (*Phytophthora sojae*) in parsley (Blume et al., 2000) has been attributed to an enhanced influx of predominantly extracellular Ca^{2+} mediated by the activation of plasma membrane channels through pathogen specific elicitors. Apart from pathogen defense and stress response pathways, however, many extracellular signals of abiotic and biotic origin elicit changes in cytosolic Ca^{2+} concentrations, and many intrinsic plant processes, such as developmental processes under phytohormonal control, are intracellularly governed by calcium signals, too (Sanders et al., 2002; Luan et al., 2002). For instance, Ca^{2+} signaling in plants is critically involved in the initiation of mycorrhizal and rhizobial symbioses (Navazio et al., 2007; Harper and Harmon, 2005), but also fungal pathogens have been reported to rely on internal Ca^{2+} signaling pathways during their infection process (Ebbole, 2007; Rispail et al., 2009). Although the role of calcium as a second messenger in prokaryotes has not yet been fully elucidated, several prerequisites for Ca^{2+} signaling, such as calcium homeostasis and Ca^{2+}-binding proteins, have been identified in some bacteria including *Bacillus subtilis* (Herbaud et al., 1998; Michiels et al., 2002; Permyakov and Kretsinger, 2009). Moreover, there is evidence that human and animal pathogenic bacteria deploy mechanisms of interference with the calcium signaling of host cells in order to suppress defense responses and precondition host tissues for the establishment of infectious diseases (Nhieu et al., 2004). In conclusion, calcium ions appear to function as ubiquitous messengers in biological systems, whereas the distinct signature of calcium signals and the specificity of their cellular receptors as part of diversified signaling pathways allow for the expression of highly stimulus-specific responses in plants (Tuteja and Mahajan, 2007; Vadassery and Oelmüller, 2009).

Magnesium

Regarding magnesium, there is limited information about its role in the pathogen resistance of plants (Dordas, 2008). Several reports suggest that magnesium supply can suppress fungal diseases such as take-all in wheat (Reis et al., 1982), clubroot (*Plasmodiophora brassicae*) in crucifers (Myers and Campbell, 1985; Young et al, 1991), or necrosis (*Phoma* sp.) in potato tubers (Olsson, 1988a). The possible mechanisms behind these findings include the degradation of fungal toxins by manganese-activated

enzymes (Colrat et al., 1999) or, similar to calcium (see above), the stabilization of tissues by forming bridges between polymer molecules of the cell wall and middle lamella (Olsson, 1988a,b). However, depending on the pathogen and other factors the influence of magnesium on pathogenesis seems to be very variable and opposite effects may occur within the same host-pathogen system (e.g. *Gaeumannomyces graminis* in cereal crops; Huber, 1989), as shown in a literature review from Jones and Huber (2007).

Sulfur

Elemental sulfur since long time is being used as a fungicide to control powdery mildews (Robertson, 1824; cited in: Vidhyasekaran, 2004; Russel, 2005) and some other pathogenic fungi (Williams and Cooper, 2004; Haneklaus et al., 2007). In contrast, the metabolic functions of sulfur in plant defense became evident more recently after the emissions of sulfur dioxide from the combustion of fossil fuels and hence the atmospheric deposition of sulfur had been drastically decreased in Europe at the end of the 1980s (Riley et al., 2000; Smith et al., 2001; Bloem et al., 2004). Among the sulfur containing metabolites that may contribute to the resistance of plants against pathogens are glucosinolates (Tierens et al., 2001), glutathione (Tausz et al., 2004), amino acids and proteins (Broekaert et al., 1995; Bloem et al., 2004), low-molecular-weight antibiotics (Pedras et al., 2000), released volatile compounds such as hydrogen sulfide (Papenbrock et al., 2007), and elemental sulfur that is formed during active defense responses (Williams and Cooper, 2004). Further research is needed, however, to clarify the sequential pathways and effectiveness of distinct sulfate metabolites in the initiation and fortification of plant defenses (Haneklaus et al., 2007).

Manganese

Enhanced manganese concentrations in host tissues have been related with enhanced resistance of plants to *Fusarium* spp. (Elmer, 1995), powdery mildews (Brain and Whittington, 1981), *Gaeumannomyces graminis* (Graham and Rovira, 1984), and numerous other fungal, but also bacterial, pathogens (Huber and Wilhelm, 1988). In relation to the low amounts of manganese required by crops (500 to 1000 g Mn ha^{-1} $cropping\ season^{-1}$; Mengel and Kirkby, 2001), however, most soils contain abundant

reserves of total manganese (20 to 3000 mg kg^{-1} soil; Reisenauer, 1988). An insufficient manganese supply to plants is, therefore, often due to its low availability rather than an absolute shortage of manganese in soils (Rengel, 2000). The balance between oxidation and reduction processes and the soil pH largely determine the plant availability of manganese in soils. Under acidic and reductive conditions manganese (Mn) is predominately found as manganous ion (Mn^{2+}), either in the soil solution or associated with exchange sites of the solid soil phase, and thus readily available for plant uptake (Ghiorse, 1988; Clarkson, 1988; Robson, 1988). On the contrary, in alkaline and oxidizing environments, hardly soluble manganese hydroxides and oxides of higher oxidation state (e.g. $Mn^{III}OOH$ and $Mn^{IV}O_2$) are precipitated (Gilkes and McKenzie, 1988; Robson, 1988; Katsoyiannis and Zouboulis, 2004). Relatively small changes in redox potential or pH can strongly affect the equilibrium concentration of manganese in the soil solution (Hem, 1972), which is governed by physical and chemical soil properties as well as by microbial and plant factors (Marschner, 1988). While manganese deficiency is common in plants grown on calcareous soils of high pH, high manganese availability on acidic soils or due to reductive conditions in waterlogged soils can lead to toxicity problems in plants (Mengel and Kirkby, 2001).

Rhizosphere microorganisms are of particular importance for the availability of manganese to plants and the abilities to oxidize and reduce manganese are widely distributed among bacterial (e.g. *Bacillus*, *Pseudomonas*) and fungal genera (Ghiorse, 1988; Marschner, 1988; Robson, 1988). In neutral and acidic soils the oxidation of manganese is mainly due to the activity of microorganisms which are able to derive energy from this process or may benefit in other ways (e.g. alleviation of Mn^{2+} toxicity) from the deposition of manganese oxides in their extracellular vicinity (Ghiorse, 1988). Both, microbial and chemical processes contribute to the reduction of manganese in soils, though the relative contribution of these two factors is difficult to separate (Ghiorse, 1988). Mn-reducing microorganisms may use manganese dioxide (MnO_2) in preference to oxygen (O_2) as terminal electron acceptor for respiration under low-oxygen supply (de Vrind et al., 1986a) as well as under aerobic conditions (Trimble and Ehrlich, 1968). Furthermore, acidic and reductive metabolites, such as protons, organic acid anions, and phenolic compounds, which are excreted by microorganisms and plant roots, or produced

during the decomposition of organic matter, may facilitate the reduction of manganese oxides in soils (Uren, 1981; Ghiorse, 1988; Marschner, 1988; Marschner et al., 2003).

The mechanisms relating to the acquisition, uptake, and internal distribution of manganese in plants remain largely to be elucidated (Yang et al., 2008; Cailliatte et al., 2010). As reported by Gherardi and Rengel (2004) lucerne (*Medicago sativa* L.) responded to a decreased supply of manganese with increased root exudation of carboxylates. Recently Yang et al. (2008) demonstrated that manganese deficient seedlings of *Arabidopsis thaliana* L. displayed stimulated root hair development. As a general conclusion, both reports indicated a need for more work in order to understand the role of physiological and morphological root responses to manganese deficiency. It is known, however, that specific adaptations for the acquisition of other sparingly available minerals, such as enhanced root growth and release of protons, carboxylates, or phenolics by phosphate starved plants or iron deficient strategy I plants (dicots and non-graminaceous monocots), can be critical for the mobilization and uptake of manganese, too (Marschner, 1988; Neumann and Römheld, 2002). In contrast, the iron deficiency induced mechanism of strategy II plants, such as barley, wheat and other *Poaceae*, to release phytosiderophores, which are highly effective chelators for ferric iron (Fe^{3+}), appears to be less effective for the mobilization of manganese, as due to the particularly low affinity of phytosiderophores to Mn^{2+} (Marschner, 1988; Zhang, 1993). As for its mobilization, manganese also shares same routes with other mineral nutrients for its uptake by plants. Recently, Pedas et al. (2005) identified two separate Mn transport systems in barley roots, mediating high-affinity Mn^{2+} influx at low Mn concentrations and low affinity Mn^{2+} influx at higher Mn concentrations in the external solution. Further investigations showed that both manganese and iron deficiency induced an up-regulation of a gene encoding a plasma membrane-localized metal transport protein able to transport Mn^{2+}, but also Fe^{2+} or Fe^{3+}, Zn^{2+}, and Cd^{2+} (Pedas et al., 2008). So far, however, no Mn^{2+}-specific transporter has been identified in plants (Pittman, 2005; Yang et al., 2008; Cailliatte et al., 2010). Notwithstanding there is little evidence for specific root responses to manganese deficiency, genotypic differences of crop plants in their ability to acquire sufficient manganese when grown on soils with low manganese availability have been related to specific alterations in the composition of the microbial community in the

rhizosphere (Timonin, 1946; Rengel et al., 1996; Rengel, 1997b). The plant availability of Mn therefore appears to depend strongly on the ratio between Mn-oxidizing and Mn-reducing microorganism (Marschner and Rengel, 2007). In particular, fluorescent pseudomonads are regarded as effective manganese reducers, which seem to be more abundant in the rhizospheres of some manganese efficient wheat genotypes (Rengel, 1997b; Rengel et al., 1998). However, the results of different investigations suggest that distinct bacterial species perform both the oxidation and reduction of manganese during different stages of their life cycles or in adaptation to changing environmental conditions (Bromfield and David, 1976; de Vrind et al., 1986b; Ghiorse, 1988; Okazaki et al., 1997).

The manganese uptake capacity of plants greatly exceeds their nutritional requirements. While the critical deficiency level for many crop species lies within a narrow range of 10 to 20 mg Mn kg^{-1} dry matter, actual uptake rates on a given soil as well as the critical toxicity concentrations in shoot tissues can be two to three orders of magnitude higher and vary widely between different genotypes (Bergmann, 1992; Mengel and Kirkby, 2001). Therefore, the uptake of manganese seems not to be actively down regulated by the plant, which may lead to toxic manganese accumulation in tissues when exposed to an excessive supply (Clarkson, 1988). Inside plants, manganese moves freely with the transpiration stream in the xylem sap (Lonergan, 1988). The concentrations of manganese in the phloem sap were found to be high in some (e.g. *Lupinus* spp.; Hocking et al., 1977), but low in several other plant species (Loneragan, 1988; Rengel, 2000). In general, the phloem mobility of manganese is regarded as rather low, because, once accumulated in the leaves, it is not effectively redistributed within the shoot or to the roots (van Goor and Wiersma, 1974; Page and Feller, 2005). Result from Hannan et al. (1985), however, indicated that manganese contained in stems, petioles and roots of *Lupinus angustifolius* can be readily redistributed to developing sinks, including seeds, while that in leaves is not retranslocated even from senescing tissues. Investigations with maize and *Vicia faba* further showed a limited translocation of foliar applied manganese, which was preferentially directed to the shoot apex, but the possibility of reversed xylem transport could not be completely excluded in this study (El-Baz et al., 1990).

In plants, manganese has important metabolic functions as structural component and cofactor of many enzymes (Burnell, 1988). It is contained in proteins of the photosystem II where a cluster of four Mn atoms is required as the catalytic centre for light-induced water splitting and in the manganese superoxide dismutase (MnSOD) participating in the defense against oxidative stress (Marschner, 1995; Millaleo et al., 2010). Reactive oxygen species, including hydrogen peroxide (H_2O_2), and their rapid accumulation, known as oxidative burst, play a central role in plant defense against biotic and abiotic stresses (Lamb and Dixon, 1997; Dat et al., 2000). This includes their direct toxicity to pathogens as well as their function as signaling molecules to control processes such as localized programmed cell death and systemic resistance responses in plants (Alvarez et al., 1998; Mittler, 2002; Liu et al., 2010). To allow for these different roles, cellular levels of reactive oxygen species need to be tightly controlled by producing and scavenging systems (Dat et al., 2000; Mittler, 2002). However, the role of manganese in this regard has not been clearly elucidated yet (Dučić and Polle, 2005; Millaleo et al., 2010). A lower content of chlorophyll and of thylakoid membrane constituents in manganese deficient leaves is attributable to the role of Mn^{2+} in the synthesis of fatty acids, carotenoids and related compounds (Marschner, 1995). As a cofactor, manganese activates enzymes of the primary and secondary metabolism in plants including key enzymes of the shikimic acid pathway and subsequent metabolic processes leading to the synthesis of important defense related compounds such as phenolics and alkaloids (Burnell, 1988; Herrmann, 1995a; Marschner, 1995). Enhanced levels of lignin and other phenolic compounds in plant tissues, have been linked with resistance to *Gaeumannomyces graminis* (Rengel et al., 1993 and 1994; Penrose and Neate, 1994), powdery mildews (Cohen et al., 1990; Dordas, 2008), and several other phytopathogenic fungi (Vance et al., 1980; Burnell, 1988; Simoglou and Dordas, 2006). To these effects, both preformed and inducible defense mechanisms may contribute. Low-molecular phenolic compounds, such as flavonoids, may function as signalling molecules in plant defense and/or can be directly toxic to pathogens as constitutive and inducible factors (Nicholson and Hammerschmidt, 1992; VanEtten et al., 1994; Treutter, 2005). The incorporation of lignin and other phenolic polymers into epidermal cell walls confers mechanical strength and resistance to enzymatic degradation (Nicholson and Hammerschmidt, 1992; Zeyen et al., 2002).

Moreover, epidermal cell walls can actively resist penetration by pathogenic fungi through the rapid formation of cell wall appositions, also called lignitubers or papillae, directly subtending the contact area of fungal infection structures (i.e. appressoria and penetration pegs) (Bhuiyan et al., 2009; Yu et al., 2010). Lignitubers, ensheathing the root invading hyphae of *Gaeumannomyces graminis* and other soil-borne parasites, may retard the colonization process and suppress disease progression (Griffiths and Lim, 1964; Yu et al., 2010). Papillae that typically develop in leaf cells upon attack by powdery mildews share common characteristics with lignitubers and can effectively block fungal ingress (Zeyen et al., 2002; Yu et al., 2010). Their formation includes the localized deposition of lignin-like phenolics, callose, silicon, and proteins with antifungal properties. Furthermore, local levels of soluble calcium, magnesium, manganese, and silicon become elevated at the sites of fungal attack during the formation of papillae (Zeyen et al., 2002). The process of papillae formation also occurs in susceptible plants but at a lower frequency or in a way that is ineffective to stop fungal growth (Li et al., 2005; Basavaraju et al., 2009). In these cases, either the defense system of the plant hosts may not recognize the pathogen early enough or the components of papillae may not be formed at sufficient rates (Conrath and Kauss, 2000). The deposition of callose, which is a polysaccharide (β-1,3 glucan), is regarded as a sensitive indicator of high manganese levels in plant tissues (Wissemeyer and Horst, 1987; Nogueira et al., 2002). Microanalytic investigations by Leusch and Buchenauer (1988b) also have proven the accumulation of silicon and manganese at the penetration sites of powdery mildew in wheat and indicated, furthermore, that appropriate silicon levels in leaf tissues were necessary for the rapid translocation of manganese. This is in accordance with other studies showing a conductive influence of silicon on the distribution (Williams and Vlamis, 1957) and mobility (Horst and Marschner, 1978a,b) of manganese in plant tissues, which may result in an enhanced tolerance towards otherwise toxic manganese levels (Rogalla and Römheld, 2002). In addition, high levels of Mn^{2+} can be directly toxic to pathogens, as shown for *Streptomyces scabies* of potato (Mortvedt et al., 1963). Manganese may also inhibit the production of pathogenesis-related enzymes. Exampes are pectolytic enzymes, especially pectin methyl esterase and polygalacturonase, which are released by pathogenic fungi during the infection process to degrade host cell walls (Subramanian, 1956; cited in:

Sadasivan, 1965; Mehta and Mehta, 1985; Kikot et al., 2009). The pathogen-induced activation of aminopeptidase, a host enzyme that is thought to supply essential aminoacids for fungal growth, is inhibited by manganese, too (Huber and Keeler, 1977; cited in: Graham and Webb, 1991). Other, more indirect, effects of manganese on the disease resistance of plants can be through its functions in photosynthesis, thereby influencing the amount and composition of root exudates and associated microflora in the rhizosphere, comprising both pathogens as well as their antagonists (Graham and Rovira, 1994; Marschner et al., 1991). In this regard it has been shown that micronutrients in the order Mn > Zn > B stimulated bacterial numbers in the rhizosphere of cotton plants, but suppressed soil populations of fungi in the same order (Varadarajan, 1953; cited in: Sadasivan, 1965).

Other micronutrients

Besides manganese (see above), also boron (Lewis, 1980), copper (Robson et al., 1981), and iron (Guerra and Anderson, 1985) are involved in the metabolism of phenolic compounds and have important roles in the synthesis of lignin. As cofactors or components of enzymes, copper, zinc, and iron contribute to the defense against reactive oxygen species in plants (Rice, 2007; Millaleo et al., 2010). Therefore, zinc, as an activator of copper-zinc superoxide dismutase (Cu-Zn SOD), can be critically involved in the protection of membranes against oxidative damage (Cakmak, 2000; Dordas, 2008). Copper, in particular, may also suppress pathogens by toxic effects due to a low demand of microbial organisms for copper, as compared to plants, and their low tolerance to excessive copper supply (Graham and Webb, 1991). Boron, similar to calcium, promotes the stability and rigidity of the cell wall and is involved with maintaining the integrity of the plasma membrane, possibly by providing structural linkages between cell wall and membrane constituents (Cakmak et al., 1995; Brown et al., 2002; Dordas and Brown, 2005; Camacho-Cristóbal et al., 2008; Wimmer et al., 2009).

Silicon

Silicon, after oxygen, is the second most abundant element in the earth's crust where it comprises about 28 % of the total mass (Epstein, 2001; Ma, 2006). In soils, however,

which can vary widely in their silicon contents (1 to 45 %), mainly silicon dioxide (SiO_2), also known as quartz, and other sparingly soluble primary and secondary silicate minerals are found (Matichenkov and Bochamikova, 2001; Sommer et al., 2006; Mitschke, 2009). Particularly in highly weathered and silicon depleted tropical soils (e.g. ultisols and oxisols), plants may suffer from insufficient silicon supply (Savant et al., 1996; Epstein, 1999). The solubility of crystalline and amorphous silicates is influenced by soil pH, but largely constant between pH 2.5 and 8.5 (Sommer et al., 2006). The soil solution contains silicon mainly as monosilicic acid $Si(OH)_4$, which at concentrations above 2 mM or under strongly alkaline conditions (pH > 9) undergoes polymerization (Sommer et al., 2006; Jugdaohsingh, 2007). The non-dissociated silicic acid $Si(OH)_4$ is a readily bio-available source of silicon, but plant species show huge differences in their silicon accumulation, ranging from 0.1 % to 15 % silicon in the shoot dry weight (Marschner, 1995; Ma, 2006). According to their capacity to take up silicon, plant species have been divided into three different groups. Silicon accumulators, such as rice, absorb silicon at higher rates than could be passively supplied through the transpiration stream, which indicates the use of active uptake mechanisms. The group of the non-accumulators, to which most other graminaceous crop species but also cucumber have been assigned, acquire silicon at rates equivalent to their water consumption. In contrast, many dicotyledonous species are considered to be excluders, as they restrict the uptake and/or transport of silicon (Jones and Handreck, 1965; Fawe et al., 2001; Ma et al., 2001; Liang et al., 2006). However, within a distinct plant species the patterns of silicon uptake can shift from exclusion to passive uptake, as shown for sunflower (van der Vorm, 1980), or from passive to active uptake, as shown for wheat (van der Vorm, 1980), barley (Rogalla, 2001), and sunflower (Liang et al., 2006), when the concentrations of silicon in the external solution are decreased. Recent results obtained with rice (Tamai and Ma, 2003; Ma et al., 2004) and cucumber (Liang et al., 2005a) have indicated that the transport of silicon from cortical cells, including those of the endodermis, to the xylem (xylem loading) is the main active process in silicon uptake and an important determinant for silicon accumulation in shoots (Ma and Yamaji, 2006). The root endodermis with its casparian strip acts as a barrier for apoplastic radial transport of silicon, also in silicon accumulators, which can lead to the deposition of silicon at the inner walls of endodermis cells (Hodson and Sangster, 1989;

Lux et al., 2002 and 2003). Nevertheless, silicon is also taken up passively, which probably includes diffusion of the uncharged $Si(OH)_4$ molecule across plasma membranes (Raven, 2001; Mitani and Ma, 2005; Liang et al., 2006). Its primary accumulation at sites of high transpiration, such as leaves or the inflorescence bracts of graminaceous species, indicates that xylem transport is the main pathway of silicon distribution within plants (Jones and Handreck, 1965; Handreck and Jones, 1968; Hodson and Sangster, 1988a). However, as shown by Hayward and Parry (1972), also the phloem contains silicon, though the role of symplastic silicon transport remains widely unknown (Raven, 2001; Ma and Yamaji, 2008; Liang et al., 2015). In shoot tissues, silicon is mainly deposited in the outer walls of epidermal cells (Hodson and Sangster, 1988a; Rogalla, 2001), but this is not a purely passive process. Rather, there is evidence that the distribution and polymerization of silicon are controlled by active transport mechanisms and the influence of preformed cell wall components (Hodson and Sangster; 1988b; Perry and Lu, 1992; Perry and Keeling-Tucker, 2000; Sangster et al., 2001; Currie and Perry, 2007). Once precipitated as amorphous silica ($SiO_2 \cdot nH_2O$) or "opal", silicon is immobile and not redistributed in plant tissues (Parry et al., 1984; Epstein, 1994; Sangster et al., 2001).

Although silicon has not been proven to be essential for most plant species except members of the *Equisitaceae* (Epstein, 1999; Wiese et al., 2007; Ma and Yamaji, 2008), it is generally regarded as a beneficial element, counteracting various biotic and abiotic stresses (Ma et al., 2001; Liang et al., 2007). This includes enhanced mechanical stability and resistance towards drought, deficiencies and toxicities of mineral elements, insect herbivores and fungal pathogens (Ma et al., 2001; Richmond and Sussman, 2003; Motomura et al., 2006; Wiese et al., 2007). In contrast, the deprivation of silicon has been reported to induce abnormalities of growth, development and reproduction in plants (Epstein, 1999). Similar to lignification, the silicification of cell walls confers enhanced compression-resistance and rigidity to plant tissues (Epstein, 1994). Due to this effect, the supply of silicon can increase the leaf erectness and lodging resistance of cereal crops and thereby counteract possible adverse influences of high nitrogen levels in intensive agriculture (Welton, 1928; Yoshida et al., 1969; Idris et al., 1975). In addition, it has been shown that silicon can increase the lignification of root cells in wheat (Jones et al., 1978; cited in: Marschner, 1995) and rice (Fleck et al., 2011) plants. This effect is attributable to

the complexation of silicic acid with phenolic compounds, facilitating the synthesis of lignin (Weiss and Herzog, 1978; cited in: Mengel and Kirkby, 2001; Dragišić Maksimović et al., 2007), the co-precipitation of silicon with lignin (Fang and Ma, 2006), and silicon-induced alterations in the expression of genes related to the metabolism of polyphenols (Fleck et al., 2011).

Besides its influence on the manganese tolerance of plants (see above), silicon alleviates stresses induced by aluminum, zinc, cadmium, and other elements, as due to its complexation or co-deposition with toxic metal ions in plants or the growth medium, and its interference with uptake processes and metabolic pathways in plants (Liang et al., 2007; Ma and Yamaji, 2008). Recent investigations from Pavlovic et al. (2016) on the remobilization of iron in cucumber indicate that silicon improves the internal distribution of iron by increasing the concentration of nicotianamine in older leaves and the expression of transporters for loaing respectively unloading of the phloem with iron-nicotianamine complexes, thus improving the iron status of younger leaves. The deposition of silicon in epidermal tissues can form an effective barrier against water loss by cuticular transpiration (Matoh et al., 1986; Agarie et al., 1998; Gao et al. 2006), insect herbivores (Massey et al., 2006), and infection by fungal pathogens (Bélanger et al., 2003). Preformed silicon deposits may hamper the infection process of the rice blast fungus *Piricularia oryzae* (Yoshida et al., 1962) or powdery mildews (Miyake and Takahashi, 1983; Adatia and Besford, 1986; Grundhöfer, 1994), which penetrate host tissues directly through the cuticle and epidermal cell wall rather than by way of the stomata (Pryce-Jones et al., 1999). Similar effects can be achieved with silicate sprays that form a superficial crust on plant surfaces (Bowen et al., 1992; Menzies et al., 1992; Liang et al., 2005b; Guével et al., 2007), which may be a useful approach for crop plants with exclusive or passive uptake mode for silicon (Ma et al., 2001). Apart from that, also the accumulation and deposition of silicon at the root endodermis has been proposed to aid in the resistance of plants towards soil-borne pathogens such as *Gaeumannomyces graminis* (Bennett, 1982), but experimental evidence to this effect is still scare (El Hiweris, 1987; Fleck et al., 2011). Recent reports have shown that in particular the inducible mechanisms whereby silicon affects the ability of plants to respond to attack by parasitic bacteria (Ghareeb et al., 2011), fungi (Fateux et al., 2005), and even insects

(Massey et al., 2007) are just beginning to be understood. Kunoh and associates (Kunoh and Ishizaki, 1975; Kunoh et al., 1975) presented first evidence for the localized accumulation of silicon, together with calcium and manganese, at the penetration sites of powdery mildews. In this regard, silicon may not only function as a barrier against invading hyphae through its local deposition in cell wall appositions (Blaich and Wind, 1989; Kim, 2002), which can be facilitated through the induction of systemic acquired resistance (SAR) (Kauss et al., 2003). Rather, the results from Leusch and Buchenauer (1988b) revealed a considerable influence of silicon on manganese-related responses (see above). More recent findings, likewise, indicate that the functions of silicon take an active part in the resistance of plants as it supports the expression of various local and systemic defense reactions (Fawe et al., 2001). To that effect, Samuels et al. (1991) observed that the availability of silicon in its soluble, mobile form at the time of infection was more important for the successful defense against powdery mildew attack by cucumber plants than the preceding deposition of high levels of silicon in host tissues. In this host-pathogen relationship, the accumulation of silicon at the sites of fungal attack did not necessarily form a physical barrier against invading hyphae, but its continuous supply decreased the time to initiation of production and/or accumulation of phenolic compounds in affected epidermal cells (Menzies et al., 1991). In another pathosystem, cucumber attacked by *Pythium ultimum*, the supply of silicon conferred enhanced resistance to the host plant and stimulated the accumulation of a material with phenolic-like properties in affected host tissues (Chérif et al., 1992b), even though no accumulation or deposition of silicon was detectable at the sites of fungal invasion (Chérif et al., 1992a). Further investigations on that aspect have shown that silicon is able to facilitate metabolic reactions in the defense system of cucumber plants that include enhanced and more rapid enzymatic responses upon pathogen attack (Chérif et al., 1994) and the accumulation of phenolic phytoalexins (Fawe et al., 1998). Related studies revealed that, like in cucumber, silicon enables active defenses also in wheat (Bélanger et al., 2003; Rémus-Borel et al., 2005), rice (Rodrigues et al., 2003 and 2004), and other plant species (Ghanmi et al., 2004; Fauteux, 2005). Furthermore, most recent findings suggest a role for silicon in signaling pathways modulating the resistance of plants at the level of gene transcription (Fauteux et al., 2006; Brunings et al., 2009). For instance, silicon-mediated resistance

against the rice blast fungus *Magnaporthe grisea* was associated with the enhanced expression of a defense related gene in a susceptible rice cultivar (Rodrigues et al., 2005). In conclusion, silicon as a passive deposit in peripheral tissues and even more so as a metabolically active solute in the liquid phase of plants has manifold functions (Epstein, 2008), but the exact mechanisms by which silicon is strengthening the defense system of plants remain to be further elucidated (Fauteux et al., 2005; Cai et al., 2009; Liang et al., 2015).

7.4 Prospects to control take-all disease in wheat by adapted rhizosphere management and fertilization strategies

In the above sections, versatile functions of mineral nutrients in the disease resistance of plants have been described in detail. The emerging picture is that each element is part of a diversity of interdependent mechanisms in a sophisticated system of defenses against pathogens (Huber and Wilhelm, 1988). Furthermore, interactions with beneficial as well as deleterious microorganisms in the rhizosphere may affect the mineral nutrition and health performance of plants in various but often highly specific ways (Rengel and Marschner, 2005; Römheld and Neumann, 2006). To facilitate healthy plant growth by an adapted rhizosphere management that optimizes the mineral nutrition of plants and suppresses harmful pathogens, therefore, appears as a promising approach for sustainable crop production. Yet, the patterns of nutrient supply or microbiological interference, which are likely to achieve targeted results, as part of an integrated disease control program, will strongly depend on the particular interactions between plants and pathogens and need to be specifically adapted for each crop in its environment (Walters and Bingham, 2007).

In the following chapter, the effectiveness of agricultural strategies to control soil-borne diseases by microbiological means and/or the purposeful application of mineral nutrients has been investigated in case studies on the take-all pathosystem of wheat under greenhouse and field conditions (Chapter 9). Take-all disease in cereals induced by subspecies of the ubiquitous soil-borne fungus *Gaeumannomyces graminis* is generally controlled by cultural practices such as crop rotation, tillage, sowing date, fertilization and

adjustment of soil pH (Huber and McCay-Buis, 1993; Freeman and Ward, 2004). Fungicides, in contrast, have limited effectiveness to control the disease and the genetic diversity of wheat varieties does not confer a sufficient degree of resistance (Weller et al., 2002). The subspecies *Gaeumannomyces graminis* var. *tritici* (Walker, 1972) is worldwide one of the most damaging root-rot pathogens of wheat crops, inducing yield losses as high as 90 % (Ballinger and Kollmorgen, 1986; Hornby et al., 1998). In an analysis of the multiple components that affect take-all severity in cereal crops, Huber and McCay-Buis (1993) concluded that the disease arises when it is favored by the interactions between the plant, the pathogen and factors of their abiotic and biotic environment, including soil and climate conditions, as well as other detrimental or beneficial microorganisms, respectively (Fig. 7.4).

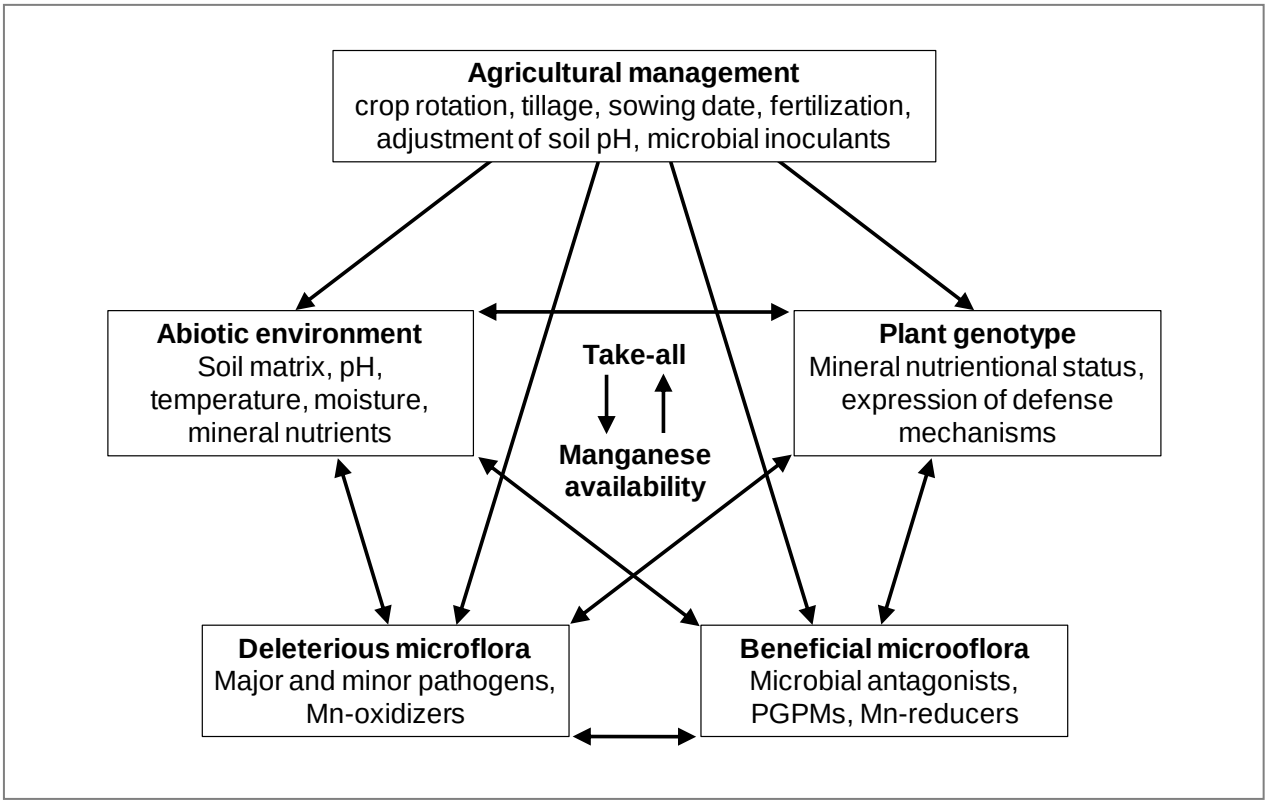

Fig. 7.4: The severity of take-all disease of cereals induced by the ubiquitous soil-borne fungus Gaeumannomyces graminis depends on the balance of interactions between plant genotype, deleterious and beneficial soil microorganisms, and components of their abiotic environment. A purposeful agricultural management of these interrelated factors may simultaneously suppress the pathogen and other deleterious microorganisms, advance the beneficial microflora, and enhance the mineral nutrition and resistance of the host plant. In particular, the availability of manganese is closely interrelated with the expression of defense mechanisms by the plant and with virulence of the take-all pathogen.

All these risk factors for take-all disease are interrelated to influence the availability of mineral nutrients, in particular manganese, which are important for the resistance of the host plant and for the virulence of the pathogen. Their appropriate modification through the agricultural management may therefore optimize the mineral nutrition and resistance of the plant and effectively control the development of take-all disease as elucidated in the following sections.

Characteritics of the pathogen determing the risk for take-all disease

Gaeumannomyces graminis (Sacc.) Arx. & D. Olivier (formerly *Ophiobulus graminis* (Sacc.) Sacc.) is a species of ascomyceteous fungi that has been sub-divided into several varieties differing in minor morphological characteristics and, more importantly, in pathogenicity (Hornby et al., 1998; Cook, 2003). Of particular importance as pathogens of wheat and other small cereal crops are *G. graminis* (Sacc.) Arx. & D. Olivier var. *tritici* J. Walker (*Ggt*) and *G. graminis* (Sacc.) Arx. & D. Olivier var. *avenae* (Turner) Dennis (*Gga*). *Ggt* is an aggressive pathogen of wheat and barley crops, also attacking rye (*Secale cereale* L.), and, occasionally, oats (*Avenae sativa* L.), as well as some other grasses (*Gramineae*). *Gga* is more virulent in oats than *Ggt*, but also has a wide host range within the *Gramineae* and is able to attack wheat and barley, too (Hornby et al., 1998; Rachdawong et al., 2002). In culture, both varieties of the pathogen look similar and are difficult to distinguish. Although the ascospores formed by *Gga* are typically longer than those of *Ggt* this is not a reliable characteristic for identification (Hornby et al., 1998). The pathogenicity of *Gga* to oats is related to its ability to produce enzymes called avenacinases that can detoxify avenacins, a group of triterpene glycoside saponins [from Latin: sapo, 'soap'] with antimicrobial properties, synthesized by oat roots (Turner, 1961; Crombie et al., 1986; Osbourn, 2003). A general factor correlated to the virulence of *G. graminis* varieties is the ability to oxidize dissolved Mn^{2+} into precipitates of Mn(IV) (Schulze et al., 1995; Pedler et al., 1996; Rengel, 1997a; Rachdawong, 1999). Where manganese is oxidized in advance of infective hyphae this appears to be a mechanism of the fungus to impair host defenses before attack (Schulze et al., 1995). Conversely, isolates of *G. graminis* defective in manganese oxidation revealed to be non-virulent or weak pathogens (Graham and Webb, 1991). While low concentrations of Mn^{2+}

(supplied as $MnSO_4$) could slightly increase the growth of *G. graminis* var. *tritici*, high Mn^{2+} concentrations had a suppressive effect on the pathogen (Wilhelm et al., 1990; Marschner et al., 1991). However, manganese concentrations commonly found in soils in general not appear to be toxic to the fungus (Wilhelm, 1991; cited in: Rengel et al., 1996).

Take-all inoculum can arise mainly from infected plant residues, where the fungus survives saprophytically, or other living hosts such as grass weeds, but also from ascospores released from perithecia, and may be spread during tillage (Hornby, 1981; Hornby et al., 1998). Conidia (phialospores) are produced by *G. graminis*, too, but their function is unclear as they are difficult to germinate *in vivo* and seem not to have roles in epidemics (Cunningham, 1981; Hornby, 1981; Hornby et al., 1998). Host roots have been shown to stimulate the production of hyphae, which grow towards the roots (Brown and Hornby, 1971). Upon reaching the root, the fungus may start to grow on all parts of the plant below or up to few centimeters above the soil surface (Hornby et al., 1998). The mycelium consists of young thin-walled micro- or infection hyphae and older parts, forming dark, thick-walled macro or runner hyphae (Skou, 1981). Infection hyphae may penetrate the epidermal cells of susceptible host anywhere, as well as root hairs and cells inside the root, by producing appressorium-like attachement structures (hyphopodia) with narrow penetration pegs (Skou, 1981; Epstein et al., 1994). As soon as the penetration peg touches and initiates decomposition of the host cell wall, a competition between defence responses of the plant and attempts by the pathogen to overcome these responses begins. In case of successful penetrations, cell contents rapidly disintegrate following cell death, whereas the infection hyphae regain their original diameter to either grow straightforward and attack the opposite cell wall, or fill the invaded cell with mycelium that develops into sclerotium-like structures. Depending on their relative resistance, compatible host tissues are more or less extensively destructed. In dead tissues, the fungus grows freely and reverts to its saprophytic state producing fruiting bodies (perithecia) that protrude from the surface of the substrate (Skou, 1981). However, in the absence of a susceptible host the survival of take-all inoculum in soils is determined by microbial antagonism, supply of nitrogen to the fungus, temperature, moisture, and other environmental factors (Mac Nish, 1973; Shipton, 1981). It has been shown that infectivity persisted longest in the corsests fragments of infected host debris in soil and that only traces of inoculum

remained infective after one year (Hornby, 1981). Cultural practices that enhance the degradation of infected crop residues or deep ploughing to remove them from the rooting zone may therefore result in less inoculum to infect subsequent crops (Christensen and Hart, 1993; Thomas, 2004). However, residue retention and decreased tillage intensity may also facilitate microbial activity including the development of antagonistic populations (Alabouvette et al., 2004).

Host plants and crop rotation as risk factors for take-all disease

The damage imposed on host plants by the take-all fungus is characterized by extensive deterioration of root tissues, including destruction of the phloem and blockage of the xylem by hyphae and plant own deposits from defense rections (Skou, 1981). Severe root rot with consequent disruption of uptake and transport processes typically leads to symptoms of water and nutrient deficiencies such as yellowing of lower leaves, stunting, reduced formation of tillers, and premature ripening or death of the plant (Deacon, 1981; Cook, 2003). Ears are often bleached (white heads) and empty or contain only a few shriveled kernels (Smiley, 1974; Manners and Myers, 1981). Maintaining adequate suppy of mineral nutrients, in contrast, may support the plant to express tolerance and resistance mechanisms. Such instances include enhanced formation of adventitious roots to compensate for the decay of infected roots, or the formation of cell wall apposistions (i.e. lignitubers) to conteract enzymatic degradation and mechanical penetration of the host cell walls by attacking hyphae (Huber, 1981; Manners and Myers, 1981; Skou et al., 1981).

Most susceptible to pathogenicity by *Ggt* infections is wheat followed by barley and rye, whereas oats are typically resistant against this variety of the take-all pathogen (Freeman and Ward, 2004). Rye is relatively tolerant to take-all disease, which has been releated to its efficiency in the acquisition of mineral nutrients, but like wheat or barley also rye increases the inoculum potential in soils to damage subsequent crops (Huber and McCay-Buis, 1993). Oats, in contrast, are not only resistant to *Ggt*, but appear to provide active mechanisms of disease control beyond the common effect of poor pathogen survival in absence of a host (Rothrock and Cunfer, 1991). The take-all suppressive effect of oats has been attributed to the release of root exudates that induce changes in the soil microflora

and increase the availability of manganese in subsequent wheat (Timonin, 1946; Huber and McCay-Buis, 1993). Furthermore, the resistance of oats to *Ggt* is associated with avenacins functioning as preformed antifunfal compounds in roots (Turner, 1953; Maizel et al., 1964; Osbourn et al., 1991). Avenacins are produced and accumulated in the epidermal cell layer of the root tips of oats to build a chemical barrier to invading pathogens such as *Ggt* (Crombie et al., 1986; Osbourn et al., 1994; Qi et al., 2004). On the contrary, an oat species (*Avena longiglumis*; Osbourn et al., 1994) or mutants of black oat (*Avena strigosa*; Papadopoulou et al., 1999; Santi et al., 2003) lacking or deficient in avenacin production revealed to be susceptible to *Ggt*. The synthesis of avenacins and/or the expression of other defences in oats might be influenced by manganese. Accordingly, oats suffering from manganganese deficiency (i.e. gray-speck disease), as they host denser populations of manganese oxidizing microorganisms in their rhizosphere or grow on manganese deficient soils, showed enhanced take-all susceptibility (Huber and McCay-Buis, 1993). A similar effect as with oats might be achieved by planting glucosinolate rich brassicas (*Brassicaceae*) such as mustard (*Brassica juncea* L.*; Sinapis spp.*) or some rape (*Brassica napus* L.) varieties. Their take-all suppressive effect has been attributed to the release of isothiocyanates, which are volatile biocidal breakdown products of glucosinolates, from root exudates or disrupted plants tissues (Angus et al., 1994; Sarwar et al., 1998; Li et al., 1999). While *G. graminis* var. *tritici* is sensitive to the toxic effect of isothiocyanates (Kirkegaard et al., 1996; Sarwar et al., 1998), populations of manganese reducing microorganisms colonizing the roots of wheat (Akter, 2007) and microbial antagonists to the take-all pathogen (Majchrzak et al., 2010) seemed to be more abundant in soils cultivated with diverse *Brassicaceae* as preceding crops. Lupines (*Lupinus* spp.) as precrops also have been reported to decrease take-all in wheat. This has been attributed to the accumulation of manganese in their biomass, which is later released during mineralization, and to their inhibitory effect on nitrification and manganese oxidation (Huber and McCay-Buis, 1993). In contrast, precrops such as alfalfa or soybean, and organic manure without nitrification inhibitor have been referred to increase nitrification, manganese oxidation, and take-all (Huber et al., 1965; Huber and Watson, 1974; Huber and McCay-Buis, 1993; Huber and Graham, 1999). Manganese reducing rhizobacteria have been shown to directly suppress the growth of *G. graminis* var. *tritici*

and increase the manganese uptake of wheat (Marschner et al., 1991). There is, however, limited evidence that manganese efficient and less take-all susceptible wheat genotypes specifically increase the proportion of manganese reducing microorganisms in their rhizospheres (Rengel et al., 1996; Rengel, 1997b; Marschner et al., 2003). It has been reported, instead, that soybeans grown after severely take-all infested wheat showed pronounced manganese deficiency and enhanced populations of manganese oxidizing organisms in their rhizosphere (Huber and McCay-Buis, 1993). Nevertheless, after severe incidence of the disease, the continous cropping of wheat or barley can induce a limited degree of soil suppressiveness known as take-all decline (Shipton, 1972; cited in Rovira and Wildermuth, 1981) that is associated with increases in soil specific populations of antagonistic microorganisms (Andrade et al., 1994; Weller et al., 2002), as already mentioned above (Chapter 7.2.1). Although the pathogen remains present in take-all decline soils, it seems to be impaired to provoke severe disease as long as monotonous cultivation of wheat or some other susceptible host (e.g. barley) continues (Cook, 1981a; McSpadden Gardener and Weller, 2001; Cook, 2003). In contrast, the introduction of non-hosts such as potatoes, oats, beans, alfalfa or rape appears to disturb the take-all suppressive composition of the soil microflora similar to chemical soil fumigation. Rotation with such break crops may therefore help to decrease the inoculum density of the pathogen, but also can make soils more conducive to the reappearance of severe disease outbreaks in subsequent series of host crops (Cook, 1981a; Hornby et al., 1998). However, the recovery of wheat yields achieved with take-all decline does usually not reach the level achieved by balanced crop rotations (Cook, 2003), and even when take-all decline has established, the pathogen can still induce considerable decreases in yield if soil and weather conditions are conducive to the disease (Freeman and Ward, 2004).

Take-all disease is a typical problem where susceptible cereal crops are grown frequently and could almost be prevented when the proportion of cereals is below 40 to 60 % of the total acreage in the crop rotation (Hornby et al., 1998). In addition, the timely elimination of graminaceous weeds, such as *Agropyron* and *Bromus* spp., can diminish the survival of *G. graminis* varieties on living hosts during rotational breaks between susceptible crops (Christensen and Hart, 1993; Cook, 2003; Chng et al., 2005; Gutteridge et al., 2005). Weed control by tillage or herbicides just few months before the sowing of susceptible

cereal crops, however, may not decrease the risk of take-all disease, as the pathogen can persist in residues of the weedy hosts (Christensen and Hart, 1993). The herbicide glyphosate, in particular, has been reported to increase the survival and pathogenicity of *G. graminis* var. *tritici* in bioassays, which has been attributed to a shift in the soil microbial community away from antagonistic populations (Mekwatanakarn and Sivasithamparam, 1987a,b). Other possible reasons for glyphosate enhanced take-all severity could be the stimulation of manganese-oxidizing organisms to decrease manganese availability for the expression of plant defenses, or the direct deterioration of resistance related metabolic pathways in plants due to its inhibitory effect on the shikimic acid pathway (Huber and McCay-Buis, 1993). These aspects have been further elucidated in recent general works on non-targeted side effects of glyphosate (Guldner et al., 2004; Neumann et al., 2006; Bott et al., 2008; Kremer and Means, 2009; Tesfamariam et al., 2009; Saes Zobiole et al., 2010), but not with particular respect to take-all disease (Johal and Huber, 2009).

Influence of the abiotic environment on the risk for take-all disease

Soil temperature, moisture, aeration, pH, and nutrient availability can have an important influence on the severity of take-all disease (Hornby et al., 1998). Although these factors are determined to a large extend by climate, weather, and the given soil conditions, they can be manipulated, or at least regarded, by agricultural practices to inhibit disease development and maintain grain yields (Christensen and Hart, 1993). The optimum temperature range for growth of *G. graminis* isolates in pure culture appears to be within the range of 20 to 25 °C (Cook, 1981b; Sivasithamparam and Parker, 1981; Irzykowska and Bocianowski, 2008) and take-all disease can develop where soil tempartures are between 5 and 30 °C (Hornby et al., 1998). Nevertheless, under non-sterile conditions, such as in field soils, severe infections are typically restricted to the lower and narrower temperature range reported as 5 to 15 °C (Cook, 1981b) or 12 to 20 °C (Hornby, 1981). This divergency has been attributed to an enhanced activity of antagonistic organisms that occurs under warmer conditions and affects the pathogen in particular before and during the infection process (Cook and Rovira, 1976; Cook, 1981b). At lower temperatures, the take-all fungus is able to maintain growth while its antagonists appear to be relatively

more inhibited (Cook, 1981b). In contrast, Huber and McCay-Buis (1993) argued that late sown winter wheat and early sown spring wheat crops may be under less manganese stress due to lower growth rates of the plants and decreased activity of manganese oxidizing organisms in cooler soils so that take-all infection is inhibited under such conditions. Although dry soil conditions generally favor the long-term survival of *G. graminis* var. *tritici* in absence of a living host, as due to a lower microbial activity that meets its weak saprophytic competitiveness, the fungus requires sufficient moisture for growth and parasitic activity (Cook, 1981b; Cook, 2003; Hornby et al., 1998). Futhermore, Mac Nish (1973) observed that warm (35 °C) and moist soil conditions were most adverse for the survival of *G. graminis* var. *tritici* in soil compared with lower temperature (15 °C) and/or dry conditions. However, growth and survival of *G. graminis* var. *tritici* also depend on sufficient soil aeration and oxygen supply (Cook, 1981b), whereas a firm seedbed, as prepared by the use of press wheels at sowing, has more anaerobic sites where manganese reduction might occur to improve the availability of manganese required for disease resistance in plants (Huber and McCay-Buis, 1993).

A particularly notable role in controlling take-all is related to the soil pH, although the disease is able to develop where soil pH is within a broad range of 5.5 and 8.5 (Hornby et al., 1998). The application of lime to increase the pH of acid soils is very likely to induce severe take-all within two or three consecutive crops of wheat or barley when soil moisture, temperature, and aeration are favorable to the disease (Cook, 1981b). In contrast, the use of ammonium (NH_4^+) fertilizers, especially when amended with nitrification inhibitors, can decrease the severity of take-all, which has been attributed to a decrease in rhizosphere pH and alterations in microbial populations, as opposed to a nitrate (NO_3^-)-dominated plant nutrition (Smiley and Cook, 1973; Huber and McCay-Buis, 1993). Soil and rhizosphere pH affect the availability of mineral nutrients, of which all are reported to interfere with take-all disease (Huber and McCay-Buis, 1993). Particularly in crops that are deficient in manganese, but also zinc or copper, the disease tends to be more severe (Cook, 1981b; Reis et al., 1982). These elements are typically more available in acid soils (Mengel and Kirkby, 2001) where the disease is seldom prevalent (Huber and Wilhelm, 1988). In addition, acid pH and anaerobic soil conditions are inhibitory to nitrification performed by *Nitrosomas* and *Nitrobacter* species (Huber

and McCay-Buis, 1993). Yet, also in neutral to alkaline soils, rhizosphere acidification induced by ammonium fertilization, particularly when supplied with nitrification inhibitors, can enhance the mobilization and plant acquisition of these elements (Thomson et al., 1993; Neumann and Römheld, 2002) and thus contribute to the suppression of take-all (Huber and McCay-Buis, 1993; Cook, 2003). The predominant importance of manganese in these processes is further indicated by reports that take-all can also be severe on acid soils when those are intensively aerated (Cook, 1981b) or deficient in total manganese (Huber and Wilhelm, 1988). Less well understood in their effect on take-all disease are the influences of different forms of nitrogen on the composition and activity of microbial communities in the rhizosphere (Cook, 2003). In contrast to nitrate, ammonium is rapidly metabolized in root tissues to form aminoacids, which may result in altered composition and patterns of root depositions with respective effects on associated microorganisms (Huber and Watson, 1974; Mahmood et al., 2005). Correspondingly, it has been shown that the proportion of antagonists towards *G. graminis* within the total population of *Pseudomonas* species isolated from wheat rhizoplanes was higher in ammonium-N than in nitrate-N amended soil (Smiley, 1978a,b). As reported by Huber and Wilhelm (1988), treatments with *Bacillus subtilis* had an additional take-all suppressive effect when combined with ammonium-N, but enhanced the disease severity when nitrate-N was supplied. Because *Pseudomonas* and *Bacillus* strains may be capable of reducing as well as oxidizing soil manganese, it is arguable that these bacteria might suppress take-all especially when ammonium supply inhibits their oxidative and favors their reductive activity (Huber and McCay-Buis, 1993; Cook, 2003). However, besides the role of managanese reduction in disease control, the antagonism exerted by various bacterial strains against *G. graminis* is likely to result from more than one mechanism (Marschner et al., 1991; Ownley et al., 2003). Although, the relationship between take-all severity and the status of mineral nutrients in plant tissues suggests the application of fertilizers for disease control (Reis et al., 1982; Huber and Wilhelm, 1988), the direct application of manganese to soils, seeds or on the foliage is likely to be of limited effectiveness due to its rapid oxidative immobilization in soils and limited phloem translocabilty within plants (Huber and Wilhelm, 1988; Huber and McCay-Buis, 1993). Because the availability of manganese in the rhizosphere and its status in root tissues are

major limiting factors for plant defense against *G. graminis*, the modification of rhizosphere processes that contribute to enhanced manganese mobilization and uptake by the plant appears to be a key strategy for the control of take-all disease (Huber and McCay-Buis, 1993).

Influence of the biotic environment on the risk for take-all disease

The suppressiveness of soils against pathogenic diseases, occurring either as consequence of repeated cropping of the host plant, as in take-all decline soils, or as a more general property of microbially balanced soils, has been widely attributed to populations of antagonistic microorganisms (Rovira and Wildermuth, 1981; Cook and Baker, 1983; Hornby, 1983; Andrade et al., 1994; Alabouvette et al., 2004). Probably the microbial group most frequently and consistently implicated with take-all decline in diverse soils throughout the world is that of fluorescent *Pseudomonas* species (Weller et al., 2002; Cook, 2003). In vitro tests with isolates from the rhizospheres of wheat plants showed that about 70 % of the fluorescent pseudomonads but only 20 % of the total aerobic bacteria growing on a non-selective medium were antagonistic to *G. graminis* (Ridge, 1976). The mechanisms by which fluorescent *Pseudomonas* may inhibit *G. graminis* var. *tritici* include the production of antibiotics such as phenazine-1-carboxylic acid (PCA) (Gurusiddaiah et al., 1986; Brisbane et al., 1987) and 2,4-diacetylphloroglucinol (DAPG) (Harrison et al., 1993), depriving the pathogen of iron by the release of siderophores (Kloepper et al., 1980a), and the mobilization of manganese required for plant resistance (Huber and Wilhelm, 1988). In various studies fluorescent *Pseudomonas* have been identified as an important group of rhizosphere microorganisms capable of reducing manganese to improve the manganese acquisition of plants (Marschner et al., 1991; Posta et al., 1994), and to suppress take-all in wheat grown under greenhouse and field conditions (Weller and Cook, 1983). Furthermore, the induction of resistance in plants (Okubara et al., 2010; Daval et al. 2011) and interactions with mycorrhizal fungi (Behn, 2008; Siasou et al., 2009) performed by strains of *Pseudomonas fluorescens* have recently obtained some attention regarding their possible effects on take-all disease. However, the microorganisms with antagonistic ability that have been isolated from diverse soils with take-all decline belong to many taxonomic groups and perform different mechanisms of

pathogen suppression (Andrade et al., 1994; Weller et al., 2002). *Actinobacteria* such as *Streptomyces* spp. (Cook and Rovira, 1976; Smiley, 1978a,b; Chin-A-Woeng et al., 2003), *Trichoderma* fungi (Simon, 1989; Dunlop et al., 1989), and *Bacillus* spp. (Andrade et al., 1994; Kim et al., 1997) are commonly found in suppressive soils and their growth inhibitory effect on *G. graminis* var. *tritici* has been mainly attributed to antibiosis. *Trichoderma* fungi, furthermore, can be strong reducers of manganese and may dissolve sparingly soluble minerals by chelation, too (Altomare et al., 1999; McBeath et al., 2005; Johal and Huber, 2009). The hyper-parasitism of *Trichoderma* spp. or other soil fungi on *G. graminis* is another possible mechanism to explain their antagonistic activity (Chet, 1987; Sivan and Chet, 1989b; Andrade et al., 1994; Zafari et al., 2008). Amoebae isolated from long-term wheat soil have been described to perforate and destruct hyphae of *G. graminis*, too (Homma et al., 1979). A sterile red fungus (Dewan and Sivasithamparam, 1988a) associated with roots of wheat has been referred to suppresses infection by *G. graminis* var. *tritici* by competition for space and nutrients, antibiosis, plant growth promotion, and induced host resistance (Rowland et al., 1994; Aberra et al., 1998). It also has been reported that non-pathogenic varieties of *G. graminis* and related fungi can induce mechanisms of resistance in wheat roots protecting them from virulent *G. graminis* var. *tritici* (Wong, 1981; Wong et al., 1996). Due to their hughe diversity of antagonistic microorganisms, take-all suppressive soils have been an abundant source of microbial isolates as bio-control agents (Hornby et al., 1998). However, approaches to control take-all disease by introducing specific bio-control agents have been hardly successful in agriculture and the level of control is typically lower than that achieved with take-all decline (Cook and Yarham, 1998; Hornby et al., 1998; Cook, 2003). A possible reason for this inconsistency is that take-all decline relies on diverse and locally adapted microorganisms, which ensures functionality of their interrelated control mechanisms over a wider range of environmental conditions than achieved by isolated and introduced bio-control agents (Hornby et al., 1998).

Conclusions

Soil-borne diseases are a major yield-limiting constraint in agricultural production and difficult to control by conventional means. The lack of genetic resistance and adequate

pesticides against many soil-borne pathogens imposes a need to develop alternative strategies for plant protection (Weller et al., 2002; Donn et al., 2015). In natural disease suppression as well as for the purposes of biological control the antagonistic mechanisms provided by microorganisms in the rhizosphere and the role of mineral nutrients in plant defense appear to be of particular importance (Cook et al., 1995; Huber and Graham, 1999; Weller et al, 2007; Hartmann et al., 2009). The research work reviewed in this chapter has elaborated many aspects of the roles that microorganisms and mineral nutrients can have in the expression of plant resistance and combat against pathogens. Less well understood in this regard are the interactions between crop plants, pathogens, other microorganisms, their abiotic environment, and the influence of agricultural practices (Cook, 2003; Alabouvette et al., 2004). Pronounced specificities occurring in these diverse interactions, however, imply that the functions of certain components need to be investigated within distinct host-pathogen relationships (Huber and Wilhelm, 1988). Such approach has been deployed by Huber and McCay-Buis (1993) in an extensive analysis of the multiple components involved in the development of take-all in cereal crops, facilitating the comprehensive understanding and integrated management of this serious soil-borne disease. A major conclusion from this work was that many of these components are interrelated to influence the plant availability of manganese in its roles for disease suppression (Table 7.1). However, to accomplish biological control of take-all disease through improved manganese availability may require a sophisticated management of the rhizosphere for the optimized mobilization and acquisition of manganese by the plant to counteract its rapid oxidation in soils and circumvent its limited translocability within plants. Other studies emphasized the exploitation of take-all decline and the use of microbial inoculants that have been isolated from disease suppressive soils and may antagonize *G. graminis* by diverse mechanisms, including the reductions of manganese for enhanced plant availability (Huber and Wilhelm, 1988; Hornby et al., 1998; Weller et al., 2002; Cook, 2003). However, these strategies revealed to be less reliable and effective to control take-all disease than balanced crop rotations (Hornby et al., 1998; Cook, 2003).

An innovative approach for disease control could be to mimic the soil amelioration produced by oats or other take-all suppressive crops preceding wheat. In general, their

effect can be mediated through the common rotation effect of poor pathogen survival in absence of the host, production of compounds with direct toxic effect on the pathogen (e.g. avenacins), influence on other microbial populations, or altered abiotic soil conditions such as the availability of nitrogen forms and manganese (Huber and McCay-Buis, 1993; Alabouvette et al., 2004). However, the importance of the individual active components in these processes has not been separated in order to exploit them as uncoupled bio-control measures independent of growing the respective crop (Alabouvette et al., 2004).

Table 7.1: The effect of plant factors and components of their abiotic and biotic environment on take-all disease reflects their influence on manganese availability. A decreased plant availability of manganese generally increases take-all severity, and vice versa (adapted from: Huber and Wilhelm, 1988; Huber and Graham, 1999).

Manganese immobilizing conditions:	**Manganese mobilizing conditions:**
- Manganese inefficient host genotype	+ Manganese efficient host genotype
- Narrow crop rotations	+ Balanced crop rotation
- Short-term monoculture of host crops	+ Long-term monoculture with take-all decline
- Precrops to wheat of alfalfa or soy bean	+ Precrops to wheat of oats, glucosinolate rich brassicas, or lupines
- Manganese oxidizing G. graminis	+ *G. graminis* defective in manganese oxidation
- Alkaline soil and/or rhizosphere pH	+ Acidic soil and/or rhizosphere pH
- Nitrate supply, nitrification	+ Ammonium supply, inhibited nitrification
- Oxidative conditions such as in dry, warm, loosely structured and aerated soils	+ Reductive conditions such as in moist, cool, densely structured and unaerated soils
- Manganese oxidizing microflora	+ Manganese reducing microflora
↳ **Increase of take-all disease**	↳ **Suppression of take-all disease**

Objectives of Part C of this study

As shown in the literature review above, intensive research on take-all in cereals worked out a considerable understanding of many aspects of the disease, but due to the complex interactions of multiple factors, effective and economic control of the disease remains

difficult. An aim of the following investigations was to investigate the effectiveness of selected crop management strategies in improving the manganese status and suppressing the severity of take-all in wheat. A main focus was to elucidate the role of different mechanisms of oat as a precrop, microbial inoculants, and ammonium nutrition compared to the direct supply of manganese in the suppression of take-all disease.

8 Bio-control of take-all disease in wheat by oat precrops or supplemental treatments with bio-effectors

8.1 Background and objectives

Wheat, besides maize and rice, is among the most important crop species and primary staples of human nutrition (Pinstrup-Andersen et al., 1999; von Braun, 2007). During recent decades, the global demand for wheat has been increasing, which has been met by higher yields per acreage and an increased proportion of the total arable land planted with wheat, rather than by the exploitation of additional land resources for agriculture (Mielke, 1998; Ramankutty et al., 2002; Hafner, 2003; von Braun, 2007; Trostle, 2008). The economic and agronomic excellence of wheat relates to its high yield potential together with its technically feasible and cost efficient production process, but with increasing cropping intensity of wheat in narrow crop rotations, severe phytopathological problems are likely to arise (Mielke, 1998; Heitefuss, 2000). Many air borne wheat diseases such as powdery mildew (*Blumeria graminis*), rusts (*Puccinia* spp.), tan spot (*Drechslera tritici-repentis*), or leaf blotch (*Septoria tritici*, *Stagonospora nodorum*) can be effectively controlled through pesticides and/or resistant varieties of wheat (Eyal, 1999; Schlüter, 2008; Börner, 2009). However, the control of soil borne diseases, such as take-all (*Gaeumannomyces graminis* var. *tritici*), eyespot (*Pseudocercosporella herpotrichoides*), or *Fusarium* root and stalk rots (*Fusarium* spp.), relies mainly on cultural methods including crop rotation and tillage to facilitate the rapid decomposition of infested crop residues, thus inhibiting the survival of the pathogens (Mielke, 1998; Schlüter, 2008; Börner, 2009). The development of take-all diseae is a major reason why subsequent wheat crops yield less than the first wheat crop in monocultural crop sequences, whereas this disease may not occur as a severe problem in balanced crop rotations (Hornby et al., 1998; Váňová et al., 2002; Kirkegaard, 2008).

Recently, efforts to control take-all disease by chemical means resulted in the release of two fungicides for seed treatment. One of them, Latitude® (active ingredient: silthiofam; Monsanto Agrar Deutschland GmbH, Düsseldorf, Germany), is regarded as specifically

toxic to *Gaeumannomyces graminis* var. *tritici* (*Ggt*) by inhibiting the export of adenosine triphosphate (ATP) from the mitochondria to the cytosol, but its exact mode of action has not yet been identified (Schoeny and Lucas, 1999; Joseph-Horne et al., 2000; Freeman et al., 2005). The other fungicide, Jockey® (active ingredients: fluquinconazole and prochloraz; BASF SE, Ludwigshafen, Germany), has a broad spectrum of activity against a range of fungal pathogens by inhibiting the biosynthesis of sterols, which are necessary components of eukaryotic cell membranes (Burden et al., 1987; Bateman et al., 1990; Dawson and Bateman, 2000; Freeman et al., 2005; Gaulin et al., 2010). Although these fungicides are able to retard the infection process and relieve the symptoms of damage to some extent by their distinct modes of actions, they may not completely eliminate the pathogen or cure the disease because of complex interactions between many factors (Wilmsmeier, 2002; Cook, 2003; Freeman and Ward, 2004; Bateman et al., 2008; Schlüter, 2008; see Chapter 7.4). Both products cannot compensate for the take-all suppressive effect of balanced crop rotations and are not recommended for application during long sequences of wheat crops for reasons such as to avoid the development of resistant pathogen populations (Hahn and Tiedemann, 2004; Freeman et al., 2005).

Among the most effective rotational break crops to control take-all disease in wheat is oat (Rothrock and Cunfer, 1991; Huber and McCay-Buis, 1993; Fig. 8.1), but oats and many other take-all suppressive crops are economically less attractive than wheat (Torán, 2001). Therefore, it would be worthwhile to mimic the effect of oat precrops by compensatory measures while cropping wheat in continuous, but well maintained, monoculture. The exact mechanisms by which oats confer control of take-all disease in subsequent wheat, however, are not completely understood and need to be further elucidated for their exploitation in sustainable systems of wheat production.

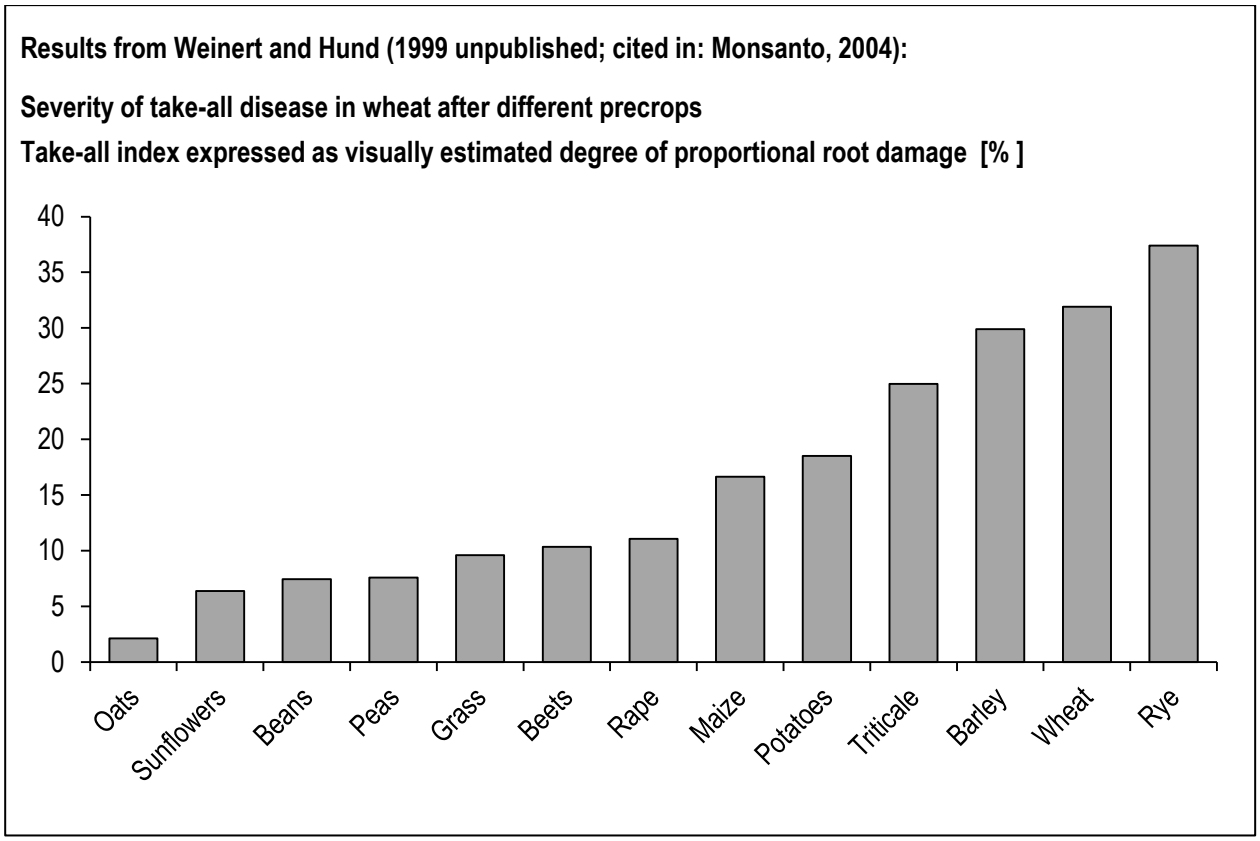

Fig. 8.1: Hund et al. (2000), who investigated the influence of different agronomic and soil physicochemical factors on the severity of take-all disease in cereal crops based on a survey conducted over four years in Germany, reported a predominant influence of the direct precrop. After personal communication with Andreas Hund and Joachim Weinert, the present figure could be reproduced from Monsanto (2004) with permission of the Monsanto Agrar Deutschland GmbH, Düsseldorf, Germany. It shows the infection rate of take-all disease in wheat grown after different precrops, expressed as visually estimated degree of proportional root damage (take-all index) according to a guideline proposed by the Biologische Bundesanstalt für Land- und Forstwirtschaft, Braunschweig, Germany in 1999 (BBA, 1999). Data were collected from 413 sites in Germany.

As indicated by the literature reviewed in Chapter 7, the suppressive effect of oat precrops against *Gaeumannomyces graminis* var. *tritici*, the variety of the take-all fungus that typically accounts for severe infections of wheat and barley plants in the field, has been associated with different mechanisms of disease control (Huber and McCay-Buis, 1993; Osbourn et al., 1994). Besides breaking the life cycle of *Gaeumannomyces graminis* var. *tritici* as a non-host crop to this pathogen, oat seems to countervail the development of take-all disease by inducing changes in the soil microflora in favor of antagonistic organisms that mobilize manganese (Mn) by reduction processes (Huber and McCay-

Buis, 1993). Bacteria of the genera *Pseudomonas* and *Bacillus*, but also *Trichoderma* fungi seem to be among the most important microbial agents in this regard (Huber and Wilhelm, 1988). Furthermore, the precrop effect of oats has been associated with an improved plant availability of manganese in subsequent wheat crops (Huber and McCay-Buis, 1993). The adequate supply of manganese in particular has been recognized as a key factor, among other mineral nutrients, related with plant defense and suppression of the take-all fungus. On the contrary, the immobilization of manganese by oxidation processes seems to be a mechanism of virulence exerted by this pathogen (Graham and Rovira, 1984; Graham and Webb, 1991; Marschner et al., 1991; Huber and McCay-Buis, 1993; see Chapter 7). Another characteristic trait that is associated with the resistance of oats to *Gaeumannomyces graminis* var. *tritici* is the ability to produce and accumulate antimicrobial saponins, named "avenacins", in epidermal root tissues (Osbourn et al., 1994; Qi et al., 2004). The fungitoxic activity of saponins has been mainly attributed to the formation complexes with sterols in fungal membranes, leading to impaired membrane integrity (Morrissey and Osbourn, 1999; Augustin et al., 2011).

In summary, the oat specific propagation of antagonistic soil microbial populations, their influence on the availability of manganese, and the direct fungitoxic effect of avenacins can be identified as three important factors by which oat crops may exert their take-all suppressive activity. However, in order to exploit these different modes of action within biological control strategies, there is a need for detailed studies on their relative importance and the interactions between them, which have not been clearly dissected yet. A central question in this regard seems to be whether an enhanced plant availability of manganese in oat-precropped soils is a cause for or a consequence of take-all suppression in subsequent wheat crops.

Working hypotheses

To elucidate the interrelationships between oat precrops, microbial populations, the availability of manganese, and the severity of take-all disease in subsequent wheat crops, Volker Römheld and Xinping Chen (pers. com., 2004) suggested the following three working hypotheses:

(1) The residues from oat crops (e.g. straw, roots) have a higher manganese concentration and therefore they provide more manganese to subsequent wheat than those from preceding wheat or barley crops upon their decomposition.

(2) Oat precrops specifically alter the population densities and/or activities of soil microorganisms in favor of manganese reducing processes. This is a lasting effect, which leads to the suppression of take-all disease by increasing the availability of manganese in the soil and the acquisition of this mineral nutrient by the following wheat crop.

(3) Due to their own mechanisms of resistance, such as the production of avenacins, oats are not hosting *G. graminis* var. *tritici*. Therefore, oat precrops are hampering the survival and carry-over of this pathogen, which already decreases the amount of its inoculum in the soil before the next wheat crop is grown.

According to the first two hypotheses (1 and 2), an increased manganese availability, induced by the oat precrop and related changes in the soil microflora, is regarded as the cause of take-all suppression by improving the defense of wheat plants against a given population of the take-all pathogen. Therefore, it should be possible to compensate the oat effect by alternative measures for improved plant availability of manganese, such as manganese fertilization, ammonium nutrition of the host plant for rhizosphere acidification, or the enhancement of manganese reducing microorganisms in the soil and especially in the rhizosphere. Distinct prospects and limitations of such approaches have been addressed in Chapter 7.

Under the third hypothesis (3), by contrast, a diminished population size of *G. gramins* var. *tritici* and, thereby, a lower manganese oxidizing activity in the soil is expected to result in enhanced manganese availability to the subsequent wheat crop as a side effect without important function in disease suppression. In this case, it would be necessary to persistently decrease the inoculum density of the pathogen in the soil in order to control take-all disease in wheat effectively.

In the literature, evidence to contradict each of the above hypotheses (1 to 3) can be found. (1) Rye, for instance, can be even more efficient in manganese acquisition and may

have higher tissue concentrations of manganese than oat, but it is known to enhance the infection potential of the take-all fungus in the soil. Despite of its low susceptibility to the disease, rye is among the most take-all conductive precrops to wheat in a rotation (Jones, 1957; Huber and McCay-Buis, 1993; Fig. 8.1). (2) Contrary to the hypothesis that the specific promotion of manganese mobilizing microorganisms by oats would effectively suppress take-all disease, oats as well as subsequent wheat are infected by *Gaeumannomyces graminis* var. *avenae* (Hornby et al., 1998). This variety of the take-all fungus is able to overcome the take-all resistance of oats by its capacity to produce enzymes (avenacinases) degrading the antifungal properties of avenacins (Turner, 1961; Walker, 1972; Crombie et al., 1986; Osbourn et al., 1991; Osbourn, 1996). Likewise, the observation that oat genotypes lacking avenacin are susceptible to *G. graminis* var. *tritici* indicates a predominant role of avenancins for the defense of oats against take-all disease regardless of associated rhizobacteria (Osbourn, 1994; Papadopoulou et al., 1999). Furthermore, rotation with oat crops seems to disturb the establishment of wheat specific microbial populations that are antagonistic to the take-all pathogen and account for the declining severity of take-all disease in prolonged wheat monocultures (Raaijmakers and Weller, 1998; Bergsma-Vlami et al., 2005). Although the specific alteration in soil microbial populations induced by oat may help to suppress the disease in the first subsequent wheat crop, an enhanced severity of take-all can arise in second or third wheat crops following oat (Cook, 1981a; Hornby et al., 1998). (3) Nevertheless, also the avenacin-related resistance of oats cannot be regarded as an exclusive and independent mechanism of take-all suppression, as supposed by the third hypothesis, because insufficient manganese supply predisposes oats to take-all infection. Reported cases of take-all disease in oats have been attributed to an absolute deficiency of this element in the soil, as well as to the inefficiency of certain oat genotypes in manganese acquisition. Such oat genotypes were found to harbor manganese immobilizing populations of microorganisms in their rhizospheres (Timonin, 1946; Huber, 1989; Huber and McCay-Buis, 1993). It has not been clearly elucidated in this regard how a deficient supply of manganese may affect the synthesis of avenacins together with the expression of other resistance mechanisms in oat plants (Huber and McCay-Buis, 1993; Osbourn et al., 2003; Haralampidis et al., 2002; Jenner et al., 2005).

Objectives of the present study

Based on the above considerations, the supply of manganese from decaying oat residues can be virtually excluded as a critical factor for achieving take-all control (1), but further research work is necessary to test the validity of the second (2) and third (3) hypothesis, as intended with the set up of the following experiments. The objective of this research work was to investigate the mechanisms of oat as a precrop in suppressing take-all disease in subsequent wheat with particular emphasis on oat-induced alterations in microbial populations, the availability of manganese, and the infection potential of *G. graminis* var. *tritici* in soils. Thereby the development of biological strategies mimicing the effect of oat precrops for take-all control was facilitated, such as by means of microbial inoculants, supply of manganese fertilizers, or an adapted rhizosphere management to achieve targeted improvements in soil-microbe-plant interactions for healthy root growth.

8.2 Materials and methods

8.2.1 Experimental approach

To dissect the role of different mechanisms of oat as a precrop in suppressing the severity of take-all disease in wheat, pot experiments with soil substrates derived from oat- and wheat-precropped plots of a take-all infested field site were conducted under controlled conditions. In one experiment, treatments with commercial preparations of *Pseudomonas* respectively *Trichoderma* strains as seed/soil inoculants for pathogen suppression through manganese reduction and/or other antagonistic mechanisms were tested in comparison with foliar or soil application of manganese sulphate. This investigation, which is described in detail here, was conducted to answer the question whether manganese fertilization can effectively suppress take-all disease, or if it is necessary to enhance antagonistic microorganisms to compensate for the effect of oat precrops. Furthermore, the discussion in Chapter 8.4 integrates results from another experiment, conducted in cooperation with Schackmann (2005) and Akter (2007) under similar conditions. In that investigation, the inoculum level of the pathogen was either decreased by application of a take-all specific fungicide (i.e. Latitude®) or elevated by inserting additional inoculum of *G. gramins* var. *tritici* into the soil. The purpose of those treatments was to elucidate the

relative importance of oat-induced decreases in the density of pathogen inoculum versus oat-induced alterations in the populations of antagonistic microorganism in the soil for the level of take-all infestation and the status of manganese nutrition in subsequently grown wheat plants.

8.2.2 Origins and properties of the experimental soil materials

On 03rd May 2006 take-all infested soil material, either wheat- or oat-precropped, was collected from the plough horizon (5-25 cm) of a field site (Longitude: 48°42'57'' N; Latitude: 9°11'42'' E) at the research station Heidfeldhof, University Hohenheim, Stuttgart, Germany. At this field site an experiment to investigate the control of take-all disease in wheat by oat-precrops, microbial bio-control agents (Vitalin T50, Vitalin SP11; see Table 4.1) or the application of mineral fertilizers (NH_4^+-nutrition, Mn-supply) had been started in the crop year 2003, as decribed by Schackmann (2005), and was still under progress in 2006. Details about this field site, its soil properties and the design of this field experiment are given in Table 8.1 and Fig. 8.2. The presented data of physical and chemical soil properties were derived from Leick (2003) and analyses performed at the Landesanstalt für Landwirtschaftliche Chemie (710) at Universität Hohenheim, Stuttgart, Germany.

Table 8.1: Physical and chemical properties of the soil material from the trial area at research station Heidfeldhof, Stuttgart, Germany

pH	Humus	N_{total}	Mineral nutrient concentrations [mg kg^{-1} dry soil]					Soil texture		
($CaCl_2$)	[%]	[%]	P (CAL)	K (CAL)	Mg ($CaCl_2$)	Mn (CAT)	Mn_{total}	Clay [%]	Silt [%]	Sand [%]
6.7	1.9	0.1	131 (D)	174 (C)	150 (C)	319 (E)	828	25	70	3

Notations in round brackets refer to the respective extraction method and indicate the level of availability according to the VDLUFA (1986) classification system (E = very high, D = high, C = sufficient) as specified in LfL (2012).

The estimation of plant available phosphorus (P), potassium (K), magnesium (Mg), and manganese (Mn) concentrations by chemical extraction methods indicated sufficient to high levels of these mineral nutrients in soil samples from the trial area. Especially high

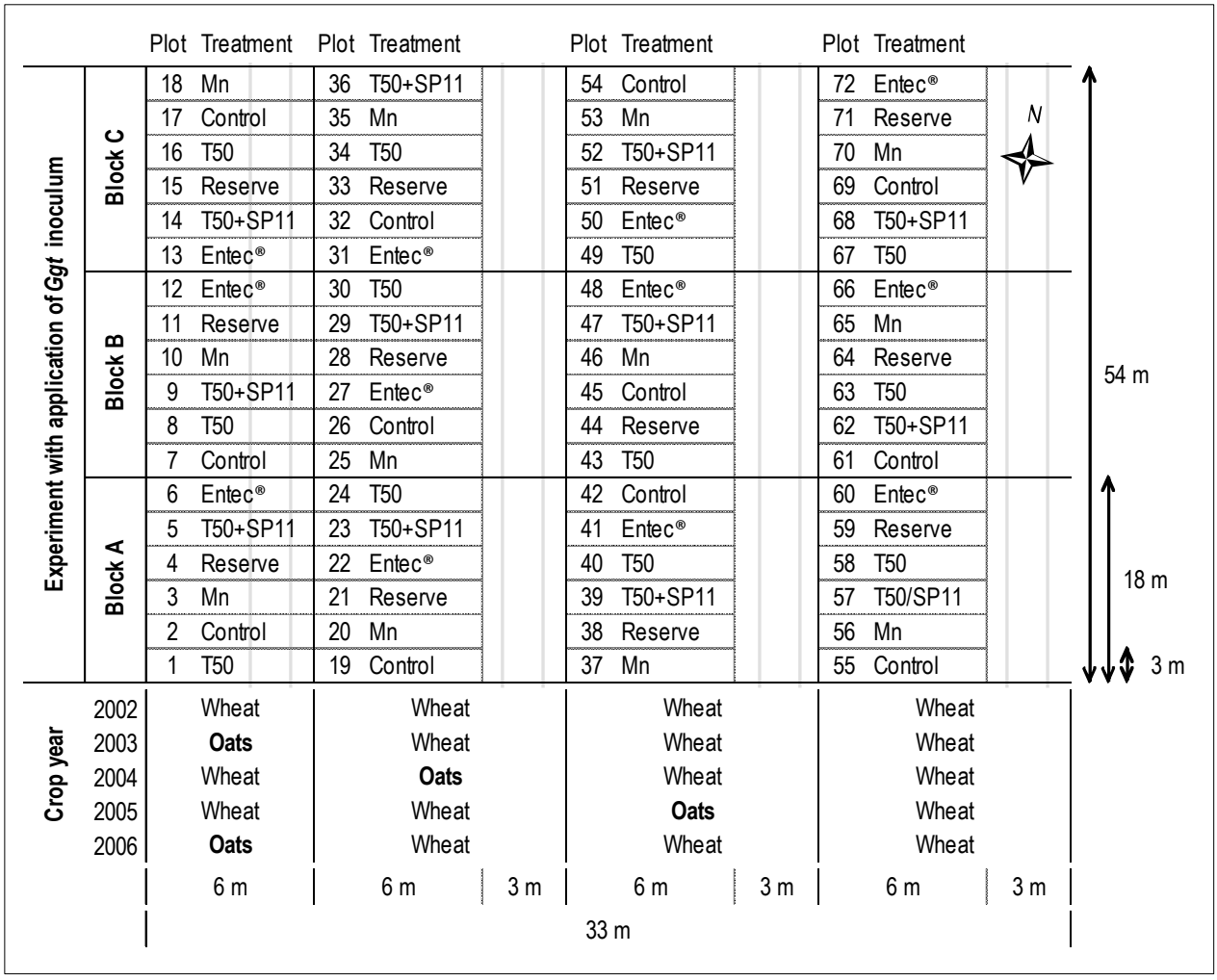

Fig. 8.2: Field plan of the experiment conducted at research station Heidfeldhof, Stuttgart, Germany in the crop years from 2002 to 2006 to investigate the control of take-all in wheat by oat-precrops or supplemental treatments. For technical reasons, the precrop factor was not randomized. Instead, the trial area was divided into four stripes cultivated with wheat or rotational oat as indicated per crop year. Each stripe was divided into 18 plots of 18 m^2 in size, which were arranged in randomized block designs with three replicates (Block A-C). Starting from the crop year 2003, as supplemental treatments stabilized ammonium sulfate plus ammonium nitrate fertilizer (Entec® 26, EuroChem Agro GmbH, Mannheim, Germany), manganese sulfate (Mn), or the microbial product Vitalin T50, either single (T50) or combined with Vitalin SP11 (T50+SP11), were tested in plots. Control plots did not receive supplemental treatments, but were, just like the Mn, T50 and T50+SP11 treated plots, fertilized with calcium nitrate (CalciNitTM Hydro, Yara, Dülmen, Germany) at same amounts of nitrogen supply than the Entec® treated plots. Reserve plots were kept available for varying treatments. To facilitate a homogenous establishment of the take-all pathogen, Ggt inoculum was distributed to the soil, in December 2002 and April 2004. The soil was tilled with a mulch cultivator in autumn to incorporate crop residues. Seedbed preparation and sowing were performed with a rotary harrow/drill combination of 3 m working width. Furthermore, the experiment was replicated in an adjacent section of the same field, but without supplementary addition of Ggt inoculum.

levels of availability were indicated for manganese (LfL, 2012), as determined by the CAT method (VDLUFA, 2002), which employes a mixture of 0.01 M calcium chloride ($CaCl_2$) and 0.002 M diethylene-triamine-pentaacetic acid (DTPA) as extractant. However, due to the dynamic influence of soil microbial and physicochemical properties as well as plant specific factors, soil extraction analyses are not fairly reliable to predict the actual availability of mineral nutrients, particularly manganese, to the plant (Browman et al., 1969; Gough et al., 1980; Marschner, 1988; Mengel and Kirkby, 2001; Mundus et al., 2012).

To facilitate a homogeneous establishment of the take-all pathogen in the soil, in one half of the trial area inoculum of *G. graminis* var. *tritici* (*Ggt*) was applied evenly to the soil during the beginning phase of the experiment. According to Weller and Cook (1983), the inoculum material was produced by propagating a mixture of four strains of *G. graminis* var. *tritici* (89/541; 90/544; 95/1/3; 95/3/5), provided by the Institute of Phytomedicine, University Hohenheim, on autoclaved wheat kernels. A first rate of inoculum (ca. 40 g m^{-2}) was distributed on 9th December 2002 and immediately incorporated into the soil before sowing of wheat (var. Drifter) and a second rate (ca. 45 g m^{-2}) was broad casted on 15th April 2003. Nevertheless, the inoculated as well as the non-inoculated part of the trial area, showed severe incidence of take-all disease with an estimated degree of proportional root damage (take-all index) of more than 50 %, as recorded in wheat-precropped wheat of the season 2005. The soil material for the present pot experiment was taken from wheat- (No. 61 and 69) and oat-precropped (No. 45 and 54) "control" plots, which means without application of bio-control agents or NH_4^+- and Mn-fertilizer treatments. In the previous four years (2002-2005), these plots had either been continuously cultivated with wheat (*Triticum aestivum* L., diverse varieties) crops only (i.e. "wheat-precropped") or, after three years of wheat cultivation, once precropped with oats (*Avena sativa* L. var. Jumbo; Nordsaat Saatzucht, Langenstein, Germany) in a wheat-wheat-wheat-oats sequence (i.e. "oat-precropped"). In autumn 2005, the distinct wheat- or oat-precropped plots had been sown with winter wheat (var. Monopol; Saatzucht Firlbeck, Atting-Rinkam, Germany), which was in the developmental stage of tillering (3 to 4 tillers per plant) at that time when the soil material for the present pot experiment was collected. To reflect site heterogeneity, the charges of wheat- or oat-precropped soil material taken from

different plots in block B and C (Fig. 8.2) of the field experiment were kept separately throughout the experiment. The desiccated top 5 cm layer of soil, however, was excluded from collection because the take-all fungus might be more likely to survive in soil layers less exposed to changing weather conditions (Hornby et al., 1998).

8.2.3 Setup of the pot experiment

Preparation of soil substrates for the pot experiment

The different charges of wheat- or oat-precropped soil material were air-dried (25 °C) sufficiently to allow sieving by 5 mm mesh without smearing it, which would disrupt the structure of micro-aggregates. After sieving, the actual water content (15-16 % w/w) of the soil material was estimated by weighing, drying until weight constancy (105 °C) and reweighing of three replicate subsamples (135 g fresh weight) per charge (Hartge, 1971). In order to produce a well aerated substrates for plant growth with optimized infection conditions for *Gaeumannomyces graminis* (Augustin et al., 1997; Hund et al., 2000; Hahn, 2004), each charge of sieved soil material was mixed in a 1:1 ratio, based on dry weights, with coarse sized quartz sand (1,6-2,5 mm grain, Nr. 5G, Dorsolit, Mannheim, Germany). Furthermore, small cut (1-2 cm pieces) and autoclaved (121 °C, 20 min) wheat straw was added to the soil/sand mixtures at rates of 1 % (w/w). The water holding capacity of the readily prepared substrate mixtures was estimated by means of a gravimetric assay according to Öhlinger (1996), as described in Chapter 5.2.2. A favorable substrate condition for take-all development, such as of being moderately moist, was further ascertained by “finger test” (Arbeitsgruppe Boden, 1994) at about 40 % water holding capacity. Proportions equivalent to 1717 g dry matter of the wheat- respectively oat-precropped substrate charges (consisting of 850 g soil material, 850 g sand, 17 g straw) were then filled into 1.5 liter pots. These pots had been constructed from polyvinyl chloride (PVC) pipes (95 mm in diameter, 210 mm in height) at one end densely closed by a PVC disc. The application rates of bio-control agents and mineral fertilizers described in the following, however, were calculated on base of the dry weight of the soil materials, as the coarse sand texture provides only a relatively small absorption capacity

per weight unit compared to the silt loam texture of the soil materials (70 % silt, 25 % clay, 1.9 % humus; see Table 8.1).

Sowing of wheat seeds and treatment with bio-control agents in the pot experiment

Eight seeds of spring wheat (*Triticum aestivum* L. var. Paragon; Dalgety Arable Limited, Ongar, Essex, UK) per pot were sown on 10th May 2006 into eight seeding holes, prepared with a small stick (6 mm in diameter), 1.5 cm deep from the substrate surface. On account of incomplete seedling appearance, the number of plantlets was to be set to four per pot at two weeks after sowing. Paragon has been described as a manganese inefficient wheat genotype (Jiang and Ireland, 2005; Jiang, 2008).

As microbial bio-control agents, the commercial products Vitalin T50 (1 g ≙ 10^8 spores of *Trichoderma harzianum* together with unidentified bacterial organisms; Vitalin Pflanzengesundheit GmbH, Ober-Ramstadt, Germany), and Proradix®WG (1 g ≙ $5*10^{10}$ cfu of *Pseudomonas* sp. *Proradix*, strain DSMZ 13134; Sourcon Padena, Tübingen, Germany) were tested. More detailed product specifications are given in Table 4.1 of Chapter 4. For seed/soil inoculation, the bio-agents were suspended in 2.5 mM $CaSO_4 \cdot 2H_2O$ at concentrations of 34 g Vitalin T50 liter^{-1} or 8.5 g Proradix® liter^{-1}. Each of eight seeds per pot received a dose of 2.5 ml of the respective product suspension added into the seeding holes immediately after seed insertion. As recommended by the producers, these application rates were calculated equivalent to concentrations of 0.4 % (w/w) for Vitalin T50 and 0.1 % (w/w) for Proradix® based on the targeted water content of the soil materials (20 % w/w; ≙ 170 ml water pot^{-1}) used for the pot culture experiment. When expressed as application rates on basis of the soil dry matter, the respective amounts were 0.8 g Vitalin T50 kg^{-1} (≙ 0.68 g pot^{-1}) or 0.2 g Proradix® kg^{-1} (≙ 0.17 g pot^{-1}). Untreated controls or treatments without microbial inoculants were consistently supplied with pure 2.5 mM $CaSO_4 \cdot 2H_2O$ only. To cover the seeds and to avoid surface aggradation due to watering, 150 g of medium sized quartz sand (0.3-1.2 mm grain, Dorsolit, Mannheim, Germany) was added over the top surface of the substrate in each pot. The moisture content of the substrate mixtures was adjusted at 40 to 50 % water holding capacity (20 to 25 % w/w of the soil material) by weighing and adding deionized water if necessary throughout the experiment. The intervals of weighing were varied in adaptation to the

water demand of the plants, starting from a minimum of less than 12 hours at the beginning of the experiment. At the end of the experiment, it was necessary to water the plants every two days.

Basal rate of mineral fertilization and treatments with manganese sulfate in the pot experiment

As nitrogen fertilizer, calcium nitrate ($Ca(NO_3)_2$) was applied in split doses. The first dose was distributed as 0.5 M solution in deionized water to the sieved soil materials by spraying under continuous mixing at a rate of 50 mg N kg^{-1} dry weight of the soil material (≙ 42.5 mg N pot^{-1}). The second dose, at a rate of 30 mg N kg^{-1} dry matter of the soil material (≙ 25.5 mg N pot^{-1}), was applied as 30 mM solution in the irrigation water at 22 days after sowing. At that time, the wheat plants had achieved the two-leaf stage and started to show symptoms of nitrogen deficiency, such as a pale green leaf color (Snowball and Robson, 1991). Measurements with a chlorophyll meter (Minolta SPAD-502, Konica Minolta Sensing, Osaka, Japan) before and 8 days after fertilization confirmed the visual observation of plant recovery after nitrogen supply. However, the nitrogen supply was kept at a moderately low level, throughout the experiment, because a high nitrogen supply could help the plant to compensate for damage from take-all disease, which could nullify the possible effects of tested bio-control agents and manganese treatments (Cook, 2003).

Manganese treatments were applied as mineral fertilizer to the soil material or onto the leaves of the wheat plants. For soil application, 0.1 M manganese sulfate monohydrate ($MnSO_4 \cdot H_2O$) in deionized water was homogenously mixed into the sieved soil material at a rate of 15 mg Mn kg^{-1} dry weight (≙ 12.8 mg Mn pot^{-1}), together with the first dose of nitrogen application, as described above. Leaf applications of manganese were performed by spraying of 12 mM $MnSO_4 \cdot H_2O$ (≙ 1.8 g $MnSO_4$ $liter^{-1}$) in deionized water at 16 (two-leaf stage), 23 (three-leaf stage), and 30 (four-leaf stage) days after sowing. To avoid manganese entry into the soil substrate by the spray applications, the top of the pots was covered by inserting stripes of tissue paper between the plants from before spraying until the spray solution was dried off. It was estimated by comparative gravimetric testing, that

in total the three-rate leaf application loaded the plant surface with 0.7 mg $MnSO_4$ $plant^{-1}$ (≙ 1.0 mg Mn pot^{-1}).

Design of and cultivation conditions in the pot experiment

The experimental design comprised two precrop levels (i.e. wheat- or oat-precropped) and five levels of supplemental treatments to compensate for the take-all suppressive effect of oat precrops (i.e. untreated control or treatments with either Vitalin T50, Proradix®, Mn-soil, or Mn-leaf application). Four replicate pots per combination of precrop and supplemental treatment (40 pots in total) were arranged in a complete randomized block pattern in order to account for variances between the different soil charges and environmental gradients (e.g. light intensity, temperature) within the growth system.

The plants were cultivated in a glasshouse at Universität Hohenheim, Stuttgart, Germany (48°42´44´´ N, 9°12´31´´ E), from 11th May until 27th June 2006. To maintain root zone temperature at 12-15 °C, the pots were placed together in a basin (120 cm in length, 75 cm in width, 25 cm in depth) with thermal insulated bottom and walls, as predefined by the block design. Plastic tubes (PVC; approx. 1.5 cm in diameter), connected with a heating/cooling system (Frigomix U plus Thermomix UB, Braun, Melsungen, Gemany) to carry circulating liquid for thermal exchange, were installed crosswise between the pots in three layers at the bottom, in the middle and near to the top level of the pots. The interstitial space between pots and the cooling tubes was filled up with moist peat/sand mixture for thermal conduction. Additional light was supplemented with 400 W mercury vapor lamps (MBF-R, Osram, Great Britain) during day time, when the weather was cloudy. The experimental growth phase ended 49 days after sowing at inflorescence emergence (Zadok´s scale Z50-59; Zadoks et al., 1974).

8.2.4 Evaluation of the pot experiment

Non-destructive measurements of plant performance during cultivation

The rate of seedling appearance was recorded by daily counts of the number coleoptiles that had reached the upper surface of the cover layer of sand until twelve days after sowing. Starting from 16 days after sowing the progress of shoot development was

characterized by measurements of leaf length and counting the number of leaves per plant at intervals of three to nine days upon experimental harvest, depending on their developmental progress. Differences in leaf color values, indicative of the plant nitrogen status, were measured in "SPAD units" (based on differences in optical density at two wavelengths) with a chlorophyll meter (Minolta SPAD-502, Konica Minolta Sensing, Osaka, Japan; Matsunaka et al., 1997) at 22, 30, and 47 days after sowing. For each pot and sampling date, eight measurements were taken at the centre of the youngest fully expanded leaf blades, which at day 47 days after sowing were the blades of the flag leaves, and mean values were calculated. At the end of the experimental growth phase, also the numbers of tillers and emerging inflorescences per plant were determined.

Experimental harvest and post-harvest measurements

The wheat plants were harvested, block by block, from 27th until 29th June 2006 (48 to 50 days after sowing) in the developmental stage of inflorescence emergence to yield samples of root, shoot and substrate material. Samples were processed for the evaluation of root and shoot biomass production, take-all induced root damage, root-associated microbial populations and their possible impact on manganese reduction and oxidation processes, root morphological characteristics, mycorrhizal root colonization, and mineral nutrient levels in plant tissues.

Collection of root and shoot samples

Plant shoots were cut off at their bases and briefly rinsed with deionized water to remove adhering dust. After drying the shoot material at 65 °C to constant weight, the weight of their dry matter was recorded. The growth substrate with roots inside was carefully poured out of the pots into sieve frames (50 x 50 cm) with 2 - 3 mm mesh size. As the different root-investigations required the separation of diverse representative subsamples from the root systems, in this section only the process of sampling is described, whereas the subsequent analyses are outlined in the following sections. At first, roots without visible necroses from take-all infection were picked out of the substrate, using sterilized forceps, and loosely adhering soil was shaken from them. The remaining more firmly adhering substrate material was considered as "rhizosphere soil" (Katznelson, et al. 1948;

Rouatt and Katznelson, 1961). For the isolation and characterization of microorganisms from the wheat rhizosphere on different agar media, root segments of about 2-3 cm in length with adhering rhizosphere soil were excised with sterilized scissors to obtain representative subsamples of about 4 g fresh weight. These subsamples were immediately transferred into autoclaved (121 °C, 20 min) 100 ml Erlenmeyer flasks plugged with cotton-wool stoppers and containing 50 ml of 0.1 % w/w proteose peptone (Difco, No. 0122-17-4) in deionized water together with 10 g glass beats (2 mm diameter). Proteose peptone had been added, because when only deionized water is used as diluent over 90 % of the bacterial population may be destructed during the isolation procedure, within one hour (Straka and Stokes, 1957). The Erlenmeyer flasks containing root pieces were cooled on ice and then kept in a refrigerator (4 °C) for about four days until further sample processing. Another subsample of roots with surrounding substrate material (about 10 g fresh weight) was taken from each replicate pot to determine the manganese-reducing capacity in the rhizosphere by an incubation test with manganese dioxide (MnO_2). For this purpose, samples were collected in sterile centrifuge tubes of polypropylene (50 ml BD Falcon™ tubes, Becton Dickinson Biosciences, Heidelberg, Germany) and stored in a refrigerator (4 °C) for about two weeks until processing in the laboratory. After subsamples for rhizosphere microbial analyses were taken, the remaining major share of the root systems was carefully brushed free from adhering substrate and the bulk of substrate was passed through the sieve frame by use of an adjustable water sprayer (Gardena, Ulm, Germany). Thereby, small root pieces, which had been accidentally detached from the root systems, were retained by the sieve mesh to be collected with forceps. Immediately after washing, the roots were assessed for the severity of damage by take-all infestation as described in the next section. Thereafter, further subsamples, each in total of about 0.5 g fresh weight per replicate pot, were collected from representative parts of the root systems to measure characteristics of the root morphology, such as root diameter, root hair length and specific root length to weight ratio, or to evaluate the intensity of mycorrhizal root colonization. For these purposes, only intact root segments without visible damage of the root cortex were selected and cut into short pieces of 1-3 cm length. Until further processing, root pieces for root-morphological measurements were stored at 4 °C in de-ionized water to keep them turgescent, whereas those for

mycorrhizal analyses were stored at 4 °C in 20 % ethanol to facilitate tissue maceration for the subsequent clearing procedure. After removal of the diverse subsamples, the remainder of roots was dried with tissue papers to measure fresh weights and then packed into paper bags for drying at 65 °C and subsequent determination of dry weights, as this material was designed for mineral nutrient analyses. Later, when root analyses were done, also the root material from different subsamples was dried at 65 °C to obtain total root dry weights per replicate pot.

Rating of the severity of take-all root rot

The severity of take-all infestation on wheat roots was rated by estimation of the "take-all index", a unit scaling the proportional degree of root damage, as proposed by the Julius Kühn-Institut, former Biologische Bundesanstalt für Land- und Forstwirtschaft, Braunschweig, Germany (BBA, 1999; see also: Monsanto, 2012). According to this, both the percentage of visibly infected roots in relation to the whole root system and the relative impairment in root development in comparison to a healthy plant were considered to estimate the extent of root damage on a scale from zero to 100 percent. Thereby, an index of zero percent represents no observable disease symptoms and an index of 100 percent indicates complete injury of the root system (Mielke, 2000). In order to introduce standardized criteria for the ranking of root damage, visual rating schemes (Moll et al., 2000; Monsanto, 2006) as well as representative examples from the present study (Fig. 8.3) were used for comparison between surveys. For reliable estimation of infection rates, it was taken care that the roots did not dry off after washing from the substrate until the evaluation was done, because this may lead to equalizing discolorations of healthy and infected root tissues (BBA, 1999).

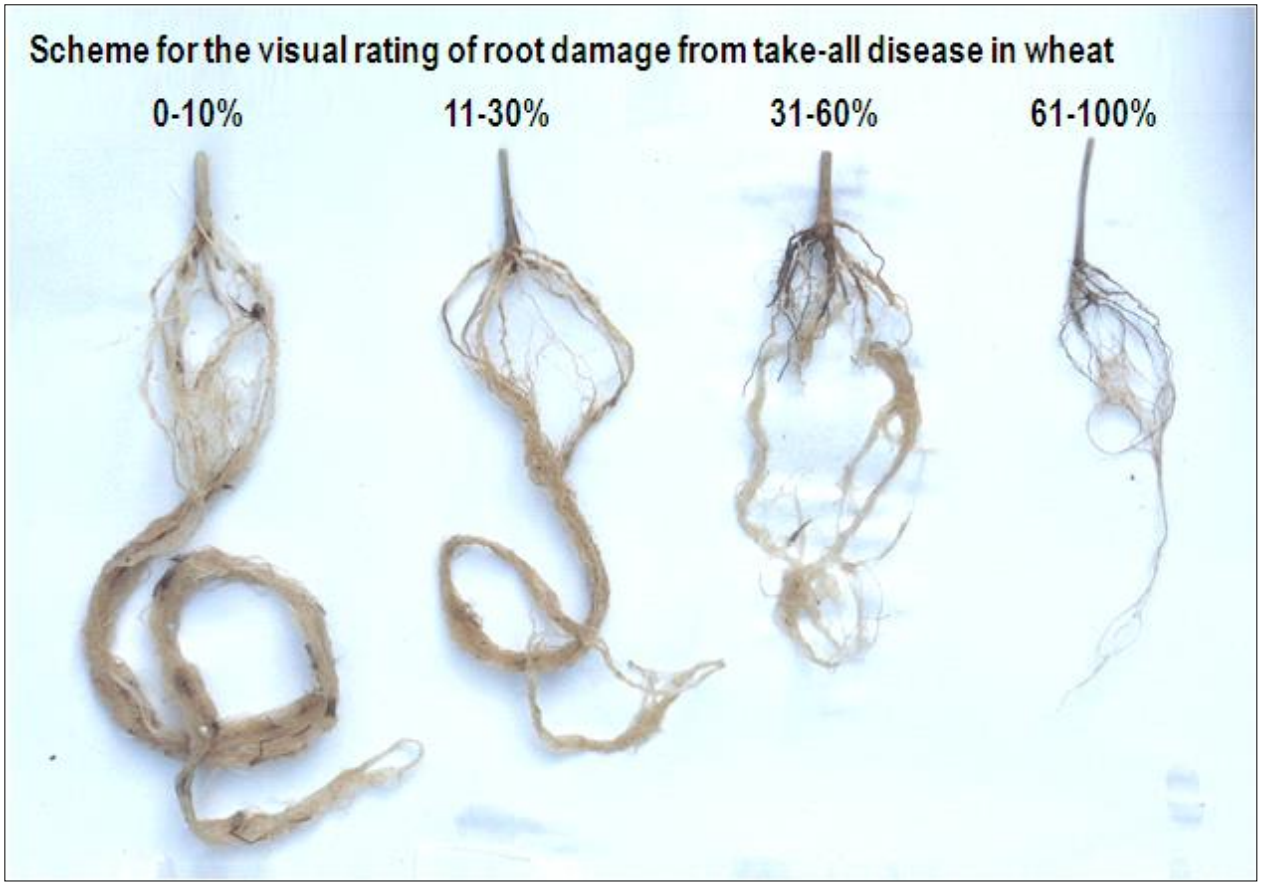

Fig. 8.3: Wheat roots obtained from the present pot experiment conducted in 2006, arranged in order of increasing severity of root damage from take-all disease to serve as a visual rating scheme.

Isolation and characterization of root-associated microorganisms

Samples of wheat roots with adhering substrate material in Erlenmeyer flasks together with peptone water and glass beads were processed according to the method from Rouatt and Katznelson (1961) for the isolation of root associated microorganisms. At first, to prepare a suspension of the root adhering substrate and microflora, the samples were placed on a rotary shaker (SM 25, Bühler, Hechingen, Germany) and vigorously shaken (250 rounds min^{-1}) for 15 minutes. The added glass beads were supposed to support the removal of microorganisms from the root surface and soil particles. After shaking, serial dilutions of the suspensions were prepared with 0.1 % proteose peptone (Difco, No. 0122-17-4) under sterile conditions (Elsherif and Grossmann, 1990). From the initial sample suspensions, 10 ml were transferred to secondary (50 ml) Erlenmeyer flasks with 25 ml of the diluent, which were again shaken (200 rounds min^{-1}) for 5 minutes to obtain a uniform mixture. Then, 0.5 ml of the diluted suspensions were removed and transferred into

reaction tubes to be mixed with 4.5 ml of the diluent by use of a vortex stirrer. This step was repeated up to four times to produce appropriately diluted samples of which then aliquots of each 50 µl were plated out on defined agar media in Petri dishes (94 mm in diameter, 16 mm in height) by use of sterilized Drigalski spatulas (Hedderich et al., 2011). The different types of agar media deployed for the quantification and qualitative assessment of root associated microbial populations were prepared as described in the following paragraphs (1-3) with reference to Tables 8.2 to 8.5.

(1) Fluorescent pseudomonads were isolated by use of a selective "NPC medium" (Table 8.2) according to Sands and Rovira (1970). This medium is based on King's medium B (King et al., 1954) supplemented by the antibiotics Novobiocin, Penicillin G, and Cycloheximide (abbreviated to NPC). In particular, the proper handling and disposal of the human toxic and persistent antibiotic Cycloheximide requires special caution for health and safety reasons (Sigma-Alrdich, 2006; Sigma-Aldrich, 2012). Cycloheximide is an inhibitor of protein synthesis in eukaryotic, but not in prokaryotic cells. Due to this selective inhibition, Cycloheximide can be used for the selection of most bacteria against many yeasts and other fungi (Obrig et al., 1971; Sigma-Aldrich, 2006). Novobiocin and Penicillin G are effective against gram-positive bacteria by inhibition of deoxyribonucleic acid (DNA) replication (Gellert et al., 1976; Cozzarelli, 1980) and cell wall synthesis (Wise and Park, 1965; Yocum et al., 1980), respectively. To induce maximum pigment production and fluorescence in fluorescent pseudomonads, the medium is kept deficient in iron (Sands and Rovira, 1970).

Table 8.2: Selective NPC medium for the isolation of fluorescent pseudomonads according to King et al. (1954) and its modification by Sands and Rovira (1970)

Components according to King's medium B to be autoclaved at 121 °C for 20 min:	
K_2SO_4, puriss., p.a. (Riedel-de Haën, No. 31270)	1.5 g
$MgSO_4 \cdot 7H_20$, puriss., p.a. (Fluka, No. 63140)	1.5 g
Proteose peptone (Difco, No. 0120-17-6; BD, No. 211684)	20.0 g
Glycerol anhydrous (Fluka, No. 49767, density 1.25 g ml^{-1})	10.0 g
Agar (Oxoid, No. LP 0028 purified agar)	12.0 g
Deionized water	940.0 ml
pH adjustment to pH 7.2 with 0.5 M NaOH	
To be mixed together in 5 ml of 95 % ethanol, then diluted with 45 ml of deionized water, and added separately by sterile filtration (< 0.45 µm) to the medium cooled to 40 °C after autoclaving:	
Novobiocin sodium salt (Sigma, No. N-1628), see Waschkies (1992)	75 mg
Penicillin G potassium salt (Sigma, No. P-7794), 1440-1680 units per mg	45 mg
Cycloheximide (Sigma, No. C-6255)	75 mg

(2) To reflect the total population density of the bacterial community in the rhizosphere, Standard-II Nutrient-Agar (Merck, Darmstadt, Germany) was used. The readily prepared medium (Table 8.3), consisting of 3.45 g peptone from meat, 3.45 g peptone from casein, 5.1 sodium chloride, and 13.0 g agar-agar per one liter of de-ionized water (Merck, 2012a), allows the growth of a broad spectrum of microorganisms with less specific substrate requirements (Waschkies, 1992). Cycloheximide was added in order to inhibit fungal growth (Waschkies et al., 1994). However, the fraction, which becomes observable by cultivation under aerobic conditions on the medium, may represent less than 10 % of the total bacterial community (Bakken, 1985; Marschner et al. 2002, Ferrari et al., 2005).

Table 8.3: Broad-spectrum medium that supports the growth of a wide range of bacteria according to Waschkies (1992)

To be autoclaved at 121 °C for 20 min:	
Standard-II Nutrient-Agar (Merck, No. 107883)	25.0 g
Deionized water	1000.0 ml
pH 7.5 after autoclaving (25 °C)	
To be dissolved in 10 ml of deionized water, and added separately by sterile filtration (< 0.45 µm) to the medium cooled to 40 °C after autoclaving:	
Cycloheximide (Sigma, No. C-6255)	200 mg

(3) Two different agar media were applicable to quantify and differentiate manganese reducing and oxidizing microorganisms.

For the determination of manganese-reducing microorganisms a modified Bromfield's medium (Bromfield, 1956) was chosen (Table 8.4; Huber and Graham, 1992; Marschner, 2003). In contrast to Bromfield (1956), no manganese sulfate ($MnSO_4$) was added to the basal medium. Rather, after autoclaving, potassium permanganate ($KMnO_4$) was admixed under stirring to form manganese oxides, mainly manganese dioxide (MnO_2), which give a brownish color to the medium (Marschner et al., 1991). As in the nutrient-poor Bromfield's medium the reduction of $KMnO_4$ to MnO_2 is a relatively slow process, the medium was kept under repeated shaking at 50 °C for about two hours before pouring into Petri dishes (Marschner et al., 1982; cited in: Römheld, 1986). Manganese-reducing microorganisms are detectable on this medium by the formation of a bright halo around their colonies where the brownish precipitates of MnO_2 have been cleared by reduction to soluble Mn^{2+}-ions (Marschner et al., 1991).

Table 8.4: Medium with manganese oxide for the determination of manganese-reducing micro-organisms according to a modified Bromfield's medium (Bromfield, 1956) described by Huber and Graham (1992)

Basal components of Bromfield's medium, to be autoclaved at 121 °C for 20 min:	
KH_2PO_4, p.a. (Fluka, No. 60220)	50 mg
$MgSO_4 \cdot 7H_2O$, puriss., p.a. (Fluka, No. 63140)	20 mg
$(NH_4)_2SO_4$, p.a. (Fluka, No. 09980)	100 mg
$Ca(H_2PO_4)_2 \cdot H_2O$ (Fluka, No. 21053)	80 mg
Yeast extract (BD, No. 212720)	50 mg
Agar (Difco, No. 0140-01)	20 g
Deionized water	995 ml
pH adjustment from initial pH 6.0 to pH 7.0 with 0.5 M NaOH	
To be dissolved in 5.0 ml of deionized water and autoclaved separately, then added under stirring to the medium cooled to 50 °C after autoclaving:	
$KMnO_4$ (Merck, No. 5080)	553 mg

The determination of manganese oxidizing microorganisms was conducted with a nutrient-rich medium according to Ridge and Rovira (1971) and its modifications proposed by Huber and Graham (1992) and Posta et al. (1994) given in Table 8.5. Manganese in its reduced form (Mn^{2+}) was admixed to the medium as manganese sulfate ($MnSO_4$) after separate autoclaving and cooling to 50 °C. On this medium, manganese-oxidizing microorganisms are becoming detectable by the formation of dark precipitates of MnO_2 underneath and around their colonies or fungal hyphae (Marschner et al., 1991; Huber and Graham, 1992; Marschner et al., 2003).

As reported by Huber and Graham (1992), the basal medium from Bromfield (1956) and that from Ridge and Rovira (1971) could be used both for the detection of manganese reducers as well as oxidizers when supplied with MnO_2 or Mn^{2+}, respectively. However, own preliminary tests showed that when the basal medium from Ridge and Rovira (1971) was charged with $KMnO_4$ after autoclaving, the solution at first turned brown rapidly within few minutes, but later after plating and cooling, the solid medium turned clear

again. This observation indicated that the primarily formed precipitate of MnO_2 was further reduced to soluble Mn^{2+} by the strong reductive capacity of the nutrient-rich medium (Son et al., 1990). The nutrient-poor Bromfield's medium, on the contrary, was not used to determine manganese oxidizing microorganisms, because it has been suspected that conditions of carbon starvation may facilitate the oxidation of manganese as an alternative source of energy for the autotrophic growth of the oxidizing organisms (Bromfield, 1956). The conditions in the rhizosphere, however, are ruled by the supply of easily available carbon sources from the root (Marschner and Timonen, 2006) and manganese oxidation in soils has been mainly attributed to actively proliferating microorganisms (Mann and Quastel, 1946).

Table 8.5: Medium with reduced manganese (Mn^{2+}) for the determination of manganese oxidizing microorganisms based on a nutrient-rich medium of Ridge and Rovira (1971) as described by Huber and Graham (1992) and Posta et al. (1994)

Basal components of the medium to be autoclaved at 121 °C for 20 min:	
KH_2PO_4, p.a. (Fluka, No. 60220)	1.0 g
$MgSO_4 \cdot 7H_2O$, puriss., p.a. (Fluka, No. 63140)	1.0 g
$FeCl_2 \cdot 4H_2O$, p.a. (Fluka, No. 44939), see Posta et al. (1994)	123 mg
$Na_2MoO_4 \cdot 2H_2O$, p.a. (Fluka, No. 71756)	10 mg
$ZnSO_4 \cdot 7H_2O$, p.a. (Merck, No. 1.08883.0100)	2 mg
$CuSO_4 \cdot 5H_2O$, p.a. (Merck, No. 2790.0100209)	1 mg
Glucose monohydrate (Fluka, No. 49159)	2.75 g
Peptone (BD, No. 211677)	5.0 g
Yeast extract (BD, No. 212720)	1.0 g
Agar (Difco, No. 0140-01)	10.0 g
Deionized water	800 ml
To be dissolved in 200 ml of deionized water and autoclaved separately, then added under stirring to the medium cooled to 50 °C after autoclaving:	
$MnSO_4 \cdot H_2O$ (Merck, No. 5963)	203 mg

The thoroughly prepared media were dispensed into Petri dishes to achieve an approximate layer thickness of 3 to 4 mm (respectively 45 ml per dish) and allowed to cool to room temperature. After plating serial dilutions of rhizosphere suspensions on the different agar media under sterile conditions, the Petri dishes were closed with sealing films (Parafilm®, Brand, Wertheim, Germany) and incubated in the dark at about 24 °C.

At 2 days after plating, the numbers of colony forming units (cfu) of fluorescent pseudomonas were counted under ultraviolet (UV) light (optimal wavelength = 365 nm; Cody and Gross, 1987) for detection of fluorescence. The total counts of colonies visible without additional equipment on the broad spectrum medium according to Waschkies (1992) were determined at 3 days after planting, whereas units appearing on the respective media as manganese oxidizing or manganese-reducing microorganisms were counted at 18 and 31 days after plating.

In order to calculate the numbers of microbial counts per weight unit of root and adhering substrate material after oven-drying at 65 °C, the root pieces were re-collected from the original Erlenmeyer flasks and washed. The substrate material was retained from the washing water and from the original suspension by filtration through pre-weighed filter papers (No. 589[1], black ribbon, Schleicher & Schüll, Dassel, Germany), with allowance being made for the amount of material that had been removed for the preparation of dilution series.

Incubation test to estimate the manganese-reducing capacity in the rhizosphere

Samples of roots with surrounding substrate material contained in 50 ml centrifuge tubes were incubated with manganese dioxide (MnO_2) as substrate in order to estimate the capacity for manganese reduction in the rhizosphere. As suggested by Petra Marschner (personal communication), a stock suspension of 27 mg MnO_2 (Merck, No. 5957) together with 2 g yeast extract (BD, No. 212720) in 1000 ml deionized water was prepared and autoclaved at 121 °C for 20 minutes. After cooling to room temperature, each 40 ml of this suspension were distributed per sample in centrifuge tubes. To avoid settling down of the insoluble MnO_2 particles, the stock suspension was kept under continuous stirring during this procedure. In addition, centrifuge tubes containing soil

material that had been left unplanted after collection from different field plots were supplied with manganese suspension at the same rate, as well as blanks with only manganese suspension were prepared. During incubation for seven days at room temperature, the centrifuge tubes were fixed in horizontal position on a rotary shaker (SM 25, Bühler, Hechingen, Germany) and shaken continuously with moderate speed (180 rounds min^{-1}). The lids of the centrifuge tubes were opened at morning, afternoon, and late evening to provide ventilation.

When the incubation time ended, sample tubes were centrifuged at 4500 rounds min^{-1}, the supernatants were discarded, and the deposited material was re-suspended for extraction. The extractant solution according to Lindsay and Norvell (1978), which was supplied at rates of 25 ml per sample tube, contained 5 mM diethylene-triamine-pentaacetic acid (DTPA), 10 mM calcium chloride ($CaCl_2$), and 100 mM triethanolamine (TEA). To prepare one liter of this solution, 1.97 g DTPA (Fluka, No. 32319) and 14.92 g TEA (Fluka, No. 90279) were dissolved in 500 ml deionized water. Separately from this, 1.47 g $CaCl_2 \cdot 2\ H_2O$ (Merck, No. 2382.1000) were dissolved in 400 ml deionized water. After sufficient time for DTPA to dissolve, both solutions were mixed together, the pH was adjusted to 7.30 ± 0.05 with 3.8 M HCl, and finally the total volume was filled up to 1000 ml with deionized water. For extraction, the samples were again shaken for two hours at 180 rounds min^{-1} and subsequently filtered through pre-weighed filter papers (No. 589[1], black ribbon, Schleicher & Schüll, Dassel, Germany). The concentration of manganese in the clear filtrates was analyzed after fifty-fold dilution by means of atomic absorption spectroscopy (ATI Unicam Solaar 939, Thermo Electron, Waltham, USA) and appropriate standards for calibration (Fluka, No. 63534). In contrast to the method described in Chapter 6.2.3, lanthanum and caesium were not added to the samples, as preliminary tests had shown that such treatment did not considerably affect the results of the spectrometric measurements in this case, probably due to low concentrations of interfering elements (Schinkel, 1984). Re-collected root, substrate and soil materials were oven dried (65 °C) to determine dry weights as basis for calculating concentrations of DTPA-extractable manganese.

Assessment of root morphological characteristics

For the measurement of root diameters and root hair lengths as well as for the determination of specific root length to weight ratios, root samples, which had been stored in deionized water, were evenly spread on a transparent plastic tray (inner size: 10 cm in length, 10 cm in width, 0.5 cm in depth). A grid pattern of each 10 horizontal and vertical lines, each of 10 cm in length, was marked on the bottom of the tray to form squares of 1 cm side length, which is of importance for the assessment of root lengths. The tray was placed under a binocular microscope equipped with digital camera and image analysis software (AxioVision, Carl Zeiss AG, Jena, Germany) to examine the root samples at about 20 to 50-fold magnification. From each four representative root segments per sample, the root diameter and the average of four measurements of the length of individual root hairs were recorded, and sample means were calculated to be used as replicate values for statistical analyses. The specific root length to weight ratio is an indirect measure of root fineness, which reflects the carbon costs per unit surface area of roots (Løes and Gahoonia, 2004). This index of root production economy is calculated from the total length of a root system or fragment divided by its dry weight (Fitter, 1976; Ostonen et al., 2007). The total length of individual root samples was estimated according to the gridline intersection method described by Tennant (1975). By systematically following every horizontal and vertical line of the grid pattern, each point where the roots intersected a line was counted. The complete number of intercepts counted per sample was converted to an estimate of the total length of the root sample with the formula:

$$Root\ length = Complete\ number\ of\ intercepts \cdot Length\ conversion\ factor.$$

The correct value of the length conversion factor depends on the mesh spacing of the grid lines and is 0.7857 for 1 cm grid squares. After root morphological investigations were completed, the samples were oven-dried at 65 °C to determine dry weights.

Rating the intensity of root colonization by arbuscular mycorrhizal fungi

To observe fungal structures of the intra- and extra-radical mycelium of arbuscular mycorrhizal fungi (Smith and Read, 1997), the root samples, which had been stored in 20 % ethanol before, were subjected to a staining procedure according to Koske and Gemma

(1989) with modifications adapted to the specific sensitivity of wheat roots (Waschkies, 1992; Neumann, 2007). During this process, cylindrical plastic vessels (27 mm in diameter, 40 mm in height) with a needle-perforated bottom were used as sample containers to facilitate the application of different liquids. For tissue clearing, root samples were placed in 10 % potassium hydroxide (KOH) and heated in a laboratory oven at 60 °C for 25 minutes. After treatment with KOH, the roots were intensively rinsed with tap water and subsequently acidified by soaking for two minutes in 2 M hydrochloric acid (HCl). For staining of fungal structures, the acidified root samples were immersed in a 0.05 % solution of trypan blue (Merck, No. 1117320025) in lactic acid and heated in a laboratory oven at 80 °C for 15 minutes. Subsequently, the samples were removed from the staining solution and again carefully rinsed in several changes of water to remove excessive stain before storage in lactic acid. Trypan blue, however, is known as a toxic compound and wastes containing trypan blue have to be collected for the adequate disposal as a dangerous material (Merck, 2012b; Akkaya et al., 2009). To avoid the use of trypan blue and other hazardous compounds, an advanced staining technique for arbuscular-mycorrhizal fungi, using 5 % ink in vinegar as staining solution, has been proposed by Vierheilig et al. (1998). As confirmed by further investigations with diverse plant species (e.g. Olatoye et al., 2012), comparable or even improved staining results can be obtained by use of certain blue inks (e.g. royal blue, Pelikan, Hannover, Germany), rendering mycorrhizal structures clearly visible.

Throughout the literature as well as in the present work, the terms “infection” and “colonization” are often used interchangeably to describe the process of invasion and successful establishment of mycorrhizal fungi in roots. Nevertheless, those authors who emphasize the mutual recognition of the mycorrhizal symbiosis might prefer “colonization” due to its less pathological connotation (St. John, 2000). For estimating the proportional root length infected (colonized) by AM-fungi, a modified gridline intersection method was used (Tennant, 1975; Giovannetti and Mosse, 1980). Therefore, the stained root samples were evenly spread on a transparent plastic tray with grid pattern and observed under the binocular microscope at about 40-fold magnification, as described above for the assessment of root morphological characteristics. By following the horizontal and vertical gridlines, at each point where the roots intersected a grid line the

presence or absence of mycorrhizal structures such as intra- and extra-radical hyphae, and arbuscules was recorded. On average about 400 intersections were scanned each in horizontal and vertical direction per sample. In addition, also the presence of vesicles or spores at the sites of intersections was recorded by use of separate counters, as these fungal structures are indicative of the developmental stage of the symbiosis (Bundrett et al., 1985; Klironomos et al., 1996). To distinguish mycorrhizal fungi from the take-all fungus or other non-mycorrhizal fungi, specific morphological characters of the different fungal groups were carefully observed. While arbuscular mycorrhizal hyphae are typically non-septate when young and ramified within the root cortex (Kormanik and McGraw, 1982; Bundrett et al., 1996; Chapter 2.3.4), the hyphae of non-mycorrhizal fungi frequently have septa at regular spacing along their hyphal strands (Walker, 1981; Kormanik and McGraw, 1982; Klironomos et al., 1996; Maron et al. 2010). *Gaeumannomyces graminis* var. *tritici*, furthermore, forms dark pigmented runner hyphae on the surface of roots. Hyaline branches from these runner hyphae pass through the root cortex and the endodermis into the stele with vascular tissues (Walker, 1981; Cook, 2003). Infected host tissues are extensively destructed by this pathogenic fungus (Skou, 1981; Freeman and Ward, 2004, Chapter 7.4). Although typically formed by mycorrhizal fungi of the order *Glomales* (Morton and Benny, 1990), arbuscules and vesicles should be interpreted carefully, because these structures can be absent or difficult to detect in mycorrhizal root tissues as well as similar structures can be formed by non-mycorrhizal fungi (Kormanik and McGraw, 1982; McGonigle et al., 1990).

After complete evaluation of all intersections between gridlines and roots, the percentage of mycorrhizal infected root length (PMIRL) in relation to the total length of the root sample can be calculated on basis of the formula from Tennant (1975), which by algebraic simplification leads to the equation:

$$PMIRL\ [\%] = \frac{Number\ of\ intercepts\ with\ mycorrhizal\ structures}{Complete\ number\ of\ intercepts} \cdot 100.$$

With regard to this calculation method, Giovannetti and Mosse (1980) argued that the mesh spacing of the grid lines would be important only for estimating the total length of infected or uninfected roots, but not for the percentage of infected roots. In this case, the

gridlines would only serve as a device for the systematic localization of observation points. Trouvelot et al. (1986; cited in: Bouamri et al., 2006) furthermore distinguished between the "colonization intensity" and the "colonization frequency", which they defined as the "proportion of cortical cells colonized by the fungi" and as the "ratio of colonized versus non-colonized root fragments", respectively. Other authors proposed advanced but intricate methods for the rating of mycorrhizal root infections, such as by using a huge (200-fold) magnification for root observation (McGonigle et al., 1990). However, root pieces of defined length (e.g. 1 to 3 cm) are likely to cross several lines when they are distributed on a grid pattern forming squares of similar or shorter side length (e.g. 1 cm). Under such conditions, a mycorrhizal infection starting from a given point of entry at the root surface will be the more likely to be detected as mycorrhizal structure at a point of intersection (on a grid of given mesh size), the larger the extension of the fungal hyphae within the colonized root tissue at the time of sampling is. Furthermore, decreasing the mesh size of the grid pattern will not affect the total length of the root sample, but it will improve the resolution power of the analysis and thus increase the probability of detecting also sporadic infections of low extension. Therefore, it is reasonable to consider the results obtained with the gridline intersection method as indicative of the intensity and not only of the frequency of mycorrhizal root infections. This is in accordance with Scagel et al. (2005), who defined the "colonization intensity" as the percentage of root length with mycorrhizal fungi.

Analyses of mineral nutrients in root and shoot tissues

The analysis of mineral nutrients in root and shoot tissues was done according to the assay from Gericke and Kurmis (1952b). For this purpose, the portion of root samples that had been oven dried immediately after experimental harvest and the total shoot dry matter per replicate pot were ground in agate disc swing mills (Fritsch, Idar-Oberstein, Germany). Aliquots of about 250 mg root or 500 mg shoot material were weighed into crucibles to be mineralized by the ashing procedure described in Chapter 5.2.3. Subsequently, the readily digested and appropriately diluted sample solutions were analyzed for magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu) concentrations by atomic absorption spectroscopy (ATI Unicam Solaar 939, Thermo Electron, Waltham, USA), as

described in Chapter 6.2.3. Before measuring iron and manganese concentrations a buffer solution containing cesium chloride and lanthanum chloride (Merck, No. 116755) was added to the samples to eliminate interferences in the air-acetylene flame of the atomic absorption spectrometer (Schinkel, 1984). The colorimetric assay according to Gericke and Kurmies (1952b) was chosen to determine phosphorus (P) concentrations by spectral-photometric measurements (U-3300, Hitachi, Tokyo, Japan) as specified in Chapter 5.2.3. Potassium (K) and calcium (Ca) concentrations were assessed by flame photometry (ELEX 6361, Eppendorf, Hamburg, Germany). Obtained values of mineral nutrient concentrations in shoot samples were compared with standard values for wheat plants reported by Bergmann and Neubert (1976) and Bergmann (1992).

Statistical analyses

Results are presented as means with associated standard errors. Statistical analyses were done with the computer software programs SigmaStat Version 2.03 (Systat Software Inc., Erkrath, Germany) and SAS® 9.4 (SAS Institute GmbH, Heidelberg, Germany). When necessary to meet the requirements of equal variance and normal distribution, the respective data sets were subjected to mathematical transformations, as described in the footnotes of figures and tables. Three-way analyses of variance (ANOVAs) were performed to test for differences between samples grouped according to the varied levels of the experimental factors precrop, supplemental treatment, and block. If the three-way ANOVAs did not show any significant effects of the block factor, in addition two-way ANOVAs were used to test for differences among the sample groups in response to the different levels of precrop and supplemental treatments as well as for interactions between these two factors. In case of significant block effects, data were further tested in two-way ANOVAs to account for the influence of block factors and to analyze the effect of supplemental treatments separately for each precrop group. ANOVAs that indicated statistically significant differences between sample groups were followed by Tukey’s test to isolate these differences by multiple pair wise comparison of individual group means, as mentioned in figure and table footnotes. The threshold level of significance for both ANOVA and post hoc tests was $p \leq 0.05$. Pearson's product-moment correlation coefficient (Pearson's r) was calculated as a measure of the strength of the association

between two variables (e.g. root and shoot biomass production of plants). Positive correlation coefficients indicate that pairs of variables increase together, whereas negative values indicate an inverse relationship. For pairs with p values below 0.05 there is a significant relationship between the two variables.

8.3 Results

8.3.1 Development of the wheat plants during the experimental growth phase

Rate of seedling appearance

Wheat seedlings started to appear at about five days after sowing. The rate of seedling emergence was heterogeneous among pots, with a maximum divergence ranging from 13 to 100 %, as observed between the sixth and eighth day after sowing. However, concerning this variable there was no significant difference between the wheat- or oat-precropped substrate as well as there was no significant effect of the different supplemental treatments in pair wise comparisons with the respective untreated control group (Tukey's test, $p > 0.05$; data not shown). From nine days after sowing, the discrepancy in seedling emergence between pots started to decrease due to the late appearance of laggard seedlings. At twelve days after sowing, an average proportion of emerged seedlings over all pots of 63 % was achieved.

Leaf length and total number of leaves per plant

By recording the length of leaves of distinct stages depending on the developmental progress of the wheat plants, two phases of growth promotion in response to the different precrop and supplemental treatments could be observed.

At 16 and 20 days after sowing the application of Proradix® induced a significantly (Tukey's test, $p \leq 0.05$) enhanced length growth in the second leaf of the wheat plants grown on the oat-precropped substrate (data not shown). When the same leafs were again measured at 23 days after sowing, this effect of Proradix® was also significant on the wheat-precropped substrate (Fig. 8.4). Congruent growth responses were still detected when the length of the third and fourth leaf was measured at 28 days after sowing (data

not shown). During this initial growth phase, however, the analyses of variance did not indicate any significant effect of the precrop factor on the variability of leaf length.

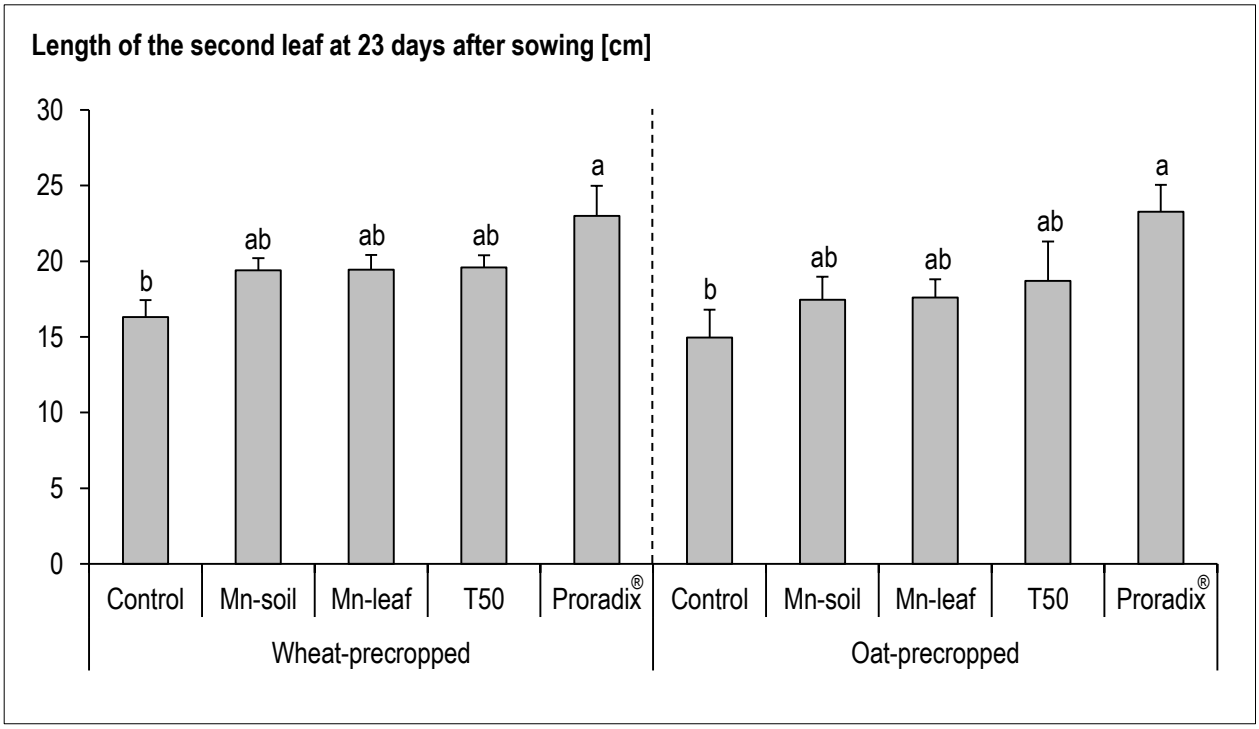

Fig. 8.4: Length of the second leaf of wheat (var. Paragon) plants at 23 days after sowing in the pot experiment with wheat- or oat-precropped substrate, treated or not (Control) with different supplemental treatments (Mn-soil, Mn-leaf, Vitalin T50, or Proradix®)

Columns represent mean values ± SEM (n = 4). Data were tested in a two-way ANOVA followed by Tukey's test comparing precrop and supplemental treatments, after a preceding three-way ANOVA did not indicate any significant effect of the block factor. Significant differences between supplemental treatments within the same precrop group are indicated by values not sharing common letters ($p \leq 0.05$). There was no significant precrop effect and no significant interaction between precrop and supplemental treatments.

During later stages of shoot development, however, the growth stimulatory effect of Proradix® concerning leaf elongation was not persistent. Rather at 37 days after sowing, length measurements in the fifth leaf indicated a growth promoting effect of Vitalin T50 and Mn-soil applications in wheat plants grown on the wheat-precropped substrate, whereas on the oat-precropped substrate Mn-soil and Mn-leaf applications induced such response (Tukey's test, $p \leq 0.05$; Fig. 8.5). Furthermore, at this developmental stage a slight but general decrease in length of the fifth leaf was recorded for plants grown on the oat-precropped compared to wheat-precropped substrate (Tukey's test, $p \leq 0.05$). Yet, this

main effect of the precrop factor was not significant at the level of pair wise comparison within sample groups of different supplemental treatments (Fig. 8.5).

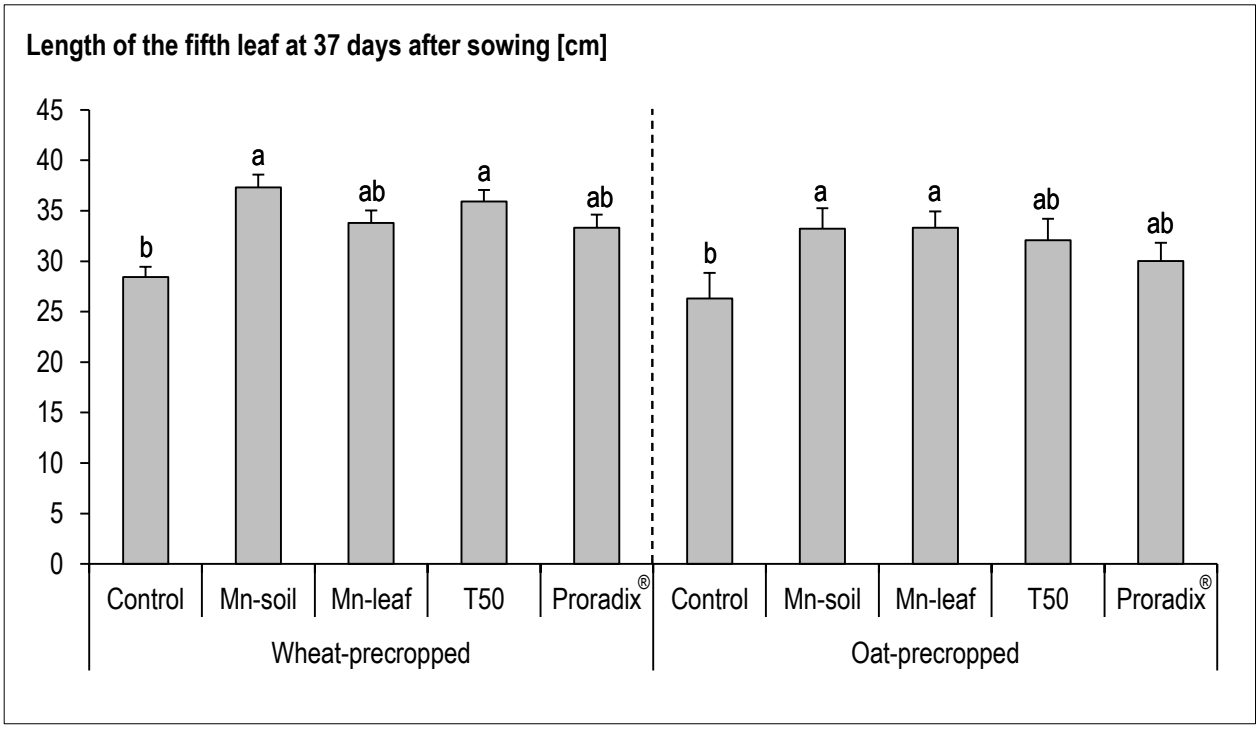

Fig. 8.5: Length of the fifth leaf of wheat (var. Paragon) plants at 37 days after sowing in the pot experiment with wheat- or oat-precropped substrate, treated or not (Control) with different supplemental treatments (Mn-soil, Mn-leaf, Vitalin T50, or Proradix®).

Columns represent mean values ± SEM (n = 4). Data were tested in a two-way ANOVA followed by Tukey's test comparing precrop and supplemental treatments, after a preceding three-way ANOVA did not indicate any significant effect of the block factor. Significant differences between supplemental treatments within the same precrop group are indicated by values not sharing common letters ($p \leq 0.05$). Tukey's test furthermore indicated a significantly ($p \leq 0.05$) higher group mean for wheat-precropped in comparison to oat-precropped samples. There were, however, no significant differences between precrop treatments when compared within groups of the same supplemental treatment, and there was no significant interaction between precrop and supplemental treatments.

During the last two assessments of leaf length before harvest, at 40 and 44 days after sowing, the developmental progress of plants had become too heterogeneous as to perform comparative measurements in leaves of a distinct developmental stage. Although the statistical analyses regarding the total number of leaves that had been formed per plant at these dates did not indicate any significant differences between precrop and supplemental treatments (Tukey's test, $p > 0.05$; data not shown), some plants had already developed seven leaves, while others were still in the five-leaf stage. Thus, besides counting the number of leaves per plant, the length of the longest leaf in each plant,

independent of its developmental ranking order, was henceforth determined. While there was no clear difference in this variable between precrop and supplemental treatments at 40 days after sowing, the data recorded at 44 day after sowing showed a significant increase in length of the longest leaf for the Mn-soil, Vitalin T50 and Proradix® treated sample groups in pair wise comparisons with the untreated control group (Tukey's test, $p \leq 0.05$). These effects of the supplemental treatments, however, were not significant when differences of means were tested within the same precrop group, although there was no significant influence of the precrop factor (Fig. 8.6).

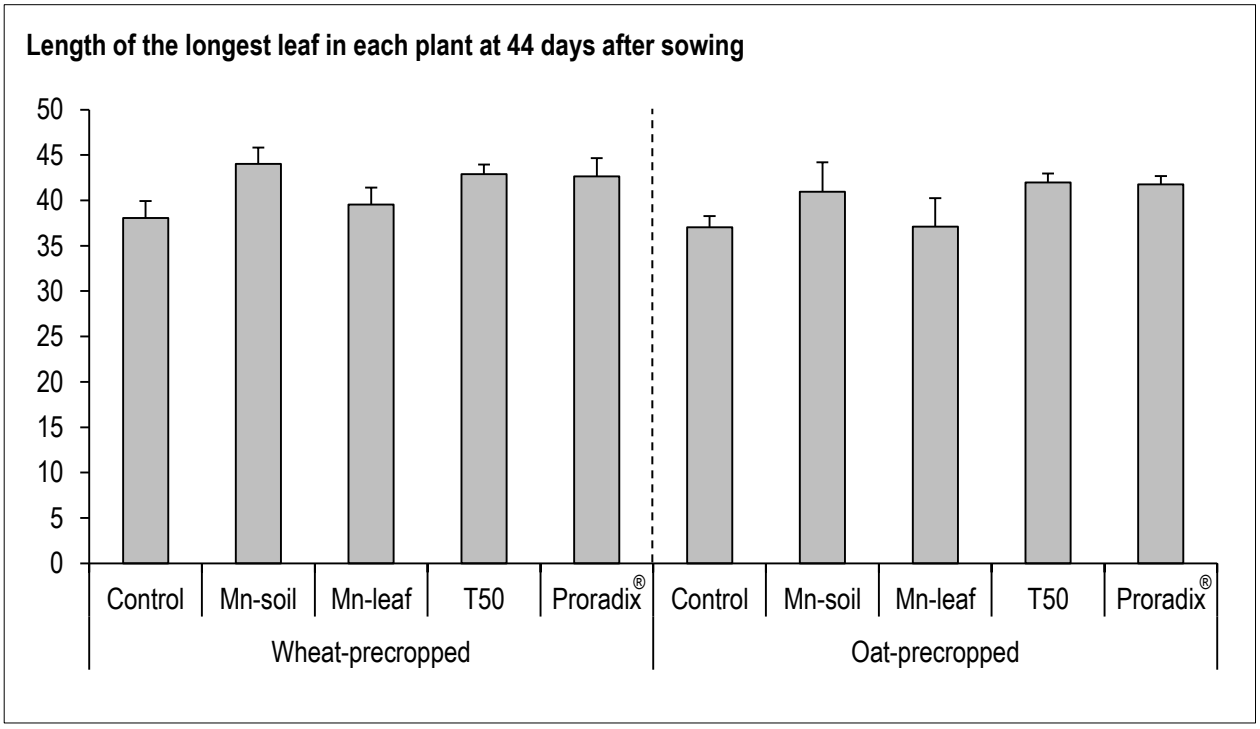

Fig. 8.6: Length of the longest leaf in each wheat (var. Paragon) plant at 44 days after sowing in the pot experiment with wheat- or oat-precropped substrate, treated or not (Control) with different supplemental treatments (Mn-soil, Mn-leaf, Vitalin T50, or Proradix®).

Columns represent mean values ± SEM (n = 4). Data were tested in three- and two-way ANOVAs followed by Tukey's test for post hoc comparisons of means. The Tukey's test after three-way ANOVA indicated significant increases in the group means of Mn-soil ($p = 0.025$), Vitalin T50 ($p = 0.025$), or Proradix® ($p = 0.035$) treated samples in pair wise comparisons with the group of untreated control samples, but no significant effect of the precrop and of the block factor. There were no significant differences when supplemental treatments were compared within the same precrop group and there was no significant interaction between precrop and supplemental treatments, as shown by Tukey's test performed after two-way ANOVAs.

Leaf color measured with a chlorophyll meter

Measurements of leaf color, indicative of the nitrogen status in plants, were first performed at 22 days after sowing when the wheat plants showed symptoms of nitrogen deficiency, such as light green leaf color (Snowball and Robson, 1991; Sharma and Kumar, 2011). At that time, the plants had achieved the two- to three-leaf stage and color values were measured in the middle of the second leaf of each plant, representing the largest and youngest completely unrolled leaf of this developmental stage. As represented by the light bars in Fig. 8.7, the values of leaf color obtained at 22 days after sowing were generally on a relatively low level ranging between about 20 and 30 SPAD units, which suggests that nitrogen concentrations were insufficient within the plants (Bergmann and Neubert, 1976; Matsunaka et al., 1997). Nevertheless, the color values of leaves were slightly enhanced in response to Proradix® ($p \leq 0.01$) and Mn-soil ($p \leq 0.05$) treatments, whereas the lowest group means were found for untreated control plants (Tukey's test after three-way ANOVA). The detailed statistical analyses furthermore showed that these treatment effects were significant in plants grown on the oat-precropped soil (Tukey's test after two-way ANOVA, $p \leq 0.05$), but not for respective comparisons within the wheat-precropped group, although there was no significance of the precrop factor.

When leaf color values were again recorded at 30 days after sowing, the wheat plants, which then were in the third- to fourth leaf stage, had recovered in response to the nitrate fertilizer that had been applied subsequently to the first measurement and showed no more symptoms of nitrogen starvation. The values of leaf color, as represented by the dark bars in Fig. 8.7, were now ranging between about 35 and 50 SPAD units, thus being indicative of an adequate but moderately low nitrogen availability to the plant (Bergmann and Neubert, 1976; Matsunaka et al., 1997). The lowest means, however, were still found within the untreated controls, whereas the general comparison over all sample groups indicated a significant increases in color values in response to the application of Mn-soil ($p \leq 0.01$), Vitalin T50 ($p \leq 0.05$), and Mn-leaf ($p \leq 0.05$) treatments (Tukey's test after three-way ANOVA). Furthermore, the comparison of samples grouped according to different levels of the precrop factor showed that these effects of Mn-soil and Vitalin T50 applications were significant within the oat- but not within the wheat-precropped group

(Tukey's test after two-way ANOVA, $p \leq 0.05$). In contrast to this, the effect of Mn-leaf treatments was not significant at the level of distinct precrop groups, although there were again no significant interferences of the precrop factor detected.

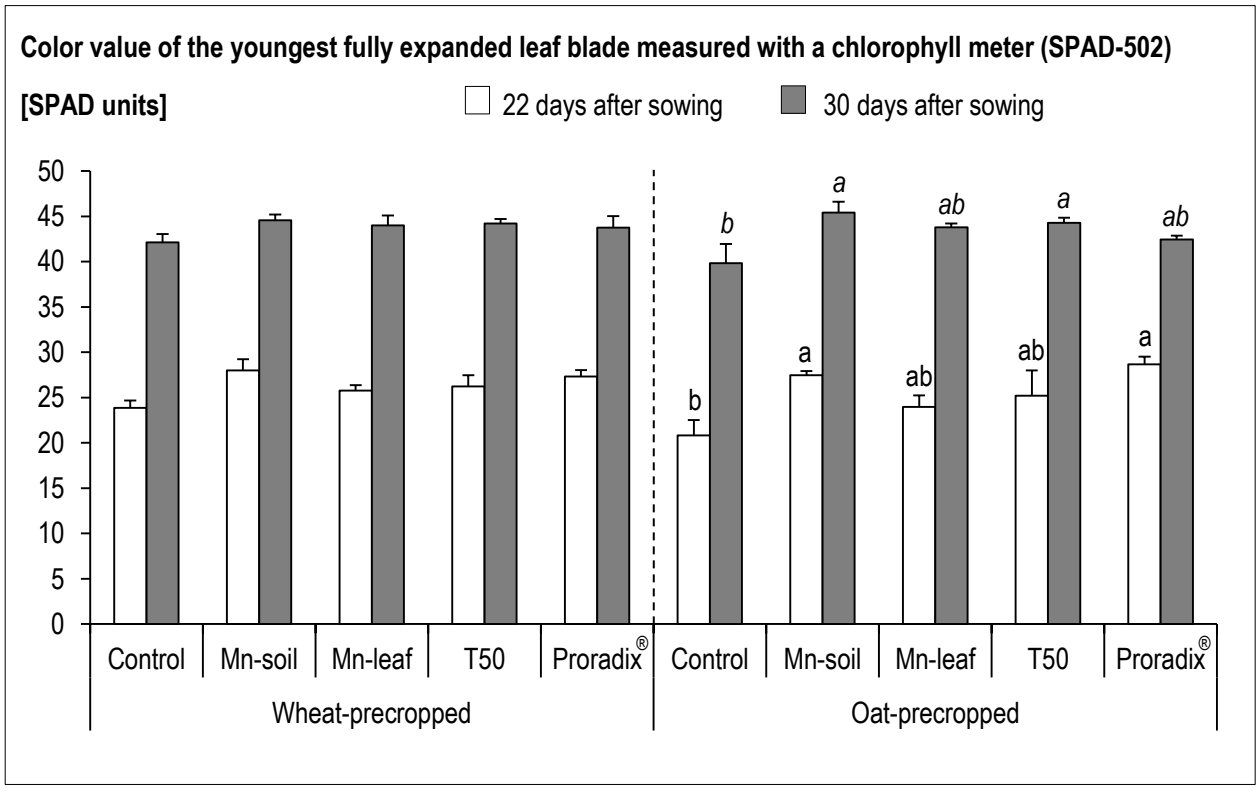

Fig. 8.7: Color value of the youngest fully expanded leaf blade of wheat (var. Paragon) measured in "SPAD units" with a chlorophyll meter (Minolta SPAD-502) at 22 and 30 days after sowing in the pot experiment. The plants were grown on wheat- or oat-precropped substrate and subjected to different supplemental treatments (Mn-soil, Mn-leaf, Vitalin T50, or Proradix®) or not (Control).

Columns represent mean values ± SEM (n = 4). Statistical analyses were performed separately for each sampling time of 22 or 30 days after sowing. Data sets were tested in two-way ANOVAs followed by Tukey's test comparing precrop and supplemental treatments, after preceding three-way ANOVAs did not indicate any significant effect of the block factor. If necessary, data were square transformed to achieve normal distribution. For both sampling times, there was no significant effect of the precrop factor found. Significant differences between supplemental treatments within the same precrop group are indicated by values not sharing common letters ($p \leq 0.05$), which are printed in non-cursive or cursive characters for data sets from 22 or 30 days after sowing, respectively. Mean values without or sharing letters are not significantly different. In addition to these statistical results, the data set from 30 days after sowing showed a significant increase in the group mean of Mn-leaf treated samples in comparison to the untreated control group (Tukey's test after three-way ANOVA, $p = 0.041$), but this effect was not significant for respective comparisons within the same precrop group.

At the end of the experimental growth phase, 47 days after sowing, the color values of leaves were still within a range of 35 and 50 SPAD units (data not shown), when

measured in flag leaves as the youngest fully expanded leaves of that time. In contrast to the previous assessments, the general comparison over all sample groups indicated significantly higher color values for Proradix® and Mn-soil in comparison with Mn-leaf treated plants (Tukey's test after three-way ANOVA, $p \leq 0.05$). There were, however, no more significant differences in comparisons with the groups of untreated control plants, as well as no significant influence of the precrop factor found.

Numbers of tillers and emerging inflorescences per plant

The emergence of ears started at about 44 days after sowing, when 5 to 7 leaves had been developed in each plant. At that time, the most noticeable differences in the number of emerging ears per plants were recorded (Fig. 8.8), whereas at 48 days after sowing the values of this variable had become more equal among the different experimental groups.

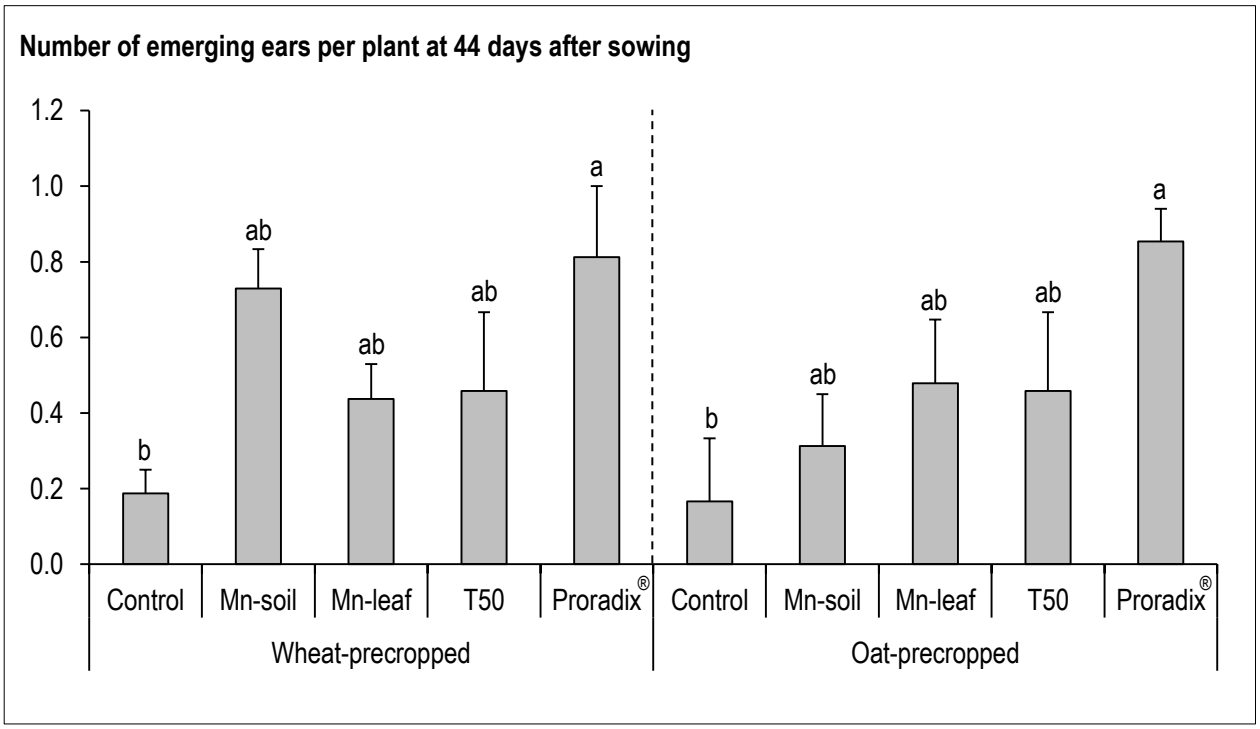

Fig. 8.8: Number of emerging ears per wheat (var. Paragon) plant at 44 days after sowing in the pot experiment with wheat- or oat-precropped substrate, treated or not (Control) with different supplemental treatments (Mn-soil, Mn-leaf, Vitalin T50, or Proradix®).

Columns represent mean values ± SEM (n = 4). Data were tested in a two-way ANOVA followed by Tukey's test comparing precrop and supplemental treatments, after a preceding three-way ANOVA did not indicate any significant effect of the block factor. Significant differences between supplemental treatments within the same precrop group are indicated by values not sharing common letters ($p \leq 0.05$). There was no significant precrop effect and no significant interaction between precrop and supplemental treatments.

As shown in Fig. 8.8, the lowest mean numbers were found in untreated control groups. In multiple comparisons, however, only the enhanced rates of ear emergence in response to the application of Proradix®, but not of other supplemental treatments, were significant against the respective control group, independent whether the plants were grown on the wheat- or oat-precropped substrate (Tukey's test after two-way ANOVA, $p \leq 0.05$). In addition, also the difference in ear emergence between Mn-soil and untreated control plants grown on the wheat-precropped substrate was significant when only these two experimental groups were compared in a separate analysis of variance followed by Tukey's test ($p = 0.023$; not indicated by superscripts in Fig. 8.8). This result, however, must be interpreted carefully due to inconsistent findings on the oat-precropped substrate.

When the experimental growth phase ended at 48 days after sowing, on average over all replicate pots 0.90 emerging ears per plant were recorded while there were no more significant differences between supplemental treatments found. However, the general comparison over all sample groups indicated that the mean number of emerging ears was slightly but significantly higher on the wheat-precropped (group mean = 0.97) compared to the oat-precropped (group mean = 0.84) substrate (Tukey's, test after tree-way ANOVA, $p \leq 0.05$; data not shown; see Fig. 8.8 for representative examples). During experimental harvest, also to the numbers of tillers, which had been formed by the plant in addition to the main shoot, were counted per plant. This evaluation, however, showed that tillers had been rarely formed by less than five percent of the plants (data not shown), which were evenly distributed among the different experimental groups.

8.3.2 Root and shoot biomass production

At the time of experimental harvest, wheat plants showed not only differences in their phenological development, but also in biomass accumulation and allocation to roots and shoots (Fig. 8.9). The numbers of emerging ears counted per plant at 44 days after sowing (Fig. 8.8) were strongly correlated with root ($r = 0.499$; $p = 0.001$) and shoot ($r = 0.698$; $p \leq 0.001$) dry matter production (Pearson's correlation coefficient). Root to shoot ratios, defined on the basis of dry matter production, were significantly higher on the oat- compared to the wheat-precropped substrate, but there was no significant influence of

supplemental treatments on biomass portioning between roots and shoots (Tukey's, test after tree-way ANOVA, $p \leq 0.05$; data not shown).

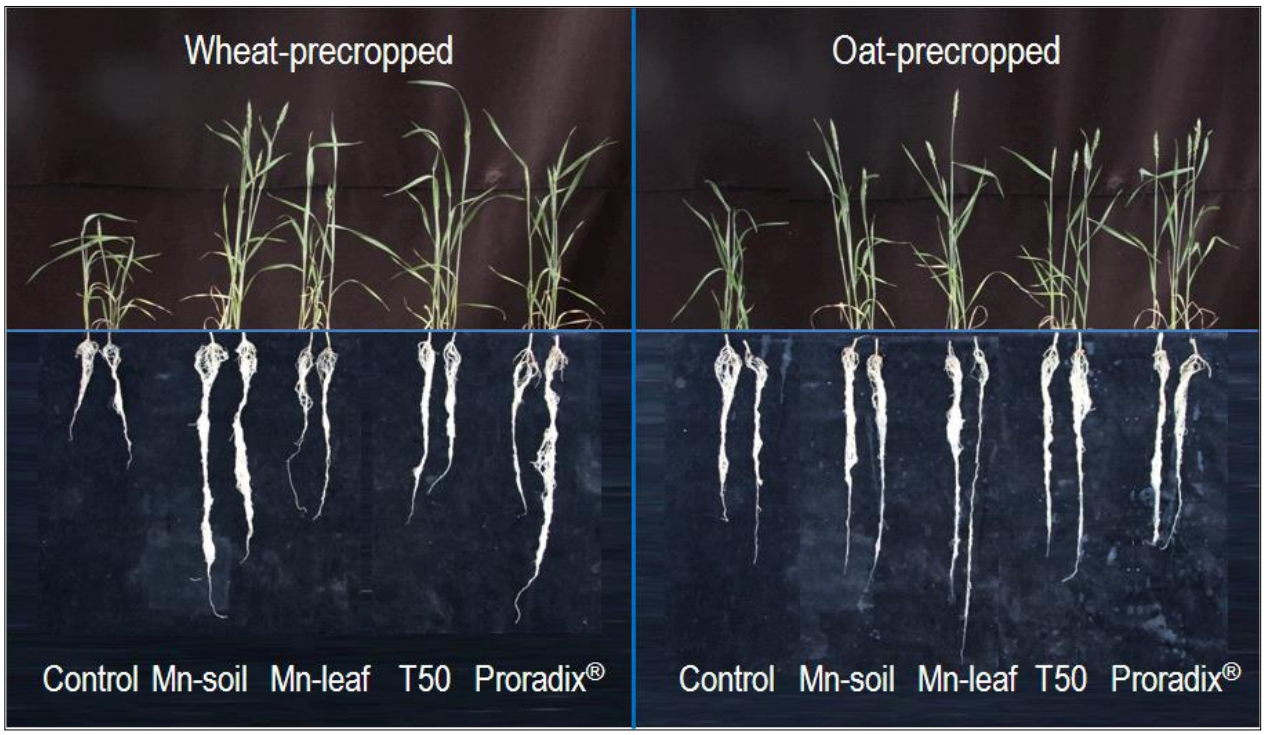

Fig. 8.9: Roots and shoots of representatively selected wheat (var. Paragon) plants grown in the pot experiment with wheat- or oat-precropped substrate, treated or not (Control) with different supplemental treatments (Mn-soil, Mn-leaf, Vitalin T50, or Proradix®). Pictures of shoots were taken before and those of shoots immediately after experimental harvest at 47 and 50 days after sowing, respectively.

Dry matter yields of root and shoot biomass production, as earned during the experimental harvested, are presented in Fig. 8.10 and 8.11. Because about 3 % of the wheat plants had shrunken before the end of the growth phase, while the remaining plants in pots that were affected in such way showed a certain degree of compensatory growth, the data of root and shoot dry weights are reported on a per pot basis. The shrunken plants were, however, evenly distributed among the different experimental groups, so there was no evidence for precrop or supplemental treatment effects on plant survival. In general, the patterns of root biomass (Fig. 8.10) produced in response to the different precrop and supplemental treatments are reflected in respective yields of shoot dry matter (Fig. 8.11; Pearson's correlation coefficient $r = 0.51$, $p \leq 0.001$), although effects in response to

precrop and supplemental treatments were more clearly expressed in root than in shoot samples.

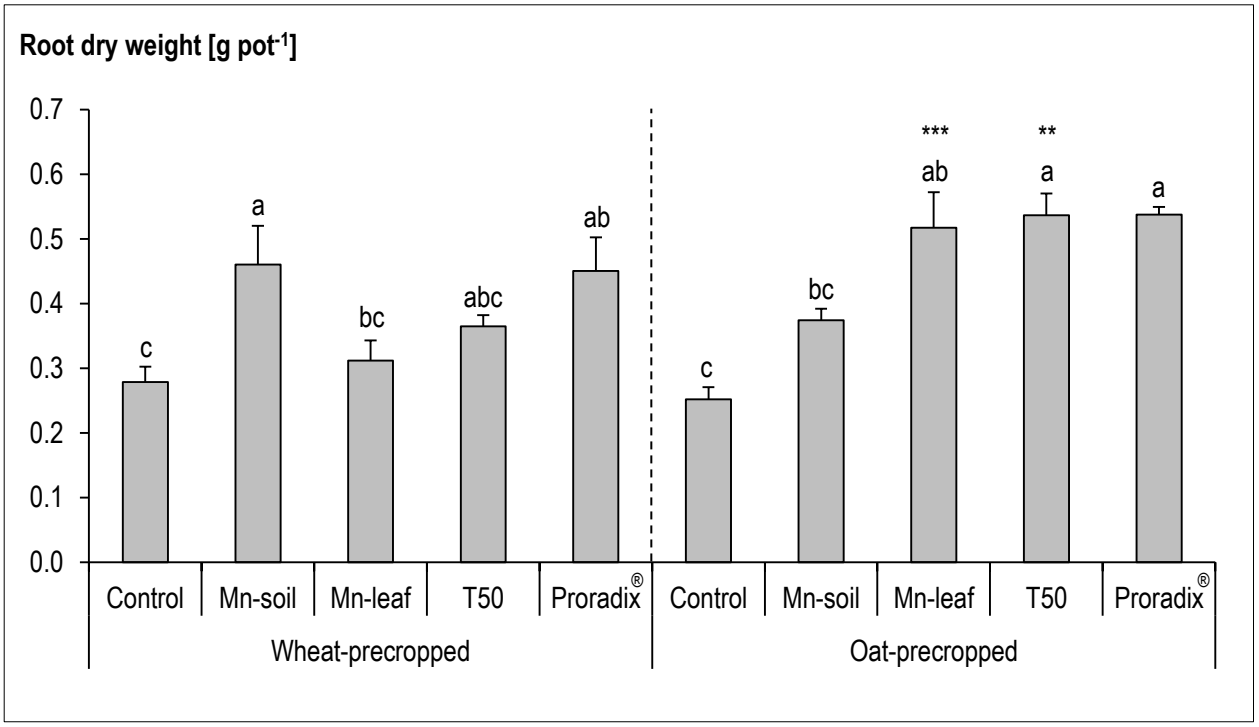

Fig. 8.10: Root dry weight of wheat (var. Paragon) at 48 to 50 days after sowing in the pot experiment with wheat- or oat-precropped substrate, treated or not (Control) with different supplemental treatments (Mn-soil, Mn-leaf, Vitalin T50, or Proradix®).

Columns represent mean values ± SEM (n = 4). Data were tested in a two-way ANOVA followed by Tukey's test comparing precrop and supplemental treatments, after a preceding three-way ANOVA did not indicate any significant effect of the block factor. If necessary, data were square root transformed to achieve equal variance. Significant differences between supplemental treatments within the same precrop group are indicated by values not sharing common letters ($p \leq 0.05$). Asterisks denote significant precrop effects within the same group of supplemental treatment (** $p \leq 0.01$, *** $p \leq 0.001$). Mean values without or sharing superscripts are not significantly different. There was a significant interaction between precrop and supplemental treatments ($p = 0.001$).

As shown in Fig. 8.10, the lowest mean values of root dry weights were found within the groups of untreated control plants on the wheat- as well as on the oat-precropped substrate. While the application of Proradix® significantly enhanced the production of root biomass on both precrop types of growth substrate in comparison to the respective control group, the effects on root growth in response to the application of other supplemental treatments were less consistent. Vitalin T50 and Mn-leaf treatments enhanced root growth significantly on the oat-precropped, but not on the wheat-precropped substrate. This finding was not only evident in comparisons with the untreated control groups, as

indicated by the letters in superscripts. There also were significant differences found when Vitalin T50 or Mn-leaf treated root samples from the oat-precropped group were compared to the respective sample groups of same supplemental treatment from the wheat-precropped substrate, as denoted by asterisks above columns in Fig. 8.10. In contrast, the enhancement in root biomass production in response to the Mn-soil treatment was significant on the wheat- but not on the oat-precropped substrate when compared with the respective control group in the multiple factorial analysis (Tukey's test after two-way ANOVA). However, there were no significant differences in root dry weights according to the different levels of the precrop factor within the groups of untreated control, Mn-soil, or Proradix® treated root samples.

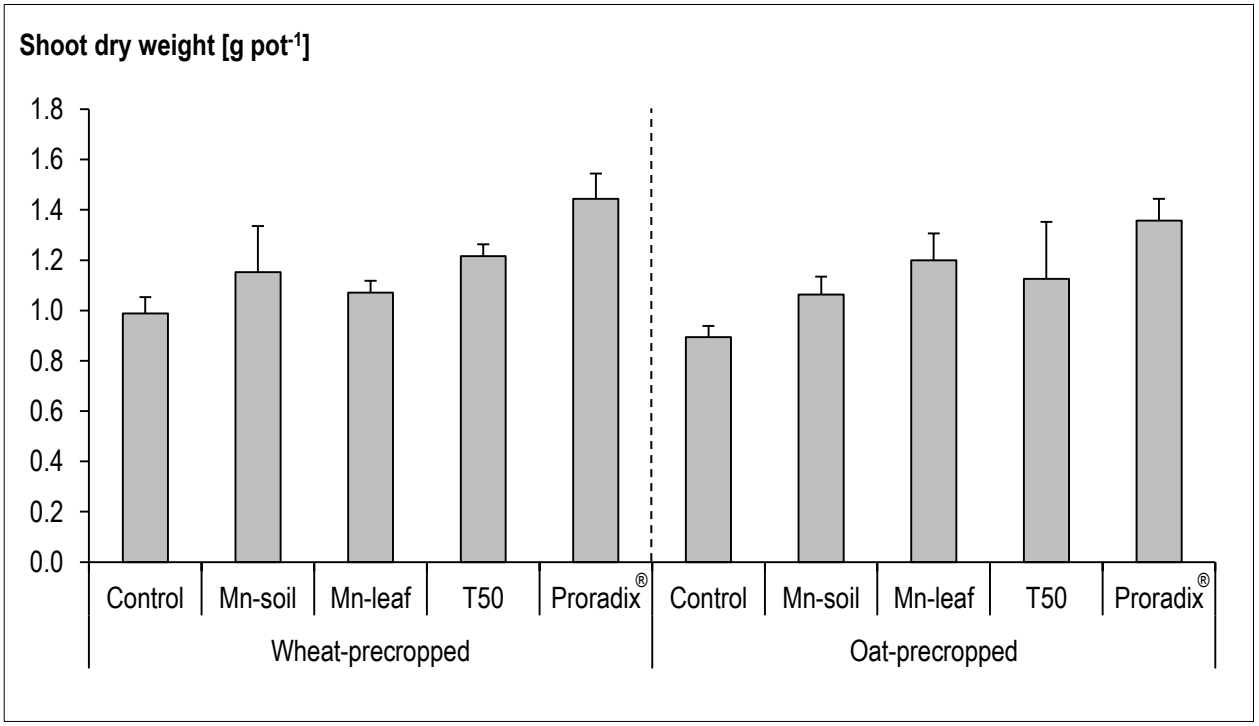

Fig. 8.11: Shoot dry weight of wheat (var. Paragon) at 48 to 50 days after sowing in the pot experiment with wheat- or oat-precropped substrate, treated or not (Control) with different supplemental treatments (Mn-soil, Mn-leaf, Vitalin T50, or Proradix®).

Columns represent mean values ± SEM (n = 4). Data were tested in a two-way ANOVA followed by Tukey's test comparing precrop and supplemental treatments, after a preceding three-way ANOVA did not indicate any significant effect of the block factor. If necessary, data were transformed to their natural logarithm to achieve normal distribution. There were no significant differences between supplemental treatments within the same precrop group and no significant effects of the precrop factor detected. In addition to these statistical results, the general comparison over all sample groups indicated that the group mean of Proradix® treated plants was significantly enhanced compared to the untreated control (Tukey's test after three-way ANOVA, $p = 0.003$), but this effect was not significant for respective comparisons within the same precrop group.

For the data set of shoot dry weights, the general comparison over all sample groups revealed a significant increase in the group means of Proradix® treated samples in comparison to untreated control plants ($p = 0.003$), but no significant differences between precrop treatments (Tukey's test after three-way ANOVA; Fig. 8.11). At the level of distinct precrop groups, however, this effect of Proradix® on shoot biomass production could only be recognized in tendency (Tukey's test after two-way ANOVA, $p \leq 0.1$).

8.3.3 Severity of take-all root rot

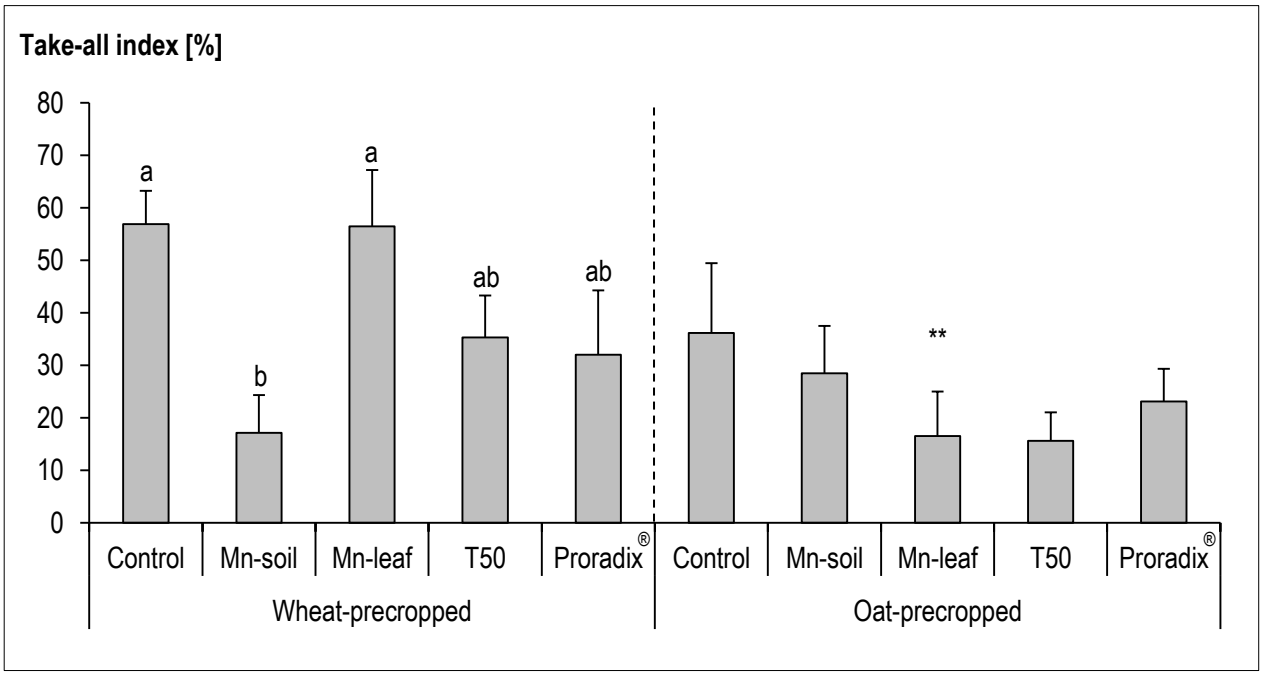

Fig. 8.12: Severity of take-all root rot expressed as visually estimated degree of proportional root damage (take-all index) in wheat (var. Paragon) at 48 to 50 days after sowing in the pot experiment with wheat- or oat-precropped substrate, treated or not (Control) with different supplemental treatments (Mn-soil, Mn-leaf, Vitalin T50, or Proradix®).

Columns represent mean values ± SEM (n = 4). Data were tested in a two-way ANOVA followed by Tukey's test comparing precrop and supplemental treatments, after a preceding three-way ANOVA did not indicate any significant effect of the block factor. Significant differences between supplemental treatments within the same precrop group are indicated by values not sharing common letters ($p \leq 0.05$). Asterisks denote significant precrop effects within the same group of supplemental treatment (** $p \leq 0.01$). Mean values without or sharing superscripts are not significantly different. There was no significant interaction between precrop and supplemental treatments. In addition to these statistical results, the general comparison over all sample groups indicated that the group mean of oat-precropped samples was significantly lower than that of wheat-precropped samples (Tukey's test after three-way ANOVA, $p \leq 0.05$).

Wheat roots were most severely damaged by take-all infestation in plants grown on the wheat-precropped substrate where no supplemental treatments (Control) or the Mn-leaf treatment were applied (Fig. 8.12). According to the general comparison over all sample groups, the group mean of oat-precropped samples was significantly lower than that of wheat-precropped samples (Tukey's test after three-way ANOVA, $p \leq 0.05$). However, when the different precrop levels were compared within the same group of supplemental treatment, this effect was only significant in Mn-leaf treated samples, but not within the untreated control group. Except from the Mn-leaf treatment within the wheat-precropped group, the severity of take-all root rot was generally lower in plants that had been subjected to any supplemental treatment when compared to the respective control group. However, the only significant effect in this regard was found in response to the Mn-soil application on the wheat-precropped soil.

8.3.4 Root-associated microbial populations and their possible impact on manganese reduction and oxidation processes

Characterization of root-associated microorganisms isolated from the rhizosphere

After incubation of microbial rhizosphere samples on different agar media, only samples of the last two replicate pots (blocks) that had been harvested at the same day were evaluated. The samples of the first two replicates, harvested one to two days earlier, gave results that were not comparable to those of the last two replicates, which likely was due to the propagation of microorganisms during the extended time of storage before plating.

Fluorescent pseudomonads and a broad spectrum of bacteria

Fluorescent pseudomonads isolated on the selective NPC medium, followed by the diverse bacterial species that appeared on the broad-spectrum medium, formed differentiated colonies within short times and were, therefore, assessed already at two and three days after plating. The recorded population densities, expressed as numbers of colony forming units per gram of root dry matter with adhering substrate material, are shown together in Fig. 8.13. Within both fractions of the rhizosphere microflora, the differences between samples grouped according the diverse precrop and supplemental

treatments were not significant, though the numbers of fluorescent pseudomonads and those of the broad bacterial spectrum showed similar patterns of distribution among the experimental groups. The highest mean population densities were generally found in Proradix® treated samples that showed about three to twelve times higher values than the respective control group, independent of the precrop type.

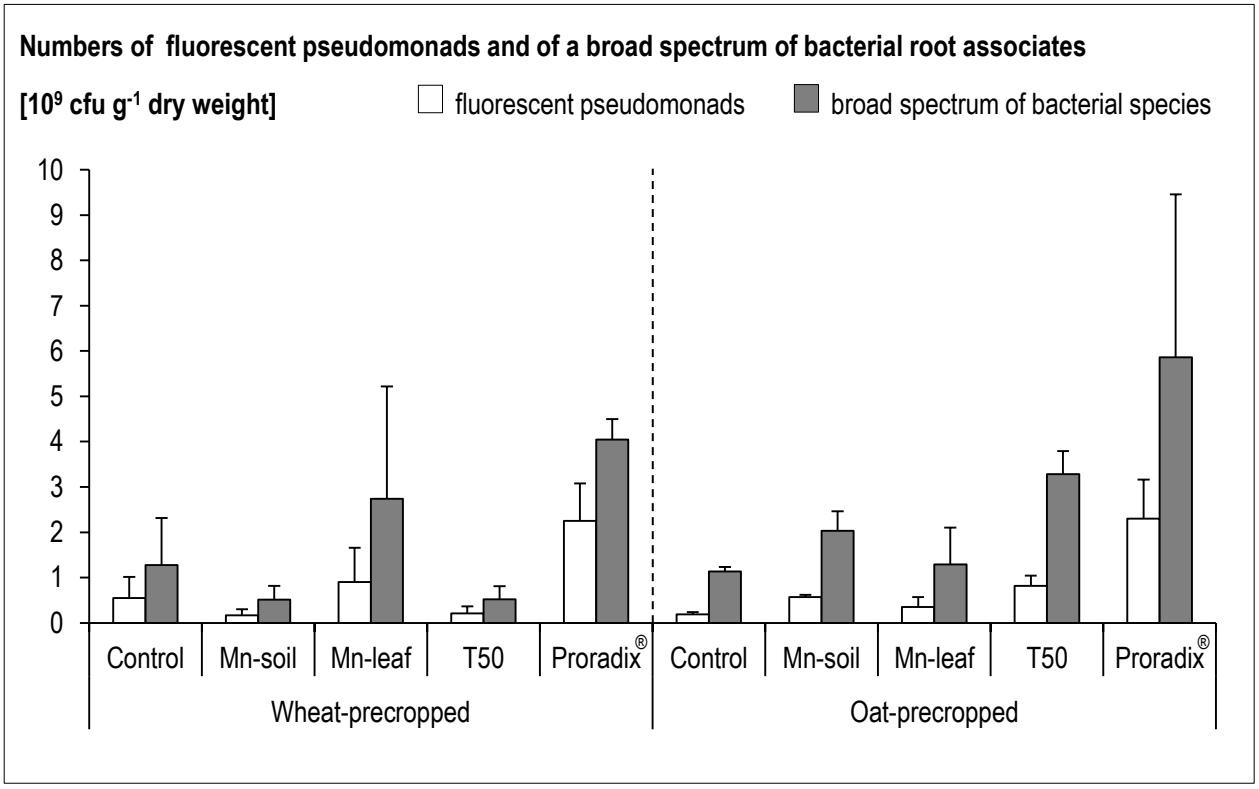

Fig. 8.13: Numbers of fluorescent pseudomonads and of a broad spectrum of bacterial species isolated from the rhizosphere of wheat (var. Paragon) plants at 50 days after sowing in the pot experiment with wheat- or oat-precropped substrate, treated or not (Control) with different supplemental treatments (Mn-soil, Mn-leaf, Vitalin T50, or Proradix®). Data are expressed as thousand millions (10^9) of colony forming units (cfu) per gram dry weight of roots with adhering substrate material.

Columns represent mean values ± SEM (n = 2). Data sets of fluorescent pseudomonads and a broad spectrum of bacterial species were tested separately in three-way ANOVAs. For both data sets, there were no significant differences among the different levels of the precrop, supplemental treatment, or block factor detected ($p > 0.05$).

Although, the proportions of fluorescent pseudomonads in relation to the broad spectrum of bacterial species were not significantly different between precrop and supplemental treatment groups (three-way ANOVA, $p > 0.05$; data not shown), fluorescent pseudomonads were at a relative frequency of about 50 % most predominant in Proradix®

treated samples. In contrast to this, their proportional abundance in untreated control samples and other groups of supplemental treatment on average was about 30 %. These findings, however, need to be interpreted carefully with respect to the huge variability among treatments and replicates.

Mn-reducing and Mn-oxidizing microorganisms

As it took several days or even weeks until manganese reducing or oxidizing microorganisms formed colonies and/or could be identified based on the visible results of their specific activities of solubilizing or precipitating manganese during incubation on the respective agar medium, their numbers were evaluated twice at 18 (data not shown) and 31 (Fig. 8.14) days after plating. In general, the numbers of manganese-reducers or -oxidizers discovered per gram of sample dry weight were on average over all sample groups by about two to four orders of magnitude (factor 10^2 to 10^4) lower than those of fluorescent pseudomonads or the broad spectrum of bacterial species. While the recorded mean population densities of manganese oxidizing microorganisms varied over several logarithmic units, those of the other three fractions of the root-associated microflora varied within more narrow ranges of about factor 10 to 60.

When the respective agar plates were assessed at 18 days after plating, manganese-reducing colonies were manly found on plates incubated with sample charges of the lowest dilution level, whereas no manganese oxidizers could be recorded (data not shown). For the numbers of manganese-reducing microorganisms obtained at that time, the general comparison over all sample groups indicated that the group mean of oat-precropped samples ($4.6*10^5$), expressed as colony forming units (cfu) per gram sample dry weight, was significantly higher (Tukey's test after three-way ANOVA, $p \leq 0.05$) than that of wheat-precropped samples ($2.2*10^5$). There were, however, no significant effects of the supplemental treatments found in this data set.

The second evaluation of the agar plates for the determination of manganese-reducing or manganese oxidizing microorganisms, performed at 31 days after plating, showed that now, particularly on plates incubated with sample charges of 100 times higher attenuation than the lowest dilution level, increased numbers of manganese-reducing colonies could

be observed. Due to this observation, the calculated mean numbers of manganese reducers per sample weight were increased by factor 64 within the group of wheat-precropped samples and by factor 19 within the groups of oat-precropped samples, as compared to the previous evaluation at 18 days after plating. In consequence of these changes, there were no more significant differences in the numbers of manganese-reducing microorganisms between samples grouped according to different levels of precrop and supplemental treatments found (three-way ANOVA, $p > 0.05$; Fig. 8.14). From the different types of colonies, it was furthermore obvious that at higher dilutions levels among others also such microbial species formed manganese-reducing colonies that were not found among the manganese reducers on plates incubated with sample charges of lower dilution levels.

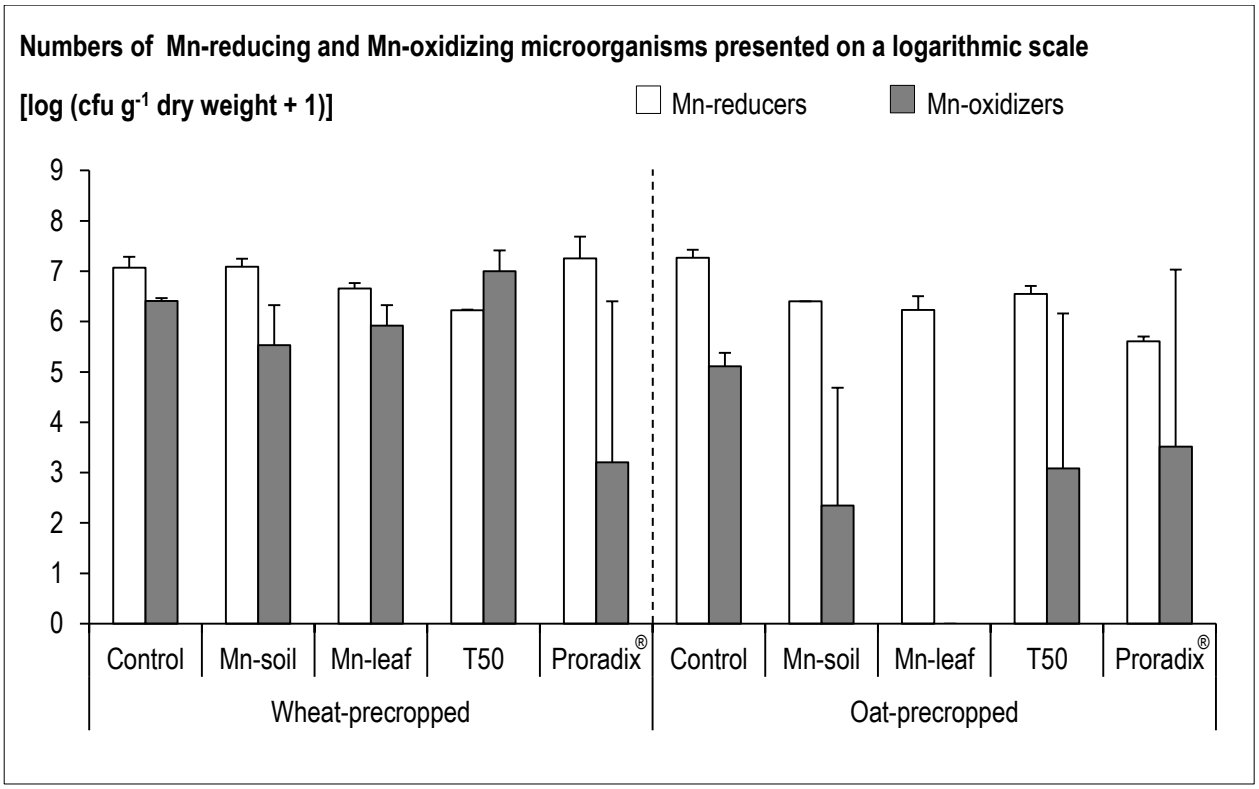

Fig. 8.14: Numbers of manganese (Mn) reducing and Mn oxidizing microorganisms isolated from the rhizosphere of wheat (var. Paragon) plants at 50 days after sowing in the pot experiment with wheat- or oat-precropped substrate, treated or not (Control) with different supplemental treatments (Mn-soil, Mn-leaf, Vitalin T50, or Proradix®). Data are expressed as colony forming units (cfu) per gram dry weight of roots with adhering substrate material and presented on a logarithmic scale by using the transformation y' = log (y + 1). Counts were performed at 31 days after plating.

Columns represent mean values ± SEM (n = 2). Data sets of Mn-reducers and Mn-oxidizers were tested separately in three-way ANOVAs, which were followed by Tukey's test in case of significant factor effects. If necessary, data were analyzed after a logarithmic transformation [y' = log (y + 1)] to achieve normal distribution. For the data set of Mn-reducers no significant differences ($p > 0.05$) according to the different levels of the precrop, supplemental treatment, or block factor were found (three-way ANOVA). For the data set of Mn-oxidizers, in contrast, the general comparison over all sample groups indicated that the group mean of wheat-precropped samples was significantly higher than that of oat-precropped samples (Tukey's-test after three-way ANOVA, $p \leq 0.05$). There were no significant effects according to the different levels of supplemental treatments, or of the block factor detected in the data set of Mn-oxidizers.

At 31 days after plating, besides from those for manganese-reducers, also the agar plates for the determination of manganese oxidizing microorganisms were evaluated, as now colonies that had formed precipitates of manganese oxide could be clearly discerned. As shown in Fig. 8.14, in sample groups with lower mean values of the numbers of manganese oxidizing microorganisms the variability between replicates was particularly high compared to those groups with higher mean values. The general comparison over all

sample groups, nevertheless, indicated that the group mean of wheat-precropped samples ($4.2*10^6$) was significantly ($p \leq 0.05$) higher than that of oat-precropped samples ($1.3*10^6$), while there were no significant differences in response to supplemental treatments (Tukey's test after three-way ANOVA; Fig.8.13).

Comparisons between the numbers of manganese-oxidizers and those of the manganese-reducers should be considered with care because of the different basal media applied for their determination as well as other reasons discussed in Chapter 8.4. However, based on the data sets recorded at 31 days after plating, there were no significant differences in the ratios of the numbers of manganese-reducing to the numbers of manganese-oxidizing microorganisms according to the different levels of the precrop, supplemental treatments and block factor detected (three-way ANOVA, $p > 0.05$; data not shown).

Manganese-reducing capacity in the rhizosphere as estimated by incubation test

The concentrations of DTPA-extractable manganese after incubation of roots with surrounding substrate material ranged from about 400 to 800 mg manganese per kg sample dry weight, while only a respective amount of about 140 mg manganese had been added as substrate in the form of manganese dioxide to the samples. The majority of DTPA-extractable manganese after incubation, therefore, originated from soil own pools of manganese. As the proportions of root dry weights in relation to the total sample dry weights were less than about 1 %, it was insignificant whether manganese concentrations were calculated on the basis of total sample dry weights or only on dry weight basis of the surrounding substrate material. In both cases, the statistical comparison of samples grouped according to the varied levels of the factors precrop, supplemental treatment and block did not indicate any significant differences in the concentrations of extractable manganese (three-way ANOVA, $p > 0.05$; data not shown). However, the analysis of DTPA-extractable manganese concentrations after incubation of soil material that had been left unplanted after collection from different field plots indicated that the manganese reducing capacity tended to be enhanced after oat compared to wheat precrops (two-way ANOVA, $p \leq 0.1$; Fig. 8.15). This effect was significant when the values obtained from incubated subsamples were not averaged for each field replicate (n of the precrop factor =

2), but computed separately (n of the precrop factor = 5; two-way ANOVA, $p \leq 0.05$; data not shown).

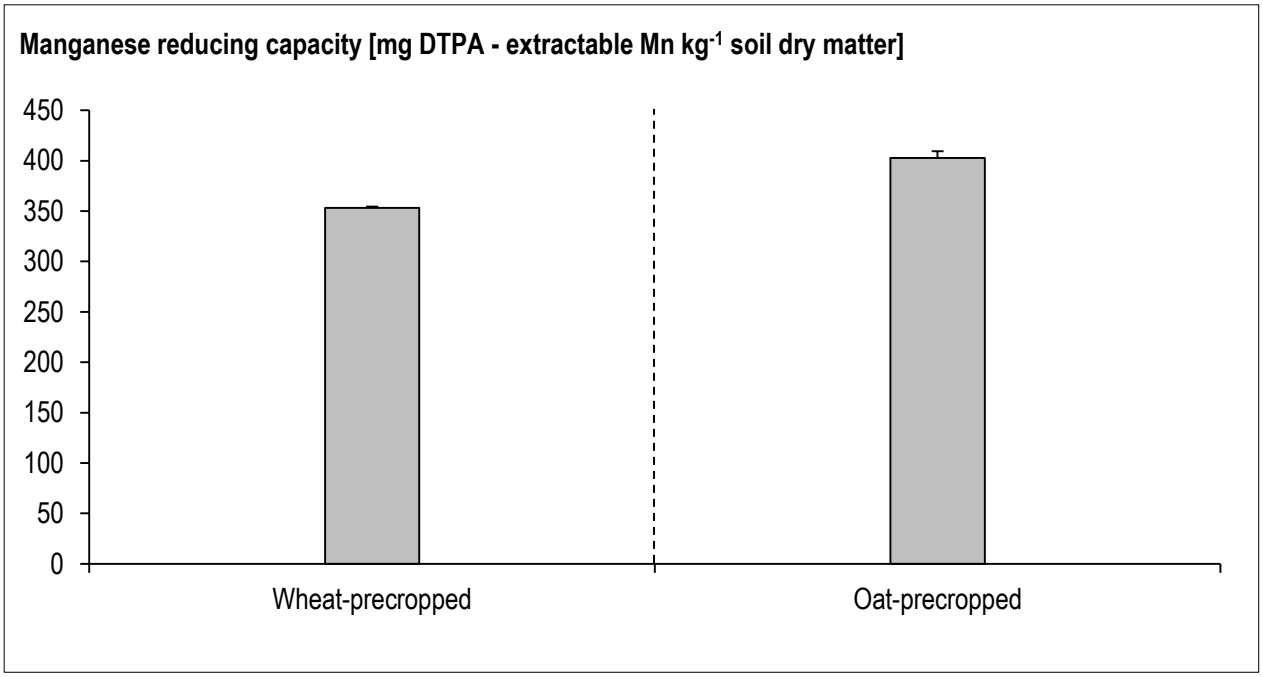

Fig. 8.15: Manganese reducing capacity in soil material that had been left unplanted after collection from wheat- or oat-precropped field plots, as measured by the concentration of DTPA-extractable manganese (Mn) per kg soil dry matter after incubation with manganese dioxide and yeast extract for seven days under shaking at room temperature.

Columns represent mean values ± SEM (n = 2). Data were tested in a two-way ANOVA for differences between subsample means grouped according to the varied levels of the factors precrop and block of a field experiment. While the mean value from oat-precropped samples tended to be enhanced compared to that from wheat-precropped samples (p = 0.068), there was no significant effect of the block factor.

8.3.5 Root morphological characteristics

The most significant effect on root morphological characteristics was induced by the foliar application of manganese (Mn-leaf). According to the general comparison over all sample groups, the Mn-leaf treatment resulted in the formation of roots with lower diameters compared to the treatment with Proradix® (p = 0.001) or Vitalin T50 (p = 0.001) as well as to untreated control plants (p = 0.045; Tukey's test after three-way ANOVA; Fig. 8.16). For pair wise comparisons between supplemental treatments within the same precrop group, however, there only was a significant difference in root diameter between Mn-leaf and Proradix®- or Vitalin T50-treated plants grown on the wheat- or oat-

precropped substrate, respectively, but not in comparison to untreated control plants (Tukey's test after two-way ANOVA; Fig. 8.16). Although there was no general influence of the precrop factor on root diameters detected, within the group of Proradix®-treated plants the mean value of oat-precropped samples in this root morphological characteristic was significantly lower compared to that of wheat-precropped samples.

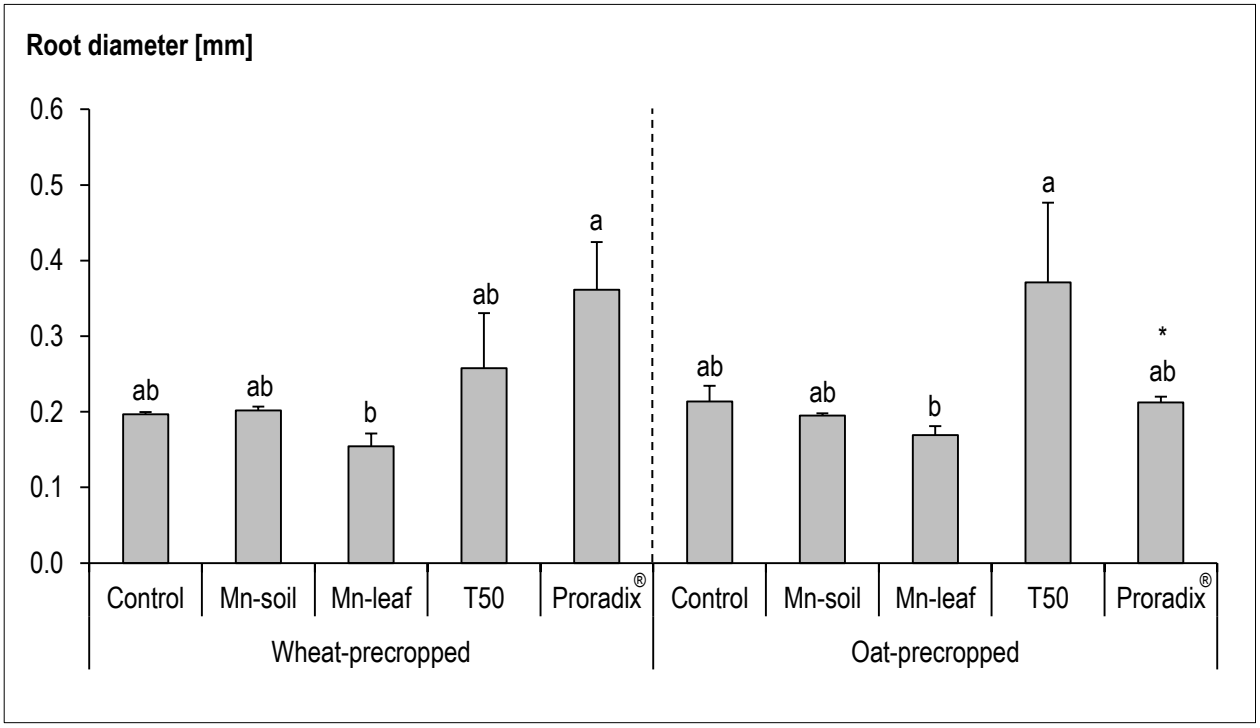

Fig. 8.16: Root diameter of wheat (var. Paragon) at 48 to 50 days after sowing in the pot experiment with wheat- or oat-precropped substrate, treated or not (Control) with different supplemental treatments (Mn-soil, Mn-leaf, Vitalin T50, or Proradix®).

Columns represent mean values ± SEM (n = 4). Data were tested in a two-way ANOVA followed by Tukey's test comparing precrop and supplemental treatments, after a preceding three-way ANOVA did not indicate any significant effect of the block factor. Data were reciprocal transformed to achieve normal distribution. Significant differences between supplemental treatments within the same precrop group are indicated by values not sharing common letters ($p \leq 0.05$). Asterisks denote significant precrop effects within the same group of supplemental treatment (* $p \leq 0.05$). Mean values without or sharing superscripts are not significantly different. There was no general precrop effect and no significant interaction between precrop and supplemental treatments. In addition to these statistical results, the general comparison over all sample groups indicated that the group mean of Mn-leaf treated plants was significantly lower compared to the treatment with Proradix® ($p = 0.001$) or Vitalin T50 ($p = 0.001$) as well as untreated control plants ($p = 0.045$; Tukey's test after three-way ANOVA).

Contrary to root diameters, specific root length to weight ratios were generally decreased ($p \leq 0.05$) by about 15 % on the oat- (group mean 209 m g^{-1} root dry weight) compared to the wheat-precropped substrate (group mean 245 m g^{-1} root dry weight). Yet, there were

no significant effects of supplemental treatments and no significant differences between precrop levels within groups of same supplemental treatments in terms of specific root length to weight ratios (Tukey's test after two-way ANOVA; data not shown).

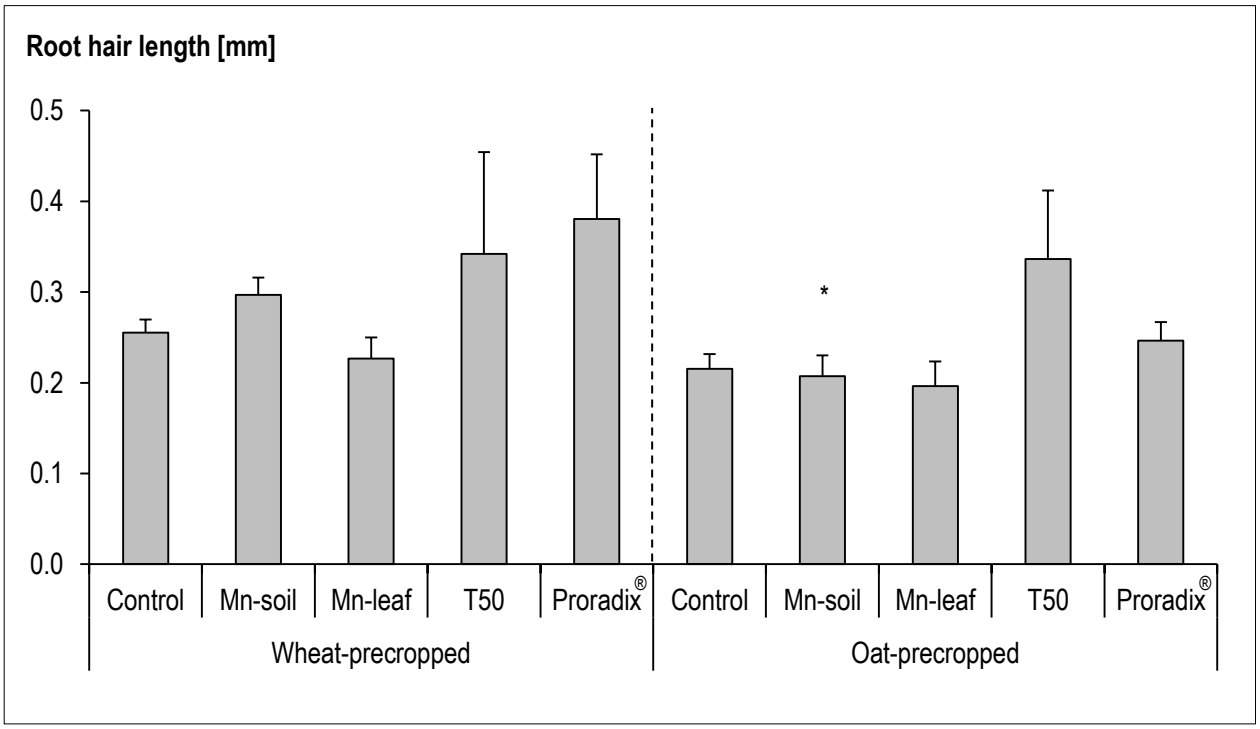

Fig. 8.17: Root hair length of wheat (var. Paragon) at 48 to 50 days after sowing in the pot experiment with wheat- or oat-precropped substrate, treated or not (Control) with different supplemental treatments (Mn-soil, Mn-leaf, Vitalin T50, or Proradix®).

Columns represent mean values ± SEM (n = 4). Data were tested in a two-way ANOVA followed by Tukey's test comparing precrop and supplemental treatments, after a preceding three-way ANOVA did not indicate any significant effect of the block factor. If necessary, data were reciprocal transformed to achieve normal distribution. There were no significant differences in pair wise comparisons between supplemental treatments found. Asterisks denote significant precrop effects within the same group of supplemental treatment (* $p \leq 0.05$). Mean values without or sharing superscripts are not significantly different. There was no significant interaction between precrop and supplemental treatments. In addition to these statistical results, the general comparison over all sample groups indicated that the group mean of oat-precropped samples was significantly lower than that of wheat-precropped samples (Tukey's test after three-way ANOVA, $p \leq 0.05$).

Similar to specific root length to weight ratios, root hair lengths were generally decreased on the oat- compared to the wheat-precropped substrate ($p \leq 0.05$; Tukey's test after three-way ANOVA; Fig. 8.17). For pair wise comparisons of precrop levels within groups of same supplemental treatment, this effect was only significant within Mn-soil treated plants ($p \leq 0.05$), although there was no clear influence of supplemental treatments on root hair lengths detected (Tukey's test after two-way ANOVA; Fig. 8.17). Nevertheless,

as with root diameters, the lowest mean values of root hair lengths were found in Mn-leaf treated plants, whereas the effects of Proradix® or Vitalin T50 on these root characteristics were less consistent.

8.3.6 Mycorrhizal root colonization

As represented in Fig. 8.18, the application of Proradix® significantly increased the proportion of root length infected by mycorrhizal fungi in comparison to the untreated control ($p = 0.001$) and other supplemental treatments ($p \leq 0.001$) that showed no effect in this regard, according to the general comparison over all sample groups (Tukey's test after three-way ANOVA). This major effect on mycorrhizal root colonization, was more clearly expressed on the oat-precropped substrate, where the effect of Proradix® was highly significant ($p \leq 0.001$) in pair wise comparison with the respective control group as well as all other supplemental treatments (Tukey's test after two-way ANOVA). On the wheat-precropped substrate, in contrast, the effect of Proradix® was only significant in comparison to other supplemental treatments, but not to the control group. Furthermore, the pair wise comparison between precrop levels within groups of same supplemental treatment showed that in Proradix® treated plants the intensity of root colonization by mycorrhizal fungi was significantly higher on the oat-precropped substrate than on the wheat-precropped substrate ($p \leq 0.01$; Tukey's test after two-way ANOVA). Within the group of untreated control plants, however, the proportion of mycorrhizal infected root length even tended to be lower on the oat- compared to wheat-precropped substrate ($p \leq 0.1$; Tukey's test after two-way ANOVA), although there was no general effect of the precrop factor on mycorrhizal root colonization detected. While vesicles were present at a density of about 55 % of the mycorrhizal infected root length, spore like structures were rarely found at less than 0.1 % of the mycorrhizal infected root length (data not shown). There was, however, no evidence for precrop or supplemental treatment effects on the relative rate of vesicle or spore formation.

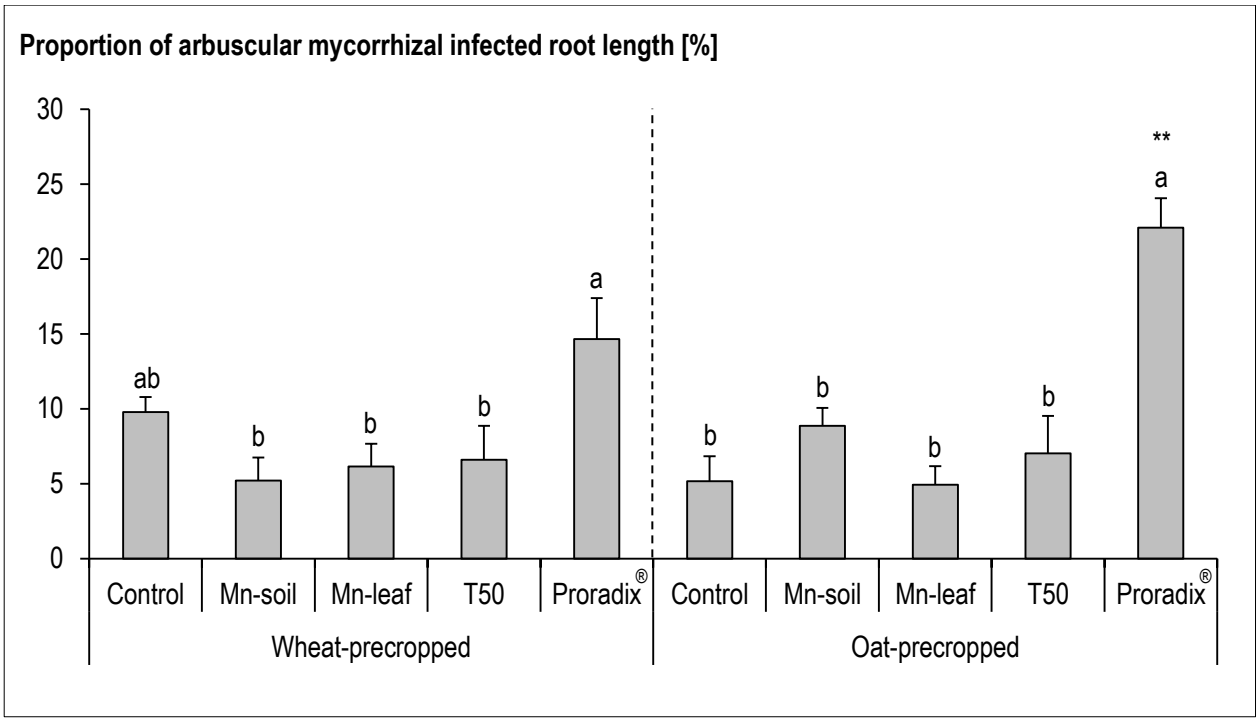

Fig. 8.18: Percentage of mycorrhizal infected root length of wheat (var. Paragon) at 48 to 50 days after sowing in the pot experiment with wheat- or oat-precropped substrate, treated or not (Control) with different supplemental treatments (Mn-soil, Mn-leaf, Vitalin T50, or Proradix®).

Columns represent mean values ± SEM (n = 4). Data were tested in a two-way ANOVA followed by Tukey's test comparing precrop and supplemental treatments, after a preceding three-way ANOVA did not indicate any significant effect of the block factor. Significant differences between supplemental treatments within the same precrop group are indicated by values not sharing common letters ($p \leq 0.05$). Asterisks denote significant precrop effects within the same group of supplemental treatment (** $p \leq 0.01$). Mean values without or sharing superscripts are not significantly different. There was a significant interaction between precrop and supplemental treatments ($p \leq 0.05$). In addition to these statistical results, the general comparison over all sample groups indicated that the group mean of Proradix® treated plants was significantly enhanced compared to the untreated control ($p = 0.001$) as well as other supplemental treatments ($p \leq 0.001$), but there was no general effect of the precrop factor (Tukey's test after three-way ANOVA).

8.3.7 Mineral nutrients in plant tissues

Of the mineral nutrients analyzed in plant tissues, the concentrations of manganese in shoots showed the most significant variations in response to the different precrop and supplemental treatments. As indicated in Fig. 8.19, the concentrations of manganese in shoot tissues were generally on a low level close to or below the critical concentration for manganese deficiency of 25 mg kg^{-1} dry matter, according to standard values reported by Bergmann and Neubert (1976). Only in Mn-leaf treated plants about four to five times enhanced values were measured, which however were excluded from statistical

evaluations because it was not discerned between manganese that had been taken up by the plant or was just adhering to the shoot surface.

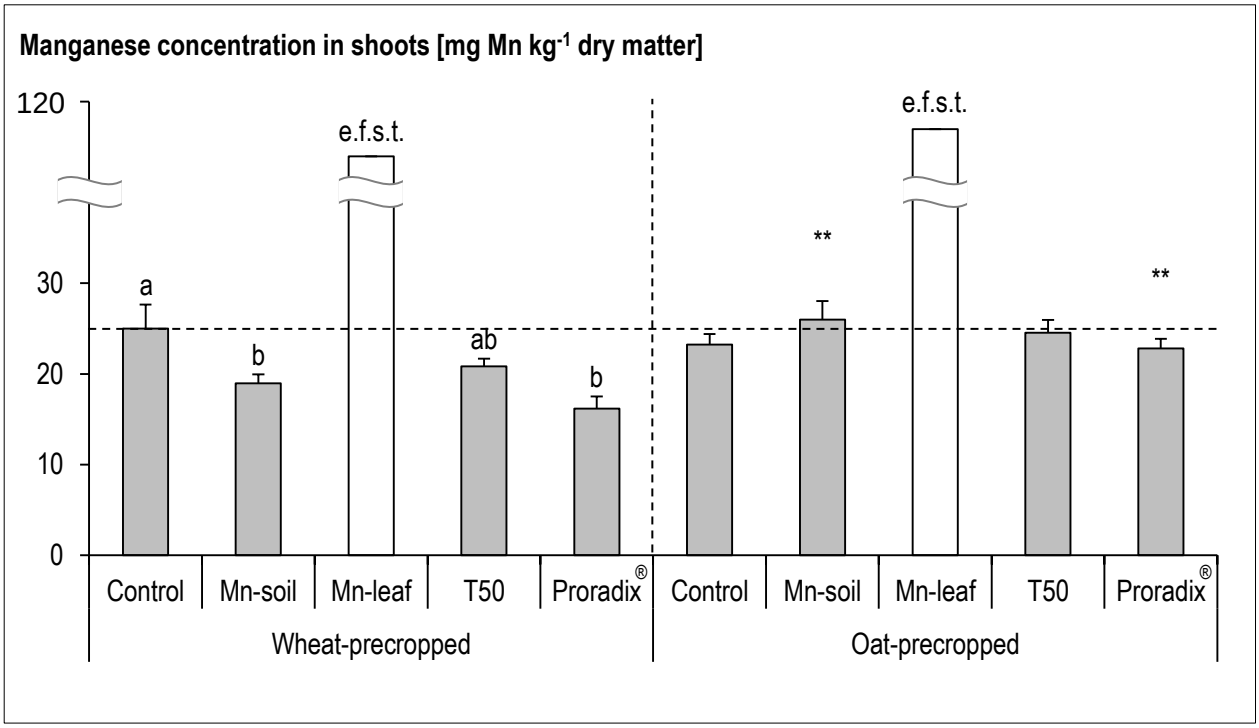

Fig. 8.19: Manganese (Mn) concentration in the shoot dry matter of wheat (var. Paragon) at 48 to 50 days after sowing in the pot experiment with wheat- or oat-precropped substrate, treated or not (Control) with different supplemental treatments (Mn-soil, Mn-leaf, Vitalin T50, or Proradix®). The dashed horizontal line (----) indicates the critical concentration for Mn deficiency in the above ground biomass of summer wheat during tillering as reported by Bergmann and Neubert (1976).

Grey columns represent mean values ± SEM (n = 4), whereas white columns represent mean values without SEM. A section of the y-axis scale between 40 and 110 has been removed to facilitate the depiction of exceptionally high values in the groups of Mn-leaf treated plants, which were furthermore excluded from statistical tests (e.f.s.t.). Data were tested in a two-way ANOVA followed by Tukey's test comparing precrop and supplemental treatments, after a preceding three-way ANOVA did not indicate any significant effect of the block factor. Significant differences between supplemental treatments within the same precrop group are indicated by values not sharing common letters ($p \leq 0.05$). Asterisks denote significant precrop effects within the same group of supplemental treatment (** $p \leq 0.01$). Mean values without or sharing superscripts are not significantly different. There was a significant interaction between precrop and supplemental treatments ($p \leq 0.05$). In addition to these statistical results, the general comparison over all included sample groups indicated that the group mean of oat-precropped plants was significantly higher than that of wheat-precropped plants (Tukey's test after three-way ANOVA, $p \leq 0.01$).

The statistical comparison over all other sample groups indicated that manganese concentrations in shoots of oat-precropped plants were generally enhanced compared to wheat-precropped plants (Tukey's test after three-way ANOVA, $p \leq 0.01$; Fig. 8.19).

When different precrop levels were compared within groups of same supplemental treatment, this effect was only significant for Mn-soil and Proradix® treated samples (Tukey's test after two-way ANOVA, $p \leq 0.01$), whereas for the untreated control group the mean value was actually slightly lower on the oat- compared to wheat-precropped substrate. Significant effects of supplemental treatments on manganese concentrations in shoots were found in plants grown on the wheat-precropped substrate, where the mean values were decreased in response to Proradix® and Mn-soil treatments when compared to the untreated control (Tukey's test after two-way ANOVA, $p \leq 0.05$). This was not the case on the oat-precropped substrate, where, aside from Mn-leaf treated plants, the highest mean value was recorded in Mn-soil treated plants, but without being significantly different from other supplemental treatment groups. As manganese concentrations were negatively correlated with biomass production in shoots (Pearson's correlation coefficient $r = -0.42$, $p \leq 0.05$), there were no significant differences between sample groups regarding total manganese contents in shoots per replicate pot, when Mn-leaf treated samples were not considered (Tukey's test after two-way ANOVA, $p > 0.05$; data not shown). Furthermore, there was no significant relationship between manganese concentrations in shoots and the severity of take-all root rot.

In root tissues (Fig. 8.20), the measured concentrations of manganese were, except for groups of Mn-leaf treated samples, several times higher than in shoot tissues. While there was no significant effect of the precrop factor, the general comparison over all sample groups indicated that the manganese concentration in Proradix® treated root samples was significantly increased in comparison with the group of untreated control samples, where the lowest mean values were observed (Tukey's test after three-way ANOVA, $p \leq 0.05$). At the level of distinct precrop groups, this effect of Proradix® was significant in plants grown on the wheat-precropped ($p \leq 0.05$), but not on the oat-precropped substrate (Tukey's test after two-way ANOVA). Apparent increases in manganese concentrations of Vitalin T50, Mn-soil, or Mn-leaf treated root samples were not significant. In contrast to shoot samples, the manganese concentrations in root samples were positively correlated with root biomass production (Pearson's correlation coefficient $r = 0.42$, $p \leq 0.01$). The pattern of differences in total root contents of manganese observed between sample groups (Fig. 8.21), therefore, was similar to that found in root biomass production (Fig.

8.10). Furthermore, there was a strong negative correlation between manganese concentrations in roots and the severity of take-all root rot (Pearson's correlation coefficient r = -0.41, $p \leq 0.01$), but there was no significant relationship between manganese concentrations in roots and those in shoots, whether Mn-leaf treated samples were considered or not. However, results from mineral nutrient analyses in root samples have to be considered carefully with respect to errors that may arise from sample contaminations by soil material (Misra, 1994). Thus, due to exceptionally high concentrations of iron found in some root samples, the measurement of iron, but also that of phosphorus, in root samples was regarded as not representative of actual tissue concentrations and excluded from further evaluations.

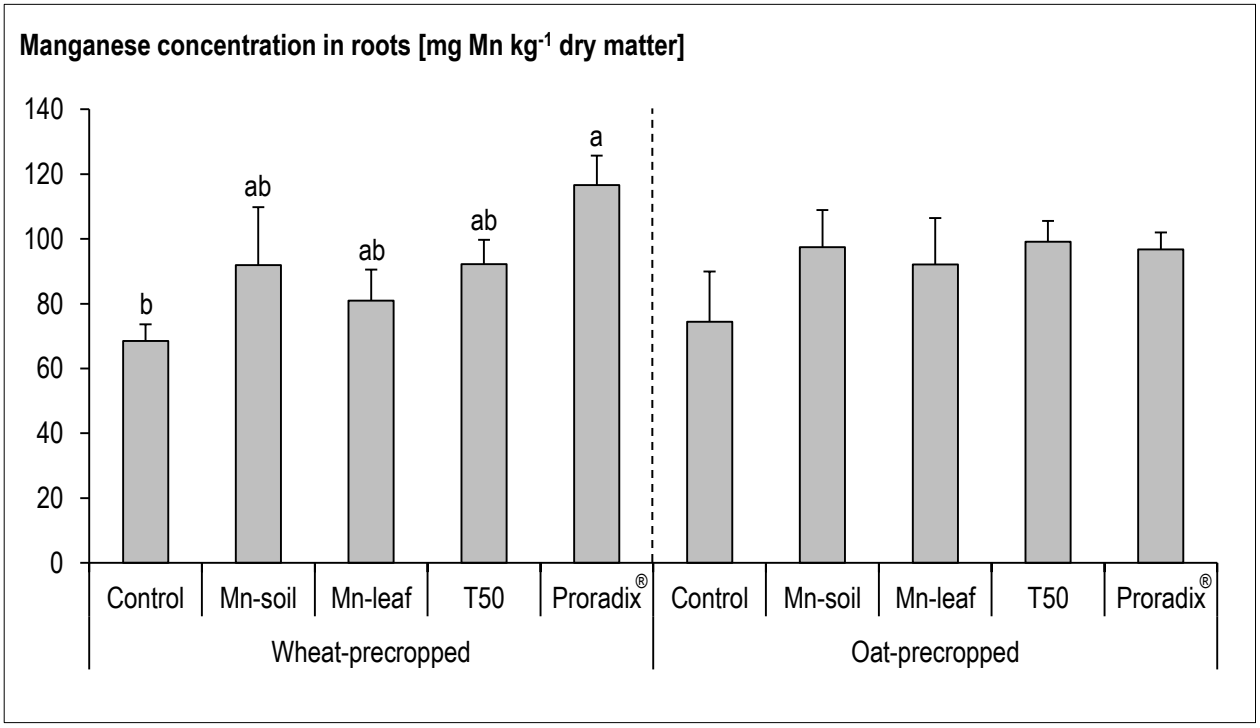

Fig. 8.20: Manganese (Mn) concentration in the root dry matter of wheat (var. Paragon) at 48 to 50 days after sowing in the pot experiment with wheat- or oat-precropped substrate, treated or not (Control) with different supplemental treatments (Mn-soil, Mn-leaf, Vitalin T50, or Proradix®).

Columns represent mean values ± SEM (n = 4). Data were tested in a two-way ANOVA followed by Tukey's test comparing precrop and supplemental treatments, after a preceding three-way ANOVA did not indicate any significant effect of the block factor. Significant differences between supplemental treatments within the same precrop group are indicated by values not sharing common letters ($p \leq 0.05$). Mean values without or sharing superscripts are not significantly different. There were no significant effects of the precrop factor detected. In addition to these statistical results, the general comparison over all sample groups indicated that the group mean of Proradix® treated plants was significantly enhanced compared to the untreated control (Tukey's test after three-way ANOVA, $p \leq 0.05$).

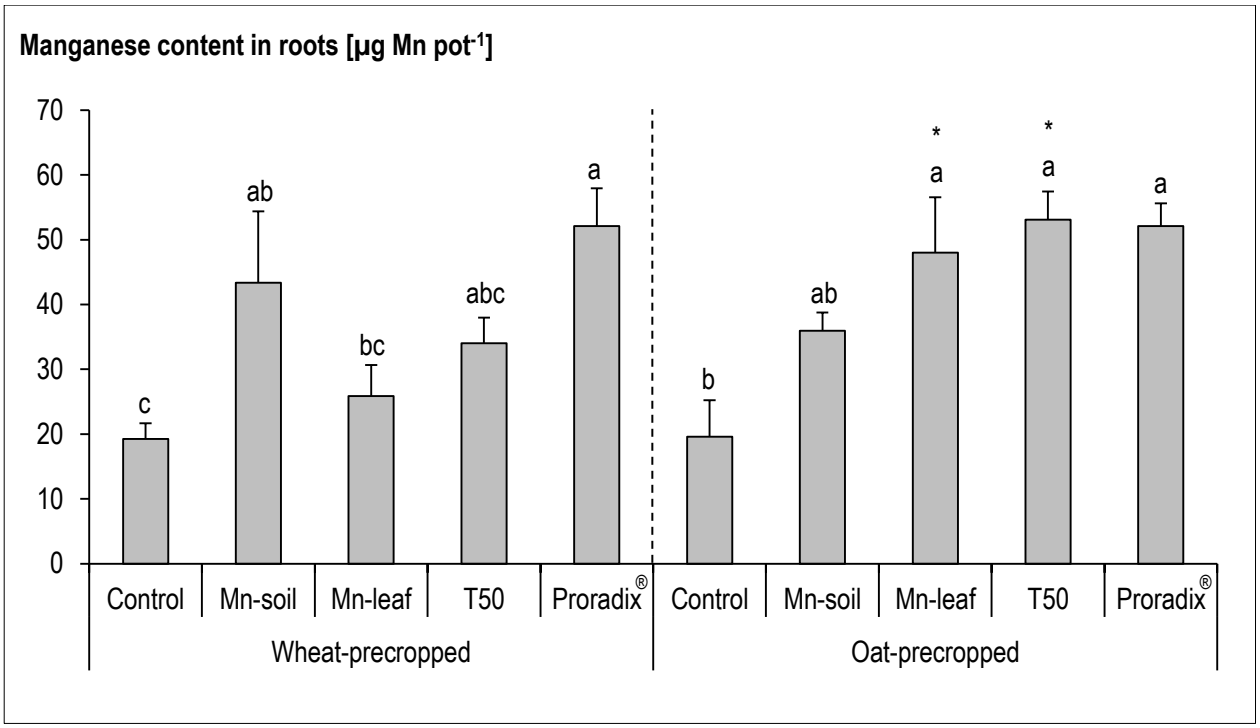

Fig. 8.21: Total manganese (Mn) content in the root tissue of wheat (var. Paragon) at 48 to 50 days after sowing in the pot experiment with wheat- or oat-precropped substrate, treated or not (Control) with different supplemental treatments (Mn-soil, Mn-leaf, Vitalin T50, or Proradix®).

Columns represent mean values ± SEM (n = 4). Data were tested in a two-way ANOVA followed by Tukey's test comparing precrop and supplemental treatments, after a preceding three-way ANOVA did not indicate any significant effect of the block factor. Significant differences between supplemental treatments within the same precrop group are indicated by values not sharing common letters ($p \leq 0.05$). Asterisks denote significant precrop effects within the same group of supplemental treatment (** $p \leq 0.01$). Mean values without or sharing superscripts are not significantly different. There was no general precrop effect and no significant interaction between precrop and supplemental treatments. In addition to these statistical results, the general comparison over all sample groups indicated that the group mean of Proradix® ($p \leq 0.001$), Vitalin T50 ($p = 0.009$), and Mn-soil ($p = 0.030$) treated plants was significantly enhanced compared to the untreated control (Tukey's test after three-way ANOVA).

Less pronounced responses to the different precrop and supplemental treatments were found in the concentrations of iron and phosphorus, analyzed in shoot samples, as well as zinc, copper, potassium, calcium, and manganese, whci were analyzed in root and shoot samples. For reasons of comprehensibility, the respective results are thus not presented in full detail, but only significant findings are briefly described in the following. According to reference values reported by Bergmann and Neubert (1976), the concentrations of these elements in shoot tissues were generally adequate to meet the requirements of wheat plants, except for magnesium in shoots, which, similar to manganese, was found at levels close to the critical value for deficiency (< 1.0 g Mg kg^{-1} dry matter). Although, the

general comparison over all sample groups indicated that shoot concentrations of magnesium were significantly decreased in response to the treatment with Vitalin T50 as compared with untreated control plants (Tukey's test after three-way ANOVA, $p \leq 0.05$), this effect was not significant for respective comparisons within groups of same precrop treatment (Tukey's test after two-way ANOVA). Asides from manganese concentrations in roots, there were no more negative correlations found that would have suggested an opposite relationship between the status of mineral nutrients in plant tissues and the severity of take-all root rot, except for phosphorus concentrations in shoots (Pearson's correlation coefficient $r = -0.40$, $p \leq 0.05$). However, the concentrations of phosphorus in shoots were not significantly affected by the different precrop or supplemental treatments (Tukey's test after three-way ANOVA, $p > 0.05$), and not significantly correlated with root biomass production or the intensity of mycorrhizal root colonization.

Furthermore, significant findings concerning the status of zinc, copper, iron, potassium, calcium, and magnesium in plant tissues were made. Zinc concentrations in shoots were significantly lower on the oat- compared to the wheat-precropped substrate (Tukey's test after three-way ANOVA, $p \leq 0.01$), but still within a sufficient range for plant growth (> 20 mg Zn kg^{-1} dry matter). For pair wise comparisons within groups of same supplemental treatment, however, this effect was significant ($p \leq 0.05$) in Vitalin T50 and Proradix® treated plants, but not within the untreated control group (Tukey's test after two-way ANOVA). As for manganese, the highest mean concentrations of zinc and other evaluated elements (Cu, K, Ca, Mg) in root tissues were found in Proradix® treated plants on the wheat-precropped substrate, but, in contrast to results obtained for manganese, these were not significantly different from the untreated control (Tukey's test after two-way ANOVA). The concentrations of iron ($r = -0.38$), copper ($r = -0.36$), potassium ($r = -0.60$), and calcium ($r = -0.50$) in shoots appeared to be negatively correlated with shoot biomass production (Pearson's correlation, $p \leq 0.05$), but only for calcium significant differences between treatment groups were found (Three-way ANOVA, $p \leq 0.05$). Thus, calcium concentrations in shoots were decreased in Mn-soil treated plants on the wheat-precropped substrate, which was significant in comparison with the respective control group of same precrop treatment as well as with Mn-soil treated plants grown on the oat-precropped substrate (Tukey's test after two-way ANOVA, $p \leq 0.05$). In roots, copper

concentrations were significantly decreased on the oat- (group mean = 14 mg Cu kg^{-1} dry matter) compared to the wheat-precropped substrate (group mean = 20 mg Cu kg^{-1} dry matter; Tukey's test after three-way ANOVA, $p \leq 0.05$). When different precrop levels were compared within groups of same supplemental treatment, however, this effect was only significant in Proradix® treated plants ($p \leq 0.01$), although the influence of supplemental treatments on copper concentrations in roots was not significant (Tukey's test after two-way ANOVA).

8.4 Discussion

8.4.1 Contrasting comparison of main results

The major findings of the above described pot experiment to investigate the effectiveness of oat precrops and supplemental treatments to improve the manganese status and suppress take-all disease in wheat can be summarized as follows:

4) Responses to supplemental treatments:

– On the wheat-precropped substrate, the soil application of manganese improved the production of root biomass and decreased the severity of take-all induced root damage, whereas the *Pseudomonas* preparation Proradix® enhanced the root growth and the concentration of manganese in roots, but was less effective to protect the roots from pathogen infection. Foliar application of manganese and treatment with Vitalin T50 did not significantly affect the biomass production or take-all severity in plants grown on the wheat-precropped substrate.

– On the oat-precropped soil substrate, in contrast, Proradix®, Vitalin T50, and manganese leaf treatments, but not the application of manganese to the soil substrate increased the formation of root biomass compared to the untreated control. Furthermore, the treatment with Proradix® strongly improved root colonization by mycorrhizal fungi.

– On the wheat- as well as on the oat-precropped substrate, the production of shoot biomass tended to be enhanced in response to the application of Proradix®, while other supplemental treatments showed no clear effect in that regard. The soil application of

manganese or microbial preparations did not improve the manganese concentrations in shoots and did not significantly affect the population densities of manganese reducing or oxidizing microorganisms detected in the rhizosphere of wheat plants. There were no manganese oxidizing microorganisms detected in the rhizosphere of Mn-leaf treated plants grown on the oat-precropped substrate.

5) Responses to the oat- versus the wheat-precropped substrate:

– Although there were no significant differences in response to the different precrop levels within the group of untreated control plants found, the general comparisons over all sample groups indicated that the severity of take-all induced root damage was lower in plants grown on the oat- compared to the wheat-precropped substrate.

– This disease suppressive effect of oat-precrops appeared to be associated with enhanced concentrations of manganese in shoots and lower population densities of manganese oxidizing microorganisms in the wheat rhizosphere.

As suggested by the contrasting comparison of these main results, supplemental treatments and oat-precrops affected the severity of take-all disease in wheat plants by different and not necessarily complementary modes of action. The specific implications of these general finding are discussed in the following sections.

8.4.2 Implications of Mn-soil treatments

The take-all suppressive effect of manganese applied as a solution of $MnSO_4$ to the soil substrate is supported by the results of Rengel et al. (1993), where an application rate of 30 mg Mn kg^{-1} soil material decreased the severity of take-all infection scored as the total length of black root lesions. These authors further reported that the resistance-enhancing effect of manganese fertilization was particularly pronounced in the genotype least efficient in manganese acquisition out of four wheat varieties grown for 28 days in a pot experiment, using highly calcareous, manganese deficient soil material as growth substrate. However, the results of Rengel et al. (1993) also suggest that manganese concentrations of about 20 or 15 mg kg^{-1} root or shoot dry weight, respectively, were

almost sufficient to achieve a maximum take-all suppressive effect, even though these values are still below the critical deficiency level (25 mg Mn kg^{-1}) as reported by Bergmann and Neubert (1976). Therefore, a low physiological requirement of manganese for the expression of pathogen defense responses in the plant, such as the biosynthesis of phenolics and lignin requiring manganese as an enzymatic cofactor only, has been assumed (Rengel et al., 1993). In contrast, Graham and Rovira (1984) observed considerable improvements in the resistance of wheat roots against take-all over the nutritional range from mildly deficient (< 13 mg Mn kg^{-1} dry weight, but no symptoms of deficiency) to adequate levels of manganese (≈ 35 mg Mn kg^{-1} dry weight) in shoot tissues of wheat after 26 days of growth in pots. These values were achieved with application rates of about 3.3 or 33.3 mg Mn per kilogram of a manganese deficient calcareous soil. To eliminate take-all infection, however, still an up to ten times enhanced application rate (333 mg Mn kg^{-1} soil) appeared to be necessary (Graham and Rovira, 1984). Recent results from Heine et al. (2011) accordingly indicated enhanced resistance of tomato against black leaf mold (*Pseudocercospora fuligena*) mediated by manganese levels above optimum values for plant growth, but below phyto- and direct fungi-toxic concentrations in leaves.

Regardless of the considerations referred to above, the critical factors of active host resistance to *G. graminis* are likely to be the concentrations of manganese, phenolic compounds and other materials at the time and point of infection for the rapid formation of inducible defense responses such as lignitubers (Rengel et al., 1993; Yu et al., 2010; see Chapter 7.3.2). Average concentrations of manganese in plant tissues, particularly if not recorded contemporaneously to pathogen attack, therefore, are of limited validity to reveal the casual relationship between manganese nutrition and take-all resistance in wheat (Rengel et al., 1994). There rather is increasing evidence that the effects of manganese on plant resistance occur mainly in the infection court during the initial stages of fungal infection. Thus, they are dependent on the mobility of manganese in plants to be rapidly allocated to the site of hyphal invasion (Leusch and Buchenauer, 1988b; Dixon et al., 2002; Johal and Huber, 2009; Underwood et al., 2012; Zhang et al., 2012). Because after uptake manganese is rapidly transported in the xylem from roots to shoots, but hardly redistributed via the phloem within the root system or within the shoot of young

wheat plants (Riesen and Feller, 2005; Page and Feller, 2005), the continuous uptake of manganese appears to be important for the effective expression of reactive defense mechanisms in roots.

As manganese in its reduced, plant available form (Mn^{2+}) is unstable at pH values above 4 with respect to oxidation, it is not expected to persist in aerated horizons of neutral to alkaline soils (Gilkes and McKenzie, 1988; Millaleo et al. 2010). In this regard, investigations from van Veen (1973) on the progress of manganese oxidation in soils showed that the oxidation of Mn^{2+} added as $MnSO_4$ did not occur in soil material of pH 4, proceeded slowly at a pH of about 5, and was much faster under neutral pH conditions, while being chiefly due to microbial activity. However, even in samples of a sandy soil material of pH 7, about 20 % of the added manganese (950 parts per million) were still detectable as water extractable Mn^{2+} after 20 days of incubation (van Veen, 1973). Correspondingly, as reported by Wilhelm et al. (1988), $MnSO_4$ applied to a highly calcareous aeolian sand (370 mg Mn kg^{-1}) at two weeks before sowing increased the root and shoot concentrations of manganese and decreased the severity of take-all infection in wheat. With respect to the results of the present study, it therefore seems to be reasonable that manganese applied to the soil substrate was available to the wheat plants particularly during early growth stages for improved resistance against the take-all pathogen. This assumption is supported by a growth promoting effect of the Mn-soil treatment on leaf length that was most pronounced at 37 days after sowing, but less distinct at later growth stages. In the following, an enhanced biomass formation by the invigorated plant, while the availability of manganese in the soil substrate was progressively decreasing due to proceeding oxidation, would have resulted in lower shoot levels of manganese during later stages of the pot experiment as a dilution effect. In accordance with this argumentation are the decreased concentrations of manganese in shoots of Mn-soil treated plants grown on the wheat-precropped substrate, as determined after experimental harvest. In this regard, a depletion of manganese stocks in the soil substrate by plant uptake can be excluded as reason for the negative correlation between biomass production and manganese concentrations in shoots. Total contents of manganese in the root (43µg) and shoot (22 µg) biomass produced per pot were only a minute fraction (< 1 %) of the application rate of manganese per pot (12.8 mg).

The observation that in wheat plants grown on the oat-precropped substrate the manganese concentrations in shoots were not decreased in response to the Mn-soil treatment, conforms to a lower pathogenic and manganese oxidizing activity of *Gaeumannomyces graminis* and other deleterious soil microorganism as compared to the wheat-precropped substrate (Huber and McCay-Buis, 1991; Marschner et al., 1991). This assumption is also supported by enhanced SPAD values in response to the Mn-soil treatment as recorded at 22 and 30 days after sowing, thus not only indicating that manganese supply was a limiting factor for chlorophyll synthesis, but also that the application of $MnSO_4$ persistently improved the plant availability of manganese in the oat-precropped substrate. As shown by Shenker et al. (2004), the excessive production of superoxide dismutase enzymes, that occurred both under extreme deficiency and excessive manganese nutrition, could led to drastic decreases in the synthesis of chlorophyll and plant growth.

The concentrations of manganese measured in root tissues are difficult to interpret as precipitated manganese oxides may adhere to the root surface or load the interior of roots, such as due to the manganese oxidizing activity of the take-all pathogen (Misra, 1994; Schulze et al., 1995). However, neither the manganese concentrations measured in root samples (< 150 mg Mn kg^{-1} dry weight ≙ 25 mg Mn kg^{-1} fresh weight) nor its application rate (15 mg Mn kg^{-1} dry weight of the soil material ≙ 75 mg Mn $liter^{-1}$ in relation to the substrate solution at 40 % water holding capacity) justify the assumption of direct toxicity to the fungus. As indicated by the results from Marschner et al. (1991), rates of $MnSO_4$ supply equivalent to more than 250 mg Mn kg^{-1} were necessary to inhibit the hyphal growth of *Gaeumannomyces graminis* var. *tritici* in a siliceous sand when no manganese reducing bacterial strains were added. Therefore, the take-all suppressive effect of Mn-soil application observed in the present study is likely to be through plant physiological mechanisms of pathogen inhibition. These may include the formation of constitutive and inducible local defenses in the plant, such as involving the synthesis of phenolics and lignin, but also quantitative and qualitative alterations in root exudation with respective consequences for the root associated microflora (Graham and Rovira, 1984; Wilhelm et al.; 1988; Graham and Webb, 1991; Marschner et al., 1991). Limited information about the effect of manganese concentrations on the activity of pathogenesis-related enzymes is

available (Huber and Wilhelm, 1988; Graham and Webb, 1991; Dordas, 2008). Results from Vares and Hatakka (1997) showed different responses among various fungal species whose lignin degrading activity was either stimulated or suppressed by elevated Mn^{2+} concentrations (7 mg Mn l^{-1}), which was attributed to the expression of different enzyme systems. In another study, diverse white-rot fungi exhibited significant lignocellulolytic enzymes activity in growth media supplemented with 0.1 mM $MnSO_4$ (55 mg Mn l^{-1}), but revealed specific requirements on the type of plant material that was provided as lignocellulosic substrate for optimal enzymatic performance (Elisashvili et al., 2009). To clarify the role of manganese as an inhibitor or cofactor of pathogenesis-related enzymes involved in the host-pathogen interactions between wheat and the take-all fungus thus requires further investigation with respect to its concentration in the rhizosphere and affected root tissues.

8.4.3 Implications of Mn-leaf treatments

In contrast to the Mn-soil treatment, foliar spays with dissolved $MnSO_4$ did not affect the severity of take-all disease on the wheat-precropped substrate, but significantly enhanced the formation of root biomass on the oat-precropped substrate. These observations confirm earlier findings made by Reis et al. (1982) as well as Wilhelm et al. (1988).

Because foliar applications of 0.1 % $MnSO_4 \cdot 7H_2O$ (198 mg Mn l^{-1}) alleviated manganese deficiency symptoms in leaves, Reis et al. (1982) concluded that the micronutrient was taken up by the leaves, but possibly not transported to the roots where it would have been necessary for take-all control. However, some controversy remains about the actual absorption and translocation of foliar-applied manganese in plants. In studies where manganese was applied to selected leaves only, El-Baz et al. (1990) found a limited uptake (1 % of supply) and distribution to other plant parts in maize and fava bean (El-Baz et al., 1990), whereas other authors detected a translocation throughout the plant, inclusive roots, in soybean, tomato (Romney and Toth, 1954) and oat (Vose, 1963). Nevertheless, the results from El-Baz et al. (1990) also showed that when applied as MnEDTA the translocation of manganese was about ten times enhanced compared to $MnSO_4$, despite of generally low rates of uptake. As reported by Single (1958), a small

but significant amount of manganese was translocated to the growing points when a 0.5 % solution of $MnSO_4 \cdot 3H_2O$ (1.34 g Mn l^{-1}) was applied to the surface of the second leaf of manganese deficient wheat plants. However, the results of that study, in which wheat was grown in solution culture, also indicated that the movement of manganese from similar leaves depended upon the conditions of supply. Thus, rewetting of the leaf surface appeared to support the uptake of residual manganese, whereas a low manganese status in treated leafs was assumed to inhibit its export to the rest of the plant due to preferential local incorporation by cell organelles such as chloroplasts (Hagen et al., 1952; Single, 1958). Recent research furthermore emphasized genotypic and phenotypic differences in the characteristics of leaf surfaces and their stomata, the surface tension of fertilizer solutions, microbial leaf colonization, and environmental conditions as critical factors for the uptake of foliar-applied mineral nutrients (Fernández and Eichert, 2009; Burkhardt et al., 2012; Will et al., 2012). The feasibility of a meaningful shoot to root translocation of manganese has been suggested by the results of Boken (1960) obtained in a pot experiment with oat grown on a sandy soil substrate of pH 9. According to these results, repeated foliar sprays with a 2 % solution of $MnSO_4 \cdot H_2O$ (6.5 g Mn l^{-1}) applied on whole shoots increased the manganese concentrations in the roots of so treated oat plants (104 mg kg^{-1} dry matter) by factor 4.33 compared to the untreated control (24 mg kg^{-1} dry matter). In that investigation, however, the possible entrainment of manganese from shoots and its subsequent entry into to the soil substrate by rainwater, hence allowing for root uptake, might not have been excluded completely (Boken, 1960).

The increased formation of root biomass in response to the foliar application of manganese on the oat-precropped substrate is probably a result of improved photosynthetic activity that in manganese deficient plants is severely inhibited (Graham and Webb, 1991). Such root growth promoting effect, but without adequate supply of manganese to the root for the local expression of resistance mechanisms, may only show to advantage under conditions of absent to moderate infection pressure from root rot pathogens like *Gaeumannomyces graminis*, which to some extend seemed to be the case in the oat-precropped substrate. In the wheat-precropped substrate, on the contrary, additional root growth might have been strongly counteracted by a high infection capacity of the take-all fungus. Although the influence of Mn-leaf applications on the chlorophyll

concentration in leaves, as estimated by SPAD readings, appeared to be less pronounced than that of soil-applied manganese, this may not be reflective of the intensity of photosynthetic activity in respectively treated plants. The most sensitive process impaired by manganese deficiency in photosynthesizing cells is the splitting of water in photosystem II, which is catalyzed by a manganese-containing enzyme, rather than the synthesis of chlorophyll (Husted et al., 2009; Broadley et al., 2012). As reported by Nable et al. (1984), a level of manganese deficiency in young leaves of subterranean clover that depressed photosynthetic oxygen evolution by more than 50 % had only a small effect on chlorophyll concentration. Kriedemann et al. (1985) obtained similar results with wheat and described a threshold concentration of 10 to 12 mg Mn kg^{-1} dry weight of leaves as a minimum requirement for photosynthetic productivity. Further studies with wheat and species of barley grass (*Hordeum glaucum* and *H. leporinum*) showed that lower proportions of biomass were allocated to the roots when the supply of manganese was decreased (Kriedemann and Anderson, 1988). In the present study, Mn-soil treatments did not induce a significant improvement of the manganese status in shoot tissues that was close to the critical deficiency value of 25 mg Mn kg^{-1} dry weight and thus far below concentrations that are sufficient for adequate growth of summer wheat (> 55 mg Mn kg^{-1} dry weight) according to Bergmann and Neubert (1976). The effectiveness of manganese applied to the soil material, therefore, might have been sufficient to induce an enhanced synthesis of chlorophyll, but not for an optimal photosynthetic activity. Contrariwise, in Mn-leaf treated plants grown on the oat-precropped substrate, an enhanced photosynthetic productivity, but without additional supply of nitrogen and other mineral nutrients might have resulted in slightly decreased chlorophyll concentrations as a dilution effect. On the wheat-precropped substrate, however, the performance of Mn-leaf treated plants was weak in general, due to the severe impairment of root functions by take-all infection.

Because foliar applications of manganese can restore the photosynthetic capacity of plants, but show week effectiveness against take-all, it has been argued that subsequent alterations in root exudation and their influence on the associated microflora are not important means by which manganese exerts its suppressive influence on this disease (Graham and Webb, 1991). The present results could not contradict this argument with statistical significance, but indicated an absence or strong repression of manganese

oxidizing microorganisms as response to the foliar application of manganese in wheat plants that were grown on the oat-precropped substrate. Conversely, there is some evidence that the efficiency in manganese acquisition and the tolerance to take-all of certain wheat genotypes depends on their specific capacity to induce chemical and microbiological alterations in the rhizosphere (Marschner et al., 1991; Rengel et al., 1996; Rengel, 1997b). Another possible mechanism that has been described in the literature to contribute to the protection of plants against fungal pathogens is the induction of local and systemic resistance by foliar sprays with solutions of diverse fertilizer salts, such as manganese chloride ($MnCl_2$) and $MnSO_4$ that were among the most effective agents (Reuveni et al., 1997; Eisa et al., 2006). In this regard, it remained unclear whether the mechanism of this protection was elicited through stress induced by the salts (Mucharromah and Kuc, 1991), or was related to an improved mineral nutrition and physiological processes like photosynthesis (Murray and Walters, 1992). However, also the successful expression of defense mechanisms characteristic of systemic resistance is likely to require adequate levels of manganese in plant tissues challenged by potential pathogens, although this aspect has not been clearly elucidated yet (see Chapter 7.3.2; Heine et al., 2011).

8.4.4 Implications of treatments with the *Trichoderma* preparation Vitalin T50

Trichoderma strains are taught to suppress fungal pathogens of plants by multiple modes of action, such as competition for nutrients and space, production of compounds that are inhibitory or toxic to the pathogen, direct hyperparasitism, and/or enhanced resistance of the plant, working in concert against disease infestation (see Chapter 2.3.3; Howell, 2003; Benítez et al., 2004). The principal ability of certain *Trichoderma* strains to antagonize other fungi can easily be demonstrated in dual culture tests, in which mycelial pieces of the test fungi are incubated together on the same agar plate prepared with a culture medium that is suitable to support the growth of each of them (Dennis and Webster, 1971; Küçük and Kıvanç, 2004). As reported by Akter (2007), the strain of *Trichoderma harzianium* contained in Vitalin T50 was able to overgrow colonies of *Gaeumannomyces graminis* var. *tritici* within a few days when both fungi were simultaneously placed on malt extract peptone agar (DSMZ, 2007b; Fig. 8.22) in an assay according to Dennis and

Webster (1971). Due to their rapid growth rates on diverse substrates and tolerance to inhibitory effects from other microorganisms, *Trichoderma* spp. are competitive saprophytes, which is considered as an important factor in retarding the development of phytopathogenic fungi in soil and the rhizosphere (Papavizas, 1985; Hjeljord and Tronsmo, 1998; Naár and Kecskés, 1998; Caihong and Quian, 2007).

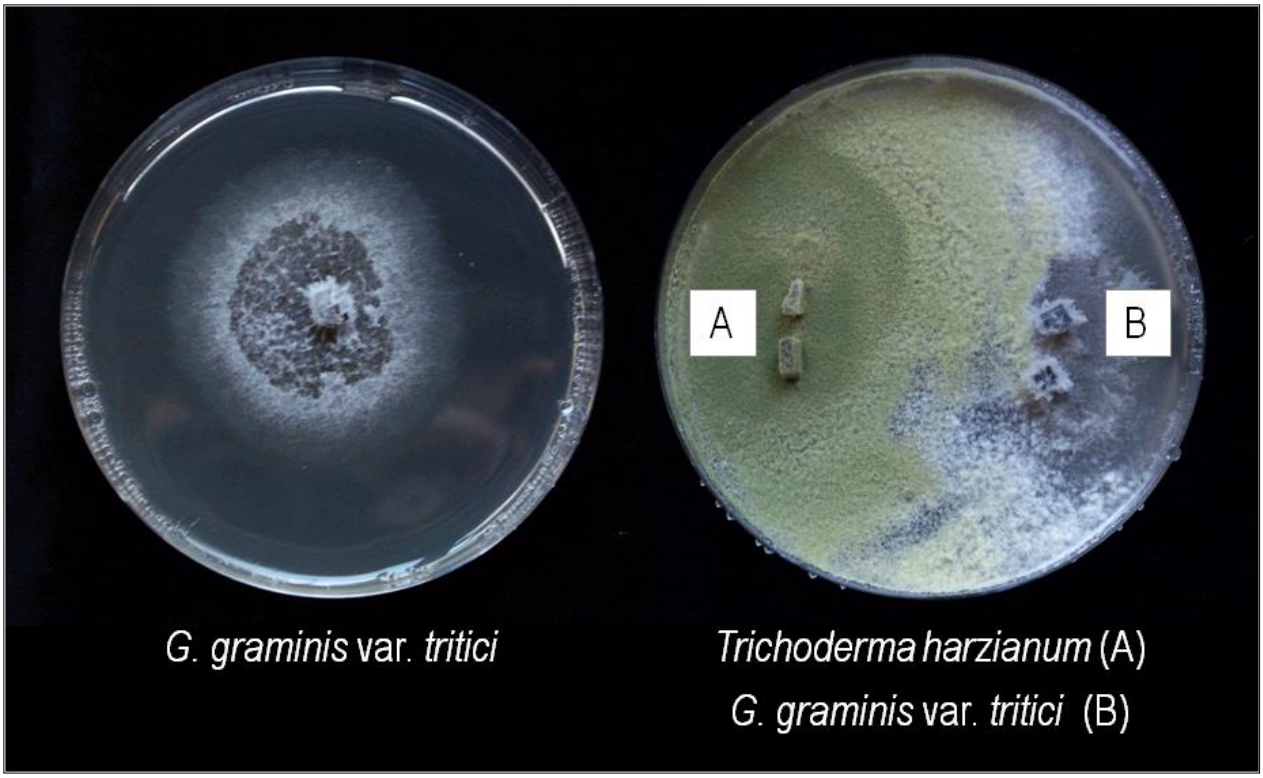

Fig. 8.22: Overgrowth and growth inhibition of Gaeumannomyces graminis var. tritici by the strain of Trichoderma harzianum contained in Vitalin T50. Mycelial pieces of the pathogenic fungus were incubated on malt extract peptone agar (30.0 g l^{-1} malt extract, 3.0 g l^{-1} soy peptone, 15.0 g l^{-1} agar, pH 5.6; DSMZ, 2007b) either alone (left side of the picture) or together with mycelial pieces of the Trichoderma fungus (right side of the picture) for seven days at about 22 °C. The mycelial pieces used as inoculants were cut from actively growing pure cultures (about 3 weeks old) prepared with the same type of medium (picture published in Akter, 2007).

The antagonistic effectiveness of diverse *Trichoderma* strains against fungal pathogens revealed by dual culture tests has been reported to be in reasonable agreement with their disease-suppressive effect observed in plants at the greenhouse or field level (Zafari et al., 2008; Ojaghian, 2011). Nevertheless, it is difficult to extrapolate results from the laboratory to the plant-soil environment (Hermosa et al., 2000). Although *Trichoderma* species are regarded as to be beneficial for a wide range of plant species, at least for

different maize lines a strong genotypic component in their response to a strain of *Trichoderma harzianum* (T22) has been reported, with some maize inbred lines even showing adverse growth reactions (Harman, 2006). On the opposite side, the ability of *Trichoderma* fungi to colonize plant roots has been associated with their strain specific capacity tolerate defense related compounds, such as terpenoids, produced by the plant in response to fungal invasion (Howell et al., 2000; Benítez et al., 2004). While each of eight *Trichoderma* isolates differentially inhibited the growth of *Gaeumannomyces graminis* var. *tritici* in dual culture tests, only two of them effectively decreased the severity of take-all disease by more than 40 % when applied to the soil substrate or as seed coating in greenhouse experiments with wheat, as reported by Zafari et al. (2008). Other strains, however, only sowed a weak to moderate effectiveness, decreasing the disease severity by less than 30 % in the same study. This discrepancy between strains and between laboratory and greenhouse results was attributed to the changing importance of certain metabolites, such as antibiotics and hydrolytic enzymes, among other factors for the expression of antagonistic activity by *Trichoderma* fungi in the different environments (Zafri et al., 2008). Thus, volatile metabolites released by the *Trichoderma* fungi and exerting a strong growth inhibitory effect against the pathogen when grown on agar medium may not be of high importance for disease suppression in the host plant. In contrast, a strain that weakly inhibited pathogen growth by different metabolites it produced, but rapidly overgrew the pathogen in dual culture tests, at least moderately decreased take-all disease. The most effective *Trichoderma* isolates in the soil environment, however, when tested under laboratory conditions, were characterized by their ability to rapidly overgrow the pathogen colony, hyper-parasitize the pathogen mycelium, and inhibit pathogen growth by specific nonvolatile metabolites. In summary, the results from Zafari et al. (2008) suggested that rapid overgrowth of the pathogen and competitive colonization of the rhizosphere are the basic functional traits to protect developing roots from pathogenic fungal infestation. Ghisalberti et al. (1990), furthermore, identified pyrone antibiotics as specific metabolites related to the superior capacity of certain *Trichoderma* strains to suppress the take-all pathogen in wheat.

Another study (Hermosa et al., 2000), comparing the antagonistic activity of 17 *Trichoderma* strains against diverse phytopathogenic fungi (*Phoma betae*, *Rosellinia*

necatrix, *Botrytis cinerea*, *Fusarium oxysporum*) in dual culture tests with three different media, revealed that their aggressiveness depended not only on the type of fungal target, but also on the composition of the culture medium. Such findings indicate a strong influence of abiotic environmental variables for the activity of *Trichoderma* strains. Accordingly, factors that are of importance for the soil physicochemical properties have been demonstrated to influence the development and the production of antagonism-related metabolites, such as antibiotics, enzymes and siderophores, in *Trichoderma* and other fungi (Moreno-Mateos et al., 2007; Trushina et al., 2013). Low soil temperatures of about 15 °C have been shown to depress the growth of *Trichoderma* strains from diverse species, including *T. harzianum*, which prefer relatively warmer temperatures of 20 to 25 °C (Dewan and Sivasithamparam, 1988b). Such warmer temperature levels were not maintained in the present pot experiment, because in non-sterile soil substrates severe take-all infections are typically restricted to the lower temperature range of 5 to 20 °C (Cook, 1981b; Hornby, 1981). Results from Ghisalberti et al. (1990), however, have demonstrated that a strain of *Trichoderma harzianium* with the ability to produce pyrone antibiotics effectively suppressed take-all of wheat even under a low temperature regime (i.e. 15 ± 2 °C). As one of the major factors affecting the activity of metabolic factors secreted by both the antagonistic and pathogenic fungi, the environmental pH is regarded (Benítez et al., 2004). Like many other fungi, *Trichoderma* species appear to be more predominant in acidic soils (Klein and Eveleigh, 1998), whereas the take-all fungus is favored by neutral to alkaline soils (Sivasithamparam, 2002). Furthermore, the activity of certain antibiotics produced by *Trichoderma* species has been reported to decrease with increasing pH (Brian and McGowan, 1945; Howell, 1998). The capacity to modify the conditions in their surroundings, therefore, seems to be another important trait for *Trichoderma* strains to prosper as bio-control agents in the rhizosphere, particularly in alkaline soils where pH is a critical limiting factor for these fungi (Benítez et al., 2004; Hanhong, 2011; Trushina et al., 2013). Results from Moreno-Mateos et al. (2004; 2007), for example, suggest that strains of *Trichoderma harzianum* are striving to tightly adjust the external pH, facilitating values for optimal activity of their own secreted enzymes. Acidification of the surrounding environment by *Trichoderma* strains can be achieved through the release of protons, which together with the secretion of carboxylates (e.g.

citrate), siderophores and reductive metabolites are able to solubilize mineral nutrients such as phosphorus, iron, manganese, zinc, and copper from sparingly available forms (Altomare et al., 1999; Olejníková et al., 2011). Where these elements are scarce, the pathogen suppressive effect of *Trichoderma* fungi has been attributed to alterations in the availability of mineral nutrients, too (Benítez et al., 2004). These changes may improve the growth and resistance of the plant, but also hamper the pathogen more directly, as due to the competitive acquisition of iron by means of *Trichoderma*-specific siderophores (Anke et al., 1991; Tripathi et al., 2010) or due to the fungitoxic activity of reduced manganese (Graham and Web, 1991).

Despite of the versatile possible activities performed by *Trichoderma* fungi in the suppression of soil borne pathogens, the results of the present pot experiment indicate a rather weak overall effectiveness of the tested *Trichoderma harzianum* contained in Vitalin T50 to combat take-all disease in wheat. Although, evidence that the application of Vitalin T50 resembled the effect of Mn-soil treatments could be observed during the fifth to sixth week of the experimental phase, such as in terms of enhanced SPAD values at 30 days after sowing on the oat-precropped (Fig. 8.7) or enhanced leaf length at 37 days after sowing on the wheat-precropped substrate (Fig. 8.5). At the time of experimental harvest, however, no significant decrease in disease severity could be approved in response to the *Trichoderma* application on the wheat-precropped substrate. On the oat-precropped substrate with lower infection pressure from the pathogen, in contrast, such treatment induced strong root growth stimulatory effects, as confirmed by measurements of dry weights. These findings indicate, that the failure of Vitalin T50 to protect wheat roots from the take-all fungus was not due to a general inactivity of this *Trichoderma* preparation under the given physicochemical soil conditions and also not due to an incompatibility with the selected wheat variety in principle. Rather, it seems that the supposable antagonistic mechanisms acting directly upon the pathogen, such as mycoparasitism, antibiotic production antibiosis, and competition for nutrients and space (Harman, 2006), were not expressed to a sufficient extent by the distinct *Trichoderma* fungus. In particular, under the regime of low soil temperatures (15 ± 2 °C) the ability of *Trichoderma* strains to produce pyrone antibiotics has been identified as a most critical feature for the successful suppression of *Gaeumannomyces graminis* var. *tritici* (Dewan

and Sivasithamparam, 1988b; Ghisalberti et al., 1990). *Trichoderma* strains that may combat this pathogen only by other modes of antagonism (e.g. mycoparasitism, competition) and/or strengthening the resistance of plant, however, showed a weak effectiveness against take-all disease of wheat grown under such conditions (Ghisalberti et al., 1990). According to these findings, it has been attested by unpublished, company internal investigations (Roland Humm, personal communication), that the *Trichoderma harzianum* strain contained in Vitalin T50 does not produce pyrones or other antibiotics.

8.4.5 Implications of treatments with the *Pseudomonas* preparation Proradix®

Similar to *Trichoderma* fungi, certain *Pseudomonas* species are known to be skilled with many features by which they can strengthen the resistance of plants or antagonize pathogens directly (see Chapter 2.3.2; Weller, 2007). Among them are the ability to induce systemic resistance in plants (Bakker et al., 2007), improve the plant growth and mineral nutrient acquisition (Vessey, 2003), and rapidly colonize the rhizosphere in aggressive competition (Saharan and Nehra, 2011; Ghirardi et al., 2012), thereby exerting toxic, inhibitory or stimulatory effects on other microorganisms (Meyer and Linderman, 1986a; O'Sullivan and O'Gara, 1992). In these processes a wide range of bacterial compounds, such as flagellins, lipopolysaccharides, siderophores (Bakker et al., 2007), carboxylic acids (Park et al., 2009), phytohormones (Vessey, 2003), antibiotics (Weller, 2007), and lytic enzymes (Diby et al., 2005) are involved. Although, these diverse modes of action and active metabolites may co-operate in the suppression of plant pathogens, their particular importance depends on the individual *Pseudomonas* strain, the antagonized pathogen, the host plant, and the environmental conditions (Duffy and Défago, 1999; Weller et al., 2002). In the case of *Gaeumannomyces graminis* var. *tritici* on wheat, the capability of certain strains of fluorescent *Pseudomonas* to produce the antibiotics phenazine-1-carboxylic acid (PCA) (Thomashow and Weller, 1990; Hamdan et al., 1991) or 2,4-diacetylphloroglucinol (2,4-DAPG) (Keel et al., 1992; Raaijmakers and Weller, 1998) has been described as a key determinant for effective disease suppression. Although the production of these antibiotics by the respective *Pseudomonas* strains appears to require sufficient availability of iron (Weller et al., 1988; Duffy and Défago, 1999; Lim et al., 2012; Almario et al., 2013), a minor role in the control of take-

all disease has been attributed to siderophores and other factors (Thomashow and Weller, 1990; Hamdan et al, 1991).

In contrast to these propositions, the application of Proradix® showed consistent improvements in plant performance that lasted throughout the course of the present pot experiment and were attributable to several modes of action different from antibiotic production. According to analyses performed by the manufacturer (Sourcon Padena, Tübingen, Germany), there are no indications that *Pseudomonas* sp. *Proradix* strain DSMZ 13134 produces antibiotics or other toxic metabolites (personal communication Karin Mai Mai and Wolfgang Vogt; EFSA, 2012). The bio-control effective modes of action performed by *Pseudomonas* sp. *Proradix* strain DSMZ 13134 rather have been attributed to the production of siderophores, in particular pyochelin and deferoxamine, and to the induction of resistance in plants (EFSA, 2012). By forming complexes with ferric iron that are unavailable to other organisms, the siderophores released by *Pseudomonas* sp. *Proradix* strain DSMZ 13134 have been supposed to competitively restrict the growth of pathogens at the plant root (EFSA, 2012). This also agrees with results from Yusran (2009) where the inhibitory effect of this bacterium against a pathogenic *Fusarium* fungus observed in dual culture tests was mainly attributable to competition for limiting nutrients, especially iron. However, Elsherif and Grossmann (1994), who tested 886 isolates of fluorescent pseudomonads, able to produce siderophores, found no relationship between their antagonistic activity against *Gaeumannomyces graminis* var. *tritici* shown under *in vitro* conditions and their efficacy *in vivo*. A pre-selection of candidate strains based on the results obtained from screening tests on artificial growth media, therefore, did not seem to be useful for biological control applications under practice conditions (Elsherif and Grossmann, 1994).

The induction of resistance in response to treatment with *Pseudomonas* sp. *Proradix* has been confirmed by the deposition of callose in roots and the activation of most pathogenesis-related (PR) plant genes (EFSA, 2012). Furthermore, salicylic acid methylether and indole-3-acetic acid (IAA) have been identified as important secondary metabolites produced by *Pseudomonas* sp. *Proradix* strain DSMZ 13134 (EFSA, 2012). The possible bio-active properties of these microbial compounds correspond well with

significant effects observed in Proradix® treated wheat plants of the present study. Thus, the enhanced length growth of leaves during the early development of the wheat plants as well as the earlier emergence of ears induced by the application of Proradix® is likely to be related with the of the phytohormone indole-3-acetic acid, the main natural auxin in plants (Napier, 2003). Similar plant growth promoting effects on leaf development and ear formation haven been reported by Spaepen et al. (2008) for a genetically modified strain of the rhizobacterium *Azospirillum brasilense* expressing enhanced production of indole-3-acetic acid in greenhouse experiments with winter wheat. Furthermore, Barbieri and Galli (1993) found that a strain of *Azospirillum brasilense* with decreased production of indole-3-acetic acid, compared to the wild-type strain, showed a lower ability to increase the root development of durum wheat in terms of number and length of lateral roots, and density of root hairs. Through such proliferation of root growth with greater surface area, these bacteria may indirectly enhance the uptake of mineral nutrients from the soil and thus the growth and stress tolerance of the host plant (Lambrecht et al., 2000; Dobbelaere et al., 2003). Although the results of the present pot experiment, as well as those of the screening tests described in Chapter 4.3.2 and 4.3.3, did not reveal clear effects of Proradix® on morphological root characteristics of wheat, the root biomass production of wheat grown on the wheat- or oat-precropped substrate was significantly enhanced in response to this treatment. These plants were obviously more enabled to tolerate the take-all pathogen and compensate root damage by additional root growth, even under severe infection pressure, while, in contrast to Mn-soil application, Proradix® did not decrease the visual proportion of take-all infected wheat roots significantly.

One important factor in this regard might be induced resistance, which, also when not completely preventing pathogen infestation, at least could enable the host plant to perform more rapid defense reactions at sites of fungal entry, which delay the infection process (Wong et al., 1975; Gindrat, 1979). This in turn would provide an extended time gap to perform further defense reactions, possibly restricting the pathogen-induced damage to the outer layers of root tissues, or to develop new roots in substitution for those destroyed by the pathogen (Gupta, 2004). Deferoxamine, also known as desferrioxamine (Chiani et al., 2010), due to its high iron binding constant has been assumed to inhibit the ferric iron catalyzed formation of harmful reactive oxygen species that are involved in plant defense

reactions against pathogens (Dellagi, 1998; Press et al., 2001). Other siderophores produced by pseudomonads, like pyochelin or salicylic acid (a precursor of the pyochelin biosynthesis), have been recognized as elicitors of induced resistance against fungal pathogens in plants (Buysens et al., 1996; Audenaert et al., 2002; Höfte and Bakker, 2007). Evidence that systemic resistance induced by strains of *Pseudomonas fluorescens* (Sari et al., 2008; Daval et al., 2011) or non-pathogenic fungi (Wong, 1975; Aberra et al., 1998; Khaosaad et al., 2007) can retard the infection of wheat roots by *Gaeumannomyces graminis* var. *tritici* has been provided in diverse studies. The specific capability of *Pseudomonas* sp. *Proradix* strain DSMZ 13134 to induce resistance in plants has been investigated by Yusran (2009) in a split-root experiment with tomato, where the application of Proradix® to one half of the root system suppressed infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici* in the other half, grown in a separate compartment. This effect was accompanied by the formation of increased protein concentrations in roots, as being indicative of an enhanced expression of resistance genes (Yusran, 2009). As reported by Fröhlich et al. (2012), the inoculation of barley roots with *Pseudomonas* sp. DSMZ 13134 not only decreased the infection of roots by *Gaeumannomyces graminis* significantly, but also induced resistance against the leaf pathogen *Rhynchosporium secalis*. Furthermore, von Rad et al. (2005) monitored changes in the gene expression of *Arabidopsis thaliana* as induced by ethanolic extract of *Pseudomonas Proradix* dissolved in water and applied by spraying on the leaves. It was found that such treatment induced the expression of genes typically activated in the salicylic acid-based pathway of systemic acquired resistance (SAR), but also of genes known to be associated with the jasmonic acid- and ethylene-regulated defense system of induced systemic resistance (ISR) (see Chapter 2.1.2). These processes resulted in a sustained activation of pathogenesis-related (PR) proteins in the plant (von Rad et al., 2005), which has been supposed to be of critical importance for a lasting resistance against pathogens (Sticher et al., 1997; cited in: von Rad et al., 2005).

Another important factor that can enable the plant to maintain root functions and growth in plants challenged by root damaging pathogens is the formation of mycorrhizal symbioses (Xavier and Boyetchko, 2002). By different authors, the take-all suppressive effect of arbuscular mycorrhizae established prior to pathogen inoculation was attributed

to improved phosphorus status and decreased root exudation (Graham and Menge, 1982) or to systemically improved resistance in the plant (Khaosaad et al., 2007), as outlined in Chapter 7.2.1. As shown by Yusran (2009), applications of Proradix® to the soil substrate enhanced the mycorrhization of tomato under exposure to as well as in the absence of the root rot pathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici*.

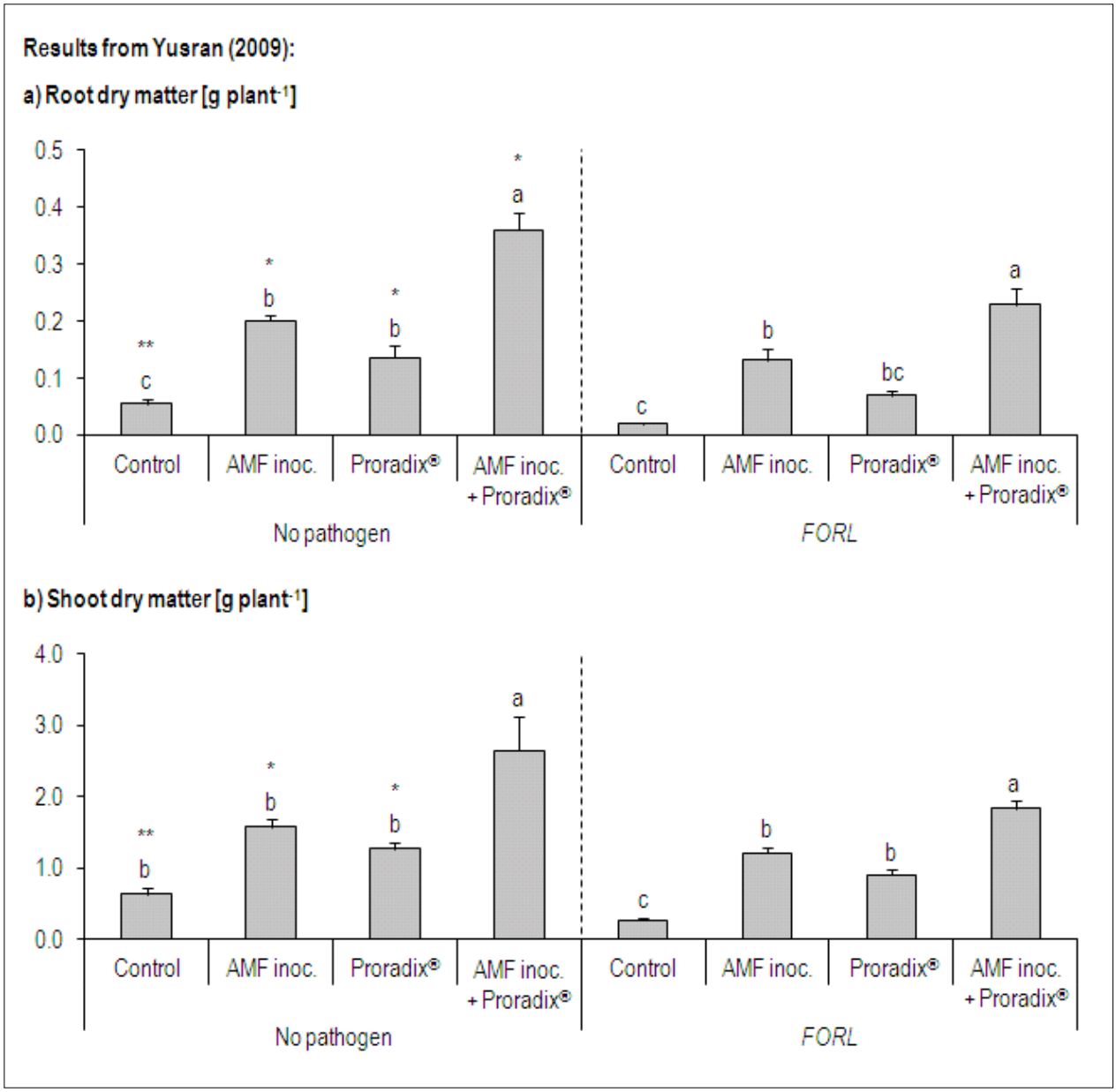

Fig. 8.23: Root (a) and shoot (b) dry weight of tomato (var. Money Maker) 9 weeks after sowing in a pot experiment described by Yusran (2009). Seeds were pre-cultured in soil substrate treated or not (Control) with inoculum of arbuscular mycorrhizal fungi (AMF inoc.; Glomus intraradices strain 510; Mycotek Biotechnik GbR, Hannover, Germany) and/or Proradix® in single or combined applications. Three weeks after sowing, the seedlings were transplanted into soil substrate where no pathogen or inoculum of the root rot pathogen Fusarium oxysporum f. sp. radicis-lycopersici (FORL) had been applied.

Columns represent mean values ± SEM (n = 5). Data sets of "no pathogen" or FORL treated plants were tested separately in one-way ANOVAs followed by Tukey's test for significant differences between the mean values of the supplemental treatment groups (control, AMF inoc., and Proradix®), as indicated by values not sharing common letters ($p \leq 0.05$). Significant differences between the mean values of "no pathogen" and FORL-inoculated plants of same supplemental treatment group, as analyzed by Student's t-test, are indicated by asterisks (* $p \leq 0.05$; ** ≤ 0.01).

Moreover, the combined application of Proradix® and mycorrhizal inoculum induced further increases in root and shoot biomass production over the values achieved with no or single application of either Proradix® or mycorrhizal inoculum (Fig. 8.23a,b). In the above cited studies, however, seeds of the given plant species were pre-cultured for two or more weeks to build up resistance by help of mycorrhizal (Graham and Menge, 1982; Khaosaad et al., 2007) and/or *Pseudomonas* (Yusran, 2009) inoculants before being exposed to pathogen attack. Such pre-treatment may explain the pronounced effects observed in consequence, but would not be practicable in wheat production under field conditions. The present pot experiment, in contrast, showed that Proradix® promoted the root growth and the colonization by soil indigenous AM-fungi of wheat that, according to agricultural practice, was directly sown into pathogen infested soil substrate, which was particularly pronounced on the oat-precropped substrate. These and other findings (see Fig. 7.2) suggest that the enhanced mycorrhization by effect of certain pseudomonads strains in diseased soils is not just the result of general helper functions assisting mycorrhiza formation, such as by enhanced spore germination and mycelial growth, inhibition of antagonists, or improved root health and plant vigor (Frey-Klett et al., 2007). It rather seems that mycorrhizal fungi in synergistic interaction with beneficial rhizobacteria are playing key functional roles in enabling the host plant to resist soil borne pathogens. Related investigations to elucidate the importance of mycorrhizal fungi in this interactive system revealed their possible influence on the activity and composition of root associated microbial populations. Results from Siasou et al. (2009), for instance, indicate that mycorrhizal root colonization may induce tremendous increases in the production of antibiotics, such as 2.4-DAPG, by strains of *Pseudomonas fluorescens* in the rhizosphere of take-all affected wheat plants. Meyer and Linderman (1986b) demonstrated quantitative and qualitative differences in microbial populations isolated from the rhizosphere of maize plants that had been treated with mycorrhizal inoculum or not. Thereby, fluorescent pseudomonads appeared to be one of most mycorrhiza-responsive bacterial groups, as identified by means of selective agar media. Further investigations of more recent date confirmed that arbuscular mycorrhizal infection affects the composition of the bacterial microflora in the rhizosphere as revealed by molecular analyses (Marschner et al., 2001; Wamberg et al., 2003).

A third root associated microbial group, besides beneficial rhizobacteria and AM-fungi, affecting the interplay between plants and major pathogenic fungi, are deleterious rhizobacteria found among *Pseudomonas* and other species (Suslow and Schroth, 1982a). Known as minor pathogens, the direct damage produced by deleterious rhizobacteria is typically restricted to juvenile tissues, such as root hairs, root tips and cortical cells (Salt, 1979; Schippers et al., 1987), but they may increase the susceptibility of roots to major fungal pathogens (Suslow and Schroth, 1982a). While the importance of root hairs as sites of entry during the initial stages of root colonization by AM-fungi needs to be further elucidated (Peterson and Farquhar, 1996; Guinel and Hirsch, 2000; Genre et al., 2005), deleterious rhizobacteria can exert inhibitory effects on the activity of AM-fungi (Waschkies et al., 1994; Miransari, 2011). On the contrary, improved mycorrhization has been reported to inhibit the proliferation of deleterious bacteria in the rhizosphere (Garcia-Garrido and Ocampo, 1988, 1989; Waschkies et al., 1994; Liousanne et al., 2009). By use of molecular characterization and tracing methods, Buddrus-Schiemann et al. (2010) have shown that *Pseudomonas* sp. *Proradix* strain DSMZ 13134 applied as seed treatment to barley, grown a loamy soil substrate taken from an agricultural field, colonized in particular the root hair zone at high cell densities in competition with seed and soil borne bacteria. Although, the inoculant strain could be detected in all parts of the root system even four weeks after sowing, the number of detectable cells decreased after three weeks while being replaced by other bacteria. Together, these findings indicate that in diseased soils the repression of deleterious rhizobacteria during the first phases of root colonization, thereby enabling the successful establishment of mycorrhizal symbiosis, can provide a mechanistic explanation for the decreased susceptibility of roots to major fungal pathogens by treatment with beneficial *Pseudomonas* strains. Indirect evidence for this assertion has also been reported by Behn (2008) who investigated the interaction between *Pseudomonas fluorescen*s strain RA56 (Phytobacter Production AG, Berlin, Germany), and arbuscular mycorrhizal fungus (*Glomus mosseae*) treatments in wheat grown on a sterile substrate with inoculum of *G. graminis* var. *tritici*. Under these conditions the single application of *Pseudomonas* strain RA56 was less effective against take-all disease, and the combined application of the bacterial and the mycorrhizal agent did not improve the mycorrhizal root colonization and the disease suppressive effect compared to the

values achieved as well with single application of the mycorrhizal fungus (Behn, 2008). When applied to soil substrate collected from replant diseased field sites, however, a commercial preparation of *Pseudomonas* strain RA56 showed strong mycorrhiza helper functions in grape vines, which was attributed to the suppression of deleterious pseudomonads of the autochthonous microflora (Weinmann, 2000; Kaließ, 2008). Deleterious microbial populations, associated with the conductivity of soils to take-all in wheat, have been characterized by their manganese-oxidizing activity as a major attribute related to disease aggravation (Marschner et al., 1991; Huber and McCay-Buis, 1993). The replacement of manganese-oxidizing microorganisms in the rhizosphere by *Pseudomonas* sp. *Proradix* strain DSMZ 13134, therefore, might explain the enhanced manganese status of wheat roots in response to the application of Proradix® in the present pot experiment. One factor of crop management that affects the activity and composition of soil microbial populations is the form of nitrogen fertilizer. As shown by Sarniguett et al. (1992b), nitrogen applied as calcium nitrate ($Ca(NO_3)_2$) enhanced the predominance of deleterious fluorescent pseudomonads in the soil, whereas ammonium sulfate ($(NH_4)_2SO_4$) favored such strains that antagonized the take-all fungus in pot cultures of wheat. Individual strains of *Pseudomonas* and *Bacillus* species that can oxidize as well reduce manganese, however, have been reported to increase or decrease the severity of take-all under conditions of nitrate or ammonium supply, respectively (Huber and McCay-Buis, 1993). Whether certain rhizobacteria may act as deleterious, beneficial or possibly indifferent root associates, therefore, also seems to alter depending on the environmental conditions. This indicates the importance of an adapted agricultural management to achieve the intended results with the application of microbial preparations (Miransari, 2011).

8.4.6 Implications of oat-precrop treatments

In the present pot experiment, the take-all suppressive effect of oat precrops did not emerge distinctively between control plants that were grown on the wheat- or oat-precropped substrate without any supplemental treatment. The plant-beneficial effect of supplemental treatments, however, was in some instances significantly stronger expressed or only apparent on the oat-precropped substrate. Thus, Mn-leaf and Vitalin T50

treatments only improved the root biomass production on the oat-precropped substrate. Proradix® enhanced the root colonization by arbuscular mycorrhizal fungi to an even stronger extent on the oat- than on the wheat-precropped substrate, as shown by multiple comparisons between precrop and supplemental treatment groups. These findings indicate that certain components contributing to the development of take-all disease were less predominant and/or could be more easily counteracted by the respective supplemental treatments after oat compared to wheat precrop. In accordance with this, the factorial analyses showed that a decreased severity of take-all root rot within the group of oat-precropped samples was associated with enhanced numbers of manganese reducing and decreased numbers of manganese oxidizing microorganisms isolated from the rhizosphere. Characterizations of microbial populations by cultivation on different agar media, however, must be interpreted carefully as the expression of traits such as manganese oxidation or reduction may depend on the type of culture media used and other environmental conditions (Marschner et al., 1991; Huber and McCay-Buis, 1993). The results obtained in this way may therefore not be representative for the behavior of microorganisms under soil conditions and are even more disputable when taking into account that most species of soil bacteria are not culturable on conventional growth media (Janssen et al., 2002; Marschner, 2007; da Rocha et al., 2009). Nevertheless, further evidence for an improved availability of manganese in response to the oat precrop can be seen in the results obtained from the incubation test to estimate the manganese-reducing capacity in soil material, the measurements of root hair lengths, and mineral nutrient analyses in shoot tissues. Thus, in soil material that had been left unplanted after its collection from field plots the manganese-reducing capacity was tendentiously enhanced after oat precrops. The root hair length of wheat plants grown on the oat-precropped substrate was generally decreased, while Yang et al. (2008) observed similar reactions to manganese supply in *Arabidopsis*. Finally, the manganese concentrations measured in shoot tissues were generally increased in the sample group of plants grown on the oat-precropped substrate, which indicates an improved acquisition of manganese by the plant, as there was no effect of the precrop factor on shoot biomass production found. An important mechanism by which improved manganese nutrition can strengthen the preformed and inducible resistance of plants against pathogens is due to the role of

manganese activated enzymes in the synthesis of lignin and other phenolic compounds functioning as cell wall strengthening materials (Graham and Rovira, 1984; Yu et al., 2010). According to Evans (1977), it can be expected that roots have thicker cell walls when their length per unit of weight is lower compared to roots of same diameter. In this regard, the generally decreased ratio of root length to root dry weight observed within the sample group of plants grown on the oat-precropped substrate, while there was no influence of the precrop factor on root diameters observed, could be interpreted as a sign of improved resistance.

However, based on the results of the present pot experiment per se, it cannot be clearly dissected whether the take-all disease-mitigating and the manganese status of wheat improving effects induced by the oat precrop were due to a decreased density of pathogen inoculum in the soil or due to the fortification of antagonistic microbial populations. To further elucidate the take-all suppressive mechanisms of oat precrops, in a pot experiment from Schackmann (2005) and Akter (2007) either the inoculum level of the pathogen in the soil substrate was increased by supplementation with additional inoculum of *G. graminis* var. *tritici* (*Ggt*) or the specific fungicide Latitude® was applied to selectively eliminate the take-all fungus. For the results obtained from that experiment, the factorial analysis indicated highly significant effects on the root dry matter production of spring wheat var. Paragon, which was generally increased in response to the oat-precrop and Latitude® treatments, but decreased in response to the supplementation with *Ggt* inoculum (Fig. 8.24). At the level of pair wise comparisons within distinct sample groups of different precrop, inoculum, and fungicide treatment, however, the adverse effect of supplementation with *Ggt* inoculum on root formation was only significant within plants grown on the wheat-precropped substrate without application of Latitude® (first versus third column in Fig. 8.24). The beneficial effect of oat-precrops on root biomass production, on the contrary, was more pronounced when the soil substrates had been charged with additional *Ggt* inoculum and, even when this was given, this effect was only significant when the plants also had been treated with Latitude® (fourth versus eighth column in Fig. 8.24).

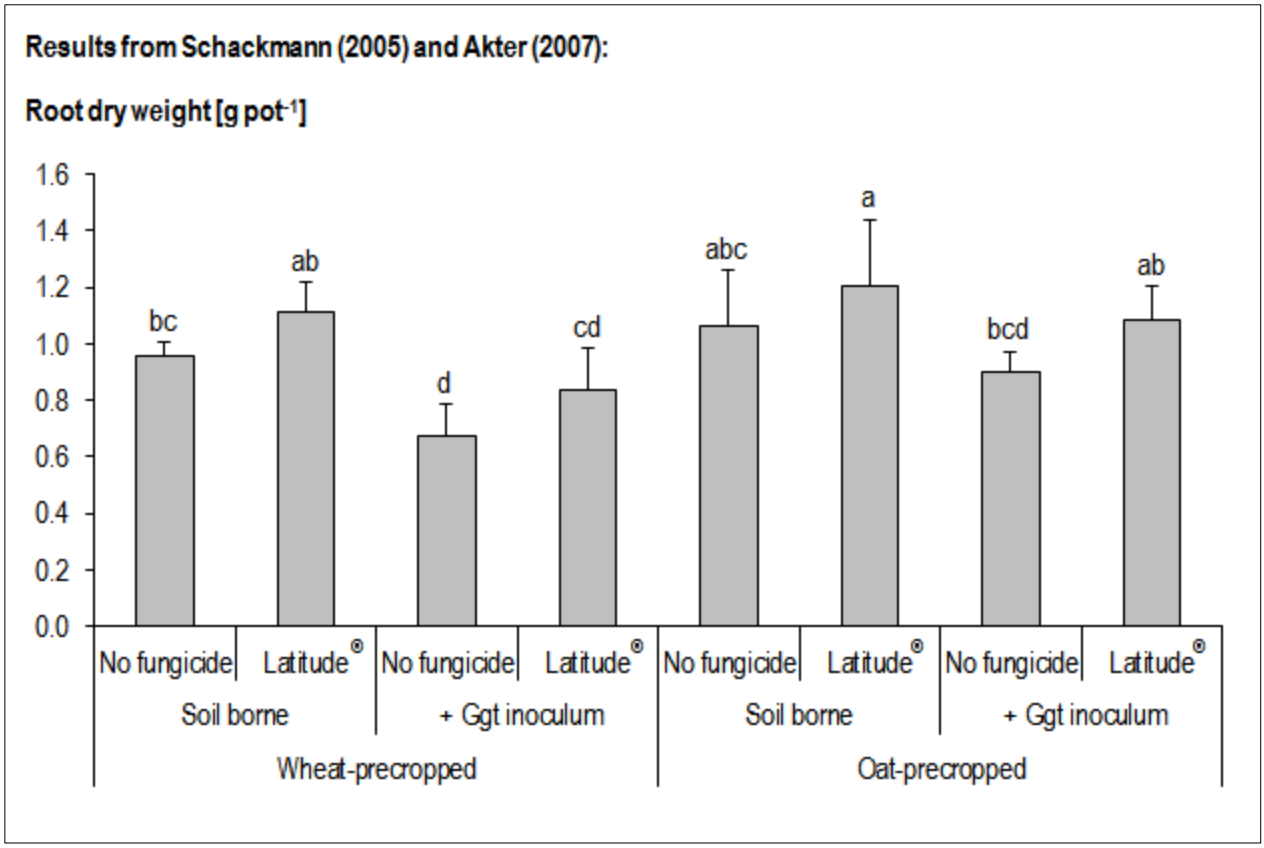

Fig. 8.24: Root dry weight of wheat var. Paragon at 42 days after sowing in a pot experiment from Schackmann (2005) and Akter (2007). The soil substrates were prepared with soil material collected on 4th October 2004 from the plough horizon (top 5-25 cm) of "control" plots in Block A of the Ggt-inoculated area of the field experiment at the research station Heidfeldhof (see Fig. 8.2) that either had been cultivated with wheat since 2002 ("wheat-precropped") or precropped with oats ("oat-precropped") in 2004. To optimize the conditions for take-all infection, the sieved soil materials (5 mm mesh) were mixed with medium sized quartz sand (1-2 mm) at 1:1 ratios. As basal fertilization $Ca(NO_3)_2$, $Ca(H_2PO_4)$, K_2SO_4, and $MgSO_4$ were supplied at rates of 100 mg N, 80 mg P,150 mg K, and 50 mg Mg kg^{-1} air-dried soil material, respectively. In addition to the soil borne infection potential, inoculum of Ggt, grown on autoclaved oat kernels, was mixed to the soil substrates at a rate of 20 mg kg^{-1} (+ Ggt inoculum) or not (Soil borne). A third treatment factor was the application of the Ggt specific fungicide Latitude® as seed dressing (50 ml kg^{-1}) or not (No fungicide). Three wheat seeds per pot were grown from August to September 2005 under similar cultivation conditions as described in Chapter 8.2.3. The moisture of the soil substrate was maintained at 20 % w/w.

Columns represent mean values ± SEM (n = 4). To facilitate the comprehensive presentation of statistical results in the graph, data were tested by two-way ANOVA considering the combination of precrop, inoculum, and fungicide treatment and the block as factors. Significant differences between mean values are represented by columns not sharing common letters in superscripts as revealed by Tukey's test ($p \leq 0.05$). In addition to these statistical results, the factorial analysis of main treatment effects indicated that root dry weights were increased in response to the oat-precrop ($p \leq 0.001$) and Latitude® ($p \leq 0.001$) treatments, but decreased in response to the supplementation with *Ggt* inoculum ($p \leq 0.001$). However, the extents of main effects were affected by a significant interaction between the levels of precrop and inoculum treatments ($p \leq 0.05$).

These variabilities in the extent of inoculum and precrop effects on the root dry matter production of wheat were associated with a significant interaction between these two factors, whereas the factor fungicide treatment did not show significant interaction effects in this regard. Together, these findings suggest that oat precrops induced lasting alterations in the soil, which protected the wheat plant against root damage by the take-all fungus even when the soil substrate was supplemented with additional *Ggt* inoculum. The take-all suppressive effect of oat precrops, therefore, could not be explained only by the non-host resistance of oats, hampering the survival and carry-over of *G. graminis* var. *tritici* and decreasing its inoculum density in the soil already before the next wheat crop is grown. Rather, there seems to be a mode of pathogen antagonism induced by the oat precrop that actively inhibits the development of take-all disease in wheat despite of high *Ggt* inoculum levels in the soil. These conclusions are also supported by the values of shoot dry weights, which were positively correlated with those of root dry weights (Pearson's correlation coefficient $r = 0.58$, $p \leq 0.001$; data not shown). As indicated by the factorial analysis of main treatment effects (Tukey's test), shoot dry matter production reflected well the growth enhancing effect of oat-precrop ($p \leq 0.001$) and Latitude® ($p \leq 0.01$) treatments, as well as the adverse effect of supplementary *Ggt* inoculum ($p \leq 0.01$).

The take-all suppressive effect of oat precrops in the pot experiment from Schackmann (2005) and Akter (2007) was also highly significant in terms of the visually estimated severity of take-all disease when determined as proportion of infected roots in relation to the total number of roots (Fig. 8.25). This variable, more clearly than root dry weights, revealed the impact of the seed treatment with Latitude® in the control of take-all disease, which, in this assessment, was particularly pronounced and only significant within the group of plants grown on the wheat-precropped substrate that had been supplemented with additional *Ggt* inoculum (third versus fourth column in Fig. 8.25). Conflicting on a first view seems to be that, in contrast to the observations made for root dry weights, supplementation with *Ggt* inoculum apparently decreased rather than increased the severity of take-all disease, when scored as proportion of take-all infected roots, as indicated by the factorial analysis of main treatment effects (Tukey's test; $p \leq 0.01$). At the level of pair wise comparisons between distinct sample groups, this effect was

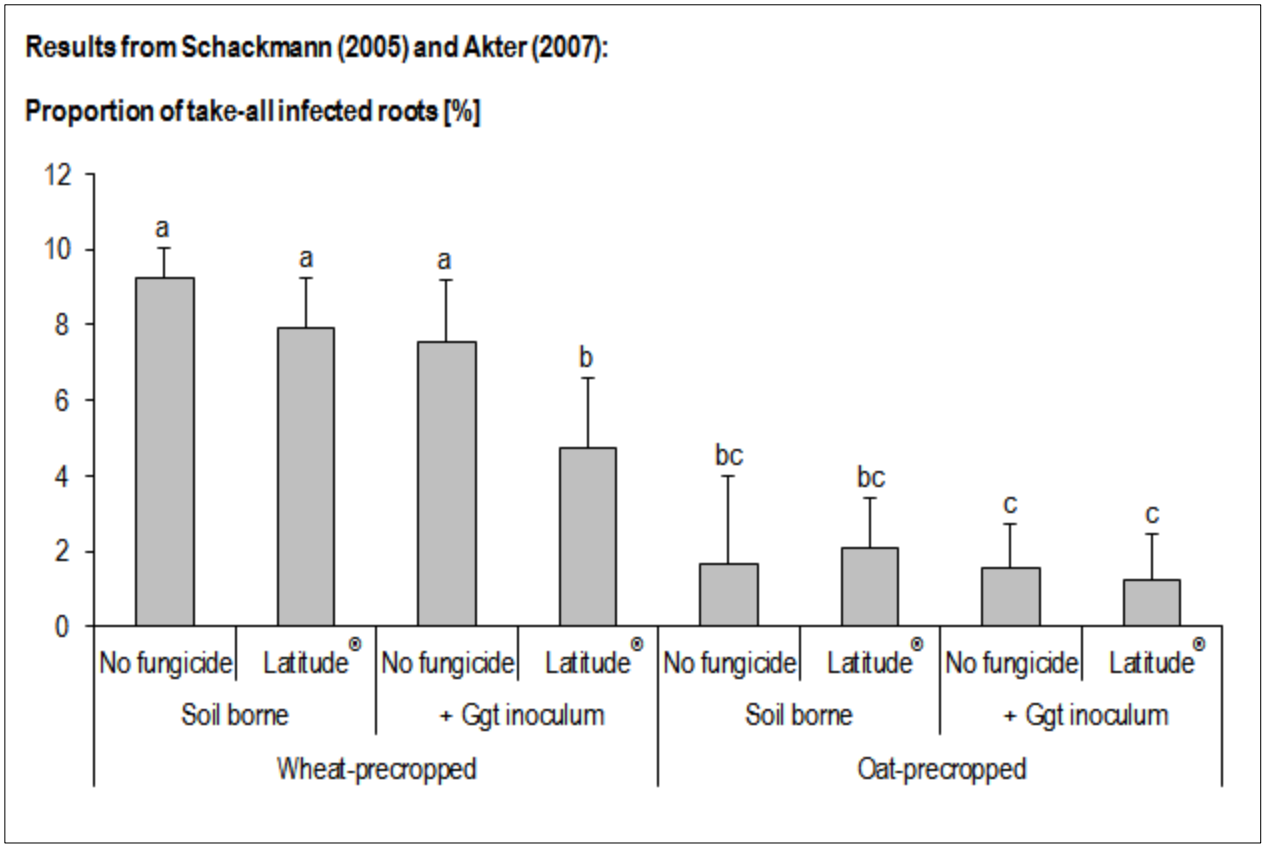

Fig. 8.25: Take-all disease severity of spring wheat var. Paragon at 42 days after sowing in a pot experiment from Schackmann (2005) and Akter (2007). As outlined in the footnote of Fig. 8.24, the plants were grown on wheat- or oat-precropped soil substrate. In addition to the soil borne infection potential these substrates were charged with additional inoculum of Ggt (+ Ggt inoculum) or not (Soil borne). A third treatment factor was the application of the Ggt specific fungicide Latitude® as seed dressing (50 ml kg^{-1}) or not (No fungicide). The severity of take-all disease was visually estimated as proportion of infected roots in relation to the total number of roots scored on a scale from 0 to 100 %.

Columns represent mean values ± SEM (n = 4). To facilitate the comprehensive presentation of statistical results in the graph, the data were tested by two-way ANOVA considering the combination of precrop, inoculum and fungicide treatment and the block as factors. Significant differences between mean values are represented by columns not sharing common letters in superscripts as revealed by Tukey's test ($p \leq 0.05$). In addition to these statistical results, the factorial analysis of main treatment effects indicated that the severity of take-all root rot was decreased in response to the oat-precrop ($p \leq 0.001$), supplementation with *Ggt* inoculum ($p \leq 0.01$), and Latitude® ($p \leq 0.05$) treatment. However, main effects were affected by significant interactions between the levels of precrop and inoculum treatments ($p \leq 0.05$), as well as between the levels of precrop and fungicide treatments ($p \leq 0.05$). Thus, the decreasing effects on take-all index observed in response to substrate supplementation with *Ggt* inoculum ($p \leq 0.001$) or the application of Latitude® ($p \leq 0.001$) were only significant within the group of wheat-precropped plants.

significant for Latitude®-treated plants grown on the wheat-precropped substrate (second versus fourth column in Fig. 8.25). This inconsistent effect of the *Ggt* inoculum, however,

must be interpreted carefully because, in contrast to an estimation of the "take-all index" as described in Chapter 8.2.4, for the data presented in Fig. 8.25 only the percentage of visibly infected roots, but not the disease induced impairment in root development was considered. Furthermore, the visible infection rate of the wheat roots was in general relatively low (maximum about 10 %) when compared to the findings of the present study (up to more than 70 %). This might have been due to differences in storage time of the soil materials and different moisture levels adjusted in the soil substrates during the experiments. While the soil materials used by Schackmann (2005) and Akter (2007) were stored for eleven months after their collection from the harvested field in October 2004 until beginning of the experiment in August 2005, in the present study soil materials collected from the cultivated field plots only one week before beginning of the pot experiment in May 2006 were used. Although persistence of the take-all pathogen in the soil without a living host plant can be for several years, a strong decrease in the amount of viable inoculum can be expected already after one year of saprophytic survival (Shipton, 1981). Schackmann (2005) and Akter (2007) maintained the water concentration of the soil substrates at 20 % w/w in relation to the readily prepared 1:1 mixtures of soil material and quartz sand. In the present work, by contrast, the moisture level of the substrates was adjusted at 20 to 25 % w/w in relation to the share of soil material only that was contained in the substrate (50:50:1 mixtures of soil material, sand, and wheat straw) and therefore by a factor of about two lower. Investigating the influence of environmental factors on disease development, Hund et al. (2000) found that the intensity of root infection by *Gaeumannomyces graminis* var. *tritici* increased with decreasing water saturation of a sand substrate over a wide range, but only extremely low moisture levels appeared to inhibit the fungal infestation of wheat roots again. From similar experiments, Augustin et al. (1997), who reported data of shoot dry weight production, but not of fungal root colonization, concluded in contrast to Hund et al. (2000) that the plant growth inhibitory effect of pathogenic isoaltes of *G. graminis* var. *tritici* was more expressed under moderately moist and wet compared to dry soil conditions. Indeed, the influence of soil moisture on take-all disease is difficult to predict and may be affected by interrelations with other factors, such as soil aeration, soil pH, or the activities of soil microorganisms, and their impact on manganese availability (Cook, 1981b; Huber and McCay-Buis, 1993;

Augustin et al., 1997). With respect to the conflicting results reported by Hund et al. (2000) and Augustin et al. (1997), therefore, it could be assumed that in roots grown under conditions of high soil moisture the formation of visible infection symptoms might be inhibited, as due to an enhanced reduction and plant availability of manganese fostering local defense reactions (Davis et al., 1976). Root growth, nevertheless, might still be suppressed considering the costs of defense against frequent attack by the pathogen, as the accumulation of defensive compounds in pathogen-challenged plant tissues depends on the supply of photosynthates and other materials (Schultz et al., 2013). This could be an explanation for the discrepancy between the adverse effect on the production of shoot and root biomass versus the apparently disease mitigating effect in terms of the visually estimated proportion of take-all infested roots, as recorded by Schackmann (2005) and Akter (2007) in response to the supplementation of the soil substrate with additional *Ggt* inoculum.

Furthermore, the results from Schackmann (2005) and Akter (2007) indicated that the take-all suppressive effect of oat precrops was associated with clear alterations in the population densities of manganese transforming microorganisms isolated from the rhizosphere (Fig. 8.26) and with corresponding changes in the manganese status of the host plant (Fig. 8.27). While the population densities of Mn-reducers were increased, those of Mn-oxidizers were decreased by factors of about three to four in response to the oat precrop, as determined by plating on agar media (Fig. 8.26). These findings support the validity of those from the present study, although in deviation therefrom Schackmann (2005) and Akter (2007) used only Bromfield's medium as basal medium for the determination of both types of microorganisms, which were counted already after two weeks of incubation at 25 °C. Supplementation with additional *Ggt* inoculum and fungicide treatments did not consistently affect the abundance of Mn-reducers or Mn-oxidizers isolated from the rhizosphere (Fig. 8.26). However, it might be that the influence of the take-all fungus on manganese oxidation in the rhizosphere was not adequately reflected by the plating assay, as Bromfield's medium has been proposed for studying manganese oxidation by pure cultures of bacteria (Bromfield, 1956). To assess the Mn-oxidizing ability of isolates of *G. graminis* var. *tritici* (*Ggt*), in contrast, Pedler et al. (1996) deployed 1:25 potato dextrose agar as basal medium.

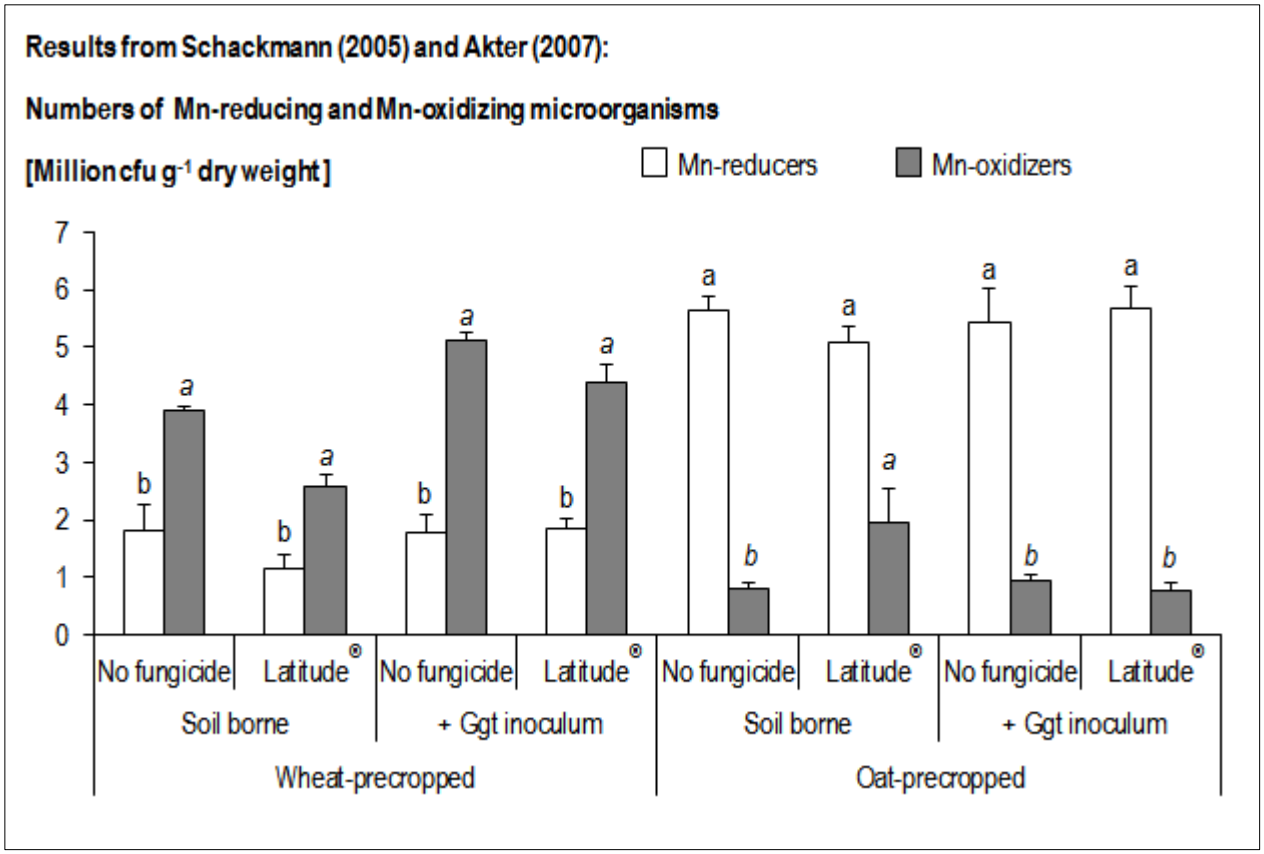

Fig. 8.26: Numbers of manganese (Mn) reducing and Mn oxidizing microorganisms isolated from the rhizosphere of wheat (var. Paragon) plants at 42 days after sowing in a pot experiment described by Schackmann (2005) and Akter (2007). As outlined in the footnote of Fig. 8.24, the plants were grown on wheat- or oat-precropped soil substrate. In addition to the soil borne infection potential these substrates were charged with inoculum of Ggt (+ Ggt inoculum) or not (Soil borne). A third factor was the application of the Ggt specific fungicide Latitude® as seed dressing (50 ml kg^{-1}) or not (No fungicide). Data are expressed as colony forming units (cfu) g^{-1} dry weight of root adhering soil substrate that were formed at 14 days after plating on a Bromfield's medium prepared with slight modification to the specifications given in Table 8.4. The basal medium was supplemented with 553 mg l^{-1} $KMnO_4$ or 203 mg l^{-1} $MnSO_4 \cdot H_2O$ for the determination of Mn reducing or Mn oxidizing microorganisms, respectively.

Columns represent mean values ± SEM (n = 4). To facilitate the comprehensive presentation of statistical results in the graph, data sets of Mn-reducers and Mn-oxidizers, after reciprocal transformation for normality, were tested separately in two-way ANOVAs considering the combination of precrop, inoculum and fungicide treatment and the block as factors. Significant differences between mean values as revealed by Tukey's test ($p \leq 0.05$) are represented by columns not sharing common letters in superscripts, printed in non-cursive or cursive characters for data sets of Mn-reducers or Mn-oxidizers, respectively. In addition to these statistical results, the factorial analysis of main treatment effects indicated that the numbers of Mn-reducers were increased in response to the oat-precrop treatment ($p \leq 0.001$). The data set of Mn-oxidizers could not be normalized for the factorial analysis by adequate transformations. Although, the numbers of Mn-oxidizers appeared to be generally decreased in response to the oat-precrop and increased in response to the supplementation with *Ggt* inoculum, the influence of the inoculum was not consistent at all combinations of the two other factors. Thus, within the group of oat-precropped and Latitude®-treated plants the supplementation with *Ggt* inoculum seemed to decrease the numbers of Mn-oxidizers.

The discerning effect of oat precrops on the counts of Mn-reducers and Mn-oxidizers isolated from the rhizosphere was associated with corresponding increases in the manganese status of the wheat plants (Fig. 8.27). Manganese concentrations in the shoot dry matter were correlated positively with the numbers of Mn-reducers (Pearson's correlation coefficient $r = 0.67$, $p \leq 0.001$) and negatively with those of Mn-oxidizers (Pearson's correlation coefficient $r = -0.68$, $p \leq 0.001$). This suggests that the improved manganese status of wheat induced by the oat precrop was due to an enhanced colonization density and activity of manganese reducing microorganisms and an opposite effect on the group of manganese oxidizing microorganism in the rhizosphere. However, also zinc concentrations in shoots were significantly increased in response to the oat precrop (Fig. 8.27) and strongly correlated with the numbers of Mn-reducers (Pearson's correlation coefficient $r = 0.85$, $p \leq 0.001$) and Mn-oxidizers (Pearson's correlation coefficient $r = -0.83$, $p \leq 0.001$) isolated from the rhizosphere. While the mobilization and immobilization of manganese in the rhizosphere is taught to be strongly governed by the proportion and activity of Mn-oxidizing bacteria (Timonin, 1946; Marschner, 1988), the plant availability of zinc has been mainly related to factors such as soil pH, the release of protons and chelators from roots, and arbuscular mycorrhizal colonization (Römheld, 1991; Marschner, 1993). The results from Schackmann (2005) and Akter (2007), therefore, suggest that in response to the oat precrop the roots of wheat were not only colonized by a more favorable microbial community increasing the supply of manganese for uptake by the plant. It rather can be assumed, that the roots were also healthier and thus allowing for an improved expression of the plant's own mechanism and its symbiotic relationship with mycorrhizal fungi that are critical in zinc acquisition.

In contrast to the observations made for manganese and zinc, Schackmann (2005) and Akter (2007) recorded no significant effect of the oat precrop on the copper status of wheat (Fig. 8.27). Because of its relative immobility in soils, a main factor determining the supply of copper to the plant is root interception, whereas iron, manganese and zinc are largely supplied through diffusion (Jarvis, 1981). Compared to these other micronutrients, the acquisition of copper by the plant is also less affected by soil pH and chemical alterations in the rhizosphere (Jarvis, 1981; Römheld, 1991). Most soils contain adequate levels of plant available copper, which is required and taken up in relatively

small quantities (Mengel and Kirkby, 2001; Yruela, 2005; Kirkby, 2012). Zinc and copper concentrations in the shoot dry matter were generally at ample to high levels (40 to 75 mg Zn kg^{-1}, 8.5 to 12.6 mg Cu kg^{-1}), well above the minimum values for adequate growth of spring wheat (25 mg Zn kg^{-1}, 7 mg Cu kg^{-1}) as reported by Bergmann (1992).

It thus appears that zinc and copper were not limiting nutrients for plant growth and resistance against the take-all pathogen, whereas manganese was found to be at a low to deficient level of about 25 mg Mn kg^{-1} shoot dry matter in the group of wheat-precropped plants without supplemental treatments (first column in Fig. 8.27).

The above described findings and considerations are leading back to the question whether the improved manganese status of the wheat plants after oat precrop was the reason for improved root health by strengthening the resistance against the take-all fungus, or whether this was just a consequence of a lower density of pathogen inoculum in the soil. Interesting in this regard is that only in the case of manganese but not zinc concentrations significant interactions between precrop and fungicide treatments ($p \leq 0.01$) and between fungicide and inoculum treatments ($p \leq 0.05$) were detected (Fig. 8.27). When comparing the effect of the fungicide treatment under different levels of the precrop factor, it was found that the application of Latitude® increased the manganese status of wheat only on the wheat precropped substrate with field borne infection potential (first versus second Mn column in Fig. 8.27). This effect was probably a consequence of killing or inhibiting the take-all pathogen, as breaking its manganese oxidizing activity may lead to an improved availability of reduced manganese for uptake by the wheat plant (Schulze et al., 1995). In contrast, the application of Latitude® did not improve the manganese status of wheat grown on the oat-precropped substrate where the severity of take-all infections was generally low (Fig. 8.25). The effect of the fungicide treatment on manganese concentrations in shoots was also not significant for the pairwise comparison within the wheat-precropped and with additional *Ggt* inoculum treated group (third versus fourth Mn column in Fig. 8.27) where the root growth was most severely inhibited (Fig. 8.24). With respect to these findings, it can be assumed that Latitude® had no direct effect on the availability of manganese in the soil substrates and was thus a suitable agent to isolate the effect of *Gaeumannomyces graminis* var. *tritici* on the manganese acquisition of wheat.

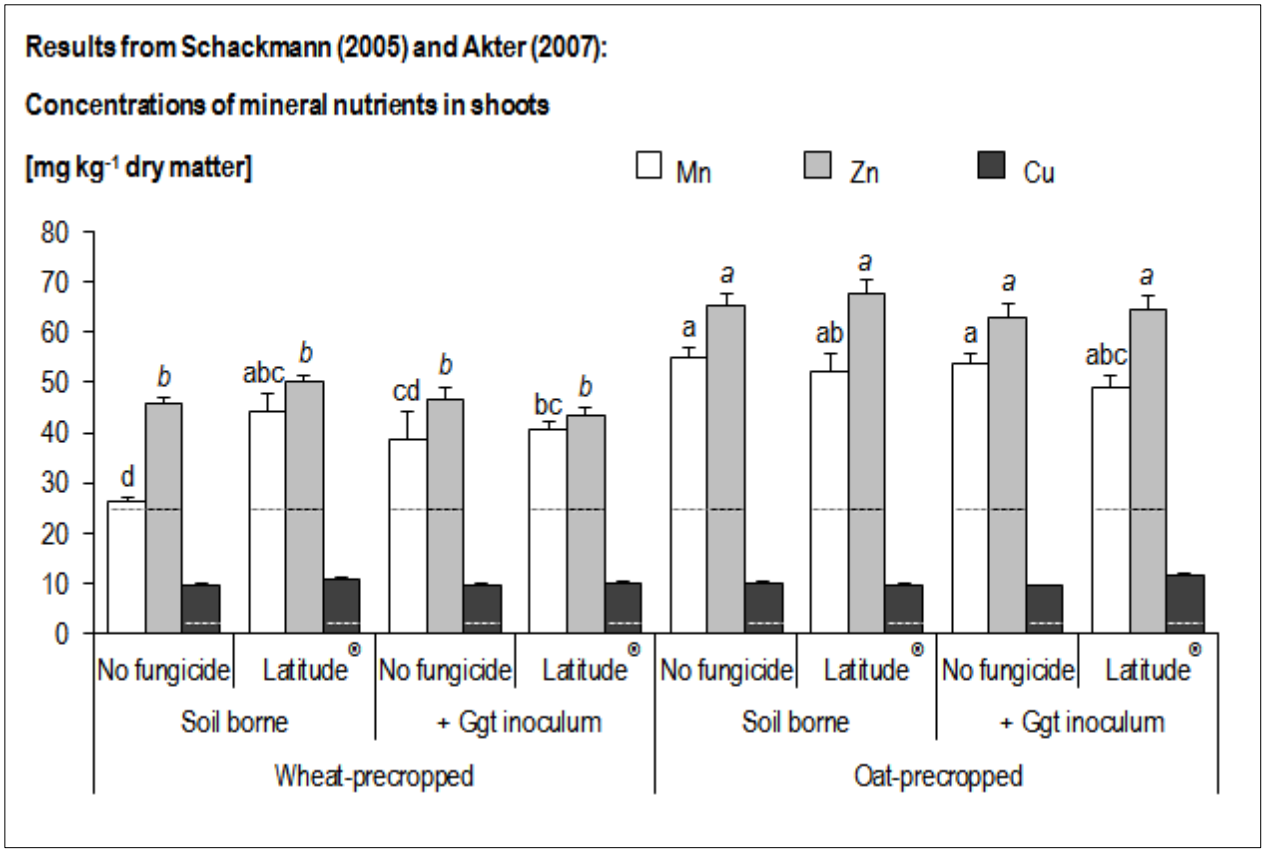

Fig. 8.27: Concentrations of manganese (Mn), zinc (Zn), and copper (Cu) in the shoot dry matter of wheat (var. Paragon) plants at 42 days after sowing in a pot experiment described by Schackmann (2005) and Akter (2007). As outlined in the footnote of Fig. 8.24, the plants were grown on wheat- or oat-precropped soil substrate. In addition to the soil borne infection potential these substrates were charged with inoculum of Ggt (+ Ggt inoculum) or not (Soil borne). A third treatment factor was the application of the Ggt specific fungicide Latitude® as seed dressing (50 ml kg^{-1}) or not (No fungicide). The dashed horizontal lines (----) within the columns indicate the concentration of the respective mineral nutrient in the above ground biomass of spring wheat that is critical for deficiency during tillering as reported by Bergmann and Neubert (1976) and Bergmann (1992).

Columns represent mean values ± SEM (n = 4). To facilitate the comprehensive presentation of statistical results in the graph, data sets of Mn, Zn, and Cu concentrations were tested separately in two-way ANOVAs considering the combination of precrop, inoculum and fungicide treatment and the block as factors. Significant differences between mean values as revealed by Tukey's test ($p \leq 0.05$) are represented by columns not sharing common letters in superscripts, which are printed in non-cursive or cursive characters for data sets of Mn or Zn concentrations, respectively. Mean values of Cu concentrations were not found to be significantly different by the two-way ANOVA. In addition to these statistical results, the factorial analysis of main treatment effects indicated that Mn and Zn concentrations were significantly increased in response to the oat-precrop treatment ($p \leq 0.001$). However, in the case of Mn concentrations main effects were affected by significant interactions between the levels of precrop and fungicide treatments ($p \leq 0.01$), as well as between the levels of fungicide and inoculum treatments ($p \leq 0.05$). For the data set of Cu concentrations, a significant interaction between the levels of precrop, inoculum and fungicide treatments ($p \leq 0.01$) indicated that the effect of one factor, i.e. fungicide treatment, was not consistent at all combinations of the two other factors. According to the evaluation of interactions, Cu concentrations were increased in response to the application of Latitude® within the group of oat-precropped and *Ggt*-inoculated plants ($p \leq 0.01$).

Furthermore, the results from Schackmann (2005) and Akter (2007) also showed that supplementation with additional *Ggt* inoculum did not decrease the abundant manganese status (Fig. 8.27) and the root biomass production (Fig. 8.24) of wheat grown on the oat-precropped substrate. These findings provide strong evidence that a decreased inoculum density of the take-all fungus after oat-precrops cannot be the only cause for improved manganese acquisition by the wheat plant. In fact, it seems that a lasting influence of oat on the composition of the soil microflora that established in the rhizosphere of the subsequently grown wheat plants was efficient to ensure an adequate manganese status and a low incidence of take-all disease in wheat even under conditions where the density of *Ggt* inoculum was additionally enhanced.

In accordance with these conclusions, oat varieties susceptible to manganese deficiency, which are typically characterized by harboring denser populations of manganese oxidizing bacteria in the rhizosphere than resistant varieties (Timonin, 1946), have been reported to be of weak take-all resistance and unable to provide significant protection against take-all disease to a subsequent wheat crop (Huber and McCay-Buis, 1993). Moreover, when grown after take-all infected wheat crops, soybeans, which may be colonized by the pathogen but are not susceptible to take-all disease (Roy et al., 1982), showed retarded growth and severe manganese deficiency, which was associated with enhanced population densities of manganese oxidizing microorganisms isolated from the rhizosphere (Huber and Mburu, 1983; cited in: Huber, 1989). Likewise, severely take-all infected wheat plants showed enhanced population densities of manganese oxidizing bacteria in the rhizosphere when compared to less diseased plants (Huber, 1989; Huber and McCay-Buis, 1993). Short sequences of wheat monocropping (over two to five seasons), thus, not only favor the propagation of the take-all fungus, but also seem to induce the establishment of a deleterious microflora where manganese oxidizing bacteria are predominant. The decline of take-all disease, which may emerge after extended monocropping of wheat, however, is thought to involve a reconditioning decrease in the population of manganese oxidizing microorganisms together with a rising prevalence of antagonistic, plant-protecting rhizobacteria (Huber and McCay-Buis, 1993; Sanguin et al., 2009).

The results from Schackmann (2005) and Akter (2007) as well as those of the present study do not imply an exact quantitative determination of *Gaeumannomyces graminis* in the soil substrates. Direct counts of fungal propagules in soil are difficult to perform and may not provide a reliable measure of inoculum levels (Herdina et al., 2004). Evidence for the aggressiveness of the additionally supplemented *Ggt* inoculum in the experiment from Schackmann (2005) and Akter (2007) can be seen in its adverse influence on root biomass production (Fig. 8.24). However, it must be considered that the pathogenicity of the take-all fungus can decrease when it is repeatedly subcultured or stored on agar media, so that it is possible that the *Ggt* inoculum supplied to the soil substrates formed a cultural variant with altered virulence compared to the natural inoculum (Chambers, 1970; Naiki and Cook, 1983). For these reasons, an exact quantification to what extent the density of field borne take-all inoculum had been decreased due to the break crop effect of oats, or to which degree the inoculum level was increased by supplementation with additional *Ggt* inoculum, could further improve the validity of the presented results. In recent years, molecular approaches based on the extraction of DNA have been further developed to estimate the quantity of the take-all fungus in soil such as by means of slot-blot hybridization (Herdina and Roget, 2000; Herdina et al., 2004) or real-time PCR (Bithell et al., 2009; Bithell et al, 2012). Certainly, also these methods have inherent biases such as concerning the collection of representative soil samples, extraction of DNA from soil, interference of co-extracted humid acids in subsequent steps of the molecular analyses, and finally the discrimination of virulent from avirulent or even dead mycelium (Herdina and Roget, 2000; Herdina et al., 2004; see also Chapter 7.1.2). Nevertheless, based on the correlation with a soil bioassay, Herdina and Roget (2000) proposed to develop a rapid DNA-based assay to estimate the level of *Ggt* inoculum in the soil with respect to the expected take-all severity in commercial wheat fields. While the correlation between the soil bioassay and DNA-based assay was high when the majority of soil samples was collected from a similar region, and therefore less diverse (Herdina et al., 1997), a poor correlation was found when the results obtained from a wider range of soil types were used as database for disease prediction (Herdina and Roget, 2000). This divergence of outcomes was likely to be attributable to variation in factors, such as soil suppressiveness or conductiveness to the growth of the fungus, soil pH, and aggressiveness of the soil

borne pathogen population, which were not considered in the linear regression model (Herdina and Roget, 2000). According to this assumption, a survey from Lebreton et al. (2004; 2007) gave evidence that models accounting for divergence in pathogenicity within a given pathogen population, rather than focussing on the total amount of *Ggt* mycelium, and integrating the influence of microbial antagonism and other environmental variables on population dynamics under cycles of successive crops are necessary for a more reliable prediction of take-all severity in wheat. The results from Lebreton et al. (2004; 2007), therefore, agree with the hypothesis that the activities of antagonistic microorganisms, such as manganese reduction or the production of antibiotics, play a critical role in take-all suppression by their inhibitory effect against the resident pathogen inoculum. Notwithstanding to this, quantitative molecular analyses (real-time PCR assays) performed by Bithell et al. (2012) also indicated that the mean concentration of *Ggt* DNA was decreased by factor ten after oat compared to the host cereals (wheat, barley, and triticale), whereas the abundance of *Gga*, the avenacin inactivating and oat infecting variety of *G. graminis* (Turner, 1961; Crombie et al., 1986), was increased. Evidence for the importance of avenacins has been provided by investigations with avenacin-deficient mutants of the diploid oat species *Avena strigosa*, also known as black oat (Santi et al., 2003), which were severely compromised in resistance to *Ggt* and a range of other fungal root pathogens (Papadopoulou et al., 1999; Trojanowska et al., 2001). In contrast, a wild type cultivar of black oat, grown as a precrop for wheat, appeared to provide similar protection against *Ggt* and induced similar increases in the population densities of Mn-reducers isolated from the wheat rhizosphere like a variety of common oats (*Avena sativa* var. Jumbo; Akter, 2007). Molecular studies to quantify the amount or, more appropriately considering that distinct strains are capable to perform reducing as well as oxidizing processes (Huber and MacCay-Buis, 1993), the expression of microbial genes involved in manganese transforming processes as induced by oat precrops in the rhizosphere of wheat have not been described in the known literature. Because the accumulation of avenacins in oats is mainly restricted to the epidermal cell layer of the root tips (Osbourn et a., 1994; Osbourn et al., 2003; Qi et al., 2004), the effect of avenacin on *Ggt* might be independent of the suppression of other Mn-oxidizers in the rhizosphere by toxic root exudates described as glycocyanides (Johal and Huber, 2009). The synthesis

of avenacins, however, might be affected by the plant availability of manganese, as oats grown on soils low in manganese concentrations, characteristically show enhanced take-all susceptibility (Huber and McCay-Buis, 1993).

Taken together, the above presented and discussed results from conventional bioassays, *in vitro* tests and molecular analyses, strongly suggest that lasting alterations in microbial populations favoring manganese reducing processes in the rhizosphere and the production of avenacins as fungitoxic defense compounds play important roles in the suppression of *Gaeumannomyces graminis* var. *tritici* by oats. Both modes of action not only due to their direct, but also due to their cumulative effect may be decisive for the capacity of oat precrops to induce a lower take-all severity in subsequent wheat. Resistance conferred by such multi-factorial traits involving layers of distinct processes is regarded as highly effective and robust against circumvention by pathogenic invaders (Holub and Cooper, 2004; Maule et al., 2007; Kou and Wang, 2012). To overcome these defensive barriers through the coevolutionary acquisition of virulence factors in individual races or strains of a given pathogen species, typically requires a considerable degree of specification, which is associated with a decrease or loss of pathogenicity to other plant species (Thordal-Christensen, 2003; Nürnberger and Lipka, 2005; Jones and Dangl, 2006). This also can be observed for *Gaeumannomyces graminis* var. *avenae* that possesses the enzymatic property to inactivate avenacin and infect oats (Turner, 1961), but, when occurring in wheat fields, is less competitive than *Ggt* (Bithell et al., 2012). Because the expression of multi-factorial disease resistance in plants is sensitive to environmental conditions and has a complex genetic basis that is not as well understood as monogenic resistance, it is difficult to be improved by breeding programs (McDonald and Linde, 2002; Koebner and Summers, 2003; St.Clair, 2012; Kou and Wang, 2012). From an agronomic perspective, it furthermore must be considered that the multi-factorial effect of oat precrops to suppress take-all disease in wheat is unlikely to be effectually compensated for by single substitutional measures in crop management. This conclusion is clearly supported by the results obtained under field conditions, as described in the following section.

8.4.7 Effectiveness of oat-precrops and supplemental treatments to suppress take-all disease in wheat under field conditions

In the fourth rotational year (2005), the field experiment conducted at the research station Heidfeldhof showed a significant decrease in proportional root damage of wheat by take-all disease from more than 60 % observed in the wheat-precropped (w-w-w-w) to less than 10 % in the oat-precropped (w-w-o-w) trial area (Schackmann, 2005; Fig. 8.28). Likewise, oats grown after wheat precrops (w-w-w-o) showed a low susceptibility to the take-all pathogen, thus indicating that *Ggt* was not a frequent genotype of the *G. graminis* population.

In contrast to the strong phytosanitary effect of oats, there was no significant bio-control effect in response to the application of different supplemental treatments on the wheat-precropped plots as shown in Fig. 8.28 (Schackmann, 2005). In detail, the application of Entec® 26 (EuroChem Agro GmbH, Mannheim, Germany), a stabilized ammonium sulfate plus ammonium nitrate fertilizer, manganese sulfate, or the microbial bio-effector product Vitalin T50, either single or combined with Vitalin SP11 (see Table 4.1), were tested. According to the literature, ammonium nutrition can be assumed to contribute to the control of take-all disease by several modes of action. A decreased rhizosphere pH induced by ammonium supply, as due to an enhanced net release of H^+-ions, may increase the plant availability of manganese and other mineral nutrients (e.g.: P, Zn, Fe) with specific functions in disease resistance (Marschner and Römheld, 1983; Römheld, 1986; Schubert and Yan, 1997; Chapter 7). Furthermore, the application of ammonium in contrast to nitrate fertilizers has been reported to induce a suppression of manganese oxidizing and deleterious, but an increase in the populations of manganese reducing and antagonistic microorganisms in the rhizosphere (Sarniguett et al., 1992b; Huber and McCay-Buis, 1993). Possible implications of manganese fertilization (Chapter 8.4.2, 8.4.3) or microbial bio-effectors, such as *Trichoderma* fungi contained in Vitalin 50 (Chapter 8.4.4) or strains of *Bacillus*, *Streptomyces* and *Pseudomonas* species (Chapter 7.4) contained in Vitalin SP11, concerning the control of take-all disease have been discussed before.

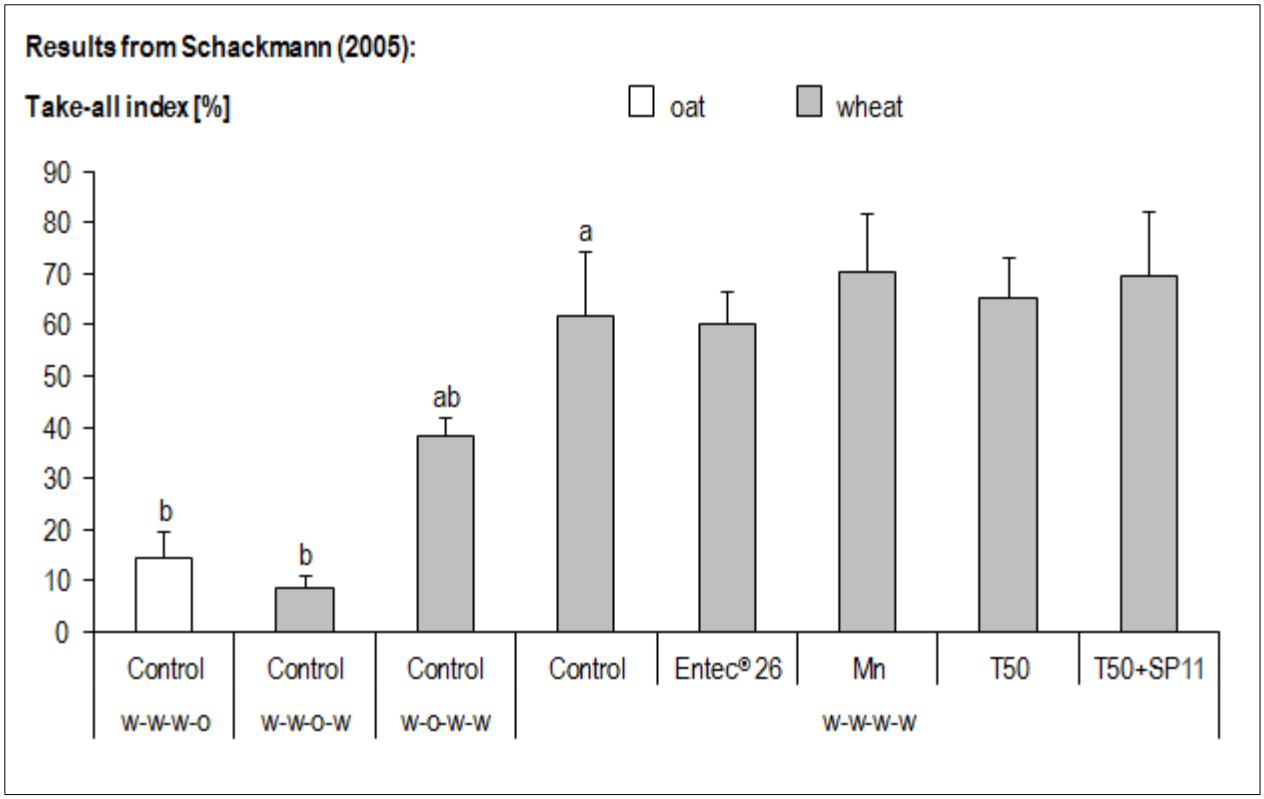

Fig. 8.28: Severity of take-all root rot expressed as visually estimated degree of proportional root damage (take-all index) in oat var. Jumbo and winter wheat var. Dekan grown in the fourth year (2005) of the field experiment conducted at the research station Heidfeldhof, University Hohenheim. Oat was grown after three years of wheat crops (w-w-w-o) and wheat after sequences of wheat-wheat-oat (w-w-o-w), wheat-oat-wheat (w-o-w-w) or three years of wheat crops (w-w-w-w). Nitrogen was fertilized in split application at rates of 80 (06th April 2005) and 60 kg N ha^{-1} (1st June 2005) in the form of ammonium sulfate plus ammonium nitrate with nitrification inhibitor (Entec® 26, EuroChem Agro GmbH, Mannheim, Germany) or as calcium nitrate (CalciNit™ Hydro, Yara, Dülmen, Germany; all other variants inclusive Control). Treatments with manganese sulfate at rates of 2 kg Mn ha^{-1}, Vitalin T50 at rates of 2 kg ha^{-1}, or Vitalin T50 in combination with Vitalin SP11 at rates of 2 kg ha^{-1} of each product were performed as spray application at tillering (22nd April 2005) and again at inflorescence emergence (2nd June 2005). For distribution on top of the plants, watering cans with spray roses and 10 liters of water per plot of 18 m^2 were used. At least 12 subsamples of roots per plot were collected from 07th June until 12th August 2005 to assess the severity of take-all root rot according to BBA (1999). The graph shows the results obtained from the area without application of take-all inoculum (see subtitle of Fig. 8.2).

Columns represent mean values ± SEM (n = 3). The effect of different precrop treatments was analyzed by a mixed model analysis of variance followed by Tukey-Kramer multiple comparison test to account for the lack of randomization of the precrop factor (Piepho et al., 2003 and 2004; personal communication Katharina Emrich). Significant differences between precrop treatments are indicated by values not sharing common letters ($p \leq 0.05$). There were no significant differences between the different levels of supplemental treatments (Control, Entec® 26, Mn, T50, T50+SP11) within the sample group of wheat grown after three years of wheat crops (w-w-w-w), as indicated by two-way ANOVA considering supplemental treatment and block as factors.

While most of the literature cited in these connections refers to experiments conducted under controlled conditions, Huber and McCay-Buis (1993) presented visual evidence that take-all disease in field-grown wheat was less severe in response to the application of anhydrous ammonia fertilizer with nitrification inhibitor to winter wheat in autumn, or seed treatments with manganese sulfate or manganese reducing bacteria. Although exact statistical data are missing, the field observations from Huber and McCay-Buis (1993) suggest that also in the experiment conducted at the research station Heidfeldhof the effectiveness of the tested supplemental treatments could have been improved by an application contemporary to the time of sowing. Epidemiological model calculations for the interaction between host plants, soil-borne pathogens and microbial antagonists suggest that the success of bio-control strategies depends on the mechanisms of antagonism and on population dynamics. Sharp transitions between total effectiveness (i.e. pathogen eradication) and total ineffectiveness can occur depending on the initial amounts of pathogen and biological control agent present in the habitat. Nevertheless, to decrease the disease severity against a high density of the pathogen, the assertiveness (bulking-up efficiency) of the antagonist is of particular importance (Cunniffe and Gilligan, 2011). Factors that can hamper the competitive rhizosphere colonization and effectiveness of microbial bio-effectors applied by seed or soil treatments under field conditions have been discussed in Chapter 6.4.

With respect to the in-effectiveness of fertilization with Entec® 26 (total nitrogen content 26 %) it furthermore needs to be considered that this product contains not only 18.5 % nitrogen in the form of ammonium, but also 7.5 % nitrogen in the form of nitrate, besides 13 % sulfur and the nitrification inhibitor 3,4-dimethylpyrazole phosphate (Zerulla et al., 2001; EuroChem Agro GmbH, 2012). Due to the high mobility of nitrate compared to ammonium in soils it can be suspected that in the short term after application, Entec® 26 mainly functions as a nitrate fertilizer, because predominately this fraction of its total nitrogen content may rapidly reach the root surface by diffusion and with the mass flow of water (Subbarao et al., 2006). Especially during early growth phases when the rooting density of wheat is still low, a fertilizer in which nitrogen is contained only in the form of ammonium with nitrification inhibitor and that is applied in close vicinity to the root might be favorable to achieve considerable responses in rhizosphere acidification (White

et al., 2013). The exact modes of action of 3,4-dimethylpyrazole phosphate on nitrifying bacteria and how this nitrification inhibitor interacts with other microorganisms in the soil are poorly described in the literature, so that a direct influence on manganese transforming, deleterious or beneficial populations can currently not be excluded (Barth, 2006; Kleineidam et al., 2011).

Likewise, the modalities of application may be decisive for the success of manganese amendments to decrease the severity of take-all disease. Effects achieved with manganese treatments under controlled conditions, such as in the present and other pot experiments (Fig. 8.12; Rengel et al. 1993), may not directly translate to field experiments (Piper, 1931) due to the influence of environmental factors, such as different rooting density, aeration and redox conditions, on manganese transforming processes in soils. Even direct applications of manganese to soils, seeds or on the foliage may be of marginal effectiveness due to the rapid microbial oxidation and limited phloem mobility of this micronutrient (Huber, 1989; Riesen and Feller, 2005). As reported by Huber and Wilhelm (1988) and Huber (1989), 0.8 kg Mn ha^{-1} applied as seed treatment in wheat fertilized with stabilized anhydrous ammonia achieved a similar mitigation of take-all severity like 1.0 kg Mn ha^{-1} coinjected with stabilized anhydrous ammonia, or 20 kg $MnSO_4$ (7.3 kg Mn) ha^{-1} broadcast distributed and disc incorporated. These findings indicated that the effectiveness of fertilized manganese could be improve by accompanying measures to facilitate its lasting availability for root uptake (Huber, 1989).

Nevertheless, more consistent results than with the application of manganese fertilizers were achieved with wheat seeds that had been selected for containing higher concentrations of manganese (Huber and McCay-Buis, 1983). The importance of an improved physiological manganese status of wheat after oat-precrops for the resistance against fungal pathogens also appeared evidently in the third year of the field experiment conducted at the research station Heidfeldhof. When in July of that year (2004) an epidemic of leaf blotch induced by species of *Septoria* and/or *Stagonospora* fungi (Eyal, 1999; Solomon et al., 2006) developed in the trial area, wheat grown after wheat precrops was highly susceptible to this disease, whereas in wheat after a precrop of oats the proportion of infested flag leaf area was lower by about 50 % (Fig. 8.29a).

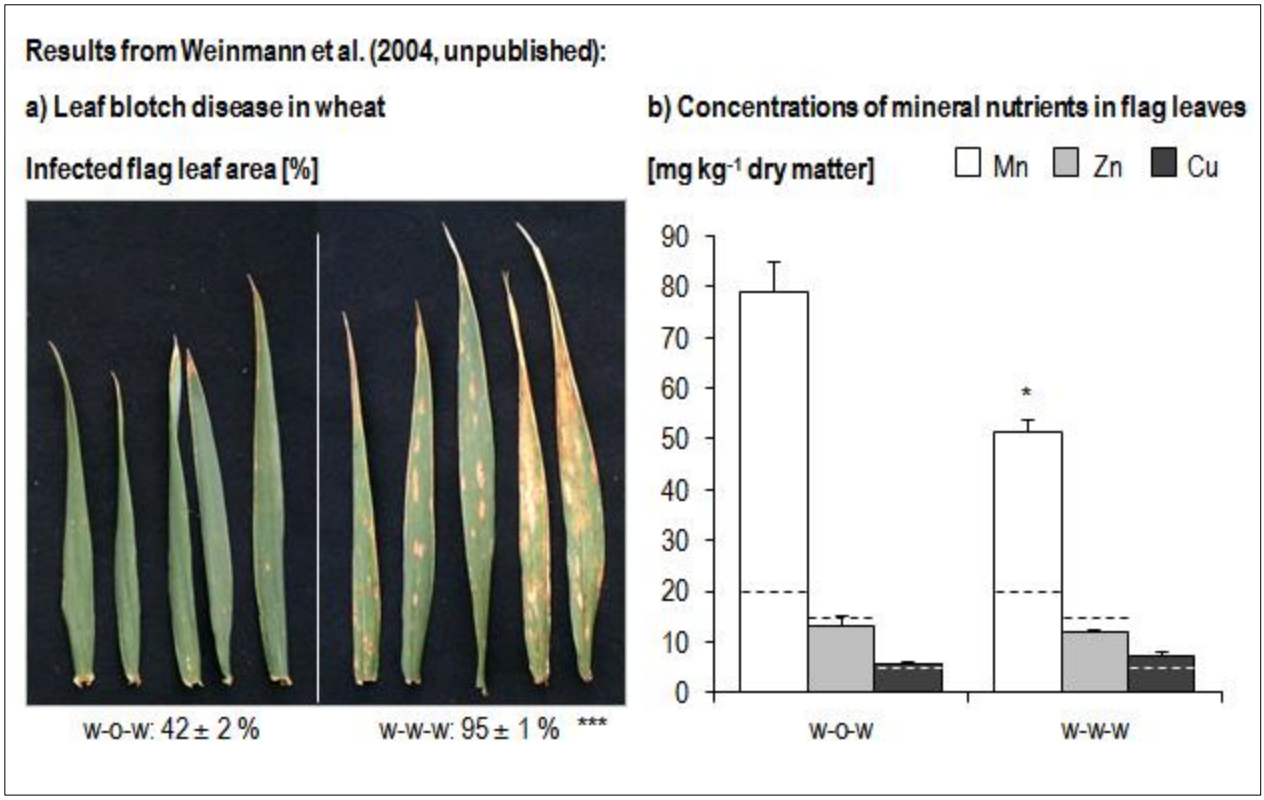

Fig. 8.29: Severity of leaf blotch disease induced by phytopathogenic fungi such as species of Septoria and/or Stagonospora (a) and concentrations of manganese (Mn), zinc (Zn) and copper (Cu) (b) in flag leaves of spring wheat var. Triso. Plants were grown on oat- (w-o-w) or wheat-precropped (w-w-w) "control" plots without supplemental treatments in the third year (2004) of the field experiment conducted at the research station Heidfeldhof, University Hohenheim. Inoculum of Gaeumannomyces graminis var. tritici had been applied homogenously to the tillage horizon of the soil in December 2002 before sowing of the second wheat crop in the rotation and again as a broad cast application in April 2003. Fifteen flag leaves per plot were collected for mineral nutrient analyses on 14th July 2004 during the milk stage of kernel development. The proportion of leaf area affected by leaf blotch disease was estimated on 22nd July 2004 according to visual rating schemes provided by Bartels and Backhaus (2000). The dashed horizontal lines (----) attendant to the columns indicate the concentration of the respective mineral nutrient in flag leaves of wheat considered as sufficient at boot to heading stage as reported by Mengel (2013). The figure shows results obtained from the area with application of take-all inoculum (see Fig. 8.2).

Percentages and columns represent mean values ± SEM (n = 3). Data sets of leaf blotch disease incidence and Mn, Zn, or Cu concentrations if flag leaves were tested separately in two way ANOVAs considering precrop treatment and block as factors. ANOVAs that indicated statistically significant differences were followed by Tukey's test to compare the mean values of oat- and wheat-precropped sample groups as indicated by asterisks (*$p \leq 0.05$, *** $p \leq 0.001$). Groups of mean values without asterisks are not significantly different ($p > 0.05$). Results must be interpreted with care, as the experiment was not completely randomized for technical reasons.

The analysis of mineral nutrient concentrations in flag leaves collected eight days before the rating of disease symptoms, furthermore showed that the manganese status of wheat

was increased by about 50 % in response to the oat precrops compared to the wheat precrops, while there was no clear influence of the precrop on zinc and copper concentrations (Fig. 8.29b). Accordingly, there was a strong negative correlation between leaf blotch infestation rates and manganese concentrations in flag leaves (Pearson's correlation coefficient $r = 0.94$, $p \leq 0.01$), but no evidence for such interdependencies with the zinc or copper status of the wheat plant. These findings support the importance of an improved manganese status of wheat after oat precrops for the host mediated resistance against fungal diseases, because oat induced improvements in rhizosphere populations of manganese reducing and antagonistic microorganisms may not directly affect the invasion of pathogens in the phyllosphere (Andrews, 1992; Sivasithamparam, 2002).

8.4.8 Conclusions

A main conclusion drawn from the results of the above presented pot and field experiments is that oat precrops decreased the severity of take-all disease in wheat by specific multi-faceted modes of action, for which distinct supplemental treatments could not completely compensate. Nevertheless, the gradual mitigation of take-all severity produced by treatments with manganese sulfate and microbial bio-control agents in the pot experiment indicated that both the availability of manganese and the microflora are important factors that depending on the environmental conditions can be manipulated effectively for disease suppression. Notably, the application of manganese sulfate to the wheat-precropped soil substrate improved the root biomass production and decreased the severity of take-all root rot, but these effects did not result in increased shoot growth. A microbial preparation based on a strain of *Pseudomonas* (Proradix®) enhanced the root and shoot growth, the mycorrhization, and the manganese concentrations measured in root samples of wheat, but was less effective to decrease the visually estimated proportion of take-all infected roots. Under field conditions, however, supplemental treatments such as applications of manganese sulfate, stabilized ammonium sulfate plus ammonium nitrate fertilizer (Entec® 26), or microbial preparations (Vitalin SP11, Vitalin T50), showed no significant effect on take-all disease.

In contrast to applications of manganese sulfate or microbial preparations, oat precrops decreased the severity of take-all disease and improved the manganese status, as analyzed in in shoot samples, of wheat plants grown under greenhouse and field conditions, which could be attributed to lasting alterations in the population of manganese transforming microorganisms in the wheat rhizosphere. The results from Schackmann (2005) and Akter (2007) furthermore revealed that the oat-induced alterations in the soil microflora supported the manganese acquisition and healthy root growth of subsequent wheat even when additional *Ggt* inoculum was added to the soil substrate. The predominance of manganese reducing antagonists in the rhizosphere of wheat, as specifically induced by oat precrops, therefore, seems to be more important for the suppression of take-all disease than a decreased level of pathogen inoculum in the soil, as due to the rotational break crop effect of oats. This conclusion supports the importance of a protective rhizosphere colonization by beneficial microorganisms as counterbalance against harmful components of the soil microflora for healthy plant growth in pathogen-infested soils (Hiltner, 1904; Brimecombe et al., 2001). A critical prerequisite in this regard seems to be that the disease suppressive microflora is already present in the soil and establishes in the rhizosphere of the host plant before the pathogen infects the root. To that point, Dixon and Tilston (2010) have argued that *Ggt* at first grows on the root surface forming ectotrophic runner hyphae before invading the root cortex, where this pathogen then could be protected from antagonism by other microorganisms.

For the oat precrop itself, however, the situation is different when it is grown initially in a *Ggt*-infested soil, because the developing oat root then first needs to procure a take-all suppressive rhizosphere population by selective proliferation of beneficial root associates out of the unbalanced microflora that is characteristic for diseased soils. During this process, providing an extended time gap where the oat root cannot yet be protected by a disease suppressive microflora in the rhizosphere, the oat plant necessarily requires an additional mechanism of defense against infection by the take-all pathogen. These considerations may explain that the production of avenacins is a critical feature for the resistance of oat plants against *Ggt*. *In vitro* experiments with oat genotypes defective in the production of avenacins (Osbourn, 1994; Papadopoulou et al., 1999), as well as with *Gaeumannomyces graminis* var. *avenae*, the variety of the take-all fungus that is able to

inactivate avenacin and infect oats (Turner, 1961; Crombie et al., 1986), have proven the defense function of these fungi toxic compounds. Nevertheless, also for oats an adequate supply of manganese, such as accomplished by manganese reducing rhizobacteria, seems to be a prerequisite for sustained resistance to infection by *Ggt*. An insufficient manganese status may not only hamper the synthesis of avenacins, which is mainly restricted to the epidermal cell layer of the root tips, but also the expression of other resistance mechanisms in the oat plant. There are no studies known which have assessed whether avenacin-deficient oats would similar like wheat benefit from the precrop effects of avenacin producing oats.

Bioassay, pot and field experiments gave different results with respect to the control of the take-all pathogen, suggesting that variable environmental factors have a critical influence on the effectiveness of supplemental treatments. In that regard, Huber and McCay-Buis (1993) concluded that the severity of take-all disease is determined by the interaction of favorable and unfavorable components of the plant, the fungal pathogen and their abiotic and biotic environment. Severe take-all infection thus appears only, but almost certainly when encouraged by disease-conductive conditions. Under take-all suppressive conditions, in contrast, the pathogen *Gaeumannomyces graminis* var. *tritici*, which is widely distributed in cereal-cropped soils worldwide, may still persist in a latent stage (Heß et al., 1998; Hund et al., 1998; Wilmsmeier, 2002; Heß, 2006).

For agricultural practice, this implies that effective control of take-all disease could be achieved when the crop management is adapted in a way to compound the disease suppressive factors through integrated approaches, thereby also supporting single treatment measures to entry into force. Direct applications of manganese fertilizers, for instance, may result in adequate take-all control in situations where the influence of microbial and/or physicochemical soil factors is favorable to keep the manganese in plant available form, whereas modification of the environmental conditions to facilitate the availability of manganese may be required in others (Huber and Wilhelm, 1988). Due to the central role of manganese in resistance mechanisms of the plant and the interrelationships with environmental factors affecting the acquisition of manganese by the plant, Huber and McCay-Buis (1993) traced the development of take-all disease

mainly to deficiency of this element. However, these authors also anticipated that especially the biotic environment through its interaction with the plant, pathogen and abiotic environment exerts a dynamic influence on manganese availability and take-all disease. This leads to the concept that, although modified by abiotic factors, the suppressiveness of soils against major pathogens as a given component of the biological diversity in soils, intrinsically results from an adequate balance between functional microbial groups. Disease incidence, on the contrary, is the expression of microbial imbalance allowing for the prevalence of deleterious populations (van Bruggen and Semenov, 2000; Alabouvette et al, 2004). To maintain healthy growth of wheat in unbalanced crop rotations and monocultures on soils infested by the take-all fungus may therefore not only require an in-depth understanding of the role of manganese and other mineral nutrients, but also of the interference of the biotic environment in host-parasite interactions. This includes beneficial and deleterious microorganisms, which by their interdependent population dynamics and activities in the soil, and particularly in the rhizosphere, can affect the pathogenesis of take-all disease through alterations in the availability of mineral nutrients as well as other intricate modes of action like the induction of resistance, phytohormonal effects, or antagonistic functions. The approach of applying microbial bio-control agents typically relies on individual strains that can be propagated and formulated in technical production processes. However, much more research is necessary not only to understand by which specific mechanisms these microorganisms can contribute to the suppression of pathogenic diseases, but also how these mechanisms are affected by factors of the physical, chemical and biotic environment (Hornby et al., 1998; Ruocco et al., 2011). Further efforts are required to translate the knowledge obtained from such research into improved crop management strategies that integrate the influence of environmental and agronomic factors. The results of present field experiments suggest that recommendations to minimize take-all disease by distinct supplemental treatments, such as the application of fertilizers or microbial bio-control agents, cannot be made with confidence without comprehending the influence environmental conditions in a given situation. Crop rotation with oats, in contrast, proved to be a reliable basis to ensure the sustainability of wheat production in terms of healthy plant growth by versatile modes of action maintaining a balanced diversity of functional

groups of soil microbes, which may also support the specific effectiveness of supplemental treatments.

D CONCLUSIVE PART

9 General discussion

9.1 General conclusions drawn from the current stage of research on the utilization of bio-effectors in a sustainable agriculture

Although the possible use of bio-effectors, such as living microorganisms and active natural compounds, to improve the mineral nutrition and healthy growth of crop plants is extensively recognized in the literature, few studies have documented the successful application of specific microbial or other biological preparations as viable alternatives to conventional fertilizers or pesticides in agricultural practice. Among the rare exceptions are rhizobial inoculants for adequate nodulation and nitrogen fixation in leguminous crops, *Bacillus thuringiensis*-based insecticides, and the use of pheromones to disturb the reproduction of insect pests, as reviewed in Chapter 1. Examples described in the following Chapters 2 to 8 show how complex the interactions with factors of the abiotic and biotic environment are that can favor or hamper the effectiveness of biological approaches to facilitate the acquisition of phosphorus and other mineral nutrients by the plant or to suppress certain soil-borne pathogens.

A main conclusion from the present work is that selected bio-effector preparations, when tested in laboratory and pot experiments, showed interesting capacities to improve the root development, mycorrhization, mineral nutrition and other growth-relevant variables in crop plants as well as to contribute to the suppression of major root-rot pathogens, whereas no such results could be reconfirmed in field experiments. Even under controlled conditions, however, the strongest effects emerged when the plants were subjected to abiotic or biotic stress factors, such as severely limited availability of phosphorus or pathogen infestation of the soil substrate, which, despite of statistically significant improvements, still restricted plant growth to agriculturally unproductive levels. According to these findings, *in vitro* tests with different agar media revealed that

microbial strains expressed potentially plant growth-promoting traits, such as the solubilization of calcium phosphates, mainly when their own growth was severely constrained by low nutrient supply, but not under adequate growth conditions (Chapter 4).

A similar weak perspective to benefit from the application of bio-effector preparations in agricultural production is provided by the current stage of research on diazotrophic rhizobacteria for associative nitrogen fixation in non-leguminous crops (Bashan et al., 2004; Bashan and de Bashan, 2010). After more than fifty years of research in that field (Döbereiner and Ruschel, 1958; cited in: Baldani and Baldani, 2005), no conclusive evidence for an agriculturally relevant supply of nitrogen by associative diazotrophs in crop production has been ascertained (Díaz-Zorita and Fernández-Canigia, 2009; Bashan and de Bashan, 2010). The results of recent investigations conducted in the frame of research projects funded by the European Union (Micro-N-fix, 2006; RHIBAC, 2010a; MICROMAIZE, 2010) have provided further evidence that inoculation with microbial bio-effectors was rarely beneficial for the growth and mineral, including nitrogen and phosphorus, nutrition of maize and wheat plants cultivated under field conditions in diverse European countries.

A general conclusion that can be drawn from the reports of these projects and the present work is that the effectiveness of the most promising microbial bio-effectors in growth promotion of non-leguminous plants is not based on single mechanisms, such as of biological nitrogen fixation or the solubilization of recalcitrant phosphates. Rather these microorganisms proliferate and establish in the rhizosphere where they interfere with the soil-plant-microbial relationships by multiple modes of action. These may include the production and/or degradation of phytohormonal compounds, improving the bioavailability of mineral elements, enhanced resistance to abiotic and biotic stresses, synergistic interactions with other beneficial soil microorganisms, and the suppression of soil borne pathogens (RHIBAC, 2010b; Çakmakçı et al., 2014). As reviewed in Chapter 2, the net effect of such processes on plant growth may be the result of cumulative impacts, synergistic interactions or even cascading feedback loops, at which the relative importance of distinct mechanisms may vary depending on the strain of microbial bio-effectors, the plant and the prevailing environmental conditions. The importance of

multiple interrelated mechanisms has likewise been emphasized by Bashan and de Bashan (2010) in a review concerned with the promotion of plant growth by *Azospirillum* species.

However, not only microbial preparations to promote plant growth by more general modes of action, such as stimulation of root growth and activity, mycorrhization, or solubilization of mineral nutrients from recalcitrant forms, often show a weak reliability to achieve improvements in crop performance under practice conditions. The influence of environmental factors may also critically affect the functioning of highly specific interactions, such as between rhizobia and leguminous plants for symbiotic nitrogen fixation. This has been shown impressively by recent results of the COMPRO project (COMPRO, 2014), which aims at the quality assurance and successful practice implementation of commercial bio-effectors products to increase the yields of crops grown by smallholder farmers in sub-Saharan Africa. In the course of this project, more than 100 commercial products from different categories have been evaluated (pers. com. Bernard Vanlauwe, 2012). These included rhizobial inoculants, free-living, associative and endophytic diazotrophs (e.g. *Azospirillum*, *Azotobacter* species), mycorrhizal inoculants, phosphate solubilizing bacteria, microorganisms for the suppression of soil-borne pathogens (e.g. *Trichoderma* fungi), and plant growth-promoting bacteria that can work by various modes of action, such as the production of root growth-stimulating phytohormones, as well as non-microbial products. After rigorous testing for quality and effectiveness in laboratory, greenhouse and on-farm trials, only four profitable products, comprising two rhizobial inoculants, one mycorrhizal inoculant and one non-microbial preparation could be identified, while for many other products no adequate performance was found under field conditions, although their potential effectiveness had been ascertained at the laboratory and greenhouse level (COMPRO, 2014). Even the most promising products, however, showed a wide variability in field performance, which was attributed to the influence of environmental factors such as rainfall, planting time, product application, and soil fertility. To achieve positive results with the application of bio-effectors, it therefore seems to be of critical importance that these agents are used as part of a well-integrated and site-specific management of crop production and soil fertility (pers. com. Bernard Vanlauwe, 2012).

9.2 Perspectives to improve the effectiveness of bio-effector applications by an integrated agro-management

9.2.1 The integrated functioning of bio-effectors

"N2Africa - Putting nitrogen fixation to work for smallholder farmers in Africa" was a large-scale project with the aim to improve the production of grain legumes and the nitrogen input from symbiotic nitrogen fixation by rhizobia in rural farming systems of sub-Saharan Africa (Giller et al., 2013). Recent studies conducted in the N2Africa project (Giller et al., 2013) confirmed the appropriateness of integrated, site-specific approaches insofar as it was possible to achieve considerable improvements in the reliability of bio-effector applications by an adequate modification of distinct environmental factors that were apparently most limiting for their effective operation. In the concrete case, the observed frequency of positive yield responses of soybean to inoculation with rhizobial strains could be clearly increased when also phosphorus was added (Fig. 9.1; Dashiell, 2012). In soils of tropical Africa, phosphorus deficiency is common and often the most limiting factor for symbiotic nitrogen fixation and legume production (Mulongoy, 1992; Giller, 2001; Nyoki and Ndakidemi, 2013).

Under such conditions, improvements in nitrogen fixation and yield of legumes have also been achieved by inoculation with arbuscular mycorrhizal fungi (AM-fungi), which was attributable to improved phosphorus nutrition of the plant on soils with a low infection potential of autochthonous mycorrhizal fungi (Barea et al., 1980; Ganry et al., 1985; Giller, 2001). AM-fungi are well adapted to scavenge phosphate from the soil solution even at such low concentrations that are virtually unavailable to non-mycorrhizal roots of certain plants (Mosse, 1973; Mosse et al., 1973). In soils with extremely low phosphorus availability, however, a major constraint to improve the phosphorus nutrition of plants by use of AM-fungi is that these organisms have very limited capabilities to mobilize phosphate by chemical processes from the usually much larger pools of sparingly soluble phosphates in soils (Lambers et al., 2008; Smith and Read, 2008). Further studies nevertheless provided evidence that the combination of AM-fungi with phosphate-solubilizing rhizobacteria can improve the effectiveness of the mycorrhizal symbiosis to

support the acquisition of phosphorus by the plant, as these bacteria release phosphate from recalcitrant forms, which than can be taken up by the mycorrhizal root (Toro et al., 1998; Dey et al., 2005).

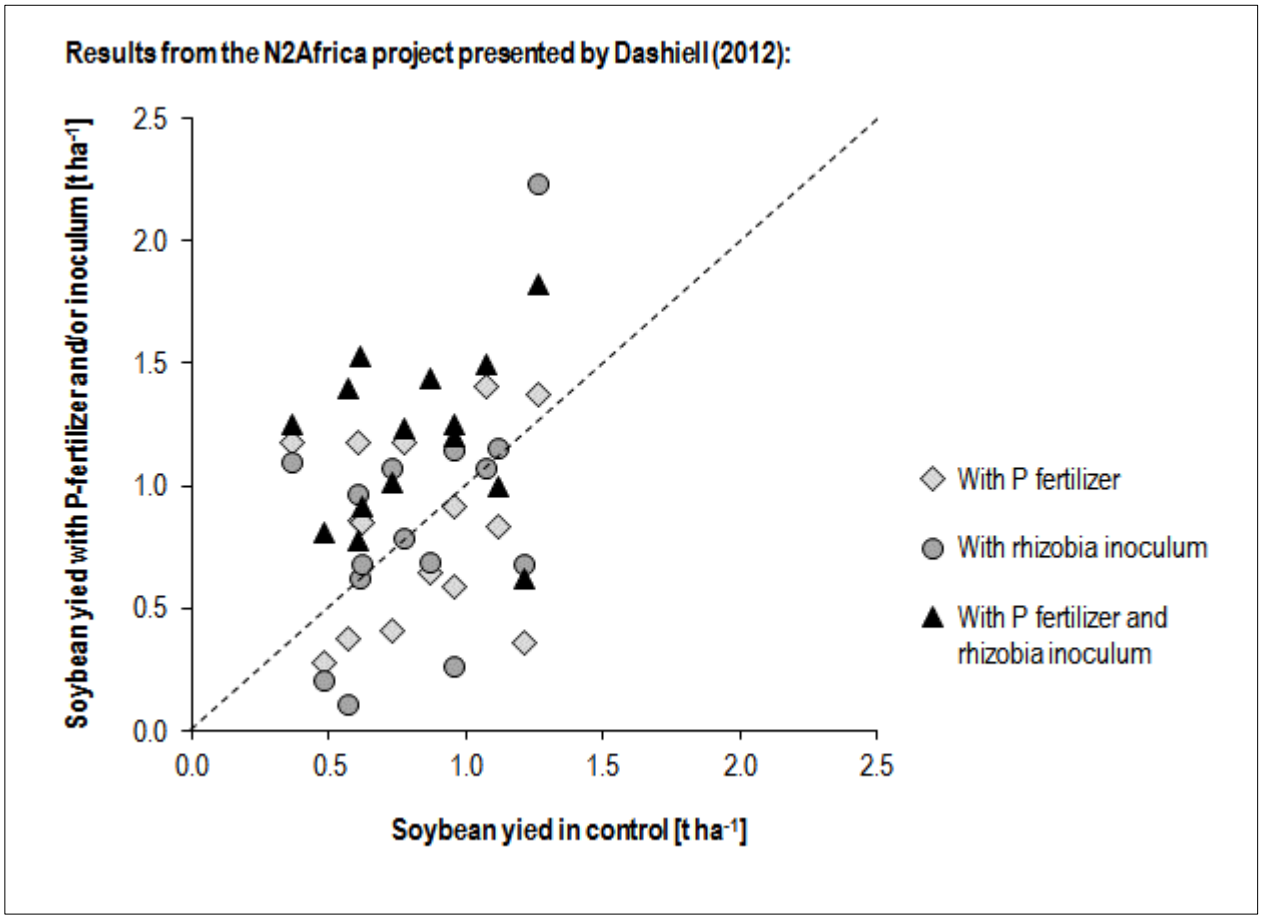

Fig. 9.1: Yield of soybeans in response to the application of phosphorus (P) fertilizer, rhizobia inoculum, or P fertilizer and rhizobia inoculum versus the yield of soybeans in untreated control plots in 15 demonstration trials of the N2Africa project conducted at different locations of Western Kenya, sub-Saharan Africa in 2010. The data shown in this figure have been reproduced from a diagram presented by Dashiell at the Global Pulse Researchers Meeting: "Transforming Grain-Legume Systems to Enhance Nutrition and Livelihoods", Kigali, Rwanda, 13th to 17th February 2012 (Dashiell, 2012). Similar results from further trials conducted in Sud Kivu, a province of the Democratic Republic of the Congo (DRC), have been reported by Giller et al. (2013).

These examples of interactive functioning between microbial bio-effectors and factors of their abiotic and biotic soil environment illustrate that beneficial effects on plant performance are not just a direct consequence of the bio-effector input, as it could be expected from the application of conventional fertilizers or pesticides. Rather, the

operation of these bio-effectors results from their integrated functioning based on the implementation of biological processes in the context of soil-plant-microbial relationships and other environmental influences.

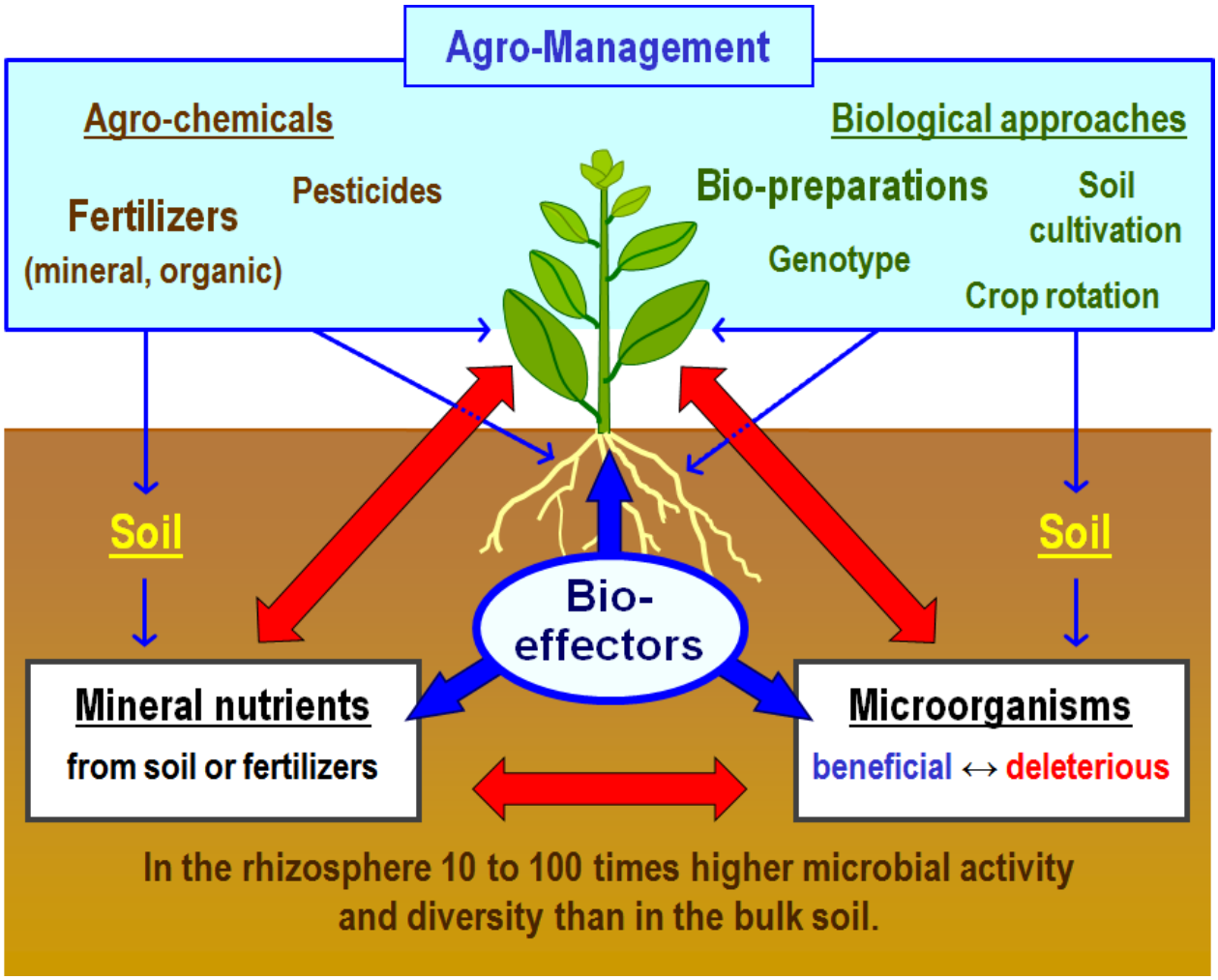

Fig. 9.2: The integrated agro-management of bio-effectors enables an adequate interplay between soil, plant and microorganisms in the rhizosphere to improve plant nutrition and health in a sustainable manner supplementing agro-chemicals by more biological approaches (modified from Römheld and Neumann, 2006).

This implies that the use of bio-effectors needs to be well integrated into the soil fertility and crop production management, based on the best knowledge on biological processes and practical capabilities, in order to achieve aspired improvements in plant growth and health with less input of agrochemicals (Fig. 9.2; Römheld and Neumann, 2006; pers. com. Bernard Vanlauwe, 2012). Such approach may include complementary applications of bio-effectors with conventional or alternative fertilizer and plant protection products,

improved crop varieties and microbial strains, as well as adequately adjusted farming practices to achieve an efficient and sustainable husbandry of natural resources that serves best for the welfare of present and future generations of the human family. With regard to the globally limited area of arable land and other constraints to feed the world population, this is an increasingly urgent challenge (Bruinsma, 2009; FAO, 2009a; Wise, 2013). In many respects, however, the complex interactions between plants, microorganisms and their environment that govern the acquisition of mineral nutrients and resistance to biotic and abiotic stresses still remain poorly understood. In most cases where bio-effector based approaches in crop production do not yield the desired effects under field conditions, it is a difficult challenge to identify the factors that are limiting their plant growth-promoting activities and to adjust the environmental conditions for an optimized functioning.

9.2.2 The delicate role of modern biotechnology for crop and bio-effector improvements

Another option to improve the productivity and sustainability of agriculture is seen in the advances of modern biotechnology to modify the genetics of plants and other organisms. Of course, concerns about how scarce resources are distributed and in which ways they are used to achieve food security and best welfare of the human society immediately not only impose questions that can be answered by natural science, but also many ethical, social, cultural, legal and economic issues requesting political decisions. In this regard, the use of genetically modified organisms (GMOs) in agriculture is among the most controversially discussed topics in Germany and worldwide (Ryan et al., 2009; McHughen and Wager, 2010), whereas other molecular breeding approaches that do not include the transfer of isolated gene sequences, such as a marker-assisted selection, are more accepted by the public (van Aken, 2009). Undoubtedly, agricultural biotechnology has a huge potential to facilitate the development of genetically improved crop varieties and microbial strains, which may increase yields or decrease production costs by enhanced resistance to abiotic and biotic stresses and a more efficient utilization of natural resources and technological inputs such as fertilizers and pesticides (FAO, 2009b). Emphasizing on the efficient utilization of nitrogen and phosphorus as two of the most critical production factors for agriculture in a world of declining natural resources, Vance

(2001) prospected that "*the world is on the brink of a new agriculture, one that involves the marriage of plant biology and agroecology under the umbrella of biotechnology and germplasm improvement*".

On the contrary, concerns about the risks of using genetically modified organisms in agriculture are raised with respect to their possible impacts on the environment, human health, and socio-economy (McHughen and Wager, 2010). In fact, herbicide resistant crops and/or insect resistant crops that are transformed to produce the *Bacillus thuringiensis* insecticidal toxin are by far the most widely planted, accounting together for virtually 100 % of the global area of transgenic crops (170 million hectares in 2012, annual growth rate 6 %; James, 2012). Herbicide resistance and toxin production, however, are traits that reinforce the use of pesticides in monocultural systems and tend to impede farmers from adopting more integrated approaches such as crop rotation, tillage, or bio-control with antagonists to mitigate weed and insect pest problems (Altieri, 2001). Contrary to claims that the invention of genetically modified crops would decrease the dependency on pesticides (Persley, 1999; Wieczorek, 2003), their implementation into farming practice has been associated with a net increase in pesticide use by an estimated 183 million kg (or about 7 %) in the United States of America between the years 1996 and 2011 (Benbrook, 2012). This was mainly a consequence of the rapid spreading of herbicide resistant weeds in management systems with herbicide resistant crops, which implied additional rates and amounts of herbicide application (Benbrook, 2012).

Besides the focus on herbicide and/or insect resistance, the biotechnological modification of other potentially useful traits that for instance may improve the resistance to abiotic stresses, nutrition, yield, or nutritive quality of plants has been receiving less attention (van Aken, 2009). Ryan et al. (2009) have reviewed the literature about attempts and limitations to engineer the rhizosphere by genetic modifications of plants and root associated microorganisms for an enhanced expression of functional traits that support the mineral nutrition and resistance of plants. One attempt, for instance, is to increase the efflux of carboxylates like citrate from roots by transforming plants with genes that either enhance the carboxylate synthesis or that encode membrane-bound proteins facilitating the transport out of the cell. In some studies, these modifications resulted in enhanced

resistance to toxic aluminum ions or a greater ability of the plant to acquire phosphorus from poorly soluble forms (de la Fuente et al. 1997; Lòpez-Bucio et al., 2000a,b; Delhaize et al., 2004; Yang et al., 2007; Ryan et al., 2009). Other studies using these strategies were less successful to effectively increase the carboxylate efflux from roots, which has been attributed to the variance of environmental factors interfering with the activation or functionality of the genetic modifications (Delhaize et al., 2001; Ryan et al. 2009). Furthermore, the carbon costs associated with the increased efflux of carboxylates from roots, especially when such traits are constitutively expressed, as well as the influence of these carbon sources on microbial populations may affect the net outcomes of such modifications on plant performance and nutrient availability, which yet need to be determined (Ryan et al., 2009).

Taken together, these outlines illustrate that also biotechnological approaches to enhance the genetic potential of plants and microorganisms need to be well integrated into agricultural production systems in order to achieve desired improvements of crop performance in adaptation to adverse environments. Biotechnology and germplasm improvement, therefore, may be considered as a tool that, given the risks have been carefully examined to prevent adverse effects on human health and the environment, can be used in concert with other aspects of an integrated agro-management, but not as a paramount solution to tackle the challenges of sustaining agriculture for food security (Altieri, 2001).

9.2.3 Needs and prospects for a more integrated agro-management utilizing the beneficial traits of bio-effectors to improve and sustain crop production

Global needs and constraints for an improved and sustainable crop production

The tremendous achievements in agricultural productivity of the past century were mainly based on modern crop varieties combined with the intensive use of mineral fertilizers and pesticides (Hazell, 2009). Wheat yields in England, for instance, ascended from two to more than seven tons per hectare on this account (Hazell, 2009) and the yields of other major cereal crops have also shown a steady growth during the second half of the 20th century (Brisson et al., 2010). Simultaneously, however, biological processes to retain soil

fertility and the closure of nutrient cycles have been widely neglected (Welbaum et al., 2004). Concerns are growing since in the latest two decades a decline in the growth trend of yields has been observed in Europe and world-wide (Pinstrup-Andersen et al., 1999; Brisson et al., 2010), while increases in food demand by 50 % from 2010 until 2030 are expected (Bruinsma, 2009; Horlings and Marsden, 2011; UNESCO-WWAP, 2012).

In a recent European study analyzing the possible reasons for the slowdown of yield growth in France, Brisson et al. (2010) found that not the progress in genetic crop improvement has declined, but rather it was counteracted by the adverse impact of climate change and agricultural management problems. Especially drought and heat stress, unsound crop rotations, and a decline in the use of nitrogen fertilizers and legumes as precrops to wheat appeared to be the most important constraints to yield increases (Brisson et al., 2010). In many Asian (Hazell, 2009), African (Agegnehu et al., 2014), and American (Boyer, 1982; Santibáñez and Santibáñez, 2007) countries the degradation of soils due to unsound agricultural practices and other deteriorations of the environment are recognized as a serious and widespread problem threatening the ecological and economic sustainability of agriculture and hampering future increases in crop productivity.

To maintain high yield levels, present agriculture strongly relies on fossil fuels and the intensive use of mineral fertilizers, whereas in unfertilized soils nitrogen and phosphorus are among the most limiting factors for plant productivity (Power, 2010). The adverse environmental impact of current production systems, local and global resource constraints, and increasing costs necessitate improved plant nutrition strategies for the sustainable maintenance of soil fertility. Especially phosphorus is regarded as a most critical natural resource to ensure global food security with respect to the limited global reserves of phosphate ores that can be mined for the production of fertilizers (Taylor, 2010). Due to the low natural delivery of phosphate by soils to plants, phosphorus is a critical factor limiting crop production on unfertilized soils worldwide (Smit et al., 2009). Yet, the availability of phosphate applied as easily soluble fertilizers is also tending to decrease with time after application, due to its proceeding conversion into sparingly soluble forms by precipitation and adsorption reactions (Smit et al., 2009). Furthermore, exports in form of crop products, surface run off, air-transported losses, and leaching can

lead to the non-sustainable depletion (mining) of soil phosphorus reserves, if there is no replacement by recycling or additional inputs (Bezama and Aomine, 1977; Gysi and Schwaninger, 1998; Pierzynski et al. 2005; Goulding et al., 2008). The careful husbanding and long-term use of this resource through improved agricultural practices, but also recycling and reuse of phosphorus from municipal and other waste products is therefore a key concern for ensuring future food security (Cordell et al., 2009; Smit et al., 2009; Dawson and Hilton, 2011; Seyhan et al., 2012).

The potential impact of bio-effectors to alleviate current constraints in crop production

The utilization of bio-effectors and other bio(-techno-)logical approaches bear a large potential for a more sustainable management of the limited natural resources, the non-renewable reserves of mineral nutrients, energy and water, to maintain soil fertility and to decrease the adverse environmental impact of agricultural production (Vance, 2001; Vessey, 2003; FAO, 2009b). However, under practice conditions most crops are cultivated in environments that are to a certain extent unfavorable to exploit their genetic yield potential and for the functioning of bio-effectors (Boyer, 1982). A major constraint are abiotic stresses, such as due to unfavorable soil and climate conditions, which on average decrease yields by about 70 %, whereas diseases, insect pests and weeds, although devastating to crops in distinct situations, only induce minor yield losses in common farming practice (Boyer, 1982; Bray et al., 2000). It is unlikely, therefore, that desired increases in crop performance are achieved by only improving the genetic yield potential of crop plants and the potential effectiveness of bio-effectors as stand-alone approaches. Not only plants and bio-effectors need to be more adapted to the environments in which they are inserted. Crop and soil management practices also need to be adjusted to support the plant's own mechanisms of resistance and the beneficial functions of bio-effectors, particularly those involved with complex rhizosphere interactions of mineral nutrient acquisition and pathogen defense, to achieve high yields under various stresses. This assumption is strongly supported by the character of improvements that can be achieved in cases of successful bio-effector applications such as: (i) stimulation of root growth, root activity and mycorrhization (Richardson et al., 2009; Chapter 4 and 8), (ii) solubilization of sparingly soluble mineral nutrients in the

rhizosphere (Jones and Oburger, 2011; Chapter 4 and 5), (iii) symbiotic and non-symbiotic biological dinitrogen (N_2) fixation (Graham and Vance, 2000), and (iv) pathogen suppression (Kloepper et al., 2004; Weller, 2007; Chapter 7 and 8).

The strategic utilization of these beneficial traits could help to alleviate many constraints of current and alternative farming systems that are associated with insufficient availability or ineffective utilization of mineral nutrients and other stresses induced by abiotic and biotic factors. In particular, this may be the case in organic and low-input farming systems, where chemosynthetic pesticides are banned or not available, when alternative fertilizers, like rock phosphate or recycling products, instead of easily soluble mineral fertilizers are used, or when innovative technologies like fertilizer placement are implemented to decrease the necessary inputs of fertilizers through their more efficient utilization. However, also in conventional agriculture, especially on less favorable land, drought stress, low root zone temperatures, soil-borne pathogens and other adverse soil and climate conditions are influential variables that can hamper root growth and mineral nutrient acquisition (Fig. 9.3). Especially certain root rot pathogens, such as *Gaeumannomyces graminis*, *Fusarium*; *Rhizoctonia*, and *Pythium* spp. are problematic to control in conventional and alternative cropping systems (van Bruggen, 1995; Tilcher, 2004; Letourneau and van Bruggen, 2006; Spence, 2007; Chapter 8). Cultural practices, including the selection of resistant crop varieties, crop rotation, tillage and other measures of soil fertility management can achieve a lot towards controlling many plant diseases and most pathogens are vulnerable to antagonists or other means of bio-control. At the current state of knowledge and implementation, however, cultural and biological plant protection strategies are not always sufficient to ensure plant health and economic production (Janisiewicz and Korsten, 2002; Pal and McSpadden Gardener, 2006).

In these situations, the stimulation of root growth, root activity and mycorrhization (Calvo et al., 2014; Barea and Richardson, 2015), the mobilization of mineral nutrients from recalcitrant pools (Altomare and Tringovska, 2011; Mohammadi, 2012), and the suppression of pathogens (Labuschagne et al., 2010; Beneduzi et al., 2012) are central targets that can be tackled by the application of bio-effectors.

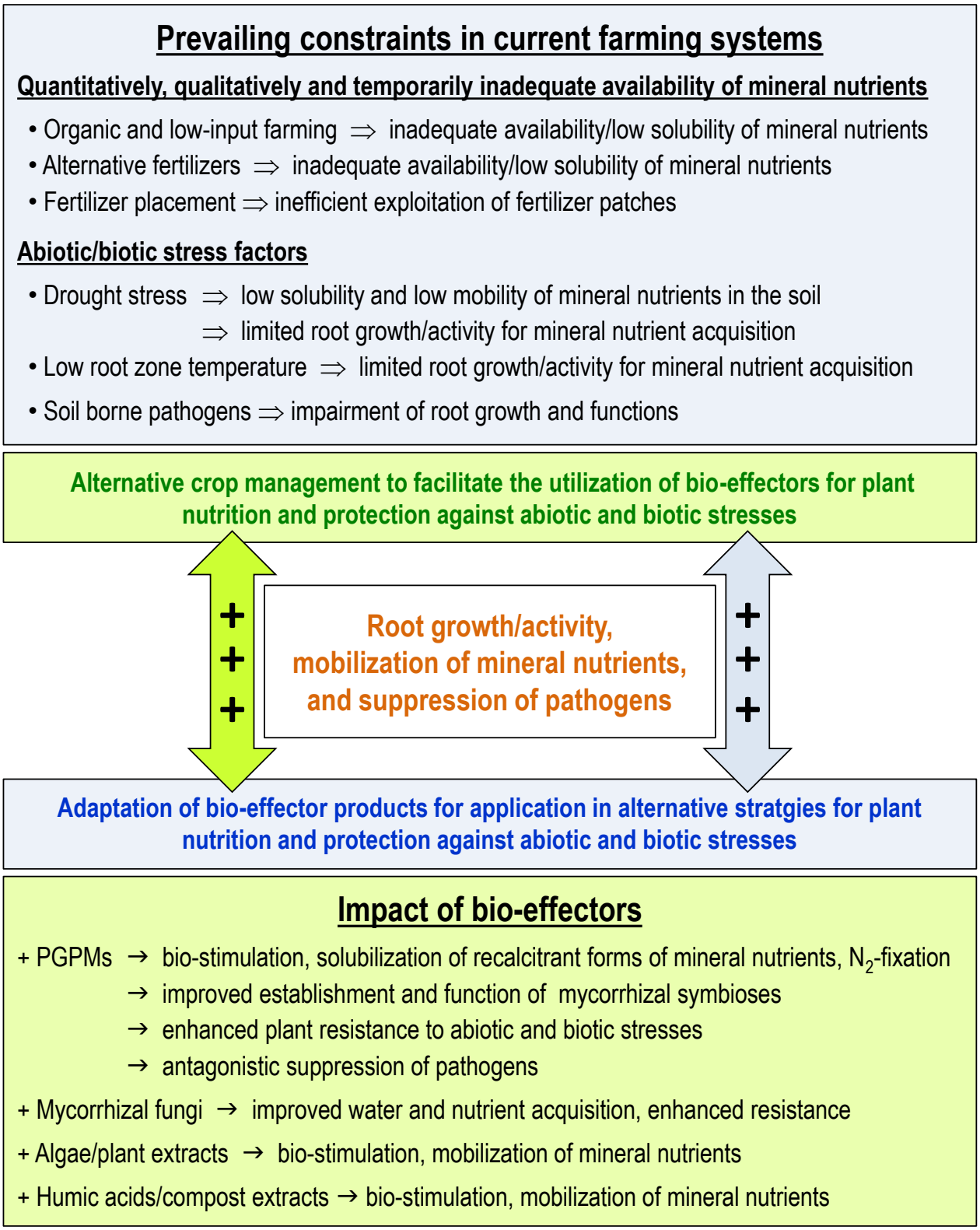

Fig. 9.3: Prevailing constraints in plant production under the current state of agricultural practice and possible impact of advanced approaches for mitigation of these constraints by alternative crop management to facilitate the utilization of bio-effectors and adaptation of bio-effector products for application in alternative plant nutrition and protection strategies (modified from BioFector, 2012).

Healthy and prosperous root development is a critical trait required for efficient nutrient acquisition from sparingly available soil reserves and alternative fertilizers, such as organic materials and industrial recycling products, which are typically characterized by insufficient rates, unbalanced composition and inappropriate timing of nutrient release instead of adequately meeting the requirements of crops (Barker, 2010). Similarly, the efficient exploitation of nutrient-rich patches in soils, such as produced by the placed application of fertilizers, depends on a sufficient rooting density and root activity (Sommer and Scherer, 2009). The limited proliferation of roots around the placed depots of mineral nutrients, as it is often observed under practice conditions, therefore is regarded as a major obstacle for the successful implementation of respective fertilization technologies (Reinhardt et al., 2010; Pfab et al., 2012; Flisch et al., 2013). For many plant growth-promoting rhizosphere microorganisms (PGPMs), such as certain *Bacillus* (Kloepper et al., 2004; Pérez-García et al., 2011) and *Pseudomonas* (Ahemad and Kibret, 2014; Sivasakthi et al., 2014) strains, but also extracts from algae (Khan et al., 2009; Calvo et al. 2014) and humic acids (Chen and Aviad, 1990; Arancon et al., 2006), the promotion of healthy root growth is among the most reliably documented traits.

Therefore, the adaptation of such bio-effectors to the conditions and requirements of specific application situations where root growth is impaired by drought stress, low root zone temperatures, high salt concentrations, soil borne pathogens and other adverse factors could be a key advance in helping to mitigate many of the prevailing constraints in current farming systems.

Challenges in formulation, quality, and application technologies of bio-effectors

A large number and diversity of bio-effectors is already available as commercial products and the interest in their agricultural use as bio-stimulants, bio-fertilizers or bio-control agents is increasing with a growing awareness of the need for biological alternatives or supplements to conventional fertilizers and pesticides (Herrmann and Lesueur, 2013; Calvo et al., 2014). For the next years from 2014, a compound annual growth rate (CAGR) of 12.5 % in the global market for bio-stimulants, containing humic acids, seaweed extracts and other natural compounds to improve plant growth, have been pre-estimated, which is expected to worth $ 2,524 million by 2019 (MarketsandMarkets,

2014a). Similar projections have been made for the markets of bio-fertilizers (CAGR of 13.9 %, projected value of $ 1,650 million by 2019; MarketsandMarkets, 2014b), comprising nitrogen fixing, phosphate solubilizing and potassium mobilizing agents, as well as bio-pesticides (CAGR of 16.0 %, projected value of $ 4,370 million by 2019; MarketsandMarkets, 2014c), based on microorganisms and other bio-logical (bio-rational) means.

However, despite of the growing markets for bio-effector products and their increasing use in agriculture, recent publications reported that many of the bio-effectors products that are currently available worldwide are of poor quality and lacking scientifically sound evaluation (Herrmann and Lesueur, 2013; Calvo et al., 2014). Among the prevalent deficits that have been found during quality control assessments of commercial bio-effector products are low levels and survival rates of propagules in microbial inoculants as well as the concomitance of contaminants that adversely affect the inoculation effectiveness (Gemell et al., 2005; Husen et al., 2007; Herrmann and Lesueur, 2013). In such cases, the improvement of quality control and assurance systems for bio-effector products surely is a major prerequisite to achieve consistent results under field conditions and to gain the confidence from farmers (COMPRO, 2014). Another very critical aspect for the successful implementation of bio-effector products is the development of formulations and application technologies that prevent the decay of the active ingredients during storage and transport, are well integrable with agricultural practices, support an optimal entry into force of the active ingredients after application, and finally ensure the sustainability and economic viability of the operations. To reach this goal, requires not only an in-depth knowledge of the functional mechanisms by which bio-effectors accomplish their beneficial effects, but it is also important to understand how their modes of action are affected by environmental factors and to consider agronomic and economic aspects for their application in real cropping situations (Hornby, 1998). This implicates the need for multidisciplinary approaches that in a holistic way can integrate the different levels of product development and adaptation of bio-effectors to specific purposes and conditions of use. Recent literature surveys over the past decades, however, indicate that research articles published on microbial species such as *Azospirillum*, *Bacillus*, *Pseudomonas*, *Rhizobia*, and *Trichoderma* have much more focused on molecular and

physiological aspects than attention has been payed to aspects of inoculum formulation and field application technologies (Xavier et al., 2004; Herrmann and Lesueur, 2013; Table 9.1).

Table 9.1: Survey of research articles published from 1980 to 2014 on microbial species such as Azospirillum, Bacillus, Pseudomonas, Rhizobia, and Trichoderma with focus on basic genetic, molecular and physiological aspects or practice related formulation and field application technologies.

Values are numbers of percentage of the total number of research articles indexed in the bibliography databases CAB Abstracts (CABI, Wallingford, Oxfordshire, UK), Scopus (Elsevier B.V., Amsterdam, The Netherlands), Google Scholar (Google Inc., California, USA) from 1980 to 2014 when searching for the indicated descriptors in the titles of the articles.

	Descriptors in article titles: *Azospirillum*, *Bacillus*, *Pseudomonas*, *Rhizobium*, and/or *Trichoderma*				
Database	total (no further specification)	genetic and/or molecular	physiology and/or physiological	formulation or formulations	field inoculation, field application, field conditions, or on-farm
CAB Abstract	49,828	1,487 (3.0 %)	269 (0.5 %)	619 (1.2 %)	156 (0.3 %)
SCOPUS	88,648	2,854 (3.2 %)	398 (0.4 %)	397 (0.4 %)	111 (0.1 %)
Google Scholar	125,000	6,016 (4.8 %)	1,006 (0.8 %)	476 (0.4 %)	265 (0.2 %)

Future approaches to increase the field efficiency of bio-effector products

The interest and market potential for the use of bio-effectors in sustainable agriculture is considerable and increasing. However, key constraints to the successful utilization of bio-effectors in crop production are the lack of adequate formulation and application technologies that ensure the viable expression of beneficial traits under field conditions. The knowledge on the conditions determining the occurrence of beneficial effects from the interactions between plants, bio-effectors and their environment is still limited. An integrated understanding of the links between abiotic and biotic factors, crop management, and bio-effectors that affect plant performance needs to be developed to improve the reliability and probability of successful field applications (Alabouvette et al., 2004; Herrmann and Lesueur, 2013).

In order that introduced microbial bio-effectors accomplish the intended activities when applied under field conditions, their ability to effectively colonize the rhizosphere at high

and persistent population densities in competition with the autochthonous microflora and despite of adverse environmental conditions is regarded as a major prerequisite (Herrmann and Lesueur, 2013; Bashan et al., 2014). The functional mechanisms involved in this "rhizosphere competence" (Ahmad and Baker, 1987) of microorganisms are incompletely understood (de Weert and Bloemberg, 2007; Ghirardi et al., 2012; Prashar et al., 2014). Nevertheless, microbial traits such as motility, high growth rates, the utilization of complex carbohydrates, the production of certain metabolites (e.g. antimicrobial compounds, amino acids, vitamins), and rapid adaptation to changing conditions (Weller, 1988; Lugtenberg and Dekkers, 1999; de Weert and Bloemberg, 2007), have been associated with competitive root colonization.

Major factors limiting the success of bio-effector applications under field conditions are constraints of the soil physicochemical properties, plant geno- and phenotype, microbial relationships and other environmental variables. An apparent difference between pot and field conditions is the strong limitation of the rooting zone in pots where roots cannot "escape" from homogeneously applied bio-effectors. This keeps the concentration of bio-effectors artificially high. In the field, however, roots may grow too fast for associated rhizosphere microorganisms to follow, and introduced inoculants may not sufficiently out-compete well-established microorganisms from the bulk soil (Haas and Défago, 2005; Raaijmakers et al., 2009; Chowdhury et al., 2013).

A current research project with the aim to address these challenges and increase the field efficiency of promising bio-effectors for the development of innovative plant nutrition strategies that can serve as viable alternatives to the current use of mineral fertilizers is the BioFector project (BioFector, 2012) funded by the European Union. The full tile of this project is "Resource Preservation by Application of BIOefFECTORs in European Crop Production", which is dedicated to the theme "Plant growth-promoting bio-effectors (microorganisms and active natural compounds) for alternative plant nutrition strategies in non-leguminous crops".

In an integrative approach, the activities of the BioFector project are structured into three interactive levels:

(1) At the basic level, the development of readily usable bio-effector products is treated. This comprises the screening, pre-selection and formulation of bio-effectors as well as the investigation of their functional mechanisms and synergistic interactions between them.

(2) A second level of the project structure is concerned with the adaptation of bio-effector products to specific application purposes in strategic combination with adapted and alternative plant nutrition strategies. To ensure strong practical relevance of this process, emphasis is set on prevailing constraints in current crop production systems. In particular, these are insufficient availability of mineral nutrients under organic and low-input farming conditions, inadequate acquisition of mineral nutrients from alternative fertilizers (e.g. organic and industrial recycling products), inefficient exploitation of placed fertilizers, and stresses from drought, low root zone temperatures, high salt concentrations and other abiotic factors. To validate the agronomic effectiveness and sustainability of the innovative bio-effector-based plant nutrition strategies, field experiments under different geographical situations and crop management systems are conducted.

(3) The third project level supports the development and implementation of the novel approaches by current and final analyses of their economic viability in comparison to the current state of sound agricultural production. Furthermore, the translation of knowledge and other project outputs into agricultural practice is facilitated by training and public dissemination activities addressed to consulting agencies, farmers and other stakeholders.

A central target of the BioFector project is to facilitate the successful performance of selected microbial inoculants and other bio-effectors by developing adequate formulation and application technologies.

Bio-effector and fertilizer placement strategies

One approach to reach this goal is the combination of bio-effectors with placed fertilization (Fig. 9.4). In contrast to broadcast fertilizer distribution, the localized application of fertilizers close to the root system of crop plants aims to decrease the input of fertilizers by increasing their use efficiency (Flisch et al., 2013). Placement of certain

mineral nutrients, such as nitrate, ammonium or phosphate, is effective to stimulate localized root growth, which can produce considerably higher rooting densities than in the surrounding bulk soil for exploitation of nutrient rich patches (Drew, 1975; Lynch et al., 2012).

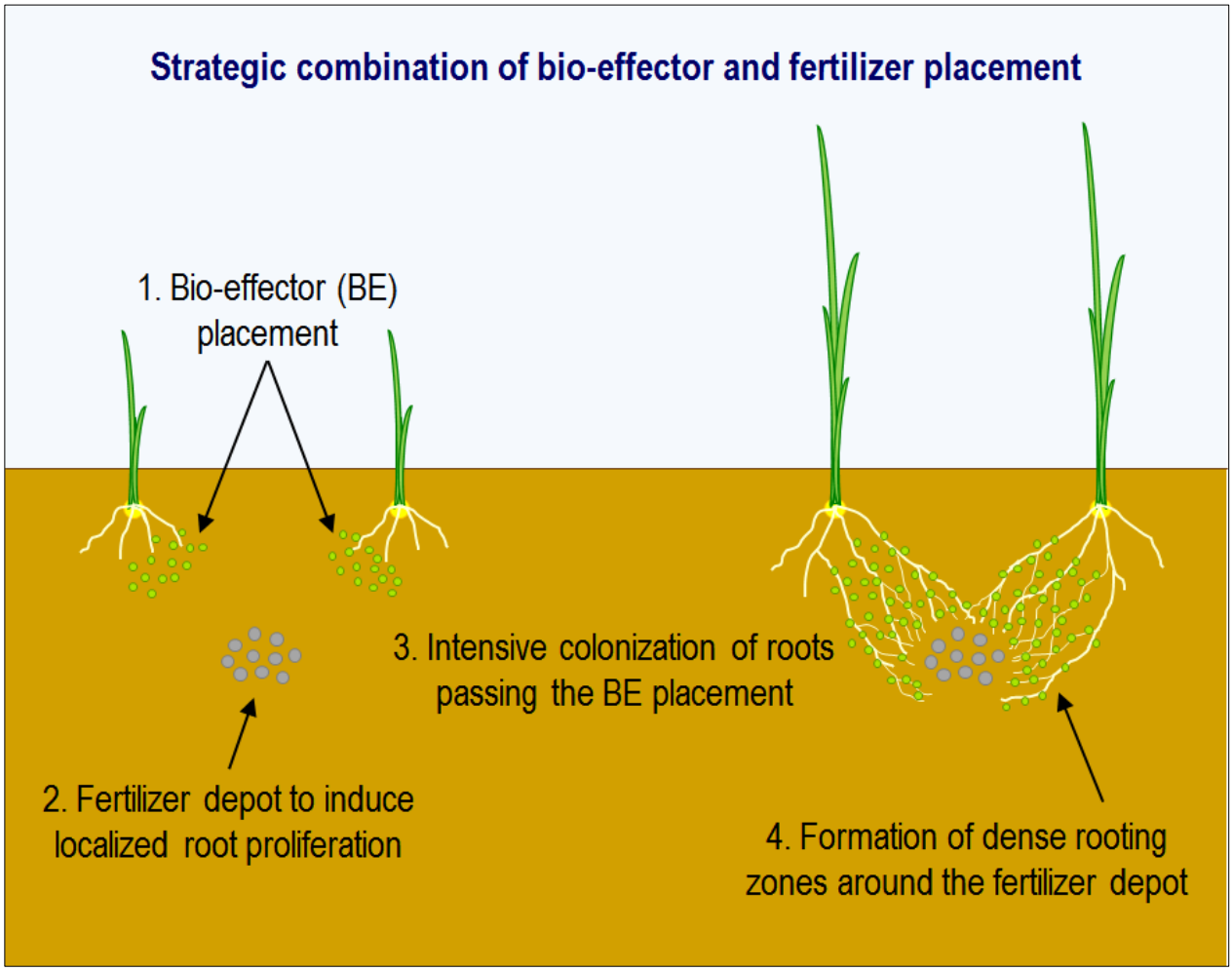

Fig. 9.4: Schematic illustration of the mutual benefits expected from the strategic combination of placed applications of bio-effectors with placed applications of fertilizers. The placement of bio-effectors close to the initial roots of young plants (1.) and the induction of localized root proliferation by fertilizer depots (2.) can produce optimized conditions for an intensive root colonization by microbial bio-effectors (3.) and the formation of dense rooting zones with a high share of rhizosphere soil around the fertilizer depots (4.). These are favorable prerequisites for the expression of beneficial effects through the introduced bio-effectors on root growth, root activity, and the mobilization of mineral nutrients, thus supporting an efficient exploitation of the fertilizer depots.

Band application of phosphate fertilizers below maize seeds at sowing, for instance, is an efficient placement method to improve the phosphorus accessibility during the early seedling development, because phosphate is largely immobile in soils and needs to be explored by the growing root system (Davis and Westfall, 2014). According to the CULTAN (Controlled Uptake Long Term Ammonium Nutrition) method, the placement of ammonium at concentrations toxic for roots and inhibiting microbial nitrification can provide a slowly releasing nitrogen depot that under ideal conditions is surrounded by zones of high rooting density (Sommer, 2008). This effect on root geometry opens options to develop rhizosphere management strategies such as modifying root mediated physical, chemical and microbial processes (Hinsinger et al., 2005; Hirsch et al., 2013). Under field conditions, however, the intended zones of high rooting density around the placed depots of mineral nutrients are often not or insufficiently developed, which is regarded as a major constraint for the effective implementation of respective fertilization technologies (BioFector, 2012; Flisch et al., 2013). The conjunction of root growth-promoting bio-effectors with fertilizer placement technologies may help to conquer this limitation, thereby improving the efficient exploitation of mineral nutrients in depots (BioFector, 2012). Conversely, the formation of densely rooted zones with a high share of rhizosphere soil may facilitate the establishment of microbial bio-effectors in association to the roots due to more concentrated distribution patterns of rhizodeposits serving as a primary carbon substrate for the microorganisms (Hirsch et al., 2013). With respect to these mutual benefits for the efficiency of bio-effector and fertilizer inputs, synergy effects are expected to result from their strategic combination in placed applications. Moreover, placement technologies can decrease the amounts of bio-effector preparations required per hectare to reach critical inoculum densities for a successful rhizosphere colonization by the introduced microbial strains in competition with the autochthonous microflora because only a confined fraction of the soil volume with intensive rooting may need to be treated. This could be an important economic advantage compared to broadcast application, especially when costly commercial bio-effector products are used.

An extreme example for placed application are seed dressing technologies, which can bring bio-effectors or other agents into the very close vicinity of germinating roots and are a convenient application method for farmers. However, seed treatments can only deliver a

limited number of propagules while multiplication and movement of the introduced microbial strains may not be sufficient to maintain adequate population densities during root growth (Caetano-Anollés et al., 1992; Leggett et al., 2011). Accordingly, investigations from Boelens et al. (1994) on the motility of *Pseudomonas* strains indicate that homogenous soil incorporation of microbial inoculants throughout the plough layer is by far more effective than seed inoculation to achieve adequate colonization densities over the whole root systems.

Placed application of microbial bio-effectors to zones of intensive rooting, such as induced by localized fertilizer depots, therefore, could be a favorable approach to provide eligible inoculum densities and bring the introduced strains in place where they are supposed to exert their beneficial effects on root development and acquisition of mineral nutrients, while keeping the inoculum costs affordable. The selection of bio-effector strains that are adapted to the specific conditions in and around the patches of placed fertilizers, like high salt concentrations and pH extremes, may further improve their competitiveness to the autochthonous microflora (Mendpara et al., 2013).

Combination of bio-effectors with organic fertilizers

Another emerging prospect to improve the field efficiency of bio-effectors is their combination with organic fertilizers, such as manure, compost and other organic amendments. In diverse recent studies, the combined use of microbial bio-effectors with organic fertilizers appeared to be superior in augmenting the productivity of crop plants compared to single applications of organic fertilizers (Chen, 2006; Akbari et al., 2011; Yildirim et al., 2011; Pathak et al., 2013). Temporary results of the BioFector project also indicate that the most significant improvements in plant growth by the application of microbial bio-effector products are achieved in combined application with farmyard manure or composted organic recycling materials. Posta et al. (unpublished), for instance, recorded strong growth improvements resulting from the application of microbial preparations in tomato seedlings that were grown on a substrate with 45 % composted cow manure during the pre-culture phase for greenhouse production (Fig. 9.5).

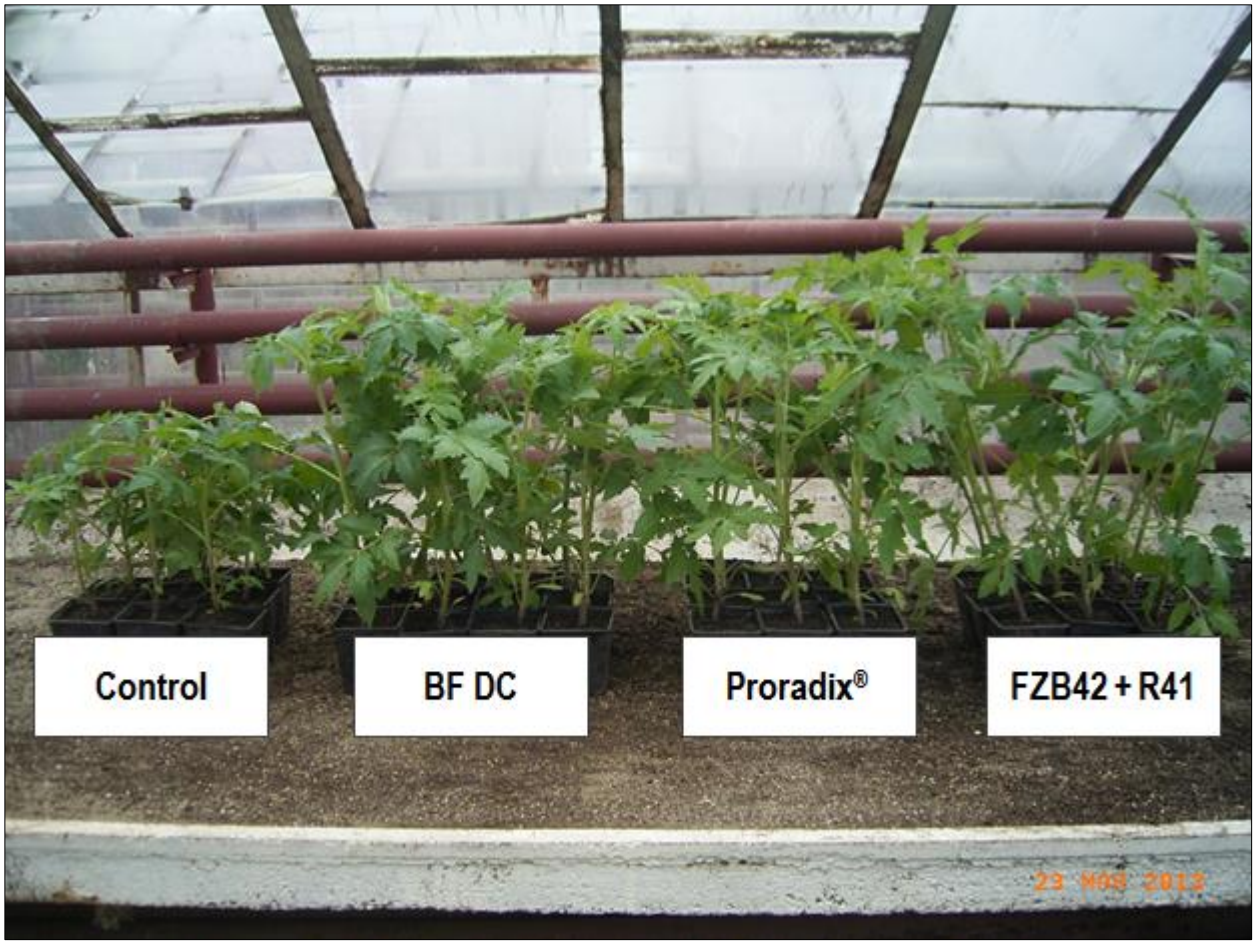

Fig. 9.5: Shoot development of tomato plantlets (Solanum lycopersicum L. var. Hellfrucht; Carl Sperling & Co. Saatzucht GmbH & Co. KG, Lüneburg, Germany) at 67 days after sowing in pots filled with a substrate mixture prepared from 45 % 2 years old cow manure, 30 % garden soil, 15 % peat and 10 % sand. At 44 days after sowing, water suspensions of different bio-effector products had been applied at a rate of 20 ml per pot as drench to the substrate mixture. The tested suspensions were the following: 0.05 % w/w Biological Fertilizer (BF) DC ($1.0 * 10^9$ vital spores of Penicillium sp. PK 112 ml^{-1} product, Bayer CropScience Biologics GmbH, Malchow/Poel, Germany); 0.02 % w/w Proradix® WP ($6.6 * 10^{10}$ colony forming units of Pseudomonas sp. DSMZ 13134 g^{-1} product, Sourcon Padena GmbH & Co. KG, Tübingen, Germany); and 0.04 % w/w RhizoVital® FZB42 fl. ($2.5 * 10^{10}$ spores of Bacillus amyloliquefaciens FZB42 g^{-1} product, ABiTEP GmbH, Berlin, Germany) plus 0.06 % w/w R41 ($1.5 * 10^{10}$ spores of Bacillus simplex R41 g^{-1} product, ABiTEP GmbH, Berlin, Germany). Control plants received only water. The plantlets were grown in a heated glasshouse (night: 12 to 14 °C; clear day: 18 to 20 °C) at the research station for horticulture of the Banat's University of Agricultural Sciences and Veterinary Medicine, Timişoara, Romania during the pre-culture phase for greenhouse production from January to March 2013 (Posta et al., unpublished).

Related studies suggest that not a single but the combination of few or many modes of action is involved in this plant growth-promoting effect:

- Das and Singh (2014) reported that combined application of farm yard manure together with a mixed preparation of *Rhizobium*, *Azotobacter*, *Pseudomonas*, and *Trichoderma* species increased the availability of nitrogen, phosphorus and potassium in soils more than did manure alone. This could be related to the ability of these microorganisms to release mineral nutrients that are bound in the organic matter (Parr et al., 1994; Jansa et al. 2013).

- As a complex carbon substrate, manure can exert a selective impact on the soil microflora (Schutter and Dick, 2001; Zhang et al., 2013) and support the establishment of introduced microorganisms for an effective expression of their beneficial traits in the rhizosphere. Especially during early phases of plant development when the root is still small and restricted in the gross production of root exudates (Gransee and Wittenmayer, 2000), the supply of additional carbon substrates may increase the survival rate of microbial inoculants and support their propagation (Kim et al., 1998b; Zhen et al., 2014).

- Furthermore, stimulatory effects on root development and colonization by arbuscular mycorrhizal fungi for an improved spatial acquisition of mineral nutrients could explain the plant growth-promoting activity of microbial bio-effectors in combination with manure amendments (Calvo et al., 2014).

Experiments to test the consistency of such effects under varied conditions have resulted in weak reproducibility of response to bio-effector applications. This indicates that the expression and impact of their possible modes of action on plant performance vary depending on the influence of environmental factors (Cummings and Orr, 2010; do Vale Barreto Figueiredo et al., 2010; Li et al, 2014). Weishaar (2009), for instance, found that the effect of plant growth-promoting bacteria (*Raoultella terrigena*) on biomass production and root morphology was dependent on the concentration and form of nitrogen applied to the growth medium in axenic *in vitro* cultures of *Arabidopsis thaliana*. Inoculation with *Raoultella* resulted in positive growth responses in Arabidopis plants grown on agar medium supplied with ammonium or ammonium plus 10 % nitrate, whereas non-inoculated plants appeared to suffer from ammonium toxicity. Plants supplied with nitrate only generally grew more vigorously and did not respond positively

to the inoculation with *Raoultella*. Recent results of the BioFector project indicate that the combination of phosphate-solubilizing rhizosphere microorganisms with ammonium nutrition of the host plant for rhizosphere acidification can yield synergistic effects in the mobilization of sparingly soluble calcium phosphates (Neumann et al., 2015). Akbari et al. (2011) tested the effectiveness of bacterial agents (*Azosporillum* and *Azotobacter* species) in a field experiment with sunflower (*Helianthus annuus* L. cultivar "Alestar") where nitrogen fertilizer (urea) and farmyard manure were applied single or in complementary combinations. The results indicated that the beneficial effect of the bacterial treatment on the seed yield of sunflower was more pronounced when only manure (100 % ratio) or only urea fertilizer (100 % ratio) was applied. The highest yield levels, however, were achieved with the combined application of manure (50 % ratio) and urea fertilizer (50 % ratio), at which the additional supply of the bacterial agents had only a marginal effect. Similar results were published by Yildirim et al. (2011), who found that root inoculation with bacterial strains (*Bacillus cereus*, *Brevibacillus reuszeri*, or *Rhizobium rubi*) increased the yield of broccoli (*Brassica oleracea* L. varietas *italica*, cultivar "Monet F1") fertilized with manure and rock phosphate fertilizer. Nevertheless, such treatments did not outperform the yield level produced with the supply of easily available mineral fertilizers (urea and triple super phosphate).

9.3 General synopsis

Status quo

When Fritz Haber in 1920 gave his Noble lecture on "The synthesis of ammonia from its elements", which together with Carl Bosch was translated into a large-scale industrial procedure named Haber-Bosch process, he stated:

"*It may be that this solution is not the final one. Nitrogen bacteria teach us that Nature, with her sophisticated forms of the chemistry of living matter, still understands and utilizes methods which we do not as yet know how to imitate*." (Haber, 1920)

Today it might be stated, that it is not only not known how to imitate the methods of bacteria and other microorganisms by which they can support the mineral nutrition and

healthy growth of plants. It is also poorly understood how to utilize the beneficial traits of such bio-effectors by an adapted crop management that facilitates their functional implementation.

As a main technology of the Green Revolution, the use of nitrogen fertilizers produced by the Haber-Bosch process has been widely adopted in agriculture during the past half century, accounting for tremendous increases in food production, together with the use of pesticides and high yielding varieties. Yet, the increasing use of fertilizers and pesticides also has been associated with environmental problems such as degradations of soil, air and water resources, which are the basis of livelihood for present and future generations (Banerjee et al., 2012; Putri et al., 2012; Subramanyachary, 2012).

In contrast, alternative biological approaches, such as the utilization of bio-effectors to improve the mineral nutrition and resistance of plants against pathogens, although proposed by Lorenz Hiltner already at the beginning of the 20th century (Hiltner, 1904; Chapter 1), have found limited implementation in agricultural practice. Only in recent decades, bio-effector based approaches have been receiving increasing interest for the development of more sustainable crop production systems (Banerjee et al., 2006; Römheld and Neumann, 2006; Herrmann and Lesueur, 2013).

Nevertheless, at the current state of science, for the vast majority of bio-effectors application or crop management strategies to successfully achieve targeted impacts under field conditions cannot be recommended with confidence. Field studies on the application of bio-effectors often show a weak reproducibility of beneficial effects. Concerning introduced microbial strains, this has been attributed to an insufficient establishment and colonization of target sites like the rhizosphere, as a critical prerequisite for the expression of their beneficial traits under variable environmental conditions (Lehman et al., 2015). However, for many microbial bio-effectors as well as active natural compounds the knowledge on their functional mechanisms and conditions that determine the occurrence of plant growth-promoting effects is still inadequate. An improved understanding of the factors involved with the successful establishment and functioning of bio-effectors for beneficial interactions with the plant and its physical, chemical, and biological environment is required to support their effective utilization in crop production (Lehman

et al., 2015). Much more research seems to be necessary to understand how the expression of beneficial bio-effector traits is affected by complex interactions with abiotic soil characteristics, the soil microflora, weather and climate conditions, soil cultivation, crop rotation, plant genotype, and the use of pesticides and fertilizers.

Where to go?

The rapid developments in analytical tools targeting on the structural and functional characterization of the microbiome as a whole and to elucidate the role and operating mechanisms of its specific components is expected to enable the detection of microbial alterations in response to the application of bio-effectors, crop management practices, and environmental factors. Correlating these shifts in the activity and composition of microbial populations to variations in soil fertility and the resistance of plants against abiotic stresses and pathogens could be helpful to detect the vital links between these descriptors (Alabouvette et al., 2004; Janvier et al., 2007).

However, the deployment of advanced analysis techniques for the detection of functional mechanisms and separation of individual effects alone will not result in advanced cultural practices to be proposed for the economic and ecological sustainability of agricultural systems. A purely reductionistic science that endeavors to dissect the mechanisms of life *in vitro* or almost restricted to the molecular and chemical level is not sufficient to understand the complexity of life and its vital interrelationships. It rather resembles closed climate chambers where plants are only exposed to artificial light sources, which produces artefacts in plant responses that are very different from those expressed under natural daylight conditions (Hogewoning et al., 2010). Especially microbial bio-effectors can affect the performance of plants by multifaceted modes of action, whose respective importance in given situations is influenced by interactions with various environmental factors under field conditions (Velivelli et al., 2014; Sessitsch and Mitter, 2014). Therefore, it is evident that the open questions how to utilize bio-effectors for improved growth, mineral nutrition and disease resistance of crops are best addressed in holistic approaches, considering the interactions and feedback loops among many variables to more appropriately understand the behavior of the complex system.

What are aspects of a holistic view?

In living organisms, "*the whole is more than the sum of its parts*" (Aristotle, 2014), and soils are living systems where plant roots connect with the soil life (Binkley and Fisher, 2013). The outcome of bio-effector applications in terms of crop performance depends on the balance of activities between many effectors, and interactions between each component of the system. Optimum levels of soil fertility and crop productivity under field conditions are likely to be achieved by practices that facilitate a rich species and functional diversity as well as a vigorous activity of the soil microbiota that may buffer against the impact of diverse and unpredictable environmental stresses (Dukes, 2001; Alabouvette et al., 2004; Creissen et al., 2013).

Recently, the plant together with its associated microbiome has been reconsidered as a superordinate entity called "meta-organism" with respect to their close relationships derived from millennia of co-evolution (Bosch and McFall-Ngai, 2011; Berg et al., 2013). Imbalances between beneficial and deleterious microorganisms in the soil and in association with the plant have been associated with the emergence of major pathogenic diseases (Gunawardena et al., 2006; Chapter 7). The importance of the microbiome for the healthiness of the host organisms does not end at the plant-microbial level. It also may favorably or adversely affect the health of humans and animals that are living in the vicinity of plants and/or nourishing on them (Berg et al., 2014). Even humans have been described as "meta-organisms", due to their symbiotic coexistence with the intestinal microbiota (Turnbaugh et al., 2007; Biagi et al., 2011; Bosch and McFall-Ngai, 2011). The bonds between soil, plants, microorganisms and humans appear to be absolutely ground-laying not only from this perspective. According to the second Genesis account of the bible, humanity was not created ex nihilo, but "*God formed man out of the clay of the ground and blew into his nostrils the breath of life, and so man became a living being* (Genesis 2,7). Like "Adam" comes from the Hebrew word "adamah [Hebrew: אדמה]", which can be translated as ground or earth, "human" is related to the Latin word "humus", meaning ground, earth, or soil. It seems that without ground and breath of life, there is no human being (Turkson, 2014).

In a holistic view, humans are not only meta-organisms at the microbiological level and they are not only recorders and processors of analytical data in the sense of reductively explainable automata, as described by Descartes (1662). They rather are meta-physical beings with the ability to distinguish between good and evil by reasoning and examination of conscience with a transcendent responsibility to use and maintain the resources of the earth in the right manner (de Aquino, 1225-1274b; cited in: Enochs, 1996; Turkson, 2014). According to reports from a recent summit conference of the Food and Agriculture Organization of the United Nations (FAO) on food security and the prospects for agriculture, the earth has sufficient resources to conquer famine and assure sustainable food security for everyone (FAO, 2009a). It cannot be overseen, however, that the access to these resources is very unevenly distributed and that the destabilizing impact of global markets and financial speculation, land grabbing, greed, the ruthless exhaustion of non-renewable resources, environmental deterioration, and climate change impose serious problems, which are primarily induced by the lifestyle of the wealthy (FAO, 2009a; Francis, 2015a; Schellnhuber, 2015). Millions of the poorest, on the contrary, are trapped in a treadmill of hunger and poverty (FAO, 2009a; Müller, 2014). Indeed, there is a lack of individual and political will to effectively implement respective corrections for the better (FAO, 2009a), but the problems of today may not be solved with the same thinking that caused them (Albert Einstein; cited in Sowada, 2003).

It was not by coincidence that Pope Benedict XVI during his speech given at the German Bundestag in Berlin, 22. September 2011, emphasized that humans as well as the rest of nature have an ecology and cannot be manipulated arbitrarily, but their inherent dignity needs to be respected. The true sources to recognize what is the right way to practice are nature and reason, reason that is open to the language of being. Decisive in these cognition processes is not only what can be seen or measured by increasingly artful analytical tools, but how human beings understand it and which conclusions, operational policies and achievements they derive therefrom (Guy Consolmagno; cited in Montemurri, 2014). In this sense, science is not a big collection of facts, that prove one thing or another, but rather a wonderful conversation with nature that is always open to learning more and changing as it learns (Guy Consolmagno; cited in Montemurri, 2014).

Can wonder help in learning from nature?

The beginning of love for wisdom is wonder (Socrates, as portrayed by Plato, *Theaetetu*s, 155d; cited in: Bollert, 2001). Beyond all notable progress in analytical technologies, our ability to wonder, as a prerequisite to learn from the language of nature, could be a key criterion to better understand the secrets of plant growth and associated microorganisms (L'Ecuyer, 2014). For that reason, Margarete von Wrangell (1877-1932), the first leader of the Plant Nutrition Institute at Hohenheim, Germany, was living with the plants and laid the ear or, as one may understand, her listening heart on the soil, as stated on a memorial stone installed at her former domain (Fig. 9.6). Albert Einstein (1879-1955) in 1951 wrote in a letter to his friend and colleague Michele Besso (1973-1955) that all the 50 years of willful pondering about the nature of light have not brought him any closer to the answer to the question „what are light-quanta" (Sillescu, 2005). However, relying on the amazing assumption that space and time are relative dimensions, but the velocity of light is a universal natural constant independent of the reference system, he was able to make very applicable predications about the impact of light (Einstein, 1905). Johanna Döbereiner (1924-2000), whose work in Brazil was foundational in the discovery of associative nitrogen-fixing bacteria in tropical grasses, at first wondered that these plants stay amazingly green without being fertilized with nitrogen (Döbereiner, 1966; Döbereiner, 1972; Coelho, 2000; Südhoff, 2006).

Such wonder approach in science, which is initiated through the perception of beauty, as the recognizable expression of truth and goodness (L'Ecuyer, 2014), realizes nature not from a purely functional perspective that is driven by the interest to gain maximum profit from the exploration and efficient exploitation of natural resources (Francis, 2015b). It rather appreciates that nature is an ordered ensemble (Greek: κόσμος kósmos) of which all creatures inclusive human beings are a part, sharing the dignity of being created nature. In listening to this cosmic harmony and ordering our intensions and practices accordingly lies the way to a respectful and really sustainable care of humanity and its natural resources for living, which are an abundant donation to be brotherly and sisterly shared with others in order to flourish (Turkson, 2014).

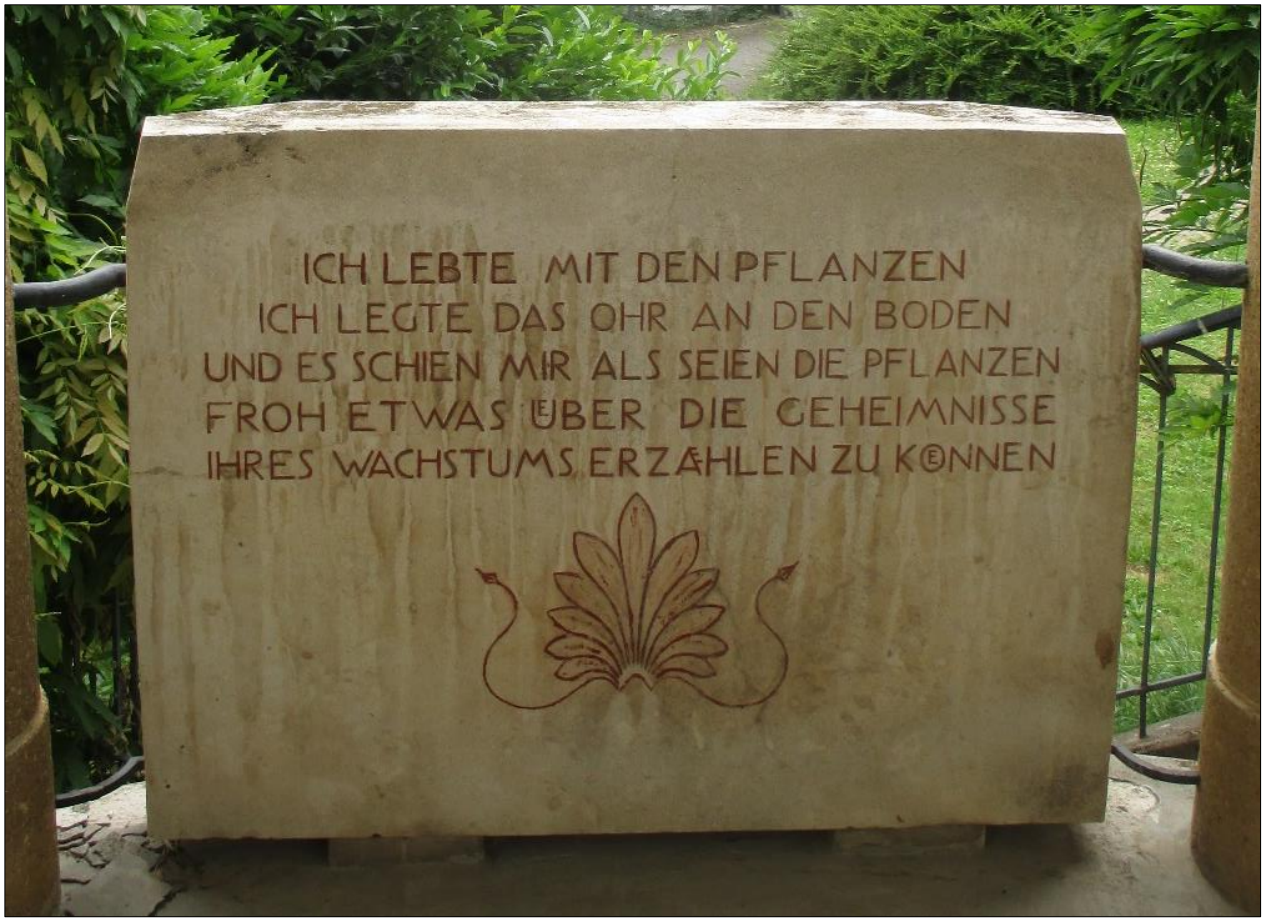

Fig. 9.6: Memorial stone for Margarete von Wrangell (1877-1932), first leader of the Plant Nutrition Institute at Hohenheim, Germany. Literally translated to English the German inscription says: "I lived with the plants. I laid the ear on the soil and it seemed to me as if the plants were pleased to be able to tell something about the secrets of their growth."

Mind-set of such kind is not simply an expression of naive romanticism. It rather is a change of view that, in contrast to the widespread reductionistic understanding of nature, refuses to regard the subjects of nature as functional objects to be simply used and controlled, which can have far-reaching consequences for the optional behavior of humans (Rohrmoser, 1996; Benedict XVI, 2011; Francis, 2015b).

In his recently published encyclical letter "Laudato Si', on care for our common home" Pope Francis wrote in this sense:

"*If we approach nature and the environment without this openness to awe and wonder, if we no longer speak the language of fraternity and beauty in our relationship with the world, our attitude will be that of masters, consumers, ruthless exploiters, unable to set*

limits on their immediate needs. By contrast, if we feel intimately united with all that exists, then sobriety and care will well up spontaneously." (Francis, 2015b)

The ability to wonder is initially present in children (L'Ecuyer, 2014), but it may shrink with an increasing adherence to the idea that nature is an aggregate of objective, observable facts functionally linked together in terms of cause and effect while excluding any meta-physical or transcendent considerations (Comte, 1994; Benedict XVI, 2011; Margarit, 2012). In consequence, the search for truth, as guiding principle for a good choice between alternative courses of action, is subordinated to practical interests, the objects of nature are regarded as a mere material to serve for purposes of use and consumption, and the economic and technical abilities of humans remain as the ultimate measure of things to solve any problem (Kant, 1781; Rohrmoser, 1996; Benedict XVI, 2008; Francis, 2015b).

What are the obstacles for wonder?

The widespread inability to wonder is to some extent promoted by the conventions of the modern society of the western world. Latest since the Age of Enlightenment, reason and experience have been increasingly accounted as the main sources of knowledge with a tendency to restrict the meaning of "reason" to human rationality and to discard anything from being regarded as scientific in a strict sense that cannot be validated or falsified by empiric data (Kant, 1781; Benedict XVI, 2011). From that perspective, all knowledge, which can be gained, finally depends on the ability of the human subject to perceive things through the senses and understand them by the structure of the mind. In this process, not the things themselves are seen, but only their appearance and meaning for the subjective human reason. Concerning this inherent bias, it is impossible to ultimately know if perception and subjective interpretation actually correspond with the external world or, generally speaking, to empirically verify any concept about the reality itself (Kant, 1781; Popper, 1935). It is obvious, that this attitude tends towards a subjective relativism that is unable to recognize any indisputable truth and, in consequence, acknowledges no other instances than the own self-centered interests (Kant, 1781; Ratzinger, 2005; Francis, 2015b).

But, does it not remain itself an unresolvable paradox that the premises and findings of empiric research are being mutual prerequisite conditions of each other in gradually proceeding recognition processes, if human reason is not inherently gifted with an authentic ability to recognize the reality (Rohrmoser, 1996)?

To transcend the bias of human subjectivism, basically necessitates the recognition of objective truth as perquisite to discern between good and evil, as to justify any moral claims or categorical imperatives for action (Hegel, 1807; 1821). This is impossible in a purely natural scientific rationality, except in the transcendent faith and ability to wonder that objective reason expressed in nature and subjective human reason correspond to one another, which presupposes that they are founded in one inventive reason (Greek: λόγος, logos; Stein, 1950; Benedict XVI, 2011). According to the Christian tradition and belief, this is the creative reason of God who created creation out of overflowing love and not out of necessity (Irenaeus, 200; Augustinus, 354-430; de Aquino, 1225-1274c; Splett, 2008; Benedict XVI, 2011).

Which conclusions concerning the beneficial use of bio-effectors for a sustainable agriculture to ensure food security for the human population can be drawn from these basic considerations?

The use of bio-effectors in agriculture is of particular interest because of their potential effectiveness to improve the heathy growth, mineral nutrition, and resistance of crop plants by biological modes of action. Their beneficial impact and successful implementation can help to decrease the dependency of current crop production systems on the intensive input of precarious agrochemicals, energy, and non-renewable resources such as phosphate rock used for the production of fertilizers, while maintaining high levels of crop productivity to ensure food security for every human being. Viable bio-effector applications are thus requested to enable a more sustainable management of biological, soil and fertilizer resources, to mitigate pollution of the environment and climate change, and to assist the maintenance of ecological resources and biodiversity (BioFector, 2012).

The present work shows that intensive and multidisciplinary research is indispensable to understand not only the functional mechanisms of bio-effectors, but also how they can improve the performance of crop plants by interactions with various environmental factors under practice conditions. Based on such knowledge that is well integrated into a holistic perspective of the natural and human ecology, the functional utilization of bio-effectors can make a vital contribution to the development of sustainable agroecosystems.

However, the problems related with environmental degradation and exhaustion of non-renewable resources, that are currently provoking an ecological crisis that threatens the basis for sustaining human life on earth, are not basically rooted in an insufficient knowledge about bio-effectors, but rather in a prevailing attitude to regard the objects of nature as mere material for use and consumption. This appears to be characteristic especially for the development of the Western society since a transcendent cosmic consciousness towards creation has been increasingly lost with a growing adherence to the ideologies of a reductionist natural science, subjective relativism, technical feasibility, and infinite economic growth, which owe their hitherto achievements to the vast abandonment of holistic questions (Rohrmoser, 1996; Francis, 2015b).

In Chapter 1 of this study, “bio-effectors” have been defined as viable organisms or active natural compounds whose direct or indirect effects on plant performance are based on the functional implementation or activation of biological mechanisms. Chapter 2 subsequently seeks to elaborate an interpretation of the prefix “bio” in a comprehensive bio-logical sense. This emphasizes the role of human’s understanding and intensions as a motive force for the purposeful implementation of bio-effector uses, aiming to integrate the most advanced knowledge on living organisms and their vital processes into the realization of optimized crop management systems. This concept is inclusive of all aspects regarding the complex nature of plant interactions with their environment and open to learning more from a respectful conversation with nature.

Nevertheless, the motivations for the development of biological approaches towards sustainable agricultural systems can be ambiguous in principle (Rohrmoser, 1996):

One attitude is to insist on anthropocentric rationality and the feasibility of technical solutions to sustain ecosystem services for the purpose of human interests such as food security, economic stability, and a high quality of living standard in a healthy environment. In this case, the aim seems to be desirable, but the way to meet the target still follows the former paradigm that nature could be entirely managed to serve for the self-centered human interests. The ecological factor is now included into the calculation to achieve maximum use efficiency insofar as it cannot be disclaimed furthermore that the ruthless exploitation of natural resources already in the medium term would lead into disaster. The attitude "after me the deluge" cannot be considered as sufficiently reliable anymore when the global consequences of environmental destruction already nowadays start to disturb the economic climate and accustomed lifestyle. Nature thus needs to be managed more ecologically in order to maintain its functionality. From that perspective, bio-effectors could be discerned as a tool besides others to be rationally used under the discipline of bio-technology for the needs of an efficient agricultural production. With these premises, however, even the most advanced knowledge and technologies will tend to reinforce the adherence to the anthropocentric ideas that are conductive to the ecological crisis.

Another view is to see nature as creation, that, although groaning in travail (John 16,21; Romans 8,22; Santmire, 1985), by reason of its origin is very good (Genesis 1,31), and to cultivate it with respect to its creatural dignity as responsible coworkers of its creator (Genesis 1,28). This relies on the faith that there is harmony between the reasonable order of nature and human reason due to their mutual foundation in the creative reason of God, who, according to the Christian belief, revealed himself as the way, the truth, the life, and the goodness itself (Luke 18,19; John 14,6; Benedikt XVI, 2008). This universal correspondence of reason not only enables the invention of technologies in the interaction of humans with nature, but also it is basic for a reason-ability to distinguish between good and evil as prerequisite to recognize the right way of practice in the concrete case (de Aquino, 1225-1274b). Because the good is also the true and the true is also the good, the self-centered thinking in categories of interest and utility can be transcended by recognizing the unselfish search for the truth as the very good for the self (Reichberg, 1999; Benedikt XVI, 2010). From this perspective, microorganims and natural

compounds could be realized as bio-logical-effectors, when humans have learned from nature, as a reasonably ordered whole, and with respect to its integrity how they can put into effect the potential activities of these agents, depending on the inherent and environmental conditions to achieve a true benefit (from Latin: *bene* ‘good’ *factum* ‘act, deed’). This, however, requests an ardent research to attain sufficient knowledge for a comprehensive survey of the nature of things and their sophisticated interrelationships, or at least the sensitivity and wisdom to see with wondering eyes what is truly important (Plato, *Republic*, Book 7, 537c; Newman, 1982; cited in: Reichberg, 1999).

How can we wonder again?

Similar to the biblical creation account, the traditions of many so called “indigenous” people, like native Americans (Cajete, 1994; Enochs, 1996), aboriginal Australians (Martin and Mirraboopa, 2003), as well as indigenous peoples of the Arctic region (Kawagley et al., 1998) and the African (Pretty, 2002) continent, are reported to comprehend nature as a created wholeness, with the understanding that spirit inhabits and interrelates all things (Cajete, 1994; Salmón, 2000). In Hinduism and Buddhism, the terms “Ṛta” (Sanskrit: ऋतं) and “Dharma” (Sanskrit: धर्म) refer to the cosmic order that makes life possible and the respective way of living (Hiltebeitel, 2011).

The modern society, in contrast, has widely disconnected the scope of natural sciences from the conception of nature as holistically ordered creation, as this all-encompassing dimension is not tangible in terms of empiric discrimination. However, the apparent objectivity of empirically based knowledge arises therefrom that the contribution of cognitive subjectivity interfering with the presuppositions, conduction and interpretation of empiric investigations is not reflected specifically in these processes (Link, 2005). If all in all is only nature, then any knowledge is principally subjective because it is impossible for the human subject, which itself is only a part of nature, to gain an unbiased perspective (Kant, 1781). Simplistically this is even due to the constraint that any self-knowledge already requires a certain ability for objective self-reflection (Stein, 1950; Buktus, 2002). In fact, the assumption of a consistent determination of reality as a self-contained aggregate of cause and effect in the sense of objective natural laws is itself a

hypothesis that cannot be verified by empirical methods (Link, 2005). In the participatory experience of life with its dynamic processes, reality rather becomes present on the basis of conditions and interrelationships as a possible but not necessarily predetermined actuality, which cannot be repeated under *ceteris paribus* conditions (Stein, 1950; von Weizsäcker, 1968; cited in: Link, 2005). Already the investigation and perception of nature is an interactive process that effectually participates in the actual becoming of reality, which hence cannot be studied from an unbiased distance. The perspective, from which nature is seen and explored, is therefore intrinsically tied to the meaning under which it will be realized (von Weizsäcker, 1968; cited in: Link, 2005). Yet, this must not necessarily be confused with the self-centered approach that human subjectivism is the ultimate measure of reality. On the contrary, the ultimate question is whether human reason is able to search and recognize the true and good in conversation with nature while being always open to learn more in hope that if the established views are fallacious they will meet purification as a critical measure for objectivity. Like the pure sunlight, the full light of truth in his brightness may not be seen directly by the senses. Faust in Goethe's drama, after all his false starts in an effort to perceive what holds the world together in its innermost marrow, states in view of a rainbow, shimmering in a waterfall, with respect to human's aspiration:

"*In the colorful reflection we have what is life.*" (von Goethe, 1832; cited in: Paoletti, 2008; Huber, 2009)

According to the bible, the rainbow is the sign of the covenant that God established with humans and every living creature after the deluge (Genesis 9,12-13). The reality becoming of being in the course of time, the transformation of potentiality into actual reality, is a process whose principal reason is basically intangible und cannot be an object of empiric research. In that light, all reality that becomes actual, while it could not, is a reason to wonder and only with wonder, this reason might be realized as the experience-able presence of creation (Stein, 1950; von Weizsäcker, 1967; cited in Link, 2005; L'Ecuyer, 2014).

All these considerations are meaningful in the faith that nature is indeed reason-able creation. If nature is seen as the ubiquitous wonder of creation, mere materialistic

approaches must appear as short-sighted attempts to decrease the reality of nature to an entirely manageable system (Weizsäcker, 1968; cited in: Link, 2005). Nevertheless, a renunciation from the anthropocentric utilitarian dealing with nature towards a cultivation with respect to its inherent dignity and holistic reasonableness would be tantamount to a cultural revolution (from Latin: *revolvo*, 'roll back') of mindset in a society that almost excluded any transcendent considerations from the sphere of natural science and economy in building of its material prosperity.

In contrast, the life histories of individual humans that are known for their sensitivity for nature, such as Francis of Assisi (Bonaventura, 1221-1274; Bonaventure, 1868; Santmire, 1985) or Ignatius of Loyola (von Loyola, 2006), show that the experience of existential threat, such as to suffer a major defeat of one's own self-involved ideas in so-called personal "Waterloo" events, can provoke an overall personal conversion. This entails not only the recognition of misconceptions and separateness from the truth, but also a desire to change towards a reconciliation with creation and the source of life (pers. com. Jörg Splett, 2015; Francis, 2015). In this process, nature seems to be recognized as an open book that testifies the universal correspondence of true reason to the enlightened eyes of human reason (Francis, 2015). The experience of existential threat initiating such conversion in the concrete cases has been reported as exceedingly harsh and the consequent changes in life style as exceptionally radical (from Latin: *radicalis* 'having roots, rooted'). The ways of conversion exemplified by Francis of Assis or Ignatius of Loyola may rather be regarded as a special grace than as a general rule, thus requesting a more modest route for the common (Benedict XVI, 2010). Nevertheless, a loving confidence into the intrinsic goodness of creation due to its very basis may be an indispensable prerequisite to recognize a comprehensive meaning in the intrinsic and transcendent relatedness of nature (de Lisieux, 1873-1897; Stein, 1950; Lee, 2000; Irsigler, 2011). To question and to go beyond the borders of the civilized society and its prevailing patterns of anthropocentric thinking, therefore, seems to be an indispensable process of critical reflection towards a more authentic vison of reality, such as to develop a relationship with nature that is sensitive for the integrity of its inner borders in which humans themselves are integrated. These inner borders are in the middle of existence, so that they cannot be crossed without disrespecting the interrelated bonds and limits that are

defined by the natural order of reality (Bonhoeffer, 2007; cited in: Link, 2015; Francis, 2014). Simple but evident instances for the validity of this argument are that the degradation of nature earlier or later impairs the live of human beings, who themselves are inextricably a part of nature, or that infinite economic growth based on the consumption of limited resources is impossible (Turkson, 2014; Link, 2015). Moreover, the inner borders of creation are corresponding with perceptible dimensions and proportions that are indicative for the maintenance of balance in the dynamics of life processes. Such a phenomenon is the beauty of nature that can be a very sensitive criterion for its ecological integrity (Link, 2015). A concrete and impressive example for this is the rapidly degrading appearance of the Great Barrier Reef in response to ecological threats like climate change and water pollution (Harriot, 2002; Schmidt et al., 2014).

But, here immediately the question raises what is beauty and how can it be known or determined what is beautiful. In a recent essay from L'Ecuyer (2014) about the attendance of children to learning, some related considerations can be found (1-4):

(1) Does the perception of beauty only emerge from subjective brain processes or is there any scientific instrument that can objectively measure beauty? – Obviously not.

(2) What if beauty is one of the essential but intangible properties of the "being" of things; the visual expression of truth and goodness, whereas evil as such is a deprivation of "being"? (de Aquino, 1225-1274a; Lee, 2000) – Beauty, obviously, is one of the properties of existing things whose perception can trigger wonder in human beings, sensing that it "is" while it could "not be".

(3) What if wonder were the conjunction between the will and the cognitive ability to understand the nature of things, the "desire to learn"? What if the ability to wonder, then would be the transcendent bridge between sensory impressions, fragmented knowledge, and a recognition of meaningful relationships? – A positive answer to these questions would request a change in the paradigms of the prevailing reductionistic (Des Cartes, 1662), positivistic (Comte, 1994), relativistic (Feyerabend, 1975), and falsificationistic (Popper, 1935; Lakatos, 1978) theories of science that

almost eliminated the claim for any authentic recognition of the true reality from their epistemology (Kuhn, 1962; Rohrmoser, 1996). Or, "must we effectively say that all these (modern) myths were true?" (Socrates, as portrayed by Plato, *Euthyphro*, 6b-c; cited in: Benedikt XVI, 2008)

(4) Could it rather be that the way back to search the true reality lies in a wonder approach, as this implies a renunciation from a "*self-centered consciousness"* towards a "*consciousness of reality-based consistence"* as a starting point to learn from nature? – In any case, the answer to this question is an issue of either dealing with nature as a strange material or striving for reasonable harmony with creation (Link, 2005).

In this regard the threat of a global ecological crisis is not only a challenge to harmonize human interests with the natural order, such as by developing more sustainable production systems, but also a chance to revise the reductionist, mere materialistic attitude towards nature in favor of a holistic integrative understanding. Nature itself does not sustain its inconsiderate exploitation as a disposable matter for use and consumption, thereby calling the human being out of its constriction in a self-centered rationality. The experience of the inevitability of the inner bonds and limitations of the natural order remembers humans of their own finiteness, thus requesting a comprehensive reasonability that is able to transcend the self-deceptive misconception of reality (Rohrmoser, 1996).

Could it therefore be that the growing interest in integrated biological approaches to sustain the prerequisites for human life on earth is not only driven by the necessity to keep house with the natural resources, but also by a growing awareness that life itself is a dynamic realization of wholeness, which cannot be reduced to a mechanical flow of interacting materials?

Howsoever, the study of bio-effector applications in cropping systems teaches that these agents show a very weak performance to improve the mineral nutrition and healthy growth of plants when applied in stand-alone approaches, such as to mitigate the collateral damage induced by unsound agricultural practices. The scope to effectively benefit from the traits of bio-effectors, especially the microbial ones, rather appears to be

bound to the implementation of their sophisticated and multifaceted modes of action as integral components of the agro-ecosystem, where they interact with the plant and its environment as an interrelated whole. This organic system of meaningful relatedness is forming a dimension of reality that is other than the sum of the isolated parts (Koffka, 1935). The nature of bio-effectors thus is to be recognized with respect to the interactive processes in which their vital activities emerge, as their modes of action may not be understood without relation to the environmental matrix where they develop (Rohrmoser, 1996). Research on bio-effectors and the secrets of their vital relationships with the plant and their environment seems to be a favorable field to learn more about the dynamic wholeness and inter-relatedness of life and biological processes, whose reality is requesting a comprehensive reason-ability for an authentic understanding.

After all a scientist may admit: The stuff of living organisms is matter, but the very essence of life is reason, whose potential actualization under the transient conditions of time and space can trigger its realization through wonder (Stein, 1950; L'Ecuyer, 2014).

Yet, the last words may be quoted from the Logos of the bible:

"*It is the spirit that gives life, while the flesh is of no avail."* (John 6,63)

10 Summary - Zusammenfassung

10.1 Summary

Bio-Effectors for improved growth, nutrient acquisition and disease resistance of crops

Recent scientific approaches to sustain agricultural production in face of a growing world food demand, limited natural resources, and ecological concerns have been focusing on biological processes to support soil fertility and healthy plant growth. In this context, the use of "bio-effectors", comprising living (micro-) organisms and active natural compounds, has been receiving increasing attention in the literature, but also by the producers of biological (bio-) preparations for application in crop production. In contrast to conventional fertilizers and pesticides, the effectiveness of "bio-effectors" is essentially not based on the substantial direct input of mineral plant nutrients, neither in inorganic nor organic forms, nor of a-priori toxic compounds. Their direct or indirect effects on plant performance are rather based on the functional implementation or activation of biological mechanisms, in particular those interfering with soil-plant-microbe interactions, as defined in Chapter 1 and discussed in Chapter 2 (Part A).

Although there is strong evidence for the functional significance of bio-effectors from laboratory experiments and detailed knowledge on their specific modes of action has accumulated, reliable data for a successful application in practice are often missing and unknown factors constrain the reproducibility of effects under variable environmental conditions. The general objective of the present research work was to improve the empirical and conceptual understanding concerning the utilization of bio-effectors in agricultural practice, following the principles of plant growth stimulation, bio-fertilization and bio-control.

One main aspect of investigation was the application of bio-effectors to improve the efficiency of phosphorus (P) acquisition by the plant (Part B). As reviewed in Chapter 3, P is one of the most common limiting mineral nutrients in crop production, regarding its low availability in soils and global resources for fertilizer production. Bio-effectors, in

contrast, may improve the P availability to plants due to diverse modes of action. Promising bio-preparations based on microbial inoculants (e.g. *Bacillus, Pseudomonas, Trichoderma* species) as well as natural compounds (e.g. algae extracts, humic acids) were tested in screening assays (Chapter 4), greenhouse (Chapter 5), and field experiments (Chapter 6) to characterize their potential effectiveness under varying environmental conditions.

In vitro tests showed a strong capacity of bacteria from three bio-preparations to solubilize sparingly available calcium phosphate when cultivated on a nutrient-poor but not on a rich medium. Aside from a product based on phytohormone active algae extracts, none of the tested bacterial bio-preparations showed a clear effect on the root development of cucumber and wheat in germination and hydroponic tests. Vitalin SP11, one of the most promising bio-preparations, was intensively tested for improved P acquisition by crop plants under greenhouse conditions. Wheat or other test plants were grown on soil substrates low in plant available P but rich in certain sparingly available P forms. Beneficial effects were possible in connection with sparingly available mineral (calcium, iron or aluminum) as well as organic phosphates. In some cases, improved plant growth was associated with enhanced P acquisition, which was attributable to mechanisms of increased spatial and chemical nutrient availability to the plant. In general, the most significant effects on plants appeared under severely low phosphate availability, but even under controlled conditions, bio-effectors required a narrow range of conductive environmental settings to reveal their potential effectiveness.

In contrast to the results obtained under laboratory and greenhouse conditions, field experiments showed that treatments with selected bio-preparations (Vitalin SP11, Vitalin T50, Bio-super, BioAktiv), were not effective to improve the P acquisition and growth of diverse crops grown in organic farming. A main reason for this ineffectiveness might be that organically managed soils with diversified crop rotations favor the establishment of beneficial populations out of the indigenous microflora, providing suitable conditions for healthy root growth and P acquisition from the soil matrix even at low P levels in the soil solution. The application of bio-effectors, or even water-soluble P fertilizers, therefore provided little additional benefit, as plants together with associated soil microorganisms

may already have expressed to a nearly optimal extent the diverse biological mechanisms involved with the mobilization of mineral nutrients.

Another focus of research was the application of bio-effectors to control soil borne pathogens, which typically appear in unsound crop rotations (Part C). As reviewed in Chapter 7, the incidence of soil borne diseases is a sign of imbalance within the soil ecosystem where antagonists are impaired and pathogens prevalent. Soil borne pathogens may induce mineral nutritional disorders in plants, whereas an adequate supply of mineral nutrients is important for resistance of plants to various stresses. Emphasis was set on take-all disease in wheat induced by the fungus Gaeumannomyces graminis. While the effectiveness of oat precrops to control take-all in subsequent wheat has been attributed to microbial changes and enhanced Mn availability in soils, the take-all fungus is known to decrease the availability of Mn by oxidation.

Against this background, the effectiveness of oat precrops and alternative crop management strategies to improve the Mn status and suppress the severity of take-all in wheat was investigated under controlled and field conditions (Chapter 8). Pot experiments with soil substrates derived from oat- or wheat-precropped plots of a take-all infested field site were conducted with wheat as test crop. While soil application of Mn decreased the proportion of take-all infected roots, a bacterial preparation enhanced root growth and mycorrhization. Oat-induced changes in the soil microflora, however, appeared to ensure an enhanced Mn status and decrease the disease severity in subsequent wheat even when additional pathogen inoculum was added. In conclusion, none of the tested supplemental treatments could fully substitute for the multiple effectiveness of oat precrops, which was further confirmed by the results of a field experiment.

Finally, some general conclusions and perspectives are summarized (Part D). Selected bio-effectors showed a strong capacity to improve the nutrient acquisition and healthy growth of crop plants under controlled conditions, but not in field experiments. However, even under controlled conditions the strongest effects occurred when plants were exposed to abiotic or biotic stresses, such as severely limited P availability or pathogen infestation of the soil substrate, still restricting plant growth to unproductive levels. Facing this situation, there is no perspective to improve the field efficiency of promising bio-effectors

applications as a stand-alone approach. The only chance to develop viable alternatives to the conventional use of fertilizers or pesticides, for an ecological intensification of agriculture that maintains high yield levels, seems to be a reasonable integration of bio-effectors into the whole crop management of sound agricultural practice. Such approach is strongly implicated by the character of improvements achieved with successful bio-effector applications that, except for biological nitrogen fixation, cannot compensate the use of mineral or organic fertilizers for the replacement of mineral nutrients that are removed from the soil. The specific modes of action employed by bio-effectors are rather recognized as key prerequisites to improve the use efficiency of mineral nutrients from the soil or fertilizers, for the closure of nutrient cycles, and to maintain a balanced diversity of microbial populations in fertile soils. Their optimized utilization to target prevailing and future constraints imposed by abiotic and biotic stresses in crop production implies further research needs and is considered as an increasingly important issues in a world of declining resources and expanding demands, even more taking into account the possible impact of global warming on agriculture.

10.2 Zusammenfassung

Bio-Effektoren zur Verbesserung des Wachstums, der Nährstoffaneignung und der Krankheitsresistenz von Kulturpflanzen

Angesichts eines weltweit wachsenden Bedarfs an Nahrungsmitteln, begrenzter natürlicher Ressourcen und ökologischer Probleme streben neuere wissenschaftliche Ansätze zur nachhaltigen Gestaltung der Landwirtschaft verstärkt die Nutzung biologischer Prozesse zur Föderung der Bodenfruchtbarkeit und eines gesunden Kulturpflanzenwachstums an. In diesem Zusammenhang hat der Einsatz von „Bio-Effektoren“, einschließlich lebender (Mikro-)Organismen und wirkaktiver Naturstoffe, wachsendes Interesse in der Fachliteratur gefunden, aber auch bei den Herstellern von biologischen (Bio-)Präparaten zur Anwendung im Pflanzenbau. Im Gegensatz zu konventionellen Düngemitteln und Pestiziden beruht die Wirksamkeit von „Bio-Effektoren“ im Wesentlichen nicht auf dem substantiellen Direkteintrag von Pflanzennährstoffen, weder in anorganischer noch organischer Form, noch von a priori toxischen Stoffen. Die direkten oder indirekten Wirkungen von “Bio-Effektoren” auf die Pflanze basieren vielmehr auf der Implementierung oder Aktivierung von biologischen Wirkungsmechanismen, insbesondere solcher die mit den Interaktionen von Boden, Pflanze und Mikroorganismen interferieren (Kapitel 1 und 2; Teil A).

Obwohl Laborversuche die funktionale Bedeutung von Bio-Effektoren belegen und detaillierte Kenntnisse über ihre spezifischen Wirkungsweisen vorliegen, fehlen in den meisten Fällen zuverlässige Daten für eine erfolgreiche Anwendung und unbekannte Einflussgrößen verhindern die Reproduzierbarkeit von Effekten unter wechselnden Umweltbedingungen. Ziel der vorliegenden Arbeit war es, das empirische und konzeptionelle Verständnis zur Nutzung von Bio-Effektoren in der landwirtschaftlichen Praxis anhand der prinzipiellen Wirkungskategorien von direkter Stimulans des Pflanzenwachstums, biologischer Pflanzenernährung (bio-fertilization) und biologischem Pflanzenschutz (bio-control) zu erweitern.

Ein Hauptaspekt galt der Anwendung von Bio-Effektoren um die Aneignungseffizienz von Phosphor (P) durch die Pflanze zu steigern (Teil B). Wie in Kapitel 3 erläutert, ist P

aufgrund geringer Verfügbarkeit in Böden als auch begrenzter Ressourcen zur Düngemittelproduktion weltweit einer der meist limitierenden Mineralstoffe in der Pflanzenproduktion. Bio-Effektoren hingegen können die Pflanzenverfügbarkeit von P durch verschiedene Wirkungsweisen steigern. Aussichtsreiche Präparate, basierend auf mikrobiellen Inokula (z.B. *Bacillus*, *Pseudomonas*, *Trichoderma* Spezies) als auch Naturstoffen (z.B. Algenextrakte, Huminsäuren), wurden in Screeningassays (Kapitel 4), Gewächshaus- (Kapitel 5) und Feldversuchen (Kapitel 6) getestet um ihr Wirkungspotential unter variablen Umweltbedingungen zu charakterisieren.

Bakterienstämme aus drei der untersuchten Präparate zeigten ein ausgeprägtes Lösungsvermögen für schwerverfügbares Kalziumphosphat wenn sie auf einem nährstoffarmen nicht aber auf einem nährstoff-reichen Agarmedium kultiviert wurden. Im Gegensatz zu einem phytohormonreichen Algenpräparat, hatte die Anwendung von bakteriellen Präparaten keine deutliche Wirkung auf die morphologische Entwicklung von Gurken- und Weizenwurzeln in Keimtests und Nährlösungsversuchen. Ein aussichtreiches Bio-Präparat, Vitalin SP11, wurde intensiv unter Gewächshausbedingungen auf seine Wirksamkeit die P-Aneignung von Pflanzen zu fördern geprüft. Weizen oder andere Kulturpflanzen wurden dazu auf Boden-substraten mit geringem pflanzenverfügbarem P-Angebot aber ausgeprägten Vorräten an bestimmen schwer verfügbaren P-Formen kultiviert. Dabei zeigten sich förderliche Effekte in Verbindung mit schwer verfügbaren anorganischen (Calcium, Eisen oder Aluminium) als auch organischen Phosphaten. In einigen Fällen, ging ein gesteigertes Pflanzenwachstum mit erhöhter P-Aneignung einher, was verschiedenen Wirkungsmechanismen zur Förderung der räumlichen und chemischen Nährstoffverfügbarkeit für die Pflanze beizumessen war. Generell traten die stärksten Effekte unter Bedingungen mit sehr geringer Phosphatverfügbarkeit auf, jedoch selbst unter kontrollierten Bedingungen war der Einfluss förderlicher und teilweise eng bestimmter Umweltfaktoren notwendig um die Wirksamkeit von Bio-Effektoren zu zeigen.

Im Gegensatz zu den unter Labor- und Gewächshausbedingungen gemachten Beobachtungen, zeigten Feldversuche keine Wirkung diverser Präparate (Vitalin SP11, Vitalin T50, Bio-super, BioAktiv) die P-Aneignung und das Wachstum verschiedener

Kulturpflanzen im ökologischen Landbau zu fördern. Ein Grund für diese Wirkungslosigkeit dürfte in der ökologischen Bewirtschaftung der Böden mit ausgewogenen Fruchtfolgen liegen welche die Etablierung nützlicher Populationen aus der indigenen Mikroflora fördert und damit günstige Bedingungen für ein gesundes Wurzelwachstum und die Aneignung von P aus der Bodenmatrix selbst bei einem geringen P-Angebot in der Bodenlösung schafft. Die Anwendung von Bio-Effektoren, oder gar von wasserlöslichem P-Dünger, war daher kaum von Nutzen, da die Pflanzen zusammen mit assoziierten Mikroorganismen im Boden die verschiedenen biologischen Wirkungsmechanismen zur Mobilisierung von Mineralstoffen und Unterdrückung von Pathogenen wohl bereits zu einem nahezu optimalen Maße ausgeprägt hatten.

Ein weiteres Thema war der Einsatz von Bio-Effektoren zur Bekämpfung bodenbürtiger Krankheitserreger, welche durch unausgewogene Fruchtfolgen gefördert werden (Teil C). Wie in Kapitel 7 beschrieben, weist das Auftreten bodenbürtiger Pflanzenkrankheiten auf eine gestörtes Gleichgewicht im Ökosystem des Bodens hin, wobei natürlichen Antagonisten beeinträchtigt sind und Pathogene überhand nehmen. Bodenbürtige Pathogene können auch den Ernährungsstatus der Pflanze beeinträchtigen, wohingegen ein angemessenes Mineralstoffangebot wichtig für die Resistenz von Pflanzen gegenüber vielfältigen Stress-faktoren ist. Die Untersuchungen befassten sich insbesondere mit der Schwarzbeinigkeit des Weizens, einer bedeutenden Wurzelkrankheit hervorgerufen durch den Pilz Gaeumannomyces graminis. Eine besonders effektive Vorfrucht zur Bekämpfung der Schwarzbeinigkeit ist Hafer, was in Zusammenhang mit mikrobiellen Veränderungen im Boden und einer erhöhten Verfügbarkeit von Mangan (Mn) für die Folgefrucht Weizen gestellt wurde. Der Erreger der Schwarzbeinigkeit dagegen kann pflanzenverfügbares Mn durch Oxidation im Boden festzulegen.

Vor diesem Hintergrund wurde die Wirksamkeit von Hafer als Vorfrucht im Vergleich mit alternativen Maßnahmen des pflanzenbaulichen Managements zur Steigerung der Mn-Versorgung und Bekämpfung der Schwarzbeinigkeit in Weizen untersucht (Kapitel 8). Zur Durchführung von Gefäßversuchen mit Weizen wurde einem von der Schwarzbeinigkeit befallenen Weizenfeld aus Parzellen mit Hafer- oder Weizen-vorfrucht Bodenmaterial entnommen. Während die Bodenapplikation von Mn den Anteil von durch

die Schwarzbeingkeit befallenen Wurzel verminderte, förderte ein bakterielles Präparat das Wachstum und die Mykorrhizierung der Wurzeln. Durch Hafer induzierte Veränderungen in der Bodenmikroflora schienen jedoch selbst dann eine höhere Mn-Versorgungen und einen verminderten Krankheitsbefall in der Folgefrucht Weizen zu gewährleisten, wenn dem Bodensubstrat zusätzliches Pathogeninokulum zugefügt wurde. Fazit war, daß keine der getesten Alternativmaßnahmen die vielseitige Vorfruchtwirkung von Hafer vollständig substituieren konnte, was auch durch die Ergebnisse eines Feldversuches bestätigt wurde.

Abschließend folgen generelle Schlussfolgerungen aus der vorliegenden Arbeit und sich daraus ergebende Perspektiven zur Nutzung von Bio-Effektoren (Teil D). Ausgewählte Bio-Effektoren zeigten unter kontrollierten Bedingungen ein hohes Wirkungspotential zur Förderung der Nährstoffaneignung und des gesunden Wachstums von Kulturpflanzen, nicht jedoch in Feldversuchen. Zudem waren die stärksten Effekte dann zu beobachten, wenn die Pflanzen abiotischen oder biotischen Stresssituationen, wie stark limitierter P-Verfügbarkeit oder einem pathogenbelasteten Bodensubstrat, ausgesetzt waren, welche das Pflanzenwachstum auch weiterhin auf ein unproduktives Maß beschränkten. Diese Ergebnisse eröffnen keine Perspektive den praktischen Nutzen von Bio-Effektoren durch Einzelmaßnahmen zu steigern. Vielmehr schein der einzig erfolgversprechende Ansatz leistungsfähige Alternativen zum konventionellen Einsatz von Düngemitteln und Pestiziden für eine ökologische Intensivierung der Agrarproduktion auf hohem Ertragsniveau zu entwickeln, eine sinnvolle Integration von Bio-Effektoren in das gesamte pflanzenbauliche Management einer fachgerechten Landwirtschaft zu sein. Solche Vorgehensweise ist bedingt durch die möglichen Wirkungsweisen von Bio-Effektoren, welche, abgesehen von biologischer Stickstofffixierung, nicht den Einsatz von mineralischen oder organischen Düngemitteln kompensieren können der zum Ersatz von Mineralstoffen notwendig ist die dem Boden entzogen werden. Die verschiedenen Wirkungsweisen von Bio-Effektoren bieten vielmehr eine Grundvoraussetzung zur Steigerung der Nutzungseffizienz von Mineralstoffen aus dem Boden oder aus Düngemitteleintrag, zur Schließung von Nährstoffkreisläufen, und zum Erhalt einer ausgewogenen mikrobiellen Diversität in nachhaltig fruchtbaren Böden. Eine optimierte Nutzung von Bio-Effektoren um vorherrschende und künftige Beeinträchtigungen durch

abiotische und biotische Stressfaktoren in der Pflanzenproduktion zu verringern impliziert weiteren Forschungsbedarf und stellt in einer Welt abnehmender Ressourcen und wachsenden Bedarfs eine zunehmend wichtige Aufgabe dar, umso mehr wenn mögliche Auswirkungen des Klimawandels auf die Landwirtschaft in Betracht gezogen werden.

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13 Tabellarischer Lebenslauf – *Curriculum vitae*

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Schulbildung:	1981–1985 Besuch der Grundschule Worms-Herrnsheim 1985-1994 Besuch des Rudi-Stephan-Gymnasiums in Worms
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Studium:	1995-2000 Allgemeine Agrarwissenschaften, Universität Hohenheim, Fachrichtung Pflanzenproduktion
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Seit 2012 Forschungsprojekt „Resource Preservation by Application of BIOefFECTORs in European Crop Production (BIOFECTOR)“, finanziert durch die Europäischen Kommission innerhalb des 7. Rahmenprogrammes, Grant agreement no: 312117

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"Microp. Microbe-Assisted Crop Production – Opportunities, Challenges & Needs", Vienna, Austria, 23. – 25. November 2015. Posterpräsentation: Weinmann M., Nkebiwe P. M., Weber N., Hasan M. M., Poşta G., Müller T., Neumann G. (2015). "Microbial bio-effectors for alternative plant nutrition: development of application strategies in the BioFector project".

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