Bioeffector products for plant growth promotion in agriculture: Modes of action and the application in the field

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Abbreviations

A95	Alga 95 (seaweed extract)
ABA	Abscisic acid (plant hormone)
ACC	1-Aminocyclopropane-1-carboxylic acid
Af	Algafect (seaweed extract)
AHL	acyl homoserine lactone
AMF	arbuscular mycorrhiza fungi
AV	AlgaVyt (seaweed extract)
AVZM	AlgaVyt Zn/Mn (seaweed extract)
BacA	*Bacillus atrophaeus*
BE	bio-effector product
BFDC	Biological fertilizer DC (BE product with fungal strain *Penicillium* sp. PK 112)
BIN	here: a functional category in MapMan gene expression analysis
Bsim	*Bacillus simplex*
BY	biomass yield
CAT	catalase
CC	climate chamber
CFU	colony forming unit
CK	cytokinin (plant hormone)
Cl	chlorosis/necrosis (or element symbol of chlorine)
Combi A	Combifector A (microbial consortia product)
CRB	completely randomized block design
CRD	completely randomized design
CRZT	controlled root zone temperature
DAS	days after sowing
DEG	differentially expressed genes
DM / DW	dry matter / dry weight
DRB	differentially regulated BIN
ECO	Ecolicitor (seaweed extract)
ER	emergence rate
Et/ET	ethylene
Ez	enzymatic measurements
FM / FW	fresh matter / fresh weight
GE	gene expression analysis
GH	greenhouse
GY	grain yield
H-NMR	Proton nuclear magnetic resonance
HR	hypersensitive reaction
Hsp	*Herbaspirillum* sp.
IAA	indole-3-acetic acid (auxin, plant hormone)
ISR	induced systemic resistance
JA	Jasmonic acid (plant hormone)
LS	latin square

MA	mineral analysis
MAD	malondialdehyd
MHB	mycorrhiza(tion) helper bacteria
Myc	mycorrhization of roots
OmG	*Trichoderma harzianum* OmG-08
P	phosphorus
P1	Product 1 (seaweed extract)
P2	Product 2 (seaweed extract)
P3	Product 3 (seaweed extract)
PCA	principal component analysis
PCR	polymerase chain reaction
PGPM	plant growth promoting microorganism
PGPR	plant growth promoting rhizobacteria
PH	plant height
Pj	*Pseudomonas jessenii*
PM	plant metabolome
POD	peroxidase
PR	pathogenesis related (genes)
PS	photosynthesis
PSM	P-solubilizing microorganism
Px	*Pseudomonas* sp. DSMZ 13134 'Proradix'
RC	root colonization by BEs (tracing)
RL	root length
ROS	reactive oxygene species
RT	room temperature
RT-qPCR	reverse transcription quantitative polymerase chain reaction
RW	root weight
RxC	row-column design
Rz	Rhizovital (containg *Bacillus amyloliquefaciens* FZB42)
SA	salicylic acid (plant hormone)
SAR	systemic aquired resistance
SD	stem diameter
SF	Superfifty / Alga 50 (seaweed extract)
SOD	superoxide dismutase
SPAD	SPAD values (chlorophyll content)
SW	shoot weight
SWE	seaweed extracts
TF	transcription factor (e.g. ERFs, WRKYs, NACs)
TH	*Trichoderma harzianum* (fungal strain)
TP	Trianum-P (containing the fungal strain *Trichoderma harzianum* T-22)
WHC	water holding capacity

A Summary

Modern agriculture faces a conflict between sustainability and the demand for a higher food production. This conflict is exacerbated by climate change and its influence on vegetation, ecology and human society. To reduce land use, the reduction of yield losses and food waste is crucial. Moreover a sustainable intensification is necessary to increase yields, while at the same time input of limited resources such as drinking water or fertilizer should be kept as low as possible. This might be achieved by improving nutrient recycling and plant resistance to abiotic or biotic stress. Bioeffectors (BE) comprise seaweed or plant extracts and microbial inoculums that may stimulate plant growth by phytohormonal changes and increase plant tolerance to abiotic stress (biostimulants), solubilize or mobilize phosphorus from sparingly soluble sources such as Al/Fe or Ca-phosphates in the soil, rock phosphates, recycling fertilizer or organic phosphorus sources like phytate (biofertilizer), or improve plant resistance against pathogens by induced-systemic resistance (ISR) or antibiosis (biocontrol).

For this study, in total 18 BE products were tested in germination, pot and field experiments for their potential to improve plant growth, cold stress tolerance, nutrient acquisition and yield in maize and tomato. Additionally, a gene expression analysis in maize was performed using whole transcriptome sequencing (RNA-Seq) after the application of two potential plant growth promoting rhizobacteria (PGPR), the *Pseudomonas* sp. strain DSMZ 13134 “Proradix” and the *Bacillus amyloliquefaciens* strain FZB42.

Seaweed products supplemented with high amounts of the micronutrients Zn and Mn were effective in reducing detrimental cold stress reactions in maize whereas microbial products and seaweed extracts without micronutrient supplementation failed under the experimental conditions.

At optimal temperature the product containing the *Pseudomonas* sp. strain was repeatedly able to stimulate root and shoot growth of maize plants whereas in tomato only in heat-treated soil substrate significant effects were observed. Results indicate that the efficacy of the product was mainly attributed to stimulation or shifts in the soil microbial community.

Additionally, the FZB42 strain was able to stimulate root and plant growth in some experiments whereas the effects were less reproducible and more sensitive to environmental conditions. Fungal BE products were less effective in plant growth stimulation and showed detrimental effects in some experiments.

Under the applied experimental conditions BE-derived plant growth stimulation mainly was attributed to biostimulation but aspects of biofertilization or biocontrol cannot be excluded, as all experiments were conducted in non-sterile soil substrates.

Root and shoot growth are stimulated in response to hormonal shifts. In the gene expression analysis only weak responses to BE treatments were observed, as previously reported from other studies conducted under non-sterile conditions. Nevertheless, some plant stress responses were observed that resembled in some respects those reported for phosphorus (P) deficiency in others those reported for ISR/SAR. Especially the activation of plant defence mechanisms, such as the production of secondary metabolites, ethylene production and reception and the expression of several classes of stress-related transcription factors, including JA-responsive JAZ genes, was observed. It also seems probable that in plants growing in PGPR-drenched soils, especially at high application rates, a sink stimulation for assimilates triggers changes in photosynthetic activity and root growth leading to an improved nutrient acquisition.

Nevertheless, due to the complexity of interactions in natural soil environments as well as under practice conditions, a designation of a distinct mode of action for plant growth stimulation by microbial BEs is not realistic.

A comparison of the overall results with those reported in literature or other working groups in a common research project (“Biofector”) supported the often-reported low reproducibility of plant growth promotion effects by BE products under applied conditions. Factors that influenced BE efficacy were application time and rates, temperature, soil buffer capacity, phosphorus sources and nitrogen fertilization, light conditions and the soil microbial community.

Results indicate that in maize cultivation seed treatment is the most economic application technique for microbial products whereas for vegetable or high-value crops with good economic benefit soil drenching is recommended. For seaweed extracts foliar application seems to be the most economic and efficient choice.

Furthermore, results emphasize the importance of a balanced natural soil microflora for plant health and yield stability. It may therefore be concluded that the stimulation and conservation of this adapted microflora should be a major concern for modern and future agriculture.

B Zusammenfassung

Die moderne Landwirtschaft steht vor einem Konflikt zwischen Nachhaltigkeit und der Forderung nach einer höheren Nahrungsmittelproduktion. Dieser Konflikt wird durch den Klimawandel und dessen Folgen noch verstärkt. Zur Verringerung der Flächennutzung ist eine nachhaltige Intensivierung erforderlich. Gleichzeitig sollte der Einsatz begrenzter Ressourcen wie Trinkwasser sowie umweltschädlicher Stoffe (Düngemittel, Pestizide) so gering wie möglich gehalten werden. Dies kann durch Verbesserungen im Nährstoffrecycling sowie durch Stärkung der Pflanzenresistenz gegenüber abiotischem oder biotischem Stress erreicht werden. Bio-Effektoren (BE) umfassen Algen- oder Pflanzenextrakte und mikrobielle Inokula, die das Pflanzenwachstum durch phytohormonelle Veränderungen stimulieren und die Pflanzenverträglichkeit gegenüber abiotischem Stress erhöhen (Biostimulanzien), Phosphor aus schwerlöslichen Quellen wie Al/Fe oder Ca-Phosphaten im Boden, Steinphosphaten, Recyclingdüngern oder organischen Phosphorquellen wie Phytat mobilisieren (Bio-Dünger) oder zur Verbesserung der Pflanzenresistenz gegen Pathogene durch induzierte systemische Resistenz (ISR) oder Antibiose (Bio-Pestizide) beitragen.

Insgesamt wurden 18 BE-Produkte in Keimungs-, Topf- und Feldexperimenten auf ihr Potenzial zur Verbesserung des Pflanzenwachstums, der Kältestresstoleranz, der Nährstoffaufnahme und des Ertrags in Mais und Tomate getestet. Zusätzlich wurde eine Genexpressionsanalyse in Mais durchgeführt unter Verwendung der vollständigen Transkriptomsequenzierung (RNA-Seq) nach der Anwendung von zwei potenziell pflanzenwachstumsfördernden Rhizobakterien (PGPR), dem *Pseudomonas* sp. Stamm DSMZ 13134 "Proradix" und dem *Bacillus amyloliquefaciens* Stamm FZB42.

Meeresalgenprodukte, die mit hohen Mengen der Mikronährstoffe Zn und Mn angereichert wurden, konnten Kältestressreaktionen bei Mais wirksam reduzieren, während mikrobielle Produkte und Meeresalgenextrakte ohne Mikronährstoffergänzung unter den Testbedingungen erfolglos waren. Bei optimaler Temperatur war das Produkt, das den *Pseudomonas*-Stamm enthält, wiederholt in der Lage, Wurzel- und Sprosswachstum von Maispflanzen zu stimulieren, während in Tomaten nur in wärmebehandeltem Bodensubstrat signifikante Effekte beobachtet wurden. Die Ergebnisse legen nahe, dass die Wirksamkeit des Produkts hauptsächlich auf Stimulation oder Veränderungen in der mikrobiellen Gemeinschaft im Boden zurückzuführen ist. Auch der FZB42-Stamm war in der Lage, das Wachstum von Wurzeln und Pflanzen in einigen Experimenten zu stimulieren, während die

Effekte weniger reproduzierbar und empfindlicher für Umweltbedingungen waren. Pilzliche BE-Produkte waren bei der Pflanzenwachstumsstimulation weniger effizient und zeigten in einigen Experimenten auch schädliche Wirkungen.

Unter den angewandten experimentellen Bedingungen scheint die BE-abgeleitete Pflanzenwachstumsstimulation hauptsächlich auf Biostimulation zurückzuführen zu sein, aber Aspekte der Bio-Düngung oder Bio-Kontrolle können nicht ausgeschlossen werden, da alle Experimente in nicht-sterilen Bodensubstraten durchgeführt wurden. Die Stimulation des Wurzelwachstums und der Sprosswachstumsrate ist eine Reaktion auf hormonelle Veränderungen. Die Genexpression zeigte nur schwache Reaktionen auf die BE-Behandlungen, wie bereits aus anderen Studien unter nicht-sterilen Bedingungen berichtet wurde. Trotzdem wurden einige pflanzliche Stressreaktionen beobachtet, die entweder für Phosphor (P)-Mangel oder aber ISR / SAR als typisch gelten. Insbesondere die Aktivierung von Abwehrmechanismen wie die Produktion von Sekundärmetaboliten, die Ethylenproduktion und -rezeption sowie die Expression mehrerer Klassen stressbedingter Transkriptionsfaktoren, einschließlich JA-responsiver JAZ-Gene, wurde beobachtet. Es scheint auch wahrscheinlich, dass in Pflanzen, die in PGPR-durchtränkten Böden wachsen, insbesondere bei hohen Aufwandmengen, eine Senkenstimulation für Assimilate die Photosyntheserate erhöht sowie Veränderungen im Wurzelwachstum auslöst, die zu einer verbesserten Nährstoffaufnahme führen können. Die Bestimmung eines speziellen Wirkungs-mechanismus ist jedoch durch die Komplexität der Interaktionen im Boden nicht möglich.

Ein Vergleich der Gesamtergebnisse mit denen, die in der Literatur oder anderen Arbeitsgruppen in einem gemeinsamen Forschungsprojekt ("Biofector") berichtet wurden, unterstützt die oft berichtete geringe Reproduzierbarkeit von Pflanzenwachstumseffekten durch BE-Produkte unter Praxisbedingungen. Faktoren, die die BE-Wirksamkeit beeinflussen, sind Applikationszeit und -rate, Temperatur, Bodenpufferkapazität, Phosphor-Quelle, Stickstoff-Düngung, Lichtbedingungen sowie die mikrobielle Gemeinschaft des Bodens. Die Ergebnisse zeigen zudem, dass Saatgutbehandlung die wirtschaftlichste Anwendungstechnik für mikrobielle Produkte im Maisanbau ist, wobei für hochpreisige Kulturen im Gemüsebau konzentrierte Bodenapplikation empfohlen ist. Für Algenextrakte scheint Blattapplikation die beste Wahl zu sein. Die Ergebnisse zeigen zudem, wie wichtig eine ausgewogene, natürliche Bodenmikroflora für die Pflanzengesundheit und Ertragsstabilität ist. Es kann daher der Schluss gezogen werden, dass die Stimulation und der Erhalt dieser angepassten Mikroflora ein wichtiges Anliegen für die moderne und zukünftige Landwirtschaft sein sollte.

1 Introduction

1.1 The need for a new 'Green Revolution'

With the words "These and other developments in the field of agriculture contain the makings of a new revolution. It is not a violet, Red Revolution like that of the Soviets, nor is it a White Revolution like that of the Shah of Iran. I call it the Green Revolution." William S. Gaud, the administrator of the U.S. Agency for International Development (USAID), praised the achievements of the industrialisation of agriculture in many developing countries of the world by modern agrotechnological approaches like high-yielding hybridized seeds, synthetic fertilizers and pesticide usage (Gaud, 1968). World agricultural net production and subsequently world average dietary supply per person (total food in kcal per person as well as protein and fat) increased since 1961 whereas undernourishment continuously decreased (FAO, 2018). Nevertheless, at the same time agricultural land use and climate gas emissions by agriculture steadily expand while forests decline. The main agricultural producer of world CO_2 equivalents is livestock production of ruminants (~ 40 %, mainly methan, + emission of 16 % by manure). However, also the production and usage of synthetic fertilizer has a major impact and accounts for up to 13 % of greenhouse gas emissions. The usage of nitrogen continuously increased in the last 15 years from 83 to 109 Mt per year. While the area of agricultural land stagnates since 1990 N use per area increased by 30 % from 65 to 86 kg ha^{-1}. Similar trends can be seen for phosphorus use (> 25 % from 26 to 33 kg ha^{-1}). Energy consumption in agriculture today is 10 times higher than in 1990. Also, world pesticide market continuously grew to almost 40 billion US$ per year. Industrialisation and intensification of agriculture also coincides with a decrease in biodiversity (CBD, 2018; McRae et al., 2017; WWF, 2016). Due to the environmental impact by industrial agriculture and the awareness raised by environmental agencies, NGOs and the scientific community worldwide (Albrecht and Engel, 2009; Ripple et al., 2017), public interest in a sustainable agriculture is growing. In Europe the area of land under organic management showed an almost exponential growth between the years 1985 to 2001 (Yussefi and Willer, 2003), then slowed down but continued to grow until it reached a size of 12.7 million ha. Worldwide about 51 million ha (1 % of the total agricultural area) is managed organically with a market size of about 82 billion US$ (Willer et al., 2017). The biggest challenges for organic or integrated agriculture are to ensure plant health and productivity while reducing the input of pesticides and fertilizers. In the following sections these aspects will be addressed in detail.

1.2 Challenges for modern agriculture

1.2.1 Plant health

The European public opinion is divided when faced with the topic of genetically modified organisms (GMOs) or synthetic pesticides. Both sectors are well established in many other regions in the world, e.g. North America, and agricultural industry as well as parts of the scientific community see no evidence for a prevention of these technologies (GTF, 2017; Wager, 2009). However, in the European public pesticides and GMOs stay unpopular, especially with the debate on the withdrawal of glyphosate or neonicotinoid insecticides from the market, due to their supposed involvement in cancer or colony collapse disorder of honey bees respectively (EFSA, 2015; Fairbrother et al., 2014; Neslen, 2016; US EPA, 2013).

In organic agriculture sulphur and copper salts are the most common treatments to fungal diseases. Nevertheless, copper is not harmless and accumulation of copper may have negative environmental impacts on soil biology, for example earthworms and microbial activity, as well as water bodies (Fishel, 2005; Husak, 2015; Van Zwieten et al., 2004; Wang et al., 2009). The topic of plant health, especially when reduced to the aspect of biotic stress, is treated only as a side issue in this thesis, but, as later on described, plant health is not only a protection from pests, but from a more general point of view also tightly connected to plant nutrition and abiotic stress, in a physiological but also economic way. A plant that is suffering from undernourishment and abiotic stress is much more susceptive to biotic stress (Huber et al., 2012).

1.2.2 Phosphorus

The second challenge is to ensure sufficient nutrient supply to agricultural crops. Efficient alternatives to soluble synthetic fertilizers, normally showing high plant availability, are rare, especially with the focus on phosphorus (P).

P is a macronutrient and is, together with N and K, the most fertilized nutrient in agricultural systems, because it is a component of several macromolecules (DNA, RNA, phospholipids), active in energy metabolism (e.g. ATP) and therefore important in plant metabolism (Hawkesford et al., 2012). Additionally to its importance for plant growth and yield stability it is of special interest due to its very low plant availability in most soils (Marschner and Rengel, 2012). Furthermore, in contrast to N that can be fixed from atmospheric N, for example by the Haber process or biological N fixation, P is mainly derived from non-regenerative rock phosphate.

P is taken up by plants mainly as orthophosphate anions. Unfortunately phosphate in solution often accounts for only 0.001 - 0.01 % of total P in the soil (Gerke, 2015). The largest part of phosphate is immobilized by adsorption to Fe/Al (hydr)oxides or humic Al/Fe complexes. In a continuous equilibrium of ad- and desorption P is transported to the root mainly by diffusion with only a low contribution by mass flow (Marschner and Rengel, 2012). Therefore, an enlarged nutrient-absorbing surface by an increased root length, root to shoot ratio, root hair length or mycorrhization by mycorrhizal fungi are commonly observed mechanisms by which plants can successfully increase their P supply. Plants with large root systems, such as wheat and other grasses, are able to reach high yields even at low P concentration in the solution of below 1.5 µM, whereas tomatoes, beans or onions, having small root systems, need 3 – 4 times more P to reach their yield potential, even when plants do not strongly differ in their P use efficiency (Föhse et al., 1988). Additional to the total root length, the architecture can be changed to exploit specific nutrient rich regions, particularly near to the soil surface, or specific root parts are strongly promoted in growth and lateral root formation for nutrient acquisition (e.g. cluster roots in white lupin) (Niu et al., 2012).

In developed countries generally a net surplus of nutrients in the environment is observed through the intensive use of mineral fertilizers and the import of nutrients from often non-developed countries, mainly via animal feed. Data from the JRC of the European Commission (Grizzetti et al., 2007) show a very positive gross balance for N and P in many European countries, including Germany, although N and P surplus on ha^{-1} basis were much lower than in Belgium and the Netherlands. A high surplus of P in the soil can cause eutrophication of water bodies, whereas traditionally surface runoff or erosion rather than leaching are considered to be the main pathway for transport of P from agricultural fields. This is due to the low solubility of P in the soil but it might differ in acidic and loose soils (Djodjic et al., 2004; Sharpley and Menzel, 1987).

To reduce negative environmental impacts in organic farming, only P fertilizers with low P-solubility are allowed. Typical sources are ground, unprocessed rock phosphates, mainly containing sparingly soluble Ca-phosphates or manure, rich in organically bound P. Many organic farms in Europe still have a relatively high P availability in their soils, probably due to a recent conversion of the farm from conventional management or due to an import of large amounts of manure from conventional farms (Cooper et al., 2018). Nevertheless, the latter practice is in conflict with organic farming principles and might therefore be forbidden in the future (Cooper et al., 2018). In the last years new and promising approaches were developed

to obtain sustainable alternatives to conventional P fertilizers, especially recycling fertilizer from urban organic household waste, sewage sludges or slaughterhouse wastes (Foereid, 2017; Meyer et al., 2017), but those approaches might be energy intensive and are not well implemented in the market yet (Hörtenhuber et al., 2017). Additionally, depending on the technology and the properties of the products, not all might be allowed for organic farming.

P-solubilization

To increase P availability in soils, plants have various mechanisms to solubilize P from sparingly soluble soil or fertilizer P sources by chemical processes. Crucial for plant derived P-solubilization is the exudation of active compounds like carboxylates, especially malate and citrate, and phenolics (also active as reductants for micronutrient acquisition) (Badri and Vivanco, 2009; Broadley et al., 2012, p. 193; Neumann and Römheld, 2012, p. 358ff.). In high pH and carbonate buffered soils, the exudation of protons (H^+) to decrease pH in the "rhizosphere" are also of importance. These processes are well studied for Proteaceae or some legumes like white lupin that are able to form cluster roots (Neumann et al., 2000). Due to the strongly increased root density of these cluster roots, in a certain spot extreme pH differences (up to 2-3 units, reflecting a 1000-fold higher proton concentration) between the rhizosphere soil and the surrounding "bulk soil" can be achieved thus reducing the soil buffer capacities (Neumann and Römheld, 2002). Together with the high concentration of carboxylates the plants are able to exploit P even from extremely P-deficient substrates. The plants are so P efficient that soil P concentrations commonly observed in our regions might cause toxicity (Hawkesford et al., 2012, p. 164).

Nevertheless, pH decrease in the rhizosphere is also observed in other plants, especially in dicotyledonous and non-graminaceous monocotyledonous Strategy I plants under Fe deficiency (White, 2012a, p. 36ff.). Cereals and grasses follow other strategies for Fe acquisition such as the release of phytosiderophores (Strategy II). Therefore pH changes in the rhizosphere under nutrient deficiency are less pronounced (Neumann and Römheld, 2002). Rhizosphere acidification is especially observed under ammonium N nutrition (Neumann and Römheld, 2012, p. 354), due to a release of protons into the rhizosphere that are produced by NH_4^+ assimilation in the root tissue (Neumann and Römheld, 2002). The decrease in pH is also a prerequisite for an optimal activity of acid phosphatases (pH 4-6) that are able to hydrolyze phosphomonoester bonds of organic compounds to liberate inorganic orthophosphate (P_i) to be taken up by the plant root (Dick et al., 2000; Lemanowicz, 2011). Although plants produce alkaline phosphatases (Kieleczawa et al., 1992) (with pH optimum >

7), they seem not to be released by roots (Dodd et al., 1987; Tarafdar and Claassen, 1988). Inside the plants Pi is incorporated into various organic forms whereas in seeds, P is mainly stored as phytic acid (*myo*-inositol hexakis dihydrogen phosphate) (Hawkesford et al., 2012, p. 162). Hence up to 80 % of the P in soils may be bound organically (González-Muñoz et al., 2015; Neumann and Römheld, 2012, p. 364), with phytate (Ca-Mg salts of phytic acid) as one of the main organic P forms due to its low solubility. Phosphatases with high substrate specificity for phytic acid (phytate) are called phytases (Mullaney and Ullah, 2003). Despite the multiple mechanisms by which plants can improve P availability from various soil P fractions, in many soils P availability remains below the needs for crop production due to adsorption of phosphatases, low solubility of recalcitrant organic P complexes (Hayes et al., 2000; Richardson et al., 2001b) and high buffer capacities in soil reducing rhizosphere acidification. Especially carboxylates may be effective to target insoluble organic P depots but most plants are not producing carboxylates in sufficient amounts. Intercropping approaches with white lupin or other legumes may be a promising approach (Gerke, 2015).

1.2.3 Nutrient recycling and use efficiency

The efficiency in nutrient solubilization and acquisition, together nutrient uptake efficiency, may differ strongly among cultivars or genotype. As shown for P-deficient maize inbred lines the higher efficiency was determined by multiple traits, such as root length and root growth, root activity, acid phosphatase activity but also hormonal changes, for example increased expression of ethylene synthesis genes (Jiang et al., 2017).

Additionally to an improved nutrient uptake higher yields can be achieved when crops are more efficient in biomass production at low internal P (or other nutrient) concentrations or are able to remobilize nutrients more efficiently from vegetative organs to generative or storage organs (or the respective organ of interest for human consumption), which is termed use efficiency (George et al., 2012, p. 412ff.). Especially in modern plant breeding one focus is to improve nutrient and water use efficiency in crops. Many definitions for nutrient use efficiency (NUE) can be found in literature, for instance “the amount of biomass or yield per amount of applied fertilizer” (agronomic efficiency (AE)), whereas the above described aspects of nutrient acquisition are included, or “biomass per amount of internal nutrient concentration” (nutrient efficiency ratio (NER)) (Baligar et al., 2001).

Remobilization capacity is highly nutrient specific. During plant or leaf senescence remobilization (or re-translocation) is commonly observed, especially in perennial plants. In annual plants these processes can be observed as well, especially under nutrient deficiencies

or in the generative phase. Nevertheless, not all nutrients are easily to remobilize from old tissues as seen by nutrient deficiency symptoms. Nutrients that can easily be mobilized, such as N, P, K and to some extend also Zn, are transported to growing tissues during nutrient starvation leading to chlorosis in old tissues whereas nutrients such as Ca, Mg, Fe, Mn and B show low capacity for remobilization and therefore deficiency symptoms may first appear in the younger leaves (White, 2012b, p. 66ff.). Also species vary strongly in their remobilization efficiency. In wheat up to 90 % of P in grains may be re-translocated from leaves. In contrast, in maize about 40 % of N was re-translocated but no remobilization at all was observed for P (Maillard et al., 2015). As indicated by soil and shoot P analysis plants were not suffering from P-deficiency in this study but results indicate that the potential of maize plants to remobilize P may be lower than in wheat. Nutrient remobilization under nutrient deficiency is tightly linked to leaf senescence regulation in plants involving a complex regulatory network of transcriptional regulators (e.g. WRKYs and NACs) and plant hormones that overlaps with plant responses to hypersensitive response (HR) or programmed cell death, also observed after pathogen attack (Lim et al., 2007). Hormones like ethylene, jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA) seem to promote leaf senescence whereas cytokinins and auxin delay leaf senescence.

1.2.4 Abiotic stress

One last challenge for modern agriculture that needs to be adressed is abiotic stress, mainly cold, drought, heat and salt stress. Although the impact of climate change on overall agricultural production is still under debate, many prognoses indicate that especially water deficiency will further spread in developing countries that already suffer from low water supply (Figure 1-1). Salt stress will further increase as a consequence of increased irradiation, water deficiency and irrigation.

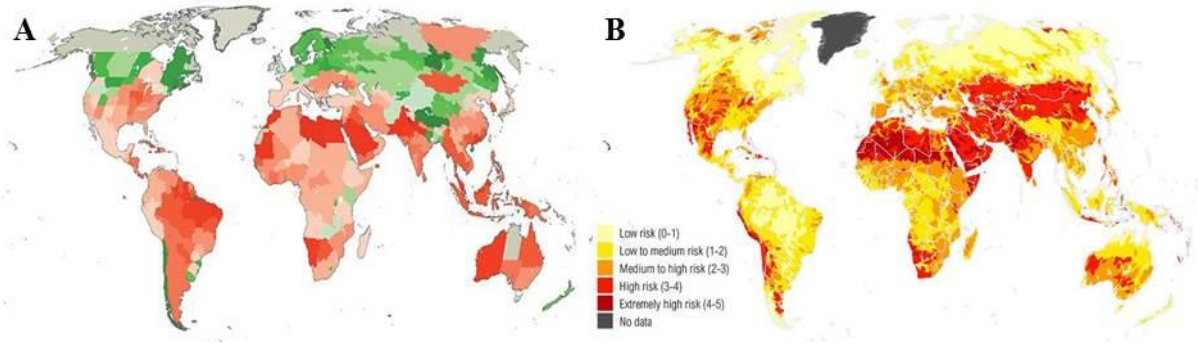


Figure 1-1 Projections for yield development in 2050 and water risk due to climate change; **Green** indicates positive change in yield (higher yields per area), **red** indicates negative change (lower yields) (A) (World Bank, 2010); from white to red increasing risk for future water stress (B) (Gassert et al., 2014)

The influence of climate change on cold stress in crops seems to be less clear, but, as suggested by the projection on yield change from the World Bank (2010), yields in the Nordic countries will further increase. This might lead to an intensified cultivation of thermophilic crops in the North and subsequently an increasing risk for cold stress in agriculture.

Cold stress can be separated into chilling stress, caused by soil or air temperatures below the optimum for plant growth, and stress by freezing, caused by ice formation and physical damage of cells (Baek, 2012). The temperature optimum for vegetative growth of maize lies between 25 – 33 °C (Duncan and Hesketh, 1968). In central or northern European countries like Germany, temperatures below 15 °C in spring may induce chilling stress that affects maize yields due to a decreased root activity which can results in limited nutrient uptake during youth development (Imran et al., 2013). This effect is increased if irradiation is high but parts of the photosynthesis inhibited. The distinct cellular localisation of photosynthetic processes in C4-plants, like maize, may further promote this problem (Foyer et al., 2002).

During the light reaction light energy (photons) is absorbed by the chlorophyll and passed to an electron transport chain, consisting of various electron acceptors such as plastoquinone or ferrodoxin, leading to the reduction of NADP. During this process a proton gradient is created by pumping proton cations (H^+) across the membrane and into the thylakoid space, thereby producing ATP by photophosphorylation. Several nutrients, like Mg, Fe, Cl, Mn and P (e.g. in ATP) and water are directly involved in these reactions as co-factors for enzymes or as part of the chemical reactions. An imbalance of the system leads to excess excitation energy and the formation of reactive oxygen species (ROS) (Engels et al., 2012, p. 88). ROS such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and the hydroxyl radical ($HO\cdot$), are common by-products of plant metabolism and furthermore seem to be important for long distant signalling in plants (Mittler, 2002) but at excessive production may cause serious oxidative damage in cells (Baek, 2012).

To scavenge ROS, plants use antioxidant enzymes. The most active and important antioxidant enzymes are catalase (CAT), superoxide dismutases (SOD), peroxidases, and enzymes in the ascorbate-glutathione cycle (Figure 1-2). SOD catalyzes the dismutation of two superoxide anions and water into H_2O_2 and O_2 whereas H_2O_2 is degraded either by CAT into H_2O and O_2 or by peroxidases, using an organic substrate ($R\text{-}H_2$) as reducing agent - as for instance ascorbate oxidized by the ascorbate peroxidase (APX) via the ascorbate-glutathione cycle (Baek, 2012). It is particularly important here that all of the three SODs have different mineral co-factors and are active at different sites in the plant cell.

MnSOD is mainly active in the mitochondria, FeSOD in the chloroplasts and the Cu-ZnSOD in the cytoplasm and in the chloroplasts.

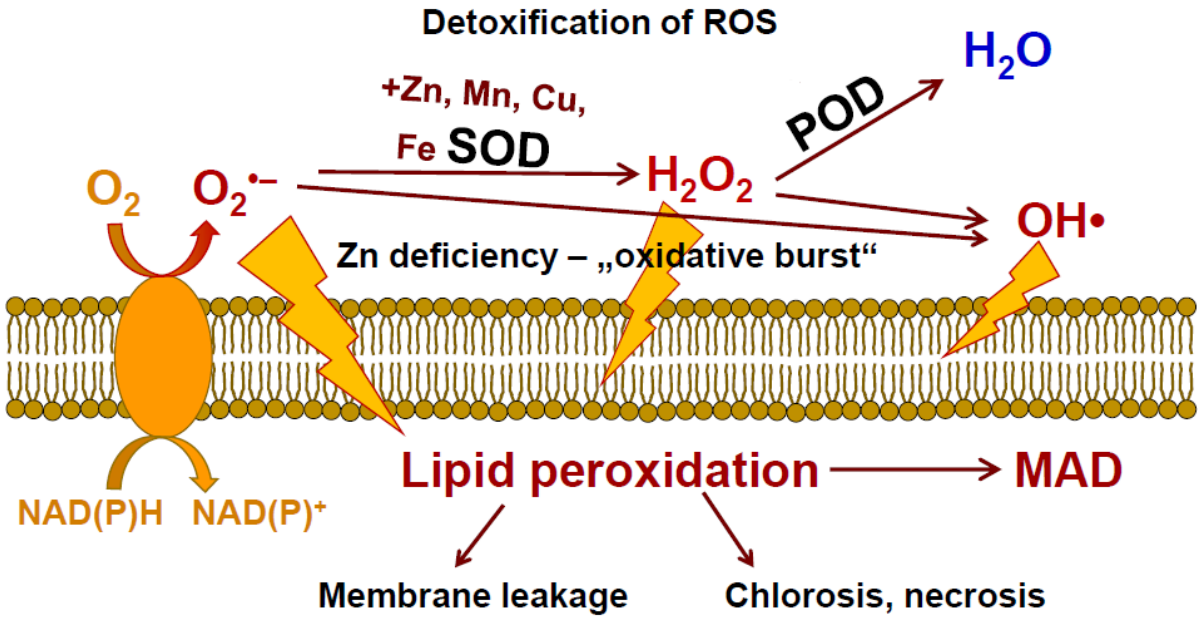


Figure 1-2 Formation and scavenging mechanisms of reactive oxygen species (ROS) (Bradáčová, 2015)

The lower nutrient uptake can be explained by a reduced root and/or shoot activity due to reduced metabolic and enzymatic activity. Depending on which part of the plant suffers from low temperature (roots in the soil or above ground organs) the sink status of the organs changes and subsequently the assimilate transport to the root or water and nutrient transport to the shoots are imbalanced (Lynch et al., 2012, p. 345; White, 2012a, p. 25). Furthermore, a shoot growth reduction at low root zone temperature is correlated with a reduction in a cytokinin production of roots and an elevated ABA export from roots to shoots (Atkin et al., 1973). Additionally, auxin transport from root to shoot seems to be inhibited by cold stress (Shibasaki et al., 2009).

1.3 Bioeffectors

1.3.1 The rhizosphere

The rhizosphere was defined by Lorenz Hiltner (1904) as the "volume of soil surrounding the roots, which is influenced by root activity" (Neumann and Römheld, 2012, p. 347). A multitude of factors is shaping the rhizosphere. First of all, the soil properties such as source rock, cation exchange capacity, pH, weathering and soil organic matter (SOM) contents are largely defining mineral contents and availability in the soil. Furthermore, texture and clay contents influence water holding capacity and the capacity and rate of plant root growth. Climate, temperature, rainfall and irradiation strongly determine biological activity in soils and mineralization of SOM but also plant performance. In addition, plant metabolism is influencing rhizodepositions (see below). The influence of plant species and phenotype on root morphology and mechanism on nutrient acquisition as well as the physico-chemical processes in the rhizosphere were already addressed in the previous sections. The next sections focus on soil biology and especially the importance of non-symbiotic microorganisms that are colonizing the rhizosphere.

1.3.1.1 Rhizodepositions

Organic rhizodepositions are the sum of all root derived sources of soil organic matter in the rhizosphere, composed of root cells, mucilage, leached assimilates and metabolites as well as compounds actively released by the root such as the already mentioned carboxylates (Neumann and Römheld, 2002). Between 10 to 40 % of total fixed carbon may be released into the rhizosphere (Badri and Vivanco, 2009; Bais et al., 2006). The rhizodeposits comprise a multitude of compound classes including low molecular weight compounds such as carbohydrates, amino and organic acids as well as more complex biomolecules like flavonols, lignins, glucosinolates or proteins (e.g. enzymes such as phosphatases) (Badri and Vivanco, 2009). These compounds thereby largely determine the composition of the rhizobiom, the sum of all microorganisms in the rhizosphere. It is well known that different groups or microorganisms have different substrate preferences (Paterson et al., 2007). Indeed, the data suggests that exudation or active release of organic compounds, e.g. flavonoids or specific amino acids, promote distinct bacterial groups but vice versa also seem to be triggered by soil microbes (Badri and Vivanco, 2009). This is best known for plant pathogens and obligate plant symbionts like rhizobia and mycorrhiza (e.g. strigolactone) (Peláez-Vico et al., 2016; Ruiz-Lozano et al., 2016).

1.3.1.2 Plant-microbe interactions

The total amount of bacteria in soil may be up to 10^{10} cells g^{-1} soil (Torsvik et al., 1996). Estimations for the rhizosphere may even exceed this number with about 10^{11} microbial cells g^{-1} root (Berendsen et al., 2012). Although bacterial diversity in agricultural soils seems to be lower than in undisturbed, natural soils, estimations from DNA re-association studies indicate that the number of species may reach several thousand per soil system. It was also proposed that only 0.1 – 1% of these species can be isolated and cultivated on media for further characterization (Torsvik et al., 1996), a view that was recently challenged by an establishment of microbial culture collections of Arabidopsis comprising the majority of the species found reproducibly in their respective natural communities (Bai et al., 2015). Nevertheless, bacterial diversity decreases in the rhizosphere of maize as compared to the bulk soil in favour of certain strongly abundant fast-growing groups such as Proteobacteria (Peiffer et al., 2013). The influence of rhizodepositions on the fungal composition seems to be more complex because fungi were generally thought to mainly decompose recalcitrant substrates with low consumption of labile C but these concepts are now under discussion (de Vries and Caruso, 2016; Hannula et al., 2017). In soils complex food webs exist with multitrophic interactions, including fungal decomposer, earthworms - feeding on litter and SOM - and collembola or nematodes - feeding on bacteria or fungi - that largely contribute to mineralisation processes in the soil. Most of these organisms do not depend on the living plant or root exudates. Only some specific groups such as rhizobia, mycorrhizal fungi (discussed below) and some plant pathogens are obligate symbionts depending on the direct interaction with the plant, comprising active root colonization and the supply with plant assimilates. Nevertheless, as described, many other free-living microorganisms benefit from root activity and therefore interact with the plant, modulate plant activity and metabolism, compete with other microbes for nutrients, thereby protecting the plant from pathogens, and may improve nutrient acquisition. The next section addresses these plant beneficial microrganisms.

1.3.2 Plant growth promoting microorganisms

1.3.2.1 PGPR

Introduced in the 1980s plant growth promoting rhizobacteria (PGPR) are a commonly used term for all free-living and plant beneficial bacteria living in the rhizosphere (Kloepper et al., 1980b). With the more general term plant growth promoting microorganisms (PGPM) plant beneficial fungi are also included. Both bacteria and fungi of this group are generally found worldwide in all natural and agricultural soils, often in high amounts, such as Pseudomonades

or Bacilli. Neither the PGPR nor the fungal species are a monophyletic group. In contrast, the plant beneficial species are not closely related and may have completely different morphological or physiological properties besides some common traits that enable the interaction with the plant such as the production of antimicrobial compounds, antibiotic resistance, capacity for root colonization, hormonal production or the release of phosphatases or chelating compounds.

1.3.2.2 Rhizosphere competence

One crucial property of all PGPR or PGPMs is the "rhizosphere competence", including activity, proliferation and vitality of the microorganism in the rhizosphere and the competitiveness of the strain in this selective environment (Compant et al., 2005). Viability and competitiveness of introduced strains are connected to the production of antibiotics or enzymes that may reduce the growth of other microorganisms, the acquisition of mineral nutrients and the ability to use root exudates as carbon sources. Additionally, the resistance to acidity, high temperature, desiccation or allelochemicals (e.g. by exopolysaccharides) - the latter present in plant and seed exudates or released by other microbes - is of importance (Deaker et al., 2004; Dutta and Podile, 2010). Furthermore, it was proposed that the ability to colonize the root is connected to the active motility of the bacteria in response to chemical attractants (chemotaxis) (Compant et al., 2005; Yaryura et al., 2008). On the other hand, bacteria might also be transported passively along the root, requiring the ability to adhere to the root. This can be achieved by active production of adhesives (e.g. exo- or lipopolysccharides) (Benizri et al., 2001; Dutta and Podile, 2010) or the agglutination by root exudates (van Peer et al., 1990). Nevertheless, in many bacteria that are abundant in the rhizosphere not all of these traits are present (Hozore and Alexander, 1991).

1.3.3 The bioeffector market

In the last 20 years an increasing amount of PGPR or fungi with plant beneficial properties were isolated from soils and cultivated, formulated and then commercially marketed (Yakhin et al., 2017). This research focussed on the investigation of different PGPR and fungal products and their potential for plant growth stimulation. Nevertheless, plant growth beneficial effects can also be observed for bioactive substances that do not necessarily contain living organisms, such as seaweed, soil and plant extracts, humic acids or vermicomposts (du Jardin, 2015). For these compounds in 1997 the term "biostimulant" was introduced that was later on defined by du Jardin as "any substance or microorganism applied to plants with the

aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content."

Due to product legislation, the aspect of "biocontrol" in which all products with biopesticidal, antimicrobial and plant protection capacity can be grouped, is excluded in this definition, as biocontrol products are categorized as pesticides and therefore are subject to governmental pesticide regulation (du Jardin, 2015; Weinmann, 2017). Nevertheless, definitions and terms differ in the literature (Lesueur et al., 2016a; Vessey, 2003; Yakhin et al., 2017) and over the time (Weinmann, 2017). Additionally, registration is hampered by the fact that multiple traits are active in bioorganic products. Therefore, the term "bioeffector" (BE) was introduced that is very close to the definition of du Jardin for "biostimulants" but does not exclude products that may exhibit "biocontrol" properties (www.biofector.info).

In this thesis the term "biofertilizer" is used for products or PGPMs that are able to enhance nutrient availability in soils, rock or recycling fertilizers and manures by solubilization or mineralisation, whereas "biostimulant" is used for products that directly interact with the plant to modulate hormonal production and metabolism, root growth or physiological activity by which plant growth and nutrient use or uptake efficiency can be enhanced. Following du Jardins recommendation for regulation, "biofertilizers" should be taken as a subcategory of "biostimulants". A detailed description of the different mode or mechanisms of action will be given in section 1.4.

The bioeffector market is continuously growing, especially due to the need for more sustainability, an increasing market for organic farming and the emergence of pesticide resistance in many target organisms (Popp et al., 2013). Recent publications estimate that the value of the European biopesticide market is about 800 million US$, representing 5 % of the European crop protection market (Weinmann, 2017). The biostimulant market was valued 800 million € in Europe, holding about 40 % of the worldwide market share, with an annual growth rate of more than 10 % (Yakhin et al., 2017).This is impressive considering the often low scientific evidence for effectiveness and therefore profitability of BE products (Yakhin et al., 2017). This may be due to the fact that an increasing number of products are available on the market but in the past the legislation of a BE product did not require the evidence for activity. This issue was addressed by adding biostimulation in the EU fertilizer regulation (Regulation (EU) 2019/1009, 2019).

1.3.4 Product categories

BE products can be categorized by their mode of action or by their taxonomy. Table 1-1 gives

Table 1-1 Taxonomic groups of BE products

Type	Phylum / Division	Selected species
Plant growth promoting rhizobacteria (**PGPR**)	Firmicutes (mainly gram(+))	Bacilli (Firmicutes, endospore forming, gram(+)): ***Bacillus amyloliquefaciens*** (e.g. FZB42 or FZB45), ***B. atrophaeus*** *GBSC56, B. megaterium, B. polymyxa, B. pumilis, B. simplex RJGP41, B. subtilis, B. thuringiensis, Paenibacillus mucilaginosus*
	Proteobacteria (gram(-))	*Burkholderia sp., Cellvibrio ostrviensis, Cellvibrio sp* Pseudomonades: ***P. fluorescens****, P. putida,* ***P. jessenii RU47*** Diazotrophs: *Azospirillum sp.,* (e.g. *A. lipoferum*), *Azotobacter sp.* (e.g. *A. chroococcum, A. vinelandii*), ***Herbaspirillum* sp.** Rhizobia: *Rhizobium leguminosarum, Rhizobium radiobacter, Bradyrhizobium japonicum*
	Cyanobacteria	***Spirulina sp.***
Plant growth promoting fungi	Ascomycota	***Penicillium*** *bilai* ***Trichoderma*** *sp.* OmG-08, ***Trichoderma harzianum****, Trichoderma virens*
	Basidiomycota	Sebacinales: *Piriformospora indica, Piriformospora williamsii, Sebacina vermifera*
	Asco- + Basidio	Ectomycorrhizal fungi: e.g., *Heboloma* sp., *Laccaria* sp.
	Glomeromycota	Arbuscular mycorrhizal fungi: *Glomus intraradices / Rhizophagus irregularis, G. Mosseae*
Plant (extracts)		*Hordeum sativa, Allium sativum, Quillaja saponaria, Sapindus mukorossi*
Algae (seaweed extracts, SWE)		***Ascophyllum nodosum, Fucus sp., Laminaria sp.***

an overview on well-known species that are used in BE products grouped by their taxonomy. Species in bold were used in our experiments. More comprehensive overviews on taxonomic groups and product classifications can be found elsewhere (Lucy et al., 2004; Yakhin et al., 2017). A categorization by mode of action is difficult because a clear separation of functional traits is often not possible as described in detail below.

1.3.5 Application techniques

Application techniques in general need to ensure best contact between the active ingredient and the plant surface. Especially for microbial products they should provide an optimal inoculum density near or directly on the seed or the plant roots. Additionally, they should promote establishment of PGPR populations in the new environment and thereby enhance root colonization. Application techniques and optimal inoculum densities are best studied for rhizobia inoculums and non-symbiotic N-fixing bacteria such as *Azospirillum* (Bashan, 1998; Deaker et al., 2004; Okon and Labandera-Gonzalez, 1994).

Various application techniques for BE products were tested and they can be grouped into four categories:

1. Treatment of seed: Seed dressing by dipping in BE solution, seed priming overnight (mainly for micronutrient priming, possible for SWE), seed coating / incrustation (e.g., with alginate or calciumhydroxide, starch etc.), seed infiltration (under vacuum)
2. Treatment of the seedling: Drenching of seedlings in BE suspensions before transplantation into growth medium, sterile substrates or soil, potato tuber dressing
3. Treatment of the substrate (drenching): Broadcast or band application, fertigation, mixture with organic substrates and manures before soil incorporation
4. Treatment of the leaves: Foliar application during the vegetation period (e.g., SWE, micronutrients or biocontrol)

All of the techniques have their advantages and disadvantages and their benefit strongly depends on the applied BE, the crop, the substrate types and the purpose of the inoculation.

1. Seed treatment

Seed treatment strongly reduces the costs for BE products and ensures the proximity of BE inoculum to the plant already in the earliest phase of plant development and is therefore widely used for rhizobia in legume production (Deaker et al., 2004). Also the establishment of PGPR root colonization may be improved. During seed germination nutrients and other compounds, such as proteins, are released. Results from germinating *Lupinus albus* seeds indicate that after a short phase of passive leakage of proteins that reflect the seed composition, seed exudation is modified to an active secretion of selected proteins that were able to inhibit fungal growth (Scarafoni et al., 2013). The inhibition of plant-pathogenic nematodes by proteins in exudates of germinating soybean seeds was observed as well (Rocha et al., 2015). Flavonoids released by Alfalfa seeds were inhibiting growth of fungal pathogens. In contrast, they did not affect *Bacillus subtilis* and were promoting growth of *Rhizobia meliloti* and *Pseudomonas putida* (Hartwig et al., 1991). These results suggest that germinating seeds already select for potential interaction partners making seeds a valuable target for BE treatments. A variety of different encapsulation techniques exist using peat carrier or alginate, a biopolymer derived from macroalgae or bacteria (Bashan, 1998). Nevertheless, the application techniques may be cost-intensive and require technical equipment for large-scale application (Bashan, 1998). Moreover, application of the treated seeds in the field using common sowing machines may be problematic and BE seed treatment needs to be coordinated with other seed treatments when used in conventional farming.

2. Drenching of seedlings

Possible one of the best procedures to ensure good root colonization is drenching or soaking of seedlings. This technique is also often used in sterile substrates and has been successfully tested in many peer-reviewed publications (Adesina et al., 2009; Fröhlich et al., 2011). Unfortunately, this technique is not applicable for crops that are directly sown without pre-cultivation phase. However, for many vegetables and greenhouse cultures this technique might be an economic option that can be easily integrated during or before transplantation. For the protection of potato against soil pathogens several *P. fluorescens* and *Bacillus sp.* based commercial products are applied as tuber dressing.

3. Soil application

Soil application is a common technique used for rhizobial strains and mycorrhizal inoculum in greenhouse or potting substrates. By mixing with manure and incorporation into the soil additional SOM and carbon-rich sources can be combined with BE inoculum.

Fertigation in greenhouse or field horticulture might also be a good option if a continuous supply of the BE product is preferred to a single inoculation. Nevertheless, here the problem might be that the fertigation system is "contaminated" by the BE product and therefore the technique is not optimal for testing of PGPR strains. Additionally, the substrate needs to be loose and porous enough to allow infiltration and transport of the BE product to the root or shoot (if not only root colonization but also endophytic shoot colonization is of interest).

For broadcast and band applications much higher inoculum amounts are necessary than in the previously described methods.

4. Foliar application

Foliar application is mainly interesting for SWE and micronutrients that can be taken up by the leaves and for application of biocontrol agents against foliar diseases or herbivorous insects (e.g., *Bt*, *B. subtilis*, entomopathogenic fungi).

1.4 Modes of action

Multiple traits probably contribute to the plant-beneficial activity of microbial but also other biological inoculants (Bashan et al., 2004). Nevertheless, bioeffector products differ in their capacities of these traits and the understanding of the possible modes of action is crucial to ensure proper usage of BE products under given circumstances and to develop better application strategies and product combinations.

1.4.1 Biocontrol

1.4.1.1 Antibiosis and antagonism

In natural systems plants are constantly confronted with the risk of pathogen infection or damage by herbivors. Nevertheless, in stable ecosystems catastrophic losses of a population are normally balanced due to the interplay of multitrophic interactions. Also, the excessive spread of a specific soil pathogen is therefore suppressed by the soil or rhizosphere microbiome. This disease suppressiveness can even be transferred to unbalanced conducive soils (Haas and Défago, 2005) and seems to be correlated with the relative abundance of specific bacterial groups (Berendsen et al., 2012). Bacterial groups that are commonly found to be enriched in suppressive soils are Proteobacteria and Firmicutes. The suppression of soil diseases, like take-all (*Gaeumannomyces graminis*) in wheat, was mainly attributed to the activity of fluorescent Pseudomonades, and the release of antibiotic and antifungal compounds (Raaijmakers et al., 1999). They release antibiotics such as phenazines, phloroglucinols, pyoluteorin, pyrrolnitrin, cyclic lipopeptides and hydrogen cyanide (Haas and Défago, 2005). Also plant growth promoting Bacilli like *B. amyloliquefaciens*, possess a multitude of genes for antibiotic production and confer resistance to pathogens (Qiao et al., 2014; Tan et al., 2013a). Disease suppressiveness is also tightly linked to plant nutritional aspects. Disease severeness was correlated with soil pH and the Mn availability, and both factors correlate with the abundance of Mn-reducing *Pseudomonas fluorescens* strains (Huber et al., 2012, p. 294). *Pseudomonas* spp. strains were also able to suppress growth of pathogens due to their competition for Fe (Haas and Défago, 2005). The strains release fluorescent compounds, named siderophores, that are chelators with high affinity to different micronutrients (Brandel et al., 2012; Kloepper et al., 1980b).

Trichoderma spp. fungi are well known for their antagonistic activity against other fungi, including the parasitism of fungal pathogens (mycoparasitism), and successful suppression of diseases such as *Rhizoctonia solani* (Howell, 2003). Furthermore, biocontrol activity of *Trichoderma* strains is connected to production of antibiotics like gliotoxin and gliovirin,

chitin degrading endo- and exonucleases and the degradation of stimulants that are released during seed germination and trigger fungal (pathogenic) spore germination.

Additionally, to the production of antibiotics, the competition for nutrients and active antagonism or parasitism (mainly fungal products), the induction of plant resistance is proposed to play a keyrole in the biopesticide activity of BE products.

1.4.1.2 Induced systemic resistance (ISR)

Disease control by PGPMs is often correlated with the induction of plant resistance (Harman et al., 2004). In the last two decades the plant immune system was intensely studied and a complex system, developed during co-evolution of plants (hosts) and pathogens, was discovered for many plant-pathogen-interactions, involving the recognition of avirulence factors (Avr) by the host, activation of immune responses and the suppression of plant defence by specific effector molecules (Jones and Dangl, 2006). The pathogen-triggered immune responses in plants were termed systemic acquired resistance (SAR). SAR depends on salicylic acid, a plant hormone that is known to be involved in many stress responses of plants (Bari and Jones, 2008). A cascade of well-studied downstream signals, such as the expression of pathogenesis-related (PR) genes, lead to hypersensitive response (HR) and programmed cell death (PCD) after infection and penetration of plant cells by biotrophic pathogens (Pieterse et al., 2009; Stassen and Van den Ackerveken, 2011). Some findings suggest that non-pathogenic fungi, including *Trichoderma* spp., produce Avr factors that trigger SAR similar to pathogens and may therefore enhance plant resistance against pathogens (Harman et al., 2004).

Interestingly, plant responses to inoculation of many PGPR differ in their signalling pathway (Pieterse et al., 1996). This induced systemic resistance (ISR) depends on the plant hormones jasmonic acid (JA) and ethylene (Pieterse et al., 1998) and does not trigger direct physiological responses such as HR but leads to a “defence priming” (Haas and Défago, 2005; Verhagen et al., 2004), increasing resistance of plants to future biotic stress. Nevertheless, the ISR and SAR pathway share certain components, most important the regulatory protein NPR1, that is crucial for SA-mediated PR activation and ISR (Pieterse et al., 1998), but suppresses JA-mediated defence pathways when SA pathway is activated (Pieterse et al., 2009). The suppression can be bypassed by ethylene (ET). Herbivors and necrotrophic fungi seem to be more susceptible to JA/ET mediated plant defence than biotrophic fungi leading to different responses in plants and also differences in the success of PGPR as biocontrol agents against pests. There is growing evidence that further plant

hormones interact in the plant defence pathways (Bari and Jones, 2008; Pieterse et al., 2009) explaining the difficulties to predict biocontrol activity of PGPR, especially if multiple biotic or abiotic stress factors are active (Mittler, 2006).

Signal compounds produced by Pseudomonades that elicit ISR are the antibiotic 2,4-diacetylphloroglucinol (Phl), the siderophores pyocyanin and pyochelin, flagellin, O-antigens (lipopolysaccharides of bacterial cell surface) and HCN (Haas and Défago, 2005). Results suggest that, similar to root exudates of plants, many microbial compounds released to the rhizosphere possess multiple functions important for PGPR activity. Interestingly, also salicylic acid (SA) is able to elicit ISR. Also 2,3-butanediol, a volatile organic compound (VOC) from *Bacillus* spp., was shown to trigger ISR. More recent findings on the induction of plant resistance by *Trichoderma virens* in maize indicate that a small protein named Sm1 is triggering ISR via a JA/ET pathway (Djonović et al., 2007).

1.4.1.3 Quorum sensing

Quorum sensing (QS) describes the phenomenon of population density dependent gene expression in bacteria populations that often trigger shifts from saprophytic to pathogenic lifestyle, biofilm formation or sporulation under adverse environmental conditions (Rutherford and Bassler, 2012). QS also regulates antibiotic production explaining why PGPR populations need to reach certain threshold densities to effectively exhibit biocontrol activity (Raaijmakers et al., 1999, 1997; Whitehead et al., 2001). QS is thereby triggered by cell-to-cell communication via the use of small signalling molecules. Many different QS signals from a wide range of different bacterial groups, including Bacilli and Pseudomonades, have so far been identified (Waters and Bassler, 2005). Some QS signals, like N-acyl-homoserine lactone (AHL), found in gram-negative bacteria like *Pseudomonas* spp., are also shown to induce diverse plant responses connected to ISR and plant growth stimulation (see below) by hormonal regulation (Hartmann et al., 2014).

1.4.2 Biofertilization

1.4.2.1 Biological N-fixation

Rhizobia

Rhizobia are probably the most widely used microbial inoculum worldwide. It is estimated that about 60 % of nitrogen in agriculture is fixed by rhizobia (Zahran, 1999). Rhizobia are a paraphyletic group of plant symbionts whereas most of the genera, such as *Rhizobium* or *Bradyrhizobium*, belong to the order of Rhizobiales. They form special root organs, named nodules, with many legume plants and are able to fix nitrogen from atmospheric N_2 via the enzyme nitrogenase. Although plant-Rhizobia symbioses can be found in natural habitats, productivity and N-fixing capacity in legume cultivation can be strongly improved by inoculation of host-specific strains, especially in areas in which legumes were newly introduced for agricultural production such as Australia but also in soils that were not cultivated with legumes for several years (Deaker et al., 2004). Due to the direct input of N into the system and their obligate symbiotic relationship with their host plants rhizobia are normally not considered as PGPR. Additionally, they are well-studied and were therefore not investigated during the thesis.

Free-living diazotrophs

There are other N-fixing bacteria (diazotrophs) that are free-living and do not require a host plant for N-fixation such as *Azospirillum* and *Azotobacter*. *Azospirillum* is probably the best-studied PGPR and a continuously growing amount of publications report on successful plant growth stimulation in field trials, especially in many developing countries (Bashan et al., 2004; Okon and Labandera-Gonzalez, 1994; Veresoglou and Menexes, 2010). The reason for its widespread use was probably the hope for a new sustainable N-fertilizer in non-legume plants. Nevertheless, it is now well-accepted that the plant growth stimulation by *Azospirillum* is not caused by its N-fixing potential but mainly due to the production of phytohormones and the stimulation of root growth thereby increasing nutrient uptake efficiency and water absorption (Bashan et al., 2004; Halpern et al., 2015; Lesueur et al., 2016a). *Azospirillum* acts therefore more as a biostimulant than a biofertilizer (see next section).

1.4.2.2 Mycorrhiza helper bacteria (MHB)

Most plants are able to establish symbiotic relationships with specialized fungal symbionts. Those fungi are actively colonizing the intercellular space of plant roots (apoplast) or directly penetrate plant root cells thereby forming new structures termed mycorrhiza (Richardson et

al., 2009). The two major groups are ectomycorrhiza (ECM), that are mainly symbionts of woody plants, and arbuscular mycorrhiza fungi (AM or AMF) that are colonizing most of the agricultural relevant plant species. Mycorrhiza are known to improve spatial P acquisition in soils due to an increase of surface, nevertheless, it seems that they are not largely contributing to solubilisation of P from fractions that are not plant available (George et al., 2012, p. 414; Richardson et al., 2009).

The efficacy of mycorrhizal symbiosis might be improved in the soil by association with soil bacteria that are able to mobilize sparingly available nutrients (Becquer et al., 2014). Several publications report on synergistic effects on plant growth for the combined application of AM fungi with PGPR or other fungal inoculants like *Trichoderma* sp. (Badda et al., 2013; Gamalero et al., 2004; Srinath et al., 2003; Yusran et al., 2009). Especially the combination of the PGPR products with mycorrhizal inoculum showed strong improvement to single inoculum. This phenomenom was previously reported and the respective PGPR were therefore termed mycorrhiza helper bacteria (MHB) (Frey-Klett et al., 2007; Garbaye, 1994). MHBs thereby may act via improved nutrient mobilization, stimulation of lateral root formation for improved mycorrhization or by antibiosis against pathogens that may compete with mycorrhizal fungi in the rhizosphere. Data suggest that associations between MHBs and fungi are specific, depending on bacterial antibiotics or carbon sources released in the fungal mycosphere (Frey-Klett et al., 2007).

1.4.2.3 Phosphate solubilizing microorganisms (PSM / PSB)

In general, many soil microorganism exhibit similar activity as plant roots in response to low P conditions in the soil, including release of protons, carboxylates and phosphatases (Khan et al., 2009; Richardson and Simpson, 2011). In contrast to plants, microorganisms additionally produce and release alkaline phosphatases in substantial amounts and may therefore contribute to P acquisition from SOM even at high pH (Sharma et al., 2013). Many PGPR or fungi are able to solubilize P from precipitated sparingly soluble inorganic Ca-phosphates in sterile media and are therefore termed phosphate-solubilizing microorganism (PSM) (Richardson et al., 2009). In those media normally buffer capacities are low increasing the efficacy of proton release and pH dependent strategies. Nevertheless, efficacy of P-solubilization is often limited in alkaline soils when buffer capacities are high (Gyaneshwar et al., 1998). Efficacy of PSMs may therefore be increased by ammonium-fertilization (Noor et al., 2017; Whitelaw et al., 1999). Organically bound P may be mobilized by extended release of phosphates, especially phytases, in the rhizosphere (Richardson et al., 2001a) and the soil

microbial community strongly contributes to overall enzyme activity (Richardson et al., 2001b). Common P-solubilizing bacteria (PSB) include *Pseudomonas* and *Bacillus* genera whereas best known P-solubilizing fungi (PSF) are *Aspergillus* and *Penicillium* spp. (Hayes et al., 2000; Khan et al., 2009; Richardson et al., 2009, 2001a). *Trichoderma* species show only low potential for P-solubilization. Nevertheless, traits for P-solubilization are conserved in a multitude of different species over a wide spectrum of bacterial and fungal groups, including potential plant pathogens (Sharma et al., 2013). Recently it was shown that Arabidopsis, belonging to the Brassicaceae plant family that is unable to establish mycorrhiza symbiosis, forms a symbiosis with the non-mycorrhizal fungi *Colletotrichum tofieldiae* at low soil P levels (Hacquard et al., 2016; Hiruma et al., 2016). Results indicate that the fungi was not only increasing surface but was also able to solubilize inorganic P (Hiruma et al., 2016). PSFs seem to be more effective in acidification and release higher amounts of organic acids such as citric, succinic and gluconic acid than PSBs (Khan et al., 2009). As described in the sections above, efficacy of PGPR applications is influenced by many environmental factors. For PSMs especially soil buffer capacity, P-source and overall P-availability are major determinants for successful P-solubilization. Therefore results on PSM application in the field are often inconsistent (Khan et al., 2009; Richardson and Simpson, 2011).

1.4.2.4 Soil food-web

It was hypothesized that P solubilized by microorganism might not always be plant available but fixed as microbial P_{mic} in the soil (Richardson et al., 2009). Nevertheless, soil ecosystems consist of complex food webs in which bacteria and fungi have also natural enemies such as protozoa or nematodes that are feeding on them (Hol et al., 2013). Therefore, the efficacy of PSMs to provide P for plants might be increased by inoculation or promotion of other organisms. Indeed, several publications report on increased P availability in the medium if P solubilizing bacteria or fungi were co-inoculated with bacterial or fungal grazing nematodes (Ingham et al., 1985; Irshad et al., 2012, 2011, 2013). Interestingly, bacterial populations were in some cases promoted and not decreased by feeding. An additional application of nematodes in agricultural practice is probably not an economic option but the results again suggest that biodiversity is important for sustainable plant production systems.

1.4.2.5 Biofertilizers and fertilization

At the end of this section, it is important to mention that it is not sufficient to increase P availability from soil only. It is necessary to recharge the soil P pools by continuous P supply in a sustainable way. A “mining” of nutrients will cause nutrient depletion and serious damage to soil fertility. This can be observed in a dramatic fashion in many countries of sub-

Saharan Africa in which low availability of manure and insufficient input of mineral fertilizer lead to soil nutrient depletion, a problem that is considered to be the major constraint for agricultural production in this area (Smithson and Giller, 2002; Stoorvogel and Smaling, 1998; Zingore et al., 2008).

1.4.3 Biostimulation

1.4.3.1 Biostimulants

Reports on plant growth stimulation by PGPR such as *Azospirillum* (Dobbelaere et al., 2001), seaweed extracts (Arioli et al., 2015; Craigie, 2011; Sangha et al., 2014) or humic acids (Chen and Aviad, 1990; Russo and Berlyn, 1991) are frequent. Biostimulation can be distinct from the other mechanisms due to its direct influence on plant physiology and signalling. Therefore seaweed and plant extracts or humic substances, containing high amounts of bioactive compounds, are also commonly termed biostimulants (Halpern et al., 2015; Yakhin et al., 2017).

1.4.3.2 Bioactive compounds

Bioactive compounds, mainly secondary metabolites, can be grouped into different categories. One category might be plant hormones or bacterial analogous including auxins, cytokinins, gibberellins, ethylene, abscisic acid, jasmonates and salicylic acid (Baca and Elmerich, 2007). Presence of most of these hormones or hormone-like activity was also found in seaweed extracts (Craigie, 2011; Sangha et al., 2014). Second category might be macromolecules such as tannins, flavonoids, phenolics, polysaccharides (e.g. laminarin or fucoidan) or humic acids, mainly present in seaweed or plant extracts, that may influence hormonal production, physiological activity or stress tolerance by their antioxidant capacities (Chen and Aviad, 1990; Craigie, 2011; Sangha et al., 2014). The third group are compounds that are important for plant-microbe signalling (e.g. strigolactone for mycorrhization or Nod factors in rhizobia-plant interaction) or for quorum-sensing such as N-acyl-homoserine-lactones (AHLs) (Ortíz-Castro et al., 2009). The last group consists of volatile organic compounds (VOCs) that partly seem to act by activation of auxin and ethylene hormonal pathways (Bailly and Weisskopf, 2012).

1.4.3.3 Root growth stimulation

Various PGPR are known to modulate root system architecture (RSA) by production of auxins such as indole acetic acid (IAA), other signalling molecules involved in auxin signalling like nitric oxide or the antibiotic DAPG, or gibberellic acid (Vacheron et al., 2013).

The production of auxin was proven for both *Bacillus* sp. (Idris et al., 2007; Talboys et al., 2014) and *Pseudomonas* sp. strains (Afzal et al., 2014; Karnwal, 2009; Khakipour et al., 2008). Also production of cytokinin, that is known to promote shoot growth and reduces root to shoot-ratio, was observed in bacterial strains, including *P. fluorescens* (García de Salamone et al., 2001). Nevertheless, production of phytohormones is often dependent on the abundance of the metabolic precursor of the biosynthesis pathway such as tryptophan for auxin, adenine and isopentyl-alcohol for cytokinin and methionine for ethylene biosynthesis (Arshad and Frankenberger, 1991). Furthermore, RSA can be modified by the reduction of plant ethylene (ET) levels via degradation of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) by the bacterial enzyme ACC deaminase (Glick, 2014). The ACC deaminase gene was found in various gram +/- bacteria, e.g. *Pseudomonas* sp., *Bacillus* sp., *Azospirillum* sp., rhizobia, but also pathogens (e.g. *Ralstonia solanacearum*) (Saleem et al., 2007). Elevated ethylene production may inhibit primary root growth and stimulate root hair formation. Nevertheless, at low concentration it also activates auxin biosynthesis, transport and signalling (Neumann, 2016). Therefore, the influence of ethylene on root growth depends on the total concentration, the ET/auxin ratio and timing. The potential of auxin to stimulate lateral root formation is connected with an increased plasma membrane H^+-ATPase activity (Canellas and Olivares, 2014; Neumann, 2016), also observed after application of humic acids (Canellas et al., 2002).

Azospirillum, one of the best-studied PGPR, shown to promote plant growth even under field conditions, increased the activity of many enzymes involved in TCA and nitrogen or amino acid metabolism (Dobbelaere et al., 2001). Additionally, levels of bioactive phytohormones or flavonoids were increased in plants due to hydrolysis of their conjugated forms, probably due to modification of β-glucosidase activity.

AHL production is a common trait in many *Pseudomonas* strains (Venturi, 2006) but is not ubiquitous (Elasri et al., 2001; Martins et al., 2014). Studies on PGPR showed that their ability to colonize the plant root correlates with the activity of the QS system (Wei and Zhang, 2006) and that AHL production is more common in plant-associated strains than in strains isolated from bulk soil (Elasri et al., 2001). AHLs were shown to modify RSA by auxin-dependent (Bai et al., 2015; von Rad et al., 2008) or auxin-independent signalling pathways (Ortíz-Castro et al., 2008) whereas their biological activity seems to be determined by the length and structure of the carbonyl chain (Bai et al., 2015; Hartmann et al., 2014; Ortíz-Castro et al., 2009).

1.4.3.4 Stress priming

Under drought, salt and cold stress it was reported that PGPR increase plant stress tolerance by elevating levels of specific metabolites, such as sugars, specific amino acids (e.g. proline) and osmoprotectants like glycine-betaine (Vacheron et al., 2013). High concentrations of these compounds are also present in seaweed extracts, explaining their potential to alleviate stress tolerance after application (Sangha et al., 2014). Some PGPR remain active under low temperature and are able to produce antifreeze proteins that may reduce plant cell damage due to ice-crystallisation (Glick, 2012; Lucy et al., 2004; Subramanian et al., 2011). Reduction of salt-stress by PGPR application due to ACC deaminase activity was reported (Lucy et al., 2004; Yang et al., 2009). Additionally, VOCs from the *B. subtilis* strain GB03 led to tissue specific differentially expression of a high affinity K^+ transporter (HKT) and subsequently lower Na^+ uptake under salt stress.

Both biotic and abiotic stress tolerance in plants can be improved by the production of tannins, flavonoids and phenolics commonly observed after application of humic acids, seaweed extracts or PGPR (Canellas and Olivares, 2014; Craigie, 2011; Vacheron et al., 2013). Already described as biocontrol mechanism, also ISR is mainly a biostimulation similar to abiotic stress priming.

1.5 The "Biofector" project

In 2012 the EU launched the 5-year research project "Resource Preservation by Application of BIOefFECTORs in European Crop Production" with the acronym "Biofector" to investigate selected products of PGPR, biostimulants and biofertilizers, grouped together under the term bioeffector, for their influence on the three major crop plants tomato (*Solanum lycopersicum*), maize (*Zea mays*) and wheat (*Triticum aestivum*) (www.biofector.org). The project and also the research conducted for this dissertation was financed by the European Union's Seventh Framework Programme (FP7/2007-2013) under Grant Agreement n°312117.

Objectives of the project were:

1. Investigation and comparison of bioeffector products from various origin and composition for their ability:
    a. To stimulate plant growth.
    b. To solubilize phosphorus from organic or inorganic sources of fertilizers and thereby increasing phosphorus availability for the plants in organic and conventional farming systems.
    c. To increase overall yields and thereby reducing mineral fertilizer input in conventional farming systems.
    d. To alleviate abiotic stress and increase tolerance in crop plants.
2. Investigation and comparison of selected bioeffector products for their mode of action, their composition and their behaviour in the environment, with special focus on:
    a. Root colonization properties of PGPR.
    b. Hormone production and hormonal stimulation of treated plants.
    c. Chemical and structural composition.
    d. Establishment in natural soil environments and influence on the natural soil microbiome, here especially with the task of risk assessment.
3. Supporting small- to middle-scale companies in the EU in aspects of research and development as well as public relation by directly including several companies in the project consortium and testing their products.
4. Establishment of a network of different companies active in the bioeffector market and representation of this network towards the lawmakers in the EU.
5. Public dissemination of knowledge to farmers, producers and lawmakers.

The project consortium consisted of more than 20 project partners from various countries in the EU and one partner from Israel. Partners were research institutes and universities, companies and product providers.

The project was structured in 11 work packages (WP) with the focus on:

1. Product development (WP01) and Synergisms and product combinations (WP02)
2. Functional mechanisms (WP03)
3. Abiotic stress (WP04), Organic farming (WP05), Recycling fertilizer (WP06) and Fertilizer placement and fertigation (WP07)
4. Field testing network (WP08)
5. Economic evaluation (WP09)
6. Public dissemination (WP10)
7. Project management and coordination (WP11)

The present work was done with the focus on the four work packages "WP02: Product combination", "WP03: Functional mechanisms", "WP04: Abiotic stress" (in our group with focus on cold stress) and "WP08: Application in the field".

Research from the "Biofector" project and related former research from the working groups involved can be found under www.biofector.org/publications.html. A meta-analysis including results from more than 100 experiments conducted during the 5-year project in different working groups will be submitted soon (Lekfeldt et al., *unpublished*).

1.6 Objectives and hypotheses

1.6.1 Objectives

This work was focussing on two major objectives. The first one was to find out under which environmental conditions plant growth stimulation by BE products can be observed and which factors are able to enhance the effectiveness of the BE products.

The second objective was to study the mode of action of different BE products to elucidate the mechanism by which the products are influencing the host plant.

Both objectives are interrelated with each other. The description of a mode of action will probably simplify the search for conditions under which a BE product is most effective, on the other hand environmental or experimental conditions might influence the mechanism and outcome of the BE-plant interaction.

Therefore, many different experimental conditions were tested and a variety of analytical methods was applied to enhance the knowledge on BE-plant relationships.

1.6.2 Hypotheses

Several specific hypotheses were formulated that are also mentioned in the results of each single experiment. The most important hypotheses that are shaping this dissertation are described shortly below.

As part of the first objective:

1. BE products are able to stimulate plant growth (shoot and root) during early plant development.
2. BE products are able to increase maize yield in field experiments under limited P supply.
3. BE products, especially bacterial and fungal strains are able to improve P acquisitions from various fertilizer sources or previously unavailable soil P.
4. BE products are able to alleviate plant stress and improve plant tolerance (especially cold stress) thereby improving plant performance and yield.
5. BE product combinations might be more effective than single inoculation of BEs.

As part of the second objective:

6. Microbial BE products are able to solubilize soil or fertilizer P also under applied conditions (PSB / PSM).

7. BE products enhance root growth thereby improving nutrient acquisition.
8. Microbial BE products enhance P acquisition by stimulation of root mycorrhization by AMF (MHB).
9. Effectiveness of PGPR depends on root colonization and application rates.
10. BE products are increasing plant growth and stress tolerance by hormonal stimulation and are able to modulate plant gene expression thereby influencing the plant metabolome, physiology and overall performance, with special focus on auxin and ethylene production and signalling.

To verify these hypotheses several pot and field experiments were conducted. Many pot experiments were performed for screening of interesting BE products or experimental conditions under which BE products might be helpful. Various types of soils, different fertilizers, fertilization rates, temperature conditions, BE application rates and techniques and different crops and cultivars (maize and tomato) were in the focus. Additionally, experiments from other working groups were repeated to reproduce BE effects and investigate conditions that might be crucial for BE activity.

Cold stress experiments were mainly conducted in the greenhouse using a system for controlled root zone temperature. Incubation experiments were conducted to assess potential product combinations *in vitro*.

One major part of the work was the analysis of maize gene expression after application of two microbial BE products. Metabolite data on these samples were provided by a partner institute in Italy and compared with the transciptome data set.

2 Material and methods

2.1 List of conducted pot and field experiments

During the doctoral thesis 23 experiments were conducted, using various bioeffector (BE) products (Table 2-11 and Table 2-12). Some of the results were already presented in master and bachelor theses whereas the results from Exp_8 and Exp_17 were published in peer-reviewed journals (Bradáčová et al., 2016; Weber et al., 2018).

2.2 Plants and BE products

Plant material

All experiments with maize (except Exp_17) were conducted with the commercially available cultivar *Zea mays* cv. "Colisee" (KWS SAAT SE, Einbeck, Germany) mainly used for silage maize. In experiments at the beginning of the project, it was found that Colisee reacted more sensitive to environmental conditions like P limitation or other abiotic stresses indicated by the red coloration in shoot due to formation of anthocyanins (Nkebiwe, 2013, *unpublished*) than other tested maize cultivars and was therefore used for product screening.

In Exp_17 the roothairless 2 (rth2) mutant of the maize inbred line B73 was used. Rth2 seeds were provided by the working group of F. Hochholdinger (INRES, Bonn).

For tomato experiments two different cultivars of *Solanum lycopersicum* were used. For experiments Exp_5 – 7 the non-hybrid cultivar "Mobil" (Kecskemét, Hungary) and for experiment Exp_20 the TMTD-treated hybrid "Primadona F1" (Hazera Genetics Ltd., Berurim M.P Shikmim, Israel). TMTD 98% Satec (Bayer CropScience, SATEC Handelsgesellschaft mbH) contains the fungicide thiram.

BE products

More than 70 BE products were provided by various companies for research purposes during the Biofector project. Of these products about 30 products were tested in our institute. Table 2-15 and Table 2-16 give further information on all BE products used during investigations described in this thesis. Focus was on microbial products, especially the products Proradix® and Rhizovital®, and their combination with various seaweed extracts, recommended for improving plant stress tolerance or as prebiotics for bacterial growth. All products were analysed for mineral composition with three replicates each by ICP-OES or ICP-MS analysis (Table 2-1). *Bacillus* strains were provided in the products as endospores, fungal products as spores, and Pseudomonades as freeze-dried cells. For pot experiments bacterial or fungal

products were suspended in 10 mM $CaSO_4$ or 0.3 % (w/v) NaCl solutions. Ctrl treatments were therefore always treated with respective amounts of the pure solutions. All other products were suspended in deionized H_2O. For field experiments BE products were suspended in normal tab water.

Table 2-1 Overview of mineral composition for all BE products

BE product	Abbr. [1]	Mineral composition [2]									
		N	**C**	**P**	**K**	**Mg**	**Ca**	**Cu**	**Fe**	**Mn**	**Zn**
		%	%	ppt	ppt	ppt	ppt	ppm	ppm	ppm	ppm
Proradix	Px	5.18	40.6	9.10	13.2	1.09	9.90	0.70	57.7	0.58	34.8
P. jessenii	PJ	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Rhizovital	Rz	0.49	11.0	1.42	0.56	0.44	1.03	0.43	17.0	21.7	12.2
B. simplex	Bsim	0.70	11.2	3.65	0.71	0.87	1.78	1.56	49.6	62.0	27.4
B. atrophaeus	BacA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Biological fertilizer DC	BFDC	0.50	52.6	0.08	0.07	NA	0.07	0.32	5.33	1.51	5.79
OmG-08	OmG	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Trianum-P	TP	0.23	40.5	0.64	0.99	0.06	NA	0.12	9.46	0.13	7.06
Herbaspirillum sp.	Hsp	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Algafect	Af	1.66	16.7	0.32	43.6	0.80	2.26	1 - 9	79.3	6661	20530
AlgaVyt	AV	9.09	11.6	0.45	41.0	0.87	2.56	14.9	455	55.7	13810
AlgaVyt Zn/Mn	AVZM	4.88	5.2	NA	4.63	0.31	0.17	2.04	27.1	61533	74426
Superfifty / Alga 50	SF	0.35	12.8	NA	70.0	2.48	0.36	10.0	60.9	11.0	9.75
Ecolicitor	ECO	0.1-0.4	8.0	0.4 - 6	12.4	2.66	2.17	0.2-9	64.1	10-25	10.7
Alga 95	A95	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Product 1	P1	0.27	11.6	1.20	27.4	3.01	5.58	0.11	63.2	8.16	5.06
Product 2	P2	0.40	25.9	1.23	6.48	4.06	5.56	1.45	375	27.3	151
Product 3	P3	0.38	26.8	0.35	109	1.17	0.66	0.66	47.5	5.36	6.13

[1] Used in this thesis

[2] Analysis done by H. Ochott (Institute of Crop Science) and LA Chemie, University of Hohenheim

2.3 Plant growth conditions

Experimental conditions

Most of the experimental conditions used for conducting the specific pot or field experiments are summed up in Table 2-13 and Table 2-14. Further detailed information on the experiments, like fertilization and treatments, is given for each single experiment in chapter 3 (results).

Soils

All plant growth experiments were performed either in pots filled with natural soils collected from various field sites or in the field. In Table 2-19 there is an overview of all soils used for the pot experiments. All soils were analysed for mineral composition and texture (Table 2-20). For optimal plant growth conditions and better harvesting of roots in most experiments soils were mixed with sand (25 % (1:3) up to 50 % (1:1) sand; Dorsilit, Gebrüder Dorfner GmbH & Co. Kaolin- u. Kristallsandwerke KG, Hirschau, Germany). In experiment Exp_20 manure (or unfertilized peat as a second experimental factor) were used in high amounts and are therefore listed as part of the substrate and not only as fertilizers. Fresh field soils were either pre-dried to reach a water content of less than 15 % or directly sieved with 5 mm mesh size to remove coarse particles and stones.

Water contents

To reach soil water contents (WC) for optimal plant growth for each soil the maximum water holding capacity (WHC_{max}) and actual water contents were determined before mixing and fertilization of the substrates. In all pot experiments plants were watered on weight, based on the optimal water contents, normally 50 - 70 % of the WHC_{max} of a soil. Because sand has a very low WHC_{max}, calculations for optimal water contents were based on dry soil weight.

Water contents and maximum water holding capacity

To determine WHC_{max} soil was filled into small glass or brass cylinders with plastic or brass meshes at the bottom and were incubated overnight in water at the height of the soil layer. After 24 h the cylinders were removed from the water and were placed on tissues for 24 h draining at RT. After draining the remaining water content was defined as the WHC_{max}. To determine the water contents, a defined amount of fresh (wet) soil was tried to constant weight at 105 °C. As an additional validation for optimal water contents of the soils, a defined amount of soil was watered until water contents of the soil reached a point that made the

formation of a soil tube with the diameter of a thick pencil possible. Nevertheless, this method is only useful for soils with relatively high clay contents.

Fertilisation

Fertilization of pot experiments was done by spraying soil or sand-soil mixtures with nutrient solutions. Nutrients used for standard fertilization are listed in Table 2-2. An overview of all organic and commercial fertilizers used during the thesis is given in Table 2-17 and Table 2-18.

Table 2-2 Standard fertilization of pot experiments

Nutrient	Mineral	Conc. (mol l^{-1})	Conc. (mg kg dry $soil^{-1}$)
N	$Ca(NO_3)_2$	0.5	100
K	K_2SO_4	0.5	150
Mg	$MgSO_4$	0.5	50
P	$Ca(H_2PO_4)_2$	0.05	50

Detailed information on fertilization strategies are given in the description of the specific experiments in chapter 3.

Climatic conditions

Pot experiments were either conducted in greenhouses or climate chambers. Because climate chambers generally had much lower light intensity (100 - 200 µE) in comparison to the greenhouse (200 – 400 µE with artificial light and up to 1 mE with sunlight), most experiments were conducted in greenhouses. Light intensity can be described as

1. Photosynthetic Photon Flux Density (PPFD) with the unit µEinstein or µmol m^{-2} s^{-1}
2. Illuminance or lumen per square meter (Lux) with the unit lx or lm m^{-2}

The units are not easily convertible as they depend on the wavelength of the light. Nevertheless, for sunlight a conversion factor of 54 lx per µmol s^{-1} m^{-2} and for cool-white fluorescent light a conversion factor of 74 lx per µmol s^{-1} m^{-2} can be assumed (Thimijan and Heins, 1983). Accordingly, 10.000 lx are equal to 135.1 µmol s^{-1} m^{-2}.

Climatic conditions in the greenhouse were only partially controllable and temperature varied from 13 °C up to 35 °C, similar to field conditions for the growth period of maize in northern Europe. More information on single experiments is given in Table 2-13 and Table 2-14.

2.4 Plant growth parameters

2.4.1 Non-destructive measurements

Plant height

Plant height in pot experiments was defined as the height of the longest leaf, measured from soil layer up to the tip of the stretched leaf. In field experiments actual plant height was measured without stretching leaves. In most cases this was the height of tassel, the inflorescence of male flowers. 5 measurements in 4 rows per plot were done.

Stem diameter

Stem diameter was found to be a valuable indicator for P-supply. Stem diameter was measured with a calliper at previously defined positions, normally the thickest stem part below the first or second leaf.

SPAD

Soil-Plant Analyses Development (SPAD) values predict leaf chlorophyll contents by measuring leaf coloration without damaging leaves. Especially as indicator for sufficient N-supply SPAD meter were used. SPAD values were measured at a previously defined leaf position, normally the middle of the leaf avoiding the midrib, of a defined developmental stage, normally the youngest fully developed leaf. In field experiments the SPAD values were measured at the leaf below and opposite to the ear. These leaves were also sampled for P, C and N-analysis of maize grown in the field. 50 measurements per plot were taken by randomized sampling using plants from different rows.

Chlorosis/ Necrosis

In pot experiments with limited P-fertilization or applied cold stress conditions, leaf chlorosis, typically a red coloration from anthocyanin formation, was quantified by visual estimations using either percentage of leaf area or an ordinal scaling system.

2.4.2 Destructive plant measurements

Plant sampling

In pot experiments always whole plant samples were analysed after harvest. For field experiments, leaf samples for nutrient analysis before harvest were collected below and opposite from the ear. Root samples for BE tracing or mycorrhization were sampled before harvest as subsamples from whole plant avoiding loss of plants for later harvest evaluations.

Plant biomass

Fresh shoot biomass from plants growing in pots was determined directly during harvest. Root fresh weight was taken after washing and quickly drying with paper tissues. If roots or shoots were not used for other purposes (e.g., metabolome or gene expression studies, root scanning) they were directly dried to constant mass at 105 °C and dry weight was measured. Because plant material for metabolome, gene expression or inorganic-P studies were directly frozen in liquid nitrogen and then later on used for RNA isolation, dry weight data could not be acquired for some experiments (Exp_11, 14, 22). In Exp_22 dry weight data were not taken due to the very low amounts of material available and the major biases that occurred due to material sticking to plastic or paper surfaces of the packing.

In field experiments, fresh weight data were taken by the plot combine (harvester) during harvesting. Subsamples from the plot harvests were subsequently dried to constant mass and dry weight per ha was calculated.

2.4.3 Root morphology

Root drawing and root hair length

In Exp_17 root morphology was determined previous to harvest by using rhizoboxes with root observation windows. Roots visible in the root observation window were drawn on transparent plastic sheets and subsequently scanned (Epson Expression 10000Xl, Epson, USA) at a resolution of 400 dpi. At the same time pictures from the root hair zone were taken with an Axio Vision 3.1 video macroscope and additional software (Carl Zeiss GmbH, Jena, Germany) at a magnification of 12.5. From these pictures root hair length was determined by taking the average length of 10 root hairs per plant.

Root length

In most of the pot experiments root length was determined after harvest. Therefore, whole roots were washed, cut into pieces if root parts were overlapping and scanned (see above). All root scans from washed roots and drawings were analysed with the WinRhizo software (Regents Instruments Inc., Canada). Root length was also analysed for specific root diameter classes, ranging from 0-0.2 mm, 0.2-0.4, 0.4-0.6, 0.6-0.8 and >0.8. Root parts with a diameter lower than 0.2 mm were defined as “fine roots”.

2.5 Mineral analysis

2.5.1 Analysis of macro- and micronutrients in plant materials

The analysis of phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na; e.g. for plants grown under salt stress) and micronutrients such as iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu) in plant materials was performed following an ashing assay (Gericke and Kurmies, 1952) or microwave digestion.

P concentration in the ash solution were measured colorimetrically (e.g. spectral-photometer U-3300, Hitachi, Tokyo, Japan) using ammonium-vanadate-molybdate as color reagent. K, Ca, and Na concentrations were measured by flame photometry (e.g. ELEX 6361, Eppendorf, Hamburg, Germany) and Mg, Fe, Mn, Zn, and Cu by atomic absorption spectroscopy (e.g. ATI Unicam Solaar 939, Thermo Electron, Waltham, USA).

The values of mineral nutrient concentrations in shoot samples obtained by this method can be compared with standard reference values for wheat, maize or tomato plants (Bergmann, 1993, 1976).

2.5.1.1 Sample preparation

Roots samples were carefully washed free from adhering soil or substrate material. Analysis of samples from pot experiments was always done for whole root or shoot samples. In field experiments the leaf below and opposite to the ear was taken as pre-harvest sample. 5 leaves from 4 maize rows were taken per plot.

After washing, root or shoot samples were oven-dried at 60°C until weight constancy.

Dry root or shoot samples were ground to powder in agate disc swing mills (e.g. Fritsch, Idar-Oberstein, Germany).

2.5.1.2 Preparation of ash solution

Reagents:

1.) 1:3 diluted nitric acid: 1 part HNO_3 65 % + 2 parts deionized water.

2.) 1:3 diluted HCl: 1 part HCl 37 % + 2 parts deionized water

Procedure:

Aliquots of 250 mg powdered plant material were weighed into porcelain crucibles to be mineralized by the ashing procedure (Gericke and Kurmies, 1952). The samples were exposed to 500 °C for at least 4 hours. After this treatment the samples were cooled to room

temperature outside of the muffle furnace, wetted with some drops of deionized water and then treated with some drops of 1:3 diluted (i.e. 65 %) HNO_3. After evaporation on a heating plate the crucibles were placed back into the furnace and kept at 500 °C for at least 1 hour. After cooling, the ashes were again wetted with some drops of deionized water. 2.5 ml 1:3 diluted HNO_3 were added to each crucible and then evaporated on a heating plate under the fume hood. This process was repeated once to clear the samples of SiO_2 by precipitation. The digested samples were then dissolved with 2.5 ml 1:3 diluted HCl and transferred into 25 ml volumetric flasks using a Pasteur pipette. The samples were boiled on a heating plate under the fume hood for 2 minutes to convert meta- and pyrophosphates formed during the evaporation with HNO_3 back to orthophosphate. After cooling to room temperature, the volumetric flasks were filled with deionized water up to 25 ml, shaken thoroughly and filtered through blue band filter paper (e.g. Rundfilterpapier 110 mm Ø, Schleicher & Schuell Nr. 589/3, Blauband; or MN 640 d; Macherey-Nagel GmbH & Co. KG).

2.5.1.3 Microwave digestion

Alternatively to the ashing method microwave digestion was performed (method 2.1.1 (VDLUFA-Fachgruppe VIII, 2011)). Here 100 – 250 mg of shoot or root material was weighed into plastic decomposition vessels. 2 ml H_2O, 5 ml HNO_3 (65 %) and 4 ml H_2O_2 were added and incubated at RT for 30 – 60 min. The volume of the acids was slightly modified depending on sample amount. After incubation samples were transferred to a microwave (MLS Maxi 44, MLS GmbH, Leutkirch, Germany) and material was digested in a three-step program (Table 2-3) optimized for maize. Pressure in the tubes reached up to 30 bars. The digestates were transferred to 20 ml volumetric flasks and filled to the mark. The solution was filtered as described for the ashing method.

Table 2-3 Microwave program

Time (min)	Watts	Temp. (°C)	Ventilation
3	1400	70	1
12	1400	210	2
50	1400	210	1

A similar method was also applied for the mineral analysis of the BE products. Here ~ 250 mg of solid or liquid BE product were used. For digestion 8 ml H_2O + 5 ml HNO_3 + 2 ml H_2O_2 were added. The digestates of the BE products were analysed externally at the former Landesanstalt für Chemie, University of Hohenheim (now Core Facility Hohenheim) by ICP-OES or ICP-MS.

2.5.1.4 P analysis ("P-yellow")

Reagents:

1.) Molybdate-vanadate color reagent
   a. 1:3 diluted HNO_3: 1 part HNO_3 65 % + 2 parts deionized water
   b. Ammonium vanadate solution 0.25 %: 2.5 g Ammonium monovanadate (NH_4VO_3) were dissolved in 600 ml boiling deionized water (under the fume hood); after cooling 80 ml conc. HNO_3 were added and the flask was filled to 1 liter with deionized water.
   c. Ammonium molybdate solution 5 %: 50 g ammonium-heptamolybdate-tetrahydrate ($(NH_4)_6Mo_7O_{24}$ x $_4H_2O$) were dissolved in 800 ml deionized water at 60 °C (under the fume hood) and filled to 1 liter with deionized water after cooling.

   The solutions a), b) and c) are then mixed to a 1:1:1 ratio.

2.) 1:30 diluted HCl: 1 part HCl 37 % + 29 parts deionized water

For P analysis an aliquot (e.g. 0.5 to 7.0 ml depending on the P concentration and adequate range for measuring) of the filtered ash solution was mixed with 3 ml molybdate-vanadate-solution and brought to a volume of 10 ml with 1:30 diluted HCl to form a yellow dye. Standard-Dilution was 1:5. Molybdate-vanadate-reagent concentration was kept constant independent of the sample dilution or the HCl concentration. Samples were measured 2 hours after staining, when color intensity of the solution is almost constant, at a wavelength of 436 nm in the spectral photometer (e.g. U-3300, Hitachi, Tokyo, Japan) and compared to a standard curve. The range for calibration solutions was 1 to 15 ppm (mg liter^{-1}).

2.5.1.5 K, Ca, and Na analysis

Samples were diluted to achieve concentrations within the adequate measuring range. Ranges for the calibration solutions, which should have the same acid concentration as the samples, were the following:

K: 10 to 100 ppm (mg liter^{-1})
Ca: 10 to 100 ppm (mg liter^{-1})
Na: 0.1 to 10 ppm (mg liter^{-1})

2.5.1.6 Mg, Fe, Mn, Zn, and Cu analysis

Before measuring Fe and Mn concentrations a buffer solution containing cesium chloride and lanthanum chloride (e.g. Merck, No. 116755) was added to the samples in a 1 : 50 ratio (1

part Cs/La-buffer + 49 parts of the sample solution) to eliminate interferences in the air-acetylene flame of the atomic absorption spectrometer (Schinkel, 1984).

Ranges for the calibration solutions were the following:

Mg: 0.1 to 1 ppm (mg liter^{-1})
Fe: 0.1 to 8 ppm (mg liter^{-1})
Mn: 0.1 to 6 ppm (mg liter^{-1})
Zn: 0.1 to 2 ppm (mg liter^{-1})
Cu: 0.1 to 5 ppm (mg liter^{-1})

2.5.2 Measurement of soluble inorganic phosphorus (Pi)

The method after (Bollons and Barraclough, 1997) was adapted in the time course of the measurement of samples from three different experiments. The last procedure is described below whereas differences to this procedure are described in the respective section of the single experiment. In contrast to the previous method plant material is not incinerated or digested under pressure but dried material is extracted gently using acetic acid.

2.5.2.1 Harvest of sample material

Shoots were cut from roots at harvest time, fresh weight was taken and then shoots were directly frozen in liquid N. Roots were washed (eventually scanned), quickly dried on paper and then also frozen in liquid N.

2.5.2.2 Drying and preparation of plant material

All samples were freeze dried. Shoot and root material was grinded in a disc mill (Scheibenschwingmühle) or small mills.

2.5.2.3 Extraction of Pi

For Pi extraction a measured amount of sample material (50 - 250 mg shoot or root material) was weighed into a 50 ml falcon tube (no Pi contamination). The sample was shaken with 25 ml of 2% w/w acetic acid at 225 rpm for 30 min. If filtrate was still coloured after filtration a small spoon of activated carbon was added directly into the extract. The extract was filtered through clean Blauband filters, discarding the first few drops.

All material was washed previously with deionized (MQ/ELGA) water, to remove any trace contaminations from P containing tensides.

2.5.2.4 Analysis of Pi

Analysis of Pi was done using the molybdate-vanadate (P yellow) or molybdate-blue method, depending on available sample amount and concentration (see below).

2.5.3 „P-blue“ measurement

2.5.3.1 Color reagent

1. All solutions described below were separately prepared in clean glass flasks (manually washed with deionized water).

Table 2-4 P-blue reagents

Reagent	Formula	V (ml)	m (ml g^{-1})
Sulfuric acid (SulfA)	H_2SO_4	200	27.8
Ammonium-molybdate (AmMo)	$(NH_4)_6Mo_7O_{24}$ x 4 H_2O	50	2.0
Ascorbic acid (AscA)	$C_6H_8O_6$	50	0.875
Antimony potassium tartrate (APT)	$KSbOC_4H_4O_6$ x ½ H_2O	100	0.275

V = Total volume of the solution; m = in weight of respective compound

Ascorbic acid and therefore all mixed colour reagents were freshly prepared every day.

2. Solutions were mixed as follows:

Total amount	Samples	SulfA	AmMo	AscA	APT
250 ml	< 60	40 ml	12.5 ml	25 ml	4 ml
500 ml	< 140	80 ml	25 ml	50 ml	8 ml
1000 ml	< 300	160 ml	50 ml	100 ml	16 ml

Solutions were filled up to 250/ 500/ 1000 ml with dest. H_2O.

2.5.3.2 Standards

Stock solution for standards (for preparation of standards for Pi measurement 2 % acetic acid instead of water was used):

- Stock I: 1.3609 g KH_2PO_4 L^{-1} (10 mM)
- Stock II: 1:10 dilution of stock I (1 mM)
- Standards in 100 ml volumetric flasks

Volume added from Stock II (ml)	Final concentration in the standard (µM)	Final conc. (ppm, mg P/L)
10	100	3.10
7.5	75	2.33
5	50	1.55
2.5	25	0.78
1.5	15	0.47
1.25	12.5	0.39
1	10	0.31
0.75	7.5	0.23
0.5	5	0.16
0.3	3	0.09
0.1	1	0.03
0.05	0.5	0.02
0	0	0.00

2.5.3.3 Measurement

For staining of plant extracts and standards the sample was mixed with colour agent 1:1. After 20 min incubation time samples were measured at a wavelength of 710 nm.

2.5.3.4 Dilution of samples

Shoot and root samples from maize were measured undiluted or as 1:5 dilutions. For total P samples that were treated with concentrated strong acids like HCl and/ or HNO_3 (ashing and microwave method) in undiluted samples no colour reaction happened. Therefore, some drops of concentrated ammonia solution (25 %) were added to induce the colour reaction.

P-values range:

Plant organ	DAS (days after sowing)	µM (~50 mg plant material)	mg/g DW
Root	6-12	50 - 200	1-1.5
Root	17-24	50 - 100	0.5
Shoot	6-12	300 - 400	3-4.5
Shoot	17-24	50 - 150	0.5-1

2.5.3.5 Calculations

$$\frac{(c(sample, Pi\ in\ \mu M) - c(Blank)) \times DF \times V(extractant\ in\ mL) \times M(P)}{1000\ mL \times m(in\ weight\ in\ mg)}$$

$$= mg\ Pi/g\ plant\ DW$$

DF= dilution factor, e.g., 1/5; c= mol. conc.; M= 31 g/mol (molar mass of phosphorus)

2.6 Microbiological methods

2.6.1 Media

2.6.1.1 PDA media (Potatoe dextrose agar media)

39 g commercial potato extract glucose agar (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) l^{-1} was used.

- 20 g dextrose
- 15 g agar
- 4 g potato starch

1 l dest. H_20 was added, pH was adjusted to 5.6 ± 0.2 at RT and medium was autoclaved at 121°C for 15 minutes.

2.6.1.2 Standard II nutrient agar

Commercial Standard II nutrient agar (Merck KGaA, Darmstadt, Germany) was used.

- 3.45 g peptone from meat
- 3.45 g peptone from casein
- 5.1 g sodium chloride
- 13.0 g agar-agar

1 l dest. H_20 was added, pH was adjusted to 7.5 ± 0.2 at RT and medium was autoclaved at 121°C for 15 minutes.

2.6.1.3 NP medium

For tracing of Pseudomonades a modified NPC medium was used (Sands and Rovira, 1970). King's agar medium or R2A agar medium were supplemented with the antibiotics novobiocin and penicillin. The highly toxic cycloheximid was omitted because fungal growth was found to be negligible if agar plates were incubated at 30 °C for optimal growth of bacterial BEs.

King's B medium (KB, Carl Roth GmbH):

- 20 g peptone
- 1.5 g K_2HPO_4
- 1.5 $MgSO_4$ x 7 H_2O
- 15 g agar
- 10 ml glycerine (has to be added to the agar medium)

990 ml dest. H_20 were added, pH was adjusted to 7.2 ± 0.2 at RT and medium was autoclaved at 121°C for 15 minutes.

R2A medium (Oxoid LTD, Basingstoke, England):

- 0.5 g proteose peptone
- 0.5 g casamino acids
- 0.5 g yeast extract
- 0.5 g dextrose
- 0.5 g soluble starch
- 0.3 g K_2HPO_4
- 0.05 g $MgSO_4$ x 7 H_2O
- 0.3 g sodium pyruvate, 0.3
- 15 g Agar

1 l dest. H_20 was added, pH was adjusted to 7.2 ± 0.2 at RT and medium was autoclaved at 121°C for 15 minutes.

After temperature of the medium decreased to 40 °C 45 mg novobiocin sodium salt (Sigma-Aldrich Chemie GmbH, Munich, Germany) and 45 mg penicillin G sodium salt (AppliChem GmbH, Darmstadt, Germany) l^{-1} were added. Antibiotics were first dissolved in 95 % ethanol and then diluted with 45 ml of water, sterile filtrated and stored at 4 °C.

2.6.1.4 LB medium

Commercial LB medium (after Lennox, Carl Roth GmbH) with or without agar was used

- 10 g tryptone
- 5 g yeast extract
- 5 g NaCl
- 15 g agar

1 l dest. H_20 was added, pH was adjusted to 7.0 ± 0.2 at RT and medium was autoclaved at 121°C for 15 minutes.

For tracing of the rifampicin (*rif*) resistant *B. amyloliquefaciens* strain LB medium was supplemented with 50 mg rifampicin (AppliChem GmbH) l^{-1} after cooling to ~ 50°C. Rifampicin was dissolved in DMSO (50 mg l^{-1}) and stored at -20°C.

2.6.1.5 Peptone

For bacterial extraction from soil sterile 0.1 % peptone solution was used (1 g Bacto™ Proteose Peptone (Difco Laboratories, Detroit Michigan, USA) l^{-1} dest. H_2O).

2.6.2 Tracing methods

2.6.2.1 Summary

To re-isolate Pseudomonades and inoculated *Bacillus* strains from soil or root samples selective media were used. A strain specific tracing of the *Pseudomonas* sp. "Proradix" (Px) strain by RT-qPCR is described in 2.7.2.2.

For plating of Pseudomonades, the semi-selective NP media was used. This medium selects mainly for Pseudomonades and is known to promote fluorescence (King et al., 1954; Sands and Rovira, 1970) but does not select specifically for the Px strain. Because in previous work in our institute (Weinmann, 2017, p. 316ff.) a significantly increased *Pseudomonas* sp. density was observed by plating assays after application of *Pseudomonas* strains, this method was first applied before using the strain specific tracing by RT-qPCR.

For tracing of inoculated *Bacillus* strains heat treatment at 80°C for 10 min was first applied with subsequent plating on LB medium. This semi-selective method selects for all endospore forming organisms in the soil, but due to a relatively low natural abundance of this "sporulation" trait, reliable quantification was already possible.

For selective tracing of the *rif*-resistent *B. amyloliquefaciens* strain (only Rz) *rif*-supplemented LB medium was used. Additional heat treatment at 80°C was applied to differentiate endospores from active (germinated *Bacillus*). The number of metabolically active cells was calculated by subtracting the number of endospores from the total cell number (without heat treatment).

2.6.2.2 Sampling

Root or soil samples from pot experiments were sampled during plant harvest at the end of the experiment. Bulk soil samples were defined as soil that was not in contact with roots at harvest time. Soil sticking to the root surface that was shaken off or scratched from root surface was defined as rhizosphere soil. Root samples were either sampled with adhering rhizosphere soil or first washed and then taken as "rhizoplane" samples for tracing of bacteria that were sticking directly to the root surface or colonized root as endophytes.

Soil samples from field experiments were taken with soil corers at 10 or 30 cm depth. Root sampling in the field was done by digging out maize roots with small shovels up to a depth of 15 cm. Samples were taken directly in the row or in between rows. The plant itself was kept in the soil. Either control plots were first sampled and subsequently inoculated plots or shovels were sterilized with alcohol and a camping gas burner in between plots.

2.6.2.3 Extraction

For isolation of bacteria from roots or soil 1 – 5 g of respective sample material was weighed into sterile 50 ml centrifuge tubes and was shaken with 25 ml pre-cooled, sterile 0.1 % peptone solution for 10 min at 225 rpm. For "rhizoplane" samples additionally sterile glass beads were added to detach bacteria from root surface. After sedimentation for further 5 – 10 min on ice, serial dilution was started.

In some cases, quantification of root colonization was based on dry soil weight. Therefore, to quantify rhizosphere soil adhering to the non-washed root samples after plating assays, roots were removed from extraction tubes and the remaining suspension was centrifuged. Suspension was decanted and the rhizosphere soil was dried at room temperature overnight.

2.6.2.4 Plating and incubation

Serial dilution of the peptone extract was done in 0.3 % NaCl, 0.1 % peptone or 10 mM $CaSO_4$ using 2 ml micro tube, 1:10 dilution steps and vortexing. Plating was done on 10 cm agar plates evenly spreading 0.1 ml from a dilution series over the complete agar surface using a sterilized spreader rod. All agar plates for tracing of bacteria were incubated at 30°C for 1 – 2 days. Especially for *Bacillus* tracing after heat treatment or by plating on LB_{rif} plates often very accurate counting was possible with clear colony separation and low amounts of contamination. Here, those dilutions were chosen that contained about 1000 CFUs ml^{-1} for accurate counting.

2.6.3 Screening for prebiotic properties

2.6.3.1 Tests on bacterial strains

Different concentrations of seaweed extracts (SWE) were tested for stimulation of bacterial growth (min. 0.0001 % - max. 3 %). 100 µl of respective SWE dilution was added to 900 µl of a BE suspension (with diluted microbial BE product in 2.5 mM $CaSO_4$) and the mixture was incubated for 4 h shaking in a 2-ml microcentrifuge tube. After the incubation 100 µl of the solution was plated on the appropriate nutrient agar and incubated for ~24 h in incubators at 27 or 30 °C. The number of cell forming units (CFU) per plate were compared to controls

without seaweed extract or to control treatments with reference nutrient sources. Bsim and Rz strains were plated on LB medium and Px strain was plated on NP medium.

2.6.3.2 Tests on Trichoderma

For *Trichoderma* product (TP) small cubes of mycelium from a pre-incubated PDA agar plate were transferred to fresh PDA plates containing different concentrations of seaweed extracts. The diameter of mycelium on the agar plate was documented.

2.6.3.3 Pasteurization of seaweed products and media preparation

Because seaweed extracts are non-sterile products, pasteurization was performed to reduce contaminations that would result in biases in the analysis of the CFU counts per agar plate:

1. Seaweed extract products were diluted 1:1 in distilled water.
2. The diluted extracts were placed in a water bath at 90-100° C.
3. The temperature in the solutions was measured with a thermometer. When temperature reaches 80°C the solutions were incubated for further 10 sec.
4. Pasteurized products were cooled at 4°C.

After pasteurization the products were diluted in sterile, distilled water. For incorporation in agar medium the diluted seaweed extracts were added to the agar medium after cooling down to ~ 60°C after autoclaving at 121°C for 20 min. For the 0-Control same amount of distilled water was added.

2.6.4 Mycorrhizal infection rate

For analysis of mycorrhization of plant roots by arbuscular mycorrhizal fungi (AMFs), a modified staining method was used (Vierheilig et al., 1998). Roots were cut into segments of 1-2 cm and incubated at 90°C for 45 min in 10% (w/v) KOH. In some cases, when roots were still dark due to humic substances or tannins in the substrate, roots were additionally bleached with H_2O_2 solution (3 ml 20% NH_40H in 30 ml 3% H_2O_2) for 10-45 min (Koske and Gemma, 1989). Cleared and bleached roots were acidified with 1 - 3% HCl and then stained in a 5 % ink-vinegar solution (5% (v/v) ink in 5 % (v/v) acetic acid) at 90°C for 10 min. De-staining was performed with acidified tap water. The grid-line intersection method of (Giovanetti and Mosse, 1980) was employed to determine the rate of mycorrhization. The method was modified in the duration of the KOH treatment or the bleaching depending on root thickness, plant species or soil substrate in which plants were growing. For short term storage and

counting of hyphal structures, spores or arbuscules under the stereo microscope (40 – 50x magnification) 1:1 diluted lactic acid was used.

2.7 Molecularbiological methods

2.7.1 RNA extraction

Sampling of plant material for RNA analysis was always done in randomized order within two hours to reduce any biases of diurnal changes in the plant transcriptome on treatment differences. Shoot samples for RNA-extraction were weighed, wrapped in aluminium sheets and then frozen in liquid-N directly after harvest. Root samples were first washed, shortly dried on paper, wrapped in plastic and aluminium sheets and then frozen in liquid-N. Samples were stored at -80°C.

For RNA extraction samples were grinded in the mortar with liquid nitrogen. ~100 mg of frozen material was used for RNA extraction using the innuPrep Plant RNA Kit (Analytik Jena AG, Jena, Germany). After homogenisation and cell lysis gDNA and non-lyzed cells are filtered out by microfilters. In a next step RNA is bound to a second filter and purified in several washing steps. RNA was eluted in RNase-free water.

RNA quality assessment

NanoDrop

RNA quality was first assessed spectrophotometrically by using a Thermo Scientific™ NanoDrop 2000c (Thermo Fisher Scientific, Waltham, USA). RNA samples with 260/280 > 2.0 and 260/230 ratios between 1.7 and 2.2 were used for RT-qPCR whereas the lower limit of the 260/230 ratios for RNA-Seq samples was set at >/= 2.0.

RNA sample quality was also checked again at the company BGI (BGI Tech Solutions (HongKong) Co., Ltd., Tai Po, Hong Kong) by using NanoDrop and RNA integrity number (RIN). Here RNA-Seq analysis was performed as described under 2.7.3.

Gel electrophoresis

For RNA gel electrophoresis a non-denaturing “bleach” gel was used (1x TAE (TRIS-Acetat-EDTA) buffer amended with 0.06 % NaClO, 1 % agar). The gel was running at 100 V for 45 min. 5 µl of RNA sample were pre-heated for 1 min at 70°C and then cooled on ice before mixing with 1 µl 5 x RNA loading dye. For size estimation 7 µl Lambda PstI ladder was added to each run. If double bands indicating 28S/18S rRNA fragments were clearly visible

and contrasted to an equally distributed comparable low intensity smear of other fragment size, minimal degradation and therefore good RNA quality / RNA integrity was assumed.

2.7.2 RT-qPCR for Proradix tracing

Strain-specific tracing of *Pseudomonas* sp. "Proradix" (Px) was done following the recently published method from a partner group at FiBL, Schwitzerland (Mosimann et al., 2017).

2.7.2.1 DNA extraction

The method was adapted to the facilities and material available in our institute. To extract DNA from fresh root tissues 1 g of washed roots (softly dried with paper) were placed in small closable plastic bags (ca. 5 x 10 cm, closable with zipper or clips). 5 ml CTAB buffer (see below) were added and the sample was smashed with a heavy round metal disc for one minute under the fume hood. The homogenate was transferred with a 5 ml pipette into 15 ml falcon tubes. 0.5 ml from this homogenate were used for the proceeding DNA extraction with the DNeasy® Plant Mini Kit (Qiagen), the rest was stored at -20°C. Before following the Kit protocol, 5 µl of linearized APA9 (8 x 10^5 copies μl^{-1}) were added and the mixture was heated to 65°C for 30 min in a thermomixer. The mixture was mixed 2- or 3-times during incubation by inverting the tubes. For elution of the DNA from the DNeasy column 50 µl pre-warmed (65°C) AE buffer were used.

Table 2-5 Composition of the DNA extraction buffer

CTAB extraction buffer	Concentration	100 ml
Tris (Carl Roth GmbH)	100 mM	1.21 g
NaCl	1.4 M	8.19 g
Na_2EDTA (Sigma-Aldrich)	50 mM	1.86 g
CTAB (Carl Roth GmbH)	2% (m/v)	2.0 g
PVP40-50G (Sigma-Aldrich)	1% (m/v)	1.0 g
SDS	0.2 % (m/v)	0.2 g
HCl to reach pH 8.0 Buffer stable for 2 years at RT		
β-Mercaptoethanol (Sigma-Aldrich) added just before use	0.2 % (v/v)	0.2 ml

2.7.2.2 RT-qPCR

Quantification of Px-specific DNA was done using the TaqMan® method. In this RT-qPCR method an increased specificity for a target sequence is reached by using a probe sequence additionally to the standard forward and reverse primers used in all PCRs. ACMV and Px-specific primers and probes were provided by Cecile Thonar at FiBL. PCR conditions were kept the same, using the KAPA Probe Fast qPCR Kit (Kapa Biosystems, Boston, USA). The CFX384 cycler was used for temperature program and data analysis.

2.7.3 Real time qPCR for gene expression

2.7.3.1 Primer selection

Primer selection was done for selected genes. Gene selection is described in 2.7.4. For primer design NCBI Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) on NCBI transcript IDs was used. Default settings were retained unless otherwise described below. Because, for most of the selected genes no exon junctions were available, Primer BLAST did not specifically search for exon junction spans. Primer pair specificity check was done in the Refseq mRNA database of *Zea mays*. Range for primer GC content was limited from min of 40 to a max of 70 %. Secondary structure alignment methods were activated. To exclude the possibility of non-specific product amplification from unintended templates like prokaryotic RNA/DNA or maize gDNA primer were also checked against those databases. Additionally, primer sequences were checked in the primer stats tool (http://www.bioinformatics.org/sms2/pcr_primer_stats.html) for GC clamps, self-annealing and hairpin formation. In Table 2-1 a list of all primers is given. For the genes LAC3 and NAC1 (Primer 21-24) primers were taken from the publications of (Caparrós-Ruiz et al., 2006) and (Lu et al., 2012) respectively. Primers were ordered from the company Invitrogen AG (Carlsbad, USA) / Thermo Fisher Scientific Inc.

2.7.3.2 Reference genes

Reference genes for maize were selected by a two-step approach. First, a literature based pre-selection for candidate genes was done using three recent publications (F. Lin et al., 2014; Y. Lin et al., 2014; Manoli et al., 2012). Using the BioMart-tool (http://plants.ensembl.org) gene IDs / transcript IDs could be converted into RefSeq mRNA accessions. Those genes were selected that showed most stable FPKM values. To further prove stability of gene expression and also search for new stable candidate genes, in a second step reference genes were searched in our own RNA-Seq database. After comparison of stability values calculated with the MS Excel add-on NormFinder (Andersen et al., 2004) as well as coefficients of variation, both based on RNA-Seq FPKM values, best candidates were selected. Given primer sequences from publications were searched in the FASTA sequences of the selected genes that were downloaded from NCBI database. Some of the primers could not be found in the sequences and therefore respective genes were excluded from the candidate list. Although all selected genes had stable expression in RT-qPCRs, as reported in the publications, the six genes (genes 19 -24) from final selection showed diverse FPKM values from 14 – 500 in the RNA-Seq analysis.

2.7.3.3 Reverse transcription

For RT-qPCR high quality plant RNA was first transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Venlo, Netherlands) according to the provided kit protocol. Potential gDNA contaminations remaining after RNA extraction were wiped out by addition of some buffer in the first step of the kit protocol. DNase treatment was performed only to check for gDNA contamination in RNA samples and to test false positive amplification of DNA fragments using 2 µl DNase I (Invitrogen, Carlsbad, USA) per 20 µl reaction mix.

2.7.3.4 Primer quality

To assess primer quality PCR with subsequent DNA gel electrophoresis was performed using a mixture of RT-PCR derived cDNA and the Genaxxon Taq DNA Polymerase S (Genaxxon bioscience GmbH, Ulm, Germany). Table 2-7 and Table 2-6 give an overview of the reaction mix and cycler program. Template concentration varied to optimize PCR for low or high expression genes. For some low expression genes optimization of annealing temperature was done to improve gene amplification. For DNA gel electrophoresis different kinds of agarose were used whereas the high-resolution agarose Roti®garose (Carl Roth GmbH) resulted in best resolution. 1.5 % agarose were dissolved in 1x TAE buffer. For size determination of PCR products, the 100 bp Gene Ruler ladder (Thermo Fisher Scientific) was added. 5 µl PCR reaction mix were mixed with 1 µl 6x DNA loading dye. Gels run for 35 - 50 min at 90 V.

Table 2-6 Program for primer check PCR

Program (Thermal cycler)	Time (min)	Temperature (°C)
Initial denaturation	3	94
3-step cycling		
Denaturation	0.5	94
Annealing	0.5	55 - 62
Extension	0.5	72
Number of cycles	35	
Final extension	10	72

Table 2-7 Reaction mix for primer check PCR

Reaction mix PCR	Volume (µl)	Final conc.
Taq-Polymerase [µl µl reaction mix^{-1}]	0.25	**0.0125**
10X amplification buffer [x]	2	**1**
Nucleotides [µM]	0.4	**200**
sterile water	8.35	**NA**
Template (cDNA, conc. varied)	5	**various**
FW and RV primer (2 µM) each [µM]	2	**0.2**
Total volume	20	/

2.7.3.5 Real time qPCR

Quantification of gene expression was done using the KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, Boston, USA). Reaction mix volume was reduced to 15 µl (Table 2-8). The cDNA concentration was calculated based on the amount of input RNA for RT reaction mix. CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, USA) was used following the cycler program described in Table 2-9. Three analytical replicates per sample/ primer combination as well as NTC-controls (non-template control), containing sterile water, and NRT-controls (no-RT), containing RNA to test for amplification of gDNA, were included. In each run two to three reference genes were included. Quantification of gene expression and quality assessment was done using the internal software Bio-Rad CFX manager 3.1. ΔΔCq values were calculated by normalization against two reference genes, whereas the combination with the lowest target stability value was chosen. Statistical analysis was done using SigmaPlot (0).

Table 2-9 RT-qPCR cycler program

Program (SYBR RT-qPCR)	Time	Temperature (°C)
Initial enzyme activation	3 min	95
2-step cycling		
Denaturation	10 s	95
Annealing + Extension	20 s	60
Number of cycles	40	
Final heating	10 s	95
Melting curve	5 s	0.5 increment, 55 - 95

Table 2-8 RT-qPCR reaction mix

Reaction mix RT-qPCR	Volume (µl)	Final conc.
2X KAPA SYBR® FAST qPCR Master Mix	**7.5**	1x
Primer pair mix (each 2 µM)	**0.75**	0.1 µM
Water for qPCR (up to 20 µl)	**2**	NA
Template cDNA (4 ng μl^{-1}) or NTC/NRT control	**5**	max. 20 ng

2.7.3.6 Quality assessment

For quality assessment of qPCR runs NRT and NTC wells are checked for expression and melting curves. Melting curves of genes were checked for the presence of double peaks, indicating non-specific template amplification or formation of primer dimers. Additionally, for each tested sample and gene, analytical replicates were checked for outliers, indicating pipetting errors or contaminations from neighbouring wells.

2.7.4 RNA-Seq

A complete transcriptome re-sequencing, including RNA-Seq library construction, sequencing and standard bioinformatics, was performed by the company BGI (BGI Tech Solutions (HongKong) Co., Ltd., Tai Po, Hong Kong).

2.7.4.1 Library construction

After DNase I treatment Oligo (dT) based enrichment of mRNA (eukaryotic) was followed by fragmentation, cDNA synthesis, addition of adapters and PCR amplification. Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System were used in quantification and qualification of the sample library. The library was sequenced using the HiSeq 4000 technology on 100 bp paired-end reads.

2.7.4.2 Bioinformatics (BGI)

Raw reads from sequencing step were filtered and cleansed from reads containing adapter sequences, high contents of unknown bases (>10%) and low-quality reads. For quality control base composition of clean reads was checked. Alignment of sequences was done using BWA and Bowtie to map clean reads to the maize B73_RefGen_v3 (NCBI) genome or gene reference. Gene annotation and functional classification was based on RefSeq, BLAST (NCBI), KEGG (pathway classification) and the Gene Ontology Consortium (GOC) databases. Gene and isoform expression levels were quantified by the software package RSEM (RNASeq by Expectation Maximization) (Li and Dewey, 2011). For normalization of gene expression the FPKM method was used:

$$FPKM = \frac{10^3 C}{NL}$$ (C = number of fragments that are uniquely aligned to the gene; N = total number of fragments that are uniquely aligned to all genes; L = number of bases on the gene)

Pearson product-moment correlation and principal component analysis (PCA) was performed for the correlation between samples based on whole transcriptome FPKM values. NOISeq procedure was used for selection of differentially expressed genes (DEGs) (Tarazona et al., 2012) in treatment comparison. In this approach log2-foldchange (M) and absolute difference (D) between two treatments is calculated for all genes to build a noise distribution model. A comparison of single gene M and D with the noise distribution models results in probability values for each gene. Genes with a log2-foldchange ≥ 1 and diverge probability ≥ 0.8 were defined as DEGs. For comparison of single samples an algorithm based on Poisson distribution was applied using FDR method for multiple comparison correction (FDR ≤ 0.001) and a log2ratio ≥ 1 as threshold. Cluster analysis, gene ontology (GO term using http://www.geneontology.org database) and pathway enrichment analysis (PEA using KEGG) were performed for DEGs to determine a shift in plant metabolism. In GO term and PEA analysis gene numbers were calculated for every term or pathway, then a hypergeometric test was used to find significantly enriched GO terms in the input list of DEGs, based on 'GO::TermFinder' (http://search.cpan.org/dist/GO-TermFinder/lib/GO/TermFinder.pm):

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

N = number of all genes with GO or KEGG annotation; n = number of DEGs in N; M = number of all genes that are annotated to certain GO term or pathway; m = number of DEGs in M. P = p-value

Pathways or GO terms with a Bonferroni corrected p-value ≤ 0.05 were defined as significantly enriched.

2.7.4.3 MapMan / PageMan

Function principle

Because PEA and GO term analysis at BGI only focused on DEGs an additional whole transcriptome functional analysis was performed using MapMan (Thimm et al., 2004). MapMan uses the KEGG, GOC, TAIR (The Arabidopsis Information Resource), TIGR (The Institute for Genomic Research) and InterPro databases for gene annotation and functional grouping in hierarchically organized BINs and subBINs. These BINs and subBINs are mapped in so-called "mapping files" specific for different plant species that are available for download on the project webpage (http://mapman.gabipd.org). As data input gene or transcript IDs with respective log2-ratios (comparison of Ctrl with BE treatments), were loaded into the program.

Statistics

Various statistical tests like hypergeometric test, Fisher's test, ChiSquare or Wilcoxon Rank sum test in the MapMan extension (Usadel, 2005) and the add-on PageMan (Usadel et al., 2006) were used for analysis of enriched functional groups. For controlling false discovery rate (FDR) due to multiple comparison the method after Benjamin Hochberg (Benjamini and Hochberg, 1995) was chosen to correct p-values from Wilcoxon Rank sum test.

Gene conversion and mapping files

RefSeq mRNA accessions and NCBI gene IDs were converted to Gramene transcript IDs (www.maizegdb.org) using the BioMart-tool (http://plants.ensembl.org). In a different approach using the Mercator tool (http://www.plabipd.de/portal/mercator-sequence-annotation) on a NCBI *Zea mays* RNA assembly (ftp://ftp.ncbi.nlm.nih.gov/genomes/Zea_mays/RNA/rna.fa.gz) a new mapping file with RefSeq mRNA sequences was created. Standard settings were used.

2.7.4.4 Manual search for DEGs and candidate genes for RT-qPCR

DEG filtering

RNA-Seq data were filtered for DEGs using MS Excel 2010. Various variables were used separately and combined such as p-values (also Bonferroni corrected), log2-ratios, FPKM-

values, pattern of gene expression (among biological replicates), results from single sample comparison and their similarity to the behavior of other genes in the same functional BIN (MapMan).

Protein-protein networks

The web-tool String (www.string-db.org) was used to detect potential protein networks that were triggered by BE application. The webpage allows searching for networks of single genes or for the interaction of a group of genes given as input on the webpage. The tool is based on information from literature search, gene co-expression studies and functional or structural similarities of genes. As protein IDs the Gramene gene IDs were given.

Selection of RT-qPCR candidate genes

For the selection of candidate genes for RT-qPCR four filter steps were applied:

1. Most candidates were DEGs. They were defined as DEGs either by the NOIseq procedure or by significant differences in a t-test and a log2-ratio > 0.75.
2. All selected genes had FPKM-values > 10, whereas most of the genes had values > 30. The threshold value was based on results from a study in which microarray and RNA-Seq datasets of maize were screened for reference genes (F. Lin et al., 2014). Here stable expression in RT-qPCR was found for genes with low FPKM-values of ~10.
3. All genes were annotated in at least two databases to classify them and give information on their cellular function.
4. They were selected as representatives of a given MapMan BIN or functional category and therefore their expression pattern was fitting to the results from MapMan analysis.

PCA and correlation analysis

Principal Component Analysis (PCA) was done using R (R Core Team, 2013) with the *prcomp* (*stats* package) and *qplot* (*ggplot2* package) procedures following descriptions from various internet sources (www.sthda.com; www.r-bloggers.com; www.cookbook-r.com; https://tgmstat.wordpress.com/). Correlation of gene expression was done using SAS 9.4 (SAS Institute Inc., Cary, NC, USA) and the *proc factor* procedure.

2.8 H-NMR

Summary

For analysis of primary metabolites in maize roots and shoots proton nuclear magnetic resonance (H-NMR) spectroscopical analysis was performed at the Interdepartmental Research Centre CERMANU (Università di Napoli Federico II, Portici, Italy) by Dr. Pierluigi Mazzei. Before H-NMR analysis water/methanol/chloroform extraction was performed, although it is generally possible to directly analyse solid compounds by NMR. The liquid extracts were transferred into the magnetic field of a 400 MHz NMR magnet in small 5 mm thick glass tube. For measurement certain steps of normalization/ standardisation have to be applied. First the spectra are normalized via the deuterium peak (deuteriumoxid (D_2O) was used as the solvent). For each sample (5 replicates per treatment), the material resulting from the extraction has been re-dissolved in a deuterated phosphate buffer (pH 6) which contained 0.05 mg mL^{-1} of Trimethylsilyl-3-Propionic Acid-d4 (TMSPA, $\delta(^1H)$ = 0 ppm), serving as internal standard. TMSPA is a water soluble derivate of trimethylsilan with a propionic acid side group in which ^1H is replaced by deuterium, making it "invisible" in the hydrogen spectra of a H-NMR analysis. A set of 1D and 2D homo- and heteronuclear NMR spectra were acquired for each experiment with the purpose to support the metabolic profiling of maize plant tissues. ^1H proton spectra were processed (Fourier Transform; phase and baseline correction; axis calibration; spectral bucketing of 0.04 ppm) and evaluated by Principal Component Analysis (PCA). To further interpret the influence of experimental conditions and BE treatments on maize metabolite profile, those metabolites were selected, that contributed significantly to the separation of treatments or experimental conditions in the performed PCA.

Detailed method description

800 µL of the extraction supernatant were dried under a flow of nitrogen, stored at -80 °C, and redissolved in 800 µL of deuterated phosphate buffer (90 mM, pH 6.0) containing 0.05 mg mL-1 3-(tri-methylsilyl) propionic-2,2,3,3-d4 acid (TMSPA, EurisoTop, France), serving as internal standard. Each sample was loaded into an NMR glass tube. The complete dissolution of each metabolic extract was ensured by a 5 min sonication prior to NMR analysis. Five replicates for each thesis were acquired.

A 400 MHz Bruker Avance spectrometer, equipped with a 5 mm BBI Bruker probe and working at the ^1H frequency of 400.13 MHz, was used to conduct all liquid state NMR measurements at a temperature of 298 ± 1 K. Monodimensional ^1H spectra were acquired by setting 5 s of thermal equilibrium delay, a 90° pulse length ranging within 8.40 and 9.05 µs

(−2 dB of attenuation), 128 transients, 32768 time domain points, and 16 ppm (6410.3 Hz) as spectral width. The signal of residual water was suppressed by an on-resonance pre-saturation during thermal equilibrium delay. NMR signals were assigned based on 2D NMR spectra and previous literature (Broyart et al., 2010; Castro et al., 2008; Gavaghan et al., 2011; Kuhnen et al., 2010; Manetti et al., 2006; Piccioni et al., 2009). 2D NMR spectra consisted of ^{1}H–^{1}H homo-nuclear experiments, such as COSY (Correlation SpectroscopY), TOCSY (Total Correlation SpectroscopY) and NOESY (Nuclear OverHauser SpectroscopY), and hetero-nuclear 1H–13C experiments, such as HSQC (Hetero-nuclear Single-Quantum Correlation) and HMBC (Hetero-nuclear Multiple Bond Correlation). All 2D spectra were acquired with spectral widths of 16 (6410.3 Hz) and 300 (30186.8 Hz) ppm for ^{1}H and ^{13}C nuclei, respectively, and a time domain of 2048 points (F2) and 256 experiments (F1). Homo-nuclear 2D spectra consisted in 16 dummy scans and 64 total transients. Additionally, a mixing time of 80 ms and a trim pulse length of 2500 ms were set for TOCSY experiment. HSQC and HMBC experiments were acquired with 16 dummy scans, 80 total transients, 0.5 µs of trim pulse length, and optimized by assuming 145 and 6.5 Hz, respectively, as the best ^{1}H –^{13}C short- and long-range J-couplings. Spectra were processed with both Bruker Topspin Software (v 2.1, BrukerBiospin, heinstetten, Germany) and MNOVA Software (v.9.0, Mestrelab Research, Santiago de Compostela, Spain), by applying phase- and baseline corrections to all mono- and bi-dimensional spectra. The free induction decays (FIDs) of 1D ^{1}H spectra were Fourier transformed with a function size of 65536 points and a 0.3 Hz apodization.

^{1}H NMR spectra were divided into symmetrical n-intervals (0.04 ppm buckets), which were then integrated and normalized with respect to the internal standard TMSPA. Each 1H NMR spectrum was integrated from 9.98 to -0.1 ppm, excluding the region of the water signal suppression (4.82-4.74 ppm). The dataset, which was composed by 250 variables, was subjected to Principal Component Analysis (PCA) by using XLStat software v.9.0 (Addinsoft). PCA represents an unsupervised multivariate method which permits to explore easily very dense datasets by reducing the dimensionality of data, while preserving most of information, expressed in terms of variable variance. The PCA outputs consist in score-plots and loading-plots, where the formers highlight the differences and the similarities existing among each sample, while the latter describe the variables (metabolites) responsible for the differentiations. The significance of treatment-related differences in metabolome content was tested by one-way ANOVA, followed by Tukey's test (significant for p-values < 0.05 at a significance level $\alpha = 0.05$).

2.9 Statistical methods

Below you can find a description of statistical methods used for evaluation of plant growth experiments, microbiological experiments and RT-qPCR. Methods used for RNA-Seq analysis and H-NMR described separately in the respective chapters. In the results and discussion part the term "significant" is used as a short form to indicate that treatments differed "statistically significant" from each other following the recommendation of One- or Two-Way-ANOVA (or in some cases Kruskal-Wallis One Way ANOVA on Ranks if data were not normally distributed or lacking equal variances).

Pot experiments with CRD

For pot experiment with completely randomized designs statistical analysis was performed using SigmaPlot 11.0 (Systat Software Inc.). One- and Two-Way-ANOVAs and subsequent Tukey's tests ($\alpha = 0.05$) were performed for pairwise comparison between treatments.

Experiments with CRB or RxC design

Analyses of experiments with block or row-column designs were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA) and the *glimmix* or *mixed* procedures. Models were optimized by using random or fixed effects. Model A included all independent variables (*model* "dependent variable" = BE SWE Block Row; *random* Block*Row). For optimization of analysis non-significant variables were reduced from models if they did not decrease AIC values (for optimization of random effects) or p-values (fixed effects). Normal distribution was tested for residuals after model adaption. To achieve normal distribution, in some cases outliers were deleted as indicated in respective analysis.

Transformation of data

Transformation of data was used for data from root colonization analysis using the log or ln transformation and for percentage data or proportions using the arcsin-square-root or square-root transformations.

Descriptive analysis

For bar charts SigmaPlot 11.0 (Systat Software Inc.) was used, using treatment mean values and standard error (SE) or standard deviation (SD) as indicated below graphs. Different letters indicate significant differences between treatments. Plotting of results from CRB or RxC designs was based on the SAS model outputs of adjusted mean values and adjusted SE values, corrected for block or row-column effects.

Table 2-10: Overview of primers used for RT-qPCR

Nr	Name	Primer	Risk[1]	Length[2]	Tm[3]	Product[4]
1	ACO1_FW	ACGTTCTACAACCCTGGCAC	s.a.	20	60.25	124
2	ACO1_RV	TGCCCTGGTAGTAGTCGAGG		20	60.4	
3	ADT_FW	GCGGTTCCTTCCTAGAGAGC		20	59.9	77
4	ADT_RV	GCGACACCGATTGGTTCAAG	s.c.	20	59.83	
5	CALS_FW	ATGATACCATGGCAGCGGAG	s.c.	20	59.96	109
6	CALS_RV	AAGCATGAGCGTGACCAGAA		20	59.96	
7	CCR1_FW	CGTCTTCACCTCCTCCTATGG		21	59.32	117
8	CCR1_RV	TACAGGTTGTCGGTTCGCTT		20	59.61	
9	CYP_FW	TGCTTAACCACCCAGACGTG	s.a.	20	60.25	145
10	CYP_RV	GGAGGGTAGAGGCGAAGAGT		20	60.4	
11	CZOG_FW	GGAGGAAGCCGTGGAGAGTA		20	60.68	71
12	CZOG_RV	ACCCGATGCCTTGAGTTTCC		20	60.32	
13	EREBP_FW	CTGTCTCTGAGCGATCGGAA		20	59.26	96
14	EREBP_RV	TATCGCGTAGTACATGGCACT	s.c.	21	59.05	
15	ERF1_FW	AAGGTGGAGGCACAGACTC		19	58.94	97
16	ERF1_RV	AAGGGATGCCGAGGAAGTTC		20	59.75	
17	JAZ1_FW	GGATCTCGCTTGCTACCCAC	s.a.	20	60.53	98
18	JAZ1_RV	TCTCCACACCAACCCCAATC		20	59.6	
19	LAC3_FW	CGCTCGATCAAACCAGCTAAT		21	58.79	205
20	LAC3_RV	TGAACTAGCAGTAGACCGACACAAA		25	61.95	
21	NAC1_FW	CCAATCACACTCGCACTCG		19	58.93	204
22	NAC1_RV	CCCTGGATGTCGTCGTAGC		19	59.93	
23	PAL1_FW	TGAACAGAGAAAATACAAGGAGCAG		25	59.3	131
24	PAL1_RV	GAAGTTGGTTACAGGGCGTTG		21	59.73	
25	pldA_FW	CCGGATCGACAGCAAGAACT	s.a.	20	60.11	91
26	pldA_RV	GTCGATGAGGTGCAGGTACG	s.a.	20	60.53	
27	TYDC1_FW	GTCGTTCGAGGACATGGTCA	s.c.	20	59.76	80
28	TYDC1_RV	GCCTAAAGCAAACGAGTGCG	s.c.	20	60.45	
29	UGT_FW	GTCAACTCGTTCCGTGACCT		20	59.97	133
30	UGT_RV	CGTATGTCTTGTCGCATGGC		20	59.7	
31	USP_FW	CGGCTTTGTCCTCTCTCCTT		20	59.39	104
32	USP_RV	TTGCAAGGGGGTGAGAACAG	s.a.	20	60.18	
33	WRKY78_FW	GTCATCTACCACGGCGAACA		20	60.11	178
34	WRKY78_RV	AGGAGGCGGAGAGGTACAAG		20	60.68	
35	CDPK_FW	CCGTCATCGCCTCACGAAGAG		21	63.09	101
36	CDPK_RV	AGAGCCTGCCTTACGGAATTGG		22	62.65	
37	DPP9_FW	TTGTGCGGTGTCTGGTGCTC		20	63.27	200
38	DPP9_RV	TTGCCGTGTGCCTGAAATGC		20	62.42	
39	DUF1296_FW	GCGGCAGTTCCCACCTCAAG		20	63.69	180
40	DUF1296_RV	AGTTGTTGTTGCTGCTGCTGTG	s.c.	22	62.65	
41	LUG_FW	TCCAGTGCTACAGGGAAGGT		20	60.18	178
42	LUG_RV	GTTAGTTCTTGAGCCCACGC		20	59.2	
43	MEP_FW	TGTACTCGGCAATGCTCTTG	s.a.	20	58.27	203
44	MEP_RV	TTTGATGCTCCAGGCTTACC		20	57.87	
45	UBCP_FW	CAGGTGGGGTATTCTTGGTG		20	57.87	97
46	UBCP_RV	ATGTTCGGGTGGAAAACCTT		20	57.62	

[1] s.a. = risk of self-annealing (Primer stats tool), s.c.: risk of self-complementarity (Primer BLAST)

[2] Length of the primer in base pairs (bp)

[3] Results from primer BLAST (because ion and dNTP concentration in KAPA master mix are not specified default settings were kept: 50 mM K/Na^+, 1.5 mM $MgCl_2$, 0.6 M dNTPs, 50 mM primers). In general, elevated $MgCl_2$ and primer concentration increases the calculated melting temperature.

[4] Length of the target product in bp

2.10 Overview tables

2.10.1 Overview on conducted pot and field experiments

Table 2-11 Overview of experiments conducted in 2014 (For description see Table 2-12)

Nr	Type	Acronym[1]	Objective of research	Title thesis[2]	SV[3]	Responsible[4]	Others[5]	Reference[6]
1	Pot	NA	Screening of various BE products	this thesis	M. Weinmann	K. Wächter	/	
2	Pot	BioF1	Interaction of sugar and BEs for plant growth promotion	this thesis	N. Weber	N. Weber	D. Reinhardt	
3	Pot	BioF2	Combination of seaweed extracts and Proradix	this thesis	N. Weber	N. Weber	/	
4	Pot	NA	Reproduction of BE effects from partner institute JKI	„Verbesserung der Phosphataneignung bei Mais durch ausgesuchte Bioeffektoren bei moderater Phosphatdüngung“, 2014	N. Weber	S. Dobczinski	/	(Dobczinski, 2014)
5	Pot	NA	Reproduction of BE effects from partner institute JKI	„Verbesserung der Phosphoraneignung bei Tomaten mit Hilfe ausgewählter Bio-effektoren in Abhängigkeit des P-Düngungsniveaus“, 2015	N. Weber	A. Lüthi	/	(Lüthi, 2015)
6	Pot	NA	Effectiveness of BEs depending on soil sterility	See 5	N. Weber	A. Lüthi	/	See 5
7	Pot	NA	Level of P availability for effectiveness of BEs	See 5	N. Weber	A. Lüthi	/	See 5
8	Pot	NA	Effectiveness of BE products for improved cold stress tolerance	“Soil Application of Microbial Bioeffectors, Algae Extracts and Micronutrients to Improve Cold Stress Tolerance of Maize”, 2015	N. Weber	K. Bradáčová	/	(Bradáčová, 2015)
9	Pot	NA	See 8	See 8	N. Weber, M. Weinmann	K. Bradáčová	/	(Bradáčová et al., 2016)
10	Pot	BioF3	Combination of seaweed extracts and Proradix	this thesis	N. Weber	N. Weber	/	
11	Pot	BioF4.1	Changes in gene expression and primary metabolism after application of BEs	this thesis	N. Weber	N. Weber	/	
12	Field	IHO2014	Combination of BEs (seaweed extracts + PGPR)	this thesis	N. Weber, M. Weinmann	N. Weber	M. Weinmann, M. Nkebiwe	
13	Field	IHO2014_Extra	Efficacy of BE applications with different inoculum densities	this thesis	N. Weber, M. Weinmann	N. Weber	M. Weinmann, M. Nkebiwe	
14	Pot	BioF4.2	Changes in gene expression and primary metabolism after application of BEs	this thesis	N. Weber	N. Weber	/	

Table 2-12 Overview of experiments conducted in 2015 / 2016

Nr	Type	Acronym[1]	Objective of research	Title thesis[2]	SV[3]	Responsible[4]	Others[5]	Reference[6]
15	Field	IHO2015	Improved P acquisition by BEs in manure and urea fertilized maize	this thesis	N. Weber, M. Weinmann	N. Weber	M. Weinmann, M. Nkebiwe	
16	Field	IHO2015_Extra	Plant growth promotion by BE foliar application in maize	this thesis	N. Weber, M. Weinmann	N. Weber	M. Weinmann, N. Morad-Talab	
17	Pot	NA	Responses of root hair mutants to PGPR	„Bedeutung der Wurzelhaarentwicklung für die wachstumsstimulierende Wirkung von *Pseudomonas* sp. Proradix bei Mais“, 2016	N. Weber	I. Herrmann	/	(Herrmann, 2016; Weber et al., 2018)
18	Pot	NA	Early growth responses to Rhizovital	„Wurzelbesiedlung und Wachstumsstimulierung durch *Bacillus amyloliquefaciens* FZB42 während Keimung und Jugendwachstum von Mais (*Zea mays* cv. Colisee)“, 2015	N. Weber	J. Brecht	/	(Brecht, 2015)
19	Pot	NA	Combination of the PGPR *Pseudomonas* sp. Proradix with nitrification inhibitor DMPP for improved P uptake from two different organic fertilizer	“Bioeffectors for improved utilization of organic recycling-fertilizers”, 2017	N. Weber	P. Cona Caniullan	/	(Cona Caniullan, 2017)
20	Pot	NA	PGPR in cold stressed tomatoes	this thesis	N. Weber	F. Kolberg	/	
21	Pot	BioF4.3	Plant responses to *B. amyloliquefaciens* LB cultures, fate of active bacteria and spores in the soil	this thesis	N. Weber	N. Weber	/	
22	Pot	NA	P-status of Proradix treated plants at different harvest times	“Interactions of microbial inoculants and ammonium nutrition for acquisition of inorganic recycling fertilizers in maize”, 2017	N. Weber	S. Kar	/	
23	Pot	BioF5	P-status of BE treated plants at different harvest times	„Einfluss von mikrobiellen Bio-Effektoren auf die P-Aufnahme und den P-Status von Maispflanzen in der Jugendentwicklung“, 2017	N. Weber	N. Burnadze	/	

[1]Acronym used during the thesis in presentations and own datasets
[2]Title of the bachelor or master thesis with date of submission, the author is mentioned under [4]
[3]Supervisor of the experiment or the bachelor/master thesis
[4]Person that conducted the experiment and author of the thesis (bachelor/master student)
[5]Additional persons that were involved in planning and organization of the experiment
[6]Reference to peer-reviewed publications or thesis

2.10.2 Overview on experimental conditions

Table 2-13 Overview of experimental conditions for pot and field experiments 2014 (For description see Table 2-14)

Nr	Type [1]	Plant	Pot [2]	Subst. [3]	Soil used	Fert [4]	WHC [5]	WC [6]	Temp. [7]	D/N [8]	Design [9]	BEs	Analysis [10]	Harvest time [11]
1	GH	Maize	1.5	soil	C-Loess	N, Mg, K, Zn, Cu, Mn, Fe	60	20.0	NA / NA/ CRZT (10)	NA	CRD	Px, Rz, Bsim, BFDC, TP, A95, Zn+Mn, OmG, Hsp	PH, SW, RW, Cl, RL	45 DAS
2	GH	Maize	1.7	s:s 1:2	Low-P	N, Mg, K, (P)	50	20.6	10 - 28 / 15.7 / CRZT (20)	14/10	CRD	Px, Rz, BFDC, TP	ER, PH, Cl, SW, RW, RL, MA+P	63 DAS
3	GH	Maize	1.9	s:s 1:2	Low-P + KH(W)	N, Mg, K, P	70	21.8	18 - 35 / 22.5	14/10	CRB / CRD	Px, Af, AV, AVZM, SF, ECO, P1, P2, P3	ER, SW, RW, RL	56 DAS
4	GH	Maize	1.8	s:s 1:2	Low-P	N, Mg, K, P	70	21.4	18 - 26 / 21	16/8	CRD	Px, Rz, TP	PH, SW, RW, RL, SD, P, Myc	47 DAS
5	GH	Tom	1.8	s:s 1:2	Low-P	N, Mg, K, P	70	21.4	18 - 26 / 21	16/8	CRD	Px, Rz, TP	see prev.	56 DAS
6	GH	Tom	3.0	s:s 1:1	Kr 1	N, Mg, K, P	60	18.6	14 - 35 / 22	16/8	CRD	Px, Rz, Pj	PH, SW, RW, (RL)	60 DAS
7	GH	Tom	3.0	s:s 1:1	Kr 1	N, Mg, K, P	50	16.0	14 - 25/ 18	16/8	CRD	Px, Rz	PH, SW, RW	56 DAS
8	GH	Maize	1.5	s:s 1:2	IHO1	N, Mg, K, P	70	21.2	13 - 35 / 19 / CRZT (14)	16/8	CRD	Px, Rz, SF, Af, AV, AVZM, Bsim, Zn/Mn	PH, Cl, SD, SW, RW, RL, MA	43 DAS
9	GH	Maize	1.5	s:s 1:2	IHO1	N, Mg, K, P	70	21.2	10- 30 / 19 / CRZT (14)	14/10	CRD	Af, AV, AVZM, Zn/Mn	PH, Cl, SD, SW, RW, RL, MA, Ez	43 DAS
10	GH	Maize	3.1	s:s 1:2	KH(a) + Ba	N, Mg, K, P	70	21.8	20 - 35 / 22.5	16/8	CRD	Px, SF, AVZM	PH, SD, SW, RW, (RL)	52 DAS
11	GH	Maize	3.0	s:s 1:1	Kr 1	N, Mg, K, P	30/ 50/ 70	9.6/ 16.1/ 22.5	13 - 34 / 22	16/8	CRD	Px, Rz	PH, SD, SW, RW, P, GE, PM	43 DAS
12	Field	Maize	NA	soil	IHO1	N, P	NA	503	0.9 - 33 / 15	NA	RxC	Px, Rz, BFDC, Af, SF	ER, PH, P, RC, Myc, GY	173 DAS
13	Field	Maize	NA	soil	IHO1	N, P	NA	503	0.9 - 33 / 15	NA	CRB	Rz, BFDC, TP	PH, P, RC, GY	173 DAS
14	CC	Maize	3.0	s:s 1:1	Kr 2	N, Mg, K, P	60	19.3	22 -26 / 24	16/8	CRD	Px, Rz	PH, SD; SW, RW, RC, PM	42 DAS

Table 2-14 Overview of experimental conditions for pot and field experiments 2015/2016

Nr	Type [1]	Plant	Pot [2]	Subst. [3]	Soil used	Fert [4]	WHC [5]	WC [6]	Temp. [7]	D/N [8]	Design [9]	BEs	Analysis [10]	Harvest time [11]
15	Field	Maize	NA	soil	IHO2	N, P, (F1)	NA	239	0.5 - 45 / 17	NA	RxC	Px, Rz	ER, PH, P, RC, BY	126 DAS
16	Field	Maize	NA	soil	IHO2	N, P	NA	239	0.5 - 45 / 17	NA	CRB	SF, BacA, Si	PH, P, RC, BY	131 DAS
17	CC	Maize	0.55	s:s 1:2	KH(b)	N, Mg, K, P	70	25.0	22 - 26 / 24	14/10	CRD	Px	PH, SW, RW, RL, MA	28 DAS
18	CC	Maize	2.7	s:s 1:3	KH(b)	N, Mg, K, P	70	25.0	23 - 27 / 25	16/8	CRD	Rz	PH, SW, RW, RL, RC	33 DAS
19	GH	Maize	3	s:s 1:2	KH(c)	N, Mg, K, (P), (F2)	60	16.9	16 - 30 / 20	16/8	CRB	Px	PH, SW, RW, RL, P, SPAD	59 DAS
20	CC	Tom	0.75	mix	KH(c)+ma-nure+peat	N, Mg, K, (P), (F2)	NA	18.9 / 23.7	18 - 24 / 21	16/8	RxC	Px, Rz, BFDC	PH, SD, Cl, SW, RW, RL, RC	42 DAS
21	GH	Maize	1.1	s:s 1:1	Kr 2	N, Mg, K, P	60	19.3	16 - 26 / 22.5	16/8	LS	Rz, P2	PH, SD, SW, RW, RC	29 DAS
22	GH	Maize	1.5	s:s 1:1	Kr 2	N, Mg, K, P	60	19.3	16 - 26 / 22.5	16/8	CRD	Px	SW, RW, RL, P	various
23	GH	Maize	2.0 / 3.1	s:s 1:1	Kr 2	N, Mg, K, P	60	19.3	19 - 30 / 24.5	14/10	CRD	Rz, Px	SW, RW, P	various

[1] GH = greenhouse (non-controlled conditions), CC = climate chamber (controlled)
[2] Amount of dry weight substrate per pot in kg
[3] Ratio of sand soil mixture
[4] Fertilization used: element symbols used, elements in () were fertilized only in some treatments, F1 = number of organic fertilizer used
[5] % of estimated maximum water holding capacity
[6] Water content in % of dry matter substrate; for field experiments rainfall during the experimental period is given (mm)
[7] Total range / average temperature (in °C) / CRZT = controlled root zone temperature
[8] Day / night rhythm (in GH experiments day light prolonged or supported by artificial light)
[9] Experimental design: CRD = completely randomized design, CRB = completely randomized block design, RxC = Row-column design, LS = latin square
[10] Measurements / Analyses performed: BY = biomass yield, Cl = chlorosis/necrosis, ER = emergence rate, Ez = enzymatic measurements, GE = gene expression analysis, GY = grain yield, MA = mineral analysis, Myc = mycorrhization of roots, P = phosphorus analysis only, PH = plant height, PM = plant metabolome, RC = root colonization by BEs (tracing), RL = root length, RW = root weight, SD = stem diameter, SPAD = SPAD values (chlorophyll content), SW = shoot weight
[11] DAS = days after sowing, for field experiments plants were harvested at full maturity

2.10.3 Overview on BE products

Table 2-15 Overview of microbial bioeffector (BE) products

BE product	Abbr. [1]	Exp [2]	Group [3]	Organisms	Active compound	Conc. [4]	Unit (conc.)	Commercial name [5]	Company	Formulation	Proposed function
Proradix	Px	Std	PGPM	gram-negative rhizobacteria *Gammaproteo-bacteria*	*Pseudomonas* sp. DSMZ 13134	6.6E+10	CFU g^{-1}	Proradix®	SP Sourcon Padena, Tübingen, Germany	powder	plant protection (competition, ISR/SAR), root growth stimulation, improved nutrient acquisition
P. jessenii	Pj	6	PGPM	gram-negative rhizobacteria (See above)	*Pseudomonas jessenii* RU47	6.6E+09	CFU g^{-1}	NA	SP + K. Smalla, Julius-Kühn-Institute, Braunschweig, Germany	powder	see previous
Rhizovital	Rz	Std	PGPM	gram-positive bacteria (spore forming)	*Bacillus amyloliquefaciens*[6] FZB42	2.5E+10	CFU g^{-1}	RhizoVital® 42	ABITEP GmbH, Berlin, Germany	liquid	plant protection, plant health, improved plant growth
B. simplex	Bsim	1, 8	PGPM	gram-positive bacteria (spore forming)	*Bacillus simplex* R41	1.5E+10	CFU g^{-1}	NA	see previous	liquid	cold tolerant strain, see previous
B. atrophaeus	BacA	16	PGPM	gram-positive bacteria (spore forming)	*Bacillus atrophaeus* GBSC56	2.5E+10	CFU g^{-1}	NA	see previous	liquid	NA
Biological fertilizer DC	BFDC	1, 2, 12, 13, 20	PGPM	saprophytic fungi	*Penicillium* sp. PK 112	1.0E+09	CFU g^{-1}	NA	Bayer CropScience Biologics GmbH (former Prophyta GmbH), Malchow/Poel, GERMANY	liquid	plant protection, plant health, improved plant growth
OmG-08	OmG	1	PGPM	mycoparasitic fungi	*Trichoderma harzianum* OmG-08	1.0E+10	CFU g^{-1}	NA	J. Geistlinger, Anhalt University of Applied Sciences, Bernburg, Germany	liquid	see previous
Trianum-P	TP	1, 2, 4, 5, 13	PGPM	mycoparasitic fungi	*Trichoderma harzianum* T-22	1.0E+09	CFU g^{-1}	TRIANUM-P	Koppert Deutschland GmbH, Insel Reichenau, Germany	powder	see previous
Herbaspirillum sp.	Hsp	1	PGPM	nitrogen-fixing rhizobacterium	*Herbaspirillum frisingense* GSF30^T	NA	NA	NA	Prof. U. Ludewig (Institute of crop science, University of Hohenheim, Germany)	liquid	N-acquisition, plant health, improved plant growth

Table 2-16 Overview of all seaweed extracts

BE product	Abbr. [1]	Exp [2]	Group [3]	Organisms	Active compound	Conc. [2]	Unit (conc.)	Commercial name [2]	Company	Formulation	Proposed function
Algafect	Af	3, 8, 9, 12	SWE	seaweed	From *Ascophyllum nodosum, Fucus spp, Laminaria spp*., free amino acids, organic acid, polysaccharides; seaweed extract, enriched with amino acids etc.	NA	NA	NA	Agriges S.r.l, San Salvatore Telesino, Italy	liquid	antioxidant, biotic and abiotic stress, cold stress, prebiotic, enhance root microorganism
AlgaVyt	AV	3, 8, 9	SWE	seaweed	From *A. nodosum, F. spp, L. spp., Spirulina spp*; enriched with chemical nitrogen;	NA	NA	NA	see previous	liquid	see previous
AlgaVyt Zn/Mn	AVZM	3, 8, 9, 10	SWE	seaweed	AlgaVyt diluted; additionally, 5 % Zn, 5 % Mn	NA	NA	NA	see previous	liquid	see previous
Superfifty / Alga 50	SF	3, 8,. 10, 12, 16	SWE	seaweed	Highly-concentrated alkaline extract from *Ascophyllum nodosum* seaweed	500	g L^{-1}	Super Fifty® 0-0-8	BioAtlantis Ltd., Clash Industrial Estate, Tralee, Co. Kerry, Ireland	liquid	Enhances performance, marketable grade, root growth & soil bacteria counts, very high antioxidant potential, Organic certification
Ecolicitor	ECO	3	SWE	seaweed	Alkaline extract from *Ascophyllum nodosum* enriched in fucoidan	NA	NA	Ecolicitor®	see previous	liquid	Enhanced seed emergence, root & shoot growth
Alga 95	A95	1	SWE	seaweed	Soluble powder (>99%) with a microgranular appearance extracted from *Ascophyllum nodosum* seaweed extract	NA	NA	Alga 95®	see previous	powder	NA
Product 1	P1	3	SWE	seaweed	SWE enriched in prebiotic compounds	NA	NA	NA	see previous	liquid	prebiotic for microbes
Product 2	P2	3, 21	SWE	seaweed	SWE partially purified and enriched in fucoidan	NA	NA	NA	see previous	powder	see previous
Product 3	P3	3	SWE	seaweed	SWE partially purified and enriched in laminarin	NA	NA	NA	see previous	powder	see previous

[1] Abbreviation used in this thesis; [2] Experiments in which the BE was used, Std = Standard-BE used in most of the experiments; [3] PGPM = Plant growth promoting microorganism; SWE = Seaweed extract; [4] Concentration of active compound; [5] If available on the market on of active compound If available on the market;
[6] *B. amyloliquefacien*s = *B. velezensis* https://www.uniprot.org/taxonomy/326423

2.10.4 Overview on fertilizers

Table 2-17 Overview of commercial and organic fertilizers

Nr	Abbr.	Name	Type	Description	Company
F1	F_{MP}	Manure pellets	org	Pelleted chicken manure	Agriges S.r.l, San Salvatore Telesino, Italy
F2	F_{MKH}	Manure KH	org	Composted cow manure	research station Kleinhohenheim, University of Hohenheim, Germany
F3	F_{Dur}	Duratec® starter	syn	Granular solid fertilizer, surface-treated, 30% polymercoated	Compo Expert GmbH, Münster, Germany
F4	F_{U}	Piagran® 46	syn	Urea, 46% N	SKW Stickstoffwerke Piesteritz GmbH, Lutherstadt Wittenberg, Germany
F5	F_{Nov}	NovaTec® Solub 21	syn	DMPP-stabilized $(NH_4)_2SO_4$ (21 % NH_4-N, 24 % S)	Compo Expert GmbH, Münster, Germany
F6	F_{MAP}	Krista™ MAP	syn	Mono-ammonium phosphate (12 % NH_4-N, 22 % P)	YARA GmbH & Co. KG, Dülmen, Germany
F7	F_{DAP}	DAP	syn	Di-ammonium phosphate (18 % NH_4-N, 20 % P)	Beiselen GmbH, Ulm, Germany
F8	F_{SP}	Superphosphat	syn	Superphosphate (7.9 % P)	Triferto B.V., Doetinchem, Netherlands
F9	F_{ES}	Easy Start® TE-Max	syn	Granulated NP complex fertilizer with high phosphate and micronutrients (11 % NH_4-N, 21 % P)	Compo Expert GmbH, Münster, Germany

Table 2-18 Overview of mineral composition of commercial and organic fertilizers

Nr	Abbr.	DM	Carbon	Total N	Mineral N (g kg^{-1})		Available P [1]	Macronutrients (g kg^{-1} DM) [2]					Micronutrients (mg kg^{-1} DM)				
		%	% of DM	% of DM	NO_3-N	NH_4-N	mg kg^{-1} DM	P	K	Ca	Mg	S	B	Cu	Fe	Mn	Zn
F1	F_{MP}	80.1	23.7	3.7	na	18.5	na	17	13.5	168	5.06	102	na	na	na	na	na
F2	F_{MKH}	69.5	na	1.5	0.46	0.004	6100	6.1	6.7	23.5	6.03	2.9	na	na	na	na	270
F3	F_{Dur}	na	na	22	80	140	69824	69.8	24.9	na	8.44	35	140	150	800	100	100
F4	F_{U}	na	na	46	na	na	/	/	/	/	/	/	/	/	/	/	/
F5	F_{Nov}	na	/	21	/	210	/	/	/	/	/	/	/	/	/	/	/
F6	F_{MAP}	na	/	12	/	120	see total P	220	/	/	/	/	/	/	/	/	/
F7	F_{DAP}	na	/	18	/	180	see total P	200	/	/	/	/	/	/	/	/	/
F8	F_{SP}	na	/	/	/	/	see total P	79	/	/	/	/	/	/	/	/	/
F9	F_{ES}	na	/	11	/	110	see total P	210	/	/	/	/	/	/	6000	1000	10000

[1] = CAL-P; [2] = total mineral nutrients from ICP-OES analysis

2.10.5 Overview on soils

Table 2-19 Overview on soils used for pot and field experiments

Nr	Name	Description	Year of collection	pH (0.01M $CaCl_2$)	P CAL	P Olsen	K CAL	Mg $CaCl_2$	N_{tot} %	S_{tot} %	C_{tot} %	C_{org} %	C_{inorg} %
1	C-Loess	subsoil loess with low organic matter and high carbonate contents	NA	7.7	6.5		47.0	200	0.03	0.10	2.80	0.16	
2	Low-P	low input grassland soil (vineyard UHOH)	2013	7.1	29.7		241	110	0.24	0.05	2.64	2.41	0.23
3	KH(W)	organic farming, arable land, research station Kleinhohenheim, UHOH, Germany	2013	6.1	69.8	49.8	166	190	0.18	0.03	1.29	1.30	0.42
4	KH(a)	see previous	2014	7.2	48.0	47.4	133	73	0.22	0.10	1.91	1.68	
5	KH(b)	see previous	2015	7.5	57.0		141	79	0.23	0.03	1.94	1.70	0.24
6	KH(c)	see previous	2014	7.0	36.7		133	170	0.15	0.10	1.31	1.28	
7	Ba	organic farming, arable land, Bavendorf, Germany	NA	6.1	14.0	19.6	257	180	0.28	0.03	1.90	1.87	0.38
8	Ba+KH(a)	mix of both soils	NA	6.9	37.6	38.9	171	106	0.24	0.08	1.91	1.74	
9	Kr 1	Soil from LTZ Augustenberg, Karlsruhe	2014	7.5	37.1		28.2	140	0.24	0.10	2.79	2.06	
10	Kr 2	see previous	2014	7.4	36.2		26.5	130	0.24	0.10	2.72	2.11	
11	IHO1	research station Ihinger Hof, Renningen, Germany	2013	6.9	122	78.7	158	220	0.12	<0.05	2.19	1.07	1.12
12	IHO2	see previous	2014	7.0	82.9	55.8	133	170	0.16	<0.03	3.00	1.07	1.93

Table 2-20 Mineral contents of soils used for pot and field experiments

Nr	Name	K	Mg	Cu	Fe	Mn	Zn	B	Ca	Cu	Fe	K	Mg	Mn	P	Zn	texture	Sand	Silt	Clay
		CAT						ICP-OES									category	%	%	%
1	C-Loess	29	226	0.4	7.8	13	0.1	105.0	54138	13	17631	2051	19138	356	344	38	Loam	40	47	13
2	Low-P	158	115	5.6	71.9	84	34.2	11.3	11034	24.1	24199	2905	3465	780	667	170	Silt loam	23	55	22
3	KH(W)	125	188	2.2	131	244	3.0	10.1	2970	15.7	22309	2875	3578	828	687	54	Silt loam	5	72	23
4	KH(a)	73	84	3.3	95.9	232	2.9	18.3	9864	29.5	44384	4982	3345	1507	1228	87	Silty clay	12	46	43
5	KH(b)	81	95	2.5	53.1	130	2.9	18.3	9592	26.6	51250	4826	3415	1539	1209	94	Silty clay	11	47	42
6	KH(c)	93	180	3.1	94.6	340	3.4	15.7	3445	20.7	37093	3828	2606	1005	881	65	Silty clay loam	15	51	34
7	Ba	206	183	5.2	69.4	213	5.0	14.7	3973	24.9	15053	3097	4170	793	484	69	clay loam	28	48	24
8	Ba+KH(a)	113	114	3.9	87.8	226	3.5	17.2	8071	28.1	35457	4408	3596	1290	1002	81	Silty clay loam	17	46	37
9	Kr 1	18	142	4.0	42.6	34	5.1	18.0	23607	22.8	20590	2779	7231	630	813	68	Silt loam	14	63	24
10	Kr 2	19	136	4.2	44.8	39	5.4	17.2	22822	22.5	20614	2858	7168	629	802	67	Silt loam	13	64	23
11	IHO1	117	248	3.6	154	230	3.6	13.4	4353	19.0	28358	3903	4789	902	953	50	Silt loam	3	69	28
12	IHO2	75	178	3.9	28.7	42	2.3	15.6	10963	24.7	29312	3976	6138	924	989	60	Silt loam	2	71	27

All values for mineral contents in mg kg^{-1} dry soil if not in %. CAT = extractable contents; ICP-OES = total mineral contents. *C_{org} = calculated from humus content *0.58

3 Results

3.1 Structure

The results part is divided into several subchapters clustering experiments with a similar experimental focus. Inside the subchapters each experiment is described separately, including the specific experimental design, results and discussion.

BE application and fertilization rates originally were calculated on kg^{-1} dry soil in most of the pot experiments but are given in most of the tables of this thesis calculated on kg^{-1} dry substrate, factoring in varying sand contents. Additionally, many experiments were done as screening experiments to screen for optimal conditions to establish plant-microbe relationship and increase efficacy of BEs. Therefore, also experimental conditions and soil properties differed strongly across experiments.

For pot experiments that are already published in Master or Bachelor theses or peer-reviewed papers only the most important results are presented.

Mean values of shoot biomass measurements at harvest for all control, Rz and Px treatments are given in Table 7-1 and Table 7-2. Due to the number of observations and measurements only those values are given. Inside the respective subchapters results are mainly presented graphically including significant differences from statistical analysis.

3.2 BEs as abiotic stress protectants

3.2.1 Screening of BE products for cold stress alleviation (Exp_1)

3.2.1.1 Introduction Exp_1

Exp_1 was conducted by K. Wächter, a previous member of the institute, before starting this PhD thesis. As the results were never published but are still of some importance for the discussion, they were included in this thesis with acceptance from K. Wächter. Exp_1 was conducted as a screening experiment for BEs that are able to improve P-acquisition from sparingly available calcium phosphates and to improve plant growth under cold stress conditions. Therefore, pots were placed into a system for CRZ temperature with an average soil temperature of 10°C.

Figure 3-1 Cooling system for controlled root zone temperature

3.2.1.2 Experimental Design Exp_1

All pots were fertilized in the same way as described in Table 3-2. 40 pots, from ten treatments with each four replicates (Table 3-1), were placed in a cooling system for controlled root zone temperature (CRZT). The system consisted of a polystyrene isolated box filled with wet peat. Inside the peat a garden hose filled with refrigerant was placed. The refrigerant was cooled and circulated by a laboratory thermostat, composed of a Frigomix 1497 with Thermomix 1480 (B. Braun Melsungen AG, Melsungen, Germany), that was connected to the garden hose (Figure 3-1). The experiment was conducted in the greenhouse in early spring with low environmental

Table 3-1 Treatments Exp_1

Trt_Nr	Treatment	Application rate
1	Ctrl	/
2	Px	2.6E+09
3	Rz	2.0E+09
4	Rz / Bsim	1.8E+09
5	Hsp	1.33 (OD = 0.6)
6	BFDC	1.0E+08
7	OmG	5.0E+08
8	TH	1.0E+09
9	A95	3.2E-01
10	Zn/Mn	4 mM Zn/ 2.5 mM Mn

Application rates: for microbial BEs in CFU kg^{-1} substrate; for Hsp 1.33 ml of bacterial culture with an OD of 0.6; for Zn/Mn seeds were soaked for 24 h in the dark in priming solutions with given concentration, washed with dest. H_20 and air dried before sowing; r = 4

temperature and a soil temperature of ~10°C. Application of BEs was done by pipetting 2 ml directly on the seed after sowing. Weekly height measurement of maize plants as well as visual evaluation of chlorosis symptoms were performed during plant growth. 45 DAS plants were harvested and shoot and root weight were measured. Root length was not determined by root scanning (0) but by manual measurement of the longest root segment and the number of lateral roots. The TH treatment was a *Trichoderma harzianum* strain from the company Prophyta (now Bayer) that was used only in this experiment and was therefore not further characterized.

Table 3-2 Fertilisation Exp_1

Element	Fertilized as (pure salt)	mg kg^{-1} substrate
N	$Ca(NO_3)_2 * 4 H_2O$	100.0
K	K_2SO_4	150.0
Mg	$MgSO_4 * 7 H_2O$	50.0
Zn	$ZnSO_4 * 7 H_2O$	2.6
Cu	$CuSO_4 * 5 H_2O$	1.0
Mn	$MnSO_4 * H_2O$	2.2
Fe	Fe-EDDHA	1.1

3.2.1.3 Results Exp_1

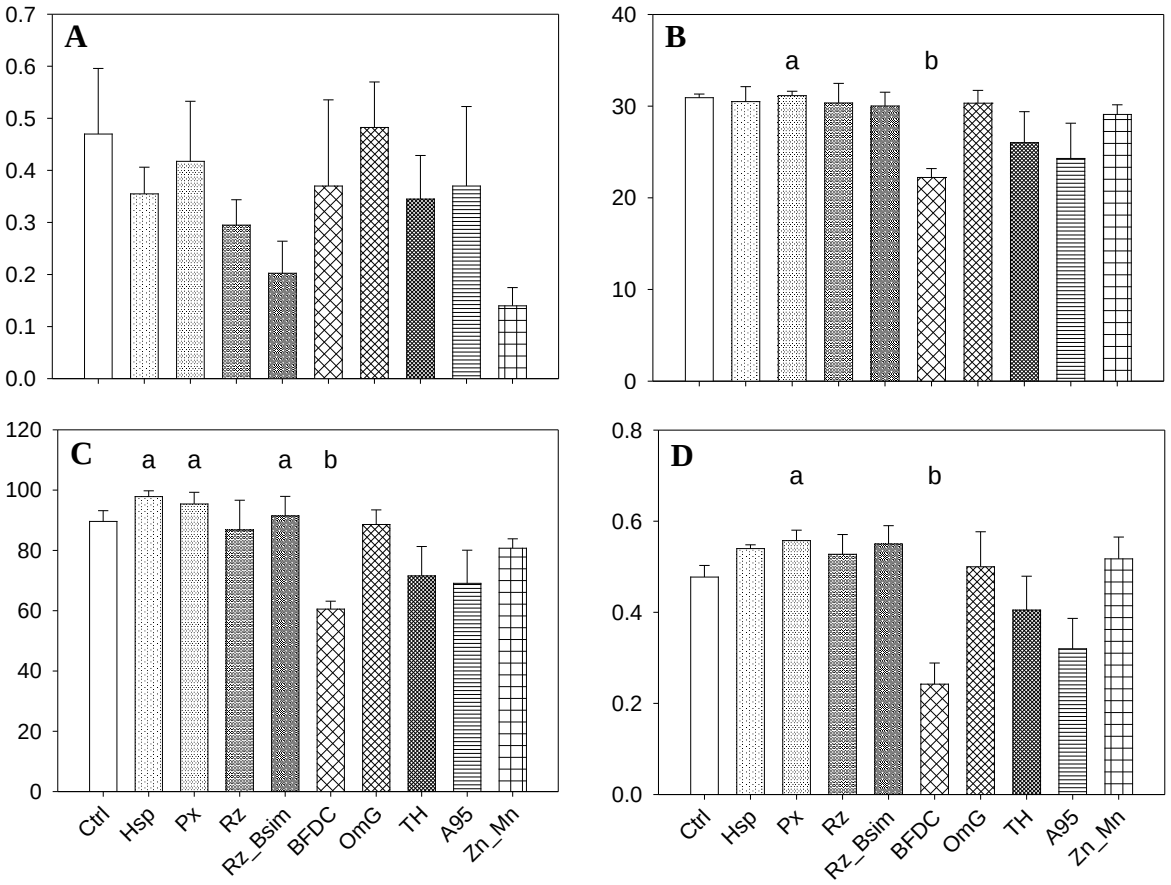


Figure 3-2 Results from maize shoot analysis Exp_1; Leaf necrosis (**A**), plant height in cm (**B**), total leaf length in cm (**C**) and shoot DW in g (**D**); Means +SE; n = 4; for A95 n = 3; different letters indicate significant difference in means of the respective treatments. All other treatments did not differ significantly from another.

Results from root and shoot analysis show strong differences among the different BE treatments (Figure 3-2). As often observed under cold stress, leaf necrosis was strongly

reduced by Zn/Mn treatment but in Exp_1 also the Rz and Rz_Bsim treatment seemed to be effective to improve cold tolerance in maize plants. Nevertheless, none of the treatments was able to significantly increase plant growth as compared to the Ctrl. Best plant growth in both shoots and roots was obtained in the *Herbasprillum* sp. (Hsp) and 'Proradix' (Px) treatments (both proteobacteria) followed by the 'Rhizovital' (Rz) treatments containing spore forming bacilli. None of the fungal products (Biological fertilizer DC (BFDC), the *Trichoderma harzianums* strains 'OmG-80' and TH) could compete with the bacterial products. Especially BFDC and TH were strongly inhibiting both shoot and root growth. Also the seaweed extract Alga 95 (A95) had a strongly growth depressing effect. Despite being effective in reducing plant stress symptoms, the Zn/Mn treatment could not contribute to an improved plant growth under these experimental conditions.

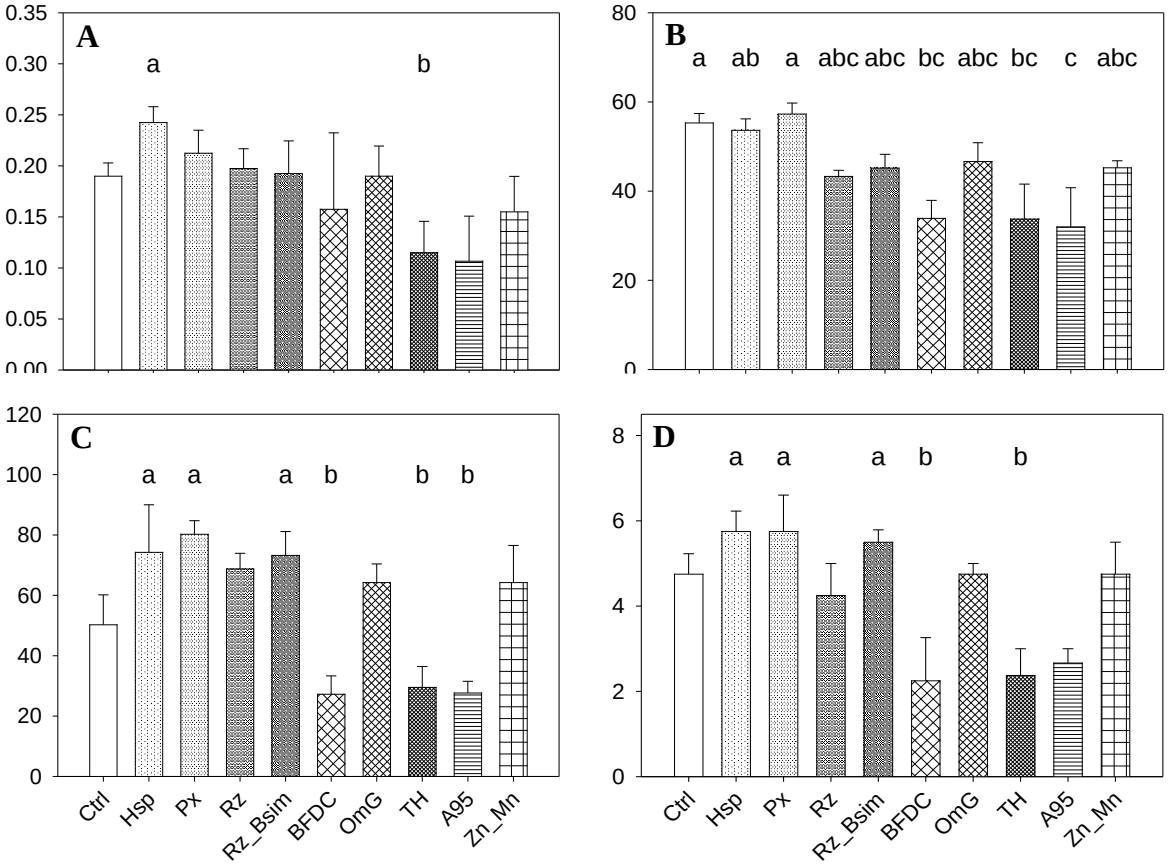


Figure 3-3 Results from maize root analysis Exp_1; Root DW in g (**A**), longest root in cm (**B**), number of lateral roots (**C**), length of lateral roots (**D**); Means +SE; n = 4; for A95 n = 3; different letters indicate significant difference in means of the respective treatments. All other treatments did not differ significantly from another

Maize plants showed very different responses to the specific BE products and the responses were not organ specific. The products always affected shoot and root growth in a similar way and even the effects on primary root and lateral roots were similar (Figure 3-3 B and C+D).

3.2.1.4 Discussion Exp_1

Can BE products alleviate cold stress?

Exp_1 was conducted to screen various BE products from different categories for their potential to alleviate cold stress. Four bacterial products (Hsp, Px, Rz, Rz_Bsim), three fungal products (BFDC, OmG, TH) and one seaweed extract (A95) were compared to pure micronutrient supply (Zn_Mn). Micronutrients like Zn, Mn and Cu are already known to play an important role in the stress alleviation and plant stress response due to their involvement in reduction of reactive oxygen species (ROS) as co-factors of the enzyme superoxide dismutase (SOD) (Baek, 2012). Therefore this treatment can be seen as a kind of positive control under these experimental conditions. The bacterial products are known to produce various plant hormones, such as auxins and GAs, that are involved in stress responses (Vacheron et al., 2013). The bacterial products can be further categorized into spore-forming *Bacillus* strains (*B. simplex*, (Bsim), *B. amyloliquefaciens* (Rz)), a *Pseudomonas* strain (P. fluorescens 'Proradix' (Px)) and a *Herbaspirillum* strain, the last two strains both proteobacteria. The Px strain is known for its siderophore production that may influence also micronutrient supply of the plants. The Bsim strain was additionally defined as "cold-tolerant" by the company ABiTEP and was therefore tested additionally to the related Rz strain. Nevertheless, no further information on specific cold-tolerant properties was given by the company. The endophytic *H. frisingense* GSF30^T strain was isolated in Germany from *Miscanthus* plants and was shown to stimulate *M. sinensis* plant growth by modulating ethylene signalling pathway (Straub et al., 2013a, 2013c).

No stress alleviation by microbial BE products

In general, none of the treatments was able to significantly improve plant growth as compared to an untreated Ctrl. As expected, the Zn_Mn treatment but also the Bsim strain indeed showed the strongest reduction in leaf necrosis as compared to the untreated Ctrl. Interestingly both treatments were not able to improve plant performance as compared to the Ctrl. Therefore, it can be assumed that another factor, probably low P supply (see below), was limiting plant growth. This is supported by the low correlation between the analysis of leaf necrosis and the biomass results. The bacterial products Hsp and Px showed a tendency to improve plant performance but were not effective in reducing leaf necrosis. Additionally, two of the fungal products (TH, BFDC) and the A95 further increased plant stress, indicated by the reduced shoot and root growth, although they showed some tendency to reduce leaf necrosis. The OmG product did not trigger any responses in the plants indicating a low

activity under these conditions. As seen later on in other experiments (Exp_8, 9, 11) bacterial products were never effective in cold stress alleviation. In contrast, especially the Px product showed the ability to improve P availability and plant growth under growing conditions without additional abiotic stress. In the field experiment 2015 maize plants were suffering from drought stress. Here the Superfifty® (SF) product, which is similar to the A95 product, led to a significantly reduced maize yield. Reasons for the lack of effects or negative outcome of the products under stress conditions could be the timing of application. Product application of these products seemed to cause problems when stress was already applied. Experiments performed in the institute in 2018 indicated that SF may promote plant biomass but reduce plant water contents (*unpublished*). Additionally, as seen in Exp_8 and 9 and also other experiments conducted in our institute by various other group members in the course of the years 2015 – 2018 (Morad-Talab et al., partly published in Bradáčová et al., 2016), only those products were really successful that were enriched in micronutrients, especially Zn. Nevertheless, later results from 2018 – 2020 showed also plant-beneficial effects under cold stress conditions for consortia products without Zn/Mn supplementation. These effects consisted mainly of changes in the plant hormonal status (ABA/CK ratio), increased levels of antioxidants and inhibition of ROS (e.g. prevention of IAA degradation). As also seen in later experiments on cold stress, an important factor for efficacy of the microbial products was their combination with stabilized ammonium nutrition (Moradtalab et al., 2020).

Phosphor limitation

As mentioned above, the limited P-supply from the low pH and Ca-P-rich soil can be assumed as an important limiting factor for plant growth. As described in 0, the C-Loess soil was strongly inhibiting plant growth in another experiment and the application of BE products was again causing additional growth depression. This effect could be conversed by mixing the soil substrate with sand, thereby decreasing buffer capacity, and by fertilization with ammonium-N.

3.2.2 Seaweed extracts and Zn/Mn as cold stress protectants (Exp_8 and 9)

3.2.2.1 Introduction Exp_8 and 9

Focus of the experiments was again the potential of various BE products to alleviate cold-stress in maize. In Exp_8 only plant growth promotion effects and plant nutrient status were investigated whereas in Exp_9 also enzymatic measurements of superoxide dismutase (SOD) and peroxidase (POD) as well as analyses of reactive H_2O_2 and malondialdehyd (MAD) levels, the later one a product of lipid peroxidation, were performed. The main results of Exp_8 were published in 2016 (Bradáčová et al., 2016) (here named Experiment 1). Results from Exp_9 were only presented in the master thesis (Bradáčová, 2015).

Table 3-3 Treatments Exp_8 and 9

Trt_Nr	Exp_8	Rate	Exp_9	Rate
1	Ctrl	/	Ctrl	/
2	Px	6.7E+08	Dura	0.79
3	Rz	6.7E+08	Zn	0.33
4	SF	11.3	Mn	0.33
5	Af	10.7	Zn/Mn A	0.33
6	Px/SF	see SA	Zn/Mn B	0.67
7	Px/Af	see SA	AV	10.7
8	Rz/SF	see SA	Af	10.7
9	Rz/Af	see SA	AV/Zn	10.7/0.33
10	Zn/Mn	0.33	AV/Mn	10.7/0.33
11	AVZM	10.7	AV/ZnMn A	10.7/0.33
12	Unc_Ctrl	/	AV/ZnMn B	21.3/0.67
13			Unc_Ctrl	/
14			Unc_Dura	/

Application rates: Px and Rz (with Bsim) in CFU, Dura using F_{Dur} in g, other products in mg kg^{-1} substrate; in Trt 6 - 9 same rates as for single applications (SA); Exp_8 three applications (0, 14 and 28 DAS); Exp_9 four applications (0, 12, 22 and 35 DAS); r = 5

Enzymatic measurements were not done by the author of this thesis and the method was established by Bradáčová and Morad-Talab in 2015 following a previously published method (Hajiboland and Hasani, 2007).

3.2.2.2 Experimental design Exp_8 and 9

Exp_8 and 9 were conducted under cold stress conditions using the system for CRZ temperature as described in Exp_1. Fertilization was done according to standard fertilization with the same rates as in Exp_3 with the exception of P that was fertilized with 80 mg P kg^{-1} soil (53 mg P kg^{-1} substrate). In both experiments some uncooled controls (Unc) were grown outside of the CRZT system. Various methods for measurement of shoot metabolites and enzymes connected to reactive oxygen species (ROS) were applied that are not described in this thesis but can be found in the publication (see below). For microbial BEs Px and Rz and also the SWE SF and Algafect (Af) low application rates of 10^9 CFU kg^{-1} soil and 16 mg kg^{-1} soil respectively, similar to the application rates in the field experiment 2014, were applied (Table 3-3). During early plant development plants were grown at about 20 - 22 °C soil

temperature. 14 days after plant emergence a two-week long cold stress period with 14 °C soil temperature started followed by a short recovery phase of 10 days before harvesting.

3.2.2.3 Results Exp_8 and Exp_9

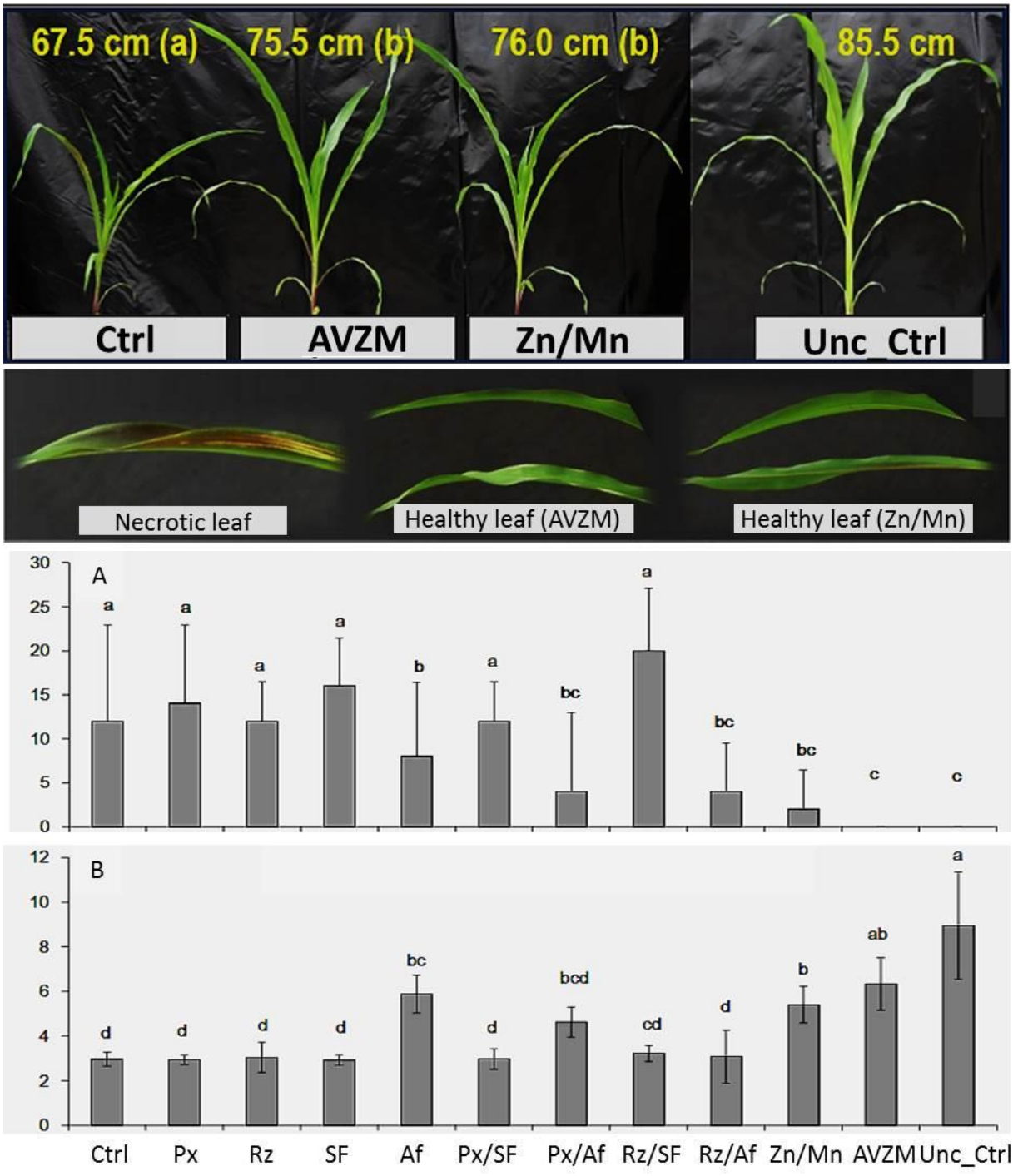


Figure 3-4 Results from Exp_8; Percentage of necrotic leaves (**A**), root length in cm cm^{-3} soil volume (**B**); Means + SD; Letter display reflects results from One-Way-ANOVA; Zn and Cu contents were measured in the leaves shown in the second picture. Necrotic leaf: Zn = 7.4 ppm, Cu = 3.2 ppm; Healthy leaf (AVZM): Zn = 23.3 ppm, Cu = 24.4 ppm; Healthy leaf (Zn/Mn): Zn = 28.2 ppm, Cu = 17.2 ppm

Plant growth measurements, observations of leaf chlorosis, analysis of the biomass at the end of the harvest, root length measurements and nutrient status of the elements P, Zn, Mn, Cu

and Fe were conducted. Plant height (*data not shown*), leaf chlorosis and root length measurements clearly showed the best results for treatments containing seaweed extracts from the company Agriges (Af, AlgaVyt (AV), AlgaVyt Zn+Mn (AVZM)) whereas SF or single application of microbial products (including the Bsim strain that was co-inoculated with the Rz strain) did not improve plant growth under cold stress conditions (Figure 3-4). Interestingly, best results for shoot and root biomass were observed for the Px/Af treatment (*data not shown*) whereas results were less significant than for the other measurements. Analysis of the P status did not show strong differences among treatments with the exception of the uncooled Ctrl that had a by far higher P content and, as observed in other experiments, a lower P concentration g^{-1} shoot due to its higher biomass. Also for the other nutrients no clear responses to treatments were observed in whole shoot biomass (*data not shown*). Nevertheless, analysis of single necrotic leaves indicated that leaf necrosis was correlated with nutrient status of Zn and Cu but not Mn (Figure 3-5). But as seen for treatments Af and Rz/Af also correlations for Zn and Cu were not perfect. Because only one leaf per treatment was analyzed data are not a strong proof but they provided a realistic explanation for the treatment effects.

In Exp_9 only those treatments were analysed that yielded best results in Exp_8. Additionally, different concentrations or additions of Zn and Mn were added to find optimal application rates and further elucidate the role of the micronutrients versus the organic compounds inside the seaweed extracts. Unfortunately, the results from Exp_8 could not be reproduced as clearly and treatment differences were less pronounced. Furthermore, the differences between uncooled Ctrl plants and the Ctrl plants under cold stress were less pronounced than in Exp_8. For none of the measurements that were conducted in Exp_8 and repeated in Exp_9 a clear treatment response was observed (*data not shown*). Some single treatments showed significant differences to the Ctrl in a single measurement, nevertheless those trends showed no consistency as in Exp_8. Also for nutrient analysis no significant differences between BE treatments and the Ctrl were observed.

Measurements of SOD, POD, H_2O_2 and MAD did not indicate significant differences between Ctrl and BE treatments. Nevertheless, the experiment was repeated in the institute and significant effects on enzymatic activity were observed that were also published (Bradáčová et al., 2016), here termed experiment 2.

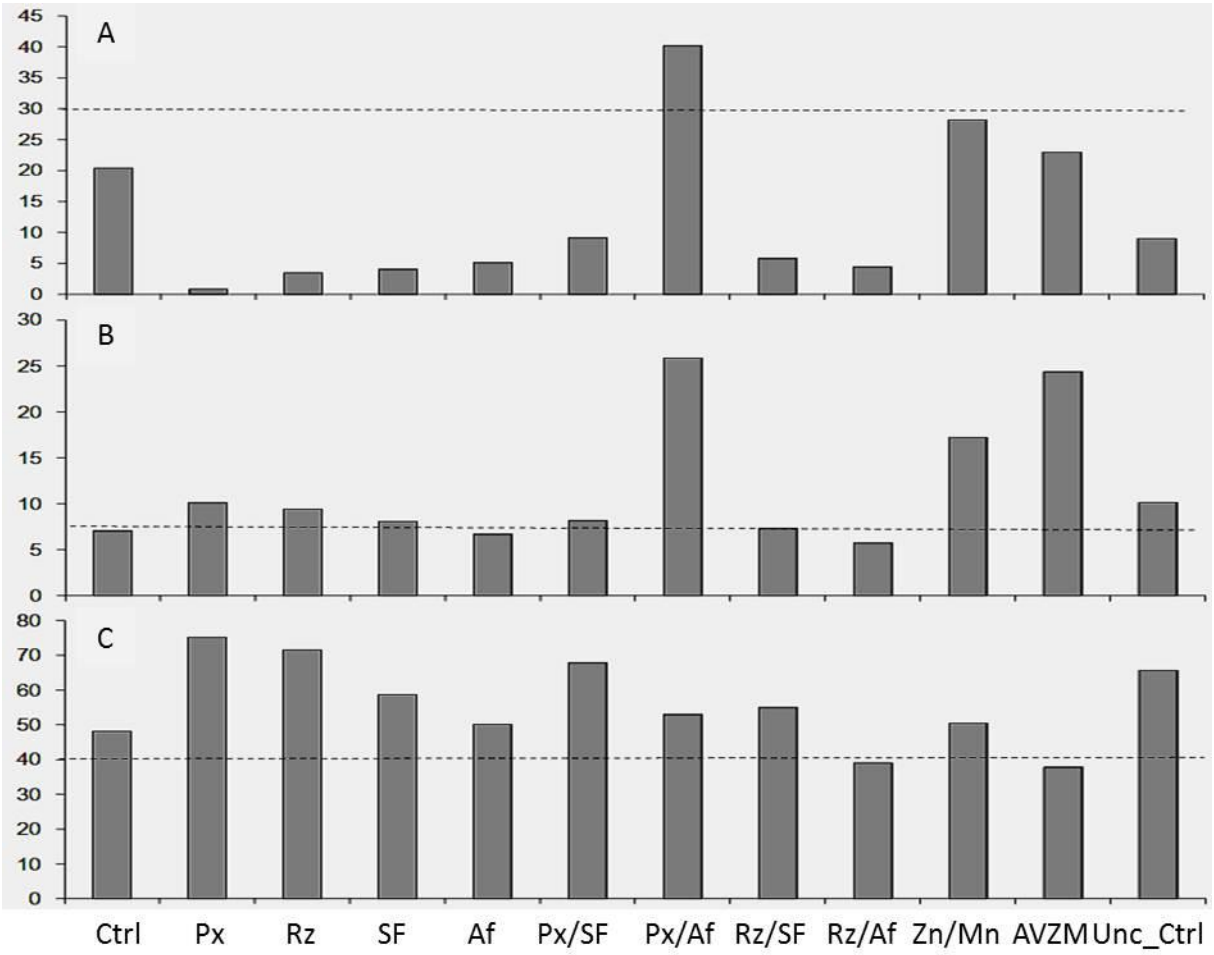


Figure 3-5 Nutrient analysis Exp_8; Zn (**A**), Cu (**B**) and Mn (**C**) concentrations in ppm in necrotic leaves of the maize plants; only one leaf per treatment was analysed

3.2.2.4 Discussion Exp_8 and Exp_9

In Exp_8 and Exp_9 BE products were again tested for their potential to increase tolerance to cold stress in maize.

Zn and Mn containing BE products alleviate cold stress

In Exp_8 some of the BE products from the company Agriges were efficacious to improve shoot and root growth and reduce leaf necrosis under cold stress conditions. An examination of the product properties shows that all successful products (Af, AV and AVZM) contained high amounts of Zn (13 – 74 ppt) and Mn (up to 60 ppt). Further support for the hypothesis that Zn and Mn were involved in the treatment differences was provided by the nutrient analysis of the necrotic leaves. During severe nutrient deficiency, in Exp_8 induced by cold stress, several nutrients are remobilized and retranslocated to other plant tissues. Although data from whole plant nutrient status did not show clear responses to the BE treatments, the nutrient status in chlorotic/necrotic leaves clearly showed the retranslocation of the micronutrients Zn and Cu in those plants that were not Zn/Mn-treated. Both nutrients are

mobile during leaf senescence whereas Mn is less mobile and may even be enriched until leaf drop in perannual plants (White, 2012b). This would explain why Mn concentration in the necrotic leaves did not show strong treatment-dependent behaviour.

Micronutrients like Zn and Mn can be used for seed priming or seed dressing in maize to improve seed germination, emergence and establishment during cold temperatures in spring (Imran et al., 2013). As described in the introduction, Zn and Mn are directly involved in stress tolerance due to their activity as co-factor of the enzyme superoxide dismutase and therefore the degradation of reactive oxygen species (ROS) (Baek, 2012).

Increased SOD activity is one mechanism of Zn-mediated stress tolerance

Therefore, in Exp_9 enzymatic measurements of SOD, H_2O_2 - the product of the SOD-catalyzed dismutation of the superoxid radical -, POD, the enzyme catalysing the peroxidation of H_2O_2 to H_2O, and MAD, a marker for excessive lipid peroxidation, were conducted. Nevertheless, in contrast to Exp_8 treatment effects in Exp_9 were far less pronounced. The reason is probably not the lack of efficacy in the treatments but the less severe damages on plants caused by the applied cold stress, as seen by the comparison between the Ctrl and the Unc_Ctrl treatment. Both experiments were conducted in the same greenhouse and the same system for CRZT but Exp_8 was conducted in summer with high light intensity and temperatures whereas Exp_9 was conducted in autumn. The growing conditions in Exp_8 reflect a more alpine climate in which extreme temperature differences between day and night and a low soil temperature together with high solar radiation are common. Due to the optimal growing conditions above ground during Exp_8, the shoots were metabolically active and grew faster whereas the cold-stressed roots could not supply the shoots with sufficient nutrients. This is suggested by the lower P and Mn contents in plants under cold stress as compared to uncooled Ctrls in both experiments. Therefore, plants suffered from heat and intense irradiation leading to production of ROS that could not be degraded due to a lack of enzymatic activity.

A repetition of the experiment with enzymatic analyses showed indeed that application of Zn and Mn significantly increased SOD activity as expressed per g FW of shoot and root tissue in maize when plants were grown under cold stress conditions (Bradáčová et al., 2016). Both, soil application and seed dressing, were successfully improving cold stress tolerance in the maize plants.

3.3 BE combinations

3.3.1 PGPR-derived plant growth stimulation in maize (Exp_2)

3.3.1.1 Introduction Exp_2

For Exp_2 it was hypothesized, that PGPR effects on plant growth and the establishment of a mutualistic plant-microbe interaction might strongly depend on the PGPR establishment in a new environment. A combined application with glucose as easily available carbon source, which might improve the ability of the inoculated microbes to compete with the already adapted microflora, was therefore tested as a second experimental factor. As it was the first experiment conducted during the PhD thesis almost all possible measurements were performed to determine the most significant indicators of PGPR effects on plants. Therefore, already during the experiment regular pre-harvest analyses were performed.

Figure 3-6 Growth system Exp_2

3.3.1.2 Experimental design Exp_2

Exp_2 was also conducted using a system for CRZ temperature (see Exp_1) but temperature was kept constantly at 20°C to ensure plant establishment in a greenhouse with non-controlled conditions. 60 pots from 12 treatments and each five replicates were used for a two-factorial experiment (Table 3-4). Fertilization was based on the standard fertilization used for most of the pot experiments described in 0. Due to N-deficiency in the plants 8 weeks after sowing 20 mg N kg^{-1} soil were added to all treatments. Artificial light was applied to have a 14h/10h day/night rhythm. Therefore, conditions were similar to conditions in the field in late spring. First application of BEs was performed directly after sowing of three seeds per pot. Second application was done 21 DAS. 9 DAS plants were thinned out to

Table 3-4 Treatments Exp_2

Trt_Nr	Treatment	BE	Appl. rate
1	Ctrl	/	/
2	Ctrl_Glc	/	/
3	P_Ctrl	/	/
4	P_Ctrl_Glc	/	/
5	TP	TP	3.79E+07
6	TP_Glc	TP	3.79E+07
7	Px	Px	9.09E+09
8	Px_Glc	Px	9.09E+09
9	Rz	Rz	9.09E+09
10	Rz_Glc	Rz	9.09E+09
11	BFDC	BFDC	3.79E+07
12	BFDC_Glc	BFDC	3.79E+07

Application rates: for microbial BEs in CFU kg^{-1} substrate; two applications with each 20 ml suspension pot^{-1}. Glc = Glucose applied with the BE suspensions: 1. 0.3 % (60 mg pot^{-1}), 2. 3 % (600 mg pot^{-1}). Fertilization rates in all treatments: 80 mg N, 100 mg K, 33 mg Mg kg^{-1} substrate; Additional 80 mg P kg^{-1} substrate in P_Ctrl; r = 5

one plant per pot. Pots were completely randomized but additionally pots were rotated every second or third day, when watered on weight, in horizontal or vertical direction to limit effects due to pot position and therefore to decrease standard deviation in between pots of the same treatment. Tracing analysis for Px was done using about 1.5 g of washed roots in 50 ml 0.1 % proteose peptone.

3.3.1.3 Results Exp_2

For Exp_2 it was hypothesized, that PGPR effects on plant growth and the establishment of a mutualistic plant-microbe interaction might strongly depend on the PGPR establishment in a

3.3.1.3.1 Pre-harvest analysis

Plants emerged 6 DAS. Here a trend for negative effects of some of the BEs on emergence rate (ER) was found. As data were not normally distributed, an ANOVA on Ranks was performed with a low P-value of 0.053. The treatment with the lowest ER was BFDC having an average ER of two plants per pot (66.6 %) only compared to the Ctrl and TP having the highest ER with almost 96.6 % (Figure 3-8 A). No effect for the glucose treatment was observed. Plant developmental stage (BBCH code), chlorosis symptoms, stem diameter (SD) and plant height (PH) were performed weekly from 14 DAS on.

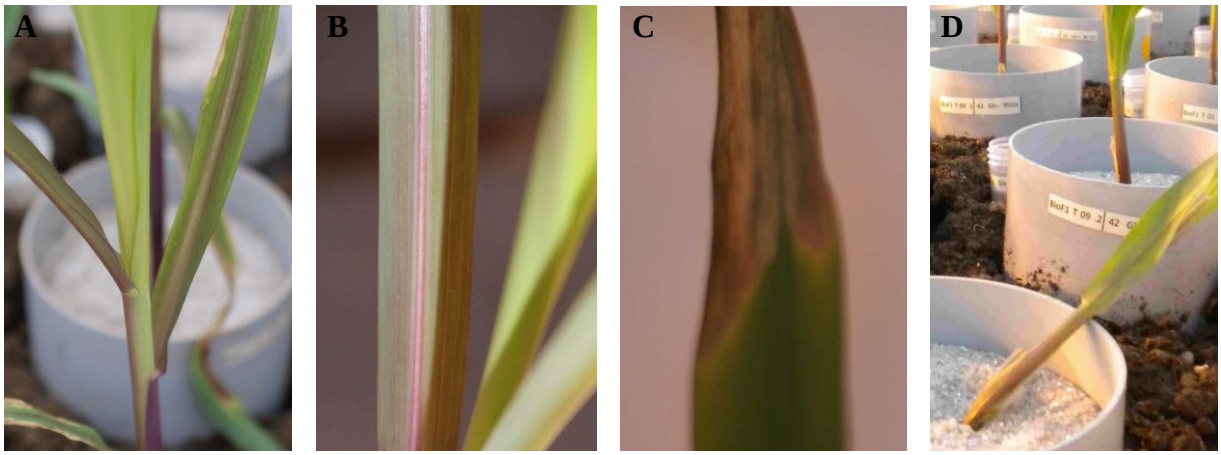


Figure 3-7 P-deficiency symptoms in maize plants Exp_2; Leaf chlorosis and necrosis (**A**, **B**, **C**) by P-deficiency, bended stem in the BFDC treatment (**D**)

Interestingly, at the first measurements of SD and leaf length 14 DAS, Px was the best performing treatment. To quantify the chlorosis symptoms the purple-coloured leaf area was estimated. A Kruskal-Wallis test with ANOVA on Ranks indicated significant difference between the P_Ctrl, showing much less symptoms, and all other plants already 28 DAS. The Px treatment showed second lowest leaf colouration whereas strongest symptoms were visible in the BFDC treatment. Already two weeks after sowing plants from P_Ctrl treatment showed much better plant growth as indicated by plant height and stem diameter measurements. First

significant differences between the Px treatment and the Ctrl were found 44 DAS for stem diameter and 49 DAS for the plant height (Figure 3-8, Table 3-5).

Table 3-5 Results from Exp_2

BE	PH 49 DAS				SD 56 DAS				PH 56 DAS				LWR			
Ctrl	77.5	±	1	**c**	8.35	±	0.21	**c**	93	±	1	**c**	5380	±	492	**b**
P_Ctrl	101	±	1	**a**	10.9	±	0.21	**a**	116	±	2	**a**	4761	±	192	**a**
TP	77.3	±	1	**c**	7.95	±	0.16	**c**	93	±	1	**c**	5523	±	280	**b**
Px	84.1	±	2	**b**	9.45	±	0.24	**b**	101	±	2	**b**	4759	±	294	**b**
Rz	76.8	±	2	**c**	8.40	±	0.22	**c**	92	±	2	**c**	5653	±	312	**b**
BFDC	64.1	±	2	**d**	6.90	±	0.18	**d**	74	±	3	**d**	7118	±	397	**b**

Results from Exp_2; Plant height (PH) and stem diameter (SD), root length-to-weight-ratio (LWR); Mean ± SE

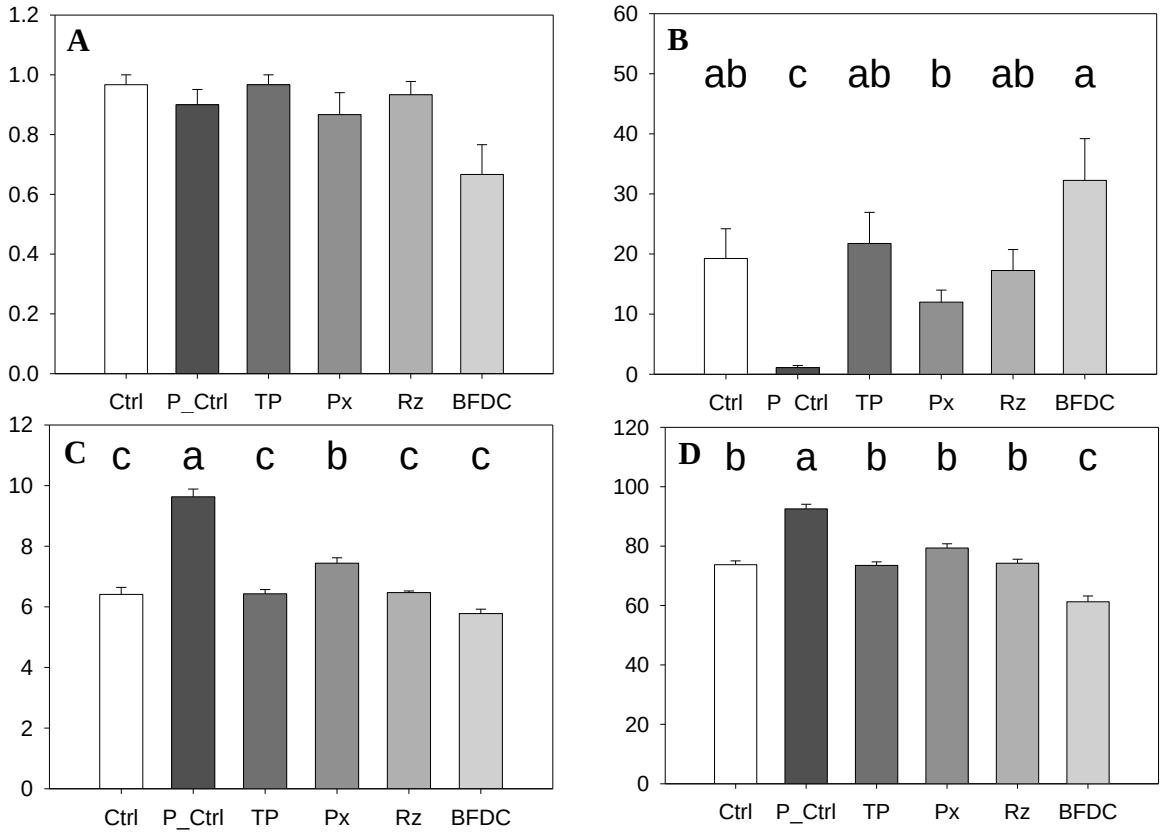


Figure 3-8 Pre-harvest analysis Exp_2; Emergence rate 6 DAS (proportion of total seeds) (**A**), leaf area with chlorosis in % 28 DAS (**B**), SD 44 DAS (**C**), PH 44 DAS (**D**); First significant difference between Px and Ctrl; Means + SE; Letters indicate differences in One-Way-ANOVA, factor glucose not significant and therefore excluded

A calculation of the stem growth rate (mm week^{-1}) for the weeks four to nine of the experiment showed a strong growth peak for the P_Ctrl plants four weeks after sowing. Interestingly, a similarly strong peak was observed for the Px treatment one week later (Figure 3-9). Both slopes declined then rapidly six weeks after sowing to the level of the other four treatments. Those treatments showed a postponed but steadily increase until the week eight when stem growth declined.

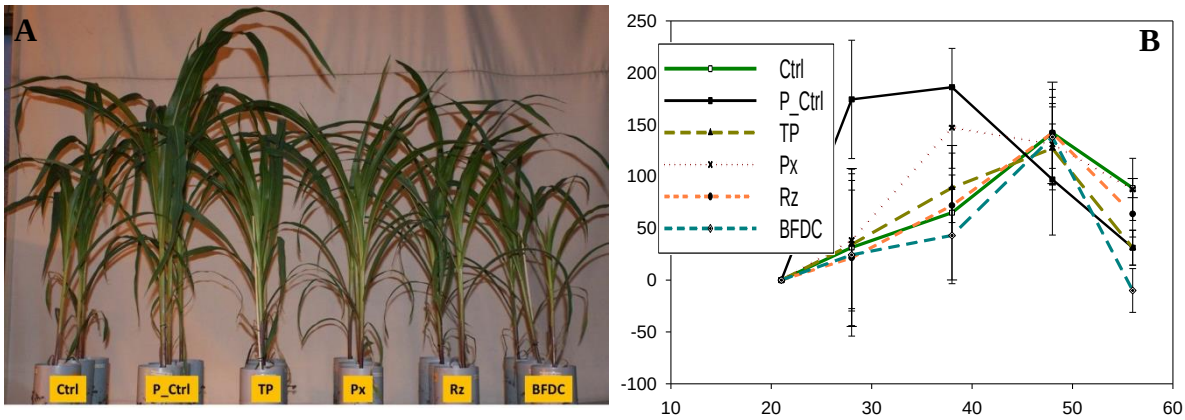


Figure 3-9 Plant growth Exp_2; P_Ctrl with obviously much stronger plant growth and BFDC with reduced plant growth, improved plant growth by Px not visible (**A**); SD growth rate in µm day^{-1} in the course of the experiment (**B**); high SEs and minus values due to the inaccuracy of the measurement (see 3.3.1.4)

Nine weeks after sowing almost no stem growth was observed anymore. 56 DAS stem diameter (SD) of the P_Ctrl plants was significantly bigger than SD of all other treatments. Nevertheless, Px treated plants also had a significantly bigger SD than the Ctrl and the other three BE treatments. BFDC plants showed the smallest SD with significant difference to all other treatments. The same results were found for the plant height (PH) 56 DAS. The thin stems of BFDC plants were also less stable resulting in more bended plants that had to be stabilized by sticks. The effects of the glucose treatment on plant performance generally were minor with no clear tendency. Nevertheless, by statistical analysis some interactions were indicated 56 DAS between the experimental factor "glucose" and a BE treatment. It was found that the BFDC treated plants were significantly higher if they were additionally treated with glucose. Additionally, repeated measurement analysis on SD using SAS proc mixed with the model BE|Glc|time, including all measurement times, indicated that SD was increased in the P_Ctrl_Glc as compared to untreated P_Ctrl (8 %, p=0.01) (data not shown).

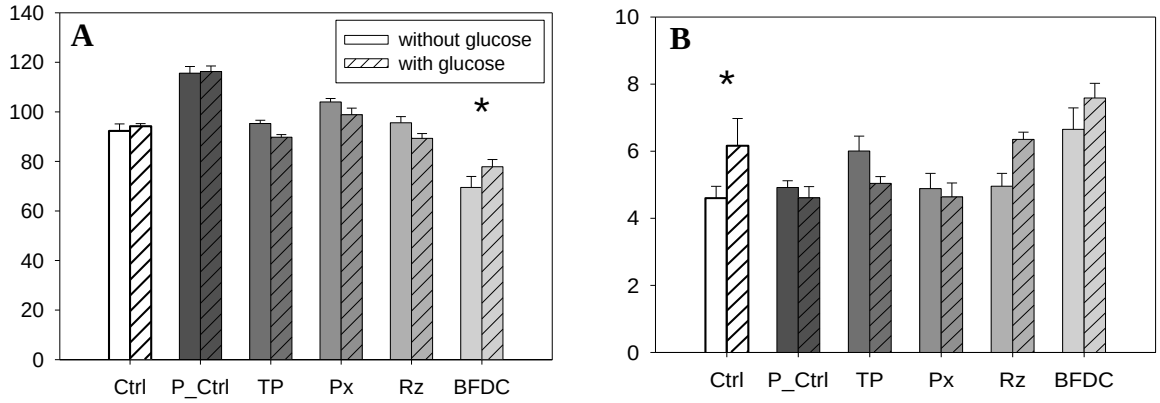


Figure 3-10 Interactions with glucose treatment Exp_2; PH 56 DAS in cm (**A**) and Length-to-weight-ratio (**B**) of roots in cm g^{-1}; significant differences between with/without glucose only for the treatments marked with *****

3.3.1.3.2 Biomass and root morphology

63 DAS plants were harvested. For root and shoot dry weight the same results were found as for SD and PH and Px differed significantly from the Ctrl, TP, Rz and BFDC (Figure 3-11). Root samples were scanned and analysed using the WinRhizo software. For this, representative subsamples of the root were taken for each pot, the weight of the subsample was measurement and then the root was scanned. Analysis of the root length did not reveal additional information. Tendencies were the same as for the other measurements, but differences were less significant with only the P_Ctrl differing significantly from all other treatments and significant difference between Px and BFDC. Interestingly, the fine root length (roots up to 0.2 mm root diameter) was also highest in the P_Ctrl treatment. This was also true for the percentage of fine roots per total root length, whereas no difference was observed among the other treatments. For the glucose treatment again some interaction could be found. Statistically significant difference was found for the length-to-weight ratio of glucose treated

Table 3-6 Results from root scanning analysis Exp_2

BE	< 0.2 mm		0.2-0.4 mm		0.4-0.6 mm		0.6-0.8 mm		> 0.8 mm	
Ctrl	1368 ± 155	**bc**	936 ± 114	**b**	238 ± 28	**b**	238 ± 46	**b**	615 ± 53	
P_Ctrl	3146 ± 168	**a**	1590 ± 95	**a**	373 ± 40	**a**	230 ± 28	**a**	759 ± 38	
TP	1350 ± 93	**bc**	691 ± 81	**b**	167 ± 20	**b**	287 ± 27	**b**	610 ± 30	**ns**
Px	1812 ± 182	**b**	1044 ± 91	**b**	275 ± 22	**b**	244 ± 24	**ab**	714 ± 40	
Rz	1500 ± 128	**bc**	933 ± 76	**b**	222 ± 26	**b**	253 ± 37	**b**	648 ± 49	
BFDC	1222 ± 121	**c**	682 ± 71	**b**	174 ± 26	**b**	274 ± 34	**b**	448 ± 42	

Results from root scanning analysis Exp_2; root length in cm for different root diameter classes; Mean ± SE

and non-treated Ctrl plants (Figure 3-10). The value was calculated as the amount of root length per dry weight. Glucose treatment significantly increased this ratio in Ctrl plants only while keeping the same root dry weight. This might indicate a growth stimulating effect by the natural microflora of the soil that was promoted by glucose application.

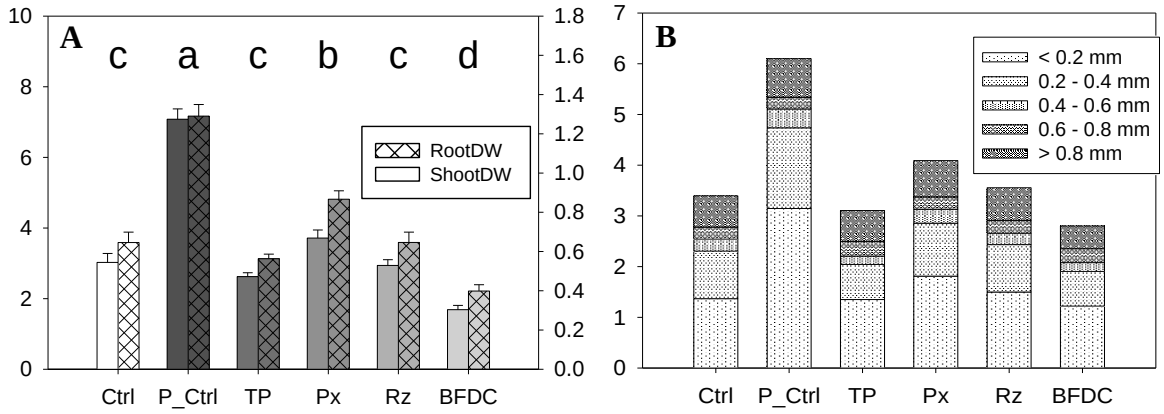


Figure 3-11 Harvest results Exp_2; Shoot and root DW in g (left axis shoot, right axis root DW)(**A**) and root length of different root diameter classes (**B**); Means + SE; significant differences for both shoots and roots

3.3.1.3.3 Microbial analysis

One day after harvest an analysis of the Pseudomonad and total bacteria population from the rhizoplane of five Ctrl and five Px treatments was done by plating on NP and StII medium. Colony counts from NP media indicated a four times higher population density (CFU) in the Px treated as compared to the Ctrl plants (7.5 vs. 1.8 x 10^5 CFU g^{-1} root). The CFU in the Ctrl pots with glucose treatment increased up to 4.0 x 10^5 CFU g^{-1} root. Nevertheless, only about 10 % of the colonies had the typical shape of the Px colonies. Counting of only Px shaped colonies indicated that the number of "real" Pseudomonades in the Ctrl was lower than in the Px treatment but increased to an even higher level as in the Px treatment when glucose was applied (Figure 3-14).

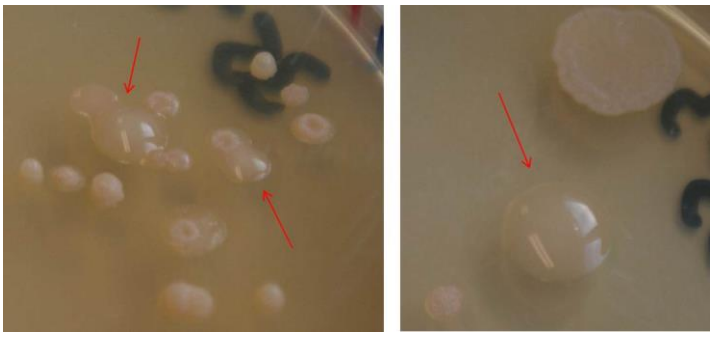


Figure 3-12 *Pseudomonas* colonies on NP medium Exp_2 CFUs with typical *Pseudomonas* sp. "Proradix" colony form indicated by the arrow

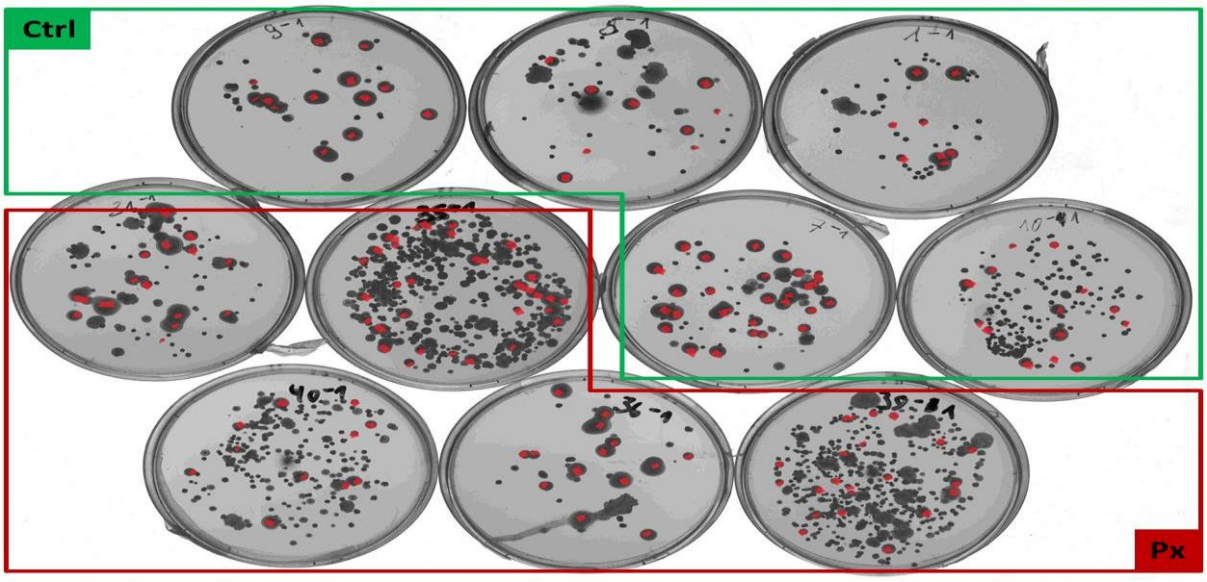


Figure 3-13 CFU counts from semi-selective NP medium; red dots indicate CFUs showing the typical colony form of the *Pseudomonas* sp. "Proradix"

CFU counts from StII medium indicated significant interactions between the BE and glucose factors. In the Px single treatment a higher total number of bacteria was found than in the Ctrl treatment (6.1 vs. 2.4 x 10^6 CFU g^{-1} root) but the opposite was found when glucose was applied. Here number of bacteria in Ctrl pots increased (5.3 x 10^6 CFU g^{-1} root) and decrease in Px pots (2.0 x 10^6 CFU g^{-1} root) twisting the ratio between the two treatments (Figure 3-14).

These results are also supporting the interpretation for the results from the length-to-weight ratio found for the roots of the Ctrl plants. Nevertheless, due to the low number of replicates and the many uncertainties for counting CFUs from plated soil extracts due to contaminations, various colony forms and clusters overlapping each other in their growth, these results should not be over-interpreted.

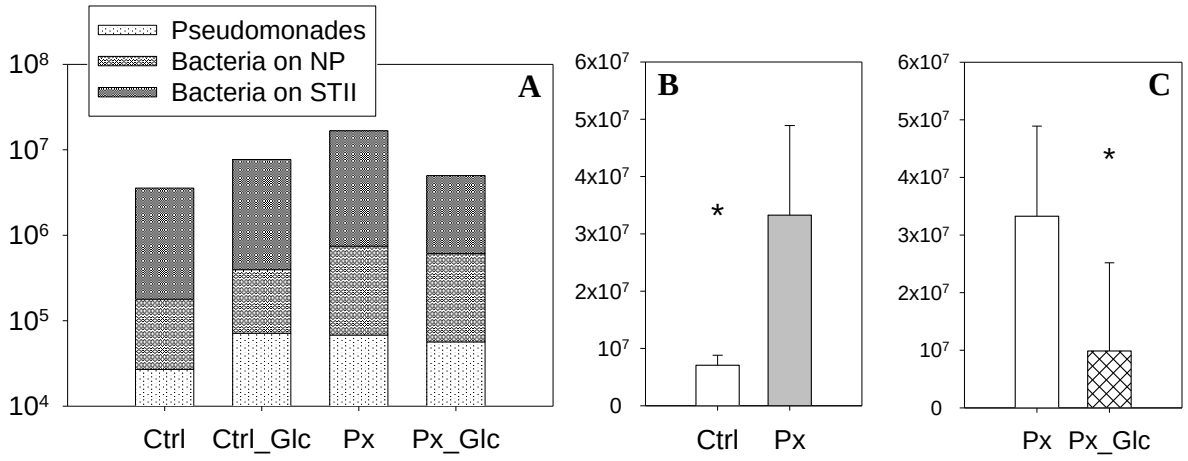


Figure 3-14 Results from microbial analysis Exp_2; Counts in CFU g^{-1} root (log-scale)(**A**); Px increased total number of CFU on STII medium significantly as compared to untreated Ctrl (**B**) but Glc treatment decreased CFU in Px treatment; Means + SE; p=0.01

3.3.1.3.4 Mineral analysis

Mineral analysis from maize shoots was done for the elements P, K, Mg, Ca, Fe, Mn and Zn. Significant differences in the concentration in maize shoots were found for the nutrients K, Mg, Ca and micronutrients Mn, Fe and Zn whereas P concentrations did not differ among treatments. Shoot P and Mn concentrations were both below the threshold for an optimal nutrient supply (Table 3-7).

Table 3-7 Mineral analysis Exp_2

Nutrient	Data	Opt	Unit
P	1.7-2	3-6	ppt
Mg	2-3	2.5-5	ppt
Ca	4-7	3-10	ppt
K	24-45	30-45	ppt
Mn	18-30	40-100	ppm
Fe	87-151	50-250	ppm
Zn	26-62	30-70	ppm

Range of concentration in own data (Data) compared with optimal values (Opt) found in Bergmann (1993)

Interestingly, despite the sincere deficiency symptoms in non-P fertilized plants P concentration was higher than in the P_Ctrl plants (Figure 3-15). Fe concentration varied strongly but similar tendencies as for the other nutrients were observed, with BFDC showing the highest concentration. Concentration of all other nutrients was the lowest in the P_Ctrl plants due to the growth improvement by P fertilization and, except for Zn, highest in the BFDC treatment (Table 3-8). Nutrient concentrations for other treatments behaved similarly in response to the plant biomass with slightly lower nutrient concentrations in the Px treatment that showed the second largest plant growth after the P_Ctrl treatment. For nutrient contents per plant, results were similar to the biomass results. Generally, P_Ctrl plants had highest nutrient contents followed by Px plants with the exception of the Zn content, which

was slightly higher in Rz than in Px plants. BFDC plants always had the lowest nutrient contents.

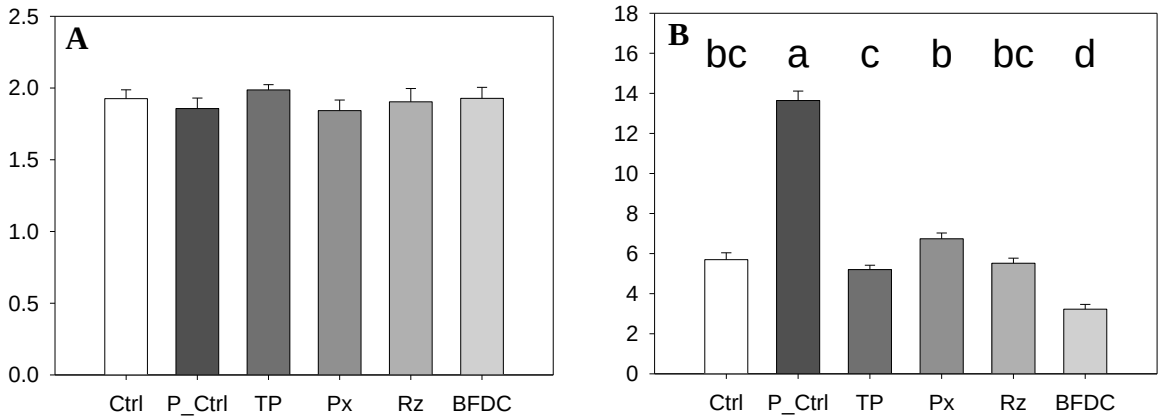


Figure 3-15 Results from phosporus analysis Exp_2; Shoot P concentration in mg g^{-1} DW (**A**) and P contents in mg $shoot^{-1}$ (**B**); Means + SE

Table 3-8 ANOVA results from shoot mineral analysis Exp_2

Concentration	P	Mg	Ca	K	Mn	Fe	Zn	Contents	P	Mg	Ca	K	Mn	Fe	Zn
Ctrl		a	b	ab	a	ab	ab	**Ctrl**	bc	bc	bc	bc	bc	bc	b
P_Ctrl		b	c	c	b	ab	c	**P_Ctrl**	a	a	a	a	a	a	a
TP	ns	a	b	ab	a	b	ab	**TP**	c	c	cd	c	cd	c	b
Px		a	b	b	a	ab	b	**Px**	b	b	b	b	b	b	b
Rz		ab	b	ab	a	ab	ab	**Rz**	bc	c	bc	bc	bc	bc	b
BFDC		a	a	a	a	a	a	**BFDC**	d	d	d	d	d	c	c

Results from One-Way-ANOVA; no significant influence for factor Glc found; Concentrations and total shoot contents for macro- and micronutrients; different letters indicate significant differences in means

3.3.1.4 Discussion Exp_2

3.3.1.4.1 Discussion on results of Exp_2

As mentioned in the introduction several PGPR are known as P-solubilizing bacteria (PSB). Therefore, several experiments were conducted with low P fertilization or in low P soils. For Exp_2 a low P grassland soil from the vineyard of the University of Hohenheim was collected and used without any further P fertilization. Furthermore, it was hypothesized that root colonization and therefore nutrient acquisition and plant growth promoting effects by PGPR can be improved by offering easily available carbon sources in the form of glucose.

Improved P acquisition and plant growth by Px

Indeed, the Px product was able to improve plant growth in Exp_2 as compared to the Ctrl treatment. Although Px plants were still much smaller than the P_Ctrl plants, biomass (root and shoot) and P contents in the Px plants both were significantly increased as compared to untreated plants. This coincided with an increased fine root growth (not significant). In

several other experiments later on similar results or tendencies for the Px product were observed whereas the P availability differed in these experiments. The mechanism by which Px was improving P acquisition could not be elucidated in this experiment. The difficulties to elucidate mechanisms under applied conditions are also seen in the results for the P_Ctrl. In contrast to the expectations, the P_Ctrl had a lower shoot P concentration than the Ctrl treatment but the highest fine root density. A comparison with the results in Exp_23, in which higher P concentrations and decreased root weights in the treatments with underfoot placement were observed, indicates that even the P_Ctrl plants in Exp_2 were suffering from limited P supply. This is also suggested by the absolute P values measured in Exp_2 that were below optimal values in all treatments (Figure 3-7). Several other experiments like Exp_11, 14, 17, 22 and 23 focussed on the mechanisms that might be involved in the observed effects. This is further discussed in section 4.1.2.1.

No BE effects on other nutrients observed

Exp_2 was one of the few experiments in which all macronutrients and some of the micronutrients were analysed. A common trend for all analysed nutrients was that contents were strongly positively correlated and concentrations were negatively correlated with the plant biomass. This is not surprising, because contents are a product of concentration and biomass and concentrations are diluted due to increasing biomass. One reason for the analysis was to elucidate if some specific nutrient may be solubilized by a specific BE. Nevertheless, most of the nutrients showed similar trends under these circumstances. Also, treatment specific effects that differed to biomass results could not be observed with the exception of Fe and P analysis. For Fe analysis a very low concentration was found in the TP treatment. Nevertheless, in Fe analysis extremely high standard deviations between biological or even analytical replicates were found making the dataset less reliable (*data not shown*). P concentration was the only analysis in which no significant differences among treatments were found. One explanation is that phosphorus was fertilized in the P_Ctrl whereas all other nutrients were not additionally fertilized and therefore decreased in the better growing P_Ctrl plants. As said before P was certainly a limiting factor for plant growth. The reason for the better growth in the Px plants might therefore be found in an improved P status. Nevertheless, it was not possible to clearly distinguish between a better P availability (e.g., solubilization) or acquisition (e.g. mycorrhiza, root growth).

As no clear nutrient specific effects were observed in Exp_2 in most of the future experiments no complete nutrient analysis was performed.

Increased root colonization by Pseudomonades

Root colonization was analysed using semi-selective and non-selective medium to calculate total bacteria and Pseudomonades only. A comparison of the colony shapes of common *Pseudomonas* bacteria like *P. fluorescens*, *P. putida*, *P. aeruginosa* with other Pseudomonadaceae like *Azotobacter* sp. showed that they form similar colony shapes. Nevertheless, diverse colony shapes were observed on the NP medium indicating that the semi-selective medium was also probably not effective to select for Pseudomonades only.

Independent of the problem of different colony shapes the taxonomical separation of the inoculated Px strain from other *Pseudomonas* sp. or *P. fluorescens* strains is only possible by using specific primers (Mosimann et al., 2017). Therefore, a mixture of soil Pseudomonades and inoculated Pseudomonades is growing on the NP agarplates. Additionally, bacteria show exponential growth rates and bacterial populations are strongly clustered and therefore single samples might differ strongly by chance.

Nevertheless, results indicate that in Px treatment Pseudomonad population was by a factor 2 - 4 (depending on which bacteria were counted from the NP medium) higher than in the Ctrl. Focussing only on the Px shaped colonies, per g root $2x10^4$ CFU more were counted on Px than on Ctrl medium. This number might represent the population density of the remaining inoculated bacteria that were still active on the rhizoplane. Additionally, a higher number of bacteria can be expected in the rhizosphere with the attached soil. Rz tracing results in Exp_14 indicate that bacterial density in the rhizosphere was by factor 100 higher than in the rhizoplane. Although *Bacillus* and *Pseudomonas* strains differ in their properties, it can be expected that a large number of cells is passively transported into the different soil compartments by inoculation and watering and therefore also distribution of both *Pseudomonas* and *Bacillus* may be similar. Nevertheless, as seen in Exp_21 the Rz strain was relatively “inert” in the soil due to its endospore form whereas the Px strain is highly depending on environmental factors and population growth will be a dynamic process. Additionally, *Pseudomonas* sp. bacteria attach to the rhizoplane due to the production of exopolysaccharids (Dutta and Podile, 2010). Therefore, the rhizoplane may have higher Px population densities than the rhizosphere soil. Population densities of Pseudomonades range from 10^4 to 10^7 CFU g^{-1} soil as commonly reported in literature (Aagot et al., 2001; Raaijmakers et al., 1997). Therefore, it is quite difficult to estimate the real survival rate of the inoculated bacteria using rhizoplane counts. The reason for washing the roots was simply a reduction of contamination, especially of fungal nature. For sure, no exponential growth of

the inoculated Px strain was observed, as about 10^7 CFU g^{-1} soil were inoculated and therefore the measured population density declined by the factor 1000. That would still be 10 times higher than an estimated factor for comparison of rhizoplane and rhizosphere. Furthermore, it is unclear if those additional bacteria are Px bacteria or other Pseudomonades that were enriched due to the Px product application. As further discussed in 4.1.4.2 the Px product with its milk power-like formulation is a prebiotic that also might stimulate natural soil microbial population.

Due to the inaccuracy and inexactness of the method, the analysis of Px root colonization was skipped in most of the later experiments.

Glucose treatment

Glucose treatment in general had little effects on plant growth. Nevertheless, three interactions were observed during statistical analysis. In the BFDC treatment the addition of glucose led to an increase in the plant height 56 DAS, in the P_Ctrl glucose significantly increased the stem diameter in repeated measurement analysis whereas in the Ctrl treatment fine root length was increased. In the case of BFDC, the glucose treatment was reducing the negative effects of the BFDC treatment, indicating that the natural soil microflora might have stimulated plant resistance mechanisms. For the Ctrl treatment the observed plant growth stimulation was also correlated with an increase in the microbial population. Glucose treatment increased the natural population of total bacteria (StII medium) and of the Pseudomonades (also Px shaped colonies only). Results indicate that the carbon source was able to increase the number of plant-beneficial soil microbes. Possibly the amount of glucose was too low to cause strong effects of growth promotion. Nevertheless, as later on observed in *in vitro* studies glucose is a relatively bad nutrition for Pseudomonades like Px as compared to full nutritional sources like milk-powder. Indeed the addition of milk-powder to the soil caused much stronger effects as discussed in 4.1.4.2.

3.3.1.4.2 Critical reflexion of the methodology

Plant height and leaf length

In Exp_2 also measurements of leaf length from all plant leaves was performed regularly. Nevertheless, the measurement of the total leaf length did not reveal additional information to the plant height measurements. Pearson-product correlation between the total leaf length and the PH (length of the longest leaf) showed high correlations with $\rho > 0.9$ and p-values < 0.001 for all measurement times. Also stem diameter and leaf length were similarly well correlated

with $\rho > 0.8$ (56 DAS $r^2 = 0.70$, indicating that 70% of the variance in stem diameter could be explained by the measurements of the leaf length). Therefore, no measurements of total leaf length were performed in future experiments. As stem diameter was found to be more sensitive to P-supply and the Px effects they were sometimes added to the measurements of the length of the longest leaf (later on termed plant height (PH)). Nevertheless, because it is difficult to regulate the pressure caused by the calliper on the stem tissue standard deviations are often high and biases sometimes resulted in negative growth rates between two measurements (Figure 3-9).

Correlation of plant height and biomass

Exp_2 also showed a good correlation between the trends observed during pre-harvest analyses and the harvest results for root and shoot biomass. Therefore, these measurements were also later on used as indicators for BE and treatment effects and if possible plants were harvested when treatment effects were most pronounced. Nevertheless, in many experiments BE effects were observed only during early plant development and vanished in the course of the experiment. In the field, where pre-harvest BE effects never could be observed, vanishing of effects during plant development was observed for different fertilization regimes, showing the potential of maize to adapt to and compensate for suboptimal growing conditions.

3.3.2 Combination of PGPRs and seaweed extracts (Exp_3)

3.3.2.1 Introduction Exp_3

Exp_3 was conducted as a screening for the potential of different seaweed extracts to promote the efficacy of Px application leading to synergistic effects on maize plants. Exp_3 was conducted after the first promising results from the *in vitro* tests on prebiotic activities (3.3.3.4). As the Px strain reacted very specific to different seaweed products and different concentrations in the incubation solution and there was no reference for the question if concentrations used in the incubation solution during the *in vitro* experiments could be a basis for the calculation of application rates in soil substrates, at first three different concentrations and products were screened for toxic or negative effects of the products.

Table 3-9 Treatments Exp_3 (A)

Trt_Nr	Treatment	c (soil)	c (susp.)
1	Ctrl	/	/
2	Px	/	/
3	AV -1	0.1	2
4	AV -2	0.01	0.2
5	AV -3	0.001	0.02
6	Af -1	0.1	2
7	Af -2	0.01	0.2
8	Af -3	0.001	0.02
9	AVZM -1	0.1	2
10	AVZM -2	0.01	0.2
11	AVZM -3	0.001	0.02
12	ECO -1	0.1	2
13	ECO -2	0.01	0.2
14	ECO -3	0.001	0.02
15	PPP -1	0.1	2
16	PPP -2	0.01	0.2
17	PPP -3	0.001	0.02
18	SF -1	0.1	2
19	SF -2	0.01	0.2
20	SF -3	0.001	0.02

All treatments except Ctrl treated with 0.02% (w/w) Px kg^{-1} soil (0.200% in solution, 10 ml pot^{-1}); PPP = equal mixture of the three BE products P1, P2 and P3; conc. in soil or suspension (% w/w or w/v) with 5 ml 100 g-1 dry soil

As the amount of variants was already difficult to handle in large scale pot experiments, no separate SWE (without Px treatment) treatment was conducted in this experiment. Additionally, the treatments were reduced to only one concentration for each SWE in the main experimental phase after the transplanting of the plants into bigger pots. Experimental conditions were more controlled than in Exp_2 trying to find optimal conditions for BE growth promotion. Therefore, standard fertilization was applied with also low amounts of soluble P fertilizer. This was an adaption following results from a partner institute at the JKI Braunschweig (Eltlbany et al., 2019; Eltlbany and Smalla, 2013) that could produce strong growth promotion effects of BEs when using additional P fertilization of the substrates.

3.3.2.2 Experimental design Exp_3

Exp_3 was conducted as a two-phase experiment. It started with a pre-culture in multi-pot seed trays with small pots containing 150 g of substrate each (1:2 sand:low-P soil). The experiment was designed as a completely randomized block design (CRB) with 20 treatments per tray and five trays as replicates (Figure 3-16). Different concentrations of six SWE were tested in combination with the microbial BE Px (Table 3-9). Two seeds per box were sown that were reduced to one plant after emergence. BE applications (Px and SWE) were done directly after sowing at the same day and 12 DAS whereas all SWE treated plants were additionally Px treated. 21 DAS plants were transplanted to bigger pots of 1.9 kg substrate using the KH(W) soil (Figure 3-17). Before transplanting plant size of the different treatments were checked for significant differences. As no difference between different dilutions of SWE treatments were found, the median of all plants treated with the same SWE was calculated and five plants were picked, that were closest to the treatment median. By this, biases due to natural variation of seeds decreased and the amount of treatments was reduced to eight treatments, only differing in the type of applied BEs. From 22 DAS four additional weekly applications of Px and SWE were done with the last application 44 DAS, using 10 ml of 1 % (v/v) SWE suspension and 5 ml 1 % Px solution, whereas the last three weeks Px treatment was done only in the treatment 7. Table 3-10 sums up the total amount of Px and SWE applied during the experiment. Pots were placed in a CRD design and were rotated regularly during watering. 56 DAS plants were harvested. For tracing of Px rhizosphere soil samples were taken by collecting soil adhering to the roots using sterile gloves.

Table 3-10 Treatments Exp_3 (big pots)

Trt_Nr	Treatment	BE	Px	SWE
1	Ctrl	/		/
2	Afect	Px/Af	4.52E+09	0.32
3	AV	Px/AV	4.52E+09	0.32
4	AVZM	Px/AVZM	4.52E+09	0.32
5	Eco	Px/ECO	4.52E+09	0.32
6	PPP	Px/PPP	4.52E+09	0.32
7	Px	Px	1.17E+10	/
8	SF	Px/SF	4.52E+09	0.32

Application rates: for microbial BEs in CFU kg^{-1} substrate; for SWE in g kg^{-1} substrate; in total six weekly BE applications. Standard fertilization (67 mg N, 33 mg P, 100 mg K, 33 mg Mg kg^{-1} substrate); r = 5

Figure 3-16 Completely randomized block design Exp_3; 20 treatments with 5 replicates blocked in racks

3.3.2.3 Results Exp_3

3.3.2.3.1 Pre-cultivation phase

Germination rate was almost 100 % in all treatments and no significant effects of the different SWE on plant emergence could be observed. Nevertheless, all BE treated pots showed a higher average emergence rate than the Ctrl treatment. Similar results were obtained during the pre-cultivation phase. No significant differences among treatments could be observed, although plant height measurement 14 DAS showed that all BE treated treatments performed slightly better than the untreated Ctrl. 21 DAS plants were transplanted into bigger pots. As no effects due to different application rates were observed, 15 plants from one SWE treatment were reduced to five plants selecting the plants nearest to the treatment median to reduce the overall amount of pots per treatment. 22 DAS plants were BE treated. Root and shoot weight of plants that were not transplanted but harvested 21 DAS did not significantly differ among treatments. Additionally, no tendency for better plant performance in the BE treatments could be observed at this time (see Figure 3-18).

Figure 3-17
Transplanting Exp_3

3.3.2.3.2 After transplantation

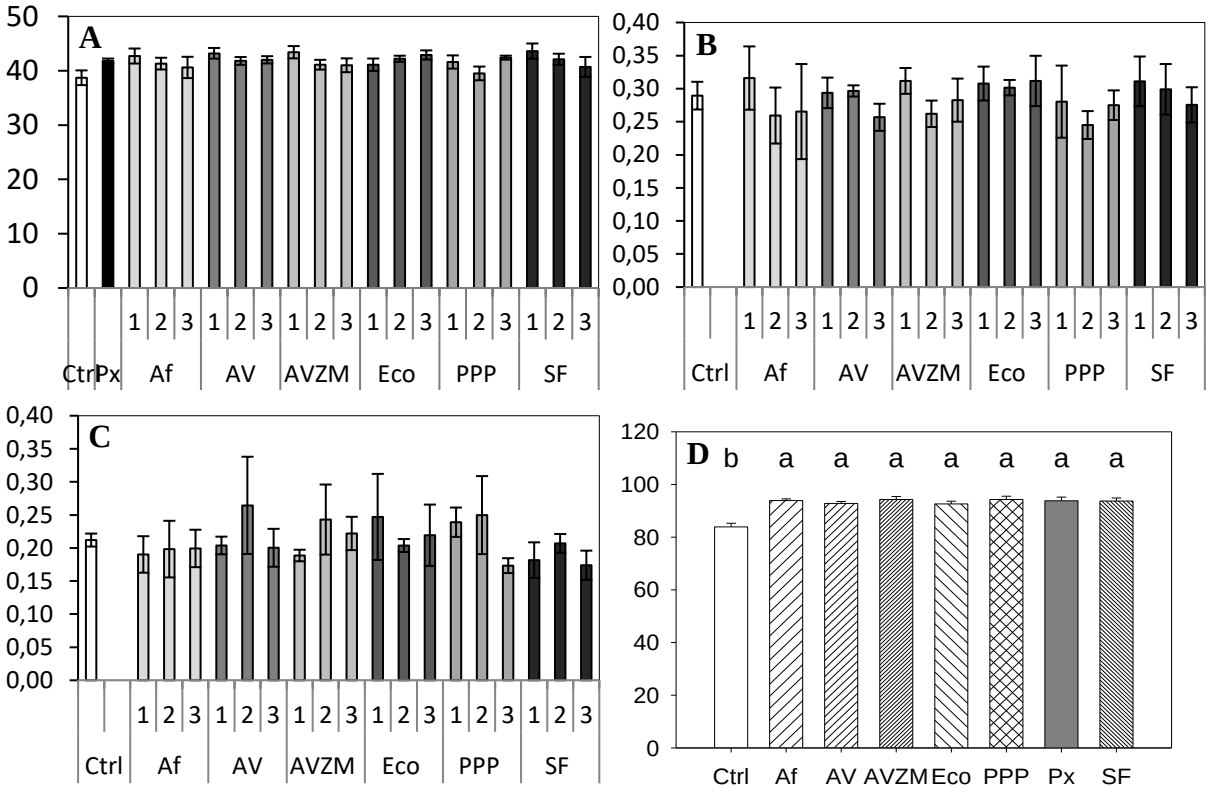


Figure 3-18 Results pre-cultivation and pre-harvest Exp_3; Plant height in cm 21 DAS (**A**), shoot and root DW 21 Das (**B+C**) (plants from Px treatment were all used for transplanting and therefore no data are available here), Plant height in cm 55 DAS (**D**); Means + SE

29 DAS plant height of BE treated plants, except for Ecolicitor (ECO), was significantly higher than height of Ctrl plants. This difference was also found 41 DAS as well as for the stem diameter 41 and 48 DAS. 55 DAS these differences decreased (Figure 3-18 D).

3.3.2.3.3 Harvest

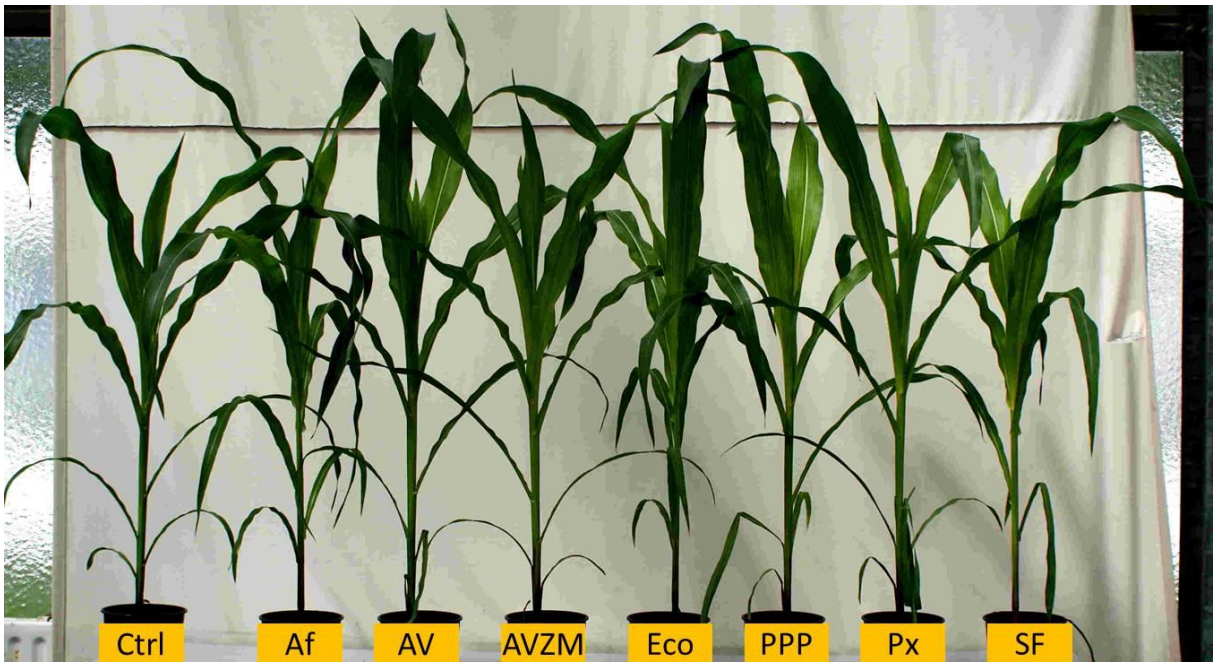


Figure 3-19 Plant growth in Exp_3 (55 DAS); Again no visible difference among treatments

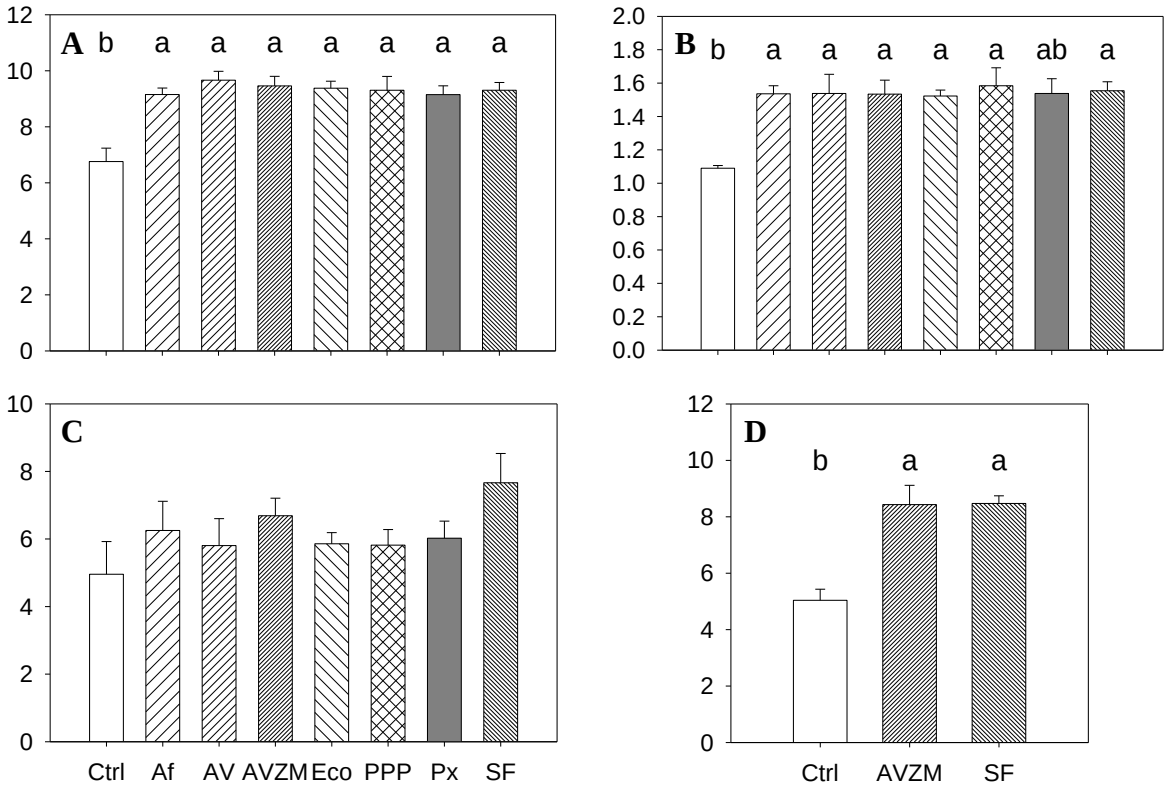


Figure 3-20 Results Exp_3 at harvest (56 DAS); Shoot (**A**) and root (**B**) DW in g, root length in m from scanning of subsamples (**C**) and whole root scanning (**D**); Means + SE; for root DW one outlier reduced (lowest value of the Ctrl treatment); root scanning data square root transformed to achieve normal distribution

Plants were harvested 56 DAS. Analysis revealed a significantly increased shoot dry weight for all BE-treated plants with an average increase of 38 % whereas no significant differences among the BE treatments could be observed. Similar results were found for the root dry weight whereas the ECO treatment had slightly less effect and did therefore not differ significantly from Ctrl treatment. Root length analysis was first performed only with representative subsamples of the roots (5-10 g root FW). Results indicated a tendency for improved root growth for all BE treatments with the largest roots in the SF treatment showing 55 % more root length than the Ctrl. Due to the high variation and the uncertainties and biases that are coming with a subsampling of roots total root scanning was performed for the most promising treatments AVZM and SF. The results showed that the roots of both BE treatments were significantly larger than the Ctrl roots with an average increased root length of more than 60 % (Figure 3-20).

3.3.2.3.4 Microbial analysis

In two pre-tests 1 g of a Ctrl soil sample and a freshly inoculated soil with Px suspension were extracted using 25 ml of two alternative extracting agents (10 mM $CaSO_4$ and 0.2 % tetra-sodium pyrophosphate) (Liu et al., 2010, p. 201). Additionally, centrifugation of the soil suspension at 300 rpm for 15 min was tested to reduce contaminations from non-target microbes. Various serial dilutions were plated on NP medium.

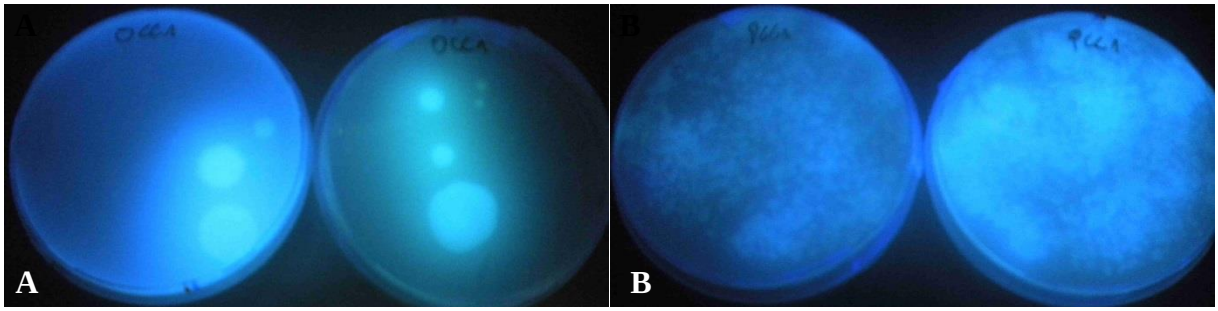


Figure 3-21 Fluorescence by *Pseudomonades*; Colonies on NP medium (KB + antibiotics), fluorescence was induced using a simple UV lamp; suspensions from non-inoculated Ctrl soil (**A**) or inoculated with Px (**B**)

Centrifugation seemed to reduce contamination but also the cell number of *Pseudomonads* and was therefore skipped in a second test. In both tests pyrophosphate extraction seemed to be less efficacious for microbial extraction due to decreasing cell number as compared to the $CaSO_4$ extracts and increasing biofilm formation, making colony counting more difficult. Although $CaSO_4$ extracts showed better extraction efficiency and promoted a separation of colonies the recovery rate from soil was only about 4 - 6 % of the inoculum. Additionally, cell numbers in the Ctrl treatments already varied from 10^5 – 10^6 CFU g^{-1} soil with a high standard deviation. To better distinguish *P*. sp. “Proradix” from other *Pseudomonas* strains

the fluorescence of the colonies from Ctrl and Px treated soils was compared using a UV light source. By this method a distinction between different species was not possible due to varying colour and intensity of the fluorescence. Because of these uncertainties in the method as well as a lack of difference for plant growth stimulation between different SWE and the Px single inoculation a further tracing in the other treatments was omitted. At this time point the Px-specific RT-qPCR method was not established in our laboratories and therefore only analysis from the 2014 field experiment were analysed by this method at FiBL institute in Switzerland.

3.3.2.4 Discussion Exp_3

Px but not seaweed extracts promoted plant growth

During the first growing phase in the small pots no significant differences among treatments could be observed. Therefore only one concentration per SWE treatment was kept to continue the experiment after transplantation of the plants in bigger pots. By using the pre-test plants it was possible to proceed faster to the next step of testing the products in bigger pots. No significant differences among the different SWE treatments were observed. All SWE extract treatments showed similar results to the Px single treatment suggesting that Px was acting in a similar way in all treatments. Some variation was observed for the root length, but due to the extremely big roots only "representative" parts could be scanned for all treatments. For whole root scan AVZM and SF were picked because they are produced by different companies and from different seaweeds. Additionally, their composition (Table 2-1) and the reaction of the Px strain in *in vitro* experiments (3.3.4.3) differed strongly among those two SWE.

Although first root scan indicated a lower root length in the AVZM treatment as compared to SF, in the second scan of the whole root the length of both treatments was almost equal. This result fits well to the often observed correlation between root weight and root length. As none of the BE treatments differed significantly from other BE treatments in root DW it can be assumed that also the root length of the other SWE treatments as well as the Px single treatment was similar to the SF treatment.

That the effects on the plant were obviously independent of the nature of the applied SWE further suggests that no synergistic interaction between a specific SWE and the Px product occurred. This aspect is further discussed from 4.3.2.2 on.

3.3.3 Combination of PGPRs and seaweed extracts (Exp_10)

3.3.3.1 Introduction Exp_10

Exp_10 was directly referring to Exp_3 testing again seaweed extracts and their potential for synergistic effects with Px. Nevertheless, various aspects were changed to test further hypotheses and to make experimental conditions more similar to field conditions:

1. To investigate the mode of action of the BE treatments additional Ctrl treatments were included with different levels of fertilization. Here it was hypothesized that the BE treatments will compensate for the lower fertilization rates by improved root growth and therefore improved nutrient acquisition.
2. As the Px single inoculation provoked similar responses in the maize plants as compared to the combined applications of Px and SWE extract the question remained in how far SWE extract application alone may contribute to the plant growth promotion. Therefore treatments with single application of SWE were included, whereas treatments were reduced to the two SWE showing best results in Exp_3.
3. Px was applied only once, similar to the conditions in the field. Additionally, the application was postponed to the time of plant emergence to ensure a high inoculum rate for the growing root.
4. Bigger pots and an additional underfoot placement of ammonium-phosphate were used to simulate field conditions.

3.3.3.2 Experimental design Exp_10

For Exp_10 a 1:2 mixture of the Ba:KH(a) soil was used. This soil mixture was again mixed with sand in a 1:2 ratio. In comparison to Exp_3 BE concentrations were drastically reduced to make a comparison to the field experiment 2014 possible (Table 3-11). Three control treatments with different fertilization levels were used to test the ability of the BEs to compensate for lower fertilization rates (Table 3-12). Directly before sowing a fertilizers placement was performed to mimic field conditions by applying a solution of mono-ammonium phosphate with a pipette in 5 – 10 cm depth in the centre of the pot. Three seeds per pot were sown that were reduced to one plant after emergence. First application of BEs was done 10 DAS when plant roots were already established by pipetting

Table 3-11 Treatments Exp_10

Trt_Nr	Treatment	Px	SWE
1	Ctrl	/	/
2	Std_Ctrl	/	/
3	P_Ctrl	/	/
4	Px	6.67E+08	/
5	Px/SF	6.67E+08	0.011
6	Px/AVZM	6.67E+08	0.010
7	SF	/	0.011
8	AVZM	/	0.010

Application rates: for Px in CFU kg^{-1} substrate, one application 10 DAS; for SWE in g kg^{-1} substrate per each of two applications 10 and 17 DAS; r = 5

30 ml of suspension on the soil surface near the plant. A second application of the SWE was performed on week later. Weekly measurement of plant height and stem diameter as well as SPAD measurements 38 DAS, were performed. Plants were harvested 52 DAS. Root scans were done for treatments 1, 7, and 8 only.

Table 3-12 Fertilisation Exp_10

Treatment	N	P	K	Mg
Ctrl	40	/	74	23
P_Ctrl	40	47	74	23
Std_Ctrl	77	47	100	33

All values as mg kg^{-1} substrate. All BE treatments fertilized like Ctrl. All treatments were additionally fertilized with a solution of MAP (20 mg P, 9 mg NH_4-N) as underfoot placement

3.3.3.3 Results

In contrast to Exp_3, weekly measurements of stem diameter and plant height did not reveal strong differences among treatments. Nevertheless, from 27 DAS on plant height of BE treatments was generally higher than in the Ctrl treatment and on the same level with the Std_Ctrl and P_Ctrl treatment. 31 and 38 DAS significant differences between the Ctrl and the SF treatment (single inoculation) occurred with SF showing the highest average plant size of all treatments (Figure 3-23). In contrast, SPAD measurements indicated that SF had the lowest average N content in leaves indicating a shoot growth simulation without improved nutrient acquisition. As plants were growing rapidly, they were harvested already 52 DAS. Analysis of the shoot fresh weight indicated for the first time significant differences between the Ctrl and the Std_Ctrl treatment with higher fertilization rates. All other treatments did not significantly differ from each other. The difference between Ctrl and Std_Ctrl for shoot dry weight was not significant anymore. Root dry weight did not differ significantly among treatments, mainly due to a high standard deviation, even though all BE treatments showed higher root weight than the Ctrl with an average growth improvement of about 24 %, similar to the Std_Ctrl. Largest roots were found in the P_Ctrl treatment with 40 % more root biomass than the Ctrl. As standard deviations were high and roots were already too big to make whole root scanning feasible, no root length analysis was performed.

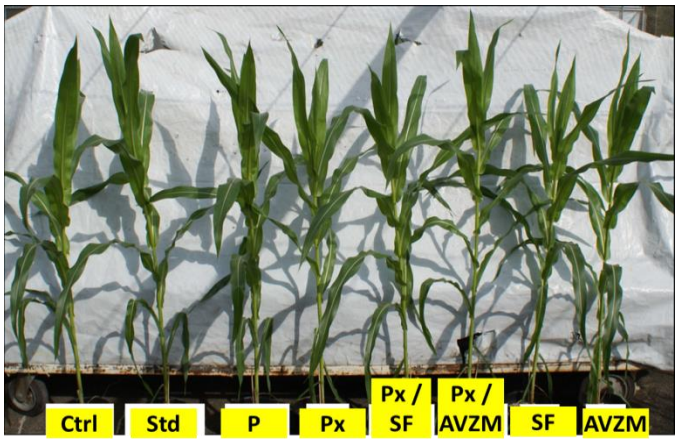


Figure 3-22 Plant habitus Exp_10 (52 DAS)

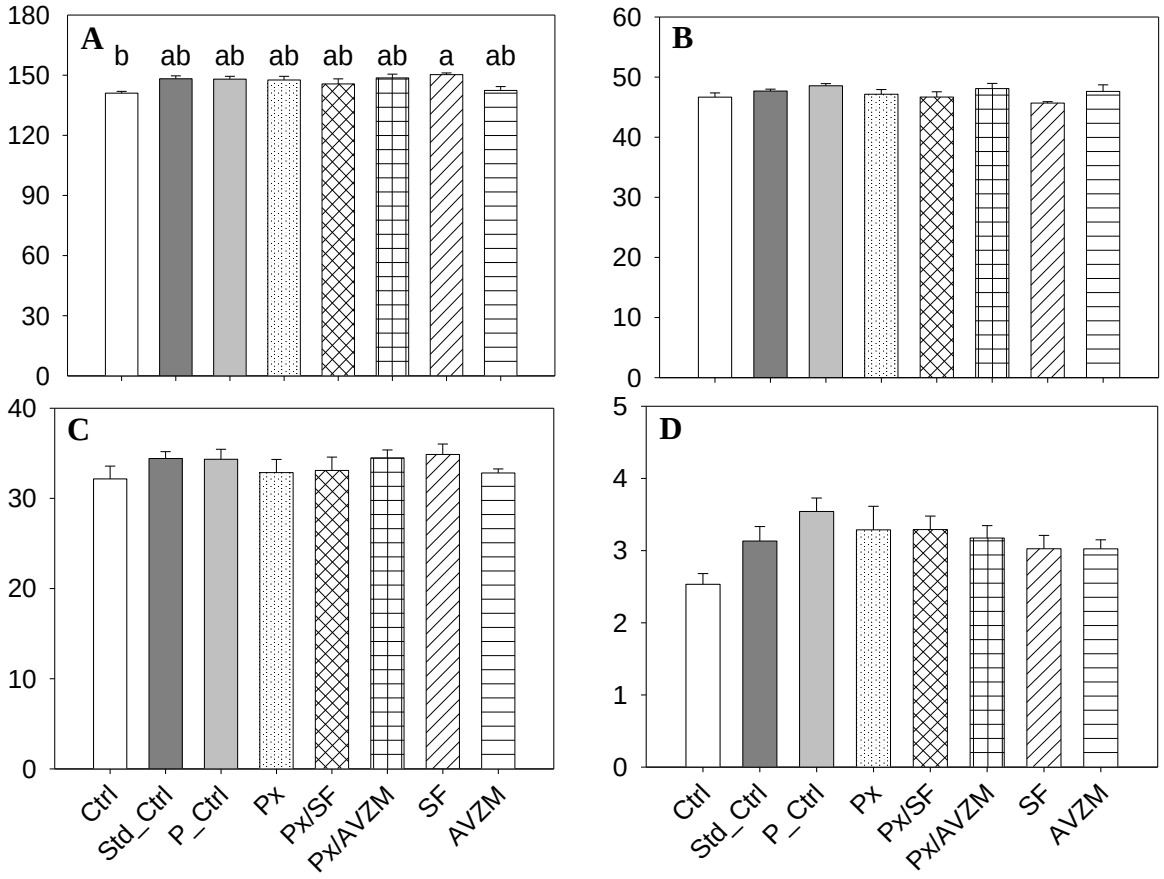


Figure 3-23 Results from Exp_10; Shoot height in cm 38 DAS (**A**), SPAD values 38 DAS (**B**), shoot (**C**) and root (**D**) DW in g pot^{-1}; Means + SE; Letter display reflects results from One-Way-ANOVA

3.3.3.4 Discussion Exp_10

Exp_10 was conducted to reproduce the effects observed in Exp_3 under more applied conditions. The strong effects of Exp_3 could not be reproduced although all BE treatments showed some tendency for growth improvement as compared to the Ctrl. Additionally, no strong differences were observed between different fertilizer regimes. Interestingly, only root weight seemed to be improved by higher fertilization rates. SF was improving shoot growth without an increase in root growth. Additionally, N status, as analysed by SPAD measurements, was decreased in the SF treatment. Results suggest that SF was stimulating plant growth without improving nutrient acquisition, probably by hormonal activity, e.g. cytokinin-like shoot growth stimulation (Craigie, 2011; Sangha et al., 2014), thereby reducing root-shoot ratio.

Loss of BE effects after changing experimental conditions

Results showed that the applied changes in conditions were reducing effectiveness of the BEs. In Exp_10 many experimental factors changed so that the analysis of possible reasons for the non-effectiveness of the BE treatments is difficult. Nevertheless, the experiment is a perfect

example for the multiple uncertainties on the way to establish a successful application and reproduction of beneficial effects when working with BEs. Various reasons for the lack of effects are possible:

1. The inoculum rate was too low. This is indeed a realistic assumption keeping in mind the high inoculum rates in Exp_2, Exp_3, Exp_11 and Exp_14 in which major effects were observed but the relatively small effects observed in the field experiments or publications on application of the Px strain in barley (Buddrus-Schiemann, 2008).

2. The inoculation happened to late. This is also supported by the results from Exp_18 (Brecht, 2015) in which single application of Rz directly at the time of sowing (Rz_single) had better effects for growth stimulation than the two applications one and two weeks after sowing (Rz_triple).

3. Underfoot placement of ammonium-phosphate is certainly a strong boost for the plant development that may interfere with BE effects on the maize plant. This is also supported by the results from field experiments in 2015 in which plants developed faster when they were supplied with additional P sources at the time of sowing (visual evaluation). Here urea fertilized treatments were delayed in their development even though this delay did not negatively affect yield at the harvest several month later.

4. As the bulk soil density was higher in Exp_10 than in Exp_3 due to the different soil substrate, it is possible that the Px inoculum did not percolate perfectly into the substrate to reach the plant roots. This might also be a reason for the lack of effects in Exp_19 (Cona Caniullan, 2017) whereas here seeds were inoculated to ensure Px penetration at least during the early plant development of the root.

5. The soil substrate was not optimal for Px establishment or nutrient status in the substrates was too high. This is supported by the fact that also the P_Ctrl and Std_Ctrl with higher fertilization rates did not significantly differ from the Ctrl treatment. Additionally, the amount of substrate is largely influencing the outcome of an experiment. In field experiments early effects of different fertilization rates and also micronutrient supply often vanished during the season due to the unrestricted root growth and therefore high overall nutrient availability (as seen in many experiments during the Biofector project).

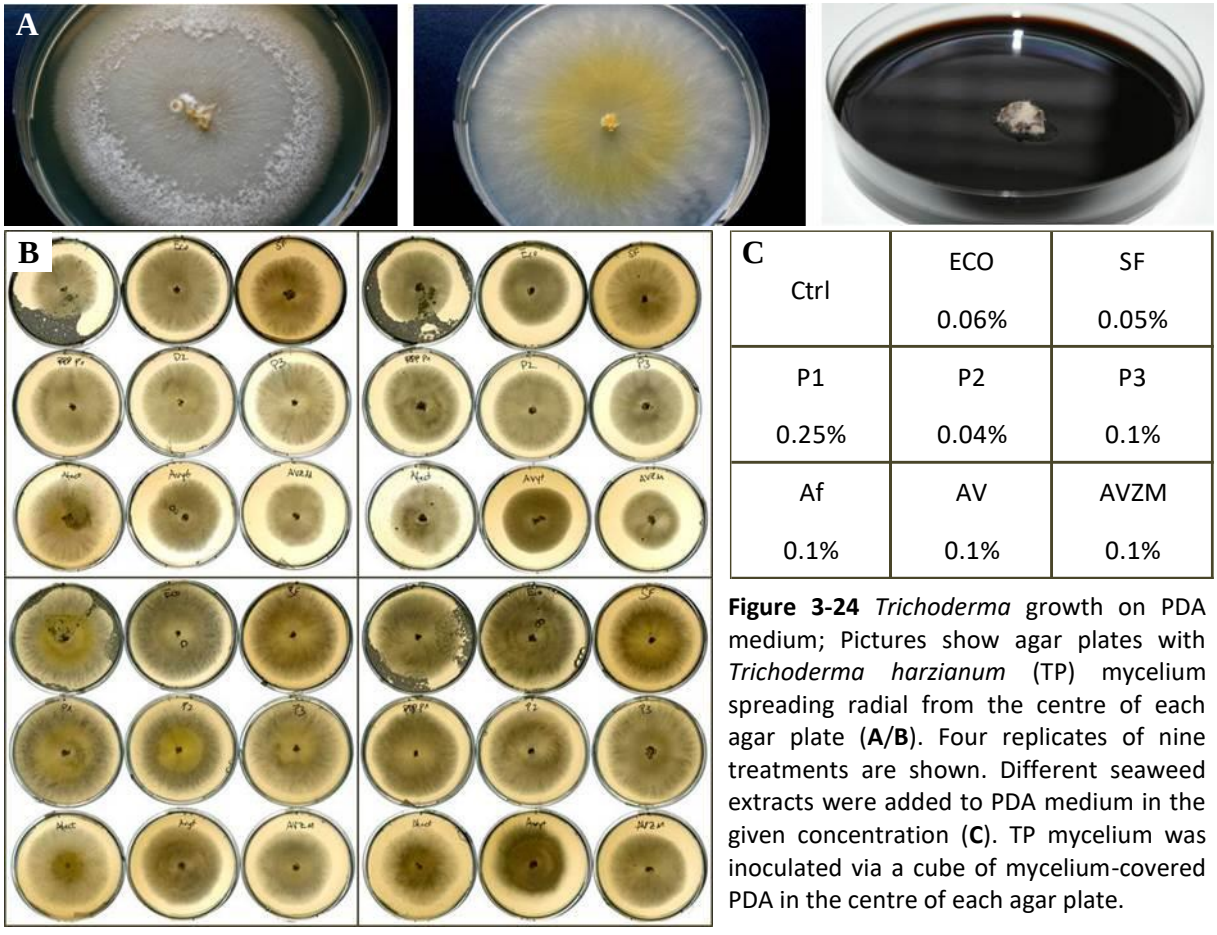
3.3.4 *In vitro* tests on prebiotic activities

In vitro tests were performed following recommendations of the product providers to test different seaweed extracts (SWE) for their prebiotic properties, for example stimulation of bacterial or fungal growth, and to screen for potential product combinations.

Products tested were sent by the companies BioAtlantis (test products 1 – 3, SF and ECO) and Agriges (AV, AVZM, Af). Tests were done after pasteurization of SWE products as recommended by the company BioAtlantis (*personal communication* with S. Krishnamoorthy, 2014).

The pasteurized products were diluted and either directly incorporated in growth media or used for incubation experiments with bacterial BE strains.

3.3.4.1 *Influence on* Trichoderma harzianum



C		
Ctrl	ECO 0.06%	SF 0.05%
P1 0.25%	P2 0.04%	P3 0.1%
Af 0.1%	AV 0.1%	AVZM 0.1%

Figure 3-24 *Trichoderma* growth on PDA medium; Pictures show agar plates with *Trichoderma harzianum* (TP) mycelium spreading radial from the centre of each agar plate (**A**/**B**). Four replicates of nine treatments are shown. Different seaweed extracts were added to PDA medium in the given concentration (**C**). TP mycelium was inoculated via a cube of mycelium-covered PDA in the centre of each agar plate.

Tests with the *T. harzianum* strain (TP) were done with SWE-treated PDA growth medium. It was investigated if mycelium growth was promoted or delayed / inhibited by the incorporated SWEs. Concentrated SWE (2% SF in the medium, Figure 3-24 A (right)) was inhibiting fungal growth whereas at lower concentrations, as recommended by the company BioAtlantis,

no strong differences among treatments were observed (Figure 3-24 B). Only for the treatments Af, AV and AVZM some growth reduction was observed. The area covered with mycelium was always lowest. Nevertheless, no clear recommendations were given from the product provider Agriges and therefore similar concentrations as for the other products were chosen. Differences in the appearance of the mycelium could not be linked to SWE treatments. Experiments on *Trichoderma* were not continued later on as differences were not strongly pronounced as for the Px product and the TP product did not show good plant growth promoting properties in the pot experiments.

3.3.4.2 Influence on Bacillus *strains*

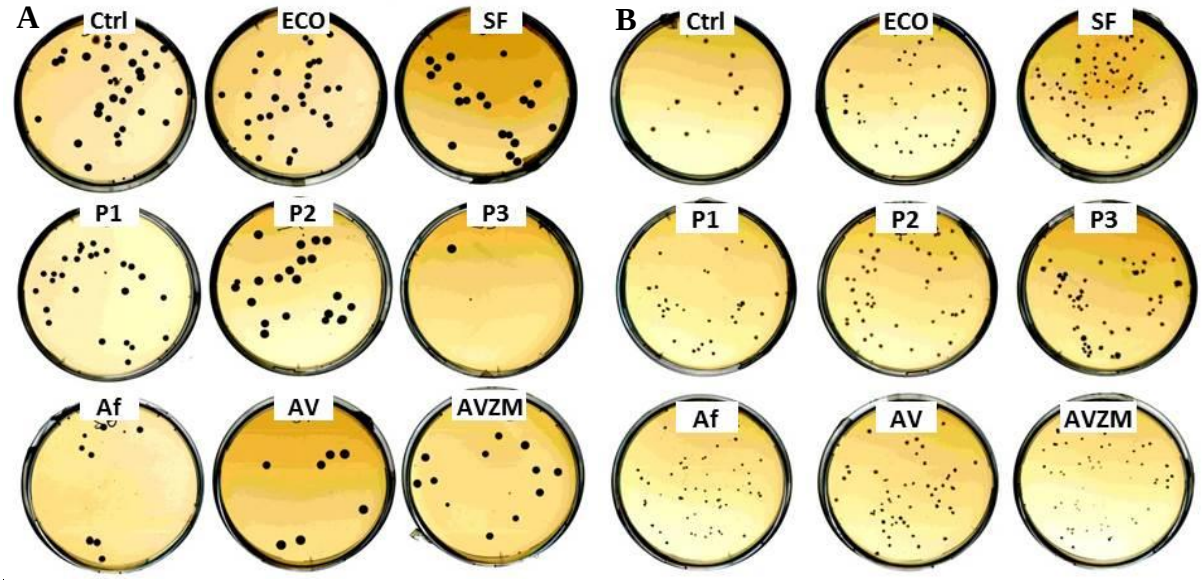


Figure 3-25 Prebiotic tests on *B. simplex* and *B. amyloliquefaciens*; Plating on LB medium after incubation for 4h in 2.5 mM $CaSO_4$ with 2 % SWE; Bsim (**A**) + Rz (**B**); r=3: only one of three replicates shown in the pictures

For the Rz strain no significant influence of SWE was observed. Interestingly, the Bsim strain reacted more specific and sensitive to the different seaweed extracts. Product P3 (Figure 3-25 A) had a toxic effect on the strain, seen on all of the three replicates. A repetition of the experiment (pictures not shown) showed the same results.

3.3.4.3 *Influence on the Px strain*

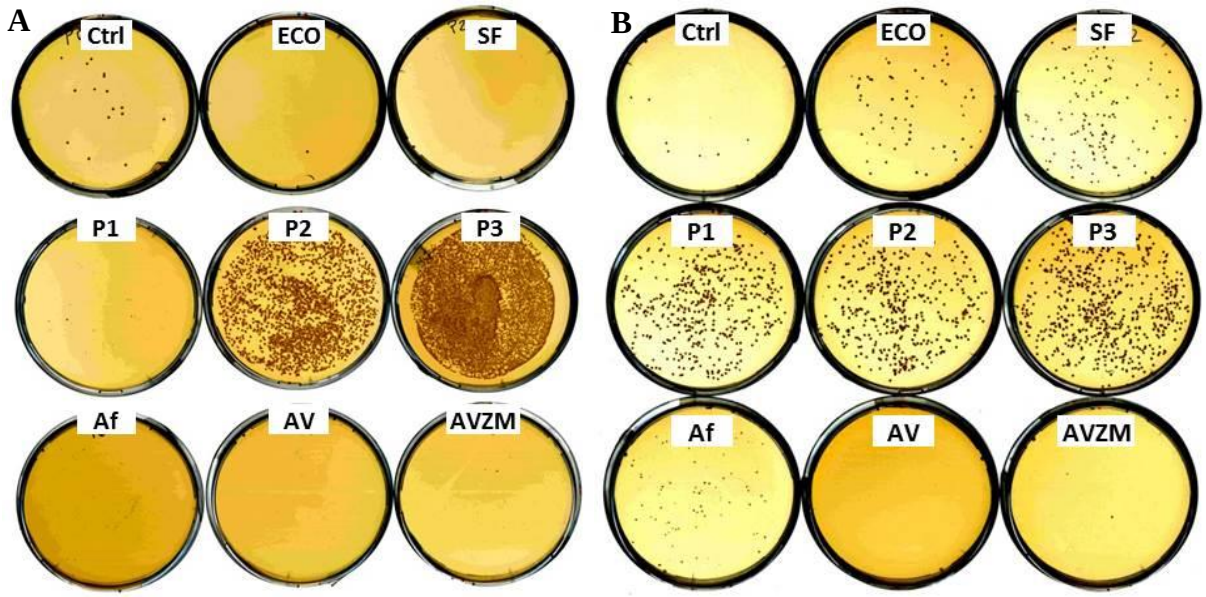


Figure 3-26 Prebiotic tests on *Pseudomonas sp.* "Proradix"; Plating on NP medium after incubation for 4h in 2.5 mM $CaSO_4$ with 2 % SWE (**A**) or 0.1 – 0.4 % SWE (Af, AV + AVZM 0.4 % SWE in medium, others 0.1 %)(**B**); r=3

Most distinct effects were seen for the Px strain. At high concentration the products ECO, SF, P1, Af, AV and AVMZ had toxic effects on the strain, not only inhibiting growth but obviously killing bacterial cells, whereas the P2 and P3 products showed strong, growth promoting, prebiotic effects leading to an exponential growth of the bacteria. Especially P3, that was toxic to the Bsim strain, was best performing for the Px strain (Figure 3-26 A). With decreasing concentrations (0.1%) also the product P1 became beneficial and growth promoting (Figure 3-26 B).

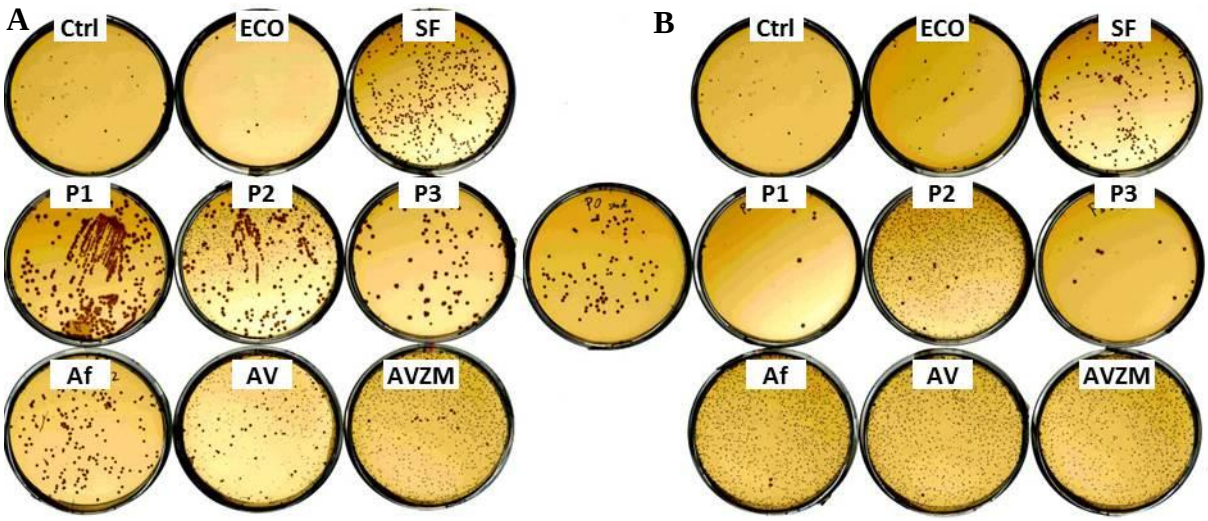


Figure 3-27 Prebiotic tests on Px strain (low concentrations); Plating on NP medium after incubation for 4h in 2.5 mM $CaSO_4$ with 0.01 % SWE (**A**) or 0.001 % SWE (**B**); agar plate in the centre shows the CFU at time 0 before starting of the incubation; cluster on the agar plates of P1 and P2 due to condense water drops; small grey dots on agar plates of P2 or Af – AVZM are air bubbles in the medium not colonies; r=2

After further decreasing the concentration in the incubation medium the beneficial effects of P1 – P3 decreased and vanished completely at a concentration of 0.001% whereas the SWEs SF and Af became beneficial (Figure 3-27). At a rate of 0.01% SF showed similar prebiotic effects like the P1 - P3 products at a 10 times higher concentration (about 4 - $5x10^3$ CFU ml^{-1}). These numbers of CFU were about 10 times higher than the CFU before incubation.

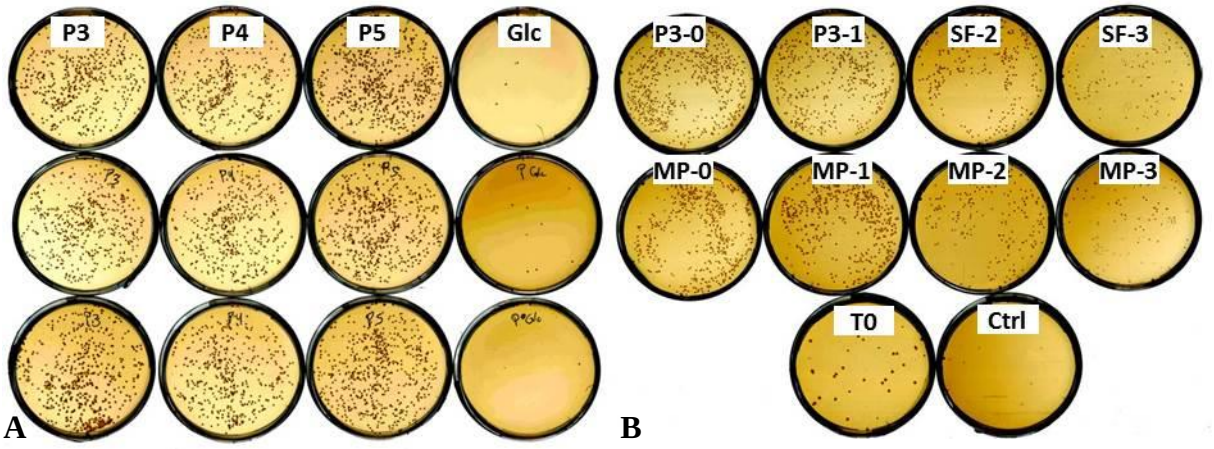
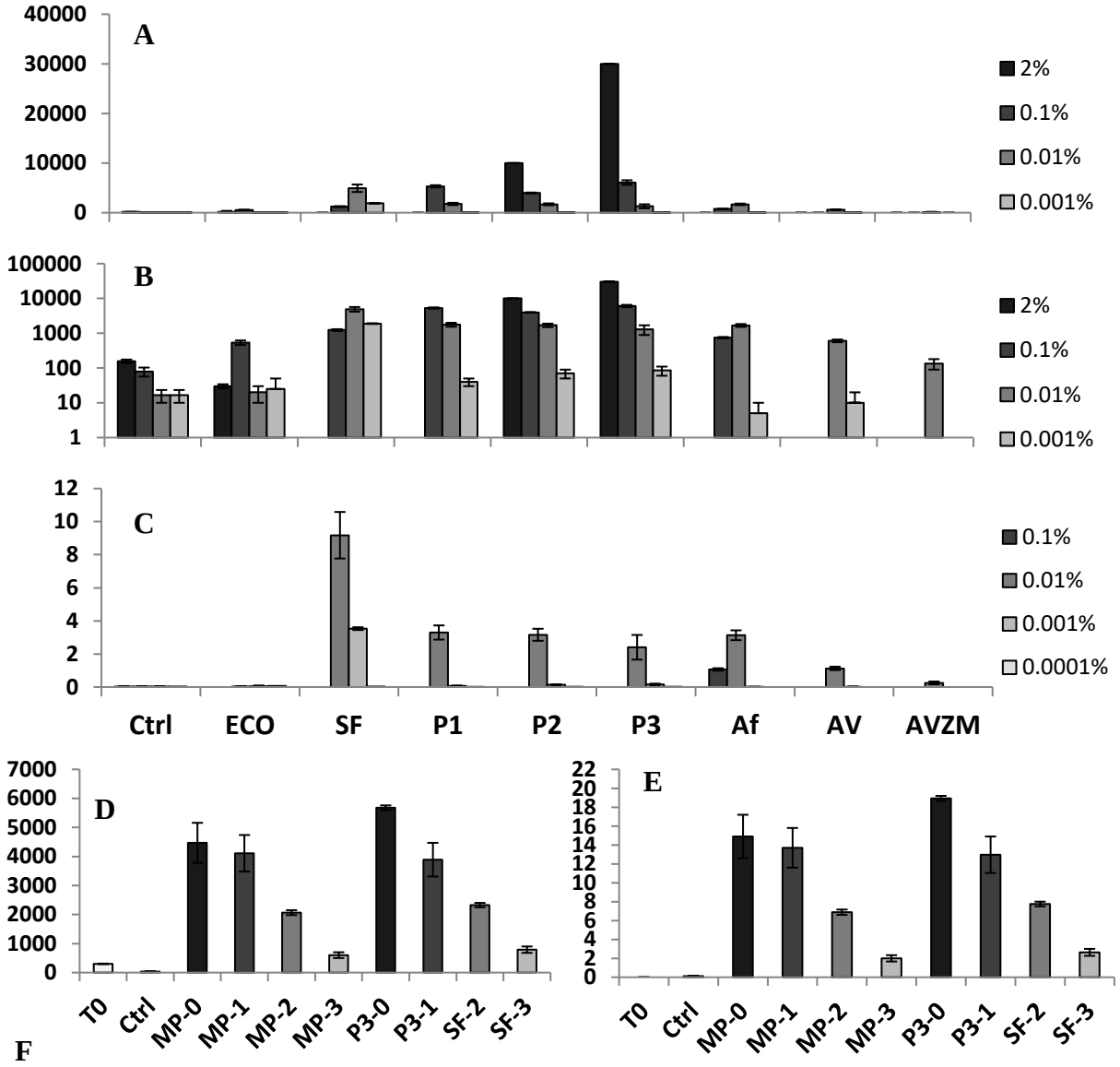


Figure 3-28 Comparison with other "prebiotic" compounds; Plating on NP medium after incubation for 4h in 2.5 mM CaSO4 with 0.1 % SWE / glucose (Glc) (**A**) or different concentrations of SWE and milk powder (MP) (**B**); Concentrations in (**B**) are given as negative power of ten (e.g. MP-2 = MP at concentration of 10^{-2} (0.01 %).

A comparison of the products with glucose or milk powder showed that the SWEs had much better prebiotic effects than pure glucose (Figure 3-28 A). Nevertheless, the usage of milk powder showed similar effects like the best performing SWEs at all tested concentrations. As SWEs performed differently depending on the concentration, P3 was replaced with SF at low concentrations. The agar plates in the first picture are the three replicates for each of the treatments. Here it is visible that the standard deviations were relatively low in comparison to the effects and therefore only a low number of replicates was accepted as sufficient for the analyses.

Results from statistical analysis for all experiments with the Px strain is given in Figure 3-29 on the next page.



F

CFU ml^{-1}	T0	Ctrl		ECO		SF		P1		P2		P3		Af		AV		AVZM	
2%	NA	157		30		0		0		~10000		~30000		0		0		0	
0.1%	540	80	e	540	d	1237	c	5320	a	3973	b	6080	a	570	d	0	NA	0	NA
0.01%	540	17	e	20	e	4925	a	1770	b	1695	b	1290	bc	1680	b	605	cd	135	de
0.001%	540	17	b	25	b	1895	a	40	b	70	b	85	b	5	b	10	b	0	b
0.0001%	540	17	NA	35	NA	15	NA	10	NA	5	NA	5	NA	0	NA	0	NA	0	NA

Figure 3-29 Prebiotic tests on Px strain (Graphs); CFU ml^{-1} in normal (**A** + **D**) and log-scale (**B**) and multiple of starting CFU at time 0 (**C** + **E**); data for P2 and P3 2% are estimations, no counting possible due to the high density; Means + letter display (**F**) (for 0.1 % and 0.01 % after square root transformation); T0 0.01% only for Af and Ctrl; no significant difference between SWE and MP found at any of the given concentrations

3.3.4.4 Product combination: Screening for prebiotic activity

3.3.4.4.1 Prebiotic effects of polysaccharides

The term prebiotic (Gibson and Roberfroid, 1995), comes from the field of human medicine and refers to substances that cannot directly be digested by humans but are fermented by the gut flora and that can provide selective advantage for one microorganism or group of microorganisms (Zaporozhets et al., 2014). Especially oligo- or polysaccharides are normally falling into this category.

In our *in vitro* studies the different seaweed extracts showed very different effects on the microbial strains, especially the Px strain. The P2 and P3 products are purified seaweed extracts enriched in polysaccharides laminarin and fucoidan that seemed to be very good nutritional sources for the Px strain. Laminarin and fucoidan are both glucan polysaccharides found at high concentrations in brown algae. Laminarin is mainly composed of (1,3)-β-D-glucopyranose residues whereas fucoidan is based on α-(1,2) linked L-fucose highly esterified with sulfate (O'Sullivan et al., 2010; Shekhar et al., 2012). Studies on prebiotic activities of seaweed extracts are mainly focussing on modulation of gut microbiomes of rats, pigs or humans. Laminarin and fucoidan both increased abundance of lactobacilli and bifidobacteria (Nguyen et al., 2016; Shang et al., 2016). Some studies could show antimicrobial properties of fucoidan against the human pathogen *P. aeruginosa* (Fitton et al., 2015; Marudhupandi and Kumar, 2013) but no reports on prebiotic effects on *P. fluorescens* were found. It was obvious that the Px strain was able to use the P2 and P3 products as nutritional sources. For the P1 product there is no information available on which components were enriched by the company.

3.3.4.4.2 Antimicrobial activity of seaweed extracts

Influence of different SWE extracts depended strongly on the specific microorganism. The low responsiveness of the Rz strain can be explained by the endospore form in the product. The prebiotic tests were one of the first experiments done in the institute and therefore experience with the spore products was limited. With the wisdom of hindsight it is recommended to perform the experiments with germinated *B. amyloliquefaciens* cells instead. Nevertheless, in the Bsim product, also containing an endospore forming *Bacillus* sp. strain, an increased sensitivity to one of the SWE was found. This is very surprising, as it was expected that the endospores should be protected against the product. It is possible that the product still induced toxicity after germination on the growth media. Another possibility

would be that the *B. simplex* endospores germinated earlier than e.g. the Rz endospores and therefore already suffered from the toxicity during the 4h incubation time. This is supported by the slightly lower CFU in the Agriges products.

Also for the fungal strain only weak responses were observed but it seems that again the Agriges products AV and AVZM had a more negative influence on the microorganisms than the Bioatlantis products. The same was found for the Px strain. The ECO and SF treatments were only toxic at highest concentration of 2 % in the medium.

A general toxicity of seaweed extracts at high concentrations can be explained by the high amounts of phlorotannins in *Ascophyllum nodosum* (Craigie, 2011; Wang et al., 2008). An explanation for the negative or toxic effects of the Agriges products on microbial BEs could be the extremely high concentration of Zn and Mn in those products (Table 2-1). A dilution of the products to about 0.1 % in the PDA medium would lead to concentrations of about 14 – 74 µg Zn g^{-1} medium. Zn toxicity on *Trichoderma viride* was observed at concentrations of 10 mM Zn in the medium (650 ppm or 650 µg Zn g^{-1} medium) whereas 1 mM Zn did not affect fungal growth (Babich and Stotzky, 1978). Af, similar to the SF product, could "stabilize" Px growth at a concentration of 0.1 and 0.01 % in the incubation medium. The positive effects of the Af product could be explained by the amino acids that were enriched in this Agriges product only. In the AVZM product the Zn concentration was about 4 times higher than in Af, nevertheless, the AV product had similar Zn concentrations. Additionally, *P. aeruginosa* was found to tolerate high Zn concentrations of 10 mM in the medium (Babich and Stotzky, 1978) and a *P. fluorescens* strain was able to tolerate Zn concentrations up to 5 mM, although stress to high Zn concentration was probably beginning already at lower concentrations between 1 and 5 mM (Alhasawi et al., 2014). Nevertheless, these concentrations are much higher than in our experiments at 0.1 or 0.01 % SWE and therefore another explanation is more probable. Only the AV and AVZM products contained *Spirulina* bacteria or extracts (concentration and extraction method unknown). *Spirulina* extracts have antimicrobial activity and were reducing growth of different *Pseudomonas* sp. strains, including one *P. fluorescens* strain, at low concentrations of 80 to 150 ppm in the medium (El-Sheekh et al., 2014; Pradhan et al., 2012).

The term "stabilization" is used for SF and Af because the population growth was not strongly increased (no exponential growth in comparison to the starting time point of incubation) but cells were kept alive although nutrient availability was as low or even lower than in the other treatments. This effect by the Af and SF treatment is interesting because they seem to positively affect and stabilize the population at extremely low concentrations whereas

in the P1 + P3 products that promoted exponential growth at higher concentrations, no effect was seen at low concentrations. Especially the SF product was, even at concentrations of 0.001%, able to act as a prebiotic. This was one of the reasons why the Af and the SF products were used later on in the field experiment 2014. No published data on concentration dependent prebiotic activity of specific seaweed extract compounds were found, therefore it can only be hypothesized that the SF product contained some additional substances, possibly with hormonal activity, that were still active at very low concentrations.

3.3.4.4.3 Prebiotic effects of the milk powder formulation

A comparison of the SWE with the milk powder-based formulation of the commercial Px product showed that in all concentrations the formulation had similar prebiotic effects on the *Pseudomonas* sp. strain than the best performing SWE. This is important because the milk powder formulation is probably more cost-effective and certainly more convenient than the SWE. This is also supported by the fact that the company Bioatlantis could not provide the institute with high amounts of the P1 – P3 products as they did not yet establish a large scale production system for these SWE. Additionally, it is of special importance, as the milk powder formulation was shown to be able to promote plant growth without the Px strain. A possible mechanism behind this effect is discussed in 4.1.4.2.

3.4 BE effects on P acquisition

3.4.1 PGPRs for improved P-acquisition (Exp_4 and 5)

3.4.1.1 Introduction Exp_4 and Exp_5

Exp_4 (like Exp_5) was conducted to reproduce the BE effects observed in maize and tomato pot experiments of a partner group at the JKI Braunschweig (Eltlbany et al., 2019). In contrast to Exp_2, the low P soil was fertilized with a low to medium dosage of soluble Ca-P fertilizer to improve the starting conditions in the youth development of the maize plants and thereby to increase the probability of successful root colonization.

3.4.1.2 Experimental design Exp_4 and 5

Exp_4 and Exp_5 were conducted at the same time using similar experimental conditions as Exp_3 (big pots) but both experiments were conducted in a neighbouring green house with less light intensity from artificial light. This was mainly due to a moving light source that did not provide light continuously for all plants but about 5 – 10 times less light intensity than in other greenhouse experiments. Temperature during day/night was set to 24°C/18°C respectively. Exp_4 was conducted with maize whereas for Exp_5 tomato plants were investigated. Ctrl and BE treatments were fertilized as described in Exp_3 but a second Ctrl treatment (P_Ctrl) with 80 mg P kg^{-1} substrate was included (Table 3-13). Three seeds per pot were sown and thinned out to one plant 7 DAS keeping the midsize plant.

Table 3-13 Treatments Exp_4 /Exp_5

Trt_Nr	Treatment	Seed	Soil appl.
1	Ctrl	/	/
2	P_Ctrl	/	/
3	Px	1.00E+09	8.60E+09
4	Rz	1.00E+09	8.60E+09
5	Rz/TP	1.00E+09	8.60E+09
		4.17E+07	7.17E+07
6	Px/Rz/TP	1.00E+09	8.60E+09
		1.00E+09	8.60E+09
		4.17E+07	7.17E+07

BE application: 1. before sowing seed soaking for 2 min in a 2.5 mM $CaSO_4$ suspension (ST), rates in CFU ml^{-1}:, 2. + 3. soil application with 15 ml of the ST suspension per pot 8 and 18 DAS, rates in CFU kg^{-1} substrate; r = 6

3.4.1.3 Results Exp_4

3.4.1.3.1 Plating assays

Plating of serial dilutions on agar media was performed for the application solutions to prove the CFU rate given by the product providers. These rates could be confirmed by this test with only minor differences (target CFU: $1x10^9$ CFUs ml^{-1}; Px: $7.4x10^8$ CFUs ml^{-1}; Rz: $2.3x10^9$ CFUs ml^{-1}). A mixture of the products did also not lead to differences in the CFU of the single BEs. A quick bioassay was performed to investigate the behaviour of the microbial BEs when combined for application. For this, dilutions of the Px and Rz suspensions (10^6

CFU ml^{-1}) were plated on PDA medium. Then a small agar cube cut from a PDA medium with *Trichoderma harzianum* (TP product) was placed in the centre of the agar plates.

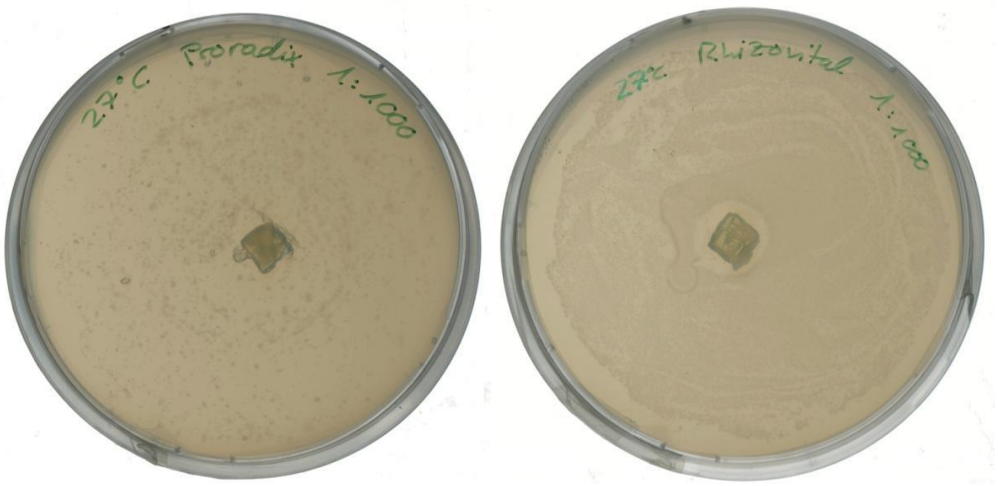


Figure 3-30 Bioassay for testing competition and co-existence of bacterial BEs; Px (left) and Rz (right) with the fungi *Trichoderma harzianum* (placed in the centre using an agar cube); 24 h after incubation

After 24 h of incubation no growth of Trichoderma was observed. Px colonies were growing everywhere but not on the cube whereas *Bacillus* colonies were visible everywhere around the cube but kept a small distance of ~1 mm from the cube. Only after 12 days of incubation at 27°C in half of the agar plates fungal growth was visible indicating a competitive relationship between bacteria and fungi but no lethal effects. This is similar to results from plating soil suspensions. Here agar plates are normally free from fungal growth if the number of bacterial CFUs is high whereas the opposite is observed if bacterial population is small or conditions for bacteria are suboptimal.

3.4.1.3.2 Pre-harvest

During the experiment stem diameter and plant height measurements showed that the P_Ctrl always performed best with significantly improved plant growth as compared to the Ctrl in most cases. Px treatment continuously showed the best results for the BEs with the tendency for growth improvement as compared to the Ctrl whereas the Rz treatment showed detrimental effects on plant growth leading to significant differences between Px and Rz treatment. The double and triple inoculation did not show clear trends in comparison to the Ctrl. All treatments, except the P_Ctrl, showed similar symptoms of leaf chlorosis indicating P-deficiency of maize plants.

3.4.1.3.3 Post-harvest

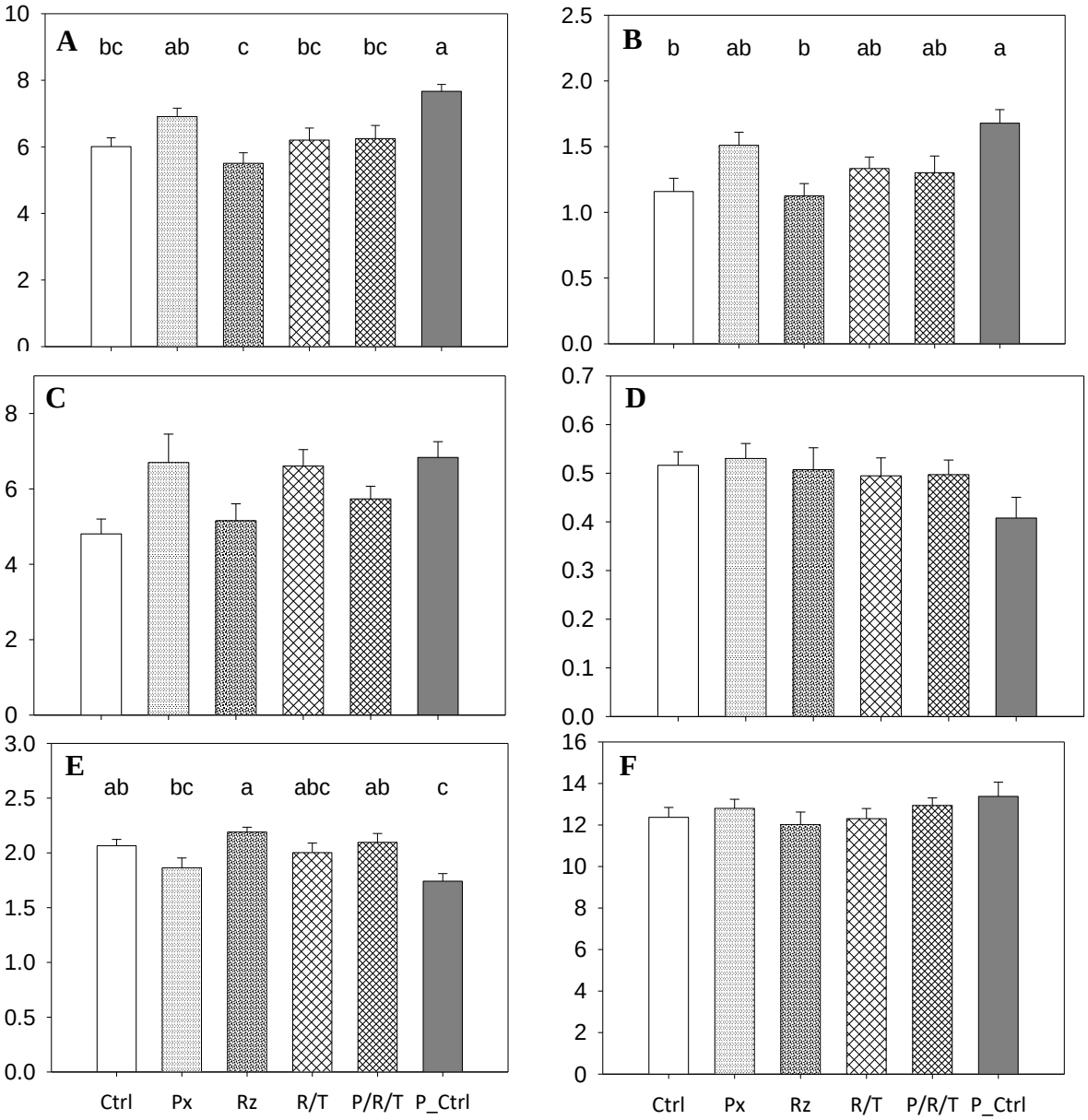


Figure 3-31 Results from post-harvest analysis of Exp_4; Shoot DW (**A**) and root DW (**B**) in g; root length in m (**C**), mycorrhization rate in % (**D**), shoot P concentration in mg g^{-1} DW (**E**), P content in mg $shoot^{-1}$ (**F**); Means +SE

Shoot DW and root DW 47 DAS differed significantly among treatments whereas for both measurements similar trends were observed. P_Ctrl was highest followed by Px treated plants. Plants of the double and triple treatments grew slightly better than the Ctrl plants whereas Rz plants showed reduced growth as compared to all other treatments. Px treatment was the only treatment in which shoot DW did not differ significantly from the P_Ctrl and Px plants performed significantly better than Rz plants. Root length was improved by Px and Rz/TP treatment as compared to Ctrl. Phosphorus analysis in shoots showed exactly opposite results as observed for the shoot DW and therefore increased plant weight correlated with reduced P

shoot concentration leading to similar shoot contents for all treatments, although P_Ctrl plants had a slightly higher P content than the other treatments.

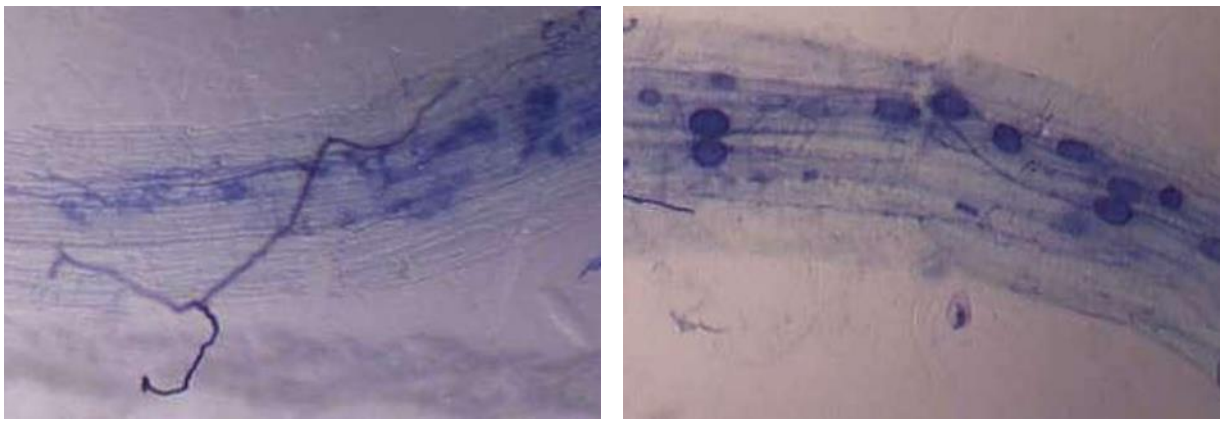


Figure 3-32 Mycorrhizal structures in maize roots after staining with blue ink as described by Vierheilig et al. (1998); External hyphae and arbuscles (left) and vesicles (right)

Mycorrhiza analysis could confirm intense maize root colonization by AM fungi with clear visual identification of mycorrhizal structures such as arbuscles, vesicles and hyphae. In the P_Ctrl mycorrhizal infection was lowest, as expected from results from literature, but not strongly reduced. Nevertheless, no clear BE effect on mycorrhization could be observed even though in the Px treatment the mycorrhization rate was slightly increased.

Both the low P contents and the relatively high mycorrhization rate suggest that the additional P-fertilization in the P_Ctrl was not completely sufficient for optimal plant growth.

3.4.1.4 Results Exp_5

Exp_5 was conducted under exactly the same conditions as Exp_4 but tomato was investigated instead of maize.

3.4.1.4.1 Pre-harvest

Measurements of plant height, total leaf number and plant developmental stages (BBCH) during the course of the experiments did not show strong differences among treatments.

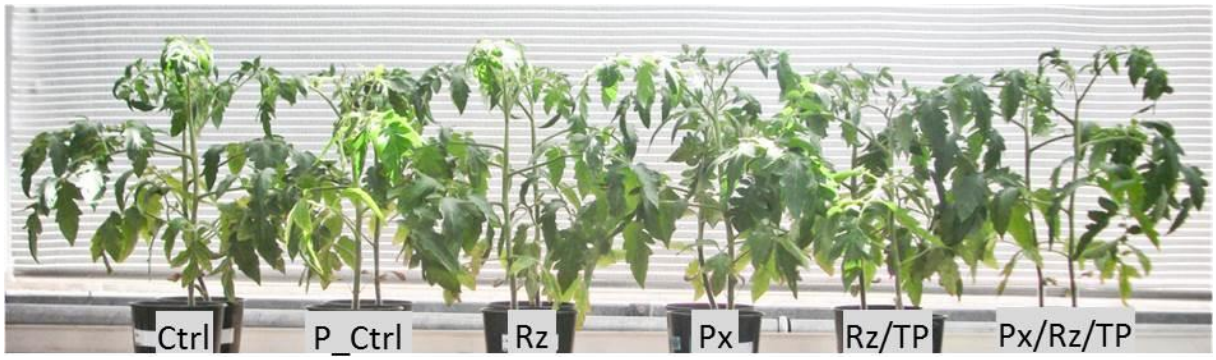


Figure 3-33 Plant habitus Exp_5 (55 DAS)

Even the P_Ctrl treatment did not always differ significantly from other treatments suggesting other growth limiting factors (see Figure 3-34). Symptoms of leaf chlorosis suggest K deficiency. Counting of the number of flowers per plant in the late stages (56 DAS) showed some more pronounced difference between Ctrl and P_Ctrl, with about 50 % more flowers in the P_Ctrl. Similar trends as in Exp_4 could be observed for the BE treatments with the Px treatment generally having the best performance with the exception of the number of flowers. Here the triple treatment had the highest average number (~20 %). No significant difference between Ctrl and BE treatments could be observed.

Figure 3-34 Chlorosis in P_Ctrl plants Exp_5 (47 DAS)

3.4.1.4.2 Post-harvest

Shoot DW was highest in the P_Ctrl but did not significantly differ to the Ctrl. The second highest shoot weight was found in the Px treatment. Lowest values were found for the Rz and Rz/TP treatments that differed significantly from the P_Ctrl. Root DW did not differ significantly among treatments but was highest in the Px and Ctrl treatments and lowest in the double and triple treatments. Nevertheless, root fresh weight of the P_Ctrl plants was much higher than root weight in the other treatments suggesting an error in the dry weights. This is supported by the results from measurement of

root length that also was increased in the P_Ctrl plants. Ctrl plants had the second longest roots.

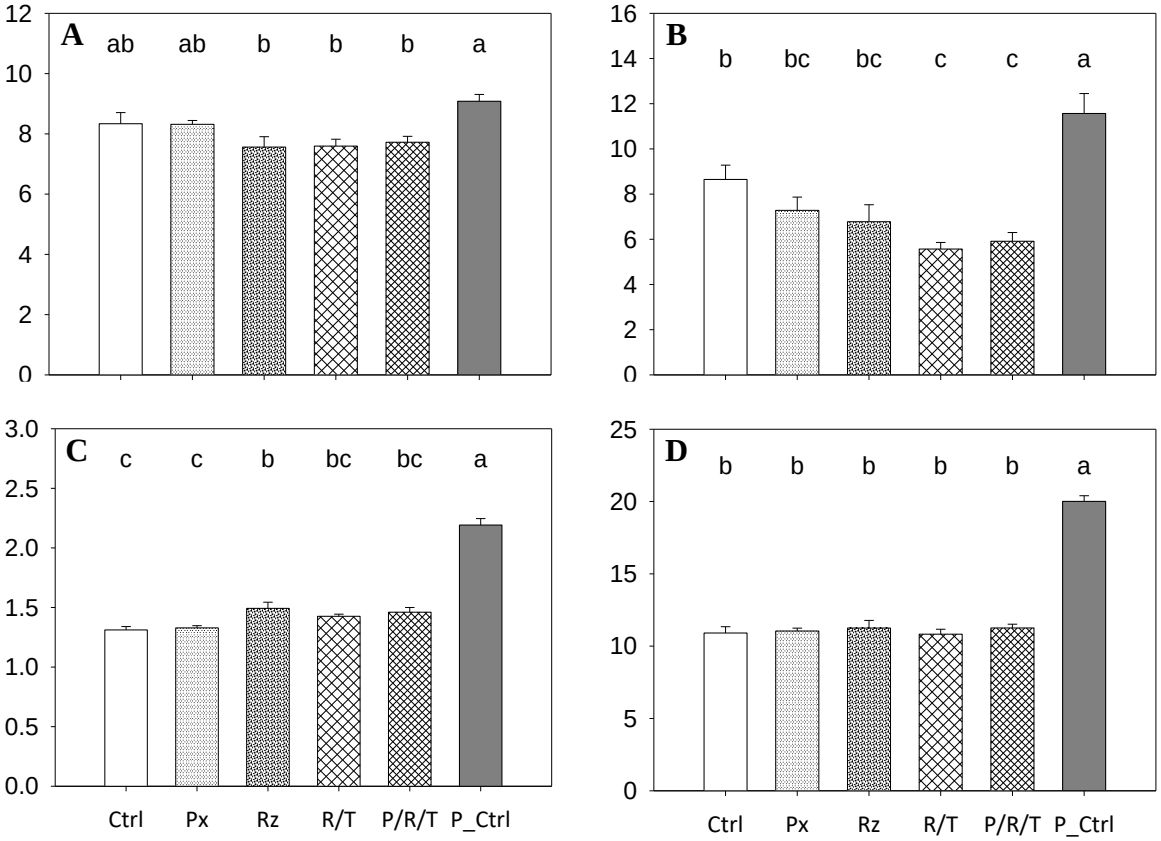


Figure 3-35 Results from post-harvest analysis of Exp_5**;** Shoot DW (**A**) in g; root length in m (**B**), shoot P concentration in mg g^{-1} DW (**C**) and P content in mg $shoot^{-1}$ (**D**); Means +SE

P concentrations in P_Ctrl were highest followed by the Rz treatment with the smallest plants. P contents per plant did significantly differ between P_Ctrl and all other treatments whereas all other treatments had almost equal contents. In contrast to the strong mycorrhization observed for the maize plants in Exp_4 no mycorrhizal structures could be found in any of the tomato roots.

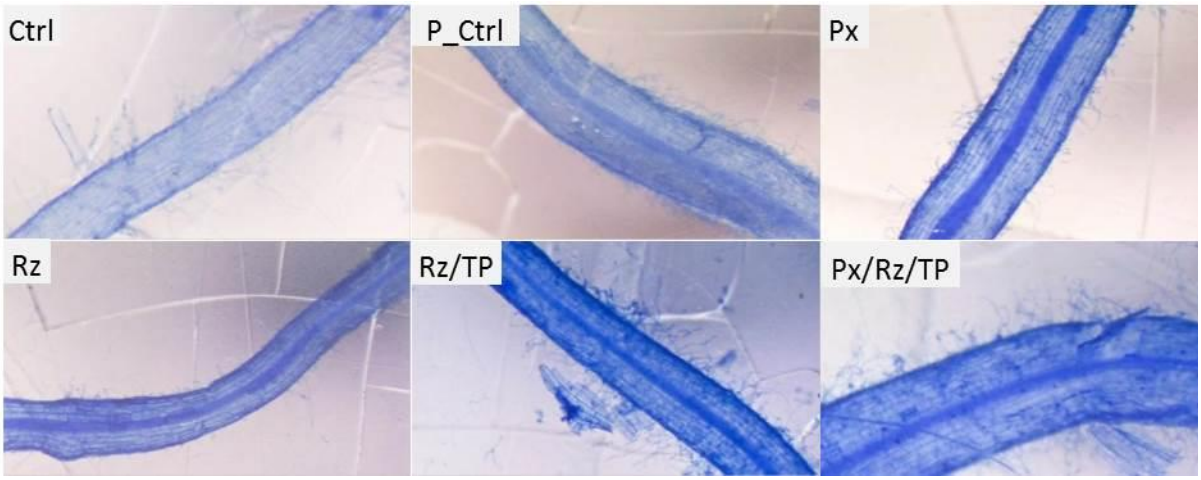


Figure 3-36 Pictures from tomato roots Exp_5 after staining with ink; no mycorrhizal structures could be found

3.4.1.5 Discussion Exp_4 and 5

Experiments at JKI Braunschweig

During experiments in the working group of Cornelia Smalla at the research institute JKI Braunschweig (Eltlbany et al., 2019) strong growth promoting effects were observed with more than 100 % more biomass in the *Pseudomonas jessenii* (Pj) and Rz treated maize and tomato plants as compared to the Ctrl (Figure 3-37).

To find out which conditions caused these tremendous effects, growth experiments were conducted applying similar conditions as at JKI. Due to a misunderstanding during the correspondence with the JKI working group, fertilizers were calculated per kg of soil whereas at JKI fertilization was calculated based on kg of substrate. Therefore only 66 % of the fertilization rates used at JKI where applied in Exp_4 and Exp_5. Additionally, sand contents were lower than at JKI.

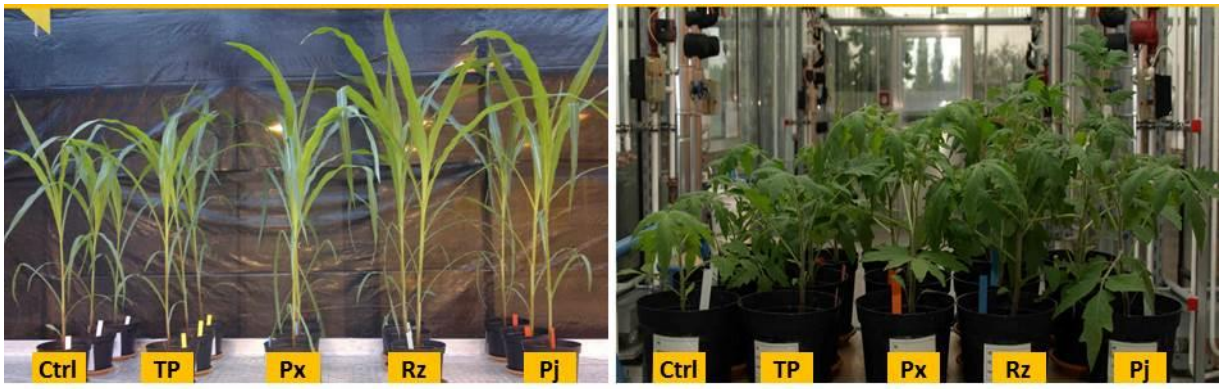


Figure 3-37 Growth promoting effects observed at JKI Braunschweig

Exp_4 (maize)

The results in Exp_4 did strongly differ from those at JKI. For the Px treatment at least similar trends were observed whereas Rz had little or negative effects on plant performance. The less pronounced effect of the Px treatment could possibly be explained by the difference in fertilization. As later on seen in Exp_11 and Exp_14, this cannot be the only explanation for the Rz treatment as adapting the fertilization rates to those applied at JKI did not lead to a reproduction of the effects seen for Rz.

In Exp_2, 3 and 4 fertilization rates and substrate mixture were the same. A comparison of Exp_2 and Exp_4 shows that the difference between the Ctrl and the P_Ctrl treatment was much more pronounced in Exp_2. Both experiments had similar fertilization rates and also the P_Ctrl was fertilized in the same way. The additional N fertilization in Exp_2 was applied very late and no N deficiency symptoms were seen in Exp_4.

Furthermore, comparing the plant heights from the three experiments it can be seen that plants were much higher in Exp_4. In Exp_2 plant height in the Ctrl treatment 49 DAS was about 78 cm and in Exp_3 plant height 55 DAS was about 85 cm whereas in Exp_4 plant height was abut 110 cm already 47 DAS (*data not shown*). At this time point in Exp_4 no difference between the P_Ctrl and the other treatments were observed.

This suggests that first, some other factor was limiting the growth of the P_Ctrl plants and that second, something was inducing an increased growth of maize plants. Tomato plants from Exp_5 growing in the same climate chamber as maize plants from Exp_4 also showed an intensified length growth suggesting that the light conditions were suboptimal for both plants. In general, plants in the greenhouse or climate chamber experiments were growing much thinner and higher than in the field. But here an additional factor plays a role. It was observed that larger pots also increase stem diameter and decrease plant height. A previous study showed that the two environmental factors low-light intensity and neighbouring plants are leading to a stem elongation in *Arabidopsis thaliana* via a change in auxin production or sensitivity to auxin signalling (Hersch et al., 2014). The environmental signals for both factors are perceived as changes in the red to far red light (R:FR) ratio. In pot experiments plants are normally closer together than in natural environments and plants may therefore exhibit different growth properties but also the mechanical sensing of the pot by the growing root might be a signal for triggering changes in plant growth (Chen et al., 2012; McConnaughay and Bazzaz, 1991). The influence of light on BE-plant interaction is discussed separately in 4.3.3.

No synergistic effect for BE product combinations

The double or triple treatments, combining different BE products, did not result in synergistic or accumulative effects. With the exception of P uptake the combined BE application was less effective than the Px single treatment. A more detailed discussion on BE product combinations and comparison with results from literature is given in 4.3.2.

The results did also not indicate that the BEs were acting as mycorrhiza helper bacteria (MHB) (Frey-Klett et al., 2007; Garbaye, 1994). The potential of the Px and Rz strain to improve mycorrhization was shown when mycorrhizal inoculum was added in the experiment (Yusran et al., 2009), nevertheless in our experiment this effect could not be reproduced for the natural mycorrhiza. The only clear difference between treatments was observed for the P_Ctrl that showed a reduced mycorrhization as compared to all other treatments.

Exp_5 (tomato)

Similar to Exp_4 the BE effects observed at JKI could not be reproduced. Additionally, tomato plants were inhibited in their growth by all BE treatments except Px. In Exp_5 the P_Ctrl plants were more strongly growth promoted than in Exp_4, but still other factors, probably also other nutrients such as K, were limited so that the full growth potential of the P_Ctrl plants could not be reached. This is strongly supported by the fact that the P concentration was strongly increased in P_Ctrl and that the plants were not able to use the P for biomass production.

Simultaneously to Exp_4 and Exp_5, negative effects by the BEs on maize growth were observed in another experiment of the working group (Kuhlmann, 2014). In this experiment the C-loess subsoil, containing mainly Ca-phosphate with low P availability, with additional P-fertilization as rock phosphate, was used. It was hypothesized that soluble P was strongly limited, leading to a competition for the nutrient between microbes and plant (Zhang et al., 2014). Although P immobilized by rhizobacteria in the form of microbial P is continuously remineralized, the growing root might not be able to take up this P as mature root tissues show less capacity for nutrient uptake (Marschner et al., 2011). Additionally, a rapid growth in the first days after application may increase immobilization. It was also observed that PGPR density often rapidly declines after application suggesting a remobilization of P from Pmic. Nevertheless, also P mineralisation is strongly repressed if C:P (carbon:phosphorus) ratio are to high (Zhang et al., 2014). Starter fertilization of soluble P or ammonium fertilization (4.3.4) are possibilities to decrease C:P ratio in the soil solution.

Interestingly, it was recently shown that *Bacillus amyloliquefaciens* inoculation in soils reduced abundance of protist – that act as predators of other microbes – thereby possibly decreasing cycling and remobilization of nutrients in the soil (Xiong et al., 2018).

Other explanations for negative plant-microbe interactions might be the additional stress for the plant, e.g. by triggering defence responses (“bad timing”, see 3.11.5.3.2 and 4.1.6.3) and the trade-offs for assimilates commonly observed for plant-microbial interactions. This is further discussed in 4.3.3.

3.4.2 PGPR effects under various P-fertilization rates (Exp_7)

3.4.2.1 Introduction Exp_7

In both previous tomato experiments (Exp_5 and Exp_6, see 3.4.1.4 and 3.5.1.3) standard deviations were high, leading to non-significant results for treatment comparison. Therefore in Exp_7 a pre-cultivation period was included to have equally sized plants for investigation of BE treatments. Additionally, different P levels were tested in combination with BE treatments to investigate optimal conditions for maximal BE effects.

3.4.2.2 Experimental design Exp_7

Tomato plants were pre-cultivated in a commercial propagation substrate "TKS1 Instant Plus Aussaat und Pikieren" (Floragard Vertriebs GmbH für Gartenbau, Oldenburg, Germany), composed of 100% peat fertilized with plant available mineral fertilizers at a concentration of 140 mg l^{-1} N, 80 mg l^{-1} P (P_2O_5), 190 mg l^{-1} K (K_2O) and 80 mg l^{-1} Mg and 50 mg l^{-1} S. The substrate was mixed with 20% sand. 17 DAS evenly sized tomato plants were selected and transplanted into bigger pots containing soil substrate. Fertilization rates were increased (standard fertilization but based on total substrate weight) and additionally three different P fertilization levels were tested (Table 3-14). At the time of transplantation first BE application was performed at a higher rate of 10^{10} CFU kg^{-1} soil. This treatment was repeated three times until 43 DAS.

Table 3-14 Treatments Exp_7

Trt_Nr	Treatment	Rate	Total
1	Ctrl_50	/	/
2	Rz_50	5.00E+09	2.00E+10
3	Px_50	5.00E+09	2.00E+10
4	Ctrl_80	/	/
5	Rz_80	5.00E+09	2.00E+10
6	Px_80	5.00E+09	2.00E+10
7	Ctrl_120	/	/
8	Rz_120	5.00E+09	2.00E+10
9	Px_120	5.00E+09	2.00E+10

BE application rates in CFU kg^{-1} substrate: Four soil applications with 15 ml of 10^9 CFU ml^{-1} solution from 17 DAS on weekly; total amount r = 5

3.4.2.3 Results

Due to the pre-cultivation standard deviations were extremely low providing good conditions for statistical analysis. Nevertheless, only limited responses by treatments were observed. Neither BE treatment nor an increased P-fertilization, did further improve plant performance. Measurements of shoot height and analysis in a two-way-ANOVA indicated a significant reduction of plant growth with higher fertilization rates 36 and 42 DAS (19 and 25 days after transplantation). Px treatment had the same effect whereas Rz treatment slightly stimulated shoot growth. Nevertheless, these pre-harvest results did not reflect the results from plant biomass after harvest. No differences or trends could be observed for the shoot dry weight but

root dry weight was enhanced (not significantly) in the Px treatments as compared to Ctrl and Rz treatments.

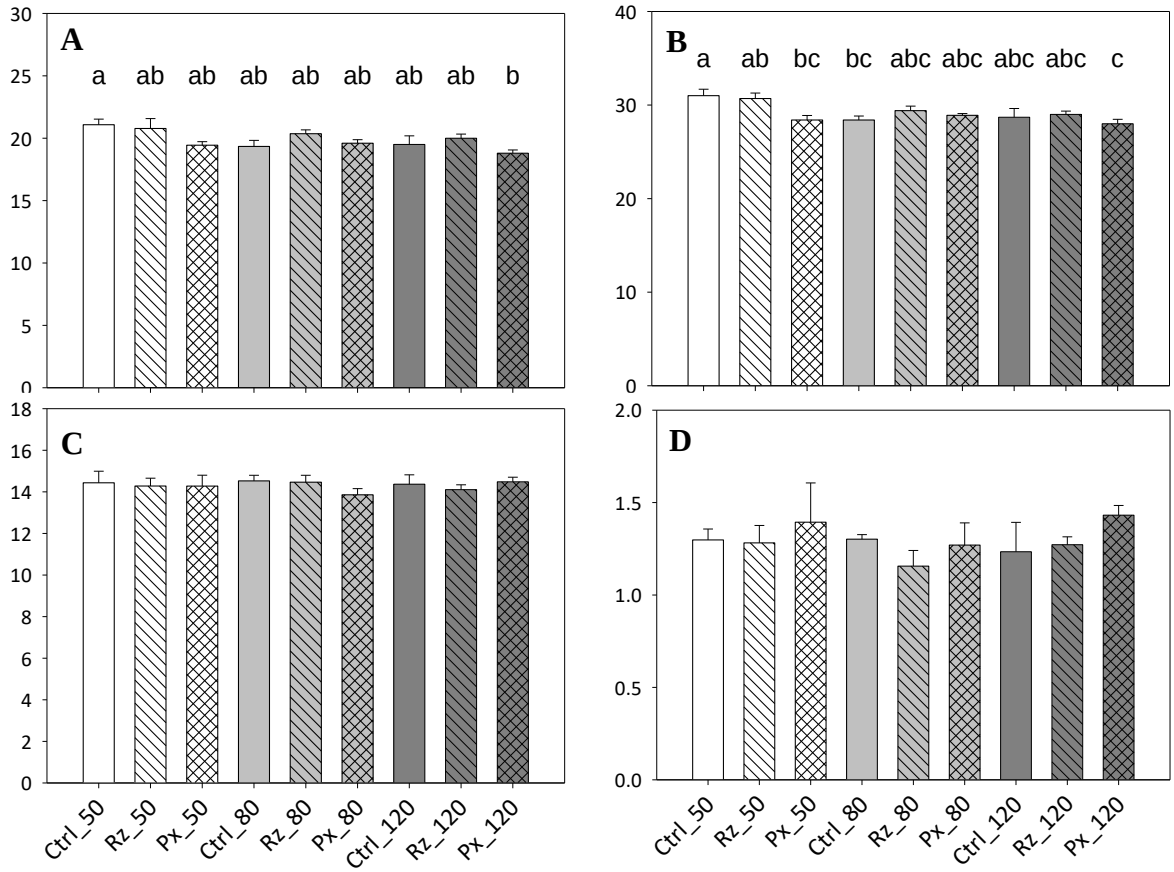


Figure 3-38 Results from Exp_7; Shoot height in cm 36 DAS (A) and 42 DAS (B), shoot (C) and root (D) DW in g pot^{-1}; Letter display reflects results from One-Way-ANOVA; In Two-Way-ANOVA treatments with 50 mg P had significantly higher plants than treatments with 80 and 120 mg P; Px treatment reduced shoot height significantly as compared to Rz treatment

3.4.2.4 Discussion Exp_7

No BE effects under high nutrient supply or late BE application

Due to a very long pre-cultivation and a growth substrate that was very rich in nutrients, plants of all treatments were developing equally independent of the P fertilization rates after pre-cultivation. This was surprising as in Exp_6 the rate of 25 mg P (+ 25 mg in a second fertilization) was too low and limited tomato growth. In Exp_7 the lowest P fertilization rate of 50 mg was already sufficient for optimal plant growth. No further increase was observed with higher fertilization rates. A review on the importance of sufficient P supply in early plant development concluded that early plant development was crucial for the biomass at harvest whereas late application often did not affect yield anymore (Grant et al., 2001). This suggests that any BE-derived plant growth promotion that was based on an improved P status of the plant would be ineffective.

Additionally, BE application was probably too late to exhibit stimulating effects. Although experiments from partner institutes in Romania showed strong effects for BE application after late application (10 - 14 DAS), late application in Exp_7 and Exp_10 did not result in effective plant growth stimulation. However, for Exp_10 other reasons are discussed that could explain the missing effects.

Similar to Exp_4 and 5, for Exp_7 suboptimal light and temperature conditions could be further explanations for the lack of effects. Exp_6 (see next page) and 7 were both conducted in the greenhouse without additional heating system but Exp_6 was conducted during late summer time with higher average temperature and irradiation whereas Exp_7 was conducted from October to mid November. At this time average temperatures and light intensity had already decreased markedly.

3.5 Microbial interaction

3.5.1 PGPR effects in a heat treated soil (Exp_6)

3.5.1.1 Introduction Exp_6

Research on PGPR is often conducted in sterile substrates, thereby controlling biotic factors that would influence the outcome of the PGPR-plant interaction and increasing the possibility for root colonization analyses. Nevertheless, those results are only of interest for basic research questions but do not give any information on the relevance of PGPR application for plant growth stimulation under practice conditions. Therefore, in most experiments of this thesis the use of sterile substrates was avoided. Exp_6 was an exception, as in previous experiments the strong effects of BE application, often reported in literature, could not be reproduced and it was hypothesized that the main reason is the difference in the substrates.

3.5.1.2 Experimental design Exp_6

For Exp_6 three bacterial BEs and two different substrates were tested on tomato plants (Table 3-15). For both substrates the Kr 1 soil was used but one substrate was "sterilized" whereas the other soil type contained a natural microflora. To sterilize soil tyndallisation – a discontinuous, fractionized heat sterilization – was used. Soil was distributed into various metal bowls and then incubated for 24 h at 85°C in a heater. After incubation the dried soil was re-wetted and incubated at room temperature for further 24 h. By this, a germination of endospores should be provoked. After incubation at RT bowls were again incubated at 85°C. The procedure was repeated another time. As it is known that heat treatment is able to cause physical and chemical changes in the soil matrix (Berns et al., 2008; Liegel, 1983), e.g. increasing Mn availability (Singh and Pathak, 1970) sometimes up to plant toxic levels (Boyd, 1971), it was decided to use the same "tyndallised" soil for both substrates but one substrate was re-inoculated with a natural microflora. For this, non-sterile soil was extracted using a 0.1 % peptone solution in a 1:4 ratio. During soil fertilization 40 ml of this suspension was applied to non-sterile "standard" treatments (odd numbers) and to 40 ml of sterile 0.1 % peptone solution was added to the "sterile" treatments (even numbers with T for tyndallisation). Additionally, in all treatments a commercial mycorrhiza product containing 17

Table 3-15 Treatments Exp_6

Trt_Nr	Treatment	Seed	Soil
1	Ctrl	/	/
2	Ctrl_T	/	/
3	Rz	1.00E+09	5.00E+08
4	Rz_T	1.00E+09	5.00E+08
5	Px	1.00E+09	5.00E+08
6	Px_T	1.00E+09	5.00E+08
7	Pj	1.00E+09	5.00E+08
8	Pj_T	1.00E+09	5.00E+08

Tyndallisation (T); BE application: 1. before sowing seed soaking for 2 min in a 2.5 mM CaSO4 suspension (ST), rates in CFU ml^{-1}; Three soil applications with 15 ml of a diluted suspension 0, 14 and 28 DAS, rates in CFU kg^{-1} substrate; r = 5

infectious units *Rhizophagus irregularis* (former *Glomus intraradices*) g^{-1} (Vitalin Pflanzengesundheit GmbH, Ober-Ramstadt, Germany) was added, using 1 g product 5 kg^{-1} soil. Substrate was standard fertilized but due to the higher sand contents fertilization rates per substrate were lower than in Exp_5 (50 mg N, 25 mg P, 75 mg K, 25 mg Mg kg^{-1} substrate). Therefore four weeks after sowing the same amount of N, K, and Mg was again fertilized due to symptoms of nutrient deficiency. Additionally to seed soaking, soil application of BEs was performed directly after sowing. Two further BE applications were done 14 and 28 DAS but BE concentrations were decreased to get application rates as in the field (10^9 CFU kg^{-1} soil).

3.5.1.3 Results Exp_6

Exp_6 was conducted using semi-sterilized soil that was treated by tyndallisation. To compare a heat-treated soil with a "standard" soil half of the soil was re-inoculated with a soil suspension from untreated soil. After plating of soil suspensions from tyndallised soil many colonies were growing on non-selective LB medium or semi-selective NP medium, showed that no sterilization was achieved by heat-treatment. Nevertheless, plating assays indicated a significant impact on the composition of the microflora as seen by comparison of the incubated agar plates from heat-treated soil and untreated soil (Figure 3-39). Diversity of the microflora was reduced, especially the fungal growth, but the amount of Pseudomonades (see NP medium) was enriched.

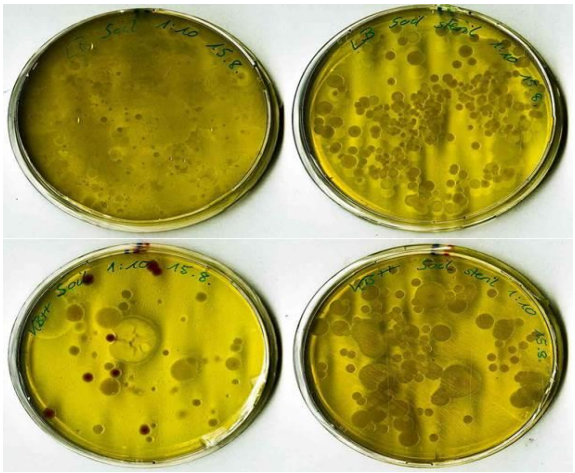


Figure 3-39 Plating assay for soil types Exp_6; Soil suspension from untreated soil on LB (top left) and NP (bottom left) and heat-treated soil on LB (top right) and NP (bottom right) medium

Figure 3-40 P-Deficiency in tomato plants of Exp_6 (59 DAS) indicated by purple leaf chlorosis

3.5.1.3.1 Pre-harvest analysis

Plant growth in the early plant development was significantly influenced by BE application. Measurements of leaf area and developmental stage were conducted but results were most significant for plant height. Significant differences in two-way-ANOVA for the shoot height,

defined as the height from soil surface to the plant meristem, were observed 25 DAS. The treatment with *P. jessenii* (Pj) led to reduced growth in comparison to the Ctrl and the Rz treatments. From there on the influence of the different soil type became more significant as seen in analysis of shoot height 31, 35, 45 and 52 DAS by two-way-ANOVA but vanished 56 DAS (Figure 3-41). No significant interactions between BE treatment and soil type were observed due to high standard deviations but graphs show that growth stimulation of the BE treatments was strongly increased in the soil lacking the re-inoculation of the natural soil microflora. No clear differences among soil types were observed in the Ctrl treatments whereas BE treatments seemed to stimulate plant growth only in the disturbed (T) substrate. Leaf chlorosis in all plants indicated P-deficiency (Figure 3-40).

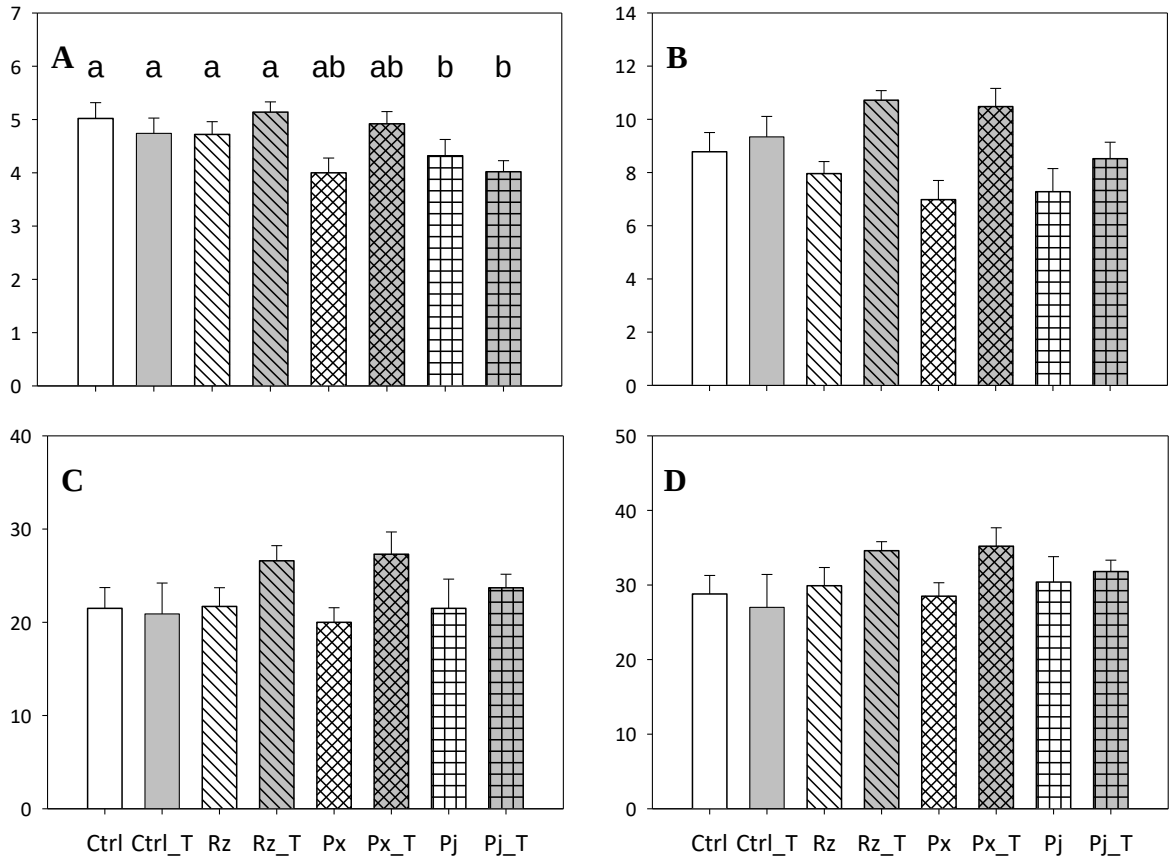


Figure 3-41 Pre-harvest results Exp_6; Shoot height (cm) measured 25 (**A**), 35 (**B**), 52 (**C**) and 59 (**D**) DAS; Significant increased shoot height in two-way ANOVA for Rz and Ctrl as compared to Pj (**A**) and in the tyndallised soil as compared to the re-inoculated soil (**B** + **C**)

3.5.1.3.2 Harvest

60 DAS plants were harvested. Root and shoot biomass differed strongly among treatments although differences were not statistically significant. Obviously, Ctrl plants suffered in the tyndallised soil, showing reduced shoot and root growth, but BE application, especially Rz and Px, could reduce these negative effects improving plant growth as compared to the Ctrl treatment up to 50 %. Root length was only measured for the treatments Ctrl_T, Rz and Rz_T

that differed most significantly in their fresh root weight. Although some growth stimulation by Rz treatments was observed, as seen for the root DW, treatments did not differ significantly. Due to the time-consuming procedure of scanning large tomato roots and a low probability to get additional information, it was decided not to continue root length analysis.

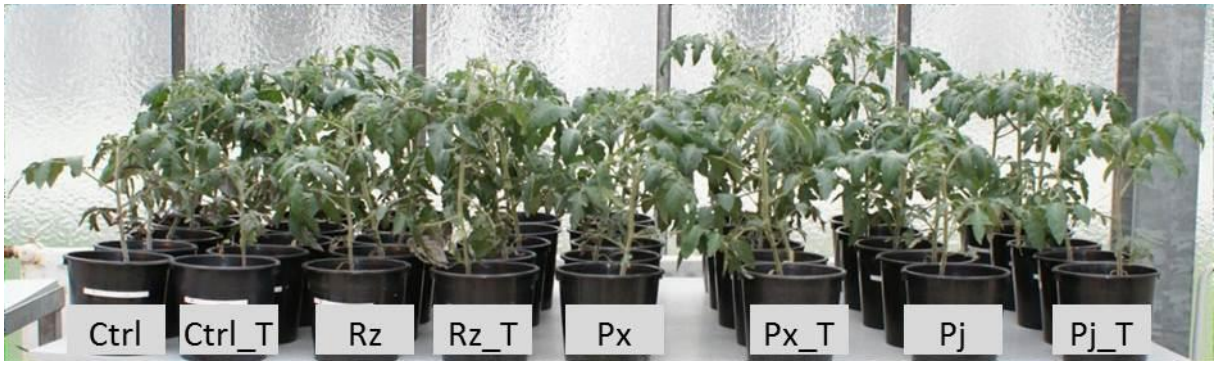


Figure 3-42 Plant habitus Exp_6 (59 DAS)

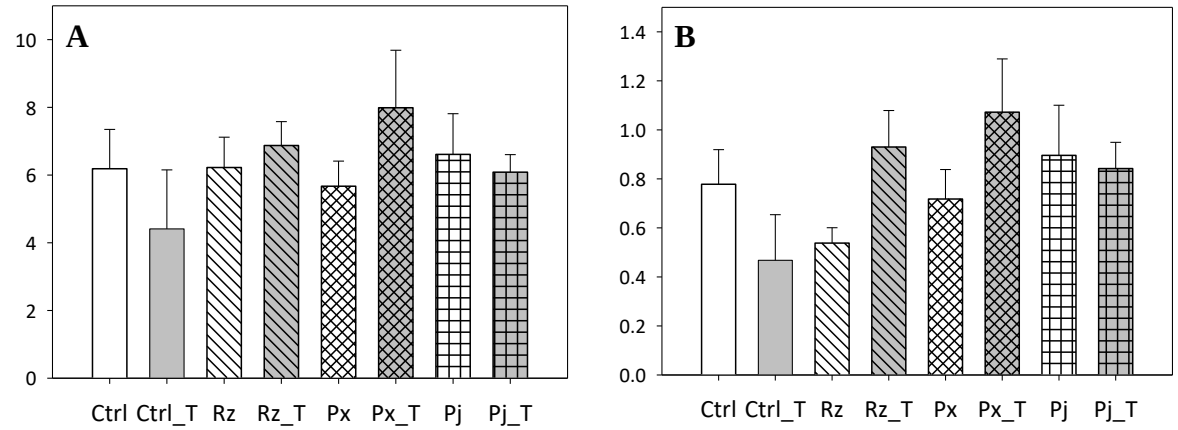


Figure 3-43 Harvest results Exp_6; Shoot (**A**) and root (**B**) DW in g pot^{-1}

During the analysis of tomato roots from Ctrl, Rz and Px treatments after staining in ink, again no mycorrhizal structures could be observed.

3.5.1.4 Discussion Exp_6

Soil sterilization affects BE-plant interaction

To prove the hypothesis that disturbed or sterile soils improve the effectiveness of BEs the substrate was manipulated by using tyndallisation. Tyndallisation was used as soil sterilization by irradiation is cost intensive and was not feasible at our facilities. Additionally, more intense methods of heating often cause disturbance of physical soil properties or a change of chemical properties, e.g. leading to the risk of Mn toxicities (Fujimoto and Sherman, 1948; Jager et al., 1969; Singh and Pathak, 1970).

Originally, the aim was an almost complete sterilisation of the soil substrate but the method was not successful as seen by the plating assays for the soil suspensions. Nevertheless, the

method was leading to a change in the microbial community. Similar to what happens when soils are naturally or manually infested by pathogens, the equilibrium of the microbial community was disturbed leading to disease conducive soils (Mendes et al., 2011). The results support this theory as strong plant growth promotion effects were observed for the BE treatments, in case of the Rz treatments for the first time during the thesis. Although treatment differences were not statistically significant due to extreme variation in between biological replicates – probably as a response to the disturbed equilibrium –, shoot and root growth in the tyndallised soils were improved by Rz and Px treatments by 56 / 81 % (shoot DW) and 100 / 130 % (root DW), respectively, as compared to the non-BE treated Ctrl.

Conversely, in the "normal soil" that was re-inoculated with natural microflora using a soil suspension, the BE (Rz and Px) inoculum was negatively affecting plant growth as compared to the Ctrl treatment. This can eventually be explained by the double stress in the re-inoculated soil caused by BE application and natural soil microflora. Both communities were probably competing with each other making a root establishment in the soil difficult.

Pj strain was not effective for plant growth stimulation

The Pj strain was only used in this experiment. Although it showed strong growth promotion effects in the JKI experiments, these effects were not observed in Exp_6. Additionally, the efforts of the company Sourcon Padena to formulate the Pj strain with the same cell density per g product as the Px strain failed (*personal communication* with K. Mai (Sourcon Padena), 2016), leading to lower concentrations by the factor 10 and therefore the need to apply higher dosages of milk powder if application rates, based on number of CFU, should be kept the same for all bacterial products. As discussed in 4.1.4.2 the milk powder formulation provided nutrients and therefore strain specific effects could not be investigated properly. An investigation of the unformulated strain, as done at JKI, was not further conducted because of a missing relevance for applications under field conditions.

3.6 Root colonization

3.6.1 Induced Growth Stimulation and Nutrient Acquisition in Maize: Do Root Hairs Matter? (Exp_17)

3.6.1.1 Introduction

The genotype of the host plant determines root morphology, root exudation and the mechanism for nutrient acquisition, factors that are crucial for compatibility with PGPR strains (Bais et al., 2006; Yang, 2016). Root hairs are important sites for water and nutrient uptake e.g. by largely increasing root surface but their contribution to root exudation and root colonization by rhizobacteria is not well understood (Neumann and Römheld, 2002). Nevertheless, investigations on endophytic root colonizing *Pseudomonas* strains indicate a pivotal role of root hairs for root colonization (Prieto et al., 2011). In this context, mutants or genotypes affected in root hair production provide a tool to study the impact of root hairs on rhizosphere processes (Gahoonia et al., 1997). Wen and Schnable (1994) found three root hairless (rth) maize mutants during a screen of mutants derived from a transposon stock. The rth2 mutant showed root hair length about $^1/_4$ to $^1/_5$ of the size of wild type maize plants but a still vigorous and healthy growth when grown in hydroponic systems with sufficient nutrient supply.

Objective of Exp_17 was to compare the rth2 mutant and the corresponding wild type to investigate the significance of root hair development for the establishment and efficiency of host plant interactions with the Px strain.

3.6.1.2 Experimental design Exp_17

For Exp_17, plants were grown in rhizoboxes to observe the root growth using a root observation window (Figure 3-44). As heterozygous rth2 mutants were used, seeds were pre-germinated on filter paper and then selected for their root hair growth via a binocular at x40 magnification. For pre-germination, seeds were first sterilized by soaking in 10% H_2O_2 for 2 min. After sterilization seeds were washed and then incubated overnight in aerated 10 mM $CaSO_4$ solution. The next day seeds were divided into two groups, whereas one group was soaked in Px suspension (10^9 CFU ml^{-1} suspension) for 1 min, and then pre-germinated separately wrapped in filter paper soaked with 10 mM $CaSO_4$. After 3 days at 24 °C in the dark, the seedlings were observed for root hair morphology. Seedlings with long root hairs were used as control plants (wildtype; W) in the following rhizobox experiment, seedlings with short root hairs were taken as rth2 mutants (M). Equally sized seedlings were selected for both treatments.

Figure 3-44 Rhizobox with opened root observation window one week after sowing

A two-factorial experiment with four treatments was designed (Table 3-16). The B73 maize was compared with one of its root hairless mutants (*rth2*) in combination with Px application. Rhizoboxes (35 cm × 10 cm × 2 cm) equipped with root observation windows were pre-filled with equal amounts of 643 g substrate. After placing one seedling into the rhizobox the surface was fixed with a transparent acrylic glass plate. Treatment with Px was performed in total three times. First inoculation was done as seed treatment as described above. Second and third inoculation were done 7 and 14 days after sowing (DAS) during watering of the rhizoboxes with a concentration of 6×10^9 CFU kg^{-1} soil. In contrast to other experiments application was not done from above on the soil surface but during the standard watering process that is performed from the backside of the rhizoboxes through tiny watering holes. By this, water but also the BE suspension were distributed more homogenously in the substrate and over the root surface. Fertilization was based on the standard fertilization with small

Table 3-16 Treatments Exp_17

Trt_Nr	Treatment	Cultivar	BE
1	u/W	B73 WT	/
2	u/M	rth2	/
3	Px/W	B73 WT	4.0E+09
4	Px/M	rth2	4.0E+09

Application rates in CFU kg^{-1} substrate; seed soaking in 10^9 CFU ml^{-1} suspension (ST); Two applications 7 and 14 DAS; Adapted standard fertilization with 87 mg N, 43 mg P, 133 mg K and 43 mg Mg kg^{-1} substrate; r = 5; u = untreated Ctrl

differences in the total amounts. Due to the very small substrate amount total fertilization was relatively low and plants started to suffer from nutrient deficiency about 20 DAS but in this experiment no additional fertilization was performed and plants were harvest already 28 DAS. Main focus of the experiment was the root growth stimulation by Px and the interaction with the plant genotype. Therefore 7, 14 and 21 DAS root length was determined by drawing the roots visible in the root observation window and subsequent scanning of the drawings at a resolution of 400 dpi. At the same time pictures from the root hair zone were taken with an Axio Vision 3.1 video macroscope and additional software (Carl Zeiss GmbH, Jena, Germany) at a magnification of 12.5. From these pictures root hair length was determined by taking the average length of 10 root hairs per plant. After harvest the whole root was washed and scanned, then roots were dried at 60°C and dry weight was measured. All root scans from washed roots and drawings were analysed with the WinRhizo software. Nutrient contents in shoots were determined for the elements P, K, Mg, Ca, Cu, Zn and Mn.

3.6.1.3 Results Exp_17

Exp_17 was conducted to investigate the importance of root hairs for effective plant growth stimulation by the Px product. The results were published in 2018 (Weber et al., 2018).

3.6.1.3.1 Growth development of roots

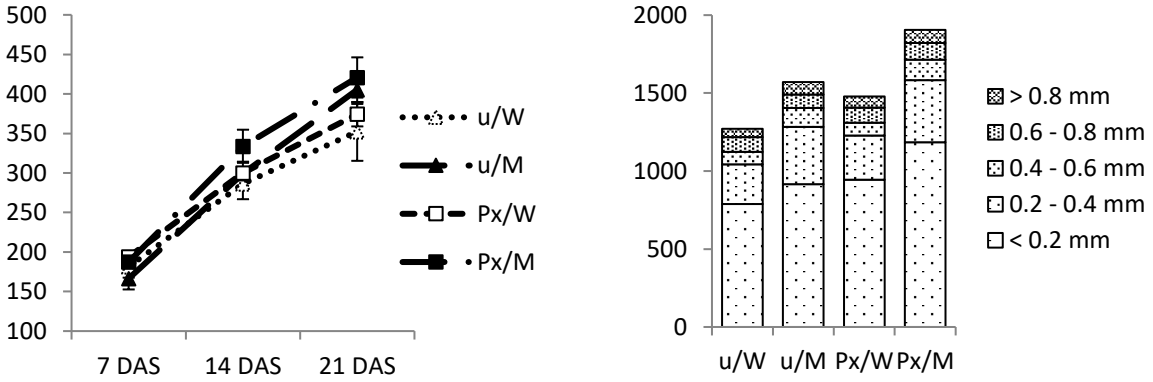


Figure 3-45 Root morphology Exp_17; Left: Root length from root window observations 7, 14, 21 DAS (cm); Right: Root length of different root diameter classes at harvest (cm)

Although root length in drawings of the root window did not differ significantly among treatments, root length of Px treated plants 7, 14 and 21 DAS was higher than that of untreated plants and root length of maize mutants was higher than that of the wildtype plants. Nevertheless the sequence of treatments sorted after root length changed between 14 and 21 DAS from Px/M>Px/W>u/M>u/W to Px/M>u/M>Px/W>u/W indicating that the Px treatment was more influencing at the beginning of the experiment whereas the genetic difference had more influence in the later plant development.

This last sequence was also found after harvest for whole root scanning. Here significant differences in One-Way-ANOVA were found between Px/M and u/W. When performing a Two-Way-ANOVA significant differences between Px treated plants and untreated plants as well as significant differences between mutant and wildtype plants were found. Additionally, Px/M and Px/W differed significantly whereas u/M and u/W differed less, indicating an increased responsiveness of the mutant to Px treatment. A closer examination of the different root size classes reveals that Px treatment significantly increased only the fine root fraction (0 - 0.2 mm diameter), whereas the genetic difference between varieties significantly influenced the first three classes from 0 – 0.6 mm. The classes 0.6 - 0.8 and > 0.8 did not significantly differ in any comparison. Root forks, as an indicator for lateral root branching, was highest in Px/W treatment and was increased by Px treatment by more than 17 % but did not differ significantly.

3.6.1.3.2 Root hairs

Root hair development of rth2 mutants was clearly impaired as already seen after the pre-germination phase. This impairment did continue during the plant development. Root hairs of mutants as compared to wildtype plants did not differ in their density but in their size and remained at a length of about $^1/_4$ to $^1/_5$ of the root hair length of the wildtype, as reported in previous research, when measured three weeks after sowing. Root hair length or density was not significantly influenced by Px treatment.

Figure 3-46 Root hairs Exp_17; Root hair length (RHL) in mm 21 DAS; WT (left); rth2 mutant (right)

3.6.1.3.3 Plant habitus and biomass

Three weeks after sowing first signs of leaf chlorosis were visible, indicating nutrient deficiency. These symptoms were visible in a similar intensity in all plants, irrespective of the treatment and may derive from various nutrient deficiencies, regarding the low fertilization rates and the low concentrations measured in the shoots (see below).

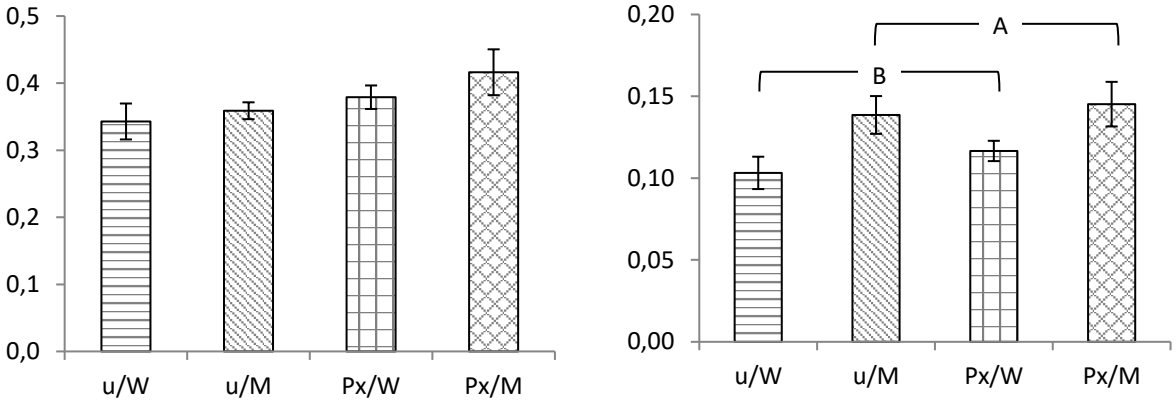


Figure 3-47 Dry weight data Exp_17; Shoot (left) and root (right) dry weight (DW) in g 28 DAS

Shoot and total plant dry biomass did not differ significantly among treatments. Interestingly trends for root weight were the same like those observed for root length but shoot weight differed in its response to treatments. Here the difference between mutants and wildtype was smaller, but Px treatments (Px/M and Px/W) both showed higher shoot biomass than the two other treatments. Root dry weight and root to shoot ratio (R/S) of mutants was significantly higher than that of the wildtype plants.

3.6.1.3.4 Macro- and micronutrients in shoots

Phosphorus

Phosphorus (P) concentration in shoots was low, as expected from the low fertilization level, with no significant difference among treatments and only Px/W showing a slight increase in P concentration as compared to the other treatments. Total shoot accumulation of P was significantly increased in the Px treated plants as compared to the untreated control when performing a Two-Way-ANOVA. One-Way-ANOVA did not indicate significant differences among treatments.

Potassium

Similar to the results found for P no differences were observed for shoot potassium (K) concentration but total shoot accumulation was significantly increased in Px treatment as compared to the untreated plants in a Two-Way-ANOVA. In contrast to the P values the average shoot K concentration was rather high when compared to values from literature (Bergmann, 1993).

Magnesium

Magnesium (Mg) concentration in shoots was low but probably sufficient. A Two-Way-ANOVA indicated significant lower concentration as well as total accumulation of Mg in the wildtype plants as compared to the mutants.

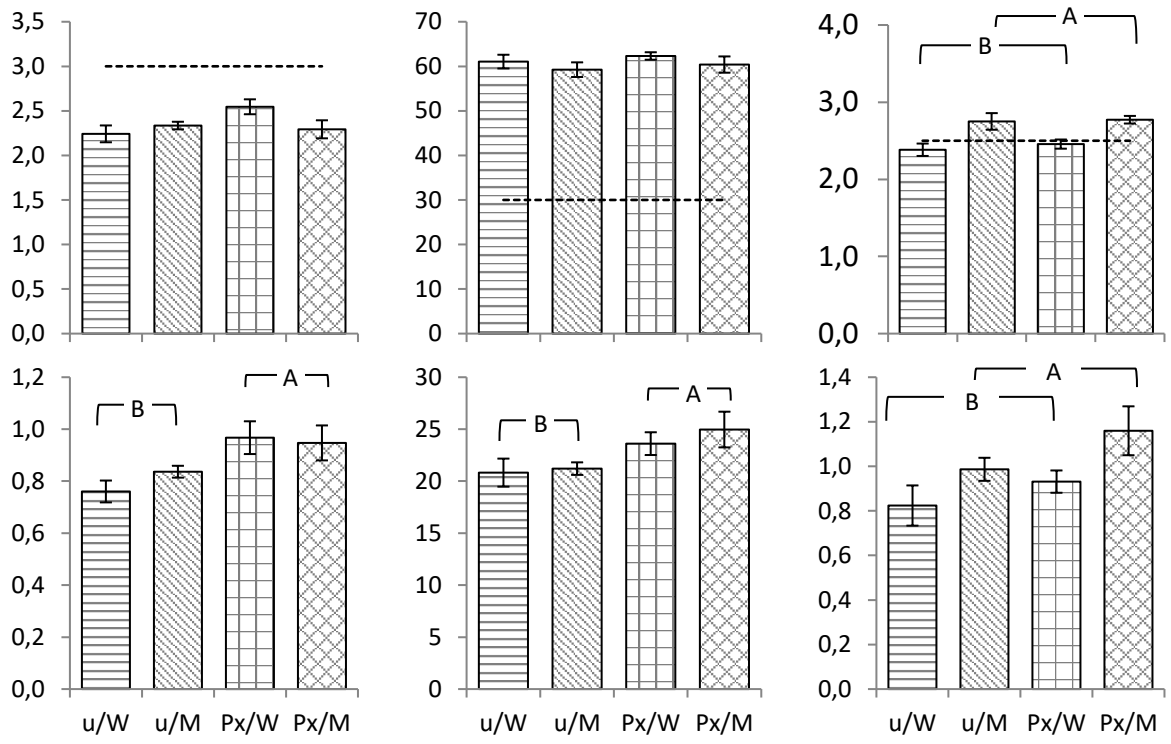


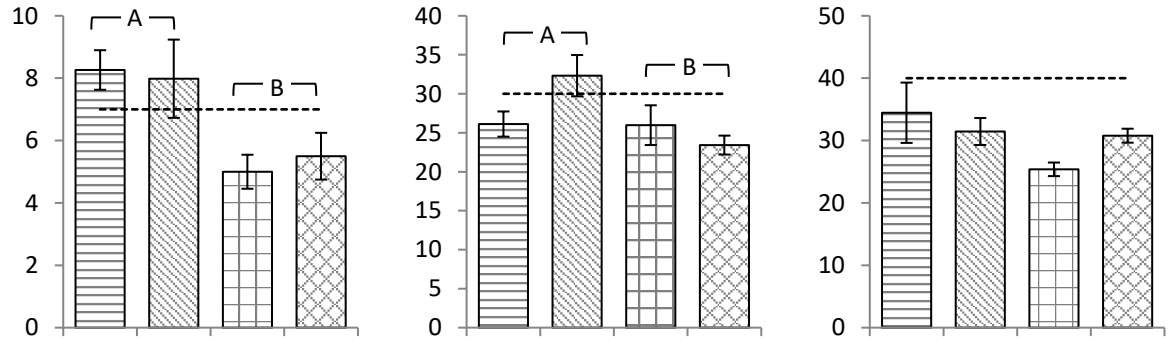
Figure 3-48 Macronutrients in shoots of Exp_17; Upper row from left to right: P, K and Mg concentration (ppt); Below from left to right: P, K and Mg content in mg shoot^{-1}.
Dashed lines indicate lower threshold level (Bergmann, 1993)

Calcium

Calcium (Ca) concentration in shoots was high due to the high amounts of $CaCO_3$ in the soil. u/W showed the highest Ca concentration and Px/M highest total accumulation but no significant differences among treatments were observed (data not shown).

Copper

In contrast to K and P, copper (Cu) concentration and shoot accumulation were significantly decreased by Px treatment. By this, shoot Cu concentration in treated plants dropped below the level that was defined as sufficient for optimal maize growth (Bergmann, 1993).



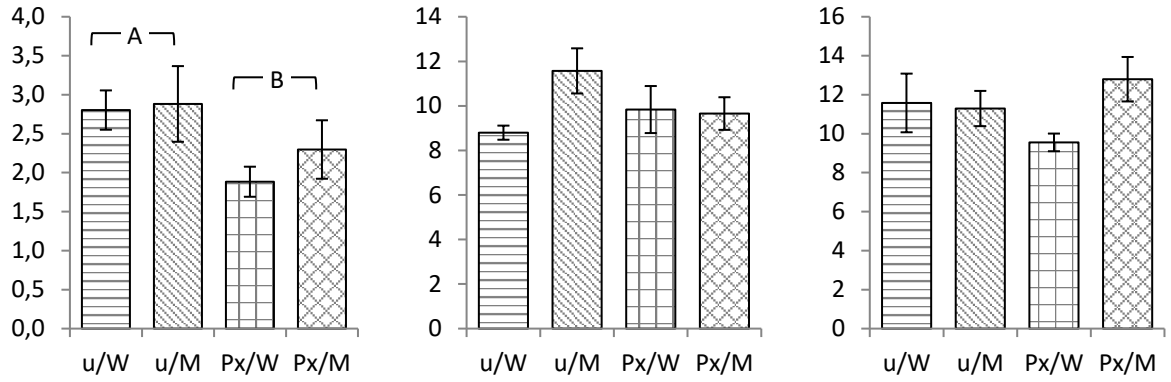


Figure 3-49 Micronutrients in shoots Exp_17; Upper row from left to right: Cu, Zn and Mn concentration (ppm); Below from left to right: Cu, Zn and Mn content in µg shoot^{-1}. For dashed lines see Fig. 5.

Zinc

For zinc (Zn) similar results as for Cu were obtained. Nevertheless, the decrease in Zn concentration by Px was only observed for the mutants, but not in the wildtype plants. One-Way-ANOVA resulted in significant difference between the u/M and the Px/M treatment. Furthermore all wildtype plants had a lower Zn concentration than the u/M treatment, indicating an increased Zn uptake by higher root length, but this effect was suppressed by Px application leading to the lowest Zn concentration in Px/M treatment. Zn accumulation did not significantly differ among treatments even though the trends were similar to the results for Zn concentration and u/W showed much lower total accumulation than u/M. The decreased concentration in Px plants was balanced due to the increased biomass.

Manganese

Trends for manganese (Mn) shoot concentration and accumulation were very similar to those found for Ca and also here no significant differences among treatments were found. But in contrast to Ca, Mn concentrations were below the plant optimum even though Mn content in the substrate was high. The reason for this is probably the high humus and carbonate content in the substrate (Broadley et al., 2012).

3.6.1.4 Discussion Exp_17

3.6.1.4.1 Root hairs and their influence on nutrient acquisition

Root hairs are known to be important sites for water and nutrient uptake into the plant root (Gilroy and Jones, 2000; Marschner and Rengel, 2012), mainly due to an increased root surface and smaller radius and therefore higher absorption capacity (Föhse et al., 1991). In our experiment a wild type maize plant was compared with the rth2 mutant that formed root hairs that were about $^1/_4$ of the size of the wild type. To investigate the influence of this impairment on nutrient acquisition from normal soil substrates the mineral contents and shoot

accumulation of these plants were analysed. In our experiments none of the investigated micro- and macronutrients was significantly reduced in mutant plants as compared to wild type plants. In contrast, in some cases mineral contents and total accumulation were even increased, especially for Mg significantly. One probable explanation is the increased root growth, reflected by a significantly increased dry root weight and total root length, found in this experiment in mutant plants. By this, the probability of a direct root contact to soil minerals is increased. Interestingly, acquisition of Mg, a mineral that is known to be transported mainly by mass flow (Marschner and Rengel, 2012), was increased most pronounced. In various studies (Föhse et al., 1991; Gahoonia et al., 1997; Gahoonia and Nielsen, 1997) a strong correlation of root hair length and P uptake was found when different cultivars of cereals were compared but maize cultivars were not tested. Additionally, correlation decreased with increasing amount of organic matter and P availability in the substrate whereas the soil used in our experiment was richer in both aspects. It is possible, that P availability as well as humus contents in our substrate was already too high for any improvement of P acquisition by longer root hairs. The relevance of root hairs for nutrient acquisition was questioned before (Wen and Schnable, 1994) but probably their influence is highly dependent on cultivar, nutrient concentration and soil type.

3.6.1.4.2 Px growth promotion and the influence of root hairs

Px was preferentially colonizing root hairs when applied as seed treatment in barley (Buddrus-Schiemann et al., 2010). Therefore the effect of impaired root hair growth on the efficacy of Px treatment for plant growth stimulation was investigated. Because no *gfp*-labelled strain was used, tracing of the bacteria was not possible in our experiment. We hypothesized, that an impaired root hair development would decrease root colonization and therefore reduce efficacy of Px treatment.

Interestingly, in our experiment growth promotion effects of Px treatment could be observed for both varieties independent of their root hair development. Furthermore, root growth promotion of Px was even more pronounced in the mutant plants, reflected by significant differences between Pr/W and Pr/M, possibly due to a higher responsiveness of mutant plants to hormonal stimulation. Root growth is known to be regulated by hormonal interplay, with special involvement of auxin (Overvoorde et al., 2010; Saini et al., 2013). Ethylene also seems to be directly involved in auxin transport, biosynthesis and downstream signalling (Li et al., 2006; Růžička et al., 2007; Song and Liu, 2015) Exp_11 indicated increased levels of ethylene production in Px treated plants. Additionally, Px and other *P. fluorescens* strains are

proposed to produce the auxin IAA (Buddrus-Schiemann, 2008; Oberhänsli et al., 1991). These findings could explain the better response of maize mutants to Px growth promotion. Interestingly, auxin is also proposed to regulate root hair growth (Neumann and Römheld, 2002), but no differences in root hair length could be observed between untreated and Px treated plants. This was also shown in other experiments (Mpanga et al., 2019a).

3.6.1.4.3 Possible mode of action for growth stimulation by Px

P. fluorescens PGPR strains are proposed to act as biofertilizer that improve plant nutrient acquisition by stimulation of root growth and solubilization of minerals via chelators (such as pyoverdine siderophores), reductants, enzymes and protons released into the surrounding environment (Richardson et al., 2009). In our experiment Px treatment was able to promote root growth, especially fine root growth, and, to a lesser extent, shoot growth. Nutrient acquisition of the macronutrients P and K was improved by Px treatment whereas not the shoot concentration but total accumulation was affected. Even though P shoot concentration was below the level for an optimal supply (Bergmann, 1976) shoot concentration was only slightly increased by Px. This is probably due to a dilution of P concentration due to higher shoot growth. P and K are both minerals known to be transported to the root by diffusion and not by mass flow (Marschner and Rengel, 2012). That is why an increased root length and formation of fine roots strongly increases the probability of their acquisition by plants. Promotion of root growth is one of the mechanisms proposed for PGPR, often explained by their ability to produce auxins or reduce ethylene levels by production of the ACC deaminase, an enzyme that degrades the ethylene precursor ACC (Glick, 2014). Nevertheless, if P and K acquisition was increased by an increased root growth, the question remains, why the mutant plants, also showing increased root growth as compared to wild type plants, did not significantly differ in their P and K shoot accumulation. This effect is only seen for Mg that was only affected by the genotype but not the Px treatment.

An alternative explanation for the increased P and K content in Px treated plants would be a decrease of rhizosphere pH due to a release of protons by Px. Many publications report an increased solubility of Ca-phosphates by PGPR when grown on growth media (Fernández et al., 2012; Fröhlich et al., 2011; Richardson and Simpson, 2011; Rodríguez and Fraga, 1999). Nevertheless, results from our lab could not prove a significantly promoted rhizosphere acidification after PGPR application even though P acquisition was dramatically improved when ammonium placement and PGPR application were combined (Nkebiwe et al., 2016b). Similar effects were observed in later experiments, performed in the institute by Mpanga et

al., but only in one of six cases significant differences in rhizosphere pH after PGPR application were measured (Mpanga, 2019; Mpanga et al., 2018; Mpanga et al., 2019). Also in experiments by Moradtalab or Bradáčová no significant pH drop after PGPR application was observed that could explain plant beneficial effects such as an improved P acquisition (Bradáčová et al., 2019b; Moradtalab et al., 2020). Another mechanism possibly explaining the improved nutrient acquisition is the release of organic acids which can be released by plants as well as microorganisms, which are known to improve P availability by complexation of sesquioxides and which may also influence K availability by cation exchange on clay minerals (Marschner and Rengel, 2012). Nevertheless, also here no further proves for this hypothesis was found in later experiments of our institute. In contrast, the level of organic acids in the rhizosphere dropped after PGPR application, probably due to an improved P status of the plant or consumption by PGPRs (Mpanga et al., 2019). Additionally, some PGPRs are known as mycorrhiza helper bacteria that might improve mycorrhization of roots and therefore uptake of P and K via mycorrhizal symbiosis (Barea et al., 2005; Frey-Klett et al., 2007). Indeed, an improvement of AMF root colonization when AMF inoculum was combined with Px was shown (Yusran et al., 2009) but results from our own studies (see 4.3.2.2) as well reports from literature (Mosimann et al., 2017) could not show these effects for a natural mycorrhiza community.

The exact mode of action by which the PGPRs were improving nutrient acquisition therefore remains unclear, although the stimulation of fine root growth seems to be part of the mechanism. A more speculative idea is the promotion of a shift in nutrient uptake, transport or recycling inside the plants (see also 3.14.4 on hormones and 4.3.3 on metabolism).

3.6.1.4.4 Limitations of Px application

Although our experiment could prove the efficacy of Px for root growth stimulation and improved acquisition of macronutrients, the acquisition of the micronutrients Zn and Cu was reduced. This reduction was not severe and did not obviously affect effectiveness of Px treatment but nevertheless shows the double sidedness of the often proposed mode of action for *Pseudomonas* sp. PGPR strains to act as biofertilizer. Px belongs to the *P. fluorescens* group, which is known to release the siderophores pyochelin, pseudobactin (Becker et al., 1985) and pyoverdine, the substance that leads to the eponymous fluorescence of the colonies on low iron medium (Meyer and and Abdallah, 1978). Whereas most investigations focus on the ability of these siderophores to form chelate complexes with iron, they are also able to bind other micronutrients like Zn and Cu (Brandel et al., 2012; Haas and Défago, 2005;

Paulsen et al., 2005). Nevertheless, unlike phytosiderophores, released by *Poaceae* for Fe uptake, these complexes seem to be a poor metal source for plants (Walter et al., 1994). Walter et al. could also not find any improvement of Fe uptake in maize plants, when they applied a pyoverdine-producing *P. putida* strain. Furthermore, the application of a mixture of soil microbes even led to severe iron deficiency in maize plants grown in hydroponic systems. Reduced iron uptake in maize plants was also reported for well aerated soils leading to the conclusion that fluorescent Pseudomonads could interfere with plant growth and functions by exacerbating iron starvation (Becker et al., 1985). Zn and Cu concentrations in our soil were extremely low. In contrast inoculation density of the strain was comparably high because previous research indicated that a certain density is necessary to establish the PGPR in non-sterile substrates (Buddrus-Schiemann et al., 2010). As reviewed in the same paper, reports of a transient shift in bacterial community after PGPR application are frequent. Because, the PGPR strain is formulated with addition of milk powder, that is known to act as a prebiotic, it is probable that rhizosphere community was additionally enriched. Having the results from investigations on iron in mind, it is possible, that, instead of improving nutrient acquisition, PGPR application could also have negative effects on micronutrient supply for plants because microbes might compete for these nutrients and even reduce their plant availability. Plant growth depression was also found in a recent study (Mosimann et al., 2017). Only in acidic soils PGPR could improve plant growth.

3.6.1.4.5 Conclusions

Purpose of our experiment was to investigate the importance of root hair formation for an effective plant growth promotion by PGPR. We conducted a pot experiment in non-sterile soil substrate with a maize mutant impaired in root hair development and analysed plant growth, root morphology and nutrient content of plants after treatment with the Px strain *Pseudomonas* sp. "Proradix". The results showed that maize mutants compensated the impaired root hair development by an increased root length and root biomass without a reduction in shoot biomass. Nutrient content of mutant plants as compared to wild type plants was similar or, in the case of magnesium, even increased, indicating that under our experimental conditions optimal root hair formation is not a limiting factor for nutrient acquisition in maize. Px treatment improved fine root growth, especially in mutant plants, and increased phosphate and potassium contents regardless of the maize genotype. These results suggest that efficacy of maize growth promotion by Px does not depend on a normal root hair formation. Nevertheless, rth2 mutants do not completely lack root hair formation. Additionally, it is also not clear in which way and how seriously exudation rates in the root hair zone might be influenced by the root hair impairment or how root colonization by Px was affected. Therefore it cannot be excluded that root hairs have some function for bacterial root colonization.

One last interesting finding was that Px treatment reduced contents of the micronutrient Zn and Cu. We hypothesized by reference to literature, that the bacteria might compete with the maize plants for these micronutrients and that biodegradation of maize phytosiderophores (Walter et al., 1994) and the release of high affinity siderophores by bacteria are mechanisms that may explain our finding.

For further investigations it is recommended to use *gfp*-labelled (Buddrus-Schiemann et al., 2010) or easily traceable PGPR strains to analyse the root colonization pattern of the bacteria. The disadvantage of the tracing method developed by Mosimann et al. (2017) is the high detection limit for the Px strain ($>10^4$ CFU g^{-1} root fresh weight), making it difficult to trace the strain from specific root compartments or later growth stages due to the often reported rapid decline in population densities of inoculated PGPR strains (Buddrus-Schiemann et al., 2010; Jacoud et al., 1998; Van Veen et al., 1997) (see also 3.10.4.1.3.2).

3.6.2 Root colonization of *B. amyloliquefaciens* FZB42 in maize (Exp_18)

3.6.2.1 Background

Exp_18 was conducted to investigate root colonization and plant growth stimulation of the Rz strain in the very early plant development of maize (6 – 18 DAS). The Rz strain was chosen because of the already established method for tracing the strain on selective media.

3.6.2.2 Experimental design Exp_18

Two different pots sizes were used for Exp_18. As described in Table 2-14 plants from pots with 2.7 kg of dry substrate were harvest 33 DAS. For various intermediate harvests a smaller pot size with 1.4 kg substrate was chosen. Five intermediate harvests were done 3, 6, 9, 12, and 18 DAS for tracing analysis of Rz from the growing maize roots. Sand contents were lower than in the previous experiments but fertilization was done on substrate basis using the same amounts as in Exp_11 and 14. Plants were grown at 25 °C with 16 h of light and 8 h in the darkness.

Table 3-17 Treatments Exp_18 (big pots)

Trt_Nr	Treatment	1. Appl	2. Appl	3. Appl
1	Ctrl	/	/	/
2	Rz_seed	ST	/	/
3	Rz_single	6.00E+09	/	/
4	Rz_triple	ST	6.00E+09	6.00E+09

Application rates in CFU kg^{-1} substrate; seed soaking in 10^9 CFU ml^{-1} suspension (ST); Applications with 18 ml of the ST suspension pot^{-1} directly at the time of sowing (1.), 8 DAS (2.) and 15 DAS (3.); r = 5

Pots for harvest 33 DAS and pots for intermediate harvest were separately randomized in completely randomized designs.

For each intermediate harvest only two treatments (Ctrl, Rz) with five replicates (r = 5) were included. For Rz treatment the maize seeds were soaked in a suspension with a spore concentration of 10^9 CFUs ml^{-1}. After seed treatment four seeds were directly sown into the pots. About 2 - 10 µl of the suspension $seed^{-1}$ were therefore transferred into the pots, making a total of about 8 – 40 x 10^6 CFUs pot^{-1}.

Additional to seed treatment as described above, for the harvest 33 DAS two more procedures were tested (Table 3-17). Plants were either directly treated with a high amount of inoculum as surface application at the time of sowing (Rz_single) or with standard treatment as performed in most of the previous experiments following the method from JKI (Rz_triple). Five seeds per pot were sown. 9 DAS (after the third harvest) plants were reduced to two plants (for harvests 12 and 18 DAS) or one plant per pot (in pots for harvest 33 DAS).

For tracing analysis 1 g root with adhering rhizosphere soil were incubated shaking in 20 ml 0.1 % tryptone solution for 10 min. Plating assays were performed using standard LB medium and heat treatment at 80°C.

3.6.2.3 Results for small pots

Two separate experimental designs were used to perform short-term experiments with intermediate harvests 3, 6, 9, 12 and 18 DAS using small cylindrical pots and a long-term experiment with harvest 33 DAS using bigger pots to prevent root growth limitation by low soil volume. Samples from each harvest time were analysed for root colonization by *B. amyloliquefaciens* (Rz) using LB_{rif} medium and plating of rhizosphere extracts. Additionally, root and shoot fresh weight of the samples were taken.

3.6.2.3.1 Tracing

Although inoculation rate per pot was low with only ~ 1 x 10^7 CFU pot^{-1} and application was done by seed treatment *Bacillus* CFUs could also be found 18 DAS at the root tips in similar amounts as in the upper parts of the root, indicating either a successful transport by water flow or an active movement of the bacteria along the growing root. Nevertheless, tracing data were not as expected, because Ctrl samples showed significant amounts of bacteria on the selective media, especially during first and second harvest, although population densities were much lower than in the Rz treatment and declined in the later harvest (Figure 3-50).

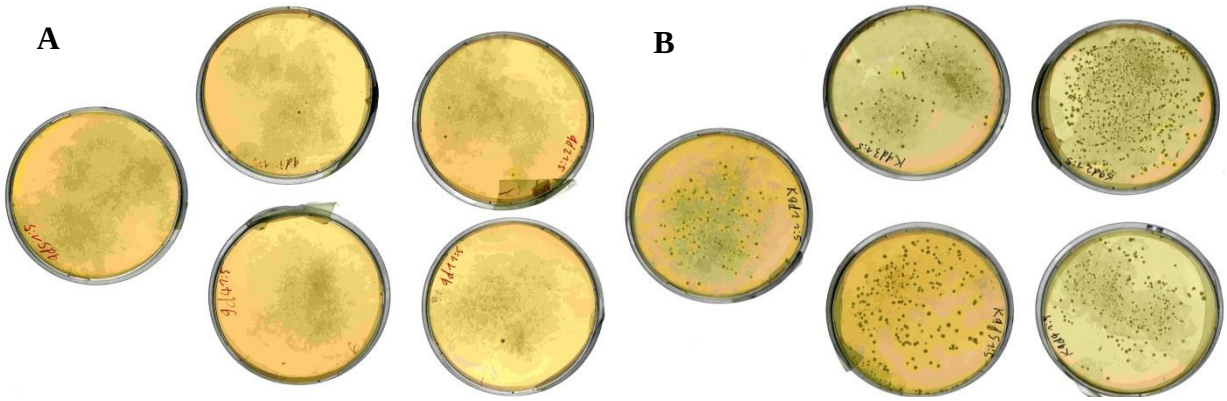


Figure 3-50 CFU on LB_{rif} medium 9 DAS (Exp_18); Plating of 1:100 dilutions of maize root sample extracts from Ctrl (**A**) and Rz treatment (**B**) 9 DAS. Only very small amounts of CFU were visible on Ctrl plates

As agar plates were relatively free from other contaminations and colony shapes were typical for Bacillus colonies it can be assumed that the rifampicin was effective. The most probable explanations for the contaminations were non-sterile spatula or non-sterile bottles as discussed further in the chapter 4. This is also supported by the fact that some Ctrl samples did not show any CFU. Therefore data are not normally distributed (even after log – transformation).

3.6.2.3.2 Biomass results

Figure 3-51 shows the average weight data of single plants (A and B) and weights per pot (graphs C and D).

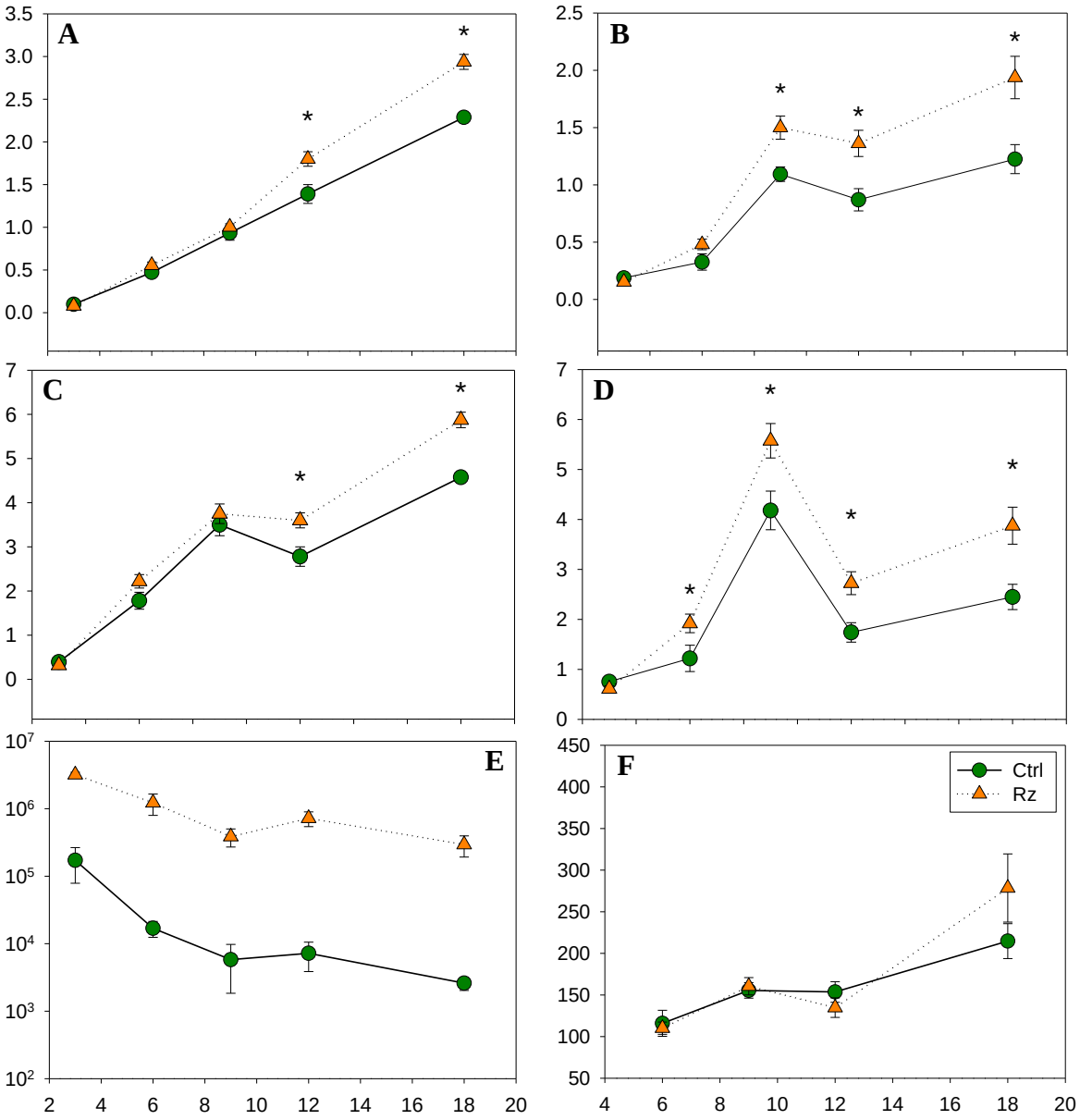


Figure 3-51 Results Exp_18 (small pots); x-axis shows the harvest time in DAS; Shoot (**A**) and root (**B**) FW per plant in g, shoot (**C**) and root (**D**) FW per pot in g, CFU g^{-1} root (**E**) and root length per plant in cm (**F**); means + SE; * indicates significant difference between Ctrl and Rz treatment for the respective harvest time. For root colonization data were not normally distributed (for further information see text)

Those graphs differ because at the harvests 3, 6 and 9 DAS four plants per pot were harvested, whereas plants were reduced to two plants per pot 9 DAS and therefore only two plants were harvested 12 and 18 DAS. The decrease in root weight and length from 9 DAS to 12 DAS (Figure 3-51 B, D, F) is probably due to the procedure of root harvesting. Here only the root segments that were clearly connected to the two remaining maize shoots were harvested whereas other root material was left in the substrate. Root and shoot growth were

both significantly stimulated by Rz application at later harvest times whereas the growth promoting effect increased over time. Data from root scanning analysis did not indicate significant difference among treatments during early harvest times. This is probably caused by the destructive harvesting procedures by which a huge amount of fine roots were lost. Nevertheless, 18 DAS fine root length (0 – 0.2 mm root diameter) was significantly increased in the Rz treatment (*data not shown*).

3.6.2.4 Results for big pots

The long-term experiment was running simultaneously with the short-term experiment in the same climate chamber with the difference that plants were growing in bigger pots. Data indicated somehow contrasting results for the seed treatment with Rz. Here seed treatment had some growth inhibiting effect. Also additional application of Rz as soil placement in the Rz_triple treatment did not improve plant growth but lead to reduced root growth as compared to the Ctrl treatment. In contrast to this, when Rz was applied in high dosage directly at the time of sowing (Rz_single) a strong growth stimulation in roots and shoots was observed correlating with the highest population density of Rz on the roots (Figure 3-52).

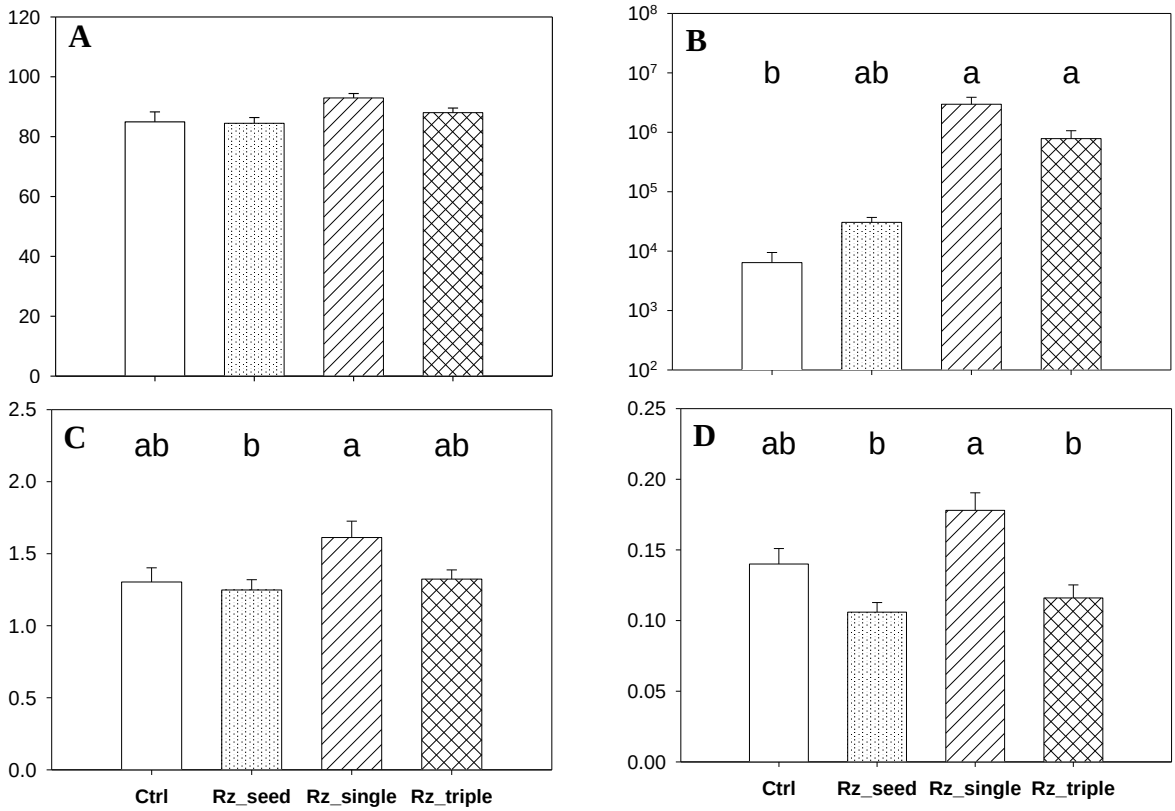


Figure 3-52 Results Exp_18 (big pots); Contrasting results in big pots. Plant height 26 DAS in cm (**A**), CFU g^{-1} root (**B**), shoot (**C**) and root (**D**) DW in g; Means + SE

These results indicated that there was an interaction between pot size and Rz application, something that is difficult to explain and could not really be elucidated during the bachelor

thesis. To sum up the results from the experiments, Rz application was indeed able to stimulate early root and shoot development and Rz CFU could be found also in the growing parts of the root. A high inoculation rate, directly at the beginning of the experiment, seemed to be the most effective strategy to have long-lasting effects on plant development and maize biomass.

3.6.2.5 Discussion Exp_18

Rz transport alongside the growing root

Main purpose of the experiment was to re-isolate the applied *Bacillus* strain at various time points during the early plant development to further investigate the ability of the PGPR to move aside the growing root. During harvest times 3 - 18 DAS relatively high numbers of CFU were counted for mixed samples from the whole root. Interestingly, counts for the root tips of 18 days old maize plants were similar. This suggests that the PGPR was indeed present in the lower soil layers and might have grown along the root. Nevertheless, investigations provide no evidence for any of the possible ways of transport for the cells/spores such as active movement, root attachment or water flow during watering.

The results for the seed treatment from the last tracing 33 DAS show a much lower CFU rate. This might either be the result of (1.) a decline of vitality and therefore population density of the PGPR, (2.) a dilution of the total amount due to the increased root growth and a higher soil volume. Instead of sampling from the Ctrl pots, sampling from bulk soil of the Rz treatment might have given more insights. But in general a sampling of a specific soil compartments is quite difficult when using pots. Therefore rhizoboxes, as used in Exp_17 might be much more suitable. Here also watering is not performed from above and therefore the risk of down washing is low.

Efficacy of Rz seed application

In previous pot experiments in our group (Hasan, 2016), *unpublished* master thesis) a zone with a stabilized ammonium depot was placed at depth of 30 cm under the soil surface. The zone was so deep that in the first experiment almost no water was reaching the lower soil layer. Nevertheless, the seed treatment resulted in a re-isolation of *Bacillus* CFU from this deep root zone.

A comparison with the tracing data from the field experiment 2015 showed very similar results. In the seed treatment a significantly higher number of CFU was found than in the Ctrl even though roots were sampled in a distance of about 10 cm from the maize row. A transport

via water flow is not probable in this case and therefore attachment to the root or active movement can be assumed. Both aspects would indeed support seed application as a promising (and for sure the most economic) application method. Contaminations were also present in the Ctrl treatment whereas these contaminations were probably derived from different sources (as discussed Exp_14).

As seen in the Exp_18 inoculum rates in the seed treatment are also sufficient to promote plant growth. That this growth stimulation is not persistent and not always reproducible under more applied conditions (big pots, field experiment) is therefore probably not a result of low application rates but shows the dependency on environmental factors and the potential of the plant to compensate a delayed early growth development in the time course of several month of vegetation period with the possibility for exploitation of large soil volumes.

Additionally, the application technique and the fertilizer sources may influence the mode of action and plant responses to PGPR. In this experiment increased root and shoot growth were observed when PGPR were applied under nitrate nutrition. In experiments with ammonium depots, root length in the sampled root zone was strongly promoted by Rz and this stimulation was increasing with higher inoculum rates (Hasan, 2016). Nevertheless, root growth stimulation in the depot zone did not increase total root weight or result in higher shoot biomass. The same effects were observed in the maize field experiment in 2014 (Nkebiwe et al., 2016b). Interestingly, in 2015, applying the same treatments and fertilizer, maize yield was improved by Px application but no increase in localized root growth in the depot zone was observed suggesting that root growth stimulation does not necessarily correlate with increased yields but depends on nutrient supply in the soil (2014: Olsen-P 78.7 kg ha^{-1}, 2015: 55.8 kg ha^{-1}).

3.7 BE effects on utilization of organic fertilizers

3.7.1 PGPRs effect under organic ammonium fertilization in maize (Exp_19)

3.7.1.1 Introduction Exp_19

The synergistic effects of Px treatment and ammonium nutrition was seen in many experiments conducted in our institute. Nevertheless, those experiments were conducted with mineral fertilizers in the form of stabilized ammonium phosphate or ammonium sulphate. Therefore, the objective of Exp_19 was to investigate if ammonium-N from organic fertilizers could be stabilized by the nitrification inhibitor DMPP and if interactions between N-source and the BE strains could be reproduced using organic fertilization.

3.7.1.2 Experimental design Exp_19

Exp_19 was conducted using two types of manures with very different amounts of available NH_4-N (Table 2-18), stabilized by a nitrification inhibitor, in combination with the microbial BE Px (Table 3-18). As control treatments, a fully fertilized treatment with nitrate-N and additional mineral P (P_Ctrl) as well as non-P fertilized nitrate (Nit) and ammonium sulphate fertilized treatments (Nov) were added.

Table 3-18 Treatments Exp_19

Trt_Nr	Treatment	N	P	K	Total BE
1	P_Ctrl	133	67	100	/
2	Nit	133	/	100	/
3	Nit_Px	133	/	100	2.0E+09
4	Nov	133	/	100	/
5	Nov_Px	133	/	100	2.0E+09
6	MP	150	70	155	/
7	MP_Px	150	70	155	2.0E+09
8	MP_NI	150	70	155	/
9	MP_NI_Px	150	70	155	2.0E+09
10	MKH	140	52	156	/
11	MKH_Px	140	52	156	2.0E+09
12	MKH_NI	140	52	156	/
13	MKH_NI_Px	140	52	156	2.0E+09

Px treatments with seed treatment (10^9 CFU ml^{-1}) and soil surface application at sowing, 7 and 14 DAS (each 6.7 x 10^8 CFU kg^{-1} substrate), total amounts in table; fertilization rates of N, P and K in mg kg^{-1} substrate; P_Ctrl with mineral P fertilization and nitrate N; Nit = $CaNO_3$, Nov = F_{Nov}, MP = F_{MP}, MKH = F_{MKH} + 20 mg N kg^{-1} soil (Nit or Nov), NI = DMPP, r = 5

The well-composted cow manure (MKH) as well as the chicken pellets (MP) used, the latter provided by the company Agriges, are both solid and porous fertilizers making grinding to fine powder possible. After grinding in a mortar the manure was homogenously mixed into the soil substrate. Mg and K were additionally fertilized in all treatments following standard fertilization rates. The fertilized amount of manure was based on the estimated P contents of the fertilizers with target value of 100 mg P kg^{-1} soil. Therefore fertilized N amounts (total N) differed slightly among treatments. As later results from mineral analysis of the manures differed as compared to the earlier results, also P fertilization rates differed among treatments. In the table the most current values are given. For stabilization of NH_4-N in the organic

fertilizers the nitrification inhibitor (NI) 3,4-dimethylpyrazole phosphate (DMPP) (Zerulla et al., 2001) was added. DMPP is also used in the ammonium fertilizer F_{Nov} and also in commercial additives for liquid manure. Following recommendations for the application of the DMPP-containing additive ENTEC® (EuroChem Agro GmbH, Mannheim, Germany) DMPP should be added according to the amounts of NH_4-N in the fertilizers or the total amount of liquid manure added. The concentrations were increased by calculating the amounts based on 1 % DMPP per total N fertilized with an average target N value of 200 mg N kg^{-1} soil (2 mg DMPP kg^{-1} soil/ 1.3 mg g^{-1} substrate). Because solid manure was used, a mixture with DMPP before fertilization was not possible. Therefore DMPP was diluted in water and then sprayed, like the other solutions, evenly on the substrate before mixing and preparation of pots. Due to the low N_{min} values in the F_{MKH} fertilizer a starter fertilization of 20 mg N kg^{-1} soil was added to treatments 10 – 13 whereas the NI treatments were supplemented with pure $(NH_4)_2SO_4$ and the other two treatments with calcium nitrate.

For BE application only Px was used. Seeds were treated by soaking in a Px suspension. Four seeds per pot were sown. Directly at sowing 1 ml of the suspension (5 x 10^8 CFU ml^{-1}) were applied on each seed. Additionally, one and two weeks after sowing diluted suspensions were applied as soil surface applications. 7 DAS the smallest of the four plants was reduced first. Of the remaining three plants the middle one was kept until harvest 59 DAS.

3.7.1.3 Results

In the course of the experiment measurements of plant height, stem diameter and SPAD values as well as documentation of the water consumption of the plants were conducted. After harvest shoot and root weight, root length as well as shoot P contents were analysed.

Most measurements before harvest, with the exception of SPAD values, correlated well with the biomass results after harvest and the results are therefore not shown.

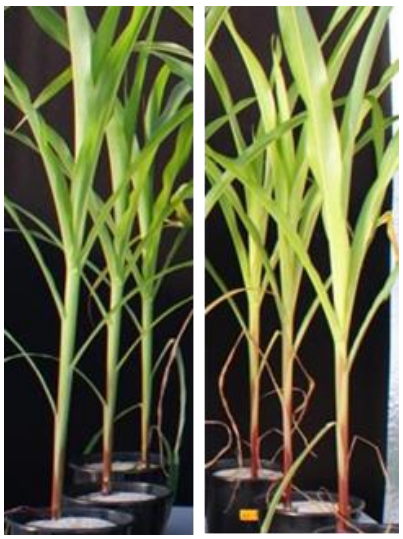


Figure 3-53 N-deficiency in Exp_19 P_Ctrl (left) and MKH_Ni_Px (right)

As seen in Figure 3-54, in Exp_19 significant differences among different fertilizers were obtained whereas BE application did not cause any significant difference among treatments. Interesting effects were observed for the mineral fertilization. Here fertilization with stabilized ammonium sulphate (Nov) strongly promoted plant growth as compared to the nitrate treatments and achieved even higher shoot DW than

observed for the P_Ctrl. P and N availability was also sufficient in the MP treatments as indicated by the high P values (Figure 3-54 C and D) and the high SPAD values (F). The opposite results were found for the MKH treatments. The manure was very low in available mineral N, as already seen by N_{min} analysis, and yellow leaf coloration 40 DAS (Figure 3-53), supported by the low SPAD values, were clearly indicating severe N deficiency in the MKH treatments. Also P availability in the MKH treatments was suboptimal, as indicated by shoot P analysis, although CAL-P values were not that low. Interestingly, despite these obvious nutrient deficiencies plant performance in the MKH treatments was good, with plants showing strong shoot growth and by far the best root growth. No effects for the added NI were observed.

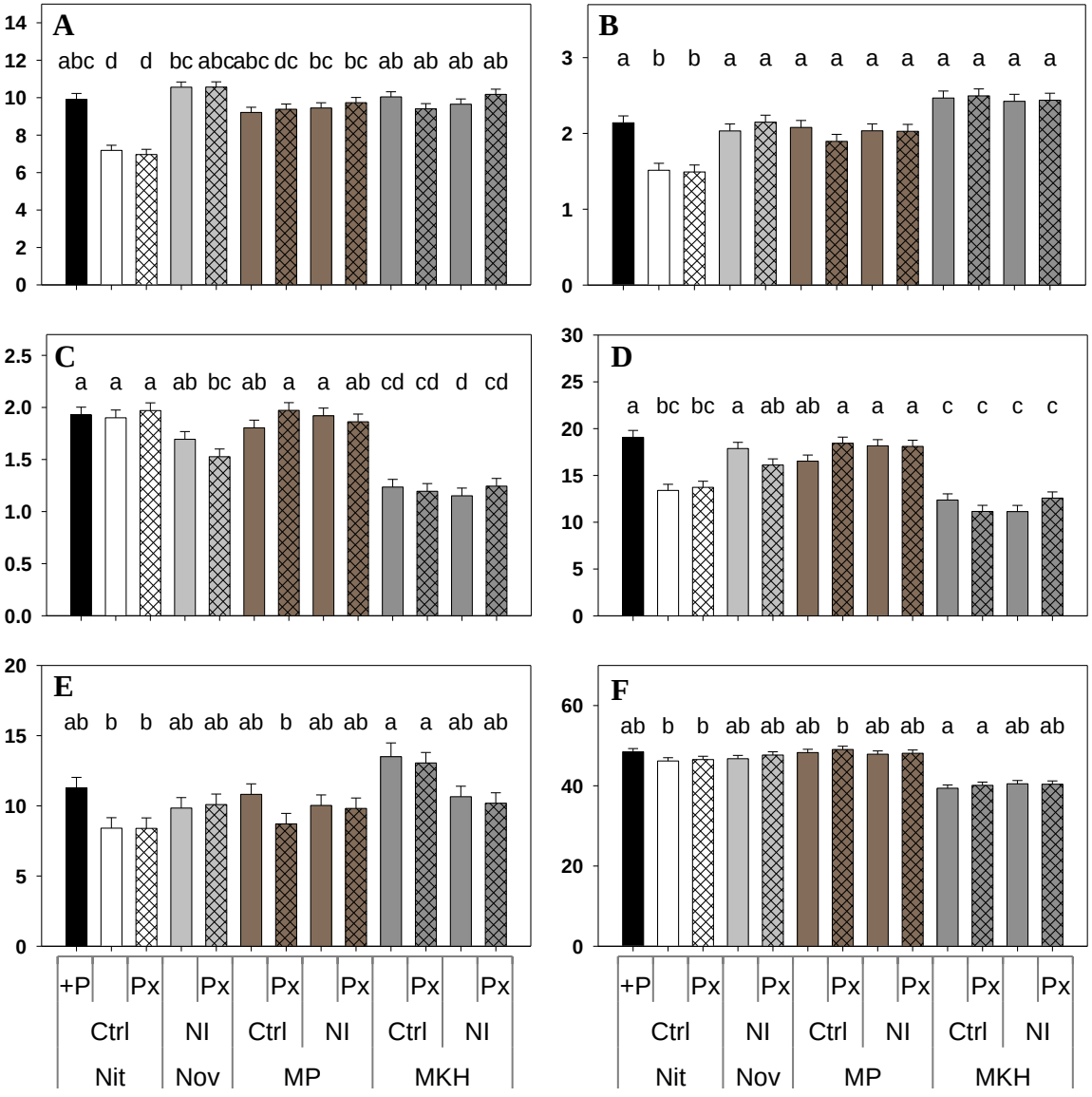


Figure 3-54 Results Exp_19; Shoot (**A**) and root (**B**) DW per plant in g, shoot P concentration in mg g^{-1} DW (**C**) and P content in g $shoot^{-1}$ (**D**), root length in m (**E**) and SPAD values 48 DAS (**F**); means + SE; for analysis of shoot DW one outlier was excluded (n=64) and for root length two outliers were excluded (n=63)

3.7.1.4 Discussion Exp_19

Using stabilized ammonium phosphate or ammonium sulphate, plant growth promoting effects of the Px product but also other microbial BEs showed better reproducibility than with nitrate fertilization (Mpanga and Nkebiwe, 2015 (*unpublished*); Morad-Talab et al., 2016 (*unpublished*); Nkebiwe et al., 2016b; from 2019 on further results, discussed in the section below or in chapter 4, were published by Mpanga, Moradtalab and Brádácova). Therefore in Exp_19 different fertilizers with stabilized ammonium were tested in combination with the Px strain.

3.7.1.4.1 Ammonium fertilization and PGPR

As seen for the F_{Nov} fertilized treatments (Nov, Nov_Px) the BE effects observed by Mpanga (2015) were not reproduced. One explanation would be the use of different P sources. In this experiment no additional P was applied whereas in the experiment by Mpanga (2015) a high amount of a sparingly-soluble mineral P source in the form of rock phosphate was applied. However, in another experiment that was conducted by Kar (Kar, 2017; Nkebiwe, 2016) and that was running simultaneously with Exp_19, no significant differences between BE treatments and Ctrl were observed, independent of the P fertilizer used, suggesting other reasons such as light conditions. Exp_19 was conducted during late autumn. Therefore, light conditions might not be optimal for plant activity, rhizodeposition and therefore establishment of BE-plant interactions. This topic is further discussed in section 4.3.3.

In contrast to the missing BE effects, strong effects were observed for ammonium fertilization. Results obtained from the F_{Nov} fertilized treatments indicate that the P content in the soil was high. The decreased rhizosphere pH, commonly observed for NH_4-N nutrition, might have improved P uptake as compared to the nitrate fertilized plants. Nevertheless, the strong growth improvement as seen by a shoot DW that was even higher than in the P_Ctrl, although shoot P concentration was lower, indicate that P was not the only limiting factor. There are several other aspects of ammonium nutrition that could explain the observed effects. First, plant availability of other nutrients, especially micronutrients, might be increased due to a decreased rhizosphere pH. Increased availability of Mn, Fe and Mo under ammonium nutrition is commonly reported (Broadley et al., 2012; George et al., 2012; Huber et al., 2012). Increased Zn availability by NH_4-N nutrition was also observed in cold-stress experiments conducted in our institute (Freytag and Wanke, 2017; Moradtalab et al., 2020) leading to improved cold-stress tolerance as also observed in Exp_8 and Exp_9. By increasing Mn-availability additionally certain groups of plant-beneficial Pseudomonades are favoured

(Huber et al., 2012). The increased proton release by ammonium nutrition is also compensated by an increased phosphoenolpyruvate carboxylase (PEPC) activity, as commonly observed for Fe and P-deficiency, resulting in an increased release of carboxylates like malate (Neumann and Römheld, 2012, 1999). The released carboxylates are not only chelating cations, thereby increasing their plant availability, but are also the most important nutritional source for rhizobacteria (Marschner, 2012a) such as the inoculated Px strain. A higher population density of the Px strain was indeed found in the rhizosphere hotspots of an ammonium-N depot (Nkebiwe et al., 2017). Nevertheless, it was also shown that the positive influence of ammonium nutrition on root colonization activity of another *P. fluorescens* strain was only observed when pH was allowed to decrease (Marschner et al., 1999). Therefore interaction could be based on the additional rhizosphere acidification by the inoculated BEs as reported for ectomycorrhizal (ECM) and arbuscular mycorrhizal fungi (AM) (Bago and Azcón-Aguilar, 1997; Li et al., 1991). Additionally, *P. fluorescens* prefers assimilation of ammonium for N-nutrition to nitrate assimilation (Betlach et al., 1981). Possible reasons for the lack of Px effects in the manure treatments are further discussed below.

Additionally, ammonium nutrition influences phytohormone production, especially of active cytokinin. However in which way phytohormone concentration changed seemed to be depending on the plant species, plant organ, developmental stage and the ratio between nitrate and ammonium uptake by the plants (Engels et al., 2012). Another factor might be the additional sulphate fertilization by the F_{Nov}. Here data on sulphate contents in plants and soil are missing.

3.7.1.4.2 Missing effects for nitrification inhibitors

The missing effects of NI application in the organic fertilizer treatments might have various reasons.

1. DMPP addition to liquid manure is tested and performed under practice conditions but it is unknown if our application technique was suitable to stabilize solid fertilizer.

2. As seen in Table 2-18 the F_{MP} contains about 1.85 % ammonium-N (~ 50 % of total N). It is unknown how fast NH_4-N in our MP treatments was nitrified to NO_3. Here intermediate soil samplings should have performed to monitor the N_{min} status of the soil. Nevertheless, no differences on plant performance were observed when NI was applied in the MP treatments. DMPP was applied in form of a pure solution with 21 % of DMPP. The applied amount of 1.3 mg kg^{-1} substrate was based on the fertilization rate of 200 mg N kg^{-1} soil and the

recommended dose of 1 % (w/w) DMPP per NH_4-N content (Zerulla et al., 2001). Nevertheless, in the publication it was also shown that in an incubation experiment with 87 mg liquid pig manure and an application rate of 3.9 mg DMPP kg^{-1} soil the ammonium was stabilized only for 2 weeks. Therefore it is possible, that the NI was not concentrated enough to stabilize the ammonium-N from the F_{MP} manure in the course of the experiment. Another explanation would be that the increased ammonium ratio did not further affect plant growth under these experimental conditions. Additionally, it is possible that P in the substrate (and probably in the manures) was mainly bound in organic form, making a decreased pH less effective. CAL-P or P-form of the F_{MP} was not assessed.

3. For the F_{MKH} NH_4-N was too low that a stabilisation by NI could have been of importance. Nevertheless, we hypothesized that BE application might promote mineralization of organic N that could then be stabilized by NI. Probably mineralized N was directly incorporated into the microbial biomass and was therefore not increasing the NH_4-N pool of the soil.

3.7.1.4.3 Px application with organic fertilizers

Independent of the efficacy of nitrification inhibitors, and therefore N-form, in most previous experiments with Px plant growth promoting effects were observed. Additionally, to the aspects discussed above, the missing effects by the PGPR application in the organic fertilizer treatments might have multiple reasons.

1. As seen for the Nov treatments the fertilizer treatments itself strongly improved plant growth as compared to the nitrate fertilized Ctrl and further effects by the BE were less significant. The differences between the P_Ctrl and –P-Ctrl treatments almost vanished if, instead of nitrate, ammonium was fertilized as N source. Focussing on the results from shoot DW, it seems conclusive that ammonium treatment alone was already providing the plant with sufficient P. Nevertheless, it might be possible that the outcome of this interaction could have been improved if higher dosages of BEs would have been inoculated.

2. Seeds and surrounding soil were inoculated directly at the time of sowing, so that at least at the beginning of the experiment the BE should have got in contact with the roots. In previous experiments seed inoculation already led to growth responses during early plant development and therefore inoculation density should have been sufficient to stimulate root growth by direct BE contact to the root, e.g. by release of auxins and AHLs or degradation of ACC.

Nevertheless, for the later top soil applications a sprayer was used so that the water stream had less pressure. Due to the low sand contents it is probable that BE infiltration into the soil

was therefore limited as also mentioned in Exp_10. Effects like the proposed interaction between ammonium source and BE inoculation, solubilization of phosphates and mineralization of nutrients from organic matter as well as the proposed mechanism of the Px product to stimulate natural soil microflora might depend on much higher inoculum densities in the substrate explaining the limited effects in this experiment (see also 4.1.4.2 and 4.3.8).

3. PGPR growth promotion under manure fertilization seems to be highly substrate specific. Under F_{MKH} fertilization also in Exp_20 no PGPR effects were obtained whereas the partner group in Romania had strong effects by PGPR application in organic substrates. Additionally, in several experiments during the Biofector project the F_{MP} was used as a standard for organic manures but no synergistic effects with the PGPR were observed whereas in the same experiments horse and cow manures were more effective. A reason could be the source and content of organic matter and the aspect of ammonia toxicity. High pH conditions may lead to quick volatilization of non-placed ammonium to toxic ammonia (NH_3) (Avnimelech and Laher, 1977; Fleisher et al., 1987). This reaction might have detrimental effects on BE population and root colonization in those treatments. Autotoxicity due to gaseous ammonia production was observed for certain *Pseudomonas* sp. strains at higher concentrations (Rogul and Carr, 1972). Although the investigated BEs were resistant to high ammonium concentrations (Nkebiwe et al., 2017), gaseous ammonia might exhibit stronger toxicity.

3.7.1.4.4 Contrasting plant growth effects in manure and nitrate treatments

In the F_{MKH} fertilized treatments plant availability of N and P was low as indicated by nutrient analysis and SPAD values. In a working group in Romania, 45 % manure in the substrate mixture was used for experiments on tomatoes with the same BEs (see also Exp_20). With this amount not only soil structure was changed dramatically but also nutrient status of P and K as well as availability of organic matter, as compared to Exp_19. Calculation of N amounts based on N_{min} values of the F_{MKH}, suggest that about 430 g of F_{MKH} would have been needed to reach the level of 200 mg N / kg substrate. This is about 40 % of substrate and therefore comparable to the amounts used in Romania. This could be one explanation why the BE effects of previous experiments on ammonium-N fertilization and organic manure were not reproduced. In fact, strongest BE effects were observed in those experiments in the course of the project, in which plants were growing under a good or high nitrogen availability.

A nutrient supply (especially N and P) up to a level that is necessary for optimal plant growth generally increases shoot but also root growth and root length. This is supported by our findings from previous experiments that P_Ctrl plants always had higher root growth than

unfertilized plants. Nevertheless, plants sensing a depletion of N or P or suffering from N or P-deficiency often show an increased root to shoot ratio (R/S) to improve spatial nutrient acquisition (Lynch et al., 2012). The nitrate control plants probably suffered from the unbalanced supply ratios between nitrate-N and P. Obviously the N signal from nitrate fertilization was stronger than the signal from P-deficiency. Due to the high nitrate availability probably cytokinin concentrations were increased (Engels et al., 2012) leading to a decreased root growth and subsequent reduction of water and P supply thereby further impairing the situation for the Nit_Ctrl and Nit_Px plants. In the manure treatments of Exp_19 both nutrients were limited, providing one explanation for the increased R/S ratio.

3.7.1.4.5 Growth promotion by humic substances

However, the observed effects observed for the F_{MKH} seem to be more complex, as not only root length but also root DW in the MKH plants was increased without reduction in shoot DW as compared to the other treatments, including the P_Ctrl. At the same time nutrient status of plants, as mentioned above, was extremely low, with P and N concentrations as well as P contents even below the values of the growth retarded nitrate controls. These results suggest that plants with manure fertilization responded positively to substances in the manure. These compounds dramatically improved biomass production and therefore nutrient use efficiency. Bioactive compounds in the soil organic matter (SOM) are often summarized under the term of humic substances (HS). Findings on properties and structure of HS have caused controversy and are still under discussion (Trevisan et al., 2010). Although some working groups have found evidence for the existence of supramolecular structures (Piccolo, 2001; Savy et al., 2016), probably newly assembled by microbial activity, these findings were rejected with the reasoning that the found HS are scientific artefacts from the extraction methods (Lehmann and Kleber, 2015). The authors therefore defined HS as mixtures of various compounds, instable polymers or degradation products of plant secondary metabolites. Also in an earlier publication it was stated that the “majority of operationally defined humic material in soils is a very complex mixture of microbial and plant biopolymers and their degradation products but not a distinct chemical category”(Kelleher and Simpson, 2006), although the authors could not rule out the possibility of the existence of additional not yet defined substances.

Indirect mechanisms by which humic substances (HS) can stimulate plant growth may be an increase in nutrient availability, cation exchange capacity or the influence on soil microbial composition (Chen and Aviad, 1990). Improved availability of selected micronutrients (Fe,

Zn, Mn) in plants by HS application was reported (Chen et al., 2004; Lee and Bartlett, 1976). Unfortunately, the micronutrient composition for the F_{MKH} is missing. However, severe macronutrient deficiencies were observed in the manure fertilized plants and therefore a growth improvement by better micronutrient supply is not probable for Exp_19.

A direct mechanism is the hormonal stimulation and therefore influence on plant metabolism, nutrient remobilization or use efficiency and root architecture (Canellas and Olivares, 2014). HS promoted lateral root growth in maize seedlings via changes in plasma membrane H^+-ATPase activity and structure analysis of the HS revealed IAA-like groups (Canellas et al., 2002). This plant growth stimulation seemed to be correlated with the hydrophobicity of the HS (Canellas et al., 2009). Mechanisms for direct plant growth stimulation by HS are still under debate but evidence for hormone-like activity were found in several studies (Trevisan et al., 2010). Nevertheless, plant responses do not always perfectly correlate to concentrations of hormone-like compounds measured inside the HS and the observation of even stronger responses by HS as compared to pure or synthetic hormone-derivatives suggest that other compounds are additionally interacting synergistically (Scaglia et al., 2015). In a recent publication ammonium-N-derived H^+-extrusion was connected to auxin translocation to lateral root primordia and auxin-mediated lateral root formation (Meier et al., 2020). Therefore the increased activity of plasma membrane H^+-ATPase by humic acids (Neumann and Römheld, 2012, p. 395) is possibly indirectly promoting root branching via translocation of root-internal auxin.

A complete characterization of the F_{MKH} manure by e.g. alkaline extraction or other fractionation techniques (Drosos et al., 2017) and subsequent structure analysis via H-NMR or LC-MS would have been interesting but was not feasible during this work.

The combination of humic substances and PGPR was proposed to be a useful approach for the production of new biofertilizers, due to the influence of HS on microbial communities and the enhancement of PGPR root colonization (Canellas and Olivares, 2014). Neither in Exp_19 nor Exp_20 synergistic effects between the F_{MKH} and the Px treatment were observed but in general BE effects in both experiments were absent. Nevertheless, there seem to be significant influences of compost fertilizers, differing in their maturation time, on the composition of soil microbial communities (Cozzolino et al., 2016). The authors observed both neutral and negative effects of HS treatments on maize growth and root mycorrhization by AMF suggesting again a highly complex interaction when combining HS and BEs.

3.7.1.4.6 Detrimental effects of DMPP application on root length

Interestingly, DMPP application combined with manure showed a detrimental effect on root length (Figure 3-54 E). We cannot provide an explanation for this observation because in the other NI treatments no detrimental effect was observed whereas in the Nov treatment, as expected for ammonium nutrition, root length was increased as compared to the Nit plants. Although several nitrification inhibitors cause phytotoxicity at higher application rates, toxicity of DMPP was not yet been reported (Slangen and Kerkhoff, 1984; Zerulla et al., 2001) and no studies on direct DMPP effects on root growth were found. Several studies report on the beneficial effects of DMPP addition to urea or ammonium fertilizer on plant growth, but they do not separate between the single DMPP effect and the effect of ammonium nutrition on plant growth (Dong et al., 2013; Ruan et al., 2007, 2000; Vitale et al., 2013; Xu et al., 2014). As described above, ammonium nutrition influences plant physiology as compared to nitrate and often promotes root growth. Therefore any underlying DMPP effect might not be obvious. In contrast, in the F_{MKH} treatments plants were in contact with DMPP without an available ammonium source, as indicated by N_{min} analysis of the manure and the observed N-deficiency symptoms of maize plants. Here further investigations would be necessary to give support of the findings from Exp_19 and elucidate the reason for the detrimental effects of DMPP on root growth observed in the manure treatments.

3.7.2 PGPRs effect under organic ammonium fertilization in tomato (Exp_20)

3.7.2.1 BE effects in Romania

Exp_20 was conducted to reproduce the strong BE effects that were repeatedly observed in tomato experiments of a working group in Romania (Banat's University, Timisoara, Posta et al. 2013 – 2017, *partly published* (Bradáčová et al., 2019a)). Here substrate mixtures with 45 % composted manure were used in the pre-cultivation phase of tomato plants before transplantation into a greenhouse (Figure 3-55).

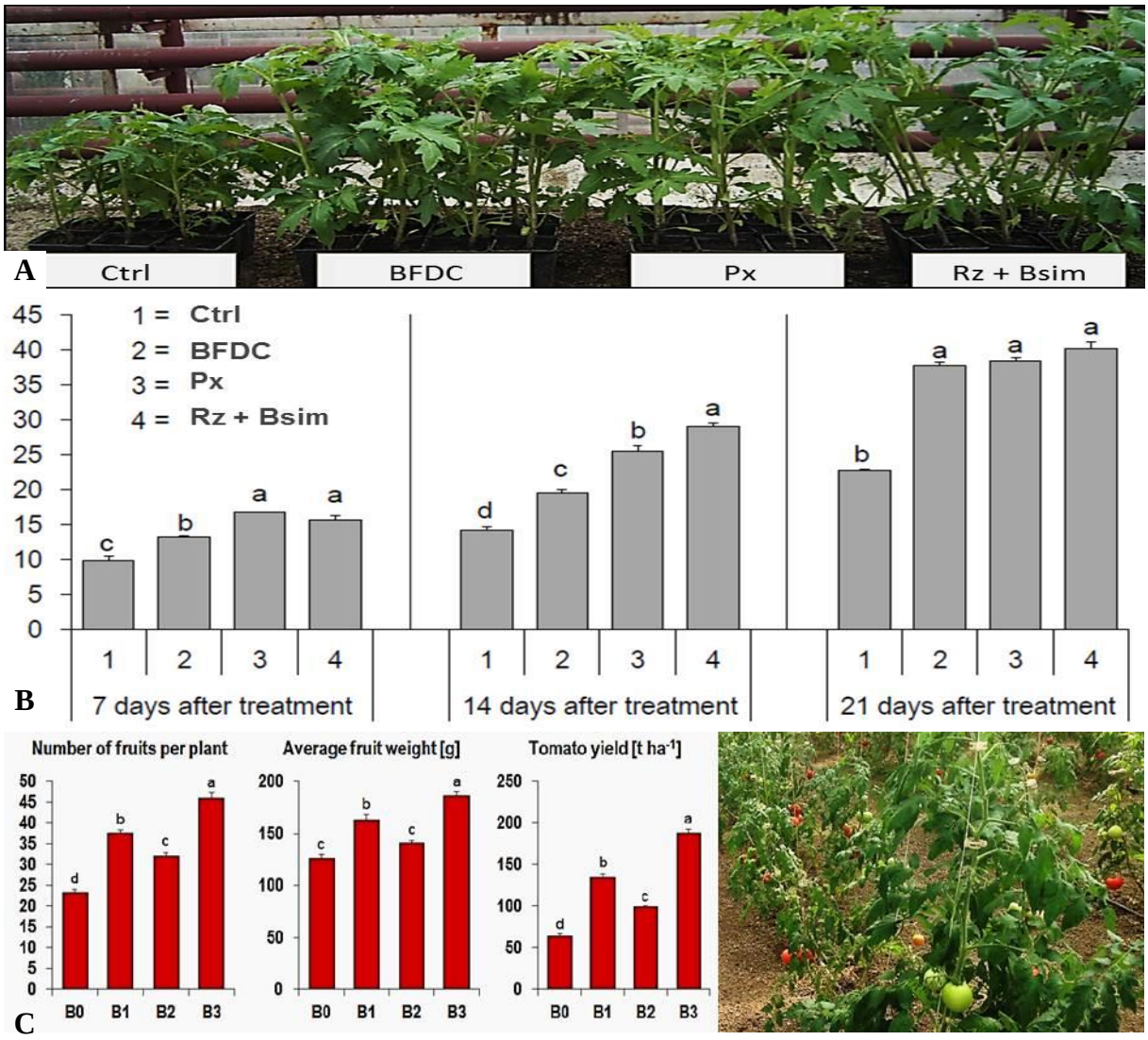


Figure 3-55 Results from tomato experiments (Posta et al.); Plant habitus of tomato plants 21 DAS (**A**) and plant height in cm (**B**) (Posta et al., 2013); Post-harvest results from greenhouse trial (**C**) (Posta et al., 2014); B0 – B3 in (**C**) indicate the same treatments like 1 - 4 in (**B**).

Although results from tomato production varied in the course of the four year project period, in general a strong plant growth promotion by BE treatments was observed in almost all trials. Growth stimulation in the pre-cultivation phase was also leading to significantly increased yields generating immense economic benefit (see 4.2.3).

3.7.2.2 Experimental design Exp_20

The design for Exp_20 was based on the experimental conditions used in the working group in Romania for pre-cultivation of tomato plants before transplanting them into a large scale greenhouse experiment. It was set up as a two-factorial experiment with three BE treatments and two types of substrate with additional Ctrl treatments reflecting conventional pre-cultivation of tomatoes with commercial substrates (Table 3-19). The organic substrate (Org) was composed of 30 % v/v soil KH(c), 45 % F_{MKH}, 15 % peat (pure Lithuanian *Sphagnum* peat) and 10 % sand. Because the substrate contained very high amounts of organic fertilizers and therefore nutrients, a second substrate was composed with similar physical properties but based on mineral fertilization (Table 3-20). This mineral substrate was composed of 25 % KH(c), 50 % peat and 25 % sand fertilized with 1.2 g of the slow-release fertilizer F_{Dur} kg^{-1} substrate. For comparison with commercial substrates the propagation substrates Pikiererde (CL P), with lower fertilizer amounts, and Topferde (CL T), higher fertilization rates (Einheitserdewerke Werkverband e.V., Sinntal-Altengronau, Germany) were used.

Table 3-19 Treatments Exp_20

Trt_Nr	Treatment	Conc. (stock)	BE rate
1	C_Org	/	/
2	Px_Org	0.02	3.5E+08
3	Rz_Org	0.04	2.7E+08
	(+ Bsim)	0.06	2.4E+08
4	BFDC_Org	0.05	1.3E+07
5	C_Min	/	/
6	Px_Min	See 2	
7	Rz_Min	See 3	
8	BFDC_Min	See 4	
9	CL_P	/	/
10	CL_T	/	/

Concentration of BE stock solutions in % w/v; 20 ml stock solution applied pot^{-1} 9 and 23 DAS; BE application rates in CFU kg^{-1} substrate; r = 5

1	3	5	4	7	9	10	8	2	6
5	4	2	6	10	8	1	3	7	9
7	9	1	3	5	4	2	6	10	8
10	8	7	9	2	6	5	4	1	3
2	6	10	8	1	3	7	9	5	4

Figure 3-56 CRB design Exp_20

Table 3-20 Fertilization Exp_20

Type	Amount pot^{-1} (g)	N	P	K	S	Mg
Org	750	2702	1099	1207	522	1086
Min	750	198	63	23	32	8
CL_P	300	172	100	190	191	668
CL_T	300	325	158	333	191	668

Amount of fertilized nutrients in mg pot^{-1} based on ICP-OES analysis of total nutrients; substrate volume always ~1 litre pot^{-1}. Calculations for mineral composition of propagation substrates based on bulk density of 0.314 kg $liter^{-1}$

BE application was first done 9 DAS as soil surface applications, similar to the experimental conditions in earlier experiments in Romania. Two weeks later the application was repeated.

At the beginning of the experiment watering of plants was not done on weight. The reason was that physical properties and composition of the substrates differed among each other and that watering in Romania also was not done on weight basis. Therefore all pots were placed in

small aluminium bowls and a standard watering was performed, whereas pots were drenched until water was seeping from the pots. Due to chlorosis symptoms that occurred during the first weeks of the experiment it was hypothesized that water contents were to high provoking anaerobic conditions in the pots and therefore it was decided to reduce the amount of water by using water amounts based on WHC_{max} of the respective substrates.

Two-factorial statistical analysis including rows and column as additional factors was done using the glimmix procedure in SAS.

3.7.2.3 Results Exp_20

3.7.2.3.1 Pre-harvest results

Five days after sowing plants emerged. Plant height, stem diameter, SPAD as well as lateral shoot measurements were performed 30 and 41 DAS. The pre-harvest analyses did not reveal strong differences among BE treatments but strong plant responses to different substrates (Figure 3-57).

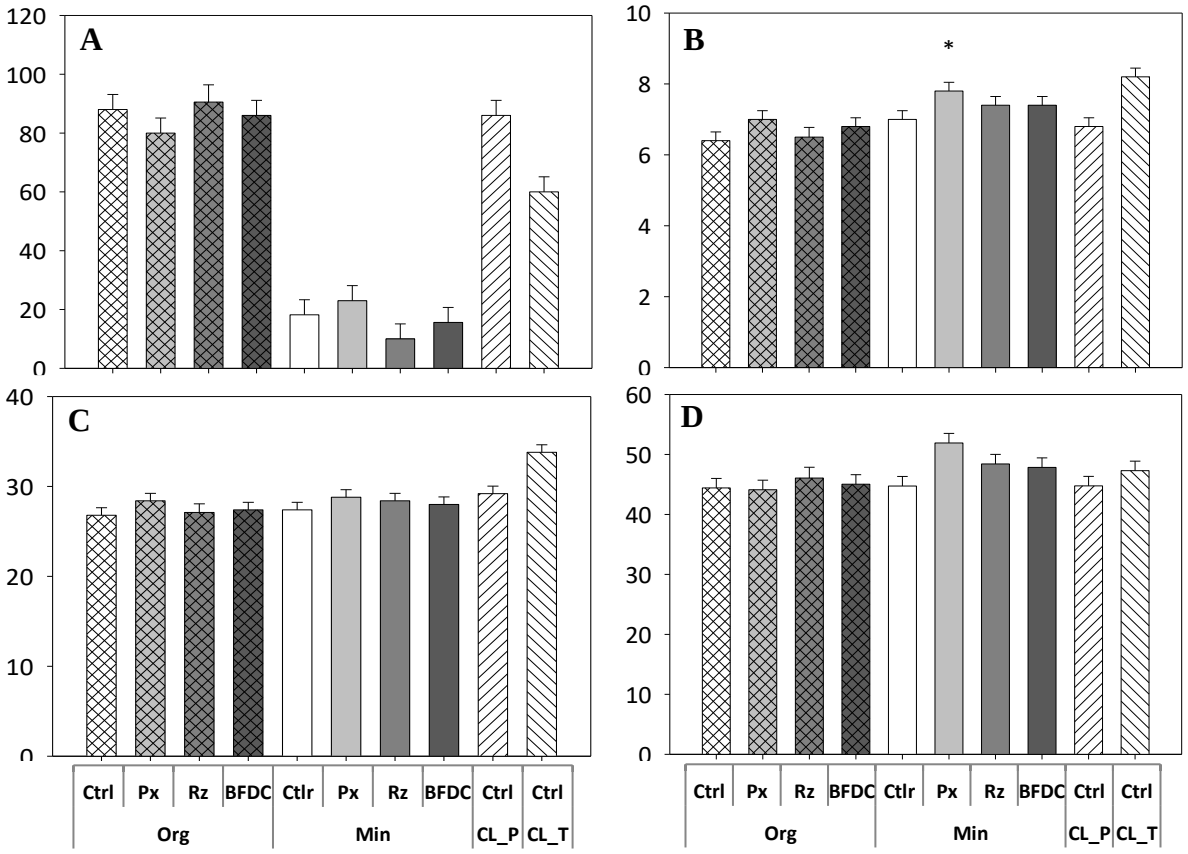


Figure 3-57 Pre-harvest results Exp_20; Leaf coloration in % (**A**), number of lateral shoots (**B**), plant height in cm (**C**) and SPAD values (**D**) 30 DAS; Means + SE; significant differences were found only in Two-Way-ANOVAs for different substrates and for the comparison of Px vs. Ctrl in (**B**)

Here the organic substrate (Org) led to the most sincere chlorosis symptoms, indicating stress and nutrient deficiency, and plant height and development of lateral shoots plant were delayed

in comparison to the other substrates (see also Figure 3-58). Leaf coloration was lowest in the Duratec treatments, with huge difference to all other treatments, indicating some micronutrient deficiency. Interestingly, stem diameter in the organic fertilized plants 30 DAS was higher than in the mineral fertilized plants ("Min"), possibly in response to a higher P supply. Plant development was strongest in the CL_T substrate containing high amounts of plant available macronutrients. CL_P treatment showed similar results as the Ctrl_Min treatment.

A closer observation of the BE effects shows a continuously better performance of the Px treated plants than all Ctrl or Rz and BFDC treatments. BFDC treatment did not show clear responses as compared to the untreated Ctrl whereas Rz treatment in the organic substrate increased stem diameter slightly as compared to the Ctrl plants. The improved growth by Px treatment was strongest in the mineral fertilized plants whereas same responses but to lesser extend were also seen the "Org" treatment. 30 DAS Px could significantly increase the number of lateral shoots when analysed in a two-way-ANOVA using *proc glimmix* in SAS.

Figure 3-58 Plant habitus Exp_20 (41 DAS); plants arranged from left to write as described in Figure 3-59.

3.7.2.3.2 Post-harvest results

Results from harvest generally reflected the results from pre-harvest measurements. A comparison of the different substrates showed that shoot and root DW of the manure fertilized plants were significantly lower than for the other substrates and the CL_T plants showed 100 % more root DW and over 200 % higher shoot DW than the organic fertilized Ctrl plants. For root length manure (Org) and Duratec (Min) fertilized plants performed more similar whereas CL_T plants still had 82 % higher root length than the Ctrl_Org plants and 63 % higher root length than the Ctrl_Min plants (see Figure 3-59 D and E).

The second highest shoot and root DW was found for the Px treated plants of the "Min" substrate. A two-way-ANOVA again revealed significant differences between the Px and the Ctrl plants for root DW whereas root length and shoot DW showed the same tendency but did not differ significantly among Px and Ctrl plants. A one-way-ANOVA did not indicate significant differences among BE treatments of the same substrate. None of the other two BEs

could significantly improve plant biomass but BFDC showed a tendency in both substrates to increase plant shoot and root DW as compared to the Ctrl.

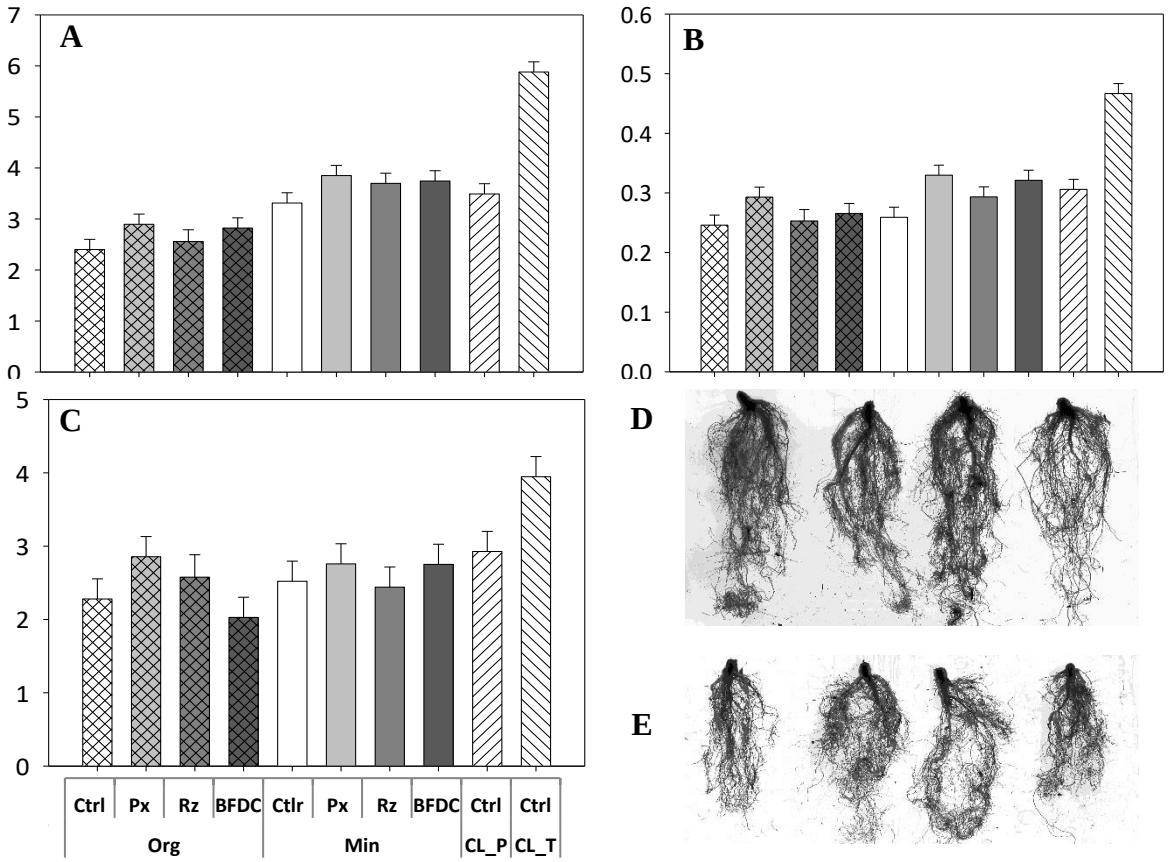


Figure 3-59 Post-harvest results Exp_20; Shoot DW (**A**) and root DW (**B**) in g plant^{-1} and root length in m (**C**), roots from CL_T (**D**) and Ctrl_Min (**E**) treatment before cutting for root scanning

3.7.2.3.3 Tracing analysis

NP agar plates for counting of *Pseudomonades* from Ctrl_Org and Px_Org treatments revealed a 2 - 5 times higher CFU for the Px treatment, as expected from previous results of tracing analyses, with a mean CFU of 7 x 10^5 CFUs g^{-1} rhizosphere soil (Figure 3-60 on the next page). This is slightly higher than the inoculum rate but the result is reasonable due to the high number of CFU from natural *Pseudomonas* population found in the Ctrl treatment and the heterogeneity in the soil population density.

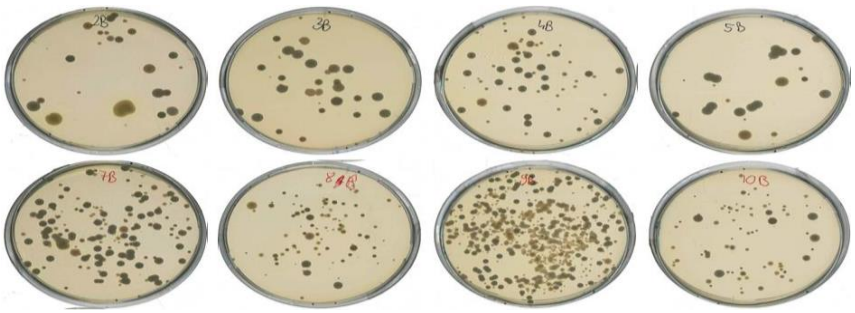


Figure 3-60 Tracing of *Pseudomonades* using semi-selective NP medium; Ctrl_Org (upper row) vs Px_Org

3.7.2.4 Discussion Exp_20

No reproduction of BE effects in Exp_20

In Exp_20 strong BE effects as observed in Romania were not observed, although the Px treatment was again the best performing treatment in both substrates. The Px effect did not seem to be substrate dependent as effects were very similar under both fertilization regimes (with the exception of the SPAD values and the number of lateral shoots in pre-harvest analysis). The other two BE treatments induced less responses in the plants, similar to other experiments in our working group.

Explanations for BE effects

It remained unclear which conditions were causing the strong BE effects in Romania until the end of the project. Several reasons could be named to explain the differences to the results from Posta et al. As discussed for Exp_4 and 5, light conditions could always influence BE-plant interaction. Our experiment was conducted in the climate chamber with much lower light intensity (120 – 200 µm) as compared to daylight (240 µm with clouds and up to 2000 µm in the sun).

Soil type

As mentioned in 3.7.2.1 BEs repeatedly showed growth stimulating and yield promoting effects for tomato plants in greenhouse cultures in Romania. Nevertheless, in 2014 the Px treatment showed less effects than the other two BE treatments. In other years (2013, 2015 - 2017) this difference was not observed. Additionally, in 2016 the results were less pronounced than in other years. In 2016 the composition of the substrate used in the working group in Romania was changed. The new mixture was also transported to our institute in Hohenheim and another tomato experiment was conducted in our greenhouse. Also here no significant BE effects were observed (Götz, 2016), *unpublished* bachelor thesis). Due to the missing effects in both working groups the results in 2016 were explained by the soil substrate used (further discussed below).

As the quality of the soil substrate was influencing the outcome of the interaction, in the following two sections some differences among substrates are discussed.

Low N-supply in Exp_20

Analysis of the growing substrate in Romania indicated even higher mineral nutrient contents than for the MKH substrate (3.8 g P, 6,7 g total N, 320 mg NO_3-N, 3.5 mg NH_4-N [kg^{-1} substrate]). Available N in Exp_20 was much lower. Calculations based on the 45 % (v/v)

manure (F_{MKH}) per pot, a bulk density of 0.63 g cm^{-3} manure, a dry matter content of 69.5 % and substrate volume of ~920 ml (750 g) lead to an amount of 180 g dry manure per pot. With this amount about 83 mg NO_3-N per pot were fertilized (110 mg NO_3-N kg^{-1} substrate), although total (non-mineralized) N was much higher. This was probably too low for the tomato plants as seen by the comparison of the plants from the CL_P (low fertilization) and the CL_T (high fertilization) treatments. P availability was very high with an estimation of ~1 g P per pot. This is also supported by the measurement of the stem diameter. Here the manure fertilized plants (Org) performed better than the Duratec plants. In other experiments stem diameter was found to be a good indicator for P availability. The difference in available N could be one explanation for the weaker effects of the BEs.

Cu deficiency or HS may induce leaf chlorosis

In Exp_20 strong chlorotic symptoms like purple coloration of the leaves occurred, especially in the manure fertilized tomato plants. This coloration by anthocyanin formation normally is a response to sincere nutrient deficiency or other stress factors like low temperature. As CL_P and the well-fertilized CL_T plants were also affected, N-deficiency was probably not the reason for the leaf chlorosis. Because the experiments were conducted following the experimental conditions from the group in Romania, temperatures at the beginning of the experiment were kept relatively low for tomato cultivation with only 20 °C. After the first two weeks temperature was increased to an average of 22 °C.

Low temperature chlorosis is often a response to ROS formation that can be inhibited by sufficient micronutrient supply. For most micronutrients like Zn, Fe and Mn a good supply can be expected from comparisons with a sheep manure used in another experiment in the institute (Riemann, 2013). Here, about 20 % of the total Zn was found for extractable Zn. Taking this value, an additional amount of 10 mg available Zn pot^{-1} can be assumed. Nevertheless, high P availability may induce Zn-deficiency due to a decreased soil availability (reduced root growth or mycorrhization), decreased solubility or lower physiological availability (Broadley et al., 2012). Although P-toxicity was reported in clover (Loneragan et al., 1979), high P concentrations of 10 ppm in the shoot, suggested as a critical level for P-toxicity in tomato (Jones, 1998), are normally not observed in calcareous soils even at high application rates of soluble P fertilizer (Orabi et al., 1982). Only Cu availability was very low in the sheep manure. Here we lack full characterization for the F_{MKH} manure but it is possible, that Cu deficiency in combination with relatively low temperatures might be the reason for the strong chlorosis symptoms. The Min treatments with F_{Dur} fertilization did not

show symptoms although supply of macronutrients was lower than in the CL_T and CL_P treatments (Table 3-20). It was the only substrate with additional Cu fertilization.

Another aspect is the composition of organic compounds that might have affected plant growth. As hypothesized in Exp_19 some kind of humic substances were influencing plant metabolism. The high concentration of these active compounds that were causing plant growth stimulation in Exp_19, might have stressed the plants in Exp_20 (Cozzolino et al., 2016). Possibly, also the peat substrates contain similar compounds.

Biological activity of bacterial BEs in Exp_20

The presence of the Px strain was analysed in a plating assay at harvest. The amount of Pseudomonades in the Px treatment was increased. The method cannot distinguish between the Px strain and other Pseudomonades from the substrate and we therefore do not have a proof for the presence of the Px strain. Nevertheless, for the Px product the establishment of the inoculated PGPR might not be crucial for its effectiveness as its biological activity is probably connected to the natural soil microflora as described in 4.1.4.2.

Biocontrol

Another working group in Hungary (Biró and Szalai, Szent István University, Department of Soil Science and Water Management) also observed strong BE effects in tomato cultivation. Here cultivation was completely different, with tomatoes growing outdoor, much lower rates of organic fertilization, following the rules for certified organic crop production. In 2016 they observed physiological changes by BE treatment, as indicated by significant differences in fruit numbers and sugar contents in the fruits (Biró et al., *unpublished*). Additionally, some microbial BE treatment that was composed of a mixture of different bacterial and fungal strains was suppressing pathogen infection thereby presenting another important mode of action in which BEs may act under applied conditions.

Also in Romania plants were infected with fungal diseases from soil making a fumigation of the greenhouse soil necessary in one year. The mode of action of the BEs in the Romanian working group could therefore also be connected to their biocontrol activity.

3.8 BE effects on germination and seedling development

3.8.1 Germination tests

Tomato and maize seeds were treated with BE suspensions to test the influence of the products on seed germination rates.

3.8.1.1 Experimental designs

Seeds were pre-selected for optimal size and shape. Then 5 seeds were placed into a 90 mm Petri dish containing two round filter papers. Depending on the test condition, seeds were surface-sterilized using a treatment with 10 % H_2O_2 for 2 min or were used non-sterilized. Seeds were germinated in the dark in a climate chamber at 22 °C/ 27°C in 12h rhythm. 3 - 5 replicates per condition were completely randomized. Various conditions were applied and tested, such as seed BE treatment, different BE concentrations and BE treatment in soil suspensions (see tables below). Controls were treated with 10 mM $CaSO_4$, 0.3 % NaCl or soil suspensions with $CaSO_4$.

➔ Treatments Germ_1 below due to formatting reasons

Table 3-21 Treatments germination experiment Germ_2 with maize

Nr	Trt	BE	Conc.	H_2O_2	Soil	Rep	Liquid
1	Ctrl	Ctrl	Ctrl	no	no	3	10 mM $CaSO_4$
2	H_2O_2	Ctrl	Ctrl	H_2O_2	no	3	10 mM $CaSO_4$
3	Px_seed	Px	$5x10^7$	no	no	3	10 mM $CaSO_4$
4	Rz_seed	Rz	$5x10^7$	no	no	3	10 mM $CaSO_4$
5	Soil	Ctrl	Ctrl	H_2O_2	soil	3	10 ml soil suspension
6	Px_soil	Px	$5x10^8$	H_2O_2	soil	3	10 ml Px $5x10^8$ CFU ml^{-1} soil susp.
7	Rz_soil	Rz	$5x10^8$	H_2O_2	soil	3	10 ml Rz $5x10^8$ CFU ml^{-1} soil susp.
8	MP_1.5	MP	1.5%	H_2O_2	no	3	10 ml MP suspension (1.5%)
9	Px_7	Px	10^7	H_2O_2	no	3	10 ml Px 10^7 CFU ml^{-1}
10	Px_8	Px	10^8	H_2O_2	no	3	10 ml Px 10^8 CFU ml^{-1}
11	Px_9	Px	10^9	H_2O_2	no	3	10 ml Px 10^9 CFU ml^{-1}
12	Rz_5	Rz	10^5	H_2O_2	no	3	10 ml Rz 10^5 CFU ml^{-1}
13	Rz_6	Rz	10^6	H_2O_2	no	3	10 ml Rz 10^6 CFU ml^{-1}
14	Rz_7	Rz	10^7	H_2O_2	no	3	10 ml Rz 10^7 CFU ml^{-1}
15	Rz_8	Rz	10^8	H_2O_2	no	3	10 ml Rz 10^8 CFU ml^{-1}
16	Rz_9	Rz	10^9	H_2O_2	no	3	10 ml Rz 10^9 CFU ml^{-1}

Each 5 seeds germinated in 10 ml liquid solution/suspension of 10 mM $CaSO_4$ with different additives described in BE; MP=milk powder based Px product formulation; Conc. = concentration in CFU ml^{-1} liquid; Seed treatments like in the field experiment 2015: Px seed infiltration ($1.2x10^8$ CFU $seed^{-1}$) and Rz seed coating ($1.4x10^8$ CFU $seed^{-1}$); H_2O_2 = surface sterilization with 10 % H_2O_2 for 2 min; Soil suspension = 1:5 soil:10mM $CaSO_4$; 3 replicates for each treatment

Table 3-22 Treatments germination experiment Germ_1

Plant	Treatment	Liquid	Replicates	Conditions
Tomato	Ctrl	10 mM $CaSO_4$	5	2 filters, 4 ml liquid, 5 seeds
Tomato	NaCl	3 g NaCl l-1 (0.3%)	5	see above
Tomato	TP	4.17E+06 CFU ml^{-1}	3	suspension in NaCl solution
Tomato	Px	1.00E+09 CFU ml^{-1}	3	see above
Tomato	Rz	1.00E+09 CFU ml^{-1}	3	see above
Maize		as for tomato but with 5 ml liquid		

Table 3-23 Treatments germination experiment Germ_3 with maize

Nr	Treatment	BE	Conc.	H_2O_2	Soil	Vol.	Description
1	Ctrl_5ml	Ctrl	Ctrl	no	no	5	5 ml 10 mM $CaSO_4$
2	H_2O_2	Ctrl	Ctrl	H_2O_2	no	5	5 ml 10 mM $CaSO_4$
3	Ctrl_10ml	Ctrl	Ctrl	no	no	10	10 ml 10 mM $CaSO_4$
4	Soil	Ctrl	Ctrl	no	soil	10	10 ml soil suspension
5	Soil_cent	Ctrl	Ctrl	no	no	10	10 ml of supernatant of soil suspension
6	Soil_sterile	Ctrl	Ctrl	no	soil	10	10 ml of heat treated-soil suspension
7	Soil_st_c	Ctrl	Ctrl	no	soil	10	10 ml of supernatant of Soil_sterile
8	Px_4	Px	10^4	no	no	5	5 ml Px 10^4 CFU ml^{-1}
9	Px_6	Px	10^6	no	no	5	5 ml Px 10^6 CFU ml^{-1}
10	Px_7	Px	10^7	no	no	5	5 ml Px 10^7 CFU ml^{-1}
11	Rz_3	Rz	10^3	no	no	5	5 ml Rz 10^3 CFU ml^{-1}
12	Rz_4	Rz	10^4	no	no	5	5 ml Rz 10^4 CFU ml^{-1}
13	Rz_5	Rz	10^5	no	no	5	5 ml Rz 10^5 CFU ml^{-1}

All suspensions/solutions in 10 mM $CaSO_4$; Heat treatment of soil was done at 105°C; Soil suspension = 1:4 soil:10mM $CaSO_4$; To get the supernatant suspension was centrifuged at low speed; 3 replicates for each treatment

3.8.1.2 Results of germination tests

First germination test was done to see if seeds treatments, as done for the experiments at the JKI, influence seed germination. For maize no differences occurred. 2 DAS almost all seeds were germinated with no difference among treatments. Tomato seeds germinated much slower than maize seeds. 2 DAS about 15 – 30 % of seeds had germinated. No pronounced differences were observed for the treatments. 5 DAS the NaCl treatment showed the highest germination rate (Figure 3-61).

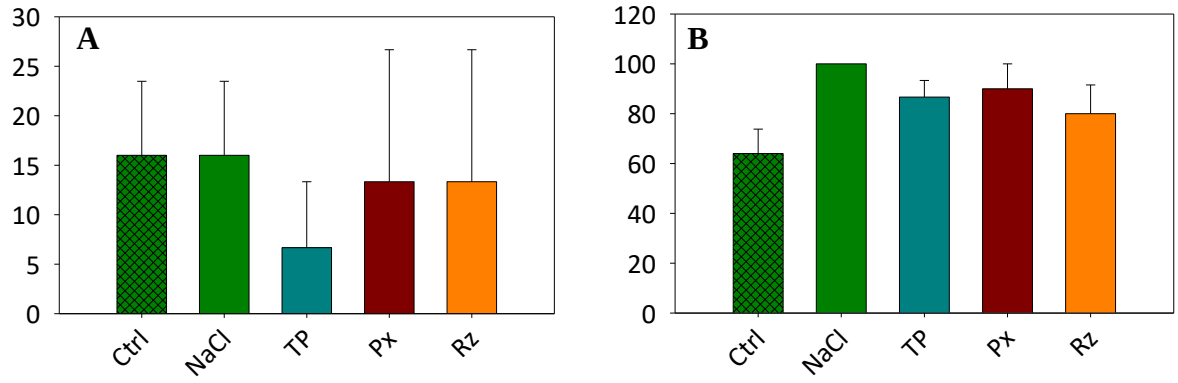


Figure 3-61 Germination rates of tomato plants Germ_1; Germination rate 2 (**A**) and 5 DAS (**B**) in % of total seeds; Means + SE

Objective of germination test Germ_2 was to investigate if BE application rates influence seed germination. The tests were only done with maize seeds.

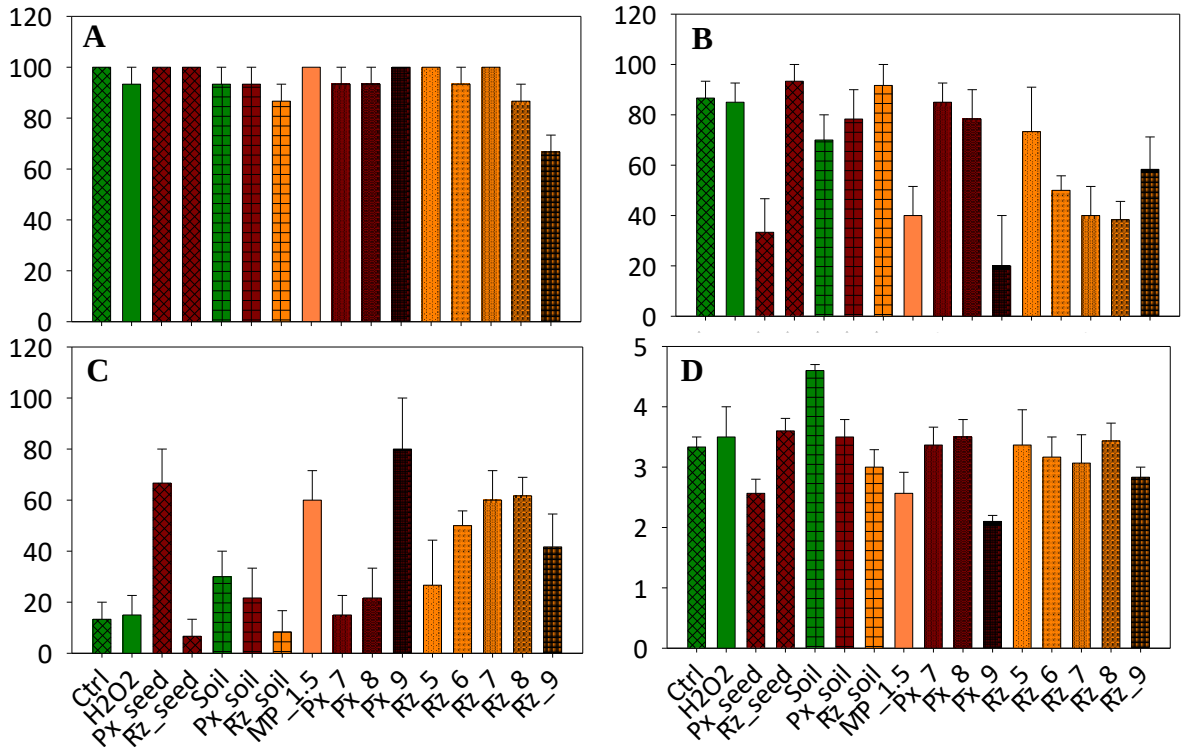


Figure 3-62 Results Germination test Germ_2; Germination rate 2 DAS in % of total maize seeds (**A**), amount of healthy seedlings (**B**) and amount of stressed seedlings (**C**) in % of germinated seeds, height of seedlings in cm 5 DAS (**D**); Means + SE

Germination rate was 100 % in almost all treatments. Only in treatment Rz_9 germination rate was reduced more pronounced. A closer examination of the germinated seedlings indicated some differences between the treatments. In some treatments seedlings had red instead of green tips. Especially the Px_seeds from the field experiment 2015, the milk powder treatment as well as the BE treatments with high concentrations showed these coloration that was interpreted as stress response. The seedling height measured 5 DAS from the table top to the seedling tip showed a good correlation with the % of healthy seedlings with exception from the soil treatments. Here the soil treatment without BEs showed best seedling growth.

In the last experiment counting of seeds was done already 1 DAS. Here treatments differed strongly. The lowest concentration of Rz (10^3 CFU ml^{-1}) seemed to promote seed germination whereas higher concentrations delayed seed germination. H_2O_2 treatment, as often seen in other experiments with pre-germination (e.g. Exp_18), led to a faster seed germination. Also the addition of soil suspension improved seed germination. For Px treatment best seed germination was observed at a concentration of 10^6 CFU ml^{-1}. Nevertheless, 2 DAS all seeds had germinated indicating that effects were not long lasting.

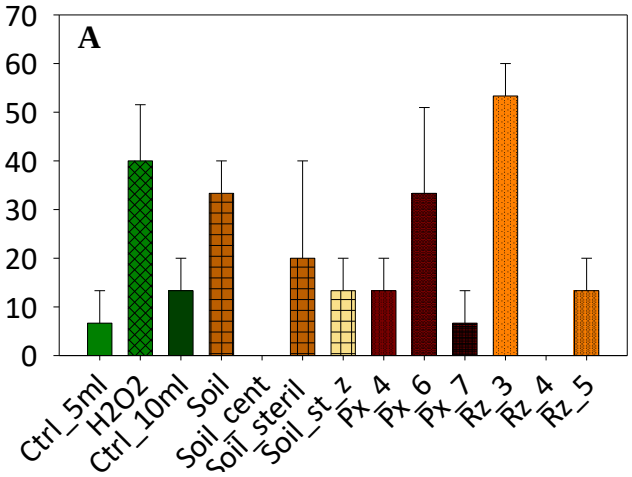


Figure 3-63 Germination rates Germ_3
Germination rate 1 DAS in % of total maize seeds; Means + SE

Additionally, in all experiments data were not normally distributed and no statistical significant differences were found.

3.8.1.3 Discussion on germination tests

An improved germination rate or faster plant emergence makes plants more competitive under field conditions and may improve yield. Also PGPR products might influence germination rate of plants (Lucy et al., 2004). In bioassays at the partner institute JKI (Eltlbany 2014, *personal communication*) application of microbial BE products also showed an improved germination rate for tomato seeds. Several short germination tests were conducted on filter papers using a variety of different treatments. The objectives were a comparison between different BE products, application techniques and rates as well as an investigation of the interaction of BE application with the soil substrate.

3.8.1.3.1 Dosage depending effects by BE treatments

As already seen for the BFDC product (Exp_2), some of the BE products may have negative influence on germination rate at high concentration. In our experiments high application rates of the Rz product caused negative effects, supporting previous observations by the company AbiTEP (*personal communication*). In the germination experiment lowest concentration in the liquid (Germ_2 and Germ_3) was most beneficial. In Germ_2 Rz application rates higher than 10^5 CFUs ml^{-1} had negative effects on germination rate as compared to the Ctrl and caused stress symptoms. A further reduction of the application rate in Germ_3 to only 10^3 CFUs ml^{-1} showed an increased germination rate as compared to the Ctrl. This was an interesting finding considering high application rates of > 10^6 CFU of Rz g^{-1} soil in most of our experiments. Nevertheless, a comparison with soil application rates is difficult because of the sorption capacities of the soil. Therefore, in the Rz_soil treatment, containing about 10^8 CFUs ml^{-1}, no plant stress responses, as seen in the Rz_7 or Rz_8 treatment, were observed. For Px detrimental effects were seen only at the highest concentration (10^9 CFU ml^{-1}) whereas this concentration was never reached in the pot or field experiments.

Interestingly, seed infiltration had different effects. Even though Rz concentration in the seed treatment was relatively high, the treatment performed well for seed germination. A reason could be the effect of the coating that may have influenced water flow to the seed. In contrast, seeds with Px seed infiltration did not perform well, as also seen in the field experiment 2015.

NaCl did not exhibit negative effects on the plants. This aspect was addressed to exclude that the differences in plant growth observed at JKI are not a response to a depressed plant growth in the Ctrl due to the salt contents in the solution.

3.8.1.3.2 Beneficial effects of the soil suspension

In general soil suspension had positive effects on seed germination. Plant growth in Germ_2 as well as germination rate in Germ_3 was promoted by soil treatment. One reason could be the natural microflora in the soils. Nevertheless, the missing effect in the treatment with supernatant (Soil_cent) and the still better germination rate in the sterilized (heat-treated) soil indicate that the reason for the better germination rate might be a physico-chemical process probably also connected to water flow, water storage or hygroscopic effects. Seed priming with water ("water priming" or "hydro-priming") is known to affect germination rate and improve seedling emergence and early plant growth (Rehman et al., 2015; Singh et al., 2015). Additionally, micronutrients in soil, such as Zn and Mn, may also contribute to the improved germination (Imran et al., 2013).

The objective of the research was to see if major differences among treatments would occur. Although differences were observed, results indicate that any advantage due to a faster germination rate vanished after a short time. The results support the observations from pot or field experiments (see Exp_2 and Exp_12), in which a strong beneficial effect of microbial BE treatment on emergence rate was absent. Therefore, it can be assumed that the significance for PGPR-derived stimulation of seed germination is rather low. Nevertheless, this observation does not deny the potential of (micronutrient) seed priming for stress alleviation or seed ball technology to support water uptake into the plant seed.

3.9 Bacterial activity

3.9.1 Soil bacterial activity of *B. amyloliquefaciens* cultures (Exp_21)

3.9.1.1 Introduction Exp_21

In all *Bacillus* products used, the bacteria are stored as endospores. Endospores are dormant, metabolically inactive structures that need to germinate to influence their environment. Endospores are formed by some members of the bacterial genera *Clostridium* and *Bacillus*. As described for the well-studied *B. subtilis*, they are formed by asymmetric cell division and encapsulation of the smaller by the bigger cell in response to adverse environmental conditions such as nutrient limitation causing starvation (Errington, 2003). They are extremely resistant against high temperature, chemical solvents like alcohol or radiation (Errington, 2003; Thomas, 2006). The resistance to alcohol was also observed in our experiments leading to contaminations of the spatula. Bleach (sodium hypochlorite) seemed to be more effective for the Rz strain (*personal communication* with AbiTEP). Interestingly, some adverse conditions, like heat treatment (e.g. 65 °C) or treatment with organic solvents such as mercaptoethanol, activate endospores and induce germination although the induction is reversible if other signals are missing (Keynan et al., 1964; Leuschner and Lillford, 1999). These signals are named germinants and are recognized by specific receptor molecules at the inner membrane of the endospores whereas it is still unknown how the germinants are able to pass through the outer layer of the endospore (Setlow, 2014). Previous heat or high pressure activation increase the responsiveness to the germinants. Potential germinants are L-alanine, L-valine, and L-asparagine but also glucose, fructose and K^+ (Black et al., 2005; Setlow, 2014) whereas D-amino acids are not effective in *B. subtilis*) (Leuschner and Lillford, 1999; Moir, 2006).

It was hypothesized that germination rates of the *B. amyloliquefaciens* FZB42 strain might be too low in our experiments to have strong plant growth promoting effects on the host plant. Purpose of Exp_21 was to investigate if pre-germination of endospores, using liquid LB medium, would lead to a better plant growth promoting activity of the Rz product. One additional control treatment was added in which soil was treated with a soil suspension enriched in Pseudomonades using liquid KB medium. The reason was that in previous experiments in the Biofector project strong growth promoting effects were observed for the Pj product but the only Ctrl was a treatment with a soil suspension (Nassal et al., 2018). We hypothesized that the soil suspension could also negatively affect plant growth (Walter et al., 1994), and therefore the BE effect would be pronounced.

3.9.1.2 Experimental design Exp_21

Exp_21 was conducted in small pots like Exp_20 and comprised only five treatments with five replicates making a Latin square design possible (Table 3-24). Substrate was prepared in exactly the same way as described for Exp_11. For Rz treatment seeds were soaked in the suspension of the commercial Rhizovital® product as in other experiments. For treatments 3 and 4 a fresh liquid LB culture of *B. amyloliquefaciens* with OD_{600} of ~ 1 was applied. To prepare the LB culture 0.5 ml Rhizovital® product were centrifuged at 4000 x g (rcf) for 5 min and washed twice with sterile LB to clean the pellet from formulation residues. Afterwards the cell pellet was re-suspended in 1 ml sterile liquid LB medium. 50 µl of this suspension were inoculated into 50 ml LB medium and incubated shaking overnight at 30 °C. To ensure that the bacterial culture had a low rate of dead cells a fresh culture was set up by inoculating 1 ml of the overnight culture into 50 ml sterile LB medium. Cell growth rate was determined by measuring the OD_{600} regularly every hour while incubating the culture at 30 °C. At about 5 - 6 h OD_{600} reached ~ 1. The LB culture was again centrifuged at 4000 x g for 5 min and the pellet was re-suspended in 50 ml sterile 0.3 % NaCl solution for application in the Ba treatment (Trt 3). For treatment 4 the NaCl-suspension was further supplemented with the fucoidan-rich product P2. Additionally, a fifth treatment was added in which plants were treated with a liquid culture of the natural soil microflora. Therefore 2.5 g of fresh soil were extracted using 25 ml 0.1 % peptone solution. 1 ml of this extract was inoculated in LB medium (used for seed treatment and first application) or KB medium. Soil cultures were treated like the cultures for Ba treatment and were applied re-suspended in 0.3 % NaCl. 7 DAS plants were thinned out from three to one plant per pot and a second BE application was done by soil surface application. A third BE application was done 14 DAS together with the

Table 3-24 Treatments Exp_21

Trt_Nr	Treatment	Seed treatment (ST)	1. Appl.	2. + 3. Appl.
1	Ctrl	0.3 % NaCl	/	6 ml of ST
2	Rz	10^9 CFU ml^{-1} in 0.3 % NaCl	/	6 ml of ST
3	Ba	re-suspended LB culture of OD_{600} ~ 1	/	6 ml of ST
4	Ba/P2	Ba supplement with 6 mg P2 ml^{-1}	6 ml of ST	Ba + 100 mg P2 ml^{-1}
5	Soil	re-suspended LB culture of OD_{600} ~ 1	8 ml of ST	re-suspended KB culture

Seed soaking before sowing for 10 min in respective treatment; 1. Application directly at sowing; 2. + 3. Application 7 + 14 DAS with 6 ml pot^{-1} of the respective solution as surface application; r = 5

application of 100 mg N as $CaNO_3$ to all pots due to symptoms of N deficiency. During harvest 29 DAS soil for tracing analysis was sampled from all treatments. Because soil was only loosely attached to the roots, no direct rhizosphere sampling was done but mainly soil

was collected that was near to or was shaken off the roots during root harvest. Soil was extracted as described above for tracing on selective *rif*-medium.

3.9.1.3 Results Exp_21

3.9.1.3.1 *Bacillus* liquid cultures

To ensure a high amount of germinated endospores and therefore metabolic active cells in the soil a fresh liquid LB culture was prepared for treatments 3 and 4. To optimize liquid cultures and to test inoculum densities plating assays of liquid cultures were performed and the results were compared to the measured OD_{600}. After 20 h incubation of the first liquid culture inoculated with the re-suspended Rz product the measured OD_{600} was ~ 2.6. Dilutions of this overnight culture resulted in equivalent reduction of OD_{600} indicating a linear relationship to cell density. Hourly measurements of the cell densities indicated a relatively slow generation time of 84 min (doubling of the OD_{600}) during the exponential phase. Also after several hours incubation a maximum OD_{600} of 2.6 did not increase further. Plating assays of bacterial cultures with OD_{600} ~ 1 indicated a cell density of about 10^8 CFU ml^{-1}. Therefore inoculation rates in treatment 2 were about 10 times higher than in treatment 3 and 4.

3.9.1.3.2 Plant performance

13, 19 and 26 DAS plant height of maize plants was measured with an average plant height of about 30, 50 and 70 cm. Soil treatment (5) followed by the Rz treatment (2) performed best 19 and 26 DAS but no significant differences among treatments were observed even though standard deviations were low. Average stem diameter 26 DAS differed significantly between the plants from the soil treatment (5) and the smaller Ctrl (1) and Ba (3) treatments (p=0.03). Plants were harvested already 29 DAS due to the small pot sizes, the lack of BE effects and the focus on tracing of *Bacillus*. Root and shoot dry weight (DW) did not significantly differ among treatments. For both measurements the Soil treatment performed best followed by treatments 4, 2, 3 and 1 for shoot DW and treatments 2, 1, 3 and 4 for the root DW (Figure 3-65). Values ranged from a maximum of 1.52 g shoot DW and 0.41 g root DW in treatment 5 to a minimum of 1.26 g shoot DW in treatment 1 and 0.33 g root DW in treatment 4.

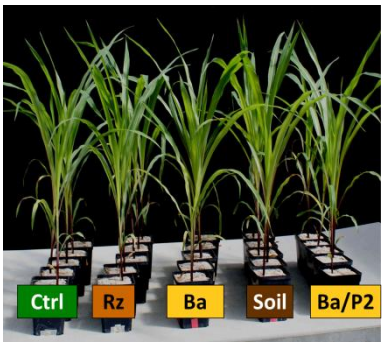


Figure 3-64 Maize 29 DAS Exp_21

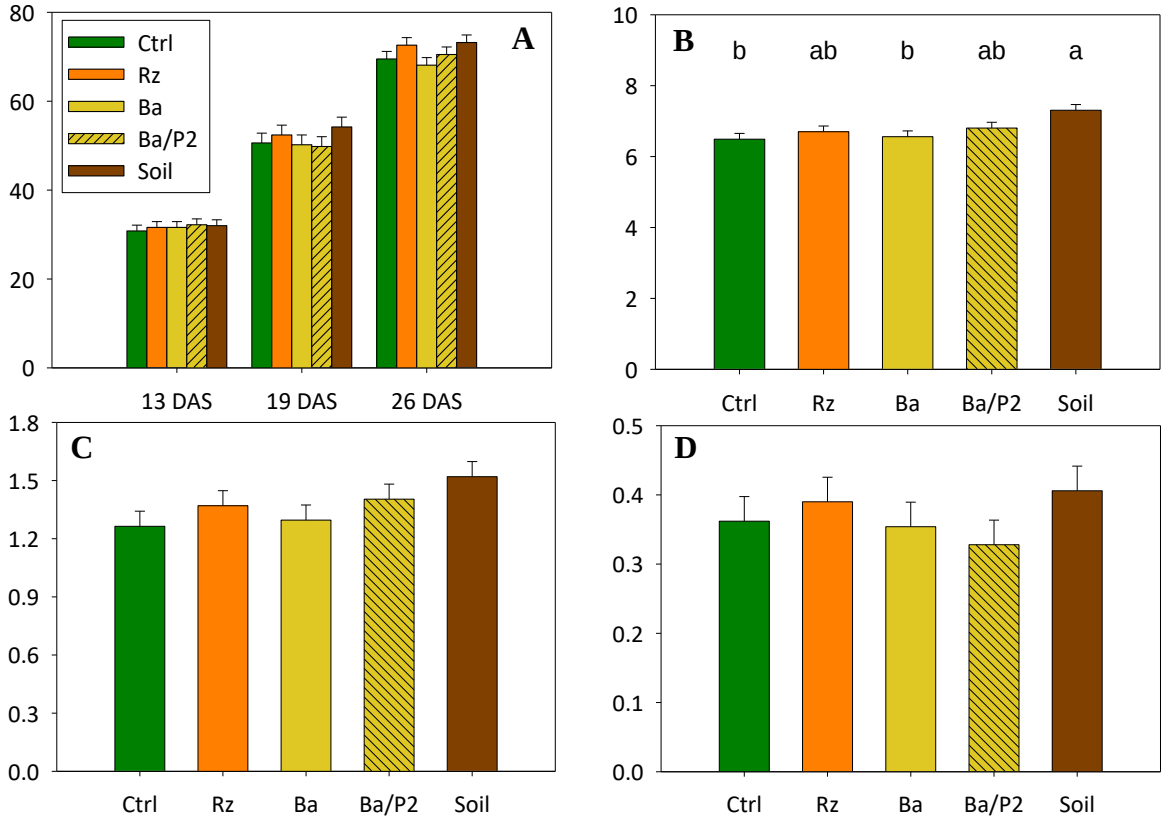


Figure 3-65 Results Exp_21; Plant height in cm at different measuring times (**A**), stem diameter in cm 26 DAS (**B**), shoot (**C**) and root (**D**) DW in g 29 DAS; Means + SE

3.9.1.3.3 Tracing of *Bacillus*

Plating on LB and LB$_{rif}$ media was performed for the BE suspensions used for seed soaking and first application as well as after harvest for extracts from rhizosphere soil. Plating of BE suspension on LB$_{rif}$ media resulted in 1.09 x 10^9 CFUs ml^{-1} for the untreated and 1.06 x 10^9 CFUs ml^{-1} for the 80°C treated Rz suspension (Trt_2), indicating about 100 % endospores in the product as expected from product description. 10 times lower and higher dilutions supported the results from counts of the optimal dilution. Plating of the untreated *B. amyloliquefaciens* (Trt_3 and 4) suspensions on LB medium resulted in the ~10^8 CFU ml^{-1}. A repetition of the plating of overnight cultures supported this value. Heat treatment at 80°C led to a decrease in the population to about 300 CFUs ml^{-1}. Plating of the soil suspension used for treatment 5 resulted in a low population density on LB$_{rif}$ media of about 500 CFUs ml^{-1}. The yellow colouration and the equally formed bubble shape of the colonies indicated that those bacteria were mainly Pseudomonades. Spontaneous rifampicin resistance is frequently reported for Pseudomonades and other soil bacteria (Bolstridge et al., 2009; D'Costa, 2006; Glandorf et al., 1992; Stubbs et al., 2014). No CFUs were found on the agar plates with heat treated soil suspension. To further enrich Pseudomonades for the later application of soil suspension KB medium was used for the cultivation of soil bacteria.

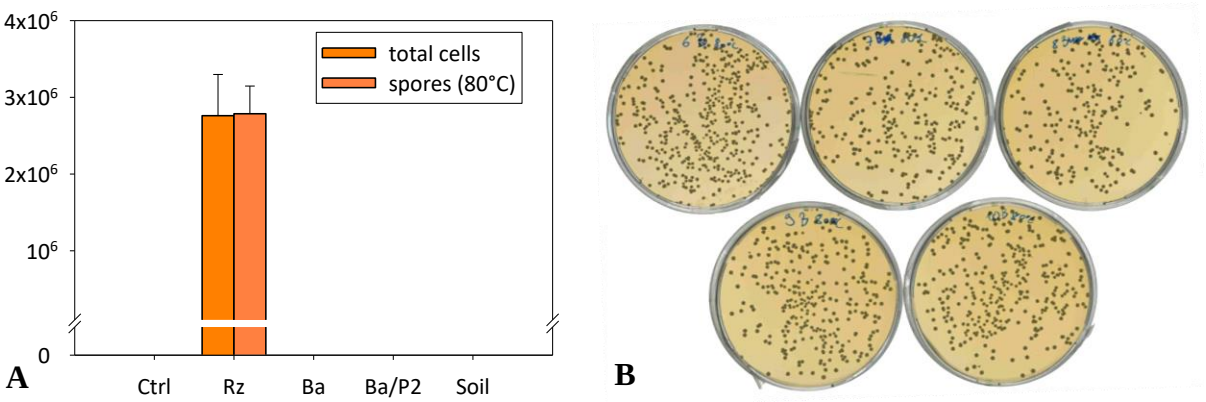


Figure 3-66 Tracing Exp_21; Number of total CFU and spore number g^{-1} rhizosphere soil 29 DAS (**A**), CFU on LB_{rif} after 1:10.000 dilution of soil suspension treated with Rz (**B**); Means + SE; outlier reduced (see text)

For tracing after harvest about 2.5 g of rhizosphere soil were incubated with 25 ml 0.1 % peptone solution for 15 min shaking. Serial dilutions with dilution from 10 – 1000 times were plated on LB_{rif} medium after or without heat treatment. In the Rz treatment counts of the highest dilution were about 20 – 50 CFUs agar $plate^{-1}$. Calculations based on volume, exact weight portion of soil and dilution factor resulted in an average of 2.8 x 10^6 CFU g $soil^{-1}$ for the untreated as well as for the 80°C heat treated extracts. This is about 50 % of the inoculum rate. For all other treatments, except for trace contaminations, no CFUs were found on both types of media. These trace contaminations were found randomly as single colonies on some agar plates and were probably the results from a not completely sterile spatula. Only for one pot of the Ctrl treatment a higher amount of 30 CFUs $plate^{-1}$ from an undiluted sample were found, indicating that maybe during harvest trace contaminations by cloves, contact with the soil sieves or other sources might have been occurred. To sum up, these results indicate that the number of endospores in the Rz treated pots was similar to the total number of cells and that in the pots treated with *B. amyloliquefaciens* liquid culture no *Bacillus* cells were able to survive either metabolically active or after sporulation as endospores.

3.9.1.4 Discussion Exp_21

3.9.1.4.1 Bacterial endospores

Tracing analysis indicated that about 50 % of the Rz population was germinating but the population of active cells declined rapidly until harvest time. This is supported by the analysis of bacterial density in the Ba treatment. By using liquid cultures the germination of endospores was artificially promoted in this treatment. Nevertheless, no *Bacillus* bacteria could be reisolated suggesting a survival rate of 0 %. This rapid decline of vegetative cells was also observed *B. subtilis* (van Elsas et al., 1986). A reason for the fast decline may be the low tolerance of the bacteria to the harsh soil environment in their exponential growth phase. Gram-positive *Bacilli* form endospores during conditions of starvation. Nevertheless, as sporulation takes up to 10 hours to be completed (McKenney et al., 2013), a sudden environmental change with nutrient starvation obviously has severe impact on the survival rate. Interestingly, also bacteria can adapt to stress. In studies on gram-positive bacteria, e.g. Pseudomonades and *E. coli*, that enter a "stationary phase" to endure adverse conditions (Navarro Llorens et al., 2010), starvation-selected mutants were isolated. They were kept in "stationary phases" with prolonged nutrient starvation and adapted to these conditions. They had lower death rates in later stress phases, indicating better resistance to environmental stress (Vasi and Lenski, 1999), although population growth was reduced under optimal nutrient supply (trade-off). Similarly, germinating endospores may be more stress tolerant than bacteria form fresh liquid cultures. Furthermore, as described in 3.9.1.1, in the rhizosphere germination is only triggered by certain signals, such as the contact with potential nutrient sources, therefore increasing survival rate of the overall population.

The maize rhizoplane was not analysed. Indeed, it was shown that bacterial density of inoculated *Bacillus* strains was higher in the rhizoplane as compared to the rhizosphere when endospores were applied (Bolstridge et al., 2009), probably due to higher amounts of root exudates. Nevertheless, when applied as pre-germinated cells, also here no *Bacillus* cells were recovered from the soil 28 days after application independently of the sampling site (rhizoplane or rhizosphere). As described in the discussion of Exp_14, *B. amyloliquefaciens* strains were also isolated from the endosphere of various host plants (Kim et al., 2016; Shu-Mei Zhang, 2012; Tan et al., 2013a; White et al., 2014; Zouari et al., 2016). Using a *gfp*-expressing B. *amyloliquefaciens* FZB42 mutant and confocal laser scanning microscopy Eltlbany et al. (2013, *unpublished*) could also detect the Rz strain inside plant root cells. Therefore it is possible that in the endosphere some bacteria survived.

3.9.1.4.2 Low activity of *B. amyloliquefaciens* in the rhizosphere

Plants were harvested 29 DAS because we assumed that the small pots were already limiting the root growth. At this time point in experiments from JKI already significant effects on plant performance were observed and also in other experiments, such as Exp_18, effects were visible in early developmental stages. Nevertheless, root and shoot DW in Exp_21 did not indicate any significant BE effects. In contrast, the soil treatment was the best performing treatment showing a significant increase in stem diameter. The increase in stem diameter was also often observed in other experiments for the Px product, containing the *P. fluorescens* strain. Obviously, in the liquid culture plant growth stimulating bacteria were enriched that resembled the Px strain. The similarity between the effects of the Px product and the soil suspension is again supporting the theory described for the Px mode of action (4.1.4.2). For Ba/P2 no plant growth stimulation was observed, suggesting that the concentration was not sufficient to exhibit prebiotic effects as observed for the Px milk powder formulation. A putative "priming" effect leading to growth promotion was also observed in tomato plants treated with dead cells of the *Pseudomonas jessenii* (Pj) strain (Nassal et al., 2018). Here *Gammaproteobacteria* were enriched and activity of acid phosphomonoesterase increased. In contrast, the application of mixed soil bacteria cultivated in LB-Lennox media resulted in growth depression and immobilization of P, probably by adsorption, as suggested by decreased soluble P and Pmic in the soil and lower plant P uptake.

3.9.1.4.3 Factors for successful BE plant growth stimulation

The question why plant growth stimulation by Rz in most of our experiments failed remains largely unanswered. Results from Exp_21 suggest that low germination rates are not a limiting factor. As already discussed in Exp_18, also inoculum densities or high root colonization rates do not seem to be crucial points for effectiveness of the Rz strain. Seed treatment in Exp_18, in the field experiment 2015 and in the germination tests were showing better results than high inoculation rates in the soil. It is more probable that the activity of certain biostimulation or biocontrol traits in the Rz strain have to be active and that the mutualistic relationship, probably depending on environmental conditions and growth stage of the host plant, is established. The potential influence of light is discussed in 4.3.3. The potential effect of ammonium N-nutrition for BE-plant interaction is addressed in 4.1.2.2.1. Especially for the Rz strain (FZB42) ammonium nutrition strongly promoted efficacy (Mpanga et al., 2019a, 2019b).

3.10 Field experiments

3.10.1 Introduction

In total four field experiments were conducted at the agricultural research station Ihinger Hof (Renningen, Germany) in the years 2014 and 2015. Purpose of the experiments was the investigation of microbial BEs and seaweed extracts in agricultural practice. Hypotheses are explained in more detail at the beginning of the respective discussions.

Exp_12 tested the combined application of microbial BEs and seaweed extracts reconnecting to the Exp_2, Exp_3 and Exp_10 as well as the *in vitro* experiments (see 3.3).

In Exp_13 two different application rates of the Rz and BFDC products as well as the fungal *Trichoderma sp.* product were investigated.

In Exp_15 the Rz and Px strain were applied under different fertilization regimes (urea, CULTAN, manure, ammonium) using different application techniques (seed treatment, granules, band and broadcast application). In Exp_16 foliar application of SF was tested.

3.10.2 Experimental designs

3.10.2.1 Research station Ihinger Hof

The more than 200 ha of farming land are cultivated conventionally with a research focus on plant protection and precision farming. Due to excellent technical equipment, such as different types of tractors, soil tillage machines, RTK-GPS (real-time-kinematic) for precision farming, small scale plot sowing and harvesting systems, an excellent technical support by the staff members and its proximity to the University of Hohenheim, the research station was chosen to test BE application methods under agricultural practice conditions. Nevertheless, as also visible in Table 2-20, soils were well supplied with phosphorus, ranging in the class of D (reduced P-fertilization recommended) according to VDLUFA (Taube et al., 2015). Average annual temperature is 7.7 °C, average temperature for the month April-September is about 14.8 °C. Average annual precipitation is 681 mm, with an average of 392 mm from April-September.

3.10.2.2 Randomisation, tillage and sowing

In each year two separate experiments were conducted with separate randomisation. Due to the large variation of environmental factors in the field, especially soil properties, all field experiments were conducted in completely randomized block or row-column designs.

Because a large amount of treatments was tested, latin-square designs were not feasible. Therefore, in Exp_12 one square of the R x C design contained five treatments, whereas in Exp_15 one square contained two treatments. Arrangement of plot plans, sowing, broadcast application of N and P fertilizers, underfoot placement of urea or the different ammonium phosphates, maize harvest and N_{min} post-harvest analyses were done GPS-based and with the technical support of the staff members and professional equipment. Application of all BEs and F_{MP}, all measurements and intermediate samplings were done manually.

Tillage in autumn was done with a plough in ca. 25 cm depth. In spring before sowing and directly after the soil application of BE products seed bed preparation was done using a Dutzi rotor tiller with a 10 cm working depth to ensure a homogenous and 10 cm deep incorporation of BE products into the soil. Sowing was done using a Kuhn pneumatic precision seed drill with a working width of 4.5 m (or 3 m in Exp_16), 6 rows á 75 cm row distance and a sowing density of 10 seeds m^{-2}. Figure 3-67 and Figure 3-87 show the experimental design and randomization used in the field experiments at Ihinger Hof.

3.10.2.3 Fertilization and treatments

Overviews on the fertilization regime of the different treatments in the field experiments 2014 and 2015 are given in Table 3-25 and Table 3-26 below. In both years, the two bigger experiments were conducted as collective experiments together with another doctoral student investigating different experimental issues. The description and results from this part of the 2014 field experiment was already published (Nkebiwe et al., 2016b). Because some of the treatments and results from 2015 are not described in the mentioned reference, data on this experimental part were included in this thesis. One example are tracing analyses to determine inoculation rates of the BEs Px and Rz in treatments with different application strategies and densities that were performed for the CULTAN treatments (see below) in the experiment 2015. N fertilization rates were calculated based on N_{min} values (38 and 61 kg N ha^{-1} in 2014 and 2015) and target N rate of 190 kg N ha^{-1}.

3.10.2.4 BE applications

Application of seaweed extracts and microbial BEs was done using water suspensions of the products that were freshly prepared on the field, then distributed with 10 l watering cans and evenly spread on the soil surface. All dilutions were done in 20 l of water $plot^{-1}$. Application rates for the bacterial products Px and Rz were 10^9 CFU kg^{-1} soil and for fungal products BFDC and TP 10^8 CFU kg^{-1} soil. All calculations were based on an assumed bulk density of 1.5 kg l^{-1} and a soil depth of 10 cm.

3.10.3 Combination of PGPMs and seaweed extracts (Exp_12 and 13)

3.10.3.1 Field experimental designs 2014

Maize in Exp_12 and Exp_13 was sown on May 21st. In this experiment multiple BE applications were performed, starting with broadcast applications one day before sowing and further applications as band application in the maize row at 6th leaf stage 27 DAS. In Exp_13 lower inoculum rates were tested (10 and 100 times lower than in Exp_12) (Table 3-25).

Table 3-25 Overview treatments IHO 2014

Trt_Nr	Treatment	Placement	Broadcast	1. Appl.	2. Appl.	3. Appl.	Total
7	0_Ctrl	/	/	/	/	/	/
9	Std_Ctrl	F_{MAP}	F_{Nov}	/	/	/	/
17	P_Ctrl	F_{MAP}	F_{Nov} + TSP	/	/	/	/
1	Easy	F_{ES}	F_{Nov}	/	/	/	/
10	Easy_Bac	F_{ES}	F_{Nov}		unknown spore concentration		
14	DuraTec	F_{Dur}	/	/	/	/	/
4	Px	F_{MAP}	F_{Nov}	10^9 (23)	10^9 (23)	/	$2x10^9$ (46)
23	Px_Af	F_{MAP}	F_{Nov}		see single inoculation		
15	Px_SF	F_{MAP}	F_{Nov}		see single inoculation		
6	Rz	F_{MAP}	F_{Nov}	10^9 (60)	10^9 (8)	/	$2x10^9$ (68)
18	Rz_Af	F_{MAP}	F_{Nov}		see single inoculation		
3	Rz_SF	F_{MAP}	F_{Nov}		see single inoculation		
16	BFDC	F_{MAP}	F_{Nov}	10^8 (150)	10^8 (20)	/	$2x10^8$ (170)
12	BFDC_Af	F_{MAP}	F_{Nov}		see single inoculation		
22	BFDC_SF	F_{MAP}	F_{Nov}		see single inoculation		
2	Af	F_{MAP}	F_{Nov}	15 (23)	15 (6)	15 (6)	45 (35)
19	SF	F_{MAP}	F_{Nov}	17 (25)	15 (6)	15 (6)	47 (37)
26	BFDC_med	F_{MAP}	F_{Nov}	10^7 (15)	10^7 (2)	/	$2x10^7$ (17)
27	Rz_med	F_{MAP}	F_{Nov}	10^8 (6)	10^8 (0.8)	/	$2x10^8$ (6.8)
28	Rz_low	F_{MAP}	F_{Nov}	10^7 (0.6)	10^7 (0.08)	/	$2x10^7$ (0.68)
29	Ctrl_E	F_{MAP}	F_{Nov}	/	/	/	/
30	BFDC_low	F_{MAP}	F_{Nov}	10^6 (1.5)	10^6 (0.2)	/	$2x10^6$ (60)
31	TP	F_{MAP}	F_{Nov}	10^8 (150)	10^8 (20)	/	$2x10^8$ (170)

Underfoot *placement*: F_{MAP} / F_{ES} = 17 kg N ha^{-1} and ca. 35 kg P ha^{-1} 5 x 5 cm from seeds; F_{Dur} = placed at a rate of 135 kg N ha^{-1} (~49 kg P ha^{-1}) in a 20 cm band under the sowing row; *broadcast before sowing*: additional fertilization of triple superphosphate (TSP) at a rate of 133 kg P ha^{-1}; *broadcast (late)*: F_{Nov} broadcast application at 3 – 4 leaf stage at a rate of 135 kg N ha^{-1}. *BE applications*: 1. Broadcast, 2. + 3. Band application (10 cm width). Px in both applications broadcast. Application rates for microbes in CFU kg^{-1} soil (and kg product ha^{-1}), for seaweed extracts (Af/SF) in mg kg $soil^{-1}$ (l ha^{-1}). Easy_Bac is the F_{ES} containing additionally 0.15 % *Bacillus subtilis E4-CDX®* endospores.

For seaweed extracts a third application as leaf application was performed at 10th leaf stage. Broadcast N-fertilization was postponed to the 6th leaf stages due to experimental design (see discussion). For Rz treatments in Exp_13 the *rif*-resistant mutant was used for making a tracing on selective media easier. This mutant was also used in 2015 in all Rz treatments. Randomization and arrangement of plots is visualized in Figure 3-67. Plot size in both experiments was 45 m², including six maize rows. Of this plot area only the four inner rows at a length of 9 m were harvested.

Emergence rate was determined 9 DAS. In each of the three inner rows plants from randomly picked 2 m length were counted. 25 DAS whole plant sampling was done to determine early effects of growth stimulation. For each sampling four plants per plot were sampled from the first four blocks. The roots of the plants were used for tracing analysis. Sampling of leaf samples for P and N analysis as well as plant height and SPAD measurements were performed 78 DAS (11 weeks after sowing, BBCH 61-75). Root sampling for mycorrhiza analysis was done 84 DAS. This time only parts of the roots were excavated. Sampling was done for the first four blocks of the Ctrl treatments (7, 9, 17) and the Px / Rz treatments (4, 6). For each plot four samples were analysed using Ø 162 intersect counts (2.6.4). A second root sampling for BE tracing was done 92 days after 1. BE application (64 days after 2. application). Harvest for forage maize was done by the technical staff of the Ihinger Hof and was performed, for organizational reasons, at the beginning of November in two separate harvest steps. Half of the plots were harvested 167 DAS, the other half 173 DAS.

	I					II					III					IV					V					
A	~~25~~	~~21~~	23	22	~~24~~	16	19	17	18	~~20~~	6	~~8~~	9	10	7	~~13~~	15	14	~~11~~	12	2	1	3	4	~~5~~	4.5 m
B	3	~~5~~	1	4	2	~~25~~	~~24~~	23	22	~~21~~	15	~~11~~	14	~~13~~	12	19	16	18	17	~~20~~	~~8~~	10	6	9	7	
C	~~20~~	19	17	18	16	12	~~11~~	14	~~13~~	15	4	1	2	~~5~~	3	7	9	6	~~8~~	10	~~25~~	~~24~~	23	22	~~21~~	
D	~~8~~	9	6	10	7	3	~~5~~	1	4	2	16	18	~~20~~	19	17	~~21~~	~~25~~	23	~~24~~	22	12	~~11~~	~~13~~	15	14	
E	14	~~13~~	15	12	~~11~~	10	9	6	7	~~8~~	~~24~~	~~25~~	~~21~~	23	22	~~5~~	1	3	4	2	18	~~20~~	19	17	16	

10 m

250 m

26	29	30	27	31	28	31	28	27	30	29	26	28	27	29	31	30	26	29	31	27	28	26	30
I						II						III						IV					

Figure 3-67 Plot plan of the field experiments 2014. Plots with 1 - 25 are part of Exp_12 and plots 26 - 31 are part of Exp_13. Crossed out numbers indicate treatments that are described elsewhere (Nkebiwe et al., 2016b). Exp_15 was divided in 5 rows and 5 blocks with each five columns. Exp_13 was divided in 4 blocks only.

The roots of the sampled plants were sent to FIBL (Switzerland, Cécile Thonar) for analysis of the Px microbial density via the qPCR method and to the company ABiTEP (Berlin, Kristin Dietel) for analysis of the Rz population via semi-selective media. Additionally, semi-selective plating of root extracts on NP medium was done in our institute to analyse the population of Pseudomonades in the treatments. For both analyses roots were washed and then the rhizoplane was analysed. The method at ABiTEP was using non-selective plating and heat treatment (at 80°C, see 2.6.2.1) for selection of spores. After plating the suspensions, colonies were analyzed via colony-PCR (communication with ABiTEP). Rhizosphere (root with adhering soil) as well as rhizoplane (bacteria sticking on the surface of the washed root) samples were analyzed.

3.10.3.2 Results Exp_12: Combination of PGPMs and seaweed extracts in forage maize

3.10.3.2.1 Plant performance

9 DAS emergence rate was determined for the BE treatments and two controls (Trt_7 and 9). At this time point not all plants had emerged as seen by the rather low average emergence rate of 58 %. The best performing plot showed an emergence rate of 84 %. Two-way-ANOVA indicated that different SWE as well as different microbial BE treatments differed significantly between each other. Af treatments showed best results in the comparison of different seaweed extracts whereas Px was the best performing microbial product. No significant interaction between microbial BEs and seaweed extracts was found.

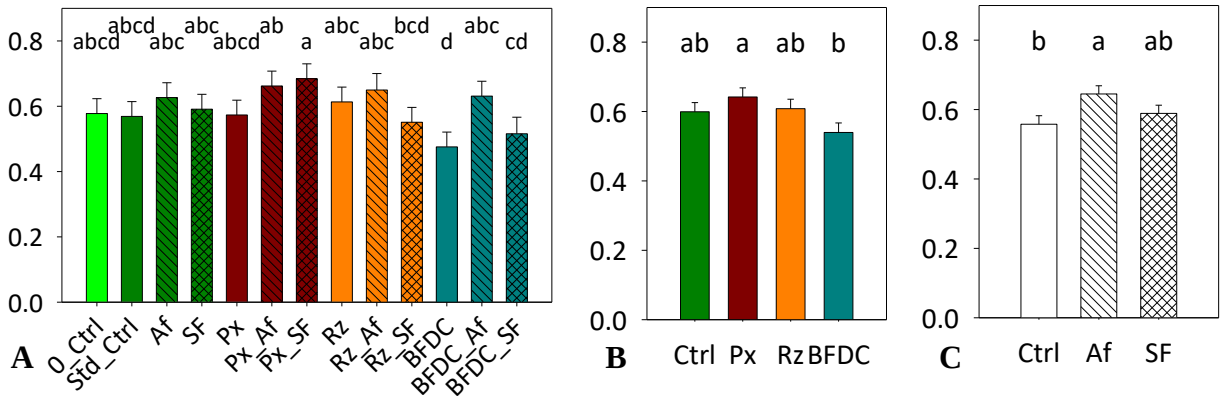


Figure 3-68 Emergence rate Exp_12; Values given as ratio not in percentage; ER of selected treatments (**A**), letters indicate significant differences in student's t-test (Tukey's-test not significant) after One-Way-ANOVA using SAS proc glimmix and the best performing model B: Trt Block; random Block*Row; Results from Tukey's test after Two-Way-ANOVA for comparison of microbial BEs over different SWE (**B**) and comparison of different SWE over different microbial BEs (**C**); Adj. means + SE; r = 5

25 DAS an intermediate sampling of whole plants from selected treatments was performed to assess early growth promoting effects of BE treatments. Sampling was done only from the first four blocks because the last block showed strong intra-block variation e.g. due to water logging, leaching and N-deficiency. After exclusion of one outlier in the Px_SF treatment the treatment differed significantly from several other treatments (Figure 3-69). Two-Way-ANOVA indicated significant interactions between microbial treatments and the SWE treatments. Among the different Px treatments SF was promoting plant growth whereas Af co-inoculation had a negative outcome on biomass as compared to the Ctrl without SWE. Among the different SF treatments Px was "benefitting" from SF application whereas Rz treated plants were depressed in growth after SF treatment. Nevertheless, without exclusion of the outlier, no significant difference among treatments was observed.

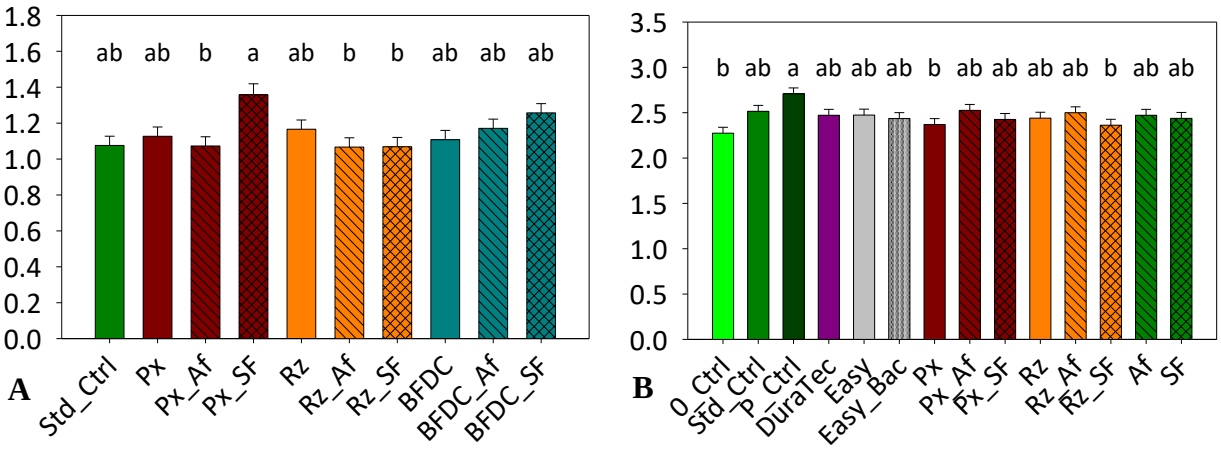


Figure 3-69 Plant growth Exp_12; Shoot dry weight per plant in g 25 DAS (**A**), Adj. means + SE; r = 4; Plant height in m 78 DAS (**B**); r = 5; BFDC plots were not measured due to oversowing (see 3.10.3.2.2); letters indicate significant differences in Tukey's test after proc glimmix model A

Plant height measurements were performed 78 DAS (11 weeks after sowing) at a BBCH of 61-75 (after N-fertilization and 2. BE application, see below). The P_Ctrl performed best among all treatments whereas the 0_Ctrl already showed a growth depression. Interestingly, also the Px and the Rz_SF treatment showed growth depression as compared to the Std_Ctrl. Af treatments showed a tendency for increased plant height, but the difference to the treatments without Af was not significant (p=0.0943).

3.10.3.2.2 Late N-fertilization and second BE application

At 6th leaf stage, together with CULTAN application in the treatments of Nkebiwe, late N-fertilization was done using F_{Nov} as leaf application. Due to the high fertilization rates and the absence of rain fall after the application, serious damage of the maize leaves was caused in all F_{Nov} fertilized treatments (Figure 3-70), making a comparison to the CULTAN treatments impossible.

Figure 3-70 Leaf damage by late N-fertilization Exp_12; Serious necrosis on maize leaves (**A**); plots from early fertilization (left) vs late fertilization with F_{Nov} (**B**); healthy plants (in the front) vs damaged plants (back) (**C**)

An additional damage was caused by BFDC leaf application. The surfactant-like formulation of the BFDC product was probably causing a membrane break in the leaf cells thereby completely destroying all treated maize plants. One week later only necrotic plants were

found (Figure 3-71). Therefore, plots were re-sown manually to ensure that neighbouring plots did not benefit from the higher light perception caused by the gaps. Nevertheless, due to the late sowing no further measurements were done in the BFDC plots. At the end of the experiment biomass of the BFDC treated late sown plots was very high with healthy looking tall maize plants but the plants did not mature in time to be harvested together with the other plots.

Figure 3-71 BFDC leaf damage Exp_12; Leaf damage several hours after leaf application of BFDC (**A – C**), dead plants one week after application (**D**); also visible is the high amount of herbs on the field, probably due to experiments by the Dep. of Weed Science in the previous years and the cold temperatures in early spring

3.10.3.2.3 Mineral analysis

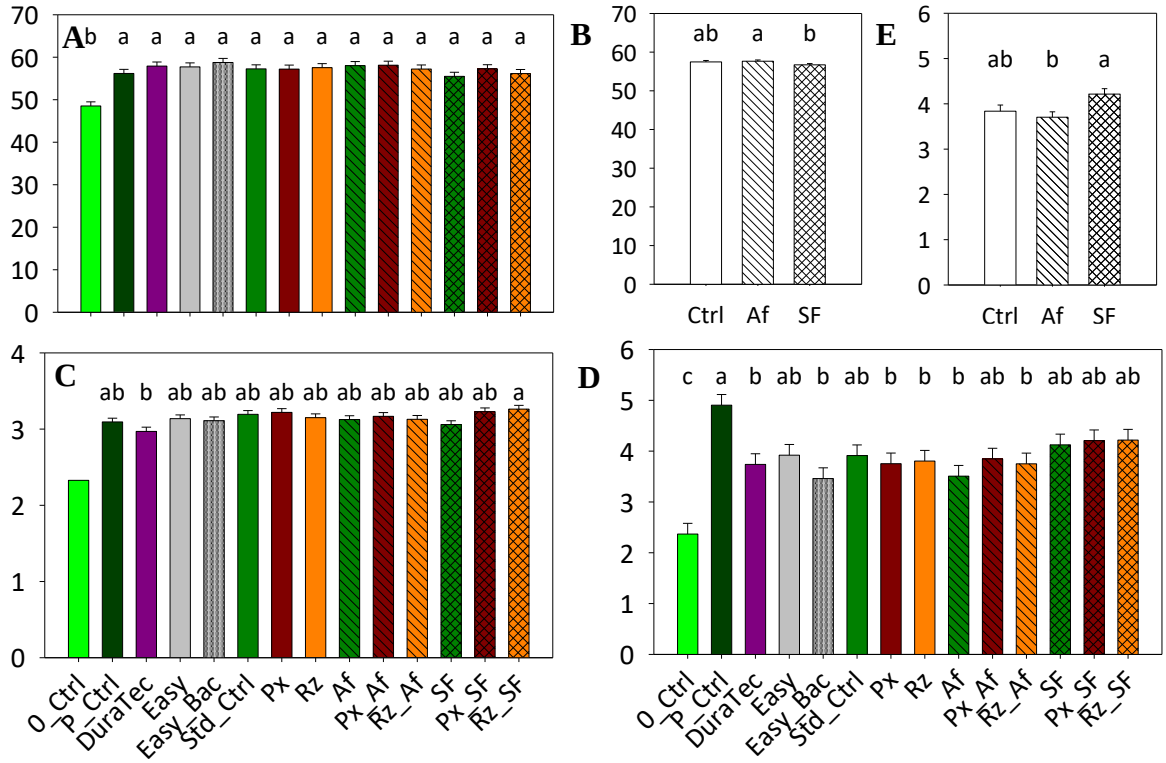


Figure 3-72 Nutrient status in maize leafs Exp_12; SPAD measurements (**A** + **B**), leaf N concentration in % (**C**) and P concentration in mg g^{-1} DM (**D** + **E**) in leaves 11 weeks after sowing; Adj. means + SE; r = 5 (DuraTec r=4); For SPAD and leaf N model A, for P model B (see Figure 3-68) + Tukey's test were used; Two-Way-ANOVA for BE treatments + Std_Ctrl only indicated significant difference between SF and Af treatments

11 weeks after sowing (see plant height measurements) SPAD measurements as well as P and N concentration of leaves opposite to the ear were determined. Significant differences in

SPAD values between the 0_Ctrl and all other treatments indicated N-deficiency in the 0_Ctrl. This was also supported by N-analysis of the leaves using a C/N-analyser. While SPAD values and N-concentrations correlated well in most cases (see 3.10.3.2.6) difference occurred for the seaweed extract treatments. SPAD analysis revealed higher SPAD values in the Af treatment but higher N-concentrations were found in the SF with Px and Rz.

For P concentration a significant difference between the 0_Ctrl and all other treatments was observed, showing the impact of the underfoot placements. Additionally, in the P_Ctrl treatment with additional P supply as TSP much higher P concentrations were measured than in all other treatments. The granulated complex fertilizers F_{Dur} and F_{ES} both showed similar effects on P nutrition like the underfoot placement of the CULTAN treatments. There were no differences or trends observed for microbial BE treatments although for the SWE treatments a trend for an improved P status in the leaves of SF treatments was found. Two-Way-ANOVA (after exclusion of the other fertilizer treatments) indicated indeed a significant difference between SF and Af treatment (p=0.0027) whereas SF treatments did not significantly differ from the Ctrl treatments without any SWE (p=0.0618).

In none of the measurements significant differences between the Std_Ctrl and any of the BE treatments were found.

3.10.3.2.4 Post-harvest analysis

Due to the huge experimental side and the diverse experimental conditions yield per plot varied strongly. Especially the plots to the north (rows A and B) as well as the plots to the east (block V) were strongly lacking nutrients as indicated by leaf chlorosis (Figure 3-73).

Figure 3-73 Maize growth Field experiment IHO 2014; Pictures from the west (**A**) and east (**B**) side of the maize field in 2014. At the right (**A**) or left (**B**) side respectively, are the plots from Exp_13.

Despite the huge variation statistical analysis of the yield data showed a significantly increased maize yield by the Af seaweed treatment over the SF treatments in Two-Way-ANOVA. Difference to the treatments without seaweed extract was not significant (p = 0.08). Average yield of Af treatments was about 73.0 dt whereas average yields of SF and Ctrl

treatments were about 67.3 and 67.6 dt, respectively. One-way-ANOVA shows only significant difference between the 0_Ctrl and three other treatments but no significant differences to the Std_Ctrl (p = 0.95 for comparison with the best performing BE treatment Px_Af). The Std_Ctrl and the microbial BE treatments Px and Rz had almost equal yields. Results for FW yield did not differ significantly from DW yield (data not shown). Exclusion of Block V (see discussion) reduced the effect of the Af treatment and no significant difference in Two-Way-ANOVA was found. Nevertheless, the Px_Af treatment remains the best treatment after DuraTec.

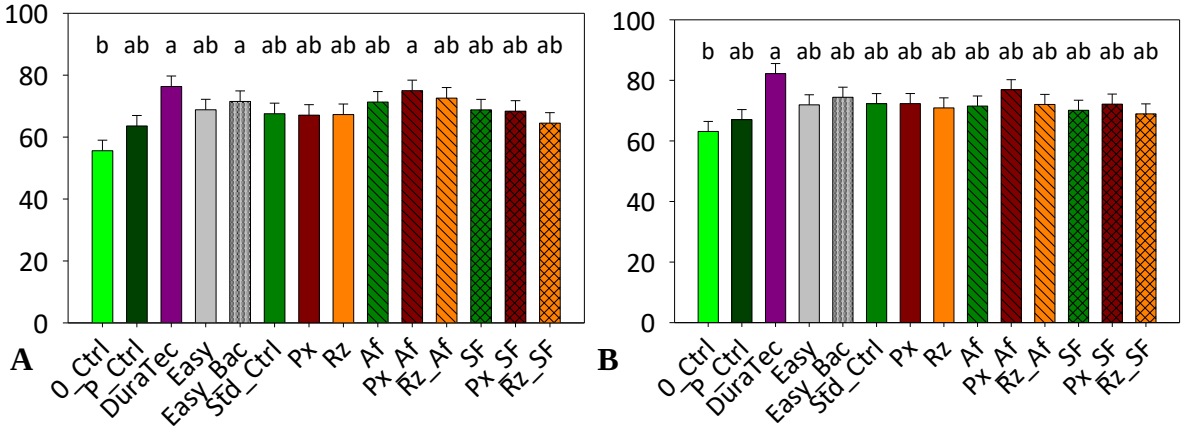


Figure 3-74 Yield data Exp_12; Dry weight corn yield in dt ha^{-1} with (**A**) and without (**B**) block V; Results from One-Way using model B in proc glimmix and Tukey's test; Adj. means + SE; r= 5; n = 70

Phosphorus analysis of the maize corn was done for different fertilizer treatments to assess the range of variation in which BE effects could occur (Figure 3-75). The block effect was only significant for the P contents but not P concentration. P concentration was highest in the P_Ctrl and lowest in the 0_Ctrl as expected. P concentrations and contents did not significantly differ between Std_Ctrl and P_Ctrl. DuraTec showed increased P contents due to the higher yield.

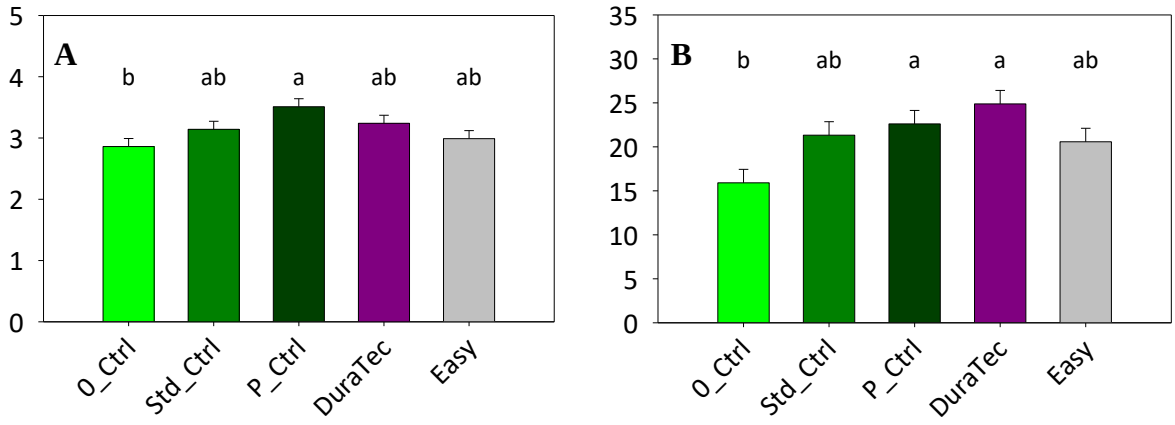


Figure 3-75 P analysis in maize corn Exp_12; P concentration in the maize corn in mg g^{-1} DM (**A**); P content of Maize corn / withdrawal of P by corn harvest in kg ha^{-1}(**B**); Results from OWA with simple model P conc. = Trt and model B P content =Trt Block; Adj. means + SE; r = 5, n = 25

3.10.3.2.5 Microbial analysis

3.10.3.2.5.1Rz tracing

For each plot sampled plant roots were mixed to one mixed sample. From this mixed sample two analytical replicates were analyzed. Cell numbers were not significantly influenced by experimental design (Block/Row) and therefore the model was simplified. For ANOVA analysis spore numbers were ln-transformed to reach normal distribution. Therefore not the adjusted means but the normal means were plotted in the diagram (Figure 3-76).

Rhizosphere and rhizoplane samples showed very similar results. The Ctrl samples show a lower number of cells, as expected. The cell counts in the Ctrl of about 10^3 to 10^4 CFU g^{-1} rhizosphere are similar to those found in pot experiments in our group using heat treatment and plating on LB medium suggesting that PCR-analysis was semi-selective and this number is representing all *Bacillus sp.* in the soil. Total number of cells was highest in the Rz_Af and Rz treatments with about $5x10^5$ CFU g^{-1} root. In the Rz_SF treatment total number was 50 % reduced to about $2.6x10^5$ CFU g^{-1} root.

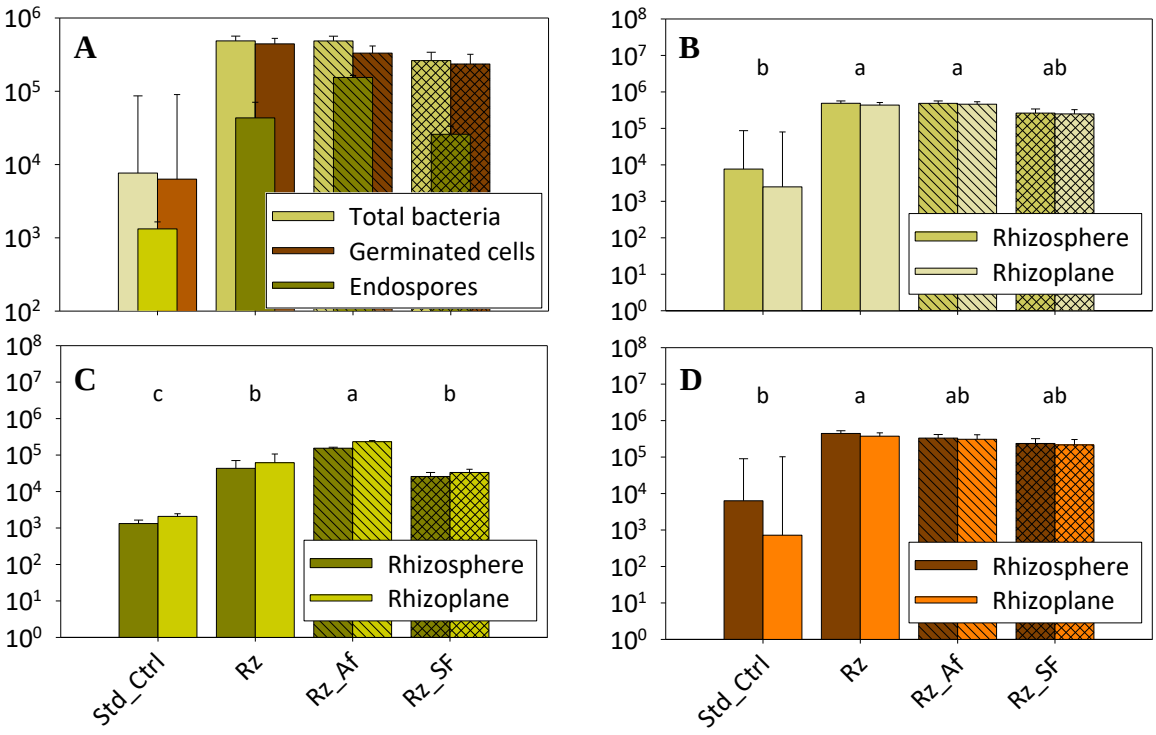


Figure 3-76 *Bacillus* root colonization Exp_12; Summary of the analysis of maize rhizosphere colonization by *Bacillus sp.* (e.g. Rz) (**A**), counts for the total number of CFU for both rhizoplane and rhizosphere (**B**), number of endospores (**C**) and calculated number of germinated / metabolically active cells (**D**) in CFU g^{-1} FW root; Adj. means or means (for spores) + SE; r = 4; glm with reduced model: Trt and Tukey's test were used; Letters indicate significant difference for both rhizosphere and rhizoplane with the exception of D (here rhizoplane samples did not significantly differ due to the high SE in the Std_Ctrl)

In the Rz product 100 % of the *Bacillus* bacteria are conserved as spores. Interestingly, root analysis indicated that most of the bacteria were in a metabolically active/ germinated state. In

the Std_Ctrl about 17 % of the cells were found as spores whereas in the Rz and Rz_SF treatments less than 10 % of spores were found. Nevertheless, in Rz_Af treatment 31.8 % of the bacteria were present in their endospore form. It seems that the Af seaweed extract was reducing *Bacillus sp.* germination significantly under the experimental conditions.

3.10.3.2.5.2Px tracing

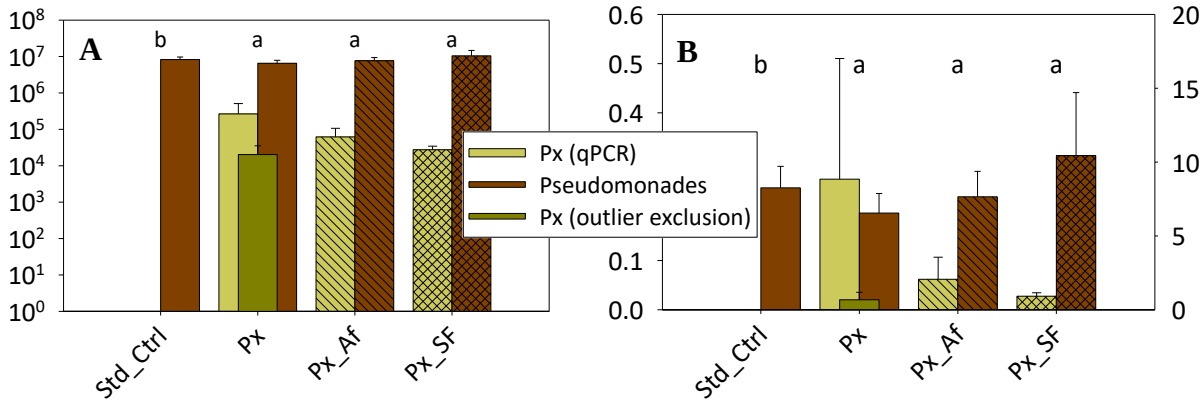


Figure 3-77 Px root colonization Exp_12 25 DAS; Summary of the analysis of maize rhizoplane colonization by *Pseudomonas sp.* "Proradix" plotted with two different scalings: Log-scale in CFU g^{-1} FW root (**A**) and linear scaling in 10^6 CFU g^{-1} FW root (**B**, Px (left y-axis), Pseudomonades (right y-axis)); green single bar represents the number of CFU found in the Px treatment after exclusion of a single extreme value; Results from OWA with simple model: Trt and Tukey's test; r = 4; letters indicate significant difference for Px cells only

25 DAS number of Pseudomonades in the samples did not significantly differ among treatments. Additionally, Px treatment showed a slightly lower total number of cells than the Std_Ctrl.

The RT-qPCR method at FIBL was optimized for specific amplification of a Px DNA segment on one of the bacterial plasmid vectors. Outcome of the analysis are number of copies g^{-1} root. This number can be calculated into CFU g^{-1} root (Mosimann et al., 2017). The calculated number of CFU varied between $2x10^4$ and $2x10^5$ CFU g^{-1} root whereas no Px DNA was found in the Std_Ctrl samples. In the Px treatment highest number of CFU was found. Nevertheless, after excluding one single extreme value of 10^6 CFU g^{-1} root (value was more than 10 x higher than all other values) CFU of Px samples was slightly lower than in the Af treatment. No significant differences among different SWE treatments were found.

A second root sampling for Px tracing was done 92 days after 1. BE application (64 days after 2. application). In the second sampling only in some (1 or 2 of 5 samples per treatment) of the root samples Px-specific DNA could be found. Therefore, a statistical analysis was not reasonable. Interestingly, in those samples in which DNA was found, the cell density was similar to the first sampling ($5x10^3$ – $5x10^4$ CFU g^{-1} FW root). For the Px_SF treatment in none of the samples Px DNA was found.

3.10.3.2.6 Mycorrhiza analysis

To test if application of PGPR is able to stimulate or improve mycorrhization of plant roots by mycorrhizal fungi in the field excavated maize roots were analyzed. Because the method is extremely time consuming only the five most promising treatments were investigated. In total 100 root samples were analyzed. Almost 16000 intersects with root fragments were checked for mycorrhizal structures. As a result the percentage of roots with mycorrhizal structures was statistically analyzed.

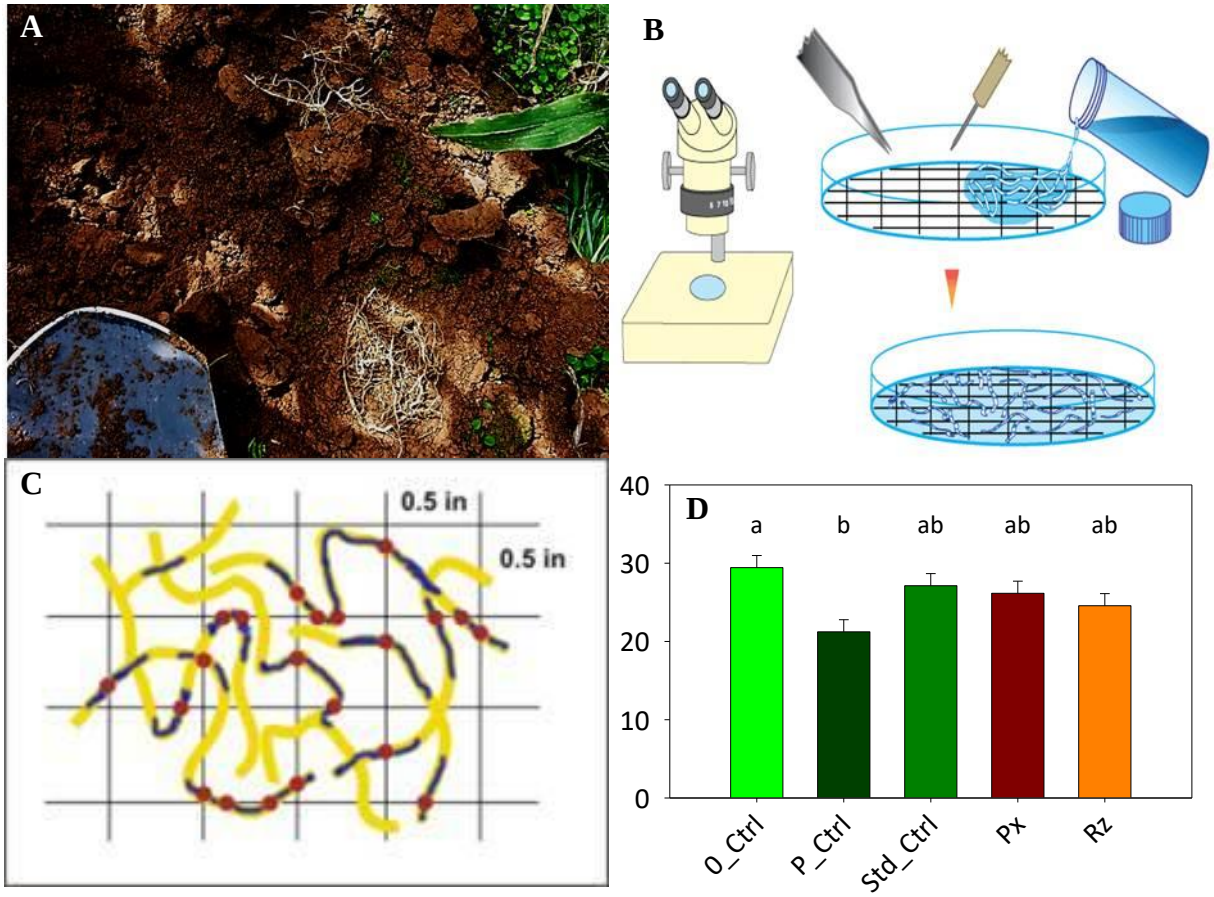


Figure 3-78 Mycorrhiza analysis Exp_12; Excavation and sampling of roots (**A**), illustration of the gridline intersect method (**B+C**, pictures taken from www.mycorrhizas.info), mycorrhization of maize roots in % intersect counts with mycorrhized roots of total counts; Adj. means + SE; r = 5; glm with reduced model: Trt and Tukey's test were used

Mycorrhizal structures were found in all plots (Figure 3-79) but results of the four root samples per plot sometimes varied strongly. Because they were analyzed at four different times it was checked if results were influenced by the time of analysis. Nevertheless, there was no significant pattern found for none of the factors "time of analysis", "block" or "row". All data were normally distributed. ANOVA indicated a significant difference between the 0_Ctrl and the P_Ctrl. No significant effect or trend for improved mycorrhization by the BE treatments as compared to the Std_Ctrl could be observed.

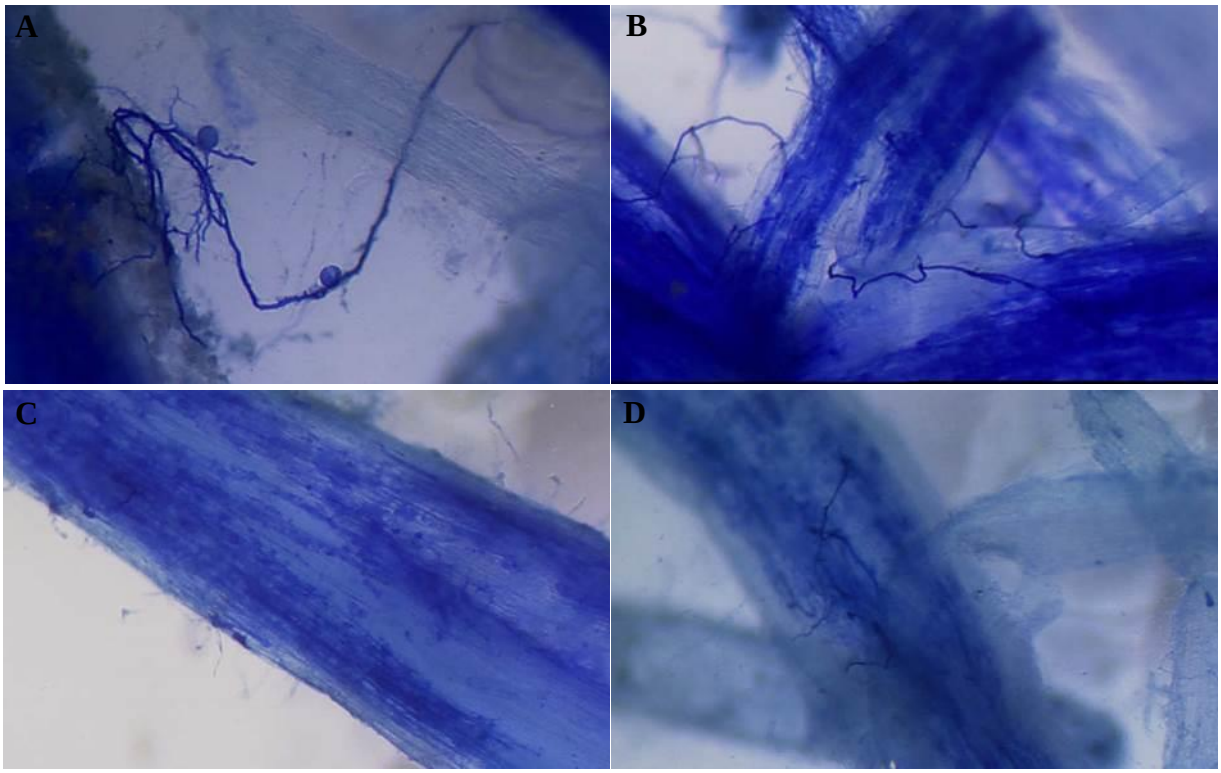


Figure 3-79 Mycorrhizal structures in maize roots Exp_12; External fungal mycelium (hyphae) and spores (**A**), internal and external mycelium (**B**), internal mycelium inside the maize root (**C**+**D**)

3.10.3.2.7 Correlation analysis

To detect variables that are tightly correlated to yield and therefore could be used as predictor variables in future experiments correlation analysis was performed using the "proc factor" procedure in SAS. Best correlation to the yield was found for plant height (ρ = 0.88) followed by the SPAD values (ρ = 0.78). C/N ratio of the leaves was negatively correlated with yield (ρ = -0.50). Leaf N concentration was even weaker correlated (ρ = 0.42) and P concentration showed lowest correlation (ρ = 0.24). As expected, SPAD measurements were relatively well correlated with leaf N (ρ = 0.66) but better correlated with C/N ratio (ρ=-0.71).

3.10.3.3 Discussion Exp_12

3.10.3.3.1 Hypotheses

In Exp_12 about 10 variables were measured. Most of them were measured only for specific treatments in which responses on these dependent variables were expected.

Main hypotheses for the research were:

1. BE treatments have the potential to improve plant performance at various time points during the season.
2. Product combinations of microbial BEs and seaweed extracts have synergistic effects on plant growth and are able to increase corn yield.

To assess possible mode of action and investigate more basic research questions it was analysed:

3. If nutritional status (N and P) in maize is improved by BE application and if BE application is able to compete with other conventional and innovative fertilization systems.
4. If PGPR are able to colonize the plant root system and maintain a high cell number and active status in the course of the growing season.
5. If PGPR root colonization and cell number in soil is influenced by co-inoculation of seaweed extracts.
6. If PGPR application is able to improve other ways of P acquisition, here with the focus on mycorrhization of maize roots by arbuscular mycorrhizal fungi (AMFs).

The seaweed extracts chosen for combination with the microbial BE products were the Af and the SF products. The products were chosen, because they were derived from different seaweeds and provided by two different project partners (Bioatlantis, Agriges). Additionally, they both showed the best prebiotic effects at low concentrations (3.3.4 and 3.3.4.4)

3.10.3.3.2 Plant growth promotion Exp_12

To assess the first hypothesis, 9 DAS emergence rate (ER), 25 DAS plant biomass, 78 DAS plant height and 167 / 173 DAS corn yield were measured. Emergence rate differed significantly among BE treatments. As mentioned in the results, it can be expected that not all plants had been emerged at the time of analysis. Corn yield at the end of the harvest also suggests that plant density in the field did not strongly differ. As also indicated by germination tests, germination rate of the maize seeds was normally about 100 %. Nevertheless, Af treatment was able to fasten plant emergence as compared to the Std_Ctrl

and the non-Af treated Rz and Px treatments. Also the combination of SF treatment with Px showed good results. Already after soil application BFDC was performing worse than the other treatments slowing down plant emergence. Only Af treatment could counteract the negative effect.

Interestingly, this negative effect vanished until the analysis of plant biomass two weeks later. Here all BFDC treatments tended to perform slightly better than the Std_Ctrl. Nevertheless, best performing was the Px_SF treatment and no significant difference between any BE treatment and the Std_Ctrl could be observed. The positive effect of the Af treatments as seen for ER were not found for the biomass sampling but re-occurred in the analysis of plant height. Besides this tendency, no BE effect was observed. In the yield data the positive effect of the Af treatment was confirmed. Best performing BE treatments was the Px_Af treatment and two-Way-ANOVA indicated significant yield improvement of Af treatment as compared to the SF treatment. However, none of the BE treatments or product combinations was able to significantly improve yield as compared to the Std_Ctrl.

BFDC led to serious damages during leaf application. Negative effects of BFDC were also seen for ER but also in the pot experiments Exp_1 and Exp_2. Nevertheless, these effects are obviously depending on application method and application rate. In field experiment Exp_13 as well as Exp_20 the product did not show these strong negative effects although no clear positive effects were observed here.

We could not observe any obvious synergistic effects. The combination of Px and SF promoted ER and shoot biomass 25 DAS was best in Px_SF treatment but the treatment did not perform especially well in later measurements. However, the Px_Af treatment was performing well or best in several measurements and the combination resulted also in the best corn yield. Even though in most cases it was mainly an Af effect that was significant, independent of the microbial BE, the good performance of the Px_Af treatment is the most promising result considering the complex experimental design as discussed under 3.10.3.3.6. Yield was increased about 11 % from 67.6 dt in the Std_Ctrl to 75 dt ha^{-1} in the Px_Af treatment. For the Rz treatments some interactions for the root colonization (discussed below) were observed but they did not strongly influence the yield.

Maize yield in Exp_12 was lower than expected from reports of the maize breeding company and the comparison with average maize yields from 2014. The variety Zea mays cv. ‘Colisee’ from the company KWS is a high yielding maize variety with an 100 – 112 % rel. corn yield

potential as compared to average corn yields in two different regions of Germany in the years 2012 – 2013 (KWS, 2014). Average corn yields in the country of Baden-Württemberg were about 116 dt ha^{-1} in 2014 (Deutsches Maiskomitee e.V. (DMK), 2016) whereas the cultivar 'Colisee' showed average yields of about 130 dt ha^{-1} in the years 2009 – 2013 in studies of the research centre LTZ Augustenberg, Karlsruhe (Amann, 2013). Fresh matter yields in Exp_12 and Exp_13 ranged from 90 – 120 dt ha^{-1}. Nevertheless, treatments in Exp_12 showed much lower yields than treatments in Exp_13 (70 vs. 85 dt ha^{-1} average DM yield). As experiments were fertilized in the same way, probably exposition and soil conditions were the main reason here. Exp_12 was conducted on a slope to the north direction whereas Exp_13 was at top of this experiment to the south. In Exp_12 the observed decrease in yield is probably due to the late fertilization and the leaf damage caused by the leaf application of the N-fertilizer. This is indicated by higher average DM yields in the CULTAN treatments (Ø 75 dt ha^{-1}) and the much better performance of the DuraTec treatment that had an average DM yield of 76 dt ha^{-1} and a FM yield of 118 dt ha^{-1}.

3.10.3.3.3 No improvement of plant nutrient status by BE application

As described in the introduction, many PGPR are known as phosphate-solubilizing bacteria (PSBs) and are able to acquire P from organic sources such as phytate but might also act as mycorrhiza helper bacteria promoting mycorrhization of host plants and thereby indirectly improving nutrient acquisition. The N and P status of plants was assessed in the leaves of the growing maize plants. The sampled leaf was the leaf opposite to the developing maize cob. There are various recommendations for which plant part should be sampled. Early sampling should be done for the whole plant whereas in later growth stages leaves near or opposite to the ear are recommended (A & L Plains Agricultural Laboratories, USA; (Kaiser et al., 2013). It was decided to sample the leaf opposite to the ear to avoid any damage to the ear.

The results for SPAD and N measurements of the Rz_SF treatment differed but in both measurements no significant differences or any trends in the comparison between the Std_Ctrl and the BE treatments were observed indicating that BE application did not have strong effects on N status. A clear N-deficiency was observed in the unfertilized 0_Ctrl. The lowest N concentration was found in the DuraTec and the highest in the Rz_SF treatment. These two results are negatively correlated with the yield data in which DuraTec performed best and the Rz_SF treatment the worst.

In SF treatments the P concentration in leaves was significantly increased. Nevertheless, as seen for the N status, the improved nutrient status did not result in a higher yield but was

probably a result of a decreased growth or nutrient translocation to the vegetative organs of the plant. Analysis of the P status in the corn was done only for different fertilizer treatments because no significant difference in yield was observed between the Std_Ctrl and BE treatments. In the DuraTec treatment P uptake was most efficient resulting in a high yield, despite much lower P fertilization rate as compared to the P_Ctrl. With only 37 % of the P_Ctrl fertilization rate and about 14 kg P ha^{-1} more than the Std_Ctrl in the DuraTec treatment the by far highest P uptake was measured. Nevertheless, in all treatments, except the 0_Ctrl, plants had P concentrations higher than 3 ppt and can therefore be considered in the range of optimal nutrient supply (Bergmann, 1993). As mentioned before, growth of the P_Ctrl and other treatments was obviously strongly limited by the suboptimal and even damaging N fertilization whereas DuraTec plants also might have benefitted from micronutrient supply.

Due to the optimal P status BE application did not have significant effects on plant P acquisition. The P status might also be a reason why mycorrhization was not improved by PGPR. It is known that the symbiosis between maize plants and AMF is depending on P status and P availability, because the plant is avoiding the costs of the symbiosis, such as supply with assimilates, if P status is sufficient (Graham et al., 1982; Marschner, 2012b). This is also supported by our data that show a significant decrease of mycorrhization in the P_Ctrl treatment as compared to the 0_Ctrl treatment.

3.10.3.3.4 Px root colonization

The ability of the PGPR for root colonization was investigated by analysis of the rhizoplane or rhizosphere of whole plant root samples. The samples were taken about four weeks after sowing to see if bacterial populations maintain stable in the course of the youth development of plants. We always hypothesized that the early growth phase of plants, in which the plant is most sensitive to environmental conditions because the root is not yet full established, is the most crucial time for plant-microbe interactions and the time when the biggest impact of BE application on growth promotion and nutrient supply can be expected. This hypothesis was supported by experiments with late application (Exp_10) and later on by early growth effects observed in experiments with intermediate harvest times (Exp_17, 18, 22).

Px population 26 days after BE application indicated a relatively high average Px population of about $1.3x10^5$ CFU g^{-1} root still present on the root surface. Excluding the single extreme value as an outlier, the average population is reduced to $3.6x10^4$ CFU g^{-1} root. This is about 13 and 4 % of the inoculum density g^{-1} soil. Obviously, four weeks after application

population had declined strongly even though there are no data for the soil itself. Soil-derived substances in the DNA extracts caused problems in PCR-amplification and therefore the strain specific tracing method was established for rhizoplane samples only (*personal communication*, Cécile Thonar). As seen before in several pot experiments, population of Pseudomonades is not correlated with the application of the BEs. Despite a relatively high inoculum density of 10^9 CFU kg^{-1} soil (but low as compared to pot experiments) the number of applied Px bacteria was about 100 times lower than that of all Pseudomonades.

The seaweed extracts did not have a significant impact on bacterial population but data also show the huge variation inside the population making analysis less stable than necessary to detect eventually minor but important changes in the population. To decrease variation and improve analysis a much higher sample amount and also amount of analytical replicates would be necessary. Unfortunately, the method is cost-intensive due to the probe qPCR technique and the Probe Fast qPCR Kit.

Another limitation concerns the tracing target. Working with the method, no direct information about active bacterial population in the soil but rather information about the abundance of bacterial plasmid DNA in the soil are gathered. DNA is relatively long-lasting in natural environments, especially if soils are dry, cold or only certain fragments are target of interest (Trevors, 1996). Therefore the data do not necessarily reflect the bacterial population at this time point but at an unknown, and probably environment depending, time point before sampling.

From literature it is known that population of applied bacteria often decline rapidly in natural soil systems (see 3.6.1.4.5). Especially the Px population is sensitive to environmental circumstances because the bacteria are not able to form protective endospores. This was also seen in our experiment at late root sampling. Only in some of the sampled roots Px DNA could be found. Nevertheless, at late root sampling roots were collected from in between maize rows. Additionally, broadcast application was already 93 days before sampling and the second application was done as band application. Therefore it is also possible that bacteria from second BE application did never reach the sampled roots in between rows.

As mentioned above, a correlation of population density and yield data was not possible due to the minimal effects in the yield.

3.10.3.3.5 Rz analysis

Due to the higher sample amount analysed and the plating method used at ABiTEP data for Rz root colonization are more reliable than the Px data. Good correlations between analytical replicates and for the comparison of rhizoplane and rhizosphere samples strongly support the outcome of the analysis. A general aspect that is obvious from the comparison of previous analyses (Exp_14 and 21) with the results from Exp_12 is the much higher amount of active cells and lower amount of spores, respectively. This is surprising given the less controlled and harsher conditions in the field as compared to the protected environment in the pot experiments. Assuming endospore germination to be a crucial pre-requisite for a PGPR to exert plant growth promoting activities this might explain why we were never able to observe strong BE effects for Rz in our greenhouse experiments (further addressed in Exp_21). Obviously, environmental conditions influence spore germination and might therefore also affect BE efficacy. In experiments at JKI, in which major effects of the Rz treatment were observed, unfortunately no data for percentage of spores of total population are available.

Nevertheless, even though spore germination in the field was much higher than in the pot experiments, no strong effects for growth promotion were observed. *Bacillus* sp. population was obviously strongly increased by the Rz application and root colonization (or at least presence and abundance of *Bacillus* bacteria in both rhizosphere and rhizoplane) was at a very high average level of $5x10^5$ CFU g^{-1} root (50 % of inoculum density), but no measurement indicated any significant effect or trend for a growth promotion or other effect on maize plants when Rz was compared to the Std_Ctrl treatment.

Interestingly, data suggest that Af application increased Rz endospore density or reduced endospore germination, respectively (a possible explanation is given in 3.10.3.5.2). Previous *in vitro* tests did not focus on the influence of seaweed products on spore germination but focussed on an increased population level only, therefore no further data are available to support this finding. Results from Exp_21 indicate that cell division rate of Rz is much slower than that of Px bacteria. Additionally, the time for endospore germination under specific environmental conditions is largely unknown. Therefore, the *in vitro* tests, probably fitting to the Px bacterium, are not easily applicable for Rz investigations. Nevertheless, due to a lack of growth promoting effects from product combinations of SWE and the Rz product these aspects were not further investigated.

A comparison of the results from root colonization with plant performance suggest that effectiveness of Rz was reduced by Af in the early plant development as compared to SF but

that later on the reduction in endospore germination might have postponed the activity and led to growth promotion in the later stages. However, due to the small differences between treatments interpretation is difficult. Also the results from Exp_13 do not provide more information, because the lower colonization rate could not be correlated with effectiveness for growth promotion.

3.10.3.3.6 Harvest

As described in the results, the Af treatments performed best among all BE treatments. The average difference between the Af treatments and the Std_Ctrl was about 6 dt. This is less than 10 % of difference in yield. Differences are not statistically significant and the treatment differences may be explained by the yield distribution in our field site. As already explained, block V showed the highest diversity in yield (Figure 3-80). Zooming in to block V the average yield decreases with each column of the experimental matrix in the direction of the east border of the maize field. All Af treatments are by chance located nearer to the west as e.g. the Std_Ctrl treatments. Especially in the Af and Rz_Af treatment (2, 18) plot yields of 70.5 and 74.7 dt ha^{-1} were harvested whereas in the Std_Ctrl and the 0_Ctrl only 48.6 and 25.5 dt ha^{-1} were harvested. These huge differences strongly influenced the outcome of the average yield per treatment. All other treatments in column 21 were CULTAN treatments and are no help in statistical analysis because they cannot be compared with the BE treatments due to a different fertilization regime. Interactions between Trt and Col or Block/Row combinations could statistically not been tested (here errors occurred in SAS due to overestimation).

Therefore the hypothesis that the Af treatments performed especially good under these suboptimal conditions as compared to Ctrl treatments is not proven.

This example shows how strongly the experimental design influenced the outcome of the analysis and how difficult interpretations are, if experimental designs are complex and yields do not differ strongly as in the case of the 0_Ctrl.

In contrast, the 0_Ctrl plants did not just have “bad luck” with their position but the comparison between Std_Ctrl and 0_Ctrl suggests that in Block V the full impact of missing N-fertilization became obvious. Therefore the unfavourable conditions were helping to detect those treatment effects. Unfortunately, it is difficult to find field sites with homogenous distribution to get valuable conclusions. This is further discussed under 3.10.3.3.7.

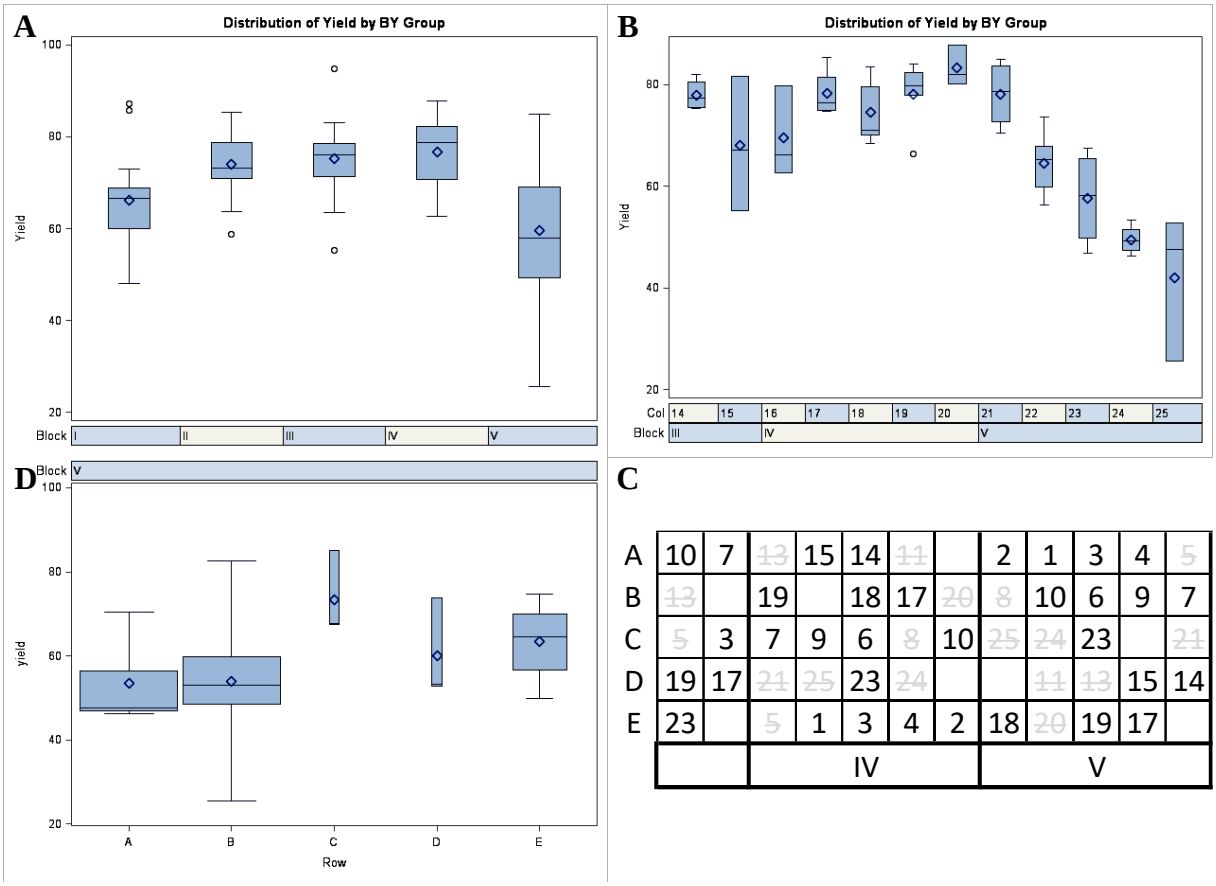


Figure 3-80 Yield distribution Exp_12; Block V showing strongest variation in yield (**A**); focus on the yield distribution per column shows a continuous decline in yield in the direction to the east border of the maize field (**B**); treatments 7 and 9 (0_Ctrl and Std_Ctrl) are in the last two columns whereas all Af treatments (2, 18, 23) are located in the columns more west (21 – 23) that also showed higher average yields for all treatments (**C**); Yield per row in Block V (**D**).

3.10.3.3.7 Methodological critique

In this section several aspects of the experiments, such as the experimental site, the experimental design, the BE application rates, the late N fertilization, sampling procedures as well as the harvest procedures will be reflected critically.

1. Experimental site

The experimental site was provided by the research station Ihinger Hof. Previous experiments from the institute of weed science on this experimental site influenced weed population and density. As seen in Figure 3-71 certain plots might have been more densely covered with weeds than others. However an evaluation was not done here. For sure the experimental site had certain slopes in both directions with a decline to the north in the direction of row A. Soil conditions and water availability in the plots, especially for those of Block V, differed strongly among each other.

.

2. Experimental design

In view of the inhomogeneity of the experimental site the experimental design of Exp_12 can be criticized. As described in the results part the site-effect strongly influenced the outcome of the analysis. Indeed the effects of the site where far bigger than the effects of the variants resulting in largely biased data. Because the inhomogeneity was known beforehand a row-column design was chosen as the experimental design. Nevertheless, due to the huge plot size and the five plots per Block/Row unit the row-column design lost its power to adjust for site specific inhomogeneity in the statistical analysis. In a latin-square design the position of each plot is exactly defined in the statistical model by its position in the block/row but there remains the bias that each unit is unique in its position and its combination with a variant. Therefore, a plot that has extremely bad conditions due to its position will negatively influence the results for the variant if the position effect is not seen everywhere in a block or a row but only as the results of the block/row combination.

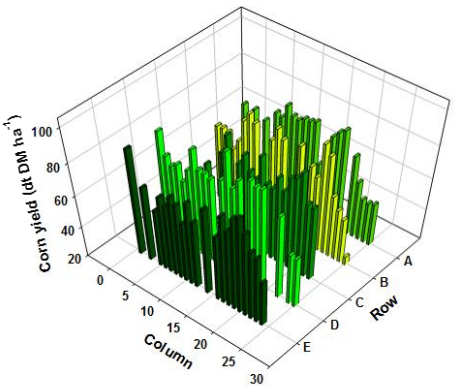


Figure 3-81 Yield distribution Exp_12

As seen for the results of Block V this was also true for our experiment. Certain row/block combinations resulted in extremely bad site-specific growing conditions (water logging, low nutrient contents, wind exposure) whereas other rows in block V provided better growing conditions for maize. This was leading to an extreme variation of yields in Block V. Owing to the fact that not each variant was present in each block/row unit the inhomogeneity inside the block influenced the variants in different ways leading to biases in the results. For this reason block V was excluded in several measurements from analysis thereby reducing the number of replicates and the power of the statistical analysis.

The problem of the row-column design is increasing, if data contain missing values. As statistical models calculate adjusted means using the inter-block and intra-block information to compensate for the missing value, an uncomplete design or sampling (see below) or high heterogeneity inside the blocks will even more influence results.

To sum up, the larger a block/ row unit, the bigger the inhomogeneity inside a unit resulting in biased data.

A. Reduction of plot size: Although a certain plot size is reasonable, especially in fertilization experiments due to border effects from neighbouring treatments, it is recommended to reduce plot size. With the focus on maize experiments at least four, better six seeding rows should be included so that the outer two rows can be excluded. Nevertheless, following the aspect of border effect it is not reasonable to have rectangular plots that are longer than their width. To increase total variant area it is better to increase replicate number.

B. Reduction of variants: To further decrease experimental area and, more important, block size the amount of variants should be reduced. This is also important in view of the feasibility of sampling. Although it is tempting to include as many variants as possible in an experiment to enable multifactorial analyses many problems are raised by this:

- Block size is increased
- Variability increases in the field but also statistically due to higher sample amount
- Under-representation of control variants
- Sampling is much more time consuming or sometimes not feasible leading to selective sampling / testing

Especially selective sampling of certain variants only, will again bias statistical analysis. By this, full-factorial analysis but also statistical evaluation including the experimental design will be disturbed.

This can also be seen in the analysis of the early plant growth 26 DAS. Due to limited time, Block V but also the variants Af and SF (2 and 19) were not sampled. This was a problem as certain block/row combinations were missing and a full-factorial analysis was not possible thereby decreasing the power of analysis and the strength of the block/row design. Partially sampling was also performed in other cases such as root sampling for tracing analysis. Therefore statistical analysis always lacks certain combinations for a proper analysis.

If more variants have to be tested they should therefore be split into separate experiments even though certain control variants would have to be included in both experiments.

This can be easily done, if hypotheses strongly differ. In both field experiments (Exp_12 and Exp_15) the CULTAN variants differed strongly from the SWE treatments or the urea and manure pellet variants. A comparison of the variants was anyhow difficult as many factors like N and P application, N and P form, sulfur application (ammonium sulfate was used in the CULTAN variants) and application technique differed among treatments. Additionally, the

analysis and interpretation of the results of these complex experiments is much more difficult and time consuming than the analysis of smaller and simpler experiments.

3. Late N fertilization

The treatments that were part of the experimental part of Nkebiwe (not listed here) were CULTAN fertilized (controlled long-term ammonium nutrition). As it was hypothesized that an early placement of ammonium fertilizers before plant root establishment would risk the loss of N due to nitrification and leaching, it was decided to postpone the CULTAN treatment. To have similar conditions for all plants therefore also the broadcast N treatments for all other standard fertilized treatments was postponed.

4. Harvest procedures

For calculation of dry matter yield one aspect has to be considered. At the research station forage maize harvest was done with a small plot harvester by the technical staff. The four inner maize rows per plot were harvested in two separate runs with the harvester harvesting only two rows each time. Half of the plots were harvested 167 DAS, the other half 173 DAS. In some plots yields of first and second run were almost equal whereas in other plots they differed strongly (up to 40 %) for unknown reasons (no influence by Trt or Block was found). After each plot subsamples were collected from which dry matter was calculated as well as the weight loss during purification of harvested corn from straw leftovers. Nevertheless, in the research station the data from the purification of the second run get lost. No correlation between the purification losses and yield (ρ = 0.3 – 0.4) were found. ANOVA for the purification losses showed no dependency on the treatment or block of the respective plots. Obviously the purification loss did not depend on known factors but possibly mechanic problems, speed of harvesting or depth of the cutting and were therefore randomly distributed. Because these unknown conditions were probably not constant between first and second run, it did not make sense to use the same amount of purification loss for both runs. The purification losses ranged from 0 – 4 % of DM yield, with only one extreme value of 11.5 %. The almost perfect correlation (ρ = 0.997) between yield calculated with or without purification loss show that the error is negligible and will probably be balanced inside one treatment. Nevertheless, the huge difference between first and second run of the harvest suggest that there are other factors involved that are not included in the analysis but that might influence the results strongly.

3.10.3.4 Results Exp_13: Screening for optimal application rates of PGPMs

All sampling and measurements were done at the same time as in Exp_12. Analyses of ER, plant biomass 25 DAS, mycorrhiza analysis and P concentration in the corn were not done in Exp_13. Additionally, no Px treatment and therefore no Px tracing was done and for Rz analysis only the plant rhizosphere was analysed.

3.10.3.4.1 Statistical analysis

None of the analyses indicated significant differences among treatments. Plant growth and performance was similar to the neighbouring plots of Exp_12 but yield differed strongly with higher average yields in Exp_13. Similar to the trends observed in block V of Exp_12, yield continuously declined in the block IV of Exp_13. Therefore, statistical analysis of all measurements was done with and without exclusion of block IV. No statistical differences between treatments were observed for both datasets but the sequence of which treatment performed best changed for some variables. In the graphs presented here in most cases block IV is excluded but for yield data both datasets are presented in graphs. If Block IV was excluded best performing treatment was BFDC_low. Here plant growth was best whereas no improvement in nutrient supply was observed. Rz treatment had a slightly negative impact on plant growth thereby leading to an increase in leaf N and P concentration.

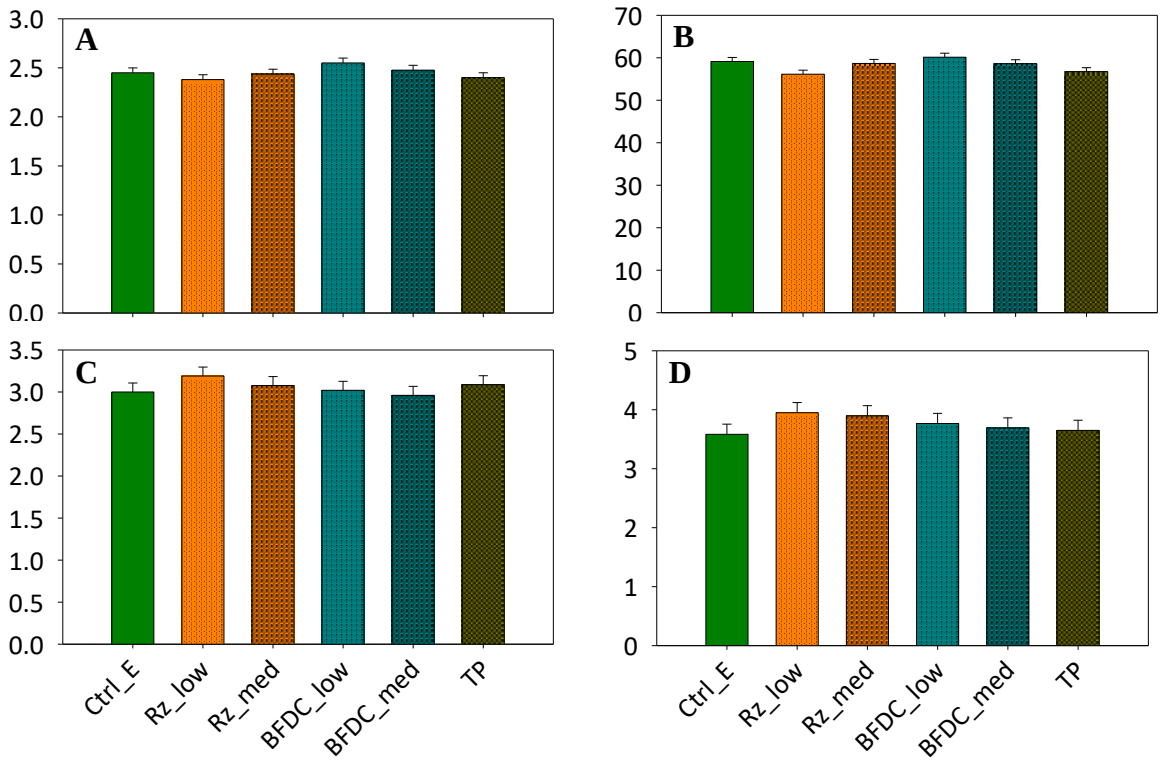


Figure 3-82 Pre-harvest results Exp_13; Plant height in m (**A**), SPAD (**B**), leaf N concentration in % (**C**) and leaf P concentration in mg g^{-1} DW (**D**) 78 DAS; Adj. means + SE; r = 3; glm with reduced model: Trt and Tukey's test was used; No significant difference observed in any of the measurements

Like the other treatments, the *Trichoderma harzianum* product (TP) that was not tested in Exp_12 did not induce any significant plant responses.

3.10.3.4.2 Harvest

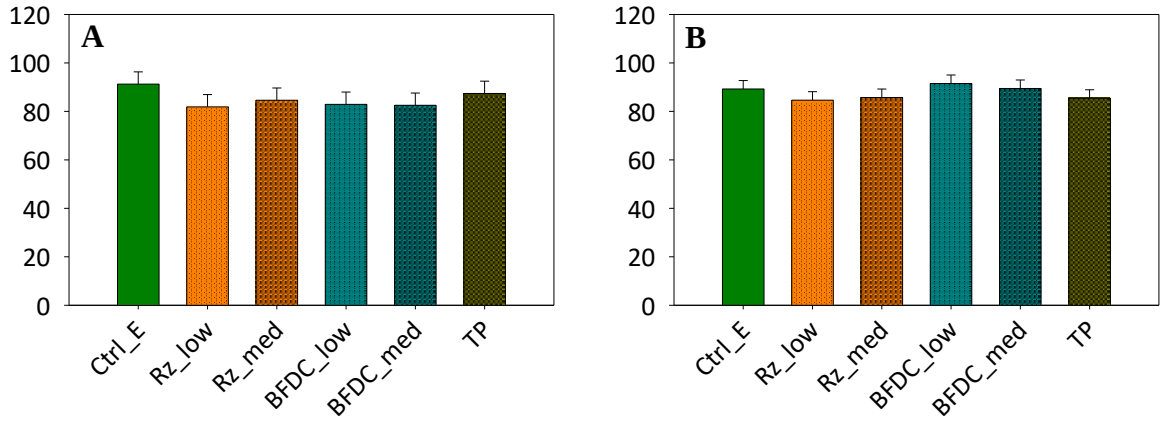


Figure 3-83 Maize corn yield Exp_13; Dry weight corn yield with (**A**) and without Block IV (**B**); Adj. means + SE; r = 4 (**A**)/ 3 (**B**) ; glm with full model (Trt Block) (**A**) or reduced model (Trt) (**B**) and Tukey's test were used

For corn yield the same tendency as for plant height and SPAD values were observed. BFDC_low was performing best when Block IV was excluded (91.5 dt ha^{-1}, Ctrl_E 89.2 dt ha^{-1}) but was performing worse than most of the other treatments when block IV was included (82.9 vs 91.2 (Ctrl_E) dt ha^{-1}). Rz_low showed in both cases the weakest performance (84.6 and 81.9 dt ha^{-1}) and the Std_Ctrl was the best treatment when Block IV was included due to the randomisation and its position nearest to Block III.

3.10.3.4.3 Microbial analysis

Similar to Exp_12 root sampling for microbial analysis was done 25 and 91 DAS. Samples from Rz plots and the Ctrl treatment were sampled and again analysed by ABiTEP with the difference that tracing was done on selective LB_{rif} media because due to the low inoculum rates in Exp_13 ABiTEP was able to provide us with the *rif*-resistant Rz strain. As expected from the lower inoculation rate cell numbers were much lower than in Exp_12. Average number of total bacteria in the Rz_low treatment was $3x10^3$ CFU g^{-1} root (~10 % of inoculum rate) and in Rz_med $2.3x10^4$ CFU g^{-1} root. As visible in plot B (Figure 3-84) background *Bacillus* population in Exp_12 (Std_Ctrl) was about the same amount like the recovery rate of Rz bacteria in Exp_13. In Exp_13 tracing was strain specific, therefore these numbers reflect the real number of the Rz strain whereas in Exp_12 this number can be estimated by subtracting the cell number in the Std_Ctrl from the cell number in the respective treatment. Because the population in treatments are 10 – 100 times higher than the background population in soil, in Exp_12 the background is negligible. Interestingly, endospore number,

respectively the amount of endospores per total bacteria, in Exp_13 is much higher than in Exp_12. Data suggest that the endospore rate decreased with increasing number of total bacteria. Only the Rz_Af treatment seemed to change this correlation.

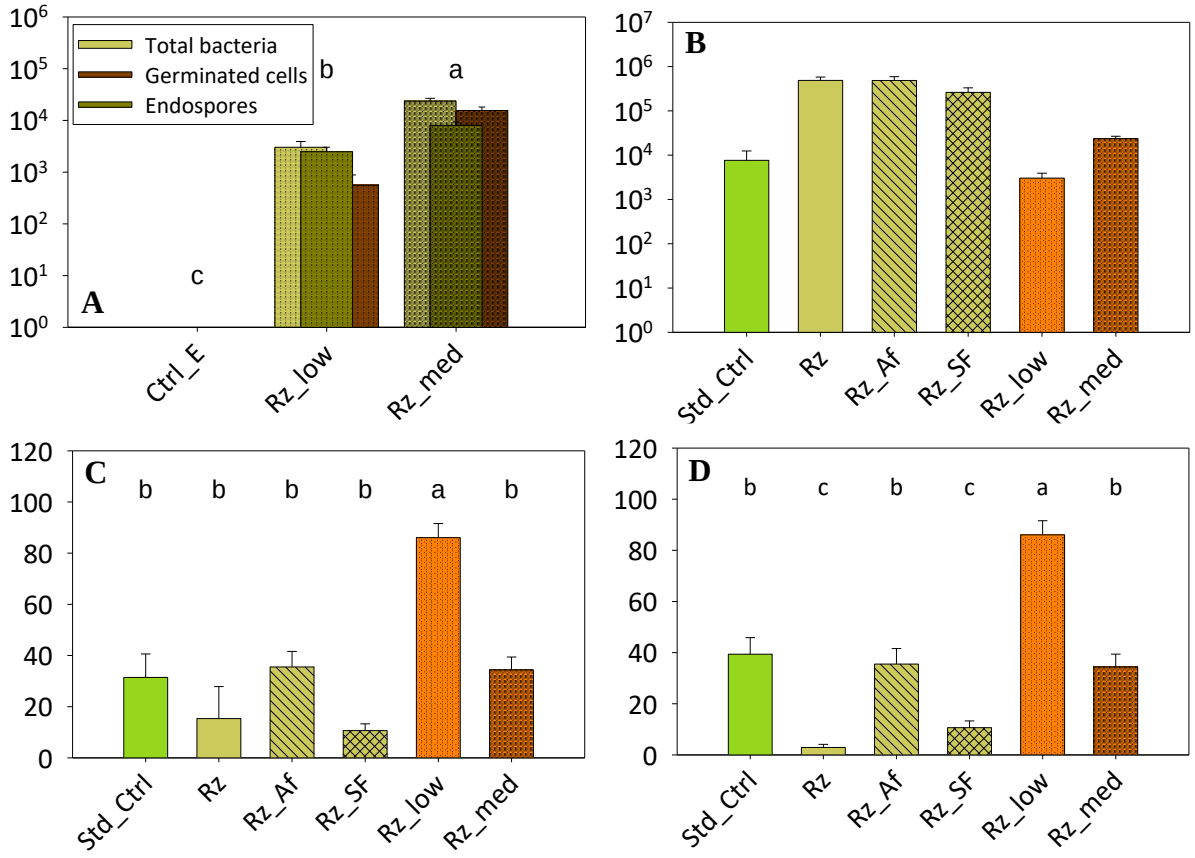


Figure 3-84 *Bacillus* FZB42 root colonization Exp_13; Summary of different bacterial forms found in the rhizosphere of the maize roots (**A**), comparison of number of total bacteria between Exp_12 and Exp_13 (**B**), comparison for the amount of endospores as percentage of total bacteria (**C** + **D**); Only in Exp_13 tracing was strain-specific; In plot D two outliers were reduced (see text); Adj. means (**A** + **B**) and means (**C** + **D**) + SE; r = 4 / n = 24 (**B** + **C**), 22 (**D**); glm with reduced model (Trt) and Tukey's test was used

3.10.3.5 Discussion Exp_13

3.10.3.5.1 Plant performance

Average plant performance in Exp_13 was much better than in Exp_12. As discussed above, one reason must be the better exposition of the plots in the upper row of the experiment. It is also possible that fertilization in previous years was better in the upper parts of the field. That would have affected especially early plant growth when plants lacked N due to the late N fertilization in the experiments. Unfortunately, in Exp_13 there are no data for plants 25 DAS to compare the experiments at this early stage. Nevertheless, average plant height 78 DAS was exactly the same in both experiments (Ø 245 cm for treatments with Std_fertilization).

3.10.3.5.2 BE application rates

In Exp_13 lower application rates were tested following suggestions by the companies providing the products. Especially for the BFDC treatment lower application rates are highly recommendable, nevertheless, also in Exp_13 none of the BE treatments was able to significantly improve plant growth or plant yield as compared to the untreated Ctrl. In general, results suggest that the environmental conditions were not optimal to establish a beneficial BE-plant interaction. Accordingly, data do not allow to draw a conclusion for the best application rate. Analysis of the microbial root colonization of the Rz strain shows some interesting pattern for the endospore number of the cells. It seems that spore germination is increased with increasing inoculum density in the soil or rhizosphere. As mentioned before quorum sensing, the population density dependent activation of bacterial traits, such as biofilm formation or pathogenesis, is commonly reported for many bacteria including PGPR. It is known that sporulation, the formation of endospores, is influenced by quorum sensing (Perchat et al., 2016; Setlow, 2014). Coordinated germination may be crucial for the survival of a bacterial population in a changing environment. Therefore it is a reasonable assumption that individual endospore germination, triggered by environmental signals, is also influenced by signals from the surrounding population.

Accordingly, this suggests a first hypothesis for the mode of action of the Af product that increased the number of spores in the Rz_Af treatment. The Af product might interfere with quorum sensing signalling or perception of the Rz strain.

3.10.3.5.3 Statistical analysis

Block IV that started in Exp_13 in Col 19 showed a continuous decline in yield to the east direction (Figure 3-85). Trt 29 (Ctrl_E) was the first Trt in Block IV having the highest yield in Block IV and therefore also highest average yield in Exp_13 whereas Trt 30 (BFDC_low) had lowest yield in Block IV leading to the second lowest average yield, although BFDC_low showed highest average yield in Block I – III (see yield data of BFDC_low with and without Block IV, Figure 3-83). Because yield decline was continuous and not abrupt, in statistical analyses also the block effect was in most cases not significant.

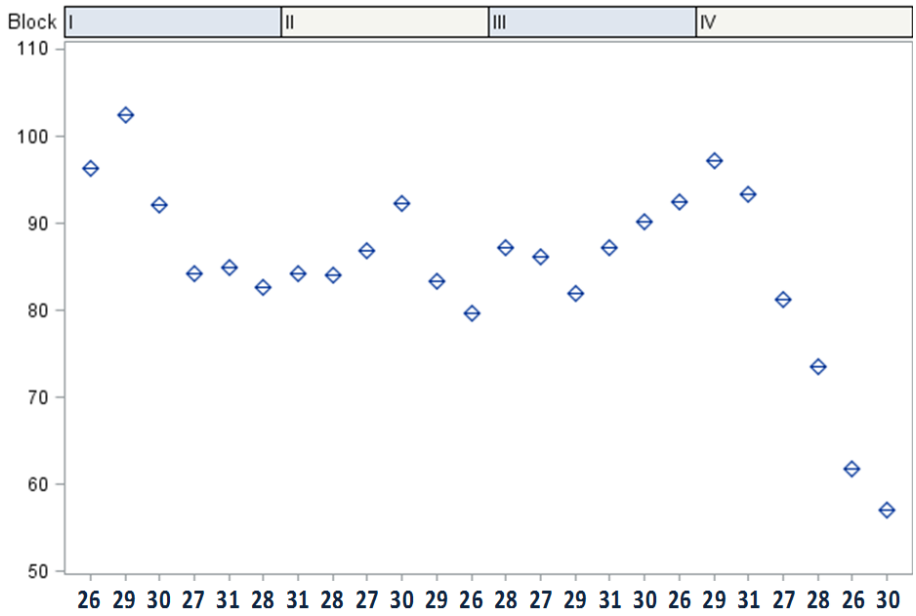


Figure 3-85 Yield distribution Exp_13; Block IV showing strongest variation in yield; Therefore differences in average yield of treatments in Exp_13 are mainly depending on the yield data of Block IV.
Trt 29 = Ctrl_E; Trt 30 = BFDC_low.

3.10.4 BE application and fertilization strategies in silage maize (Exp_15 and 16)

Introduction

Experiment 15 was focussing on BE application and fertilization strategies in silage maize. Here different fertilizers (chicken manure pellets, urea band application and stabilized ammonium fertilization with CULTAN) in combination with the two PGPR strains *Pseudomonas* sp. 'Proradix' and *B. amyloliquefaciens* FZB42 were compared to standard or optimized fertilization strategies from agricultural practice.

Experiment 16 was a smaller experiment only testing three variants on the aspect of stress alleviation (here drought stress) and application strategies, as the seaweed extract Superfifty® was applied for the first time via foliar application, as recommended by the producers.

Both experiments (Exp_15 and Exp_16) were sown on May 13th. An overview on the treatments of Exp_15 and Exp_16 is given in Table 3-26.

Table 3-26 Overview treatments IHO 2015

Trt_Nr	Treatment	Placement	Broadcast
1	0_Ctrl	/	/
2	Std_Ctrl	F_{DAP}	F_{Nov}
3	P_Ctrl	F_{DAP}	F_{Nov} + TSP
4	Std_Px	F_{DAP}	F_{Nov}
5	Std_Rz	F_{DAP}	F_{Nov}
6	CUL_Ctrl	F_{DAP} + F_{Nov}	/
7	CUL_Px_broad	F_{DAP} + F_{Nov}	/
8	CUL_Px_gran	F_{DAP} + F_{Nov}	/
9	CUL_Px_seed	F_{DAP} + F_{Nov}	/
10	CUL_Rz_broad	F_{DAP} + F_{Nov}	/
11	CUL_Rz_gran	F_{DAP} + F_{Nov}	/
12	CUL_Rz_seed	F_{DAP} + F_{Nov}	/
13	Urea_Ctrl	F_{U}	/
14	Urea_Px	F_{U}	/
15	Urea_Rz	F_{U}	/
16	Man_Ctrl	F_{MP}	/
17	Man_Px	F_{MP}	/
18	Man_Rz	F_{MP}	/
A	Ctrl	F_{DAP}	F_{Nov}
B	SF	F_{DAP}	F_{Nov}
C	BacA	F_{DAP}	F_{Nov}
D	Si	F_{DAP}	F_{Nov}

Underfoot *placement*: F_{DAP} = 28.8 kg N ha^{-1} and ca. 32 kg P ha^{-1} 5 x 5 cm from seeds; F_U/F_{MP} = Urea or MP placed at a rate of 129 kg N ha^{-1} in a 20 cm band under the sowing row; F_{Nov} = 100 kg N in a band 37.5 x 10 cm from seeds 4 weeks after sowing; *broadcast before sowing*: F_{Nov} broadcast application and soil incorporation at a rate of 100 kg N ha^{-1}, additional fertilization of triple superphosphate (TSP) at a rate of 130 kg P ha^{-1}.

Fertilization 2015

Before sowing, plots of Exp_15 were BE treated, then broadcast application of F_{Nov} and TSP as well as underfoot placements of $F_{U,}$ F_{MP} and F_{DAP} were done (also for Exp_16).

BE application Exp_15

For application of Px and Rz in the urea and manure treatments water suspensions of the products were placed in a 10 cm band on the seeding rows, previously marked mechanically by a GPS-based plot seeder, at a concentration of 10^9 CFU kg^{-1} soil using watering cans (3.1 kg Px and 8 kg Rz ha^{-1}). During fertilization on the next day this band was incorporated in the soil. Therefore calculations were based on an assumed bulk density of 1.5 kg l^{-1} and a soil

depth of 10 cm. Appropriate dilutions were prepared for 20 l water plot^{-1}. For BE application in the Std and the CUL_gran treatments pumice stones (Rotec GmbH & Co. KG, Mühlheim-Kährlich, Germany) with a diameter of 0.5 – 1 mm were sprayed with water-BE-suspensions (~ 10^8 CFU ml^{-1}) and then also applied in a ~10 cm wide band, previously engraved with rakes on the marked seeding row, at a concentration of 10^9 CFU kg^{-1} soil using plastic beakers (Figure 3-86 A – E). BE treatments in the CUL_broad treatment were also done at a target concentration of 10^9 CFU kg^{-1} soil, but due to the bigger soil surface treated total amounts were much higher than for the other BE treatments (see Exp_12, 2014). For the CUL_seed treatments seeds were either treated by seed infiltration with the Px product by the company Sourcon Padena at a concentration of 3.6 x10^{11} CFU kg^{-1} seeds (Buddrus-Schiemann et al., 2010) or by seed coating with Rz using a mixture of maize starch, milk powder and calciumhydroxid as recommended by the company AbiTEP (*personal communication with M. Nkebiwe*). The BEs were applied at a rate of 1.2x10^8 CFU Px or 1.4x10^8 CFU Rz seed^{-1} respectively (calculation based on 344 g thousand grain weight of the maize cultivar).

Figure 3-86 BE application in the field experiment 2015; Application of BE suspensions on pumice stones with sprayers or watering cans (**A** + **B**); manual placement and incorporation of pumice stones in furrows in the field plots (**C** - **E**); manual application placement of concentrated ammoniumsulfate (CULTAN) in between maize rows (**F**); foliar application of SF in Exp_16 using a backpack sprayer (**G**).

BE application Exp_16

BE treatments in Exp_16 were done one month later in June 18th at 4 – 5th leaf stage. BacA was also applied at a concentration of 10^9 CFU kg^{-1} soil using watering cans in a band around the already growing maize plants. SF was applied as foliar application at a rate of 1 l product diluted in 1000 l ha^{-1} using a backpack sprayer (Figure 3-86 G). For SF a second application was performed two weeks later on first of July at a rate of 2 l product in 500 l ha^{-1}. For silicon treatment the product Vitanica®Si (Compo Expert GmbH, Münster) was used (the treatment was done by another colleague and therefore treatment details are unknown and will not further discussed in the chapter 3 or 4).

CULTAN treatments

Fertilization of the CUL treatments (CULTAN, Controlled Uptake Long Term Ammonium Nutrition) was done at the same day together with the BE applications in Exp_16. For this a highly concentrated F_{Nov} solution (62.7 g N l^{-1}) was prepared and applied with watering cans in a band in the track between two maize rows (~37.5 cm from maize row) at a target depth of 10 cm (Figure 3-86 F). Only every second track (3 of 5 tracks) was treated to leave Ctrl tracks in which soil core sampling for root length densities could be performed later on (Nkebiwe et al., 2016b).

Randomization

Randomization and arrangement of plots is visualized in Figure 3-87 (below). Plot size in Exp_15 was 58.5 m², including six maize rows, and 39 m² in Exp_16, including four maize rows. Of this plot area only the two inner rows at a length of 9.7 m were harvested.

Analysis

Bulk soil sampling from Rz and Px treated plots was done 7 DAS using soil core samplers in 1 – 10 cm soil depth. All samples were taken within 10 cm from the sowing row where also manure pellets as well the pumice stones were placed. For each plot about 10 soil samples were mixed to one mixed sample. 20 g of the mixed sample were suspended in 50 ml 0.1 % trypton solution. For strain-specific counting of the Rz bacteria $R2A_{rif}$ medium was used. Ctrl samples were not diluted but other samples were diluted 1:10 – 1:1000.

A visual evaluation of plant performance was done 49 DAS by considering leaf coloration, plant height and vitality per plot using a scoring system from 1 - 9. Dark colour of the maize leaves was considered as positive, suggesting sufficient N supply. Ranking was done for each

of the four inner rows of a plot. For statistical analysis the mean values were taken as numerical variables.

Root tracing for Rz was done 57 DAS. Maize roots from tracks in between the maize rows were collected. Only plots from the CULTAN treatments were sampled to compare the different application strategies. For root sampling it was distinguished between tracks with or without CULTAN-depot (see 3.10.4) to assess the influence of the ammonium depot on bacterial root colonization. Roots with adhering rhizosphere were weight into vials and then suspended like the bulk soil samples. After plating of dilutions the roots were taken out, part of the solution was decanted and then the remaining rhizosphere soil was dried. For calculation of the CFU g^{-1} rhizosphere therefore two calculations were done, one per g^{-1} root (with adhering soil) or per rhizosphere soil (without root) to assess if estimations from previous root colonization assays were correct. Analysis of Px root /rhizoplane colonization 61 DAS was done following the RT-qPCR method from FIBL. The method was established using the CTAB buffer and a heavy round tool (derived from the mill used for grinding leave material) for detaching bacteria from the rhizoplane.

Plant height measurements were done 71 (Exp_15) and 84 (Exp_16) DAS. Harvest for silage maize was done 126 (Exp_15) and 131 (Exp_16) DAS.

	I			II			III			IV			V			
1	8	5		9	1		11	17		13	11		18	6		4.5
2	12	4		6	16		9	18		14	2		12	7		
3	17	14		4	3		5	4		9	5		8	5		
4	11	3		12	17		16	1		17	3		16	3		
5	6	13		14	10		14	6		1	18		17	14		40.5 m
6	10	2		11	7		13	3		6	4		9	13		
7	9	7		15	5		2	15		10	12		1	10		
8	18	16		13	2		8	7		8	16		15	4		
9	15	1		18	8		10	12		15	7		2	11		
				C	D		A	D		A	B		B	A		3m
				B	A		B	C		C	D		D	C		6 m

13 m | 11m | 15m | 9m | 9m

175 m

N

Figure 3-87 Plot plan of the field experiments 2015; Plots with numbers are part of Exp_15 and plots with letters are part of Exp_16. Exp_15 was divided in 9 rows and 5 blocks with each two columns. Exp_16 was divided in 4 blocks only.

3.10.4.1 Results Exp_15

3.10.4.1.1 Pre-harvest results

Due to the results from Exp_12 in which no significant effects of microbial BE inoculation on plant emergence were observed, in Exp_15 no detailed analysis of the emergence rate was performed. Nevertheless plots were observed for overall plant emergence. In general plant emergence was good, due to the relatively high temperatures in 2015 but major damages occurred due to crows feeding on seeds and young seedlings. Some plots were re-sown and for harvest only two rows per plot were analysed. This is further discussed in 3.10.4.2.5.

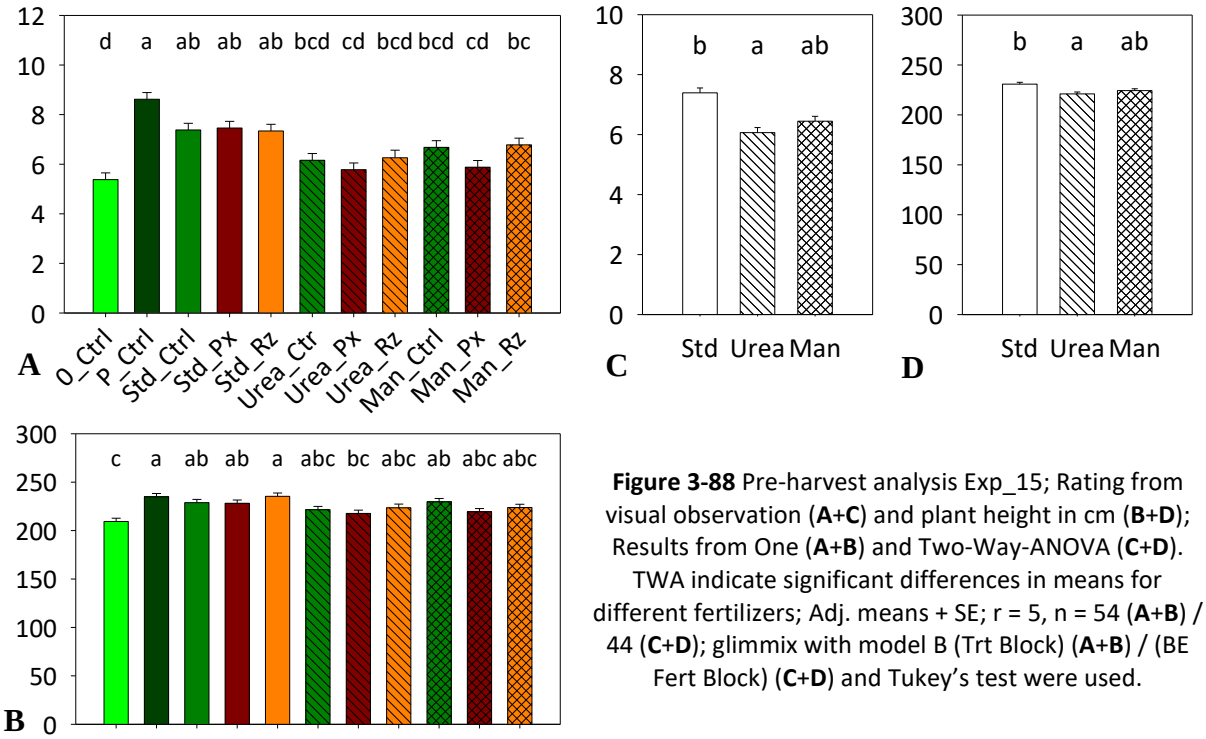


Figure 3-88 Pre-harvest analysis Exp_15; Rating from visual observation (**A**+**C**) and plant height in cm (**B**+**D**); Results from One (**A**+**B**) and Two-Way-ANOVA (**C**+**D**). TWA indicate significant differences in means for different fertilizers; Adj. means + SE; r = 5, n = 54 (**A**+**B**) / 44 (**C**+**D**); glimmix with model B (Trt Block) (**A**+**B**) / (BE Fert Block) (**C**+**D**) and Tukey's test were used.

The P_Ctrl plants clearly stood out from the other treatments, suggesting that the additional P-fertilization was beneficial for the youth development of the plants. This is further supported by the better growth development of the Std-treatments that received soluble P with the underfoot placement of $F_{DAP.}$ In a direct comparison of standard, manure and urea fertilized plots by Two-Way-ANOVA significant differences between the Std and the Urea treatments were observed. Manure treatments that also received additional P supply, although in a less available form than the Std_treatments and the P_Ctrl, performed slightly better than the Urea treatments. Obviously, supply with P was the growth determining factor here.

Similar results were also observed 71 DAS during measurement of plant height. 40 randomly picked plants per plot, 10 per each of the four inner rows, were measured. Again, treatments

with additional soluble P fertilization performed best. Nevertheless, the difference between treatments was much smaller than before.

BE products did not differ significantly from the Ctrl or from each other, nevertheless, a common trend was observed in both analyses. Here Px treated plants performed worse in those treatments that anyhow showed weaker plant growth whereas with Std-fertilization no responses were observed at all. Rz treatment did not affect plant growth as compared to the respective Ctrl treatments.

3.10.4.1.2 Harvest

Harvest of maize was done earlier than in the previous year and whole plants were harvested for silage maize. Biomass results were higher than expected considering the low rainfall in 2015 (see discussion). Results differed strongly from the pre-harvest results (Figure 3-89). None of the different fertilizer treatments, except the unfertilized 0_Ctrl, differed significantly from one another. Additionally, the Urea treatments showed the highest average yields.

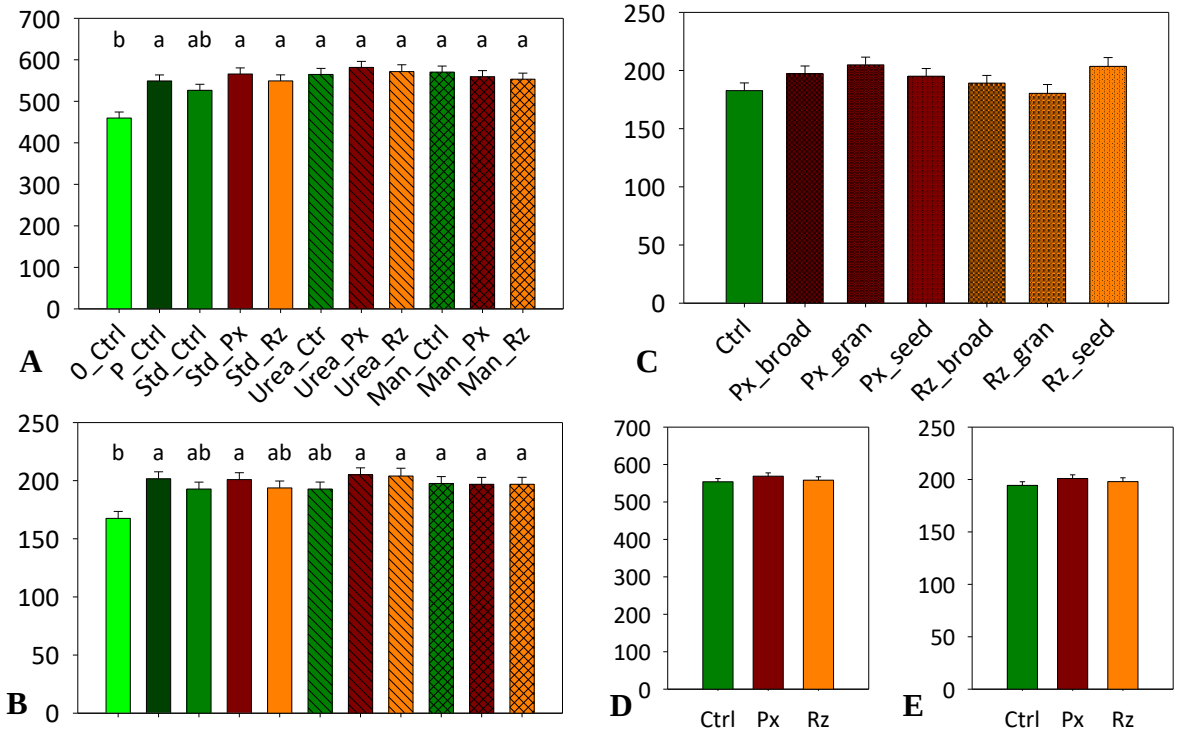


Figure 3-89 Maize yield Exp_15**;** Silage maize yield as dt fresh (**A**+**C**) or dry (**B**, **C**+**E**) weight ha^{-1}; Results from One (**A**+**B**) and Two-Way-ANOVA (**D**+**E**). CULTAN treatments showed separately (**C**); No significant differences observed in TWA for fertilizer, BE or application method; Adj. means + SE; r = 5, n = 54 (**A**+**B**) / 33 (**C**) / 44 (**D**+**E**); glimmix with model B (Trt Block) (**A**,**B**,**C**) / (BE Fert Block) (**D**+**E**) and Tukey's test were used.

The Px treatment showed best plant performance for the Std and Urea fertilized treatments whereas Rz showed a positive response in the Urea treatments only. In the manure treatments BE treated plants performed worse than the Ctrl plants. Nevertheless, none of these differences were significant in statistical analysis. Similar trends for Px were found for the

CULTAN treatments. All Px treatments improved plant growth as compared to the CUL_Ctrl (not significant, p = 0.11). Highest yield was observed in the Px_gran treatment (205 dt ha^{-1}, Ctrl = 183 dt ha^{-1}). Rz treatments differed in their effect on plant growth whereas only the seed application resulted in an increased dry matter yield (204 dt ha^{-1}). No significant differences between application methods were found in Two-Way-ANOVA. After residual analysis, four outliers were reduced (treatments CUL_Rz_gran, CUL_Rz_seed, Std_Ctrl, Urea_Rz with 4 replicates only). Reduction of outliers did not conceal or reveal further significant differences among treatments.

3.10.4.1.3 Microbial analysis

3.10.4.1.3.1Rz tracing

Even though sampling 7 DAS was done near to the sowing row in the area of granule and band application broadcast application resulted in highest bacterial density. With an average concentration of 1.0x10^6 CFU g^{-1} soil in the CUL_Rz_broad treatment exactly the amount of bacteria were found that were expected from the inoculum rate. In all other treatments only 10 – 50 % of the inoculum rate was recovered in this area. In the Std_Ctrl, where no Rz was applied, some bacteria were growing on agar plates with undiluted soil suspension.

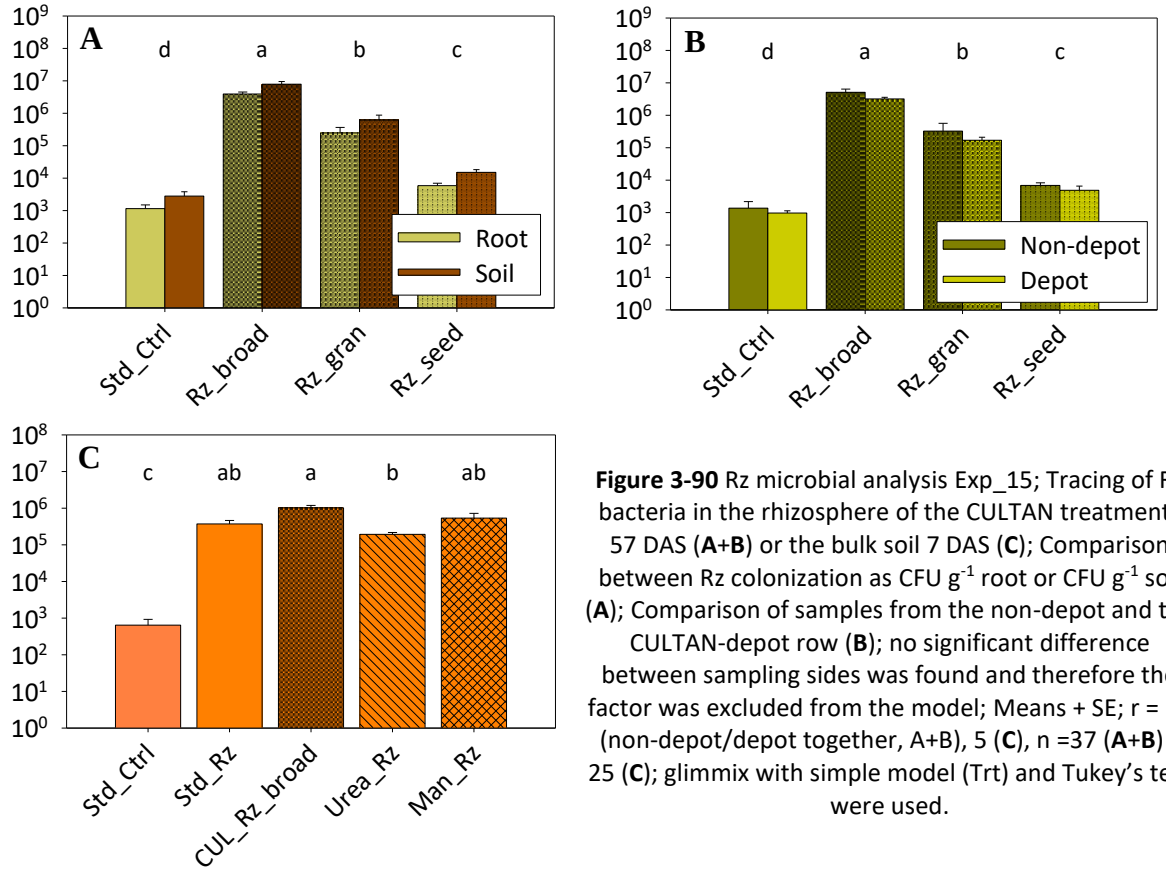


Figure 3-90 Rz microbial analysis Exp_15; Tracing of Rz bacteria in the rhizosphere of the CULTAN treatments 57 DAS (**A+B**) or the bulk soil 7 DAS (**C**); Comparison between Rz colonization as CFU g^{-1} root or CFU g^{-1} soil (**A**); Comparison of samples from the non-depot and the CULTAN-depot row (**B**); no significant difference between sampling sides was found and therefore the factor was excluded from the model; Means + SE; r = 10 (non-depot/depot together, A+B), 5 (**C**), n =37 (**A+B**) / 25 (**C**); glimmix with simple model (Trt) and Tukey's test were used.

Statistical analysis for root analysis 57 DAS did not show severe difference between the calculation methods. Furthermore, standard error was not significantly reduced by including the exact soil weight of the rhizosphere for calculations (Figure 3-90 A, ρ = 0.99). Also root colonization in the depot or non-depot tracks did not differ significantly from each. Nevertheless, in all treatments mean values in the non-depot track are higher than in the CULTAN treated track. For the Std_Ctrl similar bacterial densities as in the bulk soil were found for the rhizosphere. The number of CFU in the rhizosphere of the CUL_Rz_broad treatment was even increased as compared to the bulk soil density ($4x10^7$ CFU g^{-1} root or $8x10^7$ CFU g^{-1} rhizosphere soil). Nevertheless, an exact comparison is not possible because bulk soil analysis was done per g fresh soil whereas the rhizosphere CFU was calculated per g^{-1} dry soil. In the CUL_Rz_gran treatment, where BEs were applied in a band with previously BE-treated pumice stones, the root colonization rate in between maize rows was about $6x10^5$ CFU g^{-1} dry soil ($2.5x10^5$ CFU for the root). This is lower than the application rate in the band but not surprising, as for bulk soil sampling of the Std_Rz treatment that also received granules, a reduced cell density of $3x10^5$ CFU g^{-1} soil was found. Additionally, roots were sampled at the border of the band and therefore lower densities were expected here. For seed treatment (CUL_Rz_seed) a much lower root colonization was measured than in the other two Rz treatments that was in some samples only slightly higher than in the Std_Ctrl. Average bacterial density ($1.5x10^4$ CFU g^{-1} dry soil) still differed significantly from the Std_Ctrl ($2.8x10^3$ CFU g^{-1} dry soil).

3.10.4.1.3.2 Px tracing

To validate the RT-qPCR method root samples were inoculated with different concentrations of fresh Px suspension (10^3, 10^4, 10^6, 10^8 CFU per g root, two replicates). About 30 – 70 % of the inoculum density was found in the measurements and significant differences were observed between 10^4, 10^6 and 10^8 CFU but not between 10^3 and 10^4. At these low concentrations measured and inoculated amounts did not correlate. This supports the detection limit of about 10^4 CFU g^{-1} root mentioned in the publication (Mosimann et al., 2017). The high recovery factor (2-3) calculated from the internal standard indicated that some errors might have occurred in the calculations but this does not influence the general outcome of the analysis. For the Px tracing 61 DAS only CUL_Ctrl and CUL_Px_broad samples were analysed because here the biggest differences were expected. Nevertheless, analysis did not show a higher colonization rate in the broadcast treatment and in both cases the calculated CFU was below the detection limit (10^2 – 10^3 CFU g^{-1} root). These results are similar to the

tracing results from late second tracing in Exp_12 suggesting that there were no living bacteria present anymore.

3.10.4.1.4 Correlation analysis

Correlation analysis showed that FW and DW yield were less well correlated (ρ = 0.88) than in Exp_12 (here correlation was ρ = 0.99). Also plant height and yield data were less correlated (height/FW yield, ρ = 0.63 and height/DW yield, ρ = 0.54) than in the previous field experiment. Visual rating of treatments was only weakly correlated with yield data (FW ρ = 0.27 and DW, ρ = 0.35) showing the potential of the maize to compensate for deficiencies during the youth development.

3.10.4.2 Discussion Exp_15

3.10.4.2.1 Hypotheses

In Exp_15 focus of investigation was the interaction between different fertilizers and BE applications. Fertilizer placement was used to improve P acquisition and BE root colonization (Nkebiwe et al., 2017). In the field experiment 2014 also an increased root density was observed in the CULTAN treatments. Because mineral fertilization is not an option for organic farming, manure pellets were used as a standardized organic fertilizer product in several experiments in the Biofector project to ensure similar experimental conditions in different partner institutes. The product was later on also tested in Exp_19 due to it high concentration of NH_4-N in contrast to the F_{MKH} manure. At a working group at FiBL a similar experiment was conducted at the same time under organic farming conditions (results from a pot experiment with the BUUS soil were published elsewhere (Thonar et al., 2017)).

For comparison with farmers practice placement of urea was included as a second treatment. Urea in the soil is hydrolysed to ammonium by microbial urease (Hawkesford et al., 2012). Nevertheless, in the first steps, catalysed by urease, urea is converted to toxic ammonia and carbon dioxide. A high soil pH causes ammonia volatilization (see also discussion Exp_19). Additionally, pH may be further increased by protonation of ammonia to ammonium ($NH_3 + H_2O \rightarrow NH_4^+ + OH^-$) (Fan and MacKenzie, 1995). Following recommendations by a local farmer, who had good experience with urea placement, urea was applied at a high rate of 130 kg ha^{-1} as band application leading to a concentration of about 1 mg kg^{-1} soil (1 ppt) inside the band. At this concentration phytotoxicity due to ammonia may occur (Creamer and Fox, 1980; Fan and MacKenzie, 1995).

3.10.4.2.2 Fertilization and BEs

Indeed a growth depression in the urea and the manure treatments as compared to the Std treatment was observed during pre-harvest analysis. It is possible that in both treatments ammonia toxicity was reducing plant growth. Nevertheless, during visual evaluation no stress symptoms were observed and plant growth was still better than in the unfertilized 0_Ctrl. The good performance of the P_Ctrl indicates that P was limited and that the Std_treatments therefore might have benefitted from P starter fertilization during early plant development.

In contrast to the pre-harvest analysis, results for maize yield do not indicate any negative impact of manure or urea fertilization on plant performance and Urea_Px treatment produced the highest yield. A meta-analysis on different fertilizer placement strategies also concluded that urea (alone or with additional P) was the best N-source to be used for fertilizer placement (Nkebiwe et al., 2016a). Although the Px treatment seemed to be beneficial, slightly improving maize yield with most of the fertilizers, again no significant BE effects were observed. Therefore any conclusions on optimal BE fertilizer combinations are hypothetical. Moreover, the increased root length density in the CULTAN depots of Px treated plants in 2014 was not observed in 2015 (Nkebiwe et al., 2016b). One explanation is the high temperature in 2015 that caused drought stress in the maize plants and probably reduced survival rate of the Px strain. Additionally, the CULTAN depot was not deep enough in 2015 because the soil was too heavy for deep placement. Therefore roots, following the water to deeper soil layers, were less concentrated around the ammonium depots and the BE colonization was reduced. Interestingly, in 2015 maize yield in the BE treatments differed more from the control treatments than in 2014, suggesting that yield was less correlated with higher root length density than to P availability in the field.

At FiBL our working group performed sampling for root length density and results indicated that Px was able to promote root length in the manure depot. Here more than 100 % higher root length was measured. Nevertheless, also here no significant BE effects on maize yield were observed (Symanczik et al. 2015, *unpublished*).

3.10.4.2.3 Application methods for microbial BEs

One objective of the field experiment 2015 was to compare different BE application methods to improve efficacy of the products but mainly to reduce application rates and therefore application costs for farmers. Those application techniques were tested only in the CULTAN treatments. Only a part of these treatments and the results are published (Nkebiwe et al., 2016b). In these CULTAN treatments three BE application methods were compared:

1. Broadcast application of BEs as done in the first field experiment in 2014

2. Band application with pumice stones as performed for Trt 10 and 11.

3. Seed application using seed infiltration (for the Px treatment) and seed coating using a carbon source (for Rz treatment) at rates of about 10^8 CFU $seed^{-1}$

Tracing of the Rz roots using $R2A_{rif}$ medium revealed a still significant population density on the roots of seed treated maize sampled in a distance of about 10 cm from the seeding row.

Results from Exp_13 and Exp_21 indicate that no rif-resistant bacteria population is found in natural soils. Therefore the relatively high number of bacterial CFUs on $R2A_{rif}$ medium of the Ctrl treatments indicates that samples were contaminated. For sure, part of the contamination was coming from non-sterile conditions during sampling of roots in the field and plating of the suspensions on agar plates. Especially the cleaning process of the spatula was often not sufficient (as already mentioned in the results of Exp_18 that was performed about the same time in 2015). The problem is that the Rz spores are able to resist alcohol and high temperatures and are therefore only killed effectively if the spatula is heated for ten or more seconds. This was seen in dilution series where higher numbers of CFUs were found in the first plated high dilution than in the lower dilutions. Nevertheless, it is also possible that trace contaminations were already happening during the seed bed preparation after BE application.

As expected population densities in the band and broadcast application was much higher than in the seed treatment but yield data do not correlate with the application rates making a recommendation for optimal application rate (or population density in the rhizosphere) difficult. Initially granules were chosen because it was hypothesized that an increased surface and therefore the possibility of micro-niches for BE colonization and establishment could also promote root colonization. Px treatment was best in the granule application, although pre-tests, by plating suspensions of pumice stones on selective media for comparison between freshly sprayed and dried pumice stones, indicated that during the drying process after the application already ~ 90 % of *Pseudomonas* bacteria were dying. The sensitivity of *Pseudomonas* sp. strains to desiccation was already shown and is also a major constraint in rhizobia application (Deaker et al., 2004; Hirai, 1991). Additionally, the method is also under economic aspects criticisable. For application in the field experiment about 430 kg pumice stones ha^{-1} were used. At a sale price of 450 € t^{-1} (personal communication with the company Rotec, 2015) the farmer would need to compensate for additional costs of 190 € ha^{-1}. A further discussion on economic aspects can be found in 4.3.7. For the Rz product seed treatment had a much better influence on plant growth than the other two application

methods. These results indicate that effectiveness of application methods was BE dependent. Statistical analysis to test if there are interactions between BE treatment and application method was not significant but had a low p-value ($p = 0.078$). Analysis of root length density (RLD) further supports this theory. For RLD large soil cores were taken from CULTAN tracks and non-CULTAN tracks in the field experiments 2014 and 2015 and were analysed for their root length density. Data show that root length in the CULTAN-depot track was highest in the plot of the CUL_Rz_seed treatment (Nkebiwe et al. 2015, *unpublished*). These results are not surprising, because root colonization data do not reflect the amount of BEs that were present in the soil near the seed during germination and early root development. A concentration of 10^8 CFU $seed^{-1}$ in the seed treatments is by far higher than the expected concentration of about 10^6 CFU g^{-1} cm^{-3} soil surrounding the seed in the broad cast or band application treatments. These results again support the hypothesis that the effectiveness of BE treatments is determined in the very early plant development. Interestingly, for the Px treatments the seed application was less good performing than the other application technics (Figure 3-89 C). Germination tests that were performed later on also indicated that seeds with Px infiltration showed a delayed germination (3.8.1.3.1). It is possible that the BE concentration was too high in the Px treatment. Nevertheless, delayed germination by Px treatment was never observed before, even when high amounts of the product were applied, and therefore it is more probable that the infiltration technique used caused the inhibition.

Due to the cost intensive tracing method for Px root colonization, only samples from the CUL_Px_broad treatment were analysed. Results indicated that no bacteria were living at the time of sampling (3.10.4.1.3.2). This is not surprising, because sampling was done relatively late (61 days after application) and due to high temperatures and the very low rain fall in 2015 the dry soil conditions were probably not supportive for the inoculated bacteria. This is also supported by the results from root length density measurements. Root growth in the CULTAN depot track was strongly increased as compared to the non-depot track in both years. But only in 2014 Px application could further enhance this root growth promotion in the depot zone whereas in 2015 no such effect by Px application was observed (Nkebiwe et al., 2016b). Also no differences in plant growth among the different application techniques of the Px strain were observed. Due to the results from CUL_Px_broad treatment analysis and the lack of treatment differences in yield, analysis of Px root colonization was not further pursued.

3.10.4.2.4 Homogeneity in the field

Overall the homogeneity of the field was much better than in Exp_12. The thinner blocks resulted in a higher number of rows, but the row effect was never significant. Therefore quality of statistical analysis increased. In general, maize yields in Germany 2015 decreased as compared to the previous years. Nevertheless, in our field maize was growing relatively well with higher average yields than in the country Baden-Württemberg (541 dt vs. average yield of 393 dt ha^{-1} in BW) (Deutsches Maiskomitee e.V. (DMK), 2016).

3.10.4.2.5 Errors

As already mentioned in the results, major damages occurred due to crows feeding on seeds and young seedlings. As in 2014 no such problems occurred, a sonic blast shock device was installed too late to prevent the damage. Therefore 31 plots were slightly damaged (parts of a maize row) and 15 plots were severely damaged (almost total row eaten up) that were partly re-sown later on but still differences occurred between damaged and undamaged rows. Therefore for harvest only two rows per plot that were largely undamaged were harvested. Also one plot, in which two rows were damaged strongly, was later on excluded from analysis due to very low yield.

Several errors occurred during fertilization and sowing. Since different seeds were used in this experiment (untreated and BE-treated seeds), two plots were not sown at all due to some mistake by the technical staff at the research station. At the same time four plots were double sown with Rz-treated and untreated seeds. The wrong seeding rows were later on destroyed manually. Luckily, in none of the Ctrl plots Rz-treated seeds were sown. Nevertheless, three Px-treated plots contained therefore at least traces of Rz.

Additionally, several plots were partly treated with less or additional fertilizer. Due to the complex experimental design five different fertilizers were tested that were all applied in 2015 before sowing. One Std_Rz and one Urea_Rz plot received slightly lower fertilization rates (10 %) due to plugging in the plot fertilizer machine and one 0_Ctrl plot received a small rate of DAP from the previous plot but this did not result in a higher yield (the plot still had the lowest yield of all 0_Ctrl plots).

3.10.4.3 Results Exp_16

In Exp_16 only plant height and yield data were analysed. Plant height did not differ among treatments (data not shown). For yield data the first time significant differences between Ctrl and BE treatments were observed. Ctrl treatment had significantly higher dry matter yield than all other treatments, whereas for fresh matter yield differences where less strongly pronounced.

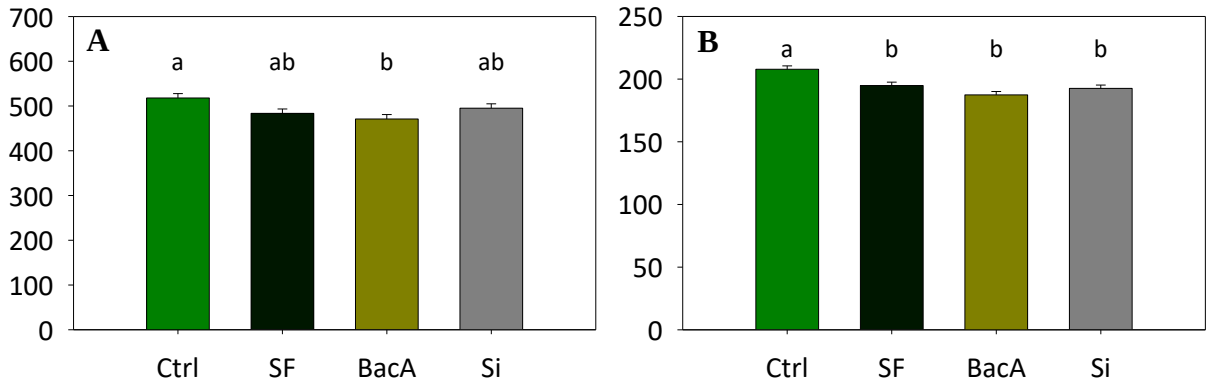


Figure 3-91 Yield data Exp_16; Fresh (**A**) and dry matter (**B**) yield in dt ha^{-1}; Adj. means + SE; r = 4, n = 16; glimmix with model B (Trt Block rnd Row*Block) and Tukey's test were used.

3.10.4.4 Discussion Exp_16

Exp_16 was done to test the SF product for maize foliar application, as recommend by the company Bioatlantis. Additionally, the *Bacillus atrophaeus* product from ABiTEP (BacA) was tested for the first time in the field. An additional treatment with Si was done as part of a product screening for another project in the institute and is here only included for the sake of completeness.

The application of all products was performed relatively late as compared to the other experiments at 4-5th leaf stage. In comparison to the 2014 experiment SF application rates were rather low but this time the SWE was directly applied to the leaves. Results from maize harvest indicate that plants from Ctrl treatment performed best. These results can be explained keeping in mind the unfavourable growing conditions of 2015. Plants obviously suffered from drought stress, also seen by withering symptoms of maize plants in both experiments. Boxplots for the yield data per block also nicely show the conditions in the field (Figure 3-92). Here block V, that was positioned at the peak of a slope, showed lowest dry and fresh matter (FM) yields but highest dry matter contents (DMC) (correlation analysis for FM/DMC ρ = -0.88). The negative correlation fits to the hypothesis that yields were decreased by low soil water contents.

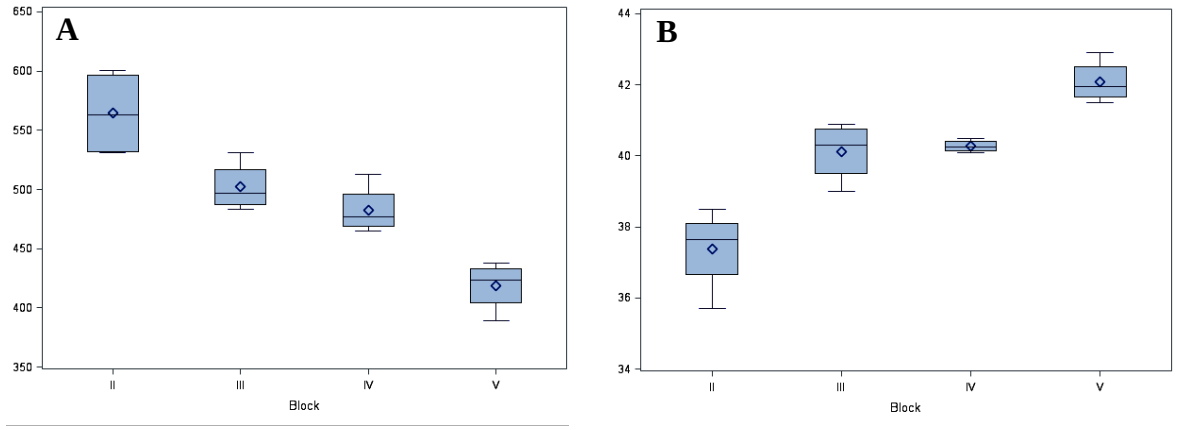


Figure 3-92 Harvest analysis per block Exp_16; Fresh matter yield in dt ha^{-1} (**A**) and dry matter content in % (**B**) as means per block; r = 4, n = 16; Block II to V (for experimental design see Figure 3-87)

It seems that the application of the BEs led to additional stress for the plant and thereby reducing the yields at the end of the growth period. This is surprising, because one of the proposed effects of seaweed extracts and Si application is an improved stress alleviation (1.4.3.42.10.3). The ability of Si to alleviate cold stress was proven in maize experiments in the institute (Moradtalab et al., 2018). Here fertigation and seed treatment with silicilic acid were highly effective in reducing ROS, increasing stress-related metabolites, SOD activity and Zn/Mn status in maize plants. In a field experiment with maize sown early in mid of April seed treatment with potassium silicate improved emergence rate of maize 41 DAS significantly as compared to the control and yield was increased by almost 80 %. Nevertheless, foliar application of Si did not result in significant difference for emergence rate or yield as compared to the control. As Si treatment seems to improve micronutrient translocation and reduce leaching in the seedling development (Moradtalab et al., 2018), foliar application was too late to improve stress tolerance of the plants by stress priming. Additionally, in Exp_16 the coincidence of two stress factors (drought and BE application) had negative impact on the maize yield. Similar results were seen in many experiments with severe nutrient deficiency (Kuhlmann, 2014; Nkebiwe, 2016; Probst, 2015) or under cold stress conditions (Exp_1 and Exp_11) when BEs were applied when other stress factors were already present. Possibly, also application rates need to be adjusted.

For SF the ability to alleviate abiotic stress was proven for *Arabidopsis* plants treated with a diluted herbicide (Omidbakhshfard et al., 2020). The seaweed extract reduced the production of ROS in herbicide treated plants. SF was applied by spraying of leaves at a concentration of 0.1 %, the same as in our field experiment (0). Nevertheless, six applications were done on two consecutive days on plants that were free from stress. 24 h later first herbicide application was done. No results for SF treatment on plant growth were described.

3.11 Plant physiological processes

3.11.1 Introduction

Four experiments with the focus on BE mode of action and plant physiological responses to BE application were conducted.

As described in 1.4.3 bioeffector products are supposed to stimulate plant growth by influencing plant metabolism. In the last 20 years various mechanisms and modes of action were already investigated, especially for bacterial BEs, and are regularly discussed in scientific reviews.

Objective was to determine the mode of action of specific BE products to possibly distinguish any specific plant-BE interactions.

As part of the overall Biofector project a gene expression analysis of maize plants, treated with the “standard” BE products (most commonly used) Rz and Px, was conducted on plant material from Exp_11. To reconnect to the results from the partner institute JKI in which experiments with the Px and Rz strain were analyzed for root colonization, plant mineral analysis and shifts in the soil microbiome (Eltlbany et al., 2019) the same experimental conditions were used as a test environment in several experiments in the course of this thesis.

Exp_14 was repeated in a very similar design as Exp_11 to reproduce the effects and generate new sample material. Exp_22 and Exp_23 were later on conducted to verify a hypothesis that came up during gene expression analysis:

Does the Proradix product influence plant metabolism and stimulate plant growth by inducing P-deficiency symptoms during early plant growth?

3.11.2 Influence of PGPR application on maize gene expression (Exp_11)

3.11.2.1 Experimental design Exp_11

Exp_11 was conducted as a two-factorial experiment with two harvest times. Three different soil water contents were tested in combination with the application of the microbial BEs Rz and Px (Table 3-27). Higher sand contents (1:1) and higher fertilization rates as in Exp_7 were used. For each of the nine treatments nine pots were prepared. Four pots were taken for intermediate harvest of maize plants designated for RNA-Seq analysis, whereas five pots were harvested 42 DAS for investigation of plant growth promotion effects. Per pot five seeds were sown. Plants from WHC_70 pots for intermediate harvest were thinned out to three plants per pot by reducing those two plants that differed most from treatment median. For late harvest all pots were thinned out to one single plant in two steps by keeping the middle sized plant. Different time points for BE application and the intermediate harvest were chosen, because major differences in the plant development of treatments with different water contents were observed. Following the experimental design from JKI the first BE treatment was done as seed treatment and the second treatment as soil surface application at full plant emergence.

Table 3-27 Treatments Exp_11

Trt_Nr	Treatment	BE	WHC	WC
1	Ctrl_30	/	30	9.6
2	Px _30	Px	30	9.6
3	Rz_30	Rz	30	9.6
4	Ctrl_50	/	50	16.1
5	Px _50	Px	50	16.1
6	Rz_50	Rz	50	16.1
7	Ctrl_70	/	70	22.5
8	Px _70	Px	70	22.5
9	Rz_70	Rz	70	22.5

WHC = % of max. WHC; WC = soil water content in % (w/w). BEs = seed soaking before sowing for 10 min in 10^9 CFU ml^{-1}, Ctrl was treated with 0.3 % NaCl solution. 1. and 2. soil application of BEs with each $5x10^9$ CFU kg^{-1} substrate; Fertilisation in all treatments: 50 mg P, 100 mg N, 150 mg K, 50 mg Mg kg^{-1} substrate; r = 4/5

After seed treatment the first BE application was done 8 DAS in treatments 7 – 9, 9 DAS in treatments 4 – 6 and 11 DAS in treatments 1 – 3 as soil surface application with the pipette. To keep the same time interval between BE application and intermediate harvest treatments where harvested 15, 16 and 18 DAS, respectively. At intermediate harvest plants for late harvest were treated with BE suspension for a second time. Intermediate harvest was done as described in 2.7.1. As climatic conditions in the greenhouse were only semi-controlled temperatures varied similarly to field conditions in late spring. Temperatures at night dropped to 14 – 15 °C during a time period of 4 – 5 days with a minimum of 13 °C 12 DAS.

3.11.2.2 Results Exp_11

3.11.2.2.1 Intermediate harvest

15 – 18 DAS the plants from the intermediate harvested were harvested without visible symptoms of cold stress. Nevertheless chlorotic purple colorations on the maize leaves, similar to the typical symptoms seen in maize in the field in late spring, could be observed 30 DAS indicating anthocyanin formation by cold stress or P-deficiency.

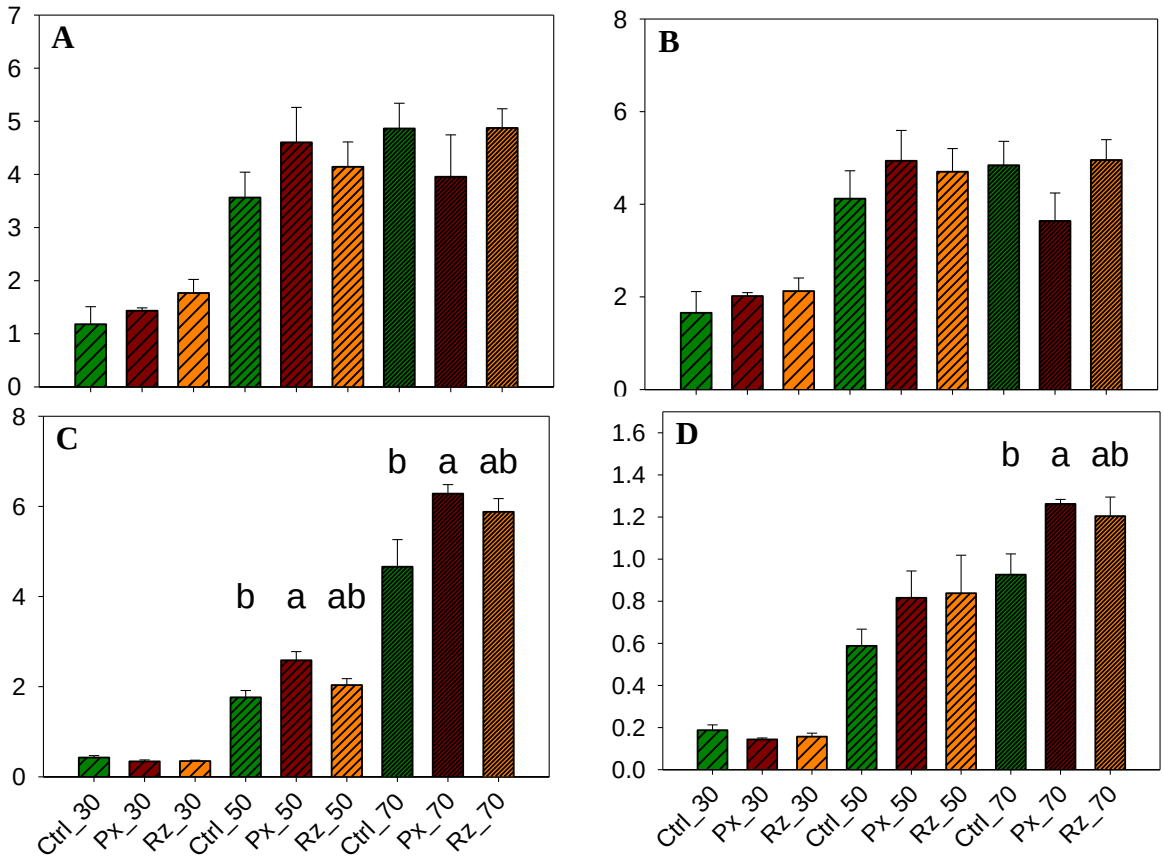


Figure 3-93 Biomass results Exp_11; Shoot (**A**) and root (**B**) fresh weight 15 – 18 DAS, Shoot (**C**) and root (**D**) dry weight 43 and 44 DAS; means + SE (n= 4 (**A+B**) / 5 (**C+D**); Letters indicate significant differences between BE treatments of the same WHC

Analysis of shoot and root fresh weights from intermediate harvest did not reveal significant differences among treatments but different trends were observed for the BE treatments depending on the applied water contents (Figure 3-93 A + B). Root and shoot weights of BE treated plants in the 30 and 50 % WHC pots tended to be larger than in the Ctrl whereas Ctrl and Rz in the 70 % WHC pots were equal and the Px treatment showed a decline in biomass.

3.11.2.2.2 Late harvest

In the plants that were kept for late harvest, first differences in plant height between treatments appeared 29 DAS. From there on Px and Rz treated plants generally showed higher plant height and stem diameter than Ctrl plants in the WHC 50 and 70 %. Plants with 30%

WHC developed extremely slow but without clear symptoms of withering. No differences among treatments were found here. Shoot and root fresh and dry weight 43 and 44 DAS differed significantly between Px and Ctrl treatment in the 70 % WHC pots, whereas Rz treatment showed a similar trend but did not differ significantly due to high standard deviations in the Ctrl and Rz treatments (Figure 3-93 C + D). Differences in the 50 % WHC treatments were smaller but Px treated plants still had a significantly higher shoot dry weight than Ctrl plants whereas no significant differences for root weight was observed. Root scanning was performed only for the 70 % treatments. Here, in the Px treatment average root length was highest but no significant differences among treatments were observed. Results from transcriptome analysis are given in 3.12.

3.11.2.2.3 Phosphorus analysis

Analysis of P status was done only for the 70 % samples from intermediate harvest that had been sent for gene expression analysis and H-NMR analysis. The analysis was performed almost one and a half years later because the samples were first analysed for gene expression and then shipped to the working group in Italy for metabolite analysis and where therefore not available for P analysis. Both inorganic P (Pi) and total P (Pt) status were analysed (described in 2.5.2 and 2.5.1.3). Pi concentrations in Px shoot samples were significantly increased in comparison to Ctrl and Rz treatments (Figure 3-94 A). This coincided with the decreased plant biomass (Figure 3-93 A). Accordingly, Pi contents were almost the same for all treatments (Figure 3-94 B). Pi and P contents were calculated by using an estimated dry weight based on the measured fresh weights divided by 10 multiplied with the concentration. In many experiments before a negative correlation between shoot biomass and P concentration was observed. Nevertheless, when comparing biological replicates shoot Pi concentrations did not differ among each other and seemed therefore treatment but not biomass dependent (see e.g. biomass and Pi concentration of S5 and S8 in Table 3-28). Additionally, in contrast to the results from Pi analysis total P concentrations as well as P contents were decreased in the Px treatment as compared to the Ctrl and Rz treatment,

Table 3-28 Total P analysis Exp_11

Trt	Pot	Rep.	Shoot FW (g)	Pt conc. (mg g^{-1})	Pi conc. (mg g^{-1})
Ctrl	S1	R1	5.64	5.92	2.86
Ctrl	S2	R2	4.97	5.77	2.26
Ctrl	S3	R3	5.35	5.97	2.99
Ctrl	S4	R4	3.5	5.76	2.95
Px	S5	R1	2.48	3.41	3.47
Px	S6	R2	3.72	3.83	3.41
Px	S7	R3	3.43	4.30	3.51
Px	S8	R4	6.19	5.65	3.27
Rz	S9	R1	5.88	3.66	2.21
Rz	S10	R2	4.83	6.22	2.95
Rz	S11	R3	4.61	4.11	3.07
Rz	S12	R4	4.18	6.29	2.88

Correlation between shoot biomass and shoot Pt concentration in the Px treatment: $\rho = 0.97$, the Ctrl treatment: $\rho = 0.75$ and the Rz treatment: $\rho = -0.69$

although differences were not statistically significant, as data failed normality test and had to be normalized. As seen in Table 3-28 shoot biomass and shoot Pt concentration in the Px treatment was highly positive correlated (ρ = 0.97).

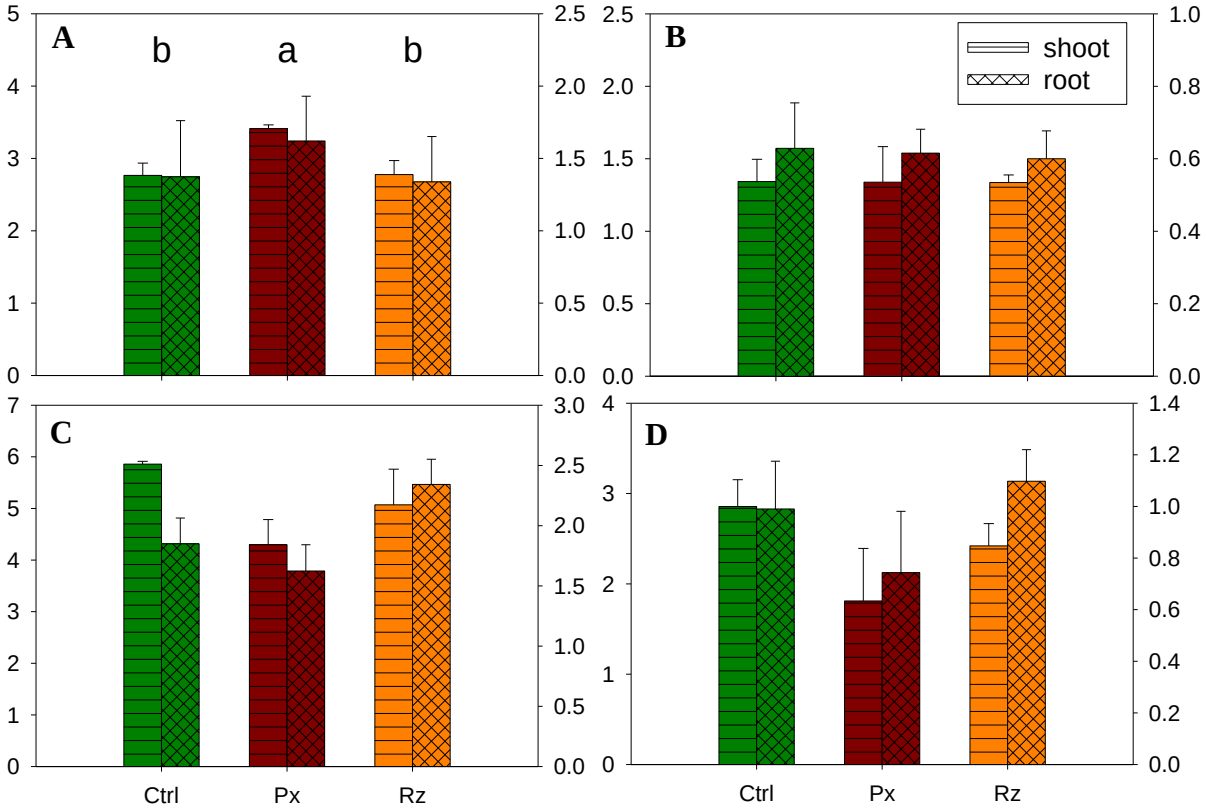


Figure 3-94 Results from phosphorus analysis Exp_11 70 % WHC; All samples from intermediate harvest; Pi concentration in mg g^{-1} DW (**A**), Pi contents in mg pot^{-1} (**B**) as well as Pt concentrations and contents (**C** and **D**) for both shoot and roots (left and right y-scale); Means + SE; Pi and Pt concentrations not normally distributed; Significant differences in Pi concentration only for shoots using Kruskal-Wallis ANOVA on Ranks

It seems that the Pi value was kept constant in the shoot to ensure a good plant development although root concentrations differed among biological replicates. The root Pi (some data missing, see) shows the best correlation with root biomass over all 12 samples (ρ = 0.74).
In the Ctrl treatment Pt concentrations were almost equal in all samples independent of the shoot biomass but also here a positive correlation was observed (ρ = 0.75) whereas in the Rz treatment a negative correlation (ρ = -0.69) was observed. Correlation between Pi and total P values also differed among treatments. Although the number of replicates is probably too small to see the data as strong evidence, the findings suggest that P acquisition in the treatments differed strongly between each other.

Some of the original plant material from the intermediate harvest of Exp_11 was later on pooled for samples that were send for gene expression analysis (see 3.12). Table 3-29 shows the calculated P status for the mixed samples. Calculation of P contents as described above.

Table 3-29 Phosphorus status of gene expression samples

Sample	Pt_conc shoot	Pt_content shoot	Pt_conc root	Pt_content root	Pi_conc shoot	Pi_content shoot	Pi_conc root	Pi_content root
C_1	5.85	3.11	1.77	0.92	2.56	1.37	1.01	0.53
C_2	5.97	3.20	2.02	1.13	2.99	1.60	0.99	0.55
C_3	5.76	2.02	.	.	2.95	1.03	2.48	0.90
Px_1	4.53	2.17	.	.	3.37	1.44	.	.
Px_2	3.83	1.43	1.41	0.51	3.41	1.27	2.08	0.75
Px_3	4.30	1.47	.	.	3.51	1.20	1.76	0.55
Rz_1	3.66	2.15	.	.	2.21	1.30	.	.
Rz_2	4.11	1.90	2.33	1.33	3.07	1.42	0.84	0.48
Rz_3	6.29	2.63	2.71	1.05	2.88	1.20	1.92	0.74

Calculated values for the sample mixtures send for gene expression analysis. For several roots samples data are missing as most of the sample material was used for H-NMR analysis. Highlighted samples were used for single sample comparison in gene expression analysis due to similar plant biomass.
Contents are given in mg plant^{-1}, concentrations in mg g^{-1} DW

3.11.2.2.4 Gene Expression

Results on gene expression and metabolite analysis are described separately in 3.12 and 3.13.

3.11.2.3 Discussion Exp_11

Exp_11 and Exp_14 were, similar to the Exp_3, 4 and 5, mainly performed to reproduce the results from the partner institute at the JKI Braunschweig. For unknown reasons the strong growth promotion effects seen at JKI could not be reproduced in previous experiments. To find explanations for the difference in the observed effects conditions were adapted in the various experiments. Due to a misunderstanding during the communication with the partner institute, in the previous three experiments substrate fertilization was based on per kg dry soil and the sand contents were only 33 %. Therefore sand contents and also fertilization rates in Exp_11 were increased (as already done in Exp_6 and 7) to keep conditions more similar. As water contents of the substrate used at JKI were unknown, different soil water contents were tested in Exp_11. Additionally, an intermediate harvest was included to isolate RNA for transcriptomic analysis from young maize roots. This early harvest was done before clear growth promotion effects were observed that would influence the metabolite concentrations and plant developmental stage and therefore would lead to “secondary effects” in a transcriptome analysis.

Again, the strong effect observed at the JKI, especially for the Rz strain, could not be reproduced. Nevertheless, both BEs were able to stimulate the growth of maize plants, in the case of the Px strain even significantly.

3.11.2.3.1 Water-deficiency and influence on BE-plant interaction

As seen from the results, 30 and 50 % of WHC_{max} were not sufficient for optimal plant growth. Obviously water was a limiting factor in both groups and especially in the 30 % pots any treatment effects were superimposed by the water-deficiency.

Interestingly, no stress symptoms were observed in the 30 % pots because plants were cultivated under these conditions already during the early development and plants were adapted to the water-deficiency by lower growth rates. This is in contrast to common experiments on drought-stress in which plants grow with optimal water supply at the beginning of the experiment but are confronted with a sudden change in environmental conditions in the course of a few days or even within hours. Therefore no hanging or withered leaves were observed, no chlorosis symptoms and probably also no other stress-symptoms on the cellular level, like formation of ROS or changes in enzymatic activity can be expected here. Additionally, it is probable that the suppression of growth due to water-deficiency influenced also photosynthesis, synthesis of assimilates and root activity. The growth depression in the BE treatments as compared to the Ctrl_30, observed 43 DAS, suggests that the BEs and the plants were either competing for nutrients and assimilates under these conditions or that the BEs were triggering metabolic shifts inside the plants that counteracted their adaptation to the water deficiency. The results are in contrast to a publication on plant growth promotion and stress reduction by *Azospirillum* in maize under water stress (García et al., 2017). Nevertheless, also contrasting species-specific PGPR effects were observed for maize plants under water stress. Here a *Pseudomonas* strain was ineffective to improve maize growth whereas *Enterobacter* and *Achromobacter* were effective, possibly due to a higher production of ACC deaminase, as proposed by the authors (Danish et al., 2020). In 2017 another experiment on drought stress was conducted in the Hohenheim institute using a microbial consortia product (Combi B, see 4.3.2.5) on maize plants. Here, nitrate fertilized plants treated with the Combi B product had a reduced root and shoot growth as compared to the untreated control although (or because, see 4.1.5) measurements on physiological parameters connected to stress responses (catalase and SOD activity, proline, protein contents) were up-regulated (Freytag and Wanke, 2017).

For the 50 % plants a significant difference in plant growth between Ctrl and Px plants could be observed at the second harvest. Nevertheless, due to the much lower total biomass as compared to the 70 % plants, it was decided to conduct gene expression analysis for samples of the higher water content. Nevertheless, in the aftermath this might have been the wrong

decision. Obviously, a certain interaction between the Px treatment and the experimental conditions influenced the 70 % plants from intermediate harvest in a way that plant growth was inhibited during early growth but promoted later on. This is in contrast to the results from the other soil water contents and also in contrast to the results from Exp_14, Exp_18, Exp_22, and Exp_23. In those experiments at least a slightly higher or equal plant biomass was observed in the Px plants as compared to Ctrl plants. The decrease in biomass in the Px treatment might be connected to the low temperatures that probably decreased root activity and nutrient uptake and thereby simulated low-P conditions. In contrast to experiment Exp_8, in which cold-stress conditions were applied in the root zone only (CRZT), in Exp_11 especially air temperatures were low. Similarly to the inhibition of plant growth by low nutrient availability and low irradiation, as discussed for Exp_4 and 5, also low air temperature directly reduces CO_2-assimilation (Savitch et al., 2011), decreases exudation of assimilates and therefore might increase the competitive activity of the microbes.

3.11.2.3.2 Px influences ratio between Pt and Pi values

In the Px treatment Pi concentrations were extremely high as compared to the Pt concentrations. Sample S5 (Table 3-28) of the Px treatment shows even a higher Pi value than total P. This is obviously not possible. It is probable that in general our estimation of Pi is higher than the real Pi concentration in the shoot because of degradation of organic P during drying and extraction (Bollons and Barraclough, 1997). Additionally, we expect the Pt values to be lower than the real Pt value because of the method used. In contrast to previous analysis it was not possible to use the P yellow method for the analysis of Pt because for some of the samples the amount of the remaining plant material was so low that the more sensitive P blue method had to be used. The same method was also used for Pi sample analysis and therefore the same standard solutions were taken. Nevertheless, the matrix of the standard solutions and the samples differed in this case because for Pt extraction much stronger acids are used. Some of the Pt samples were analysed with P yellow and P blue and therefore results could be compared. Indeed the P blue measurement had slightly lower values. The reason is that P blue colour agent is sensitive to pH changes. E.g. addition of alkaline ammonia solution was strongly increasing colour intensity whereas the acid in the Pt samples slightly decreased intensity. For Pi analysis the whole plant material was grinded and then extraction with a weak organic acid was performed. A very similar method was also used before (Wang et al., 2012). In this study Pi and total P concentrations were not directly compared but from the data we can estimate that about 50 % of total P was present as free Pi. This fits to the estimations for the control samples (C_1-3).

Nevertheless, the differences between treatments cannot be explained by the method. In the Px treatment Pt was lower whereas Pi was higher than in the other treatments. Data indeed support the findings from gene expression analysis that in the Px treatment stress responses coincided with the degradation and recycling of organically bound P. The Px treatment seemed to have influenced plant metabolism in a way that Pi status was kept higher than in untreated plants. To ensure a high Pi status plants were using metabolically “fixed” or bound phosphorus as suggested by gene expression data. At the same time Px treatment was decreasing Pt values.

3.11.2.3.3 BE-specific influence on Pt-status

Correlation between shoot biomass and shoot Pt concentration differed strongly among treatments, especially between the Rz and Px samples. Taken together with the results from gene expression, data suggest that some interaction happened in the pots in which the individual treatments were involved but the specific mode of action that caused the plant responses remained unclear.

3.11.3 Influence of PGPR application on maize gene expression (Exp_14)

3.11.3.1 Experimental design Exp_14

For Exp_14 the same fertilization and BE treatments as in Exp_11 (Table 3-27) were applied with the difference that only one soil water content was chosen (60 % WHC_{max}) and the experiment was conducted in a climate chamber with controlled air temperature. Additionally, for the intermediate harvest six pots per treatment were prepared, for the last harvest five pots as in Exp_11. For intermediate harvest the four plants sown per pot were thinned out to two plants per pot. One plant was sampled for RNA isolation whereas the other plant was sampled for tracing analysis. Tracing analysis for the Px strain was done on NP medium whereas for the Rz treatment the *rif*-resistant *B. amyloliquefaciens* strain, also provided by the company ABITEP, was used. For this 1-2 g of root with attached rhizosphere soil were incubated with 40 ml 0.1 % peptone shaking for 10 min. Dilutions, heat treatment and plating using R2A or LB medium were done as described in 2.6.2.

3.11.3.2 Results Exp_14

3.11.3.2.1 Plant performance

The effectiveness of the BEs in Exp_14 was not increased. Despite applying controlled conditions the standard deviations were similar to those in Exp_11. During the whole course of the experiment regular plant height and stem diameter measurements were performed but at no time point, including root and shoot weights from intermediate and late harvest, significant differences among treatments were observed.

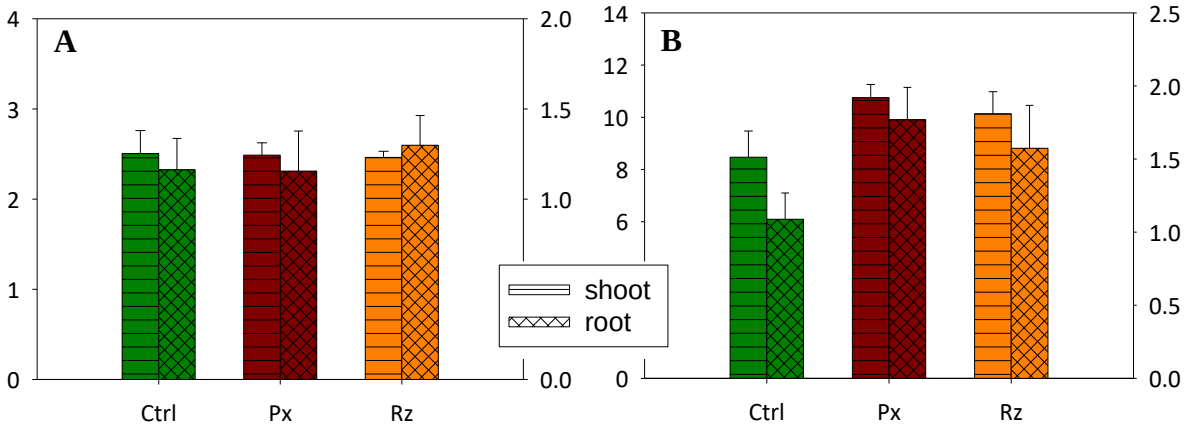


Figure 3-95 Plant biomass Exp_14; Plant fresh weight 15 DAS (A) and dry weight 42 DAS (B) in g pot^{-1}; means + SE; n = 6 (A) / 5 (B)

Nevertheless, trends were always the same with the Px treatment ahead, showing the best performance, Rz following and the Ctrl treatment performing worst. Growth improvement by

Px treatment at the end harvest was about 30 % for shoot and 60 % for root dry weight as compared to the Ctrl treatment, whereas Rz led to 20 and 40 % higher biomass respectively.

3.11.3.2.2 Tracing analysis

Tracing analysis was performed after intermediate harvest for all three treatments using root samples with attached rhizosphere soil. Plating of Ctrl and Px treatments was done on NP medium. Here again no clear separation of the Ctrl and Px treatments was possible. For the Ctrl a cell density of 1.6 x 10^6 CFU g^{-1} root and for Px a slightly higher average cell density of 2 x 10^6 CFU g^{-1} root were counted.

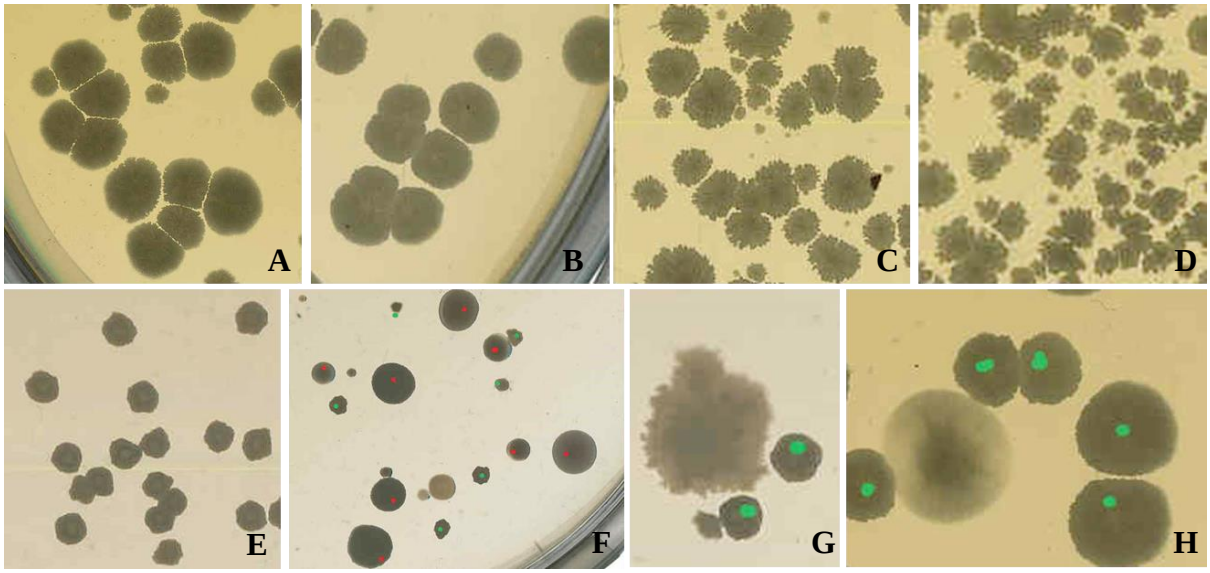


Figure 3-96 *Bacillus* sp. colonies on LB and R2A medium; *B. amyloliquefaciens* FZB42 (Rz) on $R2A_{rif}$ medium without heat treatment (**A**), after heat treatment at 80°C in high dilution (**B**), medium dilution (**C**) and low dilution (**D**), *Bacillus* sp. on LB medium after heat treatment at 80°C; contaminations on LB or R2A media (**F**–**G**); *Bacillus* sp. marked with green dots and probable *Pseudomonas* sp. colonies in (**F**) with red dots

For quantification of the *Bacillus* strain Ctrl and Rz sample extracts were plated on LB after previous heat treatment or on R2A medium containing 25 or 50 mg rifampicin l^{-1} agar medium. Both concentrations were sufficient to suppress bacterial growth of most non-target bacteria as seen by evaluation of the colony shape (Figure 3-96). Extracts were serial diluted 10 – 1000 times and different concentrations were plated. The average CFU for the untreated Rz treatments was 2.1 x 10^7 CFU g^{-1} root using the $R2A_{rif}$ media. Here a very low standard deviation was observed. Counts after heat treatment at 80°C were about 2.4 x 10^7 CFU g^{-1} root. For the LB media CFU after heat treatment was with 3 x 10^7 CFU g^{-1} root slightly higher. For Ctrl treatments only a CFU of about 10^3 CFU g^{-1} root was counted whereas some agar plates with undiluted extract did not show any CFU indicating that no bacteria were present at all. Because of a huge variation found in the Ctrl treatments some contamination

from the Rz treatments during plating procedures cannot be excluded. Nevertheless, CFU in the Rz treatments was at least 1000 times higher. Some colonies on the non-selective LB plates of the Ctrl treatment indicated that the density of the natural *Bacillus* population was about 10^5 CFU g^{-1} rhizosphere soil.

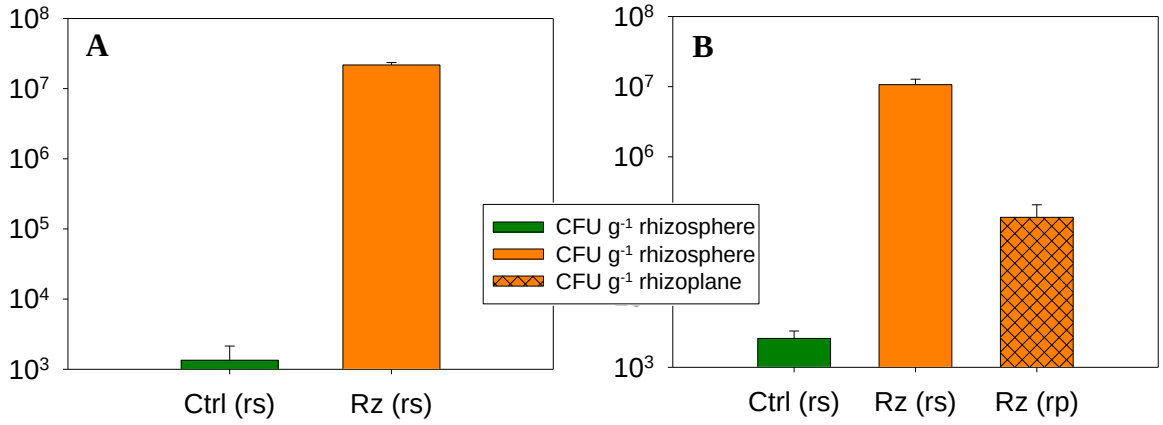


Figure 3-97 Tracing results Exp_14; *Bacillus* CFU counts at early harvest 15 DAS (A) and late harvest 42 DAS (B); for late harvest also rhizosphere samples were separately plated; means + SE; n = 6 (A) / 5 (B)

After late harvest a second tracing was performed for Ctrl and Rz treatments only. Here it was investigated where the majority of *Bacillus* spores might be found. Therefore extracts from Ctrl and Rz rhizosphere samples but also from the Rz rhizoplane (after washing of roots) were plated. For Ctrl rhizosphere samples an average of 2.6 x 10^3 CFU g^{-1} root were found. For the Rz rhizosphere 1.1 x 10^7 CFU g^{-1} root, a similar amount like in the first tracing, was counted. For the Rz rhizoplane counts declined to only 1.4 x 10^5 CFU g^{-1} root.

3.11.3.3 Discussion Exp_14

3.11.3.3.1 BE plant growth stimulation in Exp_14

Exp_14 was conducted because the growing conditions in Exp_11 were sometimes suboptimal, although they might better reflect field conditions, and we hypothesized that the temperature might be one probable reason for the fact that we still could not reproduce the BE effects observed at JKI. As seen in the results, both BE treatments were increasing plant growth but no significant differences were observed, due to high standard deviations. One reason for the high standard deviation was probably the method of sowing. Because seeds were inoculated with BE suspension directly before sowing different pipettes were used for transferring the seeds into the substrate. Therefore the contact of the seeds with the soil matrix was sometimes suboptimal, probably reducing water supply during germination. Indeed different emergence rates were observed in the different pots. To decrease variation outliers in

the pots were reduced leading to a more homogenous plant population during intermediate harvest but later on intra-treatment variation increased again.

3.11.3.3.2 Maize P status in Exp_14

Nevertheless, samples from intermediate harvest were sent for analysis of primary metabolites to the working group in Italy (for the results see 3.13). Similar to samples from Exp_11 the leftovers from H-NMR analysis were analysed for their Pi and Pt concentration (*data not shown*). The Px treatment showed a slight decrease for Pi and Pt concentrations and contents as compared to the Ctrl but differences were far less pronounced than in Exp_11 or Exp_22 and no significant differences between the treatments were observed.

3.11.3.3.3 Root colonization by Rz

CFU from 80°C treated and untreated Rz samples was almost equal and the number of counted bacteria was in the range of the amount inoculated ($1 – 3 \times 10^7$ CFU counted g^{-1} rhizosphere and 8.6×10^6 CFU inoculated g^{-1} substrate). These results indicate that only a very low amount of the spores germinated in the course of the experiment. The elevated amount of 2.1×10^7 CFU counted during analysis does not indicate an increased population density due to germination and cell growth because germinated cells would have been killed during heat treatment. It is probable that the concentration of CFU in the substrate was not equally distributed and therefore concentrations might be higher as calculated kg^{-1} of soil.

In the second tracing after the late harvest the question for the percentage of active cells to spores was not followed anymore because the hypothesis that Rz application did not result in plant growth promoting effects due to inactivity of the spores was not yet formulated.

To investigate this theory Exp_21 was conducted. Nevertheless, here no rhizoplane but rhizosphere samples were analysed. The question if the number of active *Bacillus* cells might be higher in the direct contact with the root can therefore not be fully answered. But generally there are two aspects making it anyhow difficult to answer this question:

1. The number of active cells is not measured but is calculated from the counted numbers of total cells minus the number of spores. Because these numbers are normally extremely high and variation in between samples is extreme a calculation will be always misleading and inexact.

2. Results of Exp_21 suggest that the population of active *Bacillus* cells in natural soils is extremely instable and the life time of individual *Bacillus* cells is short. Even if the

germination rate of *Bacillus* spores would be strongly promoted in the plant rhizosphere (as not shown in our data) or the rhizoplane (not yet tested), a significant difference in population density is therefore hard to detect.

It was repeatedly reported that *B. amyloliquefaciens* strains colonize roots endophytically (Kim et al., 2016; Shu-Mei Zhang, 2012; Tan et al., 2013a; White et al., 2014; Zouari et al., 2016). In contrast, the comparison of the results from rhizosphere and the rhizoplane samples showed a much lower population density in the rhizosphere than in the rhizoplane. Nevertheless, a direct comparison of the two compartments is difficult, because washed roots had higher water contents than the rhizosphere samples and therefore the original root sample was diluted and the available area for the bacteria to attach was much larger in the rhizosphere than in the rhizoplane. Additionally, Pseudomonads or total bacteria counts in the rhizosphere are generally higher than in the rhizoplane making it difficult to draw clear conclusion from this result. Furthermore, as inoculation was done from above and the inoculum was washed into the substrate by the water stream it is probable that bacteria were passively transported to the rhizoplane and hibernated there in endospore form. Differences in bacterial abundance inside the pot volume are therefore probably a result of soil depth and not preference or enrichment of the bacteria in a certain soil compartment.

Reasons for the contaminations found in the Ctrl samples may derive from spatula, non-sterile pots, sieves or plastic bottles used for soil extraction. Although spatula and the scissors for cutting roots were wiped with alcohol or burned it is not given that a full sterilization was achieved due to the extreme stress tolerance of endospores to cell toxic compounds, e.g. alcohol, or heat (Errington, 2003; Thomas, 2006).

3.11.4 Influence of *Pseudomonas* sp. Proradix on plant P status (Exp_22)

3.11.4.1 Experimental design Exp_22

Exp_22 was conducted as a repetition of Exp_11 and 14 with the same fertilization rates but six intermediate harvest times (6, 9, 10, 12, 17, 24 DAS) for analysis of root and shoot P contents. Similar to Exp_ 18 a smaller pot size was used and only two treatments per harvest (Ctrl, Px). As in Exp_11 and 14 the Px was first applied as seed treatment (with 10^9 CFU ml^{-1} suspended in 0.3 % NaCl) only and soil applications were performed 8 and 15 DAS at a concentration of 5 x 10^9 CFU kg^{-1} substrate. Plants from 9 DAS harvest were not treated with soil application and watering was stopped one day earlier for an easier removal of roots from the soil. 6, 9 and 10 DAS three plants per pot were harvested whereas the remaining plants for harvest 12, 17 and 24 DAS were reduced to one plant per pot. During harvest shoots were directly frozen in liquid nitrogen. Roots were washed, scanned and then frozen in liquid nitrogen. For analysis of the soluble inorganic P (P_i) the procedures descripted in 2.5.2 and 2.5.3 were used. Nevertheless, in Exp_22 drying of plant material was done using a domestic microwave, following a previously descripted method (Bollons and Barraclough, 1997), with subsequent drying to constant mass at 60°C. Because the method did not work optimal, for Exp_23 the method was adapted as descripted in the respective sections.

3.11.4.2 Results Exp_22

3.11.4.2.1 Biomass results

Shoot growth was increased by Px application 10, 17 and 24 DAS, root growth only 24 DAS. Because data were not normally distributed a Mann-Whitney Rank Sum Test was used. Shoot FW significantly differed 10 and 24 DAS between Ctrl and Px treated plant (Figure 3-98 A).

3.11.4.2.2 Pi concentrations in shoots and roots

The results for Pi concentration in shoots showed that Pi availability was high at the beginning and was decreasing in the course of the experiment with the lowest values 24 DAS. Pi concentrations increased 10 DAS in the Ctrl as compared to the plants from previous harvest. 12 DAS, after an increase of shoot biomass in the Px treatment 10 DAS, shoot and root Pi concentration dropped significantly as compared to the Ctrl treatment. The same was observed 12 DAS but here data for the FW are missing. 17 DAS concentrations were slightly increased in the Px treatment but decreased again, in the roots significantly, 24 DAS as compared to the Ctrl (Figure 3-98 B).

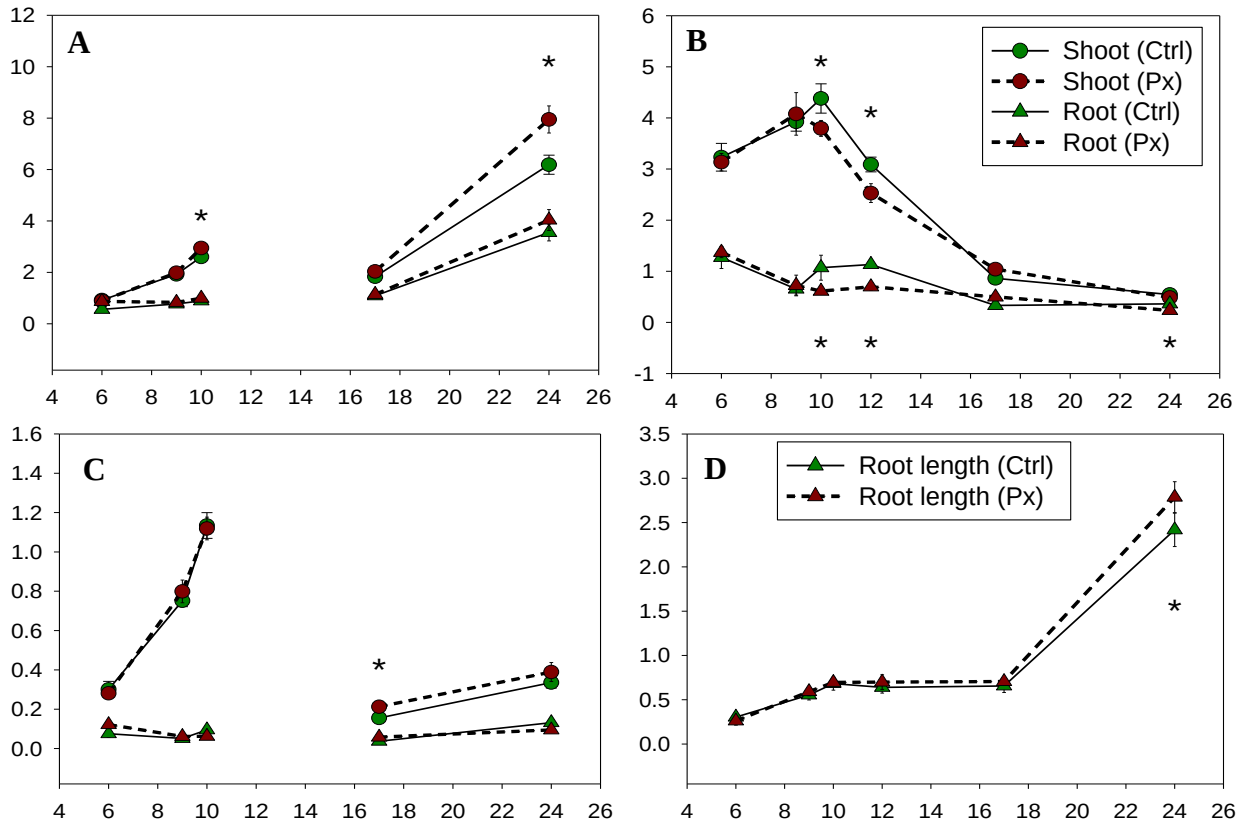


Figure 3-98 Results Exp_22; x-axis shows harvest time as DAS; Fresh weight (FW) (**A**), Pi concentration in ppt (**B**), Pi contents per pot (**C**), root length in m (**D**); P contents calculated from FW data divided by 10. Means + SE; * indicate significant difference in t-test among treatments for the respective harvest time, whereas * below the lines show significant difference for the roots and * above the lines for shoots; FW data not normally distributed

Pi contents were calculated based on fresh weight (FW) data divided by 10, giving a dry weight ratio of shoots that was found in most of our previous experiments. Pi contents did not differ between the treatments with the exception of the harvest 17 DAS. Here Px treatment significantly increased the Pi contents in the shoots (Figure 3-98 C). 24 DAS Pi contents in shoots of Px treated plants was still higher than in the Ctrl plants but dropped to a lower level in the roots. In general, Pi concentration in the roots was much lower than in the shoots and concentrations were similar in the course of the experiment. Nevertheless, during the first two harvest times Pi concentration in roots was behaving opposite to the shoot. Then, 10 DAS, root and shoot Pi concentrations in the Ctrl treatment were increased whereas in the Px treatment values dropped. Later on concentrations in the root decreased only slightly with no bigger differences.

Root length did not differ significantly between the treatments during the first harvest periods. Only at the last harvest root length in Px was found to be significantly increased as compared to the Ctrl treatment (Figure 3-98 D).

3.11.4.3 Discussion Exp_22

3.11.4.3.1 Objective

Exp_22 was conducted to give further support for the results of the gene expression analysis (Exp_11 and section 3.9.1). It was proposed that Px might induce a slight P-deficiency during early plant growth development due to competition for nutrients (as also seen in Exp_17 and in experiments by Kuhlmann and Probst (Kuhlmann, 2014; Nkebiwe, 2016; Probst, 2015, *partly published* in Mpanga et al. (2019)) that leads to the stress responses observed for the gene expression analysis. To test this hypothesis intermediate harvests, similar to Exp_18, were conducted. Instead of tracing analysis only shoot and root inorganic phosphorus (Pi) status was determined to investigate the influence of the Px treatment on the plant available Pi status in the course of the early plant development. Therefore plant samples were directly frozen in liquid N and then quick-dried using a microwave to prevent further metabolic degradation of organically bound P. For P extraction an acetic acid extraction instead of a microwave digestion or ashing procedure was used. It was expected that for Pi lower concentrations than for total P would be measured. Furthermore sample amount was very low. Therefore the P-blue method was used that is much more sensitive than the P-yellow method.

3.11.4.3.2 Changes in the plant P status

We observed a relatively high Pi status for the first three harvest times and a sharp decline from 10 DAS on. The reason is probably that in the early growth phase P is mainly provided from reserves in the maize seed (Nadeem et al., 2014, 2011; Nadeem Muhammad et al., 2012). Here the bioavailability is high and therefore Pi concentration might have been comparable to total P values. This is supported by the results from Exp_23. Although the comparison of the results from total P analysis and Pi analysis can be criticized because different methods were used, the data still give better information than a comparison with data from literature. Here not only different methods but also different experimental conditions, harvest times and cultivars were used making a comparison almost impossible. In Exp_23 Pi concentrations in shoots 10 and 13 DAS were about 75 % of the total P values. In contrast to shoot values also in this experiment Pi concentrations in the root were much lower (< 50% of total P). Root Pi concentrations did not strongly change in the course of the Exp_22. In general root Pi supply was much lower and main Pi sink was obviously the growing shoot, as often described in literature (Moussavi-Nik et al., 1998; Nadeem et al., 2011). Therefore all metabolically active P was directly transferred to the growing shoots.

The steep decline in the Pi concentration from 10 DAS on indicates that seed reserves were depleted and a boost in shoot growth led to a dilution of the Pi concentration. The increase in biomass is seen for the harvest 10 DAS.

3.11.4.3.3 Px reduces P status in response to improved plant growth

Comparison of the treatments showed indeed a significant decrease for shoot and root Pi concentration 10 and 12 DAS when Px was applied to the pots. This coincides with an elevated Pi concentration in the Ctrl 10 DAS as compared to the previous harvest time, especially seen for the root. An explanation can be given by the experimental setup. Plants from 6 and 9 DAS were not treated with Px soil application but only received seed treatment. Watering from 7 DAS on was stopped because a dry substrate for harvest and reduced water contents to apply Px 8 DAS were required. Due to the drop in the water contents Pi concentrations in the plants 9 DAS were probably slightly reduced. After Px application and re-watering 8 DAS root Pi status of the plants from 10 DAS was increased again but only in the Ctrl treatment. There are two possible explanations for the Px treatment. One reason is probably that the milk powder of the Px product caused a reduced penetration of water into the soil. This effect is only seen during application and probably does not last long but may have influenced the Pi status 10 DAS. Additionally, the observed reduction in the Pi status of the Px plants was negatively correlating with an increased shoot and root growth 10 DAS in the Px treatment. Unfortunately, data from 12 DAS get lost but a similar tendency can be expected here as the calculated Pi contents per pot show that for most harvest times contents were very similar or even increased by Px treatment. This reflects the finding that Pi concentration and plant FW are negatively correlated and that differences in Pi concentration by the Px treatment are probably a response to the growth stimulation. This is changed slightly at the end of the experiment. Here the Px treatment starts to improve total Pi contents as often seen in previous experiments. 17 DAS Pi concentrations and Pi contents in roots in shoots of Px plants were increased (only shoot Pi contents significantly). One possible mechanism might be the stimulation of root length by the Px treatment. Root length was slightly increased at later harvest times and 24 DAS significant differences were observed, that may have contributed to an increased Pi uptake from the soil substrate. Nevertheless, Pi concentrations in the Px treatment dropped again 24 DAS, probably as results of growth stimulation, because shoot and total plant (shoot + root) Pi contents were still higher than in the Ctrl. Interestingly also P contents in the root 24 DAS were lower in the Px treatment. We assume therefore, that root Pi status decreased in favour of a sufficient Pi status in the shoot. A measurement of the total P concentrations would have been useful here to further elucidate

if Px treatment may contribute to changing sink-source status of the plant but sample material was not sufficient to additionally perform this analysis.

3.11.4.3.4 Comparison with Exp_11

In contrast to the results from Exp_22, the Exp_11 plants from intermediate harvest 15 DAS treated with Px had a decreased root and shoot weight as compared to the Ctrl (Figure 3-100 A). The analysis of the Pi concentration again showed a negative correlation to plant biomass but this time with an increased Pi status in Px. Again Pi contents did not differ among treatments. This supported the hypothesis that Pi status was just a response to changes in plant growth. Nevertheless, as discussed for Exp_11, not all results can be explained by this hypothesis.

In contrast to Exp_11, in Exp_22 temperature conditions were more controlled. This may be the reason why results differed from the results in Exp_11 (see respective chapter).

3.11.5 Influence of *Pseudomonas* sp. Proradix on plant P status (Exp_23)

3.11.5.1 Experimental design Exp_23

To give further support for the results from Exp_22 a second experiment with more complex experimental design was performed. Only two harvest times (10 and 13 DAS) were included with each six treatments (Table 3-30). Pots were standard fertilized as in Exp_11/Exp_14/Exp_22 (Table 3-27) but in two treatments an underfoot placement with a mixture of F_{SP} and F_{Nov} was added during filling of pots. The powdery salts were distributed in a thin layer at a depth of ~ 10-15 cm. Then the rest of the soil was filled into the pots. Because 3 kg of substrate were used per pot about 60 mg N and 150 mg P were placed per pot. No seed treatment of Px was done. In treatments 2, 3 and 6 BEs were applied 7 DAS in a 30 ml suspension containing 10^8 CFU ml^{-1} (whereas Ctrl treatments were treated with 30 ml of 0.3 % NaCl solution). The Px2 treatment was treated with the same Px amount but directly at the time of sowing. 10 seeds per pot were sown to have sufficient plant material to perform analysis on total P (P_{tot}) and P_i. During harvest shoots and roots (after washing) were directly frozen in liquid nitrogen. Drying of samples was done using a freeze-dryer with a starting temperature of -30 °C.

Table 3-30 Treatments Exp_23

Trt_Nr	Treatment	Fertilization	BE
1	Ctrl_Low	Std	/
2	Px_Low	Std	7 DAS
3	Rz_Low	Std	7 DAS
4	Px2_Low	Std	at sowing
5	Ctrl_UFP	Std + UFP	/
6	Px_UFP	Std + UFP	7 DAS

Px and Rz treatments as soil surface treatment with 10^9 CFU kg^{-1} substrate either at sowing or 7 DAS; standard fertilization (Low) or with additional underfoot placement (UFP = 20 mg NH_4-N and 50 mg P kg^{-1} substrate); r = 5

3.11.5.2 Results Exp_23

In general, no significant differences among BE and Ctrl treatments were observed. Strong differences occurred between normally fertilized plants and plants fertilized with additional underfoot placement (UFP) of ammonium phosphate.

Dry weight (DW) of plants (Table 3-31) did not significantly differ among treatments, but a similar trend as for the other measurements was observed for the shoots. Here treatments with UFP showed higher dry weight, especially with additional Px treatment (see DW_shoot_H2). In contrast, root weight was decreased in the UFP treatments suggesting a growth stimulation of roots due to limited amounts of available nutrients in the non-UFP treatments. For the first harvest (H1) some tendency for growth stimulation as compared to the Ctrl was observed in the Px2 treatment but a growth reduction was observed in the Px treatment.

Table 3-31 Biomass results in g pot^{-1} Exp_23

Trt	DW_Shoot_H1		DW_Root_H1		DW_Shoot_H2		DW_Root_H2	
Ctrl_Low	0.77 ± 0.02		0.39 ± 0.02		0.93 ± 0.03		0.26 ± 0.01	
Px_Low	0.74 ± 0.04		0.38 ± 0.02		0.93 ± 0.02		0.26 ± 0.01	
Rz_Low	0.77 ± 0.02	ns	0.41 ± 0.01	ns	0.91 ± 0.02	ns	0.26 ± 0.02	ns
Px2_Low	0.80 ± 0.01		0.42 ± 0.00		0.92 ± 0.01		0.26 ± 0.01	
Ctrl_UFP	0.81 ± 0.01		0.38 ± 0.01		0.93 ± 0.03		0.23 ± 0.03	
Px_UFP	0.82 ± 0.01		0.39 ± 0.02		0.99 ± 0.05		0.24 ± 0.01	

Table showing means ± SE; H1 n=30; H2 n=29 (missing value); no significant differences found (ns)

Total P concentrations (Table 3-32) were increased in the UFP treatments with increasing differences in the second harvest (H2). Additionally, the lowest P values in H1 were found for the Px treatment. At H2 in the Ctrl treatment shoot P was decreased but root P increased whereas opposite results were found for the Px treatment.

Table 3-32 Total phosphorus concentrations in mg g^{-1} DW Exp_23

Trt	TP_Shoot_H1		TP_Root_H1		TP_Shoot_H2		TP_Root_H2	
Ctrl_Low	8.40 ± 0.05	abc	2.64 ± 0.06	ab	5.38 ± 0.14	b	2.30 ± 0.08	ab
Px_Low	8.14 ± 0.12	bc	2.48 ± 0.08	b	5.64 ± 0.10	b	2.18 ± 0.13	b
Rz_Low	8.18 ± 0.06	c	2.56 ± 0.02	ab	5.67 ± 0.14	b	2.29 ± 0.16	ab
Px2_Low	8.30 ± 0.10	abc	2.62 ± 0.04	ab	5.81 ± 0.13	b	2.27 ± 0.11	ab
Ctrl_UFP	9.42 ± 0.24	a	2.79 ± 0.06	a	7.96 ± 0.34	a	2.66 ± 0.12	ab
Px_UFP	9.07 ± 0.19	ab	2.74 ± 0.07	a	8.80 ± 0.23	a	2.71 ± 0.12	a

Table showing means ± SE; H2 n=29 (missing value); TP_Shoot_H1 one outlier reduced to achieve normal distribution and ANOVA on Ranks with proceeding Dunn's test for pairwise comparison; different letters indicate significant differences in means

Inorganic P (Pi) concentrations (Table 3-33) resembled the results for total P concentrations showing the much higher P values of the UFP fertilized plants but differences were more clearly pronounced, proving the high responsiveness of the Pi measurements. Nevertheless, for BE treatments no clear trends were observed. Only the lower Pi values in the shoots of the Px plants at H1 gives a slight support for hypothesis 2.

Table 3-33 Inorganic P concentration in mg g^{-1} DW Exp_23

Trt	Pi_Shoot_H1		Pi_Root_H1		Pi_Shoot_H2		Pi_Root_H2	
Ctrl_Low	6.34 ± 0.10	b	0.73 ± 0.05	b	3.97 ± 0.15	b	0.41 ± 0.02	b
Px_Low	5.97 ± 0.09	b	0.74 ± 0.08	b	4.13 ± 0.09	b	0.47 ± 0.02	b
Rz_Low	6.10 ± 0.11	b	0.73 ± 0.03	b	4.30 ± 0.19	b	0.52 ± 0.03	b
Px2_Low	6.30 ± 0.14	b	0.74 ± 0.01	b	4.36 ± 0.22	b	0.56 ± 0.05	b
Ctrl_UFP	7.85 ± 0.30	a	1.05 ± 0.04	a	7.64 ± 0.22	a	0.87 ± 0.05	a
Px_UFP	7.33 ± 0.23	a	0.90 ± 0.06	ab	8.13 ± 0.32	a	1.00 ± 0.07	a

Table showing means ± SE; H2 n=29 (missing value); Shoot_H1 and Root_H2 one outlier reduced, for Shoot_H2 two outlier reduced to achieve normal distribution; Data of Shoot_H1 were additionally square root transformed to achieve equal variance

Total P contents (Table 3-34) in shoots at H1 and H2 showed again similar trends as the other measurements. Root contents resembled the results from root weights. Due to the lower root weight higher P concentrations were equalized.

Table 3-34 Total P contents in mg pot^{-1} Exp_23

Trt	TPacc_S_H1				TPacc_R_H1				TPacc_S_H2				TPacc_R_H2			
Ctrl_Low	6.50	±	0.14	ab	1.02	±	0.04		5.01	±	0.08	c	0.61	±	0.04	
Px_Low	6.33	±	0.29	ab	0.94	±	0.05		5.24	±	0.16	c	0.58	±	0.05	
Rz_Low	6.28	±	0.14	b	1.04	±	0.02	ns	5.13	±	0.10	c	0.60	±	0.08	ns
Px2_Low	6.67	±	0.12	ab	1.10	±	0.02		5.34	±	0.20	c	0.60	±	0.05	
Ctrl_UFP	7.62	±	0.38	a	1.05	±	0.04		7.41	±	0.24	b	0.61	±	0.09	
Px_UFP	7.47	±	0.12	a	1.07	±	0.03		9.02	±	0.38	a	0.65	±	0.05	

Table showing means ± SE; H2 n=29 (missing value); Data of Shoot_H2 were additionally square root transformed to achieve equal variance; TPacc_S_H1 ANOVA on Ranks with Tukey's test

Differences in the Inorganic P (Pi) contents (Table 3-35) were, similar to the Pi concentrations, more clearly pronounced than those of the total P measurements but showed again similar trends. Like for the total P contents P status of the Ctrl plants changed in between H1 and H2. In the course of the three days BE treated plants were somehow able to improve their P acquisition as compared to the untreated Ctrl by unknown means because no differences in root weight were observed at H2.

Table 3-35 Inorganic P contents in mg pot^{-1} Exp_23

Trt	Piacc_S_H1				Piacc_R_H1				Piacc_S_H2				Piacc_R_H2			
Ctrl_Low	5.21	±	0.28	b	0.28	±	0.02	b	3.69	±	0.04	b	0.11	±	0.01	b
Px_Low	4.42	±	0.17	bc	0.28	±	0.03	b	3.83	±	0.11	b	0.13	±	0.01	b
Rz_Low	4.68	±	0.11	bc	0.29	±	0.01	b	3.89	±	0.15	b	0.14	±	0.02	ab
Px2_Low	5.06	±	0.14	bc	0.31	±	0.01	b	4.01	±	0.23	b	0.15	±	0.02	ab
Ctrl_UFP	6.35	±	0.33	a	0.39	±	0.02	a	6.70	±	0.35	a	0.20	±	0.02	ab
Px_UFP	6.04	±	0.16	ab	0.35	±	0.02	ab	7.16	±	0.38	a	0.21	±	0.03	a

Table showing means ± SE; H2 n=29 (missing value); Data of Shoot_H2 were additionally square root transformed to achieve equal variance

Similar to Exp_22, data are showing that P status is rapidly declining after the first 9 to 10 DAS, indicating the shift from seed based P nutrition to soil P acquisition combined with a rapid plant growth. The underfoot placement was efficient to stop this trend of a declined P status in the plant without further promoting of shoot growth.

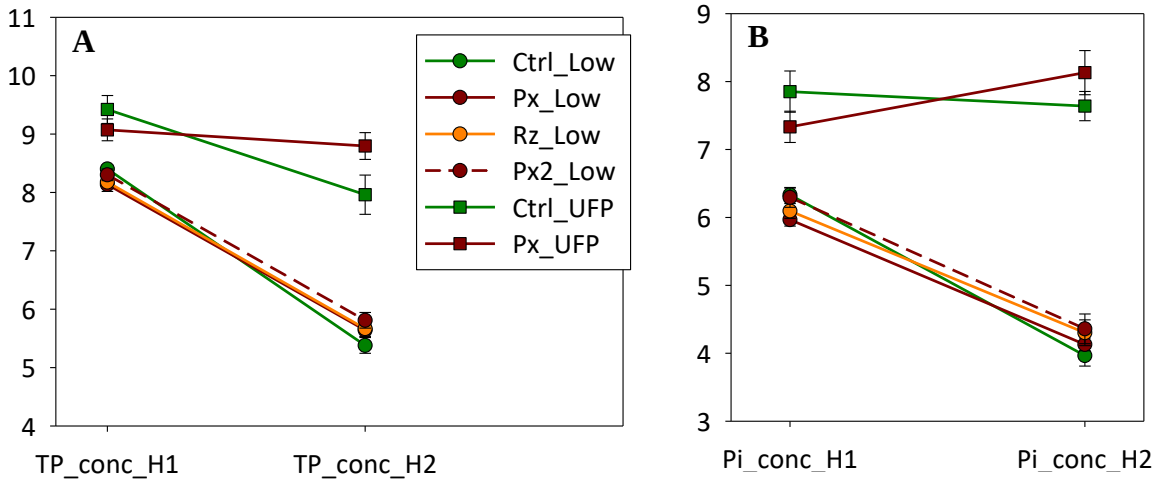


Figure 3-99 Total P and Pi in shoots Exp_23; Total phosphorus (**A**) and inorganic P (**B**) in maize shoots at both harvest time; means ± SE

Additionally, root growth was slightly suppressed by the UFP. Under field conditions these results might differ due to root growth to deeper layers for water acquisition. BE treatments did not significantly influence plant weight or P status. Nevertheless, Px treated plants (in both Px_low and the Px_UFP treatment) showed slightly reduced P values especially for shoot measurements at first harvest as compared to their respective controls, indicating that plants might suffer from stress or competition for nutrients in the very early days after application. At second harvest, places were changed and all BE treatments showed an improved P status as compared to their control (except TP status in the roots of low fertilization regime) (Figure 3-99).

3.11.5.3 Discussion Exp_23

Exp_23 was conducted directly after Exp_22 to find further support for our findings from Exp_11 and Exp_22. Results of both experiments indicated that the Px treatment may influence the P status. In Exp_22 Pi in shoots and roots 10 and 12 DAS were strongly decreased as compared to the untreated Ctrl. In Exp_11 gene expression results indicated a response to P-deficiency in the Px treatment. The conclusion that Pi concentrations in Exp_11 and Exp_22 seemed to differ only because of differences in the plant biomass could be drawn only after the analysis of Pi and Pt in the samples from Exp_11 which was performed after Exp_23.

The experimental design of Exp_23 was more complex including several control treatments and simulating field experimental conditions using underfoot placement of phosphorus. BE treatment was postponed in treatments 2 and 3 to test if the inoculation time will influence the plant responses. It was hypothesized that:

1. Growth stimulation by Px will occur in treatment 4 with early Px application (Px2_Low).
2. A competition for P without growth stimulation will occur in the days directly after application (treatment Px and Rz_Low).
3. An additional underfoot placement of P reduces P competition and the differences in P status between untreated and Px treated plants.
4. Rz will have less effect on plant performance or P status than Px.
5. Pi:Pt ratio is elevated in the Px treatment.

Plants were harvest only 10 and 13 DAS, the times that showed the strongest differences among treatments in Exp_22.

3.11.5.3.1 Underfoot placement in maize

Another objective for Exp_23 was the impact of the underfoot placement of ammonium phosphate on the youth development of maize and the interaction with the BE treatment. Placement of "starter fertilizer" is commonly done in conventional farming systems of maize, especially in cold climate (Grant et al., 2001). The efficacy of fertilizer placement seems to depend on the overall "background" availability of the respective nutrient in the soil (Cahill et al., 2008; Mallarino et al., 1999). Moreover the depth of the placement and the potential to increase nutrient availability, here NH_4^+-N nutrition in neutral or alkaline soils is of special interest, are important factors (Nkebiwe et al., 2016a). Underfoot placement was done in the field experiments and also in Exp_10. In contrast to most other experiments, in Exp_23 only weak BE effects were observed during early or later plant development. Exp_7, conducted with pre-cultured tomato plants, showed similar results. Transplantation of pre-cultured plants into soils with different fertilization rates did not affect plant growth anymore.

3.11.5.3.2 Plant stress reaction following BE application

No significant effects of growth stimulation by BEs could be observed during the experiment. Nevertheless, in the first harvest best plant growth was observed for the Px2 treatment. We hypothesized that (1.) earlier BE application will be more beneficial, and (2.) high dosage BE application in the Px treatment will cause a stress reaction in the plant. The Px treatment indeed had the lowest plant biomass of all treatments in the first harvest. Additionally, also the Pi and Pt concentrations in the Px plants were slightly decreased as compared to the untreated Ctrl. The same trend was seen for the Px_UFP treatment. Interestingly, the plants quickly recovered and in the second harvest trends were opposite. Both the Px and the Px_UFD treatment showed higher P (Pi and Pt) concentrations and contents in the shoots of the second harvest as compared to their respective controls. Only the total P concentration in the root was lower in the Px treatment but the total amount of P in both shoots and roots was higher than in the Ctrl. Also for the Rz treatment similar trends between the two harvest times were observed as also seen in Figure 3-99. Although differences were not significant (with the exception of the total P contents of the Px_UFP treatment in the second harvest as compared to the Ctrl_UFP treatment) and therefore the validity of the data can be questioned, the hypotheses 1 and 2 were somehow confirmed. Hypothesis 4 was not confirmed, the Rz treatment was not less effective than the Px treatment, but the best treatment was Px2 with the earlier BE treatment.

Also hypothesis 3 was not confirmed. In fact, differences between the Ctrl_UFP and the Px_UFP treatment were more pronounced than those between the Ctrl and Px treatment. One possible explanation is the root attraction by ammonium placement that may improve root colonization and BE activity (Nkebiwe et al., 2017, 2016b). The interaction of BEs with ammonium nutrition in general was already discussed for Exp_19. In the Px_UFP treatment about 150 mg P were added per pot, with good availability due to ammonium-derived acidification. The elevated P status of the UFP treatments clearly showed that the depot was reached by the roots. Therefore, P amount should not be a limiting factor. Still, P status of the Px_UFP plants was lower than in the Ctrl_UFP at the first harvest. Under P limiting conditions (e.g. in the C-Loess soil) plant growth depression by additional BE treatment was observed (Kuhlmann, 2014) and interpreted as competition for P but it is possible that the effects are more depending on the physiological status of the plant and the additional biotic stress that might be caused by the BE application.

3.11.5.3.3 BE application rates

The general weak effects by BE application might also be the result of the low application rates. In Exp_23 the application rate was reduced to 10^9 CFUs kg^{-1} substrate, as normally applied in field experiments. Especially for the Px treatment high inoculum rates seem to be crucial for successful BE effects as suggested by the results of Exp_10, Exp_19 and an experiment conducted by Nkebiwe 2015 (*unpublished*, see 4.1.4.2), but they are economically not feasible (4.3.7).

3.11.5.3.4 Underfoot placement changes root to shoot ratio

Interestingly, the underfoot placement was increasing shoot growth and P acquisition by the plant but it also had slightly inhibiting effects on root growth. Toxic effects or root growth inhibiting effects are often observed for fertilizer placement of ammonium leading to dense root growth around the depot avoiding direct contact with fertilizer (Nkebiwe et al., 2017, 2016b) but the concentrations of NH_4^+ in our experiment were probably to low to have any negative impact on plant growth (< 1 mg g^{-1} substrate). Therefore, it is more probable that the better nutrient supply was decreasing root to shoot ratio.

3.11.5.3.5 Pi:Pt ratio is elevated in the Px treatment.

The Pi:Pt ratio in the Px treatment was not elevated as observed in Exp_11. In Exp_23 Pi and Pt values were well correlated independent of the treatment and the harvest time. As discussed for Exp_11, probably the low temperatures were responsible for the observed effects of the Px treatment.

3.11.6 Negative influence of BEs on plant P status

As discussed in Exp_5 (0) PGPMs were probably competing for P in soils with high soil buffer capacity and strong P-deficiency. In Exp_11 and Exp_22 plants were studied during early development. Also here the decreased plant growth of Px treated plants at intermediate harvest and the transcriptomic analysis, resembling slight P-deficiency stress responses, indicated a competition effect for P. This was supported by the decreased P concentrations in shoots and roots of Px treated plants at several harvest times during the early plant development in Exp_22. P analyses of Exp_22 were performed first, because in Exp_11 samples were directly used for RNA analysis and metabolome studies and in Exp_22 biomass results were first not analysed in detail due to high standard deviations.

Nevertheless, this hypothesis on P competition in Exp_11 and Exp_22 was rejected. In Exp_11 biomass was reduced but P concentration was increased. Therefore, no differences in the P contents were observed suggesting that not a competition for P was happening. In contrast to Exp_11, in Exp_22 an increased biomass for Px treatments was leading to a "dilution" of the P concentrations but again no P competition was observed. Instead, the decrease in P concentration was following a stimulation of shoot and root growth by Px application. This early stimulation of plant growth, as often proposed by changing plant hormonal levels, was also observed in Exp_18 after Rz application.

The negative plant responses to Px application during early plant development (15 DAS) in Exp_11 are probably a result from the combination of low temperature and biotic stress due to PGPR application causing ISR similar responses. This is indicated by plant transcriptome analysis. Stress often leads to shifts in metabolism and a decrease in plant growth due to a trade-off between vegetative growth and an up-regulation of defence mechanisms such as the formation of ROS (3.14.10). Results from Exp_8 and 9 already indicated that Px is not able to improve plant tolerance to abiotic stress (Bradáčová et al., 2016). Additionally, BEs might compete for carbon. During the cold stress period root activity is reduced leading to reduced supply with assimilates. BE application might have further decreased assimilate availability in the root (trade-off hypothesis, 3.11.5.3.2, 4.1.6.3, 4.3.3).

3.12 RNA-Seq gene expression analysis of maize roots

3.12.1 Samples

RNA was extracted from maize roots of experiment Exp_11 as described in 2.7.1. As seen in Figure 3-93 the differences between the BE treatments at the time of the second harvest were most pronounced in the plants watered on 70 % WHC. Therefore, those treatments were used for RNA-Seq analysis. For each of the four pots per treatment mixed samples were obtained from three plants per pot. Because four pots per treatment were harvested two of the four mixed samples were combined after RNA extraction so that a total number of nine RNA samples, three replicates from three different treatments, were sent for whole transcriptome sequencing (2.7.4). As seen by the SE of the treatments in Figure 3-100, variation between samples of the same treatment was very high in Exp_11. To reduce variation in the RNA-Seq analysis those two mixed samples were selected for combination that differed most pronounced from each other in their root fresh weight (Table 3-36).

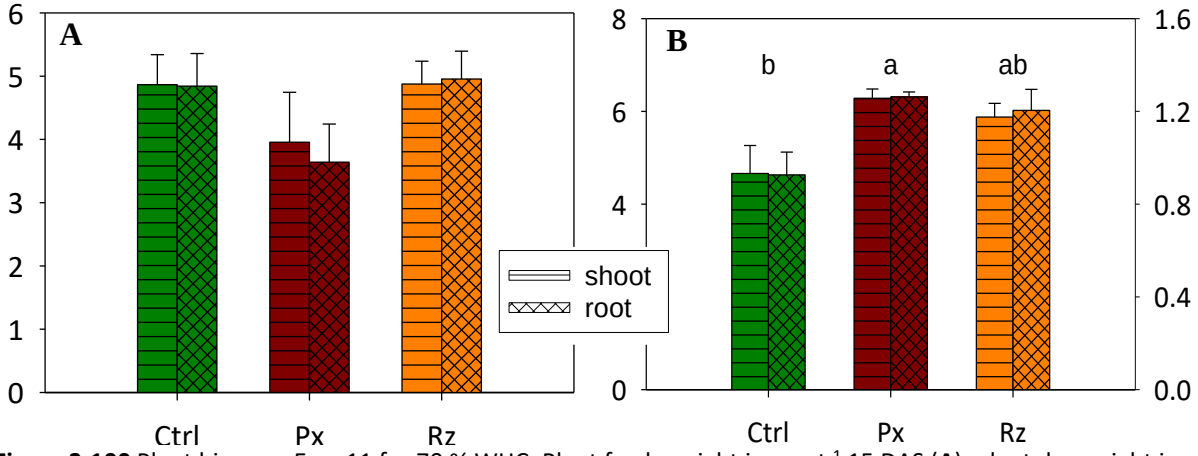


Figure 3-100 Plant biomass Exp_11 for 70 % WHC; Plant fresh weight in g pot^{-1} 15 DAS (**A**), plant dry weight in g 43 and 44 DAS (**B**); means + SE; n = 4 (**A**) / 5 (**B**); different letters indicate significant difference among treatments for both shoot and root

Table 3-36 Fresh weight data and RNA quality of Exp_11 samples

Trt	Pot	Rep.	Used	FW shoot	FW root	Conc.	Unit	260/280	260/230
Ctrl	S1	R1	C	5.64	5.77	150.0	ng/µl	2.17	2.42
Ctrl	S2	R2	C	4.97	4.38	373.2	ng/µl	2.17	2.08
Ctrl	S3	R3	S	5.35	5.61	298.6	ng/µl	2.17	2.35
Ctrl	S4	R4	S	3.50	3.61	230.6	ng/µl	2.17	2.29
Px	S5	R1	C	2.48	2.50	255.8	ng/µl	2.16	2.22
Px	S6	R2	S	3.72	3.60	550.9	ng/µl	2.15	2.35
Px	S7	R3	S	3.43	3.14	357.1	ng/µl	2.16	2.12
Px	S8	R4	C	6.19	5.32	207.7	ng/µl	2.17	2.09
Rz	S9	R1	S	5.88	5.64	204.4	ng/µl	2.15	2.10
Rz	S10	R2	/	4.83	4.59	263.0	ng/µl	2.16	2.24
Rz	S11	R3	S	4.61	5.71	223.0	ng/µl	2.16	2.20
Rz	S12	R4	S	4.18	3.88	348.3	ng/µl	2.17	2.07

Used: C = samples combined, S = send as single sample, S10 was not used at all due to low RNA quality in RNA gel; 260/280 ratio and 260/230 should be between 1.9 and 2.2. Best samples listed here after several extractions were performed to get the best RNA quality.

RNA quality was checked using the 260/280 or 260/230 ratios from spectrophotometrical measurements with the NanoDrop. Commonly, both ratios are considered to be optimal in a range between 2.0 - 2.2 whereas lower ratios indicate contaminations with proteins, carbohydrates or phenols from extraction.

To check for the RNA integrity the ratio of 28S and 18S RNA are determined either by capillary gel electrophoresis and comparison of the generated RNA Integrity Numbers (RIN) or visually by using RNA gel electrophoresis. In the gel electrophoresis the 28S and 18S RNA bands of the RNA sample S10 showed very low intensity and the smear between the bands indicated that RNA in this sample was already strongly degraded. As all RNA extractions from this root sample showed low RNA purity this pot was completely excluded from analysis. Therefore all samples from Rz treatment were single samples.

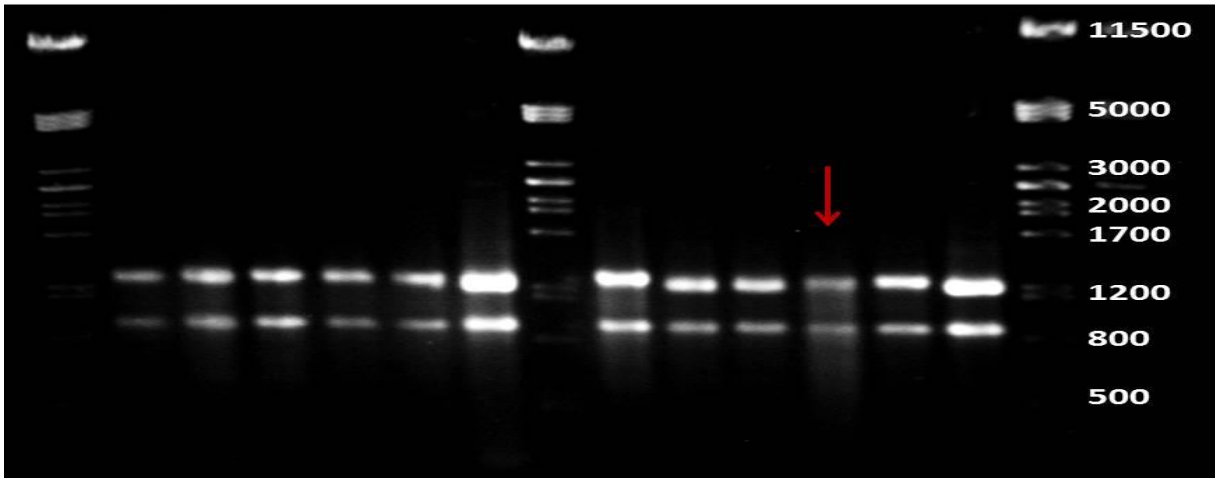


Figure 3-101 RNA gel for RNA-Seq samples; From left to right Lambda PstI Ladder samples S1- S6, ladder, S7-S12 and again the ladder; red arrow indicates S10; on the right side fragment length of the ladder in bp

Although bands were relatively short when compared to the Lamba PstI ladder, no intense smear or additional bands below 500 base pairs (bp) were observed that would indicate RNA degradation for any of the other samples.

Table 3-37 Fresh weight data and RNA quality of RNA-Seq samples

Trt	ID	Mix	Shoot (MW)	Root (MW)	Ratio	Conc.	Unit	260/280	260/230	Conc (BGI)
Ctrl	C_1	S1+S2	5.31	5.08	1:1:2	134.8	ng/µl	2.15	2.16	161
Ctrl	C_2	S3	5.35	5.61	1:1	157.8	ng/µl	2.14	2.33	178
Ctrl	C_3	S4	3.50	3.61	1:1	119.3	ng/µl	2.14	2.26	144
Px	Px_1	S5+S8	4.34	3.91	1:1:2	118.1	ng/µl	2.14	2.25	167
Px	Px_2	S6	3.72	3.60	1:1	299.5	ng/µl	2.16	2.37	411
Px	Px_3	S7	3.43	3.14	1:1	187.4	ng/µl	2.14	2.19	278
Rz	Rz_1	S9	5.88	5.64	1:1	107.5	ng/µl	2.16	2.21	160
Rz	Rz_2	S11	4.61	5.71	1:1	114.6	ng/µl	2.17	2.31	186
Rz	Rz_3	S12	4.18	3.88	1:1	188.0	ng/µl	2.16	2.18	202

Concentration of RNA samples send to BGI after mixing and/or diluting. Shoot and root FW recalculated for the mixed sample; ratio indicates the ratio of sample:RNAse-free water or sample:sample:RNAse-free water; at BGI concentrations were measured again with general higher values but same trends; grey-marked samples were picked for single sample comparison against C_3 in RNA-Seq analysis

Samples were combined and/or diluted with RNAse-free water and then send to the company BGI. At BGI the samples were again checked with capillary RNA gel electrophoresis and RIN numbers were fine for all samples.

3.12.2 RNA-Seq raw data

In all sample libraries more than 99 % of reads were defined as clean reads. All libraries contained about 56 million clean reads with genome mapping rates from 69 to 73 % and transcriptome mapping rates of 71 to 76 %. In each library about 34 – 35 thousand different expressed genes and 44 – 45 thousand expressed transcripts were mapped. In total 39634 different genes and 57796 isoforms (transcripts) were expressed in all libraries together. In each library about 2000 novel transcripts, 60 thousand alternative splicing events and 300 thousand SNPs (single nucleotide polymorphisms) were found. GC content was between 53 and 54 % in all libraries.

3.12.3 *Statistical analysis at BGI*

3.12.3.1 Whole transcriptome analysis BGI

Correlation analysis on whole transcriptome gene expression indicated a high correlation between all samples (ρ = 0.91 – 0.98) independent of the BE treatment (Figure 3-102).

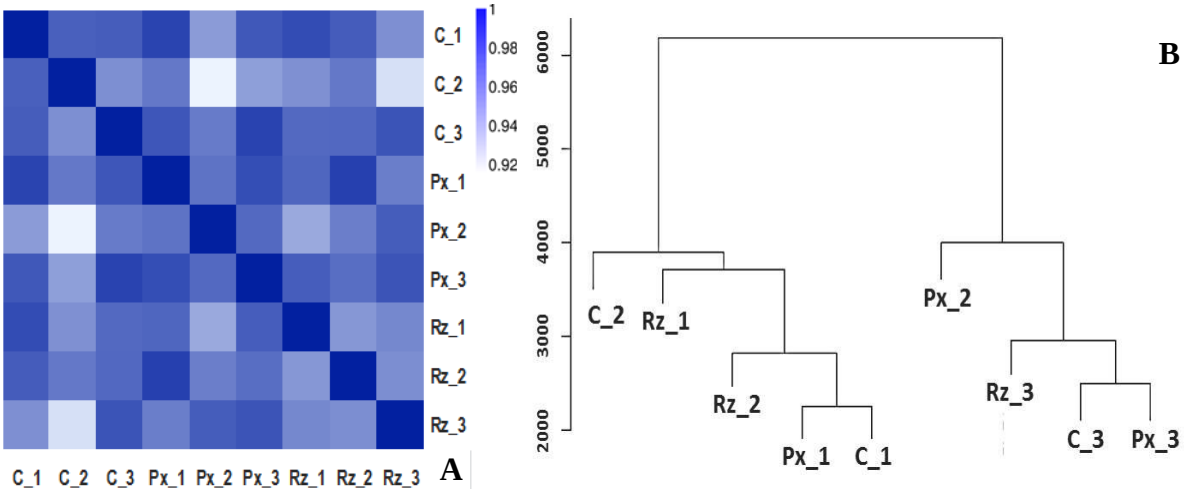


Figure 3-102 Whole transcriptome correlation analysis; Correlation matrix (**A**) and cluster dendrogram (**B**) for the transcriptome of the nine RNA-Seq libraries

No treatment-specific effects were observed on the whole transcriptome level. Especially the dendrogram gives valuable information on the factors that were influencing the gene expression most strongly. Here C_3, Px_3, Rz_3 and Px_2 are grouped together. They all share that the plants used for these samples had similar, relatively low root and shoot fresh weights. Samples C_2, Rz_1 and Rz_2 had the highest plant weights whereas Px_1 and C_1 are both mixed samples of two pots/biological replicates from the same treatment.

3.12.3.2 Differentially expressed genes

Using the NoiSeq method only 174 differentially expressed genes (DEGs) were found for the treatment comparisons (Figure 3-103). 37 DEGs are shared by both Px and Rz treatment. 117 DEGs are only found for comparison between Ctrl and Px and 15 DEGs are found only for the comparison Ctrl vs Rz. 5 DEGs were also found in the comparison between C_3 and C_1-3 (Figure 3-104).

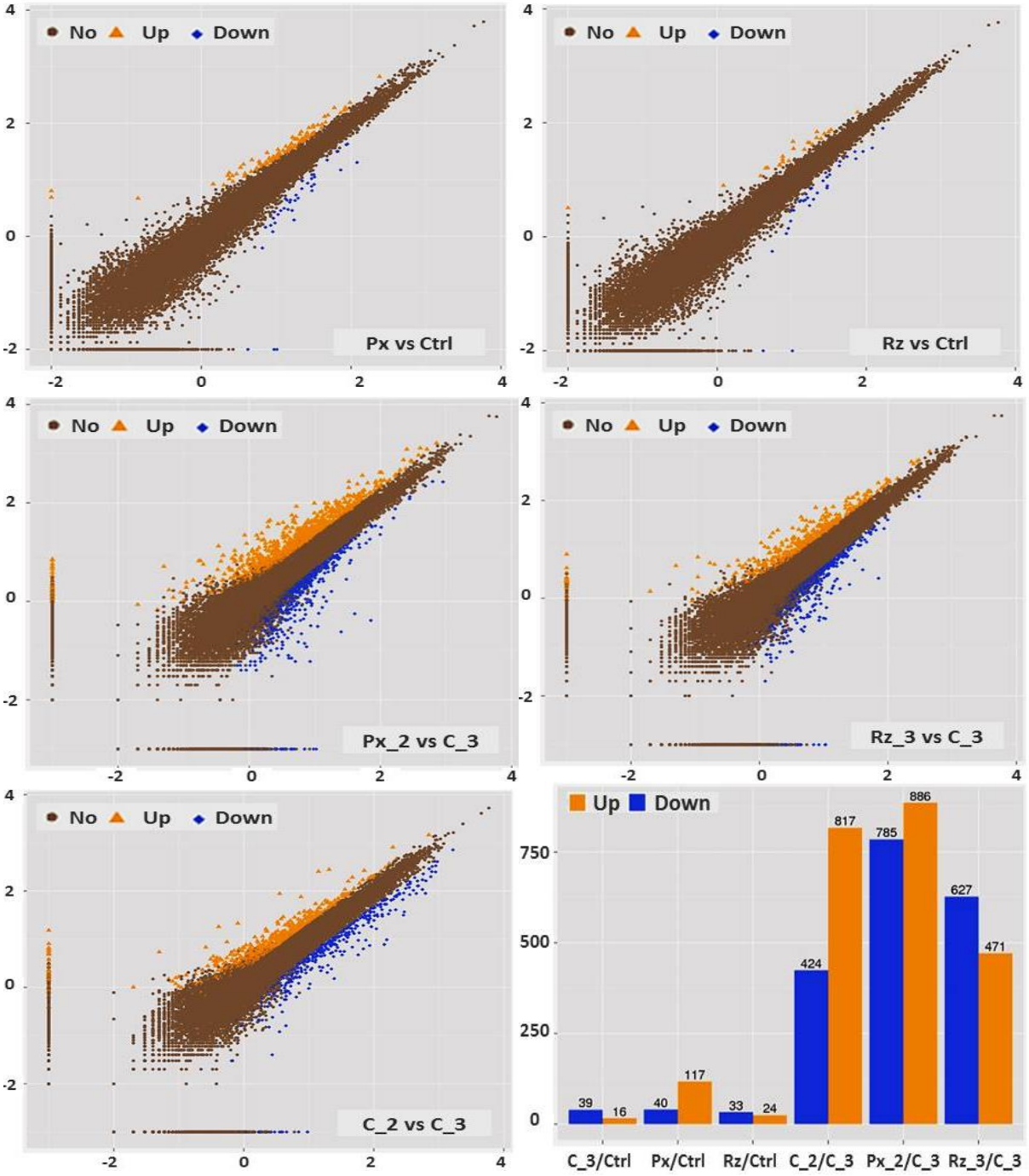


Figure 3-103 Distribution of DEGs from RNA-Seq analysis; Gene expression for all genes plotted (log10 of the FPKM values) in a 2D plot with the x-axis showing values in the Ctrl or C_3 samples and the y-axis values in the Px or Rz samples (**A** – **E**); Each gene is represented by a single dot. Yellow or blue coloured dots are DEGs in the respective comparison; Number of down- and up-regulated DEGs for all comparisons (**F**)

For single sample comparisons those Rz and Px samples were selected that differed at least in their root fresh weight from the Ctrl sample C_3 (Table 3-37). Additionally, C_3 was compared to another Ctrl sample (C_2) that differed strongly in root and shoot fresh weight to get more information on inter-sample variation in contrast to inter-treatment variation. Here a total number of 3025 DEGs was found including all single sample comparisons. Of these genes 194 were found in all comparisons (also C_2 vs C_3), 376 DEGs were found in both Rz and Px vs the C_3 sample, 152 DEGs were shared by the comparison of C_2 vs C_3 and the comparison of Px vs Ctrl (Px_2 vs C_3) and 69 were shared by the Rz (Rz_3 vs C_3) and the Ctrl comparison (C_2 vs C_3) (Figure 3-104).

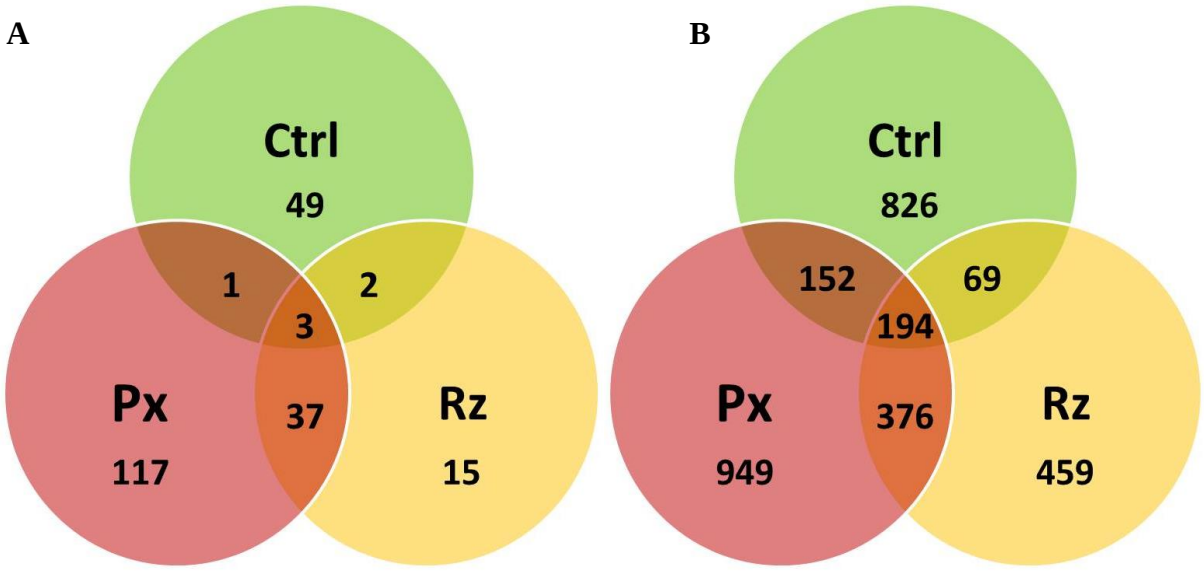


Figure 3-104 Venn diagrams showing shared DEGs; DEGs from treatment (Ctrl = C_3/C_1-3, Px = Px_1-3/C_1-3, Rz= Rz_1-3/C_1-3) (**A**) and single sample comparison (Ctrl = C_2/C_3, Px = Px_2/C_3, Rz = Rz_3/ C_3) (**B**)

As expected from the many shared DEGs in the Px and Rz treatment, results from gene ontology (GO) analysis at BGI were very similar for Px and Rz treatment. Functional groups that seemed significantly enriched in DEGs were connected to extracellular processes, cytoplasmatic processes, oxidoreductase activity and secondary, especially lignin and phenylpropanoid, metabolism. Interestingly in the comparison of C_3 vs C_1-3 none of the GO terms was enriched. Nevertheless, pathway enrichment analysis (KEGG) showed that several pathways that were highly enriched in DEGs for the Px and Rz treatment, such as primary and secondary metabolism, were also enriched in the comparison between C_2 and C_3, indicating that those pathways were not treatment specific.

Pathways that were only significantly enriched in the BE treatments were tyrosine and ascorbate metabolism, plant hormone signal transduction and plant-pathogen interaction.

3.12.4 New statistical analysis

3.12.4.1 New datasets

To support or verify the results from BGI analysis several new analyses were performed including PCAs, correlation analyses and a functional grouping using the MapMan tool on FPKM values and log2ratios of genes. In a first step some new datasets were created by applying several filters on the whole transcriptome dataset in Excel to select for different gene groups. As indicated by the correlation analysis and the dendrogram (from BGI) treatments had only minor effects on the majority of the expressed genes. Nevertheless, about 12000 genes had expression values (FPKM) lower than 1 and only 13930 genes had expression values > 10. Such low expression levels do not give reliable information on a gene expression but may rather show random fluctuations in the gene expression of those genes. Therefore data were filtered for treatment mean FPKM values > 10 (F1). Additionally, a "conservative" log2ratio dataset was created by filtering out all genes, in which two or three biological replicates of one treatment showed no expression (F2). A last dataset was created by selecting only those genes that either passed a simple t-test AND in which FPKM of all samples of one treatment where higher/up-regulated (or lower, down-regulated) as compared to all samples of the Ctrl treatment OR those genes that were indicated as DEGs (by NoiSeq method) (F3). The purpose of creating different datasets was simply to get a better impression on the reliability of the BGI and later on also MapMan results. A usage of the DEGs from NoiSeq in MapMan was not possible due to the low amounts of genes in this dataset. These genes were categorized manually using the annotations from various databases and publications.

3.12.4.2 PCA

A PCA analysis, done with R for different datasets of FPKM values, shows that the Px treatment could be separated from the Ctrl treatment by the PC1 but that the distance or variation inside the Px treatment was much higher than the distance between some of the single samples of the different treatments. A clustering of all biological replicates together, as commonly seen in PCAs, was also with other PCs not possible (see Figure 3-105 below).

3.12.4.3 Correlation analysis

A correlation analysis, as done at BGI, was repeated for the F3 dataset using SAS proc factor. Nevertheless, also after reduction of all genes with average FPKM values <10 no better correlation between single samples occurred (data not shown).

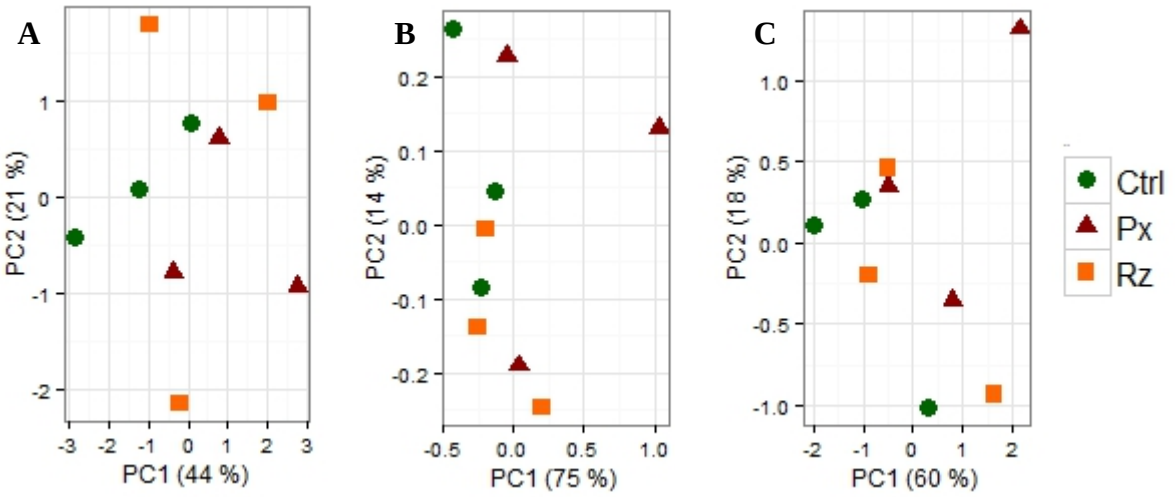


Figure 3-105 PCA scatterplot using different datasets from RNA-Seq analysis; Plotted variance for the first two PCs of each PCA, in brackets the proportion of variance explained by the respective PC. For PCAs FPKM values of all genes (n=39634) (**A**), DEGs from treatment comparison (n=174) (**B**) and DEGs from single sample comparison (n=3025) (**C**) were used as input. For Rz in none of the PCAs a separation from Ctrl samples was possible. In all PCAs Px samples could be separated from Ctrl with PC1 although differences between Ctrl and Px samples were sometimes smaller than the variation inside the treatment. In all plots Px_2 is the sample that most strongly diverges from the Ctrl samples.

3.12.5 MapMan/PageMan

3.12.5.1 Working principle

As seen from the BGI results, BE treatments had only minor effects on plant gene expression. It was hypothesized that effects were not strong enough to overcome the variation in between biological replicates so that only a low number of DEGs was found but that the effects by BE treatments, resulting in a significant higher biomass at the end of the harvest, may be visible when using functional grouping on the whole transcriptome level. In the bioinformatic tool MapMan log2ratios between Ctrl and BE treatments for the whole transcriptome are taken as input. Using the transcript IDs from the RNA-Seq analysis together with the given reference transcriptome and several online databases MapMan groups the genes into functional categories, called BINs and subBINs, and performs multiple significant tests to test if a certain functional category contains a significantly higher amount of down-regulated genes as compared to up-regulated genes and vice versa. Later on the term differentially regulated BIN (DRB) is used. In contrast to the enrichment analyses at BGI, here the single gene is not of interest and is also not checked automatically for significant down- or up-regulation. By this method, minor trends in many genes can be detected and used to indicate significant shifts in functional groups or metabolic processes.

3.12.5.2 Mapping files

MapMan provides the user with mapping files for different plant species as well as different gene or transcript IDs. Nevertheless, for maize all mapping files available for download are using gene referencing (e.g. affymetrix microarray chip IDs, Gramene transcript IDs (www.maizegdb.org)) that did not fit to the gene or transcript IDs provided by BGI (NCBI gene or transcript IDs / accession). Therefore, two possible ways were tested to make a usage of MapMan possible for the BGI derived data. First, gene IDs and transcript IDs were converted into Gramene gene IDs (often with the GRMZM sequence at the beginning). The converted IDs were compiled because conversion of transcript IDs differed in some instances from gene IDs. Furthermore, references from different databases are not always one-to-one convertible and therefore only 17055 of a total of 39635 genes could be referenced with new IDs. This resulted in 17111 mappings to BINs using the Zm_B73_5b_FGS_cds_2012 mapping file from 2012 with the latest version of Gramene ID reference.

In a second approach the Mercator tool was used to create a mapping file based on the NCBI transcript IDs. The new "NCBI" mapping file contained a total of 78577 genes. But of these genes only 43425 were assigned to a BIN with function whereas the other 35152 gene IDs were part of a big "waste" BIN for genes of unknown function or annotation. Using the new NCBI mapping file ~ 15000 genes from the RNA-Seq analysis could therefore not be grouped into BINs. In comparison to the new mapping file the Zm_B73_5b_FGS_cds_2012 mapping file contained a total of 64406 genes, but only 25797 that could not be assigned. By using the BioMart converted Gramene IDs both mapping files could be combined and ~1800 additional genes from RNA-Seq data could therefore be assigned to MapMan BINs with known function. By using the MapMan mapping files it was also possible to annotate all BGI mapped genes additionally with TAIR, TIGR and InterPro database information.

3.12.5.3 Treatment comparison (with biological replicates)

First, MapMan was used to get a visual overview on the different datasets. For this several graphical sheets with metabolic pathways or cell functions are provided by MapMan in which single gene expression is visualized by colours for down- and up-regulation. The intensity of the colour reflects thereby the magnitude of down- or upregulation, here the absolute value of the log2ratio. Also in MapMan Px and Rz showed very similar gene expression responses (Figure 3-106 A and B). They affected similar functional groups, connected to e.g. light reaction, but also primary and secondary metabolism. Figure 3-106 also shows a comparison of the different datasets for the same BINs. Although results from datasets differed they

showed some homology and common trends for several functional groups. This was supported by PageMan and statistical analysis described below.

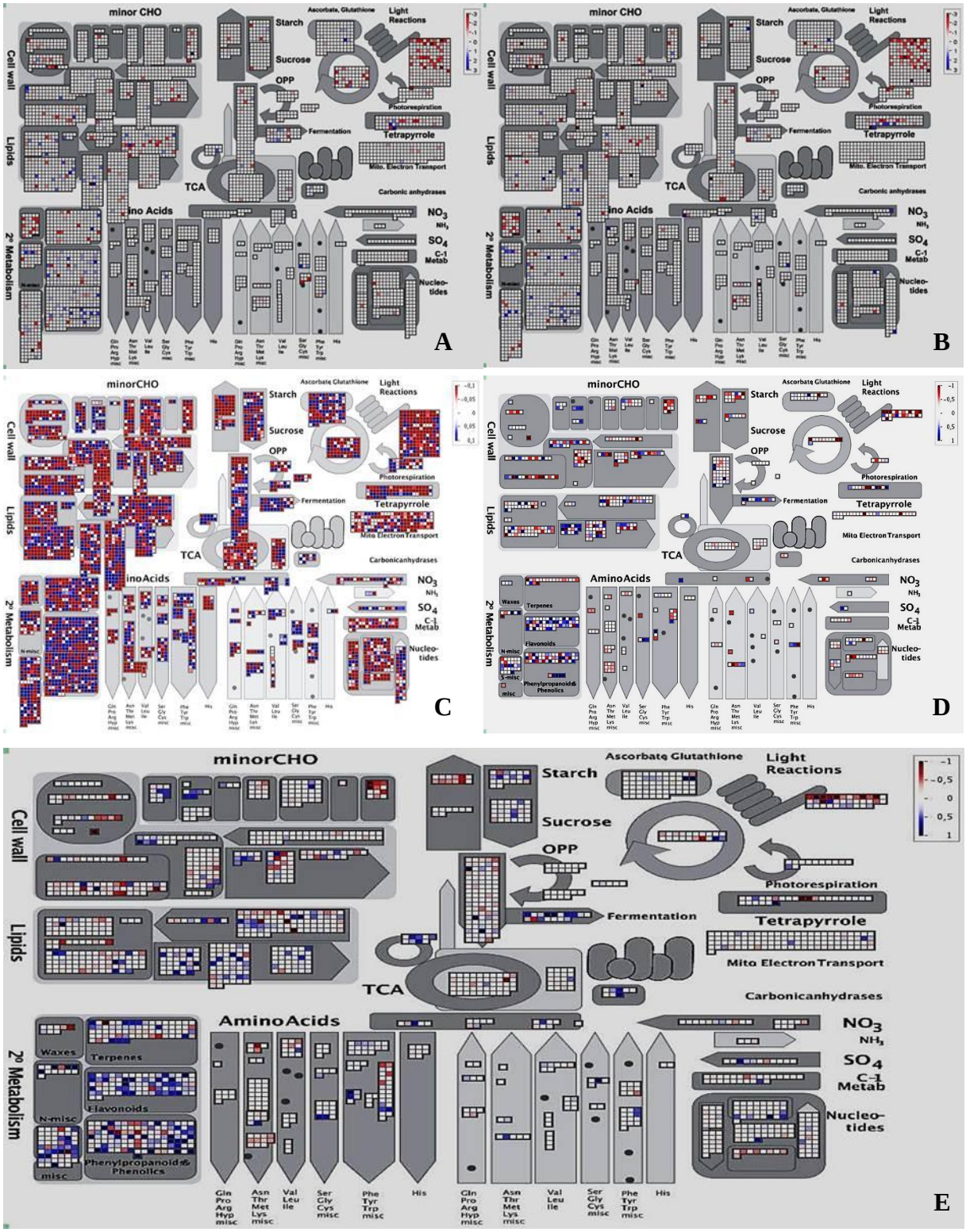


Figure 3-106 MapMan metabolism overview for various datasets; Overview of the major plant metabolism BINs for the Px/Ctrl (left) and Rz/Ctrl, scaling from log2ratio -3 to 3 (F0 dataset, **A**+**B**); **red** indicates down-regulation, **blue** up-regulation by BE; scaling from log2ratio -0.1 to 0.1 (**C**) simulating the PageMan analysis (here only + and – are of importance); F3 (**D**) and F1 (**E**) dataset with scaling -1 to 1

3.12.5.4 PageMan

PageMan is an additional option for visual analysis of the gene expression data in MapMan. Similar to the visualizing of the log2ratios of single genes in the MapMan BIN views, all DRBs are plotted with their p-values from significance tests after compression to z-scores, again using colour code. Figure 3-109 shows an example for a PageMan output that shows the BINs or subBINs that were significantly regulated (DRBs) ($p < 0.05$) as compared to the Ctrl sample. Here the results for the comparison Px_2/C_3 are shown. Z-scores (also termed standard scores) can be transformed to p-values whereas low p-values are equal to high z-scores (Table 3-38).

Table 3-38 Z-scores

p-value	z-score
0.05	1.96
0.01	2.57
0.001	3.28

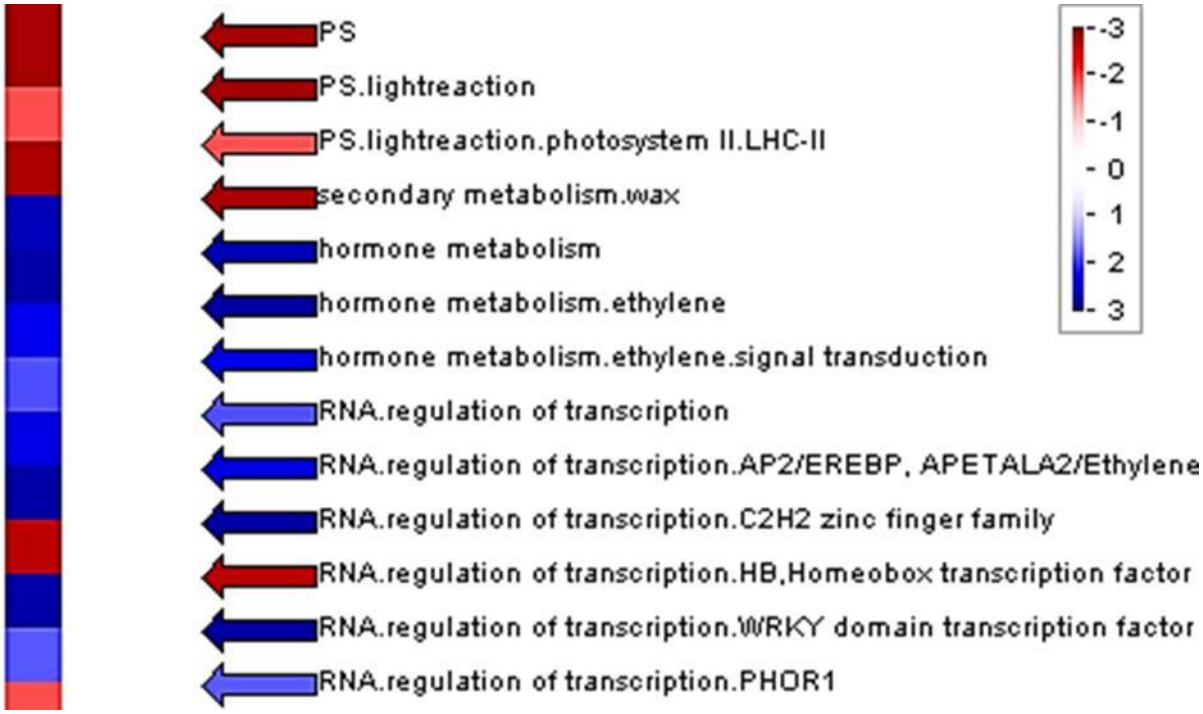


Figure 3-107 PageMan output for single sample comparison; Log2ratios of all DEGs derived from Px_2 vs C_3 single sample comparison (see 3.12.3.2 or below); **red** indicates down-regulation, **blue** up-regulation by BE treatment; scaling with z-scores, high z-scores mean low p-values in significance tests for each BIN

When using the Wilcoxon test in the PageMan options the output simply visualizes the statistical output from MapMan. Nevertheless, in the MapMan output only p-values but no information on down- or up-regulation are given. Together with PageMan and FDR (see below) adjustment of p-values in the MapMan output the p-values and "direction" of regulation for hundreds of BIN from various datasets were analysed. Additionally, different FDR methods were used and datasets from single sample comparison as well as the filtered whole transcriptome datasets were compared.

3.12.5.5 Correlation analysis in MapMan

In a second approach the Pearson product correlation coefficients for each gene of the whole transcriptome (ρ ranging from -1 to 1) were calculated using FPKM values of the respective gene in the single biological replicates as one variable and root weight as the second variable. Then the correlation coefficients were used as an input dataset for MapMan. Using all the 39364 genes no DRBs were found. Nevertheless, after using the F3 filter, reducing all genes with FPKM <10 several DRBs were found. For this dataset down-regulated DRBs indicate that most of the genes in this functional BIN were negatively correlated to root weight, whereas an up-regulation indicates a positive correlation to root weight. A comparison with the F3 dataset using the log2ratios of Px and Rz showed that ~50 % of the DRBs were shared. All of these shared DRBs were regulated opposite to the way they were regulated in the log2ratio datasets, indicating that up-regulation by Px (or Rz) treatment in these DRBs coincided with negative correlation to root weight. Together with the previous observations (e.g. the dendrogram, low amounts of DEGs) it supports again that the majority of gene expression responses observed were a secondary responses due to differences in plant biomass. A direct correlation of the single gene correlation coefficients (used in MapMan) with the log2ratios of the Px/Ctrl and Rz/Ctrl comparisons for both datasets (F0 and F3) shows a very high correlation for Px (ρ = -0.50 (F0) and -0.65 (F3)) but a much lower correlation for Rz (ρ = -0.17 (F0) and -0.40 (F3)). Nevertheless, all correlations were highly significant.

3.12.5.6 MapMan / PageMan results

3.12.5.6.1 Treatment comparison

To check for DRBs MapMan uses Benjamini-Hochberg (FDR) corrected Wilcoxon Rank sum tests. 108 significantly DRBs were found in both treatments, additionally 47 only for the Px treatment and 25 only in the Rz treatment. Table 3-39 gives an overview on the DRBs found for different datasets after filtering the transcriptome.

Table 3-39 Overview on the DRB statistics for different datasets

Comparison	Datasets	Genes	BINs	Total DRBs			Regulation DRBs			
				Both	Px	Rz	Px		Rz	
							down	up	down	up
BE/Ctrl	F0: log2ratio	35436	1443	108	47	25	82	73	78	55
BE/Ctrl	F1: FPKM>10	13932	1321	124	42	39	82	84	77	86
BE/Ctrl	F2: log2(conserv)	33386	1443	113	58	34	98	75	80	67
BE/Ctrl	F3: t-test	7369	496	29	55	41	55	30	49	21
Px/Rz	F0: log2ratio	35436	1443	19			8	11	11	8
Px/Rz	F1: FPKM>10	13932	1321	27			11	16	16	11

Results showed that many DRBs were probably false positive due to biases like different plant weight, low expression rates or non-significance in single gene expression. By adding filters like selection for higher FPKM values (F1) or t-tests for treatment comparison of single gene expression also the DRBs changed. This was expected from the correlation analyses and the dendrogram. Nevertheless, the similarity of the Px and Rz treatment was also seen in the statistical analysis using MapMan and PageMan. This is strongly supported by the fact that in all analyses performed for the BE/Ctrl comparison no single DRB was contra-regulated between the Px and Rz treatment and that the amount of DRBs in the direct comparison of the two BE treatments was much lower than in the comparison to the Ctrl.

Additionally, several DRBs from the BE/Ctrl treatment comparisons were relatively stable for all datasets. An up-regulation of genes related to ethylene, salicylic and jasmonic acid metabolism, various groups of transcription factors (TF) such as ethylene related (EREBP), WRKY, MYB and NAC TFs, secondary metabolism, especially from phenylpropanoid, terpenoid, lignin and chalcone metabolism pathways were observed.

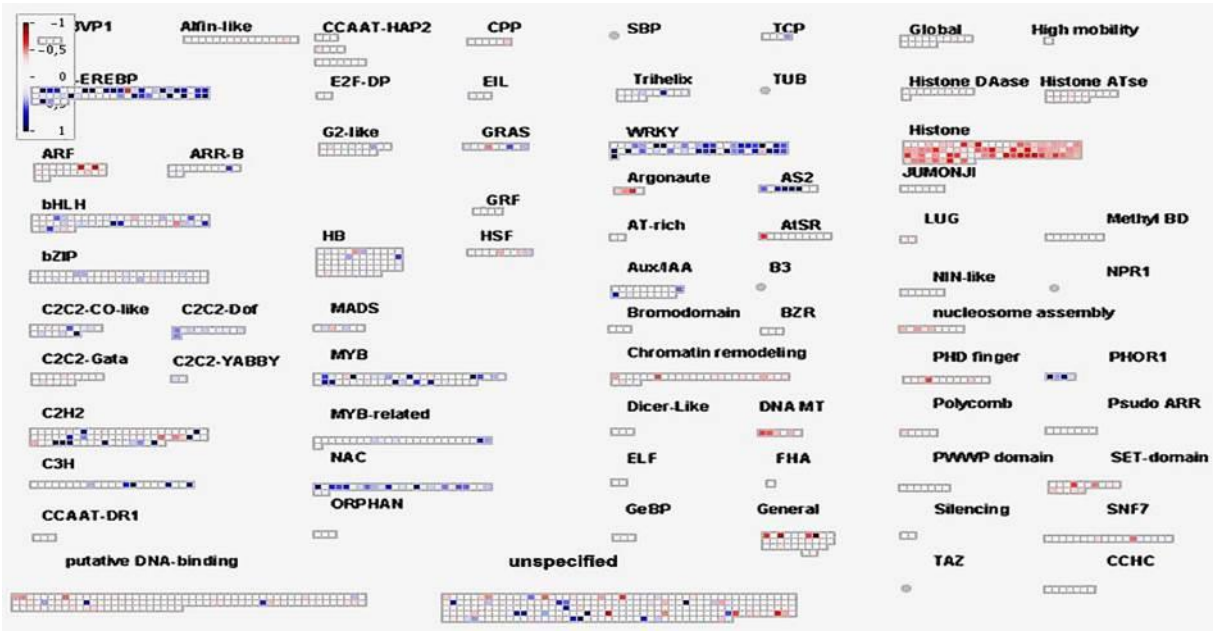


Figure 3-108 MapMan BIN view for F1 dataset; Overview on genes related to transcription factors for the Px/Ctrl dataset for all genes with FPKM>10; **red** = down-regulation, **blue** = up-regulation by BE; scaling -1 to 1

3.12.5.6.2 Single sample comparison

For single sample comparison the DEGs defined by BGI were used for MapMan and PageMan analysis. As expected from the lower number of DEGs only a few DRBs were found. For the C_2 vs C_3 comparison several subBINs connected to photosynthesis were up-regulated (enriched in up-regulated genes). Additionally, the BINs including all genes connected to RNA metabolism or RNA regulation were both weakly up-regulated. Genes

connected to gluconeogenesis as well as various subBINs for secondary metabolism (biosynthesis of flavonoids, phenylpropanoids, glucosinolates), additionally glucosyl transferases, GUS transferases and cytochrome P450 were down-regulated. It was also seen before that those BINs were well correlated to the plant biomass production. For the comparison Rz_3 vs C_3 the major BINs for secondary metabolism and hormone metabolism (also subBIN for ethylene), cytochrome P450 and the BIN for the C2H2 zinc finger TFs were up-regulated.

Table 3-40 Overview on the DRB statistics for the SS DEGs

Comparison	Datasets	Genes	BINs	Total DRBs	Regulation	
					down	up
C_2/C_3	SSDEGs (BGI)	1241	480	23	14	9
Px_2/C_3	SSDEGs (BGI)	1671	496	18	9	9
Rz_3/C_3	SSDEGs (BGI)	1098	438	6	1	5

For the Px_2 vs C_3 comparison the DRBs are listed in Table 3-41. The table gives a good overview on the different groups that were seen to be differentially regulated in the Px treatment in many datasets (highlighted in bold).

Table 3-41 MapMan output for DRBs from the Px_2/C_3 comparison

BIN	Genes	P-value	Up/Down
PS	15	8.0E-04	down
PS.lightreaction	13	0.00102	down
PS.lightreaction.photosystem II.LHC-II	5	0.04376	down
secondary metabolism.wax	7	0.00313	down
RNA.regulation of transcription.HB (Homeobox)	12	0.00418	down
DNA	36	0.04376	down
signalling.receptor kinases	50	0.05000	down
signalling.receptor kinases.leucine rich repeat XIII	5	0.03420	down
signalling.light	10	0.01137	down
hormone metabolism	**73**	**0.00418**	up
hormone metabolism.ethylene	**22**	**0.00170**	up
hormone metabolism.ethylene.signal transduction	**13**	**0.01137**	up
RNA.regulation of transcription	**232**	**0.04376**	up
RNA.regulation of transcription.AP2/EREBP	**30**	**0.00922**	up
RNA.regulation of transcription.C2H2 zinc finger	**14**	**8.0E-04**	up
RNA.regulation of transcription.WRKY	**23**	**0.00170**	up
RNA.regulation of transcription.PHOR1	6	0.05000	up
protein.degradation	72	0.05000	up

SubBINs are indicated by a "." in the BIN name; Genes are the number of genes from the input dataset that could be sorted into the respective BIN; p-value after FDR correction; RNA regulation is separated in subBINs for the different transcription factor families defined by their functional domains; BINs highlighted in bold are most representative for the Px treatment effects

Especially genes connected to ethylene, its biosynthesis as well as the ethylene responsive transcription factors (ERFs, EREBPs) were strongly up-regulated in the Px treatment.

Nevertheless, as also seen for the NoiSeq DEGs of the treatment comparison (see 3.12.3.2) biological replicates differed strongly and not all of the differences could be correlated to plant performance and plant growth. Using whole transcriptome (F0) and F1 datasets (3.12.4.1) for the comparison of the Px_2/C_3 and the Px_3/C_3 strong differences in the gene expression responses were observed, although both plant samples showed similar average biomass per pot. Out of a total of 206 DRBs that were either significant for the Px_2/C_3 or the P_3/C_3 comparison only 5 DRBs were shared by both comparisons.

3.12.6 Major functional pathways

3.12.6.1 BE treatment effects

For selection of most significant or interesting functional groups those MapMan DRBs were used that were most abundant in various datasets, had low p-values and were significant for both treatments. BINs that were correlated with root weight were not excluded if they were highly significant in both treatments suggesting that there were additional BE treatment influences that could not simply be explained by secondary effects. Lists of the most significant up- and down-regulated BINs are given in Table 3-42 and Table 3-43.

Major BINs including several subBINs that were up-regulated in both BE treatments were hormone metabolism - especially ethylene, cytokinin and salicylic acid -, ubiquitin-mediated protein degradation, several transcription factor families (AP2/EREBP, NAC, WRKY), secondary metabolism of flavonoids and phenylpropanoids, some receptor kinases and biotic stress related genes.

Down-regulation was especially seen for cell wall synthesis (also cell wall proteins), cell division and cell organisation, DNA synthesis, callose metabolism, protein synthesis, some photosynthesis-related BINs and RNA processing. Especially for DNA, protein synthesis and RNA processing extremely low p-values were found for the F1 dataset indicating down-regulation of most of the genes in these large BINs. The low p-values were also found for most other datasets.

3.12.6.2 Differences in BE treatments

A summary of the BINs that differed most significant among the two BE treatments is given in Table 3-44. BINs of several transcription factors (bzip, MYB domain, C2C2 DOF zinc, PHOR1) were not differentially regulated in the Rz treatment but were indicated as DRBs in the Px/Ctrl comparison. This regulation seemed also to be correlated with the root weight. All other BINs were indicated as DRBs in the Px/Rz comparison. Most of those BINs were DRBs for both BE treatments but they differed in their significance. For example WRKY and ethylene responsive TFs (AP2/EREBP) were much stronger up-regulated in the Px treatment than in the Rz treatment. Nevertheless, as indicated by the low p-values for the Corr dataset, those BINs were also correlated to root weight and the difference between the both BE treatment could be caused by secondary effects and not a direct stimulation by the Px treatment. Especially pronounced in the Rz treatment was the up-regulation of ubiquitin-mediated protein degradation and the down-regulation of protein synthesis as well as transport (here one subBIN for ammonium transport was weakly up-regulated).

Table 3-42 Up-regulated DRBs

BIN	Genes	Corr	Px	Rz
development.storage proteins	14	7.4E-01	4.1E-02	3.1E-02
gluconeogenesis / glyoxylate cycle	9	4.2E-02	4.2E-02	7.4E-03
hormone metabolism	356	2.7E-03	1.4E-09	3.4E-08
hormone metabolism.auxin.synthesis-degradation	8	1.1E-02	1.9E-02	3.8E-03
hormone metabolism.cytokinin	23	3.5E-03	2.2E-03	1.1E-05
hormone metabolism.cytokinin.synthesis-degradation	17	1.7E-04	1.6E-05	1.0E-05
hormone metabolism.ethylene	71	1.1E-03	1.5E-10	1.8E-03
hormone metabolism.ethylene.induced-regulated-responsive-activated	23	4.6E-01	2.7E-02	1.0E-02
hormone metabolism.salicylic acid	8	5.5E-03	5.2E-04	1.4E-04
hormone metabolism.salicylic acid.synthesis-degradation	8	5.5E-03	5.2E-04	1.4E-04
hormone metabolism.salicylic acid.synthesis-degradation.synthesis	7	1.5E-02	1.9E-03	5.0E-04
hormone metabolism.salicylic acid.synthesis-degradation.synthesis.SA glucosyltransferase ester and ether bond making SGE, SAG	5	4.2E-02	8.3E-03	6.8E-03
hormone metabolism.salicylic acid.synthesis-degradation.synthesis.SA glucosyltransferase ether bond making SAG	4	1.0E-01	2.6E-02	2.3E-02
misc	767	7.9E-06	5.5E-03	8.4E-05
misc.cytochrome P450	79	4.6E-07	9.9E-07	2.3E-05
misc.UDP glucosyl and glucoronyl transferases	104	4.8E-05	9.7E-06	1.6E-05
protein.degradation	1046	3.9E-02	4.6E-04	1.4E-07
protein.degradation.ubiquitin	695	1.2E-01	9.1E-06	1.9E-12
protein.degradation.ubiquitin.E3	435	5.7E-01	1.5E-07	5.4E-13
protein.degradation.ubiquitin.E3.RING	279	4.5E-01	9.7E-06	9.8E-09
protein.degradation.ubiquitin.E3.SCF	127	7.8E-01	8.8E-03	7.0E-05
protein.degradation.ubiquitin.E3.SCF.FBOX	110	7.0E-01	5.9E-03	6.9E-05
RNA.regulation of transcription	1270	5.6E-01	2.7E-07	4.1E-03
RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	55	4.1E-06	1.7E-12	2.6E-02
RNA.regulation of transcription.NAC domain transcription factor family	27	4.8E-05	3.1E-08	2.7E-06
RNA.regulation of transcription.WRKY domain transcription factor family	51	1.6E-07	0.0E+00	6.0E-06
secondary metabolism	313	2.9E-16	1.2E-12	0.0E+00
secondary metabolism.flavonoids	70	4.9E-07	2.0E-11	6.8E-13
secondary metabolism.flavonoids.dihydroflavonols	34	1.1E-02	4.0E-05	2.3E-05
secondary metabolism.flavonoids.flavonols	17	2.3E-03	2.5E-04	4.6E-05
secondary metabolism.phenylpropanoids	108	5.4E-06	1.1E-04	4.0E-09
secondary metabolism.phenylpropanoids.lignin biosynthesis	64	2.0E-06	4.7E-06	6.1E-10
secondary metabolism.phenylpropanoids.lignin biosynthesis.PAL	8	7.9E-02	3.6E-02	3.5E-03
signalling.receptor kinases.legume-lectin	11	1.6E-01	1.6E-03	1.6E-03
signalling.receptor kinases.thaumatin like	8	7.0E-03	7.5E-03	2.0E-02
signalling.receptor kinases.wheat LRK10 like	11	2.1E-03	9.5E-05	3.1E-03
stress	548	2.9E-01	2.5E-04	1.2E-06
stress.biotic	194	1.5E-01	3.5E-06	1.4E-09
transport.amino acids	58	1.5E-01	3.8E-03	1.0E-02

Selection of the most significantly up-regulated BINs for treatment comparison; P-values shown are from the MapMan output after FDR correction for the F1 dataset; “Corr” shows the p-value for the correlation coefficients used as input for MapMan as described in 3.12.5.5

Table 3-43 Down-regulated DRBs

BIN	Genes	Corr	Px	Rz
cell	629	3.9E-03	2.1E-12	2.3E-09
cell wall	256	1.5E-02	3.3E-04	2.6E-02
cell wall.cell wall proteins.AGPs.AGP	13	1.6E-02	5.1E-03	1.0E-02
cell.cycle	80	1.7E-01	1.5E-02	1.7E-03
cell.division	64	2.8E-01	1.9E-03	7.3E-05
cell.organisation	294	6.9E-07	2.8E-09	1.6E-05
cell.organisation.cytoskeleton	98	7.8E-06	5.9E-11	3.2E-06
cell.organisation.cytoskeleton.mikrotubuli	52	2.7E-07	1.4E-08	9.5E-05
cell.organisation.cytoskeleton.mikrotubuli.MAP65	7	2.6E-01	3.7E-03	3.9E-02
DNA	304	0.0E+00	1.2E-36	2.1E-26
DNA.synthesis/chromatin structure	190	0.0E+00	3.0E-42	5.0E-32
DNA.synthesis/chromatin structure.histone	71	3.8E-12	8.8E-26	6.9E-25
DNA.synthesis/chromatin structure.histone.core	63	2.1E-11	4.0E-25	2.5E-24
DNA.synthesis/chromatin structure.histone.core.H2A	32	4.2E-06	1.2E-12	1.7E-11
DNA.synthesis/chromatin structure.histone.core.H2B	14	5.5E-03	1.5E-07	6.1E-06
DNA.synthesis/chromatin structure.histone.core.H3	17	3.6E-02	3.7E-05	7.4E-06
DNA.synthesis/chromatin structure.histone.core.H4	14	3.8E-04	1.3E-07	5.1E-07
major CHO metabolism.synthesis.starch.AGPase	4	1.6E-01	2.6E-02	2.6E-02
minor CHO metabolism.callose	12	3.6E-01	8.8E-04	1.2E-04
misc.gluco-, galacto- and mannosidases	52	4.0E-02	2.4E-06	1.8E-03
nucleotide metabolism.deoxynucleotide metabolism	9	2.4E-01	3.8E-03	1.9E-04
protein	2656	2.6E-02	2.8E-10	6.1E-12
protein.aa activation	61	4.1E-02	9.4E-04	2.6E-04
protein.synthesis	606	2.8E-09	5.0E-42	3.5E-80
protein.synthesis.ribosomal protein	396	1.4E-06	5.4E-29	9.7E-68
protein.synthesis.ribosomal protein.eukaryotic	317	4.8E-05	4.9E-25	1.6E-61
protein.synthesis.ribosomal protein.eukaryotic.40S subunit	124	5.4E-02	4.3E-10	7.8E-27
protein.synthesis.ribosomal protein.eukaryotic.60S subunit	193	1.9E-03	1.9E-14	9.6E-34
protein.synthesis.ribosomal protein.prokaryotic	75	1.3E-02	6.4E-05	7.8E-08
protein.synthesis.ribosomal protein.prokaryotic.chloroplast	34	4.3E-02	4.9E-04	7.9E-06
protein.synthesis.ribosomal protein.prokaryotic.chloroplast.50S su	29	1.7E-01	1.1E-02	3.3E-04
protein.synthesis.ribosome biogenesis	78	3.8E-04	8.6E-09	1.5E-12
protein.synthesis.ribosome biogenesis.Pre-rRNA processing and modifications	54	1.9E-03	1.0E-08	4.4E-12
protein.synthesis.ribosome biogenesis.Pre-rRNA processing and modifications.snoRNPs	20	2.1E-02	4.4E-04	7.7E-06
protein.targeting	297	2.2E-01	6.8E-05	4.0E-03
protein.targeting.nucleus	44	9.7E-03	6.9E-05	5.9E-05
PS.lightreaction.photosystem II	11	6.5E-03	1.6E-06	1.6E-04
PS.lightreaction.photosystem II.LHC-II	5	1.6E-01	2.1E-03	2.1E-03
RNA.processing	258	1.4E-09	5.5E-14	3.5E-21
RNA.processing.RNA helicase	35	1.4E-02	1.7E-03	1.6E-04
RNA.processing.splicing	75	1.8E-04	4.6E-04	4.8E-07
RNA.regulation of transcription.Chromatin Remodeling Factors	29	7.0E-03	1.1E-02	1.0E-03
RNA.regulation of transcription.SET-domain	15	1.3E-01	2.4E-03	3.2E-02
RNA.RNA binding	187	1.7E-08	9.7E-06	1.1E-05
signalling.receptor kinases.leucine rich repeat III	15	3.3E-05	3.3E-04	1.6E-03
signalling.receptor kinases.leucine rich repeat XIII	4	1.8E-01	2.7E-02	4.0E-02

Selection of the most significantly down-regulated BINs for treatment comparison; for details see Table 3-42

Table 3-44 Differences between Px and Rz DRBs

BIN	Genes	Corr	Px	Rz	Sgn	BE/Ctrl	Px/Rz	Main
cell wall.cellulose synthesis	42	3.5E-03	3.3E-04	4.9E-01	Px	down	down	Px
cell wall.cellulose synthesis.cellulose synthase	27	3.7E-02	3.0E-04	8.6E-01	Px	down	down	Px
cell.organisation.cytoskeleton	98	7.8E-06	5.9E-11	3.2E-06	BE	down	down	Px
cell.organisation.cytoskeleton.mikrotubuli	52	2.7E-07	1.4E-08	9.5E-05	BE	down	down	Px
hormone metabolism.ethylene.signal transduction	29	4.0E-03	6.5E-09	5.3E-01	Px	up	up	Px
major CHO metabolism.degradation.starch.starch cleavage.beta amylase	5	3.0E-01	2.1E-02	7.1E-01	Px	up	up	Px
RNA.regulation of transcription.AP2/EREBP transcription factors	55	4.1E-06	1.7E-12	2.6E-02	BE	up	up	Px
RNA.regulation of transcription.AS2,Lateral Organ Boundaries	8	3.5E-01	1.1E-02	2.9E-01	Px	up		Px
RNA.regulation of transcription.bZIP transcription factors	50	6.7E-01	2.3E-02	3.5E-01	Px	up		Px
RNA.regulation of transcription.C2C2(Zn) DOF zinc finger family	11	3.2E-01	5.2E-04	2.0E-01	Px	up	up	Px
RNA.regulation of transcription.MYB domain transcription factor family	50	3.3E-01	7.7E-05	2.7E-01	Px	up		Px
RNA.regulation of transcription.PHOR1	4	5.4E-02	2.2E-02	4.9E-01	Px	up		Px
RNA.regulation of transcription.WRKY domain transcription factors	51	1.6E-07	0.0E+00	6.0E-06	BE	up	up	Px
signalling.receptor kinases.wheat LRK10 like	11	2.1E-03	9.5E-05	3.1E-03	BE	up	up	Px
transport.Major Intrinsic Proteins	28	1.5E-04	4.5E-06	1.3E-01	Px	down	down	Px
transport.Major Intrinsic Proteins.PIP	11	3.0E-03	8.3E-04	1.7E-01	Px	down	down	Px
transport.Major Intrinsic Proteins.TIP	9	1.7E-01	2.0E-02	5.9E-01	Px	down	down	Px
major CHO metabolism.degradation.starch	20	8.4E-01	7.9E-01	1.8E-02	Rz	down	up	Rz
protein.degradation.ubiquitin	695	1.2E-01	9.1E-06	1.9E-12	BE	up	down	Rz
protein.degradation.ubiquitin.E3	435	5.7E-01	1.5E-07	5.4E-13	BE	up	down	Rz
protein.synthesis	606	2.8E-09	5.0E-42	3.5E-80	BE	down	up	Rz
protein.synthesis.ribosomal protein	396	1.4E-06	5.4E-29	9.7E-68	BE	down	up	Rz
protein.synthesis.ribosomal protein.eukaryotic	317	4.8E-05	4.9E-25	1.6E-61	BE	down	up	Rz
protein.synthesis.ribosomal protein.eukaryotic.40S subunit	124	5.4E-02	4.3E-10	7.8E-27	BE	down	up	Rz
protein.synthesis.ribosomal protein.eukaryotic.40S subunit.S4	10	9.5E-01	6.0E-01	1.1E-02	Rz	down	up	Rz
protein.synthesis.ribosomal protein.eukaryotic.60S subunit	193	1.9E-03	1.9E-14	9.6E-34	BE	down	up	Rz
transport	652	7.6E-01	5.7E-01	1.8E-04	Rz	up	down	Rz

Table shows DRBs that differed between the Px and Rz treatment; BINs selected were indicated as DRBs in at least one of the BE/Ctrl comparisons and in most cases as DRBs in the Px/Rz comparison; Table sorted alphabetically and by the last column “Main” indicating the BE that was most significantly influencing the respective BIN in comparison with the Ctrl; “Corr” shows the p-value for the correlation coefficients used as input for MapMan as described in 3.12.5.5; Sgn = BE/Ctrl comparison that was significant (BE indicates significant for both BEs); BE/Ctrl = Regulation in the comparison with the Ctrl; Px/Rz = Up- or down-regulation in Px as compared to Rz, here color indicates the probability to be correlated with the root weight (the probability is decreasing if Sgn = BE)

3.12.7 Single gene analysis

3.12.7.1 Correlation analysis DEGs

Correlation analysis in SAS was done (as described in 3.12.5.5) using the 174 DEGs and the root weight as variables and the values for the nine RNA-Seq samples as data input thereby creating a correlation matrix with 30625 (175^2) Pearson's correlation coefficients. The matrix shows in each cell how well the expression values of two different genes are correlated for the nine RNA-Seq samples. This is different to the analysis at BGI where samples were compared by their expression pattern. Interestingly, by sorting the correlation matrix by correlation with the root weight in both directions of the 2D matrix, genes could be grouped into clusters with very similar gene expression (Figure 3-109). Additionally, those co-regulated gene cluster seemed to consist of genes with common and shared cellular functions.

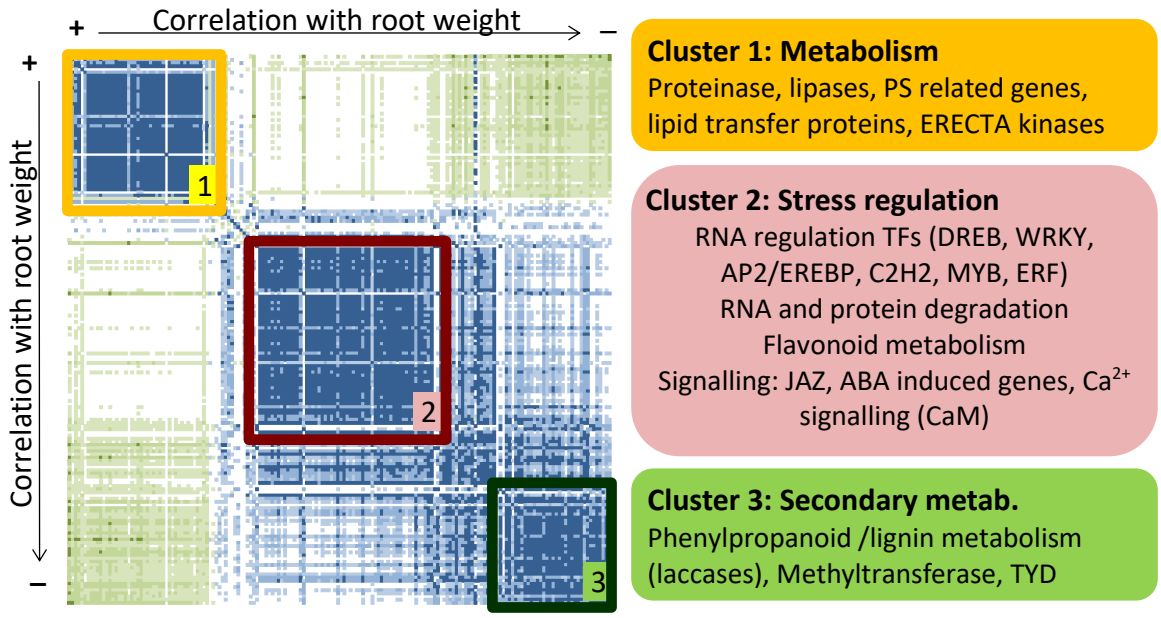


Figure 3-109 Co-regulation of maize genes; Correlation matrix using correlation coefficients from Pearson correlation of sample gene expression data for 174 differentially expressed genes (DEGs) sorted after correlation with sample root fresh weight; dark blue color indicates large positive correlation coefficients ($\rho > 0.9$) whereas green color indicates negative correlation coeffcients ($\rho < 0$)

3.12.7.2 Single gene selection

Although in MapMan several functional groups of genes seemed to be influenced by BE treatments, single gene selection that could support the findings from whole transcriptome analysis was difficult. The DEGs that were selected by NoiSeq method were checked for their FPKM values and the variation among biological replicates. Most of the DEGs (154) had FPKM values > 10. Nevertheless, only four of the Px DEGs and three of the Rz DEGs passed a simple t-test. Additionally, only 45 of the Px DEGs had higher or lower expression level in

all biological replicates as compared to all of the Ctrl replicates (for Rz only 17). Log2ratios for treatment comparison ranged from -3.4 to a maximum of 5.0. For 7 DEGs no log2ratio could be calculated because either in the Ctrl or the BE treatment expression was 0. Nevertheless, none of those genes had high expression values in the other treatment.

At the beginning it was not clear why those genes were selected as DEGs by the NoiSeq method. Then genes were checked for their specific expression pattern in the biological replicates. Interestingly, in most cases the selected genes had very similar expression values in two out of the three biological replicates. As supported by the correlation analysis the DEGs could be grouped by their expression pattern in the nine single samples and their correlation to root weight. Some of the DEGs showed a very high correlation to root weight, whereas Cluster 1 was positively and Cluster 3 negatively correlated with the root weight (Figure 3-109). A third cluster containing 54 DEGs showed an extremely defined expression pattern with always the same samples in all treatments showing the highest FPKM values. This cluster was less correlated to root weight. 52 of these DEGs were only significant for the Px treatment. In those genes Px_1 and Px_2 had very similar and much higher FPKM values than the Ctrl treatments whereas Px_3 did not differ to the Ctrl. Gene annotation indicated that most of these genes were somehow stress-related and/or hormone-responsive transcription factors.

A complete list with all of the 174 DEGs can be found in the Appendix (Table 7-3). Table 3-45 gives a list of the gene classes in which the DEGs were grouped by using the MapMan BIN classification and several subclasses based on the NCBI annotations. About 20 % of the genes were not annotated at all in none of the databases. Of those genes that were mainly up-regulated the biggest group of genes was connected to plant hormone signalling, hormone metabolism and response to stress. This group was divided into several subgroups such as plant hormone signalling (without ethylene), abiotic or biotic stress related genes (annotation does not directly refer to a specific plant hormone), pathogenesis-related genes (connection to specific pathogens, avr or HR related), ethylene related genes (Et) and genes related to hormone metabolism. One of the up-regulated genes was directly involved in ethylene synthesis (1-aminocyclopropane-1-carboxylate (ACC) oxidase). All other ethylene related genes were ethylene responsive transcription factors of the AP2/EREBP family (including DREBs (Dehydration response elements) and ERFs (Ethylene response factor). Additionally, several other hormone, drought, heat or salt stress responsive genes were up- or down-regulated. For the non-specified groups often no clear trend was seen. Especially for the PR

genes annotation was not definite because most of the annotations are based on homology to rice or *Arabidopsis* genes. Additionally, several other groups like the lectin-jacalin, the JAZ (Jasmonate (JA) zinc-finger expressed in inflorescence meristem (ZIM)-domain) proteins, WRKY transcription factors and proteins that are involved in the Ca^{2+} signalling (e.g. Ca calmodulin and interacting proteins) may be connected to stress. Therefore the table in the Appendix is sorted after the gene expression pattern of the DEGs (cluster as seen in Figure 3-109). The third biggest group was related to secondary metabolism. Here genes could be grouped into a general subgroup for secondary metabolism including oxygenases, transferases and oxidases involved in lignin and flavonoid metabolism and specific subgroups of laccases and tyrosin (or tryptophan) decarboxylases.

Many genes that were down-regulated were coding for LRR receptor kinases, lipid transfer proteins, photosynthesis related genes, e.g. for electron transport or genes that are involved in synthesis of chlorophyll or chloroplast membrane transport, and lipid metabolism.

Table 3-45 Classification of NoiSeq DEGs

Abbr.	n	Classification (by function or structure)		Abbr.	n	Classification	
LRR	**4**	**Leucine rich repeat (LRR) receptor kinases**	↓	CM	1	Cell morphology	↑
LTP	**5**	**Lipid transfer proteins**	↓	CW	2	Cell wall modification	↑
ProS	1	Protein synthesis	↓	Dev	5	Development (unspecified)	↑
TP	1	Transport of phosphate	↓	**Et**	**14**	**Ethylene signalling (+ metabolism)**	↑
Xb	1	Biodegradation of xenobiotics	↓	Ferm	1	Fermentation	↑
LJ	3	Lectin-jacalin	↙	Gly	1	Glycolysis	↑
LM	**8**	**Lipid metabolism**	↙	HM	2	Hormone metabolism	↑
PHS	**2**	**Plant hormone signalling (ABA, GA, auxin)**	↙	**JAZ**	**4**	**JAZ proteins**	↑
PS	**9**	**Photosynthesis (electrone transport, chlorophyll or chloroplast related)**	↙	Ox	2	Cytochrom redox reactions	↑
StB	4	Stress related (biotic)	↙	ProG	1	Protein glycosylation	↑
Glu	2	Glucosidases	↕	ProM	1	Protein modification	↑
PR	5	Pathogenesis related	↗	**ProU**	**4**	**Ubiquitin-mediated protein degradation**	↑
ProD	5	Protein degradation (mixed)	↗	RNA	1	RNA processing	↑
SM	**13**	**Secondary metabolism (mixed)**	↗	Sign	2	Signalling in sugar and nutrient physiology	↑
StA	7	Stress related (abiotic)	↗	TCA	1	Tricarboxylic acid cycle	↑
Unk	34	Unknown function	↗	**TF**	**11**	**Transciption factors**	↑
AAM	2	Amino acid metabolism	↑	TM	1	Transport of metabolites	↑
AsO	**4**	**Ascorbat-oxidase / Laccase**	↑	TN	1	Transport of nitrate	↑
Ca	**5**	**Ca2+ signalling**	↑	**TYD**	**4**	**Tyrosin-(Tryp)-decarboxylase**	↑

Abbreviation as used in Table 7-3 of the Appendix; n = number of genes in the respective class; colors and arrows indicate up or down-regulation: ↓ all down-regulated, ↙ more down-regulated, ↗ more up-regulated, ↑ all up-regulated

A comparison of the most abundant classes with the MapMan DRBs shows a good correlation for secondary metabolism, photosynthesis, lipid metabolism, ubiquitin-mediated protein degradation and transcriptional regulation (especially ethylene responsive genes).

3.12.8 RT-qPCR analysis

For RT-qPCR genes showing a gene expression that was representative for the functional class were selected. Most of the genes were NoiSeq DEGs, nevertheless, for some of the classes no good candidate genes were found (following the procedure described in 0) and therefore other genes were included. The Noiseq DEG annotated as ACO was not selected for RT-qPCR because expression values were low (FPKM between 5 - 40) and standard deviations high. Table 3-46 gives an overview on the selected genes. In the Appendix a second list with all FPKM values is added (Table 7-4). The two WRKY TFs are the same gene (meanwhile merged in the NCBI database) and therefore the same primers were used. Nevertheless, they were annotated differently in the RNA-Seq analysis and in a previous publication (Wei et al., 2012) and are therefore listed separately. In

Table 3-47 the most stable reference genes are listed for which primers were ordered.

Table 3-46 Candidate genes selected for RT-qPCR

Nr	Name	Func.	Functional group	Function	Transcript	KEGG
1	ACO1	Et	Ethylene biosynthesis	1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase 1)	NM_001155955	K05933
2	ADT	AAM	Amino acid metabolism	Arogenate dehydratase 6 (ADT6)	NM_001139004	K05359
3	CALS	SM	Callose metabolism	Glucan synthase-like 8 (GSL8)	NM_001143207	K11000
4	CCR1	SM	Lignin biosynthesis	Cinnamoyl CoA reductase 1 (CCR1)	XM_008680955	K09753
5	CYP	Ox	Redox reaction	Cytochrom P450	NM_001152534	K00517
6	CZOG	CYT	Cytokinin biosynthesis	*Putative* Cis-zeatin O-glucosyltransferase (CisZOG)	NM_001147693	K13495
7	EREBP	Et	Transcriptional regulation	APETALA 2/ethylene response element binding protein	NM_001152568	K09286
8	ERF1	Et	Transcriptional regulation	Ethylene response factor (ERF1)	NM_001111800	K09286
9	JAZ1	JAZ	Plant hormone signalling	Jasmonate (JA) zinc-finger expressed in inflorescence meristem (ZIM)-domain protein (ZmJAZ2)	NM_001156053	K13464
10	LAC3	AsO	Lignin metabolism	Laccase/l-ascorbat-oxidase (Lac14; ZmLAC3)	NM_001112445	K00423
11	NAC1	TF	Transcriptional regulation	NAC transcription factor (ZmNAC1)	NM_001130460	-
12	PAL1	SM	Phenylpropanoid metabolism	Phenylalanine ammonium lyase (PAL1)	NM_001174615	K10775
13	pldA	LM	Lipid degradation	Phospholipase A1	NM_001154643	K01058
14	TYDC1	TYD	Secondary metabolism	Tyrosine decarboxylase 1-like	XM_008653527	K01592
15	UGT	SA	Hormone metabolism	UDP-glucosyltransferase UGT74F1	NM_001153986	K13691
16	USP	StB	Stress response	Adenine nucleotide alpha hydrolases-like universal stress protein (USP)	NM_001139078	-
17	WRKY78	TF	Transcriptional regulation	WRKY transcription factor 53 (WRKY 33, ZmWRKY78)	XR_556057; see WRKY91	K13425
17	WRKY91	TF	Transcriptional regulation	WRKY transcription factor (WRKY 29, ZmWRKY91)	NM_001138554	K13425

Table 3-47 Selected reference genes

Nr	Name	Func.	Function	Transcript ID	KEGG	Reference
18	CDPK	Kinase	Cyclin-dependent protein kinase	NM_001147229.1, GRMZM2G149286	K02202	(F. Lin et al., 2014)
19	DPP9	AAM	Dipeptidyl aminopeptidase/acylaminoacyl peptidase (DAP2)	NM_001174461.1,XM_008645725.1, GRMZM2G174572	K01278	See CDPK
20	DUF	Unk	DUF1296; Unknown function	NM_001148217.1,XM_008649344.1, GRMZM2G163888	-	See CDPK
21	LUG	Unk	Unknown function	NM_001143648.1,XM_008656296.1, GRMZM2G425377	K12662	(Manoli et al., 2012)
22	MEP	Unk	Unknown function	NM_001137018.1, GRMZM2G018103	-	See LUG
23	UBCP	Ubi	Ubiquitin-conjugating enzyme E2	NM_001154750.1, GRMZM2G102471	K06689	See LUG

3.12.8.1 Primer quality and amplification

Primers of selected test genes and reference genes (Table 2-10) were checked for quality and specificity by PCR and DNA-gel-electrophoresis. Here cDNA from a mixture of different RNA isolates was used as a template. For the first PCR about 20 ng cDNA (RNA equivalent) were used in each reaction mix. Nevertheless, for none of the genes a visible band was seen in the gel. After using the PCR product in a second PCR for most of the genes a clear single band was visible with the size of the expected target sequence. Obviously, the concentration of 20 ng was too low for all genes (including reference genes). The ACO1 genes showed a very low intensity band. A third PCR using about 125 ng of template cDNA and various annealing temperatures was more successful and showed that the fragment could be amplified in equal amounts at all annealing temperatures. Even after increasing template cDNA concentration the ADT gene did not show a clear band. This was supported by the RT-qPCR later on. Here the melting curves of the different templates did not significantly differ to the melting curve of the non-template control (NTC). For the UGT, the WRKY and the LUG gene multiple bands were visible in the DNA-gel. The LUG genes was therefore excluded from the reference genes and not used in the RT-qPCRs. For the WRKY genes no explanation was found for the multiple bands. BLAST against the chromosomal DNA databases did not show other potential targets. For the UGT gene a 204 bp product sequence was found in the chromosomal NCBI database that fitted to the second band seen on the gel. Therefore the RNA used for cDNA was tested for gDNA contamination by using RNA as template in the PCR with UGT primers. Indeed the 204 bp product was amplified and detected, suggesting gDNA contaminations in the RNA, but gDNA contaminations could successfully be cleansed by the wipe-out buffer of the RT-PCR kit proven by the lack of bands for several tested primers on the buffer treated RNA. A test with DNAse I was not successful because also RNA was degraded during the procedure. Nevertheless, also the cleansed cDNA showed the 204 bp band for the UGT primers. The company BGI also provided data on alternative

splicing events. In the dataset of all RNA samples an intron retention event was found for the UGT gene. The position of the intron retention fitted to the position of the primers when BLASTed against the maize chromosome explaining the double bands.

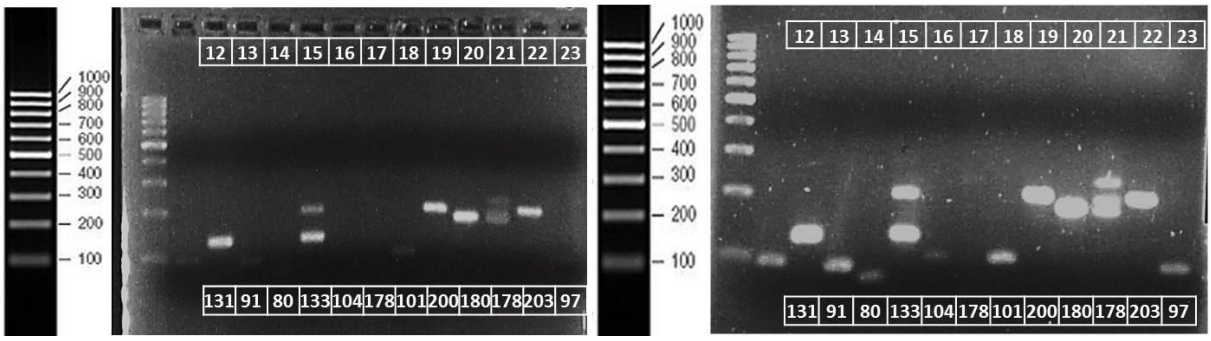


Figure 3-110 DNA-gel for testing primer quality; PCR products of genes 12 – 23 separated via gel-electrophoresis; On the left of each picture the fragment lengths in bp for the 100 bp ladder; Length of the target products in bp given in the lower lines. UGT (15) with a double band and LUG (21) with three bands. Bands of the pldA (13), TYDC1 (14), CDPK (18) and UBCP (23) genes show very low intensity only seen after increasing contrast. No band visible for the WRKY gene (17).

3.12.8.2 RT-qPCR

For RT-qPCR three separate runs with five to six candidate genes were performed. Per gene three treatments, four biological replicates (Table 3-36) and three analytical replicates were analysed. In each run two to three reference genes were added and two of them were used for normalization of the gene expression. To test the stability of the reference genes the Bio-Rad RT-Cycler program calculates the coefficient of variation (CV) and the geNorm M values for the chosen reference genes. The target stability values for the normalization of the genes differed strongly between the three qPCR runs. In Run 1 and 2 the same cDNA was used and target stability values were low whereas for Run 3 fresh cDNA had to be prepared. The last Run showed a much higher target stability value. The values reflect the variation in between the two reference genes. A high value indicates a low stability of reference genes. In all runs the combination of the MEP and UBCP genes showed the lowest target stability values and therefore they were used for normalization.

Table 3-48 Target stability values RT-qPCR

Target stability	Treshold value	Run 1	Run 2	Run 3
CV	0.25	0.0610	0.0110	0.1156
M	0.5	0.1760	0.0316	0.3342

Some analytical replicates were excluded from analysis if they strongly differed from the other two replicates or showed a strange melting curve. All candidate genes showed lower expression rates (higher Cq values) than the reference genes (Figure 3-111). This was expected for the MEP and UBCP genes that had much higher FPKM values (mean FPKM 240 and 530 respectively) than the other genes. Nevertheless, also the DPP9 and the LUG

gene, showing FPKM values of 33 and 50, showed lower Cq values than most of the test genes although mean FPKM values of several genes were higher.

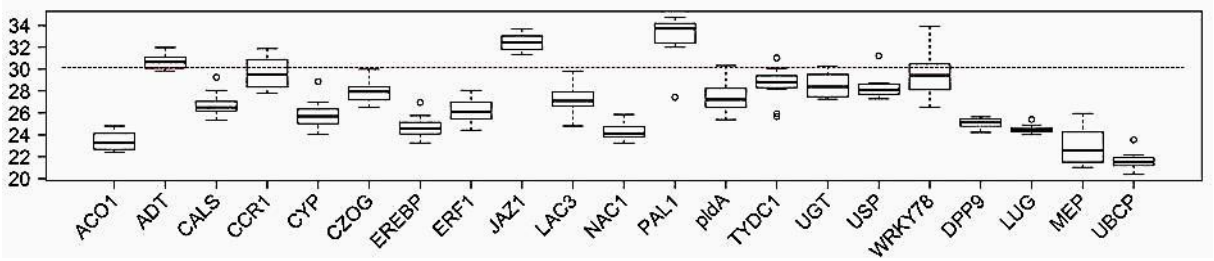


Figure 3-111 Mean Cq values for candidate and reference genes; Average number of cycles at which the amplification curve crosses the threshold value (Cq). The four tested reference genes (DPP9 – UBCP) had much lower Cq values indicating a much higher gene expression than most of the test genes. Especially the WRKY78 gene showed extremely instable expression in the different samples. Several genes had Cq value > or near to 30 indicating extremely low expression. For the PAL1 gene only one sample showed a low Cq value.

For the ACO1, ADT, CZOG, JAZ1, UGT and WRKY78 genes multiple peaks were found in the melting curve probably due to formation of primer dimers, bad annealing or weak amplification due to low expression rates. For the ACO1 and ADT primers very flat melting curves with low melting peaks were detected. For the CZOG and JAZ1 primers an intense primer peak was seen in the NTC. The UGT primers, that yielded multiple bands in the PCR-gel showed also multiple melting curves. The WRKY78 primers resulted in a very late melting peak (> 90 °C) and extremely flat curve.

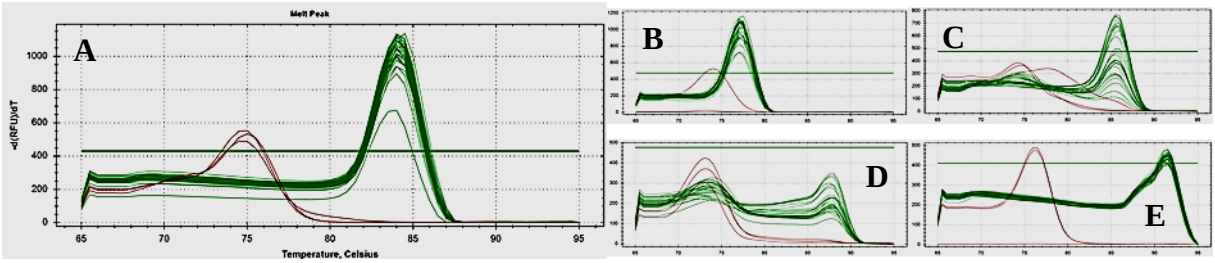


Figure 3-112 Melting curves of different test genes; Melting curves from RT-qPCR runs with the temperature on the x-axis and negative rate of change in RFU per temperature (T) change (-d(RFU)/dT) on the y-axis. The peaks represent a rapid decline of intensity due to the dissociation of the DNA strands. Red lines are NTCs, green lines are sample curves. Ideal peaks seen for the MEP (**A**) and the CALS (**B**) gene. Intense primer dimer peak and high variation in intensity between single samples seen for the UGT gene (**C**), primer dimer peaks and flat melting peaks for ACO1 (**D**) and a non-separated double peak detected for the WRKY gene (**E**).

The results for gene expression are plotted in Figure 3-113. For none of the genes significant differences were found in the treatment comparison. This was not completely surprising due to the strong variation in between biological replicates as observed in the RNA-Seq analysis. Due to the mixed samples send for the RNA-Seq analysis a direct comparison of the RT-qPCR results with the FPKM values is not possible for all single samples but a comparison for the samples C_3, Px_2 and Rz_3, used in the RNA-Seq for the single sample comparison, shows very similar trends.

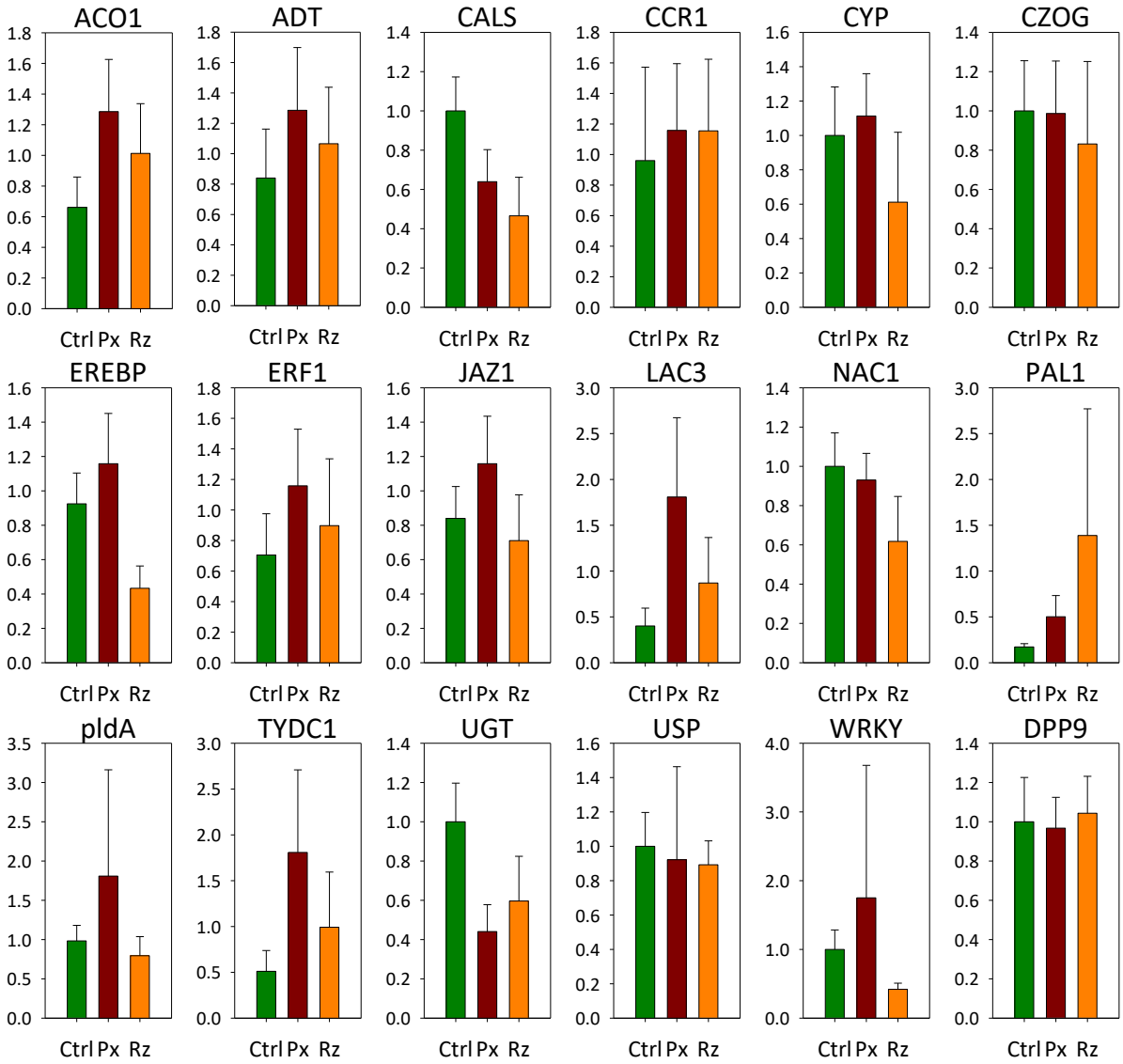


Figure 3-113 Results from RT-qPCR; Relative normalized gene expression of candidate genes; Means + SE; For none of the genes significant differences were found in the treatment comparison.

3.13 H-NMR analysis of maize metabolites

H-NMR analysis was done by Pierluigi Mazzei at the Interdepartmental Research Centre CERMANU of Professor Alessandro Piccolo for primary metabolites of the maize samples from Exp_11 and Exp_14. For analysis of roots and shoots samples S1 – S12 (Table 3-36) from Exp_11 and nine root and shoot samples from Exp_14 were send. Also the samples from Exp_14 were initially prepared for RNA-analysis and therefore combined to three samples. This was done by mixing two samples in a way that their mean weight was nearest to the total mean of the treatment to reduce intra-treatment variation (Table 3-49).

Table 3-49 Samples Exp_14 used for H-NMR analysis

Nr	Trt	Rep	Shoot FW	Root FW	Mixed	Mean shoot	Mean root
S1	Ctrl	1	3.64	0.66			
S2	Ctrl	6	2.14	1.96	C_1	2.89	1.31
S3	Ctrl	4	2.07	1.02			
S4	Ctrl	5	2.00	1.13	C_2	2.04	1.08
S5	Ctrl	3	2.40	1.08			
S6	Ctrl	2	2.78	1.12	C_3	2.59	1.10
S7	Px	4	2.22	0.46			
S8	Px	5	2.84	1.80	Px_1	2.53	1.13
S9	Px	3	2.17	0.77			
S10	Px	1	2.90	1.79	Px_1	2.54	1.28
S11	Px	2	2.19	0.97			
S12	Px	6	2.60	1.14	Px_2	2.40	1.06
S13	Rz	1	2.36	0.88			
S14	Rz	4	2.59	1.83	Rz_3	2.48	1.36
S15	Rz	3	2.64	0.96			
S16	Rz	5	NA	1.63	Rz_2	2.64	1.30
S17	Rz	2	2.28	0.99			
S18	Rz	6	2.44	1.50	Rz_3	2.36	1.25

Nevertheless, for H-NMR analysis for each experiment and treatment five samples were analysed by splitting one (Exp_11) or two (Exp_14) of the replicates into two subsamples again. This seemed to be necessary to have a higher number of analytical replicates (communication with P. Mazzei) but can be criticized from a statistical point of view.

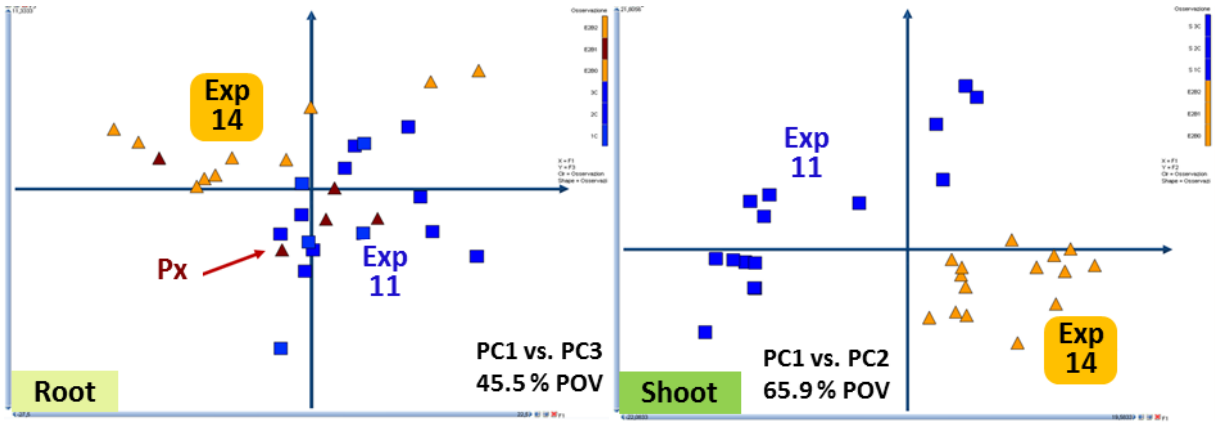


Figure 3-114 PCAs for primary metabolites (both experiments); Root = x-axis PC1 (36.3%) and y-axis PC3 (9.1 %), metabolites along PC3 are negatively correlated; Shoot = x-axis PC1 (47.6%) and y-axis PC2 (18.3%)

After measurements only those metabolites were selected that differed significantly among treatments or in the comparison between the two pot experiments. The measured concentrations of the respective metabolites were then used in PCA analyses and the PCs that were able to separate the different BE treatments or experiments from each other (Figure 3-114 to Figure 3-116) were analysed for those variables with highest proportion along the principial component (PC).

The root samples from the greenhouse (Exp_11) have been differentiated from those grown in the climate chamber (Exp_14) along PC1 because of a larger amount of fructose, caffeic acid, phenylalanine, shikimic acid, tryptophan and tyrosine. Interestingly, the Px treatment led to a change in the metabolome increasing the levels of the above-mentioned metabolites in Exp_14 and, by this, resembled the metabolome of Exp_11. A further differentiation between the experiments occurred along PC3 because of a larger amount of 4-hydroxybenzoic acid, choline, gallic acid, isocitric acid, raffinose and tyrosine in Exp_11.

The shoot samples from Exp_14 plants could been differentiated from shoot samples of Exp_11 along PC1 because of a larger amount of acetic acid, chlorogenic acid, fructose, glutamine, isocitric acid, isoleucine, malic acid, quinic acid, shikimic acid, succinic acid, sucrose, threonine and tryptophan. A further separation between the two sample groups is also observed along PC2 and is due to a larger amount of 4-hydroxybenzoic acid, aspartate, glucose, glycine-betaine, leucine, phosphatidylcholine, tyrosine and valine (Figure 3-114).

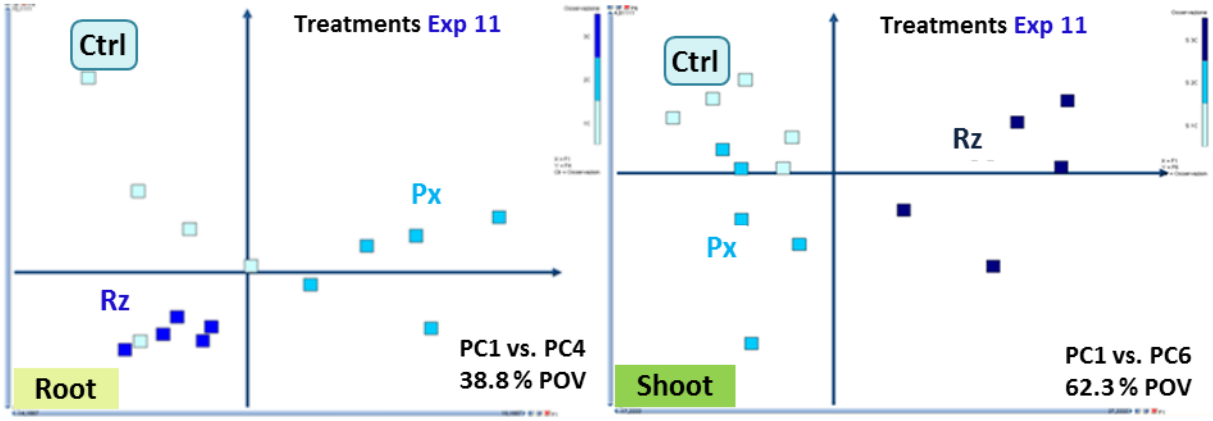


Figure 3-115 PCAs for primary metabolites (Exp_11); Root = x-axis PC1 (29.3%) and y-axis PC4 (9.4%); Shoot = x-axis PC1 (59.6%) and y-axis PC6 (2.7%)

For Exp_11 the Px root samples have been differentiated along PC1 from Ctrl and Rz samples because of their larger amount of fructose, isocitric acid, isoleucine, leucine, malic acid, proline, quinic acid, shikimic acid, succinic acid, sucrose, threonine, tyrosine and valine. Ctrl samples have been distinguished along PC4 from Rz treated roots because of the larger

amount of 4-hydroxybenzoic acid, caffeic acid, chlorogenic acid, glucose, tryptophan and tyrosine accompanied by a lower amount of 3-hydroxybutyrate.

Rz shoot samples have been differentiated along PC1 from both Ctrl and Px samples because of a larger amount of acetic acid, glutamine, isocitric acid, isoleucine, leucine, proline, quinic acid, shikimic acid, threonine, tryptophan, tyrosine and valine. Conversely, Ctrl samples have been separated from Px samples along PC6 because the former ones exhibited a larger amount of 4-hydroxybenzoic acid and shikimic acid accompanied by a lower amount of formic acid.

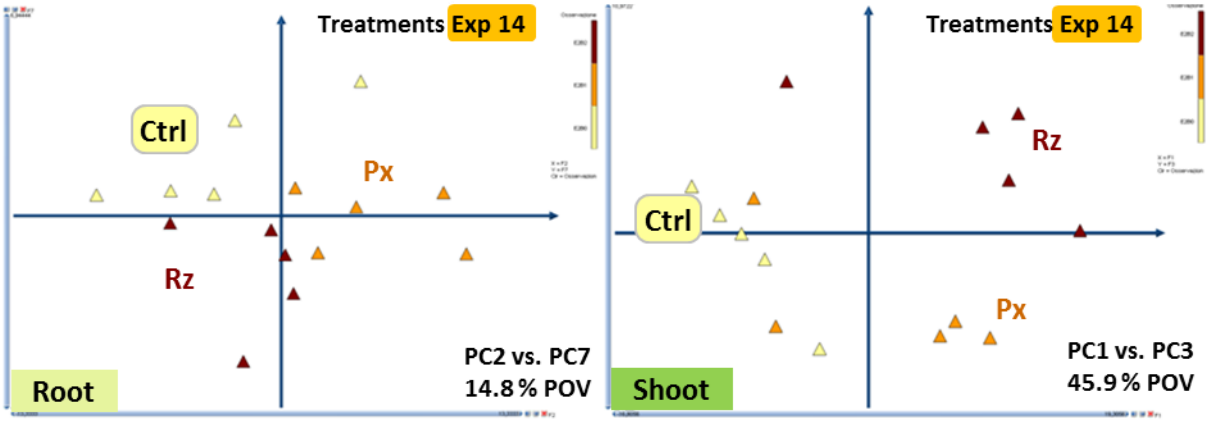


Figure 3-116 PCAs for primary metabolites (Exp_14); Root = x-axis PC2 (11.8%) and y-axis PC7 (3.0%); Shoot = x-axis PC1 (36.2%) and y-axis PC3 (9.7%)

In Exp_14 Px root samples have been differentiated along PC2 from both Ctrl and Rz root samples because of a larger amount of asparagine, aspartate, isocitric acid, malic acid, quinic acid, shikimic acid, succinic acid and tyrosine. The Rz samples have been differentiated along PC7 from Ctrl samples because of a lower amount of glucose and quinic acid.

Ctrl shoot samples are differentiated along PC1 from BE samples because of a lower amount of caffeic acid, chlorogenic acid, fructose, malic acid, phenylalanine, shikimic acid, trigonelline, tryptophan and tyrosine. Conversely, Rz shoot samples are separated from Ctrl and Px samples along PC3 because the former ones present a larger amount of 3-hydroxybutyrate, glucose, glycine-betaine, itaconic acid and sucrose.

The results are summed up in Table 3-50. For the root metabolites of the Px treatment in both experiments similar responses were observed. Additionally, the observed effects of the Px treatment on the root metabolome resembled those changes observed for the comparison of the experimental conditions (seen for aromatic amino acids, TCA, SM and sugars). Also interesting is the similarity of the changes in the metabolome seen in the Px treated roots and the Rz treated shoots of Exp_11. It also seems that Rz mainly affects shoot metabolome and not the root. For shoots of Exp_14 Px and Rz induced similar responses.

Table 3-50 Summary of the results from primary metabolite analysis

Metabolite	Roots					Shoots				
	Exp_11		Exp_14		Exp	Exp_11		Exp_14		Exp
	Px	Rz	Px	Rz	11 / 14	Px	Rz	Px	Rz	11 / 14
Amino acids										
Asparagine			up							
Aspartate			up							up
Glutamine							up			down
Isoleucine	up						up			down
Leucine	up						up			up
Proline	up						up			
Threonine	up						up			down
Valine	up									up
Aromatic amino acids										
Phenylalanine			up		up			up	up	
Tryptophan		down	up		up		up	up	up	down
Tyrosine	up	down	up		up			up	up	up
Organic acids										
3-hydroxybutyrate		up							up	
Acetic acid							up			down
Formic acid						up				
Tricarboxylic acid (TCA) circle										
Isocitric Acid	up		up		up		up			down
Itaconic acid			up		up				up	
Malic acid	up		up					up	up	down
Succinic acid	up		up							down
Osmoprotectants / Betaines										
Choline			up		up					
Glycine-betaine									up	up
Trigonelline								up	up	
Phospolipids										
Phosphatidylcholine										up
Secondary metabolism										
4-hydroxybenzoic acid		down	up		up	down				up
Caffeic acid		down	up		up			up	up	
Chlorogenic acid		down						up	up	down
Gallic acid			up		up					
Quinic acid	up		up	down			up			down
Shikimic acid	up		up		up	down	up	up	up	down
Sugars										
Fructose	up		up		up			up	up	down
Glucose		down		down					up	up
Raffinose			up		up					
Sucrose	up								up	down

Overview on the metabolites that differed in shoot or root concentration in the comparison of the two experimental conditions or the three treatments. Up = higher and down = lower concentration in the BE treatment as compared to the Ctrl or in Exp_11 as compared to Exp_14. For the Px treated root samples in Exp_14 also the metabolites were included that were mentioned in the comparison of the different experimental conditions (see Figure 3-114 Root)

3.14 Discussion of plant gene expression and metabolome

3.14.1 PGPR application induces only weak responses in gene expression

Gene expression in our experiment was only weakly affected by BE treatment and BE effects were concealed by the variation in between biological replicates. This is supported by whole transcriptome correlation analysis and the low number of DEGs. Additionally, many of these DEGs could be false positives as suggested by the RT-qPCR results. Only few DEGs had log2-ratios > 3 and those genes showed extremely low FPKM values (< 10) and where therefore not expected to be good targets for RT-qPCR. The results from RT-qPCR were not completely surprising because most of the DEGs were not significant in t-tests. Therefore RT-qPCR results reflect in most cases results from transcriptome analysis. One conclusion would be that PGPR did not have any significant influence on plant gene expression.

Nevertheless, in correlation analysis of DEGs (3.12.7.1) two kinds of shift were observed. First, effects that seemed to be correlated with plant biomass and second, effects that seemed to be independent of biomass. In both cases shifts could be a response to the BE treatment. Nevertheless, in the first case the gene expression response might be a secondary response to changes in plant development whereas in the second case gene expression might trigger a change in plant development. Therefore a differentiation seems important. Additionally, the mechanism causing the changes in plant development remains unknown, especially in the Px plants in which a growth depression was observed at first whereas at the second harvest plant growth was stimulated. It is also possible that the observed gene expression that is correlated with the plant biomass also caused the reduction in plant biomass in the Px treatment. Nevertheless, this theory cannot be proven due to a lack of gene expression data from previous harvest times. Some observations that do not support this theory are first, the obviously much higher differences between single biological replicates that differ in their biomass as compared to the differences between BEs of the same biomass and second, several genes that were related to BE treatment seemed not to be correlated with plant biomass.

The www.string-db.org tool was used to get more information on specific genes by their interaction in a network. Nevertheless, the webpage did only accept a limited amount of genes as input (up to ~ 200) and therefore mainly the DEG dataset was used. Several interaction clusters were found. Most of them were based on gene expression profiles or gene homology and did therefore not add much new information to our performed correlation analyses. One larger network included several signalling kinases involved in Ca^{2+}-signalling, genes connected to ABA signalling, a PR protein, lipases and lipid transfer proteins. One cluster

comprised ethylene biosynthesis and signalling genes, some minor clusters consisted of genes either related to pathogenesis, abiotic stress or secondary metabolism. Those results indicated an induction of abiotic or biotic stress responses in the PGPR treated maize plants of Exp_11.

Additionally, MapMan analysis was conducted to investigate minor shifts in gene expression. Several studies in which MapMan was used for analysis of gene expression in *Arabidopsis*, white lupin, barley and maize were published. Most interesting were four publications focussing on maize and white lupin under P-deficiency (Calderon-Vazquez et al., 2008; Wang et al., 2014), maize after infection of the fungal pathogen *U. maydis* (Doehlemann et al., 2008), and Arabidopsis after inoculation of the PGPR *Bradyrhizobium* and the pathogenic *P. syringae* (Cartieaux et al., 2008). In none of the studies soil substrate was used but either hydroponic culture or semi-sterile potting substrate. Additionally, in all experiments only differentially expressed genes instead of whole transcriptome dataset was used for MapMan analysis. Our whole transcriptome analysis with MapMan indicated that responses were similar to those observed under P-deficiency. Therefore also gene expression studies on different abiotic stress and nutrient deficiencies, senescence and hormonal regulation were further investigated (discussed in the next sections).

3.14.2 Drawbacks for data interpretation

In the last two decades numerous studies on plant gene expression after application of PGPR were conducted, mainly using different *Pseudomonas* or *Bacillus* strains. In total 19 studies were compared but only a part of them were using microarray and whole transcriptome sequencing approaches. Eleven of these studies were investigating the model plant *Arabidopsis thaliana*. The reason is that gene annotation is generally based on homology of the genes to *Arabidopsis* genes with known function. This is especially problematic for gene expression networks and pathways found in KEGG. The annotation of gene classes based on sequence homologies is an efficient and useful procedure but when focussing on the specific function of gene products in signalling or metabolic pathways inside the cell experimental evidence is needed, normally achieved via knock-out mutants, to prove the involvement of the specific gene. Data on PGPR-induced gene expression in other plant species are rare, whereas different legumes, rice and barley were investigated most frequently. Although the maize genome was completely sequenced the function of individual genes are largely unknown. To our knowledge in 2017 there were no data on whole transcriptome gene expression studies for the PGPR-maize interaction published but two studies were investigating expression of selected plant defence or stress-related genes (Djonović et al., 2007; Planchamp et al., 2015).

Some valuable information could be taken from publications that focussed on bioinformatic and phylogenetic analysis of gene families based on sequence homology and the comparison of their co-regulation under different abiotic stress conditions.

Another drawback for interpretation of the gene expression data was that the majority of publications on PGPR-plant interaction focusses on the induction of pathogenesis or plant defence related genes (ISR). One explanation might be that induced systemic resistance seems to induce specific pathways that are a useful starting point for investigation on PGPR (or PGPM)-specific responses and conservation of pathways among plant species (especially di- versus monocots). In contrast, general plant gene expression responses to PGPR application reported in literature differ strongly among each other. This may be due to complexity of the plant-PGPR interaction, the multitude of different combinations of plant and PGPR species tested and the difference in growing conditions. All these conditions influence hormonal status of plants and therefore the outcome of BE-plant interaction. Additionally, studies on ISR can easily refer to those conducted with pathogens. Nevertheless, in maize only few data on the regulation of genes inside SAR or ISR signalling cascades are available. Additionally, in our research no pathogen was inoculated after PGPR application and therefore no typical "defence priming" response can be expected. On the other hand, as mentioned before, pathogens were certainly present in our medium because non-sterile soil substrate was used whereas most gene expression studies are conducted under sterile conditions.

3.14.3 Recent publications on PGPR-maize interaction

Only recently two new publications on gene expression in maize were published. In both studies maize was grown in sterile substrate. Hardoim et al. (2020) reported on transcriptomic profiling after inoculation of *Azospirillum* and *Herbaspirillum* strains whereas Ahmad et al. (2019) conducted an experiment on drought stress and analysed the gene expression of selected drought related genes after inoculation of two *Pseudomonas* sp. strains. Under non-stress conditions the *Pseudomonas* strains showed only weak influence on gene expression of most of the tested genes but significantly up-regulated three genes. These genes were coding for a dehydrogenase (DHN1), a lipoxygenase (LOX6) and a hydrogen peroxidase lipase (HPL). None of the genes was differentially regulated in our gene expression analysis but in other experiments with the Px strain conducted under sterile conditions LOX genes were induced in barley and *Arabidopsis* (Fröhlich, 2008; von Rad et al., 2005).

The inoculation of *Azospirillum* had comparably weak influence on maize gene expression as compared to the *Herbaspirillum* strain. Additionally, genes connected to metabolism were differentially regulated and genes connected to cell wall membrane and cytoplasmic receptors involved in plant-pathogen interaction, as well as callose synthase and ET response genes were only repressed by *Herbaspirillum*. This was explained do to the difference in lifestyle and root colonization strategies, as *Herbaspirillum* colonizes plants endophytically and *Azospirillum* mainly the rhizoplane (Hardoim et al., 2020). However, for both strains the majority of DEGs was connected to transcription factors. Several groups were down-regulated whereas AP2-EREBP, AUX/IAA, bZIP, C2H2, MYB domain and WRKY TFs were mainly induced. This was also observed in the Px treatment although the responses were much weaker and not significant for single genes (see 3.12.6 ff. and below). Interestingly, results from Eltlbany et al. (2019) indicated that relative abundance of *Azospirillum* strains was higher specifically in the Px treatment (see also 4.1.4.3). Comparing all 4814 DEGs found by Hardoim et al. with a dataset of 360 genes that were most responsive in our analysis revealed only 52 common genes. Most of these genes were not clearly annotated. 9 genes were connected to hormonal regulation, cell membrane or immune receptors. One gene for auxin signal transduction (GRMZM2G119219), a gene for cadmium resistance (GRMZM2G151230) and three genes responsive to either auxin (GRMZM2G165133), ethylene (GRMZM2G117971) or ABA (GRMZM2G052100) stimulus were up-regulated in all PGPR treatments. In contrast, two genes coding for aquaporin membrane water channels (GRMZM2G028325, GRMZM2G082184) as well as a JA-responsive gene (GRMZM2G163406) were up-regulated by *Azospirillum* and *Herbaspirillum* but down-regulated in Rz and Px treatments. A gene connected to GA biosynthesis (GRMZM2G016922) was down-regulated by *Azospirillum* and *Herbaspirillum* but up-regulated by Rz and Px. In summary, the results indicate that there are common responses such as the activation of stress responses, stress-related genes or hormonal regulation but that different PGPRs might trigger different unique pathways as also concluded by Hardoim et al. (2020). In the following key regulation genes found in previous publications and their expression profile in our dataset are further discussed with a focus on hormonal regulation, abiotic stress and ISR.

3.14.4 Hormonal signalling

As mentioned in the introduction, PGPR-induced biostimulation correlates with hormonal shifts in plants. This may be triggered by PGPR signalling compounds that do not have

phytohormonal activity but may induce hormonal pathways (e.g. AHLs) and by production or degradation of phytohormones by PGPRs. Especially the production of auxin and the degradation of ethylene (ET) by ACC deaminase activity were shown to be important actors in PGPR-plant interaction, mainly under sterile and controlled growth conditions. We hypothesized that ethylene-response would be down-regulated and auxin response up-regulated in BE treatments. Results from MapMan analysis on different whole transcriptome datasets (filters) indicated that auxin production, transport and signalling were only weakly affected in BE treatments. In contrast, ET synthesis and signalling were up-regulated by PGPR application. Cytokinin and salicylic acid metabolism were induced in both BE treatments whereas jasmonic acid metabolism was not changed as indicated by MapMan analysis. In the Px treatment there were some minor trends for up-regulation of abscisic acid metabolism and down-regulation of brassinosteroids but due to low P-values these hormones were not further investigated. For gibberellins no difference in regulation was found.

3.14.4.1 Ethylene

Especially ethylene signal transduction was strongly induced in the Px treatment. AP2/EREBP (APETALA 2/ ethylene-responsive element binding proteins) superfamily transcription factors and its largest subfamily, the ethylene response factor (ERF) family, are involved in abiotic and biotic stress reactions in di- and monocots (Dey and Vlot, 2015; Mizoi et al., 2012). Genes of this superfamily constitute the largest group of up-regulated DEGs in our experiment. Therefore upstream regulatory elements of the ethylene signalling pathway such as EIN2 or CTR1 (Song and Liu, 2015), that are involved in activations or deactivation of downstream ERFs, were investigated but no differences in gene expression among treatments was found. Nevertheless, it seems that in maize those elements are not transcriptionally regulated as reported for Arabidopsis (Stepanova and Alonso, 2009). The ZmERF1 transcription factor that was indicated as DEG for both BE treatments and tested in the RT-qPCR was found to be induced by salt and heat stress (Q. Shi et al., 2014). Another interesting ET-responsive maize gene that was up-regulated in the Px treatment was ZmDREB2A. This gene was found to be induced by drought and heat stress (Qin et al., 2007). Nevertheless, in our transcriptome analysis not the same transcript was differentially expressed than reported in the paper and the log2ratio was < 1.

3.14.4.2 Cytokinin

In the Px treatment cytokinin metabolism was up-regulated. Nevertheless, in the MapMan BIN synthesis and degradation were not separated. In general both groups were up-regulated but a closer investigation of cytokinin-related genes indicated that mainly genes involved in

degradation and glycosylation of cytokinin showed higher expression values. Strongest up-regulation (not significant) was observed for a gene coding for a cytokinin oxidase 1 (ckx1) that is inactivating cytokinin (Bilyeu et al., 2001). Cytokinin can also be inactivated by glycosylation (Argueso et al., 2009; Behr et al., 2012).

3.14.4.3 Transcriptional regulation

Additionally to the largest family of AP2/ERF transcription factors also two other families, WRKY and NACs were strongly up-regulated, especially in the Px treatment. ERFs are clearly connected to ethylene, but also the other TFs are regulated by various plant hormones and abiotic or biotic stress conditions, including direct regulation via ethylene as seen by ERF binding sites in the cis-acting elements of some NAC factors (Voitsik et al., 2013). Many genes of NAC factors also show binding sites for WRKYs showing the interplay of those TFs. Various NAC transcription factors that were up-regulated in our data are known to be connected to ABA and abiotic stress responses (Lu et al., 2015, 2012; Vilela et al., 2013). No NACs were found in the DEG list but ZmNAC1 showed a log2ratio of 0.9 and FPKM value >100 in the Px treatment. Furthermore it seems to be involved in lateral root formation (Li et al., 2012). Seven NAC TFs were found to be differentially regulated by pathogen infection whereas especially ZmNAC41 and ZmNAC100 were up-regulated up to 40 – 200 fold by the necrotrophic pathogen *C. graminicola* (Voitsik et al., 2013). Both genes are triggered by JA and one of them also by SA, none of them triggered by ethylene precursor ACC. In our data these two NACs were not more up-regulated than other NACs suggesting that the JA pathway (ISR) was not triggered by BE treatments. All WRKY TFs were up-regulated in the Px treatment and most of them in the Rz treatment. Several of these WRKYs were up-regulated in response to *U. maydis* infection suggesting involvement in SAR (Wei et al., 2012).

3.14.5 Secondary metabolism

As described before, up-regulation of secondary metabolism is an often observed stress-response. MapMan BINs connected to phenylpropanoid and flavonoid biosynthesis were up-regulated in both BE treatments (Table 3-42). One of the key enzymes of the phenylpropanoid pathway is phenylalanine ammonia lyase (PAL) (see also KEGG pathway zma00940). Almost all PAL genes were up-regulated in both BE treatments but none of them significantly. In the RT-qPCR expression values of PAL1 were extremely low (in RNA-Seq FPKM 50 - 100) and no significant differences between treatments could be found. In our DEG list four enzymes were found up-regulated that are directly involved in lignin synthesis. They were annotated as trans-cinnamate-4-monooxygenase (C4H, KEGG K00487),

cinnamoyl-CoA reductase (CCR1, K09753) and hydroxyl-cinnamoyl-transferase (HCT, K13065). Three genes connected to flavonoid synthesis were up-regulated DEGs in the Px treatment (2OG-Fe(II) oxygenase (K05278), chalcone synthase (K00660), NAD(P)(H) oxidoreductase (K08243). Flavonoids are important for plant defense and stress protection but additionally seem to have influence on the requirement of beneficial microbes in the rhizosphere (Lakshmanan et al., 2012). Four laccase genes were found to be up-regulated in both BE treatments. ZmLac3 has a putative role in lignin biosynthesis and is up-regulated in response to wounding (Caparrós-Ruiz et al., 2006). Additionally, ZmLac3 shows high homology to the AtLAC15 gene that is involved in flavonoid biosynthesis. The ZmLac2, ZmLac4 and ZmLac5 genes are down-regulated in response to wounding. Similar trends were also observed in our dataset (no significant regulation). That implicates a connection to ISR that uses similar pathways like those triggered by attack of herbivores or necrotrophic fungi (Pieterse et al., 2014). Four tyrosine decarboxylases (TYD, K01592) were significantly up-regulated and one polyphenol oxidase (PPO, K00422) was significantly down-regulated. TYD is involved in stress and pathogen responses and the production of tyramine and dopamine, both catecholamines acting as “phytoalexins” (Kulma and Szopa, 2007; Mayer, 2006). Wounding, ABA treatment or pathogen infection increased catecholamine synthesis. PPO may have a putative function in plant stress responses and resistance against pathogens or herbivores (Mayer, 2006). The induction of PPO by stress or pathogen attack confers increased resistance and seems to be connected to JA pathway. PPO expression was increased by MeJA application but the function and mechanism of PPO as well as the regulation by JA/SA are still unclear. The observed down-regulation may therefore reflect the JA/SA interplay. Two DEGs coding for enzymes involved in DIMBOA synthesis were significantly up- or down-regulated in the Px treatment (putative O-methyltransferase ZRP4 (resveratrol synthesis)/DIMBOA-Glc O-methyltransferase and Benzoxazinone synthesis2). Benzoxazinoids (Bx) are antimicrobial compounds exuded from cereals roots and 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) is the main antimicrobial benzoxazinoid in maize (Berendsen et al., 2012). Strain-specific influence of BE treatments on maize Bx production was found after application of different *Azospirillum* sp. strains, one AM fungi and one *P. fluorescens* strain (Walker et al., 2011b, 2011a). Furthermore it was shown that *Pseudomonas putida* KT2440 is relatively tolerant to DIMBOA and chemotactically attracted by this compound (Berendsen et al., 2012).

3.14.6 Induced resistance

3.14.6.1 Jasmonic acid pathway

Jasmonic acid (JA) is often connected to ISR, response to wounding, herbivory and necrotrophic pathogens. JA biosynthesis includes the four enzymes LOX, AOS, AOC and OPR3 (Zhang et al., 2005). MapMan analysis did not indicate shifts in the expression of these enzymes by BE treatments but one ZmAOS gene (Djonović et al., 2007) with highest expression was slightly up-regulated in the Px_2 and Rz_3 samples. Nevertheless, four jasmonate ZIM-domain (JAZ) protein genes were significantly up-regulated (DEG) in the Px treatment and part of Cluster 2 (Figure 3-109). 23 JAZ genes were identified in maize (Zhou et al., 2015). Interestingly, in total 35 putative JAZ genes, indicated by KEGG orthology, were found in our transcriptome dataset. 30 of them were slightly or significantly up-regulated in the Px treatment. The four JAZ DEGs were identified as ZmJAZ2, ZmJAZ5, ZmJAZ15 and ZmJAZ21. JAZ proteins are key regulators of the JA response pathway. They suppress the MYC2 transcription factor that is an activator of downstream JA responses. However, binding of JA to a SCF^{COI1} E3 ubiquitin ligase complex leads to degradation of JAZ genes, a subsequent release of MYC2 and activation of JA responses. Furthermore MYC2 induces JAZ gene expression leading to a negative-feedback regulation in the JA signalling pathway (Chini et al., 2007). This explains why JAZ gene expression is induced by JA although they suppress JA signalling pathway. AtJAZ genes are also induced by wounding and P-deficiency (Chung et al., 2008; Khan et al., 2016). Studies on JAZ genes in maize indicate that ZmJAZ gene expression is induced by various other stimuli such as NaCl, PEG, ABA, GA, and SA but the responses seems to differ among individual genes (Zhou et al., 2015). Unfortunately, our four JAZ DEGs were not tested. But two other JAZ genes (ZmJAZ12 and 20) that were both up-regulated in comparison of the single samples (Px_2/C_3 and Rz_3/C_3) were found to be induced by salt or osmotic stress but not JA or ABA (Zhou et al., 2015). One JAZ gene was up-regulated in Arabidopsis roots after treatment with the PGPR *Bacillus amyloliquefaciens* (Sarosh et al., 2009). This coincided with the up-regulation of other genes related to the JA-response pathway such as PDF1.2 and MYC2. PDF1.2 gene expression in Arabidopsis was also up-regulated by *B. subtilis* (Lakshmanan et al., 2013) but not by *Pseudomonas fluorescens*, including the Px strain (Pieterse et al., 1998; von Rad et al., 2005). PDF1.2 together with EIN2, ERF1 and ORA59 is involved in mediation between ET and JA signalling pathways in Arabidopsis but is suppressed by the SA pathway (Pieterse et al., 2009). In our transcriptome dataset only few putative homologues with mainly

low expression values were found for PDF1.2, EIN2 and ORA59. One EIN2 gene with FPKM > 50 did not differ among treatments. As mentioned above, several ERFs were up-regulated, especially in the Px treatment.

3.14.6.2 Salicylic acid pathway

Salicylic acid (SA) is connected to abiotic and biotic stress but most publications focus on SA-mediated plant defence against (hemi)biotrophic pathogens. SA biosynthesis was slightly induced in both BE treatments. Nevertheless, most of the genes inside the slightly up-regulated MapMan SA biosynthesis BIN are UDP-glucosyltransferases that modify and deactivate SA by glucosylation (conjugation with glucose) but do not reflect *de novo* synthesis (Dempsey et al., 2011). Up-regulation of SA synthesis is a common response to (a)biotic stress but in Arabidopsis mainly the isochorismate synthase (ICS1) pathway is responsible for stress-related SA biosynthesis (Dempsey et al., 2011; von Rad et al., 2005). Nevertheless, this pathway was not in our mapping file and no ZmICS1 gene was found.

ZmOPR genes are involved either in JA or SA pathways. In our data the two OPR genes ZmOPR1 and ZmOPR2, that are induced by SA (Zhang et al., 2005), were correlated well with plant biomass but down-regulated in the comparison between Px_2/C_3. In a previous study on regulation of selected ISR/SAR response genes in maize after inoculation with the PGP fungi *Trichoderma virens*, the induction of these two ZmOPR genes coincided with up-regulation of the ZmPR1 and ZmPR5 genes, both related to SA pathway (Djonović et al., 2007). In our dataset both PR genes were highly expressed in all samples (FPKM 300 – 1200) and up-regulated (not significantly) in the Px treatment, but especially in the comparison of Px_2/C_3. JA-responsive genes were not up-regulated.

Our results indicate that the JA pathway was influenced by Px but possibly suppressed by induction of SA pathway. Therefore the transcriptome dataset was analyzed for genes that are involved in the mediation between the JA and SA pathways. One of the major mediators of JA/SA interaction is NPR1. NPR1 suppresses the JA pathway when SA pathway is activated but it seems that NPR1 itself is not transcriptionally regulated but mainly regulated by its redox status (Dempsey et al., 2011; Pieterse et al., 2014). In our data only few NPR1-like genes with very low FPKM values were found. EDS1, PAD4 and MPK4 act upstream of NPR1 (Pieterse et al., 2009). MPK4 suppresses EDS1/PAD4 and activates JA pathway whereas EDS1/PAD4 activate SA pathway. In our data two PAD4 genes and also two MPK4 genes were up-regulated (no DEGs, only significant in t-test). Additionally, two GRX480-like

genes, were slightly up-regulated in both BE treatments. GRX480 is involved in suppression of the JA/ET mediator PDF1.2 and regulated by NPR1 (Bari and Jones, 2008). In the Px treatment two NDR1-like genes (Nonspecific Disease Resistance) were significantly up-regulated (treatment DEGs) that are connected to SA and R protein mediated defence signaling (Dempsey et al., 2011; Jones and Dangl, 2006).

Although the SA-pathway seems to be induced by the PGPR the effects are not equal to those observed under pathogen infection. Maize infection by the biotrophic fungi *Ustilago maydis* was inducing secondary metabolism (PAL), repressed PS and induced the SA-pathway but additionally activated the JA pathway and auxin biosynthesis and response (Doehlemann et al., 2008). The induction of auxin and JA seem to be a mechanism by which biotrophs try to suppress host plant defense (Pieterse et al., 2009). *U. maydis* infection induced also the expression of several lipid transfer proteins (LTP) (Wei and Zhong, 2014). LTPs are a heterogeneous group of proteins involved in cellular transport of lipids (Finkina et al., 2016). They are involved in various biological processes, including plant defense against bacterial and fungal pathogens, tolerance against abiotic stress and antimicrobial activity (Finkina et al., 2016; Kader, 1997). Additionally, some LTPs seem to be involved in ISR (Petti et al., 2010) and are putative long-distance signals for SAR (Vlot et al., 2008). Their expression was also differentially regulated in AHL treated plants (von Rad et al., 2008). A genome-wide analysis of maize LTPs provided data on their expression pattern depending on plant-tissue, developmental stage and abiotic or biotic stress factors (Wei and Zhong, 2014). Several of these LTPs could be recovered in our dataset and eleven LTPs were differentially expressed (five significantly, four DEGs for both and one DEG for the Rz treatment). Nevertheless, almost all LTPs were strongly repressed by BE treatments and expression pattern differed from those observed under the different stress conditions.

Taken together with the results observed for the WRKY and NAC TFs results indicate that both JA and SA pathway were activated. This is in contrast to the SA-independent *P. fluorescens* WCS417r-mediated ISR (Pieterse et al., 1998). A suppression of SA-related genes and no regulation of ethylene-related genes was also reported for maize treated with *P. putida* (Planchamp et al., 2015). Also in an experiment with *Bradyrhizobium*, that was analyzed with MapMan, very different expression pattern as compared to our results were observed (Cartieaux et al., 2008). As reported for priming reactions single inoculation of the PGPR had only minor effects and most DEGs were down-regulated. Only additional infection with the pathogen led to an up-regulation of these genes. Nevertheless, only little overlap was found

between *Bradyrhizobium* triggered responses and our results. In contrast, our results were more similar to those observed for pathogen infection suggesting again SAR. Contrasting results were also observed in other studies on the Px strain or different *Bacillus* sp. strains (see below).

3.14.6.3 Previous studies on the Px product

In a doctoral thesis, gene expression analysis in barley leaves was conducted after application of the Px product to the roots (Fröhlich, 2008). This microarray analysis focussed on ISR-related genes. The results were only published in German inside the doctoral thesis and were not discussed in detail. However, two pathogenesis related (PR) genes, phenyl-ammonium lyase (PAL, secondary metabolism), glutathione-S-transferases (GST) and lipoxygenase genes (LOX), that are probably involved in JA-dependent responses (Wasternack and Hause, 2013), were up-regulated and several PS-related genes down-regulated. One ethylene responsive factor (ERF) and three PIN genes, involved in polar transport of auxin, were induced. Only few of the genes showed log2ratios > 1. They concluded that the Px product was inducing defence reactions in the plant. Nevertheless, experiments were conducted in sterile substrates and non-sterile potting substrates. Only under sterile conditions differentially expressed genes were detected whereas under non-sterile conditions no effects on gene expression were observed although Px was able to stimulate plant growth and increased resistance against a fungal pathogen.

Also a previous publication on plant gene expression after Px application in Arabidopsis focussed on plant defence pathways (von Rad et al., 2005). They included marker genes of the SAR pathway (PR1, PR2 and PR5), SA-biosynthesis (ICS1), the ISR /JA pathway (VSP, JIP and PDF1.2) and JA-biosynthesis (LOX2, AOS and OPR3). Px was inducing PR genes and PDF1.2, and induced levels of JA and SA but for JA only in the first hours after application whereas SA induction was longer lasting. Most of the SA was found in conjugated form and only the ICS1 pathway was activated (see above). Similar to the observation of Fröhlich (2008) GSTs and one ERF were significantly up-regulated in the Px treatment. They concluded that Px induced both defence pathways.

3.14.6.4 Studies on Bacillus *sp. strains*

There are no studies on plant gene expression after application of the Rz strain published but, as described above, previous investigations on *B. amyloliquefaciens* and *B. subtilis* indicated a typical induction of ISR (e.g. induction of PDF1.2) in treated Arabidopsis plants (Lakshmanan et al., 2013; Sarosh et al., 2009). Additionally, they observed up-regulation of

MYB, NAC and AP2/ERF transcription factors. In an experiment with tomato, treatment with a filter-sterilized cell-free filtrate of *B. thuringiensis* systemically suppressed bacterial wilt caused by *Ralstonia solanacearum* (Takahashi et al., 2013). But in contrast to the previously described studies results indicated that disease suppression was achieved by combined activation of SA and ET pathway and deactivation of the JA pathway.

3.14.7 P-deficiency

We observed down-regulation of PS genes in our root samples. This is surprising because PS-related genes are not expected to be active in root tissues. Nevertheless, down-regulation of photosynthesis (PS)-related genes in roots was also observed in other publications for maize (Calderon-Vazquez et al., 2008), this was not mentioned in the text, but visible in the MapMan overview in the supplementary material (Fig. S2 A)), *Arabidopsis* (Kang et al., 2014; Wu et al., 2003) and rice (Li et al., 2010) plants during P-deficiency. Additionally, a P-starvation related gene was found that was able to regulate PS-related gene expression and results suggest that down-regulation of PS-related genes seems to be important to prevent root growth inhibition during P-deficiency (Kang et al., 2014).

Therefore the gene expression data were analysed for other typical plant physiological responses to P-deficiency. A down-regulation of nucleotide and protein synthesis and up-regulation of several transcription factors (AP2/EREBP, MYB, WRKY, PHOR1, C2C2) was observed that was also reported for gene expression studies of P-starved cluster roots of white lupin (Wang et al., 2014). Also commonly observed is an increased phosphoenol pyruvate carboxylase (PEPC) activity (Neumann and Römheld, 2012, 1999) leading to accumulation and higher exudation rates of carboxylates. A significant up-regulation was only observed when whole transcriptome data of single samples were compared but not for treatment comparisons. Neither citrate transporter activity (MATE) (Wang et al., 2014) nor citric acid metabolite concentration were affected by BE application but malate concentration was increased in roots and shoot samples of Px and Rz treated maize plants (metabolome). Also one gene involved in the regulation of the malate transporter gene ALMT1 was up-regulated but this transporter gene had much lower FPKM values than others of the same class. The results suggest that aconitase activity, catalysing the degradation of citric acid, was not decreased as observed in white lupin (Neumann et al., 1999) and therefore no accumulation of citrate but of malate was observed. Nevertheless, also in wheat P-deficiency did not decrease aconitase activity (Neumann and Römheld, 1999). Furthermore in C_4 plants the first step in CO_2 fixation is catalysed by PEPC and not rubisco. This process is more efficient and energy

saving and malate is its first reaction product. A down-regulation of subsequent calvin-cycle due to stress conditions may therefore automatically cause malate accumulation. Malate is not only an important nutrient source for microorganisms or for chelating of mineral nutrients but has a pivotal role in shaping root system architecture as it inhibits primary root growth and promote lateral root growth through accumulation of iron in a concentration dependent manner (Canarini et al., 2019; Mora-Macías et al., 2017).

Ethylene is known to be a major regulator of P-deficiency responses, influencing root system architecture (RSA) by reducing primary root growth and stimulation of lateral root formation and root hair elongation, often in combination with auxin signalling (Neumann, 2016; Roldan et al., 2013; Song and Liu, 2015). Increased expression of ethylene biosynthesis genes (e.g. ACC oxidase) under P-limitation was observed in many legume species and Arabidopsis. Nevertheless, also contrasting results from studies on maize and tomato are published reporting a decrease in ACC and ethylene production (Drew et al., 1989) suggesting that not only biosynthesis but also ethylene signalling and sensitivity are involved in P-deficiency responses (Neumann, 2016; Roldan et al., 2013). Additionally, high ethylene concentration may inhibit lateral root growth. Especially the auxin/ethylene ratio seems to be important for the outcome of ethylene-derived changes in RSA. In our experiment the BE treatments led to an up-regulation of ethylene metabolism and signalling both on the whole transcriptome level as well as for the DEGs (here mainly Px). One of those DEGs was annotated as ACC oxidase. Especially the strong induction of ethylene-responsive transcription factors (AP2/EREBP, ERFs) contrasts with the ACC deaminase activity of many PGPR (Glick, 2014).

Table 3-51 Comparison of own RNA-Seq results with those from Schlüter et al.

Low T	Total shared genes	Co-regulated genes	Ratio	
F0: log2ratio	1880	783	41.6%	
F3: t-test	413	163	39.5%	↓
SSDEGs (only Px)	124	41	33.1%	↓
DEGs (Px)	23	9	39.1%	
Low N				
F0: log2ratio	590	254	43.1%	
F3: t-test	110	34	30.9%	↓
SSDEGs (only Prox)	42	13	31.0%	
DEGs (Px)	6	2	33.3%	
Low P				
F0: log2ratio	1430	611	42.7%	
F3: t-test	320	174	54.4%	↑
SSDEGs (only Px)	138	90	65.2%	↑
DEGs (Px)	31	25	80.6%	↑

Another study was used to see if responses where more generally related to abiotic stress. One study on maize plants focussed on differential expression and changes in metabolite profiles

after application of low temperature (low T), low nitrogen (low N) and low phosphorus (low P) stress (Schlüter et al., 2013). Although their results differed in some aspects from other reports on low P experiments (e.g. up-regulation of PS-related genes) their DEG expression profile (from the supplementary data) correlated well with our gene expression results based on transcript IDs. When filtering the data set for DEGs or SSDEGs of the Px treatment, correlation (equally regulated genes in comparison to the Ctrl) was increasing with each filter step. This was not observed in the comparison with the low N and low T DEGs suggesting that it is not a general response to abiotic stress (Table 3-51). Similar to our observation, genes related to starch synthesis were down-regulated, glycolysis and synthesis of aromatic AAs was up-regulated. In contrast to our results, P starvation responses and sucrose related genes were up-regulated and a decrease in leave metabolites contents of carbohydrates, organic acids and amino acids was observed under low P (Schlüter et al., 2013). This could be explained by the difference between roots and shoots. E.g. sucrose production is up-regulated in shoots but transported to the roots during P-starvation (Song and Liu, 2015).

Nevertheless, several key indicators of P-deficiency were not differentially regulated such as the high-affinity P transporters (PHT), phosphatases and typical P-starvation response genes that were responsive to P-deficiency in Arabidopsis and maize (Calderon-Vazquez et al., 2008; Thibaud et al., 2010). No recycling of P by ribonucleases, replacement of phospholipids or the increased production and exudation of organic acids (Song and Liu, 2015) was observed. Some key regulator genes of downstream P-starvation responses were identified that seem to be conserved in different plant species (Calderón-Vázquez et al., 2011). E.g. ZmPHR1 was also found to be key regulator of P-starvation in maize (Wang et al., 2012). Some regulators such as PHO2, SIZ1 and PHR1, seem not to be transcriptionally regulated (Roldan et al., 2013; Thibaud et al., 2010), whereas Mt4-like and PHO/SPX transcription factors seem to significantly up-regulated by P-deficiency in maize (Calderon-Vazquez et al., 2008). A list of P-starvation related regulatory genes (Calderón-Vázquez et al., 2011) was used for screening of our own dataset but none of these genes was differentially expressed in our experiment.

To clearly reject the hypothesis on P-related gene expression plants from Exp_11 were analysed for internal Pi and Pt status (3.11.2.2.3). Pt values were decreased in the Px treatment, but interestingly, Pi values increased. Pi is discussed to be involved in long-distance signalling of P related plant responses but it was assumed that external Pi instead of internal Pi levels affect P related changes in root morphology (Svistoonoff et al., 2007). In a

split-root experiment with *Arabidopsis* plants genes were isolated that were termed systemically regulated (responsive to internal shoot Pi levels) or locally regulated (responsive to local Pi levels in the medium or the root) (Thibaud et al., 2010). Systemically regulated genes were related to Pi transporters or Pi re-mobilization and translocation whereas locally regulated genes were connected to stress responses. As plants in the Px treatment had high values of internal shoot Pi it is reasonable that we did not observe changes in the systemically regulated genes. In contrast, overall gene expression response was more stress than P-deficiency related and fit therefore to the results of Thibaud et al. on locally regulated genes.

Nevertheless, it remains unclear which factors were causing the stress responses in the Px treated plants. Either plant roots were sensing a reduced soil P level or biotic stress due to sink stimulation (see 3.14.9.1.1) or induction of ISA/ SAR. Interestingly, a negative interaction was not observed in the 50 % plants and also in the Exp_23 no reduced P uptake in the Px treatments was observed. Therefore the higher humidity in the Px_70 pots was interacting with the Px treatment. As discussed in 4.2.3.2 the formulation showed clear effects on plant growth and results suggest a "soil priming" effect that enriches the plant-beneficial microflora in the soil to be the causal mode of action for this observation. These responses were seen for plants growing under optimal conditions with temperature > 20 °C. Nevertheless, growth depression effects were observed in other experiments before and it is possible that they are connected to trade-offs with the natural microbial community. Unfortunately, the observation of specific gene expression pattern were not consistent in all Px root samples, visualized by the correlation plot for the DEGs (Figure 3 108 and 3.12.7.2).

3.14.8 BE-specific differences

Both BEs showed similar tendencies for up-regulation of stress-related genes. Nevertheless, the effect of the Px treatment was more pronounced, especially when focussing on the DEGs. Px seemed to have more influence on transcriptional regulation indicated by up-regulation of various TF families in MapMan/PageMan. Px was additionally down-regulating a BIN of major intrinsic proteins. Subfamilies of these MIPs are found either in the plasma-membrane (PIPs) or in the tonoplast (TIPs). PIP aquaporins seem to determine root water uptake and some PGPR modified aquaporin abundance under various conditions (Marulanda et al., 2010). However, the mechanism behind this is not investigated yet.

Rz seemed to have more influence on protein metabolism than the Px treatment but they both show similar tendencies. Both treatments induced ubiquitin-mediated protein degradation and

repressed protein synthesis in the roots. Unfortunately, no data on shoot gene expression are available to see if responses are similar in both organs. It is possible that these shifts were a general response to stress but it could also be a way of changing sink status of the root thereby increasing P and N transport or remobilisation to the shoot.

3.14.9 Metabolome

3.14.9.1.1 H-NMR

Gene expression and metabolite data do not necessarily correlate (Amiour et al., 2012). Some proteins may not be regulated transcriptionally but via post-transcriptional changes resulting in low correlation between proteomic and transcriptomic data. Additionally, protein levels do not necessarily determine metabolite contents. This may be influenced by environmental factors such as light or nutrient availability that limit protein activity. E.g. several enzymes depend on mineral co-factors that determine their activity. A treatment that is directly influencing plant gene expression by hormonal regulation but also influences nutrient availability may affect metabolism in a contrasting way. Additionally, enzymes, receptors and transmembrane transporters often are regulated via phosphorylation by specific kinases.

Nevertheless, certain changes in metabolism were detected that fitted well to our stress hypothesis. Concentration of shikimic acid, the central substrate in the shikimic acid pathway for the biosynthesis of aromatic amino acids, was increased in most of the BE treated samples. Also the products of the pathway, phenylalanine, tryptophan and tyrosine, were up-regulated in most of the samples. As mentioned before, phenylalanine is the main precursor for phenylpropanoid biosynthesis. Induction of the pathway and increased levels of aromatic amino acids are therefore commonly observed under abiotic and biotic stress conditions (Doehlemann et al., 2008; Obata et al., 2015). Also other metabolites involved in the phenylpropanoid pathway were induced in the Px treatment. 4-hydroxybenzoic acid is a homologue of salicylic acid and is involved in stress responses. Chlorogenic acid is the ester of caffeic and quinic acid and is an intermediate metabolite in the biosynthesis of lignin. Chlorogenic acid is induced by wounding and confers resistance against herbivores in maize (Dixon and Paiva, 1995; Erb et al., 2009). It was also up-regulated by low N and P but not low temperature (Schlüter et al., 2013). Gallic acid is a precursor of tannin biosynthesis and shown to be induced by *Pseudomonas* sp. strains leading to improved plant resistance in pea and chickpea via SA-pathway (Singh et al., 2003, 2002). The induction of tryptophan biosynthesis and exudation by roots was proposed as a mechanism for elevated bacterial auxin production and subsequent root growth stimulation (Glick, 2014). Strangely, tryptophan

levels in the roots of Rz treated plants were decreased. It was shown that the Rz strain is producing higher amounts of auxin when tryptophan was supplied to the medium (Idris et al., 2007). It is possible that tryptophan was already metabolized by the Rz strain leading to depression in the root samples. The elevated concentration of malate (malic acid) was already addressed in 3.14.7. In general, all of the carboxylates produced in the TCA were up-regulated in the Px treatment. The osmoprotectants choline, glycine-betaine and trigonelline, involved in tolerance to abiotic stress (Ashraf and Foolad, 2007), were induced in some of the BE treated samples and in comparison between Exp 11 and Exp 14 (cold stress).

Sucrose is known to be transported to the root during P-deficiency (Schlüter et al., 2013; Song and Liu, 2015; Wang et al., 2015). The Px treatment was leading to increased concentrations of sucrose in the roots and a depression in the shoots. This could be explained by sink stimulation and trade-off for assimilates as already mentioned for Exp_4 and Exp_5 (0). A more detailed discussion on this topic is given in 4.3.3.1. A decrease in shoot sucrose level was also measured in Exp_11 as compared to Exp_14, possible due to lower temperatures and a lower photosynthetic activity. Glucose was only suppressed in the Rz treatment. Concentration of other sugars, such as fructose and raffinose, were increased in the Px treatment. An increased concentration of raffinose was also observed in P-deficient Arabidopsis plants (Pant et al., 2015). But again no P-deficiency specific responses, such as the reduction of phosphorylated metabolites (Pant et al., 2015), were detected. This reduction seemed to be well correlated with the soluble Pi concentration in the plant (Schlüter et al., 2013) and results from our P analysis have proven that Px treated plants did not show reduced Pi concentration. Taken together the results indicate stress responses in the plant that are probably connected to SA-pathway.

3.14.9.1.2 Phenolics

We investigated phenylpropanoids using liquid chromatography with mass spectrometry ion trap time of flight (LC/MS-IT-TOF) in the laboratory of the partner group in Italy (*data not shown*). Methanol/water extraction was used to extract metabolites from ground root or shoot material. For substance separation ultra-high pressure reverse Phase LC (UHPRPLC) with acidic H_2O and acetonnitrile as mobile phase on a non-polar octadecylsilyl (C18) stationary phase was used. Nevertheless, this was the first time that maize samples were analyzed in the lab and problems with peak separation occurred. Due to limited time a method optimization was not feasible. Additionally, no database for peak annotation was established in 2016. For manual annotation of single compounds according to their mass the program ChemSpider was

used. But in many cases single peaks contained multiple masses and the sensitivity of the IT-TOF was too low for an exact annotation of the compound (5-10 ppm). Therefore multiple candidate compounds were suggested. The formation of derivatives by hydration, glycosylation or methoxylation, formed naturally or during the process of ionisation in the IT-TOF, or polymerization (formation of dimers of the same compounds or from different compounds/ fragments or derivatives) further increases difficulties in peak interpretation. In the lab were no standards for typical maize secondary metabolites available and therefore exact annotation and quantification was not possible. Further compound fragmentation by MS/MS procedures for better annotation was also not possible due to lack of time. A rough comparison of the root and shoot spectrograms indicated clear differences but not the comparison among different treatments. Several very intense peaks in all samples but especially root samples were annotated as benzoxazinoids but they also did not show a clear response to BE treatments.

3.14.10 Conclusion

Ethylene signalling pathway was induced by BE application, together with a multitude of various transcription factors. Also JA pathway was activated indicated by the four JAZ genes that were differentially regulated. Nevertheless, both RNA-Seq analysis and H-NMR indicate that the SA-pathway was strongly involved in the plant responses. Some of our results indicated similarity to P-deficiency responses but no activation of specific P-starvation genes or reduction in phosphorylated metabolites were observed.

It is known that abiotic and biotic stress are linked via hormonal regulation. For example, induced expression of a dehydration-responsive gene in Arabidopsis plants by application of the PGPR *Paenibacillus polymyxa* coincided with increased tolerance to drought stress (Timmusk and Wagner, 1999). Conversely, P-deficiency in Arabidopsis induced the jasmonic acid (JA) pathway and enhanced their defense against insect herbivory (Khan et al., 2016). The induction of the JA signalling pathway in response to P-starvation seems to be required for inhibition of primary root growth in Arabidopsis (Chacón-López et al., 2011). Also under cold-stress parts of the SA pathway are activated (Miura and Furumoto, 2013).

The results in total are not surprising due to the multitude of environmental factors that were influencing plant gene expression under non-controlled growth conditions that were in many aspects similar to those observed in field experiments. Especially the low temperatures and the non-sterile soil substrate were factors that markedly influenced the outcome of the experiment as compared to most previously published studies. Activity of PGPR can be assumed to be decreased under these conditions and a clear distinction of signalling pathways cannot be expected in such a complex environment. Considering the Px “soil priminig” effect or studies on shifts in the soil microbial community by PGPR application, investigations on induction of strain-specific signalling pathways (e.g. ISR vs. SAR) or hormonal regulation are not feasible under these conditions. Additionally, as previously reported, plant gene expression responses to PGPR application differ strongly between plants that are free from pathogen infection as compared to those that are infected (Cartieaux et al., 2008; Verhagen et al., 2004). This is the typical “defence priming” effect only observed for ISR but not for SAR. Nevertheless, under non-sterile growth conditions any pathogen present in the soil might strongly influence the outcome of this “defence priming” and therefore strong differences among biological replicates can be expected. In conclusion the BE treatments induced stress responses with activation of various signalling pathways.

Priming for abiotic stress tolerance and plant defence may be linked and influence also plant growth. Growth depression by PGPR application might be due to a trade-off between up-regulation of plant defence and investment in growth (Karasov et al., 2017; Pieterse et al., 2009). The investment in secondary metabolites costs energy that may reduce plant growth (Straub et al., 2013b). Nevertheless, stress-derived induction of ethylene stimulates also the biosynthesis of other hormones such as auxin (Song and Liu, 2015). At intermediate harvest mainly a stimulation of the ethylene pathways was observed but it is possible that during later plant development the auxin pathway was activated. Additionally, the inactivation of cytokinin by glycosylation is reversible and may therefore trigger shoot growth later on (Argueso et al., 2009; Behr et al., 2012).

Recently, a new publication on plant gene expression and hormonal regulation in maize after application of a microbial consortia product (Combi A, see 4.3.2.5) was published (Moradtalab et al., 2020). Main differences to our experiment were (1.) the application of a more controlled temperature regime and a controlled application of cold stress (low RZT), (2.) use of a consortia BE product instead of the single inoculants Rz and Px, (3.) a postponed harvest time (analysis of plants after 6 weeks of growth).

Under these conditions the Combi A product increased ABA/CK ratio in the root and stimulated plant IAA biosynthesis in root and shoot. Additionally, elevated concentrations of JA/ SA were measured, indicating ISR. The BE treatment increased also the concentrations of typical cold stress protectants such as proline, sugar and antioxidants in roots and shoots together with an eleveated SOD and POD activity. These effects were correlated with shifts in the gene expression of the genes involved in hormonal signalling and transport. Upregulation was found for ZmTSA (auxin biosynthesis), ZmPIN1a (auxin transport and lateral root formation) and ZmARF12 (auxin signal perception). Downregulation was found for ZmIPT4 and ZmIPT5 (cytokinin biosynthesis) whereas the expression of a gene responsive to exogenous IAA supply (ZmIAA5) was not differentially regulated, suggesting that auxin production of BEs was not the mode of action.

Especially the strong influence on the whole auxin biosynthesis and signalling pathway differs from our observations. Nevertheless, this may be due to the postponed harvest time. The observed shifts in gene expression, including an activation of the ethylene signalling pathway, could also promote plant growth and influence auxin production as observed by Moradtalab et al. (2020). This was explained using a biphasic and concentration dependent model for ethylene signalling and plant growth regulation (Pierik et al., 2006).

3.14.11 Recommendations for future studies

In the gene expression study a somehow new approach was used by investigating whole transcriptome data instead of differentially expressed genes only. The results showed relatively good overlap with those obtained for DEGs, supporting the approach. Nevertheless, in RT-qPCR none of the DEGs could be confirmed leaving us with the open question on the reliability of the results. Due to the complexity of the environmental influences under non-controlled conditions an early harvest approach (with the idea of isolating primary effects) was not an effective strategy and therefore later harvest is recommendable (see 3.14.10). A clear definition of experimental hypotheses should also define the experimental conditions under which a gene expression study needs to be performed. Objective of our experiments was the investigation of PGPR-derived responses in maize plants when grown under non-controlled "applied" conditions. A more precise definition of the research objective in future investigations is therefore recommended.

It should be defined if biocontrol (especially antagonism and antibiosis against specific pathogens), biofertilization (solubilization of minerals, nutrient uptake in the plant) or biostimulation is of interest. For biocontrol or biofertilization gene expression studies are not useful to investigate the mechanism. Instead for biofertilization specific fertilization regimes should be compared.

If the research focus lies on biostimulation it should be defined previously if ISR or hormonal stimulation is the target of interest. Both aspects were investigated in detail in various plant species and for different PGPR. Nevertheless, a transcriptome-wide gene expression study for maize after application of different PGPR was not yet published. Therefore our results would be of interest but the quality is low due to the adverse environmental conditions. Several other questions were already objective of published studies:

1. Influence of PGPR derived compounds (AHLs, VOCs) and phytohormones (derivatives or chemical homologues) on gene expression and primary/secondary metabolome of different plants (Hao et al., 2016; von Rad et al., 2008).

2. PGPR-specific effects were compared directly in research articles or reviews.

3. Time-dependent studies: Plant gene expression and metabolism are differentially regulated during plant development. Additionally, PGPR application might induce changes in a time-dependent manner in response to root exudates or population density. Observed gene expression response only represent a momentary picture of the plant status but cannot explain

the mode of action for a process that causes plant growth stimulation. In several studies this problem was addressed by sequential harvest times (Fröhlich, 2008; Hiruma et al., 2016; Petti et al., 2010; Sanchez et al., 2005; Takahashi et al., 2013; Verhagen et al., 2004; von Rad et al., 2008; Weston et al., 2012; Zhang et al., 2007). Nevertheless, this procedure does not necessary reveal the key regulatory mechanism. As mentioned before, determination of a mode of action is, in contrast to synthetic pesticides or to a lesser extent symbiotic plant-microbe interactions (e.g. AM/rhizobia, biotrophic pathogens), for PGPR often not feasible (Yakhin et al., 2017).

Other aspects that were not addressed up to now are:

1. Screening of crop plants: Basic research is still focussing on the use of the model plant *Arabidopsis thaliana*. Especially with the focus on -omic studies it is therefore recommended to add the model plant in an experimental approach with a target crop plant to compare the outcome of the interactions under the same environmental conditions. Additionally, the comparison of PGPR-derived responses in different crop plants would give much higher value to the research question.

2. Screening of substrates: A direct comparison of different sterile substrates (e.g. sterile soils) would also be interesting to investigate influence of soil physico-chemical parameters on PGPR-plant interactions. In non-sterile substrates PGPR-derived plant growth stimulation should be correlated with the shift in the microbial community. This was also planned inside the Biofector project. The working group at JKI performed microbial community fingerprinting in maize and tomato after application of the Px and Rz strain. The same soil substrate was used in some of our experiments but in our institute the effects of the Rz treatment could never be reproduced. Therefore it can be assumed that conditions were different and a correlation is not reasonable.

3. PGPR-plant interaction under various environmental conditions: Biotic and abiotic stress responses are connected via hormonal pathways. Combination of multiple stress factors showed completely different results than those observed for the single stress factors (Mittler, 2006). Climate or light specific investigations are also necessary, as e.g. indicated by the fact that tryptophan (Trp) exudation is largely light dependent and that auxin production is Trp dependent. Also here sterile substrates are more valuable.

4 General discussion

In the results chapter each experiment was individually discussed. In the following discussion chapter all results from different experiments are summarized to address overall hypotheses or research questions.

As the variety of experiments conducted already indicates, this thesis aims to connect basic and applied research. The Biofector project that was conducted with several partners from the fertilizer and biofertilizer industry was clearly positioned with the scope on applied research. From the 11 work packages only one work package was focussing on mechanisms and therefore basic research. This work was done with the focus on the four work packages “WP02: Product combination”, “WP03: Functional mechanisms”, “WP04: Abiotic stress” (in our group with focus on cold stress) and “WP08: Application in the field”. Especially the work packages WP03 and WP08 differed strongly in their research focus and were therefore hard to combine. One approach was to include applied conditions in the basic research, e.g. by using non-sterile soil substrates for gene expression studies or the usage of formulated BE products instead of pure liquid cultures as normally done in published articles. The second approach was to address basic research questions, like root colonization or prebiotic properties in BE combinations, in field experiments. By this we were able to increase methodological knowledge as well as knowledge about limitations of methods. Additionally, the results may provide new insights on the relation between basic research findings and their implications or relevance for the agricultural praxis. Nevertheless, these advantages are coming with a loss in accuracy and reliability from a statistical and methodological point of view. Some recommendations for future gene expression studies were already addressed at the end of the last chapter (3.14.11).

The discussion part starts with a description on possible mechanisms of action by which BE application stimulated plant growth and increased yields. In 4.2 the results from several meta-analyses conducted for PGPM or biofertilizer application are reviewed. Thereafter biomass and plant growth results (effectiveness) are discussed. The subchapter ends with a small economic evaluation for the BE products tested in this thesis (efficiency). Subchapter 4.3 discusses environmental conditions and application techniques for successful BE applications in agricultural practice. In 4.4 overall results are summed up in a prolonged summary.

4.1 Mechanisms of action

4.1.1 BE functional traits

As mentioned in the introduction, BE products can be classified by their function or application purpose into the three categories biofertilizers, biostimulants and biocontrol agents. Nevertheless, in most cases BE functions cannot be separated clearly, especially not when products are applied in the field or in non-sterile substrates. The concept of multiple mechanisms adding up to the total effect observed was introduced as “additive hypothesis” for *Azospirillum* (Bashan et al., 2004) and is also supported by the experiments on ammonium, micronutrients and consortia products (Moradtalab et al., 2020).

Even more complex is the determination of a single mode of action that caused an observed plant growth stimulation (Richardson et al., 2009). Due to the difficulty of determining a *mode* of action it was recommended to focus on the *mechanism* of action (Yakhin et al., 2017). The difference here is that instead of focussing on a single elicitor / bioactive molecule and its respective target gene / plant receptor and signalling pathway (as often possible for synthetic pesticides), the response in the plant is observed. The information on the mechanism is also more crucial to determine the objective of BE application.

Due to the immense impact agriculture has on environmental systems, BE applications especially have implications for environmental protection and sustainable resource management. In this aspect the improvement of phosphorus use efficiency is one example for BE application that may reduce P input into agriculture and transport to environmental systems by accelerating P cycling from waste products or sparingly soluble P sources in the soil. Many agricultural soils in Europe have accumulated large amounts of P (Grizzetti et al., 2007; Raymond et al., 2020) that often are not plant available. Therefore, one major objective of the Biofector project was the investigation of BEs for their ability to improve P-acquisition from sparingly soluble P-sources. Nevertheless, for this objective not only the ability to solubilize P but also the ability to improve plant health, root growth and tolerance to abiotic stress are of importance.

In the following subchapters certain processes or BE traits that may have contributed to plant growth promotion or nutrient acquisition in our experiments will be discussed in detail.

4.1.2 Nutrient availability

4.1.2.1 BEs stimulate root growth

Probably the most common, and most important BE trait for improving nutrient acquisition (Raymond et al., 2020; Vessey, 2003; further discussed below), is the stimulation of root growth. Although root length, root density and number of lateral roots, together defining root system architecture, are probably better measurands to estimate the potential of a plant for nutrient acquisition (Richardson et al., 2009), root weight measurement was often the only feasible method and therefore the most frequently measurement for roots in this thesis. Additionally, in most experiments in which root length was determined it correlated well with root weight.

In 18 experiments and 41 treatments with the Px product root dry weight was measured. In eight treatments a reduction in root weight as compared to the control was found. In 33 treatments root weight was increased, in 23 treatments by more than 10 %. Root growth stimulation by Px was well correlated with the increase in shoot biomass and nutrient uptake, especially of phosphorus. In almost all cases in which shoot biomass was increased by more than 10 % in the BE treatment also root growth was equally or even more increased. Exceptions were the Zn/Mn priming in Exp_1 under cold stress and application of the Bsim strain in the same experiment. Both treatments were decreasing root growth although they increased shoot biomass (not significantly). Possibly other mechanisms, such as stress alleviation, were more pronounced here. No other product was able to consistently improve root growth, as results for other BEs were less reproducible (BE efficacy will be discussed in 4.2.2). However, during intermediate harvest in Exp_18 Rz treated maize plants showed a significantly increased root growth three days before shoot biomass was increased (Figure 3-51), indicating a causal relationship.

Common modes of action by which root growth may be stimulated are the production of auxins (IAA) and the reduction of ethylene by ACC deaminase activity. There is no information on ACC deaminase activity in the Px and the Rz strain and for the Pj strain no gene coding for ACC deaminase was found (Kuzmanović et al., 2018). However, the potential to produce auxin was proven for the Px, Pj and the Rz strain (Kuzmanović et al., 2018; Mpanga et al., 2019a; Talboys et al., 2014). Other compounds possibly influencing plant growth and root system architecture are bacterial AHLs (Ortíz-Castro et al., 2009), produced by some Pseudomonades for quorum sensing (Elasri et al., 2001; Venturi, 2006) or

volatile organic compounds (VOC) such as 2,3-butanediol (Bailly and Weisskopf, 2012), also produced by the Rz strain (Fan et al., 2012).

4.1.2.2 BEs for increasing soil P availability

As described in 1.4.2.3 improvement of P-acquisition is an important trait of PGPRs and the investigation of the BEs for this potential was one focus of this thesis.

Indeed in most of the 14 experiments in which plant P concentrations were analyzed Px application led to increased P contents in plants. This fits to the often proposed mechanism as phosphate solubilizing microorganism (PSM) (Richardson et al., 2009; Sharma et al., 2013).

As explained above, growth stimulation was normally the first response observed, often causing reduction in P concentrations during intermediate but also end harvests. Therefore P contents did not positively correlate with P concentrations. Nevertheless, this was also frequently observed under higher P fertilization. The increased P contents can be explained by several mechanisms. First, an increased root growth improves nutrient acquisition, especially of the sparingly soluble P. Furthermore, with increasing root length overall mycorrhization increased although colonization rate was not significantly increased. Second, the improved root growth in tomato plants treated with the microbial BEs Pj, Px and Rz coincided with an elevated release of phosphatases, especially acid monophosphoesterase that are produced by plants (Eltlbany et al., 2019), thereby targeting organic P pools. Third, an increased shoot growth, often seen at the same time and not in later stages in response to improved root growth, leads to an increased water uptake due to higher biomass and evaporation, increasing nutrient uptake. Although P uptake by plants is mainly working via diffusion, a combination of mass flow and a continuous re-supply with water (as done in pot experiments by regular watering) may improve P acquisition. This is also seen in Exp_22. Here re-watering of the plants was strongly increasing Pi concentration in the shoots of the Ctrl plants 10 DAS after a break in watering due to the planned BE application 9 DAS. In the Px treatment, nevertheless, Pi concentration did not increase in the roots. A reason is probably that the milk powder of the Px product causes a reduced penetration of water into the soil. This effect is only seen during application and probably does not last long but may have influenced the Pi status 10 DAS. Fourth, an elevated activity of alkaline phosphatases, either produced by an modified soil microbial community (see 4.1.4.3) or the inoculated PGPR, was measured in the rhizosphere of tomato plants (Eltlbany et al., 2019). Therefore direct P-acquisition by microbes from organic P pools to contribute to plant P availability cannot be excluded. Similar results were found for maize plants (Mpanga et al., 2019b). Nevertheless, when measured at rhizosphere

pH, differences in enzymatic activity as compared to the control plants vanished in the later study. Therefore it was concluded that the BEs did not directly contribute in the mobilization of P for the host plants.

Fifth mechanism is the solubilisation of P from inorganic P sources such as Ca-P. Nevertheless, in several experiments in our working group negative effects on plant P concentrations and contents after BE application were observed. Indeed, experiments on undiluted and nitrate-fertilized C-loess led to decreased plant growth after BE application (Kuhlmann, 2014; Nkebiwe, 2016; Probst, 2015). Additionally, P concentration and therefore P contents in those experiments were decreased by BE application. It seemed that BEs were competing for P in the soil causing negative effects on plant performance.

4.1.2.2.1 Influence of N-form on BE efficacy and nutrient availability

Efficacy of PSMs is known to be N-form dependent (Richardson et al., 2009). Mobilization of P could be increased by ammonium fertilization in studies with *Penicillium* and *Pseudomonas* (Noor et al., 2017; Whitelaw et al., 1999).

As often proposed in literature, under ammonium nutrition effectiveness of BE application might be increased due to an increased uptake of ammonium by the microbes and thereby an elevated exudation of H^+-ions into the rhizosphere decreasing pH. Nevertheless, as already mentioned in the discussion of experiment 17, only in few experiments in our institute a significant decrease in rhizosphere pH after BE application (Mpanga et al., 2020) was found and therefore relevance of this process is questionable (Mpanga, 2019; Mpanga et al., 2018, 2019; Bradáčová et al., 2019; Moradtalab et al., 2020). Mpanga concluded therefore: “Obviously, only in this exceptional situation, the combined effects of the acidic soil pH, low soil pH buffering and increased proton extrusion by roots and inoculants were sufficient to mediate significant additional solubilization of Rock-P in comparison with the non-inoculated control, while in all other cases the inoculant effect was not detectable.” (Mpanga, 2019, p. 148) Instead, ammonium nutrition was increasing auxin production in *B. amyloliquefaciens* (Rz), probably leading to root growth promotion and therefore higher P uptake.

This conclusion fits to the results of a recent review on PSMs (Raymond et al., 2020). The reviewed studies on P-solubilization capacities of PSMs show contrasting results and under field conditions reproducibility strongly decreases. Possible explanations that were named are the competition with other microorganisms, the soil pH and soil buffer capacities (Gyaneshwar et al., 1998) and the limited availability of C in the soil that is necessary for

microbial metabolism and production of organic acids for P-solubilization. Therefore the expression of P-solubilization traits is linked to various other aspects that influence BE-plant interaction such as the assimilate supply via root exudation (4.3.3) and the trade-off between defence activity and metabolism (shortly addressed in 3.9.1.4.1) that was also described for plants (4.1.6.3). Nevertheless, the authors emphasize also the importance of the soil microbiome for general P-cycling and therefore suggest approaches that target a diverse, healthy and active soil microbial community. This is especially interesting as PGPR application is influencing the composition of the microbial community (see 4.1.4.3).

In general, investigations on P-solubilization capacity of PSMs under applied conditions are difficult because with most experimental designs a differentiation between specific plant beneficial activities, such as P-solubilization, root growth stimulation or disease suppression, is not possible (Richardson and Simpson, 2011). A direct contribution of PSMs to P-solubilization can be determined by analysis of plant-free soil substrates after incubation with those microorganisms but any interaction with the plant, such as stimulation of population growth by root exudates, is here excluded. P-fractionation analyses can provide further information on the P-sources that were main target of PSM activity. This was done in experiments with the Px and Rz strain and different recycling fertilizer (Wollmann et al., 2017). Under the experimental conditions both strains were not able to increase plant P concentrations, contents or biomass. Some unpublished results indicated that the Px strain decreased the organic P contents in some of the P fractions but the results were not consistent over the different fertilizer (*personal communication with I. Wollmann*). Nevertheless, it still needs to be determined in how far solubilized P will be incorporated as P_{mic} or is available for the plant. Isotope labelling of nutrients is often the method of choice to study nutrient uptake efficiency and nutrient cycling but labelling of recycling or rock P sources as well as soil P are not a possibility. In a study on the Rz strain ^{33}P labelled nutrient solution in soil substrate was used for fertilization of winter wheat (Talboys et al., 2014). The Rz strain was able to stimulate root growth of the plant but decreased P uptake per cm of root length. Additionally, the expression of specific P transporters was repressed (a significant suppression of P transporters was not observed in our data set, see 3.14). Data suggest that growth stimulation of the Rz strain is probably not connected to improved P acquisition.

4.1.2.3 BEs for increasing availability of other nutrients

4.1.2.3.1 Bacterial siderophores for nutrient acquisition

Pseudomonas sp. strains are known to produce siderophores for sequestration of Fe, Cu and Zn (Brandel et al., 2012; Haas and Défago, 2005; Halpern et al., 2015; Kuzmanović et al., 2018). According to the manufacturer the *Pseudomonas* sp. DSMZ 13134 strain (Px) contains the siderophores Pseudomonine and Quinolobactine that are effective in chelating zinc, copper and iron (Sourcon Padena, n.d.). Nevertheless, as discussed for P, their ability to solubilize a mineral nutrient does not necessary translate into an increased nutrient availability for the plant, as for example siderophores are poor nutrient sources for plants (Bar-Ness et al., 1992; Walter et al., 1994). As many authors suggest that pathogen suppression by *Pseudomonades* is connected to competition for mineral nutrients (Kloepper et al., 1980a; Vessey, 2003) and siderophores and some siderophores are strong chelates (Brandel et al., 2012), a competition with the plant is also not unlikely. Furthermore PGPMs might also degrade and consume root exudates that are released by the plant for nutrient uptake, such as organic acids (Marschner et al., 2011) and phytosiderophores (Von Wirén et al., 1993).

Still, PGPRs are reported to solubilize mineral nutrients and improve nutrient acquisition in maize, especially in sterile media (Sharma and Johri, 2003). Zn acquisition in maize was increased by Zn solubilizing bacteria from various species, including one *Pseudomonas* sp. strain (Kamran et al., 2017). The strain was increasing root growth of maize and Zn acquisition from $ZnCO_3$. Nevertheless, also this experiment was conducted in sterile sand substrate and the added amount of Zn was about 6 g kg^{-1}. In comparison, the average value of Zn was about 0.1 g Zn in all soils used in this thesis. Although solubility of $ZnCO_3$ is extremely low (~10 mg l^{-1}) the high concentration might bias the outcome. Another recent research on siderophore-producing *Pseudomonas aeruginosa* strains was focusing on iron acquisition in maize. Here PGPR application was even more effective in promoting iron uptake than fertilization with added Fe (Sah et al., 2017). Nevertheless, no information on soil properties or the source of Fe is given.

Plant shoot analysis of other nutrients than P was performed for experiments 2, 8, 9 and 17. Additionally, plant samples from the JKI experiments were analyzed (Eltlbany et al., 2019). Zn concentrations in Px treated plants were decreased in all experiments except those from JKI. Zn contents of maize shoots were decreased in Exp_8, 9 and 17 but increased in Exp_2 and the JKI experiments. Similar to the observations for P, in most of the cases shoot concentrations of other nutrients were negatively correlated with the plant biomass or did not

significantly differ from each other whereas shoot contents were elevated. Clear evidence for the potential of a certain BE for improving nutrient availability of a specific nutrient was not found. Some trends for improved Mn acquisition in the Pseudomonad treatments, found for single experiments, could not be reproduced in other experiments. Cu concentrations were assessed only in Exp_8, 9 and 17. In Exp_8 no significant differences among treatments were found in shoot tissues whereas in Exp_9 no microbial BEs were investigated. While the Px strain increased root growth, K and P uptake in maize plants of Exp_17, it decreased shoot Zn and Cu concentration and Cu uptake (Weber et al., 2018). Iron, a target of microbial and plant siderophores, showed high standard deviations in most of the measurements, possibly due to contamination of metal scissors or the oven used for ashing, making data interpretation difficult. In conclusion, in most cases nutrient acquisition correlated well with root and shoot growth and therefore a clear differentiation between an increased nutrient availability (biofertilizer) or improved nutrient acquisition due to increased plant growth (biostimulation) was not possible.

4.1.2.3.2 Seaweed extracts for nutrient acquisition

There are various reports on improved nutrient acquisition by plants after application of humic substances (HS) and seaweed extracts (Halpern et al., 2015). Besides the stimulation of root growth, there are also direct influences on soil chemical parameters due to the presence of chelating compounds in the HS or SWE or the stimulation of the plant internal plasma membrane H^+-ATPase, leading to acidification.

Also recent results from greenhouse experiments with maize under water stress, fertilized with ammonium, indicate a better Zn, Mn and Cu acquisition in plants treated with the SWE Superfifty (SF) and Manek via foliar application. Status of all micronutrients was significantly increased as compared to untreated control plants although root growth was similar among treatments (Moradtalab and Wanke, *unpublished*). As seaweed extracts were not applied via soil drenching, it seems that the plant root activity or nutrient translocation from root to shoot was somehow modulated, leading to higher nutrient uptake.

4.1.3 Biocontrol

Disease suppression by PGPR application, including field experiments, is well documented especially for *Bacillus* and *Pseudomonas* strains (Kloepper et al., 2004; Vallad and Goodman, 2004). The *Bacillus amyloliquefaciens* strain FZB42 (Rz) was efficient to suppress *Rhizoctonia solani* infection in lettuce (Chowdhury et al., 2013). The Px product, containing

the *Pseudomonas* sp. "Proradix®" strain, was able to suppress *Fusarium* infection in tomato as well as *Rhynchosporium secalis* and *Gaeumannomyces graminis* root and leaf infections of barley (Fröhlich, 2008; Fröhlich et al., 2011; Yusran et al., 2009). Although no proactive application of pathogens was performed during the thesis, it is probable that in the nonsterile substrates used, biocontrol activity was more or less involved in BE-plant interaction. Pathogens and therefore biotic stress is omnipresent in natural environments. Additionally, it is well known that BEs trigger ISR or SAR as a form of "stress priming" even when pathogens are not present (see 1.4.1.2). A direct involvement of biocontrol activity was also proposed for the experiments Exp_6 and the gene expression responses observed in Exp_11 (further discussed in 0 and 3.14.6). In Exp_6 it is also possible that the PGPR were occupying ecological niches that were free after the tyndallisation process or they were actively competing with the soil microflora for nutrients and space thereby stabilizing the system and suppressing the population growth of plant detrimental organisms. For example disease-suppressiveness of soils was completely lost after heat treatment at 80°C (Mendes et al., 2011). The involvement of the Px milk powder formulation in this process was mentioned before and will be discussed in the next section.

4.1.4 Interactions with the natural soil microflora

4.1.4.1 Mycorrhiza

Improved mycorrhization or synergistic effects of the combined application of PGPR and mycorrhizal fungi (AMF and ECM) are commonly reported from various studies (MHB) (Frey-Klett et al., 2007; Garbaye, 1994). Some experiments of the Biofector project in which AMF inoculum was additionally applied also indicated that the PGPR increased mycorrhization in tomato plants (Li et al., 2014, *unpublished*). Nevertheless, in tomato experiments in this thesis no mycorrhization was observed although in maize plants that were grown in the same soil substrate for a similar period clear AMF structures in the roots were detected (compare Exp_4 and Exp_5, 0). It is possible that the natural fungal population was not adapted to tomato plants. Recent publications indicate that AMF-plant interaction might be more specific than previously thought (Torrecillas et al., 2012; Yang et al., 2012). Additionally, tomato roots formed very fine lateral roots that seem to be less colonized by mycorrhizal fungi from rice (Fiorilli et al., 2015). However, analysis of mycorrhization was also performed for maize and tomato samples from the JKI experiments (Eltlbany et al., 2019). 50 intersects per sample were checked for mycorrhizal structures. Here tomato roots were colonized. Therefore, it is probable that the adverse light conditions in our experiment

influenced the photosynthetic activity, reduced assimilate production and therefore inhibited mycorrhizal associations as previously described (Graham et al., 1982; Konvalinková and Jansa, 2016; G. Shi et al., 2014) (see also 4.3.3).

Interestingly, in BE treated tomatoes from JKI a tendency for an increased mycorrhization was observed (Eltlbany et al., 2019). Due to the increased root length and good mycorrhization rate in the tomato plants surface area for nutrient acquisition, especially for P, strongly increased, providing one explanation for better plant growth in BE treated plants.

Nevertheless, in our maize experiments (Exp_4, Exp_12) BE application did not promote mycorrhization, although an intense mycorrhization was observed. Additionally, in both experiments a decrease in mycorrhization in the P_Ctrl due to P fertilization, as often reported for mycorrhiza (Graham et al., 1982; Marschner, 2012b), was observed, suggesting that P status in our experiments was not too high to inhibit any BE-mycorrhiza plant interaction. This finding supports the reliability of the analysis and methodology applied and suggests that the BE products were not able to stimulate the natural soil AM fungal community. In maize plants of JKI BEs also decreased mycorrhization, especially the Rz product (*unpublished*). The decrease in mycorrhization of maize plants can be explained by the increase in root biomass and root length by more than 100 % in the Rz treatment but still there is no evidence that BEs were stimulating mycorrhization in this context.

It is probable that the natural soil bacteria were better adapted to the soil AMF than our inoculated strains. In contrast, if both partners are newly introduced into the soil environment or into sterile substrates, lacking any other interaction partner as done in many of the above mentioned publications, the interaction might be much more efficient.

4.1.4.2 Px soil "priming"

The formulation used in the Px product is rich in potassium and phosphorus as well as other nutrients (Table 2-1). To quantify the impact of the additional nutrients on plant performance the company Sourcon Padena, producer of the Px product, was providing the institute with the pure, Pseudomonas-free powder. A maize pot experiment with different application rates and the pure formulation powder (MP) as additional treatment factor was conducted in our institute (Nkebiwe 2015, *unpublished*). Results showed, that the effect on the maize plants was increasing with higher inoculation densities and that the effects of the Px product and the MP were equal for low to medium application rates but at very high application rates the Px product was more effective in maize growth stimulation than the MP.

These results indicate that a major reason for the effectiveness of the Px product under our experimental conditions, was a response to the formulation and not of the PGPR itself. This is especially interesting, keeping in mind, that the Px product was in most of our experiments the most effective product. Nevertheless, at low application rates nutrient input (e.g. N, P, K) by the MP, as compared to the soil nutrient levels together with the additional fertilization, was still relatively low under the aspect of plant fertilization.

Other mechanisms are therefore much more probable. 1. Milk powder is rich in sugars and amino acids, especially glutamate. Glutamate was found to influence root system architecture (Canarini et al., 2019). Sugars are involved in phosphate starvation responses and the external application in a medium promoted primary and lateral root growth in *Arabidopsis* plants (Karthikeyan et al., 2007). The direct influence on the plant might be increased under low light conditions or other stress factors causing reduced photosynthetic activity.

2. As seen for the standard deviations in many experiments, Px application was somehow reducing variability inside the treatment. Significant growth stimulation of the *Pseudomonas sp.* population by the milk powder formulation, even at very low concentrations, was shown in our *in vitro* studies (Figure 3-28, also named MP). Additionally, the impact of nutritional sources, especially glucan poly- and oligosaccharides of milk, was shown in the medical studies or studies in food technology for the gut microbiomes of humans (Pacheco et al., 2015). Data suggest that the MP is selectively increasing the cell density of, obviously beneficial, soil bacteria leading to a more stable plant growth and growth promotion. *In vitro* experiments also showed that cell division rate of the Px strain, as example for Pseudomonades, was much faster than that of the Rz strain (as also reported for Exp_21). Nutrient “priming” by MP in the soil could therefore especially promote *Pseudomonas sp.* growth. The term “priming”, when used in the context of soil organic matter (SOM), refers to an accelerated turnover of SOM due to an input of fresh organic matter (Lavelle and Gilot, 1994; Neumann and Römheld, 2012, p. 360). Priming may be caused due to an increased amount of oxygen in the system or a shift in the C/N ratio by fresh organic matter of different origin and by selective boost of microbial growth leading to changes in the composition of the microbial community. Several studies are also focussing on the rhizosphere priming by rhizodepositions, referring to any kind of root-derived input of organic matter as described in detail in the introduction. The gram(-) group of Pseudomonades shows generally much higher abundance in the rhizosphere than Bacilli (gram(+)), as supported by our analyses of root colonization. Abundance of gram(-) bacteria was increased in planted as compared to

unplanted soil because they were preferentially using the labile C-pool from rhizodepositions whereas abundance of gram(+) bacteria, using mainly carbon sources from SOM, was decreased (Bird et al., 2011). Same results were also found in other studies, especially when plants were additionally N-fertilized (Paterson et al., 2007). Similarly, easily available carbon sources like sucrose were increasing total abundance of soil bacteria causing a "priming effect" and increased turnover of SOM but, in contrast to the previous study, certain groups of gram-negative bacteria were especially using carbon sources from SOM and not the sucrose (Nottingham et al., 2009). However, both studies were using PFLA without any clear definition of the taxon and also differently grouped PFLA markers into gram(+) or gram(-).

Coming back to Nkebiwe's experiment, results indicate that an application of the Px strain at low to medium application rates was not sufficient to significantly increase the soil population and therefore was not strongly contributing to the growth stimulation effect in the plant as compared to application of MP only. Nevertheless, at high concentration rates the inoculum rate of the Px strain probably exceeded the population densities in the soil and therefore additional significant growth stimulation, as compared to MP application, was observed.

The results in Exp_21 support this theory. Here the application of the KB culture was the only treatment that could promote plant growth, at least for the measurement of the stem diameter significantly, as compared to the untreated Ctrl. This KB culture was enriched in Pseudomonades. In previous study, a soil suspension was far more efficient in phytate mobilization and plant growth stimulation than the inoculation of a single *Pseudomonas* sp. strain, previously shown high phytase activity and potential for plant growth stimulation of different grasses under sterile growth conditions (Richardson et al., 2001b). Even the glucose application in Exp_2 showed some tendencies for an increased *Pseudomonas* population and exhibited stimulating effects on P-Ctrl and Ctrl plants (0). However, as seen in our incubation experiments MP was far more successful in stimulating *Pseudomonas* population than glucose. Lowest concentration of MP tested was 0.001 % (w/w) in the suspension. About the same amount was applied in soils with the lowest application rate of 10^9 CFU kg^{-1} soil (0.02 g kg^{-1} = 0.002 %).

The growth stimulations could also be connected to fastened P cycling as labile C inputs promote microbial turnover (Raymond et al., 2020). Microbial P makes up 5 – 10 % of total P in soils. In a recent experiment of Bradáčová et al., 2019 plant growth beneficial effects by PGPR application could only be observed in freshly collected soils with high organic matter content and biological activity.

4.1.4.3 Influence of microbial BEs on the microbial community

The importance of the microbial composition of soils for plant health is reportedly shown in studies focussing on so-called disease "suppressive soils" (Berendsen et al., 2012; Haas and Défago, 2005). The importance of the *Pseudomonas* spp. group for disease suppression in these soils was previously shown (Adesina et al., 2007; Mendes et al., 2011).

The interaction between applied PGPRs and the natural microflora can be seen by the shift in the soil or rhizosphere microbiome, as shown in various studies (Adesina et al., 2009; Bradáčová et al., 2019a; Eltlbany et al., 2019), although the influence of BEs is usually transient and small as compared to soil type, host plant or plant developmental stage (Berg and Smalla, 2009; Castro-Sowinski et al., 2007; Piromyou et al., 2013, 2011; Qiao et al., 2017). This was also shown for the Px (Buddrus-Schiemann et al., 2010) and the Rz strain (Chowdhury et al., 2013).

Especially the study from Eltlbany et al. is of interest for this thesis, as experimental conditions were coordinated with the working group. Here, all microbial inoculants (in this work called TP, Px, Rz, Pj) were changing the microbial composition of the plant rhizosphere significantly. The changes were not restricted to the respective group of the inoculated BE. In contrast, application of the Rz strain did not increase relative abundance (RA) of the *Bacillus* group 43 DAS and the class *Gammaproteobacteria* was not enriched in the Px or Pj strain treatment but only in the TP treatment. As RA of *Gammaproteobacteria* was about 7 % in all treatments besides the TP, data suggest that densities of the inoculant strains 43 DAS were less than 0.1 % of the soil microbial community. Nonetheless, all bacterial products increased RA of the bacterial classes *Beta-* and *Deltaproteobacteria*, *Bacteroidetes* and *Sphingobacteria*, whereas RA of *Actinobacteria* was decreased. This coincided with an increased soil enzyme activity of alkaline and plant-derived acid phosphomonoesterases in all BE treatments, whereas the latter one was especially increased in the bacterial treatments. Although some similarity in enzyme activity and microbial composition with the bacterial BEs were observed, the fungal TP treatment influenced the RA of several other bacterial groups and did not have strong influence on the acid phosphomonoesterases. In contrast to the other two bacterial strains the Px product specifically increased RA of *Azospirillum* and *Pedobacter*.

The similarity in the changes of the microbial community and especially plant-derived enzyme activity, although the Rz strain belongs to a different phylum (*Firmicutes*) as the Px and Pj strain (*Proteobacteria*), suggests that microbial BE treatment promoted plant root

activity and exudation, thereby providing the basis for other bacterial groups in the rhizosphere to proliferate. At least some of the root exudation is due to passive loss through transmembrane carriers by diffusion following concentration gradients (Canarini et al., 2019). An increased consumption due to a "hungry" bacterial inoculum provides therefore a sink stimulation for plant assimilates (see 4.3.3) and a change in root exudation pattern, especially of primary metabolites that is interlinked with nutrient sensing and modulation of root system architecture building up a complex system of feedback loops as described by Canarini et al.

Nevertheless, the difference in the impact of the different BE treatments on shoot and root growth in the tomato experiment of Eltlbany et al., further suggest that additionally to the general and shared mechanism described above and in 4.1.7, other, not fully elucidated mechanisms were active.

4.1.5 BE-specific mode of action

4.1.5.1 Trichoderma sp.

Trichoderma strains are especially known for their biocontrol properties (Harman et al., 2004; Howell, 2003). Less is known about the mechanisms by which these fungi are able to stimulate plant growth. There are several reports on plant growth stimulation by *Trichoderma* strains, mainly in combination with PGPRs or AM fungi that were also tested in these studies (Badda et al., 2013; El-Katatny and Idres, 2014; Sandheep et al., 2013; Srinath et al., 2003). All experiments, besides Srinath et al., were conducted in sterile substrates. No mode of action for the observed growth stimulation was described by Sandheep et al. Srinath et al. proposed that *Trichoderma* was acting as a "mycorrhiza helper". In the publication from El-Katatny et al. *Fusarium* disease suppression, solubilisation of P and increased N-fixation in co-inoculation with *Azospirillum brasilense* was observed. Badda et al. measured increased acid and alkaline phosphatase activity, P and N uptake in all treatments inoculated with different AM strains, a *Pseudomonas* sp. or a *Trichoderma* sp. strain. Strong growth promoting effects on maize plants were also observed when seeds were soaked in a filtrate from *Trichoderma harzianum* liquid culture (Akladious and Abbas, 2014). Treated maize plants showed higher root and shoot growth, increased chlorophyll, carotenoid, starch, protein, RNA and DNA contents. Additionally, plant hormonal levels in IAA and GA were elevated and ABA levels decreased as compared to the control plants. Unfortunately, the authors do not give a concentration of the liquid culture but only a quantity and do not give any information on the composition of the extract. Nevertheless, higher amounts of the extract showed less beneficial results making a nutritional effect unlikely. Besides the interesting

results, again, no information on the exact mode of action was provided. Instead the authors assume production of IAA or other hormones by the fungi, as suggest for *Trichoderma virens.* This fungus is able to produce, additionally to IAA, two related substances, indole-3-acetaldehyde, and indole-3-ethanol that are all able to modulate root system architecture (Contreras-Cornejo et al., 2009). Additionally, it was shown that *Trichoderma harzianum* fungi are able to solubilize sparingly soluble minerals *in vitro* via metabolites with chelating and redox activity (Altomare et al., 1999).

Besides the various traits, the *Trichoderma* products were not successful in experiments of this thesis. However, root colonization of the *Trichoderma* strain was strongly improved by ammonium fertilization as compared to nitrate (Moradtalab et al., 2020; Mpanga et al., 2019a), suggesting that conditions were not optimal for the *Trichoderma* strain in most of our experiments (and in experiments from Eltlbany et al.).

4.1.5.2 Bacillus amyloliquefaciens

As described above, the Px product formulation largely determined the observed growth stimulation effects and is therefore one important difference to the Rz strain. In general both strains were able to suppress plant diseases (Yusran et al., 2009), promote plant growth under ammonium fertilization and produce IAA *in vitro* to a similar extent (Mpanga et al., 2019a). Both strains showed low potential to reduce cold stress (Bradáčová et al., 2016). In tomato plants, grown under nitrate fertilization and medium P supply the effectiveness of the Rz and the *P. jessenii* strain was much higher than that of the Proradix strain, for unknown reasons (Eltlbany et al., 2019). Results for maize experiments were similar. A transcriptomic profiling of Rz in response to root exudates of maize showed up-regulation of transcripts connected to synthesis of antibiotic compounds, nutrient uptake and metabolism (Fan et al., 2012). Those traits seem not be causal for the plant growth promotion. Transcripts connected to biofilm formation, chemotaxis and motility were also up-regulated. This might explain the very good root colonization potential of the Rz strain (Chowdhury et al., 2013). Additionally, transcripts encoding acetoin reductase/butanediol dehydrogenase was 1.5-fold enhanced by root exudates. 2,3-Butanediol is a volatile organic compound (VOC) released by PGPR that is able to promote plant growth (Bailly and Weisskopf, 2012). Butanediol was found in *P. chlororaphis* (Zhang et al., 2007) but not in *P. fluorescens* or the *P. jessenii* strains (Kuzmanović et al., 2018). Experiments testing various PGPR strains for biocontrol against *R. solani* in lettuce revealed that the *P. jessenii* (Pj) strain was the only one that showed consistent biocontrol activity *in vitro* and in the greenhouse (Adesina et al., 2009).

Unfortunately, also here no specific trait could be isolated that was responsible for this higher efficacy. In contrast, the *Pj* strain lacks several traits that seemed to be promising in *in vitro* screens for efficacious strains. Therefore it remains unclear which mode of action was causing the observed differences between the PGPR strains.

4.1.6 BE products for stress alleviation

4.1.6.1 Micronutrients increase abiotic stress tolerance in maize plants

In Exp_1, Exp_8 and Exp_9 plants were grown under low temperature causing symptoms of cold stress. Different BE treatments were tested for their potential to alleviate cold stress. Only those treatments could effectively reduce stress symptoms, such as leaf chlorosis, that contained Zn and Mn. The mechanism behind this is based on the Zn and Mn-dependent activity of SOD and increased levels of antioxidants and associated accumulation of IAA due to reduction of oxidative IAA degradation (see also 1.2.4). Nevertheless, as later on seen, Zn/Mn supplementation was only efficient under nitrate fertilization whereas under ammonium fertilization effects of Zn/Mn supplementation vanished due to a better availability of these micronutrients by rhizosphere acidification (Moradtalab et al., 2020). Additionally, studies on the seaweed extract SF show that the product is able to improve plant growth of lettuce under salt stress (Guinan et al., 2013) and root growth of maize under drought stress (Freytag and Wanke, 2017; Moradtalab et al., 2017), increasing plant nutrient status and reducing oxidative damage during cold stress, probably by increasing auxin/cytokinin and ABA ratios, increasing the amount of antioxidants, such as proline, influencing plant SOD activity and regulating aquaporin expression (Moradtalab and Wanke, *unpublished*). In *Arabidopsis* plants treated with a herbicide as stress inductor, SF priming (see also 3.10.4.4) was leading to the reduction of ROS-related gene expression but upregulation of carbohydrate metabolism genes, growth, and hormone signalling as well as antioxidant-related genes (Omidbakhshfard et al., 2020). Additionally, accumulation of the stress-protective metabolite maltose and the tricarboxylic acid cycle intermediates fumarate and malate were observed. Nevertheless, in none of the studies (besides Guinan et al.) a shoot growth improvement under stress was reported, as shoot growth depression might be a strategy of the plant to overcome stressful situation (see below).

4.1.6.2 PGPR for abiotic stress tolerance

Bacterial strains that are able to grow under low temperatures can be termed psychrotrophic whereas bacteria that have their optimum below 15 °C are termed psychrophile (Subramanian et al., 2011). Subramanian et al. report on various psychrotrophic bacterial strains isolated

mainly from cold regions of India that were able to promote plant growth under low temperature conditions often in a similar way as commonly reported for PGPR strains (e.g. P-solubilization, biocontrol activity, IAA or ACC deaminase production). Additionally, some strains were able to increase protein, phenolics or anthocyanin production and prevent ice nucleation in plant leaves. Increased levels of phenolic compounds were also found in plants treated with the Combifector A product in the Hohenheim institute (see 4.3.2.5). An increase in the colony size of Px colonies was frequently observed when they were stored at low temperatures of 4 °C, although growth was much slower than at 30 °C in the incubator, indicating that cells were still active. Nevertheless, none of the BE products without micronutrient supplementation was efficient in alleviating cold stress, although later experiments in the institute showed different results for microbial consortia under ammonium nutrition (4.3.2.5).

4.1.6.3 Stress priming and negative BE effects on plant growth

In many experiment in which plants were severely stressed, BE application had negative effects. This was seen in P-deficient soils (discussion Exp_5 and Exp_22), under cold stress (Exp_11) and under heat/drought stress (field experiment Exp_16). It is especially interesting to see that in the field experiment the late application of the products caused negative effects.

Depending on the stress factor plants need different strategies to survive. Under attack of biotrophic pathogens hypersensitive responses and programmed cell death are induced (Jones and Dangl, 2006), whereas mechanical damage, herbivores or necrotrophic pathogens provoke accumulation of secondary metabolites (Pieterse et al., 2014). Under abiotic stress in many cases growth reduction may be the best strategy to reduce water loss, accumulate antioxidant compounds and sugars or increase mineral nutrient concentrations to ensure enzymatic activity (Zn, Mn) - e.g. to alleviate damages by ROS - and functionality of metabolism and photosynthesis. As described, BE application was leading to the induction of stress-responses as part of the “priming” and to the accumulation of antioxidant compounds and changes in hormonal status. Therefore plant growth reduction might be a reasonable first step. In recent experiments by Moradtalab and Wanke (*unpublished*) application of seaweed extracts were done at different time points either as priming before onset of drought stress or during the stress period. Results indicate that the influence on hormonal regulation (ABA, gibberellin, cytokinin and auxin) and plant metabolism differ depending on the application time leading to different dry matter contents in maize shoots. Therefore timing is important. Although there is no general rule for the best application time (Bulgari et al., 2019), it is

reasonable to argue that a treatment that is influencing / targeting the plant and not the stress factor should be applied before the stress is active. Some common stress response for BEs is supported by the ISR mechanism, the often seen up-regulation of stress responsive metabolites (e.g. proline or sugars) and by the results from our gene expression analysis, although each product has further, more specific influences on the treated plant, especially with foliar application, as there is no transformation or feedback by the soil environment. In general, signals from BEs may prime plants to future stress increasing their tolerance by stimulation of secondary metabolism but at the same time they might reduce plant growth. This trade-off between biomass production and plant tolerance or resistance is commonly reported in literature (Caretto et al., 2015; Fritz and Simms, 1992; Neilson et al., 2013; Pieterse et al., 2009; Straub et al., 2013b). “Priming” such as ISR against pathogens or herbivores, is already a low-cost strategy, as the majority of defence reactions are only induced after infection, removing parts of the trade-off dilemma (Conrath et al., 2006). Nevertheless, with the arrival of stress the trade-off starts. In this regard BE application connects with plant breeding, as plant ecotypes or cultivars may differ in their productivity during stress (Bechtold and Field, 2018) and their strategies (Karasov et al., 2017).

4.1.7 Condition-specific traits

The results for the PGPM products indicate that the experimental conditions determined their mode of action. In most experiments in this thesis PGPM-specific traits strongly influenced outcome of PGPM-plant interaction whereas under optimal conditions, such as ammonium fertilization (4.3.4), all PGPMs were effective leading to similar reactions in the plant (Mpanga et al., 2019b). These results indicate that PGPM possess species-specific traits as well as condition-specific traits with conserved mechanisms and modes of action that are shared among various PGPMs such as stress priming, sink stimulation by degradation of root exudates (Kaschuk et al., 2009), community shifts (Eltlbany et al., 2019; Kang et al., 2013) due to sink stimulation, antibiotics, HCN (Fan et al., 2012; Kuzmanović et al., 2018) or various volatile organic compounds (VOCs) (Yuan et al., 2012; Zhang et al., 2007) and plant growth stimulation via production of phytohormones such as IAA (Kuzmanović et al., 2018; Mpanga et al., 2019a; Talboys et al., 2014) or VOCs (Fan et al., 2012; Ryu et al., 2003).

A recent publication for the Rz strain also indicated a condition-specific mode of action of the strain depending on the soil pH. Under ammonium nutrition and low soil buffer capacities the Rz decreased rhizosphere pH whereas at high buffer capacities root growth stimulation was observed (Mpanga et al., 2020).

4.2 Efficiency of bioeffector applications

4.2.1 Meta-analyses on plant-microbe interactions

Results from experiments conducted and described in this thesis as well as the spectrum of plant responses observed give further proof for the already mentioned complexity of BE-plant interaction. Astonishing effects of plant growth stimulation that are often described in peer-reviewed publications on PGPR or biostimulants were not observed in thesis. Instead, the results represent vivid examples for the often reported low reproducibility of BE effects (Dobbelaere et al., 2001; Lesueur et al., 2016b; Richardson and Simpson, 2011; Yakhin et al., 2017). In this section several meta-analyses on BE applications are discussed that present the state of the art for effectiveness of BE application in agricultural practice.

4.2.1.1 Published meta-analyses on PGPRs

In the last 20 years several meta-analyses were conducted to show the overall effectiveness of BE treatments for plant growth stimulation but also to determine the experimental conditions that could increase the probability of a beneficial plant-BE interaction. In most meta-analyses single observations of given response variables, mainly shoot biomass or yield, are used to calculate response ratios by dividing the mean value of a BE treatment by the mean value of the respective non-BE-treated control. Additionally, results from different studies are normalized using the effect size. This value reflects the proportion how much a certain observation was influencing the whole dataset and is often based on the standard deviation of the treatments. Additionally, for each observation information on the experimental conditions is collected. To elucidate certain influencing factors a so-called moderator analysis is performed. Moderators are certain experimental conditions that are shared by single observations such as crop, BE type, soil P status, soil pH, field or pot experiments or the working group that was conducting the experiment. For each moderator the dataset is splitted into subgroups, representing the level of the moderator (e.g. moderator “crop” has the three levels “maize”, “tomato” and “wheat”). Then the effect of BE addition is compared between different subgroups.

4.2.1.1.1 Early studies on N-fixing bacteria

Early studies were focussing on N-fixing bacteria. Yield increase after *Azospirillum* application for many different crops was about 5 - 30 % (Okon and Labandera-Gonzalez, 1994), and studies focussing on wheat concluded that average seed yield was increased by 6.1 (Díaz-Zorita and Fernández-Canigia, 2009) and 8.9 % (Veresoglou and Menexes, 2010). Data

also indicated that effects were in most cases positive, suggesting a relative high reproducibility making the products interesting for farmers.

4.2.1.1.2 Meta-analysis after 2010

Another search for published meta-analyses on biostimulants conducted 2021 in SCOPUS on 'biostimulants OR PGPR AND meta-analysis' (using various other spellings) revealed 12 publications but only 8 publications that were fitting. In contrast, replacing meta-analysis with 'review' resulted in 547 hits, showing the underrepresentation of reliable and quantitative analyses on the (economic) benefit of BE applications but also a lack of information on environmental and experimental conditions enabling successful BE-plant interaction. Of these 8 publications one was focussing on polymer fertilizer enhancer (Jenkins et al., 2018), another one on yeast and amino acids in corn (Da Silva et al., 2017). In one study the impact of polymer-encapsulation on PGPR application was analysed (Pacheco-Aguirre et al., 2017). A fourth study was estimating the success of AM inoculum in coffee bean production (Cogo et al., 2017). A fifth study was analysing PGPR application for alleviation of salt stress with focus on mechanisms and modes of action instead of economic evaluation (Pan et al., 2019). Only three publications were connected to economic benefit, yield effects and biostimulants as used in this thesis.

4.2.1.1.3 PGPRs are most successful under drought stress

Rubin et al. (2017) conducted a meta-analysis on PGPR plant growth stimulation under drought stress using 52 original papers, including 26 papers with 146 observations for yield. Various crops such as maize, wheat, sunflower and lettuce and PGPRs of genera such as *Pseudomonas, Azospirillum, Azotobacter* and *Bacillus* were investigated. Overall yield increase was about 19 %, whereas yield increase was significantly higher under drought (40%). Moderator analysis revealed that effects in the greenhouse were stronger than in the field, unfertilized plants were more responsive than fertilized plants, consortia were similar than single-strain treatments, legumes were most responsive (51 %), C4 grasses only 12 % yield increase, and soil treatment was most effective. In contrast, in greenhouse trials root and shoot biomass were most strongly improved by consortia and seed treatment in combination with organic fertilization, suggesting a low reproducibility from pot to field.

4.2.1.1.4 ACC activity promotes maize growth

Schmidt and Gaudin (2018) conducted a meta-analysis on economic benefit of PGPR application for biofertilization with maize as the only target crop. Using 48 publications from field experiments overall yield increase was between 13 - 18 %. Average yield increase was

highest in arid and dry climates. In the field yield increase was higher under 'high' N soil fertility (> 25 ppm / ~40 kg ha^{-1} inorganic N). In contrast, in pot experiments low N conditions resulted in far better responsiveness. Additional N fertilization did not influence the outcome of BE-plant interaction. Best performing strains were *Azotobacter* (34 % yield increase on average), *Azospirillum, Enterobacter and Pseudomonas* strains whereas *Bacillus* (2 %) strains were less efficient. Consortia products were superior in the field, but less effective in pot experiments as compared to single inoculants. Additionally, the influence of potential growth stimulation traits was investigated. Only the presence of the trait for 'ACC deaminase activity' was significantly increasing effectiveness of PGPR inoculum whereas the traits 'P solubilization', 'N-fixation', 'phytohormone' or 'siderophore' production did not have significant influence but instead slightly decreased growth promotion potential of PGPRs. The authors conclude that predicted efficacy from lab or pot experiments often does not translate into the field and recommend to reverse the lab-to-field pipeline as generally proposed (Backer et al., 2018; Basu et al., 2021). Nevertheless, soil conditions, most important P status, were not analysed or included in the moderator analyses. Additionally, biocontrol was not investigated, eventually explaining the low effectiveness observed for the *Bacillus* sp. strains. In a meta-analysis focussing on biocontrol activity of PGPRs against Bacterial Wilt Disease caused by *Ralstonia solanacearum*, *Bacillus* sp. strains most effectively reduced disease incidence and severity in various plants (Chandrasekaran et al., 2016). Nevertheless, overall yield increase was highest with *Pseudomonas fluorescens* strains.

4.2.1.1.5 Best BE effects under low to moderate P levels and in dry climates

The most comprehensive meta-analysis up to now used a large global dataset of 171 peer-reviewed publications on the application of biofertilizer in field trials, including AM fungi, PSM, rhizobia and various other PGPR and PGPM (Schütz et al., 2018). Overall yield increase by BE application was 16.2 %. Moderator analyses were conducted for climate, BE category, crop, soil organic matter and soil P levels. Dry climate was also here most responsive to BE treatments. AMF fungi and PGPR with multiple known traits, such as N-fixation and P-solubilization were most effective. Consortia products were not superior to single-strain inoculants. Nevertheless, many consortia products contain not only various strains but also micronutrients, silicate or other bioactive compounds such as HS and SWE. Schütz et al. do not provide further information on product compositions. Interestingly, 'P-solubilizer' was the least effective BE group, including some *Bacillus* sp. strains, *Penicillium* and all *Pseudomonas* sp. strains. This is in contrast to the results from Schmidt and Gaudin (2018) on PGPR application in maize. This could be explained by the different dataset. Schütz

et al. (2018) included a huge variety of crops in the meta-analysis. It is possible that the usage of *Pseudomonas* strains, excluding experiments on biocontrol activity, were less effective in vegetables or root crops. Nevertheless, categorization also lacks transparency. For example, *Penicillium* and *Pseudomonas* strains are not related at all and showed very different behaviour in all experiments of this thesis suggesting that they should not be grouped together. Different *Bacillus* strains were grouped into 'P-solubilizer', 'N-fixer', a group with multiple traits or even more than one group. Additionally, rhizobia were not separated from free-living diazotrophic bacteria that were often reported to be inefficient to contribute significantly to biological N-fixation (Bashan et al., 2004; Halpern et al., 2015; Lesueur et al., 2016a). As legumes were the most responsive crop but the overall group of N-fixer less successful, data suggest that free-living diazotrophs were not very effective. This fits to the results from previous meta-analyses on *Azospirillum* mentioned above that resulted in response ratios < 10 %. Increasing soil organic matter was decreasing BE effectiveness. This is somehow unexpected as results from Rubin et al. (2017) indicate stronger effects with organic fertilizers but the finding was explained by the lower microbial activity and therefore less competition for inoculated PGPM. However, also the outcome for the combination of PGPM with organic fertilizer is highly variable (De Corato, 2020; Thonar et al., 2017).

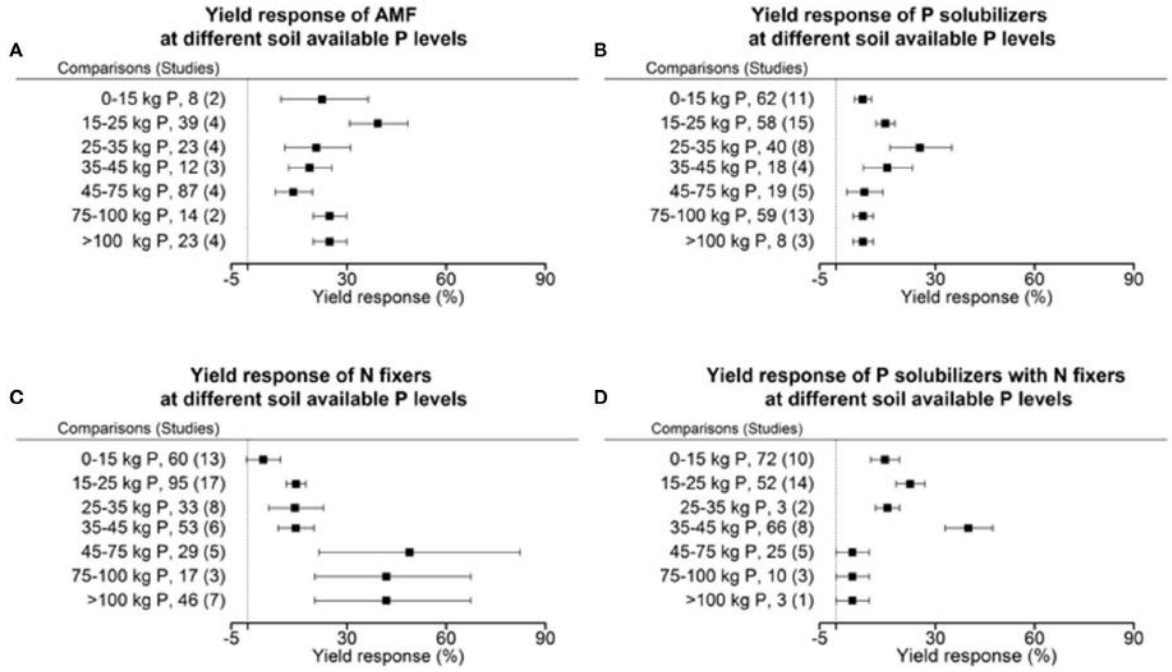


Figure 4-1 Moderator analysis on soil P levels (Schütz et al., 2018)

Interesting results were coming from the moderator analysis on soil P levels. Four BE categories were analysed: 'AMF', 'P solubilizer', 'N fixer' and 'N fixers plus P solubilizers'

(last one either multiple traits or consortia with two strains) (Figure 4-1). AMF fungi were most effective at 15 – 25 ppm P, 'P solubilizer' at 25 – 35 ppm P, 'N fixers plus P solubilizers' at 35 – 45 ppm P and 'N fixer' at all levels above 45 ppm P. These results indicate that effectiveness of individual BE strains depends variably on soil P levels. Furthermore, soils with low to moderate P levels are most responsive to BE effects of the groups used in this thesis. This is also suggested by the results from the Biofector project and the results in this thesis. In the field experiments 2014 and 2015 Olsen P values were 79 and 56 kg ha^{-1}, respectively. That is far above the values that were most responsive for 'P solubilizer' in which *Penicillium* and *P. fluorescens* strains were grouped. *Trichoderma* sp. and *B. amyloliquefaciens* strains are not named by Schütz et al.

The results for the soil P levels are in contrast to the results from a recent African large-scale screening project for biofertilizers and biostimulants, including rhizobia, AM fungi and PGPR (COMPRO). During the project eight PGPR products were tested in pot and field experiments on different crops and soils of different regions in Africa (Jefwa et al., 2014). Strongest effects were observed for tissue culture bananas grown in vertisols or ferralsols with very low soil P values (Olsen P 3 and 7 ppm) (Mwangi et al., 2013). Nevertheless, no investigation on the mode of action was conducted and soils were not fully characterized. The products tested were mainly consortia products containing strains of various taxa and other biostimulants. Interestingly, some consortia products failed completely in promoting plant growth or exhibited negative effects whereas others, especially those containing *Bacillus* sp. strains in combination with seaweed extracts and humic acids, were effective.

4.2.1.2 Biofector meta-analysis

A meta-analysis using more than 140 experimental datasets was also conducted for the Biofector project. 1111 observations were part of analysis (2016, *still unpublished*). Overall BE effect was positive, with about 5 % higher biomass or yield observed in BE treatments as compared to the control. This is slightly lower but comparable with observations from meta-analyses and reviews on the application of *Azospirillum* and results from Schütz et al. for continental climate (8.5 %) or Schmidt and Gaudin (2018) for fully humid snow climate (Köppen climate classification Df) (7.1%). The Biofector meta-analysis showed a wide spectrum of effectiveness of BE plant growth promotion depending on often unknown factors.

Many seaweed extracts improved yield in field experiments with wheat with high reproducibility. Nevertheless, for microbial products responses in the field were weak and reproducibility was low. Only in tomato cultivation effects by microbial products were

repeatedly positive. In general, seaweed extracts were less frequently tested in pot experiments than microbial products and therefore it is unclear if the products or the conditions determined the efficacy.

Analysis showed that the highest variation between subgroups was observed for the comparison of different working groups. Indeed, this result reflects our observations for the many experiments, in which we tried to reproduce the effects of the working group at JKI, as well as the results from Exp_20 in which similar experimental conditions were applied as in the working group in Romania. It seems that certain BE-effects could be reproduced when experiments were repeated by the same working group however the reproduction in other working groups was not possible, suggesting that the crucial experimental factors determining the effectiveness for BE plant growth stimulation could not be explained.

A factor that was showing relatively high reproducibility was fertilization with stabilized ammonium as N-source (as discussed in 4.1.2.2.1, 4.3.2.5 and 4.3.4), especially in experiments from 2018 on. Additionally, P status was influencing the outcome of BE application whereas low to medium soil P levels and medium soil buffer capacities were most responsive (see also 4.1.7).

4.2.1.3 Limitations of meta-analyses

Meta-analyses are crucial for estimation of overall benefits or determination of influencing factors. Nevertheless, besides statistical issues that are sometimes mentioned, they are limited due to the publication bias as studies on failed applications are less frequently published ("Publication bias," 2021). In all meta-studies cited above statistical procedures such as the funnel plot were used to test for publication bias and all studies rejected the hypothesis of publication bias. Rubin et al. (2017) additionally conducted an analysis showing that many thousands of experiments without BE effect would be needed to neglect the significance of their findings. Funnel plots seem to be a proper analysis to estimate overall quality of datasets and to ensure that datasets are not cleansed of contrasting results. However, they do not give information on how many experiments without significant effects were not published at all. Additionally, it is not only the question if BE treatments are effective or not. From the amount of publications it is obvious that they are. More important is the question under which circumstances this holds true. Additionally, BE treatments may have significantly negative effects on yield as seen in our results but also in investigations of the COMPRO project cited above.

Furthermore moderator analysis can be criticized for several reasons. One problem is the comparison of different experimental datasets with each other. This results in a nestedness of experimental factors inside tested moderators. For example in the Biofector meta-analysis tomato seemed to be more responsive to PGPR application than maize. However, maize and tomato cultivation are very different, especially in the aspect of pre-cultivation making PGPR application in tomato much easier and increasing impact due to semi-sterile potting substrate, small soil volumes and better contact with the root. Additionally, in maize more field experiments were conducted that were repeatedly shown to be less responsive than greenhouse trials and greenhouse cultivation in tomato strongly differed to maize cultivation in the field. In general, it makes sense to compare the overall system of "tomato cultivation" with "maize cultivation" but this may lead to misinterpretation of data. One example is the comparison of seed treatment versus soil drenching, as seed treatment is more often used in field experiments, maize and wheat. If responsiveness due to factors described above is lower, this will result in a lower responsiveness of seed treatment versus soil drenching although the results might be different in other context, as seen for the meta-analyses of Rubin et al. (2017).

A second problem of moderator analysis is the fact that many experimental factors that are influencing the outcome of the experiments are not included because the number of observations is too low. In 4.3.5 one example is discussed that may lead to misinterpretation of data. Here extremely strong BE effects were observed in low P soils and therefore soil P values and improved P-acquisition by PGPRs were taken as explanation for growth stimulation. In a meta-analysis this dataset would probably influence the outcome of a moderator analysis on P availability in the direction of low P soils as most responsive. Nevertheless, a more critical investigation of the experimental conditions indicated that alleviation of Al-toxicity might be a more probable reason for BE-derived growth stimulation. Such lack of important information impedes the search for suitable environmental conditions.

4.2.2 Plant growth stimulation

In this section results from this thesis are discussed with the scope on plant growth stimulation, yield and reproducibility. A short economic evaluation was conducted to show efficiency of the products and also constraints that still need to be overcome.

4.2.2.1 Pseudomonas sp.

In the overall Biofector project but also in this thesis the Px product, containing the *Pseudomonas* sp. DMSZ 13134 strain, was the most frequently used BE product, studied under various experimental conditions, as it showed the best reproducibility for plant growth promotion from the beginning on. The Px strain was used in 18 experiments and overall 50 treatments as single application or in combination with other products. 14 experiments were conducted with maize, 4 with tomato. 42 of these 50 treatments showed higher yields as the respective control without Px application and overall stimulation of grain yield or plant biomass was about 13.9 % (median 6.9 %), indicating a general positive influence of the product on plant performance. Similar results were obtained in the meta-analysis from Schütz et al. (2018) for 'P-solubilizer'. Biomass increase in Px treatments of more than 10% was observed in 21 datasets. A summary of biomass results at last harvest for the Px strain is given in Table 7-2. Especially, in Exp_11 (Px_50 and Px_70), Exp_3 (all Px treatments) and for tomato in the "sterilized" soil of Exp_6 (+81% but due to high standard deviation the results were not statistically significant; see also Figure 3-43) Px treatment strongly increased biomass of the plants as compared to the control.

No effects for the Px strain were observed in experiments with late BE application and high P fertilization (Exp_7 with tomato and Exp_10 with maize) and in experiments with low application rates (Exp_19, Exp_23). In the field experiments differences were not statistically significant (except for the CULTAN treatments) but in general outcome was positive. Highest increase in yield was observed for the Urea fertilized treatment and the placed ammonium fertilization by the CULTAN method. Additionally, overall yield increase in 2015 was more pronounced, possibly connected to lower soil P values.

In contrast, the strongest plant growth reduction as compared to the control (-21%) was observed in Exp_11 for the Px_30 treatment (30% WHC) under water deficiency.

4.2.2.2 Bacillus amyloliquefaciens

For the Rz product, containing a *Bacillus amyloliquefaciens* strain, used in 37 treatments, less positive responses were observed. 21 datasets with a higher biomass as compared to the control versus 16 datasets with biomass reduction were counted. The average increase in biomass was 4.3 % (median 0.6%). Summing up the results, Rz showed tendencies for plant growth stimulation in most experiments, but reproducibility was lower than for the Px product and the risk of negative influence was increasing. The results for the Rz strain are summed up in Table 7-1. Also for the Rz strain, best results were observed in Exp_11 and again Exp_6 (Rz_T). Additionally, good results were obtained in Exp_14 and Exp_18 (conditions similar to Exp_11 and JKI: high sand contents, medium soil P levels). Strongest biomass reduction was again observed in Exp_11 (Rz_30), followed by Exp_2 (Rz_Glc), Exp_13 in the field (Rz_low) and Exp_4 and Exp_5 (light conditions and assimilates are discussed in 4.3.3).

These results for the main PGPRs suggest that under water deficiency, as well as cold stress, the microbial BEs were not beneficial or effective. This is in contrast to the results from the meta-analysis of Rubin et al. (2017), discussed above. Nevertheless, survival rate of the Px strain under low soil water contents is reduced (see Exp_15) and the tested strains were not selected by the producers for alleviation of abiotic stress. Exp_6 showed that a disturbed or unbalanced microbiome in the substrate (see also Mendes et al., 2011) has negative effects on plant growth and therefore BE application was very effective. Here, the Px and the Rz strain both showed their potential for biocontrol.

4.2.2.3 Fungal PGPM

Several fungal products were tested whereas only two products were repeatedly used. For both products, containing a *Trichoderma harzianum* (TP) and a *Penicillium sp.* strain (BFDC), mixed results were obtained. Especially the BFDC product showed strong detrimental effects at high application rates. It was used in five experiments and in total nine treatments. In the field experiment 2014 it caused complete loss of the maize plants when used for leaf application. At lower application rates neither negative nor positive influence was observed in the field experiment. Also soil treatments showed negative effects when the product was used at application rates of 10^8 CFU per g soil. At lower application rates as used in Exp_20 the product could stimulate tomato growth by more than 10 %. In a recent study with the BFDC and another *Penicillium* strain maize seed treatment at a concentration of $7.2x10^5$ fungal spores $seed^{-1}$ resulted in a significant growth stimulation at low temperatures in a high P soil and at normal temperature in low P soil (Gómez-Muñoz et al., 2018).

Nevertheless, with optimal growth conditions, at normal temperatures in the high P soil, plant growth was significantly reduced in the fungal treatments as compared to the control. This is again linking to the trade-off dilemma of plant stress responses (4.1.6.3).

The TP strain was used in five experiments and seven treatments. Only in two treatments it had slightly beneficial effects (3 – 4 % higher biomass). In all other experiments biomass was depressed and in Exp_2 with addition of glucose maize biomass was reduced by more than 20%. In summary, both fungal products were less efficient in plant growth stimulation than the bacterial products. Suboptimal application rate and method but also the environmental conditions are possible explanations for the ineffectiveness (4.1.5.1). For instance the TP product is mainly used for biocontrol and therefore its full potential could not be exploited.

4.2.2.4 Seaweed extracts

The seaweed extracts showed mixed outcome but in general had positive effects on plant growth as compared to the fungal products or the Rz strain. Only five out of 27 treatments had a negative impact on plant performance. Only in Exp_1 a decrease in biomass of more than 10 % (-46 %) was observed for the A95 product. This product does not contain Zn or Mn supplements and therefore did not exert any positive effects under cold stress conditions. In 14 treatments an increase of more than 10 % biomass was observed. Especially under cold stress (Exp_8 and Exp_9) the Zn/Mn supplemented seaweed extracts from Agriges could improve plant growth by > 30 %. The SF product showed negative influence on maize yield in Exp_16 but a slight tendency for growth stimulation in Exp_10. Results from the overall Biofector project indicated that also products without Zn/Mn supplementation could repeatedly improve plant performance and increase yield in the field, especially in winter wheat (*unpublished*). One of the most successful products was “Manek”, an Agriges product based on processed vegetable oils and rare medicinal herbs (4.1.2.3.2). As nutrient input by the product on per ha basis was negligible, beneficial effects were obviously due to bioactive substances such as fatty acids, alkaloids, diterpenes, glucosinolates, phenols, sesquiterpenes and tannins that were enriched in this product as described by the producer. It is not yet clear why the SF product was not as efficient in plant growth stimulation. It is probable that an optimization in dosage and concentration of the products is crucial to improve efficacy of these BE products but that this optimization is far more difficult if bioactive substances are involved instead of micronutrients. The results from our prebiotic tests also indicate that lower dosages might be more effective in biostimulation than high dosages. This might be true for both microbes and plants.

4.2.3 Economic evaluation

Some of the tested BE products that are on the market are sold for specific applications such as potato tuber dressing against pathogens (Px), seed coating, fertigation or treatment in seedling cultivation (Rz). Therefore only low amounts of the products are needed on per ha basis. In this thesis these products were used in large-scale experiments for plant growth stimulation. Including the market prizes of the BE products in calculations for economic evaluation BE application would therefore cause immense deficits for the farmer (Table 4-1). Up to now only the SWE can be produced in high amounts at low costs making broadcast or foliar applications possible. Nevertheless, as seen by the cost difference between the two microbial BE products, there might be potential for an optimization and reduction of production costs.

Table 4-1 Prizes of BE products

BE	Cost in € kg^{-1}/l^{-1}	AR in $kg/l\ ha^{-1}$	Cost in € ha^{-1}
Px	1800	3	5400
Rz	125	8	1000
SF	11	6	66
AV	8	6	48

AR = Application rate for band application; Seaweed extract AV comparable with Af or AVZM

As further explained in the next chapter, high application rates were tested to ensure that everything possible was done to provide conditions for a successful BE-plant interaction without considering the economic costs. To reduce costs, band application or the use of granules and then later on seed application by seed infiltration, seed incrustation or seed coatings with alginate (in later experiment in the institute, *unpublished*) were included in field experiments to decrease inoculum amounts on per ha basis meanwhile keeping cell numbers near to the seeds high.

Results from economic evaluations conducted during the Biofector project by working groups inside the WP09 (see 1.5) indicated that only in tomato production microbial BE products were repeatedly providing an economic benefit (Zimmermann 2014 – 2017, *unpublished*). Due to the high production per ha and high tomato prizes the yield increases in tomato production by microbial BEs, as observed in partner institutes in Romania and Hungary resulted in benefits of more than 50.000 € ha^{-1}.

In maize and wheat experiments in different experimental stations in Europe (Ireland, Romania, Germany, Switzerland, Italy) highest benefits were about 500 € ha^{-1} (BE costs not included). Especially application of the low cost SWE would therefore be interesting for farmers. Also studies from farmers field trials conducted by the company ABiTEP resulted in yield improvements and substantial benefits up to 400 € ha^{-1} in maize with low application rates of only 0.25 to 1 litre of the Rz product ha^{-1}.

In our field experiments no significant differences between BE treatments and untreated controls were observed. Ignoring statistical analysis and only focussing on the yield differences between treatments, the highest benefit was found in 2014 for the Px_Af treatment with about 11 % yield increase (Std_Ctrl = 67.6 dt, Px_Af = 75 dt ha^{-1}). The difference among treatments was only 5.4 % if block V was excluded from analysis (Std_Ctrl = 72.0 dt, Px_Af = 75.8 dt ha^{-1}). In 2015 the best treatments were the Px_gran and the Rz_seed treatments in the CULTAN fertilized plots (see Table 7-1 and Table 7-2). Again, about 11 % higher dry matter yield was measured. Nevertheless, fresh matter yield of silage maize in 2015 differed less (Ctrl = 511 dt ha^{-1}, Px_gran = 547 dt ha^{-1}, 7 %). Assuming a market prize for corn maize of about 15.2 € dt^{-1} (www.agrarheute.com, 06.02.2018) and for silage maize 2.7 € dt^{-1} FM (www.agrarheute.com, 20.09.2017) the benefits by the Px application were about 113 € ha^{-1} in 2014 and 97 € ha^{-1} in 2015. Here are no costs for BE products, application and working time included.

It is quite difficult to give good recommendations for farmers. Obviously, the usage of the products in the field experiments of this thesis was not economic.

Nevertheless, the small differences in yield between positive controls and unfertilized treatments indicated a small action window for the BEs to act. In less fertile soils inside and outside of Europe or under adverse environmental conditions, for example low SOM, BE effects might be much stronger (Bashan et al., 2004; Bradáčová et al., 2019a). This is supported by results from meta-analyses. Dry climate was more responsive than humid climates, probably due to lower soil fertility and SOM contents (Rubin et al., 2017; Schmidt and Gaudin, 2018; Schütz et al., 2018). Moderate, continental and snow climate regions were least responsive to BE applications. However, contrasting results were found during the Biofector project, showing that BE products can be interesting in farmers even in Northern Europe. Furthermore, seed application techniques for the Rz product showed positive results and are cost-efficient (see 4.3.7).

With all the evidence that is provided by many publications, reviews and even some meta-analyses the question if BE application is effective can be clearly answered with yes. More challenging is the question under which circumstances, agricultural practices, application rates, market prices or technical support the application is economic for a specific farmer.

In conclusion, BE applications in the field are still an economic risk but might have potential for our region if certain aspects are taken in consideration as discussed in the following.

4.3 Factors for successful plant growth stimulation

As mentioned in the introduction, a multitude of environmental factors shape rhizosphere interaction. Factors which are important for efficacy of microbial BEs, including strain specific properties ("rhizosphere competence"), application techniques and environmental conditions, were already described in the introduction. In this research some factors such as soil P levels, soil type, temperature, soil water contents, BE active components, PGPM species and strains, application, product formulation, target crop and root hair development were addressed. At the beginning of the project it was planned to use a modelling approach for determining the outcome of BE-plant interactions but due to the complexity of the interactions and the amount of environmental factors the approach was not realised.

4.3.1 Specificity of PGPR-plant interaction

4.3.1.1 Background

Specificity of PGPR-plant interaction is often reported for disease suppression by biocontrol agents/ biopesticides (Kloepper et al., 2004), especially under controlled conditions (Beneduzi et al., 2012; Loon, 2007; Vaikuntapu et al., 2014). A growing amount of publications also reports on specific plant growth and metabolic responses depending on PGPR strain x plant cultivar combinations under applied conditions (Mosimann et al., 2017; Sandheep et al., 2013; Thonar et al., 2017; Walker et al., 2011a, 2011b). A review on this topic concluded that PGPR-plant specificity can occur due to differences in the bacterial attraction to plant signals (chemotaxis to root exudates), during root colonization and due to differences in responses by the plant (Drogue et al., 2012). The results are not surprising, having in mind that also natural communities living in the rhizosphere, rhizoplane or endophytic inside the plant differ among plant species (Becklin et al., 2012; Oh et al., 2012).

4.3.1.2 Specificity of PGPR-plant interaction

Results from the Biofector project indicated that successful interactions and responses are strongly depending on the environmental condition but less on the BE-plant combination. This was seen in experiments with ammonium (Mpanga et al., 2019a Table 1) or at JKI (Eltlbany et al., 2019). Here different PGPMs were able to promote plant growth in maize, tomato and wheat. Also results from Romania suggest that the plant beneficial effects were not BE-plant specific. This suggests that signalling compounds used by PGPMs are not plant specific. Although plants shape their specific rhizobiome on community level (Berg and Smalla, 2009), PGPM-plant interactions are not strictly host-specific as PGPM do not depend on the host plant itself but on the rhizodeposits and exudates in the rhizosphere. Host-

preference is observed for eusymbiotic interactions with mycorrhizal fungi although overall plant-mycorrhiza symbiosis is not strictly host-specific (Garbaye, 1994; Jeffries and Rhodes, 1987; Yang et al., 2012). In contrast, for obligate biotrophic pathogens host-specificity is more strict do to the complex co-evolutionary development with plant defence (Li et al., 2020). Still, compatibility and severeness of plant response may differ for induction of ISR (Pieterse et al., 1998) and due to microbial resistance against antibiotics released by the plant or responsiveness of plant genotypes to specific microbial signals (Sanchez et al., 2005; Weston et al., 2012) as also seen in tests with *Azospirillum* and various host plants (Bashan et al., 1989).

4.3.1.3 Responsiveness of crops

Indeed results from meta-analyses suggested that here is a difference in the responsiveness of crops. The meta-analysis of the Biofector project indicated that tomato was more responsive than maize. As discussed in 4.2.1.3, this is probably due to differences in cultivation. Exceptions are host-specific interactions between legumes and rhizobia and some mycorrhiza-plant interactions (see above). This hypothesis is also supported by the mixed outcome for tomato experiments in this thesis that was strongly depending on the pre-cultivation phase. Response ratio between BE treatment and control varied from – 10 % to +80 %.

4.3.2 BE product combinations

The advantage of BE product combinations, either as microbial consortia or combination between seaweed/ plant extracts and PGPR, was discussed in many review publications (Barea et al., 2005; Bashan, 1998; Canellas and Olivares, 2014; Dodd and Ruiz-Lozano, 2012; Hol et al., 2013; Vacheron et al., 2013; Van Veen et al., 1997). Due to the complexity of BE-plant and BE-BE interactions and the dependencies of BE efficacy on environmental factors it is reasonable to guess that product combinations may increase the probability that BE application might act on plants under changing or adverse environmental conditions. Results from meta-analyses generally support the application of consortia products. Nevertheless, in a meta-analyses on biocontrol strains consortia were more effective in disease suppression but less effective for yield increase (Chandrasekaran et al., 2016). In the following, results from different BE combinations are discussed.

4.3.2.1 PGPR and PGPMs

Publications on the combination of different PGPR or PGPM strains are rare. In Exp_4 and Exp_5 product combinations of fungal and bacterial strains were tested but no cumulative or synergistic effects could be observed. Publications that tested BE combination without additional mycorrhiza inoculum mainly report on the combination between *Trichoderma* sp. and bacterial inoculums like *Pseudomonas* sp. or *Azosprillum* sp. strains (Badda et al., 2013; El-Katatny and Idres, 2014; Sandheep et al., 2013). They observed synergistic or cumulative effects of plant growth promotion, but all experiments were conducted in sterilized substrates. In a previous experiment in the institute using *Fusarium oxysporum* infected soil substrates no synergistic or cumulative effects for the Px + Rz combination as compared to single inoculum was observed (Yusran et al., 2009). In an experiment using non-sterile substrates and *Ficus benjamina* plantlets the combination of AM fungi with PGPR was more efficient than single inoculations but the combination of a *Bacillus coagulans* strain with *Trichoderma harzianum* did not show a growth improvement as compared to the *T. harzianum* single inoculation (Srinath et al., 2003). Results from various studies on MHBs, indicate a high specificity for microbe-microbe interaction (Frey-Klett et al., 2007). E.g. very different responses for the interaction of two related *P. fluorescens* strains with the AM fungi *G. mossae* were observed (Gamalero et al., 2004). If two microbes do not directly benefit from each other (e.g. AM, MHBs or rhizobia) it is possible that they will compete for the same ecological niche and therefore lead to a negative outcome of the product combination. The use of pre-mixed consortia products is discussed below.

4.3.2.2 PGPR and mycorrhiza

Rhizobial and mycorrhizal inoculums were not tested in this thesis to not further increase the product spectrum of BEs although interactions between the natural mycorrhiza and the applied BE products were analyzed, as discussed in 4.1.4.1. Additionally, the usage and application of rhizobia is common practice in legumes, especially soybean (Catroux et al., 2001; Rodríguez-Navarro et al., 2011). The large-scale implementation of legumes in African countries is addressed in the N2Africa project. In contrast, the production of mycorrhiza inoculum, especially AM, is expensive due to their obligate biotrophic lifestyle, depending on living plant tissue for colonization, and therefore their large-scale field application in maize or wheat is not yet economic (Lesueur et al., 2016b; Peterson et al., 1984). As an example the company BioMyc™ Environment GmbH (Havel, Germany) sells mycorrhiza inoculum containing about $2x10^5$ spores (infectious units) l^{-1} product at prize of 16.50 €. They

recommend the use of 100 ml product per 10 litres of soil or potting substrate. On ha basis therefore about 10.000 litre of inoculum at a cost of more than 100.000 € would be needed.

4.3.2.3 PGPR, humic substances and seaweed extracts

The combination of PGPR and humic substances might have potential to improve BE activity (Canellas and Olivares, 2014). HS can be defined as assemblies of heterogeneous compounds that are insoluble in water and recalcitrant to microbial activity. Plant and seaweed extracts, as tested in our experiments, might contain substances that fall into this category such as the phenolic tannins. HS are able to promote lateral root formation, retard degradation of other organic compounds and therefore also might protect PGPR in the soil. Furthermore, an improved root colonization of *Herbasprillum sp.* and synergistic effects on plant growth in sugarcane for the combined application with HS was reported (Canellas and Olivares, 2014). Nevertheless, in none of the experiments in this thesis (Exp_3, Exp_10, Exp_12) synergistic or additive effects of PGPR/SWE combinations on plant growth were observed. Also in Exp_19 and Exp_20, using an organic fertilizer that probably contained high amounts of bioactive substances, as suggested by root growth stimulation of the substrate (3.7.1.4.5), no synergistic effects were observed.

4.3.2.4 Prebiotic effects

Viability of inoculated PGPRs in soil is a crucial prerequisite for their successful interaction with the plant (Compant et al., 2005); see also 1.3.2.2). Therefore non-spore forming bacteria such as Pseudomonades are often formulated with nutrient rich sources such as milk powder.

One research question was aiming at potential prebiotic effects of SWE stimulating PGPR population growth and the optimal concentrations for product combination. In several incubation experiments product combinations and various concentrations were tested. Results differed strongly depending on the combination of the BE products.

As the Px strain was most responsive to different SWE, first pot experiments were targeting on the combination with this strain only. In field experiments also the combination of SWE with the Rz *Bacillus* strain was investigated. Unfortunately, in pot and field experiments no synergistic effects on plant growth by combination of SWE and PGPR were found.

Only in Exp_12 an effect of the Af treatment on Rz spore number was observed (3.10.3.3.5). It is possible that Rz spores were stabilized by the seaweed extracts or environmental signal perception was disturbed by the presence of the SWE. The aspect of spore germination was addressed in Exp_21 (3.9). In incubation experiments the non-germinated Rz spores and the

low response by the Rz strain irrespective of the SWE used (3.8), indicating that the spores had not germinated until the end of the incubation period of 4 h. Therefore, in Exp_21 the SWE was combined with germinated spores of the Rz strain to stabilize the product in the soil. Nevertheless, this approach was not efficient.

Highest corn yield in 2014 was observed for the combination of the SWE Af and the Px product, but differences among treatments were not statistically significant. Additionally, no significant effect of SWE on Px root colonization was observed. One simple explanation would be the formulation of the Px product with milk powder. As shown in the *in vitro* tests the milk powder formulation of the Px product had similar prebiotic effects than the SWE and therefore the SWE could not additionally improve the root colonization.

As mentioned in 3.3.4.4 the use of the SWE is less convenient than e.g. standard compounds such as milk powder. Nevertheless, the comparison of *Bacillus* and *Pseudomonas* strains indicates specificity on the level of different bacterial divisions (phyla). The observed differences among the two *Bacillus* strains (Rz and Bsim) are even more promising as they indicated that the SWE may have a species-specificity, too. Although it is possible that different genera of e.g. Pseudomonades, plant beneficial or detrimental, will have similar responsiveness to the SWE, the results indicate that, in contrast to other product formulations, the prebiotic activities of the SWE are more specific to a potential PGPR target.

4.3.2.5 Consortia and Zn/Mn supplementation in the Biofector project

One of the most successful BE product tested during the Biofector project was the combi-product Combifector A containing a consortium of *T. harzianum* OMG08 (fungi), and the PGPR *Pseudomonas fluorescens* (Px) and *Bacillus subtilis* supplemented with Zn/Mn (produced for the Biofector project by Jörg Geistlinger, Anhalt University of Applied Sciences, Germany). The product was efficient in P-solubilization from rock phosphate and sewage sludge ash and was able to promote plant growth when ammonium was fertilized as N-source (Mpanga, 2015; Kar, 2016; Nkebiwe, 2016). Under ammonium supply the Combifector B product (*T. harzianum* OMG08 (fungi), *B. amyloliquefaciens* (Rz) supplemented with ~100 mg Zn and Mn g^{-1} product) was able to reduce leaf damage, improve root growth and increased the concentrations of phenols and proline and SOD activity under water-deficiency (Freytag and Wanke, 2017). Similar physiological effects were also observed under cold-stress, even when the Combifector A product without Zn/Mn supplementation was used (Moradtalab et al., 2020). The combination of ammonium, Zn/Mn nutrition and the BE consortium were highly efficient to alleviate cold-stress effects by

changes in hormonal balance (higher IAA/CK and ABA/CK ratios) and activation of the plant internal antioxidant and resistance system (ISR, see also 3.14). Nevertheless, also single BE applications were efficient in plant growth promotion under ammonium nutrition as mentioned before (4.1.2.2.1).

Experiments with a combi-product from the company Agrinos Inc. (USA), containing a consortium of various different bacterial and fungal strains and micronutrients, resulted in positive effects on plant performance under various experimental conditions (e.g. in tomato experiments Romania (Bradáčová et al., 2019a).

It is difficult to separate synergistic effects from effects that are exerted by a certain component in a combi-product that was not present in other products. Therefore assessment of synergistic effects is only possible if all components of a product are tested separately in the same experiment. Nevertheless, we can argue that in general a consortium or combination of micronutrients and PGPR strains further enhances the action spectrum of a product and therefore the probability that a product is efficient under many different environmental conditions. Nevertheless, PGPR or PGPM strains should not be randomly mixed without clear descriptions for synergistic interaction based on experimental evidence or ecological datasets. Additionally, product quality of consortia products is often low (Jefwa et al., 2014). This may result in mixed outcomes for consortia products. New approaches that combine –omic technologies and the establishment of large-scale isolate collections from plant rhizobiomes may further improve consortia products in future (Bai et al., 2015).

4.3.3 Influence of light for successful plant-microbe interaction

Reproduction of the BE-effects observed at JKI was not possible in our experiments. Using many different experimental conditions but also conditions that were very similar in most of the factors to those applied at JKI did not result in the expected plant growth promotion. Especially the Rz product could not promote plant growth in a way comparable with the observation at the partner institute. Also in other working groups during the project and other experiments done in our institute (Nkebiwe et al. 2013 – 2016, partly published) the effects were not reproduced. The low reproducibility is probably depending on many different factors such as temperature, irrigation and water contents, soil properties and the natural microbial community. Nevertheless, most of these factors were controlled in Exp_11 and Exp_14 and were similar (also soil types) as in the JKI experiments. One factor that could not be controlled in our facilities was the light condition.

4.3.3.1 Light and rhizodeposition

As described for Exp_4 also in Exp_5 probably light was a limiting factor that negatively influenced plant growth but possibly also the interaction with the BEs. A low light intensity reduces the production of assimilates and therefore also the production of carbon-rich root exudates (Badri and Vivanco, 2009; Rouatt and Katznelson, 1960; Rovira, 1959; Yang, 2016). It is known that amino-acid exudation and also root colonization of amino-acid dependent bacteria were reduced with reducing light intensity (see ibid). Data from studies investigating the trade-off between P or N acquisition by e.g. mycorrhizal or rhizobial symbioses and the provision of carbon-rich assimilates by the plant suggest that the microbial symbionts stimulate photosynthesis rates to increase carbon-rich nutrient supply (Dosskey et al., 1990; Mortimer et al., 2008). This "sink stimulation" also was leading to an increased rate of photosynthesis that was substantially more than the C costs of the rhizobial and AM symbioses (Kaschuk et al., 2009), a possible mode of action that could explain growth stimulation by BEs.

But what happens if photosynthesis rates are limited due to suboptimal light conditions? Early publications on legumes showed that C loss due to exudates may reach up to 50 % of the C fixed (Minchin and Pate, 1973), and C loss almost doubled from 34% to 52% of total C in wheat roots if bacteria of the strain *P. putida* were inoculated in a previous axenic culture (Přikryl and Vančura, 1980). Furthermore, it was shown that legume-rhizobia symbiosis was only plant beneficial if nutrient availability was low but light intensity high (Lau et al., 2012). Under low-light conditions but sufficient nutrient supply the costs for the plant exceeded the benefits and plant biomass was reduced. It is possible that the Rz strain was triggering root exudation and thereby further reducing the already low amount of available carbohydrates that the plants needed to invest in metabolism and biomass production, explaining the growth depression in Exp_4 and Exp_5. Also other factors such as P-deficiency (3.14.7) and cold or drought stress (1.2.4) reduce photosynthetic activity explaining BE-related plant growth depression in Exp_11.

4.3.3.2 Light and tryptophan

It was further reported, that tryptophan is highly abundant in root exudates and that bacterial growth pattern correlate with the presence of tryptophan in the rhizosphere production and exudation into the rhizosphere (Jaeger et al., 1999; Kamilova et al., 2006). Tryptophan is a precursor of IAA (auxin) production and is strongly promoting auxin production in *B. amyloliquefaciens* (Rz) and thereby influencing plant growth promoting activity of the Rz

strain (Idris et al., 2007). It is possible that a shift in the hormonal production of the Rz strain influenced the outcome of the BE-plant interaction.

Additionally, regulation of plant growth is light-dependent due to the interaction of hormonal signalling and light. It is well known that auxin is involved in phototropism in plants (Hohm et al., 2013; Whippo and Hangarter, 2006). Nevertheless, not total levels of auxin but rather auxin transport is changed in response to unilateral light conditions. As seen in gene expression analysis and reports from literature, auxin signalling and transport are involved in BE-dependent biostimulation of plants. Certain light conditions may reduce the responsibility of plants to BE application.

4.3.3.3 Light and different BEs

Nevertheless, although *Pseudomonas* sp. strains are known to produce IAA in a tryptophan-dependent manner (Karnwal, 2009; Kuzmanović et al., 2018; Mpanga et al., 2019a; Patten and Glick, 2002). Why was the Px strain more effective under these conditions? Moreover, if BEs are a sink for assimilates why did the Px treatment not have negative influence on plants in Exp_4 and Exp_5?

1. Freeze-dried Px cells are always active in the moment they get in contact with water whereas endospores need specific signals for germination, as mentioned for Exp_21 (3.9.1.1) that are also present in root exudates. Therefore the reduction of root exudates might affect Rz germination although results from Exp_21 show that germination is not sufficient for plant growth promoting activity.

2. As explained in 4.1.4.2 the Px product potentially acts on a different pathway by stimulating the natural soil microbiome. The direct mode of action and influence on the plant is thereby depending on the microbial composition in the soil and is therefore a “black box”, possibly including biocontrol activity, “sink stimulation” or other processes distinct from those of the Rz (*B. amyloliquefaciens*) or the unformulated Px (*Pseudomonas* sp.) strain.

3. The Px product is composed of a carbon-rich formulation that reduces the competition for assimilates and therefore the negative outcomes of the assimilate trade-off. Additionally, the sugars might act as signals during P-deficiency (4.1.4.2).

4.3.4 Ammonium nutrition

Ammonium nutrition was a crucial factor for successful BE applications in several experiments (Mpanga et al., 2019a). Also early publications reported on selectively increased

populations of plant beneficial and disease-suppressive Pseudomonades in the rhizosphere of ammonium fertilized wheat (Sarniguet et al., 1992a, 1992b) but it was not clear what caused this population shift. There are several explanations for the observations that probably add up to a synergistic effect between PGPR inoculation and ammonium fertilization.

First of all, ammonium N nutrition promotes rhizosphere acidification (Neumann and Römheld, 2012, p. 354), due to a release of protons into the rhizosphere that are produced by NH_4^+ assimilation in the root tissue (Neumann and Römheld, 2002). This was also observed in the experiments with BEs (Bradáčová et al., 2019b; Moradtalab et al., 2020; Mpanga et al., 2019a; Nkebiwe et al., 2016b). Rhizosphere acidification increased availability of micronutrients such as Zn, and Mn, leading to increased Zn/Mn contents and SOD activity (Moradtalab et al., 2020). Under cold or drought stress PGPRs and other BEs without Zn/Mn supplement were not effective in reducing damage by ROS in nitrate fertilized plants. Therefore the efficacy of the BEs for plant growth stimulation was limited, as for example auxins were degraded by ROS. In contrast, ammonium fertilization overcomes this limitation and enables effective BE-plant interaction. Additionally, rhizosphere acidification increases P availability for mycorrhiza and may therefore improve establishment of plant-mycorrhiza interaction if P-status is still low. It was also shown that rhizosphere acidification is not reduced to the rhizosphere but extends further into the bulk soil if mycorrhizal structures are active (Li et al., 1991). As the interaction with AM fungi brings more benefit for the plant PGPR activity as “mycorrhiza helper” is more valuable and significant.

As fungi generally prefer lower pH for growth, ammonium-derived acidification in the apoplast due to the H^+-ATPase activity might have contributed to the promotion of *Trichoderma* root colonization (Moradtalab et al., 2020; Mpanga et al., 2019a).

As mentioned before, P mineralisation from organic P sources is repressed if C:P (carbon:phosphorus) ratio is to high (Zhang et al., 2014). Starter fertilization of soluble P or ammonium fertilization are possibilities to decrease C:P ratio in the soil solution.

In 1999 Marschner et al. published a study on root colonization of Pseudomonades under different N-sources. Ammonium nutrition increased root colonization by *P. fluorescens* strain 2-79RLI at the root tip and in the lateral root zone of wheat plants (Marschner et al., 1999). This effect was only observed when soil pH was not buffered but was decreasing due to NH_4^+-fertilization. Therefore the authors suggested that population increase of Pseudomonades during NH_4^+-fertilization was a response to increased net exudation of sugars

due to impaired exudate retention as a result of high H^+ concentrations in the rhizosphere or the apoplast. This is supported by recent publication that connects ammonium fertilization and auxin-signalling (Meier et al., 2020). Auxin supports lateral root branching, as suggested due to activity of plasma membrane H^+-ATPase (Canellas and Olivares, 2014). Under ammonium nutrition auxin levels in roots are increased (Moradtalab et al., 2020; Mpanga et al., 2019a). According to the results of Meier et al., under ammonium nutrition auxin is accumulated in the root and H^+-ATPase activity leads to protonation of IAA and its diffusion to outer root cells inducing cell wall-loosening and root branching. The protonation of other compounds might also influence overall exudation rates. The increase in density of lateral roots might help to provide the 'home for soil PGPM' (Nkebiwe et al., 2017), as both Pseudomonades and *B. amyloliquefaciens* preferentially colonize the lateral root zone (Dietel et al., 2013; Marschner et al., 1999) where cell wall-loosening provides carbon sources.

The quality of root exudates influences root colonization and biofilm formation of the bacteria. Experiments on chemotaxis of *Bacillus amyloliquefaciens* indicated that the bacteria showed movement towards seed exudates but not towards root exudates of soybean (Yaryura et al., 2008). The authors suggested that the composition of root exudates of axenically grown soybean roots was not attractive to the strain. Ammonium nutrition influences PEPC activity and increases malate exudation (Neumann and Römheld, 2012, 1999). Motility and chemotaxis of *P. fluorescens* as well as *B. amyloliquefaciens* strains were both shown to be activated by specific organic acids, especially malate (de Weert et al., 2002; Tan et al., 2013b).

Additionally, ammonium is the preferred nitrogen source for bacteria as it supports a higher growth rate than any other nitrogen source (Merrick and Edwards, 1995). This might explain why auxin production of a *Pseudomonas putida* strain was increased by addition of ammonium in a tryptophan supplemented growth medium (Bharucha et al., 2013). Ammonium N-nutrition was also increasing auxin production of the Px and the Rz strain as compared to nitrate as N-source (Mpanga et al., 2019a). Also for fungi nitrogen uptake rates are higher for ammonium than nitrate, as confirmed for AM fungi (Plassard et al., 1991).

Nevertheless, the advantages of ammonium nutrition depend on soils with medium buffer capacities, as high buffer capacities inhibit ammonium-N-derived pH decrease whereas in low pH and low buffered soils the potential of PGPM to improve nutrient acquisition is reduced and ammonium-N nutrition may even lead to pH decrease below 4.5 exhibiting negative influence on plant growth (Bradáčová et al., 2019b; Mpanga et al., 2020; Mpanga, 2019).

4.3.5 Soil degradation and erosion

One explanation for the good results in tropical soils, often from Asian or African countries (Bashan et al., 2004), could be the often reported lower micronutrient availability due to leaching after heavy rainfalls, soil acidity and low input farming systems (George et al., 2012; Smithson and Giller, 2002; Stoorvogel and Smaling, 1998; Zingore et al., 2008). Additionally, the low SOM and nutrient contents of many tropical soils lead to erosion and lower biological activity thereby reducing microbial competition for inoculated PGPR (Schütz et al., 2018). Under these conditions PGPR activity might be more pronounced than in "biologically active" soils leading to stronger growth promotion effects. This hypothesis is in contrast to the results of Bradáčová et al. (2019b) that reported plant growth promotion only on freshly collected soils with high SOM and biological activity and therefore needs further investigation.

Another aspect is aluminium toxicity in tropical soils. About 70 % of the acid soils are estimated as Al-toxic as well as Mg and Ca deficient (George et al., 2012). One symptom of Al-toxicity is the inhibition of root growth that may be counteracted by biostimulation of BEs. In a publication from 2018 the effects of two PGPRs, a *P. fluorescens* strain and *Burkholderia pyrrocinia* strain, on the growth promotion of forage grass under different light conditions were investigated (Lopes et al., 2018). Interestingly, here PGPR effects were strongly promoted in grass grown in shaded conditions. The combined application of PGPRs was able to increase root growth by 600 %, single inoculations still increased plant growth by more than 100 % on average. This is, reflecting on the limited effects of PGPRs observed in this thesis, impressing. Unfortunately, the authors do not give any explanation for the observed growth effects, although the analysis of the mode of action is crucial to determine conditions under which these PGPR activities can be achieved. Nevertheless, results of the mineral analysis of the soil substrate are given in the methods part. Most important are a soil pH of 4.2, a P value of 2 mg l^{-3}, calcium concentration of 0.2 mmolc l^{-1} and aluminium concentration of 1.4 mmolc l^{-1}. These results indicate that the soil used was extremely acidic with low buffer capacities, severe P deficiency and conditions for Al-toxicity. PGPRs are able to alleviate Al-toxicity by hormonal stimulation of root growth, ACC-deaminase activity or production of organic acids (Glick, 2014; Ma et al., 2011). These traits are especially valuable under these adverse conditions. Under shaded conditions photosynthetic activity of plants and the production and exudation of organic acids that reduce Al-toxicity are decreased (Yang, 2016). Under different soil conditions plant-PGPM interaction may be inhibited. But Al

increases organic acid production of the roots improving the establishment of PGPR-plant colonization (Muhammad et al., 2019). Therefore the combination of a sensitive plant species, low light conditions, Al-toxicity resulted in optimal conditions for PGPR activity. This example shows the influence of soil fertility and other environmental factors on BE efficacy and mode of action but also the importance of proper documentation and reporting of experimental conditions in scientific publications.

4.3.6 Application rates

4.3.6.1 Application rates in the project

Pre-tests before the start of the experiments and the Biofector project indicated that high inoculum rates are crucial to ensure plant growth promoting effects (Paul Mäder, FiBL, *unpublished*). Therefore, a minimum inoculum rate of 10^9 CFU kg^{-1} soil for bacterial products and a slightly lower minimum spore number of 10^8 CFUs kg^{-1} soil for fungal products were defined at the beginning of the project. The application rates at JKI were increased later on, because better BE effects were observed with higher inoculum rates (Eltlbany 2015, *personal communication*). Competitiveness in the soil and efficient root colonization are often mentioned as crucial PGPR traits (1.3.2.2). High inoculum rates ensure that the starting population density is high. This might increase the resistance against other adapted soil microbes. Considering the importance and omnipresence of quorum sensing in microbial population it is probable that certain PGPR traits are also density dependent. Two publications concluded that threshold densities of > 10^5 CFU of antibiotic producing Pseudomonades were necessary to achieve disease-suppressiveness in soils (Raaijmakers et al., 1999, 1997). Nevertheless, as mentioned in 3.6.1.4.5 population densities of inoculated BEs often decline rapidly after inoculation. Furthermore, as seen in the previous subchapter, including product prizes in the considerations high inoculum rates will not be cost-efficient and economically feasible under applied conditions. In fact, the comparably low inoculum rates of 10^9 CFU kg^{-1} soil that were used for broadcast or band applications in the field were far beyond the margin of common fertilizer or pesticide products. Only in tomato cultivation, using the pre-cultivation phase with small substrate amounts, these application techniques might be economic. For maize, seed dressing and seed coating seems to be the only economic application technique (further discussed in 4.3.7).

4.3.6.2 Application rates for bacterial strains

Publications comparing application rates of *Azotobacter*, *Azosprillum*, *Pseudomonas* and *Bacillus* strains applied on seeds of maize or kale plants grown under aseptic conditions

indicate that optimal application rates did not differ among applied PGPRs (Piromyou et al., 2013, 2011). Additionally, bacteria inoculated at low concentrations (10^3 CFU ml^{-1}) proliferated well near the root. Colonization density therefore increased at least up to 10^6 CFU g^{-1} root in all treatments. In treatments with higher application rate highest root colonization density was 10^9 CFU g^{-1} root. At all concentrations plant growth was significantly increased as compared to untreated control and there was no correlation between inoculum or colonization density and plant growth. Also for the Rz strain various application rates were tested for disease suppression in lettuce (Chowdhury et al., 2013). Even lower application rates of $4x10^7$ CFU kg^{-1} substrate were effective, although these experiments were conducted in potting substrate with low abundance of bacterial competitors. A comparison of Rz and Px indicates that optimal application rates might differ between the two strains. The influence of the BE products on seed germination and emergence was investigated in Exp_2 and Exp_3 and the field experiment Exp_12. Additionally, germination tests were performed in the climate chamber. PGPR products showed tendencies for an improved or faster plant emergence in pot or field experiments that were not significant in most cases. The germination tests indicated that the effects were concentration dependent and that plants were sensitive to high concentrations of the Rz product but that the addition of soil substrate reduced the sensitivity of the plants and therefore no negative influence was observed in pot or field experiments. Best effects were observed for a concentration of 10^6 CFU ml^{-1} for the Px strain and 10^3 CFU ml^{-1} for the Rz strain when maize was grown without soil substrate indicating strong differences between the two bacterial BE products (3.8.1.2).

Although at JKI good results were obtained with high inoculum densities, high applications rates of Rz in Exp_4 and 5 but also other experiments were leading to growth depression in maize (4.2.2.2, Table 7-1). This effect was probably connected to experimental conditions. Similar, in a publication on *Bacillus subtilis* root colonization rates of 10^7 CFU g^{-1} fresh root were beneficial whereas higher inoculum rates seemed to depress tomato growth (Qiao et al., 2017). Interestingly, in germination tests and for seed treatment (Exp_18) the Rz strain did not exhibit strong negative effects even at higher concentrations and the best results for Rz in the field experiments (CULTAN treatments 2015) were observed for seed treatment. This indicates that Rz efficacy is more depending on environmental conditions. The company ABiTEP distributes the Rz product (RhizoVital®42) via the company Biofa with information on application rates and techniques. For maize a dosage of 0.2 l ha^{-1} as seed treatment or 5 - 15 g kg^{-1} of seeds is recommended. This is about 10^8 CFU $seed^{-1}$.

In contrast to Rz, only in few cases high soil application rates of Px had negative influence on plant growth (Exp_11 (intermediate harvest) during cold-stress). At the same time, seed treatment (infiltration) of Px exhibited negative effects on the maize plants. Some explanation for the different observations for the two bacterial products but also for the application strategies can be drawn from the different modes of action as described in section 4.1. Especially the milk powder, that was absent in infiltrated seeds, determined the observed efficacy of the Px product in soil drenching applications.

Table 4-2 below shows a summary of all application rates and the plant performance in the respective treatments of the experiments for the Px strain. The results do not indicate a correlation between application rate and yield improvement. Nevertheless, taking together all results from Px experiments, high soil application rates (of more than 10^9 CFU kg^{-1} soil) are generally increasing the chance of plant growth stimulation by the product, but the experimental conditions must be optimal to ensure that the product is effective. In Exp_5 and Exp_7, with high application rates, factors such as light, nutrient status and late application were probably reducing Px effectiveness. In Exp_6, although relatively low amounts of Px were applied, effectiveness of Px was strongly improved due to the previous tyndallisation of the soil substrate (0).

4.3.6.3 Application rates of fungal products

In general, fungal products were less efficient than bacterial products in our experiments. *Trichoderma* products at a concentrations of 10^8 spores kg $soil^{-1}$ did not cause any significant responses in most of the experiments and high inoculum rates in Exp_1 were correlated with negative impact on the plants under cold-stress (OmG and TP). The BFDC product was causing serious damage in all experiments and had negative influence on maize plant emergence with high application rates of 10^8 CFU per g soil (Exp_1, Exp_2 and Exp_12) whereas at 10 or 100 times lower application rates some tendency for growth promotion was observed (Exp_13 and Exp_20). The main reason for the negative effects observed for the BFDC product is probably not the *Penicillium* strain but the tenside-like product formulation. Surfactants are also used for synthetic pesticides to decrease water surface tension and improve soil penetration or foliar application (e.g. Tween 20 or 80). Used at high concentrations the product is destroying the plant cuticle and cell membranes damaging or killing the plants as seen in the field experiment 2014. Therefore product formulations have strong influences on BE application (as also seen for the Px product) and are framing or limiting application rates.

4.3.6.4 Application rates of seaweed extracts

The different application rates tested for seaweed extracts (SWE) had only limited impact on plant performance (Exp_3). Nevertheless, we first have to differentiate between pure SWE and SWE supplemented with micronutrients, typically Zn and Mn.

For most of the SWE without micronutrient supplementation no or little influence on plant growth was observed and for micronutrient-containing SWE plant performance was only influenced significantly under abiotic stress conditions. If micronutrients are present, application rates should be calculated according to the micronutrient concentrations and application techniques (seed priming, seed dressing, foliar application).

For the SWE SF some significant effects (although sometimes negative) were observed for very low concentrations. Together with the overall results from the Biofector project and SWE products such as Manek, the use of SWE seems to be much more economic than the application of microbial BEs (4.2.3).

A last important aspect is the usage of SWE for product combinations, as mentioned above. The incubation tests for the Px strain but also for the *Trichoderma* and Bsim strain showed that product combinations between SWE and microbial BEs should be considered carefully and the combinations should be tested beforehand. Microbes react very sensitive to higher concentrations of SWE and therefore in the field experiment 2014 SWE and microbial BEs had to be applied separately, as dilution of the products in large tanks was not feasible. Two separate applications would make this combination less attractive for a farmer but the antimicrobial activity was concentration dependent and a suspension in a large water tank might already be changing the interaction completely making a previous “toxic” SWE “prebiotic”, as shown for the SF. Successful BE product combinations could therefore be sold together but not pre-mixed.

Table 4-2 Concentrations and application rates of the Px product for all experiments and treatments

Exp_Nr	Type[1]	Crop	Trt with Px[2]	Technique	Rate	Concentrations and application rates				Comment	Yield plus[4]
						Suspension[3]	In the soil	Total	Unit		
1	GH	Maize	1	substrate	1	2.0E+09	2.6E+09	2.6E+09	CFU kg^{-1} dry substrate		16.8%
2	GH	Maize	2	substrate	2	1.5E+09	9.1E+09	1.8E+10	CFU kg^{-1} dry substrate		23.3%
3	GH	Maize	6	substrate	1	0.5 - 7.0E+09	3.0E+09	3.0E+09	CFU kg^{-1} dry substrate	concentrations in pre-culture differ from later application	38.7%
3	GH	Maize	1	substrate	1		7.8E+09	7.8E+09	CFU kg^{-1} dry substrate		35.3%
4	GH	Maize	2	seed + substrate	2	1.0E+09	8.6E+09	1.7E+10	CFU kg^{-1} dry substrate	seed and soil application in the same treatment	15.0%
5	GH	Tomato	2	seed + substrate	3	1.0E+09	8.6E+09	2.6E+10	CFU kg^{-1} dry substrate	seed and soil application in the same treatment	-0.3%
6	GH	Tomato	2	substrate	3	1.0E+08	5.0E+08	1.5E+09	CFU kg^{-1} dry substrate	see Exp_4 (in Exp_5 suspensions differ in concentration)	36.4%
	GH	Tomato	2	seed	1	1.0E+09	-	-	CFU ml^{-1}		
7	GH	Tomato	3	substrate	4	1.0E+09	5.0E+09	2.0E+10	CFU kg^{-1} dry substrate		0.8%
8	GH	Maize	3	substrate	3	1.0E+08	6.7E+08	2.0E+09	CFU kg^{-1} dry substrate		12.5%
10	GH	Maize	3	substrate	1	2.1E+08	6.7E+08	6.7E+08	CFU kg^{-1} dry substrate		4.1%
11	GH	Maize	3	seed + sub.	2	1.0E+09	5.0E+09	1.0E+10	CFU kg^{-1} dry substrate	seed and soil application in the same treatment	20.3%
12	Field	Maize	3	soil	2	2.8E+08	1.0E+09	2.0E+09	CFU kg^{-1} soil (upper 10 cm)		2.5%
14	GH	Maize	1	seed + sub.	2	1.0E+09	5.0E+09	1.0E+10	CFU kg^{-1} dry substrate	seed and soil application in the same treatment	26.9%
15	Field	Maize	1	seed	1	1.2E+08	1.2E+08	1.2E+08	CFU $seed^{-1}$	seed infiltration by company Sourcon Padena	6.7%
15	Field	Maize	3	soil	1	8.8E+09	1.0E+09	1.0E+09	CFU kg^{-1} soil (upper 10 cm); application either in the band, on manure pellets in the band or broadcast		4.7%
15	Field	Maize	2	gran	1	1.0E+08	1.0E+09	1.0E+09	CFU kg^{-1} soil (upper 10 cm); here BEs sprayed on pumice stones		6.0%
17	GH	Maize	2	seed + sub.	2	1.0E+09	4.0E+09	8.0E+09	CFU kg^{-1} dry substrate	seed and soil application in the same treatment	13.2%
19	GH	Maize	6	seed + sub.	3	1.0E+09	2.0E+09	6.0E+09	CFU kg^{-1} dry substrate	seed and soil application in the same treatment	0.2%
20	GH	Tomato	2	substrate	2	1.3E+07	3.5E+08	7.0E+08	CFU kg^{-1} dry substrate		18.5%
22	GH	Maize	1	seed + sub.	2	1.0E+09	5.0E+09	1.0E+10	CFU kg^{-1} dry substrate	seed and soil application in the same treatment	28.5%
23	GH	Maize	3	substrate	1	1.0E+08	1.0E+09	1.0E+09	CFU kg^{-1} dry substrate		1.1%

[1]GH= pot experiment in green house; [2]Number of treatments in the respective experiments with the same application rate; [3]The Px product formulated as powder was suspended in $CaSO_4$ or NaCl solution at different concentrations. These suspensions were used for seed dressing, soaking or soil application; [4]Percentage of biomass increase at harvest time as compared to untreated control averaged over all treatments of the experiment with the same BE application rate.

4.3.7 Application techniques

As described in the introduction a huge spectrum of application techniques can be used for BE products. The most commonly used strategies are soil drenching and seed application (Bashan, 1998; Chandrasekaran et al., 2016; Rubin et al., 2017). This is also because in many crops, such as maize or many other cereals, drenching of seedlings and fertigation techniques are not applicable. Therefore, broadcast and band application as soil drenchings or granules, seed dressing and coating as well as foliar application of SWE and soil incorporation with manure were used.

4.3.7.1 Seed treatment

Seed treatment is one of the most widely used application technique for PGPR (Bashan, 1998). Meta-analyses indicated that seed treatment can be effective for plant growth promotion and significantly increased yields (Chandrasekaran et al., 2016; Rubin et al., 2017).

In most studies on PGPR observed root colonization density never increased above 10^8 CFU g^{-1} root and declined over time. Although the inoculum amount on per ha basis might be low, bacterial densities reach often more than 10^8 CFU $seed^{-1}$. During seed germination a high number of organic compounds is released to the environment and therefore provides good starting conditions for the newly introduced PGPM. A preferential attraction of a *B. amyloliquefaciens* strain to seed as compared to root exudates of its host plant was shown in culture experiments (Yaryura et al., 2008). This coincided with a more intense biofilm production. Biofilm production was shown to increase PGPR potential for growth stimulation (reviewed in Backer et al., 2018). Yaryura et al. suggested that the weak response to root exudates was only transient and expected a change in composition in response to root colonization. This increases the significance of early inoculation, as primary colonizer influence their future nutrient supply and thereby possibly the microbiome of the host plant (Agler et al., 2016).

Despite of the benefits, the influence of seed treatment may be only short-term. Most PGPM modes of action, including mineral nutrient solubilization, hormonal regulation by auxins or ACC deaminase activity or more general a “sink-stimulation” (Canarini et al., 2019; Kaschuk, 2009; Kaschuk et al., 2009), depend on the close contact of the PGPM to the root. For example to promote lateral root branching auxins are released in the apoplast and taken up by lateral root primordia (Meier et al., 2020). Therefore, IAA producing bacteria should be in direct contact to root primordia. Lateral root branching is thereby a direct benefit for bacteria

as the cell wall-loosening provides higher amounts of exudates. Hot spots of bacteria were found in the lateral root zone (Marschner et al., 1999). During root elongation most bacteria from the seed coat might get lost. Also, the lateral roots will grow out of the bacterial hotspots. There are four ways bacteria can stay in contact to the root: 1. biofilm formation or lipopolysaccharide production on the root surface, 2. passive transport through the soil or along the root, 3. active movement following nutrient gradients (chemotaxis) and 4. endophytic colonization of the root. As nutrient uptake but also exudate release is highest in apical zones near the root tip, microbial density and root colonization rates of mature root parts is low (Marschner, 2012b, p. 372; Marschner et al., 2011; Neumann and Römheld, 2012, p. 350) and will probably not be contributing strongly to PGPM effects. Still, bacteria may be able to stick to root cap or parts of the elongation zone and get transported along the growing root (Yaryura et al., 2008). Passive transport and active movement depend on soil water contents, irrigation or rainfall. In pot experiments with seed treatment, we also found increased *Bacillus* colonization at the root tips (Exp_18) but in dry climate regions these processes may be strongly limited. Nevertheless, drip irrigation might allow a transport in the root channel alongside the root even with reduced soil water contents and results from field experiments and pot experiments (see 0) indicated that transport of cells does not depend on water flow from above. For the Rz strain upregulation of genes related to chemotaxis in response to maize exudates was found (Fan et al., 2012). Endophytic root colonization as shown for *Trichoderma* (Moradtalab et al., 2020; Mpanga et al., 2019a) and *Bacillus* (Tan et al., 2013a) strains may exhibit long time effects although it remains to be investigated to which extent endophytic root colonization contributes to plant growth stimulation effects as population densities of endophytes are generally much lower (>10^4 CFU g^{-1} root) than usually suggested for successful PGPR application (Munif et al., 2013). However, in experiments with different *Azospirillum* sp. strains a strain-specific endophytic colonization of wheat roots at relatively high densities of >10^5 CFU g^{-1} root over a 14 week period was found (Schloter and Hartmann, 1998). Prominent examples for effective endophytes are ascomycete fungi from the genus *Epichloë* that infect various grasses and protect their host plant against a wide variety of biotic and abiotic stresses (Xia et al., 2018). The fungi is naturally transferred via vertical transmission to successive generations and colonizes the shoots of its host plants at sufficient rates to enhance plant protection (Caradus and Johnson, 2020).

In experiments with maize seed dressing and coating seemed to be an efficient and economic option for Rz application and is also recommend by the producer for the application in maize (see 338 ff.). In our field experiment vacuum infiltration of Px seeds was not effective to

promote plant growth and in germination tests seedling development was negatively affected by this treatment. Nevertheless, as discussed for the Px product, modes of action might strongly differ between infiltration and soil drenching, as the milk powder is an active ingredient (4.1.4.2). For the Px treatment therefore soil application with high amounts of the product was most effective but is not an economic option. In previous investigations successful biocontrol activity of Px in maize against a *Fusarium* strain was shown after seed dressing (Yusran et al., 2009). Also vacuum infiltration and drenching of seedlings was efficient for plant growth promotion in barley (Buddrus-Schiemann et al., 2010; Fröhlich et al., 2011). Nevertheless, in field experiments from Fröhlich plots were not completely randomized (Fröhlich, 2008, only available in German), to prevent a distribution of the inoculated strain to neighbouring control plots. Considering the observed heterogeneity in fields (see 3.10.3.3.7), results should be interpreted carefully.

Seed treatment was repeatedly shown to be effective (Chandrasekaran et al., 2016; Rubin et al., 2017). Seed priming with water (Rehman et al., 2015; Singh et al., 2015), micronutrients (Imran et al., 2013) or silicate (Moradtalab et al., 2018) was able to improve seed germination and abiotic stress. On-farm seed priming was found to be a method with high potential for smallholder with low income in dry climates (Carrillo-Reche et al., 2018; Raj and Raj, 2019). 'Bio-priming' (Mahmood et al., 2016; Reddy, 2012), the term used for seed soaking with PGPM, has therefore high potential, especially when using consortia products with micronutrient supplementation, and is economically more efficient than soil drenching methods. Nevertheless, bio-priming is not yet well established for industrial production as seed drying after soaking is time and cost-intensive (O'Callaghan, 2016). Further methods are seed coating or pelleting using a growing variety of different carriers (Rocha et al., 2019). The development of carriers for BE seed application, such as polymer-based encapsulation (Pacheco-Aguirre et al., 2017) or various other sources is reviewed elsewhere (Deaker et al., 2004; Herrmann and Lesueur, 2013). BE application with (peat) granules might be a good alternative to seed treatments if the granules can be mechanically applied together with the seeds in a seeding machine (Bashan, 1998). Nevertheless, the distance between seeds and granules should be low to ensure contact to the root in early plant development.

4.3.7.2 Other techniques

The co-inoculation with manure was found to be efficient in previous studies but not successful in our experiments. Here it was shown that the manure source is strongly influencing the outcome of the interaction (Thonar et al., 2017). Similarly, compost

composition variably influences soil microbial community and plant performance (Cozzolino et al., 2016; De Corato, 2020). This may lead to different responsiveness of plants to BE inoculation and the shift in microbial community probably influences the establishment of PGPMs in the rhizosphere as competition may increase or decrease. The results indicate that organic fertilization is another factor that increases the complexity in BE applications. Several results from the project and from literature indicate that BE application with manure or other organic fertilizer shows high potential and should therefore be further investigated (Bradáčová et al., 2019a; Mpanga et al., 2018; Rubin et al., 2017).

Foliar application was used only for SWE. In 2016 a significantly reduced maize yield for SF as compared to the untreated Ctrl was observed. One explanation was the unfavourable timing for the treatment as plants were already suffering from drought stress and the additional stress by BE application could not be compensated later on. However, results indicate that by this application technique SF was taken up by the plant and was effectively provoking a plant response. In experiments on winter wheat, performed in 2016 – 2017 in the institute, foliar application of all SWE, including those without additional micronutrient supplements as SF, were increasing yield by 20 % on average (*unpublished*). Foliar application of SF was also successful for stress alleviation in *Arabidopsis* (Omidbakhshfard et al., 2020).

For tomato and planted cultures, a drenching method before or at the time of transplantation seems to be the most efficient option, ensuring high product concentrations (and bacterial density) close to the root in the very early plant development while keeping application rates in an economically feasible range.

4.3.7.3 Multiple application

As described for the Rz strain suppression of *Rhizoctonia solani* infection in lettuce was especially effective after a second inoculation whereas application rates were less crucial (Chowdhury et al., 2013). Also in meta-analyses on PGPRs against *Ralstonia* strongest disease suppression was reported for multiple PGPR applications (Chandrasekaran et al., 2016). To give a conclusion for application techniques: Many BE effects are concentration dependent and multiple applications help to ensure high population rates during early plant development. Therefore, combination of various application techniques and multiple applications are for sure the most effective method and should be used if economically feasible but seed treatment strategies, although less effective, can still be the more efficient choice if financial resources are limited and the financial benefit for certain crops is comparably low.

4.3.8 Redefining the objective of BE applications

4.3.8.1 Basic considerations

BE products as well as their potential mechanisms of actions are diverse. The reasons to apply a BE might differ depending on crop plants, farming conditions, soil types, form of agricultural practice (conventional or biological farming practices, integrated pest management), season (e.g., cold stress) and water supply. In contrast to chemical plant protection agents (pesticides, herbicides) or mineral and organic fertilizers, there are no predefined answers to the question "Why using a BE?". Additionally, reproducibility of the products is limited due to the fact that the exact mode of action for growth stimulation remains very often unclear (Lesueur et al., 2016b; Raymond et al., 2020; Yakhin et al., 2017). In contrast, the targeted application of synthetic hormones, as sometimes done in conventional farming and fruit cultivation, and mineral nutrients (macro- or micronutrients) is much more reproducible and therefore the commonly used strategy, whereas in organic farming strategies are more holistic, trusting in the input of organic fertilizers with high amounts of organic matter and their stimulation of the natural soil microbial community. BE-application therefore still needs to find its place on the market to contribute efficiently to modern plant nutrition and farming strategies (ibid).

4.3.8.2 The right "action window"

To exhibit an effect, a BE product, similar to other products that are applied, needs an untreated plant to overcome a stressful or suboptimal situation for development. This could be abiotic stress, such as nutrient deficiency, salt, cold and drought stress or biotic stress by pathogens or a non-adapted microflora. Furthermore, PGPR application can be used to overcome plant internal growth limitations. For example the production of tryptophan production and growth stimulation of the Rz strain was tested on *Lemna minor*, the common duckweed, one of the smallest plants on earth (Idris et al., 2007). Obviously, fast growth is not a natural strategy of this plant and therefore external hormonal stimulation had strong influence on plant growth in this plant species. Another example would be the induction of early plant growth in winter wheat increasing vegetation time (as a possible explanation for increased yields in winter wheat experiments mentioned in 4.2.2.4).

The "action window" (see also 4.2.3) is a prerequisite to observe differences between a treated and untreated plant and can be proven by including the right "Positive Control" in the experimental design. This idea refers to Liebig's law of the minimum and the concept of a limited resource / factor that finally determines plant growth (Raymond et al., 2020).

Focussing on the improved acquisition of phosphorus, in this thesis the Positive Control was in many experiments a treatment that received additional P-fertilization as compared to all other treatments. In the cold stress experiments the plants that were crowing outside the CRZ temperature were the Positive Control. If a Positive Control does not show an improved yield, there is no action window for an inoculated BE. Nevertheless, the limiting factor might be not the one that was expected. Therefore it is possible that a BE treatment reaches higher yield than a Positive Control (as observed in Exp_19 for the Novatec or the manure treatments for root growth (Figure 3-54) or in the field experiment in 2014 (Figure 3-74)).

Nevertheless, environmental conditions must provide a basis for PGPR-plant interactions. For example, as microbes depend on plant exudates for nutrition, factors strongly inhibiting photosynthesis and assimilate production (severe P-deficiency, cold and drought stress, low light intensity) therefore may lead to growth depression in BE treated plants (trade-off dilemma). Another example are highly buffered P-deficient soils that inhibit any plant interaction with the BEs (Kuhlmann, 2014; Mpanga et al., 2019a; Nkebiwe, 2016; Probst, 2015).

4.3.8.3 Inundation

One crucial consideration for the use of microbial bioeffector products is the aspect of "inoculum" density. The term "inoculum" is generally used when speaking of applying microbes to new environments. Inoculations are well known from food industry, for example yeast for baking or beer brewing, lactobacilli in dairy products, cabbage or sour bread and microbes (especially pathogens) on sterile media. After inoculation of small amounts of these microbes, they establish rapidly in their new environment, occupying this environmental niche effectively and highly resistant against intruders and therefore also increasing shelf life as important function for human nutrition.

Nevertheless, these inoculations are only efficient due to the lack of competition in the environmental niche. In contrast, the application of microbial BE products for plant growth stimulation, especially in agricultural practice, may sometimes be similar to an "inundation", a term used, for example, in biological plant control for the application of parasitoidic *Trichogramma* wasps against insect herbivores in maize (Smith, 1996). Here, high amounts of non-adapted organisms (high "inoculum densities") are released into a target environment to exhibit effects by sheer density. Population density rapidly declines and in many cases establishment of the non-adapted organisms fails due to the harsh environmental conditions or the strong competition with the natural and adapted population in the respective niches. This

is also true for many of the PGPRs applied in agriculture, especially for non-spore forming bacteria (Tabassum et al., 2017; van Elsas et al., 1986; Van Veen et al., 1997). For the Px strain we observed steep decline in root colonization rate in pot and field experiments as previously noted (Buddrus-Schiemann et al., 2010). As also seen in many publications, the influence of inoculated PGPRs or biostimulants on soil microbial community is mainly transient. The advantage is that the risk of negative influences on ecosystems on a long-term scale, as described for invasive species, is relatively low. Also, important to consider is that an inoculated PGPR showing good 'rhizosphere competence' and that is able to establish in the rhizosphere not necessarily exhibits more plant-beneficial traits as other strains of the natural soil community. The ability of the plant to shape its own rhizosphere community by root exudation is well known (Bais et al., 2006; Berg and Smalla, 2009) and the development of disease suppressive-soils is a natural process (Berendsen et al., 2012; Haas and Défago, 2005). Therefore, high application rates to ensure an efficient inoculum density, at least in the first days after application, seem almost obligatory. Nevertheless, some considerations might help to reduce economic costs for BE application.

4.3.8.4 Defining objectives

As mentioned in the introduction there are three major functions of BEs: biofertilization, biocontrol and biostimulation. This subchapter focusses on considerations and recommendations for the respective functions.

4.3.8.4.1 Biofertilization

Biofertilization, as explained in detail in the introduction as well as several experiments, can be described as the ability of microorganisms to solubilize sparingly available nutrients from organic or mineral sources to increase their plant availability. Therefore, high application rates are recommendable to ensure that a certain threshold concentration of BE product is able to solubilize high amounts of mineral nutrients from their mineral sources.

Results from this thesis as well as results from the overall Biofector project and a recent literature review (Raymond et al., 2020) give no reason to keep the hopes high to improve plant P-supply from sparingly soluble Ca-P sources by phosphate solubilizing microorganisms, especially bacteria under practice conditions. Instead, the observed growth stimulations, especially under ammonium-N-fertilization, were explained by mechanisms of biostimulation. Also the results from two meta-analyses discussed in 4.2.1.1 indicate that 'P-solubilization' is probably not a crucial trait for determining effective plant growth stimulation (Schmidt and Gaudin, 2018; Schütz et al., 2018).

Nevertheless, the term “bioeffector” generally includes mycorrhiza or rhizobia products, although these products were not addressed in this thesis. As described in the introduction, mycorrhiza inoculum was repeatedly able to improve P acquisition of host plants and their usage in greenhouse cultures is well established. Rhizobia are widely used in soybean to improve N-supply by biological N-fixation. This is also supported by the global meta-analysis (Schütz et al., 2018). Especially for mycorrhiza inoculum the combined application with microbial BEs as MHBs can still be interesting for high-value crops such as tomato or other greenhouse cultures.

There are several factors that increase probability of P-solubilization by PGPMs whereas probably only the combination of multiple factors enable efficacy (Mpanga et al., 2020; Mpanga, 2019):

1) Low C:P ratio (Zhang et al., 2014)
   This may be achieved by starter fertilization of P or ammonium (e.g., underfoot placement of ammonium phosphate). A valuable alternative might be the combination of the CULTAN method and BE application (Nkebiwe et al., 2016b; Weinmann, 2017, p. 396 ff.).
2) Low buffer capacities in the substrate
   Low pH soils or Al-rich soils might have higher potential for biofertilizer (see 4.3.5). In planting cultures buffer capacities of substrate mixtures can be reduced by using sand, peat or other substrate decreasing pH.
3) Targeted application of BEs at high inoculum rates
4) Combined application of selected BEs and organic fertilizers:
   a) Mycorrhiza inoculum and compatible mycorrhiza helper bacteria
   b) Effective PSM or PSBs
   c) Additional delayed application of bacterial-grazing nematodes (Ingham et al., 1985; Irshad et al., 2012, 2011, 2013)
   d) Organic fertilization to increase bioactivity and microbial turnover through natural bacterial and fungal predators such as protists (Xiong et al., 2018)
5) Providing time
   Using PGPMs as compost amendment (Zayed and Abdel-Motaal, 2005) provides time to mineralize organic material.

4.3.8.4.2 Biocontrol

Biocontrol is generally referring to the ability of a BE to inhibit the growth or establishment of pathogens or insect pests or to protect the plant from plant diseases. Whereas the first aspect targets the plant pests the second aspect improves plant fitness and health by biostimulation. Therefore, the second aspect is addressed in 4.3.8.4.3 (“biostimulation”).

To target crop pests by mechanisms such as antagonism or competition, high inoculum densities are necessary. In disease suppressive soils for example, the whole soil microbial community differs from non-suppressive soils (see also 4.3.6.1). It was suggested that 0.1 – 10 % of the suppressive soil needs to be added to transfer the suppressiveness to other soils (Berendsen et al., 2012; Mendes et al., 2011). Additionally, biocontrol activity that directly targets a pathogen is more species-specific and often necessitates the additional application of conventional pesticides (Leng et al., 2011; Rodgers, 1993; Tabassum et al., 2017).

Although the application strategies in the form of inundation might be effective for protection of the plant from pests, from an economic point of view the efficiency for field crops might be low and strongly depends on high market prices of the crops. Similar to the recommendations for biofertilization, the application of BEs as biocontrol agents is most interesting for high-value crops in the greenhouse (as seen for tomato cultivation in the partner group in Romania, although the mode of action remains still unclear), for potato cultivation in the form of tuber dressings (as recommended for the Px product by the company Sourcon Padena) and as a treatment of pre-cultivated vegetable or fruit plants in seedling stage before transplanting in the field. Under these conditions, targeting small soil volumes and the early developmental stage of the crop plant, application is efficient as high inoculum densities can be reached, though overall application rates are kept in an economically feasible range.

4.3.8.4.3 Biostimulation

Biostimulation is here defined as the ability of the BE to stimulate plant metabolism, generally via signal compounds changing (pre-/post-)transcriptional, metabolic or cellular processes. Possible modes of action are scavenging ROS, triggering ISR, changing sink-source relationship (shift from vegetative to generative phase) and root-to-shoot ratios of specific plant hormones improving root growth for nutrient or water acquisition and delaying or promoting senescence processes. Here the term "priming" is of importance as it describes a phenomenon that resembles vaccination in animals (Loon, 2007). The plant defence or hormonal system is triggered to respond better, faster and more appropriate to suboptimal or stressful environmental conditions. Target of the BE action is always the plant itself. Additionally, results from experiments of this thesis as well as *in vitro* experiments reported in literature indicate that effectiveness is highest during youth development of the plant.

The differentiation of the mode of action could help to find explanations why application rates, dependency of environmental factors and therefore reproducibility strongly differ among products. For example low dosages of seaweed or plant extracts sometimes were more efficient in influencing plant development than high dosage soil applications of microbial BEs and filtrates were more efficient than the PGPM itself (Akladious and Abbas, 2014).

Biostimulation is the most common mechanism for BE-plant interactions. Most of the products, if not all, can be assumed to influence plant metabolism, although the mode of action and efficacy may differ among products (Bulgari et al., 2019; du Jardin, 2015; Halpern et al., 2015; Van Oosten et al., 2017; Yakhin et al., 2017). Experimental evidence but also knowledge about the sensitivity of plant physiological processes, indicate that for this mechanism "less may be more", making the use of BE products less cost-intensive.

Nevertheless, it is also the most complex mechanism and the efficacy or effectiveness under applied conditions probably depends on many environmental factors but also on plant-BE specific signalling compounds and effective BE products are often extracts with highly concentrated bioactive substances. Another important factor is timing. As a general rule, a BE product should always be applied before the plant suffers from (a)biotic stress. Certain fertilization strategies, such as the fertilization with ammonium-N, may improve the interaction of the plant and microbial BEs. Additionally, they provide a basis for BEs to act, for example by reducing soil buffer capacities, as root growth stimulation will only contribute to nutrient uptake if nutrients, such as P, are soluble.

4.4 Synopsis

Performing research in between agricultural practice and lab-based basic research means to consider efficacy, effectiveness and efficiency. While efficacy of many bioeffector (BE) products, as well as unformulated bacterial or fungal strains, was proven under controlled conditions in the laboratory, the exact mechanism and mode of action that are effective to improve plant growth and development under applied conditions are still under discussion. Therefore, investigations of the modes of action were included in this thesis.

Objective of this research was to elucidate and determine factors for successful plant-BE interaction and to increase effectiveness of the products, with the focus on cold stress and P-acquisition. Efficiency, including economic considerations such as cost-benefit ratios, were not focus of this research but were addressed briefly in the discussion. In short, the efficiency of many products, especially of those based on the activity of microbes, is still not at the level for large-scale usage.

The investigated BE products could be classified into **three classes** by their effectiveness, closely linked to their composition and mode of action. Products of the first class were repeatedly effective in alleviating plant stress responses mainly due to their increased contents of the micronutrients Zn and Mn. The class comprised only seaweed extracts form the company Agriges that were enriched with the micronutrients in mineral form and could therefore be defined as **micronutrient fertilizer**. Investigations on specific modes of action indicated that superoxide dismutase (SOD) activity seemed to be positively correlated with the effectiveness of the Zn/Mn-enriched seaweed extracts for plant growth promotion and stress tolerance of maize plants under cold stress conditions.

The product **Proradix**® (Px), containing the PGPR *Pseudomonas sp.* “Proradix”, as the only member of the second class, was repeatedly able to stimulate plant root and shoot growth under non-stress conditions when the product was applied at high application rates, mainly due to a shift in the **natural soil microflora** by its carbohydrate-rich product formulation.

Products of the third and largest class were only in some cases effective to stimulate plant growth whereas in most cases products did not have any significant effect on plants and in few cases, when application rates were high (***Bacillus* and the *Penicillium sp.* products**), application techniques not optimal (e.g. *Penicillium sp.* in field experiments) or environmental conditions suboptimal for BE-plant interaction (very low soil-P availability or abiotic stress),

exhibited negative effects on plants. This group consists of all fungal products, seaweed extracts without additional micronutrients, and the tested *Bacillus* products.

The tested microbial BE products did not significantly stimulate root mycorrhization per g of root by soil-derived AM fungi, as proposed for "**mycorrhiza helper bacteria**". A solubilisation of Ca-phosphates from selective media by the tested BEs was observed by other group members. Nevertheless, under applied conditions no direct experimental evidence could be provided that PGPRs were able to contribute to plant P-uptake by **solubilizing P** in a significant way. Instead, **root growth stimulation**, for example due to auxin production or nutrient translocation, an increased activity of phosphatases, elevated microbial turnover or sink stimulation could explain improved P acquisition by plants after BE application.

Root and shoot growth stimulation is often proposed in response to hormonal shifts. Common mechanisms are the reduction of ethylene in the roots by microbial ACC deaminase activity or the direct root stimulation by bacterial auxin-derivatives or segregation of AHLs. In our **gene expression study** many plant stress-related genes were differentially expressed and some of the responses resembled those observed under P-deficiency whereas analysis of the plant P status did not reveal clear evidence for a BE-plant competition for P. Instead, it seems that in BE treated plants, especially in those with the Proradix product, the levels of internal inorganic P were increased and several plant defence mechanisms, such as the production of secondary metabolites, ethylene production and reception and the expression of several classes of stress-related transcription factors, including JA-responsive JAZ genes, were triggered. This "**stress priming**" may have increased root growth but also overall plant performance three weeks later. Links in plant signalling pathways in response to abiotic (e.g., nutrient depletion or cold stress) and biotic stress were reported before and actual studies support that the activation of ISR by PGPRs might also be involved in abiotic stress alleviation.

Although studies on similar products like those categorized in the third class, have shown the growth promoting potential of these products, data of this thesis reflect the mixed outcome of the meta-analysis conducted for the Biofector project on several hundred data sets from different research groups in Europe. **Low reproducibility** of microbial BE products still limits the large-scale implementation of BE products into agricultural practice although a huge potential is seen by many scientists and agricultural industry.

By using screening approaches under various conditions and by comparison of the results with experiments performed in other working groups we tried to elucidate environmental factors that determine **BE efficacy**. Best reproducibility of BE products is commonly found for products in sterile substrates and **erosive or disease conducive soils** and many BE products are on the market as biocontrol agents or biopesticides due to their potential to suppress plant pathogens. Also, in our studies strongest BE effects were observed under controlled conditions and in **semi-sterile soils**, mainly during early plant development, whereas for none of the products of the three classes significant increases in corn yield in field experiments could be observed although BE application repeatedly showed positive trends.

Another important factor was the average **nutrient availability**, especially of phosphorus, which should be at low to medium level to ensure that early plant development and therefore root colonization are not inhibited. High **buffer capacities**, as seen in Ca-rich soils with extremely low phosphorus availability, often produced negative BE-effects on plant growth whereas reduction of the buffer capacities by sand-mixture or **ammonium nutrition** strongly improved BE efficacy. Later experiments in the working group showed that the combination of BE products with ammonium nutrition leads to the most stable plant-BE interactions and showed best reproducibility. Additionally, low **temperature** (below 14 °C) was reducing efficacy of most microbial products if not applied in combination with Zn/Mn or ammonium fertilization. We speculate furthermore that factors such as light condition (intensity and quality), temperature, water and soil organic matter (SOM) contents and biological activity in the soil are additional factors that determine BE effects. Here also the aspect of Al-toxicity might be important. The results are supported by **global meta-analyses** that indicate average yield increase by PGPMs between 10 to 40 % with best results in dry climates, in soils with low to medium soil P levels and low SOM contents.

Similar to basic research, outcomes from applied research are **condition-specific**. Economic aspects should therefore be more often considered when designing pot experiments, especially with the focus on application rates that need to be reduced. In agricultural practice, such as field and greenhouse experiments, control treatments should be included that test those approaches that are economic and already applicable for farmers, as the mode of action may change with changing applications strategies (soil vs. foliar or seed application, high vs. low dosages, early vs. late-stage application). For commercial products recommendations of the producers can be used as references for the control treatments.

Especially **seed treatments** for maize have a high potential to decrease inoculum rates on per ha basis while ensuring that a high bacterial density is present at the time of seed germination. Here the challenge will be to implement BE products into seed coating technologies and seed industry. For **greenhouse** or planting culture **drenching of seedlings** is a recommended application method as effectiveness of PGPRs often was increasing with higher inoculum rates and **multiple applications**. Germination tests for bacterial products but also the strong detrimental effects for the BFDC product at high application rates indicated that optimal application rates need to be determined at a species or product level whereas a simplified classification as fungal, bacterial or seaweed products is not sufficient for successful application strategies.

Tests for toxic or **prebiotic properties** of seaweed extracts showed the sensitivity of some microbial products to high amounts of bioactive substances but also the potential of certain product combinations for stabilization or stimulation of bacterial populations, findings that should be considered for future development of BE products.

To **improve BE** products the isolation of adapted strains, compatibility tests for microbial consortia, new product formulations and application strategies in combination with organic or ammonium fertilizers should be in the focus of future research.

Observations for the Proradix product and the higher efficacy of microbial consortia emphasize again the importance of a balanced natural soil microflora for plant health. One conclusion is therefore that the stimulation and **protection of this adapted microflora** should be a major concern for modern agriculture.

5 Outlook

5.1 Diversity and stable systems

Climate change and the decline in biodiversity are severe threats to human society and both threats are interrelated. For example deforestation of rainforests is seen as one tipping element in climate change (Lenton et al., 2008). Modern biological and ecological research provides growing evidence for the complexity of ecosystems and their interrelated functions. Biodiversity is important to stabilize ecosystems against excessive propagation of invasive species especially under environmental changes (Levine et al., 2004), for example due to the reduction of trade-off costs against herbivores or pathogens via tritrophic interactions in which one species depends on another to defend itself against a third one (Karasov et al., 2017), or due to microbial hub species that shape microbial communities (Agler et al., 2016). In forestry with the massive economic losses by windthrow in spruce-monocultures it was first realized that monoculture systems and cultivation of unadapted species might not always be lucrative (Felton et al., 2016; Klimo et al., 2000). The increasing risk of species extinction is mentioned since decades, but the massive decline of populations and reduction of animal biomass, meanwhile observed and quantified, is assumed to be the "Earth's sixth mass extinction" that will largely affect the totality of life and human civilization on this earth (Ceballos et al., 2017, 2015). About 41 % of insect species are declining, a third of all insect species are threatened (Sánchez-Bayo and Wyckhuys, 2019). For protected nature areas in Germany an even higher decline of 76% in just 27 years for airborne insect biomass was estimated (Hallmann et al., 2017). Publications on the decline in biodiversity urgently recommend a diversification of agriculture and a reduction of pesticides in agriculture as changes in land-use due to agriculture are the main drivers for species loss (Sánchez-Bayo and Wyckhuys, 2019).

At the same time, agricultural systems in many countries in the world stagnate or decline due to climate change or misuse of resources. Recent publications indicate that agricultural production of many key resources has already reached its peak, meaning that the potential to further increase yields declines (Seppelt et al., 2014). Main attention should be given to alterations in human nutrition, alternative food sources, reduction of food waste but also to a sustainable intensification of agriculture possibly combining organic farming, biotechnology and socio-economic approaches for market intensification, especially in developing countries (Godfray and Garnett, 2014). To achieve intensification some publications suggest a spatial reallocation of crops to the most suitable locations and an increased cropping intensity,

meaning multiple annual cropping to prevent further land use and detrimental effects for biodiversity (Mauser et al., 2015). The highest potential for intensification lies thereby in developing countries, especially West to South Africa, India and Central America but also in Eastern Europe. Promotion of organic farming was one of the recommendations given in the final report of the International Assessment of Agricultural Knowledge, Science and Technology for Development (IAASTD) ("Weltagrarbericht") to achieve a worldwide sustainable agriculture and land use system (Schmidtner and Dabbert, 2009).

Conservation agriculture and no-tillage strategies both focus on the conservation and protection of soils and their complex microbial network. As an important part of soil ecosystems, the microbial community needs to be preserved to provide resistance to abiotic and biotic stress that will accumulate with climate change. For disease-suppressiveness of certain soil pathogens replanting and long-term monoculture seems to be a prerequisite (Berendsen et al., 2012), although, many other diseases are caused due to continuous replanting. Also mycorrhiza (Jeffries and Rhodes, 1987; Siddiqui and Pichtel, 2008) but also soil bacteria biodiversity and abundance is negatively affected by tillage (Castro-Sowinski et al., 2007). Similarly, the "Permaculture" movement focusses on soil conservation but at the same time on increased crop diversity depending on the environmental conditions. Therefore, different systems need different solutions.

5.2 Potentials and constraints

5.2.1 Potentials for the new 'Green Revolution'

As mentioned in 1.1 and further expounded above a new and greener revolution is necessary to preserve the Earth's ecosystem for future generations. Unfortunately, further intensification and protection of the environment seem to be irreconcilable contradictions. Therefore the "New Green Revolution" seems to be "diversification". Biostimulants are certainly one component of this diversification to a greener revolution. The aspect of biocontrol was also mentioned in the IAASTD report as one important aspect of organic farming. The application of PGPR or other plant stimulants as biostimulants or biofertilizers cannot be separated from their potential activity as biocontrol agents. Biopesticides but also biological pest control are already frequently used in organic farming and therefore new products are more easily adapted.

BE products could additionally be interesting for remediation (Lopes et al., 2018; Ma et al., 2011) and restoration agriculture in degraded, disturbed, unbalanced or deserted systems as

shown by experiments in the Negev desert, Israel (Bradáčová et al., 2019a) or in arid or semiarid conditions of Mexico (Bashan et al., 2004). Nevertheless, securing the survival of the inoculum under these conditions will be a major challenge (Bashan, 1998).

As simulations from the MARS crop yield forecasting system of the European Joint Research Centre (EU Commission) indicate, the increase in temperature and prolonged vegetation period in autumn due to climate change may delay winter hardening and reduce frost tolerance of winter wheat leading to more frost-kill events with rapid temperature drop even in cold adapted species (van den Berg et al., 2020). Under these changing conditions targeted and well-timed application of biostimulants may improve tolerance of crops to cold and freezing stress.

Meta-analyses, large-scale research projects and reviews clearly show the potential of PGPRs and other biostimulants for plant growth stimulation especially under medium and low P conditions suggesting that P-acquisition is one important mechanism for BEs, although the mode of action behind this is probably not P-solubilization.

5.2.2 Constraints

There are many constraints limiting effectiveness of PGPR or biostimulant application including shelf-life, specificity of biocontrol activity and optimal application strategies (Basu et al., 2021; O'Callaghan, 2016; Tabassum et al., 2017). Development of efficient seed treatment technology is a major challenge. Another important aspect is the rhizosphere competence. As mentioned before, the introduction and establishment of new bacterial strains with high potential for growth promotion or N-fixation is often limited due to the competition with well-adapted autochthonous strains (Rodríguez-Navarro et al., 2011). This is also reflected in research of PGPRs under more natural experimental conditions such as non-sterile soils in pot and especially field experiments (Raymond et al., 2020).

Although PGPM application is still not at a level to have significant ecological impact and actual data on shifts in microbial communities suggest only transient effects of BE inoculation, a risk assessment to prevent biodiversity loss in the soil microflora by large-scale usage of inoculants that are able to establish in the soil, should be considered in the future (Castro-Sowinski et al., 2007) as negative effects of invasive species certainly need to be avoided to protect biodiversity (Sánchez-Bayo and Wyckhuys, 2019).

5.3 Research fields

5.3.1 Microbial community and hub species

Basic research on PGPR or biostimulants is highly valuable to understand the function of bioactive substances and molecules in our environment and create scientific evidence for ecosystem properties and the importance of biodiversity and plant-microbe interactions.

The activity of a *Pseudomonas* sp. containing BE product tested in this thesis was probably connected to selective stimulation of the microbial community. For several PGPR products the influence on microbial community in the rhizosphere was shown. However, there is more information needed on the question if shifts in microbial community are a response to changes in plant growth after PGPR application or one causal mode of action by which plant growth is stimulated. Probably both aspects are somehow true, but the extent may differ among PGPRs (Kang et al., 2013). Another interesting question concerns the influence of rhizosphere exudation on functional traits. It is known that the plant is shaping its rhizosphere and that specific taxa are enriched, nevertheless, less clear is the abundance or dominance of certain traits in the rhizosphere that fulfil plant growth relevant functions (Mendes et al., 2013). This may be useful to isolate traits a PGPR should possess to stimulate the plant growth under the respective situation.

In the last years the concept of keystone species that strongly shape and influence their ecosystem (Cardinale et al., 2012; “Keystone species,” 2021), receives increasing interest in soil and plant microbiology (Backer et al., 2018; Caradus and Johnson, 2020; Hannula et al., 2017). In this context also the term microbial “hub” is important. One feature of a hub species is the significant correlation between its own presence and the abundance of many other taxa. For *Arabidopsis* several hub species were identified by analysing community structures and co-occurrence networks of endo- or epiphytically living fungi, bacteria and oomycetes (Agler et al., 2016). Especially the presence of the obligate biotrophic oomycete pathogen *Albugo laibachii* was strongly influencing the endo- and epiphytic community of all taxa. This was explained by its lifestyle that depends on the control of the host plant metabolism. Nevertheless, also other taxa, including Proteobacteria from the taxa *Caulobacter* sp., a genus of *Comamonadaceae* and a genus of *Burkholderiales*, that are less closely associated to the host plant, were important hubs. The relevance of these finding for BE products is not yet clear. First, the identification at family level is not sufficient but needs further research. Second, hub species need to be identified separately for each plant species or crop cultivar.

Third, it is possible that the most hub taxa are not plant beneficial. Fourth, as described above, the influence or agronomic relevance of the community structure on crop performance has to be further investigated (Heijden and Hartmann, 2016).

For disease-suppressive soils research results indicate that plants can recruit certain bacterial taxa for their protection against pathogens (Mendes et al., 2011). In contrast to the concept of few hub species that stabilize the microbial community (Agler et al., 2016), the relative abundance of various taxa seems to be important for stabilization against the massive invasion of a specific plant pathogen that would cause a disease in the host plant (Mendes et al., 2013, 2011). Interestingly, research of Mendes et al. (2011) also identified a specific *Pseudomonas* sp. strain with a trait - the production of an unknown lipopeptide - that seemed to be responsible for the suppression of the fungal disease. Together the results suggest that microbial communities or consortia with different traits allow the activity of a specific trait that is active for biocontrol or biostimulation in a certain condition. Therefore, consortia reduce the pressure to isolate one 'super strain' with all necessary plant beneficial and root colonization relevant traits. The knowledge on specific hub species could improve the development of new biocontrol or biofertilizer products, identify conditions that modulate community structure, decrease competition in the rhizosphere and increase the probability of new PGPMs to successfully colonize the plant root.

In another research hundreds of strains from naturally occurring leaf, root and rhizosphere communities of *Arabidopsis* were isolated and synthetic communities imitating the natural once were created by using data from 16S rRNA sequencing (Bai et al., 2015). By inoculation into sterile clay substrates, they created artificial soils. The company AgBiome, Inc. uses such data and culture collections to develop new biocontrol products composed of complex microbial consortia. Large-scale characterizations of soil microbiomes become more easily feasible due to the fast development of –omics technologies and big data computing (as seen in fungal metacommunity studies from Toju et al., 2018). It might be possible that in future a world map based on soil microbiome data will give valuable recommendations to create soil inoculums with hub species, following ideal examples of healthy soils for the ecosystem of interest, to manage and conserve or restore soils in combination with input of organic matter and planting of adapted pioneer species. Indeed, it was already shown that agricultural management practices such as organic farming versus conventional farming strongly influences bacterial communities and abundance of taxa (De Corato, 2020; Heijden and Hartmann, 2016).

5.3.2 Light and PGPM-plant interactions

The importance of light conditions for the establishment of symbiotic interactions was shown for the intimate symbiotic relationship of plants with mycorrhiza and rhizobia (Kiers and Heijden, 2006; Lau et al., 2012). Nevertheless, more research is needed to quantify the impact of light on PGPR-plant interaction. Conversely, the hypothesis of a sink stimulation by PGPM application, as reported for mycorrhiza and rhizobia (Kaschuk et al., 2009), as a general mode of action should be investigated.

5.3.3 Emphasis on applied research and field experiments

Besides the importance of basic research, activities of BE products must be validated more frequently under applied conditions (Schmidt and Gaudin, 2018). As seen for screening experiments on P-solubilization or biocontrol of pathogens, *in vitro* results often are not reproducible in practice. On the other hand, BEs that seem to be less effective under *in vitro* conditions might exhibit stronger effects in greenhouse experiments (Adesina et al., 2009; Basu et al., 2021; Schmidt and Gaudin, 2018). In this aspect also the use of terms such as “soil” and “non-sterile conditions” is important. Many publications still mix up substrates such as potting soil or peat substrates with natural soils and try to validate bioactivity and effectiveness of BEs under these conditions. Nevertheless, these substrates are far from a fertile soil in which billions of organisms in every gram shape and determine the processes and the starting conditions for newly introduced strains. Although more and more reviews on modes of action and successful plant growth stimulation emerge there is a lack of quantification by reliable meta-analyses. Results from the Biofector project suggest that the environmental condition, application strategy and product formulation are important determinants for effectiveness of BE products under applied conditions. It is therefore crucial to conduct meta-analyses that elucidate the role of environmental conditions that allow successful plant growth stimulation.

5.4 Product development

5.4.1 Strain selection and consortia

Although some results from the Biofector project indicate common modes of action and similar PGPM effectiveness under optimal experimental conditions and with high inoculum rates, many publications indicate a strong influence of PGPM species or isolate, probably depending on the rhizosphere competence of the PGPM including abiotic stress tolerance, antibiotic resistance and production, production and reception of signal compounds for PGPM-plant specific communication and quorum sensing, chemotaxis, biofilm formation, preference for root exudate quality or quantity and the expression rate of plant growth promoting traits such as organic acid, phosphatase, ACC deaminase or auxin production. To increase product efficiency and to reduce application rates effectiveness and reproducibility of PGPMs under applied condition still has to increase. Therefore the re-isolation of adapted strains, possibly using data from studies on microbiome, co-occurrence networks and hub species (as done by Zheng et al., 2021 for isolation of Pseudomonas strains against Ralstonia), and the development of consortia products (Mendes et al., 2013; O'Callaghan, 2016; Rocha et al., 2019; Van Veen et al., 1997) and their combined application may increase efficiency of products. A diverse mixture of natural strains is often superior to single strain inoculations (Jeffries and Rhodes, 1987; Weinmann, 2017). The superiority of composed products and consortia as compared to single BE treatments was also shown in Biofector and the COMPRO project, although this finding not always holds true (Bradáčová et al., 2019a, 2019b) and global meta-analyses have mixed outcomes. This may be due to low product quality often seen in consortia products (Jefwa et al., 2014).

Schmidt and Gaudin (2018) suggested to overthink the general lab-to-field pipeline as lab and pot screenings often do not reflect the effectiveness of BE products in the field. Probably the development of product formulations is too cost and time-intensive and is therefore conducted only for high-potential strains. However, if these strains are not effective in practice the strategy might finally not be the best. In some cases, it might be a possibility to simply replace various strains using the same formulations once basic conditions for taxa are established.

5.4.2 Formulations and application strategies

The development of product formulations is certainly one crucial aspect and a major challenge for the BE market (Tabassum et al., 2017). Nevertheless, there is an increasing

knowledge base on proper formulations and a continuous research for better carriers (Bashan, 1998; Herrmann and Lesueur, 2013). Here microgranules, combining organic material with different C/N ratios, mineral nutrients and microbial consortia (e.g., https://minigran.com/de/) seem to have potential.

Ammonium fertilization might play an important role to increase rhizosphere competence of microbial inoculum and to establish stable plant-PGPR-relationships (Mpanga et al., 2019a, 2019b) whereas in organic farming the combination with different organic and recycling fertilizers is of special interest. BE application in combination with manures showed highest yield responses during the Biofector project and in other meta-analyses (Rubin et al. (2017). As extensively reviewed by De Corato et al. (2020), agricultural practice and fertilization strategies using organic amendments (OA), specific composts and organic waste materials can strongly and reproducibly improve soil fertility and plant health due to the specific microbial composition in the OAs and the influence on soil microbial community. Nevertheless, the efficacy of the composts is disease or pathogen specific and cannot be generalized.

5.5 Bioeffector databases

The still increasing market for organic farming and production reflects the interest in society for more sustainable food production. Additionally, there is growing evidence for the importance and significance of plant-microbial interactions, indicated by the amount of described modes of action, signalling molecules, and active compounds but also successful applications of BEs, reported in many research articles on biostimulants and PGPRs. About 20.000 articles were found in Google Scholar searching for “plant growth promotion AND PGPR OR biostimulant” in the last 40 years. SCOPUS search shows an almost exponential increase in publications on PGPRs in the last 20 years. Nevertheless, alternative strategies for fertilization and plant protection are still not well integrated in the agricultural market or in farmers practice (Raymond et al., 2020). Reasons are low reliability of products and reproducibility of effects (Jefwa et al., 2014; Lesueur et al., 2016b), difficulties in the legislation of the products (du Jardin, 2015; Weinmann, 2017; Yakhin et al., 2017), e.g. due to undefined modes of action, active compounds or antibiotic resistance of certain PGPRs, such as *Pseudomonas* (see DART 2020 - Deutsche Antibiotika-Resistenzstrategie) resulting in a lack of transparency and clarity for farmers. Some reviews present roadmaps for product development and recommendations how to achieve reliability and quality control for both producers as well as users (Backer et al., 2018; Basu et al., 2021). These steps are important for quality of single products but do not improve clarity about the variety of BE products for

users. A multitude of models is visualizing PGPR-plant interactions and modes of action of biostimulants allowing us to understand specific concepts. In total they reflect the complexity of the overall processes and the dependency of a successful PGPR application on many environmental factors. The detection or formulation of a single cure-all product, although from farmer's point of view quite welcome, is not probable. Modern research projects such as "SolACE - Solutions for improving Agroecosystem and Crop Efficiency for water and nutrient use" (www.solace-eu.net) therefore try to use interdisciplinary approaches to combine the knowledge of different research fields. Here biostimulants are integrated in a more holistic management system. Nevertheless, as research approaches and scientific knowledge become more and more complex, clarity is reduced.

Therefore the importance of dissemination is increasing (Backer et al., 2018). The establishment of a reliable and consistent database, for example by global organisations such as the FAO, could be a valuable project for future generations and for the transformation to a more sustainable agriculture. Such databases should contain information from reliable sources on strains and active components, formulations, optimal environmental conditions, target crops, applications / modes of action (e.g., biocontrol against specific target pests or pathogens), application strategies, and best timing for application. Experimental evidence from lab and field experiments, including proper experimental designs and statistical analysis, should be regularly complemented by reports from farmers' practice. During the Biofector project a similar database was developed but was not maintained as its maintenance and continuous update is associated with immense effort. There are commercial databases such as https://biostimulants.online/ online. Nevertheless, as the protection of the environment is of interest for society the establishment of such databases need to be funded for long-term and should be administrated by public institutions. Due to new legislations, lists from the German research Julius-Kühn institute (JKI) are not updated and lack accessible information (https://web.archive.org/web/20130917080340/http://pflanzenstaerkungsmittel.jki.bund.de/array1.php, https://archiv-pflanzenstaerkungsmittel.julius-kuehn.de/). On the long-term the establishment of reliable databases with public access may allow decentralization of the fertilizer and pesticide market. This may promote technological development in fermentation processes and product formulation thereby reducing costs for bioeffector products. Trainings for multipliers working in the field of agricultural consulting as well as the integration of bioeffector application in multidisciplinary approaches will help to develop the potential of bioeffector products in achieving transformation processes to a more sustainable agriculture.

6 Literature

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7 Appendix

Table 7-1 All treatment results for the Rz product

Nr	Exp	VarName	Type	Stage[1]	Organ[2]	Rep[3]	Unit	Ctrl_M[4]	Ctrl_SD[5]	Rz_M	Rz_SD	Y+[6]
1	Exp_01	Rz	Pot	Veg	Shoot	4	g/pot	0.48	0.05	0.53	0.09	10.5%
2	Exp_02	Rz_Glc	Pot	Veg	Shoot	5	g/pot	3.20	0.68	2.68	0.26	-16.1%
3	Exp_02	Rz	Pot	Veg	Shoot	5	g/pot	2.85	0.95	3.20	0.61	12.1%
4	Exp_04	Rz	Pot	Veg	Shoot	6	g/pot	6.01	0.65	5.51	0.78	-8.3%
5	Exp_05	Rz	Pot	Veg	Shoot	6	g/pot	8.34	0.90	7.56	0.84	-9.3%
6	Exp_06	Rz	Pot	Veg	Shoot	5	g/pot	6.19	2.60	6.22	2.00	0.6%
7	Exp_06	Rz_T	Pot	Veg	Shoot	5	g/pot	4.41	3.90	6.87	1.58	55.8%
8	Exp_07	Rz_80	Pot	Veg	Shoot	5	g/pot	14.28	0.84	13.86	0.66	-2.9%
9	Exp_07	Rz_120	Pot	Veg	Shoot	5	g/pot	14.27	1.17	14.10	0.53	-1.2%
10	Exp_07	Rz_50	Pot	Veg	Shoot	5	g/pot	14.43	1.24	14.52	0.60	0.6%
11	Exp_08	Rz	Pot	Veg	Shoot	5	g/pot	3.52	0.41	3.44	0.30	-2.3%
12	Exp_08	Rz/SF	Pot	Veg	Shoot	5	g/pot	3.52	0.41	3.96	0.48	12.5%
13	Exp_08	Rz/Af	Pot	Veg	Shoot	5	g/pot	3.52	0.41	4.04	0.59	14.8%
14	Exp_11	Rz_30	Pot	Veg	Shoot	5	g/pot	0.43	0.09	0.35	0.04	-18.6%
15	Exp_11	Rz_50	Pot	Veg	Shoot	5	g/pot	1.76	0.34	2.04	0.32	15.9%
16	Exp_11	Rz_70	Pot	Veg	Shoot	5	g/pot	4.66	1.35	5.88	0.66	26.2%
17	Exp_12	Rz_SF	Field	Mat	Grain	4	ton/ha	7.20	0.64	6.89	0.64	-4.3%
18	Exp_12	Rz	Field	Mat	Grain	4	ton/ha	7.20	0.64	7.06	0.64	-1.9%
19	Exp_12	Rz_Af	Field	Mat	Grain	4	ton/ha	7.20	0.64	7.24	0.64	0.6%
20	Exp_13	Rz_low	Field	Mat	Grain	4	ton/ha	9.15	1.01	8.22	1.01	-10.2%
21	Exp_13	Rz_med	Field	Mat	Grain	4	ton/ha	9.15	1.01	8.48	1.01	-7.2%
22	Exp_14	Rz	Pot	Veg	Shoot	5	g/pot	8.47	2.24	10.14	1.89	19.7%
23	Exp_15	Std_Rz	Field	Mat	Shoot	5	ton/ha	20.08	1.32	19.38	1.31	-3.5%
24	Exp_15	CUL_Rz_gran	Field	Mat	Shoot	5	ton/ha	18.26	1.47	18.00	1.63	-1.5%
25	Exp_15	Man_Rz	Field	Mat	Shoot	5	ton/ha	19.76	1.31	19.69	1.31	-0.4%
26	Exp_15	CUL_Rz_broad	Field	Mat	Shoot	5	ton/ha	18.26	1.47	18.86	1.50	3.3%
27	Exp_15	Urea_Rz	Field	Mat	Shoot	5	ton/ha	19.28	1.31	20.41	1.32	5.9%
28	Exp_15	CUL_Rz_seed	Field	Mat	Shoot	5	ton/ha	18.26	1.47	20.38	1.73	11.6%
29	Exp_18	Rz_seed	Pot	Veg	Shoot	5	g/pot	1.30	0.22	1.25	0.16	-4.3%
30	Exp_18	Rz_single	Pot	Veg	Shoot	5	g/pot	1.30	0.22	1.61	0.25	23.6%
31	Exp_18	Rz_ triple	Pot	Veg	Shoot	5	g/pot	1.30	0.22	1.32	0.14	1.5%
32	Exp_20	Rz_Org	Pot	Veg	Shoot	5	g/pot	2.40	0.45	2.56	0.51	6.6%
33	Exp_20	Rz_Min	Pot	Veg	Shoot	5	g/pot	3.31	0.45	3.70	0.45	11.7%
34	Exp_21	Ba	Pot	Veg	Shoot	5	g/pot	1.26	0.08	1.30	0.17	2.5%
35	Exp_21	Rz	Pot	Veg	Shoot	5	g/pot	1.26	0.08	1.37	0.17	8.4%
36	Exp_21	Ba/P2	Pot	Veg	Shoot	5	g/pot	1.26	0.08	1.40	0.17	11.1%
37	Exp_23	Rz_Low	Pot	Veg	Shoot	5	g/pot	0.93	0.03	0.91	0.02	-3.0%

1: Stage = Plant stag, Veg = Vegetative stage, Mat = Maturity (full fruit and grain development)

2: Organ: Harvested plant organ

3: Rep = Replicates for each treatment; if outliers were reduced, in some cases replicates for Ctrl and Rz differ, e.g. in Exp_15; data shown for Exp_12 with only 4 replicates (Block V excluded)

4: Ctrl_M/ Rz_M = Mean value for the treatment (average of all replicates)

5: Ctrl_SD/Rz_SD = Standard deviation for the mean value of the treatment

6: Y+= Yield plus; increase in biomass or yield of the Px treatment as compared to control in % of the control

Table 7-2 All treatment results for the Px product

Nr	Exp	VarName	Type	Stage[1]	Organ[2]	Rep[3]	Unit	Ctrl_M[4]	Ctrl_SD[5]	Px_M	Px_SD	Y+[6]
1	Exp_01	Px	Pot	Veg	Shoot	4	g/pot	0.48	0.05	0.56	0.05	16.8%
2	Exp_02	Px_Glc	Pot	Veg	Shoot	5	g/pot	3.20	0.68	3.70	0.68	15.8%
3	Exp_02	Px	Pot	Veg	Shoot	5	g/pot	2.85	0.95	3.73	0.84	30.8%
4	Exp_03	Px	Pot	Veg	Shoot	5	g/pot	6.76	1.06	9.15	0.70	35.3%
5	Exp_03	Af	Pot	Veg	Shoot	5	g/pot	6.76	1.06	9.15	0.52	35.4%
6	Exp_03	SF	Pot	Veg	Shoot	5	g/pot	6.76	1.06	9.30	0.62	37.6%
7	Exp_03	PPP	Pot	Veg	Shoot	5	g/pot	6.76	1.06	9.30	1.10	37.6%
8	Exp_03	ECO	Pot	Veg	Shoot	5	g/pot	6.76	1.06	9.38	0.56	38.7%
9	Exp_03	AVZM	Pot	Veg	Shoot	5	g/pot	6.76	1.06	9.46	0.76	39.9%
10	Exp_03	AV	Pot	Veg	Shoot	5	g/pot	6.76	1.06	9.66	0.71	42.9%
11	Exp_04	Px	Pot	Veg	Shoot	6	g/pot	6.01	0.65	6.91	0.62	15.0%
12	Exp_05	Px	Pot	Veg	Shoot	6	g/pot	8.34	0.90	8.32	0.31	-0.3%
13	Exp_06	Px	Pot	Veg	Shoot	5	g/pot	6.19	2.60	5.67	1.66	-8.3%
14	Exp_06	Px_T	Pot	Veg	Shoot	5	g/pot	4.41	3.90	7.99	3.80	81.2%
15	Exp_07	Px_50	Pot	Veg	Shoot	5	g/pot	14.43	1.24	14.46	0.74	0.2%
16	Exp_07	Px_80	Pot	Veg	Shoot	5	g/pot	14.28	0.84	14.37	1.00	0.6%
17	Exp_07	Px_120	Pot	Veg	Shoot	5	g/pot	14.27	1.17	14.48	0.49	1.5%
18	Exp_08	Px	Pot	Veg	Shoot	5	g/pot	3.52	0.41	3.60	0.51	2.3%
19	Exp_08	Px/SF	Pot	Veg	Shoot	5	g/pot	3.52	0.41	3.80	0.55	8.0%
20	Exp_08	Px/Af	Pot	Veg	Shoot	5	g/pot	3.52	0.41	4.48	0.41	27.3%
21	Exp_10	Px	Pot	Veg	Shoot	5	g/pot	32.16	3.17	32.86	3.26	2.2%
22	Exp_10	Px/SF	Pot	Veg	Shoot	5	g/pot	32.16	3.17	33.10	3.31	2.9%
23	Exp_10	Px/AVZM	Pot	Veg	Shoot	5	g/pot	32.16	3.17	34.48	2.00	7.2%
24	Exp_11	Px_30	Pot	Veg	Shoot	5	g/pot	0.43	0.09	0.34	0.07	-20.9%
25	Exp_11	Px_70	Pot	Veg	Shoot	5	g/pot	4.66	1.35	6.28	0.45	34.8%
26	Exp_11	Px_50	Pot	Veg	Shoot	5	g/pot	1.76	0.34	2.59	0.43	47.2%
27	Exp_12	Px	Field	Mat	Grain	4	ton/ha	7.20	0.64	7.23	0.64	0.4%
28	Exp_12	Px_SF	Field	Mat	Grain	4	ton/ha	7.20	0.64	7.32	0.64	1.8%
29	Exp_12	Px_Af	Field	Mat	Grain	4	ton/ha	7.20	0.64	7.58	0.64	5.4%
30	Exp_14	Px	Pot	Veg	Shoot	5	g/pot	8.47	2.24	10.75	1.13	26.9%
31	Exp_15	Man_Px	Field	Mat	Shoot	5	ton/ha	19.76	1.31	19.69	1.31	-0.4%
32	Exp_15	Std_Px	Field	Mat	Shoot	5	ton/ha	20.08	1.32	20.09	1.31	0.0%
33	Exp_15	Urea_Px	Field	Mat	Shoot	5	ton/ha	19.28	1.31	20.51	1.31	6.4%
34	Exp_15	CUL_Px_broad	Field	Mat	Shoot	5	ton/ha	18.26	1.47	19.73	1.53	8.0%
35	Exp_15	CUL_Px_gran	Field	Mat	Shoot	5	ton/ha	18.26	1.47	20.46	1.55	12.0%
36	Exp_15	CUL_Px_seed	Field	Mat	Shoot	5	ton/ha	18.26	1.47	19.48	1.52	6.7%
37	Exp_17	Px/W	Pot	Veg	Shoot	5	g/pot	0.34	0.06	0.38	0.04	10.5%
38	Exp_17	Px/M	Pot	Veg	Shoot	5	g/pot	0.36	0.03	0.42	0.08	15.9%
39	Exp_19	MKH_Px	Pot	Veg	Shoot	5	g/pot	10.04	0.74	9.40	0.74	-6.4%
40	Exp_19	Nit_Px	Pot	Veg	Shoot	5	g/pot	7.18	0.74	6.96	0.74	-3.1%
41	Exp_19	Nov_Px	Pot	Veg	Shoot	5	g/pot	10.56	0.74	10.57	0.74	0.1%
42	Exp_19	MP_Px	Pot	Veg	Shoot	5	g/pot	9.21	0.74	9.38	0.74	1.8%
43	Exp_19	MP_NI_Px	Pot	Veg	Shoot	5	g/pot	9.45	0.74	9.73	0.74	3.0%
44	Exp_19	MKH_NI_Px	Pot	Veg	Shoot	5	g/pot	9.65	0.74	10.18	0.74	5.5%
45	Exp_20	Px_Min	Pot	Veg	Shoot	5	g/pot	3.31	0.45	3.85	0.45	16.2%
46	Exp_20	Px_Org	Pot	Veg	Shoot	5	g/pot	2.40	0.45	2.90	0.45	20.7%
47	Exp_22	Px	Pot	Veg	Shoot	5	g/pot	6.19	0.83	7.95	1.18	28.5%
48	Exp_23	Px2_Low	Pot	Veg	Shoot	5	g/pot	0.93	0.03	0.92	0.01	-1.7%
49	Exp_23	Px_Low	Pot	Veg	Shoot	5	g/pot	0.93	0.03	0.93	0.02	-0.6%
50	Exp_23	Px_UFP	Pot	Veg	Shoot	5	g/pot	0.93	0.03	0.99	0.05	5.7%

1: Stage = Plant stag, Veg = Vegetative stage, Mat = Maturity (full fruit and grain development)

2: Organ: Harvested plant organ

3: Rep = Replicates for each treatment (If outliers were reduced, in some cases replicates for Ctrl and Px differ)
Data shown for Exp_12 with only 4 replicates (Block V excluded)

4: Ctrl_M/ Px_M = Mean value for the treatment (average of all replicates)

5: Ctrl_SD/Px_SD = Standard deviation for the mean value of the treatment

6: Y+= Yield plus; increase in biomass or yield of the Px treatment as compared to control in % of the control

Table 7-3 List of all 174 DEGs found by NoiSeq method

Putative function or protein structure	DEG	Func.	Cluster	Px/Ctrl	Rz/Ctrl	FPKM	Gene	NCBI	Gramene
BETA GLUCOSIDASE 13	BE	Glu	Cl_1	-2.05	-2.11	9.63	100281760	NM_001154680	GRMZM2G014844_T01
Putative Jacalin-like plant lectin	Px	LJ	Cl_1	-1.04	-0.68	45.62	100502041	NM_001196600	GRMZM2G163406_T06
Unknown; salt and JA induced gene	Px	LJ	Cl_1	-1.05	-0.88	58.19	100274518	NM_001148875	GRMZM2G050412_T01
Acyl-desaturase 6	Px	LM	Cl_1	-2.31	-1.20	3.93	103638337	XM_008661278	GRMZM2G003368_T01
GDSL-like Lipase	BE	LM	Cl_1	-1.82	-1.47	8.52	100192615	NM_001137830	GRMZM2G374475_T01
GDSL-like Lipase	Px	LM	Cl_1	-2.02	-1.33	6.31	100274304	NM_001148667	GRMZM6G799230_T01
GDSL-like Lipase	BE	LM	Cl_1	-2.06	-1.36	9.71	100280267	NM_001153195	GRMZM2G044882_T01
GDSL-like Lipase	BE	LM	Cl_1	-2.03	-1.62	6.68	100281363	NM_001154281	GRMZM2G062577_T01
ERECTA kinase	BE	LRR	Cl_1	-2.91	-2.68	3.87	100383941	NM_001176559	GRMZM2G463904_T01
ERECTA kinase	BE	LRR	Cl_1	-2.49	-2.36	4.43	103504710	NM_001294283	GRMZM2G463904_T01
Protein serine/threonine kinase	BE	LRR	Cl_1	-1.92	-1.77	7.89	100193598	NM_001138701	GRMZM5G809695_T02
Putative LRR signal receptor kinase; Receptor-tyrosin-kinase (RTK)	Px	LRR	Cl_1	-1.81	-1.78	4.98	100279811	NM_001152767	GRMZM2G082855_T01
Lipid-transfer protein ZmLTPg24 (Wei and Zhong 2014)	Rz	LTP	Cl_1	-1.37	-1.76	5.60	100282960	NM_001155865	GRMZM2G379035_T01
Lipid-transfer protein ZmLTPg1 (Wei and Zhong 2014)	BE	LTP	Cl_1	-2.27	-1.75	7.23	100194231	NM_001139272	GRMZM5G850455_T01
Lipid-transfer protein ZmLTP1.1 (Wei and Zhong 2014)	BE	LTP	Cl_1	-2.28	-1.63	37.01	100280743	NM_001153664	GRMZM2G126397_T01
Putative Lipid transfer protein	BE	LTP	Cl_1	-1.51	-1.41	17.12	100273286	NM_001147727	GRMZM2G331518_T01
Hybrid proline-rich proteins, ZmLTP (Wei and Zhong 2014)	BE	LTP	Cl_1	-1.60	-1.46	14.87	100281790	NM_001154710	GRMZM2G372074_T01
MFS18 protein	BE	PR	Cl_1	-2.57	-1.73	58.87	542405	NM_001111976	EF517601.1_FGT016
Putative cysteine proteinase EP-B 2 precursor	Rz	ProD	Cl_1	-0.60	-1.11	39.00	100191670	NM_001137099	AC209810.3_FGT002
Putative cysteine proteinase EP-B 2 precursor	Rz	ProD	Cl_1	-0.87	-1.05	112.54	100272949	NM_001147401	GRMZM2G035045_T01
Light harvesting complex binding protein 1 (LHCB1)	BE	PS	Cl_1	-2.12	-1.87	5.32	100193833	NM_001138915	GRMZM2G402936_T01
Light harvesting complex binding protein 1 (LHCB1); CAB1	BE	PS	Cl_1	-1.95	-2.05	14.85	100281248	NM_001154167	AC207722.2_FGT009
Light harvesting complex binding protein 2 (LHCB2)	BE	PS	Cl_1	-2.51	-1.75	8.02	103643653	XM_008666820	GRMZM2G018627_T01
Plastocyanin	BE	PS	Cl_1	-1.66	-1.90	11.61	103629356	XM_008650497	GRMZM2G071450_T01
Protochlorophyllide reductase1 (pcr1)	BE	PS	Cl_1	-2.23	-1.94	14.75	100194154	NM_001139204	GRMZM2G084958_T01
Psb P, PSII	BE	PS	Cl_1	-1.52	-1.61	7.41	100273117	XM_008653782	GRMZM2G047954_T01
Zea mays ferredoxin 1 (FDX1)	BE	PS	Cl_1	-2.44	0.00	3.72	101027099	NM_001279470	GRMZM2G122337_T01
Putative 3-ketoacyl-CoA synthase	Px	SM	Cl_1	-1.28	-0.92	19.32	100283031	NM_001155933	GRMZM2G445602_T01
Putative Polyphenol oxidase (Catechol oxidase)	BE	SM	Cl_1	-1.37	-1.14	19.56	100275424	NM_001149499	GRMZM2G319062_T01
Major latex protein 22 / 423; Bet v I allergen	Px	StA	Cl_1	-1.54	-1.09	19.63	542195	NM_001111809	GRMZM2G102356_T01
Putative Responsive to dehydration 22 (RD22)	Px	StA	Cl_1	-1.68	-1.31	7.98	100194238	NM_001139279	GRMZM2G446170_T01
Dirigent protein	BE	StB	Cl_1	-3.16	-2.30	7.60	100283263	NM_001156165	GRMZM2G112210_T01
Putative Defensin-like protein	Rz	StB	Cl_1	-0.95	-1.17	46.06	100280611	NM_001153529	GRMZM2G153488_T01
Ricin precursor (rRNA N-glycosidase)	Px	StB	Cl_1	-1.10	-0.89	31.96	103647312	XM_008671855	GRMZM2G047713_T01

Carbohydrate/phosphate transmembrane transporter/ sugar:hydrogen symporter	Px	TP	Cl_1	-1.06	-0.34	67.72	100502494	NM_001196972	GRMZM2G070087_T01
Unknown	BE	Unk	Cl_1	-1.78	-1.36	10.31	103630195	XR_554911	GRMZM2G306216_T01
Unknown (Armadillo-type fold (ARM repat superfamily), VAC14 homologue)	BE	Unk	Cl_1	0.00	0.00	3.40	103625753	XM_008646149	GRMZM2G143989_T01
Unknown (maybe meiosis related)	BE	Unk	Cl_1	-1.96	-1.39	17.57	100283935	NM_001156833	GRMZM2G033222_T01
Unknown function	Px	Unk	Cl_1	-2.14	-1.65	4.73	100304045	NM_001165558	GRMZM2G314667_T01
Unknown function	Px	Unk	Cl_1	0.00	-1.85	1.79	103650412	XR_564137	na
Zinc finger CCCH domain-containing protein	Px	Unk	Cl_1	-1.22	-0.93	14.51	103643935	XM_008667108	GRMZM2G070998_T01
Putative carboxylesterase	Px	Xb	Cl_1	-3.40	-1.72	3.05	100502249	NM_001196726	GRMZM2G391795_T01
EXORDIUM (EXO)	Rz	PHS	Cl_2	0.29	-1.31	38.04	100501091	NM_001195912	AC220927.3_FGT007
Unknown function	Rz	Unk	SM	-1.24	-3.32	2.78	103639172	XR_559278	GRMZM2G348167_T01
Cysteine-type endopeptidase	Rz	ProD	Unk	-0.42	-1.52	63.23	100501549	NM_001196245	GRMZM2G099765_T01
DEAD-box ATP-dependent RNA helicase 18-like	Rz	ProS	Unk	0.00	0.00	1.73	103652213	XM_008677845	GRMZM2G099978_T01
Benzoxazinone synthesis2 (bx2)	Px	SM	Unk	-1.07	-0.38	49.90	100192631	NM_001137845	GRMZM2G085661_T02
Heat- and acid-stable phosphoprotein	Px	Unk	Unk	0.00	0.00	6.33	100282957	NM_001155862	GRMZM2G081615_T01
Unknown function	BE	Unk	Cl_2	1.57	-1.08	65.47	100280483	NM_001153403	GRMZM2G355572_T01
AtEXO70A1, member of EXO70 gene family	Rz	CM	Cl_1	-0.07	1.58	12.49	103645743	XM_008670445	GRMZM2G074530_T01
Calmodulin like; EF hand calcium-binding protein family	Px	Ca	Cl_2	1.25	-0.52	13.67	100284346	NM_001157241	GRMZM2G340807_T01
CBL-interacting serine/threonine-protein kinase 11	Px	Ca	Cl_2	1.04	0.09	30.24	100280939	NM_001153859	GRMZM2G177050_T01
EF-hand Ca2+-binding protein CCD1	Px	Ca	Cl_2	1.07	-0.61	100.32	100285110	NM_001158005	AC225718.2_FGT006
Polcalcin EF hand calcium-binding protein	Px	Ca	Cl_2	1.22	-0.85	14.24	100280482	NM_001153402	GRMZM2G474755_T01
Putative calcium-binding protein CML19	Px	Ca	Cl_2	1.08	-0.14	39.46	103650717	XM_008676282	GRMZM2G426046_T01
Polygalacturonate 4-alpha-galacturonosyltransferase	Px	CW	Cl_2	1.28	-0.65	14.60	100283040	NM_001155942	GRMZM2G149024_T01
APETALA 2/ethylene response element binding protein	Px	Et	Cl_2	1.03	-0.43	50.57	100279571	NM_001152568	GRMZM2G164591_T01
APETALA 2/ethylene response element binding protein	Px	Et	Cl_2	1.82	-1.26	9.77	100272507	NM_001146976	GRMZM2G069082_T01
DREB (Dehyrdration response element)	Px	Et	Cl_2	1.21	-0.61	29.90	100280778	NM_001153700	GRMZM2G069126_T01
DREB subfamily A-1 of ERF/AP2 transcription factor family (CBF3)	Px	Et	Cl_2	1.26	-0.51	29.60	103633049	XM_008654734	GRMZM2G069146_T01
Ethylene response factor (ERF)	Px	Et	Cl_2	1.15	-0.46	30.66	100281797	NM_001154717	GRMZM2G010555_T02
Ethylene response factor (ERF)	Px	Et	Cl_2	1.22	-0.57	54.57	100304148	NM_001165619	GRMZM2G089995_T01
Ethylene-responsive factor ERF-like	Px	Et	Cl_2	1.35	-0.14	9.68	100286307	NM_001159194	GRMZM2G025062_T01
Ethylene-responsive transcription factor ERF109-like ; RRTF1 ({REDOX RESPONSIVE TRANSCRIPTION FACTOR 1) ERF1	Px	Et	Cl_2	2.50	-1.63	5.48	103647485	XM_008672016	GRMZM2G138396_T01
Putative AP2-EREBP-transcription factor 54	Px	Et	Cl_2	1.61	-0.44	33.12	100278463	NM_001151735	GRMZM2G020054_T01
Putative Ethylene responsive; AP2 domain protein	Px	Et	Cl_2	1.49	-1.05	10.49	100283357	NM_001156258	GRMZM2G369472_T01
JAZ protein (Jasmonate (JA) zinc-finger expressed in inflorescence meristem (ZIM)-domain); ZmJAZ15 (Zhou et al. 2015)	Px	JAZ	Cl_2	1.40	-0.18	15.99	100286212	NM_001159100	GRMZM2G173596_T01
JAZ protein ZmJAZ2 (Zhou et al. 2015)	Px	JAZ	Cl_2	1.08	-0.22	95.77	100283151	NM_001156053	GRMZM2G445634_T01
JAZ protein ZMJAZ21 (Zhou et al. 2015)	Px	JAZ	Cl_2	1.50	0.41	14.05	100284433	NM_001157328	GRMZM2G036351_T01

JAZ protein ZmJAZ5 (Zhou et al. 2015)	Px	JAZ	Cl_2	1.20	-0.17	30.88	100284894	NM_001157789	GRMZM2G145412_T01
Dirigent protein	Px	LJ	Cl_2	1.07	0.47	29.98	100304441	NM_001165874	GRMZM2G002630_T01
Triacylglycerol lipase like protein	Px	LM	Cl_2	1.19	-0.13	28.60	100281723	NM_001154643	GRMZM2G097704_T01
ABA induced protein phosphatase2 (PP2C)	Px	PHS	Cl_2	1.16	0.02	21.17	100192073	NM_001137496	GRMZM2G010855_T01
Proline-rich receptor-like protein kinase PERK7	Px	PR	Cl_2	1.46	-0.21	8.61	103654965	XM_008681778	GRMZM2G374074_T01
Putative Avr9/Cf-9 rapidly elicited protein 137	Px	PR	Cl_2	1.42	0.03	24.88	100191889	NM_001137313	GRMZM2G054807_T01
Mitogen-activated protein kinase 16 (or 2-like)	Px	ProM	Cl_2	1.39	-0.12	10.23	103636208	XM_008658561	GRMZM2G173965_T01
E3 Ubiquitin-protein ligase (RING zinc finger protein)	Px	ProU	Cl_2	1.12	-0.20	32.21	100384058	NM_001176660	GRMZM2G478553_T01
E3 Ubiquitin-protein ligase (RING zinc finger protein)	Px	ProU	Cl_2	1.21	-0.39	21.66	100501341	NM_001196091	GRMZM2G567897_T01
U-box domain-containing protein 16	Px	ProU	Cl_2	1.51	-0.34	28.15	103630485	XM_008651535	GRMZM2G125034_T01
Putative Ribonuclease	Px	RNA	Cl_2	1.26	-0.30	31.05	100193751	NM_001138837	na
protein chaperone protein dnaJ	Px	StA	Cl_2	1.53	-0.36	20.94	103634596	XM_008656987	GRMZM2G144997_T01
Putative src2-like protein with C2 protein domain	Px	StA	Cl_2	1.13	-0.69	26.32	100382513	NM_001175250	GRMZM2G425004_T01
C2H2 zinc finger protein 1-like	Px	TF	Cl_2	1.08	-0.54	41.10	103625861	XM_008646258	GRMZM2G035103_T01
cpm7 (MYB145); MYB domain transcription factor family	Px	TF	Cl_2	2.10	-0.55	5.88	100285675	NM_001158566	GRMZM2G158700_T01
Putative C2H2 zinc finger protein	Px	TF	Cl_2	2.36	-0.11	8.62	100286206	NM_001159094	GRMZM2G002815_T01
Putative WRKY transcription factor with zinc-finger domains	Px	TF	Cl_2	1.21	-0.07	18.79	100193434	NM_001138554	GRMZM2G025895_T01
WRKY transcription factor (WRKY40/60/71)	Px	TF	Cl_2	1.44	-0.90	22.76	100281342	NM_001154260	GRMZM2G036711_T01
ZmWRKY78 (Wei et al. 2012)	Px	TF	Cl_2	1.20	-0.05	15.06	103632605	XR_556057	GRMZM2G025895_T01
ATNRT2.5 (nitrate transporter2.5)	Px	TN	Cl_2	1.35	0.59	18.90	103636218	XM_008658573	GRMZM2G455124_T01
Unknown function	Px	Unk	Cl_2	1.07	-0.31	34.58	100191551	NM_001136981	GRMZM2G037015_T01
Unknown function	Px	Unk	Cl_2	2.12	-0.31	4.88	100193251	NM_001138400	GRMZM2G381404_T01
Unknown function	Px	Unk	Cl_2	1.28	-0.47	12.24	100194319	NM_001139357	GRMZM2G479529_T01
Unknown function	Px	Unk	Cl_2	1.05	-0.49	24.56	100274748	NM_001149040	GRMZM2G343317_T01
Unknown function	Px	Unk	Cl_2	1.10	-0.37	40.26	100274968	NM_001149201	GRMZM2G454056_T01
Unknown function	Px	Unk	Cl_2	1.11	0.39	45.75	100275437	NM_001149509	GRMZM2G133430_T01
Unknown function	Px	Unk	Cl_2	1.58	-0.45	12.74	100278922	NM_001152042	GRMZM2G405017_T01
Unknown function	Px	Unk	Cl_2	1.37	-0.14	16.06	100279381	NM_001152395	GRMZM2G131055_T01
Unknown function	Px	Unk	Cl_2	1.39	-0.39	37.79	100285353	NM_001158245	GRMZM2G357631_T01
Unknown function	Px	Unk	Cl_2	1.28	-0.61	11.88	100383996	NM_001176608	GRMZM2G031580_T01
Unknown function	Px	Unk	Cl_2	1.71	0.87	12.39	103630124	XM_008651211	GRMZM2G317428_T01
Unknown function	Px	Unk	Cl_2	0.00	0.00	2.92	100278702	XM_008655103	GRMZM2G431885_T01
Unknown function	Px	Unk	Cl_2	1.58	-0.57	7.23	103644045	XM_008667247	GRMZM2G047422_T01
Unknown function; similar to ATHSPRO2 | HSPRO2	Px	Unk	Cl_2	1.44	-0.01	16.56	100382754	NM_001175469	GRMZM2G123394_T01
Arogenate dehydratase	Px	AAM	Cl_3	1.07	0.44	44.92	100193934	NM_001139004	GRMZM2G437912_T01
Laccase/l-ascorbat-oxidase	BE	AsO	Cl_3	1.59	1.33	38.26	100501671	NM_001196348	GRMZM2G094375_T03
Laccase/l-ascorbat-oxidase (lac3)	BE	AsO	Cl_3	1.72	1.34	49.10	732839	NM_001112445	GRMZM2G169033_T01

Laccase/l-ascorbat-oxidase (laccase-25-like)	BE	AsO	Cl_3	1.61	1.23	58.38	103640860	XM_008664318	GRMZM2G094375_T01
Laccase/l-ascorbat-oxidase (laccase-9)	BE	AsO	Cl_3	1.77	1.45	39.40	103627746	XM_008648054	GRMZM2G320786_T01
Putative Hairpin-induced gene (HIN1)/ non-race specific disease resistance gene (NDR1)/ YELLOW-LEAF-SPECIFIC GENE 9 (YLS9)	Px	Dev	Cl_3	1.43	0.78	16.82	103650969	XM_008676564	GRMZM2G074248_T01
AP2/EREBP	Px	Et	Cl_3	1.31	1.07	15.96	100193143	NM_001138301	GRMZM2G169382_T01
Ethylene-responsive factor ERF1; 97% identity with ZmERF1 (AY672654; Shi et al. 2014)	BE	Et	Cl_3	1.27	1.09	45.71	542184	NM_001111800	GRMZM2G053503_T02
Putative Ent-kaurene synthase B	Px	HM	Cl_3	1.39	0.67	16.42	100383607	NM_001176255	GRMZM2G016922_T01
ATGPAT2, GPAT2 | GPAT2 (GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE 2)	Px	LM	Cl_3	1.41	0.85	22.50	103648202	XM_008672689	GRMZM2G070304_T01
Sphinganine C(4)-monooxygenase 1-like	Px	LM	Cl_3	1.65	1.35	13.50	103651403	XM_008677038	GRMZM2G003526_T01
Cytochrom b5 isoform A-like	Px	Ox	Cl_3	1.03	0.73	134.25	103627487	XM_008647779	GRMZM2G135385_T01
LURP-one-related 8-like/ Tubby C 2; Tub-2 superfamily	Px	PR	Cl_3	1.26	0.49	14.60	103654295	XM_008681126	AC190885.4_FGT006
protein degradation; protease nephentesin-1, similar to pepsin, digesting protein to smaller peptids	Px	ProD	Cl_3	1.16	0.59	34.54	100274575	NM_001148928	GRMZM2G468657_T01
CMP-N-acetylneuraminate-poly-alpha-2,8-sialyltransferase-like	Px	ProG	Cl_3	1.48	0.81	11.79	103646246	XM_008670973	GRMZM2G111975_T01
E3 Ubiquitin-protein ligase	Px	ProU	Cl_3	1.61	0.68	7.10	100285280	NM_001158173	GRMZM2G123212_T01
RLK DUF26; protein kinase	Px	Sign	Cl_3	1.18	0.44	129.04	100281111	NM_001154030	GRMZM2G334181_T01
Putative 2OG-Fe(II) oxygenase / gibberelin 20 oxidase/ DOWNY MILDEW RESISTANT 6 (DMR6)	Px	SM	Cl_3	1.09	0.44	31.80	100284800	NM_001157695	GRMZM2G099467_T01
Putative Cytochromes P450	Px	SM	Cl_3	1.08	0.54	46.12	100382386	NM_001175131	GRMZM2G135387_T01
Putative HYDROXYCINNAMOYL TRANSFERASE / Shikimate O-hydroxycinnamoyltransferase	BE	SM	Cl_3	1.44	1.15	61.79	100383097	NM_001175768	GRMZM2G114918_T01
Putative NAD(P)(H) oxidoreductases	Px	SM	Cl_3	1.08	0.91	29.36	100191526	NM_001136957	GRMZM2G132875_T03
Putative O-methyltransferase ZRP4 (Resveratrol synthesis)/DIMBOA-Glc O-methyltransferase	Px	SM	Cl_3	1.19	0.86	77.57	100281319	NM_001154237	GRMZM2G127418_T01
Putative Pyridoxal phosphate (PLP)-dependent transferases	Px	SM	Cl_3	1.15	0.41	24.44	100383587	NM_001176235	GRMZM2G181135_T01
Trans-cinnamate 4-monooxygenase	BE	SM	Cl_3	1.47	1.11	37.71	100284998	NM_001157893	GRMZM2G028677_T01
Late embryogenesis abundant (LEA); harpin-induced protein (HIN1) related	Px	StB	Cl_3	1.26	0.64	24.75	100384448	NM_001176984	GRMZM2G449094_T01
ADP/ATP carrier 1 (AAC1)	Px	TM	Cl_3	1.07	0.95	52.13	100274442	NM_001148801	GRMZM2G420988_T01
Putative Tyrosine decarboxylase	BE	TYD	Cl_3	1.74	1.61	9.46	100285936	NM_001158825	GRMZM2G093125_T01
Tyrosine decarboxylase 1-like	BE	TYD	Cl_3	1.99	1.77	21.13	103631817	XM_008653527	GRMZM2G056469_T01
DUF716; maybe response-related	Px	Unk	Cl_3	1.17	0.47	40.56	100272958	NM_001147410	GRMZM2G429617_T01
Unknown function	Px	Unk	Cl_3	2.00	1.45	5.17	103633690	XM_008655397	GRMZM2G355499_T01
Unknown function	Px	Unk	Cl_4	1.18	0.29	26.76	100191536	NM_001136967	GRMZM2G426336_T01
Unknown function	Px	Unk	Cl_4	2.39	0.59	5.86	100274623	NM_001148964	GRMZM2G050384_T01
Unknown function	Px	Unk	Cl_4	2.17	0.93	6.45	100274767	NM_001149055	GRMZM2G026780_T01
Unknown function	Px	Unk	Cl_4	2.54	1.16	4.35	100501879	NM_001196519	GRMZM2G050384_T01
Unknown function	Px	Unk	Cl_4	1.47	0.34	8.86	103633539	XM_008655220	GRMZM2G435986_T01
Asparagine synthase (ASN)	Rz	AAM	SM	0.35	2.71	3.57	100192349	NM_001137541	GRMZM2G093175_T02
B12D protein	Px	Dev	SM	1.37	0.64	61.81	100280945	NM_001153865	GRMZM2G045155_T01
Early nodulin 93	BE	Dev	SM	1.35	1.20	28.87	100193182	NM_001138337	GRMZM2G147399_T01

Unknown function	BE	Dev	SM	1.50	1.23	18.79	100275040	NM_001149240	GRMZM2G009080_T01
1-aminocyclopropane-1-carboxylate oxidase (ACO)	Rz	Et	SM	1.26	1.35	13.90	103641391	XM_008664747	GRMZM2G166616_T01
Putative AP2/EREBP	BE	Et	SM	1.23	1.67	47.45	100216626	NM_001143039	GRMZM2G129674_T01
6-phosphofructokinase 2	Px	Gly	SM	1.20	0.66	18.35	100281688	NM_001154608	GRMZM2G059078_T01
Universal stress protein (USP)	Px	HM	SM	1.55	0.87	59.94	100194015	NM_001139078	GRMZM2G009719_T01
ATP-dependent zinc metalloprotease FTSH 5	Px	ProD	SM	1.15	0.21	31.54	103626091	XM_008646470	GRMZM5G819464_T01
Chlorophyllase-2	BE	PS	SM	2.24	1.78	14.48	103632406	XM_008654237	GRMZM2G170734_T01
Chalcone synthase CHS, TT4, ATCHS	Px	SM	SM	2.30	0.53	8.21	100282642	NM_001155550	GRMZM2G151227_T01
Cinnamoyl CoA reductase	BE	SM	SM	1.70	1.74	26.70	103654125	XM_008680955	GRMZM2G099420_T01
Putative Osmotin, thaumatin precursor	Px	StA	SM	1.65	0.66	113.63	100284970	NM_001157865	GRMZM2G006853_T01
Putative Osmotin, zeamatin precursor	Px	StA	SM	1.25	0.50	155.27	100281135	NM_001154054	GRMZM2G039639_T01
Thaumatin-like protein precursor; osmotin 34 (OSM34)	Px	StA	SM	1.48	0.56	419.52	103640644	XM_008664129	GRMZM2G136372_T01
C2H2 zinc finger family protein ZAT6-like	Px	TF	SM	1.54	0.83	10.42	103639074	XM_008661867	GRMZM2G105092_T01
CCCH transcription factor	Px	TF	SM	1.24	0.93	17.00	100280983	NM_001153903	GRMZM2G004795_T02
Putative C2H2 zinc finger family protein	Px	TF	SM	1.80	-0.52	4.59	100502060	NM_001196607	GRMZM2G361210_T01
Aromatic-L-amino-acid decarboxylase (DOPA decarboxylase) (Tryptophan decarboxylase)	Rz	TYD	SM	1.02	1.43	14.53	100279950	NM_001152900	GRMZM2G021388_T01
Putative Tyrosine decarboxylase	Rz	TYD	SM	1.28	1.42	13.97	100383025	NM_001175704	GRMZM2G021277_T01
Unknown function	BE	Unk	SM	1.32	2.17	27.79	100275953	NM_001149872	GRMZM2G034623_T01
Unknown function	Px	Unk	SM	1.32	0.14	11.75	103632651	XM_008654405	GRMZM2G162396_T01
Unknown function TSJT1; AILP1	Rz	Unk	SM	0.43	1.02	111.03	100283989	NM_001156887	GRMZM2G119219_T01
Xyloglucan endotransglucosylase/hydrolase protein 1	Px	CW	Unk	1.14	0.07	33.32	100284097	NM_001156995	GRMZM2G110299_T01
SULPHUR DEFICIENCY-INDUCED 1 (ATSDI1)	Rz	Dev	Unk	-0.18	1.84	8.15	100194250	NM_001139291	GRMZM2G075563_T01
Pyruvate decarboxylase 1	Px	Ferm	Unk	1.37	0.95	12.93	542376	NM_001111952	AC197705.4_FGT001
Glucan endo-1,3-beta-glucosidase homolog1 (geb1)	Px	Glu	Unk	1.50	0.24	81.97	100037765	NM_001112477	GRMZM2G065585_T01
Putative cytochrome P450 superfamily protein	Px	Ox	Unk	1.04	0.83	36.98	100279536	NM_001152534	GRMZM2G087875_T02
Subtilisin-chymotrypsin inhibitor CI-1C	Px	PR	Unk	1.22	0.71	23.37	100280776	NM_001153698	GRMZM2G058358_T01
TIC20 (Protein import to Chloroplast)	Px	PS	Unk	0.00	0.00	1.63	103628942	XM_008650132	GRMZM5G840946_T03
Unknown function	Px	Sign	Unk	1.45	1.09	21.43	103651368	XM_008676992	GRMZM2G062121_T01
Agmatine coumaroyltransferase-1-like / Shikimate O-hydroxycinnamoyltransferase	Px	SM	Unk	1.13	0.80	43.99	103639152	XM_008661943	GRMZM2G030436_T01
Isocitrate dehydrogenase [NAD] catalytic subunit 5	Rz	TCA	Unk	0.00	0.00	1.82	103637948	XM_008660949	GRMZM2G404855_T01
Putative aspartic proteinase nepenthesin-2 precursor	Px	TF	Unk	1.32	-0.01	12.62	100382179	NM_001174939	GRMZM2G089506_T01
Putative VOZ1-like transcription factor	Px	TF	Unk	5.03	4.13	2.45	542193	NM_001111807	GRMZM2G111696_T01
Unknown function	Rz	Unk	Unk	0.81	1.16	56.04	100277849	NM_001151310	GRMZM2G468111_T01

Table 7-4 FPKM values of the candidate genes selected for RT-qPCR

Gene name	NoiSeq DEG	BE/Ctrl	Log2 Px/Ctrl	Log2 Rz/Ctrl	Cluster	FPKM values C_1	C_2	C_3	MW_C	Px_1	Px_2	Px_3	MW_Px	Rz_1	Rz_2	Rz_3	MW_Rz
ACO1	no	up	0.84	0.66	Unk	123.7	53.2	147.0	108.0	135.1	248.0	198.4	193.8	148.2	112.8	252.3	171.1
ADT	Px	up	1.07	0.44	Cl_3	32.8	29.3	28.6	30.3	44.4	101.5	44.2	63.4	18.5	31.1	73.8	41.1
CALS	no	down	-1.07	-0.52	Unk	18.9	19.8	25.5	21.4	14.0	10.7	6.0	10.2	20.2	9.6	15.1	15.0
CCR1	BE	up	1.70	1.74	SM	5.6	1.5	24.6	10.6	27.7	50.8	24.3	34.2	45.9	31.0	29.0	35.3
CYP	Px	up	1.04	0.83	Unk	25.2	17.8	25.7	22.9	32.3	50.2	59.2	47.2	7.2	30.1	85.1	40.8
CZOG	no	up	1.00	0.73	Unk	14.8	11.8	13.1	13.2	16.5	25.3	37.6	26.5	5.9	23.8	36.1	21.9
EREBP	Px	up	1.03	-0.43	Cl_2	54.5	37.0	29.0	40.2	108.3	108.5	28.5	81.8	43.7	14.3	31.3	29.8
ERF1	BE	up	1.27	1.09	Cl_3	24.7	11.8	37.7	24.7	35.3	87.2	56.6	59.7	38.3	24.1	95.7	52.7
JAZ1	Px	up	1.08	-0.22	Cl_2	87.6	85.2	44.5	72.4	178.5	240.9	39.0	152.8	55.5	18.5	112.3	62.1
LAC3	BE	up	1.72	1.34	Cl_3	30.3	6.7	27.7	21.6	37.3	124.2	51.5	71.0	35.6	15.4	113.3	54.8
NAC1	no	up	0.90	0.15	Cl_1	70.4	67.9	46.7	61.7	125.2	152.8	68.1	115.3	50.2	59.9	95.5	68.5
PAL1	no	up	0.82	0.69	SM	67.7	31.1	85.8	61.6	55.2	165.0	105.0	108.4	49.8	53.9	193.5	99.1
pldA	Px	up	1.19	-0.13	Cl_2	26.8	19.9	14.7	20.4	52.0	72.4	15.5	46.7	18.0	13.1	25.1	18.7
TYDC1	BE	up	1.99	1.77	Cl_3	9.7	2.3	10.7	7.6	11.6	52.3	26.3	30.1	5.3	5.1	66.8	25.8
UGT	no	up	0.75	0.69	Unk	20.7	12.8	17.4	16.9	25.5	34.1	26.1	28.5	21.8	24.4	35.5	27.3
USP	Px	up	1.55	0.87	SM	36.1	22.8	34.7	31.2	65.3	138.0	70.8	91.3	68.0	40.7	63.0	57.3
WRKY78	Px	up	1.20	-0.05	Cl_2	12.3	11.7	7.8	10.6	25.2	39.0	9.0	24.4	10.2	6.8	13.6	10.2
WRKY91	Px	up	1.21	-0.07	Cl_2	14.9	15.9	8.9	13.2	37.3	43.0	11.4	30.6	13.7	9.2	14.9	12.6

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