Novel concepts in biological plant protection on the basis of the biological control agent *Serratia plymuthica* HRO-C48

Neue Konzepte für den biologischen Pflanzenschutz am Beispiel des Biological Control Agents *Serratia plymuthica* HRO-C48

Dissertation zur Erlangung des akademischen Grades doctor rerum naturalium
(Dr. rer. nat.) der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität Rostock

Henry Müller
geb. am 17.08.1976
aus Rostock

Oktober, 2006

urn:nbn:de:gbv:28-diss2008-0119-6

Gutachter:

Prof. Dr. Gabriele Berg
Technische Universität Graz
Institut für Umweltbiotechnologie
Petersgasse 12
A-8010 Graz

Prof. Dr. Hubert Bahl
Universität Rostock
Institut für Biowissenschaften Mikrobiologie
Albert-Einstein-Straße 3
D-18059 Rostock

Abstract

For ecological and economical reasons the management of soil-borne diseases in agriculture by conventional pesticides is limited. Environmentally friendly alternatives such as the employment of microbial antagonists to control phytopathogens are of growing interest. Inconsistent performance of the microorganisms, however, has hampered commercial application of biological control agents (BCAs). One of the main problems is to achieve an active and stable colonization of the plant root by the introduced microorganism.

The results of the present work revealed the special significance of adequate cell densities for the efficiency of the biological control agent *Serratia plymuthica* HRO-C48, which has already been successfully commercialized for strawberry cropping under the name of RHIZOSTAR®. Constitutively produced and secreted *N*-acyl homoserine lactones (AHLs) allow Gram-negative bacteria to monitor their own population density and to coordinate the regulation of gene expression; a mechanism referred as quorum sensing. In *S. plymuthica*, particularly, these signalling molecules are essentially involved in the suppression of Verticillium wilt in oilseed rape. *In vitro* analyzes of two AHL-deficient derivates of HRO-C48 indicate the responsibility of AHLs for the regulation of antagonistic traits including the excretion of the fungal cell-wall degrading chitinases and proteases as well as the production of the antifungal compound pyrrolnitrin. Additionally, AHL-mediated control was demonstrated for behaviours characteristic for the plant-microbe interaction such as the biosynthesis of indole-3-acetic acid, biofilm formation and motility.

Growers of oilseed rape and olive trees are faced to severe yield losses caused by the Verticillium wilt; the control of which by chemical measures is nearly impossible. The application of *S. plymuthica* to control *Verticillium*-mediated premature ripeness of oilseed rape demands a seed inoculation technique which allows the BCA to effectively establish on the developing root from the moment of germination. Using bio-priming, which was newly developed in order to apply the BCA on oilseed rape seeds, HRO-C48 was located in the seed interior. Situated within the seed, the deleterious impact from external influences on the bacterial cells is limited. This advantage resulted in an enhanced shelf-life of the bacterial cells during the storage at 20°C. Bio-priming, which is suitable for the integration into the seed production framework, was employed for experiments under field conditions. Results confirmed the usability of the bio-priming procedure to support the establishment of the antagonistic bacterium in the rhizosphere of oilseed rape.

Integrated management of Verticillium wilt in olive involves the delivery of pathogen-free planting material by nurseries. The introduction of *S. plymuthica* to seven-month old olive trees by root dipping resulted in the colonization of the olive root, disease suppression and enhanced plant growth over a period of five months.

The knowledge of the mode of action of bacterial antagonists and its regulatory backgrounds allows the development of profound concepts for biological control. The AHL-based quorum sensing system of *S. plymuthica* HRO-C48 is involved in the population density-dependent expression of beneficial traits. From the practical point of view, the objective to obtain high cell densities of the BCA in the rhizosphere demands reliable application procedures. Considering plant health and plant growth, the utilization of bio-priming to inoculated oilseed rape seeds and the bacterial treatment of young olive plant by root dipping resulted in effective population densities of HRO-C48.

Zusammenfassung

Die effiziente Bekämpfung bodenbürtiger Schadorganismen in der Landwirtschaft mit Hilfe von konventionellen Agrochemikalien ist sowohl aus ökologischen, als auch aus ökonomischen Gründen limitiert. Die Nutzung des Potenzials mikrobieller Antagonisten zur Kontrolle von Phytopathogenen stellt eine bedeutende und gleichzeitig umweltfreundliche Alternative dar. Die derzeit geringe Akzeptanz biologischer Pflanzenschutzpräparate wird mit der fehlenden Wirksicherheit begründet, die oftmals die Folge von zu geringen Populationsdichten des applizierten Organismus an der zu schützenden Pflanze ist.

Mit der vorliegenden Arbeit konnte die Signifikanz adäquater Zellzahlen für die Wirksamkeit des Biological Control Agents (BCA) *S. plymuthica* HRO-C48, auf dessen Basis bereits das Präparat RHIZOSTAR® zur Abwehr von pathogenen Pilzen und Ertragsteigerung im Erdbeeranbau entwickelt wurde, belegt werden. Die von HRO-C48 produzierten *N*-Acyl-Homoserinlaktone (AHLs), deren Konzentration in der Zelle und in dessen Umgebung eine direkte Funktion der Zelldichte ist, sind bei der Suppression der Verticillium-Welke am Raps von essentieller Bedeutung. AHLs fungieren als Autoinduktoren in bakteriellen Quorum sensing-Systemen und sind in *S. plymuthica* sowohl in der Regulation antagonistischer Wirkmechanismen, als auch in der Steuerung der Interaktion mit der Pflanze involviert. Die *in vitro* Analysen von zwei AHL-defekten Mutanten von HRO-C48 belegen, dass die Signalmoleküle die Exkretion von extrazellulären Enzymen, die Synthese des Antibiotikums Pyrrolnitrin sowie die Auxinproduktion, Biofilmbildung und Motilität beeinflussen.

Die Verticillium-Welke konfrontiert die Produzenten von Winterraps und Oliven mit steigenden Ernteausfällen und fehlenden Bekämpfungsmöglichkeiten. Der Einsatz von *S. plymuthica* zur Schadensminimierung im Pathosystem Winterraps-*Verticillium longisporum* erfordert eine Saatgutbehandlung, die den BCA befähigt, sich mit Beginn der Wurzelentwicklung zu etablieren. Mit Hilfe der Methode des Bio-primings, die während dieser Arbeit für die Applikation von HRO-C48 an den Rapssamen entwickelt wurde, war es möglich, das Inokulum direkt im Inneren des Saatkorns zu positionieren. Der daraus resultierende Schutz vor äußerlichen Einwirkungen wirkte sich signifikant positiv auf die Lagerstabilität der Zellen bei 20 °C aus. Das Bio-priming, welches sich potenziell großtechnisch realisieren lässt, kam im Rahmen eines Feldversuches zum Einsatz, mit dem die Eignung dieser Inokulationsprozedur für eine stabile Etablierung des antagonistischen Bakteriums an der Rapswurzel validiert wurde.

Im Rahmen einer integrierten Bekämpfung der Verticillium-Welke im Olivenanbau ist die pathogenfreie Vermehrung von Pflanzenmaterial von primärer Bedeutung. *S. plymuthica* HRO-C48, appliziert durch ein Wurzeltauchbad, besiedelte die Olivenwurzel und zeigte sowohl eine Hemmwirkung auf das Pathogen als auch eine Pflanzenwachstumsförderung an vier Monate alten Olivenbäumen über einen Zeitraum von fünf Monaten.

Das Wissen über die Wirkmechanismen von bakteriellen Antagonisten und dessen Regulation erlaubt eine gezielte Entwicklung neuer Konzepte für den biologischen Pflanzenschutz. Ein auf AHL-Moleküle basierendes Quorum sensing-System koordiniert die Expression nützlicher Eigenschaften in *S. plymuthica* HRO-C48 in Abhängigkeit von der Zelldichte. Eine wesentliche technische Vorraussetzung für das Erreichen hoher Zellzahlen des BCAs in der Rhizosphäre ist eine geeignete Applikationsprozedur. Hinsichtlich der Pflanzengesundheit und des Pflanzenwachstums resultierte die für die Inokulation von Rapssamen entwickelte Methode des Bio-primings sowie die Behandlung von jungen Olivenpflanzen mit der Wurzeltauchbadtechnik in eine wirksame Populationsdichte von *S. plymuthica* HRO-C48.

Table of contents

Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
AHLs	*N*-acyl homoserine lactones
Aqua dest.	distilled water
ASS	compound fertilizer containing ammonia, sulfur and saltpeter
AUDPC	area under disease process curve
BCA	biological control agent
bp	base pairs
CFU	colony forming unit
CLSM	confocal laser scanning microscopy
D	defoliating
dH_2O	distilled water
ddH_2O	double distilled water
DSI	disease severity index
DSMZ	Deutsche Stammsammlung für Mikroorganismen und Zellkulturen
EMBL	European Molecular Biology Laboratory
EPS	exopolysaccharide
et al.	lat.: *et alteri*
IAA	indole-3-acetic acid
ISR	inducing systemic resistance
KAS	compound fertilizer containing potassium, ammonia and sulfur
LPS	lipopolysaccharides
LSD	least significant difference
n. d.	not determined
ND	non-defoliating
N-GlcNAc	*N*-Acetyl-D-glucosamine
NPZ	Norddeutsche Pflanzenzucht Hans-Georg Lembcke KG
PRN	pyrrolnitrin
PRs	pathogenesis-related proteins
PCR	polymerase chain reaction
PGPR	plant growth promoting bacteria
ppm	parts per million
PPO	polyphenol oxidase
qRT-PCR	quantitative real-time PCR
QS	quorum sensing
SA	salicylic acid
rfm	root fresh mass
rfw	root fresh weight
Rif^r	rifampicin resistance
rpm	rounds per minutes
SSCP	single-strand conformation polymorphism analysis
UPGMA	unweighted-pair group methods using averages
UV	ultraviolet
VBNC	viable but not culturable
VOCs	volatile organic compounds
v/v	volume per volume
w/v	weight per volume

1 Introduction

World agricultural production is threatened by a variety of harmful biotic and abiotic factors. Annual yield losses caused by weeds and by viral, bacterial and fungal pathogens resulting from increasingly intensive commercial agriculture are estimated to be around 30% worldwide (Oerke 2006). So far, this problem has been countered by the extensive use of chemical-based pesticides. Since the efficiency of agrochemicals is limited, and additionally, concerns over food quality and food safety have risen, the future-looking plant protection industry is searching for novel strategies to meet the public demands: efficiency, economic compatibility, sustainability and protection of natural resources.

Verticillium wilt is a prominent example of a soil-borne disease where the control possibilities are affected by new environmental constraints. Three species in the phylogenetic class Deuteromycetes of the genus *Verticillium*, namely *V. albo-atrum* REINKE & BERTHOLD, *V. dahliae* KLEBHAHN, and *V. longisporum* KARAPAPA, BAINBRIDGE & HEALE (synonym *V. dahliae* var. *longisporum* STARK), are the major inducers of vascular wilting for a wide range of dicotyledonous plants, including economically important field crops, horticultural crops and trees (Pegg and Brady 2002; Barbara and Clewes 2003). The Verticillium wilt fungus, preferring moist soils and a temperature range of 21-27°C, is a typical monocyclic disease divided into a dormant, a parasitic and a saprophytic phase (Fradin and Thomma 2006). The fungus remains in the soil as asexual microsclerotia (*V. dahliae*, *V. longisporum*) or melanized mycelium (*V. albo-atrum*) which is stimulated to germinate by plant root exudates (Olsen and Nordbring-Hertz 1985; Mol and Vanriessen 1995). Directed by nutrient gradients, the fungal hyphae grow actively towards the root system and infect the plant by penetrating the roots or via wounds (Beckman 1987). The pathogen systemically colonizes the plant by forming conidia, which spread throughout the vascular tissue using the water transport system of the plant (Beckman 1987). The infested plant reacts by isolating the fungus via a protective plugging of the water conducting system with a phenol-pectin mixture, preventing the water from reaching the upper parts of the plant (Beckman and Talboys 1981). Exhibited symptoms in plant parts deprived of water resemble those of water stressed plants including wilting and foliar chlorosis and necrosis. In the later stage of the pathogenesis, the fungus enter into the saprophytic stage by colonizing non-vascular plant parts, and form microsclerotia in the more senescent plant (Mol and Scholte 1995).

This work is centered on the protection of the *Verticillium* hosts oilseed rape (*Brassica napus* L. *oleifera*) and olive (*Olea europaea* L.), which are among the most affected crops. In the last 20 years, an increase of 260% in oilseed production has been recorded worldwide, driven by the enormous economic significance of oilseed crops as a renewable raw material used for a variety of applications in food and non-food areas (Kimber and McGregor 1995; UFOP 2006; FAO 2006). However, concurrently with intensified oilseed cropping in regions such as Northern Europe and North America, Verticillium wilt has become a significant problem (Heale and Karapapa 1999; Zeise and Steinbach 2004; Johansson *et al.* 2006). The Verticillium threat to one of Germany's major crops, with acreage of 1.43 million, has caused yield losses of 10 to 50% annually (Garbe 2000). Great care is needed to differentiate the disease-causing fungi, as there exists a number of contradictions in published works concerning the phylogenetic relationship of the main contributor to Verticillium wilt in *Brassicaceae* (Collins *et al.* 2003; Barbara and Clewes 2003; Zeise and von Tiedemann 2001, 2002; Fahleson *et al.* 2004). Stark (1961) reported a *Verticillium* isolate with long spores which was termed *V. dahliae* var. *longisporum*. In 1997, based on molecular approaches, long-spored isolates were recognized as an individual species *V. longisporum* (Karapapa *et al.* 1997). For both *V. dahliae* and *V. longisporum,* a high similarity exists in the ITS sequences as well as for the whole genome (Fahleson *et al.* 2004). A known today, the long-spored *Verticillium* is an amphihaploid interspecific hybrid between *V. dahliae* and another unknown *Verticillium* species (Collins *et al.* 2003). This hybrid has a narrow host range, which preferentially includes cruciferous crops such as cabbage (Zeise and Tiedemann 2002). Furthermore, *V. longisporum* exhibits very distinct morphology (Karapapa *et al.* 1997), genetics (Steventon *et al.* 2002) and pathogenicity (Zhou *et al.* 2006) in comparison to short-spored *V. dahliae* isolates. Both fungal species can be distinguished by shape and the size of the conidia as well by their polyphenol oxidase (PPO) activity (Karapapa *et al.* 1997). The DNA content in *V. longisporum* is usually twice as high as in other non-long-spored *Verticillium* isolates (Steventon *et al.* 2002). Additionally, Zhou *et al.* (2006) have found that *V. longisporum* penetrates the roots via lateral roots and root hairs whereas *V. dahliae* infects via the main roots. Taking into consideration the current level of knowledge and following the prevailing suggestions, *Verticillium* strain ELV25, a long-spored isolate originated from oilseed rape, is used as model pathogen in the present work (Messner *et al.* 1996), referred to as *V. longisporum*.

Verticillium wilt, caused by *V. dahliae*, is one of the most serious diseases affecting olive trees worldwide and is responsible for severe yield losses and plant death (Al-Ahamad and Mosli 1993; Jiménez-Díaz *et al.* 1998). In the last two centuries, the disease has spread throughout the olive-growing regions of Europe due to the establishment of new orchards in already infested soil and the use of infected plant material (Blanco-Lòpez *et al.* 1984; Thanassoulopouslos 1993). Moreover, high tree density and irrigation techniques support the proliferation of the pathogen (Rodrígez-Jurado 1993). *V. dahliae* isolates infecting olive plants can be classified into defoliating (D) and non-defoliating (ND) pathotypes based on their ability to defoliate shoots and twigs or not, respectively (Mercado-Blanco *et al.* 2003). Olive trees infected by the ND pathotype show only mild symptoms and can recover from the disease, whereas the D pathotype induces more severe symptoms, which include the sudden defoliation of green leaves of one or more branches and branch dieback. In susceptible cultivars, such as Arbequina and Picual, the attack by the defoliating pathogen can be lethal (Jiménez-Díaz *et al.* 1998). The management of the Verticillium disease based on an integrated strategy, which involves primarily the choice of planting sites with low inoculum densities and increasing certainty that pathogen-free plants come from the nurseries (Tjamos 1993).

V. dahliae and *V. longisporum* form resistant, long-term surviving microsclerotia (Wilhelm 1955). They can infect plants throughout the growing period (Zeise and Seidel 1990) and establish themselves within the interior of the plant. Therefore, chemical control is nearly impossible. Until recently, effective control of *V. dahliae* was achieved by fumigating the soil with methyl bromide. However, in developed countries the application of methyl bromide and related substances was ultimately banned in 2005 and will be forbidden in developing countries in 2015 (Duniway 2002). With the impending worldwide phase-out of methyl bromide and related substances, disease management strategies must be reconsidered (Martin *et al*. 2003). Alternative chemical soil disinfection methods include the use of alternative fumigants such as methyl amid and ozone, but they have been shown to be inadequate in reducing microsclerotia levels in soil (Hamm *et al.* 2003). Effective non-chemical methods exist, but their application is often limited to certain cropping systems. Steaming the soil requires a large investment and is therefore only applicable for high value crops (Runia 2000). Soil solarization and flooding reduce inoculum levels of soil-borne pathogens, but solarization is only an option in warmer areas, and flooding is not feasible in many locations (Tjamos

1992; Blok *et al.* 1998). Moreover, some soil amendments, e.g. incorporation of broccoli residue, lignin or liquid swine manure into field soil reduced the infection potential of *Verticillium* (Subbarao *et al.* 1999; Conn and Lazarovits 2000; Debode *et al.* 2002).

An interesting environmentally friendly strategy sought to use naturally occurring microbial antagonists to control soil-borne pathogens in the rhizosphere (Weller 1988; Emmert and Handelsman 1999; Whipps 2001). In the classic publication of Hiltner (1904), the rhizosphere is defined as the soil layer surrounding the root with a high microbial activity which is influenced by metabolic compounds released by the plant root (termed as rhizodeposition). Plant species, plant site and growth stage are the major determinants of the composition of the bacterial and fungal community of plant-associated microhabitats (Grayston *et al.* 1998; Berg *et al.* 2005a; Berg *et al.* 2006; Mougel *et al.* 2006). Plant-associated microbial communities consisting of microorganisms can be classified as either neutral, beneficial or deleterious regarding their relationship with the host plant (Lynch *et al.* 1990). Deleterious microorganisms include pathogens which colonize the host plant causing chronic damage (slowed growth, plant decline during reproductive stage) or acute damage (sudden wilting, plant death), respectively. Resistance mechanisms to air-borne pathogens are widespread among plants, but curiously resistance to most phytopathogens attacking via roots is lacking (Cook *et al.* 1995). This disability may be compensated through a completely novel strategy, which involves the selective enrichment of plant-beneficial microbes (de Weger *et al.* 1995). These microorganisms interact with the plants by preventing them from experiencing the deleterious effects of the pathogens, inducing systemic resistance (ISR) or promoting plant growth through the production of phytohormones and the delivery of nutrients. Mechanisms involved in direct inhibition of a pathogen's growth by antagonistic microorganisms comprise antibiosis, parasitism or competition (Whipps 2001). Parasitism relies on lytic enzymes for the degradation of cell walls of pathogenic fungi (Chet *et al.* 1990). In particular, the extracellular enzymes such as proteases, chitinases and 1,3-beta-glucanases were reported to act as hydrolytic agents in the rhizosphere (Chernin and Chet 2002). Furthermore, it is commonly known that rhizobacteria produce antibiotics which interfere with the biological functions of the pathogens (Fravel 1988; Weller and Thomashow 1999; Dowling and O'Gara 1994). Competing for nutrients and colonization space, evidenced by the production of iron-chelating agents (siderophores), has also been recognized as a pathogen repressing factor (Chet *et al.* 1990; Fravel and Keinath 1991; Winkelmann 2002). Another group of beneficial

microorganisms have been termed plant growth promoting bacteria (PGPB) (Kloepper 1992). The mechanisms for plant growth promotion and ISR by PGPR have been extensively studied in the past decade. There are several determinants for mechanisms of growth promotion that include bacterial synthesis of the plant hormones indole-3-acetic acid (IAA), cytokinin, and gibberellin, breakdown of plant-produced ethylene by bacterial production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and increased mineral and nitrogen availability in the soil (Kloepper 1992; Glick 1998; Costacurta and Vanderleyden 1995). For mechanisms of ISR, previous works demonstrated that several bacterial determinants such as siderophores, salicylic acid (SA), and lipopolysaccharides (LPS) contributed to ISR (van Loon *et al.* 1998).

Plant-associated bacteria, both pathogenic and beneficial, were found to regulate specific genes involved in a variety of phenotypes in respect to cell density. In particular, expression of virulence factors in phytopathogens such as *Erwinia carotovora, Agrobacterium tumefaciens* and *Burkholderia cepacia* are under QS control. In beneficial bacteria, the production of antibiotics and siderophores, the excretion of exoenzymes (chitinases, proteases, and lipases), the exopolysaccharide (EPS) synthesis as well motility issues are coordinated with regard to the population density. The cell density-dependent coordination of bacterial behaviour, termed quorum sensing (QS), was first described by Nealson *et al.* (1970) and Eberhard (1972) as a phenomenon in bioluminescent marine bacteria. Since then, it has emerged that a increasing number of bacteria regulate a set of physiological processes in response to their population size (see reviews Fuqua *et al.* 1994; Eberl 1999; Whitehead *et al.* 2001; Bassler 2002; Visick and Fuqua 2005). Bacterial cell-to-cell communication utilizes small diffusible signal molecules called autoinducers, which the bacteria both produce and perceive. One class of chemical signals widely used by Gram-negative bacteria consist of *N*-Acyl homoserine lactones (AHL) which differ in the length of the *N*-acyl side chain (four to 14 carbons), the presence and absence of double bonds or side chain substituations (keto, hydroxyl) (Swift *et al.* 1999). The AHLs known so far are the product of a protein related to the AHL-synthase LuxI of *Vibrio fisheri.* These signal molecules accumulate in the cytoplasm and bacteria-surrounding environment. When the autoinducer concentration reaches a critical threshold, the bacteria sense and respond by activating the cognate LuxR-receptor, which promote the transcription of specific genes or set of genes.

In recent studies, a few bacterial isolates antagonistic to *V. dahliae* have been identified, some of which provided some degree of Verticillium wilt suppression in several host plants (Berg *et al.* 2001; Graner *et al.* 2003; Tjamos *et al.* 2004; Mercado-Blanco *et al.* 2004). Those strains belong to the genera *Bacillus, Burkholderia, Erwinia, Flavobacterium, Pseudomonas, Serratia, Sphingomonas, Strenotrophomonas,* and *Streptomyces* and were isolated from oilseed rape (*Brassica napus* L.), strawberry (*Fragaria x ananassa* Duchesne), and mosses (*Bryophyta*) (Berg and Ballin 1994, 2000b; Berg 1996; Opelt and Berg 2004). For example, *Serratia plymuthica* HRO-C48, a Gram-negative rhizosphere-associated bacterium originally isolated from oilseed rape, was shown to suppress Verticillium wilt on strawberry plants and to enhance the yield under field conditions (Kurze *et al.* 2001). A product called RhizoStar® (e-nema GmbH, Raisdorf, Germany) which is based on *S. plymuthica* HRO-C48 was developed, and its application to strawberry roots by root dipping as well the overall effect on the inoculated plants were patented (EU patent 98124694.5) (Berg *et al.* 1999). The identification of HRO-C48 as a bacterium with a high potential to act as commercial biocontrol agent (BCA) was derived from a sophisticated screening procedure by which numerous plant-associated bacteria were analyzed *in vitro* on their ability to inhibit growth of certain pathogens and to promote plant growth (Berg *et al.* 2001). *S. plymuthica* HRO-C48 shows high antagonistic activity against various fungal pathogens including *V. dahliae, V. longisporum, Sclerotinia sclerotium, Fusarium* spp*.,* and *Rhizoctonia solani* (Kalbe *et al.* 1996). The following phenotypical analyzes have contributed to advancement in knowledge regarding the mode of its inhibitory effect on fungi. Frankowski *et al.* (2001) identified and characterized the chitinolytic system of HRO-C48 consisting of at least three chitinases. When the chitinolytic activity was abolished by chemical-induced mutagenesis, HRO-C48 failed in suppression of *V. longisporum* in both *in vitro* and *ad planta* assays. Additionally, the synthesis of the antifungal compound pyrrolnitrin by the BCA was demonstrated (Meincke pers. comm.). In addition to the antagonistic ability, *S. plymuthica* was found to enhance root length and the fresh weight of strawberry and lettuce seedlings (Kurze *et al.* 2001; Richter 2001) which is probably a result of the excretion of the plant growth hormone indole-3-acetic acid (IAA) (Kalbe *et al.* 1996). In respect to commercial and legal requirements, other relevant properties were taken into account. Importantly, the biocontrol strain *S. plymuthica* HRO-C48 belongs to the risk class one according to German legislation (BiostoffV: BGBI I pp. 50-60), meaning that the isolate is harmless to humans and the environment. This fact was underpinned by predicting the non-pathogenic character of HRO-

C48 using the model nematode *Caenorhabditis elegans* (Westendorf 2006). Additionally, HRO-C48 exhibits a low level of antibiotic resistance (Berg 2000).

Experiments on oilseed rape performed by Frankowski (2002) suggest that HRO-C48 is able to delay the development of the Verticillium wilt and to promote plant growth under greenhouse conditions. In contrast, the application of the BCA to oilseed rape seeds by means of pelleting prior to sowing at naturally *Verticillium*-infested field sites resulted in low population densities. No disease suppression and only slight increase in crop yield were observed (Frankowski 2002). These results underline a problem generally recognized concerning the application of microbial agents to control soil-borne pathogens under field conditions. Many biocontrol agents with a promising degree of disease suppression in greenhouse approaches have failed in consistency and efficiency in controlling the target pathogen on field-grown crops. Many reasons have been suggested for the often unreliable and unpredictable field effect, but it has been hard to demonstrate the course of the events behind this variations. Pierson and Weller (1994) listed four reasons for inconsistency in biocontrol effect against phytopathogens: i) variability in colonization; ii) variable production or inactivation *in situ* of bacterial metabolites iii) occurrence of disease of non-target pathogens, spatial and temporal variability in disease incidence and severity. Fourthly, much of the inconsistency has been attributed to variability in the physical and chemical properties within the niches occupied by the biocontrol agents, as well as the plants which affect both colonization and expression of beneficial traits (Smith and Goodman 1999). In addition to the ecological argues, some technical problems are in charge of the small number of living microorgansims which are directly used for biocontrol in practice (Whipps 2004). The main reasons for the limited success in commercial use of biocontrol agents for plant disease control are: often narrow activity spectrum, usually less effective than chemical standards, and short shelf-life to be able to survive storage and transportation. (Lumsden *et al.* 1995; Whipps 1997).

According to the factors mentioned, the present study aimed to:

1. Develop and evaluate seed treatment techniques suitable for the application of *S. plymuthica* HRO-C48 to oilseed rape. The technical development should consider the compatibility with present commercial seed processing equipment. Three different seed treatment methods (pelleting, film coating, and bio-priming) were evaluated with regard to their impact on root colonization ability, degree of suppression of the Verticillium wilt, and plant growth promotion effect under controlled greenhouse conditions. The stability of formulated bacteria during the storage at room temperature is of primary interest and usually determines the potential of an application procedure for commercial use.
2. Evaluate the effect of *S. plymuthica* HRO-C48 on field-grown winter oilseed rape in two consecutive years at different sites in Germany. In the first field trial, HRO-C48 was applied by pelleting, whereas bio-priming was used for the second field experiment. The population dynamics of the introduced BCA, the effect on the plant health and yield as well as the impact on the structure of the autochthonous bacterial rhizospheric community was determined.
3. Exploit the potential of *S. plymuthica* HRO-C48 acting as biological control agent to suppress Verticillium wilt in micro-propagated olive plants. Seven-month old olive plants were inoculated with the biocontrol strain by root dipping. The establishment of HRO-C48 in the rhizosphere and endorhiza and the effect on plant growth as well on the development of symptoms caused by *V. dahliae* was monitored for a period of 169 days.
4. Clarify the relevance of AHL signaling molecules in the biocontrol activity of *S. plymuthica* HRO-C48. For this purpose, genetically modified derivates of the wild-type strain impaired in AHL excretion were applied to the pathosystem *V. dahliae* oilseed rape to determine the role of the signaling compounds on the disease suppression and plant growth promotion effect. Additionally, various phenotypes of HRO-C48 assumed to be involved in both plant-microbe and bacteria-fungi interaction were analyzed *in vitro* on their regulation by AHLs. Those phenotypes comprised the biosynthesis of auxin, the production of hydrolytic enzymes and pyrrolnitrin as well as the rhizosphere competence, biofilm formation, and motility.

2 Material and Methods

2.1 Sources of supply

Unless indicated specifically, chemicals used in this study were purchased from Boehringer (Mannheim, Germany), FLUKA (Buchs, Switzerland), Merck (Darmstadt, Germany), Carl-Roth (Karlsruhe, Germany), SERVA (Heidelberg, Germany), and Sigma-Aldrich (St. Louis, USA). Cultivation media and media components were provided by Difco (Detroit, MI, USA), Gibco (Eggenstein, Germany), Oxoid (Basingstoke, Great Britain), and SIFIN (Berlin, Germany).

2.2 Organisms and culture conditions

Fungal strains used in this study and the media, in/on they were routinely grown for seven to ten days at 20°C, are listed in Table 1. *Verticillium dahliae* ELV25 was preserved by adding 15% (v/v) sterile glycerine to the liquid culture. To prepare stock cultures of *V. dahliae* V138 and *Rhizoctonia solani*, three agar blocks containing mycelium of the fungus were deposited in preservation medium. Preparations were store at −70°C.

Bacterial strains used in this study are listed in Table 2. Cells were routinely grown at 30°C in nutrient broth (NB) and nutrient agar (NA), respectively. Where required, growth media were supplemented by antibiotics in concentrations noted in Table 3. For long-term storage, stock cultures were prepared by adding sterile glycerine (final concentration 15% (v/v)) to a 24-h culture and stored at −70°C. At regular intervals, bacteria were reconstituted by transferring cell material from cryoconserves onto NA. Liquid cultures to a certain volume of 100.0 ml were directly inoculated with 0.01 volumes unfrozen stock culture, more voluminous approaches by 0.01 volume of a 24-h pre-culture. Identity of strains was continuously checked by means of BOX-PCR according to Rademaker and De Bruijn (1997).

Table 1: Fungal strains used in this study

Strain	Relevant characteristics	Preferred growth media	Reference
Verticillium longisporum ELV25[1]	Diploid, long-spored, isolated from *Solanum tuberosum* L.	Waksman agar Czapek-Dox broth	Messner *et al.* 1996
Verticillium dahliae V138[2]	Defoliating strain, isolated from *Olea europea* L.	Potato dextrose agar Potato dextrose broth	Bejarano-Alcázar *et al.* 1996
Rhizoctonia solani Kühn[1]	Anastomosis group 3, isolated from *Solanum tuberosum* L.	Potato dextrose agar Potato dextrose broth	Ogoshi 1987

[1] Culture collection of Graz University of Technology, Institute of Environmental Biotechnology, Graz, Austria
[2] Culture collection of culture collection of Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible, CSIC, Córdoba, Spain

Table 2: Bacterial strains used in this study

Strain	Relevant characteristics	Reference
Serratia plymuthica		
HRO-C48 WT[1,2]	Wild-type, isolated from the rhizosphere of *Brassica napus* var. *oleifera* f. *biennis*	Kalbe *et al.* 1996
HRO-C48 Rif^r	Spontaneous rifampcin-resistant mutant of the wild-type; Rif^r	This study
HRO-C48 pME6863	AHL-negative mutant of the wild-type obtained after transformation with pME6863 encoding AHL-lactonase (AiiA) of *Bacillus subtilis*; Tc^r, Rif^r	Chernin, unpublished
HRO-C48 AHL-4	AHL-negative mutant impaired in *splI* gene obtained after insertion of mini-Tn5 transposon into *splI*; Km^r	Chernin, unpublished
HRO-C48 Chi$^-$	Chitinase-negative mutant of the wild-type impaired in *chiA* obtained after insertion of mini-Tn5 transposon into *chiA*; Km^r	Chernin, unpublished
HRO-C48 PRN$^-$	Pyrrolnitrin-negative mutant of the wild-type impaired in *prnD* obtained by insertion of *aphII* gene cartridge from pUC4K (conferring kanamycin resistance) into *prnD*; Km^r	This study
Pseudomonas fluorescens		
PICF7[3]	Wild-type, isolated from the endorhiza of *Olea europea* cv. Picual	Mercado-Blanco *et al.* 2004
PICF7 Rif^r	Spontaneous rifampicin-resistant mutant of the wild-type; Rif^r	Mercado-Blanco *et al.* 2004
Escherichia coli		
DH5α	Isolate used for routine cloning experiments; φ80d*lac*ZΔM15 marker provides α–complementation of the β-galactosidase gene from pGEM	Hanahan 1983
Chromobacterium violaceum		
CV026	AHL reporter strain; violacein-negative mutant of *Chromobacterium violaceum* ATCC 31532 obtained by mini-Tn5 transposon mutagenesis	McClean *et al.* 1997

Km^r kanamycin resistant, Rif^r rifampicin resistant, Tc^r Tetracyclin resistant

[1] Deposited as DSM 8571 in the German collection of Microorganisms and Cell Cultures (DSMZ): Strain collection of Antagonistic Microorganisms (SCAM), Braunschweig, Germany

[2] Culture collection of Graz University of Technology, Institute of Environmental Biotechnology, Graz, Austria

[3] Culture collection of culture collection of Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible, CSIC, Córdoba, Spain

Table 3: Antibiotics used for selective cultivation of bacterial strains

Antibiotic	Solvent (100.0 µg ml^{-1})	Working concentration (µg l^{-1})
Rifampicin	Methanol	100
Ampicillin	dH_2O	100
Kanamycin	dH_2O	50
Tetracyclin	dH_2O	40

2.3 Media

Unless otherwise indicated, growth media were adjusted to a pH of 7.0 and autoclaved for 20 min at 121°C.

2.3.1 Bacterial growth media

5. ABC medium / ABG medium (Clark and Maaløe 1967)

Solution 1:	
$(NH_4)_2SO_4$	20.0 g
Na_2HPO_4	60.0 g
KH_2PO_4	30.0 g
NaCl	30.0 g
Aqua dest.	*ad* 1000.0 ml
Solution 2:	
1.00 M $MgCl_2 \cdot 6\ H_2O$	2.0 ml
0.50 M $CaCl \cdot 2\ H_2O$	0.2 ml
0.01 M $FeCl_2 \cdot 6\ H_2O$	0.3 ml
Aqua dest.	*ad* 900.0 ml
Citrat solution:	
Citrate	19.2 g
Aqua dest.	*ad* 100 ml
Glucose solution:	
Glucose	20.0 g
Aqua dest.	*ad* 100.0 ml

Solution 1, solution 2 and C-sources were autoclaved separately. After cooling, 100.0 ml of solution 1 was added to solution 2. For ABC medium 10.0 ml citrate solution, and for ABG medium 10.0 ml glucose solution was added.

- **Luria-Bertani medium (LB)**

Tryptone	10.0 g
NaCl	10.0 g
Yeast extract	5.0 g
Aqua dest.	*ad* 1000.0 ml

- **Standard I nutrient agar (NA) (SIFIN, Berlin, Germany)**

  Instant medium: Preparation according manufacturer's instructions

- **Standard I nutrient broth (NB) (SIFIN, Berlin, Germany)**

  Instant medium: Preparation according manufacturer's instructions

- **Tryptic soy agar (TSA) (Gibco, Eggenstein, Germany)**

  Instant medium: Preparation according manufacturer's instructions

- **Tryptic soy agar, half-concentrated**

Tryptic soy broth (Gibco, Eggenstein, Germany)	15.0 g
Agar-Agar	15.0 g
Aqua dest.	*ad* 1000.0 ml

- **φ-broth**

Tryptone	20.0 g
$MgSO_4$	10.0 g
Yeast extract	4.0 g
KCl	0.74 g
Aaua dest.	*ad* 1000.0 ml

- **Chitin minimal agar**

Solution 1:

NaCl	0.5 g
Nutrient broth	1.6 g
M9 Salts	6.0 g
Agar-agar	15.0 g
Colloidal chitin	2.0 g
Aqua dest.	*ad* 1000.0 ml

Solution 2:

0.1 M $CaCl_2$	1.0 ml
1.0 M $MgSO_4$	1.0 ml
0.05 M Thiamine-HCl solution	1.0 ml

After autoclaved solution 1 was cooled to 50°C, sterile-filtrated solution 2 was added.

- **Growth medium for detection of indol-3-acetic acid (Sawar and Kremer 1995)**

Glucose	5.0 g
Yeast extract	25.0 mg
L-Tryptophane	0.2 g
Aqua dest.	*ad* 1000.0 ml

- **Tween80 agar**

Solution 1:

Tryptone	10.0 g
NaCl	10.0 g
Yeast extract	5.0 g
Agar-agar	15.0 g
Aqua dest.	*ad* 1000.0 ml

Solution 2:

Tween80	10.0 g
Aqua dest.	*ad* 1000.0 ml

Solutions 1 and 2 were autoclaved separately. After cooling to 55°C, both solutions were mixed.

- **Skim milk agar**

Solution 1:		
Skim milk powder	100.0	g
Aqua dest.	*ad* 1000.0	ml
Solution 2:		
Tryptic soy broth (Gibco, Eggenstein)	10.0	g
Agar-agar	40.0	g
Aqua dest.	*ad* 1000.0	ml

Solution 1 was autoclaved for 5 min and solution 2 for 20 min at 121°C. After cooling down to 55°C both solutions were mixed.

- **Chrome azurol S agar (Schwyn and Neilands 1987)**

Solution 1:		
$FeCl_3 \cdot 6\ H_2O$	2.7	mg
10 mM HCL	*ad* 10.0	ml
Solution 2:		
Chrome azurol S	60.5	mg
Sterile *Aqua dest.*	50.0	ml
Solution 3 (pH 6.8):		
Cetyl Trimethyl Ammonium Bromide (CTAB)	72.9	mg
Sterile *Aqua dest.*	*ad* 40.0	ml
Solution 4 (pH 6.8):		
KH_2PO_4	0.3	g
NaCl	0.5	g
NH_4Cl	1.0	g
Piperazin-N, N'-bis (2-ethanesulfonic acid) (PIPES)	30.0	mg
Agar-agar	20.0	g
Aqua dest.	*ad* 800.0	ml
Solution 5:		
Glucose	2.0	g
Aqua dest.	*ad* 70.0	ml
Solution 6:		
Casamino acids	3.0	g
Aqua dest.	*ad* 30.0	ml

Solutions 4, 5 and 6 were autoclaved separately. Sterile filtrated solution 1 was added to solution 2. This mixture was slowly added to solution 3 under stirring. Solution 4 was mixed with solution 5 and 6, and adjusted to pH 6.8 using 50% (w/v) KOH. Mixed solutions (4, 5, and 6) were cooled to 50°C and solutions 1 through 3 were added.

2.3.2 Fungal growth media

- **Czapek Dox Broth (CDB) (Gibco, Eggenstein, Germany)**

  Instant-medium: Preparation according manufacturer's instructions

- **Potato dextrose agar (PDA)**

Glucose	20.0	g
Agar-agar	15.0	g
Aqueous potato extract	*ad* 1000.0	ml

- **Potato dextrose broth (PDB)**

Glucose	20.0	g
Aqueous potato extract	*ad* 1000.0	ml

- **Preservation medium**

Glycerine	60.0	ml
Glucose	10.0	g
Peptone	2.0	g
Yeast extract	2.0	g
Aqua dest.	*ad* 1000.0	ml

- **Synthetic nutrient-poor agar (SNA) (Nirenberg 1976)**

Glucose	0.2	g
Sucrose	0.2	g
KH_2PO_4	1.0	g
KNO_3	1.0	g
KCl	0.5	g
$MgSO_4 \cdot 7\ H_2O$	0.5	g
Agar-agar	22.0	g
Aqua dest.	*ad* 1000.0	ml

  Adjusted to pH 5.5 using 1 M KOH.

- **Waksman agar (WA)**

Tryptone	5.0	g
NaCl	5.0	g
Meat extract	3.0	g
Glucose	10.0	g
Agar-agar	20.0	g
Aqua dest.	*ad* 1000.0	ml

2.4 DNA manipulations

2.4.1 Extraction of genomic DNA from bacteria

Chromosomal bacterial DNA was prepared following the protocol developed by Anderson and McKay (1983) modified for genomic DNA and applied to an initial volume of 10.0 ml bacterial culture (Figure 1).

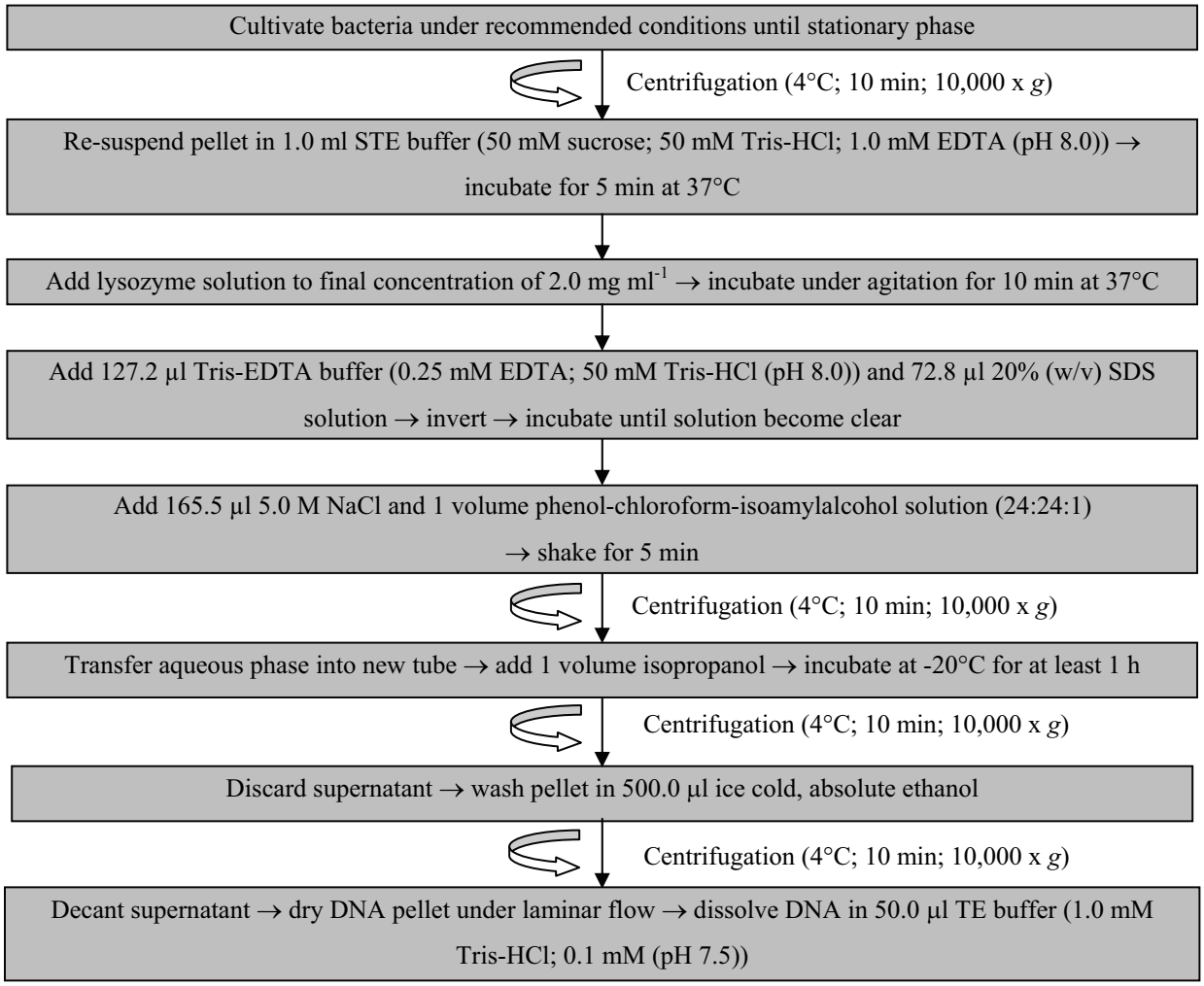

Figure 1: DNA extraction protocol for bacteria (Anderson and McKay 1983, modified)

2.4.2 Plasmid isolation

Plasmid DNA was extracted by applying QIAprep Spin miniprep Kit (Qiagen, Hilden, Germany) (Birnholm and Doly 1979) according to the manufacturer's protocol. Isolated plasmid DNA was used for restriction analyzes, cloning experiments and for PCR reactions.

2.4.3 Extraction of genomic DNA from fungi

Isolation of whole genomic DNA from fungi was executed as follows (Figure 2).

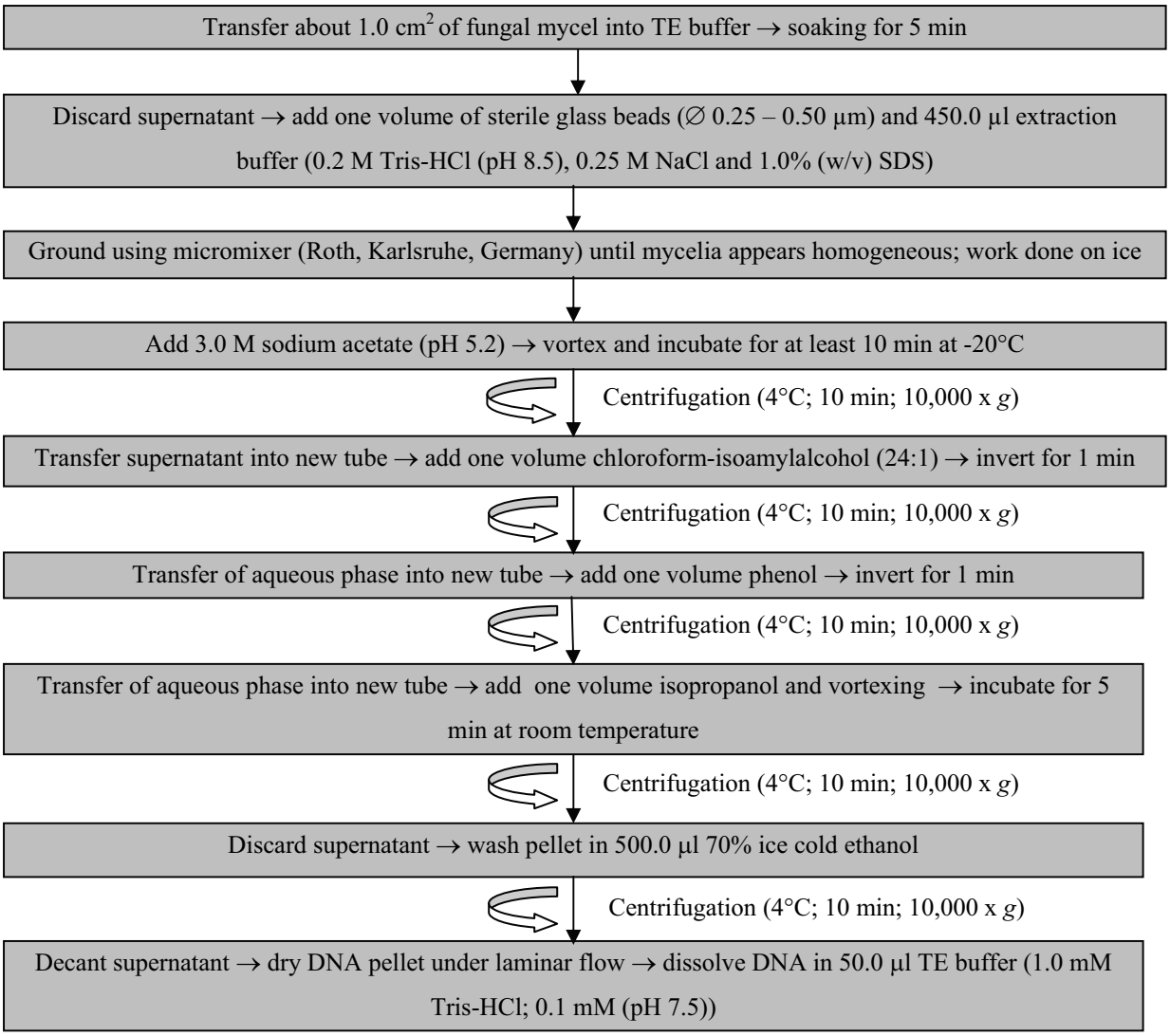

Figure 2: DNA extraction protocol for fungi

2.4.4 PCR approaches

In vitro amplification of DNA was done in a TGradient or Tpersonal thermocycler from Biometra (Göttingen, Germany). Oligonucleotides used in this study are displayed in Table 4, and were synthesised by biomers.net (Ulm, Germany). PCR experiments were conducted using reaction mix containing 0.02 U μl^{-1} Taq DNA polymerase; 100 µM each dNTP; 10.0 mM Tris-HCl (pH 9.0); 50.0 mM; 50.0 mM KCl; 1.5 mM $MgCl_2$; 0.2 mg ml^{-1} BSA; 0.1% (v/v) Triton X-100; 1.8 % (v/v) glycerine (Taq&Go, Qbiogene, Carlsbad, CA, USA); 1.0 µM each primer, and about 20-50 ng DNA template. Conditions under which the PCR was performed are specified in Table 4. Each reaction was started with an initial denaturation step for 5 min at 94°C and finished, except for BOX-PCR, with a final elongation step for 10 min at 72°C.

Table 4: Oligonucleotides used in this study: Target regions, sequences and PCR conditions

Target region	Primer	Sequence* (5´-3´)	Reaction cycles	Reference
S. plymuthica HRO-C48-specific site within *prnD* gene	PRND-C48f PRND-C48r	gcagaacgccacccacctt catccagtgcccgtttcat	94°C 1 min 65°C 1 min 72°C 2 min } 34 x	This study
V. dahliae-specific site within ITS region	VD1 VD2	ctcataaccctttgtgaacc ccgaggtcaaccgttgccg	94°C 1 min 58°C 1 min 72°C 2 min } 34 x	Volossiouk *et al.* 1995
Universal site within eubacterial 16S rDNA	Unibac-II-515f Unibac-II-927r[P]	gtgccagcagccgc cccgtcaattymtttgagtt	95°C 20 sec 53°C 30 sec 72°C 1 min } 35 x	Lieber *et al.* 2002
BoxA1-subunit within box-sequence	BoxA1	ctacggcaaggcgacgcttgaccg	94°C 1 min 53°C 1 min 65°C 8 min 65°C 16 min } 35 x	Martin *et al.* 1992
Specific site within pGEM vectors	usp rsp	gtaaaacgacggccagt caggaaacagctatgacc	Corresponding to the insert, which was obtained using one of the primer pair listed in this Table	Promega (MI, USA)
Universal binding site within *prnD* gene	PRND1 PRND2	ggggcgggccgtggtgatgga ycccgcsgcctgyctggtctg	94°C 1 min 65°C 1 min 72°C 2 min } 30 x	De Souza and Raaijmakers 2003

[P] 5´ phosphorylated primer

* IUPAC-code: y = t/c; m = A/C

2.4.5 Extraction and purification of DNA fragments from solutions and agarose gels

For purification of DNA solutions as well as for extraction of DNA fragments from agarose gels GENECLEAN Turbo Kit (Qbiogen, Carlsbad, CA, USA) was utilized. DNA obtained according to the manufacturer's protocols was suitable for restriction analyzes, cloning experiments and for PCR reactions.

2.4.6 DNA quantification

The concentration and purity of DNA in solutions was determined spectrophotometrically by measuring the absorption spectrum at 260 and 280 nm using photometer Spekol 1200 (Carl-Zeiss, Jena, Germany). A second method was utilized to estimate DNA quantities. Samples with unknown DNA concentration were separated in an agarose gel and stained with ethidium bromide. Under UV light signal intensity was compared with MassRuler™ DNA Ladder, high range and low range, respectively (Fermentas, St. Leon-Rot, Germany).

2.4.7 Restriction experiments

Restrictions analyzes of plasmid and genomic DNA was routinely done in a 20.0 µl reaction mix (1.0 to 2.0 µg DNA, 10 to 20 units enzyme) under conditions recommend by the manufacturer (buffer, temperature). Resulting DNA patterns were analyzed by agarose gel electrophoresis. Restriction enzymes and buffer were purchased from New England Biolabs (Beverly, MA, USA) and Fermentas (St. Leon-Rot, Germany).

2.4.8 DNA gel electrophoresis

DNA was routinely separated by means of horizontal gel-electrophoresis. Samples were mixed with 0.6 volume loading dye (Fermentas, St. Leon-Rot, Germany), loaded along with a length standard (GeneRuler™ 1kb DNA Ladder, Fermentas, St. Leon-Rot, Germany) on 0.8% (w/v) agarose gels in 1 x TAE and separated at constant voltage of 90.0 V. Molecular fingerprints from bacteria were produced by separating amplicons obtained by BOX-PCR in a 1.5% (w/v) agarose gel in 0.5 x TBE. Subsequently, gels were stained in ethidium bromide solution (1.0 µl ml ^{1}) for 20 min, washed for 10 min in dH_2O and documented using Gelprint 2000i (MWG Biotech AG, Ebersberg, Germany).

- **50 x TAE-Buffer (pH 7.5) (Sambrook *et al.* 1989)**

Tris	242.0 g
Acetic acid	57.0 ml
0.5 M EDTA (pH 8.0)	100.0 ml
Aqua dest.	*ad* 1000.0 ml

- **5 x TBE-Buffer (Sambrook *et al.* 1989)**

Tris	54.0 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20.0 ml
Aqua dest.	*ad* 1000.0 ml

2.4.9 Ligation

Plasmid vectors pGEM-T and pGEM7 were used to clone DNA fragments of interests after PCR amplification or restriction. Ligation was done using following reaction mix: 250.0 ng DNA; 50.0 ng pGEM-T/pGEM7 vector; 5.0 µl 2 x ligation buffer, 1.0 unit T4 DNA ligase and ddH_2O to a final volume of 10.0 µl (pGEM® vector systems, Promega Cooperation, Madison, MI, USA). Incubation was done for 3 h at room temperature, followed by 12 h at 4°C. Ligated plasmids were immediately used for transformation of *E. coli* DH5α cells.

2.4.10 Transformation of *Escherichia coli* DH5α

$CaCl_2$-mediated competence of *E. coli* DH5α was obtained by the protocol published by Hanahan (1983). First, *E. coli* was pre-cultured in 3.0 ml φ-broth at 37°C over night. Pre-culture served as inoculum for 100.0 ml main culture, which were incubated at 37°C (150 rpm) as long as an optical density (OD_{600}) of 0.48 was achieved. Then, culture was cooled down on ice for 10.0 min and centrifuged (5 min, 4°C, 5,000 x *g*). Pellet was re-suspended in 30.0 ml ice cold TJB buffer (100.0 mM RbCl; 50.0 mM $MnCl_2 \cdot 4\ H_2O$; 10.0 mM $CaCl_2 \cdot 2\ H_2O$; 30.0 mM potassium-acetate; 15.0% (v/v) glycerine (pH 5.8)) and incubated on ice for 5 min. After an additional centrifugation step (5 min, 4°C, 5,000 x *g*) 20.0 ml ice cold PIPES (10.0 mM RbCl; 10.0 mM MOPS; 75.0 mM $CaCl_2 \cdot 2\ H_2O$; 15.0% (v/v) glycerine (pH 7.0)) buffer amended with 1.4 ml DSMO was used for re-suspension of the pellet. Suspension was gently mixed, incubated on ice (10 min), dispensed in 100.0 µl aliquots and stored at -70°C. Before transformation, cells were slowly thawed on ice and intermixed gently with 10.0 µl of ligation reaction mix. The transformation approach was incubated on ice for 30 min without agitation, followed by heat shock for 100 s at 42°C and 5 min incubation on ice. Next, 900 µl of pre-warmed LB was added to the cells, which were incubated at 37°C for 1.0 h at 250.0 rpm. Transformation efficiency was checked by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) assay. The application of the pGEM® vector systems (Promega Corporation, Madison, MI USA) allows the selection of *E. coli* clones carrying recombinant plasmid-DNA based on hydrolysis of X-Gal catalysed by β-galactosidases, followed by oxidation to indigo dye. Cloning strain *E. coli* DH5α impaired in β-galactosidases gene generates after induction with IPTG (Isopropyl-β-D-thiogalactopyranosid), an inactive form of this enzyme. PGEM−cloning vectors carry the *lacZ* gene encoding a protein, which restore the gene defect. Complementation of this gene can be impeded by insertion of DNA fragments into the cloning site located within the *lacZ* gene. Agar plates (LB) amended ampicillin (100.0 µg ml^{-1}) were coated with 50.0 µl 100.0 mM IPTG and 50.0 µl X-Gal (20.0 mg ml^{-1}) allowing differentiation between clones carrying vectors with insert (white) and without insert (blue). 50.0 to 200.0 µl of transformed *E. coli* cells were spread onto agar plates and incubated at 37°C for 24 h. Cell material of white colonies were confirmed to carry recombinant plasmid and used for plasmid DNA preparation and sequencing.

2.4.11 DNA sequencing

DNA fragments to be sequenced were obtained by PCR amplification from plasmid DNA using primer pair USP/RSP. Sequencing was performed using the Beckham Coulter system (University of Rostock, Department of Animal Physiology, Rostock, Germany) or commercial facilities of GATC Biotech AG (Konstanz, Germany).

2.4.12 Southern blot analysis

2.4.12.1 DNA extraction for Southern blot analysis

For the isolation of chromosomal DNA *S. plymuthica* strain HRO-C48 WT and HRO-C48, PRND were grown in 200.0 ml NB for 24 h at 30°C and 150 rpm. Pelleted cells obtained after centrifugation at 10,000 x *g* were re-suspended in 3.0 ml lysis buffer (50.0 mM glucose, 25.0 mM TrisHCl (pH 8.0), 10.0 mM EDTA (pH 8.0)), followed by incubation in a water bath at 60°C for 30 min. 500.0 µl lysis buffer containing 26.0 mg lysozym were added. Cell suspension was shaken gently and incubated for 1.5 h at 37°C. In the next step, the suspension was supplemented with 750.0 µl NaCl (5.0 M), 1.0 ml EDTA (0.5 M) and 4.0 ml SDS solution (1.0% SDS, 0.7 M NaCl) and incubated for 10 min at 37°C. Another incubation period was performed for 1 h at 37°C after addition of protease K solution (20.0 mg ml ^{1}). For the last step of cell lysis, to the preparation CTAB solution (10.0% cetyl-tri-methyle ammonium bromide, 0.7 M NaCl) was added and incubated for 20 min at 65°C. Protein removal was performed using 10.0 ml of chloroform/isoamyl alcohol (24:1, v/v). The mixture was shaken thoroughly, followed by centrifugation for 30 min at 10,000 x *g* at room temperature. Again, 5.0 ml of chloroform/isoamyl alcohol (24:1, v/v) was admixed to supernatant, which had been transferred into a clean tube. Aqueous phase was separated by centrifugation for 10 min (10,000 x *g*) and transferred into a clean tube. To precipitate DNA, two volumes of absolute ethanol were added to the supernatant, followed by incubation over night at 4°C and centrifugation for 20 min at 10,000 x *g* at 4°C. After discarding supernatant, the precipitate was air dried. RNA removal was done by the following steps: DNA pellet was re-suspended in 2.0 ml 10 x TE buffer (10 mM EDTA, 100 mM Tris) and dispensed to 2.0 ml microcentrifuge cups. To each of these tubes 10.0 µl of RNases (10 mg ml ^{1}) was added. RNA was degraded during an incubation time of 25 min at 37°C. For final purification step 1.0 ml phenol was added. Preparation was centrifuged for 10 min at room temperature (10,000 x *g*) and supernatant was transferred into a clean tube. Then, supernatant was mixed with 1.0 ml of a phenol/chloroform mixture (1:1, v/v) and centrifuged for 10 min at room temperature (10,000 x *g*). Transferred aqueous phase was combined with 1.0 ml of chloroform/isoamyl

alcohol (24:1, v/v). The blend was shaken and centrifuged again for 10 min (10,000 x *g*). 200.0 µl sodium acetate (3.0 M, pH 6.0) and 4.0 ml of absolute ethanol was added to the transferred supernatant, followed by overnight incubation at 4°C. Precipitated DNA was pelleted by centrifugation for 20 min at 4°C and 10,000 x *g*. Finally, after washing with 1.0 ml of 80% ethanol followed by drying under aseptic air flow, pellet was re-suspended in 500 µl 10 x TE buffer (1.0 mM Tris-HCl; 0.1 mM (pH 7.5)). Quality and quantity of extracted DNA was controlled by electrophoresis in agarose gel (0.8% in 1 x TAE).

2.4.12.2 Agarose gel separation and blot transfer

Genomic DNA was subjected to restriction digestion using *pst*I in a 20.0 µl reaction mix (5.0 µg DNA; 2.0 µl 0.1% BSA; 2.0 µl 10 x reaction buffer; 20 units *pst*I) at 37°C over night. Whole reaction mix was loaded on 0.8% agarose gel (1 x TAE) to separate digested DNA. After staining and documentation, agarose gel was incubated in denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 30 min, followed by 30 min incubation in neutralisation buffer (1.0 M Tris, 2.0 M NaCl, pH 7.5) and 5.0 min in 2 x SSC buffer (20 x SSC, 3.0 M NaCl, 0.3 M Na-Citrat, pH 7.0). DNA was transferred to a nylon membrane (Roti®-Nylon plus, Roth, Karlsruhe, Germany) by semi-dry blotting. Semi-dry blot apparatus was constructed of several layers of filter paper on which two slides of cellulose paper (3MM Chr, Whatman, Dassel, Germany) were placed, followed by a nylon membrane equilibrated in 2 x SSC buffer. Agarose gel was directly positioned onto nylon membrane covered by a glass plate and weights (about 200 g). Transfer of DNA fragments was realised within 12 h. To immobilise DNA, nylon membrane was exposed to UV light at 312 nm for 3 min.

2.4.12.3 Digoxigenin-labelling of the DNA probe

A linear DNA fragment obtained by PCR amplification of *prnD* gene from *S. plymuthica* HRO-C48 WT (final size 750 bp) served as probe labelled with digoxigenin (PCR DIG Probe Synthesis kit, Roche Diagnostics, Mannheim, Germany). For random priming of the DNA with dioxigenin, 2.0 µl reaction mixture (containing hexanucleotide mixture in 10 x reaction buffer) was added to 1.0 µg DNA to a final volume of 12.0 µl. Preparation was denaturated at 95°C for 10 min and immediately cooled down on ice. The labelling reaction was started by adding 2.0 µl dNTP stock mix and 1.0 µl Klenow enzyme (1.0 unit) to the freshly denaturated DNA, followed by over night incubation at 37°C. Reaction was stopped by adding 2.0 µl EDTA (0.2 M, pH 8.0), 2.5 µl LiCl (4.0 M) and 75.0 µl absolute ethanol. DNA was precipitated for at least 30 min at -70°C and centrifuged (15 min, 4°C, 10,000 x *g*). The pellet

was washed with 80% ethanol, dried under laminar flow and dissolved in 50.0 µl TE buffer (1.0 mM Tris-HCl; 0.1 mM (pH 7.5)).

2.4.12.4 Hybridisation of the probe target

A nylon membrane harbouring immobilised DNA was pre-hybridised at 42°C for at least 1 h by immersion in 5.0 ml hybridisation solution (5 x SSC, 50.0% (v/v) formamide, 2.0% (w/v) blocking reagent, 0.1% (w/v) sodium-N-laurylsarcosin and 0.02% (w/v) SDS). The dig-labelled probe was mixed with 5.0 ml hybridisation solution, denaturated for 10 min in boiling water and immediately poured over the nylon membrane. After overnight incubation at 42°C DNA probe was discarded and filter was washed twice with 2 x SSC buffer containing 0.1% (w/v) SDS for 15 min.

2.4.12.5 Immunological detection of digoxigenin-labelled DNA

DNA homologous to the applied DNA probe were detected using DIG DNA and detection kit (Roche Diagnostics, Mannheim, Germany). After washing with TN buffer (1 min, 100.0 mM Tris/HCl, 150.0 mM NaCl, pH 7.5), the filter was incubated in TN buffer supplemented with 0.5% (w/v) blocking reagent for 30 min. Filter was washed in TN buffer for 5 min and incubated for 30 min in 20.0 ml alkaline phosphatase- conjugated antibody solution (conjugated antibody was 1:5000 diluted in TN buffer). Membrane was immersed in buffer 3 (100 mM Tris/HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9,5) for equilibration and probe-target hybrids were detected by applying 10.0 ml staining solution (buffer 3 amended with 45.0 µl nitro blue tetrazolium solution and 35.0 µl 5-bromo-4-choro-3-indolyl-phosphate solution). Staining was performed in the absence of light until bands became visible, and was stopped by rinsing with dH_2O.

2.4.13 *In silico* DNA manipulations

Sequence data were analyzed using the database provided by NCBI (http://www.ncbi.nlm.nih.gov) using BLAST algorithms (Altschul *et al.* 1997). Nucleotide sequences received from databases and obtained by sequencing were handled by means of ClustalX for multiple sequence alignment (Thompson 1997), pDRAW (AcaClone Software, Carlsbad, Denmark) for restriction and cloning analyzes, and Primer Premier 5.0 (Premier Biosoft International, Palo Alto, USA) for primer design suitable for PCR applications.

2.5 Genetical modifications of *Serratia plymuthica* HRO-C48

2.5.1 Generation of a spontaneous rifampicin-resistant mutant of *Serratia plymuthica* HRO-C48

Mutants of *Serratia plymuthica* HRO-C48 resistant to rifampicin were selected by culturing wild-type isolate in nutrient broth amended with 20.0, 60.0, 80.0, 100.0, or 150.0 µg ml^{-1} rifampicin at 30°C for 24 h at 150 rpm. Bacteria grown in the presence of 150.0 µg ml^{-1} rifampicin were plated on NA (100.0 µg ml^{-1} rifampicin). After 24 h resistant colonies were selected on the basis of their morphology and growth being similar of those of the wild-type isolates on NA. To stabilise spontaneous mutation of selected isolates, an alternating cultivation on NA with and without the antibiotic was conducted. The rifampicin-resistant isolates were confirmed on their antifungal activity *in vitro* against *V. longisporum* using dual culture assay.

2.5.2 Generation of a pyrrolnitrin-deletion mutant of *Serratia plymuthica* HRO-C48

2.5.2.1 Construction of a modified *prnD* fragment

For the generation of mutagenesis in specific genes the *aphII* gene cartridge (conferring kanamycin resistance) from pUC4K (Vieira and Messing *et al.* 1982) was inserted in a unique restriction site within the *prnD* gene, which was cloned into *E. coli* vectors (Table 5). In general, cloned constructs were isolated from transformed *E. coli* DH5α cells by using QIAprep spin miniprep (Qiagen, Hilden, Germany). *PrnD* gene of *S. plymuthica* HRO-C48 obtained by PCR using primer pair PRND1/PRND2 was cloned into pGEM-T vector; resulting in pPRND-T. Next, *prnD* was subcloned as a *Nsi*I/*Apa*I fragment into pGEM7; resulting in pPRND. Plasmid pPRND was subjected to restriction digestion with *Sal*I, before kanamycin resistance cassette *aph*II was ligated into *Sal*I restriction site within *prnD*. The modified gene was obtained by PCR from pPRND::Km using HRO-C48-specific primer pair PRND-C48f/PRND-C48r. About 1.0 µg of the amplicon was used for electroporating *S. plymuthica*.

2.5.2.2 Transformation of *Serratia plymuthica* HRO-C48

Electrocompetent cells of *S. plymuthica* HRO-C48 were obtained as follows. Erlenmeyer flask containing 100.0 ml LB was inoculated with 5.0 ml of an overnight culture (30°C, 150.0 rpm) and incubated (30°C, 150 rpm) until mid-log phase (OD of 0.5 to 0.7) was reached. Culture was chilled on ice for 15 min before centrifugation (4°C, 15 min, 5,000 x *g*).

Harvested cells were re-suspended in 10.0 ml ice-cold bidistilled water and centrifuged again (4°C, 15 min, 5,000 x *g*). Bacterial sediment was washed by re-suspending in 20.0 ml 10.0% (v/v) glycerine, followed by centrifugation under conditions mentioned above. Finally, pellet was re-suspended in 300.0 µl 10% (v/v) glycerine, aliquoted in 40.0 µl volumes in 1.5 ml microcentrifuges tubes and stored at -70°C.

Table 5: Plasmids used to generate pyrrolnitrin-deletion mutant of *S. plymuthica* HRO-C48

Plasmid	Description
pPRND-T	pGEM-T containing *prnD* gene as a 769 bp fragment obtained by PCR with PRND1 and PRND2 primer, final size: 3.8 kb
pPRND	pGEM7 containing *prnD* gene as a 769 bp fragment obtained by restriction of pPRND-T with *Nsi*I/*Apa*I, final size: 3.8 kb
pPRND::Km	pPRND containing inactivated *prnD* gene as 2.0 kb fragment (*aphII* gene is inserted in the unique *Sal*I site); final size: 5.0 kb

Cells to be transformed were thawed on ice and intermixed with 1.0 µg DNA. Preparation was transferred into pre-chilled 0.2 µl electroporation cuvette. Electroporator (Gene Pulser II, Bio-Rad, Hercules, CA, USA) was set to 2.5 kV voltage, 25.0 µF capacitor and 20.0 Ohm resistance. After addition of pre-warmed LB medium, cell suspension was transferred into microcentrifuge tube and incubated for 1.0 h at 30°C without agitation. *S. plymuthica* cells were plated onto LB agar amended with 50.0 µg ml ^{1} kanamycin and cultivated for 24.0 h at 30°C. Antibiotic-resistant (Km^r) clones were selected after 24 h incubation. Successful recombination of the DNA fragment into the genome of HRO-C48 was confirmed by PCR using primer pair PRND-C48f/PRND2-C48r as well as Southern Blot analysis. The loss of pyrrolnitrin activity was confirmed by bio-assay described in chapter 2.7.4.

2.6 *Ad planta* experiments

2.6.1 Seed treatment

2.6.1.1 Preparation of the bacterial inoculum

Serratia plymuthica HRO-C48 cells used for all seed treatment procedures were prepared by harvesting bacteria from 24 h culture (NB, 30°C, 150 rpm) by centrifugation at 7,500 x *g* for 10 min and re-suspending in treatment-specific agent mentioned below. All suspensions were adjusted to $\log_{10}$ 10.0 CFU ml ^{1}. Investigations on oilseed rape were exclusively done with the cultivar Talent (NPZ, Hohenlieth, Germany).

2.6.1.2 Pelleting

Pelleting is commonly used to coat seeds with pesticides and other additives. In this study, seed treatments were performed using laboratory-scaled machine, where an inclined, rotating 10 ml Erlenmeyer flask served as pelleting drum with a capacity of 2.0 g seeds. Figure 3 shows both the protocol for conventional seed treatment according to the procedure routinely performed by Northern German Plant Breeding Hans-Georg Lembke KG (Hohenlieth, Germany) (A), and the protocol for bacterial treatment (B). Seeds were treated in the conventional way by wetting with the insecticide Chinook (20.0 ml kg ^{1}) (Bayer, Leverkusen, Germany), followed by adding a mixture consisting of the fungicide DMM (Dimethomorph) (5.0 g kg ^{1} seed) (Bayer, Leverkusen, Germany) and 25.0 g kg ^{1} seed talcum. Bacterial treatment was done according to formulation protocols applied by Richter (2001), which were modified and adapted to present conditions. 500.0 µl of cells suspended in 1.5% methyl cellulose (Roth, Karlsruhe, Germany) solution were pipetted to seeds. To bind remaining liquid 2.0 g talcum was added. Seeds continued rotating in the flask until the seed coating was uniform. When the combination of standard and bacterial pelleting was attained, first standard protocol and subsequent bacteria were applied. Treated seeds were stored in water- and gas-proofed bags.

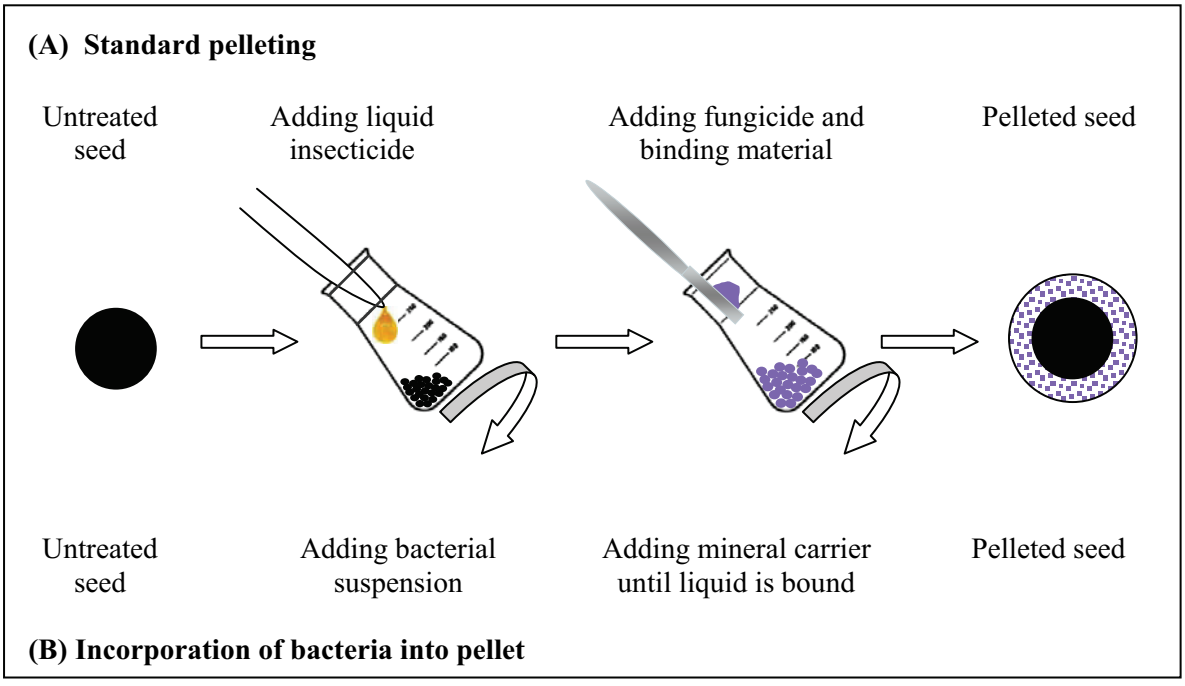

Figure 3: Schematic protocol for pelleting of oilseed rape seeds

2.6.1.3 Film coating

Using film coating preliminary experiments were conducted to assess appropriate concentrations of sucrose and *N*-Acetyl-D-glucosamine (D-GlcNAc), which are assumed to

be protective of bacterial vitality during lyophilization and long term storage (Frankowski, 2001, Leslie *et al.* 1995). 0.4 g seeds were wetted for 5 min in 1.0 ml of cell suspension containing the oligosaccharides sucrose (1.0%, 2.0% 4.0%, 6.0% (w/v)) and D-GlcNAc (3.0%, 5.0%, and 7.0% (w/v), respectively (Figure 4). Afterwards inoculated seeds were lyophilised for 5 h in a SpeedVac concentrator (Savant Instruments, Farmingdale, NY, USA). Film coated seeds were stored in water- and gas-proofed bags.

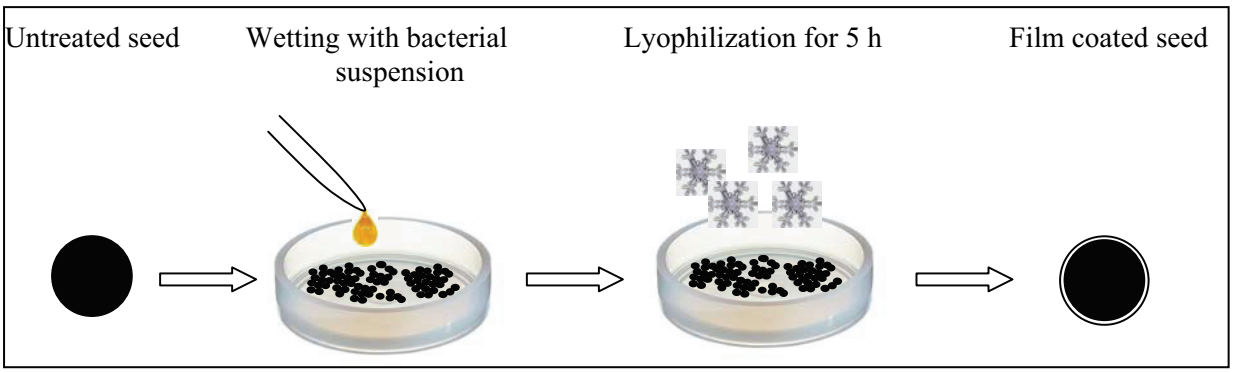

Figure 4: Schematic protocol for film coating of oilseed rape seeds.

2.6.1.4 Bio-priming

Seed priming techniques for physiological enhancement of germination is in widespread commercial use. Depending on seed batches, the course of germination may differ, and therefore, priming procedure had to be specified for each seed batch used in the present research. To specify the time course of germination of seeds, 1.0 g of seeds were covered by 5.0 ml 0.85% sodium chloride. The increase of water content over the time was determined by Moisture Analyser MB 35 (Roth, Karlsruhe, Germany). After decanting the remaining sodium chloride solution, adherent liquid was removed using filter paper. Water content of seeds was recorded after drying for 30 min at 125°C. The development of cell counts correlative to the time course of germination according to Bradford (1995) were carried out by seed treatment described below (chapter 1.6.1.4). Samples were taken after 4, 12, and 20 h. To detect *S. plymuthica* cells within the seed exclusively prior grounding, seed surface was disinfected with NaClO (2.5% available chlorine) for 5 min, and rinsed with sterile demineralised water three times for 1 min.

In the present study two seed batches were used for bio-priming. For inoculating seeds from the year 2003, 0.4 g seeds were incubated in 2.0 ml of cell suspension containing 0.85% sodium chloride for 12 h at 20°C. Using seeds harvested in the year 2004, incubation time had to be reduced to 5 h. During incubation seeds were agitated. Infiltrated seed were dried for 24 h at 20°C to the desired moisture content of about 5.0% and stored in water- and gas-proofed bags (Figure 5).

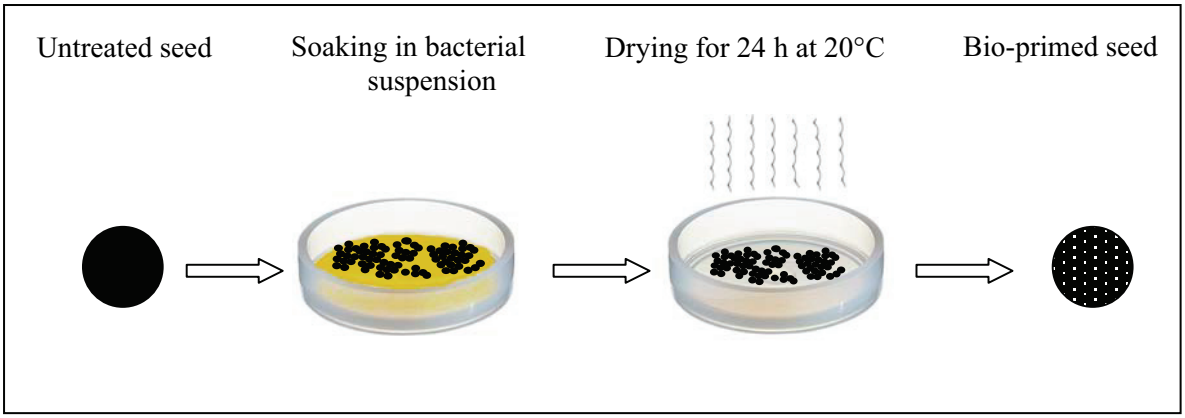

Figure 5: Schematic protocol for bio-priming of oilseed rape seeds.

2.6.1.5 Re-isolation of *Serratia plymuthica* HRO-C48 from inoculated seeds

For determination of cell counts 20 seeds were transferred into sterile 1.0 ml 0.85% sodium chloride solution. Pelleted and film coated seeds were vortexed until the coatings came off the seeds completely. Primed seeds were ground for 1 min using an autoclaved mortar and pestle. Suspensions were serially diluted with sterile 0.85% NaCl and plated onto NA medium. Plates were incubated for 24 h at 30°C and colony forming units (CFU) were counted to calculate the means.

2.6.1.6 Seed germination assay

Germination rate of treated oilseed rape seeds were tested in 100 replicates each treatment. Seeds were arranged on a wetted cellulose filter which was placed in a phytochamber. Germinated seeds were enumerated after 3 d incubation at 20°C in the absence of light.

2.6.2 Effect of *Serratia plymuthica* HRO-C48 on oilseed rape under greenhouse conditions

Altogether, four greenhouse experiments were performed to evaluate seed treatment techniques, and to investigate the mechanisms involved in biocontrol activity of *Serratia plymuthica* HRO-C48 in regard to disease suppression, plant growth promotion and rhizosphere colonization. Table 6 gives an overview of studies which were done to investigate the influence of seed treatments. Pelleted seeds applied in field trial 2003/2004 were evaluated in a parallel greenhouse experiment (experiment ST I). In a second approach (ST II), the influence of respectively pelleting, film coating and bio-priming on the performance of the biological control agent *ad planta* was obtained. To exclude the unilateral impact of the treatments, seeds treated with sterile 0.85% (w/v) NaCl solution instead of bacterial suspension were included in the experiment. The second test, analyzing the mode of action of HRO-C48, comprised two experiments (Table 7). M II was an independent replication of

experiment M I except for strains HRO-C48 PRN , and HRO-C48 pME6863, which were included for the first time.

All experiments were set as described by Zeise (1992). For root colonization studies, strain HRO-C48 WT was replaced by spontaneous rifampicin mutant HRO-C48 Rif^r allowing selective re-isolation from the rhizosphere.

Table 6: Greenhouse experiments to evaluate the influence of seed treatment techniques suitable for application of *Serratia plymuthica* HRO-C48 on oilseed rape on disease suppression, plant growth promotion effect and root colonization.

Experiment	Variants	Applied seed treatment technique
ST I	Standard (Control) Standard + HRO-C48 Rif^r (Diamol GM) Standard + HRO-C48 Rif^r (talcum)	Pelleting (see chapter 2.6.1.2)
ST II	Pelleting (Control) Pelleting (HRO-C48 WT) Film coating (Control) Film coating (HRO-C48 WT) Film coating (Control) Bio-priming (HRO-C48 WT)	Pelleting, Film coating, Bio-priming (chapter 2.6.1.2 – 2.6.1.4.)

Table 7: Greenhouse experiments to investigate biocontrol-related mechanisms possessed by *Serratia plymuthica* HRO-C48 in oilseed rape.

Experiment	Variants	Applied seed treatment technique
M I	Untreated control HRO-C48 WT HRO-C48 AHL$^-$ HRO-C48 Chi$^-$	Bio-priming
M II	Untreated control HRO-C48 WT HRO-C48 AHL$^-$ HRO-C48 Chi$^-$ HRO-C48 PRN$^-$ HRO-C48 pME6863	Bio-priming

2.6.2.1 Preparation of the fungal inoculum

Microsclerotia of *V. longisporum* strain ELV25 (Messner *et al.* 1996) were produced in Czapek-Dox vermiculite medium. 200.0 ml of Czapek-Dox broth was added to four litre of vermiculite (Vermiculite Dämmstoffe, Spröckhövel, Germany) and inoculated with a two-week old culture of *V. longisporum* (Czapek-Dox broth, 20°C at 120 rpm) and incubated at room temperature for four weeks.

2.6.2.2 Experimental design

For plant growth promotion and root colonization experiments, seeds were sown in pots with a volume of 250.0 ml containing propagation compost (Einheitserdewerk, Uetersen, Germany) mixed with vermiculite (4:1, v/v). For evaluation of biocontrol activity against *Verticillium*, plants were grown in pots containing a propagation compost/vermiculite mixture supplemented with the fungal inoculum (14:5:1, v/v/v). The final pathogen density was 50 to 60 microsclerotia g^{1} soil. Plants were greenhouse-grown over a period of 9 weeks at a temperature of 25 ± 10°C and assimilation light (12 h dark/light period). They were watered every second day.

2.6.2.3 Assessment of disease development

After appearance of first symptoms, the disease reaction of plants was assessed by severity of symptoms on a 1 9 scale (1 no symptoms, 2 few dark coloured wires, 3 oldest leave with strong symptoms, 4 loss of the oldest leave, 5 about 50% of leaves with strong symptoms, 6 loss of about 50% of the leaves, 7 loss of over 50% of the leave, 8 only the vegetation conus left, 9 dead plant) at weekly intervals. Data on disease severity was used to calculate area under disease process curve (AUDPC) determined as AUDPC $\Sigma((S_i+S_{ti+1})/2)*(ti + 1 - ti)$, where S_i is the symptoms severity, and t_i is the date of assessment of symptoms severity (Zeise 1992).

2.6.2.4 Assessment of plant growth parameter

When the experiments were finished, aerial parts of infested and non-infested plants respectively were separated from roots and weighed for the purpose of analyzing the effects of *S. plymuthica* HRO-C48 on biomass production.

2.6.2.5 Sampling for root colonization assays and cultivation-independent analyzes

Roots with adhering soil from six plants of each variant and sampling time were sampled into sterile bags. Material of two plants were combined (5.0 g) to extract rhizosphere-associated *Serratia* cells by adding 50 ml of distilled water and homogenising in a Stomacher laboratory blender for 180 s (BagMixer, Interscience, St. Nom, France). Samples were serially diluted with sterile 0.85% NaCl and plated onto NA supplemented with 100 µl ml^{1} rifampicin; for re-isolation of HRO-C48 PRN rifampicin was substituted by kanamycin. Plates were incubated for 24 h at 30°C and colony forming units (CFU) were counted to calculate the means of colonies (log_{10} CFU) based on root fresh weight. Soil dispersions obtained after homogenising

were also taken for extraction of microbial fraction to analyse bacterial community structure by means of SSCP analysis (see chapter 2.6.4).

2.6.2.6 Plant tissue preparation for DNA isolation and ELISA assay

Oilseed rape plants treated with *S. plymuthica* HRO-C48 WT using pelleting, film coating and bio-priming, and an untreated control were grown in *Verticillium* infested soil and non-infested soil, respectively (experiment ST II, Table 9). The experiment was finished 21 days after sowing; at this time no symptoms of Verticillium wilt were visible. Each variant was grown in 12 replicates. Aerial parts (stems and leaves) of four plants each treatment were pooled for further proceedings. Plant material were washed under running tap water, surface sterilised for 1 min in 1.0% NaOCl and stored at -70°C. Frozen samples were ground in sterile mortar and pestle in the presence of liquid nitrogen, and then plant material was lyophilised over night. Dry samples were subsequently used for DNA extraction and Enzyme-linked immunosorbent assay (ELISA). Total DNA from plant powder was extracted using DNEasy DNA extraction kit for plants (Qiagen, Hilden, Germany) according to manufacturer's manual and applied as a template for qualitative Real-time PCR. Quantification of *V. dahliae* in oilseed rape plants via ELISA were performed by a commercial company (PLT Pflanzen-Testlabor, Oestrich-Winkel, Germany).

2.6.2.7 Real-time PCR approach for quantification of *V. longisporum* DNA in plant tissue

For PCR detection of *V. longisporum*, primer pair VD1/VD2, which is directed to a specific site of the ITS region, was used (Volossiouk *et al.* 1995). First, PCR assay was conducted to confirm the specificity and the presence of *V. longisporum* DNA in oilseed rape plants obtained from greenhouse trial ST II. Reactions included negative controls (no DNA, DNA from non-inoculated plant) and positive controls (DNA of *V. longisporum* ELV25 extracted from pure mycelia). The amplification was performed under conditions listed in Table 4.

The real-time PCR approach for quantification of *V. dahliae* DNA was conducted using an iCycler apparatus (BioRad, Hercules, CA, USA), and results were analyzed with the software Optical System Software v 3.0a provided by BioRad. Reaction mixtures (30.0 µl) consisted of 100.0 nM each primer, 1.2 mM each dNTP, 2.5 mM $MgCl_2$, 3.0 µl of 10 x reaction buffer (50.0 mM KCl, 10.0 mM Tris HCl pH 9.0, 1% v/v Triton XC-100), 0.9 U EcoTaq DNA polymerase (Ecogen, S.R.L., Barcelona, Spain), 3.0 µl Sybr-Green (Molecular Probes, Inc., Eugene, OR, USA) 1:15000 v/v in water (1:100) and DMSO (1:150), and 100 ng of template DNA. Amplification reactions were performed in 96-well microtiter plates (BioRad, Hercules,

CA, USA). Every reaction was performed using aliquots of the same master mix. Three simultaneous, replicated amplifications were carried out for each DNA sample, using 30.0 µl aliquots from a 90.0 ml mixture. Thus, all DNA samples (and their replicates) were submitted to the same experimental conditions. Additionally, a plate contained *V. dahliae* DNA samples with known DNA concentrations which were used to develop the standard curve (see below), as well as DNA samples from non-inoculated oilseed rape plants and a negative control (no template DNA). The real-time PCR program involved an initial step of denaturation (4 min, 95°C) followed by 40 cycles of 1 min at 94°C, 45 s at 54°C, 45 s at 72°C, and 25 s at 90°C. Fluorescens emission of the target amplicon (T_m 92.5°C) was measured at 90°C. A final extension step of 4 min at 72°C was added. After that, a melting curve program was run for which measurements were made at 0.5°C temperature increments every 10 s within a range of 60 100°C. Finally, PCR products were also visualised in an ethidium bromide-stained agarose gel (1.0% in 1 x TAE buffer) under UV light, as an additional check of amplification.
A standard curve was developed by plotting the logarithm of known concentrations (10-fold dilution series from 1.0 pg to 10.0 ng per 30.0 µl reaction) of *V. longisporum* ELV25 against the Ct value. Ct is the cycle number at which the fluorescence emission of the PCR product is statistically significant from the background.

2.6.3 Effect of *Serratia plymuthica* HRO-C48 on oilseed rape under field conditions

2.6.3.1 Experimental design

Effect of *S. plymuthica* HRO-C48 on winter oilseed rape cv. 'TALENT' under field conditions was evaluated in two consecutively years. The first field trial was conducted in the growing period 2003/2004 at three sites located in Northern Germany. Field sites of the second experiment were located in the South (Moosburg), West (Hovedissen) and North (Hohenlieth) of Germany. Important data describing experimental field sites, plot design and plant protection events are noted in Table 10 and 11. Plants were grown in a completely randomised block design. Each block was 12.0 m^2 in area and separated from other blocks by four lines of untreated plants of the same cultivar. Weather data for the second field trial was obtained from observation stations of the German Weather Service (DWD, Offenbach, Germany) near to the field sites. Variants for the field trial 2003/2004 comprised conventionally treated seeds and two variants with an additional layer of a mineral compound carrying *S. plymuthica* HRO-C48 Rif^r cells (Table 8). After the procedure was up-scaled from laboratory scale (see chapter 2.6.1.2), seeds were prepared using a pilot machine applicable for pelleting of 5.0 kg seeds per batch (Northern plant breeding company Hans-Georg

Lembke NPZ, Hohenlieth, Germany). Seeds used for the second field study were treated using the procedure of bio-priming described in chapter 2.6.1.4. Table 9 contains seed treatment variants sowed for field evaluation in the growing period 2004/2005. Seeds coated with standard compounds and seeds primed with sterile NaCl-solution served as control variants. Two seed variants were infiltrated with *S. plymuthica* HRO-C48 Rif^r, whereby variant 2 was additionally pelleted with Chinook, DMM and talcum.

Table 8: Seed treatment variants included in field trial 2003/2004

	Seed treatment variant		
	1	2	3
Coatings	1. Standard pelleting	1. Standard pelleting 2. Incorporation of *S. plymuthica* HRO-C48 Rif^r using pelleting **Mineral carrier:** Diamol GM	1. Standard pelleting 2. Incorporation of *S. plymuthica* HRO-C48 Rif^r using pelleting **Mineral carrier:** Talcum
Log_{10} CFU seed^{-1}	-	2.5	5.0

Table 9: Seed treatments variants included in field trial 2004/2005

	Seed treatment variants			
	1	2	3	4
Seed treatment	1. Standard pelleting	1. Bio-priming with *S. plymuthica* HRO-C48 Rif^r 2. Standard pelleting	1. Bio-priming with sterile 0.85% (w/v) NaCl	1. Bio-priming with *S. plymuthica* HRO-C48 Rif^r
Log_{10} CFU seed^{-1}	-	5.5	-	5.5

Table 10: Characterisation of field sites, experimental design and plant protection plan for evaluation of *S. plymuthica* HRO-C48 in oilseed rape (2003/2004)

Location	Hohenlieth		Poel Island
Field designation	Hamm	Rothenstein	Malchow
Position	54°24′46.65″ N 09°53′06.53″ E	54°26′06.56″ N 09°55′52.46″ E	53°59′25.00″ N 11°28′11.76″ E
Soil texture	Loamy sand	Loamy sand / Sandy loam	Sandy Loam
Relative soil fertility[1]	51	54	58
Crop order	Spring wheat Legumes Spring wheat Winter barley	Oilseed rape Winter Wheat Fallow land Sugar beet Winter wheat Barley	Winter wheat Phacelia
Field design	Randomised blocks	Randomised blocks	Randomised blocks
Replicates per treatment	5	5	6
Initial fertilisation	K_2O, P_2O_5	K_2O, P_2O_5	Application of the fungicide Contans WG before sawing
Before winter dormancy period:			
Herbicides	Butisan Top, Gallant super	Butisan Top, Gallant super	Butisan, Fusilade Max
Insecticides	Karate Zeon	Karate Zeon	
After winter dormancy period			
Fertilization	N, S (KAS, ASS)	N (KAS, ASS)	N, P_2O, K_2O, MgO, S, CaO
Insecticides	Karate Zeon, Fastac SC, Trafo	Karate Zeon, Fastac SC, Trafo	
Fungicides	Konker R	Konker R	

[1] The relative soil fertility indicates the productivity of a given soil compared to the most fertile soil in Germany (Magdeburger Börde) for which the value is set to 100.

2.6.3.2 Sampling for root colonization assays and cultivation-independent analyzes

Rhizosphere samples, two (field trial 2003/2004) or four replicates (field trial 2004/2005) per treatment, were taken at six-leaf stage, rosette stage, flowering stage, and fully-ripe stage. For each sample four plants were combined, resulting in 5.0 to 10.0 g root material with adhering soil which were placed into sterile bags, supplemented with 50.0 ml sterile distilled water and treated in a Stomacher laboratory blender for 180 s (BagMixer, Interscience, St. Nom, France). Soil suspension were serially diluted with sterile 0.85% NaCl and plated onto NA medium containing 100 µg ml ^{1} rifampicin. Plates were incubated for 24 h at 30°C and colony forming units (CFU) were counted to calculate the means of colonies ($\log_{10}$ CFU) based on root fresh weight.

Soil dispersions obtained after blending were used for extraction of the microbial fraction for community structure analysis by means of SSCP described in chapter 2.6.4.

Table 11: Characterisation of field sites, experimental design and plant protection plan for evaluation of *S. plymuthica* HRO-C48 in oilseed rape (2004/2005)

Location	Hohenlieth	Fehmarn Island	Bielefeld	Moosburg
Field designation	Stilland	Burg auf Fehmarn	Hovedissen	Grünseiboldsdorf
Position	54°24′31.38″ N 09°52′27.05″ E	54°25′57.34″ N 11°12′59.70″ E	52°01′22.35″ N 08°39′00.83″ E	48°26′39.80″ N 11°54′27.21″ E
Soil texture	Slight loamy sand	n.d.	Sandy loam	Sandy loam
Relative soil fertility[1]	48	n.d.	62	75
Crop order	Winter wheat Legumes Winter wheat Winter barley	n.d.	Winter wheat Winter barley	Corn Spring wheat Oat Spring wheat
Field design	Randomised blocks	Randomised blocks	Randomised blocks	Randomised blocks
Replicates per treatment	8	2	3	6
Initial fertilisation	140 kg K_2O 90 kg P_2O_5	n.d.		
Before winter dormancy period				
Fertilization	N (ASS)			
Herbicides	Reglone, Butisan Top, Gallant super		Butisan, Agil	Cirrus, Butisan, Butisan Top
Insecticides	Fastac SC			
After winter dormancy period				
Fertilization	N, S (ASS, KAS)		N, P_2O_5, K_2O, CaO, MgO (org. manure)	Bor
Insecticides	Fastac SC, Karate Zeon, Calypso			Trafo, Fury
Fungicides	Cantus			
Weather observations stations	Ostenfeld	n.d.	Bad Salzuflen	Erdinger Moos

n.d. - no data available

[1] The relative soil fertility indicates the productivity of a given soil compared to the most productive soil in Germany (Magdeburger Börde) for which the value is set to 100.

2.6.4 Effect of *Serratia plymuthica* HRO-C48 on olive under greenhouse conditions

2.6.4.1 Preparation of the bacterial inocula

Two biocontrol strains, namely *Serratia plymuthica* HRO-C48 WT and *Pseudomonas fluorescens* PICF7 WT, were used to investigate their effect on plant growth and the development of Verticillium wilt in olive trees. *P. fluorescens* PICF7 was already shown to

suppress Verticillium wilt in olive under greenhouse conditions (Mercado-Blanco *et al.* 2004). Inocula of *Serratia* and *Pseudomonas,* both wild types and rifampicin resistant mutants*,* were grown on NA at 30°C for two days, scraped from the medium with sterile Drigalski spatula, and suspended in 10.0 mM $MgSO_4 \cdot 7H_2O$. Bacterial suspensions were centrifuged twice in 10.0 mM $MgSO_4 \cdot 7H_2O$ to remove residual metabolites (10,500 x *g*, 20 min) and re-suspended in physiological $MgSO_4$ solution. Bacterial concentration in the suspension were adjusted to $\log_{10}$ 8.7 CFU ml^{-1} each strain. Combining both strains in suspension was adjusted to a total cell number of $\log_{10}$ 8.7 CFU $ml^{-1,}$ each strain in equal shares. In a parallel experimental approach, spontaneous rifampicin resistant mutant of both BCAs, designated as *S. plymuthica* HRO-C48 Rif^r and *P. fluorescens* PICF7 Rif^r respectively, were employed to enumerate root colonizing bacteria. The preparation of these isolates was performed as described for wild-type strains.

2.6.4.2 Preparation of fungal inoculum

Monoconidial *V. dahliae* isolate V138 used in this study is a representative of the defoliating pathotype originated from diseased cotton plants (Bejarano-Alcázar *et al.*, 1996). For experiments, inoculum consisted of conidia from cultures in potato-dextrose broth (PDB) incubated in the dark for seven days at 24°C and 125 rpm. Conidia were harvested by filtering liquid culture through a layer of sterile cheesecloth and inoculum concentration were adjusted to $\log_{10}$ 7.3 condia ml^{1} by dilution with sterile water.

2.6.4.3 Experimental design

A greenhouse trial was conducted in order to evaluate the ability of the biological control strains *S. plymuthica* HRO-C48 and *P. fluorescens* PICF7 to suppress Verticillium wilt caused by the defoliating pathotype of *V. dahliae*, to promote plant growth, and to colonize the rhizo- and endosphere of olive plants (Figure 6). The experiment was arranged in a two-stage setup. For the first 90-day stage, seven-month old, micropropagated olive plants cv. Arbequina, provided by Cotevisa (Valencia, Spain), were carefully uprooted from the substrate, their roots thoroughly washed in tap water, and dipped into a bacterial suspension for 10 min. The suspensions contained respectively *S. plymuthica* HRO-C48, *P. fluorescens* PICF7 and the combination of both strains. For the control treatment, plants were dipped into 10.0 mM $MgSO_4 \cdot 7H_2O$. Plants applied for plant growth promotion (16 per treatment) and root colonization assay (15 per treatment) were transplanted (one per pot) into clay pots with a diameter of 15 cm containing an autoclaved (twice on consecutive days) soil mixture (sand/loam, 2:1, v/v).

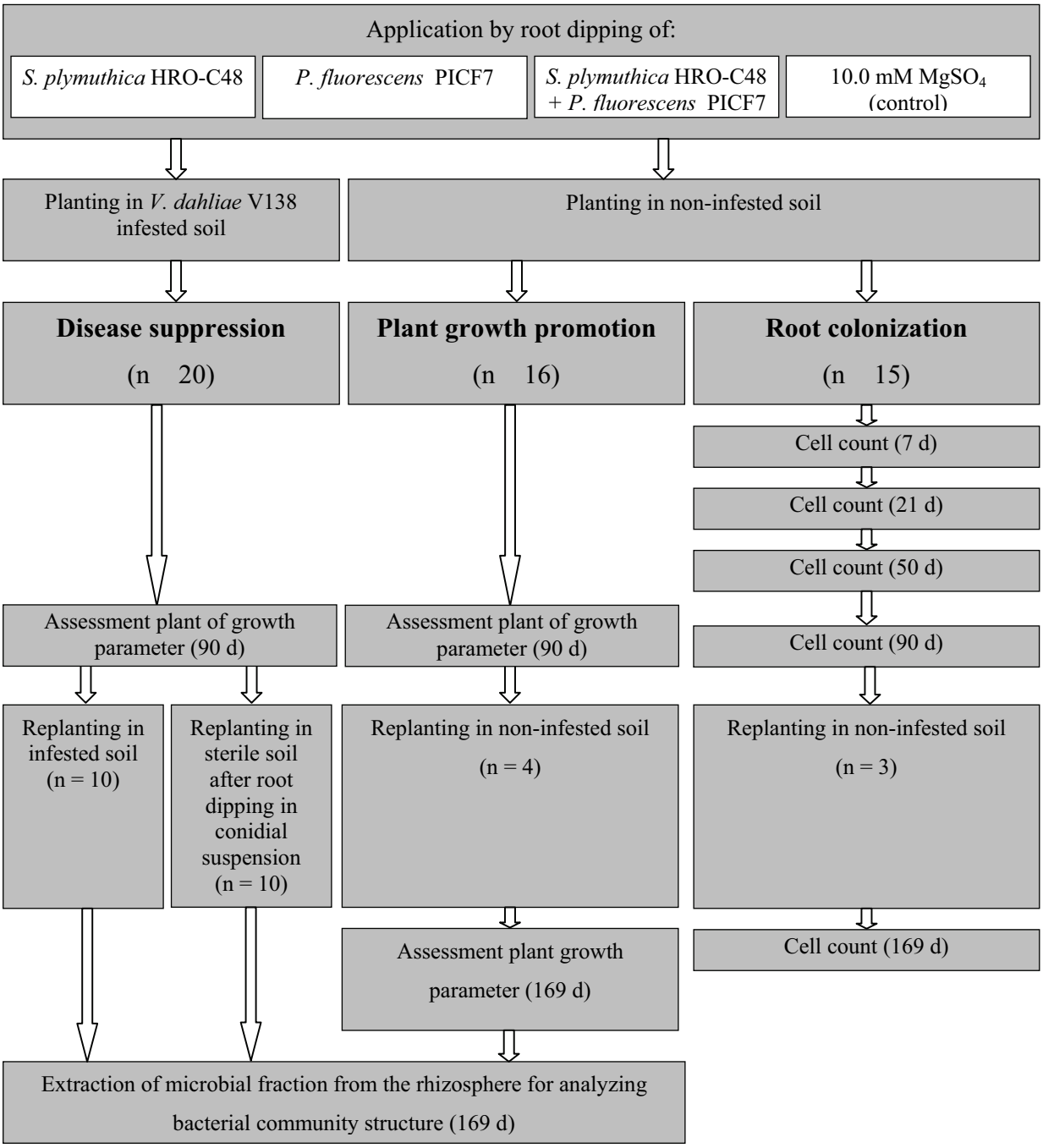

Figure 6: Experimental setup to investigate effect of *S. plymuthica* HRO-C48 and *P. fluorescens* PICF7 on olive plants. For root colonization experiments plant roots were treated with spontaneous rifampicin resistant mutants instead of wild type strains. Number of replicates noted in the brackets indicating observed plants per bacterial treatment.

For the biocontrol experiment, 20 plants were grown in soil which was infested by thoroughly mixing 100.0 ml of conidial suspension of *V. dahliae* V138 with 1.0 kg of soil mixture to obtain a final concentration of $\log_{10}$ 6.3 conidia g^{1} soil. The experiment was conducted in a randomized block design. Plants were incubated under greenhouse conditions at 23±1°C, 60 to 90% relative humidity, and received a 14 h photoperiod for three months. Plants were

watered as needed, and fertilized weekly with a hydro-sol fertilizer 20-5-32+microelements (Haifa Chemicals, LTD, Haifa, Israel).

During 90 days no symptoms of Verticillium wilt could be observed. Thereafter, plants which were grown in infested soil were again exposed to the pathogen. Ten plants per treatment were uprooted and replanted into 20-cm-diameter clay pots filled with autoclaved sand/loam mixture (2:1, v/v) infested with $\log_{10}$ 7.0 conidia g ^{1}. Soil free root bales of another ten plants each treatment were trimmed using sterile scissors, dipped into conidial suspension $\log_{10}$ 7.0 conidia ml ^{1}) and transplanted into 20-cm-diameter containing sterile sand/loam mixture. Additionally, four plants each treatment from the plant growth promoting and root colonization experiment were also replanted into 20-cm-diameter containing sterile sand/loam mixture after they were uprooted. Olive plants were arranged in a randomized block design and were grown for another 79 days under same condition mentioned before.

2.6.4.4 Assessment of disease development

Disease reaction were assessed by the percentage and severity of symptoms on a 0-4 rating scale according to the percentage of affected leaves and twigs (0 no symptoms, 1 1 33%, 2 34-66%, 3 67-100%, and 4 dead plant) at weekly intervals after the first symptoms occurred. Data were subjected to analysis of variance. Percentage values were arcsine transformed $(Y / 100)^{0.5}$ before analyzes. Data on disease severity were used to calculate the following: (i) a disease severity index (DII) determined as DII (Σ Si * Ni)/(4 * Nt), where Si is the symptoms severity, Ni is the number of plants with Si symptoms, and Nt is the total number of plants; (ii) the incubation period (IP) established as the number of days from the inoculation with the pathogen until DII > 0; (iii) the final disease incidence (percentage of affected plants) (final DI); and (iv) the standardised area under disease progress curve of DII plotted over time (AUDPC) calculated according to Campbell and Madden (1990). Treatment means were compared with those of the control using the Dunnett's test at P 0.05.

2.6.4.5 Assessment of plant growth parameter

Plant growth parameter comprised stem diameter about 1.0 cm over soil level and total plant length, calculated by summing stem and branch length. Initial measurements were done after transplanting treated olives. Plant growth promotion experiment was terminated after 90 days, and stem diameter as well as total plant length were recorded again to calculate relative plant growth. Four plants were grown another 79 days after replanting into a new pot and measured once again.

2.6.4.6 Enumeration of root colonizing bacteria

Population densities of introduced strains *S. plymuthica* HRO-C48 Rif^r and *P. fluorescens* Rif^r in the rhizosphere and the endorhiza were determined after 7, 21, 50, 90, and 169 days. About 5.0 g of roots with adhering soil from three plants were sampled into sterile 100 ml-containers. After adding 50.0 ml distilled water samples were treated by shaking vigorously for 3 min. Soil suspension was serially diluted and plated onto NA amended with 100.0 µg rifampicin ml^{-1} and incubated at 30°C for 48 h. Cell numbers of *S. plymuthica* were counted after 24 h and those of *P. fluorescens* after 48 h.

After root-surrounding soil was sampled for determining rhizospheric abundances, roots were further processed to determine bacterial counts in the endorhiza. Olive roots were washed under running tap water, surface-disinfested in 1.0% NaOCl for 3 min, washed three times in sterile distilled water, and cut into 1 cm long pieces, before they were weighed and ground in 10.0 ml 10.0 mM $MgSO_4 \cdot 7H_2O$ using an sterile mortar and pestle. Serial dilutions of the macerates were plated onto NA amended with 100.0 µg rifampicin ml^{-1} and incubated at 30°C for 48 h. Cell numbers of *S. plymuthica* were counted after 24 h and those of *P. fluorescens* after 48 h to calculate the means of colonies (log_{10} CFU) based on root fresh weight.

2.6.4.7 Sampling for cultivation-independent analyzes

At the end of the experiments total microbial fractions from the rhizosphere were yielded by extraction of root-surrounding soil and fractional centrifugation (chapter 2.6.4). Depending on plant conditions, roots were sampled from at least two plants each bacterial treatment and manner of fungal inoculation. Only plants appearing to be in a similar physiological condition (disease severity, plant height) were considered for sampling.

2.6.5 Investigations on bacterial community by single-strand polymorphism analysis

2.6.5.1 Extraction of DNA from rhizosphere samples

Rhizosphere samples of oilseed rape and olive trees collected from experiments performed in this study (chapters 2.6.2 - 2.6.4) were dispersed in 50.0 ml distilled water and treated using Stomacher laboratory blender for 180 s (BagMixer, Interscience, St. Nom, France). Microorganism fraction was obtained after serial centrifugation steps. Soil dispersion was centrifuged for 5 min at 500 x *g* to remove larger soil particles. The supernatants were transferred into clean tubes and centrifuged again at 10,000 x *g* for 20 min to get microbial fraction. Sedimented microorgansims were re-suspended in sterile 0.85% (w/v) NaCl solution.

Aliquots of the suspensions were pipetted into 2.0 ml eppendorf cups to perform a final centrifugation step at 10,000 x *g*. After discarding, supernatant microbial extract were stored at -20°C. Total genomic DNA of rhizospheric samples was isolated according to the protocol described by Martin-Laurent (2001) and schematically illustrated in Figure 7. Subsequently, extracted DNA was purified using Geneclean Turbo Kit (Qbiogene, BIO101® Systems, Carlsbad, CA, USA) which includes buffer containing guanidine thiocyanate for elimination of humic acids.

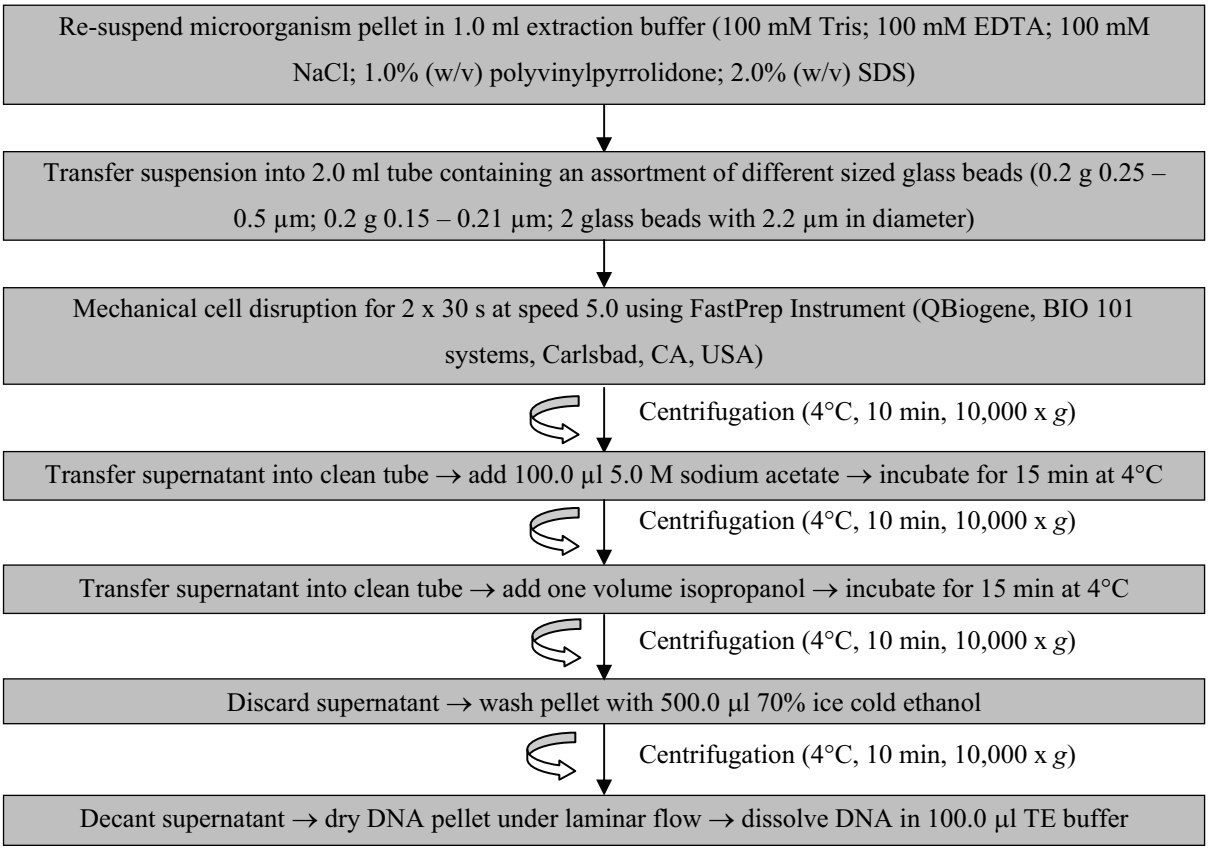

Figure 7: DNA extraction protocol from soil (Martin-Laurent *et al.* 2001, modified)

2.6.5.2 Single-strand conformation polymorphism analysis

The procedure of single-strand conformation polymorphism analysis (SSCP analysis) was done according to Lieber *et al.* (2002). Bacterial communities were analyzed using the universal eubacterial primer pair Unibac-II-515f/Unibac-II-927rP listed in Table 5. The reaction mixture was comprised of 60.0 µl in which the primer concentration was decreased to 0.5 µM each primer. The PCR products were purified by the GeneClean Turbo Kit (Qbiogene, BIO101®) and eluted in 30.0 µl elution buffer. To obtain single strand DNA, amplicons were subjected to a nuclease digestion for 1 h at 37°C after adding 2.4 µl of λ-exonuclease (12 U) and 3.6 µl of 10 x λ-exonuclease buffer (New England Biolabs, Beverly,

MA, USA). Preparation was supplemented with 30.0 µl loading buffer consisting of 95% (v/v) formamide, 0.025% (w/v) bromphenol blue and 10.0 mM NaOH. Single strand DNA was denatured for 2 min at 95°C followed by folding on ice for at least 5 min. Samples were separated by means of native polyacrylamide gel electrophoresis, performed with TGGE MAXI system from Biometra (Göttingen, Germany) in a gel matrix of 0.7 x MDE stock solution (Biozym Scientific GmbH, Oldendorf, Germany) at a constant temperature of 26°C and 400 V for 26 h. Gels were silver-stained according to the procedure published by Bassam *et al*. (1991), which was executed as follows: fixation in 10.0% (v/v) acetic acid for 30 min, washing in distilled water (three times) for 5 min, silver staining in 0.1% (w/v) silver nitrate solution amended with 0.5 volumes 37% (v/v) formaldehyde for 30 min, developing in 3.0% (w/v) sodium hydroxide solution amended with 0.5 volumes 37% (v/v) formaldehyde until bands were visible, fixation in 10.0% (v/v) acetic acid for 30 min. Finally, stained gel was immersed in conservation solution containing 10% (v/v) ethanol and 13% (v/v) glycerine, covered by cellophane, and air-dried.

Numerical analyzes of digitized fingerprints of bacterial communities were performed using GelCompar 4.0 (Applied Math, Gent, Belgium) according to Rademaker *et al.* (1999). For comparison of electrophoresis patterns, Pearson correlation coefficient was used as a pairwise similarity measure of two lanes, which resulted in a similarity index represented in dendrogram using average linkage between group method (UPGMA). Computer-assisted pattern analyzes of molecular fingerprints of rhizospheric communities resulted in a correlation matrices which were subjected to significance test by applying permutation test with 10^5 random permutations of sample elements (Kropf *et al.* 2004).

2.7 Phenotypical characterization of the AHL-deficient derivates of *Serratia plymuthica* HRO-C48

2.7.1 Detection of *N*-Acyl homoserine lactones

Excretion of *N*-Acyl-homoserine lactones (AHLs) by *S. plymuthica* HRO-C48 strains were routinely checked by a dual culture assay using *Chromobacterium violaceum* CV026 suitable for detection of C_4- and C_6-AHLs (McClean *et al.* 1997). Isolates to be tested were placed perpendicular to the sensor strain on NA and cultivated for 24 h at 30°C. Violet pigmentation of *C. violaceum* indicated the presence of AHLs formed by the test strain.

2.7.2 Dual culture assay

The ability of bacterial isolates to inhibit fungal growth was tested utilizing dual-culture assay, which were performed in petri dishes containing Waksman agar. To test bacteria in relation to *V. longisporum* ELV25, 200.0 µl of the fungal culture (14 d, 20°C, 120 rpm) were spread onto the medium and dried for 30 min. Using *R. solani* as fungal test strain, four mycelium containing agar plugs (WA, 20°C, 7 d) were repeatedly placed onto the agar plate. A maximal number of four bacteria were transferred to the inoculated plate. Zones of inhibition were measured after 4, 7, and 10 days of incubation at 20°C.

2.7.3 Detection of exoenzymes

2.7.3.1 Chitinases

Chitin-degrading abilities of *S. plymuthica* HRO-C48 strains were tested by a qualitative chromogenic enzyme assay. For quantitative detection of extracellular chitinolytic activity, strains to be tested were cultivated for 2 d at 30°C and 150 rpm in respectively ABC and ABG medium supplemented with 1.0% (v/v) colloidal chitin solution (10.0 g l^{1}). Cell-free supernatant was prepared by centrifugation (15,000 x *g*, 30 min), sterile filtration (pore size 0.25 µm), and addition of protease inhibitor. To 150.0 µl culture filtrate, 450.0 µl substrate (0.2% carboxymethyl-chitin-remazol brilliant violet (CM-chitin-RBV)), 100.0 mM succinate buffer (pH 6.0) (1:2, v/v)) were added, filled up with corresponding medium to a final volume of 600.0 µl and incubated at 30°C over night. After adding 100.0 µl 2.0 M HCl, preparation was incubated on ice for 1 h. Precipitate was removed by centrifugation for 10 min at 15,000 x *g* prior supernatant was directly measured photospectrometrically at 550 nm. Measurements were done in three replicates each strain, as control served 150.0 µl of sterile medium.

2.7.3.2 Proteases

To investigate proteolytic activity of *S. plymuthica* HRO-C48 WT and its derivates a quantitative chromogenic enzyme assay was conducted. For detection of extracellular proteases formed by *S. plymuthica* HRO-C48 strains, a quantitative enzyme assay was applied. Bacteria were grown for 1 d at 30°C and 150 rpm in LB broth. 150.0 µl of cell-free supernatants obtained by centrifugation (15,000 x *g*, 30 min) and subsequent sterile filtration (pore size 0.25 µm) were mixed with 250.0 µl substrate solution (2.0% (w/v) azoalbumin (pH 7.2)), and filled to 400.0 µl with corresponding media. After overnight incubation at 30°C, the preparation was admixed with 1.2 ml 10% (v/v) trichloroacetic acid, followed by 15 min incubation at room temperature and centrifugation for 10 min at 15,000 x *g*. 750.0 µl 1.0 M

NaOH was added to the preparation before absorption maximum at 440 nm was measured. Measurements were performed on three replicates each strain, where 150.0 µl of medium served as the control.

2.7.3.3 Lipases

An on-plate assay was carried out to detect lipid-degrading enzymes produced by *S. plymuthica* HRO-C48. Bacteria were grown in three replicates on solid medium containing Tween80 for 72 h at 30°C before diameter of hydrolysis zone was measured.

2.7.4 Detection of pyrrolnitrin

Excretion of the antifungal compound pyrrolnitrin by bacteria was studied as followed. Three agar plates (NA supplemented with 2.0% (v/v) glycerine) of each isolate to be investigated were seeded with 200.0 µl of a pre-culture (NB, 30°C, 12 h, 150 rpm). Bacterial film of each plate was scrapped from the medium with sterile Drigalski spatula after cultivation (3 d, 30°C), and suspended in 6.0 ml distilled water. Suspended bacteria were sedimented by centrifugation (10,000 x *g*, 5 min, 4°C) and re-suspended in 6.0 ml acetone/water mixture (4:1, v/v). Acetone was removed using rotating evaporator (Heidolf instruments, Schwabach, Germany). One volume chloroform was added, the suspension was shaken and the chloroform removed prior to dissolving the extract in 2.0 ml acteton. A volume of 400.0 µl of the cell extract-aceton solution was fractionated by thin-layer chromatography (TLC) on K60 F_{254} plates (Merck, Darmstadt, Germany). Synthetic pyrrolnitrin served as analytical standard. After separating in chloroform/acetone (9:1, v/v), antibiotic absorption was monitored at 366 and 254 nm, the wavelengths commonly used for detecting pyrrolnitrin. Finally, TLC plate was coated with a thin layer of Waksman agar (40°C) and inoculated with eighter 1.0 ml liquid *V. longisporum* ELV25 culture (CDB, 20°C, 120 rpm, 14 d) or mycel-containing agar plugs from *R. solani* culture (WA, 20°C, 7 d). Inhibition zones caused by bacterial-borne antibiotics were analyzed after incubation period of seven days for *V. longisporum* and three days for *R. solani* at 20°C in the absence of light.

2.7.5 Detection of siderophores

Detection of siderophores was performed under iron-limited conditions using universal siderophore assay developed by Swyn and Neilands (1987). Iron (III) ions form a complex with chrome-azurol-S dye. When a strong bacterial-produced chelator removes the iron from the dye, its colour turns from blue to orange. For testing, cell material was transferred onto

chrome azurol-S medium and cultivated for 3 to 5 d at 30°C. Diameter of discoloured zone represented the degree of siderophore excretion and was measured metrical.

2.7.6 Detection of indole-3-acetic acid

Indole-3-acetic acid (IAA) excretion by *S. plymuthica* HRO-C48 strains was determined using the colorimetric analysis developed by Sawar and Kremer (1995), a commonly applied method to quantify and qualify extracellular IAA and its precursors. First, bacteria were grown on half-concentrated TSA at 30°C for 24 h. 5.0 ml of IAA-growth medium was inoculated with cell material from the pre-culture to an OD_{500} of 0.5. After 72 h cultivation at 20°C and 150 rpm in absence of light, 1.0 ml of the culture was centrifuged (10,000 x *g*, 10 min, 4°C). Afterwards, 90.0 µl of supernatant was pipetted into a microtiter plate, mixed with 60.0 µl Salkowski reagent (50.0 mM $FeCl_3$, 35.0% (v/v) perchloric acid) and incubated for 30 min in absence of light. IAA concentration was measured photospectrometrically using the microplate-reader Spectramax-250 (Molecular devices, Union City, CA, USA) at 530 nm and quantified using standard curve. The standard curve was developed by plotting the extinctions of known concentrations (dilution series from 5.0 to 60.0 µg ml^{-1}). Experimental setup consisting of three replicates each strain was repeated three times.

2.7.7 Biofilm formation assay

Biofilm formation capacity of *S. plymuthica* HRO-C48 WT and pME6863 on a polystyrene surface was analyzed under static conditions in presence of different media: ABC-, ABG- and LB-medium (Pratt and Kolter 1998). Prior to experiments, a pre-culture (LB, 30°C, 24 h, 150 rpm) was adjusted to OD 0.1. 200.0 µl of this suspension was added to 20.0 ml test medium and re-grown for 1 h (30°C, 150 rpm) to overcome the lag-phase. Cavities of a 96-well plate made of polystyrene with round bottoms were filled with 100.0 µl of sterile test medium and inoculated with cells from 1-h-culture using a toothpick. Altogether, five multi-well plates, one per sampling (3, 6, 24, 48 and 72 h), were prepared, each containing four replicates per isolate and medium. The plates were sealed and incubated at 30°C. To monitor biofilm formation, medium was removed from the cavities, thoroughly rinsed with dH_2O, dried and covered with 100.0 µl crystal violet solution. After 30 min of incubation at room temperature crystal violet solution was discarded. After a washing step, cavities were dried, and adsorbed colour dye was dissolved in 120.0 µl DSMO. Solution was mixed with 800.0 µl absolute ethanol and subjected to spectrophotometrical measurement at 570 nm using absolute ethanol as reference.

2.7.8 Motility assay

The swarming motility assay was conducted on ABC medium amended with 0.4% agar. A whit of cell material was transferred onto test plates using sterile toothpicks. After sealing, plates were incubated for at least 24 h at 30°C. *S. plymuthica* isolates were tested in three replicates. For investigating swimming motility, the same experimental setup described above was applied, except the agar concentration was decreased to 0.3%.

2.7.9 Phytochamber assay

Lettuce seeds (*Lactuca sativa* L. cv. Daguan (S 5601), Syngenta Seeds GmbH, Bad Salzuflen, Germany) were surface-sterilised with 1.0% NaOCl for 5 min. Sterility of seeds was confirmed by imprinting them on nutrient agar, before they were pre-germinated in moist chambers for 2 days at 20°C. One pre-germinated seed was deposited in each well of a standard 24-well microplate containing 1.0 ml water agar followed by addition of 10.0 µl of bacterial suspension close to the seed. For control treatment 10 µl sterile 0.85% NaCl solution was applied instead of bacteria. The bacterial inoculum was obtained by harvesting cells from a 24 h culture (24 h, 30°C, 150 rpm) by centrifugation at 5,000 x *g* for 10 min and re-suspension in 0.85% NaCl solution. *S. plymuthica* isolates were evaluated at $\log_{10}$ 5.0 CFU ml ^{1}. Two weeks after incubation (16 h of artificial light, 22°C) in a climatic chamber (Percival Scientific, Boone, IA, USA), total fresh mass and total plant length were measured to determine effects of bacterial treatment on plant growth. Each isolate was tested in 24 replicates.

2.7.10 Effect of volatile organic compounds on fungal growth

In contrast to the above mentioned dual culture assay, inhibitory effect of bacterial volatile compounds on the fungi *R. solani* and *V. longisporum* ELV25 was investigated exclusively. For this purpose half-divided petri dishes containing fungal and bacterial growth medium respectively, were utilized. Testing towards *R. solani* either Waksman agar or SNA, and towards *V. longisporum,* Sabouraud agar was used. *S. plymuthica* HRO-C48 isolates were cultivated on NA. 5.0 mm-diameter agar plugs from fungal culture served as inoculum. Bacterial isolates were applied simultaneously by seeding 200.0 µl pre-culture (NB, 30°C, 24 h, 150 rpm) on the half-site containing NA. Plates were double-sealed with Parafilm and incubated at 20°C in the absence of light. Three replicates each fungus and bacterial strain was performed. Fungi grown in presence of sterile NA served as control.

2.8 Statistical analyzes

Obtained data were statistically analyzed using Statistical Service and Product Solution for Windows (SSPS Inc., Chicago). Means were compared for significance applying Fisher's protected least significant difference (LSD) test at $P \leq 0.05$. Root colonization data were $\log_{10}$ transformed before statistical analysis.

3 Results

3.1 Evaluation of seed treatment techniques

3.1.1 Optimization of the procedure of film coating

Utilization of protective additives to enhance the vitality of bacterial cells during dehydration by lyophilization is a common practice in cryobiology. Sucrose and *N*-Acetyl-D-glucosamine (GlcNAc) were chosen because both carbohydrates were shown to positively influence the cell physiology of *Serratia plymuthica* HRO-C48 during lyophilization (Frankowski 2002; Palmfeldt *et al.* 2003). The effect of different concentrations on the survival of HRO-C48 during lyophilization and following storage was investigated (Figure 8). After wetting with any of the bacterial suspensions on average $\log_{10}$ 7.0±0.15 CFU remain attached to the surface of each seed. The level of decline of the cell number during the freeze-drying process depended on the additive. Cell numbers of $\log_{10}$ 6.4±0.20 CFU seed ^{1} survived the process in presence of sucrose, compared to $\log_{10}$ 5.5±0.13 CFU seed ^{1} in presence of GlcNAc. No differences were observed between the different concentrations. There was a further drop of viable cell counts during the storage at 4°C for 40 days. On average, 89.1±6.72% more bacteria were re-covered from the seeds if sucrose served as protective agent, and not GlcNAc. After 120 days, survival rate of cells which were freeze-dried in presence of GlcNAc ($\log_{10}$ 3.2±0.68 CFU seed ^{1}) were lower than that of cells lyophilized with sucrose ($\log_{10}$ 2.1±0.66 CFU seed ^{1}). Sucrose at the concentration of 1.0% provided the best degree of protection. Thus, the freeze-drying procedure using 1.0% sucrose was applied for inoculating seeds for following studies.

3.1.2 Optimization of the procedure of bio-priming

The bio-priming procedure has to be aligned with the time course of seed germination. Preliminary experiments suggested that the germination process strongly depends on the cultivar and the quality of the seed lot (data not shown). Seeds of the cultivar ‘TALENT’ used in this study were immersed in 0.85% NaCl solution. The time course of germination of the seeds is presented in Figure 9. Seeds went through the phase of imbibition (phase I) in the first four hours, the activation phase (phase II) was located between 4 h and 16 h, and the last phase of germination (phase III) began after 16 h. According to these three phases of germination, development of the number of *S. plymuthica* HRO-C48 cells during the process of bio-priming was measured. After 4 hours, an abundance of $\log_{10}$ 3.4±0.40 CFU seed ^{1} were found inside the seed. Despite water uptake and imbibition was completed, a higher level of

bacteria was achieved after 12 h (log_{10} 6.2±0.26 CFU seed ^{1}). No further increase of cell counts was observed after 20 h (log_{10} 6.3 ±0.21 CFU seed ^{1}).

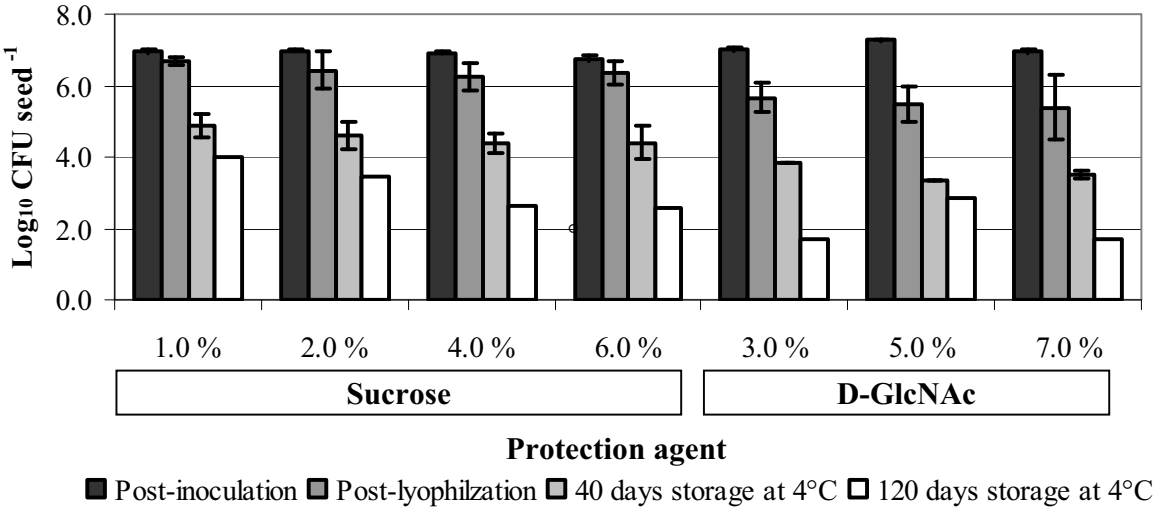

Figure 8: Effect of sucrose and D-GlcNAc at different concentrations on the survival of *S. plymuthica* HRO-C48 cells at the surface of oilseed rape seeds during lyophilization and following storage at 4°C. Error bars indicate confidence intervals at $P \leq 0.05$.

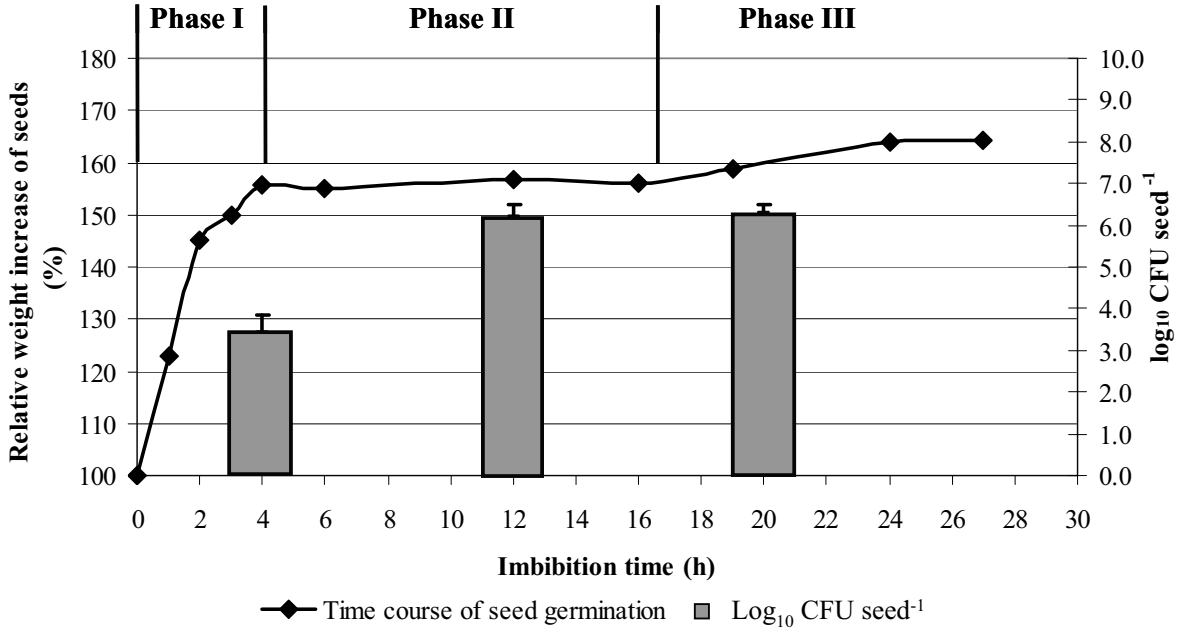

Figure 9: Optimization of the bio-priming procedure for seeds of oilseed rape cv. 'Talent'. Diagram shows time course of germination and correlative cell number of *S. plymuthica* HRO-C48 inside the seeds. Phase I: imbibition phase; phase II: activation phase, and phase III: growth of radicals. Error bars indicate confidence intervals at $P \leq 0.05$.

3.1.3 Root colonization assays

A greenhouse trial was performed to study the influence of different concentrations of *S. plymuthica* HRO-C48 applied to seeds of oilseed rape on the ability of the BCA to colonize the rhizosphere. Therefore, film coating was used as application method because this treatment resulted in most accurate cell number. Initial bacterial cell densities from $\log_{10}$ 3.0 to 7.0 CFU seed^{-1} of spontaneous rifampicin resistant mutants of *S. plymuthica* HRO-C48 were applied. Data in Figure 10 demonstrate that no statistically significant differences ($P \leq 0.05$) in plate counts of re-isolated bacteria were found. After 30 days, a mean abundance of $\log_{10}$ 4.7 ± 0.04 CFU g^{-1} root fresh mass (rfm) was estimated.

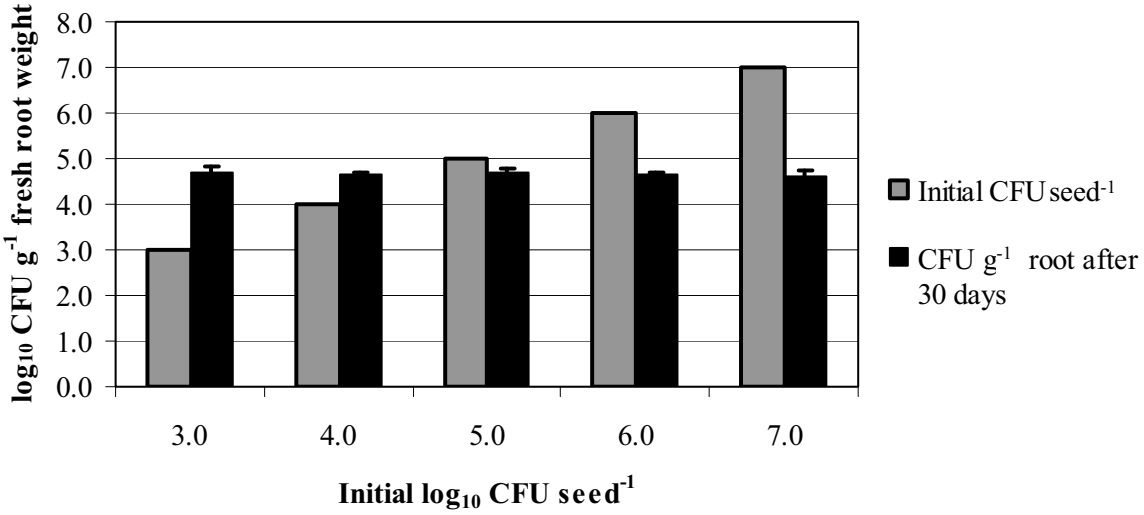

Figure 10: Influence of the initial cell concentration on the cell number of *S. plymuthica* HRO-C48 Rif^r colonizing the rhizosphere of oilseed rape. Seeds were treated with spontaneous rifampicin resistant mutants using film coating. Bacteria were re-isolated after 30 days of plant growth under greenhouse conditions. Error bars indicate confidence intervals at $P \leq 0.05$.

In a second trial, the influence of different seed treatments on the colonization ability of *S. plymuthica* HRO-C48*Rif*^{r} was examined. Different strategies, which were used to apply *Serratia* cells on/in rape seeds, are shown in Figure 11. To analyse the impact of the application method, for all treatments identical initial bacterial cell densities of $\log_{10}$ 6.0 CFU seed^{-1} were used. After 15 days of plant growth, a significant higher number of *S. plymuthica* HRO-C48*Rif*^{r} cells was established in the rhizosphere using bio-priming ($\log_{10}$ 4.8±0.28 CFU g^{-1} rfw) than using pelleting ($\log_{10}$ 4.2±0.32) and film coating ($\log_{10}$ 4.1±0.12), respectively. In the course of time the abundances decreased successively and no differences between the methods of treatment were found. After 30 days, on average $\log_{10}$ 4.1±0.04, after 45 days $\log_{10}$ 3.6±0.07 and after 60 days $\log_{10}$ 3.2±0.09 CFU g^{-1} rfw could be re-isolated.

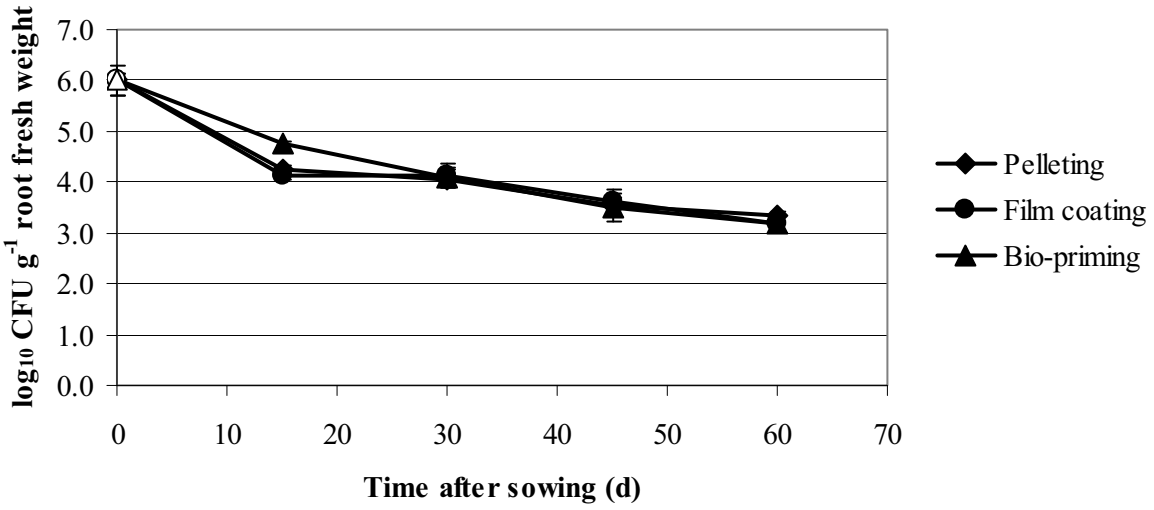

Figure 11: Influence of the seed treatment technique on the cell number of *S. plymuthica* HRO-C48 Rif^r colonizing the rhizosphere of oilseed rape. Seeds were treated with spontaneous rifampicin resistant mutants at a concentration of $\log_{10}$ 6.0 CFU seed^{-1} (open symbols). Bacteria were re-isolated after 15, 30, 45 and 60 days of plant growth under greenhouse conditions (solid symbols). Error bars indicate confidence intervals at $P \leq 0.05$.

3.1.4 Disease development

The ability of *Serratia* cells to reduce disease incidence caused by *V. longisporum* was analyzed in greenhouse trials using an artificial inoculation of the pathogen. In general, all *Serratia* treated plants had a significantly lower disease severity expressed by AUDPC compared to the untreated control (Figure 12). However, different application methods resulted in different biocontrol effects. Whereas film coating led in a disease suppression of 5.2±2.32%, plants treated by pelleting and bio-priming showed a higher reduction of disease severity of 13.4±2.48% and 14.3±1.34%, respectively.

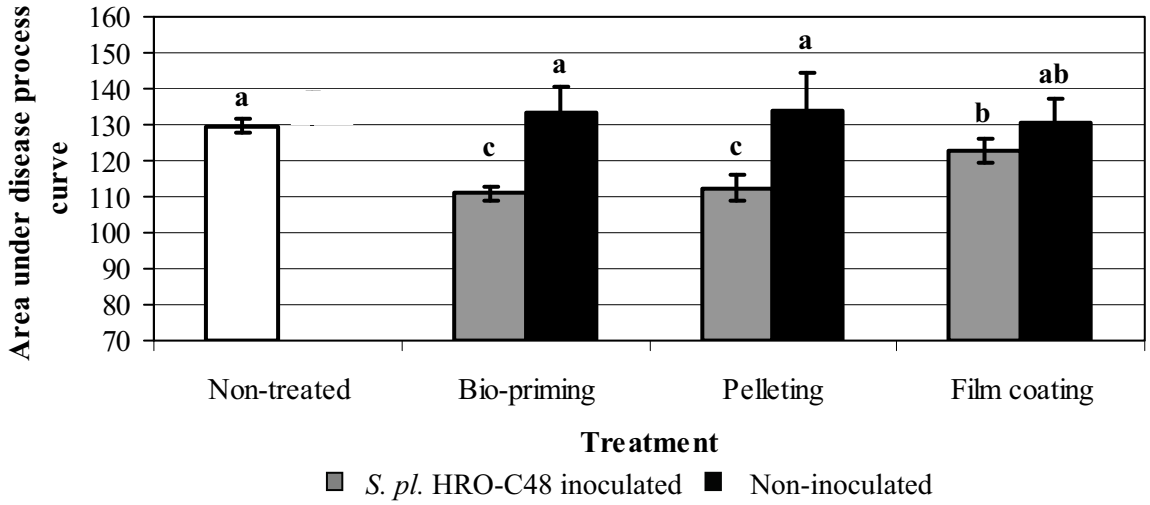

Figure 12: Influence of seed treatment technique on the ability of *S. plymuthica* HRO-C48 to suppress the development of Verticillium wilt expressed by the AUDPC in comparison to the untreated and non-inoculated controls. Oilseed rape seeds were treated with bacteria at concentrations of $\log_{10}$ 6.0 seed^{-1} (bio-priming) or 7.0 (pelleting and film coating). Same letters above bars symbolize no statistically significant differences at $P \leq 0.05$. Error bars indicate standard deviations.

In addition to the visual assessment of disease development, *V. longisporum* colonizing the plant tissue was quantified prior appearance of symptoms. Four weeks after sowing, aerial parts of plants grown in infested soil were sampled, grinded and lyophilized. The powdery plant material was used for detection of fungal biomass by means of ELISA approach which was conducted by a company (PTL Pflanzentestlabor, Oestrich-Winkel, Germany). Applying *V. longisporum*-specific antibodies, in none of the samples signals associated with the presence of the fungus could be detected. A more sensitive method to quantitatively monitor the presence of the pathogen within the plant material is the quantitative real-time PCR (qRT-PCR). Whole genomic DNA, extracted from the prepared plant tissue, was subjected the qRT-PCR using primer pair VD1/VD2 which bind to *Verticillium*-specific nucleotide sequences (Volossiouk *et al.* 1995). For each of the samples a distinct signal was obtained. The average of values of four replicates each treatment are displayed in Figure 13. In non-inoculated plants, the highest proportion of fungal DNA was detected (36.9 ppm) compared to DNA extracted from plants inoculated with *S. plymuthica* HRO-C48 using bio-priming (19.7 ppm), film coating (9.9 ppm) and pelleting (13.3 ppm). A correlation factor of 0.96 using Pearson correlation coefficient were calculated to correlate the relative *Verticillium*-DNA contents of differently treated plants and the values from the visual disease assessment (Figure 12).

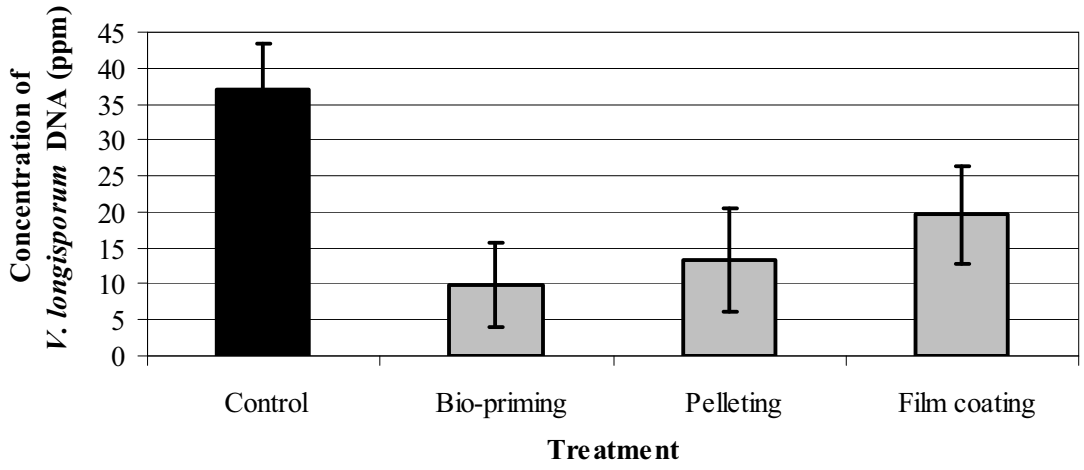

Figure 13: Influence of the mode of seed treatment with *S. plymuthica* HRO-C48 on the degree of *V. longisporum* colonizing oilseed rape plants. The amount of *V. longisporum* DNA in stem and leaves of non-symptomatic plants was monitored by quantitative real-time PCR using primer pair VD1/VD2 (Volossiouk *et al.* 1995). Error bars indicate standard error.

3.1.5 Storage stability

The survival of *S. plymuthica* cells in dependence on the seed treatment was determined over a period of 30 d at storage temperatures of 4°C and 20°C in a climate chamber (Figure 14). At the beginning of the experiment, about $\log_{10}$ 7.0 CFU seed ^{1} could be re-isolated from the seed surface using pelleting and film coating. In contrast, after bio-priming, less cells of *S. plymuthica* in comparison to the other seed treatments were found on the seeds with about $\log_{10}$ 6.0 CFU seed ^{1}. The important difference is that after bio-priming these cells were found inside the seed whereas using the other treatments cells were located on the surface. After storage of 30 days at 4°C, bacteria on pelleted seeds showed the highest stability at $\log_{10}$ 5.9±0.07 seed ^{1}. On coated seeds a cell number of $\log_{10}$ 4.2±0.15 CFU seed ^{1}, and in primed seeds $\log_{10}$ 4.8±0.18 CFU seed ^{1} could be re-isolated. Cells of *S. plymuthica* applied to the surface of seeds showed less stability during storage at 20°C than bacterial cells in infiltrated seeds. After 30 days, the following plate counts of *Serratia* were determined: $\log_{10}$ 2.6±0.18 using pelleting, $\log_{10}$ 2.5±0.64 using film coating, and $\log_{10}$ 4.7±0.29 CFU seed ^{1} applied by bio-priming. For the latter, statistically significant more *Serratia* cells survived than using the other treatments.

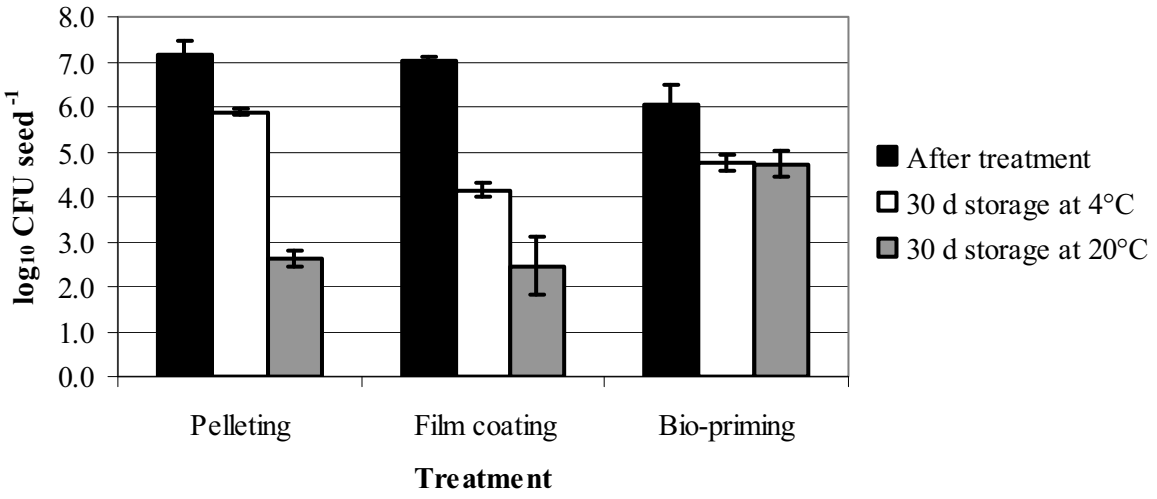

Figure 14: Stability of *S. plymuthica* HRO-C48 applied to oilseed rape seeds using different treatments during storage at 4°C and 20°C. Error bars indicate confidence intervals at $P \leq 0.05$.

3.1.6 SSCP analyzes

In order to investigate the influence of either *V. longisporum* or *S. plymuthica* on the composition of the bacterial populations in the rhizosphere of oilseed rape grown under greenhouse conditions SSCP profiles were generated (Figure 15). The eubacterial community structure in the rhizosphere of non-bacterized as well *Serratia*-treated oilseed rape plants grown in *V. longisporum*-infested soil were clearly distinguishable from that of plants equally treated but grown in pathogen-free soil. The resulting dendrogram, generated by computer-assisted cluster analyzes, is separated into two clusters revealing a significant influence of the fungus at P 0.09 (Figure 16). One band corresponding to *S. plymuthica* HRO-C48 (arrows), unexceptionally occurred in the rhizosphere of infested plants. Re-isolation approaches confirmed the increased population densities of HRO-C48 (Figure 18). After 60 days, mean cell densities of the BCA colonizing the root of diseased were higher ($\log_{10}$ 4.5 CFU g ^{1} rfw) compared to the plate count of *S. plymuthica* on healthy plants ($\log_{10}$ 3.0 CFU g ^{1} rfw).

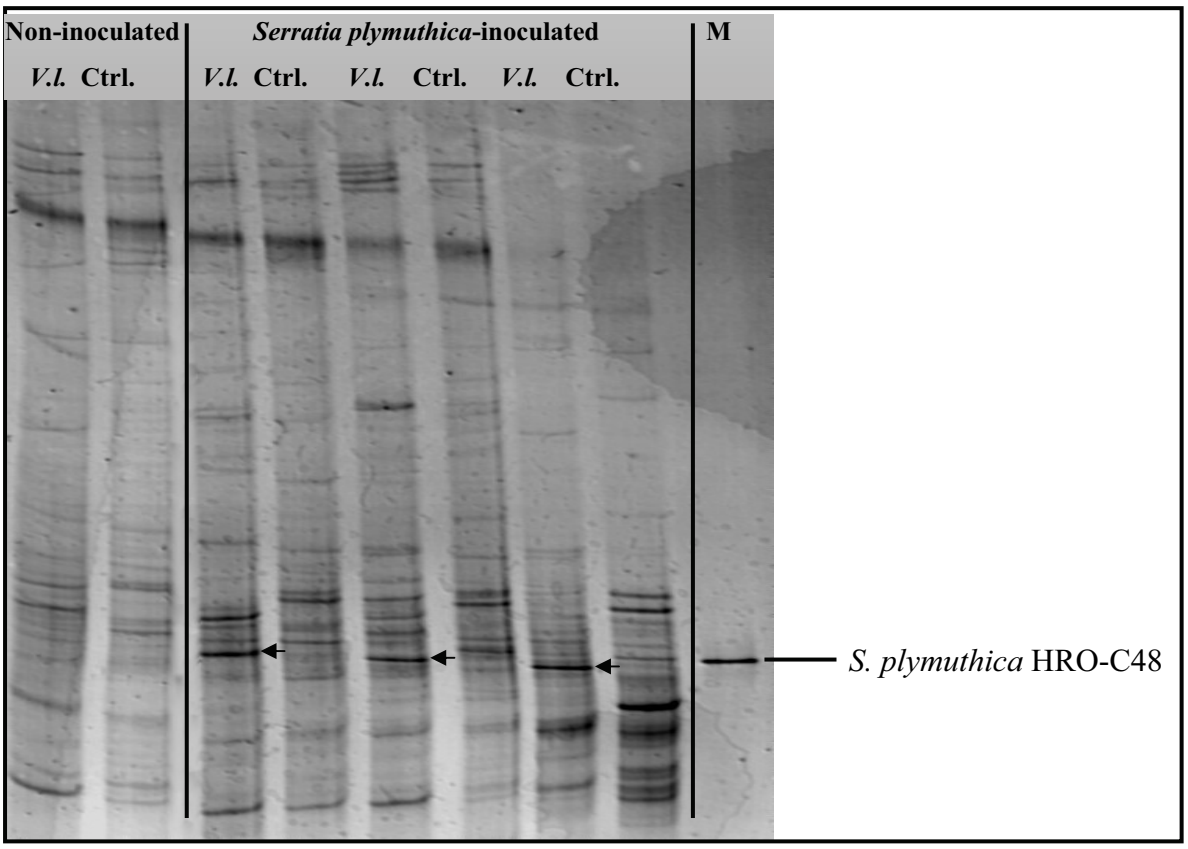

Figure 15: SSCP patterns of the eubacterial communities associated with the rhizosphere of non-treated oilseed rape plant and plants inoculated with *S. plymuthica* isolates and grown for 60 days under greenhouse conditions in *V. longisporum* infested (*V.l.*) and non-infested soil (Ctrl.), respectively. As reference marker (M) served the PCR product from a pure culture of *S. plymuthica* HRO-C48.

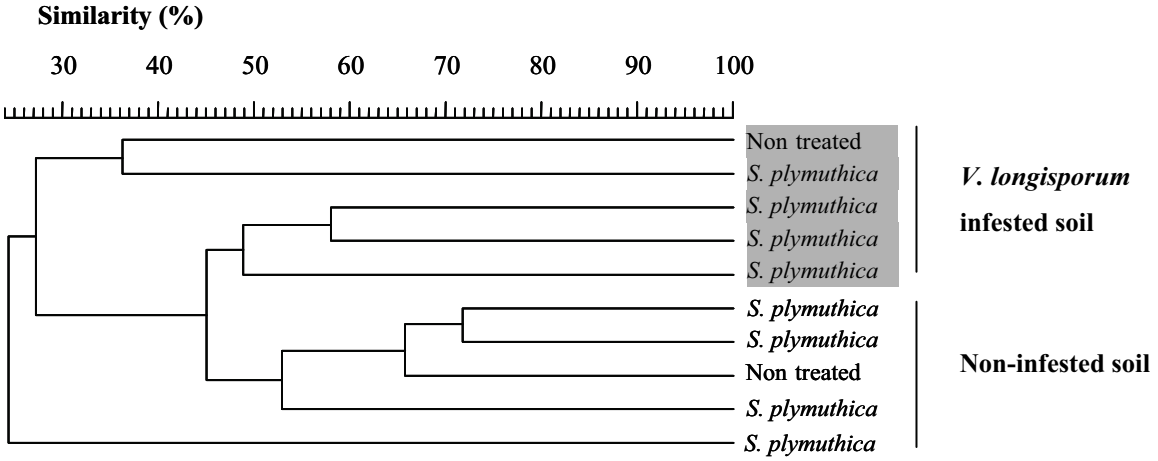

Figure 16: Dendrogram generated from the SSCP profiles within the gel illustrated in Figure 15 using the unweighted-pair group methods using average linkages (UPGMA). Similarity indices were calculated by the Pearson correlation index.

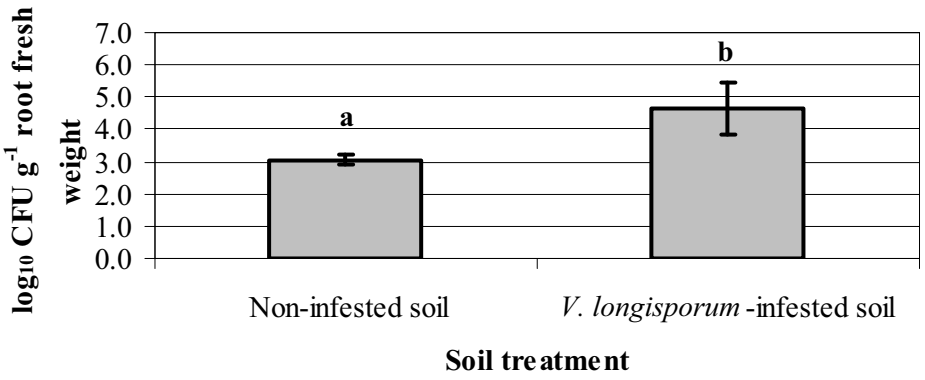

Figure 17: Colony forming units of *S. plymuthica* HRO-C48 Rif^r re-isolated from the rhizosphere of oilseed rape grown for 60 days under greenhouse conditions in *V. longisporum*-infested and non-infested soil, respectively. Same letters above bars symbolize no statistically significant differences at $P \leq 0.05$. Error bars indicate standard deviations.

3.2 Evaluation of *Serratia plymuthica* HRO-C48 under field conditions

3.2.1 Field trial 2003/2004

3.2.1.1 Root colonization assays

For the first of out of two field experiments, seeds were inoculated by means of pelleting. It is assumed that the low inoculum density after the seed treatment further declined during the storage until the time point of sowing. Treated seeds were sown at three locations in Northern Germany, and at four growth stages the population densities of *S. plymuthica* HRO-C48 were recorded. Table 12 display cell numbers associated with oilseed rape roots at the six-leaf and rosette stage. At the sampling times after the winter dormancy, no cells of HRO-C48 could be recovered. In general, the cell counts strongly fluctuate in a range between $\log_{10}$ 0.0 and 6.9 CFU g^{1} rfw. Particularly, in Rothenstein in most samples no *Serratia* cells could be re-isolated. The formulation of the bacteria using Diamol resulted in population densities of $\log_{10}$ 4.6±1.55 and $\log_{10}$ 2.5±0.49 CFU g^{1} rfw on average at the field sites Hamm and Malchow, respectively.

Table 12: Population densities of *S. plymuthica* HRO-C48 Rif^r in the rhizosphere of oilseed rape of the field trial 2003/2004 in Hamm, Rothenstein, and Malchow. No *S. plymuthica* cells could be detected at the flowering and fully-ripe stage.

Sampling time	Mineral carrier	log_{10} CFU g^{-1} root fresh weigth		
		Hamm	Rothenstein	Malchow
Six-leaf stage	Diamol	3.4 – 4.1	0.0 – 2.7	2.1 – 2.9
	Talcum	0.0 – 5.5	0.0 – 6.2	0.0 – 3.1
Rosette stage	Diamol	4.1 – 6.9	0.0 – 4.7	2.4 – 3.2
	Talcum	0.0 – 3.9	0.0 – 5.7	1.8 – 3.0

3.2.1.2 Effect on plant condition, plant height, premature ripeness and yield

The parameter plant condition is a parameter of the general plant development at the rosette stage. In Hamm both inoculated variants and in Rothenstein the variant treated using Diamol were rated lower compared to the control plants (Figure 18). The experimental plots in Malchow were found to be in the best condition. The plant height measured at the flowering stage was 166±1.9 cm on average in Hamm and Rothenstein and 196±0.5 cm in Malchow. There were no differences between the treatments. Severe disease incidences, mostly caused by the pathogen complex *Leptosphaeria maculans*/*V. longisporum*, and forced by the attack of cabbage maggots, resulted in a high degree of premature ripeness at both field sites near Hohenlieth (Hamm, Rothenstein). In contrast to Rothenstein, in Hamm *Serratia*-inoculated plants show slightly reduced degree of premature ripeness. No indications of Verticillium wilt were observed in Malchow, but pathogens such as *Sclerotinia sclerotiorum* caused infection and some degree of premature ripeness. Considering the cropping yield, no notable differences were recorded. Interestingly, despite the overall better condition of oilseed rape in Malchow, the yield was marginal reduced compared to the plots in Hohenlieth.

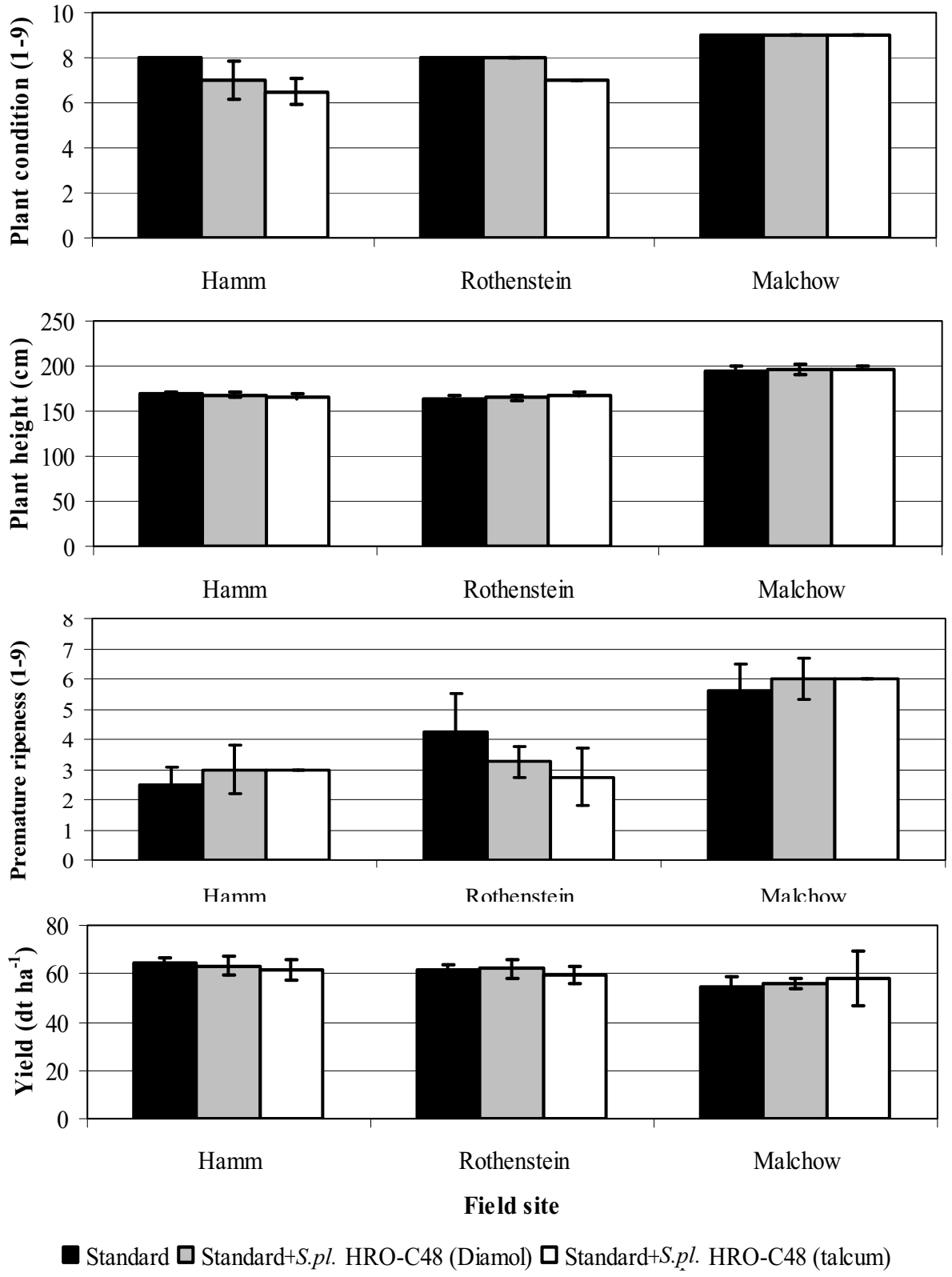

Figure 18: Result of the field trial 2003/2004. Influence of differently formulated *S. plymuthica* HRO-C48 Rif^r was accessed by monitoring plant condition before winter, plant height, premature ripeness, and yield. Data derived from four plots each variant and field site. Error bars indicate confidence intervals at $P \leq 0.05$.

3.2.1.3 SSCP analyzes

Figure 19 exemplarily illustrates the eubacterial community profiles of the rhizosphere of plants grown in Hamm. No differences caused by any of the seed treatments were found. Furthermore, a band indicating the presence of HRO-C48 was found neither in any of the variant nor growth stage. Obviously, remarkable shifts in the banding patterns were observed associated with the development stage. These observations were supported by the cluster analysis generated by using UPGMA after calculating the Pearson correlation index for each pair of lanes within a gel of the locations Hamm, Rothenstein and Malchow (Figure 20). In general, the SSCP patterns grouped according the growth stage of the plants. Particularly, the clusters of the community patterns at the six-leaf stage were strongly separated from the groups consisting of the profiles of the other three sampling times ($P \leq 0.0001$). Compared to this, the similarities between the bacterial populations of the rosette stage, flowering stage and fully were higher. However, a statistically significant grouping according the development stages at $P \leq 0.05$ was found at all field sites.

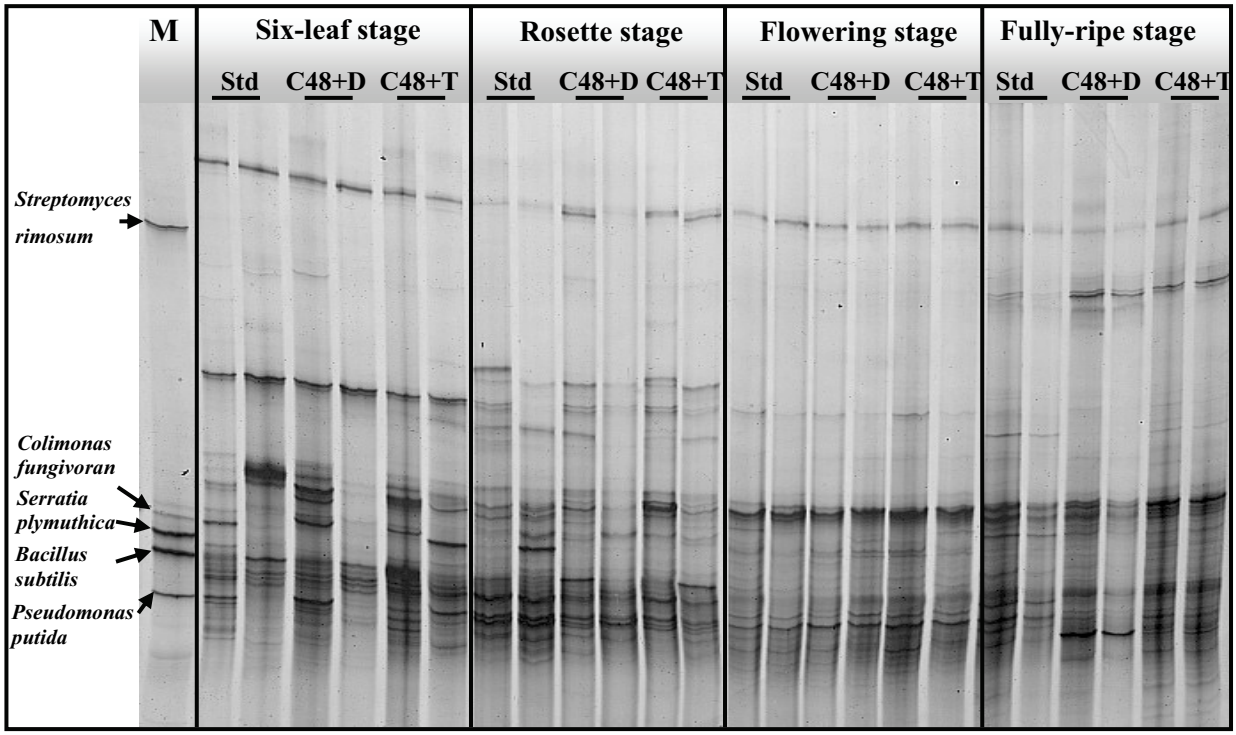

Figure 19: Eubacterial community profiles of the rhizosphere of oilseed rape plants from the field trial 2003/2004 in Hamm at four development stages obtained by SSCP analysis. Prior sawing, seeds were treated with standard pesticides (Std) and with standard pesticides combined with *S. plymuthica* HRO-C48 cell incorporated in an additional layer consisting of Diamol (C48+D) or talcum (C48+T). As reference marker (M) served PCR products from cultures.

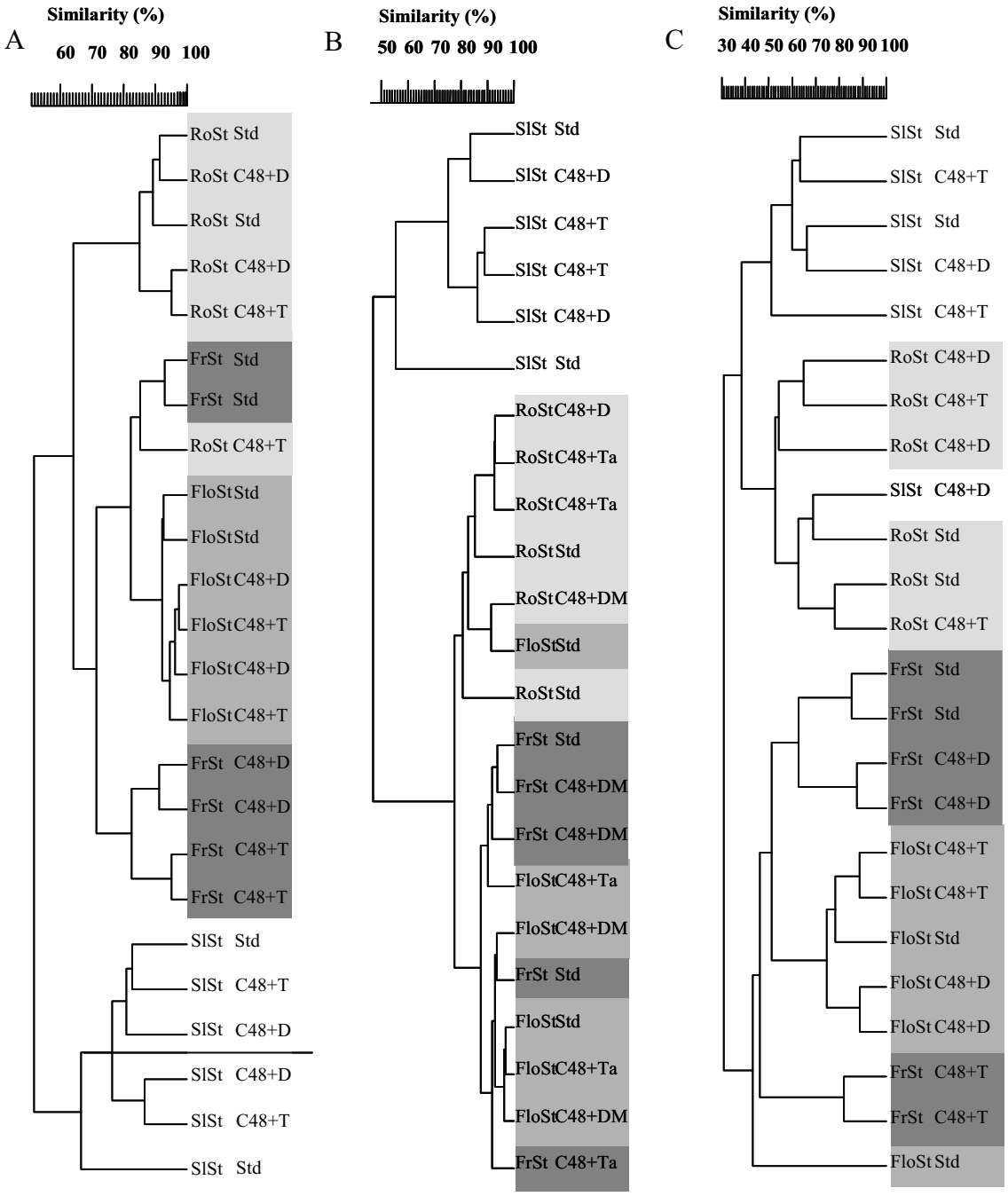

Figure 20: Dendrogram generated from the SSCP profiles of the eubacterial community in the rhizosphere of oilseed rape plants at the six-leaf (SlSt), rosette (RoSt), flowering (FloSt) and fully-ripe stage (FrSt) grown at the field site in Hamm (A), Malchow (B) and Rothenstein (C) after calculating Pearson correlation index and applying the unweighted-pair group method using average linkages (UPGMA). Prior sawing, seeds were treated with standard pesticides (Std) and with standard pesticides combined with *S. plymuthica* HRO-C48 cell incorporated in an additional layer consisting of Diamol (C48+D) or talcum (C48+T).

3.2.2 Field trial 2004/2005

3.2.2.1 Root colonization assays

The second field experiment was performed with bio-primed seeds inoculated with about $\log_{10}$ 6.0 CFU seed ^{1}. Figure 21 shows the mean population density of *S. plymuthica* HRO-C48 associated with the roots of oilseed rape throughout the growing season. In the rhizosphere of plants grown from seeds inoculated with the BCA, but not treated with standard pesticides, $\log_{10}$ 7.5±1.05 CFU g ^{1} rfw was found at the six-leaf stage. The plate counts declined to $\log_{10}$ 5.4±1.02 CFU g ^{1} rfw on average until the rosette stage. After the winter dormancy, the population of HRO-C48 was found to be established at $\log_{10}$ 4.8±0.79 CFU g ^{1} rfw at flowering and fully-ripe stage. In contrast, if the BCA were applied in combination with the pesticides, a very low number of *Serratia* cells could be re-isolated from the oilseed rape roots at the six-leaf stage ($\log_{10}$ 1.5±2.89 CFU g ^{1} rfw on average). From rosette stage, cell counts resemble those of the population when HRO-C48 applied alone.

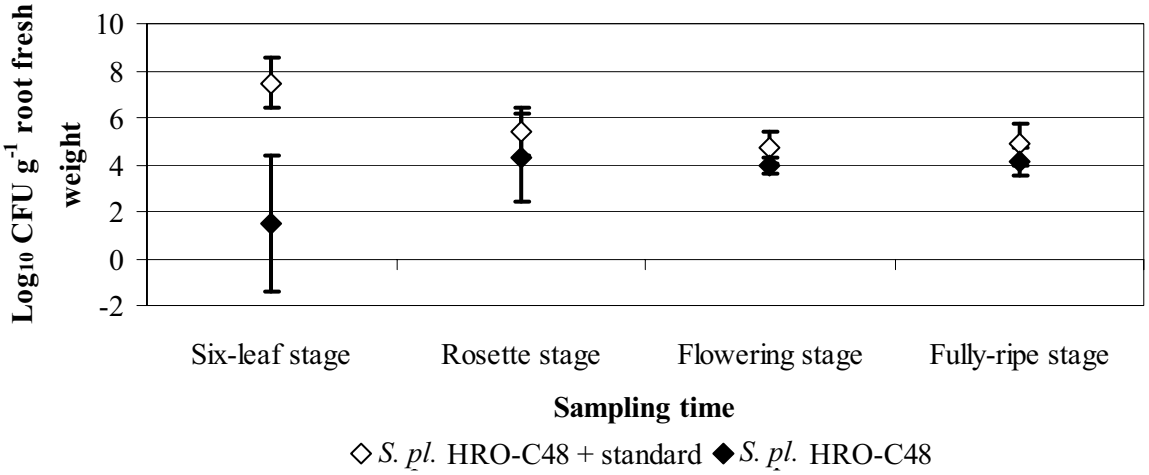

Figure 21: Population densities of *S. plymuthica* HRO-C48 Rif^{r} in the rhizosphere of oilseed rape of the field trial 2004/2005 in Hohenlieth. Error bars indicate confidence intervals at $P \leq 0.05$.

3.2.2.2 Effect on plant condition, plant height, premature ripeness and yield

Before winter, the plant condition was monitored only in Hohenlieth and Hovedissen, and was estimated at 7.5 and 8.2 on average, respectively (Figure 22). No differences of the plant height were found between the variants at the field sites in Hohenlieth and Moosburg. Compared to non-inoculated plants, an increase of the height of 9.5% of *Serratia*-inoculated plants was recorded in Hovedissen. At all locations, premature ripeness resulted mainly from infection by the pathogen complex *Leptosphaeria maculans*/*V. longisporum*. In Hohenlieth as well in Hovedissen minor increase of plant health were observed, if plants were grown from

bacterized seeds. Crop yields differ only slightly, but an increase of about 2.0% may be decisive in commercial cropping. Mean yield enhancements of 4.9% were monitored in Hovedissen for the bio-primed variants. Furthermore, control plants grown from seeds primed with sterile NaCl solution were shown to provide higher yields (6.6% on average). In Moosburg, the plots were damaged by hails and caused yield losses of about 30%. However, a yield increase of 4.6% was demonstrated for the inoculated variants.

3.2.2.3 SSCP analyzes

Results from the cultivation-independent profiling of the eubacterial community in the rhizosphere revealed a strong influence of the growth stage (Figure 23). The development stage of the plant was found to be the major determinant of the plant-associated populations ($P \leq 0.002$) as the threshold similarity was set to 30%. At a similarity of 50% no distinct groups were found. Although HRO-C48 was highly abundant, neither a corresponding band nor an impact of *S. plymuthica* were observed.

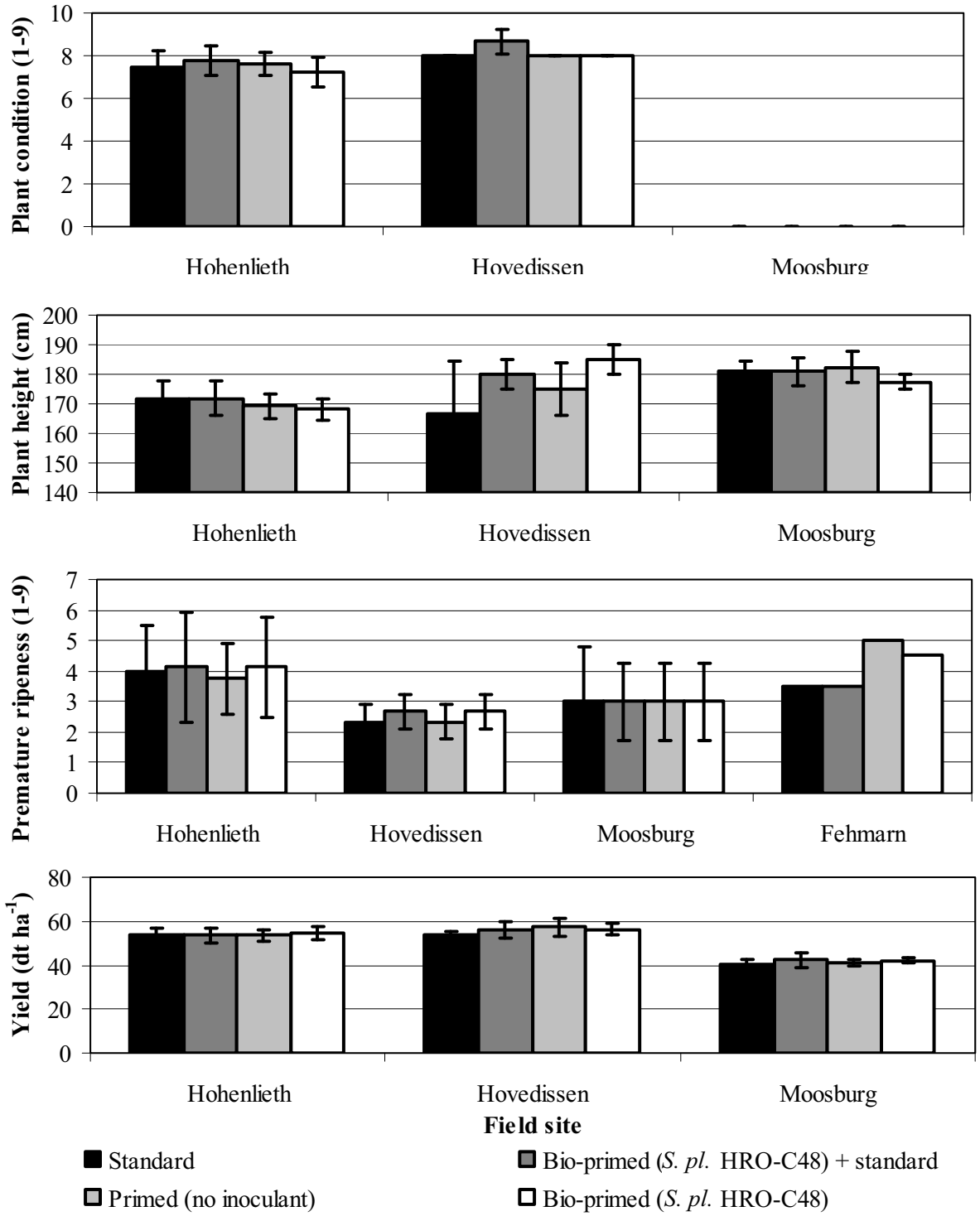

Figure 22: Result of the field trial 2004/2005. Influence of differently formulated *S. plymuthica* HRO-C48 Rif^r was accessed by monitoring plant condition before winter, plant height, premature ripeness, and yield. Data derived from eight (Hohenlieth), six (Moosburg), and three (Hovedissen) plots each treatment. Only the degree of premature ripeness of two plots at the location Fehmarn was scored. Error bars indicate confidence intervals at $P \leq 0.05$.

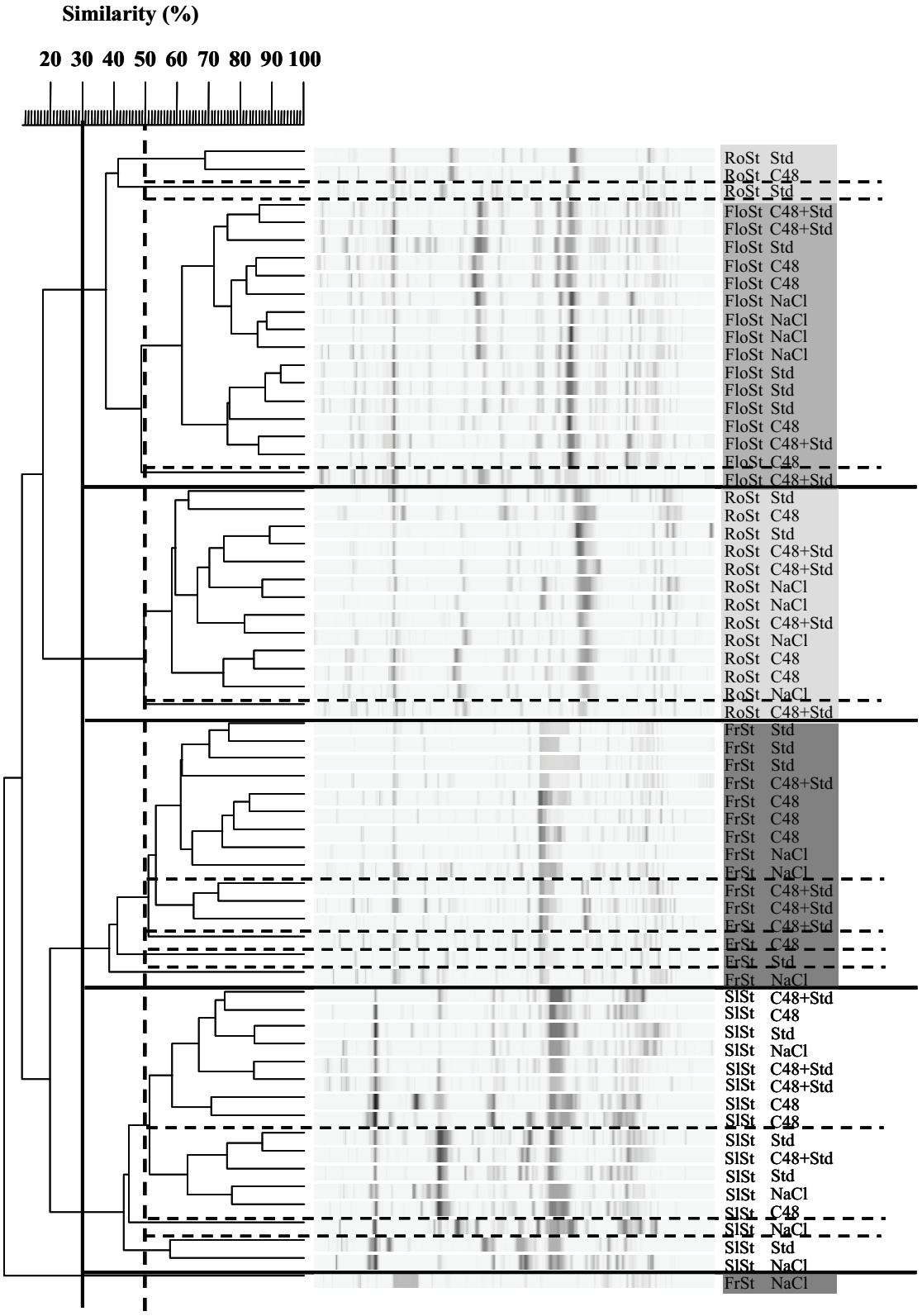

Figure 23: Dendrograms generated from the SSCP patterns of the eubacterial community in the rhizosphere of oilseed rape plants at the six-leaf (SlSt), rosette (RoSt), flowering (FloSt) and fully-ripe stage (FrSt) grown at the field site in Hohenlieth after calculating Pearson correlation index and applying the unweighted-pair group method using average linkages (UPGMA). Prior sawing, seeds were treated with standard pesticides (Std), bio-primed with HRO-C48 and additional coated with standard pesticides (C48+Std), primed with sterile NaCl solution (NaCl) and bio-primed HRO-C48 (C48).

3.2.2.4 Climatic conditions

Several abiotic factors were accessed in line with the field experiment 2004/2005. In addition to soil characteristics, which are listed in the Material and Methods section, climatic parameters were purchased by the German Weather Service (www.dwd.de). In Figure 24 the soil temperature and soil moisture are displayed over the time of the growing period. Averages of temperatures and rainfalls of the vegetation periods before and after the winter dormancy are summarized in Table 13. Comparing the courses of the soil temperatures, no distinct differences were detected. Considering only the vegetation periods, at the more southern field sites in Hovedissen and Moosburg higher mean temperatures than in Hohenlieth (1.4°C on average) were recorded. In Moosburg, low levels of rainfalls resulted in highly reduced soil moisture during the vegetation period before the winter time (61.8% nFK on average). In contrast, in Hohenlieth and Hovedissen, during the ripening stage in June and July, the soil moisture droped down to 32.3% nFK and 52.8% nFK, respectively, and were lower compared to Moosburg (69.0% nFK at the minimum).

Table 13: Climatic conditions for field trial 2004/2005. Mean data received from observation stations of German Weather Service nearest to the experimental field sites. Soil moisture is represented by plant-available water of the field capacity (% nFK).

		Hohenlieth	Hovedissen	Moosburg
Summary vegetation period before winter dormancy (6^{th} of Sept. – 6^{th} of Nov.)	Air temperature (°C)	11.2	11.8	11.6
	Sum rainfall (mm)	161.4	124.7	115.4
	Soil temperature (°C)	11.6	12.7	13.2
	Soil moisture (% nFK)	94.6	101.3	61.8
Summary vegetation period after winter dormancy (15^{th} of March – 6^{th} of July)	Air temperature (°C)	12.1	13.6	14.0
	Sum rainfall (mm)	292.1	266.4	368.2
	Soil temperature (°C)	15.3	16.6	16.7
	Soil moisture (% nFK)	92.0	88.0	94.7

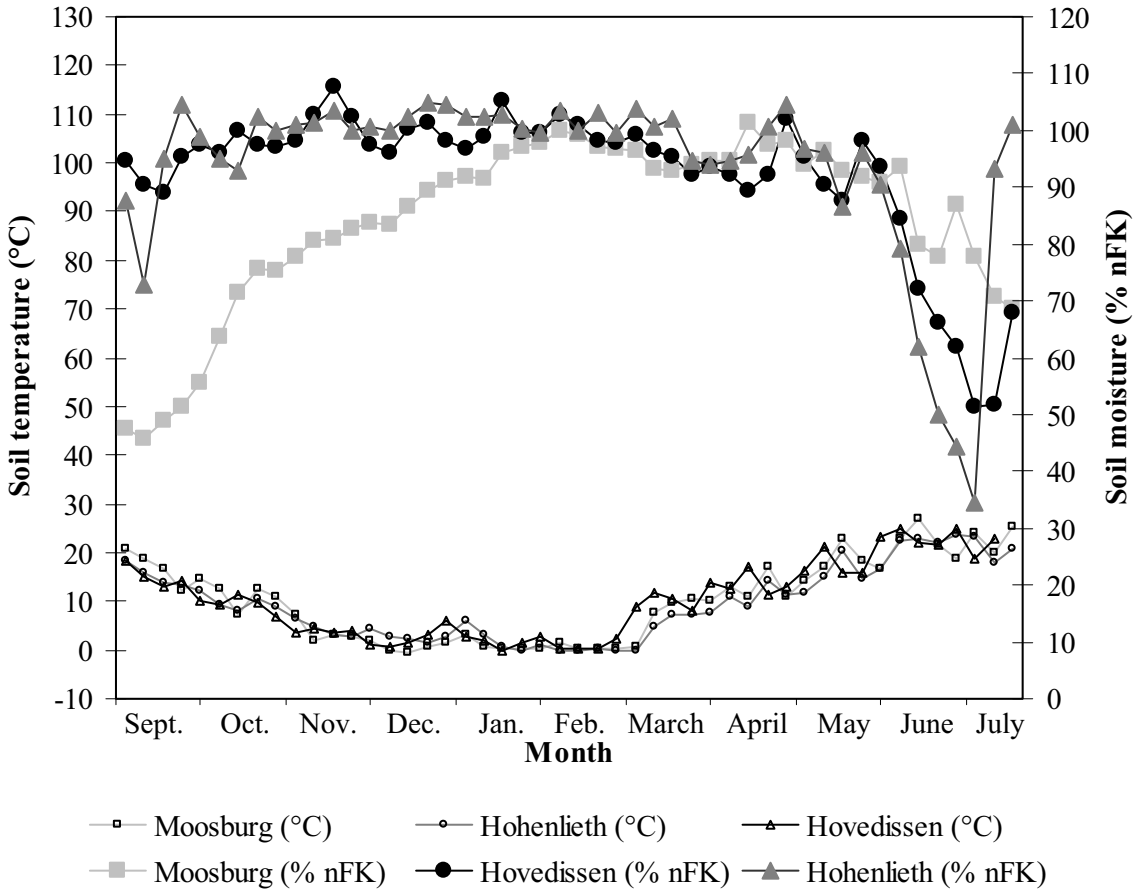

Figure 24: Soil temperature and soil moisture for trial 2004/2005 plotted in a weakly interval. Data received from observation stations of German Weather Service nearest to the experimental field sites. Soil moisture is represented by plant-available water of the field capacity (% nFK).

3.3 Effect of *Serratia plymuthica* HRO-C48 on olive

3.3.1 Root colonization assays

An experiment was conducted to determine the ability of *S. plymuthica* HRO-C48 Rif^r and *P. fluorescens* PICF7 Rif^r, applied individually and in combination, to colonize olive roots. Mean population sizes of both inoculants in the rhizosphere and in the endorhiza over the period time of 169 days are shown in Figure 25. The cell densities of HRO-C48, which were re-isolated from the rhizosphere, were comparable to those of the introduced *Pseudomonas* strain. After seven days, $\log_{10}$ 7.1±0.31 CFU g^{1} rfw on average were recovered. The population size consecutively decreased to $\log_{10}$ 6.0±0.43, 4.5±0.51 and 3.2±0.28 CFU g^{1} rfw after 21, 43 and 90 days, respectively. Furthermore, 169 days post-inoculation the mean population densities of HRO-C48 and PICF7 were $\log_{10}$ 2.1±0.47 CFU g^{1} rfw, when the bacterial isolates were inoculated alone. In contrast, the combined approach resulted in a cell density below the detection limit of $\log_{10}$ 1.0 CFU g^{1} rfw.

Bacteria colonizing the endorhiza were re-isolated after sterilization of the root surface. The efficacy of the procedure was tested by printing sterilised roots on nutrient agar; no bacterial cells could be detected. The mean population sizes of the endophytic strain *P. fluorescens* PICF7 were shown to be stable at $\log_{10}$ 5.8±0.37 CFU g^{1} rfw during the first 43 days after inoculation and decreased to $\log_{10}$ 4.2±0.49 and 1.2±1.54 CFU g^{1} rfw after 90 and 169 days, respectively. No differences between single and combined inoculation were observed. When applied separately, after 7, 21 and 43 days the abundances of HRO-C48 were lower than those of PICF7, and were comparable to the cell number of PICF7 after 90 and 169 days. In presence of PICF7, *S. plymuthica* was found to be less abundant during the first three weeks, compared to HRO-C48 introduced as a pure culture.

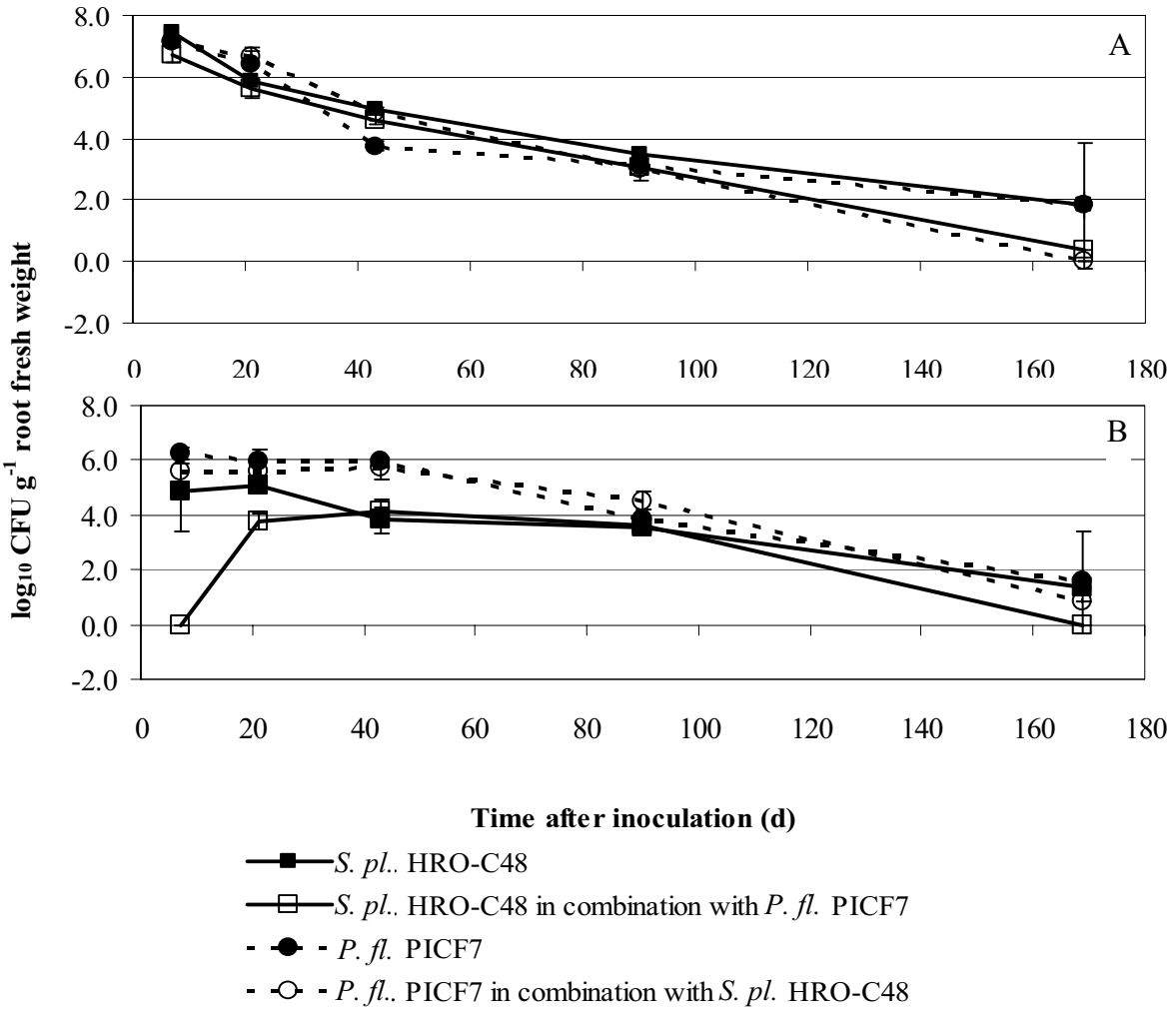

Figure 25: Population density of *S. plymuthica* HRO-C48 Rif^{r} in the rhizosphere (A) and endorhiza (B) of olive plants grown under greenhouse conditions. Cell counts at each sampling time and inoculant represent average of four replicates. Error bars indicate confidence intervals at $P \leq 0.05$.

3.3.2 Disease development

After inoculation with the biocontrol strains and planting in *Verticillium*-infested soil, no symptoms caused by *V. dahliae* V138 developed in olive plants. Thus, after the first period of growing, the experimental plants were re-planted and were once more exposed to the pathogen by means of soil infestation and cutting root prior dipping into conidial suspension, respectively. Figure 26 illustrate the course of development of the disease from the moment of the second infestation and Table 14 gives numbers of relevant parameters including the first day of occurrence of symptoms (IP), final disease intensity index (DII) and the area under the curve of DII (AUDPC). Using soil infestation, all plants evolved typical symptoms characteristic to those caused by the D pathotype. In control plants, Verticillium wilt developed by 32.9 days reaching a final DII of 0.64 and an AUDPC of 27.8. No differences were observed for *P. fluorescens* PICF7, compared to the control. Bacterization with HRO-C48 delayed the IP by 3.4 days and reduced the DII by 23.4%, and AUDPC by 40.6%. In contrast, treatment of olives with HRO-C48 and PICF7 resulted in an IP of 26.1 and the final DII was increased by 37.5% and AUDPC by 42.4 %, compared to the control. Compared to the double inoculation, the disease suppression of individually applied *S. plymuthica* significantly increased.

Results obtained by plant infestation by immersing olive roots into conidial suspension were different to those of soil infestation. In non-inoculated control plants, first symptoms appeared by 25.7 days post-inoculation reaching the final DII of 0.71 and AUDPC of 31.2. The disease development in *S. plymuthica*-treated plants started by 24.9 days, and the final DII (0.81) as well the AUDPC (38.6) were slightly increased compared to the control. Bacterization of plants with PICF7 statistically significantly decreased the final DII by 64.8% and AUDPC by 68.9%.

3.3.3 Plant growth promotion effect

At the beginning of the experiment, after 90 days and 169 days, the length of the plant and the diameter of the stem were recorded (Figure 27). After 90 days, the relative length growth of the control plants amounted to 312.9±78.94% compared to 339.3±71.49%, 290.1±25.77% and 299.1±68.28% of plants inoculated with HRO-C48, PICF7 and both bacteria, respectively. Compared to the other treatments, the bacterization of olives with HRO-C48 resulted in a statistically significant increase of length growth of 181.4±19.56% on average after 169 days. The diameter of the stems of control plants increased by 35.4±5.40% after 90 days, and 177.4±15.77% after 169 days. No statistically significant differences at any time and for any treatment were found in terms of stem broadening.

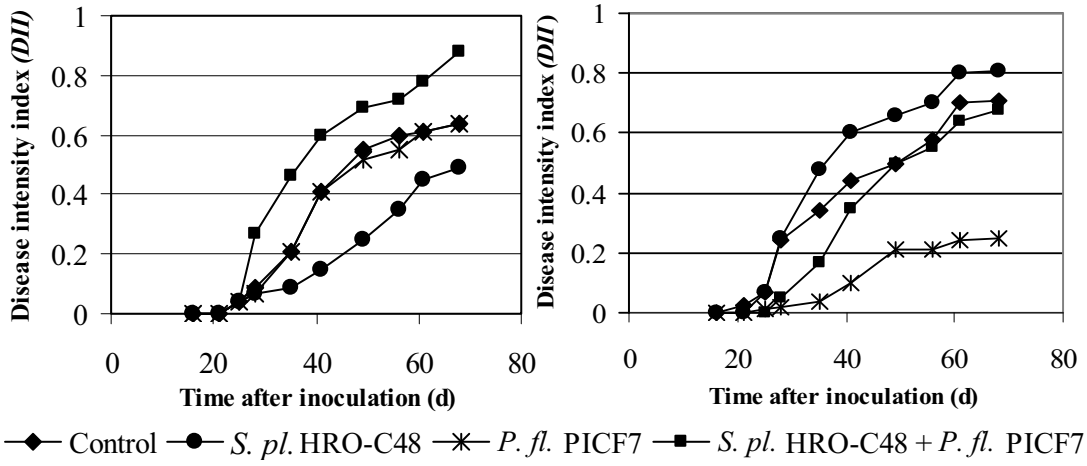

Figure 26: Effect of *P. fluorescens* PICF7 and *S. plymuthica* HRO-C48 on the development of Verticillium wilt of olive cv. Arbequina. *V. dahliae* was inoculated by either soil infestation (A) or root dipping. The disease intensity index (DII) ranging from 0-1 was calculated with data on incidence and severity of symptoms recorded at 7-days intervals.

Table 14: Effect of *P. fluorescens* PICF7 and *S. plymuthica* HRO-C48 on the development of Verticillium wilt of olive cv. Arbequina. DII represents the disease intensity index; IP indicates incubation period (number of days until DII > 0) and AUDPC the area under the curve of DII increase over time. Mean values followed by same letters were not significantly different according to Fisher's protected LSD test at $P \leq 0.05$.

	IP (days)		Final *DII*		SAUDPC	
Treatment	**Root dip**	**Infested soil**	**Root dip**	**Infested soil**	**Root dip**	**Infested soil**
Control	25.7 ab	32.9 ab	0.71 a	0.64 ab	31.2 a	27.8 ab
P. fl. PICF7	34.7 a	29.6 ab	0.25 b	0.64 ab	9.7 b	27.5 ab
S. pl. HRO-C48	24.9 b	36.3 a	0.81 a	0.49 b	38.6 a	16.5 b
HRO-C48 + PICF7	31.9 ab	26.1 b	0.68 a	0.88 a	26.1 a	39.6 a

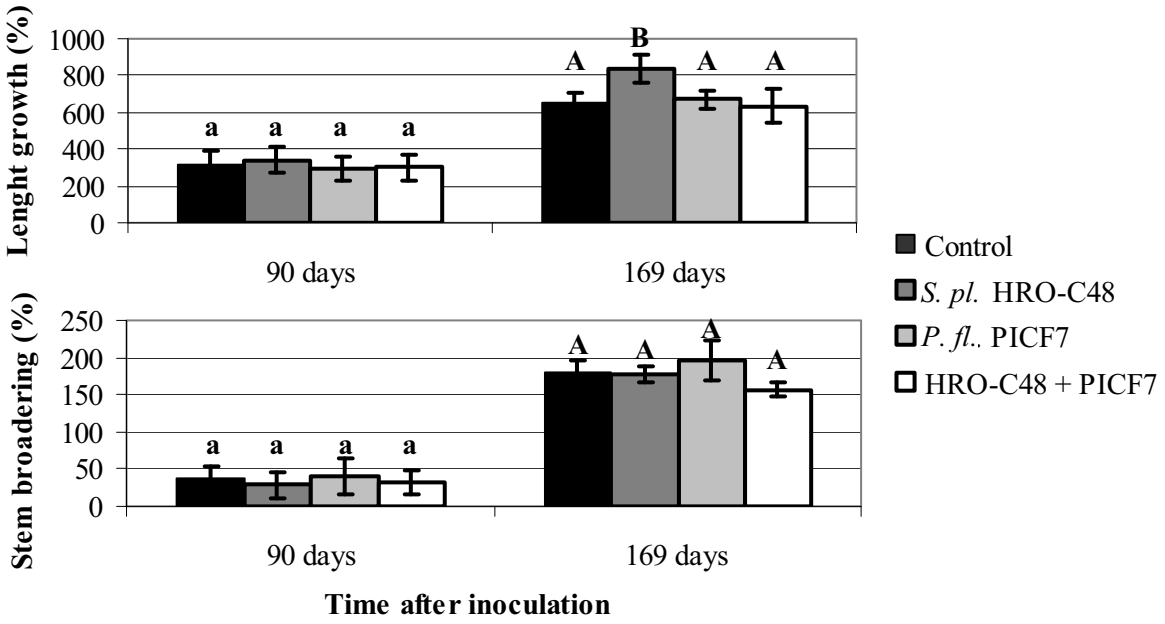

Figure 27: Plant growth promotion effect of *S. plymuthica* HRO-C48 and *P. fluorescens* PICF7 on olive cv. Arbequina accessed by monitoring total plant length (A) and stem diameter (B). Bars with same letters were not significantly different at $P \leq 0.05$. Error bars indicate standard deviations.

3.3.4 SSCP analyzes

The rhizosphere-associated eubacterial community of olive plant from the biocontrol and plant growth promotion experiment was studied by means of SSCP analysis. The structure of the bacterial community of plants infested with *V. dahliae* by root dipping was significantly distinguishable from that of plants grown in non-infested soil (P 0.002) (Figure 28A). At a threshold similarity of 45.0% two distinct clusters were found for *Verticillium*-infested plants (cluster II) and non-infested plants (cluster I). An arrangement according to the bacterial treatment was not observed. The dendrogram calculated from the SSCP patterns of plants grown in *Verticillium*-infested soil and the analogically treated control plants was more separated than the profiles of plants treated by root dipping (Figure 28B). Cluster IV contains the majority of non-infested control plants. Setting the threshold similarity at 40.0%, in the cluster III, V and VI the rhizosphere patterns of plants affected by *V. dahliae* were grouped. Each of those groups consists of either non-bacterized plants (V) or plants inoculated with HRO-C48 (III) or with PICF7, and with both strains (VI). Those groups were observed to be statistically significantly separated from each other ($P \leq 0.001$). In both experimental setups, for the compositions of the bacterial microflora in the rhizosphere of olive plants a high degree of dissimilarities was found. The SSCP patterns clustered according to the presence or absence of *V. dahliae*, respectively. Secondly, using soil infestation, the bacterial profiles grouped in correlation of the bacterial inoculant.

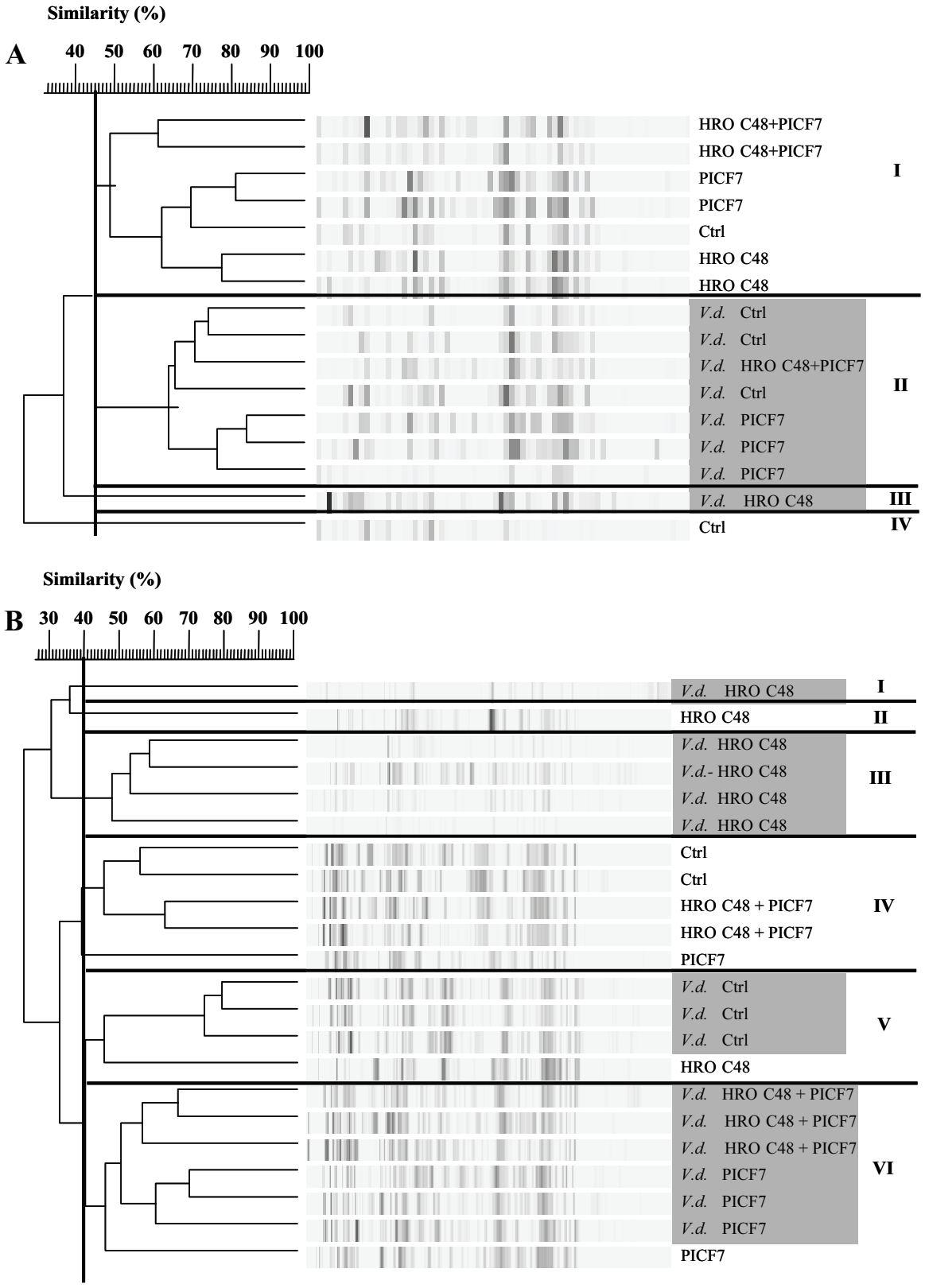

Figure 28: Dendrogram generated from the SSCP profiles of olive plants inoculated with bacterial strains and infested with *V. dahliae* by root dipping (A) or via soil preparation (B) using the unweighted-pair group methods using average linkages (UPGMA). Similarity indices were calculated by the Pearson correlation index. Control plants (inoculated and non-inoculated) were treated analogue to the plants infested with the fungus, but using sterile 10.0 mM $MgSO_4$ solution.

3.4 Phenotypical characterization of the AHL-deficient derivates of *Serratia plymuthica* HRO-C48

Two AHL-deficient derivates of *S. plymuthica* HRO-C48, namely HRO-C48 AHL-4 and HRO-C48 pME6863 were investigated on their ability to suppress Verticillium wilt of oilseed rape and to promote plant growth compared to the wild-type strain. Additionally, the impact of the disruption of the AHL-mediated QS system on a set of phenotypes, which are supposed to be involved in either plant-bacteria or fungi-bacteria interaction, were analyzed *in vitro*. Both mutants were provided by Prof. Leonid Chernin (Hebrew University of Jerusalem, Israel). The AHL-synthase gene *splI* of *S. plymuthica* strain HRO-C48 AHL-4 was disrupted by insertion, resulting in an almost totally decline of AHL release. The strain HRO-C48 pME6863 carrying a plasmid bearing the *AiiA* gene, which encodes for an AHL-lactonase. This mutant was shown to be completely impaired in AHL excretion.

3.4.1 Biocontrol activity

The role of AHLs produced by *S. plymuthica* HRO-C48 in its biocontrol activity was investigated in the pathosystem oilseed rape *V. longisporum* under greenhouse conditions. Two experiments were performed testing the wild-type strain and the transposon mutant HRO-C48 AHL-4 in the first trial (Figure 29), and the wild-type strain, HRO-C48 AHL-4 and HRO-C48 pME6863 in the second one (Figure 30). Seeds from winter oilseed rape cv. Talent were inoculated with bacterial strains by bio-priming and grown in *V. longisporum*-infested soil. The area under disease process curve (AUDPC), indicating disease severity, was recorded in weekly intervals from appearance of the first symptoms for five weeks. Symptoms caused by the pathogen developed in all plants included in these experiments. In greenhouse trial MI, plants grown from seeds inoculated with the wilt-type strain of *S. plymuthica* HRO-C48 showed a statistically significantly delayed of the development of the Verticillium wilt compared to the non-inoculated control; resulting in a reduction of AUDPC of 25.9±6.15% on average. In contrast to the wilt-type strain, the level of the disease development in plants treated with strain HRO-C48 AHL-4 was significantly increased. However, compared to the non-bacterized control variant the AUDPC was still significantly lower (7.9±6.43%). In the second greenhouse trial, the degree of disease suppression by HRO-C48 WT was similar compared to the experiment I (14.9±7.32% in average). The disease incidence and the calculated AUDPC of plants grown from seeds inoculated with AHL-deficient mutants HRO-C48 AHL-4 and HRO-6863, respectively, were as high as for the control plant. The biocontrol activity against *V. longisporum* in oilseed rape of both derivates was found to be reduced or even abolished.

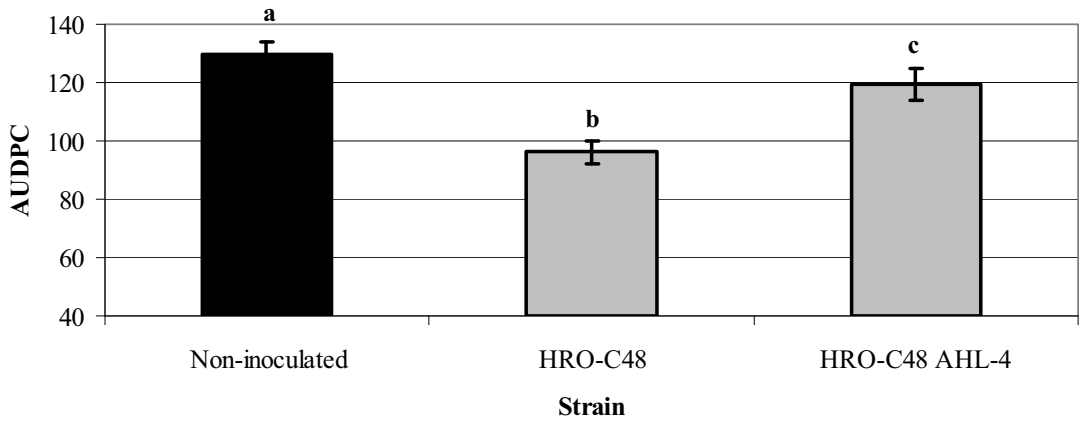

Figure 29: Results from greenhouse trial I: Effect of *S. plymuthica* HRO-C48 AHL-4 impaired in AHL production on the development of Verticillium wilt on oilseed rape expressed by the Area under disease process curve (AUDPC) in comparison to the wild-type strain and the non-inoculated control. Same letters above bars indicate no significant differences at $P \leq 0.05$. Error bars symbolize standard deviations.

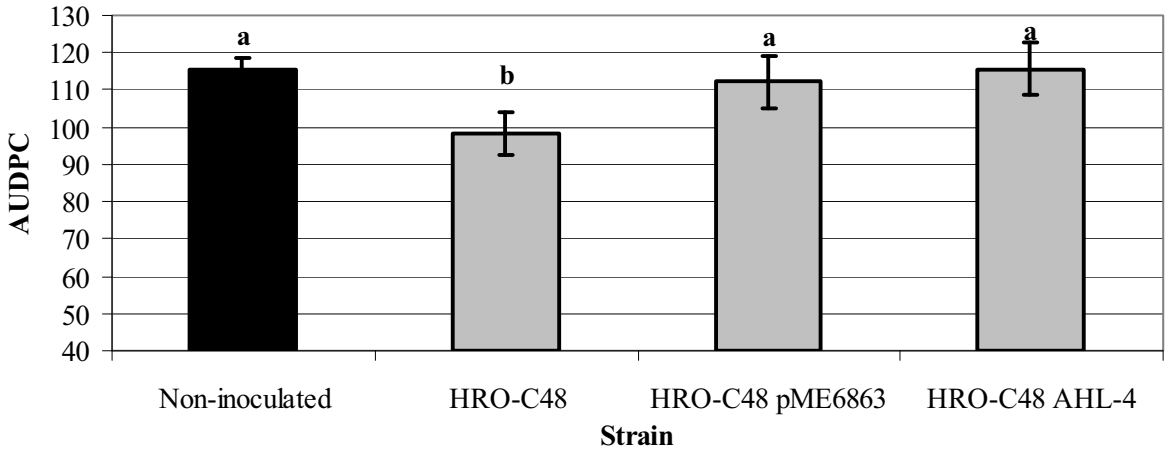

Figure 30: Results from greenhouse trial II: Effect of *S. plymuthica* HRO-C48 derivates impaired in AHL release on the development of Verticillium wilt on oilseed rape expressed by the Area under disease process curve (AUDPC) in comparison to the wild-type strain and the non-inoculated control. Same letters above bars indicate no significant differences at $P \leq 0.05$. Error bars symbolize standard deviations.

3.4.2 Plant growth promotion ability

S. plymuthica HRO-C48 was shown to enhance plant growth of numerous plants. Here, the effect of HRO-C48 WT and HRO-C48 AHL-4 on the plant growth of oilseed rape and lettuce was tested. Results from greenhouse trial showed a statistically significant increase of the weight of fresh stems and leaves of oilseed rape plants, which were inoculated with the bacterial strains (Figure 31). Compared to the non-inoculated control the wild-type strain promoted the plant growth by 41.1±14.80%. The fresh weight of plants, which were colonized

by the miniTn5-transposon mutant, was even higher (77.4±21.58%), and was significantly different compared to the control and the wild-type strain.

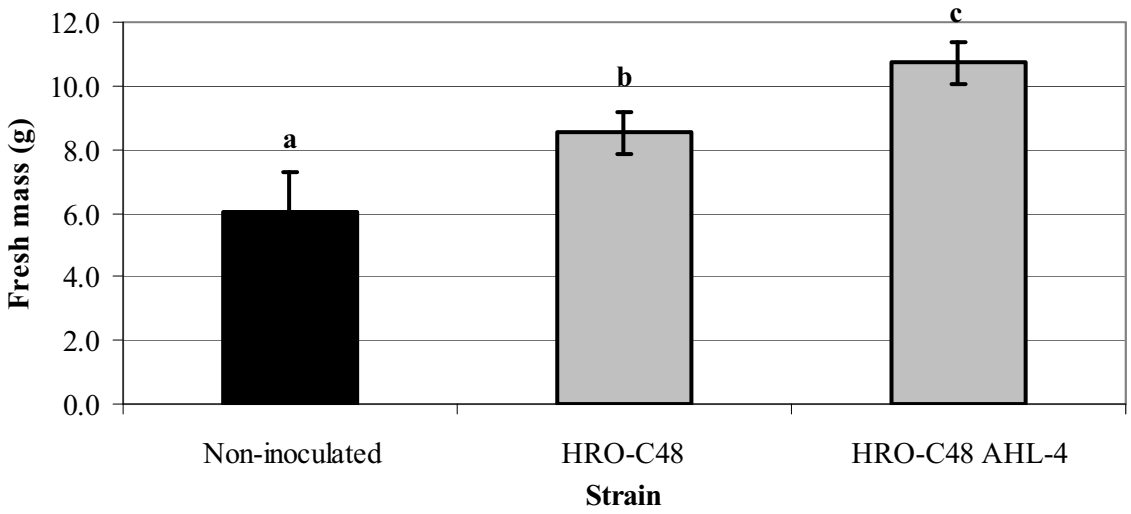

Figure 31: Results from greenhouse trial I: Effect of *S. plymuthica* HRO-C48 AHL-4 impaired in AHL production on the fresh mass of oilseed rape plants grown for 60 days in comparison to the wild-type strain and the non-inoculated control. Same letters above bars indicate no significant differences at $P \leq 0.05$. Error bars symbolize standard deviations.

In vitro seedlings assays using lettuce seeds indicate the plant growth promotion effect of both applied strains (Figure 32). Compared to the non-inoculated plants, the fresh weight of seedlings after 14 days of cultivation in presence of *S. plymuthica* HRO-C48 and HRO-C48 AHL-4 was statistically significantly increased by 34.1% and 52.2%, respectively. Additionally, the total plant length was enhanced by 18.7% (HRO-C48 WT) and 40.7% (HRO-C48 AHL-4) on average, compared to the control. On both, lettuce and oilseed rape, the positive impact of *S. plymuthica* HRO-C48 on plant growth were shown. The disruption of the AHL-synthase gene in HRO-C48 AHL-4 did not affect the plant growth promotion ability or even positively.

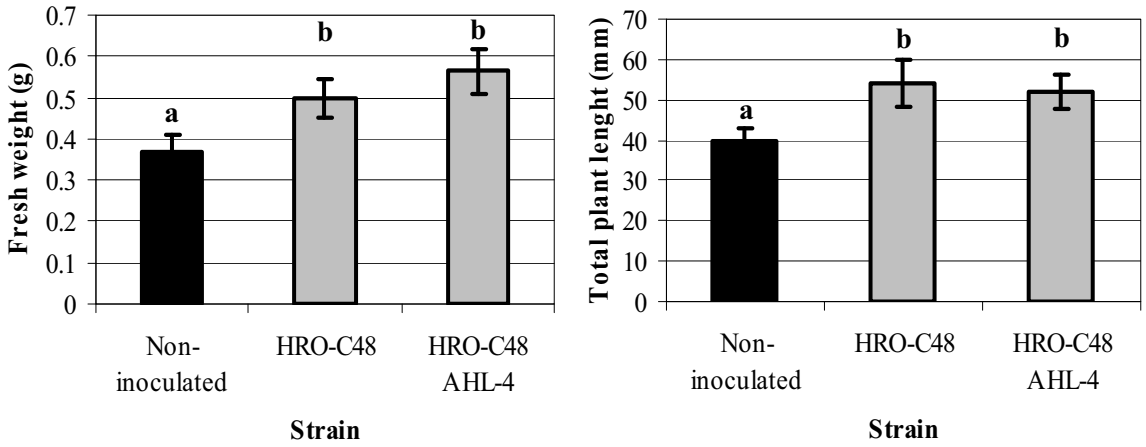

Figure 32: Plant growth promotion effect of *S. plymuthica* HRO-C48 AHL-4 on lettuce seedlings in comparison to the wild-type strain and the non-inoculated control. Using an *in vitro* assay the fresh mass and total length of 24 seedlings each variant was recorded. Same letters above bars indicate no significant differences at $P \leq 0.05$. Error bars symbolize standard deviations.

3.4.3 Root colonization assay

Root colonizing experiments were conducted using plants grown from bio-primed seeds inoculated with $\log_{10}$ 6.0 CFU seed^{-1}. To re-isolate *S. plymuthica* from the rhizosphere, the wild-type strain of HRO-C48 was represented by the spontaneous rifampicin mutant HRO-C48 Rif^{r}. No differences in their ability to colonize the rhizosphere of oilseed rape were observed for all *Serratia* strains for a period of time of 31 d from the day of sawing (Figure 33). After 10 d of the greenhouse trial, the population size of *S. plymuthica* HRO-C48 Rif^{r}, HRO-C48 AHL-4, and HRO-C48 pME6863 was $\log_{10}$ 5.4±0.57 CFU g^{-1} root fresh weight on average. In the following 10 days, the cell density decreased to $\log_{10}$ 3.5±0.33 CFU g^{-1} in all treatments and was stable until 31 d post-inoculation at $\log_{10}$ 3.4±0.07 CFU g^{-1} root fresh weight. Altogether, no differences in root colonization were found between the derivates of *Serratia*, which imply that this important factor for biocontrol is not influenced by presence of AHLs.

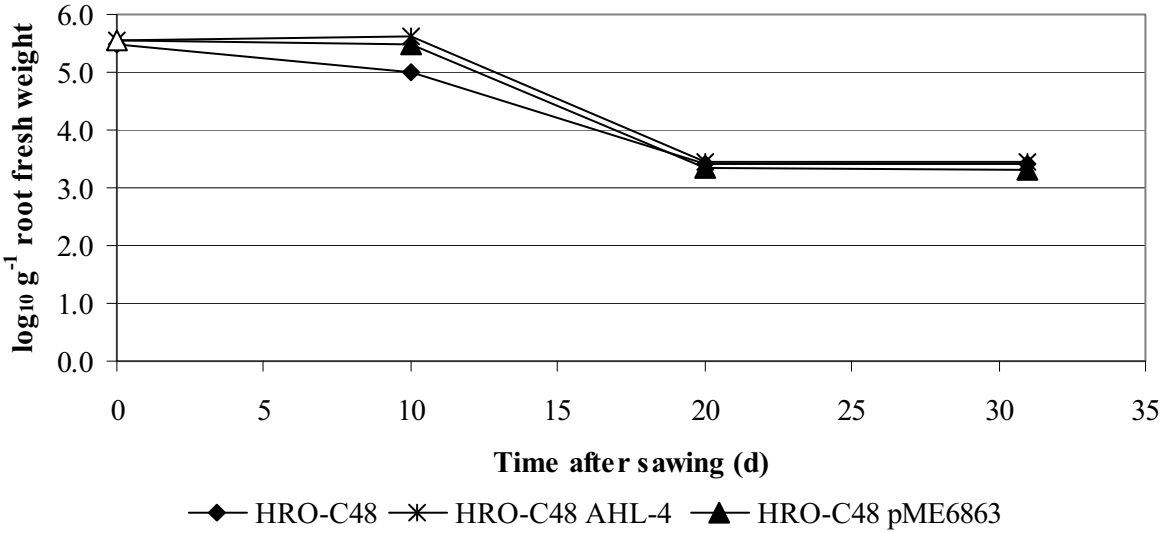

Figure 33: Population densities of HRO-C48 Rif^r, HRO-C48 AHL-4, and HRO-C48 pME6863 in the rhizosphere of oilseed rape grown under greenhouse conditions. Open symbols indicate cell density applied to seeds prior sawing by means of bio-priming.

3.4.4 Biofilm formation

The ability to form biofilms was tested in a microtiter-plate assay using AB minimal medium supplemented with respective citrate and glucose, and the LB complex medium as test media. In none of the applied media the AHL-negative mutant of HRO-C48 showed a significantly different behaviour than the wild-type strain. Nevertheless, a medium-specific effect on the level of biofilm formation could be measured; in glucose containing minimal medium *Serratia* cells form a denser biofilm compared to AB medium containing citrate and in LB medium (Figure 34).

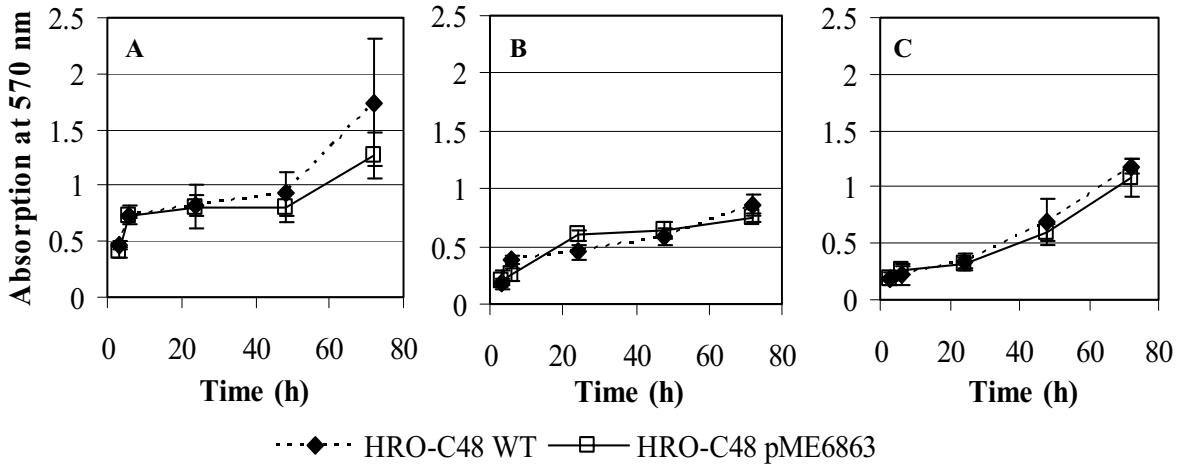

Figure 34: Capacity to form biofilm formation of *S. plymuthica* HRO-C48 wild-type strain and its transconjugant HRO-C48 pME6863 in ABG medium (A), ABC medium (B) and LB medium (C) examined using static microtiter plate assay and crystal violet staining. Error bars indicate standard deviations.

3.4.5 Motility assay

Both the swarming and swimming motility, of *S. plymuthica* strains were studied by on-plate assays using LB medium containing 0.4% and 0.3% agar-agar, respectively. Whereas *Serratia* cells were not able to swarm over the agar surface, all tested isolates were shown to be motile on/in a medium containing 0.3% agar-agar. The wild-type strain colony covered a mean area with a diameter of 52.5±9.03 mm (Figure 35). The mean colony diameters of HRO-C48 pME6863 and HRO-C48 AHL-4 were 68.0±4.55 mm and 75.3±5.74 mm, respectively, meaning a statistically significant increase of 29.5% and 43.3% in comparison to the wild-type strain.

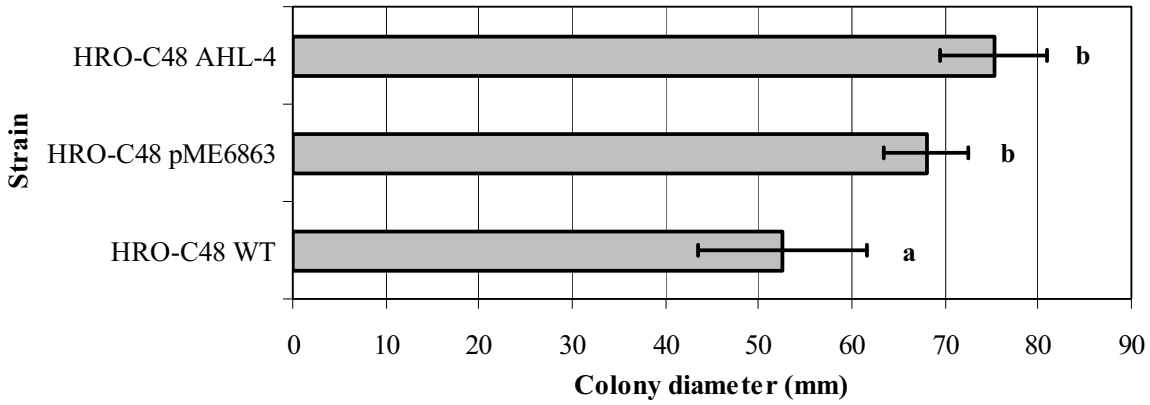

Figure 35: Swimming motility of *S. plymuthica* HRO-C48 and its AHL-deficient derivates using medium containing 0.3% agar-agar. Same letters above bars indicate no significant differences at $P \leq 0.05$. Error bars symbolize standard deviations.

3.4.6 Indole-3-acetic acid production

In vitro production of phytohormones such as indole-3-acetic acid (IAA) indicates the capacity of a plant-associated bacterium to promote plant growth. *S. plymuthica* HRO-C48 is known to synthesize IAA (Kalbe *et al.* 1996). The IAA concentration in the supernatant of the bacterial cultures was determined using a colorimetric method established by Sawer and Kramer (1996). In Figure 36 the amounts of auxin produced by *S. plymuthica* HRO-C48 WT and HRO-C48 pME6863 are shown. The culture filtrate of the wild-type strain contained 15.9±6.36 µg ml^{1} IAA. Interestingly, a statistically significantly increased IAA formation was found for transconjugant strain HRO-C48 pME6863 as well as for HRO-C48 AHL-4. Compared to the wild-type for the latter strains an almost threefold higher concentration of the phytohormone was measured in the supernatant of isolate HRO-C48 pME6863 (48.2±18.35 µg ml^{1}) and HRO-C48 AHL-4 (43.8±18.56 µg ml^{1}), which suggest that the production of IAA is negatively regulated by QS.

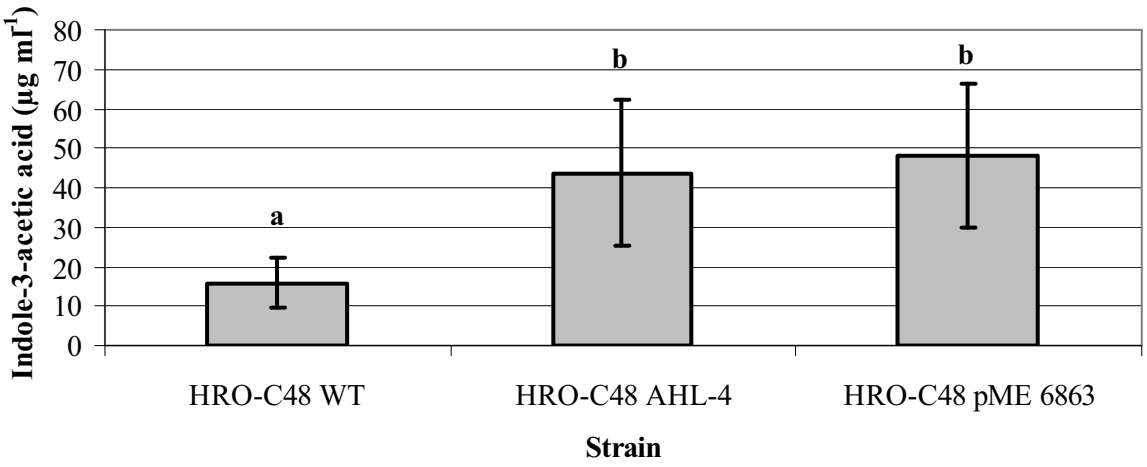

Figure 36: Indole-3-acetic acid synthesis of *S. plymuthica* HRO-C48 and its AHL-deficient derivates. Same letters above bars indicate no significant differences at $P \leq 0.05$. Error bars symbolize standard deviations.

3.4.7 Analysis of extracellular enzyme activity

The role of AHL in the regulation of chitinolytic, proteolytic and lipolytic activity was investigated via chromogenic enzyme assays and on-plate assay, respectively. Figures 37, 38 and 39 show the specific activities of extracellular enzymes of the analyzed *S. plymuthica* strains. The presence of bacterial proteases in the spent culture supernatant was found for both, wild-type strain and mutant (Figure 37). Compared to the wild-type strain, in strain HRO-C48 pME6863 the proteolytic activity was reduced by about 70%. Analogically, the specific chitinolytic activity was almost completely abolished (Figure 38). Therefore, the production of both analyzed exoenzymes is influenced by QS. Furthermore, the affect of the ability to hydrolyse lipids was studied using plate approaches. All tested strains were able to utilize Tween80, there were no differences in the diameter of hydrolysis zone (Figure 39).

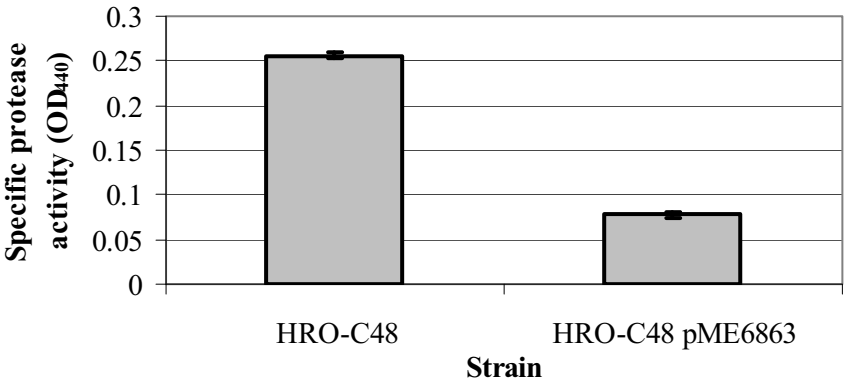

Figure 37: Specific proteolytic activity of *S. plymuthica* HRO-C48 and its transconjugant HRO-C48 pME6863 in LB medium analyzed by means of quantitative enzyme assay. Error bars indicate confidence intervals at $P \leq 0.05$.

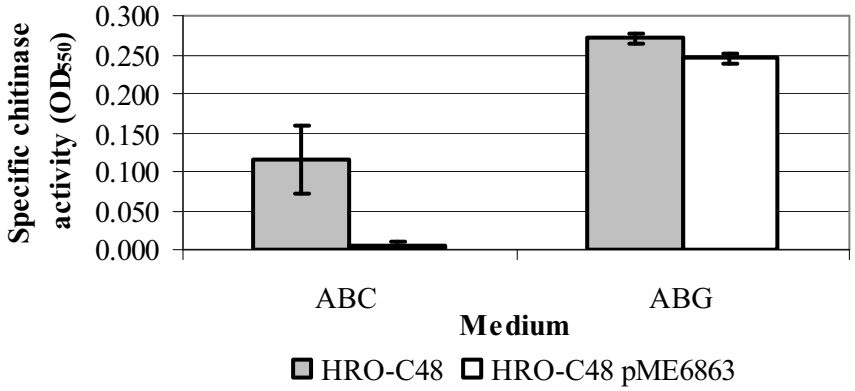

Figure 38: Specific chitinolytic activity of *S. plymuthica* HRO-C48 and its transconjugant HRO-C48 pME6863 in ABC and ABG medium analyzed by means of quantitative enzyme assay. Error bars indicate confidence intervals at $P \leq 0.05$.

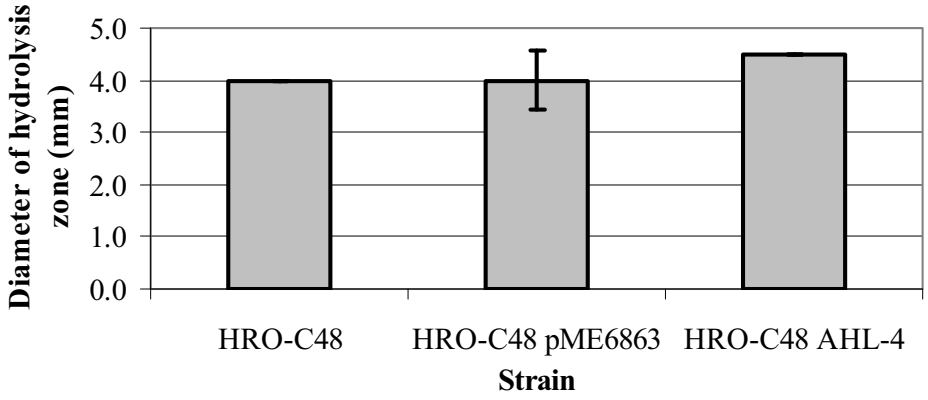

Figure 39: Lipase activity of *S. plymuthica* HRO-C48 and its transconjugant HRO-C48 pME6863 on Tween80 medium Error bars indicate confidence intervals at $P \leq 0.05$.

3.4.8 Pyrrolnitrin synthesis

Pyrrolnitrin (PRN) is an antifungal substance produced by wide range of rhizosphere-associated bacteria. To detect pyrrolnitrin produced by bacterial cells, aceton crude extracts of HRO-C48 cells were separated by thin-layer chromatography. The inhibitory effect of migrated compounds on mycelial growth of *Rhizoctonia solani* was recorded seven days post-inoculation. For the wild-type strain an inhibition zone at R_f of 0.89 was observed (marked by open circles), which corresponds with the migration and antifungal properties of the purified PRN standard (Figure 40). The amount of PRN extracted from transconjugant strain HRO-C48 pME6863 correlating with smaller inhibition zones was significantly reduced compared to the wild-type strain. The biosynthesis of PRN of HRO-C48 AHL-4 was found to be

completely eliminated. The affect of disrupted of AHL release in HRO-C48 on the PRN production indicated the AHL-based regulation of pyrrolnitrin in *S. plymuthica* HRO-C48.

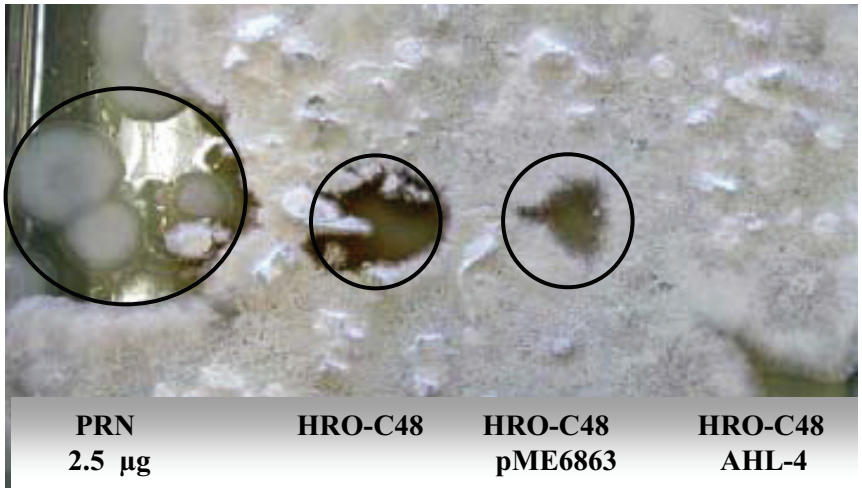

Figure 40: Pyrrolnitrin production of *S. plymuthica* HRO-C48 wild-type strain and its AHL-deficient mutants was examined by thin-layer chromatography combined with a bioassay using *R. solani* as test strain.

3.4.9 Siderophore production

S. plymuthica HRO-C48 was shown to produce high levels of iron-chelating compounds. Performing plate-assays using iron-limited medium the ability to excrete siderophores was analyzed. Wild-type strain and both AHL-negative mutants were indistinguishable in mobilizing iron from the crome azurol blue/iron(III) complex (data not shown).

3.4.10 Inhibition of fungal growth by volatile organic compounds

A two component Petri dish was used for studying the effect of volatile organic compounds (VOCs) released by *S. plymuthica* HRO-C48 and its derivates on fungal growth. Antifungal volatile activity was observed for all bacteria on both fungi. Data of inhibition of *V. longisporum* are shown in Figure 41 and Table 15. When the fungus was exposed to head space volatiles of HRO-C48 derivates, a repression of mycelial development was found for all strains; the final diameter of the fungal colony was reduced by all tested bacteria. Compared to the control, statistically significant reduction of AUFGC was observed for the wilt-type strain of HRO-C48 (9.1% on average) as well as for the AHL-deficient strains HRO-C48 AHL-4 (18.8%) and HRO-C48 pME6863 (12.1%). The miniTn5 mutant possessed the highest degree of inhibition, which was of statistically significance compared to the wild-type strain. Morphological characteristics such as the melanisation were non-affected by the bacterial volatiles.

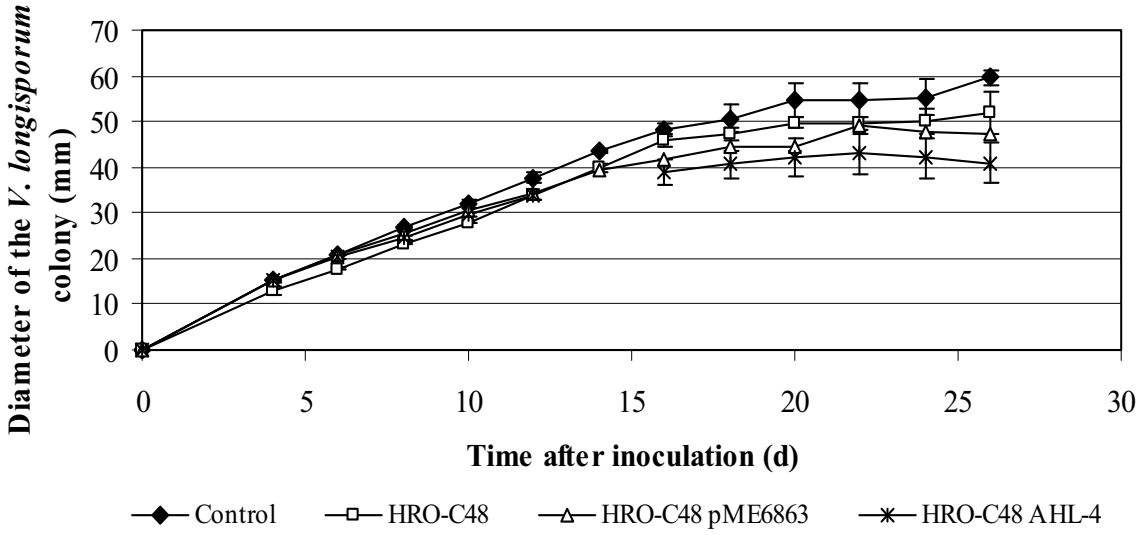

Figure 41: Mycelial inhibition effect of volatiles emitted by *S. plymuthica* HRO-C48 WT and its derivates on the growth of *V. longisporum* ELV25 using divided plate assay. Bacteria and the fungus were grown on nutrient agar and Waksman agar, respectively. Error bars indicate standard deviations.

Table 15: Effect of volatile compounds emitted by *S. plymuthica* HRO-C48 WT and its derivates on the growth of *V. longisporum* ELV25 using divided plate assay. After 26 days of cultivation at 20°C the final diameter of the fungal colony was measured and the area under fungal growth curve was calculated. Mean values followed by same letters were not significantly different at $P \leq 0.05$.

Treatment	Final diameter		Area under fungal growth curve	
	Mean	Standard deviation	Mean	Standard deviation
Control	59.7 a	± 1.73	922.0 a	± 33.53
S. pl. HRO-C48	52.0 ab	± 2.40	838.0 b	± 28.34
S. pl. HRO-C48 pME6368	47.3 bc	± 9.76	810.7 bc	± 25.02
S. pl. HRO-C48 AHL-4	41.0 c	± 5.19	749.0 c	± 59.65

Because of the rapid growth of *R. solani*, the monitoring of mycelial growth was limited to five days. Within this period, volatile compounds emitted by bacterial strains have a negative impact on the growth of the fungal colony (Figure 42; Table 16). Compared to the control, the AUFGC was statistically significantly reduced by 17.7% (HRO-C48), 24.9% (HRO-C48 pME6863) and 24.2% (HRO-C48 AHL-4) Further incubation of the experimental plates revealed an affect of bacterial VOCs on the formation of the microsclerotia. Photograph series in Figure 43 illustrates that in presence of the bacteria, the sclerotial formation is repressed within 21 days. The size as well as the structure of the sclerotial complexes, indicated by

arrows, appeared to be different, particularly if *R. solani* was incubated in presence of strain HRO-C48 AHL-4.

Overall, volatile substances produced by *S. plymuthica* HRO-C48 were demonstrated to inhibit the growth of the phytopathogenic fungi *V. longisporum* and *R. solani*, and to repress sclerotial formation of the latter fungus. A higher degree of suppression was observed for the strains impaired in AHL release.

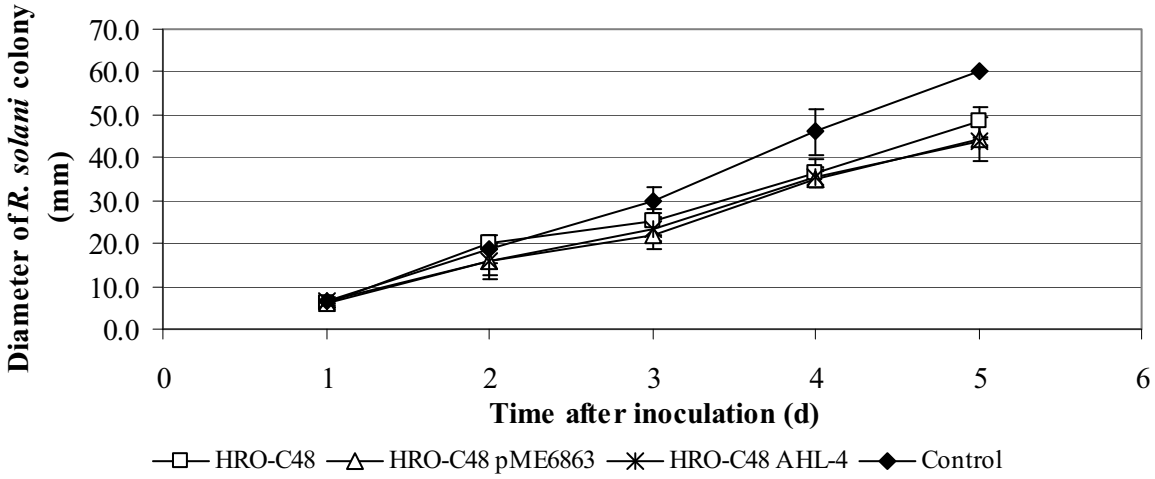

Figure 42: Mycelial inhibition effect of volatiles emitted by *S. plymuthica* HRO-C48 WT and its derivates on the growth of *R. solani* AG2/2 using divided plate assay. Bacteria and the fungus were grown on nutrient agar and SNA, respectively. Error bars indicate standard deviations.

Table 16: Effect of volatile compounds emitted by *S. plymuthica* HRO-C48 WT and its derivates on the growth of *R. solani* using divided plate assay. After five days of cultivation at 20°C the final diameter of the fungal colony was measured and the area under fungal growth curve was calculated. Mean values followed by same letters were not significantly different at $P \leq 0.05$.

Treatment	Final diameter (mm)		Area under fungal growth curve	
	Mean	Standard deviation	Mean	Standard deviation
Control	60.0 a	± 0.00	145.33 a	± 9.54
S. pl. HRO-C48	48.3 b	± 3.51	119.67 b	± 3.79
S. pl. HRO-C48 pME6368	44.3 bc	± 5.13	109.17 b	± 11.81
S. pl. HRO-C48 AHL-4	43.7 c	± 0.58	110.17 b	± 6.29

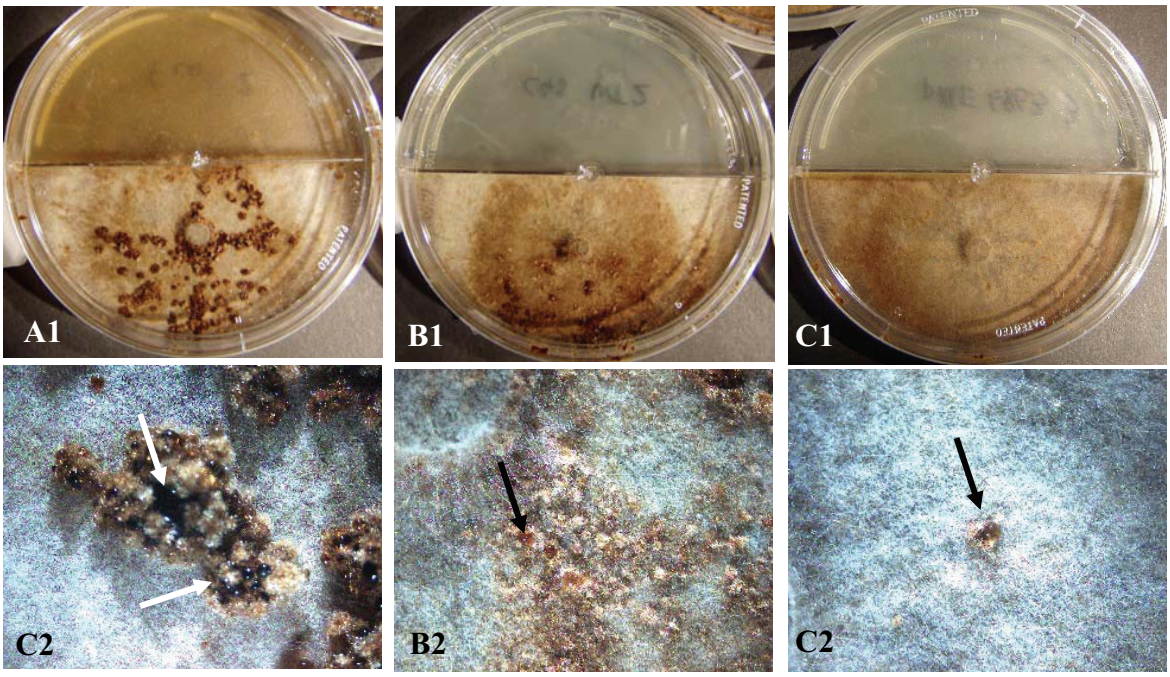

Figure 43: Effects of volatile compounds emitted by *S. plymuthica* HRO-C48 and its AHL-negative mutant on the sclerotia formation of *R. solani.* In the upper line divided plates assays inoculated *R. solani* alone (A), with HRO-C48 WT (B) and HRO-C48 pME6863 (C) and incubated for 30 d at 20°C are shown. The The upper compartment of the Petri dishes (upper row) were either non-inoculated (A1) or seeded with the bacterial strain (B1, C1); the lower compartment contains *R. solani.* The lower row shows microslerotia magnified by a binocular microscope.

3.5 Generation of a pyrrolnitrin-negative mutant of *S. plymuthica* HRO-C48

Results from TLC analyzes indicated that the synthesis of the antifungal compound pyrrolnitrin is impaired in AHL-deficient strains. Hence, the role of PRN in the biocontrol activity of *S. plymuthica* HRO-C48 was investigated characterising a PRN-negative mutant. To destruct the gene responsible for pyrrolnitrin synthesis, *S. plymuthica* HRO-C48 was subjected to insertion mutagenesis. To do this, an *aphII* resistance gene cassette conferring kanamycin resistance was introduced in a unique restriction site into the *prnD* gene. Prior transformation, the *prnD* gene of *S. plymuthica* was amplified using primer pair PRND1/PRND2 (de Souza and Raaijmakers 2003) and subsequently sequenced (Figure 44). Similarity searches in the EMBL database performed with the BLASTN search algorithms show sequence homologues with *prnD* genes of various bacterial species (*Burkholderia pyrrocinia, B. cepacia, Pseudomonas fluorescens*). The modified *prnD* gene fragment was introduced by electroporation into strain HRO-C48. DNA from kanamycin resistant mutants was extracted to confirm homologue recombination by means of PCR analyzes (data not shown). One transformant (PRND-3), in which the wild-type fragment was completely absent, was used for extended molecular and phenotypical studies. Southern blot analyzes revealed fragments that were larger than the fragments obtained with wild-type DNA (Figure

32). The size differences corresponded exactly to the increase in size expected if an *aphII* gene cassette was inserted (1.2 kb). Furthermore, the wild-type fragment was not detectable. The results of the Southern blot analyzes indicated that the constructed fragment used for transformation of *S. plymuthica* HRO-C48 in order to obtain the mutants were correctly integrated by homologous recombination and that the *prnD-aphII* allele completely replaced the wild-type copy of the *prnD* gene.

```
  1 GCCTGCCTGG TCTGCAGCCC AAACAGCACG TAGTCGGCCG CGCGGCGCAG
 51 AACGCCACCC ACCTTTTTGA TCGAGATGAG CATGTGCATG ACGTTCTTGC
101 CGTCGCTCAC CGGCGTCACA CACTGGAGCA GTTTGTATTT GAAGTCTCCG
151 TCCAGCGAGA CGGTCATGAC GCACCCGCCG GGGTAGCCAT CGAAGTGCAG
201 GTTCATCTGC GACATGTTCA GGCCGAGCAC GCGTGCCAGC ATGCCGGAGG
251 CCCCGAAATA CCGGTCCACG GTAAAGTCGA TCCCGGCACC GAACCACGCG
301 CCCGCCTGTG CCAGAGACTC AACCTCCGGC CACCGGCGCC AATCGTCGAA
351 GAGCTTGAGC TCGAAGGCCG AGATCGGGAG CTCGTGCACG GGGGACGCGT
401 GTTGCGCGTC GTAGAAGTTC TCTACGATCC GCAAGACCGC CGTCGTCGTC
451 TCGAACGCGA AGTGCAGGTG CATAAAGTCG CCGTTGTCGA CGTCGGCTGC
501 GGCGATTTCG GGCAGCGGGT GCAGCGGCTC CGGGGAGCCG TACCAGACCC
551 ACACGTAGCC GTATCGCTCG GCGGTGACCA ACGTCGGCTG GCGTACCGAT
601 CGCGGCACGG GCTCCAGCCG GCGCACCGTC TGGTTGTGAC CGGGGATGTG
651 AACGCACCGG CCCTGCTCGT CGTACCGCCA GTGATGAAAC GGGCACTGGA
701 TGCACCCGTC CTTGACCTGC CCGTCGGCCA GGTTCGCACC GAGGTGCGAG
751 CAGTGGCGGT CCATCACCA
```

Figure 44: Nucleotide sequence of *prnD* gene of *S. plymuthica* HRO-C48.

As expected, the mutant was confirmed to be abolished in pyrrolnitrin production by TLC analyzes (Figure 45) and was designated as HRO-C48 PRN . Moreover, *S. plymuthica* HRO-C48 PRN was studied for *in vitro* inhibition of *V. dahliae* and *ad planta* in the pathosystem *V. longisporum* oilseed rape (greenhouse experiment MII). The extent of suppression of the fungus *in vitro* was indistinguishable compared to the wild-type strain (data not shown). On the other hand, in comparison to HRO-C48 WT, the ability to suppress Verticillium wilt of oilseed rape was slightly, but not statistically significantly affected (data not shown).

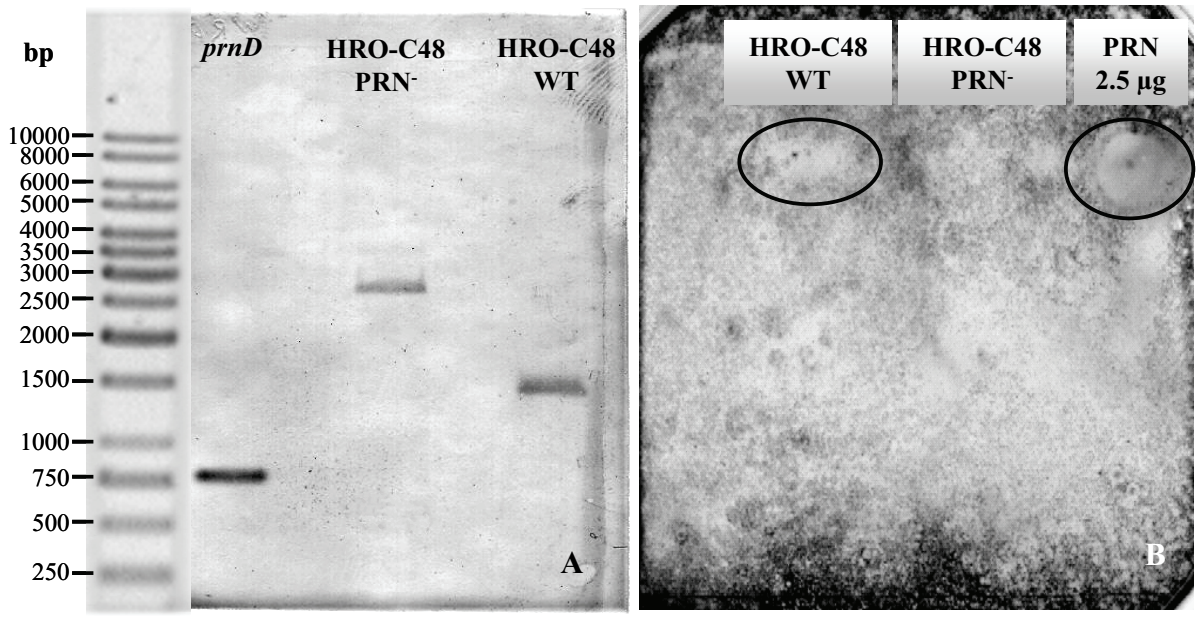

Figure 45: Confirmation of the recombination of gene *prnD* in *S. plymuthica* HRO-C48. A: Detection of the insertion of the kanamycin-resistant cartridge in gene *prnD* of *S. plymuthica* HRO-C48 by Southern blot hybridisation. Genomic DNA of HRO-C48 WT and HRO-C48 PRN$^-$ was digested using endonuclease *pst*I prior separation by gel-electrophoresis. Wild-type gene *prnD* obtained by PCR served as probe (left lane). B: Pyrrolnitrin production of wilt-type strain and the insertion mutant was examined by thin-layer chromatography combined with a bioassay using *V. longisporum* as test strain.

4 Discussion

Biological control using either a single strain or a mixture of organisms to protect crops from the damage caused by pathogens is generally considered to be an environmentally friendly alternative to conventional, chemical-based plant protection strategies (Weller 1988; Emmert and Handelsman 1999; Whipps 2001). In the present study, the biological control agent *Serratia plymuthica* HRO-C48, which was formerly evaluated as a BCA of *V. dahliae* in strawberry plants (Kurze *et al.* 2001), was employed in the suppression of Verticillium wilt in oilseed rape and olive plants. In the last decade, Verticillium wilt in both of these crops has become a serious problem worldwide. Due to intensive farming systems, the inocula of soil-borne pathogens reach high densities and have caused severe yield losses in these crops (Jiménez-Diáz *et al.* 1998; Zeise und Steinbach 2004). When investigated under greenhouse conditions, HRO-C48 was shown to suppress the development of symptoms associated with *Verticillium* of both oilseed rape and olive. With regard to the commercial application of the BCA for oilseed rape, potential seed treatment techniques were compared and the reliability of the methods was evaluated. Results from two field experiments using different means of seed inoculation underline the importance of an adequate method for the application of these beneficial bacteria in order to yield successful biocontrol. Understanding the mechanisms by which *S. plymuthica* HRO-C48 inhibits fungal growth and stimulate plant growth, and its expression in an ecological context, promotes the development of novel control strategies. *In vitro* and *ad planta* analyzes of relevant phenotypes revealed that the AHL-mediated quorum sensing system of HRO-C48 represents an essential tool in the regulation of beneficial traits. The relevance of the AHL-regulated phenotypes in the plant-bacterium and plant-pathogen interaction is discussed later.

4.1 Application of *Serratia plymuthica* to oilseed rape

4.1.1 Evaluation of seed treatment techniques

Numerous beneficial members of the genera *Pseudomonas* sp., *Bacillus* sp., *Enterobacter* sp., and *Rhizobium* sp. have been used for biological seed treatment in the control of pathogens and to enhance plant growth (Whipps 1997). The employment of bacterial control agents, which are intended to operate in the rhizosphere of the plant to be protected, demands very specialized application techniques. Inoculation techniques must deliver the BCAs in an adequate number, at the right place and at the right time (Lumsden *et al.* 1995). In contrast to the introduction of beneficial bacteria to strawberry plants by root dipping, the application of

the BCA to seeds should be conducted prior to sowing via an appropriate treatment method. Overall, three seed treatment methods were selected for assessment, and were evaluated according to the following industrial and environmental demands posited by Rhodes (1993): The inoculation process must be easily practicable and to the treated seeds must endure various outside influences during formulation, storage, transportation and sowing procedure without dying or loosing effectiveness (Rhodes 1993). Additionally, the utilized seed treatment procedure has to be compatible with existing technologies, as well as being feasible for large-scale utilization (Powell 1993).

Pelleting is a commonly used method to apply BCAs to seeds (McQuilken *et al.* 1998). The procedure is simple and easy to integrate into common commercial seed treatment practices. For this reason, the method is well practiced and growers have experience with the materials used. Pelleting has an additional advantage: It is possibility to apply other additives during the procedure. After pelleting, seeds are generally larger than prior to treatment, so it is often necessary to adapt the available sowing techniques. The efficacy of pelleted seed in disease suppression and plant growth promotion has already been shown, though both parameters could be significantly enhanced. After the pelleting procedure, seeds must be re-dried in order to prevent germination, and thus the inoculant must survive a period of low water activity (Fravel *et al.* 1998). This is one reason that the storage of pelleted seeds is problematic, as the normal storage temperature of 20°C promotes germination. In contrast, at 4°C germination is almost entirely prevented and the survival rate of *Serratia* cells in the pellet is much higher, but storage at this temperature is costly. In summary, the introduction of *Serratia* by pelleting onto oilseed rape seed is possible and suitable for experiments completed under greenhouse conditions. However, the storage of treated seeds is problematic and/or expensive. This procedure can be further enhanced by adding further beneficial substances. For example, osmoprotectans are one promising category, as they are able to protect bacterial cells against fluctuating salinities and dryness. *S. plymuthica* HRO-C48 produces two different osmoprotective substances, proline and glycin betain (Meincke 2004). These two substances can be employed in two ways: i) to enhance the content of autochthonous osmoprotectans by cultivation in high-saline media, or ii) to add extrinsic substances such as trehalose or glucosylglycerol. The incorporation of bacterial cells by encapsulation to protect them physically against dehydration offers a promising mode of seed inoculation, but the technique is still under development (Patel *et al.* 2004; Tilcher *et al.* 2004)

The second seed treatment procedure investigated was film coating, in which *Serratia* cells were lyophilized directly on the seed coat, resulting in a thin layer surrounding the seed. Lyophilization is a method for the stabilisation of bacterial cells, and is commonly used in both research and industry (Souzo 1992). Freeze-drying bacterial culture has the advantage that the dried material can be stored at room temperature. Several papers have focused on the optimisation of the freeze-drying process to prevent cell damage during processing and storage. Among other affected parameters, the lyophylization medium determines the stress tolerance of treated cells. *Serratia* cells resuspended in a medium containing 1.0% sucrose prior to lyophilization were found to be more stable than those lyophilized at higher sucrose concentrations and those processed in presence of GlcNAc at any concentration. Non-reducing disaccharides like sucrose have been recognized as efficient protective agents for the preservation of many Gram-negative bacteria including the biocontrol strain *P. chlororaphis* MA 100 (Palmfeldt *et al.* 2003). The protective effect has been ascribed to the interaction between proteins and membranes during dehydration (Leslie *et al.* 1995). Nevertheless, in order to improve the survival rates of *S. plymuthica* exposed to freeze-drying, the influence of the physical condition of the cells, freezing rate, freeze-drying conditions, rehydration conditions, and initial cell concentration deserve further investigated. After the procedure of lyophilization, bacteria form a very thin layer around the seed, and the resultant seeds are comparable in size to those left untreated. No new machines or strategies would be required if coated seeds are to be used in agricultural practice. Additionally, only a little additional material is necessary in order to implement this procedure. The major disadvantage is that the freeze drying itself is too expensive. In comparison to the other treatments, *Serratia* cells did not show a higher survival rate on coated seeds. In addition, a negative impact was found in plant growth rates and disease suppression ability compared to cells of HRO-C48 modified by pelleting or bio-priming. A possible reason for this is the fact that lyophilized and re-activated bacterial cells could suffer diminished activity. The rehydration of lyophilized bacteria is vital and is mainly influenced by the medium used (Costa *et al.* 2000). Once the seed is placed into the soil, a controlled rehydration of the cells is impossible. In conclusion, this method proved to be unfeasible for the application of *Serratia* on rape seeds.

The third procedure which was used to apply *Serratia* cells on seeds is referred to as bio-priming. Seed priming is a well established procedure, especially in vegetable cultivation. It is used to get a faster and more uniform germination of seeds (Gray 1994). In addition, bio-priming was used to apply BCAs on seeds for the biological control of *Pythium ultimum*

preemergence damping-off in sweet corn (Callan *et al.* 1990). This procedure is practicable and integrable into current agricultural methods. However, the effectiveness of bio-priming depends on the cultivar as well as on the quality of seeds. To prevent loss in germination capacity, a preliminary experiment is necessary for the optimisation of the bio-priming process. To obtain an optimal cell density in the seed, the procedure should be stopped in the middle of the activation phase of germination, as has been shown to be most effective in the present research. At this time, water uptake is finished and the maximum number of bacterial cells has been integrated. The survival of *Serratia* cells within rape seeds was excellent in the experiments done. At 4°C, survival rates were comparable to pelleted seeds, but at 20°C the survival rate surpassed that of the other treatments. In contrast to other methods where the bacteria are entirely on the surface of the seed, bio-priming establishes bacterial presence inside the seeds, meaning they are more protected against unfavourable abiotic and biotic conditions. Moreover, despite re-drying after infiltration, the degree of moisture within the seed remains quite constant at 4.0 to 6.0%. Seeds of oilseed rape are also naturally colonized by bacteria, and an impressive proportion of them were antagonistic towards *Verticillium,* as shown in a study published by Graner *et al.* (2003). *S. plymuthica* belongs to the autochthonous inhabitants of the oilseed rape rhizosphere (Berg *et al.* 2002, Berg *et al.* 2006). Another advantage of this method is that no negative interaction occurred between the bacteria and insecticides and fungicides, which were often applied to the seed surface of oilseed rape. For example, during the study it was found out that the insecticide Chinook (Bayer, Dormagen, Germany), which is strictly used for rape seeds, has a lethal effect on *Serratia* cells when it was applied in combination (data not shown). Concluding, bio-priming is an excellent and innovative method to apply *Serratia* to oilseed rape seeds.

In summary, it is possible to apply an effective number of *Serratia* cells to oilseed rape seeds to promote an establishment in the rhizosphere of oilseed rape roots under greenhouse conditions. In addition, suppressive effects on *V. longisporum* as well as an enhancement in plant growth resulted. However, the three different formulation procedures for *S. plymuthica* HRO-C48 which were compared yielded in quite different results. The evaluation scheme for seed treatment procedures focused on the requirements defined by Rhodes (1993) and Powell (1993), and is shown in Table 17. To summarize, the present study demonstrates the potential for *S. plymuthica* HRO-C48 applied by bio-priming to be an environmentally friendly alternative for the protection of oilseed rape against Verticillium wilt. Nonetheless, the results of these greenhouse trials were used to compare the seed treatments and do not reflect the real efficiency of *S. plymuthica* under field conditions. In the next step of the investigations, the

results must be verified in soils naturally infested with *V. dahliae*. The field performance of HRO-C48 applied by pelleting and bio-priming has also been assessed, and the results are discussed in the next chapter.

Table 17: Evaluation scheme of the seed treatment techniques pelleting, film coating and bio-priming derived from experiments under laboratory and greenhouse conditions

Evaluated parameter	Pelleting	Film coating	Bio-priming
Principle	HRO-C48 embedded in a matrix consisting of a mineral carrier and binding compound applied around the seed by using rotating drum	HRO-C48 applied as a thin layer around the seed by wetting and stabilizing by lyophilization using sucrose as protective agent	Seeds are infiltrated by HRO-C48 during seed imbibition
Cell density per seed (log_{10})	7.0	7.0	6.0
Shelf-life at 20°C	Insufficient	Insufficient	Sufficient
Impact of Chinook on viability	Yes	Yes	No
Impact on the ability of HRO to			
- Colonize the rhizosphere	No	No	No
- Promote plant growth	No	Negative	No
- Suppress Verticillium wilt	No	Negative	No
Influence on seed germination	No	No	No
Practical feasibility/integration into organizational framework	Means available/ integration requires minor modifications	Means not available/ integration may be expensive	Means available/ Priming is not commonly used for oilseed rape and has to be integrated as an additional process

4.1.2 Effect of *S. plymuthica* HRO-C48 on oilseed rape under field conditions

Field experiments under near-commercial conditions have been indispensable in the evaluation of a biological control agents and their optimal formulation. To test the effect of *S. plymuthica* HRO-C48 on oilseed rape cultivar 'TALENT', field trials were conducted over a two-year period. The seed material for the first field trial was inoculated with HRO-C48 utilizing small-scale pelleting equipment capable of handling batch sizes of up to 5 kg. The three variants applied in the experiments were initially dressed with a liquid insecticide (Chinook), powdery fungicide (DMM) and water adsorbing mineral (talcum) representing standard coating. Two variants were additionally coated with a layer consisting of either talcum or diatomite carrying *S. plymuthica* cells. The composition of the bacteria-carrier matrix was linearly up-scaled from the laboratory-proven formulation. In contrast to the cell numbers achieved in the laboratory, only few bacteria survived the pelleting process. Instead

of $\log_{10}$ 7.0 cells per seed, mean bacterial counts of 10 to 100 could be recovered from the coatings of seeds treated with the pilot machine. The consequent one-week storage resulted in even higher loss rates which continued to increase until the point of sowing. It can be argued that the low initial inoculum density was not sufficient for a stable establishment of HRO-C48 in the rhizosphere of the germinated plant, which was confirmed by low and inconsistent cell counts at the two-leaf and six-leaf stage. No *Serratia* cell endured the winter dormancy period. As far as biocontrol activity is concerned, a certain population density of the introduced BCA is necessary for accurate assessment (Chin-A-Woeng *et al.* 2000). Hence, data gathered from this field trial in relation to the effect of HRO-C48 on plant condition, plant health and yield should be considered with care. The problems during the seed treatment procedure and the storage merit discussion. There were several factors which probably negatively influenced the vitality of the inoculum: i) occurrence of mechanical stress, especially shear forces, within the pelleting drum, which ii) generate temporary high temperature; iii) intimate contact between the bactericide (Chinook) and the bacteria; and iv) drying of the pelleted seeds using warm air flow during subsequent storage. Apart from the conventional procedure, seed treatments which include the incorporation of living cells face new demands. Although existing industrial pelleting equipment could be easily adjusted for bacterial treatment, the management of bacteria requires gentle handling. Particularly, the physical stress on the bacteria caused by dynamic friction should be minimized. An additional critical factor is the moisture level in the bacterial environment. The water content may not exceed 10%, above which seeds start to germinate during storage. On the other hand, bacteria are very sensitive to dehydration. For Gram-negatives, this is especially the case. Certain strategies, such as the choice of appropriate carrier substrates and the employment of osmoprotective additives, have been discussed in chapter 4.1.

The inoculation of seeds by bio-priming provides a method by which bacterial cells are placed inside the seeds; protected against outside influences: dryness, mechanical forces, and bacterizial additives. This technique was utilized for the inoculation of seeds applied in the 2004/2005 field experiment. At four field sites across Germany, which are different in soil characteristics and climatic conditions, the performance of *S. plymuthica* HRO-C48 on plant condition before winter dormancy. In this trial, plant height at the flowering stage, and yield as well as premature ripeness was monitored. In addition, the population densities of HRO-C48 in the rhizosphere and its influence on indigenous microflora were assessed at the field site in Hohenlieth. Compared to the previous field trial, *Serratia* cells were recovered throughout the growing season, indicating that bio-priming is a very effective and applicable

technique. On plant roots grown from bio-primed, non-coated seeds, high colonization rates of *S. plymuthica* were observed. With a cell count of $\log_{10}$ 7.4 and 5.0 CFU g^{1} rfw on average at the six-leaf sand rosette stage, respectively, and with a stable population density of 5.0 after the winter dormancy through the fully-ripe stage, HRO-C48 could be considered to be well established. Scher *et al.* (1994) proposed that $\log_{10}$ 3.5 CFU g^{1} rfw represents the minimal cell number required of the introduced bacterium to be recognized as a root colonizer. *S. plymuthica* strain HRO-C48 were shown to be competent in long-term colonizing of the rhizosphere of other plants as well, such as olive (this study) and strawberry (Kurze *et al.* 2001; Scherwinski *et al*. 2006). Isolates of species *S. plymuthica* were found in various host plants and habitats (Kloepper *et al.* 1992; Berg 2000; Kamensky *et al.* 2003; Berg *et al.* 2005b). Thus, this species can be generally considered as a typical member of plant associated habitats (Berg 2000).

Conversely, low counts of re-isolated HRO-C48 cells applied in combination with pesticides were observed at the at the six-leaf stage, which is believed to be attributable to the presence of the insecticide Chinook. The lethal action of Chinook on *S. plymuthica* was already confirmed from agar diffusions tests. Astoundingly, the *Serratia* population recovered through the rosette stage and the numbers resembled those in the rhizosphere of plants from seeds which were not treated with Chinook. Now the question arises as to whether the population at the rosette stage developed from a few cells which withstood the impact of Chinook, or conversely that the majority of bacterial cells enter a state termed viable but not culturable (VBNC) as they came into contact with the agrochemical substances. Troxler *et al.* (1997) have shown that *Pseudomonas fluorescens* strain CHA0 enter a dormant state in which they were viable but not culturable in order to withstand environmental stress. The authors of this study suggest that the occurrence of nonculturable cells of *Pseudomonas* in the rhizosphere was influenced by climatic factors including water availability and soil temperature. No studies about the persistence of Chinook in the rhizosphere have been performed. However, the population of HRO-C48 recovered, probably because *Serratia* overcome the state of VBNC in more beneficial conditions (Nilsson 1991).

Despite no significant effect of *S. plymuthica* HRO-C48 on oilseed rape being observed, the field trial 2004/2005 revealed an overall positive influence of *Serratia* on plant condition at all field sites. In general, none of the inoculated plants were negatively affected by either the priming process or the BCA. A high incidence of premature ripeness was observed at all field sites, obviously caused by a mix of pathogens including *V. longisporum*, *Lepthospheria*

maculans, Sclerotinia sclerotiorum and *Alternaria brassicae*. The parameter of premature ripeness was scored without regard to the pathogenic cause. However, Verticillium wilt was reported to be very evident at all field sites (Frauen and Baer, pers. comm.). In contrast to Moosburg, at the field sites in Hohenlieth and in Hovedissen the disease severity was slightly less. The spatial distribution of the control agent on the root surface plays an important role in the relationship with soil-borne fungus and may explain the discrepancies between the biocontrol efficiencies under greenhouse and field condition. Bacteria adhere to root surfaces in biofilm-type structures that vary from small clusters of cells to extensive biofilms (Götz *et al.* 2006). Among other things, the surface and morphological properties of the plant root, nutrient and water availability, and the proclivities of the colonizing bacteria strongly influence the resulting biofilm structure. The underground part of oilseed rape is characterized as a tap root system. In fact, the specific development of the root morphology depends on the soil structure. The roots of plants grown in propagation compost, which was used for greenhouse trials, can be described as a nearly fibrous root system with a less prominent tap root and many longer branch roots. In contrast, oilseed rape growing under field condition shows a strong tap root with fewer small branch roots. Investigations on spatial distribution of HRO-C48 on the surface of oilseed rape roots by means of fluorescence techniques and confocal laser scanning microscopy (CLSM) are necessary to align the compatibility of *Serratia* and the pathogenicity of *V. longisporum*, particularly at which location HRO-C48 establishes itself and where *V. longisporum* attacks (Zhou *et al.* 2006). Additionally, soil-borne fungal pathogens are known to enter via damaged root tissue. Recently, the cabbage maggot (*Delia brassicae bouche*) became of increasing relevance in oilseed cropping in Germany. In both field trials, the experimental plots were threatened by the insect, whose larvae cause damage both in the autumn and the spring. Resulting lesions represent likely locations for the entry for soilborne fungal pathogens (SeedQuest 2005). More generally, root tissue damaged done by various animal pathogens provides a preferential zone for fungal infection. Interaction of *V. dahliae* with nematodes has been observed by Martin *et al.* (1982) and Wheeler *et al.* (1994).

Improvements in crop yield of 2 to 5% were recorded at the more southern locations in Hovedissen and Moosburg. From an economical viewpoint, the achievement of such levels of yield increase, the application of biocontrol agents to seeds is a viable option. However, crop yield enhancement did not take place in Hohenlieth. Variation in environmental factors is an important reason for inconsistent biocontrol and plant growth promotion in the field. Therefore, a thorough understanding of the influence of environmental factors on biocontrol

agents is essential for successful biocontrol. The variables can be abiotic or biotic in nature. The survival and activity of the BCA can be influenced by numerous abiotic factors such as soil quality, soil temperature and soil moisture (Dupler and Baker 1984; Davis and Whitbread 1989; O'Callaghan *et al.* 2001; Landa *et al.* 2004; Schmidt *et al.* 2004). Furthermore, the stimulation of plant growth caused by the bacteria was often related to the nutrient status of the plant. A decrease in the content of nutrients such as P, K, S, Mo, and Ba in shoots resulted in a significant growth-promoting effect of ACC-utilizing rhizobacteria on oilseed rape (Belimov *et al.* 2002). Several environmental parameters were recorded in the context of the field experiment 2004/2005, including climatic conditions (soil moisture, soil temperature), soil characteristics and agricultural data (crop history, application of pesticides and soil fertilizer). In particular, the soil type and the soil temperature of Moosburg and Hovedissen were clearly different compared to the plots in Hohenlieth. The soil at the latter field site consisted of loamy sand, whereas the soil type 'sandy loam' was dominant at the other locations. Moreover, the soil temperature in Moosburg and Hovedissen during the vegetation periods before and after winter dormancy was between 1.5°C and 2.0°C higher than at the more northern field site. The low significance of outcomes in terms of the plant health and crop yield does not lend itself to speculation about the impact of the abiotic environmental factors on the biocontrol activity of *S. plymuthica* HRO-C48.

On the other hand, biotic factors clearly impact the efficacy of beneficial bacteria. For example, the population size of the BCAs may be decreased by predatorial bacteria (*Bdellovibrio* sp.) (Elsherif and Grossman 1996; Wilkinson 2001), bacterial phages (Keel *et al.* 2002), protozoas and nematodes (Elsherif and Grossman 1996). Non-predatorial but competing microorganisms hinder the establishment of the introduced bacterium in the rhizosphere, and therefore limit the expression of beneficial traits (Pierson and Weller 1994).

The technique of applying *S. plymuthica* HRO-C48 oilseed rape seeds determines the quantity of establishment of the BCA in the rhizosphere. Pelleting, applied for the first field experiment, was shown to be unsuitable for the inoculation of seeds, because the procedure *per se*, the low water potential in the formulation and the intimate contact with the insecticide Chinook extremely stressed the bacterial cells. Low cell numbers found on the seeds at the point of sowing resulted in an inadequate colonization of the plant root. On the other hand, the inoculation method of bio-priming, by which the bacteria located inside the seed, possesses the advantage that the *Serratia* is less exposed to environmental impacts such as mechanical forces and drought. Starting from the inner seed *S. plymuthica* effectively colonize the

rhizosphere. The colonization ability of the BCA was proved to be impended by standard pesticides additionally applied to the seed. The active substances DMM and Chinook provide fundamental advantages in oilseed cropping and are not called into question. Thus, biological seed treatment has to be compatible with commercially standardized measures. Overall, positive effects of *S. plymuthica* on disease incidence and yield of oilseed rape was found. In the future, further field trials are necessary to confirm the performance of HRO-C48, particularly on different cultivars.

4.1.3 Impact of the introduced *S. plymutica* HRO-C48 on the indigenous microbial community in the rhizosphere of oilseed rape

As the BCA was artificially introduced to the oilseed rape roots, the bacterium occupies an ecological niche and may therefore suppress other organisms via competition, antibiose and/or lyses. The impact of *S. plymuthica* HRO-C48 may evoke changes in the non-target soil microflora and affect the microbial balance in the soil ecosystem. The single strand conformation polymorphism (SSCP) analysis provides an excellent tool for the cultivation-independent study of the composition of the dominant members of the microbial community. This method was utilized to assess the influence of HRO-C48 on the rhizosphere of oilseed rape. In general, the development stage of the plant was demonstrated to be the major determinant of the structure of the microbial community. Different stages of the plant vegetation cycle are associated with distinct composition of the rhizodeposits which modulate the root-associated microbial populations (Mougel *et al.* 2006). In neither of the two field trials an impact on the introduced BCA was found. Although *S. plymuthica* was highly abundant in the 2004/2005 growing season, no distinct band corresponding to the PCR-product of HRO-C48 was observed in the community profiles. Furthermore, no effect of *Serratia* on the eubacterial community of oilseed rape could be detected during the growing period. This is in agreement with studies carried out by Scherwinski *et al.* (2006), who investigated the impact of *S. plymuthica* HRO-C48 on the rhizospheric microflora associated with strawberry plants. Investigation of closely related bacterial groups such as *Pseudomonas* or the group of beta-proteobacteria by using group-specific primers can deliver more detailed information about the influence of HRO-C48. Additionally, Lieber *et al.* (2002) have generated an oligonucleotid, which specifically amplifies the 16S rDNA of bacteria belonging to the genera *Klebsiella* and *Serratia*.

4.2 Effect of *S. plymuthica* HRO-C48 applied to olive trees

Management of Verticillium wilt in olive plants is important and should be based on an integrated strategy (Tjamos 1993). That necessarily includes the distribution of healthy olive plants by nurseries as a pre-planting measure. Although olives can recover from a mild Verticillium wilt, this recovery is of minor value for tree nurseries, because die-back causes high losses and the recovered trees still carry the infectious *V. dahliae* (Jiménez-Díaz *et al.* 1998). Mercado-Blanco *et al.* (2004) have described the first-time employment of antagonistic bacteria to protect nursery-plants against *V. dahliae*. In planting material of the highly susceptible olive cultivar 'Picual', the introduction of a root-associated pseudomonad resulted in suppression of the defoliating pathotype of *V. dahliae*. Engaging a similar experimental design, but using the susceptible cultivar 'Arbequina', the capacity of *S. plymuthica* HRO-C48 to protect young olive plants against *V. dahliae* was investigated. For comparison, the endophytic, olive-root associated *P. fluorescens* strain PICF7, efficient in controlling *Verticillium* in 'Picual', was also employed (Mercado-Blanco *et al.* 2004).

Both, the autochthonous PICF7 and HRO-C48, were shown to be able to colonize the rhizosphere as well the endorhiza until 168 days post-inoculation. The cell densities of *Serratia* and *Pseudomonas* in root-surrounding soil declined throughout the experiment as was already described for HRO-C48 in oilseed rape (this study) and strawberry (Kurze *et al*. 2001). When applied in combination, colonization of the rhizosphere of neither of the BCAs was influenced by the other one. Despite the microbial community structure in the rhizosphere being plant-specific (Garbeva *et al.* 2004), the successful introduction of the allochthonous HRO-C48 was possible. For example, *P. fluorescens* strain NBRI2650 isolated from chickpea, effectively colonized the roots of several other crops (tomato, cotton, and cucumber) (Nautiyal *et al.* 2002). Focussing on population dynamics within the endorhiza, *P. fluorescens* PICF7 cells were found to be more competent than HRO-C48. However, when *S. plymuthica* was inoculated separately, the strain migrated into the internal root tissue to a similar extent to PICF7. Surprisingly, the colonization behaviour of HRO-C48 was negatively affected by the presence of *P. fluorescens,* indicating that PICF7 is the better competitor compared to HRO-C48 which provide an advantage in colonizing the habitat. The environmental conditions in the endosphere are more specific than those in the rhizosphere, and therefore favour the establishment of specifically olive-adapted bacteria (Raaijmakers and Weller 2001).

The ability to promote plant growth in the absence of the pathogen was determined by recording plant length and stem diameter. After 90 days, these parameters were measured. No statistically significant differences between the bacterial treatments and the non-inoculated control were observed. This is in agreement with the study of Mercado-Blanco *et al.* (2004) who could not observe any enhancement in plant growth within this time period. In contrast, for plants monitored for 168 days, a statistically significant increase in the plant length was observed. Woody plants such as olives are known to grow relatively slowly. Accordingly, effects originating from bacterial activity ought to manifest in a long-term scale. Hence, experiments to evaluate plant growth promotion should be of duration longer than five months.

In order to evaluate the ability of *S. plymuthica* HRO-C48 and *P. fluorescens* PICF7 to suppress the D-pathotype of *V. dahliae*, the fungus was applied twice. After the first infestation with fungal conidia by soil inoculation, no visible symptoms caused by the pathogen were observed during the first 90 days of the experiment, probably due to the reduced physiological activity of the olive trees. For the second inoculation *V. dahliae* was either incorporated into the soil or applied directly to the root by dipping in a solution containing fungal condidia. The ability of the bacteria to delay the development of symptoms caused by the defoliating pathotype of *V. dahliae* depended on the inoculation procedure. For *S. plymuthica* HRO-C48, a suppression of the Verticillium wilt was found when *V. dahliae* was applied by soil inoculation rather than by root dipping. Conversely, PICF7 was more effective when the fungus was applied by root dipping. This is likely due to the fact that the mechanism of both bacteria is different and takes place in different microhabitats. PICF7 was isolated from the endosphere of olives, and studies on the antagonistic mechanisms suggested the involvement of siderophores in the suppression of *Verticillium* (Mercado-Blanco *et al.* 2004). *P. fluorescens* PICF7 were established in both rhizosphere and endorhiza, but were more abundant in the latter habitat. In contrast, HRO-C48 appeared to be more rhizosphere-competent. When compared to the soil infestation method, it is apparent that the rhizosphere of plants dipped into the conidial suspension was disturbed by this procedure. It could be speculated that the cells of HRO-C48, which function from the outer surface, were washed off by the immersion and were thus unable to prevent the root from penetration by fungal hyphaes. The endophytic population of PICF7 was not affected by the inoculation method; and was subsequently active in plant protection. Incorporation of the fungal inoculum into the growth substrate represents the more realistic form of *Verticillium* infestation. In that case, the rhizosphere and its bacterial community remain intact. The putative ‘protective shield’ formed

by *S. plymuthica* was able to impede the fungal attack. Surprisingly, the initial inoculation with both BCAs in combination didn't result in an enhanced disease suppression level in any of the infestation approaches. Quite the contrary, plants inoculated with both BCAs suffered Verticillium wilt at rates comparable to the control or even worse.

In conclusion, under conditions similar to those in the olive-nursery industry, the effect of *S. plymuthica* HRO-C48 on disease development and plant growth was demonstrated. The biocontrol strain was singularly applied at the start of the experiment by root dipping and was shown to colonize the root during the experiment period of 169 days. Importantly, even three months post-inoculation, HRO-C48 was shown to provide some degree of disease protection. It can be suggested, that once HRO-C48 is introduced, the BCA may provide a long-term protection against soil-borne fungi, even when the plant is replanted into new, potentially infested soil.

4.3 Significance of AHL-signalling molecules in the biocontrol activity of *S. plymuthica* HRO-C48

The production of extracellular signalling molecules, which mediate cell-to-cell communication, is widespread among Gram-negative bacteria (Eberl *et al.* 1999). Operating as autoinducer in response to the population density, those molecules are involved in the regulation of specific set of genes encoding products, which are presumably of benefit to the bacteria in particular habitats. In particular, AHL activity within plant-associated bacterial communities was demonstrated to be a commonplace event (Cha *et al.* 1998; Berg *et al.* 2002; Loh *et al.* 2002a; 2002b). Almost all isolates from different host plants of the genera *Agrobacterium*, *Pantoea* and *Rhizobium*, and more than half of the *Erwinia* and *Pseudomonas* species were AHL-producers (Cha *et al.* 1998). About 10% of bacteria colonizing the rhizosphere of wheat and tomato were identified to produce signalling compounds (Pierson *et al.* 1998; Steidle *et al.* 2001). Berg and associates (2002) analyzed 32 members of the family of *Enterobacteriaceae* originated from the rhizosphere of strawberry, oilseed rape and potato on their ability to release AHL-like signalling compounds. Studies done by Steidle *et al*. 2001 and Schuhegger *et al*. 2006 have proved *in situ* activity of signalling molecules in the rhizosphere of tomato plants. *S. plymuthica* HRO-C48 was also shown to produce of a set of AHLs including *N*-butanoyl homoserine lactone (BHL), *N*-hexanoyl homoserine lactone (HHL) and *N*-3-oxohexanoyl homoserine lactone (OHHL) (Westendorf 2006). These

components are also dominantly present in the AHL profiles of several members of the genus *Serratia* (Wei and Lai 2006).

In order to exploit in which extent the AHL signalling molecules are involved in the biocontrol activity and the expression of plant-beneficial features, two AHL-defective derivates of *S. plymuthica* HRO-C48 were studied. One of the derivates, designated as HRO-C48 AHL-4, carries a miniTn5 transposon in the gene *spI,* which encodes an AHL-synthase. The AHL synthesis of HRO-C48 AHL-4 is strongly impaired (Westendorf 2006). Nevertheless, the *splI* mutant release residual levels of AHLs, tentatively being a 3-hydroxy-substituted AHL, indicating that *S. plymuthica* possesses another QS system beside the SplIR system (Chernin pers. comm.). The introduction of cloned *splI* genes into the HRO-C48 AHL-4 mutant has been demonstrated to completely restore its AHL pattern to that of the parental strain HRO-C48 (Chernin pers. comm.). The transformation of HRO-C48 with the hybrid plasmid pME6863 bearing the AHL-lactonase gene *aiiA* of *Bacillus* sp. strain A24A resulted in a derivate that was totally deficient in AHL release. The transconjugant was referred as HRO-C48 pME6863. The QS quenching effect of the genetically introduced lactonases provides a commonly used strategy to investigate AHL-regulated phenotypes in bacteria, but the method lacking possibilities to verify the obtained results by, for example, complementation experiments (Reimmann *et al.* 2002).

Both mutants HRO-C48 AHL-4 and HRO-C48 pME6863, exhibit a similar antifungal activity against *V. longisporum in vitro* compared to the wild-type strain. Contrarily, the AHL-deficient derivates were unable to suppress Verticillium wilt in the pathosystem *V. longisporum* oilseed rape. Although several antagonistic traits have been identified to be regulated by QS, only a few studies have demonstrated that disruption of AHL-dependent means of communication correlates with reduced suppression of plant pathogens *ad planta*. The present study indicates that AHL-mediated QS is essentially involved in the biocontrol activity of *S. plymuthica* HRO-C48. A similar observation was reported by Zhou *et al.* (2003). An AHL-deficient mutant of biocontrol strain *Burkholderia ambifaria* BC-F loss its ability to suppress damping-off in cucumber caused by *Pythium ultimum*. Wei and Zhang (2006) showed the involvement of AHLs in the biocontrol of wheat take-all disease by *P. fluorescens* strain 2P24. The AHL-negative mutants were less effective in controlling *Altenaria alternata ad planta*.

Many plant-associated bacteria possess quorum sensing systems to control specific biological functions (Pierson *et al.* 1998). Depending on the bacterial species, phenotypes regulated by quorum sensing are extremely diverse and specific. Recent advances revealed the regulatory function of AHLs in coordinative behaviour of rhizobacteria includes biofilm formation, motility, biosynthesis of antibiotics, and excretion of biosurfactants, siderophores and exoenzymes (Whitehead *et al*. 2001). In *S. plymuthica* strain HRO-C48 the control of various phenotypes were shown to be influenced by AHLs (Table 18). Particular investigations on strain HRO-C48 AHL-4 (biofilm formation, chitinolytic and proteolytic activity) were done by collaborators and were not included in the experimental work of the present thesis. Nevertheless, the results of these studies were included in the discussion.

Table 18: Summary of tests of phenotypes of AHL-defective strains HRO-C48 AHL-4 and HRO-C48 pME6863 compared to the wild-type strain. Symbols indicate no differences (0); an increase (+); a decrease; and type of differences unclear (+/-).

Phenotypes	HRO-C48 AHL-4	HRO-C48 pME6863	Strains in which the phenotype was reported to be QS-regulated
Root colonization	0	0	*P. fluorescens* 2P24 (Wei and Zhang 2006)
Swimming motility	+	+	*P. syringae* (Quinones *et al.* 2005); *Vibrio fischeri* (Lupp and Ruby 2005)
Biofilm formation	+[1]	0	*S. liquefaciens* MG1, *P. putida* IsoF (Steidle *et al.* 2002)
IAA production	+	+	Unknown
Proteolytic activity	-[1]	-	*Burkholderia cepacia* H111 (Huber *et al.* 2002), *P. aeruginosa* (Winson *et al.* 1995)
Chitinolytic activity	-[1]	-	*B. cepacia* H111 (Huber *et al.* 2002); *P. aeruginosa* (Winson *et al.* 1995)
Lipolytic activity	0	0	*S. proteomaculans* B5a (Christensen *et al.* 2003)
Siderophore production	0	0	*B. ambifaria* BC-F (Zhou *et al*. 2003)
Pyrrolnitrin production	-	-	Unknown
Emission of VOCs	+/-	+/-	Unknown

[1] Chernin, pers. comm.

Recent studies highlighted the importance of biofilm structures in initiating and maintaining contact with the plant host representing an intrinsic component of plant microbe interactions. (Stanley and Lazazzera 2004). Plant-associated bacteria interact with host tissue surfaces during pathogenesis and symbiosis, and in commensal relationships. Regulation by QS has

been implicated in the formation of biofilms by various bacteria including plant-associated members of the genera *Pseudomonas*, *Burkholderia* and *Serratia* (Davies *et al.* 1998, Eberl 2006; Wei and Lai 2006). Applying microtiter plate assay to study the capacity to form biofilms, the absolute biomass attached to the polystyrene surface was recorded, and no differences between the wild-type strain and strain HRO-C48 pME6863 were observed. In contrast, in HRO-C48 AHL-4 statistically significant increase in the extent of biofilm formation was stated by Chernin (pers. comm.). The explanatory power of the crystal violet staining is limited because only the absolute coverage of surface by the bacterial cells is monitored, but not the structure of the biofilm. The AHL dependency of the structural biofilm development of *P. putida* IsoF has been observed by Steidle *et al.* (2002). An isogenic AHL-negative derivate of IsoF formed more structured biofilms compared to the wild-type strain. Clearly, to monitor structural differences in biofilms formed by HRO-C48 WT and the AHL-deficient derivates, advanced microscopic techniques has to be employed. Changes in biofilm formation are generally a consequence of differently expressed genes responsible for synthesis of biosurfactans, surface adherence and motility (Deziel *et al.* 2001).

S. plymuthica HRO-C48 does not produce biosurfactans and exhibits no swarming motility. However, the ability of the BCA to actively spread in/on a medium containing 0.3% agar was shown and was recognized as swimming motility mediated by chemotaxis (Eberl *et al.* 1996). Studies carried out by de Weger *et al.* 1987 indicated that flagella are required for colonization of potato roots root. Turnbull *et al.* (2001) suggested recently a dual role for flagellum-mediated motility in *E. coli* and *P. aeruginosa* biofilm formation in which flagella promote initial cell-to-surface contact and also contribute to the spreading of a growing biofilm along an abiotic surface. Our results pointed out that swimming motility in HRO-C48 is negatively controlled by the splI/splR QS system meaning that *Serratia* cells were agiler in absence of an adequate concentration of AHL molecules. Similar observations were reported by Atkinson *et al.* (2006): In *Yersinia pseudotuberculosis* and *Y. enterocolitica* AHL-dependent quorum sensing is involved in the control of swimming motility via the flagellar regulatory cascade. In contrast, the positive regulation of swarming motility by quorum sensing was demonstrated for *Burkholderia cepacia* and *S. liquefaciens* (Daniels *et al.* 2004). Besides swarming all of the tested strains possess flagella-mediated swimming motility. Mutants defective in AHL synthesis were negatively affected in its swarming ability when exposed to a surface, whereas the flagella-driven swimming motility was throughout the mutants indistinguishable from the wild-type strains.

The relevance of bacterial-borne IAA in the plant-microbe interaction implies primarily the stimulation of plant growth conferring a well known mode of beneficial relationship between rhizobacteria and plants (Costacurta and Vanderleyden 1995). Besides the function of IAA as a plant growth regulator, the phytohormone has also been implicated as secondary metabolite, which is involved in induction of bacterial genes (Broek *et al.* 1999). The plant growth hormone indole-3-acetic acid stimulates growth of root hairs, a location with increased levels of root excudates (Cambell and Greaves 1990). Enhanced root development potentially extents colonization space and generate additional nutrient sources which can be acquired by the rhizobacteria. In favour of this, the biosynthesis of IAA of *S. plymuthica* HRO-C48 is up-regulated in the mutants deficient in releasing AHLs. It can be speculated that the regulation of IAA biosynthesis is intimately connected with the requirements and receptivity of the host plant. Among others, the effect of IAA on plant growth strongly depends on the present concentration (Libbert 1987). In addition, the IAA uptake by plants depends on specific binding sites, which are found to be inducible by AHLs by tissue-specific activation of beta-glucuronidase reporter fusions to auxin-responsive promoters (Napier and Venis 1995, Mathesius *et al.* 2003). Less abundant bacteria, correlated with low levels of AHLs, excrete higher amounts of IAA to provide effectual amounts of the hormone which are necessary to achieve positive plant response. As the bacterial population reach a critical size the IAA production is reduced avoiding enrichment of IAA in plant environment and reacting on increased receptivity of the plant tissue. The AHL-mediated quorum sensing system represses IAA synthesis in *S. plymuthica* HRO-C48. This in particular and the involvement of AHLs in plant growth hormone activity in general, is firstly demonstrated.

Chitinases and proteases are exoenzymes assumed to play a key role in antifungal activity (Chernin and Chet 2002). *S. plymuthica* HRO-C48 employs a chitinolytic system consisting of three chitinases (Frankowski *et al.* 2001). In addition, extracellular proteolytic activity was found. Both hydrolytic enzymes appear to be controlled by quorum sensing in HRO-C48. This is in agreement with results obtained for *P. aeruginosa*, *B. cepacia* and *S. liquefaciens* in which regulation of both chitinases and proteases have been demonstrated (Winson *et al.* 1995; Huber *et al.* 2004; Eberl *et al.* 1999).

Pyrrolnitrin is a common antifungal compound which has been considered as the key factor in the antagonistic activity of various rhizobacteria (van Pee and Ligon 2000; Ligon *et al*. 2000) is synthesized by enzymes encoded in the operon *prnABCD* (Hammer *et al*. 1997). In the cell

extracts of the wild-type of *S. plymuthica* HRO-C48 the accumulation of bioactive PRN was detected. Analyzing the AHL-deficient mutants, in HRO-C48 pME6863 a reduced level of PRN production was observed, and in HRO-C48 AHL-4 the biosynthesis of PRN was completely abolished. That indicates that PRN synthesis is positively regulated by QS. Quorum sensing systems operating as regulator in biosynthesis of antibiotics was stated for phenazine in *P. aureofaciens* (Wood *et al.* 1997). Members of the genus *Serratia* are shown to possess antibiotic activity which is organized in a cell density manner. In *S. marcescens* the red pigment prodigiosin, and in another *Serratia* spp. the carbapenem production is regulated by an autoinducer-2 dependent QS system (Coulthurst *et al*. 2004). However, the regulatory function of AHLs in the production of the antibiotic PRN is a novel matter of fact in the research of bacterial communication systems. In order to prove whether the deficiency in PRN synthesis is correlated with the impaired biocontrol activity of the AHL-defective strains, a PRN-negative derivate of *S. plymuthica* was generated by site-directed mutagenesis. Despite the degree of disease suppression was slightly reduced compared to the wild-type strain, the *prnD* mutant still provided a control activity. On the other hand, the insertion of the kanamycin-resistance gene destructed the gene *prnD* which encodes the enzyme catalyzing the formation of pyrrolnitrin from the precursor aminopyrrolnitrin. Kirner *et al.* (1998) have reported, however, that the pathway of synthesis from tryptophan to aminopyrrolnitrin is still intact, when *prnD* is knocked out. Aminopyrrolnitrin have a lower antifungal activity than PRN, but the excretion of that compound by HRO-C48 may recover its antifungal activity.

In summary, AHL signalling molecules essentially involved in the regulation of mechanisms responsible for the biocontrol activity of *S. plymuthica* HRO-C48. The mode of action comprises a complex of mechanisms acting in two directions: towards the pathogenic fungus and towards the host plant. Results of the present study indicate the involvement of QS in both the expression of antifungal traits and the plant-bacteria interactions. Remarkably, AHLs activate the expression of antifungal factors (chitinases, proteases and PRN), whereas the signalling molecules supposed to repress the IAA synthesis, the swimming motility and the biofilm formation. In search of the critical point in which AHLs are essentially in charge in the suppression of fungal pathogens, several phenotypes exhibited by HRO-C48 were analyzed on their QS regulation. Some of them, including the production of chitinases and PRN, are potentially responsible for the inhibition of *V. longisporum*. However, mutants impaired in the production of either chitinases or PRN were still able to control Verticillium wilt in oilseed rape. So far, complex regulatory networks which take place in the rhizosphere

are not fully enlightened. In addition to the direct antagonism and plant growth promotion abilities, it is assumed that the AHL molecules excreted by HRO-C48 directly interact with the host plant. Plant response to bacterial signal molecules involves activation of defense mechanisms and changes in primary and hormone metabolism (Bauer and Mathesius 2004). An important hint was recently given by Schuhegger and colleagues (2006) who stated the direct effect of bacterial-borne AHLs by inducing systemic resistance in tomato plants. In particularly, the wild-type strains of *S. liquefaciens* MG1 und *P. putida* IsoF induce systemic resistance in tomato by the activation of salicylic acid- and ethylene-dependent genes.

4.4 Influence of *Verticillium* spp. on the root-associated bacterial community

In both investigated pathosystems, oilseed rape - *V. longisporum* and olive - *V. dahliae*, a significant influence on the pathogenic fungi on the eubacterial community structure was demonstrated. Cultivation-dependent and mostly cultivation-independent methods revealed an affect of the pathogens on the composition of bacteria associated with roots of infected plants. The SSCP patterns, taken from the rhizosphere of plants grown in infested soil and non-infested soil, were clustered by computer-assisted analyzes according to their similarities. Resulting dendrograms showed distinctly clustered groups. The clear separation originated not only from the appearance or disappearance of single bands, but primarily from a general shift within the community. In addition, the effect of *V. longisporum* on the population of the introduced *S. plymuthica* HRO-C48 on oilseed rape roots was selectively monitored by recovering rifampicin-resistant mutants. The cell density of the BCA increased in the presence of the pathogen by approximately one magnitude. Accordingly, a distinct band corresponding to HRO-C48 became clearly visible in the SSCP profiles from the diseased plants. Distinct compositions of bacterial communities in the rhizosphere of pathogen-stressed plants have also been reported for the pathosystems strawberry - *V. longisporum* (Wolf 2004), potato - *R. solani* (Scherwinski unpublished) and avocado - *Phytophtora cinnamomi* (Yang *et al.* 2001). In those studies, clearly different bacteria colonized roots of infected plants compared to healthy roots. Changes in the composition of endophytic communities in potato stems induced by an *E. carotovora* infection were shown by Reiter *et al.* (2001).

Chitin is a common structural element of fungal cell-walls. Vigorous growth of *V. longisporum* in highly infested soil used for biocontrol experiments on oilseed rape allegedly raised the chitin content in the rhizosphere. It can be deduced that the fungal biomass served as nutrient source for chitin-degrading bacteria. In agreement with higher numbers of *S.*

plymuthica HRO-C48 in the presence of the fungus in the pathosytem oilseed rape - *V. longisporum* (Figure 17), Adholia and Alström (2003) have found an increased number of chitinolytic bacteria associated with the root of oilseed rape when chitin was amended to the soil. In general, soil amendments such as chitin were proven to stimulate the microbial populations of different crops (Hallmann *et al.* 1999; Lazarovits *et al*. 2000).

On the other hand, the modification of the composition of rhizosphere-associated bacteria may be caused by general plant response to the infection. Plants stressed by phytopathogens response by a cascade of local and systemic reactions leading to synthesis of pathogenesis-related proteins (PR), stress metabolites (H_2O_2, phytoalexins) and stress signals (jasmonic acid, salicyclic acid and ethylen) (Lichtenthaler 1998). The important role of rhizosphere bacteria in protecting plants against soil-borne pathogens has been emphasised by several authors. It has been suggested that the interaction between a host plant and its rhizospheric microflora is one mechanism by which disease suppression operates (Bauer and Teplitski 2001). However, it is still a matter of speculation to what extent the specificity of the microbial population is involved in the plant's protection and whether the plant actively modulates the composition of bacterial communities. Root exudate release patterns of healthy plant differ from those of infected hosts and may cause shifts in the microbial community. One may expect that variations in rhizodeposits influence root-inhabiting microorganisms (Graystone *et al.* 1998). Reiter *et al.* (2001) proposed that mobilization of specific nutrients facilitating conditions for growth of disease suppressing bacteria during infection. Selective enrichment of plant growth by genetically encouraging bacteria to release increased amounts of opines was noted by Savka *et al*. (2002).

In the course of plant-bacteria interaction, plant-associated bacteria can detect various host-released chemicals, to which they respond in ways that allow them to more effectively colonize their host (Brencic and Winans 2005). In particular, plant reaction to the attack of pathogens comprises the release of signals such as quorum sensing mimicry molecules, which are detected by plant-beneficial bacteria and induce expression of traits necessary for the inhibition of pathogen growth.

Analyzes of the bacterial communities colonizing the rhizosphere of oilseed rape and olive grown under greenhouse conditions revealed that the presence and/or the phytopathogenic action of *Verticillium* cause structural changes in the composition of the root-associated populations. Alterations in the rhizospheric community of infected plants compared to healthy

ones are assumed to be attributable to two factors. There is either a direct effect of the massive abundance of the fungus which represents an effective member of the microbial community or an indirect effect mediated by a specific plant response during the infection by the pathogen. It can only be speculated that modified bacterial rhizosphere populations in diseased plants provides improved protection against plant pathogens. In fact, the population density of *S. plymuthica* HRO-C48, a demonstrably beneficial bacterium, was increased in *Verticillium*-infested plants.

4.5 Outlock

In the present study, an appropriate way to apply *S. plymuthica* HRO-C48 to seeds of oilseed rape was developed and the effect of the BCA on plant health and plant growth under field conditions was assessed. However, the future integration of bio-priming into the seed producing frameworks requires consistent results obtained from additional field trials which support the observations of the experiments performed in this research. Following studies should address the efficacy of the beneficial strain at different environmental conditions using different cultivars.

S. plymuthica were demonstrated to colonize both the rhizosphere and the endorhiza of olive plants for more than five months. Furthermore, the allochthonous bacterium showed some degree of disease suppression as well plant growth promotion. Importantly, the BCA appeared to be generally competent in colonizing the root of various host plants and to exhibit biocontrol activity. Therefore, the employment of *S. plymuthica* to protect other economically relevant *Verticillium* hosts including hop, pepper and maple trees should be considered.

The control activity of *S. plymuthica* HRO-C48 relies on an AHL-based cell-cell communication system. Despite several biocontrol-related phenotypes of HRO-C48 were shown to be QS-regulated, the mechanism particularly responsible for the plant protection effect is not fully clarified. Additional investigations should stress the interaction between the BCA and the host plant. In particular, the ability of *S. plymuthica* to induce systemic resistance in plants is a worthwhile object for future research. Moreover, *in vitro* assays suggest an influence of AHL signalling compounds on the VOC patterns of HRO-C48. Subsequently, it is important to know, in which extent the signalling molecules involved in the emission of VOCs. There are some indications revealing that *S. plymuthica* possesses more than the already identified LuxI/LuxR QS system. The hypothesis of the presence of another QS systems operating in HRO-C48 being in charge in the regulation of plant beneficial traits should be verified.

5 References

Adholia, T. D. and S. Alström. 2003. Impact of culturing conditions on microbial populations associated with two different cultivars of oilseed rape *Brassica napus*. 6th International PGPR Workshop. Calicut, India.

Al-Ahmad, M. A. and M. N. Mosli. 1993. Verticillium wilt of olive in Syria. *Bulletin OEPP/EPPO Bulletin* 23: 521–529.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Jinghui Zhang, Zheng Zhang, Miller, W. and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25: 3389-3402.

Anderson, D.G. and L. L. McKay. 1983. A simple and rapid method for isolating large plasmid DNA from lactic Streptococci. *Applied and Environmental Microbiology* 46: 549-552.

Atkinson, S., Chang, C.-Y., Sockett, R. E., Cámara, M. and P. Williams. 2006. Quorum Sensing in *Yersinia enterocolitica* controls swimming and swarming motility. *Journal of Bacteriology* 188: 1451-1461.

Barbara, D. J. and E. Clewes. 2003. Plant pathogenic *Verticillium* species: how many of them are there? *Molecular Plant Pathology* 4: 297-305.

Bassam, B. J., Caetano-Anolle, G. and P. M. Gresshoff. 1991. Fast and sensitive silver 9 staining of DNA in polyacrylamide gels. Anal. Biochem. 80: 81-84.

Bassler, B. L. 2002. Small talk: cell-to-cell communication in bacteria. *Cell* 109: 421-424.

Bauer, W. D. and M. Teplitski. 2001. Can plants manipulate bacterial quorum sensing? *Australian Journal of Plant Physiology* 28: 913-921.

Bauer, W. D. and U. Mathesius. 2004. Plant responses to bacterial quorum sensing signals. *Current Opinion in Plant Biology* 7: 429–433.

Beckman, C. H. 1987. The nature of wilt disease in plants. The American Phytopathological Society, American Phytopathology Society. St. Paul, Minnesota, USA.

Beckman, C. H. and P. W. Talboys. 1981. Anatomy of resistance. In: Mace, M. C., Bell, A. A. and C. H. Beckman (eds.). Fungal wilt diseases in plants. Academic Press. New York: pp. 487-521.

Bejarano-Alcázar, J., Blanco-López, M. A., Melero-Vara, J. M. and R. M. Jiménez-Díaz. 1996. Etiology, importance and distribution of Verticillium wilt of cotton in southern Spain. *Plant Disease* 80: 1233-1238.

Belimov, A. A., Safronova, V. I. and T. Mimura. 2002. Response of spring rape (*Brassica napus* var. oleifera L.) to inoculation with plant growth promoting rhizobacteria containing 1-aminocyclopropane-1-carboxylate deaminase depends on nutrient status of the plant. *Canadian Journal of Microbiology* 48(3): 189-99.

Berg, G. and G. Ballin. 1994. Bacterial antagonists to *Verticillium dahliae* Kleb. *Journal of Phytopathology* 141: 99-110.

Berg, G. 1996. Rhizobacteria of oil seed rape antagonistic to *Verticillium dahliae*. *Journal of Plant Diseases Protection* 103: 20-30.

Berg, G., Kurze, S. and R. Dahl. 1999. Isolated rhizobacteria for treatment of phytopathogenic fungal diseases. European Patent Nr. 98124694.5.

Berg, G. 2000. Diversity of antifungal and plant-associated *Serratia plymuthica* strains. *Journal of Applied Microbiology* 88: 952-960.

Berg, G., Fritze, A., Roskot, N. and K. Smalla. 2001. Evaluation of potential biocontrol rhizobacteria from different host plants of *Verticillium dahliae* Kleb. *Journal of Applied Microbiology* 156: 75-82.

Berg, G., Roskot, N., Steidle, A., Eberl, L., Zock, A. and K. Smalla. 2002. Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different *Verticillium* host plants. *Applied and Environmental Microbiology* 68: 3328-38.

Berg G., Zachow, C., Lottmann, J., Götz, M., Costa, R. and K. Smalla. 2005a. Impact of soil type and plant species on rhizosphere associated fungi antagonistic to *Verticillium dahliae* Kleb. *Applied and Environmental Microbiology* 71: 4203-4213.

Berg, G., Krechel, A., Ditz, M., Sikora, R. A., Ulrich, A. and J. Hallmann. 2005b. Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. *FEMS Microbiology Ecology* 51: 215-229.

Berg, G., Opelt, K., Zachow, C., Lottmann, J., Götz, M., Costa, R. and Smalla, K. 2006. The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus *Verticillium* differs depending on plant species and site. *FEMS Microbiology Ecology* 56: 250-261.

Birnbolm, H. C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* 7:1513-1523.

Bloemberg, G. V. and Lugtenberg, B. J. J. 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. *Current Opinion in Plant Biology* 4: 343–350.

Blanco-López, M. A., Jiménez-Díaz, R. M. and J. M. Caballero. 1984. Symptomatology, incidence and distribution of Verticillium wilt of olive trees in Andalucia. *Phytopathologia Mediterranea* 23: 1-8.

Blok, W. J., Slomp, C. P., Termorshuizen, A. J. and J. G. Lamers. 1998. Control of soilborne pathogens by induction of soil anaerobiosis. 7th International Congress of Plant Pathology, Edinburgh, Scotland. Invited Papers Abstracts Vol. 2: 2.7.10.

Bradford, K. J. 1995. Water relations in seed germination. In: Kigel, J. and G. Galili (eds.) Seed Development and Germination, Marcel Dekker, Inc., New York. pp. 351-396.

Brencic, A. and S. C. Winans. 2005. Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. *Microbiology and molecular biology reviews* pp. 155-194.

Broek, A. V., Lambrecht, M., Eggermont, K. and J. Vanderleyden. 1999. Auxins upregulate expression of the indole-3-pyruvate decarboxylase gene in *Azospirillum brasilense*. Journal of Bacteriology 181: 1338-1342.

Callan, N. W., Marthre, D. E. and J. B. Miller. 1990. Bio-priming seed treatment for biological control of *Pythium ultimum* preemergence damping-off in sh-2 sweet corn. *Plant Disease* 74, 368-372.

Campbell, C. L. and L. V. Madden. 1990. Introduction to Plant Disease Epidemiology. John Wiley & Sons, New York.

Campbell, R. and M. P. Greaves. 1990. Anatomy and community structure of the rhizosphere. In: J. M. Lynch (ed.) The rhizosphere. John Wiley & Sons, Chichester, England. pp. 11-34.

Cha, C., Gao, P., Chen, Y. C., Shaw, P. D. and S. K. Farrand. 1998. Production of *N*-acyl-homoserine lactone quorum-sensing signals by Gram-negative plant-associated bacteria *Molecular Plant-Microbe Interaction* 11: 1119-1129.

Chernin, L. and I. Chet. 2002. Microbial enzymes in biocontrol of plant pathogens and pests In: Burns, R. and Dick, R. (eds.). Enzymes in the Environment. Marcel Dekker Inc., New York, NY. pp. 171-225.

Chet, I. 1990. Biological control of soil-borne plant pathogens with fungal antagonists in combination with soil treatments. In: Hornby, D. (ed.) Biological control of soil-borne plant pathogens. Wallingford: CAB International, pp. 15-26.

Chin-A-Woeng, T. F., Bloemberg, G. V., Mulders, I. H., Dekkers, L. C. and B. J. Lugtenberg. 2000. Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. *Molecular Plant-Microbe Interaction* 13: 1340-1345.

Christensen, A. B., Riedel, K., Eberl, L., Flodgaard, L. R., Molin, S., Gram, L. and M. Givskov. 2003. Quorum-sensing-directed protein expression in *Serratia proteamaculans* B5a. *Microbiology* 149: 471–483.

Clark, J. D. and O. Maaloe. 1967. DNA replication and the division cycle in *Escherichia coli*. *Journal of Molecular Biology* 23: 99-112.

Cook, R. J., Thomashow, L. S., Weller, D. M., Fujimoto, D., Mazzola, M., Bangera, G. and D. S. Kim. 1995. Molecular mechanisms of defense by rhizobacteria against root disease. *Proceedings of National Academy of Sciences USA* 92: 4197-4201.

Costa, E., Usall, J., Teixido, N., Garcia, N. and I. Vinas. 2000. Effect of protective agents, rehydration media and initial cell concentration on viability of *Pantoea agglomerans* strain CPA-2 subjected to freeze-drying. *Journal of Applied Microbiology* 89: 793-800.

Coulthurst, S., Kurz, C. L. and G. P. Salmond. 2004. LuxS mutants of *Serratia* defective in AI-2-based "quorum sensing": strain dependent impacts on virulence, prodigiosin and antibiotic production. *Microbiology* 150: 1901-1910.

Collins, A., Okoli, C. A. N., Morton, A., Parry, D., Edwards, S. G. and D. J. Barbara. 2003. Isolates of *Verticillium dahliae* pathogenic to crucifers are of at least three distinct molecular types. *Phytopathology* 93: 364-376.

Conn, K. L. and G. Lazarovits. 2000. Soil factors influencing the efficacy of liquid swine manure added to soil to kill *Verticillium dahliae. Canadian Journal of Plant Pathology* 21: 400-406.

Costacurta, A. and J. Vanderleyden. 1995. Synthesis of phytohormones by plant-associated bacteria. *Critical Reviews in Microbiology* 21: 1-18.

Crowe, L. 1995. Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Applied and Environmental Microbiology* 61: 3592-3597.

Daniels, R., Vanderleyden, J. and J. Michiels. 2004. Quorum sensing and swarming migration in bacteria. *FEMS Microbiology Reviews* 28: 261-289.

Davies, D. G., Parsek, M.R., Pearson, J.P., Iglewski, B.I., Costerton, J.W. and E.P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 280: 295 –298.

Davies, K. G. and R. Whitbread. 1989. *In vitro* studies of siderophore production by wild type and rifampicin resistant strains of fluorescent pseudomonads. *Plant and Soil* 116: 123-125.

Debode, J., Spiessens, K., De Rooster, L. and M. Hofte. 2002. Verticillium wilt of cauliflower in Belgium. *Meded Rijksuniv Gent Fak Landbouwkd Toegep Biol Wet*. 67(2): 241-9.

De Souza, J. T. and J. M. Raaijmakers. 2003. Polymorphisms within the prnD and pltC genes from pyrrolnitrin and pyoluteorin-producing *Pseudomonas* and *Burkholderia* spp. *FEMS Microbiology Ecology* 1454: 1-14.

De Weger, L. A., van der Vlugt, C. I., Wijfjes, A. H., Bakker, P. A., Schippers, B. and B. Lugtenberg. 1987. Flagella of a plant-growth-stimulating *Pseudomonas fluorescens* strain are required for colonization of potato roots. *Bacteriology* 169: 2769-73.

De Weger, L. A., van der Bij, A. J., Dekkers, L. C., Simons, M., Wijffelman, C. A., and B. J. J. Lugtenberg. 1995. Colonization of the rhizosphere of crop plants by plant-beneficial pseudomonads. *FEMS Microbiological Ecology* 17: 221–228.

Deziel, E., Comeau, Y. and R. Villemur. 2001. Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpiliated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. *Journal of Bacteriology* 183: 1195-1204.

Dowling, D. N. and F. O'Gara. 1994. Metabolites of *Pseudomonas* involved in the biocontrol of plant diseases. *Trends in Biotechnology* 12: 133–141.

Duniway, J. M. 2002. Status of chemical alternatives to methyl bromide for pre-plant fumigation of soil. *Phytopathology* 92: 1337-1343.

Dupler, M. and R. Baker. 1984. Survival of *Pseudomonas putida*, a biological control agent, in soil. *Phytopathology* 74:195−200.

Eberhard, A. 1972. Inhibition and activation of bacterial luciferase synthesis. *Journal of Bacteriology* 109: 1101-1105.

Eberl, L. 1999. *N*-acyl homoserinelactone-mediated gene regulation in gram-negative bacteria. *Syst Appl Microbiol*. 22: 493-506.

Eberl, L. 2006. Quorum sensing in the genus *Burkholderia. International Journal of Medical Microbiology* 296: 103-110.

Elsherif, M. and F. Grossmann. 1996. Role of biotic factors in the control of soil-borne fungi by fluorescent pseudomonads. *Microbiol Res.* 151: 351-357.

Emmert, E. A. B. and J. Handelsman. 1999. Biocontrol of plant disease: a ($Gram^+$) positive perspective. *FEMS Microbiological Letters.* 171: 1-9.

Fahleson, J., Hu, Q. and C. Dixelius. 2004. Phylogenetic analysis of *Verticillium* species based on nuclear and mitochondrial sequences. *Arch Microbiol*. 181: 435-442.

FAO 2006. Oilseed, oils and oilmeals. In: Food Outlook – Global market analysis. Web source: http://www.fao.org/docrep/009/j7927e/j7927e00.htm.

Fradin, E. F. and B. P. H. J. Thomma. 2006. Physiology and molecular aspects of Verticillium wilt disease cause by *V. dahliae* and *V. albo-atrum. Molecular Plant Pathology* 7: 71-86.

Frankowski, J., Lorito, M., Schmid, R., Berg, G. and H. Bahl. 2001. Purification and properties of two chitinolytic enzymes of *Serratia plymuthica* HRO-C48. *Archieves of Microbiology* 176: 421-426.

Frankowski, J. 2002. Das chitinolytische Sytem von *Serratia plymuthica* HRO-C48 und seine Bedeutung für die biologische Kontrolle der Verticillium-Welke. PhD thesis. University of Rostock, Germany.

Fravel, D. R. 1988. Role of antibiosis in the biocontrol of plant diseases. *Annual Review of Phytopathology*. 26: 75-91.

Fravel, D. R. and A. P. Keinath. 1991. Biocontrol of soilborne plant pathogens with fungi. In: Kleister, D. L.; Cregan, P. B. (eds.): The rhizosphere and plant growth. Kluwer Academic Publishers, Dordrecht, Niederlande. pp. 237-243.

Fravel, D. R., Connik, W. J. and J. A. Lewis. 1998. Formulation of microorganisms to control plant diseases. In: Burgues, H. D. (ed.) Formulation of Microbial Pesticides. Beneficial Microorganisms, Nematodes and Seed Treatments. Kluwer Academic Publisher, London. pp. 187-202.

Fuqua, W. C., Winans, S. C. and E. P. Greenberg. 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *Journal of Bacteriology* 176: 269-275.

Garbe, V. 2000. Rapswelke oder Verticillium-Stängelfäule. In: Bartels, G. and G.F. Backhaus (eds.). Testing of crop cultivars for resistance to noxious organisms at the Federal Biological Research Centre, Part 2: Testing of resistance of field and horticultural crops to fungi, bacteria and viruses. *Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft* 373: 46-47.

Garbeva, P., van Veen, J. A. and J. D. van Elsas. 2004. Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. *Annual Review of Phytopathology* 42: 243–270.

Glick, B. R., Penrose, D. M. and J. Li. 1998. A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. *Journal of Theoretical Biology* 190: 63-68.

Götz, M., Gomes, N. C. M., Dratwinski, A., Costa, R., Berg, G., Peixoto, R., Hagler, L. M. and K. Smalla. 2006. Survival of gfp-taggedantagonistic bacteria in the rhizosphere of tomato plants and their effects on the indigenous bacterial community. *FEMS Microbiology Ecology* 56: 207-218.

Graner, G., Persson, P., Meijer, J. and S. Alstrøm. 2003. A study on microbial diversity in different cultivars of *Brassica napus* in relation to its wilt pathogen *Verticillium longisporum*. *FEMS Microbiological Letters* 29: 269-276.

Gray, D. 1994. Large-scale seed priming techniques and their integration with crop protection treatments. In: Martin, T. (ed.) Seed treatment: Progress and prospects, Monograph 57. British Crop Protection Council, Farnham. pp. 353-362.

Grayston, S. J., Wang, S., Campbell, C. D. and A. C. Edwards. 1998. Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biology and Biochemistry* 30: 369-378.

Gunderson, L. H. 2000. Ecological resilience in theory and application. *Annual Review of Ecology and Systematics* 31: 425-439.

Hallmann, J., Rodriguez Kabana, R. and J. W. Kloepper. 1999. Chitin-mediated changes in bacterial communities of the soil, rhizosphere and within roots of cotton in relation to nematode control. *Soil Biology and Biochemistry* 31(4): 551-560.

Hamm, P. B., Ingham, R. E., Jaeger, J. R., Swanson, W. H. and K. C. Volker. 2003. Soil fumigant effects on three genera of potential soilborne pathogenic fungi and their effect on potato yield in the Columbia Basin of Oregon. *Plant Disease* 87: 1449-1456.

Hammer, P. E., Hill, D. S., Lam, S. T., Van Peee, K. H. and J. M. Ligon. 1997. Four genes from *Pseudomonas fluorescens* that encode thee biosynthesis of pyrrolnitrin. *Applied and Environmental Microbiology* 63: 2147-2154.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* 166: 557-580.

Heale, J. B. and V. K. Karapapa. 1999. The *Verticillium* threat to Canada's major oilseed crop: Canola. *Canadian Journal of Plant Pathology* 21: 1-7.

Hiltner, L. 1904. Über neuere Erfahrungen und Probleme auf dem Gebiet der Bodenbakteriologie und unter besonderer Berücksichtigung der Gründüngung und Brache (On recent insights and problems in the area of soil bacteriology under special consideration of the use of green manure and fallowing). *Arb. Dtsch. Landwirt. Ges*. 98: 59-78.

Huber, B., Feldmann, F., Köthe, M., Vandamme, P., Wopperer, J., Riedel, K. and L. Eberl. 2004. Identification of a novel virulence factor in *Burkholderia cenocepacia* H111 required for efficient slow killing of *Caenorhabditis elegans. Infection and Immunity* 72: 7220-7230.

Jiménez-Díaz, R. M., Tjamos, E. C. and M. Cirulli. 1998. Verticillium wilts of major tree hosts: olive. In: Hiemstra, J. A. and Harris, D. C. (eds.) A compendium of Verticillium wilts in tree species. CPRO-DLO/HRI-EM, Wageningen/West-Malling. pp. 13-16.

Johansson, A., Goud, J. K. C. and C. Dixelius. 2006. Plant host range of *Verticillium longisporum* and detection of microsclerotia in Swedish soils. *European Journal of Plant Pathology* 114: 139-149.

Kalbe, C., Marten, P. and G. Berg. 1996. Members of the genus *Serratia* as beneficial rhizobacteria of oilseed rape. *Microbiological Research* 151: 4433-4400.

Kamensky, M., Ovadis, M., Chet, I. and L. Chernin. 2003. Soil-borne strain IC14 of *Serratia plymuthica* with multiple mechanisms of antifungal activity provides biocontrol of *Botrytis cinerea* and *Sclerotinia sclerotiorum* diseases. *Soil Biology & Biochemistry* 35: 323-331.

Karapapa, V. K., Bainbridge, B. W. and J. B. Heale. 1997. Morphological and molecular characterization of *Verticillium longisporum* comb. Nov. pathogenic to oilseed rape. *Mycological Research* 101: 1281-1294.

Keel, C., Ucurum, Z., Michaux, P., Adrian, M. and D. Haas. 2002. Deleterious Impact of a Virulent Bacteriophage on Survival and Biocontrol Activity of *Pseudomonas fluorescens* Strain CHA0 in Natural Soil. *Molecular Plant-Microbe Interaction* 15: 567–576.

Kimber, D. S. and D. I. McGregor. 1995. The species and their origin, cultivation and world production. In: Brassica Oilseeds. Production and Utilization. Kimber, D. and K. McGregor (eds.) CAB International, Cambridge. pp. 1-7.

Kirner, S., Hammer, P. E., Hill, D. S., Altmann, A., Fischer, I., Weislo, L. J., Lanahan, M., van Pee, K.-H. and J. M. Ligon 1998. Functions Encoded by Pyrrolnitrin Biosynthetic Genes from *Pseudomonas fluorescens. Journal of Bacteriology* 8: 1939-1943.

Kloepper, J. W. 1992. Plant growth-promoting rhizobacteria as biological control agents. Soil microbial ecology: applications in agricultural and environmental management. ed by Metting, F. B. Jr. Marcel Dekker Inc., NY, USA. pp. 255-274.

Kropf, S., Heuer, H., Gruening, M. and K. Smalla. 2004. Significance test for comparing complex microbial community fingerprints using pairwise similarity measures. In: Journal of Microbiological Methods 57(2): 187-195.

Kurze, S. Dahl, R., Bahl, H. and G. Berg. 2001. Biological control of soil-borne pathogens in strawberry by *Serratia plymuthica* HRO-C48. *Plant Diseases* 85: 529-534.

Landa, B. B., Navas-Cortés, J. A. and R. M. Jiménez-Díaz. 2004. Influence of temperature on plant-rhizobacteria interactions related to biocontrol potential for suppression of Fusarium wilt of chickpea. *Plant Pathology* 53: 341-352.

Lazarovits. G., Conn, K. and M. Tenuta. 2000. Control of *Verticillium dahliae* with soil amendments: Efficacy and mode of action. In: Advances in *Verticillium* research and disease management. Tjamos, E. C., Rowe, R. C., Heale, J. B. and D. R. Fravel. (eds.) APS Press. pp. 274-291.

Leslie, S. B., Israeli, E., Lighthart, B., Crowe, J. H. and L. M. Crowe. 1995. Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Applied and Environmental Microbiology* 91: 3592-3597.

Libbert, E., 1987. Lehrbuch der Pflanzenphysiologie, Fischer Verlag, Jena, Germany.

Lichtenthaler, H. K. 1998. The stress concept in plants: an introduction. *Annals of New York Academy of Science* 851: 187-198.

Lieber, A., Kiesel, B. and W. Babel. 2002. Microbial diversity analysis of soil by SSCP fingerprinting technique using TGGE Maxi System. Borkheider Seminar zur Ökophysiologie des Wurzelraumes. Merbach, W., Hütsch, B. W., Augustin, J. and B. G. Teubner. Stuttgart, Leipzig, Wiesbaden.

Ligon, J. M., Hill, D. S., Hammer, P. E., Torkewitz, N. R., Hofmann ,D., Kempf, H. J. and K. H. van Pee. 2000. Natural products with antifungal activity from *Pseudomonas* biocontrol bacteria. *Pest Management Science* 56: 688-695.

Loh, J., Dasharath, J. L., Lohar, P., Andersen, B. and M. G. Stacey. 2002a. Quorum sensing in plant-associated bacteria. *Journal of Bacteriology* 184: 1759-1766.

Loh, J., Pierson, E.A., Pierson, L.S. III, Stacey, G. and A. Chatterjee. 2002b. Quorum sensing in plant-associated bacteria. *Current Opinion in Plant Biology* 5: 285-290.

Lumsden, R. D., Lewis, J. A. and D. R. Fravel. 1995. Formulation and delivery of biocontrol agents foruse against soilborne plant pathogens. In: Hall, F. R. and Barry, J. W. (eds.): Biorational pest control agents. Formulation and delivery. Washington. pp. 166-182.

Lupp, C., and E. G. Ruby. 2005. *Vibrio fischeri* uses two quorum-sensing systems for the regulation of early and late colonization factors. *Journal of Bacteriology* 187: 3620–3629.

Lynch, J. M. 1990. The rhizosphere. Wiley. New York, USA

Martin, M. J., Riedel, R. M. and R. C. Rowe. 1982. *Verticillium dahliae* and *Pratylenchus penetrans*: Interactions in the early dying complex of potatoes in Ohio. *Phytopathology* 72: 640-644.

Martin, B., Humbert, O., Camara, M., Guuenzi, E., Walker, J., Mitchell, T., Andrew, P., Prudhomme, M., Alloing, G., Hakenbeck, R., Morrison, D. A., Boulnois, G. J. and J. P. Claverys. 1992. A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. *Nucleic Acids Research* 20: 3479-3483.

Martin, F. N. 2003. Development of alternative strategies for management of soilborne pathogens currently controlled with methyl bromide. *Annual Review of Phytopathology* 41: 325-350.

Martin-Laurent, F., Philippot, L., Hallet, S., Chaussod, R., Germon, J. C., Soulas, G. and G. Catroux. 2001. DNA extraction from soils: old bias for new microbial diversity analysis methods. *Applied and Environmental Microbiology* 67(5): 2354-2359.

Mathesius, U., Mulders, S., Gao, M., Teplitski, M., Rolfe, B. G. and D. W. Bauer. 2003. Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals. *Proceedings of the National Academy of Sciences*, USA 100: 1444-1449.

McClean, K. H., Winson, M. K., Fish, L., Taylor, A., Chhabra, S. R.., Camara, M., Daykin, M., Lamb, J. H., Swift, S., Bycroft, B. W., Stewart, G. S. and P. Williams. 1997. „Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones." *Microbiology* 143(12): 3703-3711.

McQuilken, M. P., Halmer, P. and D. J. Rhodes. 1998. Application of microorganisms to seeds. In: Burgues, H. D. (ed.) Formulation of Microbial Pesticides. Beneficial Microorganisms, Nematodes and Seed Treatments. Kluwer Academic Publisher, London. pp 255-286.

Meincke, R. 2004. Analyse der Wirkung von *Serratia plymuthica* im Pathosystem *Verticillium dahliae* Kleb. – Raps unter verschiedenen Gewächshausbedingungen. Diploma thesis. University of Rostock, Germany.

Mercado-Blanco, J., Rodríguez-Jurado, D., Parrilla-Araujo, S. and R. M. Jiménez-Díaz. 2003. Simultaneous detection of the defoliating and nondefoliating *Verticillium dahliae* pathotypes in infected olive plants by duplex, nested polymerase chain reaction. *Journal of Plant Disease* 87: 1487-1494.

Mercado-Blanco, J., Rodríguez-Jurado, D., Hervás, A. and R. M. Jiménez-Díaz. 2004. Suppression of Verticillium wilt in olive planting stocks by root-associated fluorescent *Pseudomonas* spp. *Biological Control* 30: 474-486.

Messner, R., Schweigkofler, W., Schweigkofler, M., Berg, G. and H. Prillinger. 1996. Molecular characterization of the plant pathogen *Verticillium dahliae* Kleb. using RAPD-PCR and sequencing of the 18S rRNA-gene. *Journal of Phytopathology* 144: 347-354.

Mol, L. and H. W. Vanriessen. 1995. Effect of plant-roots in the germination of microsclerotia of *Verticillium dahliae*. *European Journal of Plant Pathology* 101: 673-678.

Mol L. and K. Scholte. 1995. Formation of microsclerotia of *Verticillium dahliae* Kleb. on various plant parts of two potato cultivars. *Potato Research* 38: 143-50.

Mougel, C., Offre, P., Ranjard, L., Corberand, T., Gamalero, E., Robin, C. and P. Lemanceau. 2006. Dynamic of the genetic structure of bacterial and fungal communities at different developmental stages of *Medicago truncatula* Gaertn. cv. Jemalong line J5 *New Phytologist* 170: 165-175.

Napier, R. M. and M. A. Venis. 1995. Auxin action and auxin-binding proteins. *New Phytologist* 129: 167-201.

Nautiyal C. S., Johri, J. K. and H. B. Singh. 2002. Survival of the rhizosphere-competent biocontrol strain *Pseudomonas fluorescens* NBRI2650 in the soil and phytosphere. Canadian Journal of Microbiology/Reviews Canadian microbiol. 48(7): 588-601.

Nealson, K. H., Platt, T. and J. W. Hastings. 1970. Cellular control of the synthesis and activity of the bacterial luminescent system. *Journal of Bacteriology* 104: 313-322.

Nilsson, L., Oliver, J. D. and S. Kjelleberg. 1991. Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. *Journal of Bacteriology* 173: 5054-5059.

Nirenberg, H. I. 1976. Untersuchungen über die morphologische und biologische Differenzierung in der *Fusarium*-Sektion Liseola. Mitteilungen der Biologischen Bundesanstalt für Land- u. Fortswirtschaft, Berlin, Heft 169.

O'Callaghan, M., Gerard, E. M., and V. W. Johnson. 2001. Effect of soil moisture and temperature on survival of microbial control agents. *New Zealand Plant Protection* 54: 128-135.

Olsen, S. and B. Nordbring-Hertz. 1985. Microsclerotial germination of *Verticillium dahliae* as affected by rape rhizosphere. *FEMS Microbiology Letters* 31: 293-299.

Oerke, E. C. 2006. Crop losses to pests. *The Journal of Agricultural Science* 144: 31-43.

Ogoshi, A. 1987. Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani* Kühn. *Annual Review of Phytopathology* 25: 125-43.

Opelt, K. and G. Berg. 2004. Diversity and antagonistic potential of bacteria associated with bryophytes from nutrient-poor habitats of the Baltic Sea Coast *Applied and Environmental Microbiology* 70: 6569-6579.

Palmfeldt, J., Rodström, P. and B. H. Hägerdal. 2003. Optimisation of initial cell concentration enhances freeze-drying tolerance of *Pseudomonas chlororaphis. Cryobiology* 47: 21-29.

Patel, A. V., Bilgeshausen, U., Vorlop, K. D. and W. Beitzen-Heineke. 2004. Mikrokapseln zur Formulierung von biologischen Schädlingsbekämpfungsmitteln – Technologietransfer. *Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft* 396: 460-461.

Pegg, G. F. and B. L. Brady. 2002. Verticillium Wilts. CAB International.

Pierson, E. A. and D. M. Weller. 1994. Use of mixtures of fluorescent pseudomonads to suppress take-all and improve the growth of wheat. *Phytopathology* 84: 940-947.

Pierson, E. A., Wood, D. W., Cannon, J. A., Blachere, F. M., Pierson, L. S. 1998. Interpopulation signaling via *N*-acyl-homoserine lactones among bacteria in the wheat rhizosphere. *Molecular Plant-Microbe Interaction* 11: 1078-1084.

Pouzet, A. 1995. *Brassica* oilseeds: production and utilization Part I: Field Crop, Agronomy, Kimber, D. S. and D. I. McGregor. (eds.) pp. 65-92**.**

Powell, K. A. 1993. The commercial exploitation of microorganisms in agriculture. In: Jones, D. G. (ed.) Exploitation of microorganisms. Chapman and Hall, New York. pp. 441-459.

Pratt, L. A. and R. Kolter. 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Molecular Microbiology* 30: 285-293.

Quinones, B., Dulla, G. and S. E. Lindow. 2005. Quorum sensing regulates exopolysaccharide production, motility, and virulence in *Pseudomonas syringae*. *Molecular Plant-Microbe Interactions* 18: 682–693.

Raaijmakers, J. M. and D. M. Weller. 2001. Exploiting genotypic diversity of 2,4- diaceylphloroglucinol-producing *Pseudomonas* spp: Characterization of superior root-colonizing *Pseudomonas fluorescens* strain Q8r1-96. *Applied and Environmental Microbiology* 67: 2545-2554

Rademaker, J. L. W. and F. J. De Bruijn. 1997. Characterization and classification of microbes by REP-PCR genomic fingerprinting and computer-assisted pattern analysis. In: Caetano-Anolle´s, G. and Gresshoff, P. M. (eds.) DNA markers: protocols, applications and overviews. Wiley & Sons, Inc., New York, N.Y. pp. 151-171.

Rademaker, J. L. W., Louws, F. J., Rossbach, U. and F. J. de Bruijn. 1999. Computer assisted pattern analysis of molecular fingerprints and data base construction. In: Akkermans, A. D. L., van Elsas; J. D. and F. J. DeBruijn (eds.). Molecular microbial ecology manual. Kluwer Academic Publishers. Dordrecht, The Netherlands. pp. 1-33.

Reimmann, C., Ginet, N., Michel, L., Keel, C., Michaux, P., Krishnapillai, V., Zala, M., Heurlier, K., Triandafillu, K., Harms, H., Défago, G. and D. Haas. 2002. Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1. *Microbiology* 148: 923-932.

Reiter, B., Pfeifer, U., Schwab, H. and A. Sessitsch. 2002. Response of endophytic bacterial communities in potato plants to infection with *Erwinia carotovora* subsp. *atroseptica*. *Applied and Environmental Microbiology* 68: 2261-2268.

Rhodes, D. J. 1993. Formulation of biological control agents. In: Exploitation of Microorganisms. Jones, D.G. (ed.) London: Chapman & Hall. pp. 411-439.

Richter, I. 2001. Entwicklung und Bewertung verschiedener Formulierungen von Rhizobakterien für den biologischen Pflanzenschutz. Diploma thesis. University of Rostock, Germany.

Rodríguez-Jurado, D. 1993. Interacciones huésped-parásito en la Verticilosis del olivo (*Olea europaea* L.) inducida por *Verticillium dahliae* Kleb. Ph.D. Thesis, University of Córdoba, Spain.

Runia, W.T. 2000. Steaming methods for soils and substrates. Proceedings of the fifth international symposium on chemical and non chemical soil and substrate disinfestation. In: Gullino, M.L., Katan, J., and A. Matta (eds.). *Acta Horticulturae* 532: 115-123

Sambrook, J., Fritsch, E. F. and T. Maniatis. 1989. Molecular cloning: A laboratory manual, Second Edition. Cold Spring Harbour Laboratory, NY.

Savka, M. A., Oger, P., Dessaux, Y. and S. Rossbach. 2002. Engineered bacterial competitiveness and persistence in the phytosphere. *Molecular Plant-Microbe Interactions* 159: 866-874.

Sawer, M. and R. J. Kremer. 1995. Determination of bacterially derived auxins using microplate method. *Letters of Applied Microbiology* 20: 282-285.

Scherwinski, K., Wolf, A. and G. Berg. 2006. Assessing the risk of biological control agents on the indigenous microbial communities: *Serratia plymuthica* HRO-C48 and *Streptomyces sp*. HRO-71 as model bacteria. *BioControl*. Online publication: DOI10.1007/s10526-006-9006-8

Scher, F. M., Ziegle, J. S. and J. W. Kloepper. 1994. A method for assessing the root-colonizing capacity of bacteria on maize. *Canadian Journal of Microbiology* 30: 151-157.

Schmidt, C. S., Agostini, F., Simon, A.-M., Whyte, J., Townend, J., Leifert, C., Killham, K. and C. Mullins. 2004. Influence of soil type and pH on the colonization of sugar beet seedlings by antagonistic *Pseudomonas* and *Bacillus* strains, and on their control of *Pythium* damping-off. *European Journal of Plant Pathology* 110: 1025-1046.

Schuhegger, R., Ihring, A., Gantner, S., Bahnweg, G., Knappe, C., Vogg, G., Hutzler, P., Schmid, M., van Breusegem, F., Eberl, L., Hartmann, A. and C. Langebartels. 2006. Induction of systemic resistance in tomato by *N*-acyl-homoserine lactone-producing rhizosphere bacteria. *Plant, Cell and Environment* 29: 909-918.

SeedQuest. 2005. Projekt zur Bekämpfung von Pilzkrankheiten - Union zur Förderung von Oel- und Proteinpflanzen fördert Untersuchungen. *News realease* 13: 119.

Smith, K. P. and R. M. Goodman. 1999. Host variation for interactions with beneficial plant-associated microbes. *Annual Review of Phytopathology* 37: 473-491.

Souzo, H. 1992. Freeze-drying of Microorganisms. *Encyclopedia of Microbiology* Vol. 2, Academic Press, New York.

Stark, C. 1961. Das Auftreten der Verticillium-Tracheomykose in Hamburger Gartenbaukulturen. *Gartenbauwissenschaft* 26: 493-528.

Steidle, A., Sigl, K., Schuhegger, R., Ihring, A., Schmid, M., Gantner, S., Stoffels, M., Riedel, K., Givskov, M., Hartmann, A., Langebartels, C. and L. Eberl. 2001. Visualization of *N*-acylhomoserine lactone-mediated

cell-cell communication between bacteria colonizing the tomato rhizosphere. *Applied and Environmental Microbiology* 67: 5761-5770.

Steidle, A., Allesen-Holm, M., Riedel, K., Berg, G., Givskov, M., Molin, S. and L. Eberl. 2002. Identification and characterization of an *N*-acylhomoserine lactone-dependent quorum-sensing system in *Pseudomonas putida* strain IsoF. *Applied and Environmental Microbiology* 68: 6371-6382.

Steventon, L., Fahleson, J., Hu, Q. and C. Dixelius. 2002. Identification of the casual agent of Verticillium wilt of winter oilseed rape in Sweden as *Verticillium longisporum*. *Mycological Research* 106: 570-578.

Subbarao, K. V., Hubbard, J. C. and S. T. Koike. 1999. Evaluation of broccoli residue incorporation into field soil for Verticillium wilt control in cauliflower. *Plant Disease* 83: 124-129.

Swift, S., Williams, P., G. S. A. B. and Stewart. 1999. *N*-acylhomoserine lactones and quorum sensing in proteobacteria. In: Cell-Cell Signalling in Bacteria. Dunny, G. M. and S. Winans, (eds.) American Society for Microbiology, Washington DC, USA. Pp. 291-313.

Schwyn, B. and J. B. Neilands. 1987. Universal chemical assay for the detection and determination of siderophores. *Anal Biochem*. 160: 47-56.

Thanassolopoulos, C. C. 1993. Spread of Verticillium wilt by nursery plants in olive growes in the Chalkidiki area (Greece). Bulletin OEPP/EPPO Bulletin 23: 517–520.

Thompson, J. D., Gibson,T. J., Plewniak, F., Jeanmougin, F. and D. G. Higgins. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25: 4876-4882.

Tilcher, R., Beitzen-Heineke, W. and A. V. Patel. 2004. Applikation von freien und mikroverkapselten Zellen bakterieller Antagonisten in Zuckerrübenpillen. . *Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft* 396: 462.

Tjamos, E. C. 1992. Selective elemination of soilborne plant pathogens and enhancement of antagonists by steaming, sublethal fumigation and soil solarisation. In: Tjamos, E. C., Papavizas, G. C. and R. J. Cook. (eds.) Biological Control of Plant Diseases. Progress and Challenges for the Future. NATO ASI Series, Series A, Vol. 230, Plenum Press, New York. pp. 1-17.

Tjamos, E. C. 1993. Prospects and strategies in controlling Verticillium wilt of olive. Bull. OEPP/EPPO Bull. 23: 505-512.

Tjamos E. C., Tsitsigiannis, D. I., Tjamos, S. E., Antoniou, P. P. and P. Katinakis. 2004. Selection and screening of endorhizosphere bacteria from solarized soils as biocontrol agents against *Verticillium dahliae* of solanaceous hosts *European Journal of Plant Pathology* 110: 35-44.

Troxler, J., Zala, M., Moënne-Loccoz, Y., Keel, C. and G. Défago. 1997. Predominance of nonculturable cells of the biocontrol strain *Pseudomonas fluorescens* CHA0 in the surface horizon of large outdoor lysimeters. *Applied and Environmental Microbiology* 63: 3776-3782.

Turnbull, G. A., Morgan, J. A. W., Whipps, J. M. and J. R. Saunders. 2001. The role of bacterial motility in the survival and spread of *Pseudomonas fluorescens* in soil and in the attachment and colonization of wheat roots. *FEMS Microbiology Ecology* 36(1): 21-31.

Union zur Förderung von Oel- und Proteinpflanzen E.V. 2006. Winterrapsaussaat 2006. UFOP-Information.

Van Loon, L. C., Bakker, P. A. H. M. and C. M. J. Pieterse. 1998. Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology* 36: 453-483.

Van Pee, K. H. and J. M. Ligon. 2000. „Biosynthesis of pyrrolnitrin and other phenylpyrrole derivates by bacteria." Natural product reports 17: 157-164.

Vieira, J. and Messing, J. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* 19(3): 259-268.

Visick, K. L., and C. Fuqua. 2005. Decoding microbial chatter: cell-cell communication in bacteria. *Journal of Bacteriology* 187: 5507-5519.

Volossiouk, T., Robb, E. J. and R. N. Nazar. 1995. Direct DNA extraction for PCR-mediated assays of soil organisms. *Applied and Environmental Microbiology* 61(11): 3972-3976.

Wei, J.-R. and H.-C. Lai. 2006. *N*-Acylhomoserine lactone-dependent cell-to-cell communication and social behavior in the genus *Serratia. International Journal of Medical Microbiology* 296: 117–124.

Wei, H. L. and Zhang, L. Q. 2006. Quorum-sensing system influences root colonization and biological control ability in *Pseudomonas fluorescens* 2P24. *Antonie Van Leeuwenhoek* 89(2): 267-80.

Weller, D. M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annual Reviews of Phytopathology* 26: 379-407.

Weller, D. M. and L. S. Thomashow. 1999. The role of microbial metabolites in biological control of plant pathogens, pp. 415-422. In Macías, F. A., Galindo, J. C. G., Molinillo, J. M. G. and H. C. Cutler (eds.) Recent Advances in Allelopathy. Vol. 1, A Science for the Future. Universidad De Cádiz, Cadiz, Spain.

Westendorf, C. 2006. Analyse der Interaktion des antagonistisch wirksamen *Serratia plymuthica* HRO-C48 und verschiedene Mutanten mit bodenbürtigen Schaderregern. Diploma thesis. University of Rostock, Germany

Wheeler, T. A., Madden, L. V., Riedel, R. M. and R. C. Rowe. 1994. Distribution and yield-loss relations of *Verticillium dahliae, Pratylenchus penetrans, P. scribneri, P. crenatus*, and *Meloidogyne hapla* in commercial potato fields. *Phytopathology* 84: 843-852.

Whipps, J. M. 1997. Ecological considerations involved in commercial development of biological control agents for soil-borne diseases. In: Van Elsas, J. D., Trevors, J. T. and E. M. H. Wellington (eds.) Modern soil microbiology. Marcel Dekker, Inc., New York, N.Y. pp. 525-545.

Whipps, J. 2001. Microbial interactions and biocontrol in the rhizosphere. *Journal of Expermental Botany* 52: 487-511.

Whipps, J. M. 2004. Prospects and limitations for mycorrhizas in biocontrol of root pathogens. *Canadian Journal of Botany* 82: 1198–1227.

Whitehead, N. A., Barnard, A. M. L., Slater, H., Simpson, N. J. L and G. P. C. Salmond. 2001. Quorum sensing in Gram-negative bacteria. *FEMS Microbiological Reviews* 25: 365-404.

Wilhelm, S. 1955. Longevity of the Verticillium wilt fungus in the laboratory and field. *Phytopathology* 45: 180-181.

Wilkinson, M. H. F. 2001. Predation in the presence of decoys: an inhibitory factor on pathogen control by bacteriophages or bdellovibrios in dense and diverse ecosystems. *Journal of Theoretical Biology* 208: 27-36.

Winkelmann, G. 2002. Microbial siderophore-mediated transport. *Biochemical Society Transactions* 30: 691-696.

Winson, M. K., Camara, M., Latifi, A., Foglino, M., Chhabra, S. R., Daykin, M. 1995. Multiple *N*-acyl-L homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences, USA* 92: 9427-9431.

Wolf, A. 2004. Bewertung des Einflusses verschiedener Nutzorganismen auf die Nutzorganismen der Rhizosphären. Final report: Project 00HS029, Federal Agency for Agriculture and Food. Bonn, Germany.

Wood, D. W., Gong F., Daykin, M., Williams, P. and L. S. Pierson. 1997. *N*-acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *Journal of Bacteriology* 179: 7663-7670.

Yang, C., Crowley, D. E. and J. A. Menge. 2001. 16S rDNA fingerprinting of rhizosphere bacterial communities associated with healthy and *Phytophthora infected* avocado roots. *FEMS Microbiology Ecology* 35: 129-136.

Zeise, K. and D. Seidel. 1990. Zur Entwicklung und Schadwirkung der Verticillium-Welkekrankheit am Winterraps. *Raps* 8: 20-22.
Zeise, K. 1992. Screening for resistance to *Verticillium dahliae* Kleb. on oilseed rape (*Brassica napus var. oleifera* METZGER) under greenhouse conditions. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* 44: 125-128.

Zeise, K. and A. von Tiedeman. 2001. Morphological and physiological differentiation among vegetative compability groups of *Verticillium dahliae* and *V. longisporum*. *Journal of Phytopathology* 149: 469-475.

Zeise, K. and A. von Tiedemann. 2002. Host specialization among vegetative compatibility groups of *Verticillium dahliae* in relation to *Verticillium longisporum*. *Journal of Phytopathology* 150: 112-119.

Zeise, K. and P. Steinbach. 2004. Schwarze Rapswurzeln und der Vormarsch der Verticillium-Rapswelke. *Raps* 4: 170-174.

Zhou H, Yao, F., Roberts, D. P. and T. G. Lessie. 2003. AHL-deficient mutants of *Burkholderia ambifaria* BC-F have decreased antifungal activity. Current Microbiology 47(3): 174-9.

Zhou, L., Hu, Q., Johansson, A. and C. Dixelius. 2006. *Verticillium longisporum* and *V. dahliae*: infection and disease in *Brassica napus. Plant Pathology* 55: 137-144.

6 Acknowledgements

First and foremost, my deepest gratitude to my supervisor Professor Gabriele Berg for her support, advice and encouragement throughout the last years.

I would also wish to acknowledge Professor Hubert Bahl from the Department of Microbiology, University of Rostock, for his willingness being co-referee and for his support in administrative issues.

My greatest acknowledgement is expressed to the Deutsche Bundesstiftung Umwelt that founded my doctorate study. In particularly, I would like to thank Dr. Maximilian Hempel for the uncomplicated conversation.

The thesis as it stands would not have been possible without the significant input of Christian Westendorf and Remo Meincke during their diploma thesis.

I am grateful to Dr. Martin Frauen (Norddeutsche Pflanzenzucht Hans-Georg Lembke KG) for the provision of experimental fields and financial support. Prof. Leonid Chernin from the Hebrew University of Jerusalem in Israel, my gratitude for giving scientific inputs regarding the exploitation of the microbial smalltalk. It is a pleasure to thank PD Dr. Martin Hagemann (Plant Physiology, University of Rostock), who spared me a lot of his valuable time and gave me constructive suggestions in genetically modifying *Serratia plymuthica*. I would like to express my thanks to Prof. Birgit Piechulla (Biochemistry, University of Rostock) for the impulse to investigate the smelly site of HRO-C48. I also wish to express my greatest gratitude to Prof. Raphael Jiménez-Días, Dr. Blanca B. Landa, Dr. Jesús Mercado-Blanco and all of the colleagues from the Institute for Sustainable Agriculture in Córdoba, Spain, for the hospitality within and apart from the laboratory.

I also wish to thank all the busy and lovable Microbiology Group members in Rostock and in Graz with whom I shared lab, conversations and laughter. Special thank goes to Anja, Arite, Christin, Gerit, Hella, Jana, Katja O. and Katja, S.!

At last, I would like to thank my mother, Ingolf and my sister. I want them to know that I am very grateful for their unreserved affection, encouragement and moral support throughout my studies.

Cirriculum vitae

Henry Müller

Born on the 17th of August 1976 in Rostock

Career	
Oct. 2005 to present	Research assistent at the Institute of Environmental Biotechnology; Graz University of Technology, Austria
Dec. 2002 to Sept. 2005	PhD student, fellowship of German Federal Environmental Foundation Subject: Strategies in developing an efficient commercial product for biological control of soil borne fungal pathogens by *Serratia plymuthica* HRO-C48
2001	Diploma thesis at the Department of Microbiology; University Rostock; Final result: excellent (1.0) Subject: Influence of transgenic T4 lysozyme-producing potato on antagonistic bacteria and fungi in the geocaulosphere
1997 to 2001	Study of Food technology at the University of Applied Sciences of Neubrandenburg; Final: result excellent (1.2)
1995 to 1997	Military service in Husum
1995	University-entrance diploma at advanced secondary school in Rostock
Experience	
2000	Five-month practical at the Food Refrigeration and Process Engineering Research Centre; University of Bristol, UK
2002	Six-month practical at the Department of Fluid Mechanics and Process Automation; Technical University of Munich
2004	Two-month research period at the Institute of Sustainable Agriculture in Cordoba, Spain

Publications:

Müller, H., Westendorf, C., Chernin, L., Schmidt, S., Eberl, L. and G. Berg. The activity of the biocontrol agent *Serratia plymuthica* HRO-C48 is under quorum sensing control. *FEMS Microbial Ecology*. in press

Müller, H. and G. Berg. Impact of formulation procedures on the effect of the biocontrol agent *Serratia plymuthica* HRO-C48 on Verticillium wilt in oilseed rape. *BioControl.* Published Online: 10.1007/s10526-007-9111-3

Müller, H., Tejedor-González, E., Mercado-Blanco, J., Rodríguez-Jurado, D., Jiménez-Díaz, R. and G. Berg. Effect of the biological control strain *Serratia plymuthica* HRO-C48 on Verticillium wilt of olive trees cv. Arbequina. *IOBC Bulletin*. 30: 173-176.

Müller, H., Kurze, S., Richter, I. and G. Berg. 2004. Strategies in developing an efficient commercial product for biological control of soil borne pathogens by *Serratia plymuthica* HRO-C48. *IOBC Bulletin* 27: 199-203.

Wolf, A., Scherwinski, K., Müller, H., Golly, A., Smalla, K. and G. Berg. 2004. Impact of application of biocontrol agents to plant root on the natural occurring microbial community. *IOBC Bulletin* 27: 297-300.

Müller, H., Meincke, R. and G. Berg. 2004. *Serratia plymuthica* HRO-C48: Strategy to control *Verticillium dahliae* in oilseed rape and effects on the microbial community. IOBC Bulletin. *IOBC Bulletin* 27: 399-404.

Oral presentations:

Müller, H., Meincke, R., Westendorf, C., Chernin, L. and G. Berg. 2005. Regulation of the antagonistic activity of the biocontrol agent *Serratia plymuthica* HRO-C48. VAAM Annual Conference. Göttingen, Germany.

Müller, H., Meincke, R., Mercado-Blanco, J., Jiménez-Díaz, R., Liu, X., Chernin, L. and G. Berg. 2005. Effects of *Serratia plymuthica* HRO-C48 treatment in the pathosystems oilseed rape *V. dahliae* and olive - *V. dahliae.* The 9th International Verticillium Symposium. Monterey, CA, USA.

Müller, H., Meincke, R., Mercado-Blanco, J., Jiménez-Díaz, R., Chernin, L. and G. Berg. 2005. Wirkung und Etablierung von *Serratia plymuthica* C48 in den Pathosystemen *V. dahliae Brassica napus* und *V. dahliae Olea europea*. Deutsche Phytomedizinische Gesellschaft: Tagung des Arbeitskreises „Biologische Bekämpfung von Pflanzenkrankheiten". Kleinmachnow, Germany.

Müller, H., Dahl, R. and G. Berg. 2005. RHIZOSTAR®: Ein biologisches Produkt zur Ertragssteigerung der Erdbeere und zum Schutz vor bodenbürtigen Schadpilzen. Fördergemeinschaft Ökologischer Obstbau: Arbeitstagung „Ökologischer Anbau von Stein- und Beerenobst". Weinsberg, Germany.

Müller, H., Meincke, R. and G. Berg. 2004. Strategie zur biologischen Kontrolle der Verticillium Welke im Rapsanbau mit *Serratia plymuthica*. 54. Deutsche Pflanzenschutztagung. Hamburg, Germany.

Müller, H., Kurze, S., Richter, I. and G. Berg. 2003. Strategies in developing an efficient commercial product for biological control of soil borne pathogens by *Serratia plymuthica* HRO-C48. IOBC Meeting: Multitrophic Interactions in Soil for Integrated Plant Protection. Bonn, Germany.

Poster presentations:

Müller, H., Liu, X., Westendorf, W., Bimerew M., Voloshina, P., Khmel, I., Eberl, L., Chernin, L. and G. Berg. 2006. Bidirectional mode of action of the biological control agent *Serratia plymuthica* HRO-C48 and the regulation by quorum sensing. IOBC Meeting: Biological control of fungal and bacterial plant pathogens: Fundamental and practical approaches to increase biocontrol efficacy. Spa, Belgium.

Müller, H., Meincke, R. and G. Berg. 2004. *Serratia plymuthica* HRO-C48: Strategy to control *Verticillium dahliae* in oilseed rape and effects on the microbial community. IOBC Meeting: Management of plant diseases and arthropod pests by BCAs and their integration in agricultural systems. S. Michele all' Adige, Italy.

Müller, H., Meincke, R. and G. Berg. 2004. *Serratia plymuthica* HRO-C48: Using its highly antifungal potential to control *Verticillium dahliae* in oilseed rape. VAAM Annual Conference, Braunschweig, Germany.

Selbständigkeitserklärung:

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Henry Müller Graz, 01. Oktober 2006