Studies on the antagonistic effect of rhizobacteria against soilborne *Phytophthora* species on strawberry

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Dedicated to my respected parents

Abstract

The present work is divided into five chapters. The first chapter gives a comprehensive introduction, its contents include a brief literature review on soil borne *Phytophthora* fungal pathogens; *Phytophthora cactorum* causing crown rot disease and *Phytophthora fragariae* var. *fragariae* causing red stele disease in strawberry.

In the second chapter different *in vitro* (dual culture and culture filtrate tests), greenhouse and field studies were conducted to evaluate the potential of selected rhizobacteria against crown rot (*Phytophthora cactorum*) and red stele disease (*Phytophthora fragariae* var. *fragariae*) of strawberry. Three antagonistic bacterial isolates viz.: *Raoultella terrigena* strain G-584, *Bacillus amyloliquefaciens* strain G-V1 and *Pseudomonas fluorescens* strain 2R1-7 proved to be potential biocontrol agents in dual culture, culture filtrate and greenhouse studies. In greenhouse experiments following bacterial treatment, the disease symptoms of crown rot (*Phytophthora cactorum*) and red stele disease (*Phytophthora fragariae* var. *fragariae*) of strawberry were significantly reduced after 90 days. In all field experiments, tested rhizobacteria such as *R. terrigena* G-584, *B. amyloliquefaciens* G-V1 *and P. fluorescens* 2R1-7 showed different level of biocontrol efficacy and in some cases efficacies were similar to the chemical control Aliette.

The third chapter describes the spread of bacteria in the rhizosphere of strawberry. The investigations revealed that the applied rhizobacterium *R. terrigena* G-584 labeled with Green Fluorescent Protein (GFP), could successfully colonize strawberry plant roots.

The fourth chapter deals with the mode of action of the antagonistic bacteria. Results revealed that the three tested rhizobacteria have direct effect on morphology of both *Phytophthora* spp. with hyphal distortion. Moreover, productions of different extracellular enzymes have been observed from the three antagonistic bacteria as an indication for the antifungal effect.

The fifth chapter describes the detection of specific genes from the antagonistic bacteria. 2,4-diacetylphloroglucinol gene from *P. fluorescens* 2R1-7, cellulase gene from *B. amyloliquefaciens* G-V1, phytase gene from *R. terrigena* G-584 were identified.

Additionally genes were partially sequenced. Finally, 2,4-diacetylphloroglucinol gene expression pattern from *P. fluorescens* 2R1-7 was studied under different pH conditions and results revealed that this gene is pH dependent.

Keywords: Biological control, *Phytophthora fragariae* var. *fragariae*, *Phytophthora cactorum*, Rhizobacteria

Kurzfassung

Die vorliegende Arbeit gliedert sich in fünf Abschnitte. Der erste Teil gibt eine umfassende Einführung zum Thema mit einer kurzen Literaturübersicht über die bodenbürtigen pilzlichen Pathogene, *Phytophthora cactorum*, den Erreger der Rhizomfäule und *Phytophthora fragariae* var. *fragariae*, den Erreger der Roten Wurzelfäule der Erdbeere.

Im zweiten Kapitel sind verschiedene *in vitro* Untersuchungen (Dualkultur und Kulturfiltrattests), Gewächshaus- und Freilandversuche wiedergegeben, um das antagonistische Potential der ausgewählten Rhizobakterien gegen die beiden bodenbürtigen Pilzkrankheiten näher zu charakterisieren. Dabei zeigten die drei eingesetzten Bakterienisolate *Raoultella terrigena* Stamm G-584, *Bacillus amyloliquefaciens* Stamm G-V1 und *Pseudomonas fluorescens* Stamm 2R1-7 jeweils einen hohen Hemmeffekt gegenüber den Pathogenen in Dualkultur und Kulturfiltrat wie auch in den Gewächshaustests. In den Gewächshausversuchen waren die Krankheitssymptome von Rhizomfäule und Roter Wurzelfäule in signifikanter Weise reduziert (90 Tage nach Behandlung). In den Freilandversuchen zeigten die drei eingesetzten bakteriellen Antagonisten unterschiedliche Wirkungsgrade, wobei in einigen Fällen ein gleicher Effekt wie der chemische Standard Aliette erzielt wurde.

Im dritten Abschnitt der Arbeit ist die Verbreitung der bakteriellen Antagonisten in der Rhizosphäre der Erdbeerpflanze. Diese Untersuchungen ergaben, dass das eingesetzte Rhizobakterium *Raoultella terrigena* G-584 nach Markierung mit GFP (Green Fluorescent Protein) die Wurzeln der Erdbeerpflanzen erfolgreich kolonisieren konnte.

Der nächste Teil der Arbeit befasst sich mit Untersuchungen zum Wirkungsmechanismus der antagonistischen Bakterien. Aus den Ergebnissen geht hervor, dass die drei Rhizobakterien jeweils einen direkten Effekt auf die morphologische Struktur der Hyphen von beiden *Phytophthora*-Arten ausüben. Darüber hinaus konnte von den drei Antagonisten die Produktion von unterschiedlichen extrazellulären Enzymen nachgewiesen werden, was als ein Hinweis für den antifungalen Effekt herangezogen werden kann.

Das fünfte Kapitel befasst sich mit molekularen Untersuchungen zum Nachweis von spezifischen Genen der antagonistischen Bakterien, die zur Aufklärung des Antagonismus durchgeführt wurden. Zunächst wurden das 2,4-Diacetylphloroglucinol-Gen von *P. fluorescens* 2R1-7, das Cellulase-Gen von *B. amyloliquefaciens* G-V1, das Phytase-Gen von *R. terrigena* G-584 identifiziert und anschließend partiell sequenziert. Zum Abschluss wurde das 2,4-Diacetylphloroglucinol-Gen von *P. fluorescens* 2R1-7 bei unterschiedlichen pH-Bedingungen untersucht und eine pH-Abhängigkeit des Gens nachgewiesen.

Schlagwörter: Biologische Bekämpfung, *Phytophthora fragariae* var. *fragariae*, *Phytophthora cactorum*, Rhizobakterien

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List of Abbreviations

bp	=	base pairs
°C	=	degree Celsius
cDNA	=	complementary deoxyribonucleic acid
CFU	=	colony forming units
CM	=	carboxymethyl
cv	=	cultivar
DNA	=	deoxyribonucleic acid
dNTP	=	deoxyribonucleotide triphosphate
et al.	=	and others
Fig.	=	figure
g	=	gram
GFP	=	green fluorescent protein
h	=	hour(s)
µl	=	microlitre
µM	=	micromolar
mg	=	milligram
min	=	minute(s)
ml	=	millilitre
mM	=	millimolar
PCR	=	polymerase chain reaction
pp.	=	page(s)
RNA	=	ribonucleic acid
sp.	=	species (single)
spp.	=	species (plural)
Tab.	=	table
%	=	percent

I. General Introduction

The strawberry (*Fragaria* x *ananassa* Duchesne) is a herbaceous, perennial member of the *Rosaceae*, subfamily *Rosoideae*, along with blackberries and raspberries. There are about 34 other species of *Fragaria* found in Asia, North and South America and Europe, of which two are cultivated commercially for their fruit: *Fragaria moschata*, the Musky or Hautboy strawberry, and *F. vesca*, the Wood or Alpine strawberry (Ellinger, 1995). These species were cultivated for centuries, but there is very little production of them today, due to the success of *Fragaria* x *ananassa.* The name "Strawberry" may have derived from the practice of using straw mulch for cultivation many years ago. Alternatively, it may have come from the Anglo-Saxon word "strew" meaning to spread, as strawberry plants spread by runners. "Strewbery" or a similar word was changed to strawberry in English.

Strawberries are produced in 71 countries worldwide on 506,000 acres. Strawberries are among the highest yielding of all fruit crops. Average yields worldwide are just under 14,000 lbs/acre, but approach 40,000 lbs/acre in the USA, the most productive country. Many tree crops with much greater leaf area per unit of land cannot produce fruit yields as high as strawberry.

Medicinal Properties and Non-Food Usage: Strawberries are higher in vitamin C than many citrus fruits. Roots and leaves were made into lotions and gargles in England, and used for fastening loose teeth. Fruit juice was used for mouth ulcers. Using a different species, Indians in Western Washington made a tea from leaves and used it against diarrhea. Fruit of the parent species (*F. virginiana* and *F. chiloensis*) were considered folk remedies for diarrhea, gout, stomach ache, and kidney stones. They were used as astringents, diuretics, and mild laxatives. Strawberries are symbolic of "perfect excellence". (http://www.uga.edu/fruit/strawbry.htm).

Top 10 Countries (% of world production, 2002 FAO)
1. USA (27%)
2. Spain (10%)
3. Japan (6%)
4. Korea, Rep of (6%)
5. Poland (5%)
6. Italy (5%)
7. Mexico (4%)
8. Russia (4%)
9. Turkey (4%)
10. Germany (3%)

Tab. 1: World production of strawberries in the top 10 countries

The yield of fruits depends on soil conditions, photoperiod, temperature and losses caused by pests and diseases. Both biotic and abiotic factors play an important role in the growth and yield of the plant. The genetically different clones and cultivars of strawberry vary in their reactions to many pathogens. Viral, bacterial and fungal diseases are common to strawberry. Of the fungi attacking strawberries the most important pathogens are *Botrytis cinerea*, *Colletotrichum acutatum*, *Verticillium dahliae*, *Phytophthora fragariae* var. *fragariae* and *Phytophthora cactorum*.

Soilborne *Phytophthora* diseases of strawberry

The genus *Phytophthora* is grouped in the class oomycetes and covers over 40 species, which worldwide cause a large number of plant diseases. The genus *Phytophthora* is distinguished from the related oomycete genus *Pythium* by the complete differentiation of motile zoospores within sporangia before expulsion (Smith *et al*., 1988). The *Phytophthora* pathogens of strawberry, *Phytophthora fragariae* var. *fragariae* causing red stele and *Phytophthora cactorum* causing crown rot, are morphologically very similar, soilborne and facultative or obligatory parasites. However, they vary in their ability to attack the plant

hosts. Infections are predominantly initiated by asexually formed zoospores, which are differentiated in zoosporangia. A prerequisite for formation of sporangia and zoospores is high soil moisture content. Root exudates and particularly the root tips exercise a strong attraction on the zoospores.

Phytophthora isolates were obtained from either roots or soil, using both selective culture media, plant baiting and were identified on the basis of morphological and cultural features (Ribeiro and Olaf, 1978), the electrophoretic pattern of mycelial proteins (total proteins and isozymes), the polymorphism of DNA sequences amplified by RAPD-PCR as well as the PCR amplification of all or part of the Internal Transcribed Spacers (ITS) of rDNA with restriction digests (ITS-RFLP) of the resultant products combined with limited DNA sequencing (Bonants *et al.*, 1997; 2004).

Phytophthora cactorum has a wide spectrum of host plants. It occurs in areas with a moderate climate and predominantly attacks plants from the *Rosaceae* family (Nienhaus, 1960) including apple, apricot, cherry and peach, in addition to numerous shrubs e.g. Rhododendron as well as ornamental plants e.g. lilies and tulips (Hoffmann *et al*., 1985). In Germany the disease was first observed in Hamburg (Deutschmann, 1954) and later in South Germany (Schmidle, 1961). Since the 1950's, *P. cactorum* has spread from Germany to several other European countries and became a major hazard to the field crops in Southern France (Molot and Nourrisseau, 1966). A warm period with prolonged wetness favours infection by *P. cactorum*. Especially high temperatures, water stress and day lengths less than 13 h favour disease development (Lederer and Seemüller, 1992). The temperature that favours disease spread in the field lies between 17 °C and 25 °C (Ribeiro and Olaf, 1978). The minimum, optimum and maximum temperatures are 4°, 25° and 30 °C. In the field, oospores germinate at temperatures ranging from 10–20 °C and zoospores are produced via sporangia. The infection is caused mainly by zoospores; however, deciduous sporangia may also cause infection. The disease symptoms are first observed in the above ground plant parts by sudden withering and dying of the plant. A further characteristic symptom is the red-brown necrosis in the rhizomes. At first, small defined areas are affected. At later stages the entire rhizome may be destroyed. Penetration through wounds (Werres, 1987; Lederer, 1990) and leaf sheaths has been described. Cultivation of

the crop in the same field over many years can increase the severity of the disease.

The red stele or red core disease caused by *P. fragariae* was first described in 1920 in Scotland (Montgomerie, 1977). By 1940 the disease had spread in the United Kingdom and since the end of the seventies, the disease has also been observed in Germany (Smith *et al*., 1988). Different from *Phytophthora cactorum*, *Phytophthora fragariae* var. *fragariae* is host-specific and found only in strawberry (Duncan, 1990). However, other genera of the *Rosaceae* family can also be infected with *P. fragariae* under experimental conditions. Montgomerie (1967) reported that a British race of *P. fragariae* attacked all cultivars and clones on which it was tested. The root rot caused by this fungus is known as red core in Europe and red stele in North America (Smith *et al*., 1988). Red stele (*Phytophthora fragariae*), a very serious fungus disease of strawberries, attacks plants during the cool part of the year, but above-ground symptoms are most apparent from March to July. The fungus persists for many years in the soil, and it occurs most frequently in poorly drained areas. The causal fungus spreads from one area to another in the roots of infected plants and within an area in surface water or in soil carried on farm implements. Red stele affected plants become stunted and wilt in dry weather. Older leaves turn yellow or red particularly along the margin. The symptom that helps to identify red stele is the brick red discoloration in the center (stele) of live white roots. The red color may extend the length of the root, or it may show up for only a short distance above the dead root tip. This symptom is obvious only during winter and spring. The discoloration does not extend into the crown of the plant. Infected plants usually die by June or July.

These two pathogens can cause substantial economical damage in strawberry production (Seemüller, 1998). As a result in areas, where *P. fragariae* var. *fragariae* and *P. cactorum* are a major problem, cultivation of strawberry is mainly depending on the use of chemicals. In most countries, two different fungicides viz. Ridomil with Metalaxyl and Aliette with Aluminium-Fosetyl as active substances are used against these pathogens. However, in some fields where Metalaxyl was used for many years, resistant strains of *P. fragariae var. fragariae* have been found (Seemüller and Sun 1989, Nickerson, 1998). The problems assoicated with the use of agrochemicals have promoted researching in the field of biological control of plant diseases as an alternative method of control.

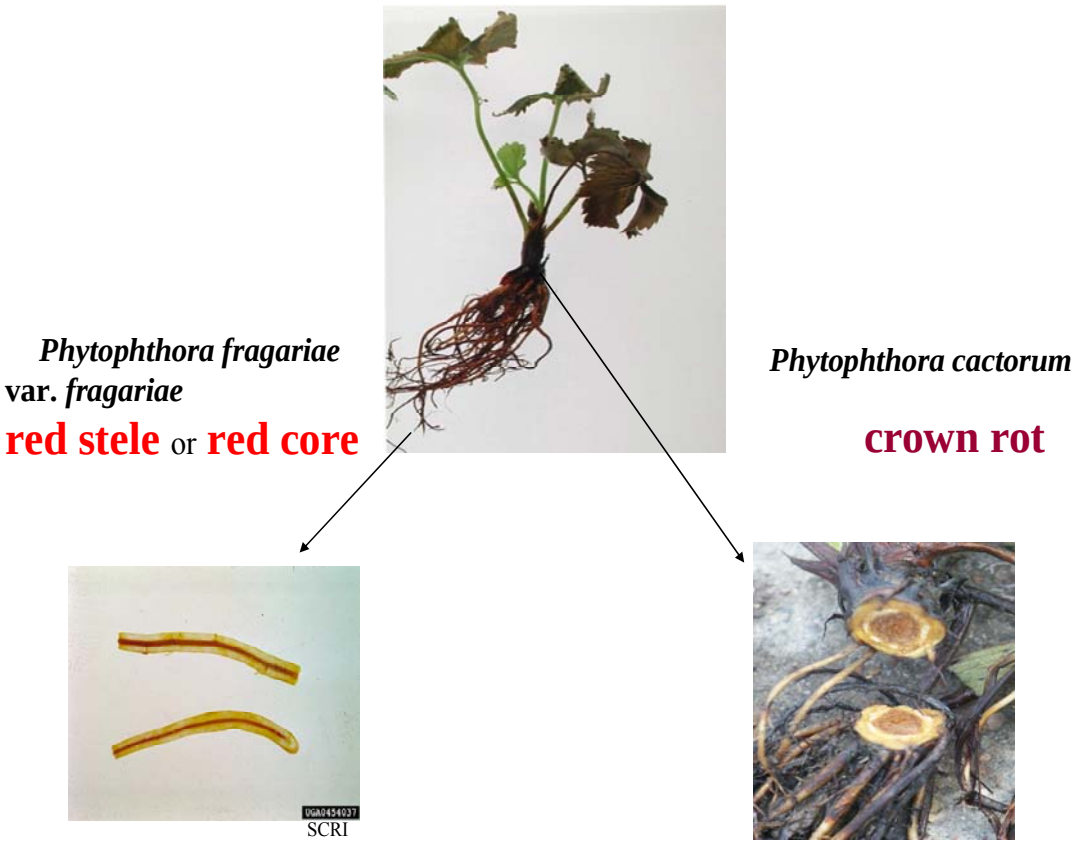


Fig. 1: Typical disease symptom of red stele (*Phytophthora fragariae* var. *fragariae*) and crown rot (*Phytophthora cactorum*) in strawberry

Biological control has been known since 1874 when Roberts showed the suppressive activity of *Penicillium glaucum* against bacteria and regarded this phenomenon as antagonism. The biological control of root pathogens is known over 80 years (Deacon, 1991). At the end of 19th century, Russian agriculturists began showing that the application of bacteria increased growth parameters and yield in different culture crops. However, many of these experiments were conducted with unspecific species of bacteria (Mithustin and Naumova, 1962).

One of the most intensively studied fields of biological control is the use of bacteria and fungi against soilborne pathogens (Weller, 1988; Cook, 1993; Weller *et al*., 2002). Becker and Schwinn (1993) described the different advantages of biocontrol over the conventional agrochemical. The effect of biocontrol may be prolonged, there is less chance for the development of resistance against antagonists and most importantly there is no evidence that biocontrol has any negative effect on nature. Biological control measures are generally well accepted by the authorities responsible for certifying pesticides. Commercialization of a number of microbial biocontrol products (Fravel and Larkin, 1996) has further intensified research in biological control.

Interest in biological control of soilborne diseases has increased in the past two decades. Bacteria have been applied to soil and on the roots of different plants in order to increase plant growth and yield (Suslow, 1982; Suslow and Scrroth, 1982; Kloepper, 1991; Gupta *et al*., 2000; Kloepper *et al*., 2004) and to reduce the disease intensity (Sikora, 1992; Hoffmann-Hergarten, 1994; Keuken, 1996; Gulati, 1997; Koch, 1997). Species of *Bacillus* are among the most intensively studied biocontrol agents (Schisler *et al*., 2004). Treatment of seed or plants with bacilli promoted plant health of different crops viz. grains (Merriman, 1974), vegetables (Merriman and Birkenhead, 1977), onions (Reddy and Rahe, 1989) peanuts (Turner and Backmann, 1991), apple (Utkhede and Smith, 1992) and tomatoes (Keuken, 1996; Gulati, 1997; Hoffmann-Hergarten *et al*., 1998). Among the gram-negative bacteria, *Pseudomonas* species were the most commonly used biocontrol agents against soilborne pathogens (Weller, 1988; Hemming, 1990; O' Sullivan and O'Gara, 1992; Lemanceau and Alabouvette, 1993; Keel *et al*., 1996; Weller *et al*., 2002; Haas and Défago, 2005).

Plant growth promoting rhizobacteria (PGPR) are naturally occuring soil microorganisms that colonize roots and stimulate plant growth. Such bacteria have been applied to a wide range of agricultural species for the purposes of growth enhancement, including increased seed emergence, plant weight, and disease control. Yield increases between 10 % and 20 % with PGPR applications have been documented for several agricultral crops (Kloepper *et al*.,1991).

When compared to the crops mentioned above, efforts to control diseases of strawberry with bacteria or fungi are limited. There are a number of studies related to biological control of *Botrytis cinerea* (Elad, 1994; Freeman *et al*., 2004) on aboveground plant parts on strawberry. Tronsmo (1976) showed that application of spores of the antagonistic fungus *Trichoderma pseudokoningii* to strawberries at flowering could considerably reduce the disease. Similar results were obtained with *Bacillus pumilus* and *Pseudomonas fluorescens* (Swadling and Jeffries, 1998). Liu (1993) reported that different isolates of actinomycetes, fungi and bacteria (*Bacillus* and *Pseudomonas* spp.) caused significant reductions in mycelial growth of *B. cinerea* in dual culture tests. Studies on biocontrol of soilborne diseases of strawberry are rare. Kurze *et al*. (1998) reported that three different isolates of *Serratia plymuthica* showed antifungal effects against different soilborne pathogens of strawberry viz *Verticillium dahliae* Kleb, *Rhizoctonia solani* Kühn and *Phytophthora cactorum* (Lebert and Cohn) J. Schröt in *in vitro* experiments. Previous studies have shown that dip treatment of roots with *Bacillus licheniformis*, *Enterobacter agglomerans* and *Pseudomonas fluorescens* (Hessenmüller and Zeller, 1996) and *Pseudomonas chlororaphis* (Koch *et al*., 1998) resulted in reduction of disease index of red stele and crown rot disease of strawberry.

Hence in this study, an attempt has been aimed to study the antagonistic effect of rhizobacteria against both *Phytophthora* spp. in strawberry.

II. Biological control of crown rot (*Phytophthora cactorum*) and red stele (*Phytophthora fragariae var. fragariae*) disease of strawberry with rhizobacteria

1.0 Introduction

Strawberry is one of the important berry cultures in Germany. The yield of fruits depends on the soil conditions, photoperiod, temperature, and losses caused by pests and diseases. The most important fungal pathogens of strawberry are *Botrytis cinerea*, *Colletotrichum acutatum*, *Verticillium dahliae*, *Phytophthora fragariae* var. *fragariae* and *Phytophthora cactorum*. Losses in crops due to root rots caused by various *Phytophthora* species are well documented (Scheer van der, 1971; Seemüller and Schmidle, 1979). Red stele (*P. fragariae* var. *fragariae*) and crown rot (*P. cactorum*) disease of strawberry can cause substantial economic damage (Seemüller, 1998). Rijbroek *et al*. (1997) reported that *Phytophthora cactorum*, causal agent of crown rot of strawberry, caused serious losses in the Netherlands and described the difficulty in management of the disease. Therefore, in areas where *P. fragariae* and *P. cactorum* are present cultivation of strawberry depends directly on the use of chemicals. The number of fungicides available for the control of *P. fragariae* and *P. cactorum* are limited. In Germany, Aliette is the only registered fungicide against crown rot and red stele disease of strawberry (Anonymous 1999). In some fields where Metalaxyl was used for years, resistant strains of *P. fragariae* var. *fragariae* have been found (Nickerson, 1998). As resistance of *Phytophthora* species to Metalaxyl has been documented (Seemüller and Sun, 1989). Biological control methods proved in recent years as an alternative to chemical treatments for disease management. Thus, bacteria have been applied to soil and on roots of different plants in order reduce the disease intensity (Sikora, 1992; Koch, 1997).

In the present study, after a screening of more than 100 bacterial isolates out of the rhizosphere of fruit orchards and potato crops in dual culture test, three with the best inhibition effect against the two *Phytophthora* pathogens, were further used for greenhouse

and field experiments. The following bacterial isolates were used: *Raoultella terrigena* (formely *Klebsiella terrigena*) strain G-584, *Bacillus amyloliquefaciens* strain G-V1 and *Pseudomonas fluorescens* strain 2R1-7.

Taxonomic identification of the used bacterial strains: *Raoultella terrigena* strain G-584, *Bacillus amyloliquefaciens* strain G-V1 was carried out by the German Collection of Microorgansims and Cell Cultures (DSMZ), Braunschweig, based on 16S rDNA sequencing and physiological test. *Pseudomonas fluorescens* strain 2R1-7 was from the University of Rostock.

The aims of the present study were to:

Test the efficacy of *Raoultella terrigena* strain G-584, *Bacillus amyloliquefaciens* strain G-V1 and *Pseudomonas fluorescens* strain 2R1-7 against *Phytophthora cactorum* and *Phytophthora fragariae* in *in-vitro* experiments.

Evaluate the effect of these strains on crown rot (*Phytophthora cactorum*) and red stele (*Phytophthora fragariae* var. *fragariae*) disease of strawberry in greenhouse and field experiments under artificially and naturally infested soils in different parts of Germany.

2.0 Materials and methods

a) Media

King's B medium (KBB)

10 g Protease Peptone No.3

10 g Glycerine

1.5 g $K_2HPO_4x3H_2O$

1.5 g $MgSO_4x7H_2O$

1000 ml distilled H_2O (pH 7.2-7.4)

Potato Dextrose Broth (PDB)

24 g PDB (Difco)

1000 ml distilled H_2O (pH 6.5)

Tryptic Soy Broth (TSB)

30 g TSB (Difco)

1000 ml distilled H_2O (pH 7.2-7.4)

Vegetable Broth (V-8)

200 ml V-8 vegetable juice (albi)

3.0 g $CaCO_3$

1000 ml distilled H_2O (pH 6.5)

For agar plates, 15 g agar (Difco) was added to the respective media. Media were autoclaved for 20 minutes at a temperature of 121°C.

Cultivation of antagonistic bacteria

The bacterial isolates were prepared by growing in Tryptic Soy Broth for 24 h at 26 °C, cells were harvested by centrifugation and diluted to an optical density of 0.20 at 660 nm; 10^9 to 10^{11} cfu/ml were used for the experiments.

Cultivation of the pathogens

Inocula of *P. fragariae* var. *fragariae* and *P. cactorum* were cultured in 1-L Erlenmeyer flasks on autoclaved vermiculite media composed of vermiculite (250 g),

wheat-bran (100 g), rye seeds (100 g), Vegetable juice 100 ml, and aqua dest. (250 ml) for 4 weeks at 18 °C and 3 % (w/w) of inoculum used for artificial infestation of soil.

2.1 *In vitro* experimental design

2.1.1 Influence of culture media on growth of bacteria and fungi

To determine a suitable medium for the co-cultivation of fungi and bacteria, three different media were tested: Potato Dextrose Agar (PDA), Tryptic Soy Agar (TSA) and Vegetable-8 Agar. A loop full of bacteria from 2 days old TSA media culture was streaked on agar plates and growth of bacteria was recorded (*** = excellent growth, ** = good growth, * = almost no growth). A 5 mm disk of fungal mycelial was placed in the centre of agar plates and mycelial growth was measured. The number of replications was six. The growth of bacteria was assessed after 24 h. The fungal growth was rated 72-96 h after inoculation using a scale of 1-3. The best media for both organisms was then used for *in vitro* tests.

2.1.2 *In vitro* tests for antagonistic activity of rhizobacteria

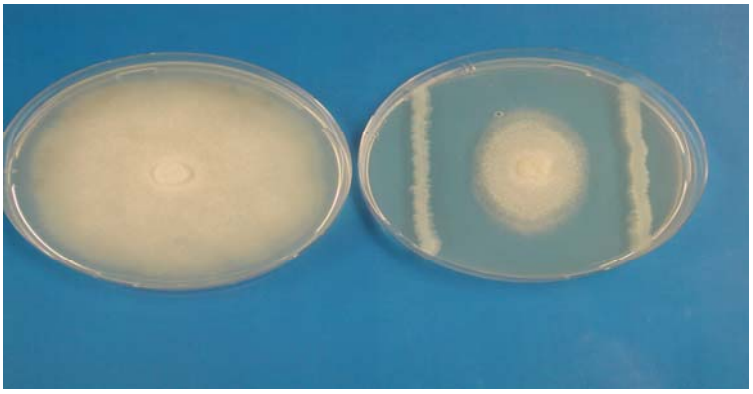
The antagonistic effect of the *Raoultella terrigena* G-584, *Bacillus amyloliquefaciens* G-V1 and *Pseudomonas fluorescens* 2R1-7 was evaluated by the dual culture test and culture filtrate test.

Dual culture test: Agar disks carrying actively growing mycelium of the different fungi were placed in the centre of V-8 agar plates and cells of bacteria were streaked 3 cm away at both sides. Plates without bacteria served as control (Fig. 2). The number of replications was twelve. The growth of mycelium (average of width and length of mycelium) on control plates was taken as reference for computing the antagonistic activity of bacteria with the following equation:

$$\text{Reduction in mycelium growth \%} = \frac{\text{Control-Treatment}}{\text{Control}} \times 100$$

Treatment = (mycelial growth of fungus in plate with streaked bacteria)

Control = (mycelial growth of fungus in plate without bacteria)



(a) Control (b) *B. amyloliquefaciens* G-V1 treated

Fig. 2: Dual culture test (b) shows the reduction of *P. cactorum* mycelial growth by *Bacillus amyloliquefaciens* G-V1

Culture filtrate test: Two loops of bacteria from 2 day old TSA culture were transferred into a 200 ml glass flask with 50 ml of sterile TSB medium. The medium with bacteria was shaken (96 rpm) on a rotary shaker for 24 h in an incubator at 24 ± 2 °C. One ml of this culture was added to a 200 ml Erlenmeyer glass flask with 50 ml of sterile medium and was shaken (96 rpm) on a rotary shaker for 24 h in an incubator at 24 ± 2 °C.

In order to separate cells and culture filtrate the culture was centrifuged at a speed of 6000 rpm at 6-8 °C for 20 – 25 min. The bacterial pellet was discarded and the supernatant solution was filtered through 0.2 µm nitrocellulose filters. The filtrate was added to V-8 agar at a concentration of 30 %. TSB culture medium (30 %) was mixed into V-8 agar as a control. One week old agar disks carrying actively growing mycelium of fungi were placed in the centre of agar plates. The plates were incubated at 20 °C in darkness. The number of replications was twelve. Mycelial growth of the fungi was measured at regular intervals and percent reduction of mycelial growth on test plates was computed in relation to mycelial growth on control plates at the termination of experiment.

2.2 Greenhouse experiments on biological control of red stele (*Phytophthora fragariae var. fragariae*) and crown rot (*Phytophthora cactorum*) diseases of strawberry

Tests were performed with strawberry (*Fragaria* x *ananassa* Duchesne) plants of cv. " Elsanta "(Frigo) and "Honeoye". Both varieties are highly susceptible to red stele and crown rot disease.

Roots of young strawberry ("Elsanta" Frigo) plants were individually dipped for 15 min. into the bacterial suspension in order to have direct exposure of the roots to the antagonistic bacteria. Roots of control plants were either dipped in tap water or 0.5 % Aliette. Plants were then transferred to 14 cm plastic pots with artificially infested soil using 3 % of inoculum. 90 days after planting, roots were carefully washed and symptoms scored using a 1-6 scale (1: root healthy, 2: 20 % root rot, 3: 40 % root rot, 4: 60 % root rot, 5: 80 % root rot, 6: plant dead) and disease severity was recorded on 25 plants for each treatment. Disease index was calculated with the following scheme as follows:

$$\% \text{ Disease index} = \frac{\text{Sum of the rating value}}{\text{Total no. of plants x 6 (Highest rating value)}} \times 100$$

Biological control efficacy was calculated using the formula according to Abbot (1925):

$$\% \text{ Disease control} = \frac{(\text{Disease index of control} - \text{Disease index of treatment})}{\text{Disease index of control}} \times 100$$

Moreover plant growth promotion effect was determined in the absence of pathogen, three months after treatment of the strawberry plants by measuring (20 plants per treatment) fresh weight of shoots and roots .

2.3 Field trials on biological control of red stele (*Phytophthora fragariae var. fragariae*) and crown rot (*Phytophthora cactorum*) disease of strawberry

Field trials were conducted at four different locations in Germany under artificially (Darmstadt: South Hessia) and naturally (Jork: North Germany, Grossostheim: North West of Bavaria and Denzlingen: South Germany) infested soil conditions in the season 2003/2004. In the season 2004/2005, field trials were conducted at two different locations (Darmstadt and Jork). The experimental design was a completely randomized.

2.3.1 Experiments under artificially infested soil conditions: 2003/2004

The experiment at Darmstadt was carried out in the research field of the Institute for Biological Control, Federal Biological Research Centre for Agriculture and Forestry (BBA), (plot size: 13 m in length and 8 m in width); it started in June, 2003, roots of strawberry (Elsanta (Frigo)) plants were dipped in three antagonistic preparations of *R. terrigena* strain G-584, *B. amyloliquefaciens* strain G-V1 and *P. fluorescens* strain 2R1-7, Alliete (0.5 %) or tap water for 15 minutes. Then treated plants were planted in artificially infested soil (*P. fragariae var. fragariae* and *P. cactorum* inoculum was applied to each planting hole). The plants were harvested 3 months after planting, disease severity recorded on 80 plants for each treatment.

2.3.2 Experiments under naturally infested soil conditions: 2003/2004

The trial at Jork carried out in the research field of Fruit-growing laboratory (Obstbau-Versuchs-und Beratungszentrum-OVB) under naturally infested soil conditions started in August, 2003. Five treatments i.e. suspensions of *B. amyloliquefaciens* G-V1, *R. terrigena* G-584, mixture of G-V1 and G-584, Aliette (0.5 %) and a water control were used. Treated strawberry (Elsanta) plants were planted in soil naturally infested with *Phytophthora* spp. (plot size: 15 m x 10 m). The plants were harvested in May, 2004, disease severity was recorded on 85 plants for each treatment.

The trial at Grossostheim carried out on a commercial Farm (Hans Ballman) under naturally infested soil conditions, started in August, 2003. Four treatments i.e. suspension of *B. amyloliquefaciens* G-V1, *R. terrigena* G-584, Aliette (0.5 %), and a water control were used. Then strawberry (Elsanta) plants were planted in soil naturally infested with *Phytophthora* spp. (plot size: 15 m x 10 m). Rating of disease, the number of dead and infected plants (based on shoot symptoms) was noted in July, 2004, disease severity was recorded on 100 plants for each treatment.

The trial at Denzlingen carried out on a commercial organic Farm (Christof Höfflin), started in August, 2003. Four treatments, i.e. suspensions of *B. amyloliquefaciens* G-V1, *R. terrigena* G-584, Aliette (0.5 %) and a water control were used and treated strawberry (Honeoye) plants were planted in soil naturally infested with *Phytophthora* spp (plot size: 15 m x 10 m). Rating of disease: the number of dead and infected (based on shoot symptoms) were noted in August, 2004, disease severity was recorded on 100 plants for each treatment.

2.3.3 Experiments under artificially infested soil conditions: 2004/2005

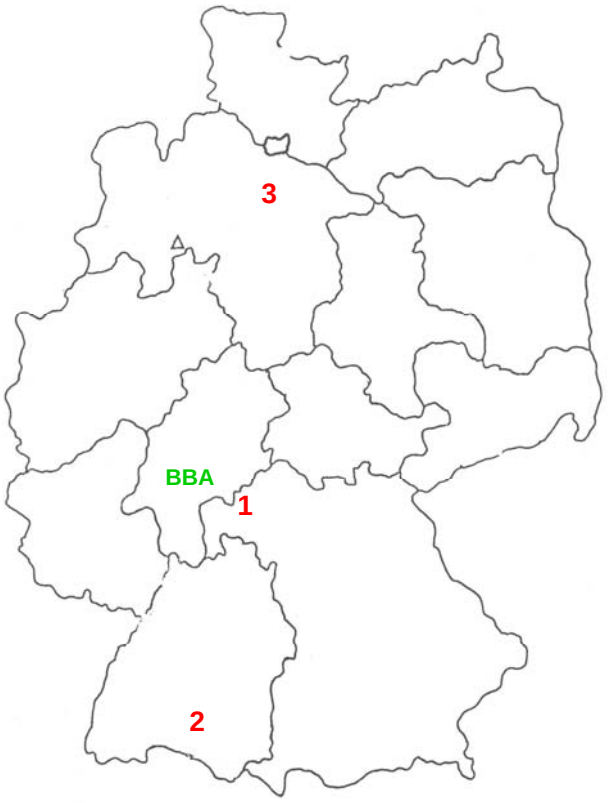
The experiment at Darmstadt was carried out in the research field of Institute for Biological Control, BBA, (plot size: 13 m x 8 m); it started in May, 2004, roots of strawberry (Elsanta (Frigo)) plants were dipped in three antagonistic preparation of *R. terrigena* G-584, *B. amyloliquefaciens* G-V1 and *P. fluorescens* 2R1-7, Alliete (0.5 %) or tap water for 15 min. Then treated plants were planted in artificially infested soil (*P. fragariae var. fragariae* and *P. cactorum* inoculum was applied to each planting hole). The plants were harvested 3 months after planting, disease severity recorded on 80 randomly selected plants for each treatment

2.3.4 Experiments under naturally infested soil conditions: 2004/2005

The trial at Jork carried out in the research field of Fruit-growing laboratory (Obstbau-Versuchs-und Beratungszentrum -OVB) under naturally infested soil conditions started in August, 2004. Six treatments i.e. suspensions of *B. amyloliquefaciens* G-V1, *R. terrigena* G-584, *Pseudomonas fluorescens* 2R1-7, mixture of G-V1 and G-584, Aliette (0.5%) and a water control were used. Treated strawberry (Elsanta) plants were planted in soil naturally infested with *Phytophthora* spp (plot size: 15 m x 10 m). The plants were harvested in May, 2005; disease severity was recorded on 100 plants for each treatment.

2.4 Statistical analysis

Statistical significance was computed with SAS (SAS Institute Inc., NC, USA) using the Tukey Test.



Under naturally Infested soil conditions

1: Grossostheim

2: Denzlingen

3: Jork

Under artificially infested soil conditions

BBA Darmstadt

Fig. 3: Field experiment locations in Germany

Greenhouse experiments

Field experiments

Darmstadt

Grossostheim

Denzlingen

Fig. 4: Greenhouse and Field experiments on different locations in Germany

3.0 Results

3.1 *In vitro* studies

Influence of culture media on growth of bacteria and fungi

Bacteria and fungi were cultured on different media to find a suitable medium for simultaneous cultivation of both groups of organisms. On tryptic soy agar and in vegetable 8 agar (V-8 agar) all tested bacterial isolates showed excellent growth (Tab. 2). However, the growth of fungi was best on V-8 agar. Vegetable 8 agar was therefore taken as a standard media for dual culture and culture filtrate tests.

Bacteria and Fungi	Potato dextrose agar	Tryptic soy agar	Vegetable-8-agar
Bacillus amyloliquefaciens G-V1	***	***	***
Raoultella terrigena G-584	**	***	***
Pseudomonas fluorescens 2R1-7	**	***	***
Phytophthora cactorum	**	**	***
Phytophthora fragariae	**	*	***

*** = excellent growth, ** = good growth, * = almost no growth

Tab. 2: Influence of culture media on growth of bacteria and fungi in petri dishes

Dual culture test: The mycelial growth of *P. cactorum* and *P. fragariae* var. *fragariae* was reduced up to 60 % in dual culture tests with the three rhizobacteria (Fig. 5). The tested three antagonistic strains showed nearly the same level of effect against the both *Phytophthora* spp.

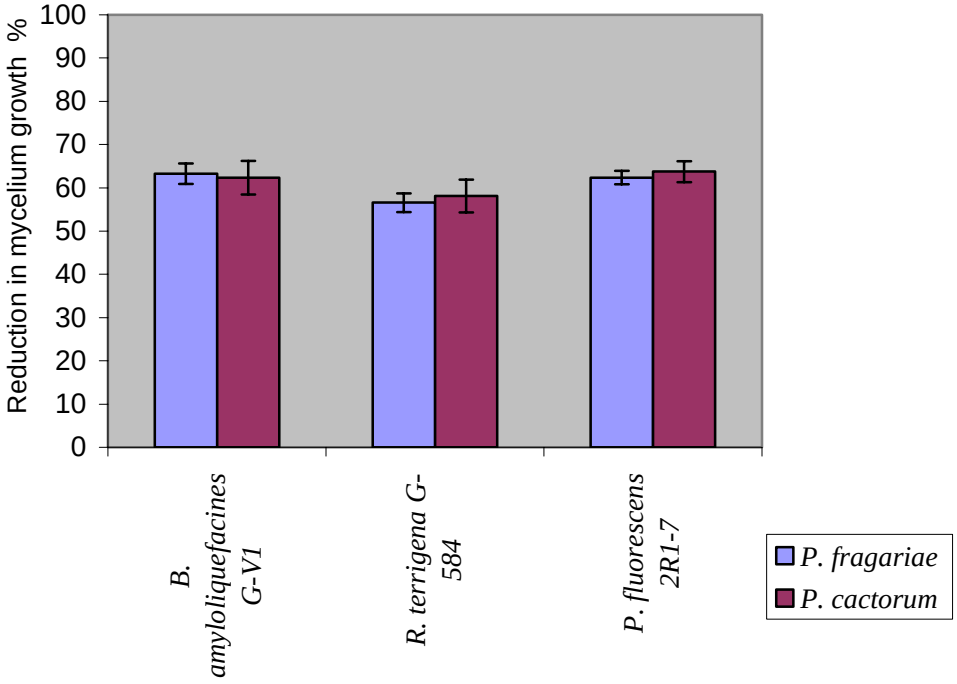


Fig. 5: Effect of *B. amyloliquefacines* G-V1, *R. terrigena* G-584 and *P. fluorescens* 2R1-7 on mycelial growth of *Phytophthora* spp. in dual culture test. Results are presented as reduction in mycelial growth compared to control, n=12, (± standard deviations)

Culture filtrate test: In culture filtrate test the mycelial growth of *P. fragariae* was reduced about 61 % by *P. fluorescens* 2R1-7, followed by *B. amyloliquefaciens* G-V1 with 29 % and 25 % from *R. terrigena* G-584. The tested three strains showed same level of mycelial growth reduction against *P. cactorum* (Fig. 6).

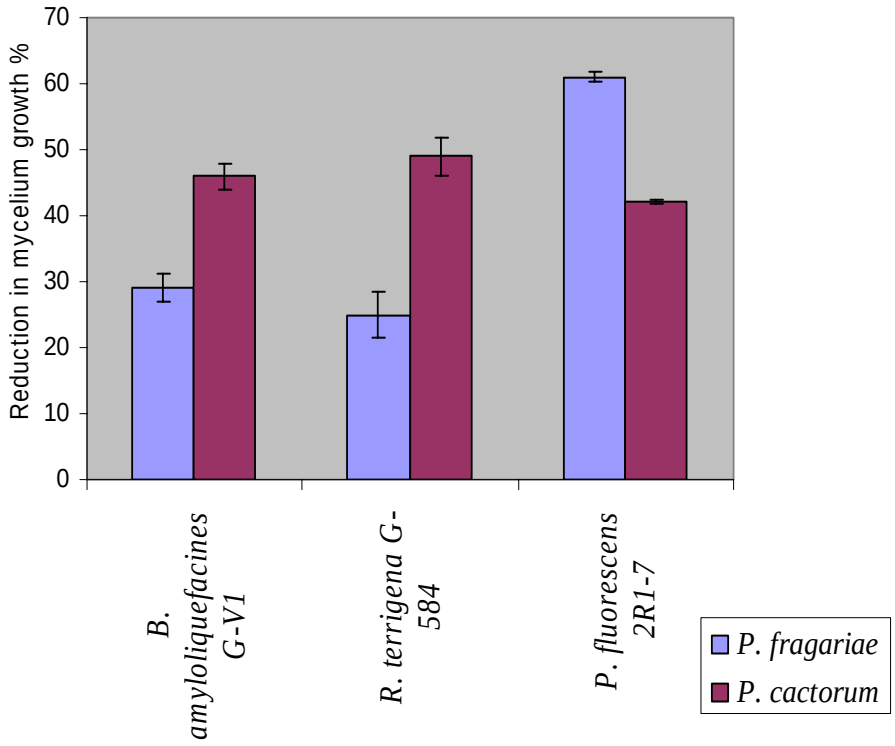


Fig. 6: Effect of *B. amyloliquefaciens* G-V1, *R. terrigena* G-584 and *P. fluorescens* 2R1-7 on mycelial growth of *Phytophthora* spp. in culture filtrate test. Results are presented as reduction in mycelial growth compared to control, n=12, (± standard deviations)

3. 2 Greenhouse experiments on biological control of red stele (*Phytophthora fragariae var. fragariae*) and crown rot (*Phytophthora cactorum*) diseases of strawberry

3.2.1 Test on biocontrol activity of the three rhizobacteria

Under greenhouse conditions, the three rhizobacteria showed nearly same level of biocontrol activity towards *P. fragariae var. fragariae* (red stele) and *P. cactorum* (crown rot). All bacterial treatments gave a significant effect compared to infected control (Tab. 3). The higher percentage of biological control was obtained against red stele disease by using *Pseudomonas fluorescens* 2R1-7 with 59.3 %. In the case of crown rot disease with 56.9 % using G-584 of *Raoultella terrigena*, these results were nearly comparable with the chemical control Aliette of red core and crown rot diseases of 61 % and 66.1 % respectively.

Treatments	Red stele disease		Crown rot disease	
	Disease index %	Disease control %	Disease index %	Disease control %
Control	47.2 a		52.0 a	
R. terrigena G-584	26.4 b	44.0	22.4 b	56.9
B. amyloliquefaciens G-V1	28.0 b	40.6	25.6 b	50.7
P. fluorescens 2R1-7	19.2 b	59.3	26.4 b	49.2
Aliette	18.4 b	61.0	17.6 b	66.1

Tab. 3: Influence of rhizobacteria in controlling red stele and crown rot diseases of strawberry under artificially infested soil conditions in greenhouse. Disease index followed by different letters are significantly different according to Tukey Test, $P<0.05$, n=25

3.2.2 Test for plant growth-promoting properties

The effect of root bacterization on plant growth in the absence of the pathogens was evaluated based on production of plant fresh matter. All the treatments with rhizobacteria showed nearly equal fresh-shoot weight of strawberry plants with that in the control. Data in Table 4 show that fresh weight of root systems of strawberry plants treated with *P. fluorescens* 2R1-7 decreased compared to control.

Treatments	Shoots fresh weight (g)	Roots fresh weight (g)
Control	23.55 a	6.01 a
R. terrigena G-584	24.98 a	6.48 a
B. amyloliquefaciens G-V1	24.57 a	5.59 ab
P. fluorescens 2R1-7	20.11 a	3.93 b
Aliette	23.62 a	5.78 ab

Tab. 4: Effect of rhizobacteria on fresh shoot- and root- systems of strawberry plants, different letters are significantly different according to Tukey Test, $P<0.05$, n=20

3.3 Field trials on biological control of red stele (*Phytophthora fragariae var. fragariae*) and crown rot (*Phytophthora cactorum*) diseases of strawberry

3.3.1 Experiments under artificially infested soil conditions 2003/2004

The trial at Darmstadt: Data in Table 5 showed that the application with the three rhizobacteria gave a significantly considerable degree of protection against both pathogens under field conditions. The percentage of biological control was obtained for the control of red stele disease using the isolate 2R1-7 of *P. fluorescens* with 45.0 % followed by of *B. amyloliquefaciens* strian G-V1 and *R. terrigena* strain G-584 with 37.6 % and 35.0 % respectively. On the other hand, the three genera of bacteria *R. terrigena*, *P. fluorescens* and *B. amyloliquefaciens* with nearly the same percentages of control effect (35.1 %, 39.7 % and 29.6 %, respectively) against crown rot. Aliette showed 49.6 % control efficacy against red stele and 52.5 % on crown rot.

Treatments	Red stele disease		Crown rot disease	
	Disease index %	Disease control %	Disease index %	Disease control %
Control	37.7 a		32.7 a	
R. terrigena G-584	24.5 b	35.0	21.2 bc	35.1
B. amyloliquefaciens G-V1	23.5 b	37.6	23.0 b	29.6
P. fluorescens 2R1-7	20.7 b	45.0	19.7 bc	39.7
Aliette	19.0 b	49.6	15.5 c	52.5

Tab. 5: Efficacy of rhizobacteria in controlling the red stele and crown rot diseases of strawberry under artificially infested soil conditions in the field at Darmstadt, June, 2003. Disease index followed by different letters are significantly different according to Tukey Test, $P<0.05$, n=80

3.3.2 Experiments under naturally infested soil conditions 2003/2004

Under natural conditions the infection pressure with the pathogen was different in the locations mentioned before (Fig. 3) and also a differentiation of both *Phytophthora* diseases could not be evalauted. Therefore it is described as the general *Phytophthora* incidence of the infested field.

The trial at Jork (OVB): The level of control based on number of infected or dead plants at the termination of experiments is shown in Fig. 7. Through the treatment with the rhizobacteria a reduction of disease could be observed about 37.5 % at this location in North West Germany. Fig. 7 indicated that the rating of the diseases of strawberry grown in naturally infested field was reduced compared with control (23.2 %), when each of *B. amyloliquefaciens* G-V1 and *R. terrigena* G-584 was together (14.5 %). However, individual application with *B. amyloliquefaciens* G-V1, *R. terrigena* G-584 and Aliette showed no significant effect.

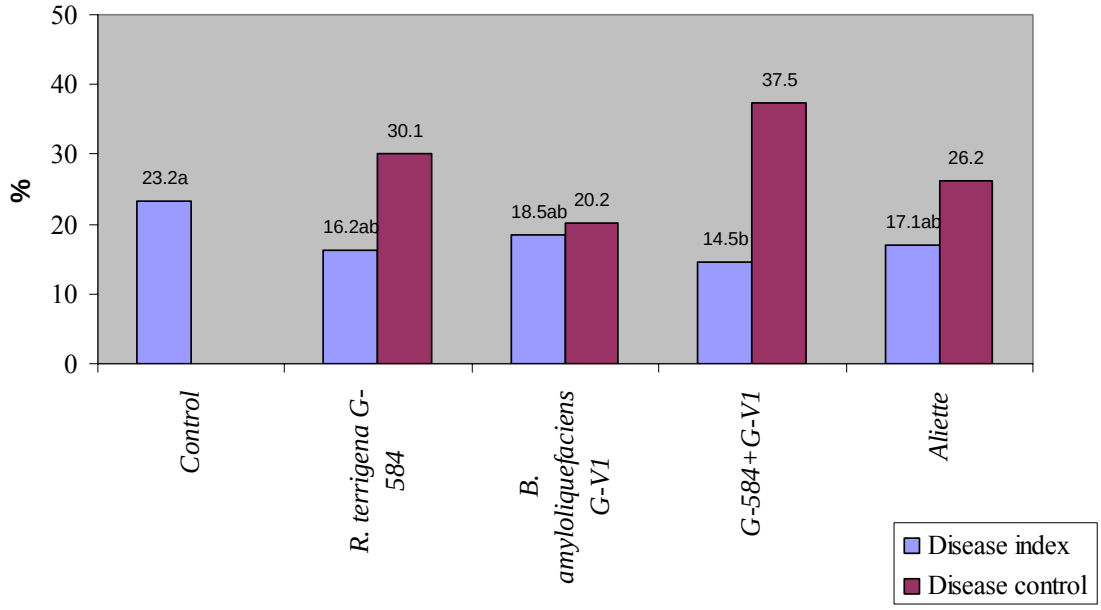


Fig. 7: Efficacy of rhizobacteria in controlling *Phytophthora* spp. of strawberry grown in naturally infested soil at Jork, 2003/2004. Different letters show statistically significant differences among treatments according to Tukey Test, $P<0.05$, n=85

The trial at Grossostheim: Fig. 8 showed the reduction on the rating of disease caused with *Phytophthora* spp. of strawberry by the applied bacterial antagonistic strains, grown in a commercial farm. Disease control efficacy in this naturally infested field reached nearly to 50 % in all treatments either using rhizobacteria or Aliette when it was compared with that in the control (Fig. 8).

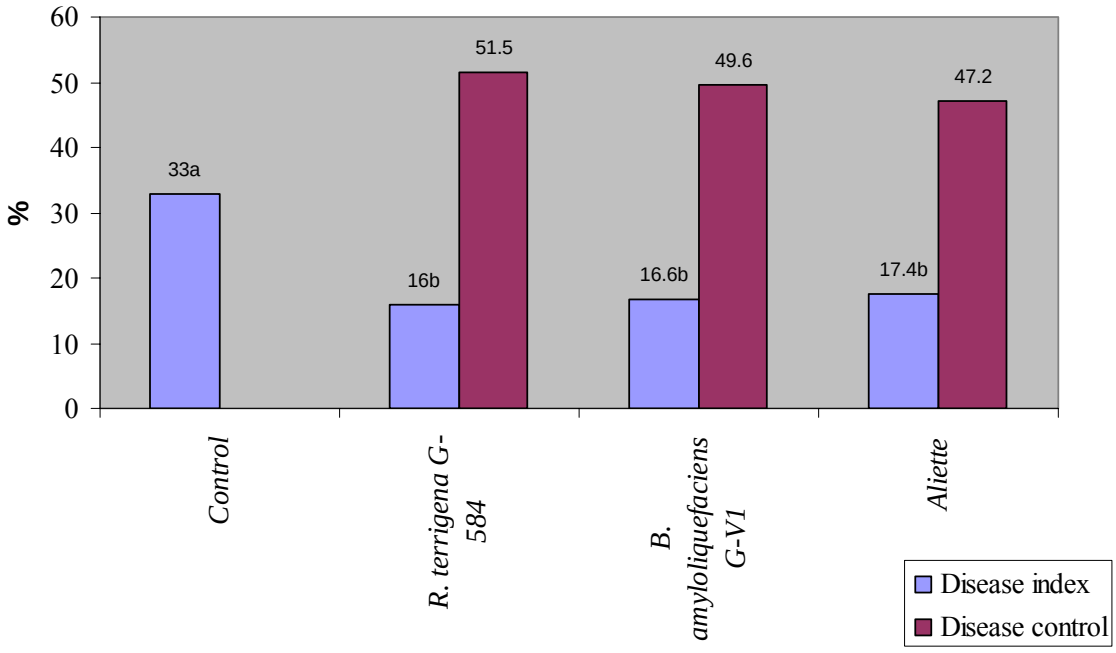


Fig. 8: Efficacy of rhizobacteria in controlling *Phytophthora* spp. of strawberry grown in naturally infested soil at Grossostheim, 2003/2004. Different letters show statistically significant differences among treatments according to Tukey Test, $P<0.05$, n=100

The trial at Denzlingen: In the last experiment under naturally infested soil conditions in a commercial organic farm in South West Germany (Fig. 9), the incidence of the fungal disease was with 25 % in control. At this site none of the rhizobacterial antagonists significantly reduced disease index compared to the control except the chemical Aliette (Fig. 9). Disease reduction by Aliette was 56.8%.

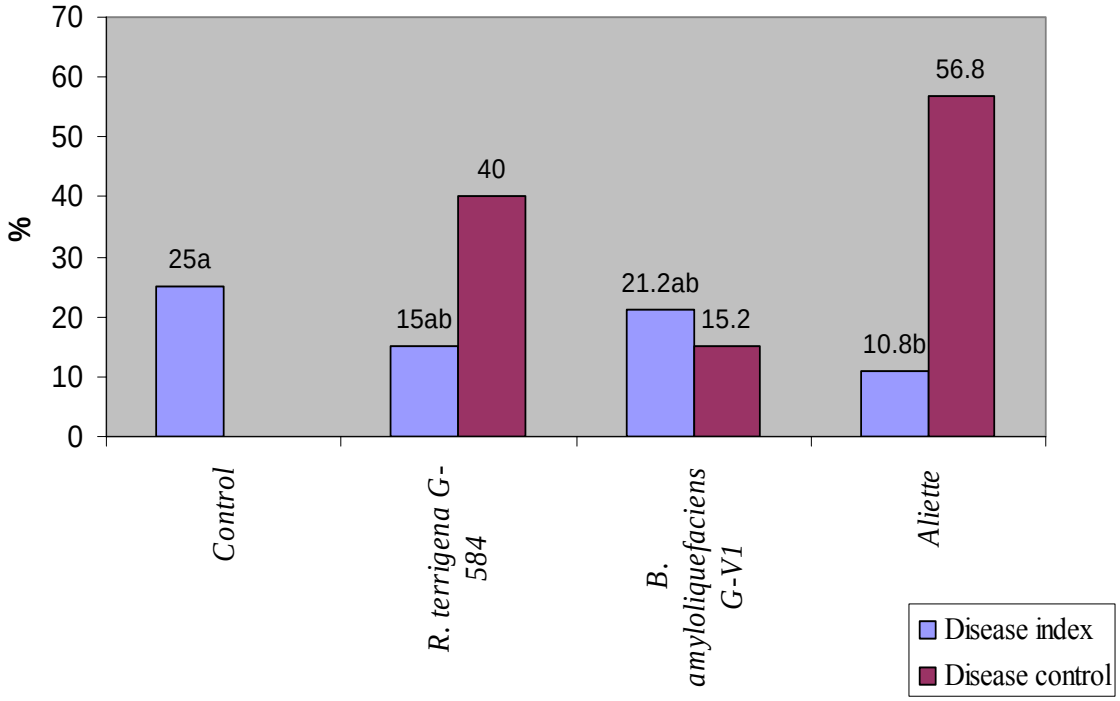


Fig. 9: Efficacy of rhizobacteria in controlling *Phytophthora* spp. of strawberry grown in naturally infested soil at Denzlingen 2003/2004. Different letters show statistically significant differences among treatments according to Tukey Test, $P<0.05$, n=100

3.3.3 Experiments under artificially infested soil conditions 2004/2005

The trial at Darmstadt: Data in Table 6 showing that the application with the three rhizobacteria showed efficacy in different to field trail 2003 (Tab. 5). Significant percentage of biological control was obtained for control of red stele disease using the isolate of *B. amyloliquefaciens* G-V1 with 27.2 %, other treatments showed no significant effect compared to control. On the other hand, treatment with Aliette only showed significant control effect of 36.7 % against crown rot compared to other rhizobacterial treatments.

Treatments	Red stele disease		Crown rot disease	
	Disease index %	Disease control %	Disease index %	Disease control %
Control	52.9 a		61.2 a	
R. terrigena G-584	46.4 ab	12.2	47.7 ab	22.0
B. amyloliquefaciens G-V1	38.5 b	27.2	49.7 ab	18.7
P. fluorescens 2R1-7	44.1 ab	16.6	57.0 a	6.8
Aliette	40.4 ab	23.6	38.7 b	36.7

Tab. 6: Efficacy of rhizobacteria in controlling of red stele and crown rot diseases of strawberry under artificially infested soil conditions in the field at Darmstadt, June, 2004. Disease index followed by different letters are significantly different according to Tukey Test, $P<0.05$, n=80

3.3.4 Experiments under naturally infested soil conditions 2004/2005

The trial at Jork (OVB): Fig.10 showing that the application with *R. terrigena* G-584 showed significant percentage of biological control effect with 45.1 %. However, the other treatments, mixture of G-584 & G-V1, *B. amyloliquefaciens* G-V1, *P. fluorescens* 2R1-7 and Aliette showed no significant effect compared to control which is in different to field trail 2003 (Fig. 7).

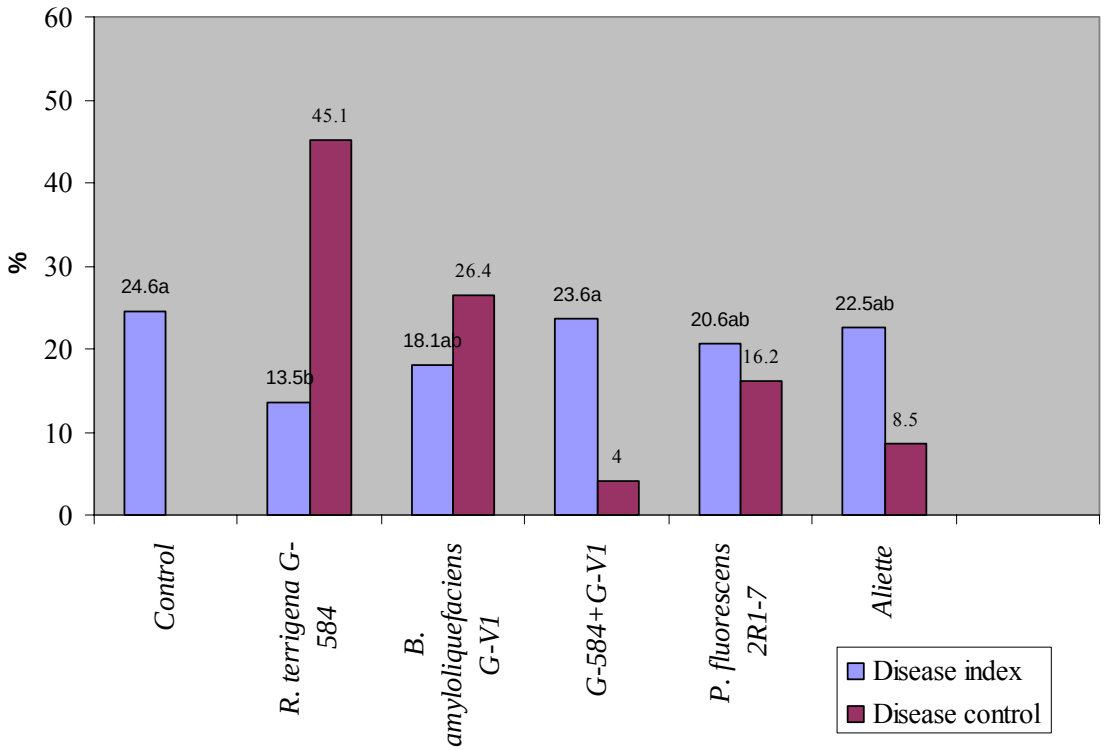


Fig. 10: Efficacy of rhizobacteria in controlling *Phytophthora* spp. of strawberry grown in naturally infested soil at Jork, 2004/2005. Different letters show statistically significant differences among treatments according to Tukey Test, $P<0.05$, $n=100$

4.0 Discussion

In vitro studies

The aim of the present study was to test three rhizobacteria from different genera for their efficacy against the crown rot and red stele disease of strawberry. In the study here, *in vitro*, greenhouse and field experiments were conducted. In comparing experiments the growth of bacteria was similar on almost all the selected media. However, the V-8 media was most suitable for the growth of fungi. Therefore for dual culture and culture filtrate tests V-8 media was determined as the suitable nutrient media. Cultivation media play an important role in biological control of plant pathogens (Dickie and Bell, 1995; Borowicz and Omer, 2000) and a suitable environment is the best for the performance of experiments (Dhingra and Sinclaire, 1985).

In vitro experiments described in this part of study have been used by other authors for testing the antagonism of rhizobacteria against fungal pathogens (Koch *et al*., 1998; Velahzazan *et al*., 1999). The positive results achieved *in vitro* tests systems, however, may not always be the same in the field (Harman and Lumsden, 1990). The antagonistic potential of a biocontrol agent not can be successfully explored with *in vitro* experiments. However, *in vitro* methods are a quicker means of testing the antagonistic potential of the selected isolates than greenhouse tests (Kempf, 1988; Renwick *et al*., 1991) or field studies. They also save time and material in selection of potential antagonists (Kloepper, 1991).

Greenhouse experiments on biological control of crown rot (*Phytophthora cactorum*) and red stele (*Phytophthora fragariae* var. *fragariae*) disease of strawberry

Bacterial isolates: The used antagonistic bacteria, *Raoultella terrigena* G-584, *Bacillus amyloliquefaciens* G-V1 originating from apple orchards and *Pseudomonas fluorescens* 2R1-7 from potato crop proved to be good antagonists in the test system used. Strains of *Bacillus* and *Pseudomonas* spp. have in the past also been reported to be very good biological control agents of plant diseases (Bochow, 1992; Cook, 1993; Raaijmakers *et al*., 1995; Koch *et al*., 1998; Zeller, 1999). Cook and Weller (1987) reported that an isolate should be selected from the same location where it is to be used as an antagonist due to the fact that rhizobacteria are often relatively host specific, cultivar specific which effects root colonization and plant growth promotion (Schroth and Becker, 1990). However, generally isolates are selected from different crops and then used to control diseases in other cultures (Harman and Lumsden, 1990).

Bacteria treatment: The method of application or treatment of biological agents can play an important role in the efficacy of the applied agent on the target pathogen. Biological control agents can be applied prior to sowing, on transplants, tissue culture plantlets or as drenches. In previous studies root bacterization or application of bioagents has been done at the time of planting (Koch *et al*., 1998) or at sowing (Hoffmamm-Hergarten, 1994; Keuken, 1996; Gulati, 1997). Tu and Zheng (1996) showed that a single treatment of *Bacillus subtilis* or *Pseudomonas fluorescens* could significantly control *Verticillium* wilt of tomato caused by *Verticillium dahliae*. In the experiments conducted here strawberry plants were used therefore the roots of the plants were dipped in bacteria cell solution (10^9 - 10^{11} for 15 minutes) before planting. Similar concentrations have also been used in root dip treatments of other plants with positive results (Sikora, 1988; Gulati, 1997; Lottmann *et al*., 1998; Koch *et al*., 1998; Hoffmann-Herrgarten *et al*., 1998). The advantage of this method in treatment of plant roots is that the roots are directly exposed to the bacterial inoculum free of soil. The other advantages are that the pre-treatment insures higher levels of activity, reduces production costs, simplifies formulation and application, reduces extension costs and minimizes impact on the environment (Sikora, 1997). The experiments were done in a greenhouse at a temperature of 20 °C as this is regarded as an optimum temperature for the

growth of the two species of *Phytophthora* and favours infection (Duncan and Kennedy, 1995).

Disease rating: The aim of this part of the study was to measure the antagonistic potential of *Raoultella terrigena* G-584, *Bacillus amyloliquefaciens* G-V1, *Pseudomonas fluorescens* 2R1-7 or in comparision with standard fungicide Aliette against crown rot and red stele disease of strawberry. Antagonistic root-associated bacteria are an important functional group of benificial bacteria responsible for the control of soilborne pathogens (Weller, 1988). As a result of the first screening, three genera of bacteria were found to produce detectable inhibition zones against *Phytophthora* spp. The production of clear inhibition zones in dual culture screens is due to the production of antibiotics, toxic metabolites or siderophores as mechanisms for biological control (Swadling and Jeffries, 1998).

In the present study an antagonistic potential of three selected rhizobacteria *Raoutella terrigena* G-584*, Bacillus amyloliquefaciens* G-V1 *and Pseudomonas fluorescens* 2R1-7 against *Phytophthora* spp. the causal organism of crown rot and red stele diseases of strawberry was observed. *Bacillus* spp. and *Pseudomonas* spp. are well known antagonists of different plant pathogens (Bochow, 1992; Cook, 1993; Koch *et al*., 1998; Mansour and Farag, 1999; Zeller, 1999; Duffy *et al*., 2004). But until now, no data on the control of *Phytophthora* spp. by *Raoultella terrigena*, a Gram negative enteric bacterium, have been reported. This species was first isolated from soil and water (Izard *et al*.,1981). Another species, *Klebsiella oxycota* have been used on a large scale to improve plant growth of several important crops in China (Lin, 2000). In this study, *Raoultella terrigena* strain G-584 had moreover a high inhibitory activity against the *Phytophthora* spp. The results of dipping the plants in bacterial cell suspensions at the time of planting are in agreement with that of Koch *et al*, (1998). In previous studies Hessenmüller and Zeller (1996) showed that following plant treatment with *A. radiobacter*, *B. licheniformis* and *P. fluorescens* the disease rate of *P. cactorum* was reduced to 68 % and *P. fragariae* to 41 % in strawberry plants after four weeks in soils artificially inoculated with the pathogen. Koch *et al*. (1998) also showed that in greenhouse experiments following treatment with *P. chlororaphis* isolate I-112 the severity of infection by *P. fragariae* was significantly reduced compared to untreated control and also in field experiments considerable control was achieved in

artificially inoculated soils. Bacteria and fungicide treatment must have provided protection to the young strawberry plants from *P. cactorum* and *P. fragariae* after plantation. Reduction of mycelial growth *in vitro* experiments and reduction of disease rate of plant roots show that there is a correlation between the reduction of the mycelial growth of the fungi in *in vitro* and greenhouse studies. Similar results were also obtained in studies in the past with *Bacillus*, *Erwinia* and *Pseudomonas* species (Sauer and Zeller, 1992; Hessenmüller and Zeller, 1996; Koch *et al*., 1998).

Plant growth promoting activites: The goal of this part of the study was to determine if strawberry growth would be influenced by bacterization of the plant root. Treatment with selected rhizobacteria was observed with no direct plant growth promotion. Plant growth-promoting bacteria can have an impact on plant growth and development in two different ways: indirectly or directly. The indirect promotion of plant growth occurs when these bacteria decrease or prevent some of the deleterious effects of a phytopathogenic organism by any one or more of several different mechanisms. On the other hand, the direct promotion of plant growth by plant growth-promoting bacteria generally entails providing the plant growth with a compound that is synthesized by the bacterium (Glick, 1994). In other studies it has also been documented that bacteria play a positive role in the rhizosphere that results in improved plant health (Weller, 1988; Kempf *et al*., 1993). Sikora (1988) differentiated the bacterial isolates according to their mode of action as either plant growth or plant health promoting. Nieto and Frankenberger, (1990) observed that growth promotion in bacterial treatments could be due to the production of phytohormones such as cytokinins. Previous investigations have also shown that bacterization can increase root growth through increased lignin content (Boller and Metraux, 1988).

Field trials on biological control of crown rot (*Phytophthora cactorum*) and red stele (*Phytophthora fragariae* var. *fragariae*) diseases of strawberry

Field Experiment: Field experiments gave a real picture of the antagonistic potential of isolates under the abiotic and biotic conditions existing in the ecosystem (Merriman and Russel, 1990; Pusey, 1990). It is known that the results are often reproducible in *in vitro* or in greenhouse experiments however not in the field (Mahaffee and Backmann, 1993). In the present study field experiments were conducted in two successive years (2003-2005) in different locations in Germany under artificially infested field and natural densities of *Phytophthora cactorum and Phytophthora fragariae*.

In all field experiments, the plants were dipped in bacterial cell suspension at the time of planting. Tested rhizobacteria such as *Raoutella terrigena* G-584, *Bacillus amyloliquefaciens* G-V1 and *Pseudomonas fluorescens* 2R1-7 showed different level of biocontrol efficacy against *Phytophthora* spp. the causal organism of crown rot and red stele diseases of strawberry. The results are in agreement with that of Koch *et al*. (1998). They documented that following treatment of *P. chlororaphis* isolate I-112 a considerable degree of protection against *P. fragariae* in the field in soils artificially inoculated with the pathogen occured after 8 weeks of planting. Variability among biocontrol experiments is not uncommon and the inability to obtain repeatable results in realistic conditions is a major problem in the development of biological control (Weller, 1988; Campbell, 1989). The poor results recorded may be due to different reasons. The variation in results can be due to different abiotic or biotic factors. Abiotic factors such as soil type, moistures and temperature as well as variation in application technology may play a direct role in the potential of the biocontrol agent. It is well known that *P. fragariae* var. *fragariae* exists as oospores in soil and on plant debris and as mycelium in infected roots. Wet or moist soils, where the soil water level is very near the saturation point favours the production and release of zoospores and red stele is more severe when the preceding winter has been cold and wet (Nickerson, 1998). The warm climatic conditions favour the development of the *P. cactorum* infection and colder conditions in the winter favour the development of *P. fragariae* infection (Nickerson, 1998). Koch (1999) also reported that the weak performance of the bacteria in his experiments might be due to the high level of the disease

pressure. Biotic factors such as competition in root colonization with the soil-specific microbial community may have been responsible for the variation in two years field experiement. The intensity of the pathogens may have been higher in this field due to favourable weather conditions for the development of the disease. The virulence of naturally occurring strains could have also been another factor for the limited control of the disease in the fields.

Another aspect investigated was to test if mixtures of bacteria species give a better control against the crown rot and red stele disease of strawberry than one bacterium alone. From previous studies it is known that combinations of different isolates may lead to improved antagonistic activity (Weller and Cook, 1983; Weller, 1988). Application of mixtures of antagonistic micro-organisms, preferably with different modes of action, has been proposed as a strategy to increase the efficacy and to improve the consistency of disease control (Thomashow *et al*., 1990, Pierson and Weller, 1994; Schisler *et al*., 1997). In this study, field trial (2003-2004) at Jork with root bacterization of strawberry with dual mixtures of *B. amyloliquefaciens* G-V1 and *R. terrigena* G-584, observed with improved control compared to individual isolates. However, the results of mixture of *B. amyloliquefaciens* G-V1 and *R. terrigena* G-584, treatment in 2004-2005 could not be confirmed. A combination of different isolates also may result in negative results (Hadar *et al*., 1983). Sikora *et al*. (1990) also showed a negative effect by combining antagonistic bacteria against *Pythium ultimum*. Koch *et al*. (1998) also observed that seed bacterization of cucumber with dual mixtures of isolates did not improve control of *Pythium ultimum* and *Pythium aphanidermatum* on cucumber compared to single isolate treatment. In lettuce the root length of plants treated with *B. sphaericus* isolates A-1 and A-2, *B. amlyloliquefaciens* isolate VM-1-1 was decreased (Gulati, 1997). Decreased root length and root weight has also been reported after *B. subtilis* isolates S-20 and S-26 treatments of tomato (Keuken, 1996). The application of a certain strain may also have a direct effect on the existence of other strains of bacteria or vice versa (Cook, 1993). Some bacteria have the capability of producing antibiotics *in vitro* and *in vivo* (Weller and Thomashow, 1993) that may not only be toxic to microorganism in the soil but also have a negative effect on the plant growth (Kim *et al*., 1995).

Despite intensive research on biological control of soil borne diseases, practical methods of biological control of soil borne pathogens are limited. Future research to increase the efficacy of the bacterial antagonists should concentrate on application methods, different formulations and frequent sprays. To improve biological control agents there is a need to combine the biological control agents with fungicides of lower doses thereby reducing the quantity of the chemicals needed and the risk that the pathogen may become resistant to a certain chemical.

III. Characterization of microbial colonization following root bacterization

1.0 Introduction

In biological control of soil borne pathogens the rhizosphere plays an important role. Plant roots release an enormous amount of root exudates, leading to a significant stimulation of the microbial density and activity. The rhizosphere was defined in 1904 by Hiltner, as the contact zone between soil, roots and microorganism. Rhizosphere is also known as the component of intensive microbial activity and is affected by the activity of the plant roots such as exudates and due to lysis of plant cells (Lynch *et al*., 1990). Rhizosphere colonization following the introduction of bioagents is considered to be an important factor for successful biological control of plant diseases. Strong colonization of roots is generally necessary for disease suppression (Parke, 1991). Weller (1988) reported that insufficient root colonization has been one of the limiting factors in the use of rhizobacteria as biocontrol agents. Similarly, Lemanceau and Alabouvette (1993) reported that inefficient root colonization of introduced pseudomonads is often responsible for the inconsistent performance. Gamalero *et al.* (2003) described that there are different methods for investigating root colonization or for measuring colonization of plant roots by applied rhizobacteria.

Different techniques have been used for investigating root colonization and for measuring rhizobacteria colonization of plant roots for example:

Antibiotic resistance

Antibiotic resistances have been widely used as markers in microbial ecology. Although various plasmids and transposons have been used (Prosser, 1994; Van Overbeek *et al*.,1997), most of the studies on bacterial survival kinetics are based on the use of spontaneously occurring antibiotic-resistant mutants. Rifampicin resistant strains have been frequently used in re-isolation of applied rhizobacteria from the rhizosphere (Kloepper and Beauchamp, 1992). Hoffmann-Hergarten (1994) and Saddlers (1996) used

this technique for studying the population dynamics of applied rhizobacteria from tomato plants and Hessenmüller and Zeller (1996) in strawberry roots. Kanamycin and streptomycin resistance obtained by Tn5 mutagenesis with the suicide plasmid method of Simon *et al*, (1983) was also described as a possible marker (Van Elsas *et al*., 1986). The maintenance of Tn5 in the mutant JM218 was ascertained by comparing bacterial densities of this mutant in root suspensions, estimated by serology, with bacterial density estimated by plate count on King's B medium supplemented with kanamycin (Lemanceau *et al*., 1992). However, possible genetic changes associated with chromosomal- mediated antibiotic resistance may affect several ecologically important traits (Blot *et al*., 1994; Mahaffee *et al*., 1997). Moreover, the use of antibiotic tagged bacteria carries with it the risk of contributing to the spread of antibiotic resistance in nature (Jansson, 1995).

Serological markers

Immunological techniques are relevant especially for the detection, enumeration and localization of introduced bacterial strains in the soil and rhizosphere. Among the serological methods, the enzyme-linked immunosorbent assay (ELISA) is a very sensitive immunoassay for the detection of antigens. ELISA is based on direct or indirect sandwich methods. The ELISA method has been used to study and quantify the external and internal root colonization of maize by two *P. fluorescens* strains (Benizri *et al*., 1997) and the distribution of two diazotrophic enterobacterial strains, *Pantoea agglomerans* and *Klebsiella pneumoniae*, on cereal shoots and roots (Remus *et al.,* 2000). The critical aspect of serological methods is the specificity of the antibodies used. Polyclonal or monoclonal antibodies may be applied according to their specificity. Monoclonal antibodies are obviously more expensive to raise but are more specific. The specificity of the antibodies, especially polyclonal ones, should be checked to decrease the occurrence of possible cross-reactions. Usually, a high enough specificity may be obtained for fluorescent pseudomonad strains with polyclonal antibodies raised against membrane proteins (Glandorf *et al.*, 1992).

Fluorescent markers

Stable and unstable green fluorescent proteins are another attractive marker system for monitoring bacterial cells in the environment is the green fluorescent protein (GFP). The GFP is a 27 KDa polypeptide which converts the blue chemiluminescence of the Ca2+-sensitive photoprotein (aequorin from the jellyfish *Aequorea victoria*) into green light (Chalfie *et al*., 1994). A series of red shifted GFP mutants, 20–35 times stronger than the wild type, with various excitation and emission wavelengths such as the ECFP (enhanced cyan), EGFP (enhanced green) and EYFP (enhanced yellow), have been recently developed (Tsien, 1998). The advantages and disadvantages of this marker have been extensively discussed by Errampalli *et al*., (1999). Some of the most relevant advantages are that GFP is extremely stable and resistant to proteases, is easily detectable, does not require exogenous substrate and allows the monitoring of single cells even in real time. Moreover, GFP is continuously synthesized and there is no background in indigenous bacterial populations. However, the interference of soil particles, the variability of GFP expression in different species, the inability to work in anaerobic conditions and the instability of the plasmid should be considered. In order to overcome the latest limitation and reduce the risk of a plasmid transfer to other microorganisms, bacterial strains used are preferentially chromosomally marked. For that purpose, several Tn5 transposon suicide delivery vectors have been developed (Suarez *et al*., 1997; Tombolini *et al*., 1997). The stability of the GFP varies according to the variants and plasmid constructs in the range of hours or days (Jansson *et al*., 2000).

Specific primers and oligonucleotidic probes

Introduced bacteria can be monitored using primers or probes that allow amplification or hybridization of sequences which are strain-specific. Specific probes can be used to hybridize bacterial colonies after *in vitro* growth (Werner *et al*., 1996) or bacterial cells for *in situ* studies. Probes are usually covalently linked to a fluorochrome such as fluorescein, rhodamine, Texas red, Cy3 and Cy5 (Amann *et al*., 2001). Specific sequences may be introduced by a genetic construction. As an example, a specific primer amplifying across

nptII-lacZ junctions on the Tn5B20 construct was used to follow the survival kinetics in the soil and rhizosphere of the strain *P. fluorescens* R2f tagged by the lacZ-nptII marker gene (Van Overbeek *et al*., 1997). However, as stressed before, genetic constructs may affect the ecological behavior of bacterial strains. Another strategy consists of identifying sequences specific to the strains in order to design primers and probes. Different approaches have been proposed to develop this identification. One is to compare homologous nucleic acid sequences of ribosomal RNA (rRNA) to sequences available in databases. Since rRNA are present in all living microorganisms in high copy number and are quite stable, oligonucleotidic probes can be applied (Amann *et al.,* 2001). They are either species-specific or even strain specific in some cases (Assmus *et al*., 1995). *Pseudomonas* specific primer has been designed by Braun-Howland *et al* (1993). This PSMg primer was applied to describe the dynamic of indigenous populations of *Pseudomonas* in soil hot-spots (Johnsen *et al*., 1999) and to characterizing the succession of *Pseudomonas* on barley root in a perturbed environment (Thirup *et al*., 2001). Analysis of the 16S rDNA of the *Paenibacillus azotofixans* strain with that of 2000 bacteria also enabled (Rosado *et al*., 1996) to identify the presence of three highly variable regions that were used to design primers for studying the kinetics of this bacterial strain in the soil and wheat rhizosphere. Monitoring introduced bacteria on the basis of its specific RAPD-PCR pattern has also been proposed but is very time consuming (Latour *et al*., 1999).

Culture-dependent methods

These methods are based on the suspension-dilution of soil and/or root samples and on inoculation of growing media (solid or liquid) with adequate dilutions. The culture-dependent methods differ according to the type of marker used giving the specificity to the growing media. This type of method is quite simple to perform, not too expensive and quite sensitive (10^2–10^3 cfu per g), but labor-intensive and shows some limitations (Jansson *et al*., 2000). This type of method underestimates the number of bacteria present in soil or in the rhizosphere. Bacteria may remain physically attached to the soil particles, may be killed in the dilution medium or may fail to grow on growth media (Kloepper *et al*., 1992). Some of them may remain aggregated even during the dilution process in such a way that a cfu may be originated by more than one cell. Suspension dilution can either be plated on solid

media or introduced into liquid media with various dilutions in order to determine from which dilution there is no more bacterial growth. This last method, named Most Probable Numbers (MPN), requires the use of probability tables to process data that contribute to reducing the sensitivity of the analysis compared with plating (Mac Crady, 1915). The most basic method consists of plating mutants resistant to antibiotics on solid growth medium supplemented with the corresponding antibiotic and with an anti-eucaryotic compound such as cycloheximide. This method is widely used, especially for survival kinetics of introduced bacteria and for competition studies between wild-type strains and mutants impaired in specific phenotypes (Orvos *et al*., 1990; Mavingui *et al*., 1992).

The aim of the present study was:

To study the root colonization properties of *Raoultella terrigena* G-584 on strawberry root, the GFP marker system was used in order to make the strain visible on the root system.

2.0 Materials and methods

Bacterial strains and growth conditions

Raoultella terrigena G-584 was routinely cultured in KB at 28 °C. Donor strain (*E. coli* DH5α) and Helper strain (*E.coli* HB 101) was routinely cultured in Luria-Bertani medium (LB) supplemented with kanamycin (final concentration of 30 µg/ml).

2.1.1 GFP Transformation

GFP-tagged strain

Helper strain: *E. coli* HB 101 carrying kanamycin-resistance plasmid pRK2013 provided by Prof. M. Ullrich, International University Bremen.

Donor strain: *E.coli* DH5α containing GFP plasmid pEGFP-c1/1 carrying kanamycin resistance was provided by Dr. Vladimir Benes, Embl, Heidelberg.

Triparental matting method (Cohen *et al*., 1994) was used to transfer the GFP plasmid to *Raoultella terrigena* G-584 by using Donor and Helper strains.

Triparental conjugation

Triparental conjugations on solid media were carried out using overnight cultures grown in 10 ml LB (with antibiotic selection) at 30 ºC and 100 rpm. Aliquots of 400 µl of both donor (*E.coli* DH5α) and recipient (*Raoultella terrigena* G-584) strains were transferred to a sterile eppendorf and 300 µl of *E. coli* HB 101 (helper plasmid) was transferred. The eppendorf was centrifuged for 5 min at 14,000 rpm. The supernatant was removed and the pellet was re-suspended in 200 µl of LB. The 200 µl was vortexed and plated onto LB and incubated at 30 ºC. The plate was washed with 5 ml of NaCl solution (0.85 %) into a sterile universal bottle. A 100 µl aliquot of the conjugation wash suspension was used to prepare spread plates on selective media and incubated for 24-48 h at 30 °C. Selection of

transconjugants was done on minimal media supplemented with kanamycin (30 μg/ml) and Kings B media.

2.1.2 Greenhouse experiments

Tests were performed with strawberry (*Fragaria* x *ananassa* Duchesne) plants of cv. Elsanta (Frigo). Roots of young strawberry plants were individually dipped for 15 min into the bacterial suspensions in order to have direct exposure of the roots to the antagonistic bacteria. Roots of control plants were dipped in tap water. Plants were then transferred to 14 cm plastic pots with soil.

Bacteria harboring plasmids with GFP genes were examined using fluorescence microscopy. Filter block L3 were used (Excitation: Band pass filter 450-490 nm & Emission: Band pass filter 525/20 nm)

Strawberry roots colonized by *Raoultella terrigena* G-584 were observed from 3 to 35 days after inoculation with intervals. Triplicate samples of plant roots were removed and fixed for 30 min in 4 % para formaldehyde in phosphate-buffered saline (PBS).

3.0 Results

Visualization of GFP-tagged strain in strawberry root system

Bacterial colonies on the roots were typically seen in the primary root system and frequently in the root tip (Fig. 11a, b, c d & e). They were also often detected at sites of emergence of lateral roots. Bacterial colonies were found in the root system up to two weeks, although visualization of bacteria in the root after approximately 4 weeks of growth was problematic, probably due to plasmid loss during the course of experimentation. No fluorescent bacterial colonies were detected on roots of untreated plants (Fig. 11f). Occasionally, roots of strawberry plants in the untreated control were covered with auto fluorescing dust particles.

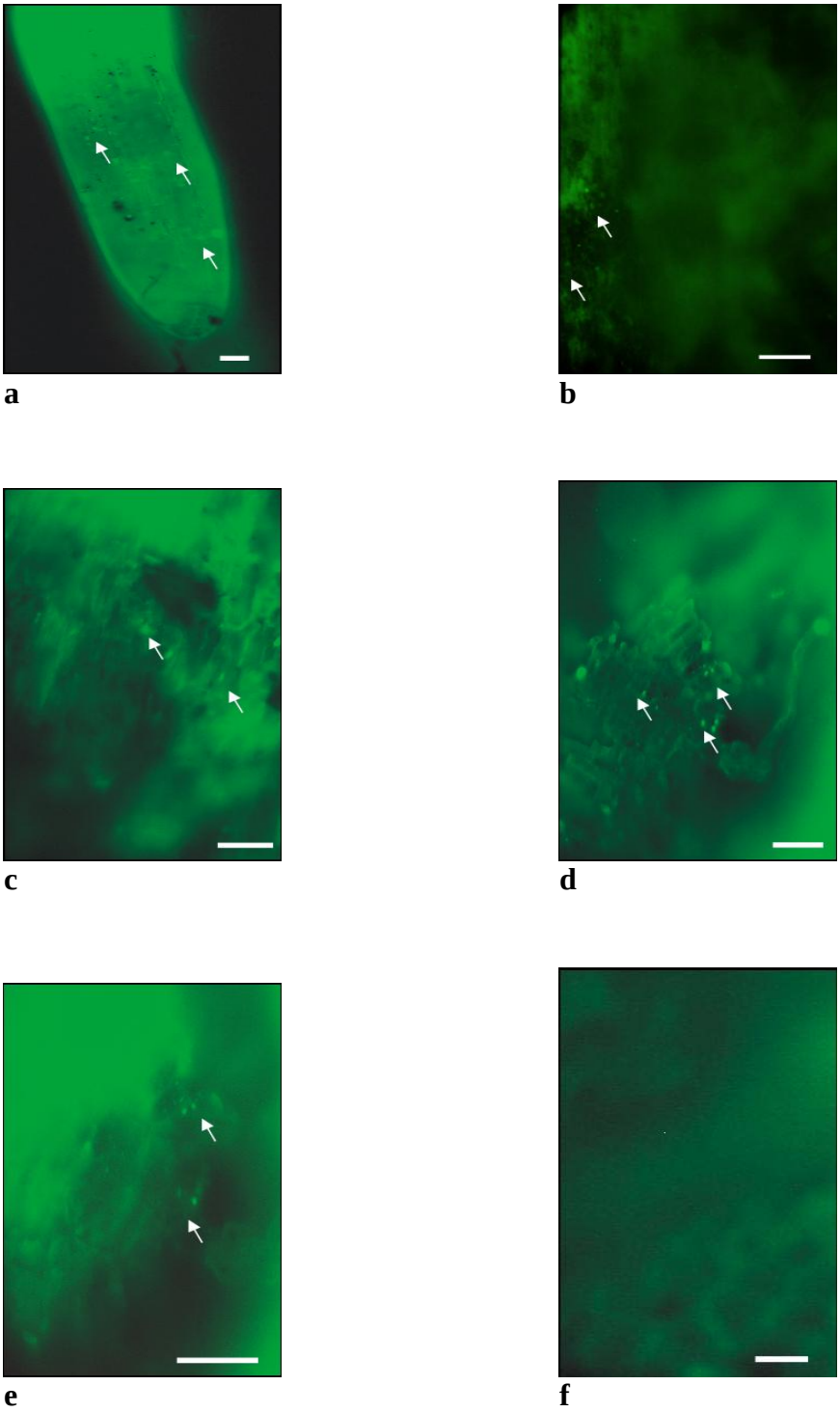


Fig. 11: Surface of strawberry plant roots treated with GFP-labelled *Raoultella terrigena* G-584, a: root tip & b, c, d, e: primary root with fluorescent bacterial colonies, f: control, Bar= 100µm

4. 0 Discussion

The use of GFP as a marker is a useful tool for studying plant-microbe interactions (Andersen *et al*., 1998, Chapon *et al*., 2002; Chitarra *et al*., 2002). This marker is well suited to study the colonization patterns of bacteria within plants because there is no requirement for exogenous substrate or co-factors. In addition, GFP can be detected in single cells with no concern for its presence in the background. A potential disadvantage of using fluorophore markers is the deterioration in contrast generated by autofluorescing plant tissue. The stability of the marker plasmid is also a cause for concern. However, here the abundance of GFP-tagged bacteria colonizing the plant root did not suggest significant marker loss. In this study, colonization of strawberry root by *Raoultella terrigena* G-584 was distinct in that it was typically found in the primary root and root tip. Colonization events by diazotrophs have been studied in several grass species. Cells of *K. pnuemoniae* colonization was studied on stem and root system of maize (*Zea mays*), bacteria were detected in both the plant roots and stems. Bacterial colonies on the roots were typically seen in the regions of elongation and more frequently in the root hair region of maturation **(**Chelius and Triplett, 2000). Bacteria were also detected at the point of lateral root emergence**.** *Herbaspirillum seropedicae* colonizes the sugarcane root cortex intra- and intercellularly, as well as the xylem vessels (Björklöf and Jørgensen 2001). *Azoarcus* sp. colonizes rice and kallar grass in the root cortex, root cap, epidermis, exodermis, and xylem (Burlage and Kuo, 1994; Benizri *et al*., 1997). *Pantoea agglomerans* has been found in the intercellular spaces of wheat roots (Burr *et al.,* 1978).

Dijkstra *et al*., 1987 showed that when bacteria were applied on the root tips, they were re-isolated from all the root parts. Kluepfel (1993) in microscopic studies reported that bacteria dominate in colonization of rhizosphere. The passive spreading of bacteria on roots may be due to the precolating water (Parke *et al*., 1986). The presence of bacterial colonies towards the root tips in this study may also be due to the precolating water, which may have helped the bacteria to move. The capability of gram-negative *Pseudomonas* to colonize plant roots make them potential candidates in biological control (Howell and Stipanovic, 1980; Weller and Cook, 1983). From the present study it is found that *R. terrigena* G-584 has the potential to colonize the strawberry plant root system. However,

further studies are needed to determine the colonization patterns of applied rhizobacteria quantitatively, this could be done with some of the methods mentioned before.

IV. Mode of action of the antagonistic bacteria

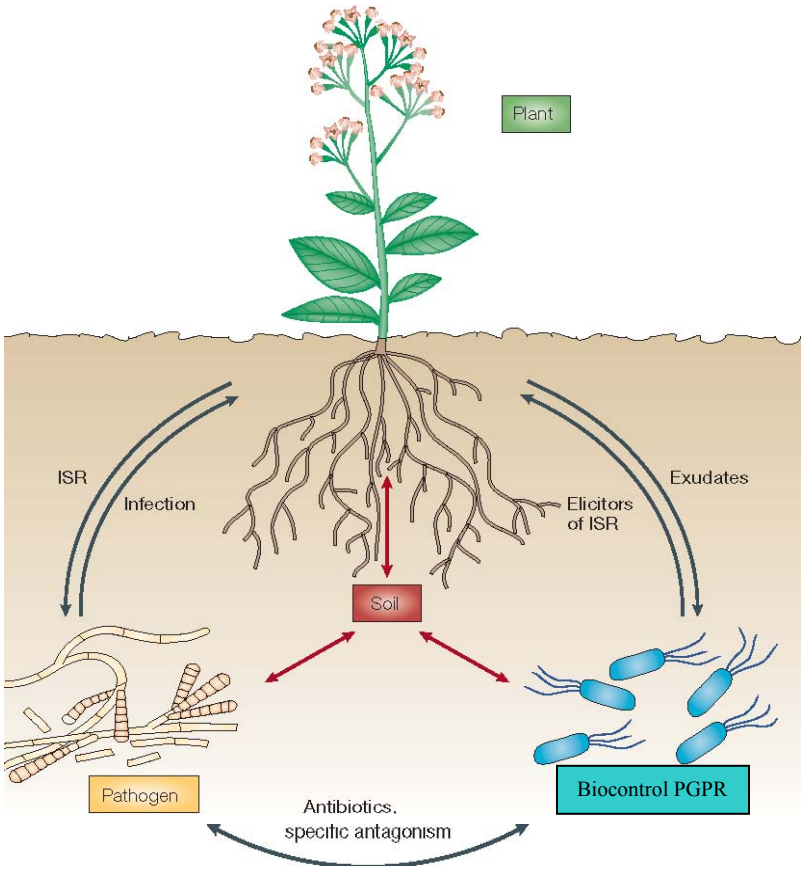
1.0 Introduction

In general mode of action include: Inhibition of the pathogen by antimicrobial compounds (antibiosis); competition for iron through production of siderophores; competition for colonization sites and nutrients supplied by seeds and roots; induction of plant resistance mechanisms; inactivation of pathogen germination factors present in seed or root exudates; degradation of pathogenicity factors of the pathogen such as toxins; parasitism that may involve production of extracellular cell wall-degrading enzymes, for example, chitinase and ß-1,3 glucanase that can lyse pathogen cell walls (Kloepper *et al*., 1980; Keel and Défago, 1997). None of the mechanisms are necessarily mutually exclusive and frequently several modes of action are exhibited by a single biocontrol agent. Indeed, for some biocontrol agents, different mechanisms or combinations of mechanisms may be involved in the suppression of different plant diseases. A few specific examples of the modes of action involved with bacterial biocontrol of fungal pathogens in the rhizosphere are given below.

Antibiosis

There are numerous reports of the production of antifungal metabolites (excluding metal chelators and enzymes) produced by bacteria *in vitro* that may also have activity *in vivo*. These include 2,4-diacetylphloroglucinol (Ph1), pyoluterin (Plt), pyrrolnitrin (Pln), viscosinamide, HCN, kanosamine, Oligomycin A, Oomycin A, phenazine-1-carboxylic acid (PCA), xanthobaccin, zwittermycin A, ammonia, butyrolactones, as well as several other uncharacterized moieties (Milner *et al*., 1996; Nielsen *et al*., 1998; Kim *et al*., 1999; Thrane *et al*., 1999; Nakayama *et al*., 1999, Mansour and Farag, 1999, Haas and Défago, 2005). To demonstrate a role for antibiotics in biocontrol, mutants lacking production of antibiotics or over-producing mutants have been used (Bonsall *et al*., 1997; Chin-A-Woeng *et al*., 1998). Alternatively, the use of reporter genes or probes to demonstrate production of antibiotics in the rhizosphere is becoming more common place (Kraus and Loper, 1995; Raaijmakers *et al*., 1997). Indeed, isolation and characterization of genes or gene clusters responsible for antibiotic production has now been achieved (Kraus and Loper, 1995; Bangera and Thomashow, 1996; Hammer *et al*., 1997). Significantly, both Phl and PCA have been

isolated from the rhizosphere of wheat following introduction of biocontrol strains of *Pseudomonas* (Thomashow *et al*., 1990; Bonsall *et al*., 1997; Raaijmakers *et al*., 1999), finally confirming that such antibiotics are produced *in vivo*. Further, Ph1 production in the rhizosphere of wheat was strongly related to the density of the bacterial population present and the ability to colonize roots (Raaijmakers *et al*., 1999). PCA from *Pseudomonas aureofaciens* Kluyver Tx-1 has even been used as a direct field treatment for the control of dollar spot (*Sclerotinia homeocarpa* F. T. Bennett) on creeping bentgrass (*Agrostis palustris* Hudson) (Powell *et al*., 2000).



Hass & Défago., 2005

Fig. 12: Interactions between biocontrol plant growth-promoting rhizobacteria (PGPR), plants, pathogens and soil. These elements interact with one another through biotic and abiotic signals, many of which are still unknown. ISR, induced systemic resistance

Interestingly, signalling between pathogenic fungi and potential biocontrol bacteria has also been detected. In one case, trehalose derived from *Pythium debaryanum* Hesse up-regulated genes in its biocontrol strain *Pseudomonas fluorescens* ATCC 17400 (Gaballa *et al*., 1997) and yet in another example *Pythium ultimum* Trow caused a down-regulation of five gene clusters of *P. fluorescens* F113 which provides biocontrol of this pathogen in the rhizosphere of sugar beet (*Beta vulgaris* L.) (Fedi *et al*., 1997). These findings may be of considerable significance for bacterial–fungal interactions in general and has major implications for the control of gene expression in complex microbial communities.

Parasitism and production of extracellular enzymes

The ability of bacteria, especially actinomycetes, to parasitize and degrade spores of fungal plant pathogens is well established (El-Tarabily *et al*., 1997). Assuming that nutrients pass from the plant pathogen to bacteria, and that fungal growth is inhibited, the spectrum of parasitism could range from simple attachment of cells to hyphae, as with the *Enterobacter cloacae* (Jordan) Hormaeche & Edwards–*Pythium ultimum* interaction (Nelson *et al*., 1986), to complete lysis and degradation of hyphae as found with the *Arthrobacter–Pythium debaryanum* interaction (Mitchell and Hurwitz, 1965). If fungal cells are lysed and cell walls are degraded then it is generally assumed that cell wall-degrading enzymes produced by the bacteria are responsible, even though antibiotics may be produced at the same time. Considerable effort has gone into identifying cell wall-degrading enzymes produced by biocontrol strains of bacteria even though relatively little direct evidence for their presence and activity in the rhizosphere has been obtained. For example, biocontrol of *Phytophthora cinnamomi* Rands root rot of *Banksia grandis* was obtained using a cellulase-producing isolate of *Micromonospora carbonacea* Luedemann & Brodsky (El-Tarabily *et al*., 1996) and control of *Phytophthora fragariae* var. *rubi* Hickm causing raspberry root rot was suppressed by the application of actinomycete isolates that were selected for the production of ß-1,3-, ß-1,4- and ß-1,6-glucanases (Valois *et al*., 1996).

Induced resistance

Perhaps the greatest growth area in biocontrol in the last few years has been concerned with induced resistance defined as 'the process of active resistance dependent on the host plant's physical or chemical barriers, activated by biotic or abiotic agents (inducing agents)' (Kloepper *et al*., 1992; 2004). Most work has focused on the systemic resistance induced by non-pathogenic rhizosphere-colonizing *Bacillus* and *Pseudomonas* species in systems where the inducing bacteria and the challenging pathogen remained spatially separate for the duration of the experiment, and no direct interaction between the bacteria and pathogen was possible (Sticher *et al*., 1997; van Loon, 1997). Such split root or spatial root inoculation experiments were used to demonstrate the phenomenon in radish (*Raphanus sativus* L.) and *Arabidopsis* against *Fusarium oxysporum* (Leeman *et al*., 1996*a*; van Wees *et al*., 1997) and in cucumber (*Cucumis sativus* L.) against *Pythium aphanidermatum* (Edson) Fitzp. (Chen *et al*., 1998). Various combinations of timing and position have indicated that induced resistance also occurs in carnation (*Dianthus caryophyllus* L.) (van Peer *et al*., 1991), tobacco (*Nicotiana tabacum* L.) (Maurhaufer *et al*., 1994) and tomato (*Lycopersicon esculentum* Mill.) (Duijff *et al*., 1997). Bacteria differ in ability to induce resistance, with some being active on some plant species and not others; variation in inducibility also exists within plant species (van Loon, 1997). The full range of inducing moieties produced by bacteria is probably not yet known, but lipopolysaccharides (Leeman *et al*., 1995) and siderophores (Métraux *et al*., 1990; Leeman *et al*., 1996*b*) are clearly indicated.

Changes that have been observed in plant roots exhibiting ISR include: (1) strengthening of epidermal and cortical cell walls and deposition of newly formed barriers beyond infection sites including callose, lignin and phenolics (Benhamou *et al*., 1996, 2000; Duijff *et al*., 1997; M'Piga *et al*., 1997); (2) increased levels of enzymes such as chitinase, peroxidase, polyphenol oxidase, and phenylalanine ammonia lyase (M'Piga *et al*., 1997; Chen *et al*., 2000); (3) enhanced phytoalexin production (van Peer *et al*., 1991; Ongena *et al*., 1999); (4) enhanced expression of stress-related genes (Timmusk and Wagner, 1999). However, not all of these biochemical changes are found in all bacterial–plant combinations (Steijl *et al*., 1999). Similarly, the ability of bacteria to colonize the internal tissue of the roots has been

considered to be an important feature in many of the bacterial–root interactions involving ISR, but is not a constant feature of them all (Steijl *et al*., 1999).

The aim of this present study was to find out some further indication to the antagonism of the 3 rhizobacteria. As mentioned before the antagonistic activity can be caused by volatile compounds, in the following aerated plate method was used, to find out, if these compound(s) could play a specific role. Moreover the influence of extracellualr enzyme was analysed with Microplate and API ZYM kit methods.

2.0 Materials and methods

2.1 Aerated plate method

Five mm wide central strip of agar was removed aseptically from V-8 agar medium plates to provide physical separation between the fungus and the bacterium cultured on either half. Each of *Bacillus amyloliquefaciens* G-V1, *Pseudomonas fluorescens* 2R1-7, and *Raoultella terrigena* G-584 were individually spread over one side of the V-8 agar medium, other side was individually inoculated with 5 mm disk of *P.cactroum* and *P.fragariae*. Similar plates were prepared without bacteria served as a control. Incubation was made at 25 °C up to two weeks. Fungal growth was recorded on 12 plates for each treatment and microscopically examined.

2.2 Enzyme assays

Preparation of bacterial culture filtrates

Two loops of bacteria from 2 days old culture on KB medium culture were transferred into a 200 ml glass flask with 50 ml of sterile KB medium. The medium with bacteria was shaken (96 rpm.) on a rotary shaker for 24 h in an incubator at 24 ± 2 °C. One ml of culture was added to a 200 ml Erlenmeyer flask with 50 ml of sterile medium and was shaken at 96 rpm on a rotary shaker for 24 h in an incubator at 24 ± 2 °C in darkness. In order to separate the cells and culture filtrate, the culture was centrifuged at a speed of 6000 rpm at 4 °C for 20 – 25 min.

Two different methods were used. Firstly, Microplate assay was carried out according to the method of Wirth and Wolf (1992), bacterial culture filtrate (100 µl), substrate (100 µl) (CM-Cellulose-RBB for Cellulase, CM-Curdlan-RBB for Glucanase, CM-Chitin-RBV for Chitinase and Casein-RBB for Protease) and sodium acetate (0.5 M; pH 5) buffer (100 µl) were added in an eppendorf tube and kept in a water bath at 37 °C for 1 h. The enzyme reaction was terminated by adding 1 N HCL (100 µl) then the sample was centrifuged (5000 rpm at 4 °C for 5 min) and the supernatant (100 µl) was measured at 595 nm. Secondly, API ZYM kit (Head and Ratnam, 1988) was employed, a semi quantitative micromethod consisting of 20 micro cupules, 19 of which contain dehydrated chromogenic substrate for detecting 19 performed enzyme activities. The test strips are inoculated with bacterial culture filtrates (50 µl) and incubated aerobically at 37 °C for 1 h, and the two

reagents (supplied with kit) are added to develop the chromogenic substrates. The resultant colorimetric reactions are indicative of the degree of enzyme activity and they are compared with the control well and a colour chat.

3.0 Results

3.1 Aerated plate method

Aerated plate method showed that the tested rhizobacteria have reduction effect (Fig. 13) on the mycelial growth of *P. fragariae* var. *fragariae* and *P. cactorum*. This strongly indicates that the antagonistic bacteria are producing some diffusible or volatile secondary metabolites. *B. amyloliquefaciens* G-V1 showed higher inhibiton activity compared to other two antagonistic strains, 43.5 % against *P. fragariae* and 48.3 % on *P. cactorum.* *R. terrginea* G-584 produced 25 % of mycelial growth inhibiton on *P. fragariae* and 10 % on *P. cactorum.* *P. fluorescens* 2R1-7 showed 18.5 % against *P. fragariae* and 21.6 % on *P. cactroum.* The microscopic examination of the plates showed physiological abnormalities of the hyphae, including hyphal distortion and vacuolation (Tab. 7, Fig. 15 and 16).

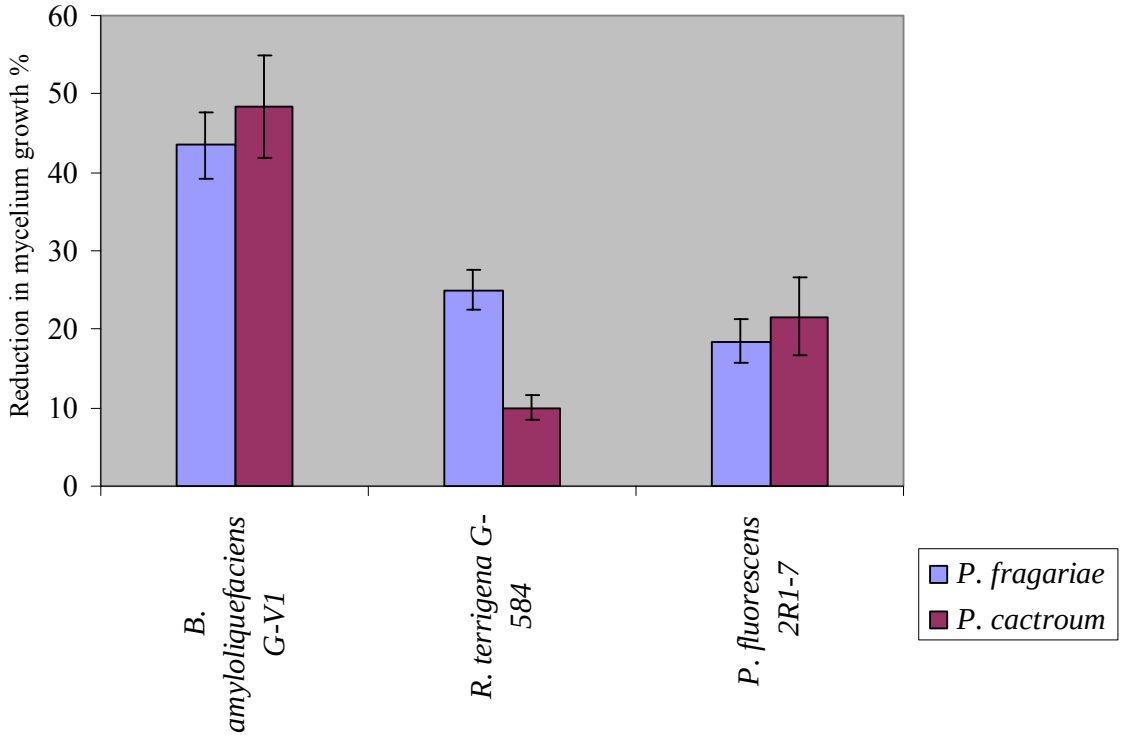
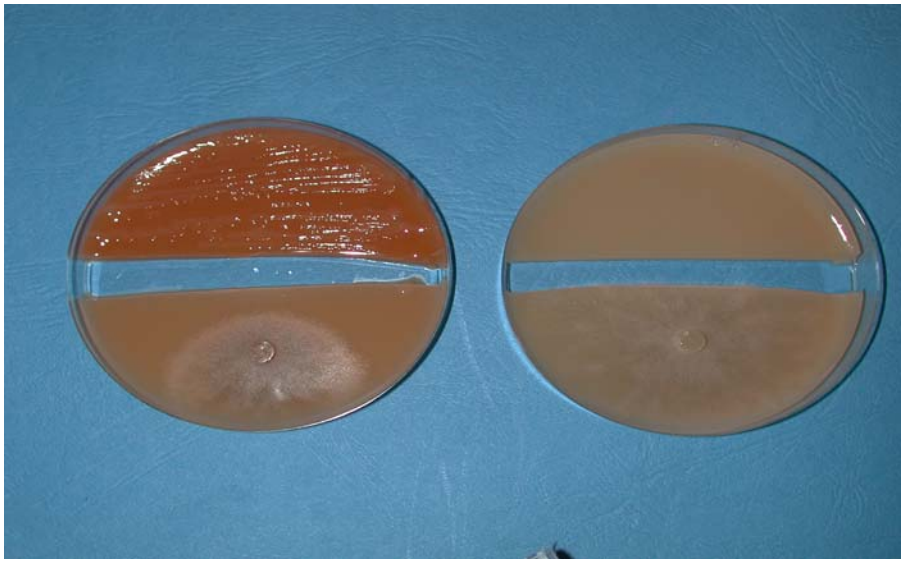


Fig. 13: Effect of *B. amyloliquefacines* G-V1, *R. terrigena* G-584 and *P. fluorescens* 2R1-7 on mycelial growth of *Phytophthora* spp. in aerated plate. Results are presented as reduction in mycelial growth compared to control, n=12, (± standard deviations)

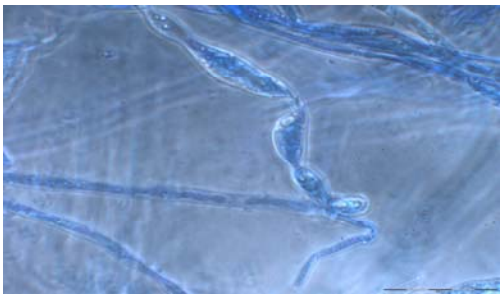


a) *P. fluorescens* 2R1-7 treated b) Control

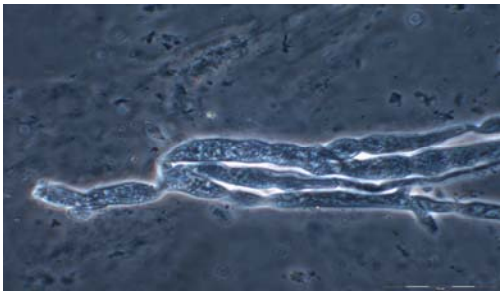
Fig.14: Aerated plate method (a), shows the reduction effect of *P. fluorescens* 2R1-7 on the growth of *Phytophthora cactorum*

Bacteria	*P. fragariae* var. *fragariae*	*P. cactorum*
B. amyloliquefaciens G-V1	15.8 a	15.9 a
P. fluorescens 2R1-7	16.1 a	10.4 b
R. terrigena G-584	16.9 a	12.4 b
Control	5.8 b	6.1 c

Tab. 7: Width of hyphae (µm) of *Phytophthora fragariae* var. *fragariae* and *P. cactorum* exposed to three genera of bacteria, n=10, Different letters show statistically significant differences among treatments according to Tukey Test, $P<0.05$



a



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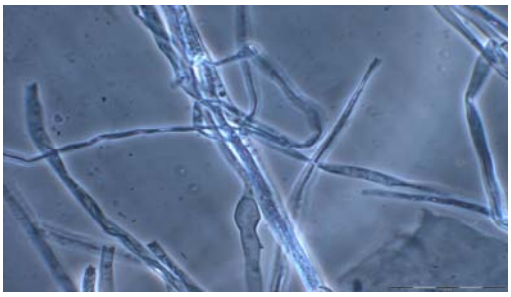

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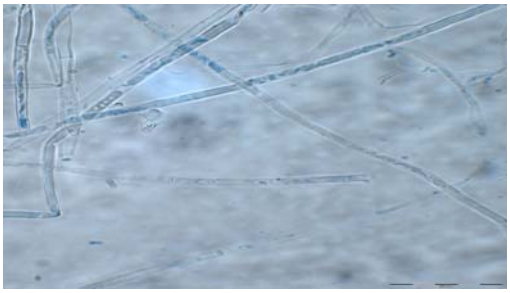
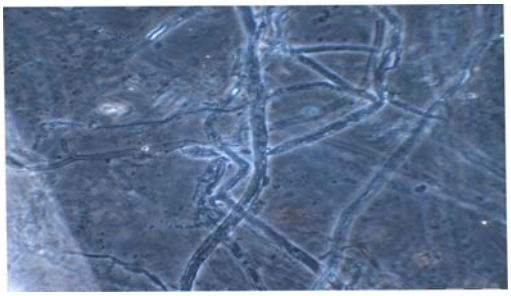
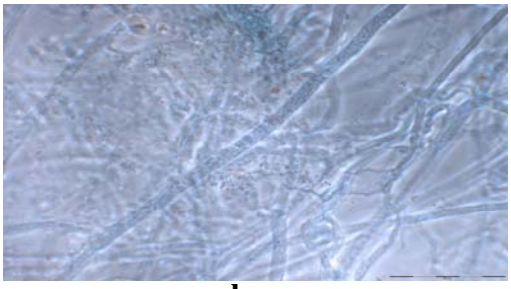


d

Fig. 15: Hyphal abnormalities of *Phytophthora fragariae* var. *fragariae* treated with *B. amyloliquefaciens* G-V1 (a), *R. terrigena* G-584 (b), *P. fluorescens* 2R1-7 (c) and normal hyphae: untreated control (d), Bar=50µm



a



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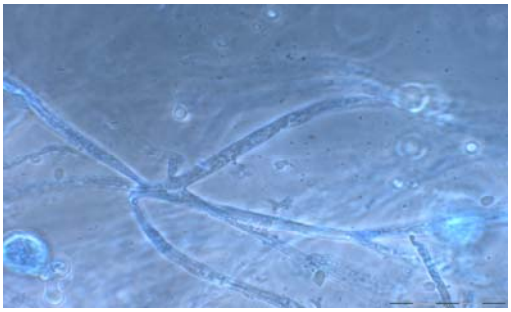

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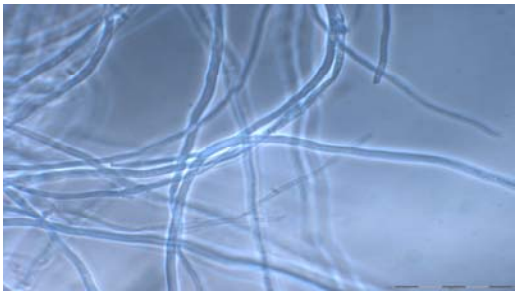

d

Fig. 16: Hyphal abnormalities of *Phytophthora cactroum* treated with *B. amyloliquefaciens* G-V1 (a), *R. terrigena* G-584 (b), *P. fluorescens* 2R1-7 (c) and normal hyphae: untreated control (d), Bar=50µm

3.2 Enzyme assays

Table 8 shows, the enzyme profile of the *R. terrigena* G-584, *B. amyloliquefaciens* G-V1 and *P. fluorescens* 2R1-7 were recorded according to two different methods of enzyme assay. Presence of cellulase, glucanase, alkaline phosphatase, esterase (C4) and esterase lipase (C8) were found from *B. amyloliquefaciens*. *R. terrigena* culture filtrate showed glucanase, alkaline phosphatase, leucine arylamidase and acid phosphatase activity. The chitinase enzyme activity only was found from culture filtrate of *P. fluorescences.* In general, cellulase, glucanase and chitinase have been reported as part of biocontrol activity of bacterial antagonists.

Enzymes	Bacterial antagonists		
	R. terrigena G-584	*B. amyloliquefaciens* G-V1	*P. fluorescens* 2R1-7
Micro plate assay			
Cellulase	-	+	-
Glucanase	+	+	-
Chitinase	-	-	+
Protease	-	-	-
Test kit API ZYM			
Alkaline phosphatase	+	+	-
Esterase (C 4)	-	+	-
Esterase Lipase (C 8)	-	+	-
Lipase (C 14)	-	-	-
Leucine arylamidase	+	-	-
Valine arylamidase	-	-	-
Cystine arylamidase	-	-	-
Trypsin	-	-	-
α-chymotrypsin	-	-	-
Acid phosphatase	+	-	-
Naphthol-AS-Bl-phosphohydrolase	-	-	-
α-galactosidase	-	-	-
ß-galactosidase	-	-	-
ß-glucuronidase	-	-	-
α-glucosidase	-	-	-
ß-glucosidase	-	-	-
N-acetyl-ß-glucosaminidase	-	-	-
α-mannosidase	-	-	-
α-fucosidase	-	-	-

+ = Positive reaction, - = Negative reaction

Tab. 8: Determination of enzymes by Micro-plate assay (4) & Test kit API ZYM (19) from bacterial antagonists

4.0 Discussion

Aerated plate method

The described aerated plate method in this part of study has been used by other authors for testing *Bacillus subtilis* volatile compound(s) against fungal pathogens (Fiddaman and Rossall, 1993; Mansour and Farag, 1999). The results found in this study are similar to Fiddaman and Rossall (1993), who found that a strain of *Bacillus subtilis* which produce a volatile compound(s) was antifungal to *Rhizoctonia solani* and *Pythium ultimum.* Growth of the fungi was severely impaired in the presence of the volatiles and physiological abnormalities of the hyphae were observed, including hyphal distortion and vacuolation. So it can be concluded also from this study that volatiles of *B. amyloliquefaciens* G-V1 has a similar effect.

The morphological changes, which were observed by using *P. fluorescens* 2R1-7 in the study, can be an indication for a possible production of Hydrogen cyanide (HCN), which is a broad-spectrum antimicrobial compound involved in biological control of root diseases by many plant-associated fluorescent pseudomonads. Production of HCN by biocontrol fluorescent pseudomonads is implicated in suppression of diseases caused by phytopathogenic fungi, such as *Thielaviopsis basicola* on tobacco (Voisard *et al*., 1989; Laville *et al*., 1998), *Septoria tritici*, and *Puccinia recondita* f. sp. *tritici* on wheat (by recombinant HCN-producing *P. putida* strains) (Flaishman *et al*., 1996). Direct inhibition of the fungi by HCN is thought to be the main mechanism of action (Blumer and Haas 2000), in which case, the effect of the bacterium would be comparable to the HCN-mediated plant defense mechanism (Luckner 1990).

First report of *R. terrigena* G-584 volatiles on mycelial reduction of *Phytophthora* spp. was observed here, but compared with *B. amyloliquefaciens* G-V1 on a lower level.

Cyanide of microbial origin has not been measured in the rhizosphere (Hass and Défago, 2005). However, in one historical experiment (Timonin, 1947), the effect of added cyanide was tested directly in the field. This treatment killed fungi *en masse*, significantly reduced 'grey speck' disease of oats, and tripled oat grain yields. No side effects on the fauna were recorded.

In general, from the present study there is an indication that volatiles of the three antagonistic bacteria have an influence on the morphological structure of the two tested *Phytophhtora* spp. Identification of the volatile compound(s) can elucidate more their importance.

Enzyme assay

Certain enzymes excreted by bacteria are suspected to play an important role in suppression of pathogens (Buchenauer, 1998). If fungal cells are lysed and cell walls are degraded then it is generally assumed that cell wall-degrading enzymes produced by the bacteria are responsible, even though antibiotics may be produced at the same time. In the present study (Tab. 8), the production of cellulase from *B. amyloliquefaciens* G-V1, glucanase from *B. amyloliquefaciens* G-V1 and *R. terrigena* G-584, and chitinase from *P. fluorescens* 2R1-7 were found, which are known as cell wall degrading enzymes.

Considerable effort has gone into identifying cell wall-degrading enzymes produced by biocontrol strains of bacteria. For example, biocontrol of *Phytophthora cinnamomi* Rands root rot of *Banksia grandis* was obtained using a cellulase-producing isolate of *Micromonospora carbonacea* (El-Tarabily *et al*., 1996) and control of *Phytophthora fragariae* var. *rubi* causing raspberry root rot was suppressed by the application of actinomycete isolates that were selected for the production of ß-1,3-, ß-1,4- and ß-1,6-glucanases (Valois *et al*., 1996). Fridlender *et al*. (1993) demonstrated biocontrol of *Rhizoctonia solani* by an isolate of *Burkholderia cepacia* and showed hyphal damage presumed to be due solely to the production of ß-1,3-glucanase activity. Lim *et al*. (1991) showed that growth inhibition and cell wall lysis of *Fusarium solani* by *Pseudomonas stutzeri* was caused by a combination of extracellular ß-1,3-glucanase and chitinase activities.

The other enzymes found in this study like alkaline and acid phosphatases which were found from *R. terrigena* G-584 and *B. amyloliquefaciens* G-V1, could involve in phosphate solubilization in the soil. Plant meets their phosphorus requirement through the uptake of phosphate anions from the soil. To be available to plants, organic form of soil phosphorus

must be mineralized by those processes which are mediated by phosphatase enzymes (Bieleski and Ferguson, 1983). Plant-stimulatory effects exerted by PGPR might also be due to an enhanced availability of limited plant nutrients such as nitrogen, phosphorus, B-vitamins and amino acids in the rhizosphere caused by phosphate-solubilizing and diazotrophic bacteria (Rozycki *et al.*, 1999; Nautiyal *et al.*, 2000).

Generally, the enzyme amino acid arylamidase catalyzes the hydrolysis of an N-terminal amino acid from peptides, amides, or arylamides. Arylamidase may play an important role in nitrogen mineralization in soil (Acosta-Martínez and Tabatabai, 2000). Direct role of esterase and esterase lipase (C8) could not be correlated in some antagonistic activity.

Summarizing the relationship of extracellular enzyme production of the three studied antagonistic bacteria, results presented are first indication of the cell wall degrading enzymes and other enzymes in the antagonistic activity of the tested rhizobacteria.

V. Polymerase Chain Reaction (PCR) analysis for detecting 2,4-diacetylphloroglucinol, cellulase and phytase gene from the bacterial antagonists

1.0 Introduction

Biological sources for plant disease control remain an important potential alternative to the use of chemical pesticides. Biological controls that are based on introduced microbes could be an alternative, but however, have been slow to develop due to inconsistencies in their performance (Weller, 1988). Such inconsistencies often result from a lack of understanding the mechanisms by which individual microorganisms function to control disease. It is often difficult to gain a complete understanding of how biocontrol agents control diseases, since many functions through a variety of mechanisms. In such cases, identifying contributing mechanisms often requires a systematic approach that directly evaluates individual traits and their contributing roles to the overall operating mechanisms.

DNA technology and biochemical research techniques have led to an improved understanding of the mechanism of antibiosis (Fravel, 1988; Défago and Hass, 1990). The hypothesis that secondary metabolites produced by rhizobacteria play a vital role in the biocontrol activity has been confirmed by constructing and testing mutants deficient in production of these metabolites (Thomashow and Weller, 1988; Voisard *et al*., 1989; Keel *et al*., 1992; Maurhofer *et al*., 1994; Chin-A-Woeng *et al*., 2001; Huang *et al*., 2004). By using PCR-based techniques Raaijmakers *et al*. (1997) concluded that a population of *Pseudomonas* spp. that produced phloroglucinol was responsible for decline of take-all disease. Different known metabolites produced by *Pseudomonas* species include:

i) Biosurfactants: e.g. rhamnolipids (Stanghellini and Miller, 1997) and hydrogen cyanide (Voisard *et al*., 1989; Schippers *et al*., 1991)

ii) Lytic enzyme: e.g. β-1,3 glucanase (Friedlender *et al*., 1993)

iii) Plant hormones and other plant growth promoting substances e.g. auxins, indole-3-acetic acid (Loper and Schroth, 1986), gibberellins (Lubczynka *et al*., 1997) and 1-aminocyclopropane-1-carboxylate deaminase (Jacobson *et al*., 1994)

iv) Siderophores e.g. pyoverdin, pseudobactin and pyochelin (Teintze *et al*., 1981; Van der Hofstad *et al*., 1986; Duijff *et al*., 1994)

v) Antibiotics e.g. phenazine (Brisbane *et al*., 1987; Thomashow and Weller, 1988; Whistler and Pierson III, 2003) pyoluteorin (Howell and Stipanovic, 1980; Kraus and Loper, 1995), pyrrolnitrin (Elander *et al*., 1968; Lambert *et al*., 1987) and phloroglucinols (Keel et al., 1992: Landa *et al*., 2003)

Bacillus species have been shown to control phytopathogenic fungi (Bochow, 1990; Mansour and Farag, 1999; Alippi *et al*., 2000) and bacterial diseases (Schmiedeknecht *et al*., 1998) in greenhouse and under field conditions (Douville and Boland, 1992). There are a number of antifungal compounds produced by *Bacillus* species e.g. Bacylysin, Bacillomycin, Fungistatin, Mycosybtilin and Iturin (Katz and Demain, 1977, Ohno *et al*., 1992), Rhizoctin (Kugler *et al*., 1990). In later studies production of antibiotic Kanosamine was identified from *Bacillus cereus* UW85 (Milner *et al*., 1996). Sadlers, (1996) also reported that *Bacillus* spp. isolate S-18 reduced the growth of various phytopathogenic fungi. Other antifungal substances identified from *Bacillus* species include Fengomycin (Vanaittanakom *et al*., 1986) and Rhizoctin A (Kugler *et al*., 1990).

Determining the exogenous environmental signals that modulate the biosynthetic regulation of antifungal compounds has been comparatively slow, largely because isolating and quantifying metabolites produced in the soil and rhizosphere is tedious (Thomashow and Weller, 1996). Numerous reporter systems for gene expression have been described which ultimately may help identify conditions triggering antibiotic biosynthetic genes. Reporter systems in biocontrol pseudomonads have also been used as a preliminary investigative tool to examine the influence of iron availability on the expression of pyoverdine genes (Loper and Henkels, 1997) and the influence of *Pythium* culture filtrates on the expression of trehalase genes (Gaballa *et al*., 1997) and genes thought to be involved in rhizosphere competence (Fedi *et al*., 1997).

Understanding the environmental factors that regulate the biosynthesis of antimicrobial compounds by disease-suppressive strains, is an essential step towards improving the level and reliability of their biocontrol activity.

The goal of the following set of experiments was:

- Identification of 2,4-diacetylphloroglucinol gene from *Pseudomonas fluorescens* 2R1-7 and studying the influence of pH on 2,4-diacetylphloroglucinol gene expression. The polyketide antimicrobial metabolite, 2,4-diacetylphloroglucinol (DAPG), has emerged as one of the most important antimicrobial compounds produced by biocontrol strains of *Pseudomonas fluorescens* (Keel *et al*., 1992, Thomashow and Weller, 1996)

- Identification of cellulase gene from *Bacillus amyloliquefaciens* G-V1. Certain enzymes excreted by bacteria are suspected to play an important role in suppression of pathogens (Buchenauer, 1998), moreover cellulase enzyme production was found from this strain (in chapter IV, see table. 8)

- Identification of phytase gene from *Raoultella terrigena* G-584. In *R. terrigena* comb.nov., as phytase activity is known to increase after cells reach the stationary phase (Greiner *et al.*, 1997) and the role of extracellular phytase activity on plant growth promotion has been reported (Elsorra *et al*., 2002)

2.0 Materials and methods

2.1 Polymerase Chain Reaction (PCR) for gene amplification

Sequence alignment & Primer designing

Database Searching for 2,4-diacetylphloroglucinol gene from *Pseudomonas* spp., cellulase gene from *Bacillus* spp. and phytase gene from *Raoultella* spp. and other bacterial spp. All database searching was done through the website of the National Center for Biotechnology Institute (NCBI) at http://www.ncbi.nlm.nih.gov/. Sequences collected were catalogued by accession number, length, DNA or protein, bacterial species, and the type of enzyme within the family. Nucleotide sequences from NCBI were saved as GenBank and FASTA files. Multiple Sequence alignments (MSA) were performed using the ClustalX algorithm (Chenna *et al*., 2003) (Fig. 17). Stringency was varied to achieve an alignment with the smallest number of gaps and mismatches. Altering the stringency was also done to yield as many regions with a high degree of sequence similarity as possible. Primers were designed manually.

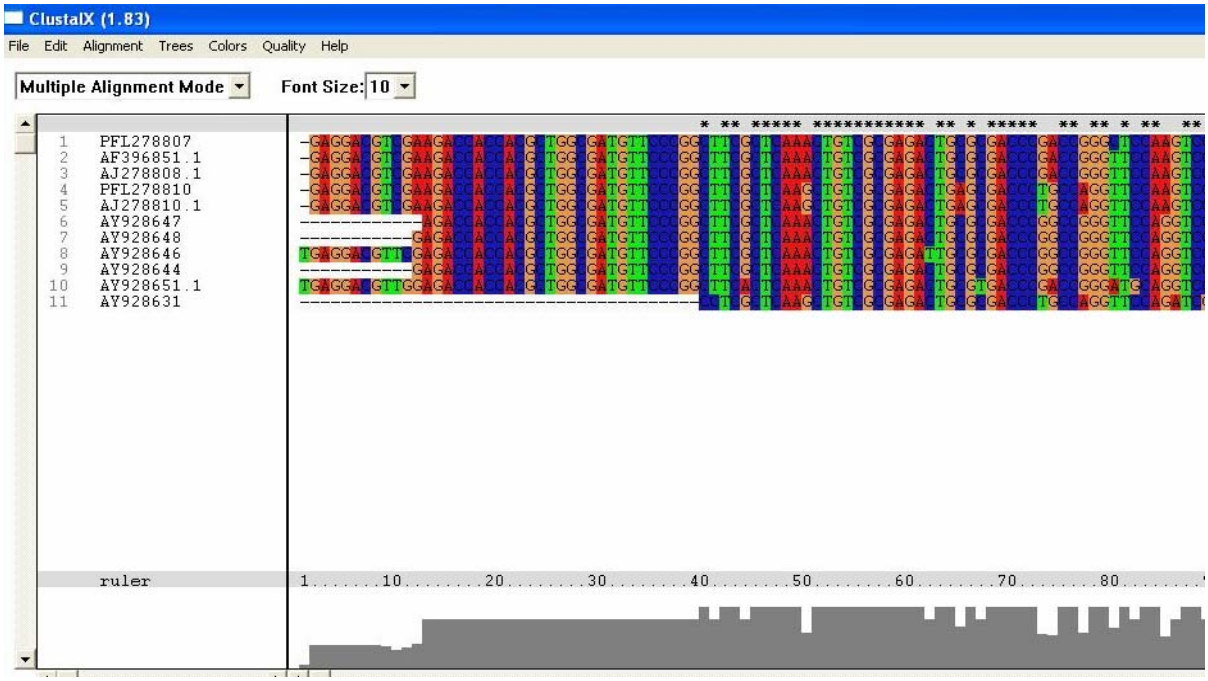

Fig.17: ClustalX algorithm- Multiple Sequence Alignments

Isolation of genomic DNA

Overnight grown culture of *Bacillus amyloliquefaciens* G-V1, *Pseudomonas fluorescens* 2R1-7 and *Raoultella terrigena* G-584 in LB medium was centrifuged at 10,000 rpm at 4 ºC for 5 minutes. The pellet was re-suspended in 200 µl TE Buffer. The following purification protocol was used (using Fermentas Genomic DNA purification kit**)**.

Purification Protocol

The 200 µl sample was mixed with 400 µl lysis solutions and incubated at 65 °C for 5 min. After incubation, 600 µl chloroform were added, gently emulsified by inversion and centrifuged at 10,000 rpm for 2 min. Precipitation solution were prepared freshly (mix 720 µl water nuclease-free, with 80 µl of the supplied 10X concentrated solution). The upper aqueous phase containing DNA were transferred to a fresh tube, 800 µl precipitation solution added, mixed at room temperature for 1-2 min and centrifuged at 10,000 rpm for 2 min. Supernatant were completely removed, DNA pellet were dissolved completely in 100 µl 1.2 M NaCl solution. Finally, 300 µl cold ethanol added to the sample, incubated at -20 °C for precipitation and centrifuged for 3-4 min at 10,000 rpm, the ethanol were poured off. Lastly, the pellet was washed with 70 % cold ethanol, dissolved in 100 µl nuclease free water.

Reagents and Primers

a) Detection of 2,4-diacetylphloroglucinol gene from *Pseudomonas fluorescens* 2R1-7

Reagents for one reaction volume of 25 µl

2.5 µl Taq buffer (1X)
1.5 µl $MgCl_2$ (1.5 µM)
0.5 µl dNTPs (200 µM of each dATP, dCTP, dGTP, dTTP)
0.625 µl forward primer (0.25 µM)
0.625 µl reverse primer (0.25 µM)
0.125 µl Taq polymerase (Hot Start)
1 µl DNA (100 ng/µl)
18.2 µl Sterile distilled water

PCR condition:
Step 1: 94 ºC for 15 minutes
Step 2: 94 ºC for 30 seconds
Step 3: 63.2 ºC for 30 seconds
Step 4: 72 ºC for 1 minute
Step 5: repeat from step 2 for 35 times
Step 6: 72 ºC for 3 minutes

Forward Primer: 5' CTCAARCTGTCGCGAGA*
Reverse Primer: 5' CACSGGKTTCATGATGCC*

b) Detection of cellulase gene from *Bacillus amyloliquefaciens* G-V1

Reagents for one reaction volume of 25 µl

2.5 µl Taq buffer (1X)
1.5 µl $MgCl_2$ (1.5µM)
0.5 µl dNTPs (200 µM)
0.625 µl Forward primer (0.25 µM)
0.625 µl Reverse primer (0.25 µM)
0.125 µl Taq Polymerase (Hot Start)
1 µl DNA (100 ng/µl)
18.2 µl Sterile distilled water

PCR condition:
Step 1: 94 ºC for 15 min
Step 2: 94 ºC for 30 sec
Step 3: 45 ºC for 30 sec
Step 4: 72 ºC for 1 min
Step 5: repeat from step 2 for 35 times
Step 6: 72 ºC for 3 min

F Primer: 5' GAYGAARTYGGY TTYATGGT*
R Primer: 5' CCDACY TCYTCYTGMACVGTTSC*

c) Detection of phytase gene from *Raoultella terrigena* G-584

Reagents for one reaction volume of 25 µl

2.5 µl Taq buffer (1X)
1.5 µl $MgCl_2$ (1.5 µM)
0.5 µl dNTPs (200 µM)
0.625 µl forward primer (0.25 µM)
0.625 µl reverse primer (0.25 µM)
0.125 µl Taq Polymerase (Hot Start)
1 µl DNA (100 ng/µl)
18.2 µl Sterile distilled water

PCR condition:
Step 1: 94 ºC for 15 min
Step 2: 94 ºC for 30 sec
Step 3: 45 ºC for 30 sec
Step 4: 72 ºC for 1 min
Step 5: repeat from step 2 for 35 times
Step 6: 72 ºC for 3 min

F Primer: 5’ GACTGGCAGCTGGAGAAAG*
R Primer: 5’ CGCCTGTTTCAATAGCTGG*

* IUPAC-IUB SYMBOLS FOR NUCLEOTIDE NOMENCLATURE

Extended DNA / RNA alphabet: (includes symbols for nucleotide ambiguity)

Symbol	Meaning	Nucleic Acid
A	A	Adenine
C	C	Cytosine
G	G	Guanine
T	T	Thymine
U	U	Uracil
M	A or C	
R	A or G	
W	A or T	
S	C or G	
Y	C or T	
K	G or T	
V	A or C or G	
H	A or C or T	
D	A or G or T	
B	C or G or T	
X	G or A or T or C	
N	G or A or T or C	

Assay of amplification

Primers were tested for efficacy by doing PCR with *B. amyloliquefaciens* genomic DNA, *P. fluorescens* genomic DNA, *R. terrigena* genomic DNA and a negative control. The PCR reaction was carried using PTC-thermo cycler with the above mentioned PCR program.

Samples were loaded into a 2 % agarose gel. DNA ladders were loaded in 2 µl volumes, while 5 µl of sample was loaded with 2 µl of loading dye. The gel was allowed to run for ~45minutes. Test results were visualized with a Alphamanager ™ 2200 &1220 Documentation & Analysis system and Ethidium Bromide (EtBr) staining.

TOPO® Cloning

TOPO® Cloning (Invitrogen) method was used to clone the PCR DNA fragment. This was done by cloning the PCR fragment into a vector, transforming into *E. coli* cells, and using blue/white selection to determine transformants.

The key to TOPO® Cloning is the enzyme DNA topoisomerase I, which functions as both a restriction enzyme and a ligase. Its biological role is to cleave and rejoin DNA during replication. To harness the religating activity of topoisomerase, over 30 vectors are provided linearized with topoisomerase I covalently bound to each 3' phosphate. This enables fast ligation of DNA sequences with compatible ends. After only 5 minutes at room temperature, the ligation is complete and ready for transformation into *E. coli*.

Ligation and Transformation

4 µl of DNA was mixed with 1 µl of TOPO Vector (It is important to keep the TOPO vector on ice at all times. The Topo vector self-catalyzes the ligation reaction via topoisomerases, when left at room temperature these enzymes lose activity) in an eppendorf tube and ligation was carried out at room temperature for 5 minutes.

After ligation, chemically competent *E. coli* cells (TOP10 competent cells supplied with the kit) were added to the Topo mixture and gently mixed (do not mix by pipetting up and down). They were kept on ice for 20 minutes followed by heat shock at 42 ºC for 30 sec and suddenly the TOPO mixture were kept on ice. Finally 250 µl of SOC medium was added to the Topo mixture and incubated for 1 h at 37 ºC with shaking horizontally (200 rpm).

Plating cells: Selective LB plates (100 µg/ml, ampicillin) were spread with 40 µl of X-gal (5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside) is a chromogenic substrate used to identify recombinant plasmids. In the presence of X-Gal, bacterial colonies appear blue, whereas recombinant colonies appear white) (40 mg/ml) and prewarmed for 1 h at 37 ºC. From the TOPO mixture, 20 and 50 µl were spread on LB plates and incubated for 37 ºC for overnight.

Selection of Clones: Colonies that have acquired the cloned PCR product/topo vector construct were selected by Blue/white colonies. Blue colonies are ones that have most likely not contaning the insert/vector. White colonies were acquired the insert/vector, selected for sequencing & further studies (Fig. 18).

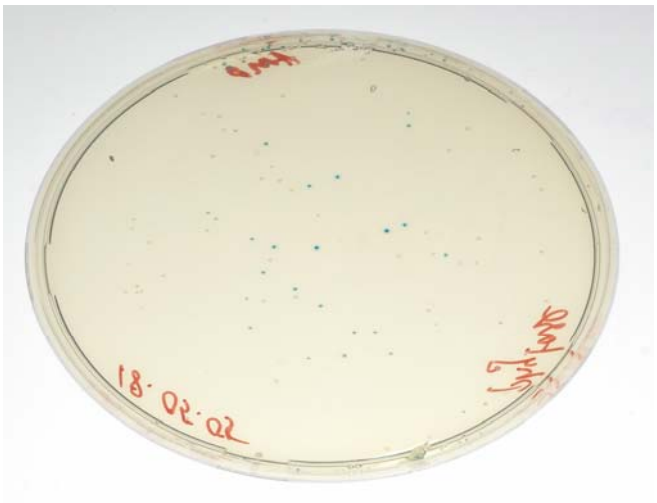

Fig. 18: Selection of recombinants

2.2 DNA sequencing

The recombinant plasmid from the clone was sequenced using Sanger's dideoxy chain terminator method (Sanger *et al*., 1977) in a MegaBace 1000 automated sequencer at the European Molecular Biology Laboratory, Heidelberg, Germany.

2.3 2,4-diacetylphloroglucinol gene expression

Growth condition: *P. fluorescens* 2R1-7 was grown on LB media for 24 hours at 25°C and the pH of the media was adjusted to 7.2, 6.5, 6, 5.5, 5.

RNA Isolation

Total RNA was isolated using the Qiagen RNeasy kit according to the manufacturer's instructions. To isolate total RNA, the following procedure was used. The cells in a 1-ml suspension were pelleted by centrifugation at 5000 rpm for 5 min at 4 °C. Remaining steps were done at room temperature. The pellet was thoroughly resuspended in 100 µl of lysozyme (400 µg/ml)-containing TE buffer by vortexing and incubated for 3-5 min at room temperature. 350 µl buffer RLT (Lysis buffer) were added to the sample and mixed thoroughly by vortexing. To the sample 250 µl ethanol (96-100 %) were added and mixed thoroughly by pipetting. The sample mixture were added to the RNeasy mini column

placed in a 2 ml collection tube and centrifuged for 15 sec at 8000 rpm and flow-through were discarded. 700 µl buffer RW1 (Wash buffer) were added to the RNeasy column and centrifuged for 15 sec at 8000 rpm and flow-through were discarded. The RNeasy column was transferred into a new 2 ml collection tube. To wash the column 500 µl buffer RPE (Wash buffer) were added onto the RNeasy column, centrifuged & flow-through were discarded. Again 500 µl buffer RPE were added to the RNeasy column and centrifuged for 2 min. After centrifugation, the RNeasy column was transferred into a new 1.5 ml collection tube for eluting the RNA. 30-50 µl RNase-free water were added directly onto the RNeasy Silica-gel membrane and centrifuged for 1 min at 8000 rpm and finally flow-throw collected (containing RNA).

To eliminate carryover DNA, DNase I digestion was performed about 20 min at 30 °C. The amount of RNA was determined using spectrophotometer and quality of RNA checked in 1 % Agarose gel.

cDNA synthesis

Reverse transcription into cDNA was performed using the StrataScript kit (Stratagene). Reaction volume of 20 µl containing 200 ng of RNA, 2.0 µl of first strand buffer (10x), 3.0 µl of random primers (0.1 µg/µl), 0.8 µl of dNTP mix (25 mM each dNTP), RNase-free water incubated at 65 °C for 5 min. The mixture were cooled at room temperature and 1 µl StrataScript RT (50 U/µl) added and they were kept at 42 °C for 60 min. Finally the reaction were terminated by incubating at 70 °C for 15 min, chilled rapidly on ice and stored at -20 °C.

Real-time quantitative RT-PCR

Reagents and Primers

Reaction volume (25 µl)

1 µl cDNA
1.25 µl Forward Primer (10 µM)
1.25 µl Reverse Primer (10 µM)
12.5 (2x) SYBR Green PCR Master Mix (Eurogentec)
9 µl distilled water

Primers for 2,4-diacetylphloroglucinol

Forward Primer: 5` GTCATCGGCGCGCATAA
Reverse Primer: 5` CGTTCATATCAGCCGCGTTA

Primers for 16S RNA (Internal control: reference gene)

Forward Primer: 5` TCCACGCCGTAAACGATGT
Reverse Primer: 5` TGCGTTAGCTGCGCCACTA

Real-time quantitative RT-PCR (relative quantification) was done with ABI Prism 7500 detection system (Applied Biosystems). The value used for comparison and quantitation was the threshold cycle (C_T), defined as the cycle number at which the fluorescence emission exceeds an arbitrarily set baseline or threshold level**.** This threshold level reflects a midpoint in the linear range of amplification**.** During amplification, the amount of amplified target is directly proportional to the input amount of target**.** Thus, the higher the initial amount of specific template in each reaction mixture, the fewer the cycles that are required to exceed the threshold value**.** Expression levels for gene in each environment are presented as fold induction relative to expression levels determined for RNA isolated (Pfaffl *et al*., 2002)**.**

3.0 Results

3.1 Identification of 2,4-diacetylphloroglucinol gene from *P. fluorescens* 2R1-7 and partial sequencing

A fragment of 585 bp (Fig. 19) length was observed after PCR with genomic DNA from *P. fluorescens* strain 2R1-7 with primers designed by multiple sequence alignment. In order to identify the sequence of the PCR fragment, the PCR product was cloned into Topo Vector and transformed into *E. coli*; positive clones were selected by Blue/White colonies (Fig. 18). The partial gene sequence was identified after DNA sequencing reaction (Fig. 20 & Fig. 21). NCBI Blast was used to analyse the homology with other prokaryotic system. As high as 99 % identity was found with *Pseudomonas fluorescens* and most relevant results presented (Fig. 22).

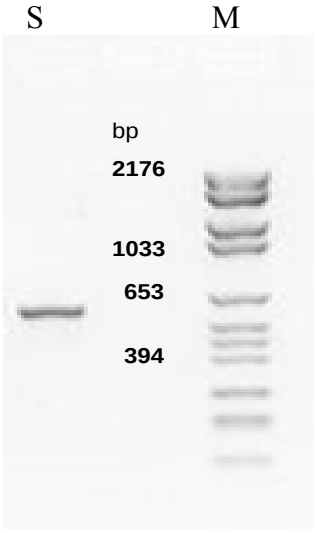

Fig. 19: Amplification product in agarose gel after PCR of genomic DNA of *Pseudomonas fluorescens* 2 R1-7 for 2,4-diacetylphloroglucinol gene (M= DNA Marker, S= Sample)

```
CTCAAGCTGT CGCGAGACTG CGCGACCCGA CCGGGTTCCA AGTCCAGTTG
CAGGACCAGT TCATCAAGAA TTTTCCGTCC GCCGGTATGG AAGATGAAAA
AGTCATTTTG AGCGCAATGT TGATTGAAGG TCTCGTAGTT CAATTCCTCC
ATCATCGGTG CGACATCTTT AATGGAGTTC ATGACAGCCT TGTCCAAGGT
GAAATGAAAG CCGCTGTCTT TAACGTCGTA TTTAATGTAG TGCTCGCTAT
CAGGCAGGAA GTAAGACCCG GTATTGGCGA TCTTGAAACC AGGCGCCTGG
TCATCGGCGC GCATAACGCG GGCCGATACG GCATCGCCGA ATAACGCGGC
TGATATGAAC GCGTGCAACT TGGTGTCCTG GGGTTGATAG CAGAGCGATG
AGAACTCCAG GGAGACGATG AGGACATGGT TGTCCGGCGC CCGGCTGGCG
AAGTCATTGG CTCGATTGAT CGCCGCAGCG CCTGCCACGC AGCCCAGTTG
AGCGATGGGC AGTTGTACGG TCGACGTTCG CAGGCCCAGG TCATTGATCA
AGTGGGCTGT CAGCGAGGGC ATCATGAAAC CGGTG
```

Fig. 20: Partial 2,4-diacetylphloroglucinol gene sequence

T G F **Met Met** P S L T A H L I N D L G L R T S T V Q L P I A Q L G C V A G A A A I
N R A N D F A S R A P D N H V L I V S L E F S S L C Y Q P Q D T K L H A F I S A A
L F G D A V S A R V **Met** R A D D Q A P G F K I A N T G S Y F L P D S E H Y I K Y
D V K D S G F H F T L D K A V **Met** N S I K D V A P **Met Met** E E L N Y E T F N Q H
C A Q N D F F I F H T G G R K I L D E L V L Q L D L E P G R V A Q S R D S L

Fig. 21: Partial 2,4-diacetylphloroglucinol gene sequence translation

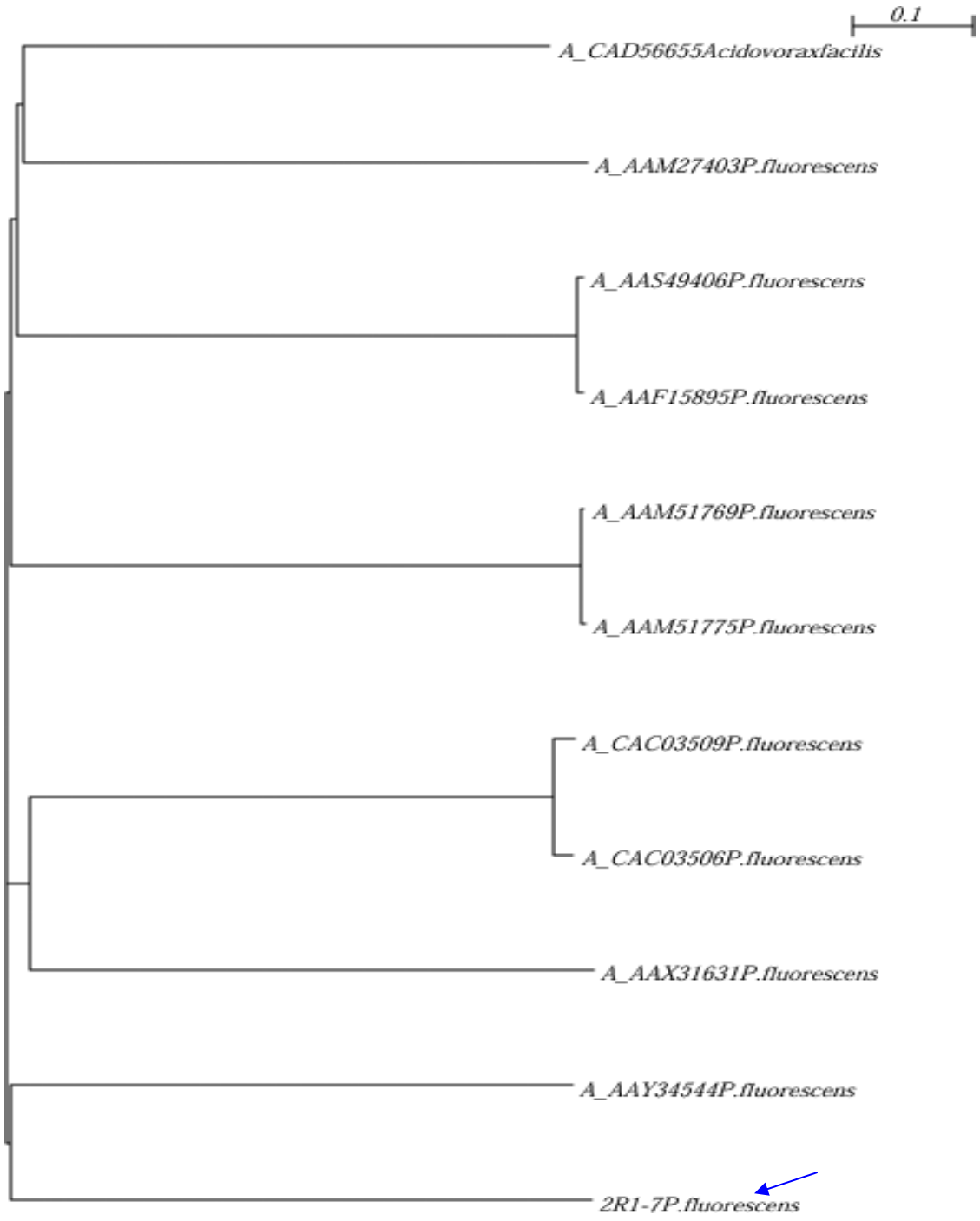

Fig. 22: Homology analysis of partial 2,4-diacetylphloroglucinol gene of *Pseudomonas fluorescens* 2R1-7

3. 2 2,4-diacetylphloroglucinol gene expression from *P. fluorescens* 2R1-7

To investigate the influence of pH on 2,4-diacetylphloroglucinol gene expression from *P. fluorescens* 2R1-7, relative quantification showed the expression of this gene is depended on pH (Fig. 23). The data in Fig. 23, showed the gene expression in fold change, when compared to the pH range 7.2, the maximum 13.5 fold change of upregulation of this gene is observed in pH 6.5 and fold change decrease against decreasing pH range.

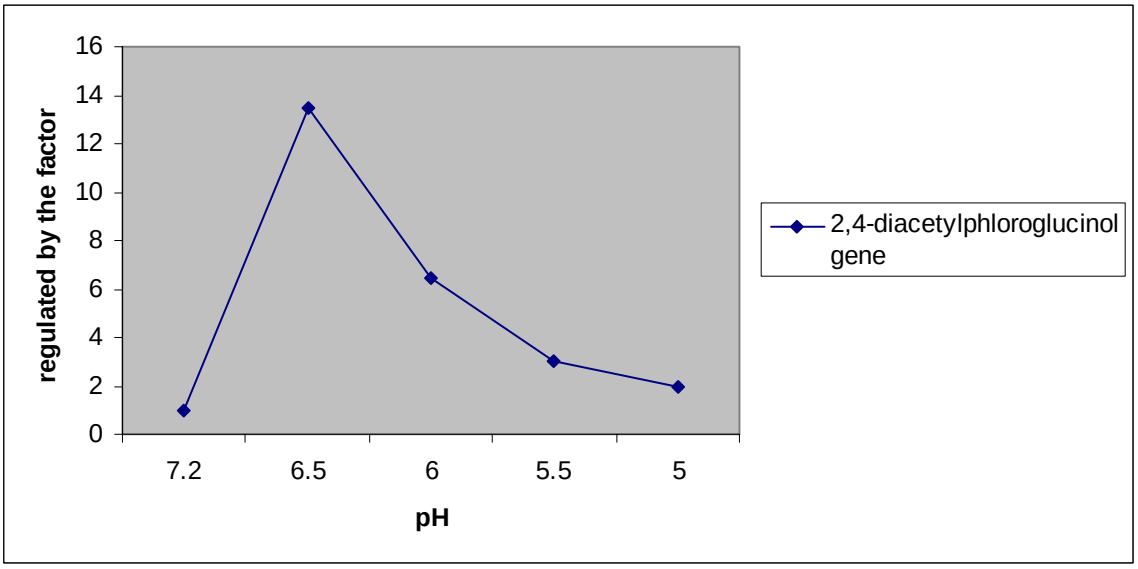

Fig. 23: 2,4-diacetylphloroglucinol gene expression pattern in *P. fluorescens* 2R1-7 in response to different pH conditions

3.3 Identification of cellulase gene from *B. amyloliquefaciens* G-V1 and partial sequencing

The multiple sequence alignment led to the determination of two primer sequences for cellulase gene in *B. amyloliquefaciens* G-V1. PCR with genomic DNA of G-V1 strain with designed primers was detecting the gene, resulted in amplification of a gene with 444 bp length (Fig. 24). The PCR fragment was cloned in Topo Vector and transformed into *E. coli*, positive clones were selected by Blue/White colonies (Fig. 18). Partial cellulase gene sequence was identified when the clones were subjected to DNA sequencing (Fig. 25 & Fig. 26). The partial nucleotide sequence was analysed for homology with other prokaryotic system using NCBI Blast. Most relevant results presented (Fig. 27) and as high as 81 % of identity was recorded with *Bacillus licheniformis* DSM 13.

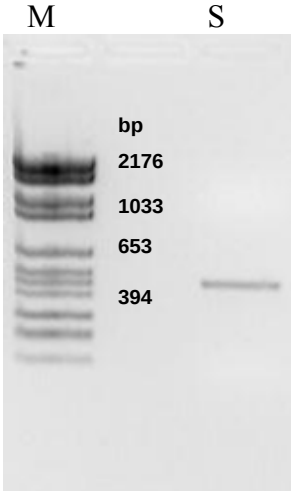

Fig. 24: Amplification product in agarose gel after PCR of genomic DNA of *Bacillus amyloliquefaciens* G-V1 for cellulase gene (M= DNA Marker, S= Sample)

```
TGATGAAGTT GGTTTCATGG TGACGCAAAT CACTGATAAA GGCTTTCTCC
GCTTCCAAAC GGTCGGCGGC TGGTGGTCTC AGGTGATGCT GGCCCAGCGC
GTCACCGTCG TGACAAAAAA AGGAGACACC ACGGGAATCA TCGGTTCGAA
GCCGCCGCAT ATTTTACCGC CTGACGCCAG AAAAAAAGCC GCCGATATCA
AAGAGATGTT CATCGATATC GGGGCGTCCA GCCGTGAAGA AGCAATGGAA
TGGGGCGTTC TTCCGGGTGA CCAGGTTGTG CCGTATTTTG AATTTACAGT
GATGAACAAT GAAAAACATT TATTGGCGAA AGCATGGGAC AATCGTATCG
GCTGTGCGAT TGCCATCGAT GTATTAAAAA ATCTGAAAAA CAGTGATCAT
CCGAATGAAG TATACGGAGT GGGAACCGTT CAGGAAGAAG TAGG
```

Fig. 25: Partial cellulase gene sequence

D E V G F **Met** V T Q I T D K G F L R F Q T V G G W W S Q V **Met** L A Q R V T V V
T K K G D I T G I I G S K P P H I L P P D A R K K A A D I K E **Met** F I D I G A S S R
E E A **Met** E W G V L P G D Q V V P Y F E F T V **Met** N N E K H L L A K A W D N R
I G C A I A I D V L K N L K N S D H P N E V Y G V A T V Q E E V

Fig. 26: Partial cellulase gene sequence translation

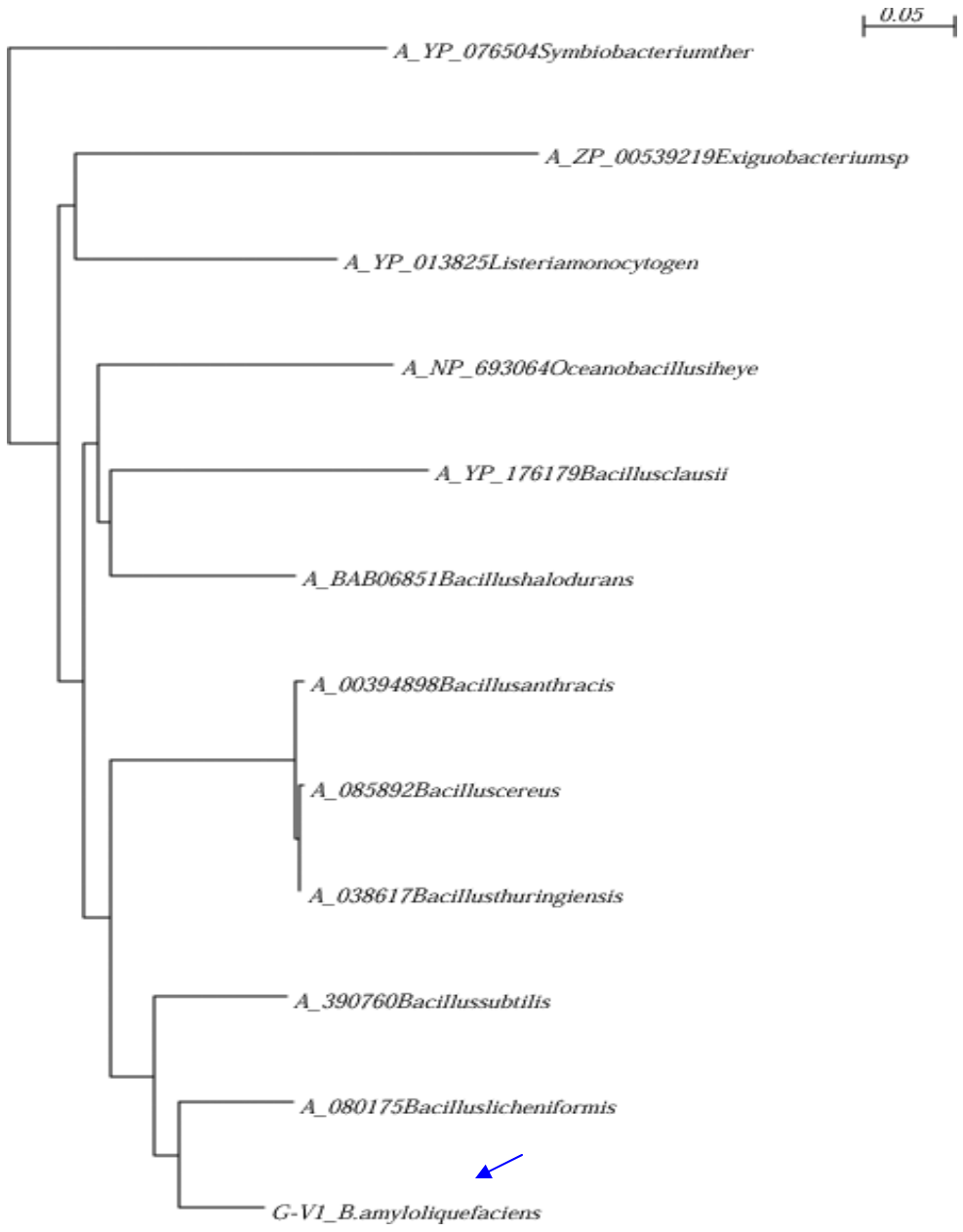

Fig. 27: Homology analysis of partial cellulase gene of *Bacillus amyloliquefaciens* G-V1

3.4 Identification of phytase gene from *R. terrigena* G-584 and partial sequencing

PCR with genomic DNA of *Raoultella terrigena* G-584 with primers for detecting the phytase gene, resulted in amplification of a gene with 486 bp length (Fig. 28). When the PCR product was cloned and transformed into *E.coli*, positive clones were selected by Blue/White colonies (Fig. 18). Partial phytase gene sequence was identified using DNA sequencing reaction (Fig. 29 & Fig. 30). As high as 86% of identity was recorded with *Klebsiella pneumoniae* when the partial nucleotide sequence was analysed for homology with other prokaroyotic system using NCBI Blast, most relevant result presented (Fig. 31).

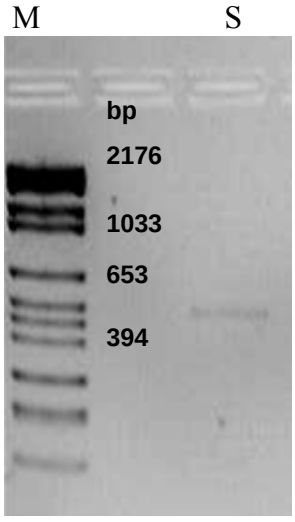

Fig. 28: Amplification product in agarose gel after PCR of genomic DNA from *Raoultella terrigena* G-584 for phytase gene (M= DNA Marker, S= Sample)

CGCCTGTTTC AATAGCTGGA TGGTGGGCTC CAGGGCCCGG CGGCGCAGCG
CCAGGTCGCC GGCTTTTTTT TGCACCTCCG CCAGCTGCCG GGCAGGATCG
GTCTGGGTTG TCGCGAACCG GTCGGTCTGG AACAGCGGGT CAGCGTCGCC
GGCGACATAG TGAATGCGCG TCCCGCATCC CGGAAACGCG CCGTCAACCA
GGGCCTCAGC GGTGGCTCGC GTGCGCTGCA GCGGGCTGGC GCGGACGTAG
ATGTCGCCCG GGGTCGGGCA GCCGGCGCTC AGCAGCCCGA GCGCGCGGTA
GTGCGCGCCC TCGGCCCGCC CTTTGTTCAC GACCGCCGCG TAGCCGTGGC
CGGTTAATTC CCCGTCGCGG GTTGTCCACT GCGTCCATGG GCGTTGGGTG
GCGGCCTCGA TGGCTTCGCG GTTGCCCGCC GTCGGCGGGC GGATCCCGTG
GCGACTGAGC TCAACCACTT TCTCCAGCTG CCAGTC

Fig. 29: Partial phytase gene sequence

D W Q L E K V V E L S R H G I R P P T A G N R E A I E A A T Q R P W T Q W T T R
D G E L T G H G Y A A V V N K G R A E G A H Y R A L G L L S A G C P T P G D I Y
V R A S P L Q R T R A T A E A L V D G A F P G C G T R I H Y V A G D A D P L F Q
T D R F A T T Q T D P A R Q L A E V Q K K A G D L A L R R R A L E P T I Q L L K
Q A

Fig. 30: Partial phytase gene sequence translation

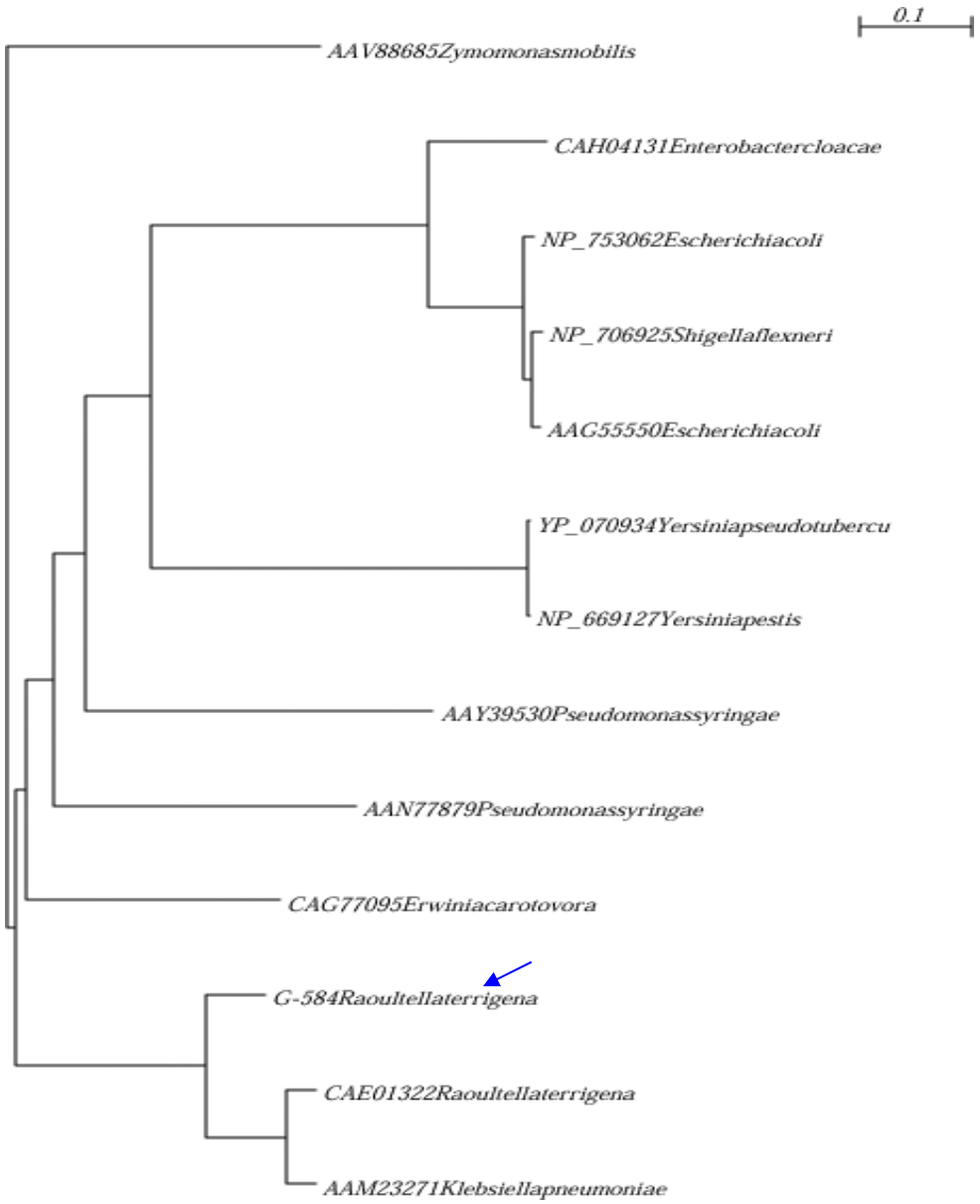

Fig.31: Homology analysis of partial phytase gene sequence of *Raoultella terrigena* G-584

4.0 Discussion

The *in vitro* test in chapter II showed that the production of antifungal substances by the three antagonistic rhizobacteria was one of the mode of action responsible for the reduction of mycelial growth. In the present study, PCR analysis led to the amplification of genes such as 2,4-diacetylphloroglucinol (from *P. fluorescens* 2R1-7), cellulase (from *B. amyloliquefaciens* G-V1) and phytase (from *R. terrigena* G-584). Additionally genes were partially sequenced; NCBI Blast analysis revealed the homology with other identified gene sequence in prokaryotic system.

The identification of 2,4-diacetylphloroglucinol-encoding gene in strain 2R1-7, will be instrumental in determining the overall contribution of 2,4-diacetylphloroglucinol metabolite to biocontrol by means of site-directed mutagenesis for the sequential inactivation of gene. To demonstrate a role for antibiotics in biocontrol, mutants lacking production of antibiotics or over-producing mutants have been used in other studies (Bonsall *et al*., 1997; Chin-A-Woeng *et al*., 1998). For example, introduction of gene(s) phlx encoding a monoacetylphloroglucinol acetyl transferase into a wild-type strain (M114) of *Pseudomonas* sp., which is unable to synthesize the more active antifungal metabolite 2, 4-diacetylphloroglucinol, has resulted in an enhanced biocontrol ability of strain M114 against *Pythium ultimum* both in the laboratory and in greenhouse experiments (Fenton *et al*., 1992). *P. fluorescens* Pf-5 with a mutation in the *apdA* sensor gene lost the ability to produce pyoluterin (Plt) and pyrrolnitrin (Pln) (Hrabak and Willis, 1992; Corbell and Loper, 1995) and *P. fluorescens* CHA0 with a defect in the *gacA* response gene lost the ability to produce pyoluterin (Plt) as well as protease and phospholipase C (Laville *et al*., 1992; Sacherer *et al*., 1994). Further, the development of a constitutively siderophore-producing mutant improved siderophore-mediated biocontrol under condition of high iron in *in vitro* (O' Sullivan and O' Gara, 1991). Moreover in the present study, 2,4-diacetylphloroglucinol gene expression from *P. fluorescens* 2R1-7 under different pH conditions showed this gene expression depends on the pH range, it could directly influence the production of antifungal metabolites. The results achieved in present study could be similar to results reported by Dickie and Bell (1995) who showed a small change in the pH value can alter the inhibition of pathogen growth. Hultberg *et al*. (2000)

documented that a *P. fluorescens* strain 5.014 known to produce phloroglucional compounds suppressed *Pythium ultimum* damping-off of tomato seedlings significantly when compared to the mutant strain. 2,4-diacetyl-phloroglucional (Phl) is a major determinant for the protection of wheat against take-all diseases in Washington State soils (Raaijmakers *et al*., 1997) and this can also be an indication for the biocontrol activity of the studied *P. fluorescens* strain 2R1-7.

The PCR analysis with genomic DNA of the antagonistic strain of *B. amyloliquefaciens* G-V1 amplified the cellulase gene and in chapter IV cellulase enzyme prouduction from this strain was confirmed by enzyme assay, makes it evident that this strain in excreting this enzyme. Lytic enzymes excreted by bacteria are suspected to play an important role in suppression of pathogens (Buchenauer, 1998). The identification of cellulase-encoding gene in strain G-V1, will be ideal in determining the overall contribution of cellulase enzyme to biocontrol against the tested *Phytophthora* spp. by means of site-directed mutagenesis for the sequential inactivation of gene. For example, Tn*5* mutants of *E. agglomerans* (Beijerinck) deficient in chitinolytic activity were unable to protect cotton (*Gossypium barbardense* L.) and expression of the *chiA* gene for endochitinase in *Escherichia coli,* allowed the transformed strain to inhibit *R. solani* on cotton seedlings (Chernin *et al*., 1997). Similar techniques involving Tn*5* insertion mutants and subsequent complementation demonstrated that biocontrol of *Pythium ultimum* in the rhizosphere of sugar beet by *Stenotrophomonas maltophila* W81 was due to the production of extracellular protease (Dunne *et al*., 1997). The biocontrol activity of a genetically manipulated strain of *Trichoderma virens* was enhanced against cotton seedling disease incited by *R. solani* (as compared with wild-type strain) due to the overexpression of a chitinase gene (Cht 42) (Back *et al*., 1999). Biocontrol of *Phytophthora cinnamomi* Rands root rot of *Banksia grandis* was obtained using a cellulase-producing isolate of *Micromonospora carbonacea* (El-Tarabily *et al*., 1996) and control of *Phytophthora fragariae* var. *rubi* Hickm causing raspberry root rot was suppressed by the application of actinomycete isolates that were selected for the production of ß-1,3-, ß-1,4- and ß-1,6-glucanases (Valois *et al*., 1996).

Plants producing phytases (Greiner and Larsson , 2001) display only low activity in roots and other plant organs, and occurrence of plant-secreted phytase within the rhizosphere is not documented. This suggests that plant roots may not possess an innate ability to acquire phosphorus directly from soil phytate. The possible role of microbial phytases produced by PGPR in supporting plant growth under phosphate limitation is limited and recently the role of extracellular phytase activity on plant growth promotion has been reported (Elsorra *et al*., 2002). Phytase has been isolated and characterized from a few Gram-positive and Gram-negative soil bacteria, e.g. *B. subtilis* (Kerovuo *et al.*, 1998), *Bacillus amyloliquefaciens DS11* (Kim *et al.*, 1998), *Klebsiella terrigena* (Greiner *et al.*, 1997), *Pseudomonas* spp. (Richardson and Hadobas, 1997) and *Enterobacter* sp. 4 (Yoon *et al.*, 1996). Besides other factors, the ability of some root-colonizing bacteria to make the phytate phosphorus in soil available for plant nutrition under phosphate-starvation conditions might contribute to their plant-growth-promoting activity. Another beneficial effect due to bacterial phytase activity in the rhizosphere is elimination of chelate-forming phytate, which is known to bind nutritionally important minerals (Reddy *et al*., 1989). The identification phytase-encoding gene in strain G-584, will be useful in determining the contribution of phytase enzyme to biocontrol activity of *Raoultella terrigena* strain G- 584 which has been observed in this study against the *Phytophthora* spp. by means of site-directed mutagenesis for the sequential inactivation of gene.

VI. General Discussion

Phytophthora pathogens and other soilborne pathogens can cause severe damage to plants. Biological control offers an environmental friendly alternative to chemical control of plant diseases. The knowledge of mechanisms contributing to plant protection by biocontrol agents can facilitate more effective use of existing agents and identification and development of new ones.

In the present study, three rhizobacteria were tested against the crown rot (*Phytophthora cactorum*) and red stele disease (*Phytophthora fragariae* var. *fragariae*) of strawberry. In *in vitro* tests the mycelial growth of the fungus was significantly reduced by *Bacillus amyloliquefaciens* G-V1, *Raoultella terrigena* G-584 and *Pseudomonas fluorescens* 2R1-7. In greenhouse experiments an antagonistic potential of three selected rhizobacteria against *Phytophthora* spp. was observed. *Bacillus* spp. and *Pseudomonas* spp. are well known antagonists of different plant pathogens (Bochow, 1992; Cook, 1993; Koch *et al*., 1998; Mansour and Farag, 1999; Zeller, 1999). But until now, no data on the control of *Phytophthora* spp. by *Raoultella terrigena*, a Gram negative enteric bacterium, have been reported. In this study, *Raoultella terrigena* G-584 had moreover a high inhibitory activity against the *Phytophthora* spp.

In all field experiments, tested rhizobacteria such as *Raoutella terrigena* G-584*, Bacillus amyloliquefaciens* G-V1 *and Pseudomonas fluorescens* 2R1-7 showed different level of biocontrol efficacy and in some cases with similar effect compared to the chemical control Aliette against the two *Phytophthora* diseases on strawberry. Another aspect was to test if mixtures of these bacterial species give a better control against both diseases than the bacterium alone, as in previous studies has been shown that combinations of different isolates may lead to improved antagonistic activity (Weller and Cook, 1983; Weller, 1988). Application of mixtures of antagonistic micro-organisms, preferably with different modes of action, has been proposed as a strategy to increase the efficacy and to improve the consistency of disease control (Pierson and Weller 1994; Schisler *et al*. 1997). In the field, field trial (2003-2004) at Jork with root bacterization of strawberry with dual mixtures of

B. amyloliquefaciens G-V1 and *R. terrigena* G-584, an improved control effect compared to individual isolates could be observed. However, the results of mixture of *B. amyloliquefaciens* G-V1 and *R. terrigena* G-584, treatment in 2004-2005 could not be confirmed. A combination of different isolates also may result in negative results (Hadar *et al*., 1983). Sikora *et al*. (1990) also showed a negative effect by combining antagonistic bacteria against *Pythium ultimum*. The inconsistent results achieved in the field experiment of the present study may be related to the different environmental conditions from year to year and site to site.

The capability of gram-negative *Pseudomonas* to colonize plant roots makes them potential candidates in biological control (Howell and Stipanovic, 1980; Weller and Cook, 1983). From the present study, it is found that *R. terrigena* G-584 has the potential to colonize the strawberry plant root system, which has been found by using the GFP-marker in the microscopic studies of chapter III. Therefore this strain can be considered as a good root colonizer.

Out of the enzyme assays of the tested antagonistic bacteria, excretion of different extra cellular enzymes could be observed. This can be considered as a further antagonistic effect, as this capability has been also detected in biological control from other antagonistic strains in many studies (Lim *et al*., 1991; Fridlender *et al*., 1993; El-Tarabily *et al*., 1996, Valois *et al*., 1996).

In the molecular studies in chapter V, specific genes could be detected from the three antagonistic bacteria, as for instance 2,4-diacetylphloroglucinol gene from *P. fluorescens* strain 2R1-7. 2,4-diacetylphloroglucional (Phl) is a major determinant for the protection of wheat against take-all diseases in Washington State soils (Raaijmakers *et al*., 1997). Hultberg *et al*. (2000) documented that a *P. fluorescens* strain 5.014 known to produce phloroglucional compounds suppressed *Pythium ultimum* damping-off of tomato seedlings significantly when compared to the mutant strain. Morover in the present study, 2,4-diacetylphloroglucional gene expression from *P. fluorescens* 2R1-7, found to be pH dependent, it could directly influence the production of antifungal metabolites. This could be similar to results reported by Dickie and Bell (1995) who showed a small change in the

pH value can alter the inhibition of pathogen growth.

The identification of 2,4-diacetylphloroglucinol-encoding gene in strain 2R1-7, will be instrumental in determining the overall contribution of 2,4-diacetylphloroglucinol metabolite to biocontrol by means of site-directed mutagenesis for the sequential inactivation of gene. Also the information of cellulase- encoding gene in strain G-V1 and phytase-encoding gene in strain G-584, will be useful in determining the overall contribution of each enzyme to biocontrol by means of site-directed mutagenesis for the sequential inactivation of each gene. Another important method would be in modifying the known antifungal metabolite producing strains genetically so that the bacteria produce more antifungal substances. Ligon *et al*., (2000) reported that by modifying pyrrolnitrin genes within a *P. fluorescens* strain, a significant increase in the production of this metabolite over the wild-type strain was achieved.

In summarizing, the three antagonistic bacteria against the *Phytophthora* diseases in strawberry, a reduction of the disease could be detected from the antagonists. The studies on the mode of action and genetic basis could demonstrate that the antagonistic effect was correlated with the production of specific enzymes (cellulase and phytase) and 2,4-diacetylphloroglucinol compound. More studies in molecular biological aspects should be done to improve the strains efficacy for bio-control of the *Phytophthora* diseases based on the present results.

VII. References

Abbot, W. S. (1925) A method for computing the effectiveness of an insecticide. J. Econ. Entomol. 18, 265–267

Acosta-Martínez, V., Tabatabai, M.A. (2000) Arylamidase Activity of Soils. Soil Science Society of America Journal 64: 215–221

Alippi, A. M., Perello. A. E., Sisterna, M. N., Greco, N. M., Cordo, C. A. (2000) Potential of spore-forming bacteria as biocontrol agents of wheat foliar diseases under laboratory and greenhouse conditions. Journal of Plant Diseases and Protection 107, 2: 155–169

Amann R., Fuchs B., Behrens S. (2001) The identification of microorganisms by fluorescence in situ hybridization. Curr. Opin. Biotech. 12, 231–236

Andersen, J. B., Sternberg, C., Poulsen, L. K., Bjørn, S. P., Givskov, M., Molin, S. (1998) New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl. Environ. Microbiol. 64, 2240–2246

Anonymous (1999) Pflanzenschutzmittel-Verzeichnis Teil 2 1999 Gemüsebau – Obstbau – Zierpflanzenbau 47. Auflage 1999, ISSN 0178-0603 Biologisches Bundesanstalt für Land-und Forstwirtschaft Bundesrepublik Deutschland

Assmus, B., Hutzler, P., Kirchhof, G., Amann, R., Lawrence, J.R., Hartmann, A. (1995) In situ localization of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labeled rRNA-targeted oligonucleotide probes and scanning confocal microscopy. Appl. Environ. Microbiol. 61, 1013–1019

Back, J. K., Howell, C. R., Kenerley, C.M. (1999) The role of an extracellular chitinase from *Trichoderma virens* GV 29-8 in the biocontrol of *Rhizoctonia solani*. Curr Genetics 35: 41-50

Bangera M. G., Thomashow L. S. (1996) Characterization of a genomic locus required for synthesis of the antibiotic 2,4-diacetylphloroglucinol by the biological control agent *Pseudomonas fluorescens* Q2-87. Molecular Plant-Microbe Interactions 9, 83–90

Becker, J. O., Schwinn, F. J. (1993) Control of soilborne pathogens with living bacteria and fungi: status and outlook. Pesticide Science 37, 355–363

Benhamou N, Bélanger RR, Paulitz TC. (1996) Induction of differential host responses by *Pseudomonas fluorescens* in Ri T-DNA-transformed pea roots after challenge with *Fusarium oxysporum* f. sp. *pisi* and *Pythium ultimum*. Phytopathology 86, 1174–1185

Benhamou, N., Gagné, S., Quéré, D. L., Dehbi, L. (2000) Bacterial-mediated induced resistance in cucumber: beneficial effect of the endophytic bacterium *Serratia plymuthica* on the protection against infection by *Pythium ultimum*. Phytopathology 90, 45–56

Benizri, E., Schoeny, A., Picard, C., Courtade, A., Guckert, A. (1997) External and internal root colonization of maize by two *Pseudomonas* strains: enumeration by enzyme–linked immunosorbent assay (ELISA). Curr. Microbiol. 34, 297–302

Bieleski, R.L., I. B. Ferguson. (1983) Physiology and metabolism of phosphate and its compounds, pp. 422-449. *In* R. Läuchi and R.L. Bieleski (eds). Inorganic Plant Nutrition. Springer Berlin

Björklöf, K., Jørgensen, K.S. (2001) Applicability of non antibiotic resistance marker genes in ecological studies of introduced bacteria in forest soil. FEMS Microbiology Ecology 38, 179–188

Blot, M., Hauer, B., Monnet, G. (1994) Tn5 bleomycin resistance gene confers improved survival growth advantage in *Escherichia coli*, Mol. Gen. Genet. 242, 595–601

Blumer, C., Haas, D. (2000) Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. Arch. Microbiol. 173: 170–177

Bochow, H. (1990) Synergistic actions of biocontrol agents – bacterial antagonists and fungicides against soilborne plant mycoses. Tages-Bericht. Akademisches Landwirtschaft.-Wissen. DDR, Berlin. 291, 257–267

Bochow, H. (1992) Phytosanitary effects of *Bacillus subtilis* as biocontrol agent. – Meded. Faculity Landbouwwissenschaft. Universität Gent 57: 2b

Boller, T., Metraux, P. (1988) Extracellular localization of chitinase cucumber. Physiology Molecular Plant Pathology 33: 11–16

Bonsall R. F., Weller D. M., Thomashow L. S. (1997). Quantification of 2,4-diacetylphloroglucinol produced by fluorescent *Pseudomonas* spp. *in vitro* and in the rhizosphere of wheat. Appl. Environ. Microbiol. 63, 951–955

Borowicz, J. J. and Omer Z. S. (2000) Influence of rhizobacterial culture media on plant growth and on inhibition of fungal pathogens. BioControl 45: 355–371

Bonants, P. J. M, Hagenaar D. V. M., van Gent-Pelzer, M. P., Lacourt, I., Cooke D. E. L., Duncan J. M. (1997) Detection and Identification of *Phytophthora fragariae* Hickman by the Polymerase Chain Reaction. European Journal of Plant Pathology 103, 345–355

Bonants, P. J. M., van Gent-Pelzer, M. P., Hooftman, R., Cooke, D. E. L., Guy, D. C., Duncan, J. M. (2004). A combination of baiting and different PCR formats,

including measurement of real-time quantitative fluorescence, for the detection of *Phytophthora fragariae* in strawberry plants. European Journal of Plant Pathology 110: 689–702

Braun-Howland, E. B., Vescio P.A., Nierzwicki-Bauer, S.A. (1993) Use of a simplified cell blot technique and 16S rRNA-directed probes for identification of common environmental isolates, Appl. Environ. Microbiol. 59, 3219–3224

Brisbane, P. G., Janik, J. L., Tate, M. L., Warren, R. F. O. (1987) Revised structure for the phenazin antibiotic from *Pseudomonas fluorescens* 2-79 NRRL B-15132. Antimicrobial agents in Chemotherapie 31, 1967–1972

Buchenauer, H. (1998) Biological control of soilborne diseases by rhizobacteria. Journal of Plant Diseases and Protection105 4: 328–348

Burlage, R. S., Kuo C.-T. (1994) Living biosensors for the management and manipulation of microbial consortia, Ann. Rev. Microbiol. 48, 291–309

Burr, T. J., Schroth, M. N., Suslow, T. (1978) Increased potato yield by treatment of seed pieces with specific strain of *Pseudomonas fluorescens* and *Pseudomonas putida.* Phytopathology 68 1377–1383

Campbell, R. (1989) The use of microbial inoculants in the biological control of plant diseases. *In* Microbial Inoculation of Crop Plants. MacDonald, R. and Campbell R. (eds.), IRL Press, Oxford 67–77

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., Prasher, D.C. (1994) Green fluorescent protein as a marker for gene expression. Science 263, 802–805

Chapon, A., Guillerm, A.Y., Delalande, L., Lebreton, L., Sarniguet, A. (2002) Dominant colonization of wheat roots by *Pseudomonas fluorescens* Pf29A and selection of the indigenous microflora in the presence of the take-all fungus. European Journal of Plant Pathology108, 449–459

Chelius, M. K., Triplett, E. W (2000) Immunolocalization of Dinitrogenase Reductase Produced by *Klebsiella pneumoniae* in association with Zea mays L. Appl. Environ. Microbiol. 66, 2, 783–787

Chen, C., Bélanger, R. R,, Benhamou, N., Paulitz, T. C. (1998) Induced systemic resistance (ISR) by *Pseudomonas* spp. impairs pre- and post-infection development of *Pythium aphanidermatum* on cucumber roots. European Journal of Plant Pathology 104, 877–886

Chen, C., Bélanger, R. R., Benhamou, N., Paulitz, T. C. (2000) Defence enzymes induced in cucumber roots by treatment with plant growth-promoting rhizobacteria (PGPR) and *Pythium aphanidermatum*. Physiological and Molecular Plant Pathology 56, 13– 23

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., Thompson, J. D (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. Jul 1; 31(13): 3497–500

Chernin, L.S., de la Fuente, L., Sobolev, V., Haran, S., Vorgias, C.E., Oppenheim, A.B Chet, I. (1997) Molecular Cloning, Structural Analysis, and Expression in *Escherichia coli* of a Chitinase Gene from *Enterobacter agglomerans*. Appl. Environ. Microbiol. 63. 3, 834-839.

Chin-A-Woeng, T. F. C., Bloemberg, G. V., van der Bij, A. J. (1998) Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL 1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Molecular Plant-Microbe Interactions 11, 1069–1077

Chin-A-Woeng, T. F. C., Thomas-Oates, J. E. Lugtenberg, B. J. J., Bloemberg, G. V. (2001) Introduction of the *phzH* gene of *Pseudomonas chlororaphis* PCL1391 extends the range of biocontrol ability of phenazine-1-carboxylic acid-producing *Pseudomonas* spp. strains. Molecular Plant-Microbe Interactions 14, 1006–1015

Chitarra, L. G., Langerak, C. J., Bergervoet, J. H. W., Van der Bulk, R. W. (2002) Detection of the plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris* in seed extracts of *Brassica* sp. applying fluorescent antibodies and flow cytometry. Cytometry 47 118–126

Cohen, M. F., Wallis, J. G., Campbell, E. L., Meeks, J. C. (1994) Transposon mutagenesis of *Nostoc* sp. strain ATCC 29133, a filamentous cyanobacterium with multiple cellular differentiation alternatives. Microbiology 40: 3233–3240

Cook, R. J. (1993) Making greater use of introduced microorganisms for biological control of plant pathogens. Annual Review of Phytopathology 31, 53– 80

Cook, R. J., Weller, D. M. (1987) Management of take-all in consecutive crops of wheat or barley. *In* Innovation approaches to Plant Disease Control Chet (ed.). Wiley Series in Ecological and Applied Microbiology

Corbell, N., Loper, J.E. (1995) A global regulator of secondary metabolite production in *Pseudomonas fluorescens* Pf-5. Journal of Bacteriology 177, 6230–6236

Deacon, J. W. (1991) Significance of ecology in the development of biocontrol agents against soilborne plant pathogens. Biocontrol Science and Technology 1, 5–20

Défago, G., Haas, D. (1990) *Pseudomonads* as antagonists of soilborne plant pathogens. Mode of action and genetic analysis. *In* Soil Biochemistry. Bollag, J. M. and Strotzky, G. (eds.). Marcel Dekker, New York, Basel 6: 249–291

Deutschmann, F. (1954) Eine Wurzelfäule an Erdbeeren, hervorgerufen durch *Phytophthora cactorum*. Nachrichtenbl. Deutsche Pflanzenschutzdienst 6: 7–9

Dhingra, O. D., Sinclaire, J. B. (1985) Basic plant pathology methodes. CRC Press, Boca Raton, Florida

Dickie, G. A., Bell, C. R. (1995) A full factorial analysis of nine factors influencing *in-vitro* antagonistic screens for potential biocontrol agents. Canadian Journal of Microbiology 41, 284–293

Dijkstra, A. F., Scholten G. H. N.; Van Veen J. A. (1987) Colonization of wheat seedling (*Triticum aestivum*) roots by *Pseudomonas fluorescens* and *Bacillus subtilis*. Biological Fertile Soils Vol. 4. 41– 46

Douville, Y., Boland, G. J. (1992) A note on the antibiotic properties of *Bacillus subtilis* against *Colletotrichum trifolli*. Phytoprotection 73. 1: 31–36

Duffy, B., Keel, C., Défago, G. (2004) Potential Role of Pathogen Signaling in Multitrophic Plant-Microbe Interactions Involved in Disease Protection. Appl. Environ. Microbiol. 70. 3, 1836–1842

Duijff, B. J., Gianinazzi-Pearson V., Lemanceau P. (1997) Involvement of the outer membrane lipopolysaccharides in the endophytic colonization of tomato roots by biocontrol *Pseudomonas fluorescens* strain WCS417r. New Phytologist 135, 325–334

Duijff, B. J., de Kogel, W. J., Bakker, P. A. H. M., Schippers, B. (1994) Influence of pseudobactin 358 on the iron nutrition of barley. Soil Biology and Biochemistry 26: 1681–1688

Duncan, J. M. (1990) *Phytophthora* species attacking strawberry and raspberry. Bulletin OEPP/EPPO Bulletin 20: 107–115

Duncan, J. M., Kennedy D. M. (1995) Effect of temperature and host genotype on the production of inoculum by *Phytophthora fragariae* var. *fragariae* from the roots of infected strawberry plants. Plant Pathology 44: 10–21

Dunne, C., Crowley, J. J., Moënne-Loccoz, Y., Dowling, D. N., de Bruijn, F. J., O'Gara, F. (1997) Biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* W81 is mediated by an extracellular proteolytic activity. Microbiology 143, 3921–3931

Elander, R. P., Mabe, J. A., Hamill, R. H., Gorman, M. (1968) Metabolism of tryptophans by *Pseudomonas aureofaciens*. VI. Production of pyrrolnitrin by selected *Pseudomonas* species. Applied Microbiology 16, 753–758

Elad, Y. (1994) Biological control of grape grey mould by *Trichoderma harzianum*. Crop Prot. 13:35-38

Ellinger, W. (1995) Der Obstmarkt: 1995 mehr Erdbeeren. Obstbau 6/95, 296–300

Elsorra, E. I., Oliwia, M., Abdelazim, F., Kristin., Ralf, G., Helmut, B., Thomas, R., Rainer B. (2002) Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth-promoting effect. Microbiology 148, 2097–2109

El-Tarabily, K. A., Hardy, G. E. StJ., Sivasithamparam, K., Hussein, A. M., Kurtböke, D. I. (1997) The potential for the biological control of cavity-spot disease of carrots, caused by *Pythium coloratum* by streptomycete and non-streptomycete actinomycetes. New Phytologist 137, 495–507

El-Tarabily, K. A., Sykes, M. L., Kurtböke, I. D., Hardy, G. E. StJ., Barbosa, A. M., Dekker, R. F. H. (1996) Synergistic effects of a cellulase-producing *Micromonospora carbonacea* and an antibiotic-producing *Streptomyces violascens* on the suppression of *Phytophthora cinnamomi* root rot of *Banksia grandis*. Canadian Journal of Botany 74, 618–624

Errampalli, D., Leung, K., Cassidy, M. B., Kostrzynska, M., Blears, M., Lee, H., Trevors J.T. (1999) Applications of the green fluorescent protein as a molecular marker in environmental microorganisms. J. Appl. Microbiol. Methods 35, 187–199

Fedi, S., Tola, E., Moënne-Loccoz, Y., Dowling, D. N., Smith, L. M, O'Gara, F. (1997) Evidence for signaling between the phytopathogenic fungus *Pythium ultimum* and *Pseudomonas fluorescens* F113: *P. ultimum* represses the expression of genes in *P. fluorescens* F113, resulting in altered ecological fitness. Appl. Environ. Microbiol. 63, 4261–4266

Fenton, A. M., Stephens, P. M., Crowley, J., Callagahan, M. O., O` Gara, F. (1992) Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. Appl. Environ. Microbiol. 58: 3873-3878

Fiddaman, P. J., Rossall, S. (1993) The production of antifungal volatiles by *Bacillus subtilis*. J. Appl. Bacteriol. 74, 119 –126

Flaishman, M. A., Eyal, Z., Zilberstein, A., Voisard, C., and Haas, D. (1996) Suppression of *Septoria tritici* blotch and leaf rust of wheat by recombinant cyanide-producing strains of *Pseudomonas putida*. Molecular Plant-Microbe Interactions. 9: 642–645

Fravel, D. R. (1988) Role of antibiosis in the biocontrol of plant diseases. Annual Review of Phytopathology 26, 75–91

Fravel, D. R., Larkin, R. P. (1996) A vailability and application of biocontrol products. Biological and Cultural Tests. 11: 1–7

Freeman, S., Minz, D., Kolesnik, I., Barbul, O., Zveibil1, A., Maymon, M., Nitzani, Y., Kirshner, B., Rav-David, D., Bilu, A., Dag, A., Shafir, S. and Elad, Y. 2004. *Trichoderma* biocontrol of *Colletotrichum acutatum* and *Botrytis cinerea* in strawberry, population survival, and identification of biocontrol isolates according

to ITS sequence analysis. European Journal of Plant Pathology 110: 361–370

Friedlender, M., Inber, J., Chet, I. (1993) Biological control of soilborne plant pathogens by a ß-1,3 glucanase-producing *Pseudomonas cepacia*. Soil Biology and Biochemistry. 25, 1211–1221

Gaballa, A., Abeysinghe, P.D., Urich, G., Matthijs, S., de Greve, H., Cornelis, P., Koedam, N. (1997) Trehalose induces antagonism towards *Pythium debaryanum* in *Pseudomonas fluorescens* ATCC 17400. Appl. Environ. Microbiol. 63, 4340– 4345

Gamalero, E., Lingua, G., Berta, G., Lemanceau, P. (2003) Methods for studying root colonization by introduced beneficial bacteria. Agronomie 23, 407– 418

Glandorf, D. C. M., Brand, I., Bakker, P. A. H. M. (1992) Stability of rifampicin resistance as a marker for root colonization studies of *Pseudomonas putida* in field. Plant and Soil 147, 135–42

Glick, B. R. 1994. The enhancement of plant growth by free-living bacteria. Can. J. Microbiol. 41: 109-117

Greiner, R., Haller, E., Konietzny, U., Jany, K. D. (1997) Purification and characterization of a phytase from *Klebsiella terrigena*. Arch Biochem Biophys 341, 201-206

Greiner, R., Larsson, A. M. (2001) Stereospecificity of *myo*-inositol hexakisphosphate dephosphorylation by phytate-degrading enzymes of cereals. J Food Biochem 25, 229–248

Gulati, M. K. (1997) Influence of rhizobacteria on biological control of *Melidogyne incognita* in lettuce and tomato and on yield M.Sc. Thesis. Universität Bonn

Gupta, V. P., Bochow, H., Dolej, S., Fischer, I. (2000) Plant growth-promoting *Bacillus subtilis* strain as potential inducer of systemic resistance in tomato against *Fusarium* wilt. Journal of Plant Diseases and Protection 107, 2: 145–154

Hadar, Y., Harman, G. E., Taylor A. G., Norton J. M. (1983) Effects of pregermination of cucumber seeds and seed treatment with *Enterobacter cloacae* on roots by *Pythium* spp. Phytopathology 73: 1322–1325

Hammer, P. E., Hill, D. S., Lam, S. T., van Pée, K-H., Ligon, J. M. (1997) Four genes from *Pseudomonas fluorescens* that encode the biosynthesis of pyrrolnitrin. Appl. Environ. Microbiol. 63, 2147–2154

Haas, D., Défago, G., (2005) Biological control of soil-borne pathogens by fluorescent *Pseudomonads*. 01 Apr Review. Nature Reviews Microbiology 3, 307–319

Harman, G. E., Lumsden R. D. (1990) Biological disease control. *In* The Rhizosphere. Lynch J. M. (ed.). John Wiley and Sons, New York

Head, C. B., Ratnam, J. (1988) Comparision of API ZYM System with API AN-Ident, API 20A, Minitek Anaerobe II, and RapID-ANA Systems for Identification of *Clostridium difficile*. Journal of Clinical Microbiolgy, Jan. 144–146

Hemming, B. C. (1990) Bacteria as antagonists in biological control of soilborne plant pathogens. *In* New Directions in Biological Control. Baker, R. R. and Dunn, P. E. (eds.). Alan R. Liss, Inc.; New York. 223–242

Hessenmüller, A., Zeller, W. (1996) Biological control of soilborne *Phytophthora* species on strawberry with bacterial antagonists. Journal of Plant Diseases and Protection 103, 6: 602–609

Hiltner L. (1904) Über neuere erfahrungen und problem auf dem gebeit der bodenbakteriologie und unter besonderer berucksichtigung der grundungung und brache, Arb.Dtsch.Landwirt.Ges. 98, 59–78

Hoffmann, G. M., Nienhaus, F., Schönbeck, F., Weltzien, H. C., Wilbert H. (1985) Lehrbuch der Phytomedizin, Verlag Paul Parey

Hoffmann-Hergarten, S. (1994) Untersuchungen zum Einsatz von *Pseudomonas fluorescens* (Migula) gegen den Zystennematoden *Heterodera schachtii* (Schmidt) an Zucker- rueben unter Beruecksichtigung von Einflussfaktoren bei der Saatgutapplikation. Dissertation Universität Bonn

Hoffmann-Hergarten, S., Gulati, M. K., Sikora, R. A. (1998) Yield response and biological control of *Meloidogyne incognita* on lettuce and tomato with rhizobacteria. Journal of Plant Diseases and Protection 105, 4: 349–358

Howell, C. R., Stipanovic, R. D. (1980) Suppression of *Pythium ultimum* induced induced damping off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic pyoluterorin. Phytopathology 70, 712–715

Hrabak, E. M., Willis, D.K., (1992) The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. *syringae* on bean is a member of a family of two-component regulators. Journal of Bacteriology 174, 3011–3020

Hultberg, M., Alsanius, B., Sundin, P. (2000) *In vivo* and *in vitro* interactions between *Pseudomonas fluorescens* and *Pythium ultimum* in the suppression of damping-off in tomato seedlings. Biological Control 19: 1– 8

Huang, Z., Bonsall, R. F., Mavrodi, D. V., Weller, D. M., Thomashow, L. S. (2004) Transformation of *Pseudomonas fluorescens* with genes for biosynthesis of penazine-1- carboxylic acid improves biocontrol of rhizoctonia root rot and *in situ* antibiotic production. FEMS Microbiology Ecology 49, 243–251

Izard, D., Ferragut, C., Gavini, F., Kersters, K., DeLey, J., Leclerc, H, (1981) *Klebsiella terrigena*, a new species from soil and water. Int. J. Syst. Bacteriol. 31 116-127

Jacobson, C. B., Pasternack, J. J., Glick, B. R. (1994) Partial purification and characterization of 1-aminocyclopropane-1-carboxylate deaminase from the plant growth promoting rhizobacterium *Pseudomonas putida* GR 12-2. Canadian Journal of Microbiology 40: 1019–1025

Jansson, J. K., Molin, S., Romantschuk, M., (2000) Methods for monitoring genetically modified microorganisms (GMMs) in nature. NT Tech Report 455

Jansson, J. K., (1995) Tracking genetically engineered microorganisms in nature. Curr. Opin. Biotechnol. 6, 275–283

Johnsen, K., Enger, O., Jacobsen, C.S., Thirup, L., Torsvik, V. (1999) Quantitative selective PCR of 16S ribosomal DNA correlates well with selective agar plating in describing population dynamics of indigenous *Pseudomonas* spp. in soil hot spot, Appl. Environ. Microbiol. 65, 1786–1789

Katz, E., Demain, A. L. (1977) The peptide antibiotics of *Bacillus*: chemistry, biogensis, and possible functions. Bacteriological Reviews 41: 449– 474

Keel, C., Défago, G., (1997) Interactions between beneficial soil bacteria and root pathogens: mechanisms and ecological impact. In: GangeAC, Brown VK, eds. Multitrophic interactions in terrestrial system. Oxford: Blackwell Science, 27–47

Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, U., Wirthner, P., Haas, D., Défago, G. (1992) Suppression of root diseases by *Pseudomonas fluorescens* CHA0: Importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. Molecular Plant-Microbe Interactions. 5: 4–13

Keel, C., Voisard, C., Berling, C. H., Kahr, G., Défago, G. (1989) Iron sufficiency, a prerequisite for the suppression of tobacco black root rot by *Pseudomonas fluorescens* strain CHA0 under gnotobiotic conditions. Phytopathology 79:584–589

Keel, C., Weller, D. M., Natsch, A., Défago, G. Cook, R. J., Thomashow, L. S. (1996) Conversation of 2,4-diacetophloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. Appl. Environ. Microbiol. 62: 552–563

Kempf, H. J. (1988) Biologische Bekämpfung von pflanzenpathogenen Pilzen, insbesondere *Fusarium culmorum*, durch *Erwinia herbicola* und andere mikrobielle Antagonisten. Dissertation, Universität Göttingen

Kempf, H. J., Bauer, P. H., Schroth, M. N. (1993) Herbicolin A associated with crown and roots of wheat after seed treatment with *Erwinia herbicola* B267. Phytopathology 83, 213–216

Kerovuo, J., Lauraeus, M., Nurminen, P., Kalkkinen, N., Apajalahti, J. (1998) Isolation, characterization, molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis*. Appl. Environ. Microbiol. 64, 2079–2085

Keuken, O. (1996) Interaktion zwischen dem rhizosphärebacterium *Bacillus cereus*, dem Wurzelgallennematoden *Meloidogyne incognita* und Tomate. Dissertation Universität Bonn.

Kim, B. S., Moon, S. S., Hwang, B. K. (1999) Isolation, identification and antifungal activity of a macrolide antibiotic, oligomycin A, produced by *Streptomyces libani*. Canadian Journal of Botany 77, 850–858

Kim, D. S., Bonsall, R. F., Thomashow, L. S., Weller, D. M. (1995) Construction of transgenic *Pesudomonas fluorescens* Q-69c-80 for improved biocontrol activity to take all. *In* APS Annual Meetings. Aug 12-16, 1995 Pittsburg, Pennsylvania. USA

Kim, Y. O., Lee, J. K., Kim, H. K., Yu, J.H., Oh, T.K. (1998) Cloning of the thermostable phytase (Phy) from *Bacillus* sp. DS11 and its overexpression in *Escherichia coli*. FEMS Microbiol Lett 162, 185–191

Kloepper, J., Tuzun, S., Ku, J. (1992) Proposed definitions related to induced disease resistance. Biocontrol Science and Technology 2, 347–349

Kloepper, J. W., Beauchamp, C. J. (1992) A review of issues related to measuring colonization of plant roots by bacteria. Canadian Journal of Microbiology 38, 1219–1232

Kloepper, J. W. (1991) Development of *in vivo* assays for prescreening antagonists of *Rhizoctonia solani* on cotton. Phytopathology 81: 1006–1013

Kloepper, J. W., Leong, J., Teintze, M., Schroth, M. N. (1980) Enhancing plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286, 885–886

Kloepper, J. W., Ryu, C.-M., Zhang, S. (2004) Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology 94, 1259–1266

Kluepfel, D. A. (1993) The behaviour and tracking of bacteria in the rhizosphere. Annual Review of Phytopathology 31: 441– 472

Koch, E. (1997) Screening of rhizobacteria for antagonistic activity against *Pythium ultimum* on cucumber and kale. Journal of Plant Diseases and Protection 104. 4: 353–356

Koch, E. (1999) Evaluation of commercial products for microbial control of soilborne plant diseases. Crop Protection 18: 119–125

Koch. E., Kempf, H. J., Hessenmüller, A. (1998) Characterization of the biocontrol activity and evaluation of potential growth-promoting properties of selected rhizobacteria. Journal of Plant Diseases and Protection 105, 6: 567–580

Kraus, J., Loper J. E. (1995) Characterization of a genomic region required for production of the antibiotic pyoluteorin by the biological control agent *Pseudomonas fluorescens* Pf-5. Appl. Environ. Microbiol. 61, 849–854

Kugler, M., Loeffler, W., Rapp, C., Kern, A., Jung, G. (1990) Rhizoctin A, an antifungal phosphono-oligopeptide of *Bacillus subtilis* ATCC 6633: Biological Properties. Arch. Microbiology 153: 276–281

Kurze, S., Schroeder, M., Berg, G., Bahl, H. (1998) Evaluierung verschiedener Isolate von *Serratia plymuthica* als Biological Control Agent. *In* Mitteilungen aus der Biologischen Bundesanstalt fuer Land- und Forstwirtschaft, Berlin-Dahlem. 51.Deutsche Pflanzenschutztagung ISBN 3-8263-3241-5 No. 357. 360

Lambert, B., Leyns, F., van Rooyne, F., Gossele, F., Papon, Y., Swings, J. (1987) Rhizobacteria of maize and their antifungal activities. Appl. Environ. Microbiol. 53, 1866–1871

Landa, B. B., Mavrodi, D. M., Thomshow, L. S., Weller, D. M**.** (2003) Interactions between strains of 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens* in the rhizosphere of wheat. Phytopathology 93: 982–994

Latour X., Philippot L., Corberand T., Lemanceau P. (1999) The establishment of an introduced community of fluorescent *Pseudomonads* is affected both by the soil-type and the rhizosphere. FEMS Microbiology Ecology 30, 163–170

Laville, J., Blumer, C., von Schroetter, C., Gaia, V., Défago, G., Keel, C., and Haas, D. (1998) Characterisation of the hcnABC gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHA0. Journal of Bacteriology. 180: 3187–3196

Laville, J., Voisard, C., Keel, C., Maurhofer, M., Défago, G., Haas, D. (1992) Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. Proc. Natl Acad. Sci. USA 89, 1562–1566

Lederer, W. (1990) Untersuchungen zur Prädisposition der Erdbeerpflanze für die Rhizomfäule (*Phytophthora cactorum* (Leb. et Cohn (Schroet.) und zum Antagonismus von *Trichoderma* Pers. gegenüber dem Erreger. Dissertation Univ. Heidelberg

Lederer, W., Seemüller, E. (1992) Untersuchungen zur Prädisposition der Erdbeere für die Rhizomfäule (*Phytophthora cactorum*). Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz. 99: 225–233

Leeman, M., den Ouden, F. M., van Pelt, J. A., Cornelissen, C., Matamala-Garros, A., Bakker, P. A. H. M., Schippers, B. (1996*a*) Suppression of *Fusarium* wilt of radish by co-inoculation of fluorescent *Pseudomonas* spp. and root-colonizing fungi. European Journal of Plant Pathology 102, 21–31

Leeman, M., den Ouden, F. M., van Pelt, J. A., Dirkx, F. P. M., Steijl, H., Bakker P. A. H. M, Schippers B. (1996*b*). Iron availability affects induction of systemic resistance to *Fusarium* wilt of radish by *Pseudomonas fluorescens*. Phytopathology 86, 149–155

Leeman, M., van Pelt, J. A., den Ouden, F. M., Heinsbroek, M., Bakker, P. A. H. M., Schippers, B. (1995). Induction of systemic resistance against *Fusarium* wilt of radish by lipopolysaccharides of *Pseudomonas fluorescens*. Phytopathology 85, 1021–1027

Lemanceau, P., Bakker, P. A. H. M., De Kogel, W. J., Alabouvette, C., Schippers, B. (1992) Effect of pseudobactin 358 produced by *Pseudomonas putida* WCS358 on suppression of fusarium wilt of carnations by non pathogenic *Fusarium oxysporum* Fo47. Appl. Environ. Microbiol. 58, 2978–2982.

Lemanceau, P., Alabouvette, C. (1993) Suppression of *Fusarium*-wilt by fluorescent pseudomonads: mechanisms and applications. Biocontrol Science and Technology. 3: 219–232

Ligon, J. M., Hill, D. S., Hammer, P. E., Torkewitz, N. R., Hofmann, D., Kempf, H. J., Pée, K. H. (2000) Natural products with antifungal activity from Pseudomonas biocontrol bacteria. Pest Management Science 56, 8. 688-695

Lim, H.-S., Kim, Y.-S., Kim, S. D. (1991) *Pseudomonas stutzeri* YPL-1 genetic transformation and antifungal mechanism against *Fusarium solani*, an agent of plant root rot. Appl. Environ. Microbiol. 57: 510–516

Lin, M., Smalla, K., Heuer, H., Van Elsas, J. D. (2000) Effect of an *Alcaligenes faecalis* inoculant strain on bacterial communities in flooded soil microcosms planted with rice seedlings. Applied Soil Ecology 15 (2): 211–225

Liu, S. D. (1993) The screening of antagonistic microorganisms against gray mold disease of strawberry and their biocontrol effect. Plant Protection Bulletin Taipei. Vol. 35. 2: 105–115

Loper, J. E., M. D. Henkels. (1997) Availability of iron to *Pseudomonas fluorescens* in rhizosphere and bulk soil evaluated with an ice nucleation reporter gene. Appl. Environ. Microbiol. 63: 99–105

Loper, J. P., Schroth, M. N. (1986) Influence of bacterial sources of indole-3-acetic acid on root elongation of sugar beet. Phytopathology 76: 386–389

Lottmann, J., Heuer, H., Smalla, K., Berg, G. (1998) Antagonistic bacteria of potato as a basis for biological control of soilborne pathogens. 7th International Congress of Plant Pathology Edinburgh, Schottland 9-16 August 1998. Vol. 3, 6:144

Lubczynka, J., Pietr, S. J., Dordzik, M., Bogo, K. (1997) Possibilities of gibberellin secretion by rhizosphere bacteria from the genus *Bacillus* and *Pseudomonas*. *In* Microorganisms in the environment: Appearance, activity and importance. Barabasz, W. (ed.) Krzeszow, 375–381

Luckner, M. (1990). Secondary products derived from amino acids. Pages 246-249 in: Secondary Metabolism in Microorganisms, Plants, and Animals. Springer-Verlag, Berlin, Germany

Lynch, J. M., Harman, G. E., Lumsden, R. D. (1990) Biological disease control. The rhizosphere. 259-280

MacCrady, M. H. (1915) The numerical interpretation of fermentation tube results, J. Infect. Dis. 17, 183–212

Mahaffee, W. F., Bauske, E. M., Van Vuurde, J. W. L., Van der Wolf, J. M., Van der Brink, M., Kloepper, J.W. (1997) Comparative analysis of antibiotic resistance, immunofluorescent colony staining, and a transgenic marker (bioluminescence) for monitoring the environmental fate of rhizobacterium, Appl. Environ. Microbiol. 63, 1617–1622

Mahaffee, W. F., Backman P. A. (1993) Effects of seed factors on spermosphere and rhizosphere colonization of cotton by *Bacillus subtilis* GB03. Phytopathology 83: 1120–1125

Mansour, M. S., Farag, M. M. (1999) Effect of volatile(s) produced by *Bacillus subtilis* (Cohen) on the common bean (*Phaseolus vulgaris* L.) wilt fungus. Egyptian Journal of Phytopathology 27: 47 – 64

Maurhofer, M., Keel, C., Haas, D., Défago, G. (1994) Pyoluteorin production by *Pseudomonas fluorescens* strain CHA0 is involved in the suppression of *Pythium* damping-off of cress but not of cucumber. European Journal of Plant Pathology 100. 3-4: 221–232

Mavingui, P., Laguerre, G., Berge, E., Heulin, T. (1992) Genetic and phenotypic diversity of *Bacillus polymyxa* in soil and in the wheat rhizosphere, Appl. Environ. Microbiol. 58, 1894–1903.

Merriman, P. R. (1974) Effects of seed inoculation with *Bacillus subtilis* and *Streptomyces griseus* on the growth of cereals and carrots. Australian Journal of Agriculture Research 25: 215–219

Merriman, P. R., Birkenhead, W. E. (1977) Effect of *Bacillus subtilis* and *Streptomyces*

griseus on the growth of vegetables. Australian Plant Pathological Newsletter, 6/24

Merriman, P. R., Russel K. (1990) Screening strategies for biological control. *In* Biological control of soilborne plant pathogens. Hornby, D. (ed.). CAB International, Wallingford UK. 427– 435

Métraux, J-P, Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K, Schmid, E., Blum, W., Inverardi, B. (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science 250, 1004–1006

Milner JL, Silo-Suh L, Lee JC, He H, Clardy J, Handelsman J. (1996) Production of kanosamine by *Bacillus cereus* UW85. Appl. Environ. Microbiol. 62, 3061–3065

Mitchell R, Hurwitz E. (1965) Suppression of *Pythium debaryanum* by lytic rhizosphere bacteria. Phytopathology 55, 156–158

Mithustin, E. N., Naumova, A. N. (1962) Bacterial fertilizers, their effectiveness and mode of action. Mikrobiologia 31: 543–545

Molot., Nourrisseau. (1966) Dessechement printainer du fraisier provoque par *Phytophthora cactorum*. Compte Rendu Hebdomadaire des Seances de l' Acadamie d' Agriculture de France 52. *In* Oomycetes. European Handbook of Plant Diseases. Smith, I. M.; Dunez, J.; Lelliott, R. A.; Phillips, D. H. and Archer, S. A. (eds.) 8: Blackwell Scientific Publications Ltd. 199–239

Montgomerie, I. G. (1967) Pathogenicity of British isolates of *Phytophthora fragariae* and their relationship with American and Canadian races. Transactions of the British Mycological Society 50: 57–67

Montgomerie, I. G. (1977) Red core disease of strawberry. Horticulture Review 5. Commonwealth Bureau of Horticulture and Plantation Crops, East Malling Research Station, Nr. Maidstone, Kent, U.K. 47

M'Piga, P., Bélanger, R. R., Paulitz, T. C., Benhamou,. N. (1997) Increased resistance to *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato plants treated with the endophytic bacterium *Pseudomonas fluorescens* strain 63–28. Physiological and Molecular Plant Pathology 50, 301–320

Nakayama, T., Homma, Y., Hashidoko, Y., Mizutani, J., Tahara, S. (1999) Possible role of xanthobaccins produced by *Stenotrophomonas* sp. strain SB-K88 in suppression of sugar beet damping-off disease. Appl. Environ. Microbiol. 65, 4334–4339

Nautiyal, C. S., Bhadauria, S., Kumar, P., Lal, H., Mondal, R., Verma, D. (2000) Stress induced phosphate solubilization in bacteria isolated from alkaline soils. FEMS Microbiol Lett 182, 291–296

Nelson, E. B., Chao, W-L., Norton, J. M., Nash, G. T., Harman, G. E., (1986) Attachment of *Enterobacter cloacae* to hyphae of *Pythium ultimum*: possible role in biological control of *Pythium* pre-emergence damping-off. Phytopathology 76, 327–335

Nickerson, N. L. (1998) Red stele root rot. *In* Compendium of Strawberry Diseases, Second Edition. Maas, J. L. (ed.). APS Press 48–50

Nielsen, M. N., Sørensen, J., Fels, J., Pedersen, H. C. (1998) Secondary metabolite- and endochitinase-dependent antagonism toward plant-pathogenic microfungi of *Pseudomonas fluorescens* isolates from sugar beet rhizosphere. Appl. Environ. Microbiol. 64, 3563–3569

Nienhaus, F. (1960) Das Wirtsspektrum von *Phytophthora cactorum* (Leb. et Cohn) Schroet. Phytopathology Zeitung 38: 33–68

Nieto, K. F., Frankenberger, W. T. Jr. (1990) Microbial production of cytokinins in Soil Biochemistry. Bollag, J. M. and Stotzky E. (eds.). Drekker, M. INC. New York and Basel

O' Sullivan, D. J., O'Gara, F. (1992) Traits of fluorescent *Pseudomonas* spp. Involved in suppression of plant root pathogens. Microbiology Review 56: 662–676

O' Sullivan, D. J., O' Gara, F. (1991) Genetic improvement of siderophore production aimed at enhancing biocontrol in *Pseudomonas* strains. In: DL Keister, Cregam, eds. The Rhizosphere and Plant Growth. Beltsville Symposia in Agricultural Research. PB No. 14

Ohno, A., Ano, T., Shoda, M. (1992) Production of antifungal antibiotic iturin in a solid state fermentation by *Bacillus subtilis* NB 22 using wheat bran as a substrate. Biotechnology Letters 14, 9: 817–822

Ongena, M., Daayf, F., Jacques, P., Thonart, P., Benhamou, N., Paulitz, T. C., Cornelis, P., Koedam, N., Belanger, R. R. (1999) Protection of cucumber against *Pythium* root rot by fluorescent pseudomonads: predominant role of induced resistance over siderophores and antibiosis. Plant Pathology 48, 66–76

Orvos, D. R., Lacy, G. H., Cairns, J. Jr. (1990) Genetically engineered *Erwinia carotovora*: survival, intraspecific competition and effects upon selected bacterial genera. Appl. Environ. Microbiol. 56, 1689–1694

Parke, J. L., Moen, R., Rovira, A. D., Bowen G. D. (1986) Soil water flow affects the rhizosphere distribution of a seed borne biological control agent *Pseudomonas fluorescens*. Soil Biology and Biochemistry 18: 583–588

Parke, J. L. (1991) Root colonization by indigenous and introduced microorganisms.-*In* The rhizosphere and plant growth Keister, D. L., Cregan P. B. (eds.). Kulwer Acacdemic Publishers. 33–42

Pfaffl, M.W., Horgan, G. W., Dempfle, L. (2002) Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. May 1; 30(9): e36

Picard, C., Ponsonnet, C., Paget, E., Nesme, X., Simonet, P. (1992) Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction, Appl. Environ. Microbiol. 58, 2717–2722

Picard, C., Cello, F. D., Ventura, M., Fani, R. Guckert, A. (2000) Frequency and biodiversity of 2,4-Diacetylphloroglucinol producing bacteria isolated from the maize rhizosphere at different stages of plant growth Appl. Environ. Microbiol. 66. 3: 948-955

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

Powell, J. F., Vargas, J. M., Nair, M. G., Detweiler, A. R., Chandra, A. (2000). Management of dollar spot on creeping bentgrass with metabolites of *Pseudomonas aureofaciens* (TX-1). Plant Disease 84, 19–24

Prosser, J. I. (1994) Molecular marker systems for detection of genetically engineered microorganisms in the environment. Microbiology140, 5–17

Pusey, P. L. (1990) Control of pathogens on aerial plant surfaces with antagonistic microorganisms. Special Report, Biological and Cultural Tests. 5: 5–7

Raaijmakers, J. M., Bonsall, R. F., Weller, D. M. (1999) Effect of population density of *Pseudomonas fluorescens* on production of 2,4-diacetylphloroglucinol in the rhizosphere of wheat. Phytopathology 89, 470–475

Raaijmakers, J. M., Weller, D. M., Thomashow, L. S. (1997) Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. Appl. Environ. Microbiol. 63, 881–887

Raaijmakers, J. M., Leeman, M., van Oorschot, M. M. P., van der Sluis, I., Schippers, B., Bakker P. A. H. M. (1995) Dose-response relationship in biological control of *Fusarium* Wilt of radish by *Pseudomonas* spp. Phytopathology 85. 10: 1075–1081

Raaijmakers, J. M., Weller D. M., Thomashow L. S. (1997) Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. Appl. Environ. Microbiol. 63: 881-887

Reddy, M. S., Rahe, J. E. (1989) Growth effects associated with seed bacterization not correlated with populations of *Bacillus subtilis* inoculant in onion seedling rhizosphere. Soil Biology Biochemistry 21: 373–378

Reddy, N. R., Pierson, M. D., Sathe, S. K., Salunkhe, D. K. (1989) Phytases in Cereals and Legumes. Boca Raton, FL: CRC Press

Remus, R., Ruppel S., Jacob H. J., Hecht-Bucholz, C., Merbach, W. (2000) Colonization behavior of two enterobacterial strains on cereals. Biol. Fertil. Soils 30, 550–557

Renwick, A., Campbell, R., Coe, S. (1991) Assessment of *in vivo* screening systems for potential biocontrol agents of *Gaeumannomyces* graminis. Plant Pathology 40, 524–532

Ribeiro., Olaf, K. (1978) A source Book of the Genus *Phytophthora*

Rijbroek, P. C. L., Meulenbroek, E. J., Lindeloof, C. P. J., Lindeloof, C. P. J., Scheer, H., Lieten, F., Dijkstra, J. (1997) Development of a screening method for resistance to *Phytophthora cactorum*. *In* Proceedings of the third international strawberry symposium, Veldhoven, Netherlands, 29 April-4 May 1996. Volume I. Acta-Horticulturae. 439: 181-183

Richardson, A. E., Hadobas, P. A. (1997) Soil isolates of *Pseudomonas* spp. that utilize inositol phosphates. Canadian Journal of Microbiology 43, 509-516

Rosado, A. S., Seldin, L., Wolters, A.C., Van Elsas, J.D. (1996) Quantitative 16S rDNA-targeted polymerase chain reaction and oligonucleotide hybridization for the detection of *Paenibacillus azotofixans* in soil and the wheat rhizosphere. FEMS Microbiology Ecology 19, 153–164

Rozycki, H., Dahm, H., Strzelczyk, E., Li, C. Y. (1999) Diazotrophic bacteria in root-free soil and in the root zone of pine (*Pinus sylvestris* L.) and oak (*Quercus robur* L.). Appl Soil Ecol 5, 29–56

Sadlers, H. M. (1996) Wirkung eines selektierten Rhizosphärenbakterienstammes auf das Wachstum und die Pflanzengesundheit von Tomate. Dissertation Universität Bonn

Sacherer, P., Défago, G., Haas, D. (1994) Extracellular protease and phospholipase C are controlled by the global regulatory gene *gacA* in the biocontrol strain *Pseudomonas fluorescens* CHA0. FEMS Microbiology Letters 116, 155–160

Sanger, F., Nicklen, S., Coulson, A. R. (1977) DNA Sequencing with chain-terminating inhibitors. Proc. Natl Acad. Sci. USA 74, 5463–5467

Sauer, H., Zeller, W. (1992) Biological control of collar rot on apple (*Phytophthora cactorum* (Leb. et Cohn) Schroet.) by bacteria of soil and bark. Acta Phytopathologica et Entomologica Hungarica. Vol. 27 1-4 part II, 571–575

Scheer van der, H. A. T. (1971) Isolation of *Phytophthora cactorum* from soil in orchards and strawberry fields and differences in pathogenicity to apple. Netherlands lands Journal of Plant Pathology. 77: 56-72

Schippers, B. A., Bakker, A. W., Bakker, P. A. H. M., van Peer, R. (1991) Beneficial and deleterious effects of HCN-producing *Pseudomonas* on rhizosphere interactions. *In* The rhizosphere and plant growth. Kleister, D. L. and Cregan, P. B. (eds.). Kluwer Academic Publishers, The Netherlands, 211–219

Schisler, D. A., Slininger, P. J., Bothast, R. J. (1997) Effect of antagonist cell concentration and two-strain mixtures on biological control of *Fusarium* dry rot of potatoes. Phytopathology 87: 177–183

Schisler, D. A., Slininger, P. J., Behle, R. W., Jackson, M. A. (2004) Formulation of *Bacillus* spp. for biological control of plant diseases. Phytopathology 94, 1267–1271

Schmidle, A. (1961) Über eine Rhizom- und Wurzelfäule der Erdbeere. Bad. Obst. Gartenbau 19: 401– 418

Schmiedeknecht, G., Bochow, H., Junge, H. (1998) Use of *Bacillus subtilis* as biocontrol agent. II. Biological control of potato diseases. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz. 105. 4: 376–386

Schroth, M. N., Becker, J. O. (1990) Concepts of ecological and physiological activities of rhizobacteria related to biological control and plant growth promotion. *In* Biological control of soilborne plant pathogens. Hornby, D.(ed.). CAB International, 389–414

SCRI-Dundee Archives, Scottish Crop Research Institute, www.forestryimages.org

Seemüller, E. (1998) Crown rot. *In* Compendium of Strawberry Diseases, Second Edition. Maas, J. L. (ed.). APS Press. 50–51

Seemüller, E., Schmidle A. (1979) Einfluss der Herkunft von *Phytophthora cactorum*-Isolates auf ihre Virulenz an Apfelrinde, erdbeerrhizomen und Erdbeerfrüchten Phytopathologische Zeitschrift 94: 218-225

Seemüller, E., Sun, C. (1989) Auftreten von Metalaxyl-Resistenz bei *Phytophthora fragariae*. Nachrichtenblatt Deutsche Pflanzenschutzdient. 41: 71–73

Sikora, R. A (1992) Management of the antagonistic potential in agricultural ecosystems for the biological control of plant parasitic nematodes. Annual Review of Phytopathology. 30: 245–270

Sikora, R. A (1997) Biological system management in the rhizosphere: an inside-out/outside-in perspective. *In* International Symposium on Crop Protection. Gent (Belgium). Mededelingen-Faculteit-Landbouwkundige-en-Toegepaste-Biologische-Wetenschapp-en-Universiteit-Gent (Belgium). Vol. 62(2a) NT: 4 ill. 105–112

Sikora, R. A. (1988) Interrelationship between plant health promoting rhizobacteria, plant parasitic nematodes and soil microorganisms. Med. Fac. Landbauww. Rijksuniv.

Gent 53/2b: 867–878

Sikora, R. A., Bodenstein, F., Nikolay, R. (1990) Einfluß der Behandlung von Rübensaatgut mit Rhizosphärebakterien auf den Befall durch Pilze der Gattung *Pythium*. I. Antagonistische Wirkung verschiedener Bakterienisolate gegenüber *Pythium* spp. Journal of Phytopathology 129: 111-120

Simon, R., Priefer, U., Pühler, A. (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. Biotechnology 1: 784–791

Smith, I. M., Dunez, J., Lelliott, R. A., Phillips, D. H., Archer, S. A. (1988) Oomycetes *In* European Handbook of Plant Diseases. Blackwell Scientific Publications LTD, 199–239

Stanghellini, M. E., Miller, R. M. (1997) Biosurfactants: their identity and potential efficacy in the biological control of zoosproic plant pathogens. Plant Diseases 81: 4–12

Steijl, H., Niemann, G. J., Boon, J. J (1999) Changes in chemical composition related to fungal infection and induced resistance in carnation and radish investigated by pyrolysis mass spectrometry. Physiological and Molecular Plant Pathology 55, 297–311

Sticher, L., Mauch-Mani, B., Métraux, J. P. (1997) Systemic acquired resistance. Annual Review of Phytopathology 35, 235–270

Suarez, A., Guttler, A., Stratz, M., Staendner, L. H., Timmis, K. N., Guzman, C. A. (1997) Green fluorescent protein based reporter systems for genetic analysis of bacteria including monocopy applications. Gene 196, 69–74

Suslow, T. V. (1982) Role of root-colonizing bacteria in plant growth. Phytopathogenic Prokaryotes. Mount, S. and Lazy, J. H. (eds.). New York Academic Press Inc. 187–223

Suslow, T. V., Schroth M. N. (1982) Rhizobacteria of sugarbeets: Effects of seed application and root colonization on yield. Phytopathology 72: 199–206

Swadling, I. R., Jeffries, P. (1998) Antagonistic properties of two bacterial biocontrol agents of grey mould disease. Biocontrol Science and Technology 8: 3, 22. 439 – 448

Teintze, M., Hosssain, M. B., Baines, C. L., Leong, J., Van der Helm, D. (1981) Structure of ferricpseudobactin, a siderophore from a plant growth promoting *Pseudomonas*. Biochemistry 20: 6446–6457

Thirup, L., Johnesn K., Winding A. (2001) Succession of indigenous *Pseudomonas* spp. and Actinomycetes on barley roots affected by the antagonistic strain *Pseudomonas fluorescens* DR54 and the fungicide imazalil. Appl. Environ. Microbiol. 67, 1147–1153

Thomashow, L. S., Weller D. M., (1988) The rhizosphere and plant growth *In* Beltsville Symposia in Agricultural Research. Keister, D. L. and Cregan, P. B. (eds.)14: 245-251

Thomashow, L. S., Weller D. M., Bonsall, R. F., Pierson III, L. S. P. (1990) Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. Appl. Environ. Microbiol. 56, 908–912

Thomashow, L. S., Weller, D. M. (1996) Current concepts in the use of introduced bacteria for biological disease control: mechanisms and antifungal metabolites. In: Plant-microbe interactions, vol 1. Chapman and Hall, N. Y. Stancey, G. and Keen, N. (ed.). 187–235

Thomashow, L. S., Weller, D. M. (1998) Role of phenazin antibiotics from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. Journal of Bacteriology 170: 3499–3508

Thrane, C., Olsson, S., Nielsen, T. H., Sörensen, J., (1999) Vital fluorescent stains for detection of stress in *Pythium ultimum* and *Rhizoctonia solani* challenged with viscosinamide from *Pseudomonas fluorescens* DR54. FEMS Microbiology Ecology 30, 11–23

Timmusk, S., Wagner, E. G. H. (1999) The plant-growth-promoting rhizobacterium *Paenibacillus polymyxa* induces changes in *Arabidopsis thaliana* gene expression: a possible connection between biotic and abiotic stress responses. Molecular Plant-Microbe Interactions 12, 951–959

Timonin, M. I. (1947) Microflora of the rhizosphere in relation to manganese-deficiency disease of oats. Soil Sci. Soc. Am. Proc. 11, 284-292

Tombolini, R., Unge, A., Davey, M. E., de Bruijn, F. J., Jansson J. K. (1997) Flow cytometric and microscopic analysis of GFP-tagged *Pseudomonas fluorescens* bacteria. FEMS Microbiology Ecology 22, 17– 28

Tronsmo, A. (1976) Experiments with biological control of grey mould on strawberries. (Forsoek pa biologisk kontroll av grasskimmel pa jordbaer). Gartneryrket., Vol. 66: 20: 414

Tsien R.Y. (1998) The green fluorescent protein. Ann. Rev. Biochem. 67, 509–544

Turner, J. T., Backman, P. A. (1991) Factors relating to peanut yield increase after seed treatment with *Bacillus subtilis*. Plant Diseases 75: 347-353

Tu, J. C., Zheng, J. M. (1996) Effect of *Bacillus subtilis* and *Pseudomonas fluorescens* on plant growth and *Verticillium* wilt development of tomatoes. *In* 48th International Symposium on Crop Protection. Gent (Belgium). Mededelingen Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen Universiteit Gent (Belgium). Vol. 61 (2b). 475–481

Utkhede, R. S., Smith, E. M. (1992) Promotion of apple tree growth and fruit production by the EBW-4 strain of *Bacillus subtilis* in apple replant disease soil. Canadian Journal of Microbiology 38: 1270-1273

Valois, D., Fayad, K., Barbasubiye, T., Garon, M., Déry, C., Brzezinski, R., Beaulieu, C. (1996) Glucanolytic actinomycetes antagonistic to *Phytophthora fragariae* var. *rubi*, the causal agent of raspberry root rot. Appl. Environ. Microbiol. 62, 1630–1635

Van der Hofstad, G. A. J. M., Marugg, J. D., Verjans, G. M., Weisbeek, P. J. (1986) Characterization and structural analysis of the siderophore produced by the PGPR *Pseudomonas putida* strain WCS 385. *In* Iron, siderophores and plant diseases. Plenum, N.Y. Swinburne, T. A. (ed.). 71–75

Van Elsas, J. D., Dijhstra, J. M., Govaert, J. M., Van Veen, J. A. (1986) Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into soil of different texture in field microplots. FEMS Microbiology Ecology 38, 151–160

Van Loon, L. C. (1997) Induced resistance in plants and the role of pathogenesis-related proteins. European Journal of Plant Pathology 103, 753–765

Van Overbeek, L.S., Van Veen, J.A., Van Elsas, J.D. (1997) Induced reporter gene activity, enhanced stress resistance and competitive ability of a genetically modified *Pseudomonas fluorescens* strain released into a field pot planted with wheat, Appl. Environ. Microbiol. 63, 1965–1973

van Peer, R., Niemann, G. J., Schippers, B. (1991) Induced resistance and phytoalexin accumulation in biological control of *Fusarium* wilt of carnation by *Pseudomonas* sp. WCS417. Phytopathology 81, 728–734

van Wees, S. C. M., Pieterse, C. M. J., Trijssenaar, A., Van't Westende, Y., Hartog, F., van Loon LC. (1997) Differential induction of systemic resistance in *Arabidopsis* by biocontrol bacteria. Molecular Plant-Microbe Interactions 10, 716–724

Vanattanakom, N., Loeffler, W., Koch, U., Jung, G. (1986) Fengycin-a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. Journal of Antibiotics 39: 888–901

Velahzazan, R., Samiyappan, R., Vidhyasekaran, P. (1999) Relationship between antagonistic activities of *Pseudomonas fluorescens* isolates against *Rhizoctonia solani* and their production of lytic enzymes. Journal of Plant Diseases and

Protection 106. 3: 244–250

Voisard, C., Keel, C., Haas, D., Défago, G. (1989) Cynamid production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. EMBO Journal 8: 351–358

Weller, D. M., Cook, R. J. (1983) Suppression of take-all of wheat by seed treatments with fluorescent *Pseudomonads*. Phytopathology 73: 463–469

Weller, D. M., Thomashow, L. S. (1993) Use of rhizobacteria for biocontrol. Current Opinion in Biotechnology 4: 306–311

Weller, D. M., Raaijmakers, J. M., McSpadden Gardener, B. B., Thomashow, L. S. (2002) Microbial populations responsible for specific suppressiveness to plant pathogens. Annual Review of Phytopathology 40: 309–348

Weller, D.M. (1988) Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annual Review of Phytopathology 26: 379–407

Werner, D., Batinic, T., Feder, I. S., Kosch, K., Redecker, D., Schulz, U., Streit, W., Fleischman, P.V. (1996) Competitiveness of symbiotic and antagonistic soil bacteria-Development of reporter gene fusions and specific gene probes, Proceedings of the Fourth International Workshop on Plant Growth Promoting Rhizobacteria, pp. 44–48

Werres, S. (1987) Untersuchungen zur Rhizomfäule (*Phytophthora cactorum* (Leb. et Cohn) Schroet.) und zur Roten Wurzelfäule (*Phytophthora fragariae* Hickman) bei Erdbeerpflanzen und zum serologischen Nachweis von P. *fragariae*. Dissertation Universität Hannover.

Whistler, C. A., Pierson III, L. S. (2003) Repression of phenazine antibiotic production in *Pseudomonas aureofaciens* strain 30-84 by RpeA. Journal of Bacteriology 185: 3718-3725

Whitelaw, M. A. (2000) Growth promotion of plants inoculated with phosphate-solubilizing fungi. Advances in Agronomy 69, 99–151

Wirth, S. J., Wolf, G. A. (1992). Micro-plate calorimetric assay for endo-acting cellulase, xylanase, chitinase, ß-1,3-glucanase and amylase extracted from forest soil horizons. Soil. Biol. Biochem. 24: 511–519

Yoon, S. J., Choi, Y. J., Min, H. K., Cho, K. K., Kim, J. W., Lee, S. C., Jung, Y. H. (1996). Isolation and identification of phytase-producing bacterium, *Enterobacter* sp. 4, and enzymatic properties of phytase enzyme. Enzyme Microb. Technol. 18, 449– 454

Zeller, W. (1999) Zum Stand des Einsatzes von möglichen Alternativen zum Plantomycin gegen den Feuerbrand *In* 2. Symposium Phytomedizin und Pflanzenschutz im Gartenbau. 27-30 Sept. 1999 Wein. 55–56

Publications

Anandhakumar, J., Zeller. W. (2004) Studies on efficacy and mode of action of rhizosphere bacteria against *Phytophthora* spp. in strawberry. IOBC/wprs Bulletin Vol.27 (8), 261-264

Anandhakumar, J., Zeller. W. (2005) Biological control of red core (*Phytophthora fragariae var. fragariae*) and crown rot (*P. cactorum*) disease of strawberry by bacterial antagonists. Phytopathology. Vol. 95, (6), S4, June(supplement)

Paper presentation

Anandhakumar, J., Gulati, M. K., Zeller, W: Biocontrol of *Phytophthora* diseases on strawberry with antagonistic bacteria- 4. Symposium Phytomedizin und Pflanzenschutz im Gartenbau. 22-25.09.2003, 105-106, ISSN 1728-9564 Vienna, Austria

Anandhakumar, J., Zeller, W: Field studies on the efficacy and mode of action of Rhizosphere bacteria against *Phytophthora* spp. in strawberry-54. Deutsche Pflanzenschutztagung: 20-23.09.2004, p-375, ISSN 0067-5849 Hamburg, Germany

Poster presentation

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Declaration

I hereby declare that the Ph.D. thesis entitled: **Studies on the antagonistic effect of rhizobacteria against soilborne *Phytophthora* species on strawberry,** submitted to the University of Hannover is an independent work carried out by me and the thesis has not formed previously the basis for the award of any degree.

Yours sincerely

(Mr. Anandhakumar Jayamani)