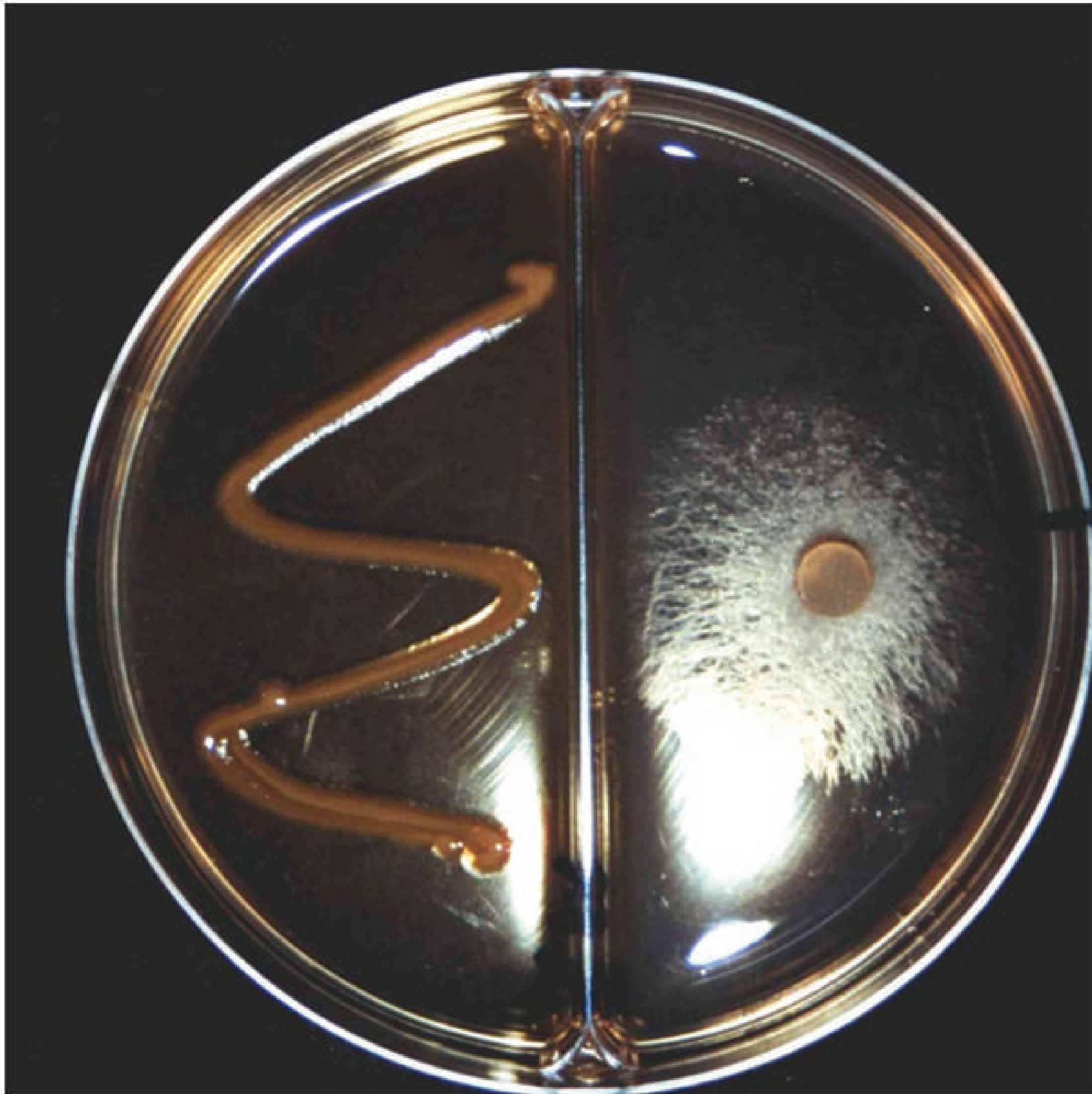
Suryo Wiyono

Optimisation of Biological Control of Damping-Off of Sugar Beet (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima* Doell) Caused by *Pythium ultimum* Trow by Using *Pseudomonas fluorescens* B5



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Optimisation of Biological Control of Damping-Off of Sugar Beet (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima* Doell) Caused by *Pythium ultimum* Trow by Using *Pseudomonas fluorescens* B5

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Suryo Wiyono

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Nonnenstieg 8, 37075 Göttingen
Telefon: 0551-54724-0
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LIST OF ABBREVIATIONS

AFM	Antifungal Metabolites
Am Mo	Ammonium Molybdate
ANOVA	Analysis of Variance
APE	*ad planta* efficacy
CA	Casamino Acids
CF	Culture Filtrate
cfu	colony forming unit
DMRT	Duncan Multiple Range Test
DRMO	deleterious rhizosphere microorganisms
DS	Disease Suppression
DT –LB	Doubling Time in media LB
DT-KB^+	Doubling Time in Media KB^+
g	gram
g	gravitation
h	hours
HCN	Hydrogen Cyanide
IAA	Indole- 3- Acetic Acid
IBA	Indole-3-Butyric Acid
IPA	Indole-3-Propionic Acid
NAA	Naphthalene Acetic Acid
GA	Gibberelic Acid
IZ-KBA^+	Inhibition Zone in KBA^+
IZ-TSA	Inhibition Zone in TSA
KB	King's B
KBA	King's B Agar
LB	Luria Berthani Broth
MEA	Maize Extract Agar
MEB	Maize Extract Broth
ml	mili liter
mM	mili Molar
MWR-CF KB	Mycelial Weight Reduction by KB+-Based Culture Filtrate
MWR-CF LB	Mycelial Weight Reduction by LB-Based Culture Filtrate
MYEA	Malt Yeast Extract Agar

ns	not significant
OD	Optical Density
P	Probability of Error
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
Pf B5	*Pseudomonas fluorescens* B5
P. ultimum	*Pythium ultimum*
Ps. fluorescens	*Pseudomonas fluorescens*
ppm	part per million
REL HCN	Relative HCN Production
REL IAA	Relative IAA Production
SEA	Soil Extract Agar
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
w/v	weight per volume
WT	Wild Type
g	gram
G	gravitation
rpm	rotation per minute
ppm	part per million
µg	micro gram
ml	mili liter
mm	mili meter
KB-CF	King's B based Culture Filtrate
LB-CF	LB-based culture filtrate
Pf B5 WT	*Pseudomonas fluorescens* B5 wild type
PGPR	plant growth promoting rhizobacteria
Str	Streptomycin-Resistant
Trp	Tryptone
V	Variety (es)
W1	adherence type 1
W2	adherence type 2

I. INTRODUCTION

Sugar beet is one of the most important sugar-producing crops in the world, with a contribution of 40 % on world sugar production. Germany, with 415.000 ha and an average productivity of 54.68 ton/ha during the last decades is ranking on fourth position in sugar beet production in the world, next to USA, China and France (Wirtschaftliche Vereinigung Zucker-Verein der Zuckerindustrie, 2003).

Problems of pests and diseases substantially are limiting factors in sugar beet production. In Germany, damping-off is one of the most important soil borne diseases caused by a complex of pathogens and mainly by *Pythium ultimum* and *Aphanomyces cochlioides.* It is an important sugar beet disease in Germany since 1880'es (Koble, 1987). Damping-off contributes in substantial amount to yield losses due to fungal diseases, *e.g.* 16 % and 16.5 % in USA and worldwide respectively (James, 1982). Until now, disease management is mainly based on chemical control by applying fungicides which are incorporated into pelleted seeds. Other control strategies, such as the use of resistant varieties and other agronomical practices, do not provide effective control. However, decreasing of public acceptance of the use of chemicals and their environmental risks makes it important and relevant to develop novel biological control strategies by using microbial antagonist.

One of effective bacterial antagonists against *P. ultimum* is *Pseudomonas fluorescens* B5 (Pf B5) which had been studied intensively in the Institute of Plant Pathology and Plant Protection Georg-August University Germany (Heupel 1992, Maass, 1996; Schulz *et al.*, 1994; Schulz and Wolf, 2002). The antagonist showed a biocontrol efficacy of 44% against damping-off in sugar beet seedlings in controlled environment under high inoculum pressure of pathogen, and has also been proven effective in field experiments (Heupel, 1992).

Some important features associated with the antagonistic activity of *Ps. fluorescens* B5 have been characterized. Heupel (1992) described that under *in vitro* conditions, besides the production of cyanide (HCN) and the

ferric iron binding siderophores, hydrolytic enzymes such as glucanases, xylanases and amylases were detected in the culture filtrate of B5. Further investigations with transposon (Tn 5) mutants had been done to identify the antagonistic mechanisms of Pf B5 (Schulz *et al.*, 1994; Schulz and Wolf, 2002). The study showed that siderophore production plays a minor role in biocontrol in the system *Pythium ultimum* - sugar beet seedlings – *Ps. fluorescens B5*. Moreover, no strong correlation between adhesion to roots, motility and colonisation in the rhizosphere was observed. Even though all examined traits of Pf B5 *i.e.* production of antibiotics and siderophores, adhesion, motility and colonization ability contributed to the antagonistic activity of Pf B5, it was apparent that antibiosis is one of the most important mechanism of its biocontrol performance (Schulz and Wolf, 2002).

Application of mutants that overproduce antifungal metabolites is one alternative to improve the antagonistic activity of an antagonistic strain (Walsh *et al.,* 2001). For instance, biocontrol performance of *Ps. fluorescens* CHAO against *P. ultimum* in cotton seedlings could be improved by applying antibiotics overproducing mutants (Maurhofer *et al.*, 1992; Maurhofer *et al.,* 1995). Moreover, Delany *et al*. (2001) could found a similar effect using *Ps. fluorescens* F113 against *Pythium ultimum* in sugar beet by altering the production of 2,4-diacetylphloroglucinol. The possibility to enhance the biocontrol activity of *Ps. fluorescens* B5 by applying its antibiotic overproducing mutants was studied in this research.

It is well known that some biocontrol strains of the pseudomonads produce regulators of plant growth such as indole acetic acid (IAA) (Nautiyal *et al.*, 1997; Zhao, 2001; Bano and Mussarat, 2003), and therefore could act as plant growth-promoting rhizobacteria (PGPR) (Xie *et al.,* 1996; Barazani and Friedman, 1999; Asghar *et al.*, 2002). Studies on IAA production with fluorescent pseudomonads are mostly related to its role as growth regulator, excluding studies on its fungicidal activity.

Ps. fluorescens B5 produces IAA in a considerable amount *in vitro* and preliminary research showed that IAA inhibits also the growth of *P. ultimum in*

vitro. Indeed, the required concentration of IAA to suppress the disease *ad planta*, and the relation of IAA production *in vitro* and biocontrol activity *ad planta* are still unknown. Therefor the role of IAA in biocontrol and growth promotion by *Ps. fluorescens* B5 was investigated in this study.

Host properties are components often neglected in the development of biological control. In some studies, host varieties had no effect on the activity of antagonistic pseudomonads (Hebar *et al.,* 1998), while Smith and Goodman (1999) could find an affect of host plant genotypes on biocontrol efficiency. Regarding the application of *Ps. fluorescens* B5 in the system sugar beet – *Pythium ultimum*, the role of sugar beet varieties on biocontrol is poorly understood. But this aspect was found to be essential to optimise biocontrol activity of *Ps. fluorescens* B5 and hence was integrated into the studies.

An important step to establish biocontrol of plant disease with microbes is the development of appropriate formulation techniques. It plays a crucial role to guarantee success of the biocontrol strategy in the field. There are two approaches for the optimisation of an antagonist formulation *i.e.* the screening for appropriate carrier materials like wood–flour (Vidyasekaran, 1997; Imam Ali *et al.* 2001, Krishnamurthy and Gnanamanickam, 1998b), and formulation additives (Schmidt *et al.*, 2001). Wood flour-based materials seem to be suited for the formulation of fluorescent pseudomonads as seed pellet (Heupel, 1992; Tilcher, 2002). Since fluorescent pseudomonads do not produce spores, the development of appropriate carrier materials, which protect cells from desiccation and sustain long-lasting survival and antagonistic activity of microorganisms, is essential. For that reason, the study to find suitable pelleting materials was enforced.

Moreover, a further technique to improve an antagonist formulation is the use selective additives. Ideal formulation additives should provide the advantages for disease control in antagonist-pathogen-host plant system. This means, additives should improve the biocontrol efficacy of pseudomonads without enhancing the growth and activity of pathogens and

should not have detrimental effects on host plants. Studies on formulation additives of fluorescent pseudomonads are very limited. Some foregoing research indicated the importance of formulation additives for the use of *Bacillus mycoides* against *Cercospora beticola* under green house and field conditions by adding 1 % 1,3-ß-glucan (Kiewnick and Jacobsen, 1996). Moreover, addition of glycine into the seed pellet could improve rhizosphere colonization of *Ps. fluorescens* B5, and calcium gluconate increased the efficacy of B5 against *P. ultimum* in sugar beet seedlings (Schulz and Wolf, 1998).

With the exception of Schulz and Wolf (1998), who incorporated the addities directly into the seed pellet, nutrients were applied as fertilizer or as soil applications in most of the studies to improve activity of fluorescent pseudomonads. For instance, soil applications of nitrogen fertilizers containing mixtures of NO_3^- and NH_4^+ enhanced the capacity of *Pseudomonas fluorescens* strains to promote plant growth and to inhibit *Fusarium* growth on rye (Kurek and Jaroszuk-Sciseł, 2003). Glucose and zinc applied into the soil were reported to enhance the biocontrol ability of *Pseudomonas fluorescens* CHAO and *Pseudomonas aeruginosa* IE-6S+, both *in vitro* and *ad planta* against *Macrophomina phaseolina* in soybean (Shaukat and Siddiqui, 2003). Moreover, Hamid *et al.* (2003) stated that soil application of ammonium molybdate mediated the enhancement of biocontrol efficacy of *Ps. fluorescens* CHAO against the root knot nematode *Meloidogyne javanica* in soybean. On the contrary, information on the use of nitrogen compounds and trace elements as formulation additives to improve biocontrol performance of fluorescent pseudomonads is very limited or even not available. Therefore, in the last part of this work the possibility of the use of nitrogenous compounds and trace elements as formulation additives was examined.

Based on the above reasons, a series of experiments was done to achieve the following objectives:

1. To optimise biological control activity of *Ps. fluorescens* B5 against *Pythium ultimum* in sugar beet by means of mutants overproducing antifungal metabolites.
2. To study the role of indole-3-acetic acid (IAA) in the biological control activity of *Ps. fluorescens B5* and its growth promoting effect on sugar beet seedlings.
3. To study the role of sugar beet varieties in antagonistic activity of *Ps. fluorescens* B5 against *P. ultimum*.
4. To optimise biological control activity of *Ps. fluorescens* B5 by improving formulation techniques through a) appropriate pelleting materials which support long term survival and antagonistic activity of B5 and, b) formulation additives from nitrogen sources and trace elements which enhance biocontrol activity of *Ps. fluorescens* B5 against *P. ultimum*.

2. MATERIALS AND METHODS

2.1. Location and Time

Experiments were done in the Institute of Plant Pathology and Plant Protection, Georg-August University Göttingen, Germany from August 2000 until July 2003.

2.2. Materials

2.2.1. Chemicals

All chemicals are purchased from Merck, Darmstadt, Germany except the following ones:

- IAA (indole-3-acetic acid), Sigma Aldrich Steinheim, Germany
- IBA (indole-3 butyric acid) Sigma Aldrich Steinheim, Germany
- IPA (indole-3-propionic acid) Sigma Aldrich Steinheim, Germany
- Streptomycin sulphate, Serva Heidelberg, Germany
- Kanamycin acetic sulphate, Serva, Heidelberg, Germany
- Tetracyclin hydrochloride, Fluka, Germany
- Casamino Acid, Becton Dickinson France, le Pont de Clax, France
- Yeast Extract, Becton Dickinson France, le Pont de Clax, France
- Tryptone, Scharlau, Barcelona, Spain
- Bacto Casitone Peptone (pancreatic digest of casein), Becton Dickinson, le Pont de Clax, France
- Glycerin, Carl Roth GmBH and Co, Karlsrühe, Germany
- Agar AG 002 (for microbiological purpose), Scharlau, Barcelona, Spain
- Proteose Peptone, Scharlau, Barcelona, Spain
- Tryptic Soy Broth (TSB), Scharlau, Barcelona, Spain
- Kieselgur (diatomaceous earth) , KWS, Einbeck, Germany
- Wood flour 1, KWS Einbeck, Germany
- Wood flour 2, KWS Einbeck, Germany
- Cotton flour, KWS Einbeck, Germany
- Peat, KWS Einbeck, Germany

- Potato Dextrose Broth, Scharlau, Barcelona, Spain
- Wheat Oil, Weizenkeimöl, Dr Ritter GmbH, Germany

2.2.2 Media

Soil Extract Agar (SEA)

660 g sieved field soil were autoclaved in 100 ml tap water and filtered up to clear after cooling.

Soils extract	500 ml
Agar	8 g
aqua bidest	up to 1000 ml

pH adjusted to 7.0

King's Medium B (KB)

Proteose Peptone	20 g
$K_2HPO_4 \cdot 3\ H_2O$	2g
$MgSO_4 \cdot 7\ H_2O$	1.5 g
Glycerin	10 g
Agar	16 g
Aqua bidest	up to 1000 ml

pH adjusted to 7.0

King B^+ Agar (amended with Fe^{3+})

Proteose Peptone	20 g
$K_2HPO_4 \cdot 3\ H_2O$	2g
$MgSO_4 \cdot 7\ H_2O$	1.5 g
Glycerin	10 g
Agar	16 g
Aqua bidest	up to 1000 ml

$FeCl_3$ is amended in a final concentration of 200 nM

pH adjusted to 7.0

Phosphate Buffer

25 mM Na_2HPO_4	3.45 g
11 mM KH_2PO_4	1.49 g
Aqua bidest	up to 1000 ml
pH adjusted to 7.0	

Kings B Agar$^+$ (KB$^{+)}$ for HCN determination

Proteose Peptone no 3.	20 g
$K_2HPO_4 \cdot 3\ H_2O$	2g
$MgSO_4 \cdot 7\ H_2O$	1.5 g
Glycerin	10 g
Glycin	4.4 g
Aqua bidest	up to 1000 ml
pH adjusted to 7.0	

Mineral media for screening of formulation additives *in vitro* (modified from succinic medium)

KH_2PO_4	3 g
K_2HPO_4	6 g
$(NH_4)_2SO_4$	1 g
Glucose	15 g
$Mg\ SO_4 \cdot 7H_2O$	0.2 g
$CaCl_2$	10 mg
Agar	16 g
Aqua bidest	up to 1000 ml

Tryptic Soy Broth (TSB)

TSB	20 g
Aqua bidest	up to 1000 ml
pH adjusted to 7 or 6.5	

Tryptic Soy Agar (TSA)

TSB	20 g
Aqua bidest	up to 1000 ml
Agar	16 g

pH adjusted to 7 or 6.5

Water Agar (WA)

Agar	16 g
Aqua bidest	up to 1000 ml

pH adjusted to 7.0

Luria Berthani (LB) Medium

Glucose	5 g
Tryptone	10 g
Casamino Acids	5 g
NaCl	1 g
Aqua bidest	up to 1000 ml

pH adjusted to 7.0

Maize Extract Agar (MEA)

10 g of corn meal was mixed with 200 ml tap water, boiled, and sieved. After cooling, it was centrifuged with 4000 g for 10 min.. The supernatant was filled up to 1000 ml with aqua bidest,

Agar	16 g

pH adjusted to 5.5

Maize Extract Broth (MB)

10 g of corn meal was mixed with 200 ml tap water, boiled, and sieved. After cooling, it was centrifuged with 4000 g for 10 min.. The supernatant was filled up to 1000 ml with aqua bidest,

Agar	16 g

pH adjusted to 5.5

100 ml of corn broth were filled in 250 ml erlenmeyer flask and supplemented with 5 drops of wheat germ oil.

Potato Dextrose Broth (PDB)

Potato Dextrose Broth	20 g
Aqua bidest	up to 1000 ml

pH adjusted to 5.5

Potato Dextrose Agar (PDA)

Potato Dextrose Broth	20 g
Aqua bidest	up to 1000 ml
Agar	16 g

pH adjusted to 5.5

Tryptone Agar (Semi Liquid Agar) for evaluation of motility

Trypton	5 g
Agar	5 g
Aqua bidest	up to 1000 ml

pH adjusted to 7

Malt Yeast Extract Agar (MYEA)

Malt Extract Agar	10 g
Yeast Extract	5 g
Aqua bidest	1000 ml

Pelleting Materials

Kieselgur (Diatomaceous Earth) Fluka GmBH

Peat	KWS
Bentonite	KWS
Wood flour 1	KWS
Wood flour 2	KWS
Cotton flour	KWS

2.2.3. Organisms

Fungal and bacterial isolates were obtained from departmental culture collection, Prof. Wolf, Institute of Plant Pathology and Plant Protection, Georg-August Universität, Göttingen.

Fungi

Pythium ultimum var. *ultimum*

Bacteria

Pseudomonas fluorescens B5 WT (wild type)

Pseudomonas fluorescens B5 $^{Str\ s}$ (Streptomycin resistant strain)

Escherichia coli Tn 5 (*E. coli* S 17-1 with Plasmid pSUP 5011) (pBR 325-Tn5-Mob) from Simon (Simon *et al*, 1983)

Sugar beet

Cultivar EVA-MSV as pelleted seeds used for most of the experiments from KWS Einbeck, Germany and the following varieties as unpelleted seeds:

300-0132B (V1)
300-0088B (V2)
300-0364B (V3)
300-0483B (V4)
300-0448B (V5)
300-0520 A (V6)
300-0226B (V7)
300-0493B (V8)

Sugar beet seeds were supplied by Dr. Ralph Tilcher (KWS Einbeck, Germany)

2.3. Methods

2.3.1. Production and testing of antifungal metabolite-overproducing mutants

2.3.1.1 Strain maintenance

Bacteria:

For long time storage bacteria were stored on soil extract agar (SEA) media, pH 7.0, at 5°C supplemented with antibiotics, if needed. Every 6 months the bacteria were recultured on TSA medium. In addition, bacteria in 75% glycerin in Eppendorf tubes were stored in refrigerator at -20° C. For *ad planta* experiments one day old luria berthani broth (LB)-cultures of bacteria were used and supplemented with antibiotics, if needed. The bacterial cultures were prepared by transferring 1% (v/v) of the pre-cultures.

***Pythium ultimum*:**

P. ultimum was stored on maize extract agar (MEA, pH 5.5) at 5° C. For *in vitro* treatments a two-day-old potato dextrose agar (PDA) culture was used and for *ad planta* experiments a 5 d old MEA culture.

2.3.1.2. Production of antifungal metabolite - overproducing mutants

Pseudomonas fluorescens B5^{Str} (Schulz and Wolf, 1998) was provided by the culture collection of Prof. Wolf, Institute of Plant Pathology and Plant Protection, Georg August University Göttingen. This spontaneous mutant that has similar properties (vitality and antifungal activity) compared to its wild type strain. *Ps. fluorescens* B5^{Str} was cultured in LB containing 400 ppm streptomycin sulphate at 100 rpm and 20°C for 24 h. The donor strain *Escherichia coli* Tn 5^{Kan} was cultured in LB medium supplemented with 150 ppm kanamycin sulphate at 28°C and 100 rpm for 24 h.

Ten milliliters of the bacterial cultures were centrifuged at 5 °C and 18.000 g, washed twice with 10 ml of tryptone soy broth (TSB), pH 7.0, to remove the antibiotics. Finally, the bacterial pellets were concentrated 10 fold by dissolving it in 1 ml TSB, pH 7.0.

The transmission of transposon was carried out by the 'spot-agar-mating' technique according to Friedrich *et al.* (1981). 0.5 ml of a concentrated bacterial suspension was vigorously mixed in a sterilized Eppendorf tube and 0.4 ml was plated on TSA, pH 7.0 and incubated at 25°C for 24 h. Bacteria were washed off the agar with sterilized 5 ml phosphate buffer.

A serial dilution of the bacterial suspension was made from 10^0, 10^{-1}, 10^{-2} and 10^{-3} in phosphate buffer and plated on TSA amended with 150 ppm kanamycin sulphate and 400 ppm streptomycin sulphate. *Pseudomonas fluorescens* $B5^{Str.}$ and *Escherichia coli* $Tn5^{Kan}$ were plated together on the same media. Controls of the bacterial strains were plated separately on antibiotic agar and incubated at 25°C for 24 h.

2.3.1.3. Screening for antifungal metabolites – overproducing mutants

Prescreening

5000 transconjugants from transposon mutagenesis were selected for a first screening towards overproduction of antifungal metabolites. Two transconjugants and *Ps. fluorescens* B5 WT were streaked out in triangle on 9 cm petri dishes containing TSA, pH 6.5, and were incubated at 20°C for 24 h. An agar plug of a one day old MEA based culture of *P. ultimum* was placed in the centre and the petri dishes were incubated for another 24 h. Evaluation was carried out by comparing the inhibition zone of transconjugants with the standard wild type strain *Ps. fluorescens* B5. Promising transconjugants were tested in a second assay more in detail.

Screening

Always only one of the selected transconjugants was tested per petri dish. Transconjugants were streaked out on TSA pH 6.5 in the middle of the petri dish. After incubation for 24 h an agar plug of *P. ultimum* was placed on both sides 2 cm apart from the growing bacterial colony. After further incubation at 20° C for 48 h the inhibition zones of each side were measured.

Treatments were done with six replications with two repeatings of the whole experiment. *Ps. fluorescens* B5 WT and Pf $B5^{Str}$ were used as standards.

2.3.1.4. Characterisation of mutants *in vitro*
2.3.1.4.1 Antifungal activity of culture filtrate produced by mutants

Selected mutants were grown in 20 ml LB and KB^+ (KB amended with $FeCl_3$ in a final concentration of 200 nM) at 20°C in rotary shakers with 100 rpm. Culture filtrate (CF) was harvested at 24 h, 48 h, 72 h after inoculation. The culture was adjusted its pH to 6.0, centrifuged at 20,000 g and filter sterilized (membrane filter Φ 0,2 µm, MinisArt, Sartorius, Göttingen, Germany). LB based culture filtrates (LB-CF) and KB^+-based culture filtrate (KB^+-CF) were obtained. PDB, pH 5.5, containing 10 % (v/v) of the filtrate was inoculated with an agar plug of a one day old culture of *Pythium ultimum* and incubated at 20°C at 100 rpm for 48 h. The mycelium of the fungus was harvested and its dry weight determined. Treatments were carried out with 5 replications each with two repeatings of the whole experiment.

2.3.1.4.2. Growth

Selected mutants were grown in Luria Berthani (LB) medium and King's B^+ (amended with 200 nM $FeCl_3$), pH 7.0, and cultivated at 20 °C and 100 rpm. Bacterial growth was measured by determining the OD at λ=592 nm (Spectra II, Fa Tecan, Belgium) at 0, 3, 6, 9, 12, 24, 36, 48, 72, 90 h after inoculation. The experiment was carried out with three replications. A regression line was made between the measured values at OD_{592nm} and the viable cells by plating the bacterial suspension on LBA. Moreover, the generation time of each mutant was determined according to the formula:

$$G = \frac{t}{3.3 \log N_2 / N_1}$$

Where
G = generation time
t = time between two measurements
N_2 = OD at second measurement
N_1 = OD at first measurement
N_2 and N_1 were determined during exponential growth

2.3.1.4.3. Bioassay of volatile antifungal metabolites

The bioassay of volatile antifungal metabolites was done in split plate petri dishes, one side containing PDA, pH 5.5, and the other with KBA supplemented with glycine. The two parts of the plate are separated, but air and other volatile substances can pass the barrier (Figure 1). Mutants were grown on KBA^+ (KBA amended with glycine) and incubated for 24 hour at 20 °C. Then an agar plug of a 24 h old PDA based culture of *Pythium ultimum* was placed on the other side on PDA and the petri dishes were sealed with parafilm an incubated for 48 hours.

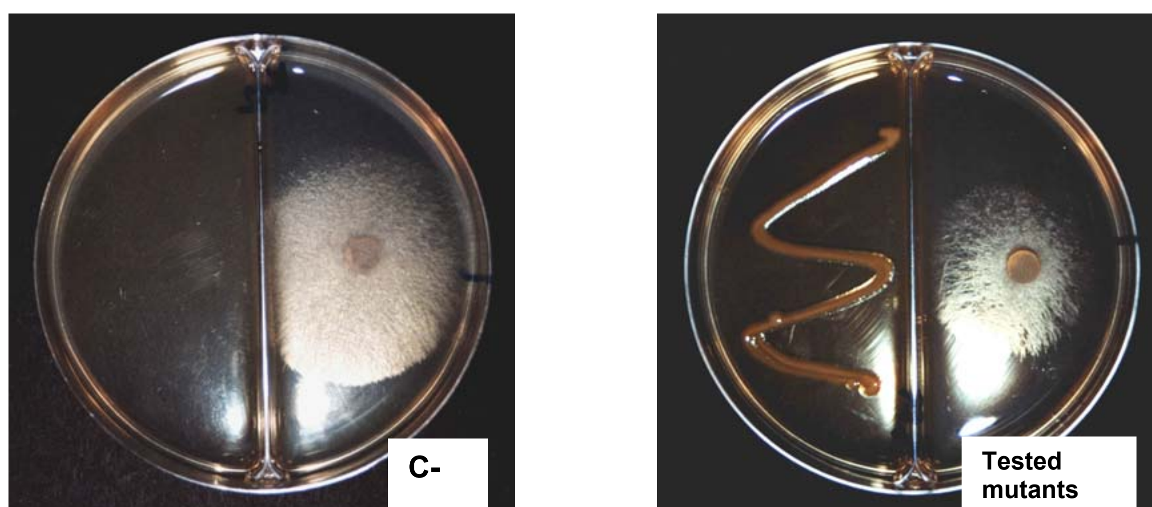

Figure 1. Bioassay of volatile antifungal metabolites produced by mutants (left: control; right: inhibition of *P. ultimum* by a mutant)

The effect of volatile antifungal metabolites was assessed by comparing the colony diameter of *P. ultimum* treated with bacteria and its diameter in absence of bacteria. The experiment was set in completely randomised design with four replications.

2.3.1.4.4. HCN production

The determination of HCN was conducted by colorimetric technique using Spectroquant ^{R} rapid test (Merck, Darmstadt). Culture of mutants grown in KB^+ (KB amended with glycine) were harvested 24h, 48h and 72 h after inoculation, centrifuged at 10,000 g and filter sterilized (membrane filter, pore diameter 0.2 µm, Fa. Sartorius, Göttingen). The determination of HCN is based on the colorimetric reaction performed by Asmus and Garschagen (1953). Cyanide and chlor salt (reagent CN-1A) build chlorcyan, and this reacts with pyridin (reagent CN-3A) and forms glutacondialdehyde, that afterward reacts with 1,3 dimethyl barbituric acid (reagent CN-2A) to form a violet polymethin.

The sample were collected in microplates with 350 µl wells, then measured at λ=592 nm (Spectra II, Fa Tecan, Belgium). HCN-containing samples change their colour from yellow-brown to pink, with intensity proportional to the HCN content (Figure 2). Each treatment was replicated 4 times.

2.3.1.4.5. Indole-3-acetic acid (IAA) production

The production of indole acetic acid (IAA) was determined by colorimetric methods (PC Methods that was firstly perfomed by Pilet and Chollet) (Glickmann and Dessaux, 1995). Transconjugants were grown in KB medium containing 0.5 g/l tryptophan for 24 h, centrifuged at 10,000 g and the supernatant filter sterilized (membrane filter, pore diameter 0.2 µm, Fa. Sartorius, Göttingen). Reagent consists of 12 g of $FeCl_3$ per litre in 7.9 M H_2SO4. One millilitre of reagent was added to 1 ml of sample solution in 1,5 ml Eppendorf tube and mixed vigorously. The mixture was left in the dark for

30 minutes at room temperature. The samples were filled into microplates with 350 µl wells and the OD was measured at λ=550 nm using a spectrometer (Spectra II, Fa Tecan, Belgium). IAA content was calculated from a regression made with indole -3- acetic acid in concentrations from 0, 6.25, 12.5, 25, 50, 75 and 100 µg/ml (Figure 3).

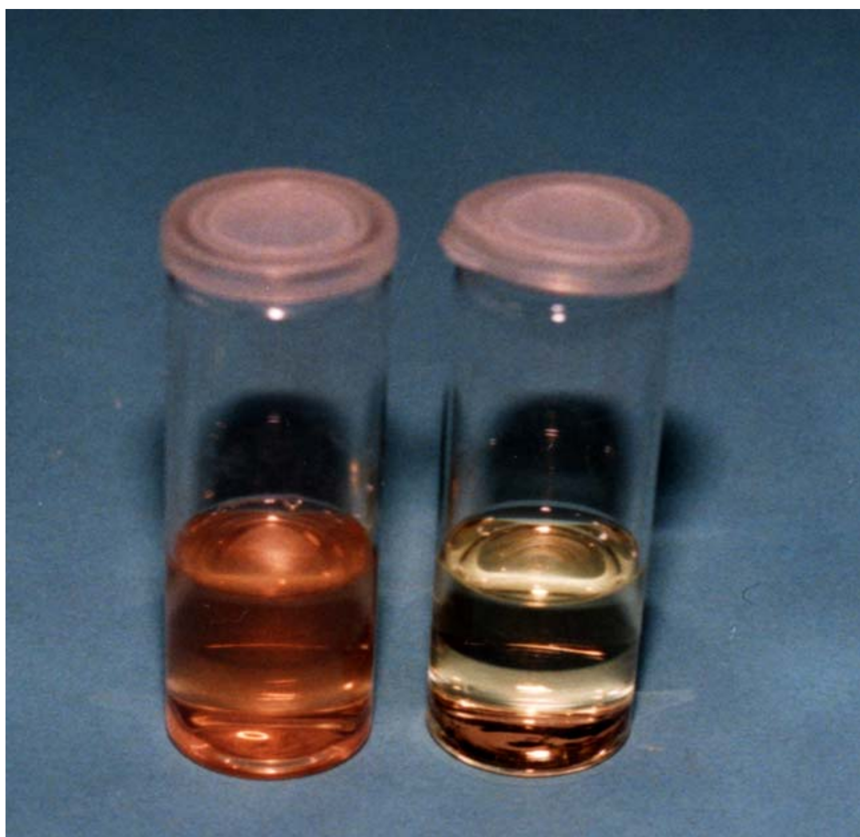

Figure 2. Cyanide determination using Spectroquant R. Dark violet brown colour on the left indicates a HCN positive samples, clear yellow colour on the right indicates a HCN negative sample.

2.3.1.4.6. Motility

The method of Scher *et al.* (1988) was used to determine the motility of mutants. 10 µl of a bacterial suspension was transferred onto petri dishes containing semi liquid tryptone agar (1% tryptone and 0.5 % agar) and incubated for 24 h at 20 C. Motility was determined by measuring the

diameter of the swarming colony. Each treatment was done with four replications.

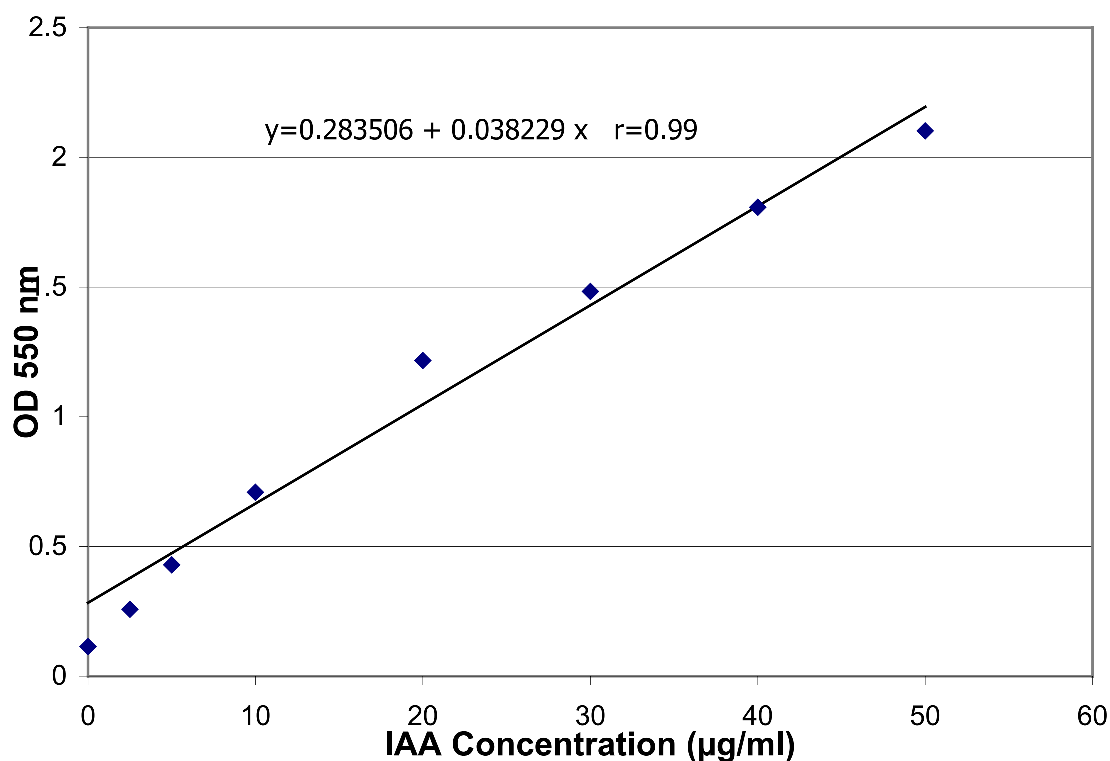

Figure 3. Regression line for IAA production (PC Methods)

2.3.1.5. Root Colonisation

Colonisation experiments were done in microcosm in controlled environment under light intensity of 3000 lux, photoperiods of L:D=18:6, temperature of 18°C, and soil water content of 60% of field capacity. Seeds were dipped for 20 minutes in the suspension of one-day old-LB-based culture of *Ps. fluorescens* B5 ^{Str} with an OD_{592} of 0.25. Excess of water or bacterial suspension was removed. Six seeds were planted into each microcosm. At the end of experiment, 13 day after sowing, plants with roots were removed carefully. Soil adhering to the roots was used for estimation of rhizosphere colonization. Sugar beet plants were put on a wet sheet of paper towels to keep them fresh. Two equally grown plants with acceptable root recovery were taken from each of 2 columns to make 3 replicates with 2

plants each. Roots were measured and an 8 cm long root section beginning with the hypocotyl was used for the determination of rhizoplane colonisation. They were put into a universal, weighed, and kept cold at 5°C (overnight). Next day, universals were filled with 3 parts of saline solution (of root fresh weight with soil). Dilutions were made in Eppendorf cups to estimate colonization in the rhizosphere (soil). Roots were washed twice gently with sterile Ringer solution to remove adhering soil and grinded in a mortar with 10 parts of saline. Dilutions till 10^{-6} were made in Eppendorf cups and 10 µl drops were plated onto petri dishes containing TSA with 400 ppm streptomycin to estimate the rhizoplane colonization of antagonists.

2.3.1.6. Efficacy of antifungal metabolite - overproducing mutants *ad planta*

The inoculum was produced by growing one-day-old PDA-culture of *P. ultimum* in maize broth at 20°C for 5 days on a rotary shaker at 90 rpm. The mycelium was harvested with a sieve, rinsed with sterile water, weighted and homogenized in a blender at low speed. The water suspension containing mycelia and oospores of the required dry weight (100 mg dry weight ~2.6 g fresh weight/ treatment) containing 2000 oospores /g soil, was filled to 200 ml with water and mixed with 2.8 kg of a sand-compost mixture for each seed treatment (six pots).

Monogerm-seeds (cultivar EVA-MSV) were obtained from Kleinwanzlebener Saatzucht AG (KWS, Germany). Seeds were planted in a mixture of 2.6 kg steamed compost soil sieved to particle size of 4mm with 0.4 kg fine sand and 200 ml of tap water or water containing the homogenized mycelium and oospores of the pathogen respectively to give a soil moisture content of 60 % field capacity determined according to Laermann (1972). 16 seeds were sown 2-cm deep in each 10*10 cm plastic pot containing 500 g of infested soil. Six replicates were planted for each seed treatment in a completely randomised experimental design. 3 kg of compost soil were infested with 1.25 g fresh weight of mycelia of *P. ultimum* corresponding to 100 mg dry weight. Pots were covered with foils and set at

15°C until emergence. Further incubation carried at a temperature of 18°C with illumination from mercury vapour lamps (3000 Lux) for 16 h per day. After emergence pots were watered to maximum field capacity for 2 days. Thereafter the soil moisture contents of the pots were adjusted gravimetrically daily to maintain 60 % of maximum field capacity

The disease suppression [DS] was evaluated 2 weeks after seedling emergence by number of healthy plants:

$$DS\ [\%] = \frac{X - C+}{C^{-} - C^{+}} \cdot 100\%$$

Where:

X = number of healthy plants in the treatments

C-=number of healthy plants in non infected control

C+= number of healthy plants in infected control

2.3.2. The role of indole-3-acetic acid (IAA) in biocontrol activity of *Ps. fluorescens* B5 against *Pythium ultimum* in sugar beet seedlings

2.3.2.1. Relation between IAA - production of mutants *in vitro* and their efficacy a*d planta*

The total *in vitro* production of IAA in the whole culture and the production per 10^9cells were plotted against biocontrol efficacy *ad planta* of the corresponding mutants. Pearson’s correlation coefficient was calculated between the two variables (N=18) using the Statistica package program. A value of P<0.05 shows a significant correlation.

2.3.2.2. Antifungal activity of IAA and some indole derivatives against *P. ultimum in vitro.*

Stock solutions of Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and indole-3- propionic acid (IPA) were prepared in aqua bidest. The solutions were filter sterilized and added to PDB, pH 5.5, to provide a final

concentration of 12,5; 25; 50; 75 and 100 ppm (µg/ml) respectively. An agar plug (diameter 5 mm) of a one-day-old PDA-culture of *P. ultimum* was used as inoculum and incubated for 48 h at 20°C and 100 rpm. Sterilized aqua bidest instead of indole derivative in PDB served as a control. The mycelium of the fungus was harvested for dry weight estimation after drying mycelium overnight in an oven at 121°C. The experiment was done in a completely randomised design with four replications per treatment.

2.3.2.3. Effect of IAA and other indole substances and its combination with *Ps. fluorescens* B5 in suppressing *P. ultimum ad planta*

The fungicidal effects of IAA and of other indole growth regulators i.e. IBA and IPA against P. ultimum ad planta were tested in concentrations of 0, 12.5; 25; 50; 75; and 100 µg/ml (w/v). Sugar beet seeds were dipped in the solution for 20 minutes, than sown in plastic boxes with 16 seeds per box containing compost-soil and pathogen as used in the standard *ad planta* assay (Chapter 2.3.1.6). The experiment was set in a completely randomised design with 5 replications.

The effect of exogenous IAA application and its combination with *Ps. fluorescens* B5 in controlling *P. ultimum* in sugar beet seedlings was studied in the standard biocontrol assay except for the seed treatment. Experiment was set in a completely randomised design with the concentration of IAA *i.e* 12.5; 25; 50; 75; and 100 µg/ml as a first factor and the application of Pf B5 (Pf B5 absent or in combination with IAA derivatives) as the second factor. Naked unpolished seeds, variety 300-0132B (KWS), were dipped in aqueous IAA in appropriate concentrations for 20 minutes, with or without *Ps. fluorescens* B5 $OD_{592nm,}$ depending on treatment of 0.25 for 20 minutes.

2.3.2.4. Bioassay for the effect of mutants on the root growth of sugar beet seedlings *in vitro*

The effect of IAA on the growth of sugar beet seedling *in vitro* was determined by a modified technique of Barazani and Friedman (1999). Surface

sterilized unpelleted sugar beet seeds were grown on NA and incubated for 48 hours. Pf B5 and the mutants were grown on three different media *i.e.* MYEA, KBA, and TSA in petri dishes equally for 48 h. The seeds were transferred to these petri dishes parallel to the colony of the test bacteria (Figure 4). Root length was measured at 48 h.

2.3.2.5. Effect of IAA and *Ps. fluorescens* B5 on the growth of sugar beet seedlings

Naked unpolished sugar beet seeds were dipped in aqueous IAA concentrations of 12.5; 25; 50; 75; and 10 µg/ml for 20 minutes. Seeds inoculated with *Ps. fluorescens* B5 OD_{592} =0.25 served as a positive control, noninoculated seeds as a negative control. The seedlings were grown under controlled conditions in the standard microcosm assay in absence of the pathogen. Seedlings were harvested 20 days after sowing and shoot height and shoot weight were estimated. The experiment was done in a completely randomised design with six replications per treatment.

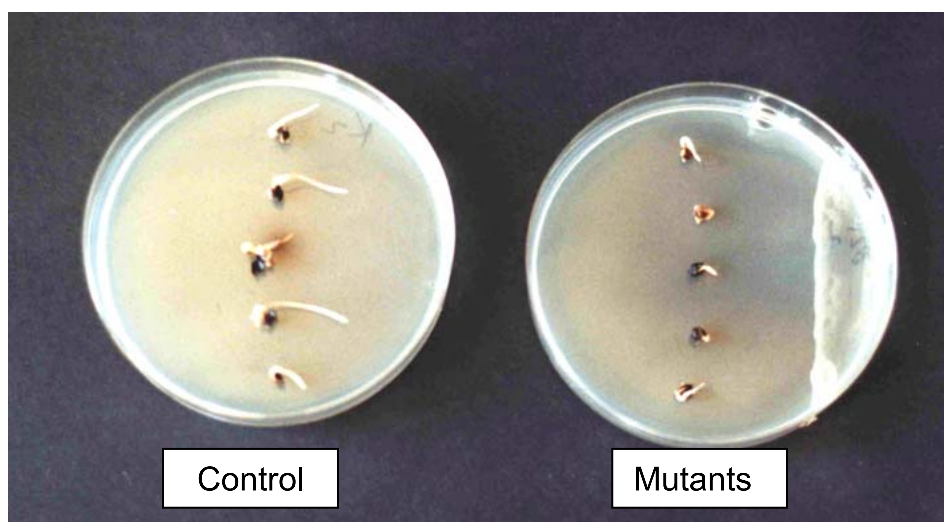

Figure 4. Effect of IAA produced by tested mutants on the growth of sugar beet seedlings *in vitro* (modified from Barazani and Friedman, 1999; MYEA, pH 7.0, 20° C)

2.3.3. The role of sugar beet varieties in biological control of *P. ultimum* by using *Ps. fluorescens* B5

2.3.3.1. Antagonistic activity of *Ps. fluorescens* B5 in different sugar beet varieties *ad planta* under controlled conditions

The standard biocontrol assay (2.3.1.6) was used in this experiment Experiment was set in a completely randomised design with the varieties as the first factor (*i.e.* 300-0132B (V1), 300-0088B (V2), 300-0364B (V3), 300-0483B (V4), 300-0448B (V5), 300-0520 A (V6), 300-0226B (V7), 300-0493B (V8)), and the application of Pf B5 (Pf B5 absent or in use with the varieties) as the second factor. Two controls (without and with *Pythium* inoculation) were used for each tested variety. Each treatment was replicated four times and the whole experiment was repeated three times.

2.3.3.2. Antagonistic activity of *Ps. fluorescens* B5 in different sugar beet varieties with two inoculum concentrations of the pathogen under controlled conditions

The antagonistic activity of *Ps. fluorescens* B5 was estimated for eight sugar beet varieties and two concentrations of the pathogen. Experiment was set in completely randomised design with three factors. The first factor was the sugar beet varieties, the second the application of PF B5, and third the pathogen inoculum, *i.e.* high inoculum at ~2000 oospores /g soil (equivalent to mycelial dry weight of 33 µg/g soil) and low inoculum at 400 oospores/g (equivalent to mycelial dry weight of 6.2 µg/g soil). The first and second factors were the same as in chapter 2.3.3.3. The whole experiment was repeated twice.

2.3.3.3. Root colonisation

The experiment was carried out with the methods described in 2.3.1.6 except seeds were not pelleted. Each treatment (eight varieties) was replicated 4 times and the experiment was repeated twice, with two control *Pythium*-infected and healthy control.

2.3.3.4. Root adherence

Eight varieties of sugar beet, 300-0132B (V1), 300-0088B (V2), 300-0364B (V3), 300-0483B (V4), 300-0448B (V5), 300-0520 A (V6), 300-0226B (V7) and 300-0493B (V8) were used in this experiment with 6 replications, and three seedlings (roots) per replication. Adherence test was carried out using the method of Glandorf *et al.* (1994). Seeds were surface sterilized in 5 % NaOCl and rinsed two times with sterilized distilled water. Then they were incubated in moist paper in petri dishes (Ø 15 cm) for 5 days. The roots were cut and weighed and immersed in a bacterial suspension of *Ps. fluorescens* $B5^{Str}$ with an OD_{592nm} = 0.25 for 15 minutes in a ernlemeyer flask and agitated on shaker at 100 rpm for 15 minutes. Roots were removed and gently shaken to remove excess of bacterial suspension. Roots were washed with 0.1 M $MgSO_4$ one times and shaken vigorously in universal glass with 5 ml 0.1 M $MgSO_4$ in the ratio of 1:25 (w/v). The washing solution was considered as W1. Roots were macerated in 0.1 M $MgSO_4$ in the ratio of 1:5 (w/v) by using pestle and mortar. The resulting suspension was considered as W2. W1 was used to characterize adherence type 1 after determination of the cfu in the washing solution on TSA, pH 7.0 containing 400 ppm streptomycin sulphate. W2 was used to characterize adherence type 2.

2.3.4. Optimisation of the biocontrol activity of *Ps. fluorescens* B5 by improving its formulation technique

2.3.4.1. Screening for appropriate pelleting materials

2.3.4.1.1. Growth of Pf B5 in different materials

In order to obtain a pelleting material, which support long-term survival, different materials *i.e.* wood flour 1, wood flour 2, kieselgur (diatomaceous earth), cotton flour, bentonite and peat were tested. Materials (5% w/v) were add to 100 ml TSB in 250 ernlemeyer flasks and the pH was adjusted up to 7.0 after autoclaving. TSB containing materials were inoculated with 1 % of one-day old culture of *Ps. fluorescens* $B5^{Str}$ and incubated at 20°C with 100

rpm for 48 hours. The density of bacteria in each culture was determined by serial dilutions followed by plating onto TSA supplemented with 200ppm streptomycin sulphate 6h, 12h, 24h, 36h, and 48 h after inoculation. At 48 h, bacterial culture was filtered through a fibreglass filter paper (Ø 90 cm, Scheichel-Schuell, Germany) using a vacuum pump. The residues of the pelleting materials were than mixed with aqua bidest to remove remaining nutrients and filtered again. The liquids passing the filter paper after the first and second filtration are considered as liquid part and washing water respectively. In order to compare the binding capacity of each material, the number of bacteria in the residue of pelleting materials, in the liquid part and in the washing water as well, was determined by serial dilution followed by plating onto TSA containing 400 ppm streptomycin sulphate.

2.3.4.1.2. Survival of *Ps. fluorescens* B 5 in different materials

Residues of pelleting materials, except bentonite, after vacuum filtration had moisture content of ~60% (determined gravimetrically). Bentonite with 60 % moisture was received by air-drying for 24 h. Two moisture contents of the residues were used in this experiment *i.e.* 60 % and 10 %. 10 % of moisture in the residues was obtained by air drying at 20°C for 24 h, except bentonite (48 h). The residues of various pelleting materials containing bacteria were stored in parafilm sealed petri dishes at 5 °C until use.

The survival of pseudomonads was assessed monthly from zero up to 12 month. 100 mg of pelleting material was diluted up to 10^{-6} with 0.1 M saline solution (0.9 g NaCl/l aqua bidest) and plated onto TSA, pH 7.0, amended with 400 ppm streptomycin. The experiment was set in completely randomised design with six replications of each treatment. The whole experiment was repeated twice.

2.3.4.1.3. Antagonistic activity *in vitro* of *Ps. fluorescens* B5 after storage in different materials

To investigate the influence of different materials on the antagonistic activity during storage *Ps. fluorescens* B5 was recovered from each material after 12 months storage and grown on TSA, pH 7. A one-day-old colony was used to test antagonistic activity using the methods described in chapter 2.3.1.4. The experiment was set in complete randomised design with five replications, and whole experiment was repeated twice.

2.3.4.2. Screening for additives to improve formulation of *Pseudomonas fluorescens* B5

Nitrogen sources and trace elements should serve as additives to improve the formulation of antagonists, to stimulate the production of antifungal metabolites *in vitro* and increase the efficacy of *Ps. fluorescens* B5 *ad planta*, but do not favour the pathogen and do not give detrimental effects on host plant.

2.3.4.2.1. Screening for nitrogen sources and trace elements as formulation additives *in vitro*

Urea, $(NH_4)_2SO_4$, NH_4NO_3, casamino acids and tryptone were used as nitrogen sources in three concentrations *i.e.* 0.5 %; 0.1 %; 0.02 % (w/v) in modified succinic media, where succinic was replaced by glucose, pH 7.0. Trace elements were: H_3B0_4, $CuSO_4$ 5 H_20, $FeCl_3$ 2 H_20, $ZnSO_4$ 7 H_2O, $MnSO_4$ H_2O, $CoCl_2$ 12 H_2O, $H_{24}Mo_7N_6O_{24}$. The used concentrations of trace elements were 1 mM, 0.2 mM, and 0.05 mM. Because of the toxicity of Cu and Co to the growth of *Ps. fluorescens* B5, *i.e.* 0.05 mM of these elements inhibited the growth of PF B5, the substances were not further tested. One percent of one-day old culture of PF B5 was used as inoculum at 20°C and 100 rpm. The culture was harvested after 4 days (stationary growth phase). Growth was determined by measuring the OD_{592nm} after 24 h, 48 h, 72 h and 96 h. The culture was centrifuged at 9.600 rpm at 5°C for 20 minutes. The pH of supernatant was adjusted to 6.5, then filter sterilized using a sterile

membrane filter (Ø 0.2 μm, Minisart, Sartorius, Germany). Culture filtrate was add to 100 ml erlenmeyer flasks containing 20 ml of PDB, pH 5.5, at the rate of 10 % (v/v) and inoculated with an agar plug of a one day culture of *Pythium ultimum.* Controls were made by amending standard media with the solution of the tested nitrogen source or trace element in appropriate concentration. After incubation for 48 h mycelium of fungus was harvested and dry weight was determined after drying in an oven at 121 °C for one day. The experiment was carried out in a completely randomised design with 5 replications each.

2.3.4.2.2. Screening for nitrogen sources and trace elements as formulation additives *ad planta*

Selected trace elements (H_3BO_3, $MnSO_4$ and $ZnSO_4$) and nitrogen compounds (ammonium nitrate and casamino acids) in two concentrations for trace elements (0.05 and 0.2 mM) and for nitrogen compounds (0.1% and 0.5%) were further tested *ad planta*. Concentration optimisation of tested trace elements *ad planta* was investigated by testing at the concentration of 0.2 mM, 0.05 mM, 0.01 mM and 0.005 mM. Combined treatment of MnSO4 and ZnSO4 with concentration of each substance of 0.01 mM and 0.05 mM was also tested. Pelleted sugar beet seeds were dipped in a mixture of *Ps. fluorescens* B5 WT (OD_{592nm} = 0.25) with an appropriate concentration of the additive for 20 minutes, and excess of liquid was removed. Biocontrol activity of Pf B5 was than tested in the standard pot assay (2.3.1.6). Experiment was set in completely randomised design with five replications each and the whole experiment was repeated twice.

A direct effect of $MnSO_4$ and $ZnSO_4$ on the disease *ad planta* was also tested. Seeds were dipped into the corresponding solution containing the additives and planted in *Pythium* infested soil but without Pf B5 treatments. The experiment was carried out with five replications.

Effect of $MnSO_4$ and $ZnSO_4$ on the emergence and growth of sugar beet in absence of *Pythium ultimum* and Pf B5 was also investigated. Seeds

were dipped with the solution of $MnSO_4$ or $ZnSO_4$ at the concentrations of 0.05 and 0.2 mM were grown under controlled conditions without Pf B5 and *Pythium ultimum*. Emergence, total and individual fresh weight of seedlings were determined 27 days after sowing.

2.3.5. Statistical analysis

Obtained data were organized by Excel (Microsoft), than statistically analysed using Statistica program package. Before analysed, data distribution was tested its normality by a nonparametric test of Kolmogorov-Smirnov (K-S). Data passing K-S test were directly analysed using Analysis of Variance (ANOVA). Data, which were not normally distributed, were transformed mostly by log (x+1), and arc sin (x) for the variety antagonist interaction. If the calculated F-value according to the ANOVA showed a significant difference, mean comparison was made by Duncan Multiple Range Test (DMRT) at $P < 0.05$ (Gomez and Gomez, 1984). Correlation analysis (Pearson's) was also performed among physiological and antagonistic traits among mutants in mutant experiment (N=18), and among colonisation-adhesion-antagonistic activity of Pf B5 in different varieties (N=24). The resulting correlation coefficients were furthermore tested their significance at $P < 0.05$, and $P < 0.01$.

3. RESULTS

3.1. The use of antifungal metabolite-overproducing mutants to enhance antagonistic activity of *Ps. fluorescens* B5

3.1.1. Production and screening of antifungal metabolite-overproducing mutants

In order to create antibiotic-overproducing mutants, transposon mutagenesis was done with *Escherichia coli* Tn5 containing the resistance gene for kanamycin as donor strain while the streptomycin resistant *Ps. fluorescens* B5 was used as recipient. The resulting transconjugants were resistant to streptomycin and kanamycin. Table 1 shows that transposon mutagenesis with *E. coli* Tn 5 was efficient to produce transconjugants. It was revealed by the very few number or almost negligible spontaneous mutants. Totally, about 5000 mutants were obtained.

Table1. Numbers of spontaneous and Tn5-mutants resistant to streptomycin + kanamycin in the conjugation experiment with *Ps. fluorescens* B5 ^{Str}

Spontaneous mutants in the dilution			Mutants after conjugation with *E.coli* Tn 5 in the dilution		
10^0	10^{-1}	10^{-2}	10^0	10^{-1}	10^{-2}
2	0	0	>500	75	5

Mutants were screened *in vitro* towards overproduction of antifungal metabolites effective against *P. ultimum*. There was a first indication that 3 out of 5000 mutants (No. 010, No. 029, No. 311) were antifungal metabolite-overproducing mutants. This was indicated by a bigger inhibition zone in dual culture against *P. ultimum* compared to wild type of *Ps. fluorescens* B5 (Pf B5 WT) (Figure 5, Table 2). Four other antifungal metabolites-overproducing mutants *i.e*. N 24, Tn 12, Tn XII and No. 825 obtained by similar technique (transposon mutagenesis) were supplied by Dr. Dietmar Schulz. Antagonism test *in-vitro* of those seven mutants against *P. ultimum* on TSA shows that only No. 825 had a significant larger inhibition zone compared to Pf B5 WT.

Figure 5. Screening for antifungal metabolite - overproducing mutants of Pf B5 (in Figure No 311 and Tn 12) by dual culture technique. Both mutants produced bigger inhibition zone than wild type.

Table 2. Inhibition zone of antifungal metabolite-overproducing mutants of Pf B5 against *P. ultimum* on TSA (pH 6.5) and King's B Agar (pH 6.5; 24-h pre-incubation, evaluation 24 h after fungus inoculation).

Mutants	Inhibition Zone (mm)	
	TSA	KBA
P. fluorescens B5 WT	13.25 b	11.25 A
P. fluorescens B5 ^{Str}	12.42 ab	11.00 A
No. 010	14.00 b	13.17 B
No. 029	11.83 a	13.00 B
No. 311	14.25 bc	12.50 B
N 24	13.00 ab	12.50 B
Tn 12	14.58 bc	13.00 B
Tn XII	12.83 ab	11.42 A
No. 825	15.41 c	12.08 AB

Values in the same column followed by the same letter are not significantly different with DMRT test (P < 0.05). Each treatment has five replications (the whole experiments was repeated twice).

While on KBA^+(KB containing 200 nM $FeCl_3$) except for Tn XII, all tested mutants had a significant greater inhibition zone than Pf B5 WT (Table 2).

3.1.2. Characterisation of antifungal metabolite - overproducing mutants

3.1.2.1 Antifungal activity of the culture filtrate *in vitro*

Antagonism test was carried out with culture filtrate from different media (LB and KB^+) with different culture age (24 h and 48 h). The results obtained in this experiment are not parallel to that obtained in dual culture test on solid media (TSA and KBA^+). With the exception of No. 010 and No. 825, LB-based culture filtrate of the remaining mutants inhibited the growth of *P. ultimum* more effective compared to Pf B5 WT. LB-based culture filtrates of mutants No. 311 and Tn 12 appeared to be the most strongest ones. Except for No 010, and No 311, KB^+-based culture filtrates of the mutants provided higher inhibitory effects against *P. ultimum* than of Pf B5 WT.

The antifungal activity of some culture filtrates was influenced by its culture age. A 24 h old LB-based culture filtrate of the mutants No. 311, N 24 and Tn 12 showed higher antifungal activity compared to 48 h old LB-based culture filtrate (Figure 6). In contrast, the effect of culture age was not observed for other tested mutants. Except for No. 010 and No. 029, a 24 h old KB^{+-}based culture filtrate of all mutants had a higher antifungal activity than a 48 h old filtrate (Figure 7).

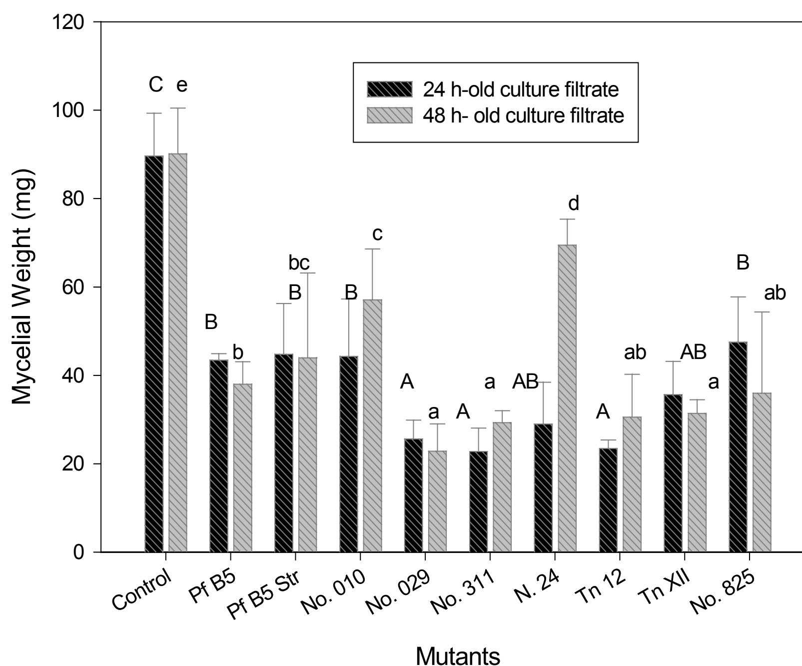

Figure 6. Effect of culture filtrate of antifungal metabolites-overproducing mutants of Pf B5 against *P. ultimum in vitro* (bacteria grown in LB medium pH= 7.0, 20°C, 100 rpm, harvested 24 h and 48 h after inoculation, 5 replications each, whole experiment repeated 2 times. Same shaded bars with the same symbols are not significantly different at P< 0.05).

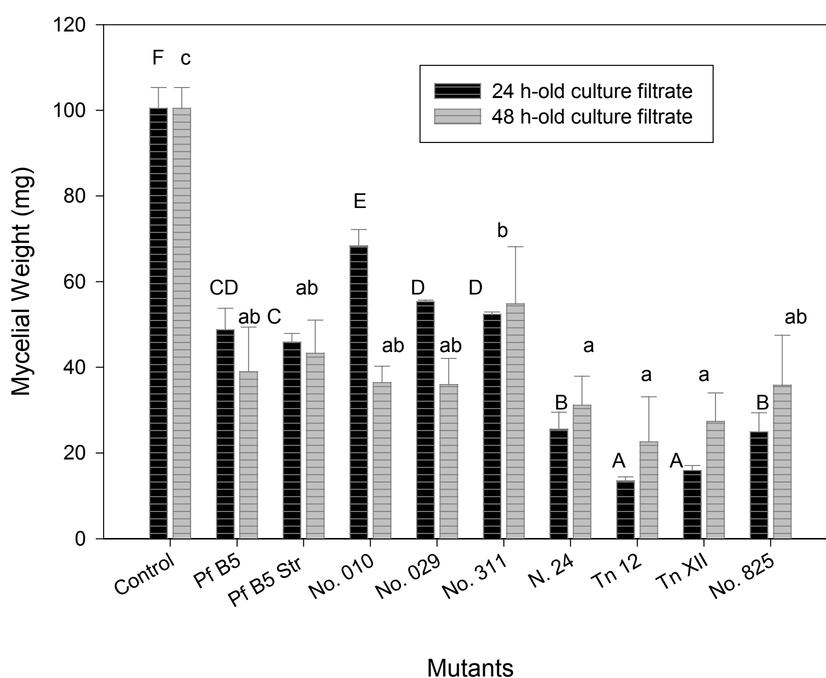

Figure 7. Effect of culture filtrate of antifungal metabolite-overproducing mutants of *Ps. fluorescens* B5 against *P. ultimum in vitro* (bacteria grown in KB^+ medium pH 7.0, 20°C, 100 rpm, harvested 24 h and 48 h after inoculation, 5 replications each, whole experiment repeated 2 times. Same shaded bars with the same symbols are not significantly different at P< 0.05).

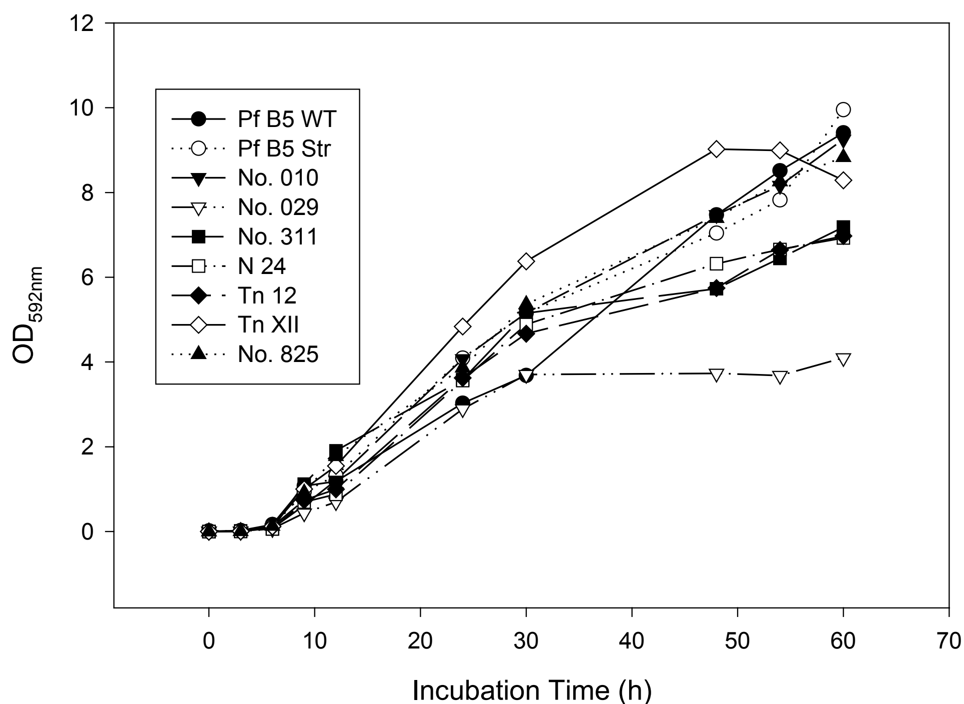

Figure 8. The growth of antifungal metabolite- overproducing mutants of *Ps. fluorescens* B5 (LB, pH 7.0, 20°C, 100 rpm).

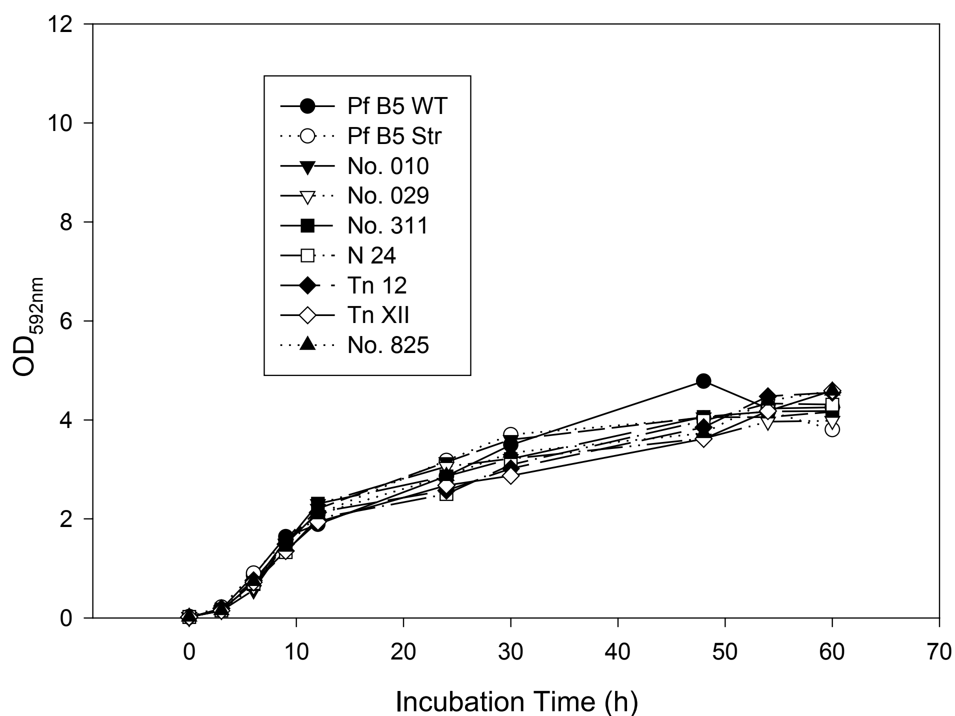

Figure 9. The growth of antifungal metabolite-overproducing mutants of *Ps. fluorescens* B5 (KB $^{+}$, pH 7.0, 20°C, 100 rpm).

Table 3. Doubling time of the antifungal metabolite - overproducing mutants

Mutants	Doubling Time (h) in	
	LB	KB^+
Ps. fluorescens B5 WT	1.07	1.86
Ps. fluorescens B5 ^{Str}	1.01	2.11
No. 010	0.93	1.76
No. 029	0.68	1.85
No. 311	0.95	1.87
N 24	0.76	1.92
Tn 12	0.70	2.04
Tn XII	0.86	1.87
No. 825	0.88	1.87

3.1.2.2. Growth

Great inhibition zones in dual culture and high antifungal activity of culture filtrates may also be due to the better growth of mutants, therefore growth was evaluated as well. All tested mutants grew faster in LB media and cfu in stationary growth phase was higher than in KB^+ media. In KB^+ media, no difference in growth behaviour among tested mutants was detected. In LB, except for No. 029 and Tn XII, the growth of most mutants was similar to wild type of *Pseudomonas fluorescens* B5. Mutant No. 029 grew slower and cfu in stationary phase was lower than cfu of Pf B5 WT in LB media. On the contrary, mutant Tn XII grew faster and growth estimated during stationary phase was better than that of Pf B5 WT in LB media (Figure 8 and 9). In LB media, all mutants reached stationary phase of growth after 30 h, whereas in KB^+ media this phase started already after 20 h.

Bacterial growth can also be characterised by comparing doubling or generation time of the mutants during exponential growth phase. Doubling time of wild type strain of *Ps. fluorescens* B5 and tested mutants are shown in

Table 3. The results indicated that doubling times of all mutants in LB are slightly shorter compared to Pf B5 WT. In KB^+ media, doubling time of the mutants and Pf B5 WT, except for Tn 12 and Pf B5 ^{Str}, are nearly the same.

3.1.2.3. Bioassay of volatile antifungal substances

It is well known that antagonistic fluorescent pseudomonads can produce volatile antifungal substances such as HCN and ammonia, therefore a bioassay of volatile antifungal substances produced by mutants against *Pythium ultimum* was conducted.

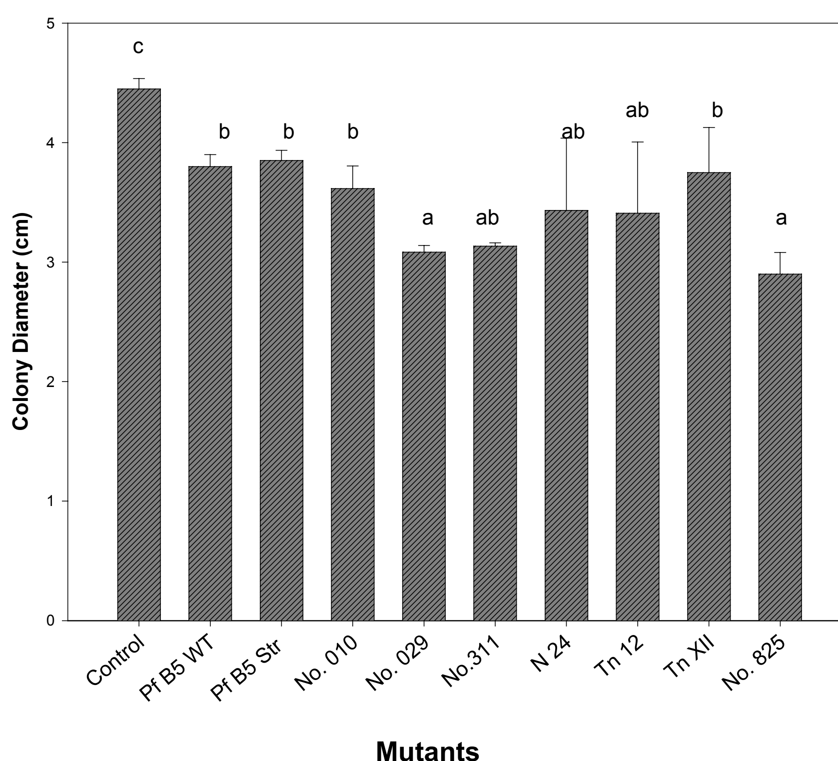

Figure 10. Effect of volatile antifungal substances produced by mutants of *Ps. fluorescens* B5 on the growth of *P. ultimum in vitro* (split plate petri dishes, 20°C, bacteria on KB^+, *P. ultimum* on PDA, pH 5.5) Bars with the same symbols are not significantly different at $P<0.05$.

The bioassay, indicated by the growth of *P. ultimum*, shows that only the mutants No. 029, No. 311 and No. 825 showed a significantly stronger production of volatile antifungal substances compared to Pf B5 WT.

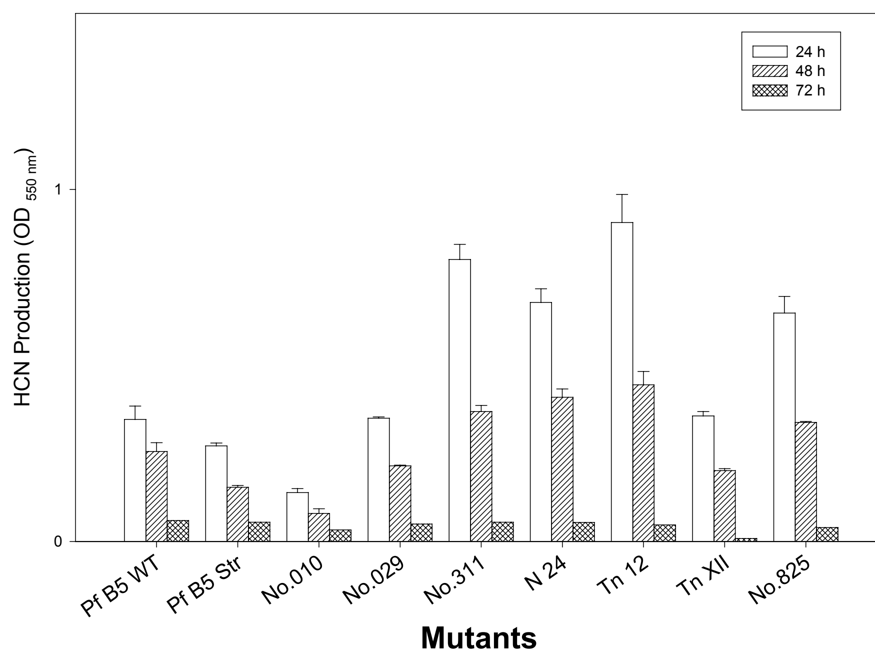

Figure 11. HCN production of antifungal metabolite-overproducing mutants of *Ps. fluorescens* B5 detected in 24 h, 48 h, and 72 h old culture filtrate (HCN detection kit, Merck, Germany).

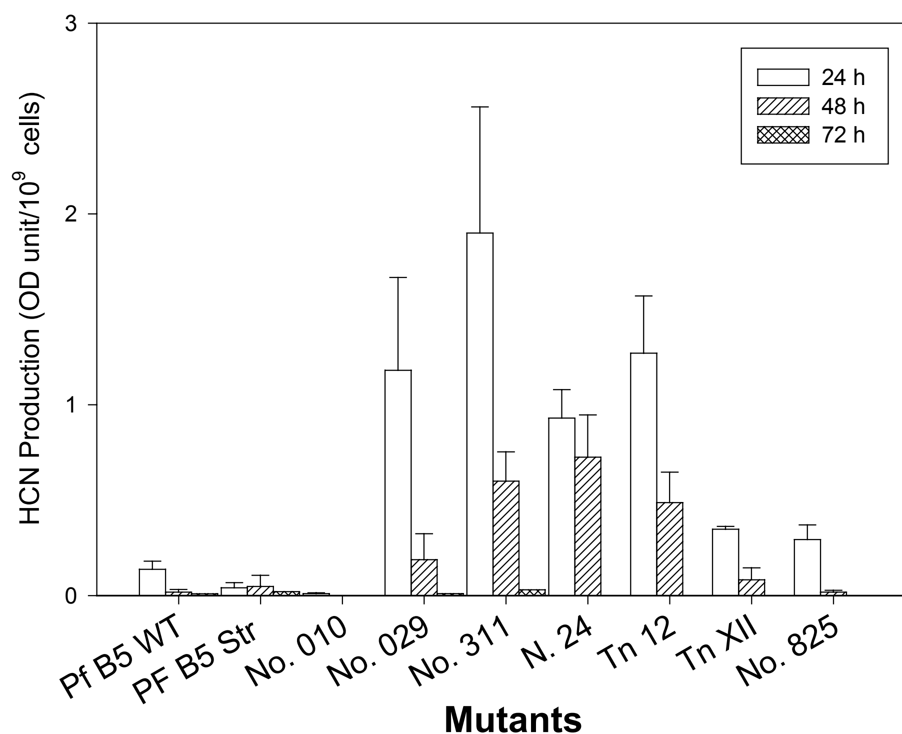

Figure 12. HCN production per 10^9 cells of antifungal metabolite-overproducing mutants of *Ps. fluorescens* B5 detected in 24 h, 48 h, and 72 h old culture filtrate (HCN detection kit, Merck, Germany).

3.1.2.4. HCN production

HCN is a known antifungal substance produced by various biocontrol strains of fluorescent pseudomonads and therefore HCN production of mutants production was investigated *in vitro.* It was obvious from Figure 11 that mutants No. 311, N 24, Tn 12 and No. 825 produced HCN *in vitro* to a greater extent than wild type strain of *Ps. fluorescens* B5. The highest level of total HCN *in vitro* was achieved by Tn 12 and followed by No. 311, N 24, 825, Tn XII, and No. 29 respectively.

However, a ranking among the tested mutants is quite different, when HCN production is related to cell number. The highest level of HCN per 10^9 cells was achieved by mutant No. 311 followed by Tn 12, No. 029, No. 24, Tn XII, No. 825, Pf B5 WT, Pf B5 ^{Str} and No. 010, respectively (Figure 12).

3.1.2.5. Indole-3-acetic acid (IAA) production

Indole-3-acetic acid (IAA) is one of the substances commonly produced by antagonistic and PGPR strains of fluorescent pseudomonads and production by mutants was also studied in the present work. IAA production *in vitro* as measured by colorimetric method (Figure 15) was variable among the tested mutants ranging from 13.7 µg/ml to 76.1 µg/ml (Figure 13). Only the mutants No. 029 and No. 311 produced more total IAA than wild type strain of *Ps. fluorescens* B5.

IAA production per 10^9 cells was higher for the mutants No. 029 No. 311, N 24 and Tn 12 compared to *Ps. fluorescens* B5 and the other tested mutants. But the level of IAA production per 10^9 cells of No 010 and No. 825 was lower compared to Pf B5 WT (Figure 14).

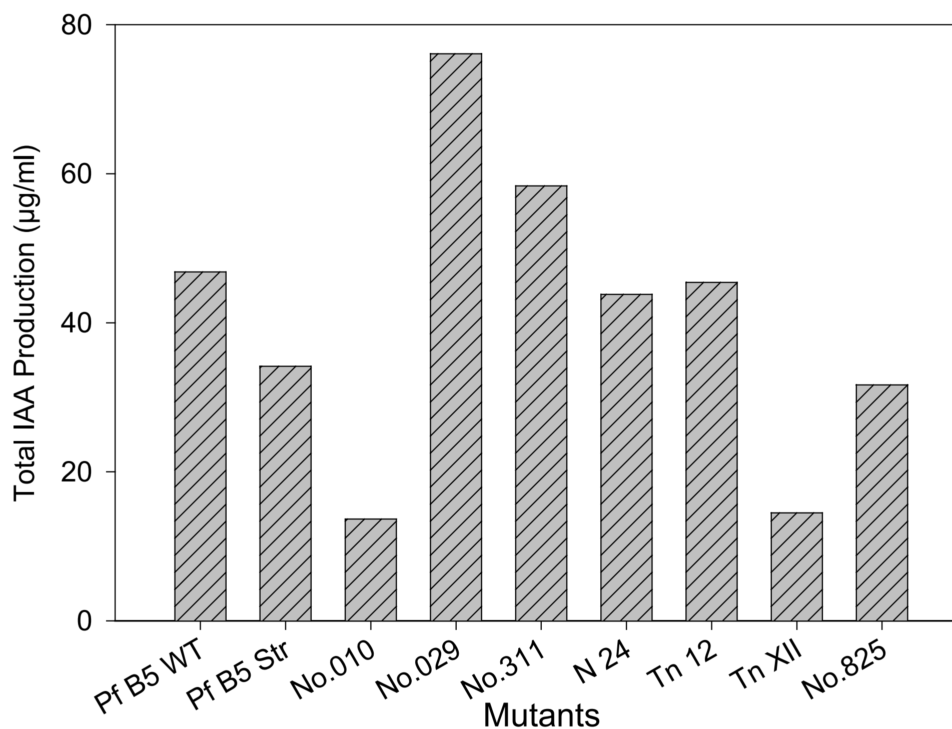

Figure 13. Total production of IAA *in vitro* of the antifungal metabolite-overproducing mutants and wild type of *Ps. fluorescens* B5.

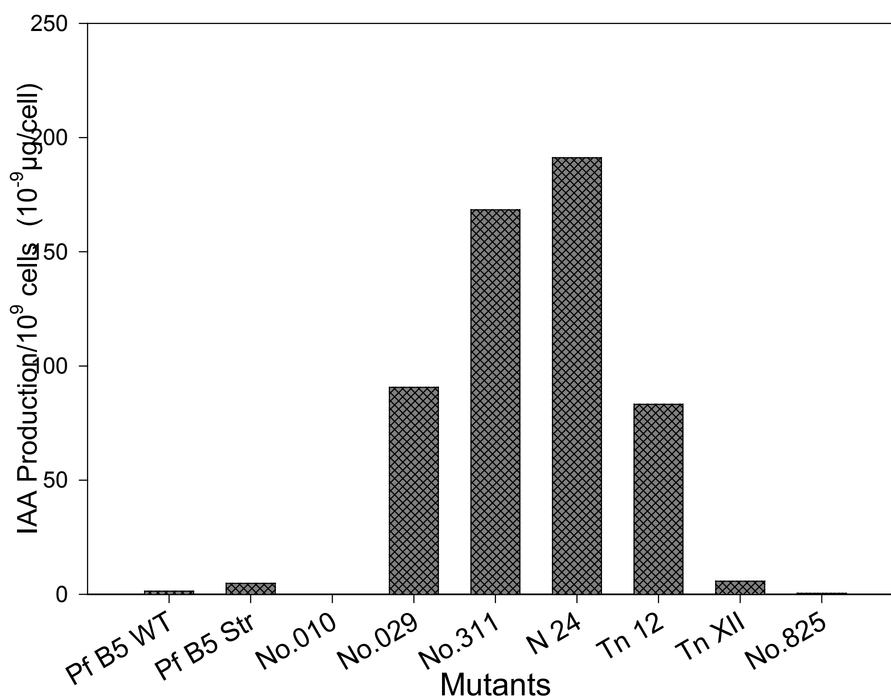

Figure 14. IAA production *in vitro* per 10^9 cells of the antifungal metabolite-overproducing mutants and wild type of *Ps. fluorescens* B5.

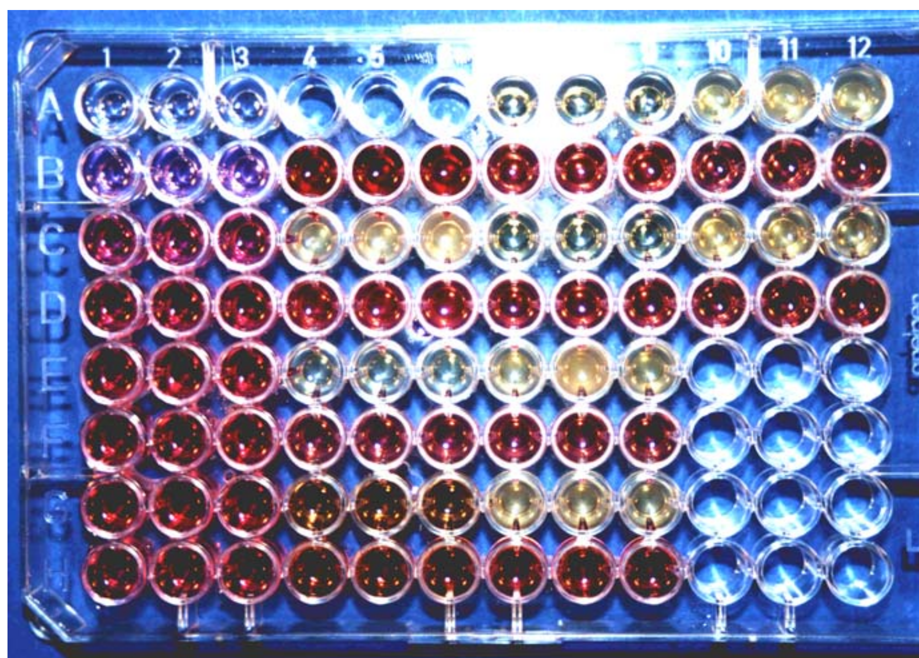

Figure 15. Determination of IAA production of antifungal metabolite-overproducing mutants of *Ps. fluorescens* B5. Red colour of the reaction medium indicates positive samples and yellow colour a negative sample of IAA.

3.1.2.6. Motility

Motility of mutants was also characterised in this study. Figure 16 shows the performance of non-motile and motile mutants on semi-liquid tryptone agar.

The result shows the variability of motility of the tested mutants compared to wild type strain of *Ps. fluorescens* B5 (Table 4). The motility of the mutants No. 825 and Tn XII were not significantly different to Pf B5 WT, but N 24 and Tn 12 showed a significantly higher motility.

Table 4. Motility of antifungal metabolites - overproducing mutants

Mutants	Motility [1 day-old swarm diameter (in mm) on semi liquid medium, pH 7, at 20°C]
Ps. fluorescens B5 WT	19.25 c
Ps. fluorescens B 5 ^{Str}	26.25 d
No. 010	10.87 b
No. 029	6.25 a
No. 311	9.88 ab
N 24	25.25 d
Tn 12	25.25 d
Tn XII	19.25 c
No. 825	22.75 c

Values in followed by the same symbols are not significantly different with DMRT test (P<0.05), 5 replications.

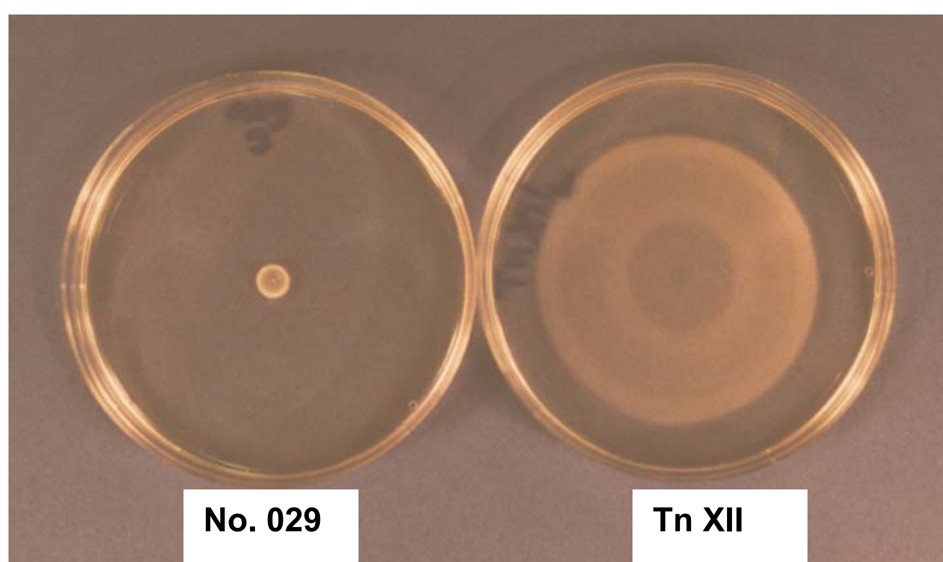

Figure 16. Swarm diameter on semi liquid agar showing motility (No 029:non motile, Tn XII: motile).

3.1.3. Root colonisation

The mutants were also characterised for their colonisation ability both in rhizosphere and rhizoplane, under controlled conditions without pathogen inoculation. Concerning to the rhizosphere colonisation, there was no significant difference among the tested mutants (Figure 17).

There was only a tendency of higher rhizoplane colonisation of mutant Tn 12, Tn XII and No. 825. Mutant No. 029 was found to be a very weak rhizosphere and rhizoplane coloniser.

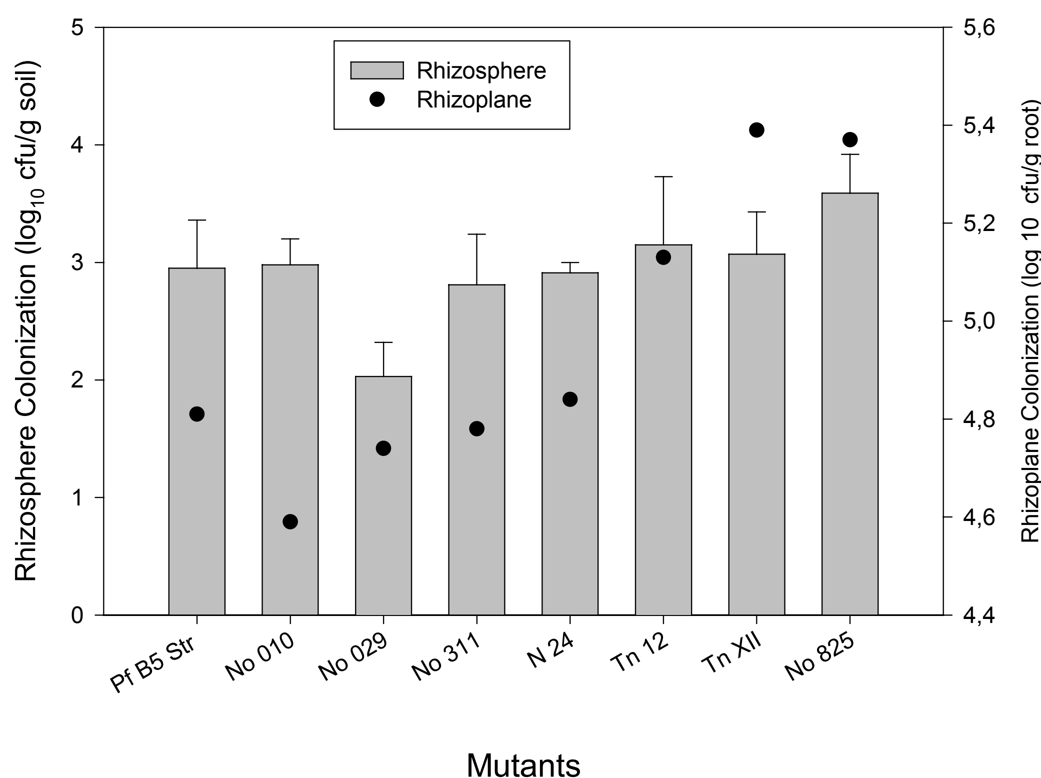

Figure 17. Colonisation of antifungal metabolites-overproducing mutants of *Ps. fluorescens* B5 under controlled conditions (T=18°C, photoperiod of L:D = 18:6, soil moisture = 60% of field capacity, without *Pythium* infestation). Each treatment was four times replicated with the whole experiment repeated two times.

3.1.4. Antagonistic activity of mutants *ad planta*

The antifungal metabolites-overproducing mutants were tested in biocontrol assay in the system *Pythium ultimum* – sugar beet – antagonist (mutant) under controlled conditions. To obtain more reliable data, *ad planta* experiments were repeated twice. The results of the *ad planta* test are shown in Figure 18 and 19. Only three mutants (Tn 12, No. 311 and N 24) showed a significantly higher ($P < 0.05$) antagonistic activity than Pf B5 WT and Pf B5 ^{Str} The mutants Tn 12, No. 311 and N 24 could suppress the disease to an extent of 74 %, 64 % and 60 %, respectively, while suppression achieved by Pf B5 WT and Pf B5 ^{Str} was only 45 % and 43 % respectively.

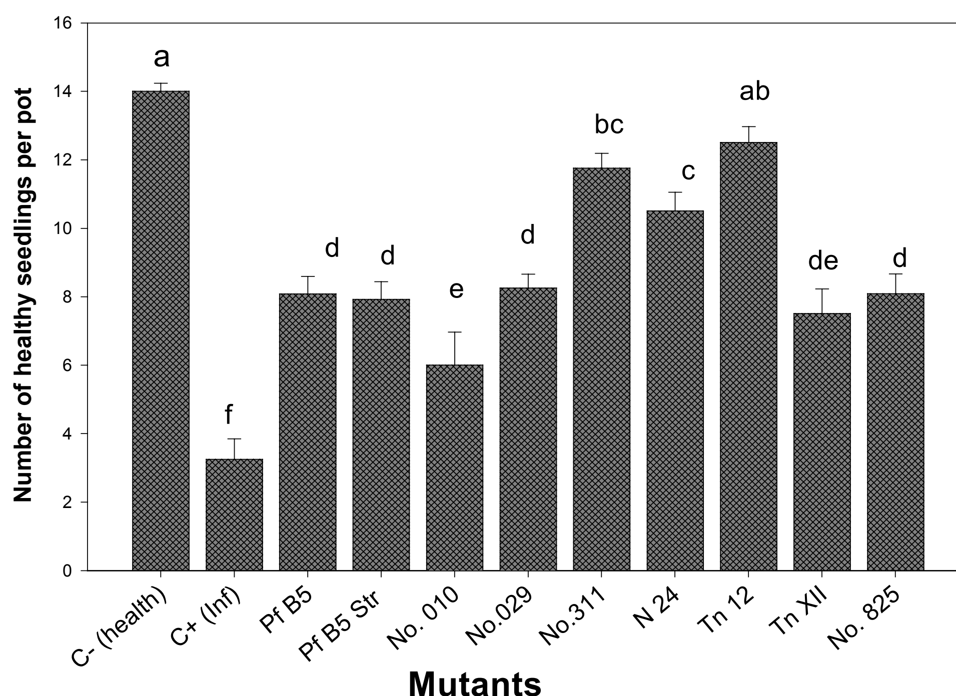

Figure 18. Efficacy of antifungal metabolites-overproducing mutants of *Ps. fluorescens* B5 against damping off caused by *P. ultimum* *ad planta* under controlled conditions (T = 18°C, photoperiods of L:D = 18:6, soil moisture = 60% of field capacity, six replications each and the whole experiment was repeated two times). Number of sown seeds per pots =16. Bar with the same symbols are not significantly different at $P < 0.05$ according to DMRT test.

Figure 19. Improvement of biocontrol activity of *Ps. fluorescens* B5 *ad planta* by applying antifungal metabolite-overproducing mutants under controlled condition in pot test (Tn 12 show a higher control than Pf B5 WT, C-=healthy control, C+= Pythium-inoculated control)

Mutants No. 825, Tn XII, and No. 029 showed no significant difference in their antagonistic activity *ad planta* compared to Pf B5 WT. The mutant No. 010 led to lower biocontrol *ad planta* than both standard strains Pf B5 and Pf B5 ^{Str}.

3.1.5. Relationship of mutant's *in vitro* physiological traits and their efficacy *ad planta* of tested mutants

An important result of the present study was the development of a correlation matrix of *in vitro* physiological traits of tested mutants and antagonistic activity *ad planta* (Table 5). The analysis identified *in vitro* physiological traits of mutants, which have a close relationship to the antagonistic activity *ad planta.* Among 9 tested mutants (including Pf B5 WT and Pf B5 ^{Str}), with 13 characterized traits *in vitro*, antagonistic activity *ad planta* correlated significantly with total HCN production *in vitro* ($r=0.75$, $P<$

0.001), HCN production/10^9 cells (r=0.69, P< 0.001), total IAA production i*n vitro* (r=0.47, p< 0.05), IAA production/10^9 cells (r=0.60, P <0.05) and mycelial growth reduction *in vitro* in KB^+-based culture filtrate (r=0.64, P< 0.01) (Table 5).

Regarding to the colonisation both in rhizosphere and rhizoplane, the result shows there was no significant correlation between the two types of colonisation and the antagonistic activity *ad planta*. Two types of colonisation correlated significantly to antibiosis - related properties. Rhizosphere colonisation correlated positively and significantly with the inhibition zone on TSA medium, mycelial growth reduction with LB-culture filtrate and motility. Rhizoplane colonisation had a positive siginificant correlation with mycelial weight reduction in LB-based culture filtrate and motility. Both colonisation types correlated to each other.

Table 5 also shows a significant correlation among *in vitro* physiological traits of mutants. For instance, total HCN production *in vitro* correlated significantly to mycelial growth reduction of *P. ultimum* in bioassay with volatile substances (r=0.55, P< 0.05), and inhibition zone in TSA test (r=47, P< 0.05). Moreover, total IAA production *in vitro* and IAA production/10^9 cells correlated significantly with HCN production/10^9 cells (each with r=0.68, P< 0.01 and r=0.63, P < 0.01), and fungal growth reduction in KB^+-based culture filtrate (r=0.67, P< 0.01).

Table 5. Correlation matrix of *in vitro* physiological traits of mutants and antagonistic activity and colonisation *ad planta*

	APE	DT-LB	DT-KB	IZ-TSA	IZ-KBA	MWR-CF-LB	MWR-CF-B	HCN	REL HCN	IAA	REL IAA	V-B	Motility	RC
Ad Planta Efficacy (APE)	-													
Doubling Time LB(DT-LB)	ns	-												
Doubling Time KB (DT-KB)	ns	ns	-											
Inhibition Zone –TSA (IZ-TSA)	ns	ns	ns	-										
Inhibition Zone –KBA (IZ-KBA)	ns	-0.64**	ns	ns	-									
Mycel. Weight Red. CF-LB (MWR-CFLB)	ns	ns	0.71**	ns	ns	-								
Mycel Weight Red. CF-KB (MWR-CFKB)	0.64**	-0.65**	ns	ns	ns	ns	-							
Total HCN Production (HCN)	0.75***	ns	ns	0.47*	ns	ns	ns	-						
HCN/10^9 cells(RELHCN)	0.69***	-0.50*	ns	ns	ns	ns	ns	0.74**	-					
Total IAA Production (IAA)	0.47*	ns	ns	ns	ns	ns	0.67**	ns	0.68**	-				
IAA/10^9 cells (REL IAA)	0.60*	ns	ns	ns	ns	ns	ns	ns	0.75**	0.48*	-			
Fungal Growth reduction-Volatile Bioassay (V-B)	ns	ns	ns	ns	ns	ns	ns	0.55*	ns	ns	ns	-		
Motility	ns	ns	0.72**	ns	ns	ns	ns	ns	ns	ns	ns	ns	-	
Rhizosphere Colonisation (RC)	ns	ns	ns	0.72**	ns	0.53*	ns	ns	ns	ns	ns	ns	0.66**	-
Rhizoplane Colonisation	ns	ns	ns	ns	ns	0.75***	ns	ns	ns	ns	ns	ns	0.50*	0.61**

Note: N=18, Pearson's coefficient is used. ns=non significant, * significant at P< 0.05, ** significant at P< 0.01, *** significant at P< 0.001

3.2. The role of indole-3- acetic acid (IAA) in the biocontrol activity of Ps. *fluorescens* B5 against damping-off of sugar beet seedlings caused by *P. ultimum*

3.2.1. Correlation between IAA production *in vitro* and antagonistic activity a*d planta* of tested mutants

As mentioned in chapter 3.1 that wild type of *Ps. fluorescens* B5 and its mutants produced considerable amounts of IAA *in vitro*. Further experiment conducted in controlled environment shows that total IAA production *in vitro* was significantly correlated (r= 0.47, P< 0.05) to antagonistic activity of corresponding mutants *ad planta* (Figure 20). IAA production/10 ^{9} cells *in vitro of the mutants co*rrelated significantly to their antagonistic activity ad planta (r=0.60, P<0.05). In addition, total IAA production *in vitro* of mutants correlated to HCN production/10 ^{9} cells *in vitro*. This seems to be an indication for the importance of IAA production *in vitro* in biocontrol activity of Pf B5.

3.2.2. Antifungal activity of IAA in *vit*ro

A further study was conducted to assess the role of IAA in the biocontrol of *P. ultimum* of sugar beet seedling by using *Ps. fluorescens* B5. Biossay *in vitro* using solid media (PDA) and liquid media (PDB) demonstrated that IAA alone is able to inhibit the growth of *P. ultimum* (Table 6, Figure 22; Figure 23). Fungicidal effect of IAA against *P. ultimum in vitro* was obviously detected at the concentration of 50 µg/ml in PDA and 12.5 µg/ml in PDB. Moreover antifungal activity against *P. ultimum in vitro* was also provided by other indole plant growth regulators such as indole-3-butyric acid (IBA) and indole-3-propionic acid (IPA) (Figure 22, 23; Table 6). Two of later indole substances even resulted in significantly stronger antifungal effect compare to IAA (Table 6).

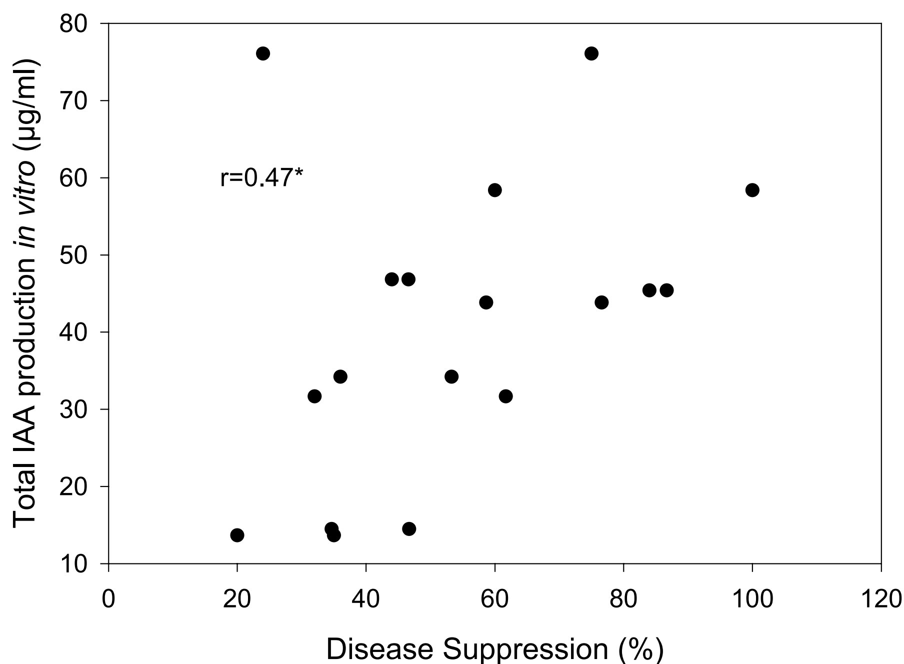

Figure 20. Correlation between total IAA production of mutants in *vitro* and their biocontrol activity against damping-off caused by *Pythium ultimum ad planta (*N=18, * significant at $p< 0.05$*)*

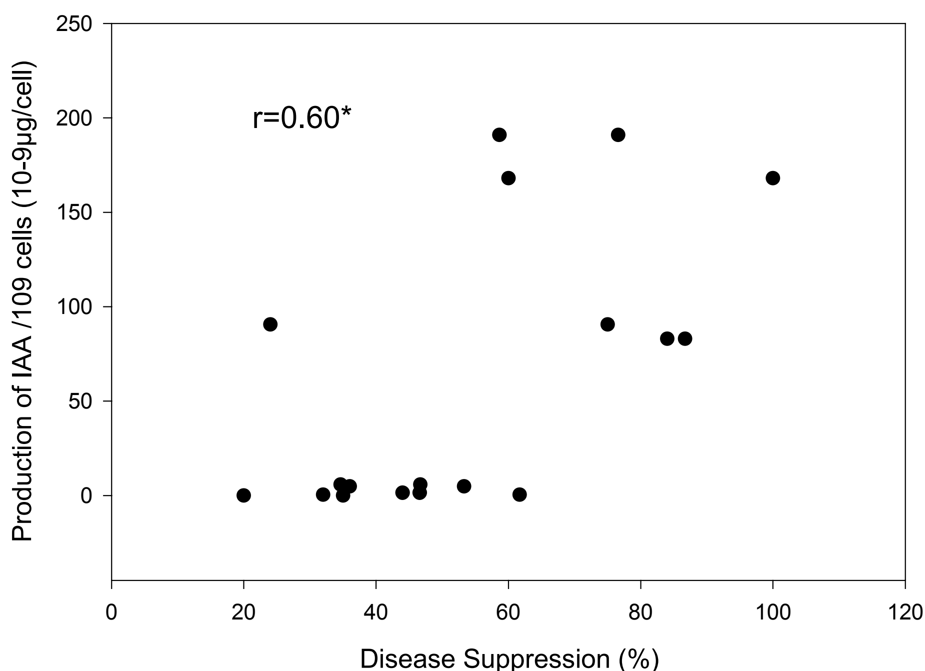

Figure. 21. Correlation between IAA production per 10^9 cells of mutants *in vitro* and their biocontrol activity *ad planta (*N=18, * significant at $p< 0.05$)

3.2.3. Antifungal activity of IAA *ad planta*

Experiment *ad planta* with seed dipping shows that IAA alone at the concentration of 50 µg/ml could suppress damping-off of sugar beet seedling caused by *P. ultimum* (Figure 24, Table 7). In addition, other indole plant growth regulators *i.e.* IBA and IPA with certain concentration can also directly suppress damping off *ad planta* (Table 7).

Table 6. The antifungal effect of indole -3 -acetic acid (IAA), indole-3- butyric acid (IBA), and indole -3- propionic acid (IPA) against *P. ultimum in vitro* (PDB pH 5.5, 20° C, 100 rpm, 48 hours)

Concentration (µg/ml)	**Mycelial Weight (mg)**		
	IAA	**IBA**	**IPA**
0	89.78 j	89.78 j	89.78 j
6.25	81.25 i	22.49 f	26.25 f
12.5	63.00 hi	11.33 e	10.45 e
25	49.25 ghi	5.35 cd	9.25 de
50	34.50 fg	3.70 c	3.47 c
75	37.25 fgh	1.25 b	4.53 c
100	15.00 fg	0.00 a	5.10 cd

Values in followed by the same symbols are not significantly different with DMRT test ($p < 0.05$)

Treatment of exogenous IAA together with *Ps. fluorescens* B5 at the low concentration (12.5 µg/ml) tended to enhance the protection of sugar beet seedlings by Pf B5 against *P. ultimum* (Figure 24). On the contrary, in higher concentration, IAA reduced antagonistic activity, and reduction starting to be significant ($P < 0.05$) at 100 µg/ml.

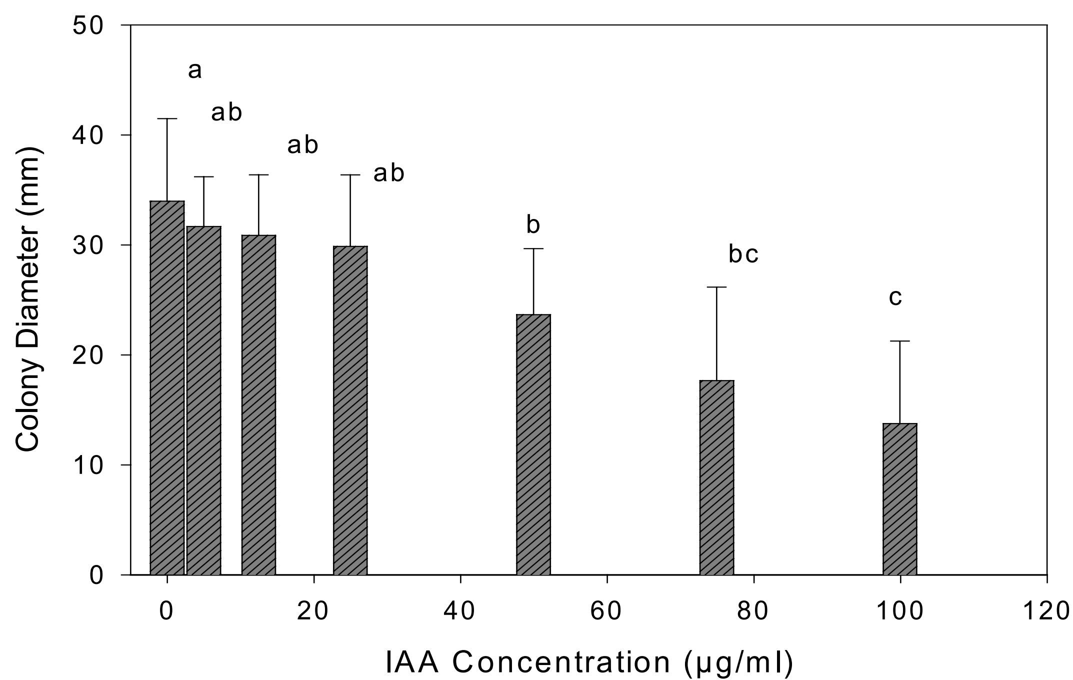

Figure 22. Antifungal effect of IAA against *P. ultimum in vitro* (PDA *)*

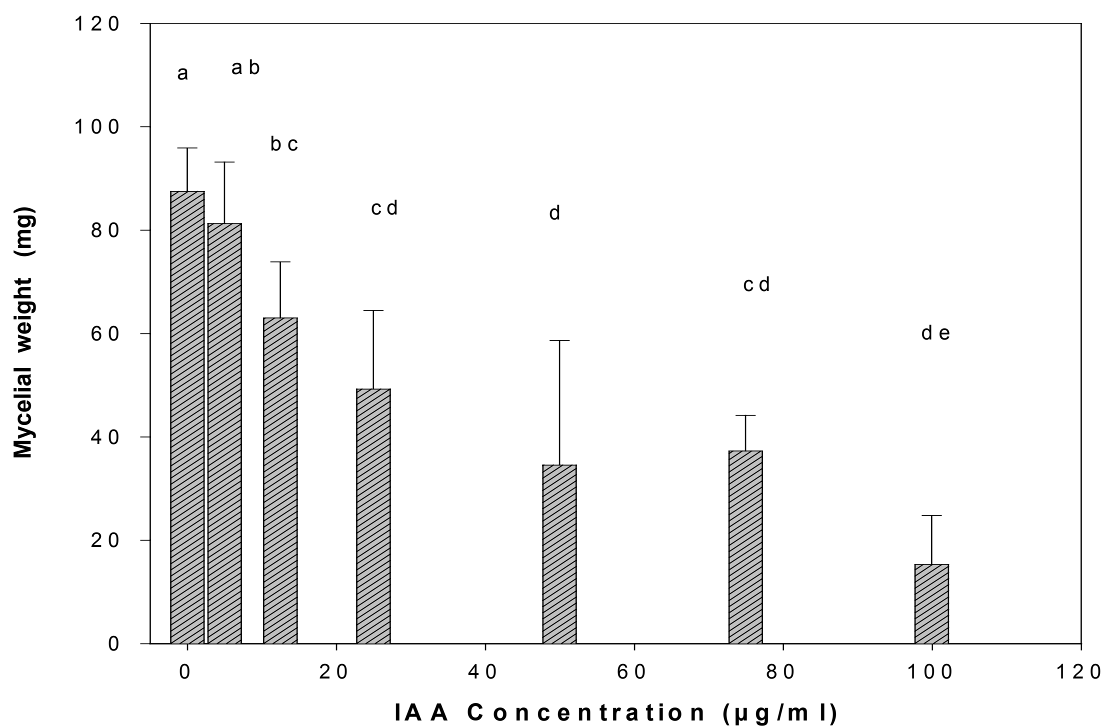

Figure 23. Antifungal effect of IAA against *P. ultimum in vitro* (PDB*)*

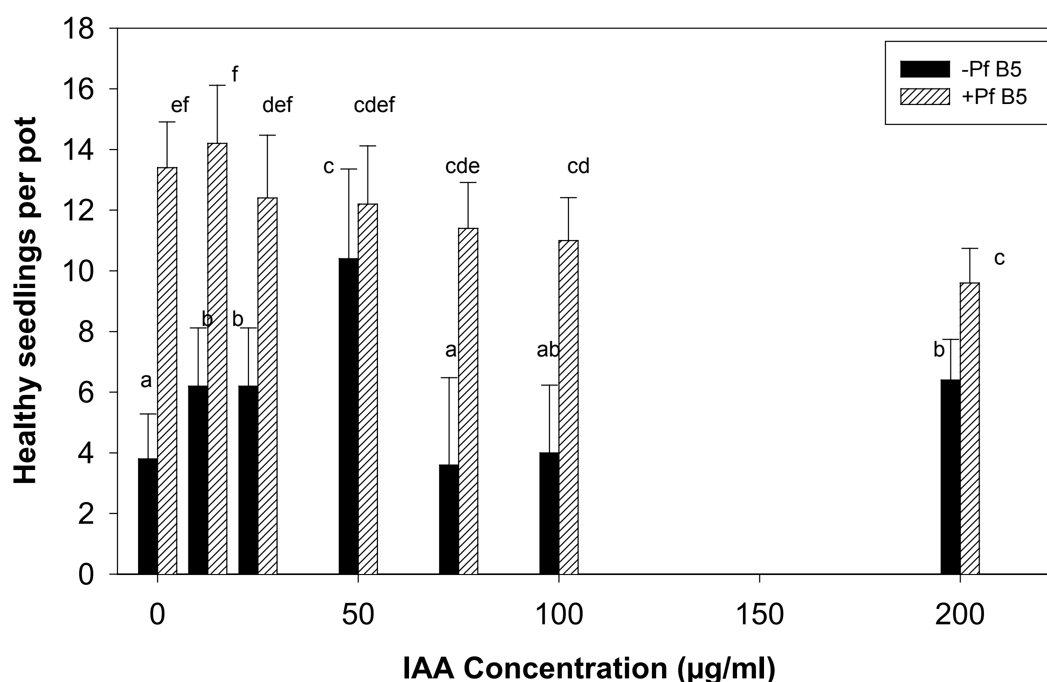

Figure 24. Effect of exogenous IAA treatment and its combination with *Ps. fluorescens* B5 on the damping- off of sugar beet caused by *P. ultimum ad planta* (number of seedlings per pot=16)

3.2.4. Effect of IAA on the seedling's emergence and growth

IAA is a famous plant growth regulator; hence its effect on sugar beet seedling's growth was also investigated. Experiment *ad planta* without pathogen infestation shows that addition of exogenous IAA, applied by seed dipping technique provided different response of different growth traits of sugar beet seedling. All of tested range of IAA treatment did not influence the germination rate and seedling's height of treated seedlings (Figure 25 and 26). In contrast, Figure 27 demonstrates the increase of fresh weight of seedling's shoot due to exogenous IAA at the rate of 25 µg/ml, and no more effect at higher tested concentration. Pf B5 WT treatment alone did not increase significantly seedling's height, but it increased significantly seedling's shoot weight. Generally combination treatment of Pf B5 WT and IAA 100 µg/ml did have any effect on the observed growth parameters of seedlings.

Table 7. Effect of IAA, IBA and IPA on the damping- off of sugar beet *ad planta* under controlled environment

Concentration (µg/ml)	**Number of healthy seedlings (n=16)**		
	IAA	**IBA**	**IPA**
0	0.00 a	0.00 a	0.00 a
6.25	2.25 bc	1.25 ab	1.75 b
12.5	0 a	0 a	0 a
25	0.50 a	2.00 b	1.00 ab
50	3.00 c	1.25 ab	0.50 a
75	0 a	2.25 b	0.75 a
100	0.75 a	0.5 a	0.25 a

Numbers followed by the same symbols are not significantly different according DMRT test ($p<0.05$)

Table 8. Effect of application of *Pseudomonas fluorescens* B5 WT and its mutants on the emergence and growth of sugar beet seedlings (without pathogen infestation, controlled environment) [1,4]

Mutants	**Emergence (%)[3]**	**Height (cm) [3]**	**Total Weight (g) [3]**	**Individual Seedling's Weight (g) [3]**	**Root Length [2] (cm)**
Control	96.21 b	4.14 a	2.83 b	0.27a	1.27 b
Pf B5 WT	81.25 a	4.29 a	2.34 b	0.34 bc	0.77 a
No. 010	96.71 b	4.33 a	2.79 b	0.29 ab	1.30 b
No. 029	91.33 ab	4.36 a	2.58 ab	0.30 ab	0.67 a
No. 311	79.79 a	4.17 a	2.18 a	0.38 c	0.60 a
N 24	91.50 ab	4.48 a	2.94 b	0.31 b	0.41 a
Tn12	87.33 a	3.99 a	2.72 b	0.29 ab	0.67 a
Tn XII	91.33 ab	4.95 b	2.54 ab	0.34 bc	0.69 a
No 825	88.54 ab	3.87 a	2.47 ab	0.27 a	0.60 a

1) Values followed by the same symbols in the same column are not significantly different according DMRT test ($P < 0.05$), 2) determined in petridish-bioassay according modified technique of Barazani and Friedman (1999), 3) Pot experiment under controlled environment, 4) Each treatment was six times replicated with the number of seed per pot=16

3.2.5. Effect of antifungal metabolites-overproducing mutants on the emergence and growth of seedlings

Since some strains of fluorescent pseudomonads promote or inhibit the growth of associated host, the effect of mutants on this aspect was also studied. Application of wild type *Pseudomonas fluorescens* B5 on sugar beet seeds, without *Pythium* infestation significantly reduced germination rate (Table 8). Some of the mutants also had the similar or even stronger inhibitory effect (No. 311 and Tn 12) than wild type. Tn XII treatments enhanced significantly seedling's height, and no observed influence of other mutants.

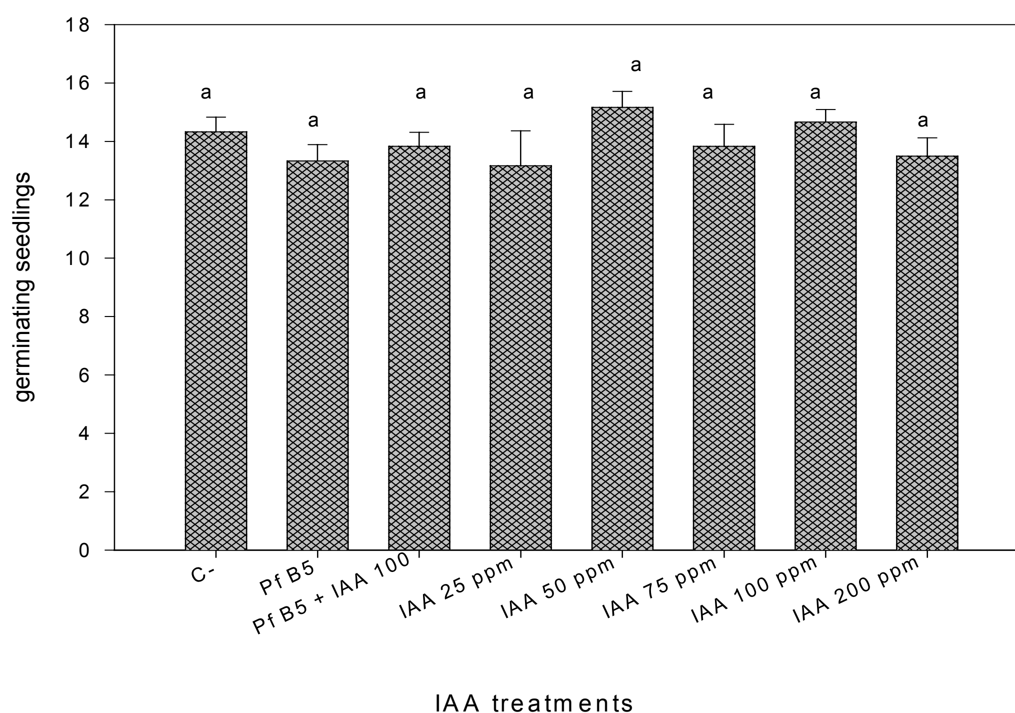

Fig 25. Effect of exogenous IAA application on the germination rate of sugar beet seedlings *ad planta* (without pathogen inoculation)

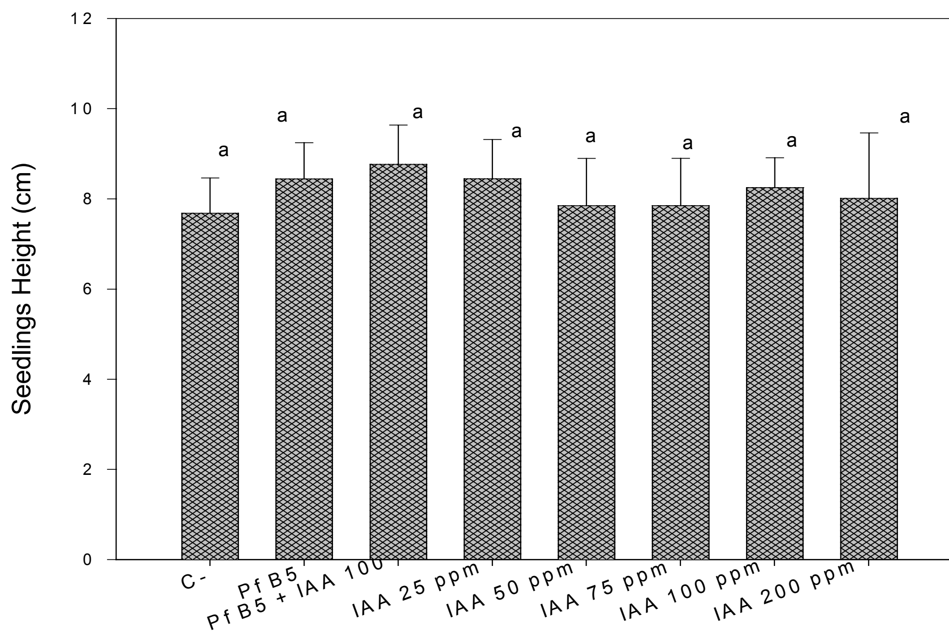

Figure 26. Effect of exogenous IAA application on the seedling's height of sugar beet *ad planta* (without pathogen inoculation)

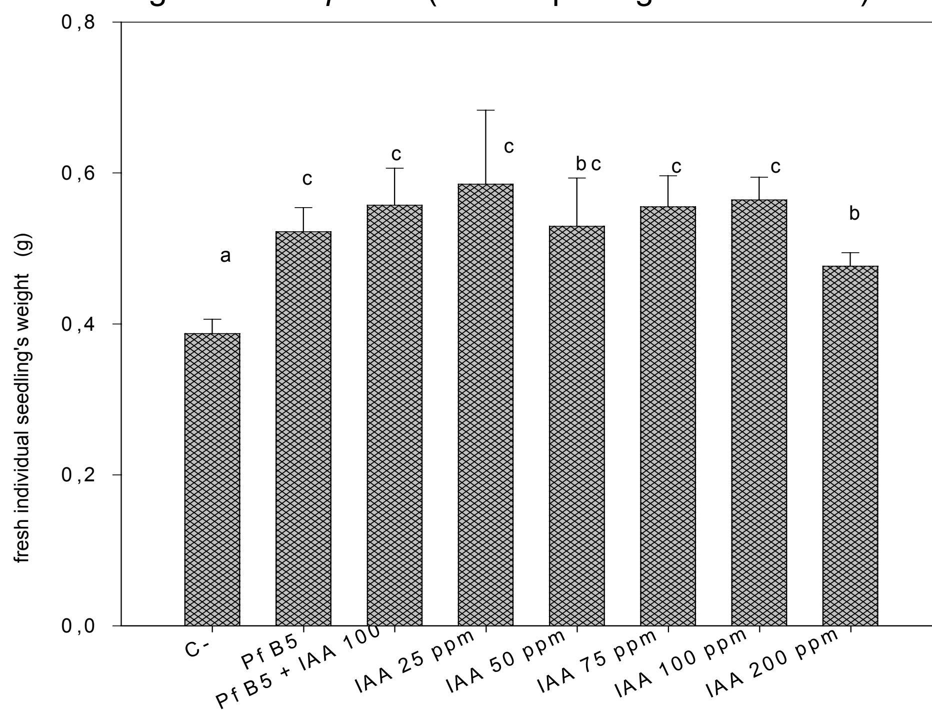

Figure 27. Effect of exogenous IAA application on the fresh individual seedling's weight (without pathogen inoculation)

Table 9. Correlation matrix of the growth of sugar beet seedlings treated with various mutants and IAA and HCN production of mutants *in vitro*

	Emergence	Height	Total Weight	Indiv. Weight	Root Length	Total IAA Prod. *in vitro*	IAA/ 10^9 cells *in vitro*	Tot HCN prod. *in vitro*
Emergence [1]								
Height [1]	ns							
Total Weight [1]	0.81*	ns						
Individual weight [1]	-0.77*	ns	-0.66*					
Root length [2]	ns	ns	ns	ns				
Total IAA Production *in vitro*	ns	ns	ns	ns	ns			
IAA/10^9 cells *In vitro*	ns	ns	ns	ns	-0.55*	ns		
Tot HCN production *in vitro*	-0.51*	ns	ns	ns	-0.57*	ns	ns	
HCN/10^9 cells *in vitro*	-0.60*	ns	ns	ns	ns	0.70*	ns	0.70*

Note : N=18, Pearson correlation coefficient is used, 1*)*determined under controlled environment in pot without *Pythium 2)*determined by bioassay in petri dish , *significant at $P < 0.05$

3.2.6. Correlation of some *in vitro* physiological features of mutants and the growth of treated seedlings *ad planta*

Correlation matrix of growth parameters of sugar beet seedlings treated with various mutants and total IAA production *in vitro* was performed (Table 9). This table shows that germination rate was negatively (significant at $p<0.05$) correlated to the total production of HCN *in vitro* and production of HCN/10^9 cells *in vitro*. It did not correlate to the total IAA production and production of IAA per 10^9 cells *in vitro.* Furthermore, negative significant correlation existed between the root length of sugar beet seedlings in petridish-bioassay and IAA production/10^9cells and total production of HCN *in vitro.*

3.3. The role of sugar beet varieties in biological control of *P. ultimum* by using *Pseudomonas fluorescens* B5

There is an existing phenomenon that host genotype can influence antagonist activity. The aim of the following studies was to investigate the role of sugar beet varieties on the antagonistic activity of *Ps. fluorescens* B5 and the involved mechanisms.

3.3.1. Efficacy of *Ps. fluorescens* B 5 against *Pythium ultimum ad planta* in different sugar beet varieties under controlled condition

To study the antagonistic activity of *Ps. fluorescens* B5 in different varieties, an *ad planta* experiment under controlled conditions was carried out. Analysis of variance (ANOVA) of the effect of sugar beet varieties on the activity of Pf B5 based on direct observed variables, *i.e.* the number of healthy seedlings, shows a very high significant interaction ($P<0.01$) between the application of Pf B5 and sugar beet varieties (Table 10). ANOVA on variables based on biocontrol activity or relative disease suppression (Table 11) gave a similar result, *i.e.* a high significant effect of the varieties ($P<0.01$). The results show that biocontrol activity of *Ps. fluorescens* B5 against *P. ultimum ad planta* depends on the sugar beet varieties.

Without antagonist (Pf B5) treatment, the tested varieties showed a variable susceptibility to *P. ultimum* (Figure 28). According to their resistance against damping-off, the sugar beet varieties can be classified into two groups. The first group consists of the fairly resistant varieties V6, V2, V4 and V7. The more susceptible varieties V1, V3, V5 and V8 form the second group. There was a significant difference in disease incidence between varieties in the two different groups, but not within the group. Because of the different resistance against damping-off detected among the tested varieties, the number of healthy plants is not an appropriate parameter to assess biocontrol activity of Pf B5. Disease suppression, which include the contribution of resistance of each variety in the calculation is more reliable.

Table 10. Analysis of variance of the effect of sugar beet varieties on biological control performance of *Ps. fluorescens* B5 against *P. ultimum ad planta* (based on number of healthy plants; 3 experiments with 4 replications each).

Effect	Degree of freedom	Mean square	Degree of freedom error	Mean square of error	F	P
Varieties	7	46.25	144	1.41	32.85	0.0000***
Pf B5	1	2707.51	144	1.41	1922.96	0.0000***
Time	2	0.69	144	1.41	0.49	0.6124
Varieties x Pf B5	7	9.42	144	1.41	6.69	0.0000***
Varieties x Time	14	0.95	144	1.41	0.68	0.7929
Pf B5 x Time	2	3.76	144	1.41	2.67	0.0728
Varieties x Pf B5 x Time	14	0.74	144	1.41	0.53	0.9132

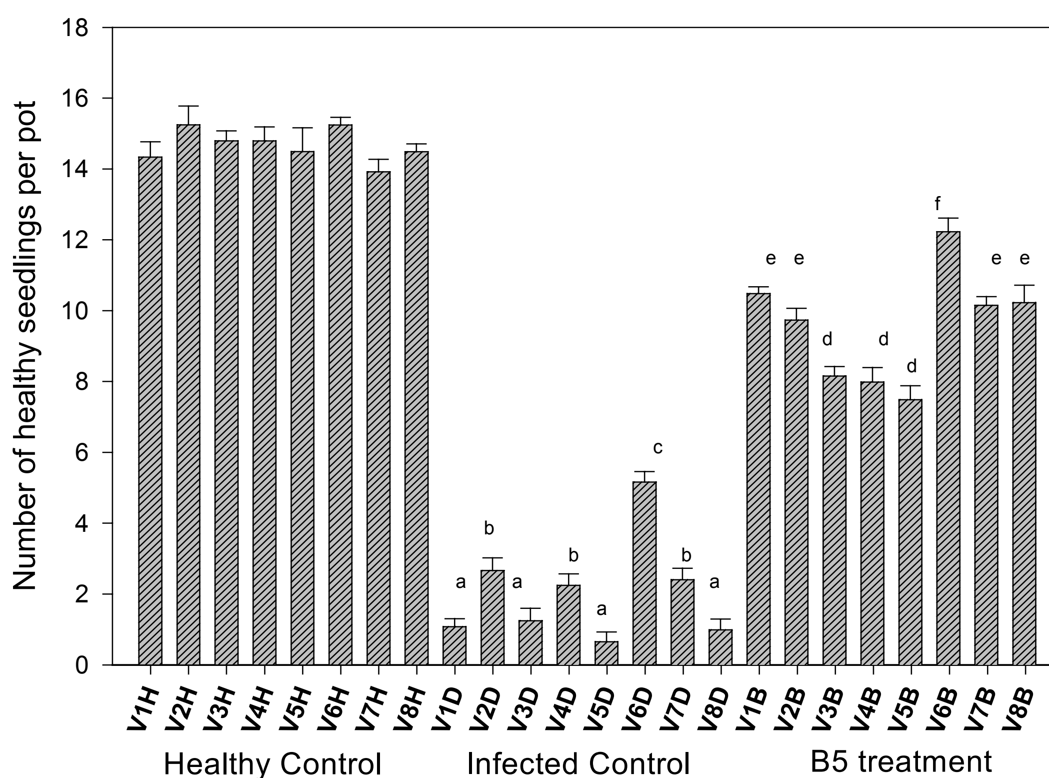

Figure 28. Biocontrol activity of *Ps. fluorescens* B5 against damping-off of sugar beet seedlings in different sugar beet varieties (based on total number of healthy seedlings, N=16). H=healthy control, D=only with pathogen, B=with Pf B5 with pathogen

Table 11. Analysis of variance of the effect of sugar beet genotypes on biological control performance of *Ps. fluorescens* B5 against *P. ultimum ad planta* (based on disease suppression, 3 experiments with 4 replications each, data are transformed to arc sin x)

Effect	Degree of freedom	Mean Square	Degree of freedom error	Mean square of error	F	p-level
Varieties	7	0.270	72	0.0229	11.795	0.0000****
Time	2	0.023	72	0.0229	1.036	0.3600
Varieties x Time	14	0.018	72	0.0229	0.784	0.6824

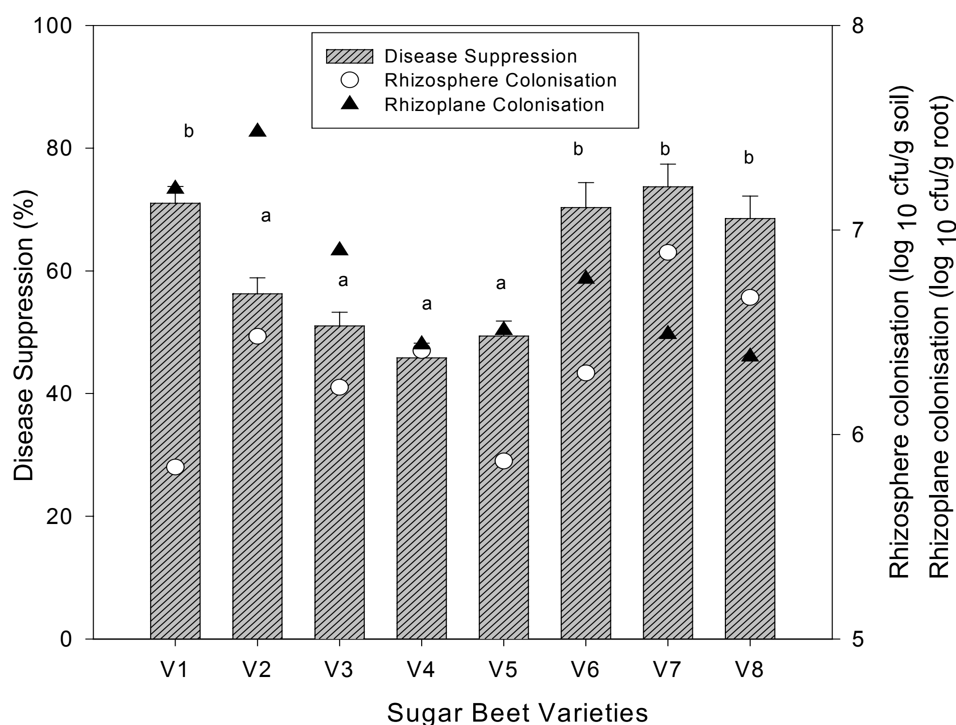

Figure 29. Biocontrol activity of *Ps. fluorescens* B5 against damping-off of sugar beet seedlings in different varieties (based on disease suppression calculated as DS = $((X-C^+/C^-C^+)x\ 100\%)$* and bacterial colonisation. Inoculum: mycelial dry weight of 100 µg/g soil. * X = number of healthy seedlings in the treatment, C^- = number of healthy seedlings in uninoculated control, C^+ = number of healthy seedlings of pathogen inoculated control

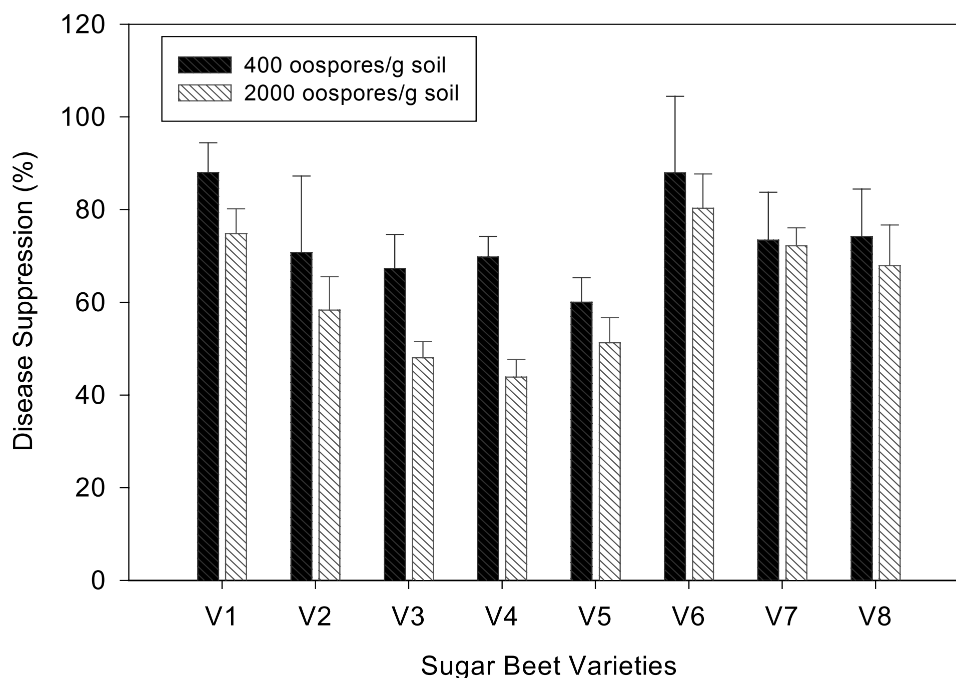

Figure 30. Biocontrol activity of *P. fluorecens* B5 in different sugar beet varieties with two concentration levels of pathogen inoculum

Biocontrol activity of Pf B5 with the eight tested varieties could be equally classified into two groups (Figure 29-30). The first group comprises varieties in which the biocontrol activity of Pf B5 is more than 65% (V1, V6, V7, V8). The second group comprises the varieties in which biocontrol activity of Pf B5 is less than 55% *i.e.* V2, V3, V4 and V5. There was a significant difference for biological control between the groups but not within the groups. Grouping of biocontrol activity is not always parallel to resistance grouping, as can be seen for variety V1, grouped into the susceptible group but supported high biocontrol activity of Pf B5.

3.3.2. Efficacy of *Ps. fluorescens* B 5 against *Pythium ultimum ad planta* in different sugar beet varieties with two levels of pathogen inoculum under controlled conditions

To investigate the influence of pathogen concentration on biocontrol performance of Pf B5 in different sugar beet varieties an *ad planta* experiment with two rates of *Pythium ultimum* inoculum (mycelial dry weight of 100 µg/g soil and 20 µg/g soil) was conducted. The effect of disease pressure levels and varieties acted separately and additively. There was no significant

interaction between pathogen concentration and sugar beet varieties on biocontrol activity of *Ps. fluorescens* B5 (Table 12). However, with low pathogen inoculum, the antagonistic activity of Pf B5 in the varieties V3 and V4 tend to be much higher than the additive effect. The experiment reveals that biocontrol activity of *Ps. fluorescens* B5 was higher with lower concentrations of the pathogen and *vice versa,* and this was valid for all of the tested varieties (Fig. 30).

Table 12. Analysis of variance of the effect of varieties and inoculum level of pathogen on biocontrol activity of *Pseudomonas fluorescens* B5

Effect	Degree of freedom	Mean Square	Degree of freedom error	Mean square of error	F	p-level
Varieties	7	0.3048	48	0.0848	3.59	0.003448***
Pathogen concentration	1	0.8481	48	0.0848	9.99	0.002718***
Varieties x Path. conc.	7	0.0202	48	0.0848	0.23	0.973374

*** significant at $P < 0.005$

3.3.3. Root colonisation

Colonisation is believed to be an important feature of antagonists related to its efficacy *ad planta*. Therefore a study on the colonisation of *Ps. fluorescens* B5 with eight sugar beet varieties was carried out. The result of the experiment as described in Figure 31 shows that colonisation both of the rhizosphere and rhizoplane was variable among the tested varieties. Rhizosphere colonisation is defined as the number of cells isolated from root-adhering soil. Rhizoplane colonisation refers to the number of bacteria in the root tissue isolated by root tissue maceration. With regard to the rhizosphere, best colonisation of Pf B5 was found with variety V7 and the worst with V5 and V1. A significant difference was found only between varieties V7 and V5/V1 ($P<0.05$). Moreover, the highest rate of rhizoplane colonisation was found for V2 and the lowest for V8, which was significantly different ($P<0.05$).

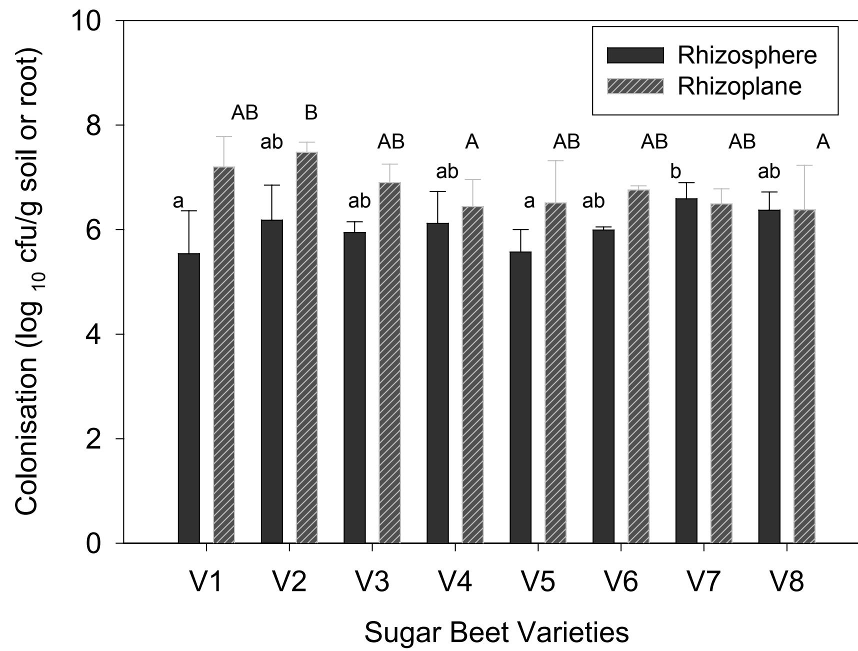

Figure 31. Rhizosphere and rhizoplane colonisation of *Ps. fluorescens* B5 on different sugar beet varieties (assessed at 13 days after sowing).

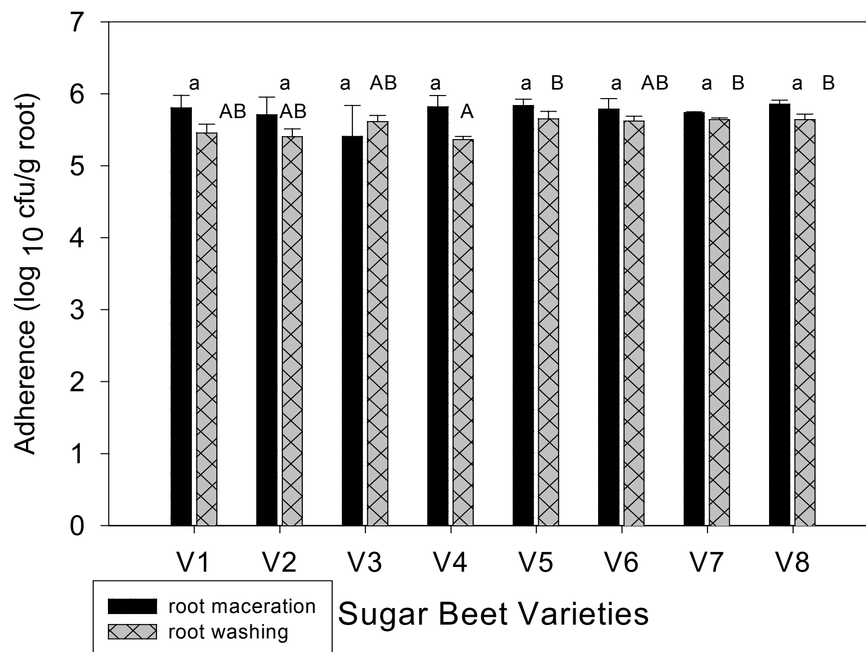

Figure 32. Adherence of *Pseudomonas fluorescens* B5 to the seedling roots of different sugar beet varieties.

3.3.4. Root adherence

Root adherence of antagonistic bacteria is considered as an important trait in root colonising ability. The experiment was done to study the root adherence of *Pseudomonas fluorescens* B5 on eight sugar beet varieties. Root adherence was classified into two groups according to Glandorf *et al.* (1994), *i.e*. type 1 (cell number obtained by gently washing of roots), type 2 (cell number isolated by root maceration). There was no significant difference in root adherence of type 1 among the tested varieties for *Ps. fluorescens* B5 (Figure 32). In contrast, a significant difference was found for the adherence type 2, where a significantly higher root adherence was found for variety V4 compared to V8.

3.3.5. Relation between antagonistic activity *ad planta of Pseudomonas fluorescens* B 5, colonisation and adherence on different sugar beet varieties

To assess the relation of adherence, colonisation, biocontrol activity *ad planta* of Pf B5 in different sugar beet varieties, correlation analysis was applied (Table 13). The correlation was made on 32 (N) pairs of data set of tested variables. There was no significant correlation of antagonistic activity and colonisation of different sugar beet varieties. In addition, adherence did not correlate to both types of colonisation.

Table 13. Correlation matrix of adherence, colonisation and antagonistic activity of *Ps. fluorescens* B5 *ad planta* against *P. ultimum* in eight sugar beet varieties. [1) 2)]

	Antagonistic activity	Rhizosphere colonisation	Rhizoplane colonisation	Adherence 1	Adherence 2
Antagonistic activity					
Rhizosphere colonisation	**0.31 ns**				
Rhizoplane colonisation	0.19 ns	-0.02 ns			
Adherence 1	0.26 ns	-0.08 ns	0.27 ns		
Adherence 2	**0.34 ns**	0.13 ns	**0.28 ns**	0.14 ns	

Note:(1) correlation value depicted in the table are Pearson`s correlation coefficient. (2) N=32, ns= not significant

3.4. Optimisation of the antagonistic activity of *Pseudomonas fluorescens* B5 by improving formulation techniques

Sugar beet seeds are commercially available in pelleted form with wood flour as the main ingredient. Incorporation of *Pseudomonas fluorescens* B5 into the sugar beet pellet is one of the most promising formulation of this bacterium. The success of such kind of formulation depends on the suitability of pelleting materials and pelleting process. In addition, the use of selective defined nutrients as formulation additives is another approach to improve the antagonistic activity of bacteria. For this purpose, the study was conducted to obtain: 1) pelleting materials which favour survival and activity of *Ps. fluorescens* B5, 2) nitrogen compounds and trace elements which can be used as formulation additives.

3.4.1. Effects of different pelleting materials on the growth, adhesion, survival and antagonistic activity of Pf B5

3.4.1.1. The growth of *Pseudomonas fluorescens* B5 in different pelleting materials.

Six seed pelleting materials *i.e.* two kinds of wood flour, diatomaceous earth, bentonite, cotton flour and peat were evaluated. Growth of PF B5 in TSB amended with various material as depicted in Figure 33 reveals that in most of the TSB media supplemented with pelleting materials *Ps. fluorescens* B5 grew slower than in TSB alone, but growth during stationary phase, estimated as cfu, was nearly always the same, except for wood flour.

3.4.1.2. Adhesion of *Pseudomonas fluorescens* B5 in different pelleting materials

Ps. fluorescens B5 adhere in different rates to the tested materials. Wood flour 2 and cotton flour gave highest adhesion rates, in contrats to bentonite and diatomaceous earth with the lowest adhesion. The rates for peat and wood flour 1 were in the middle (Figure 34).

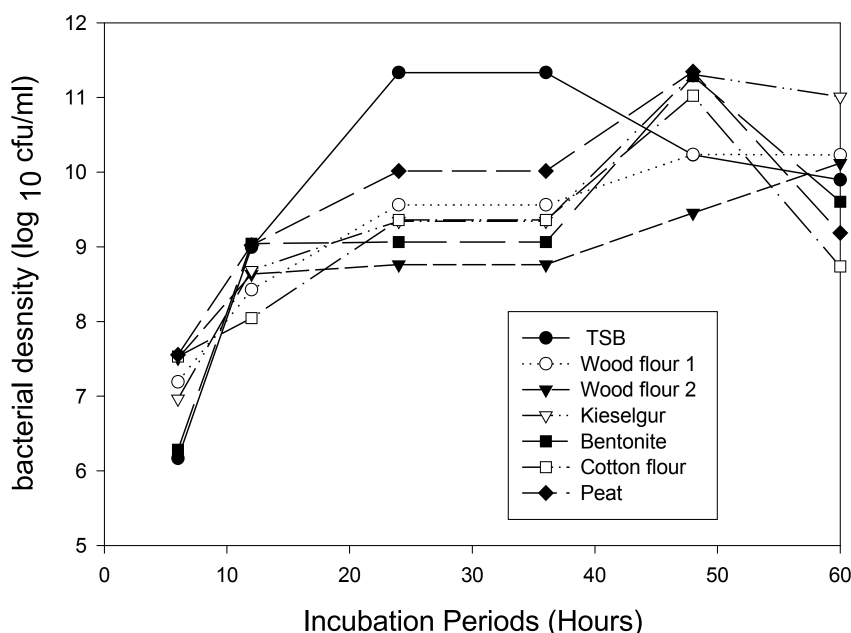

Figure 33. The growth of *Pseudomonas fluorescens* B5 in TSB suplemented with different pelleting materials (5% w/v, pH after autoclaving adjusted to 7.0, 20°C, 100 rpm). Bacterial density was assessed by serial dilutions followed by plating on TSA containing 400 ppm streptomycin.

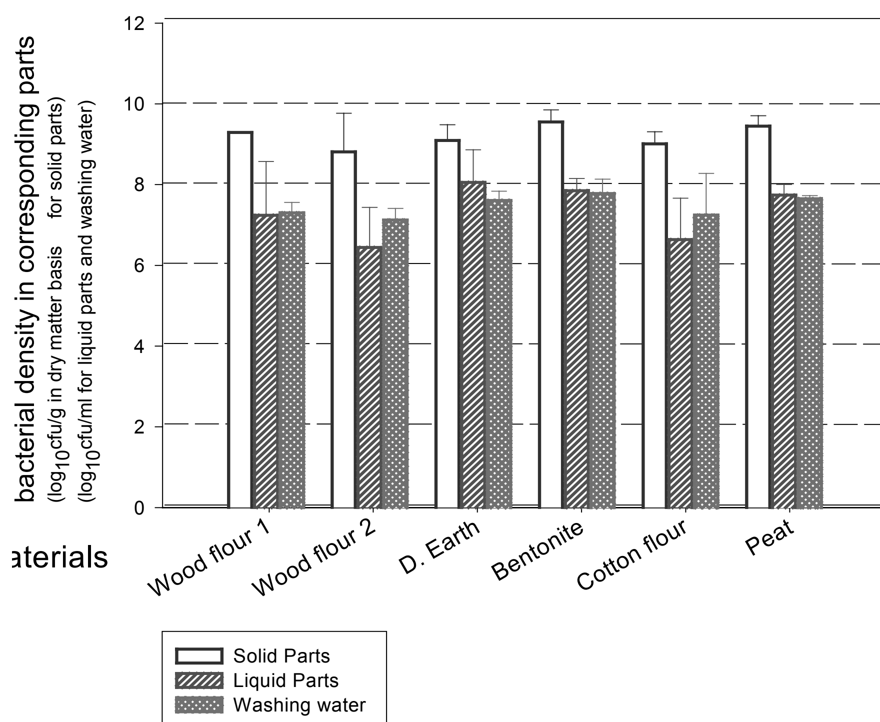

Figure 34. The density of *Pseudomonas fluorescens* B5 in different parts of pelleting materials (bacteria grown in TSB with 5 % (w/v) of material, pH 7, harvested 48 h after inoculation. Solid and liquid parts of the medium were separated by vacuum filtration, remaining residues were washed out using saline solution).

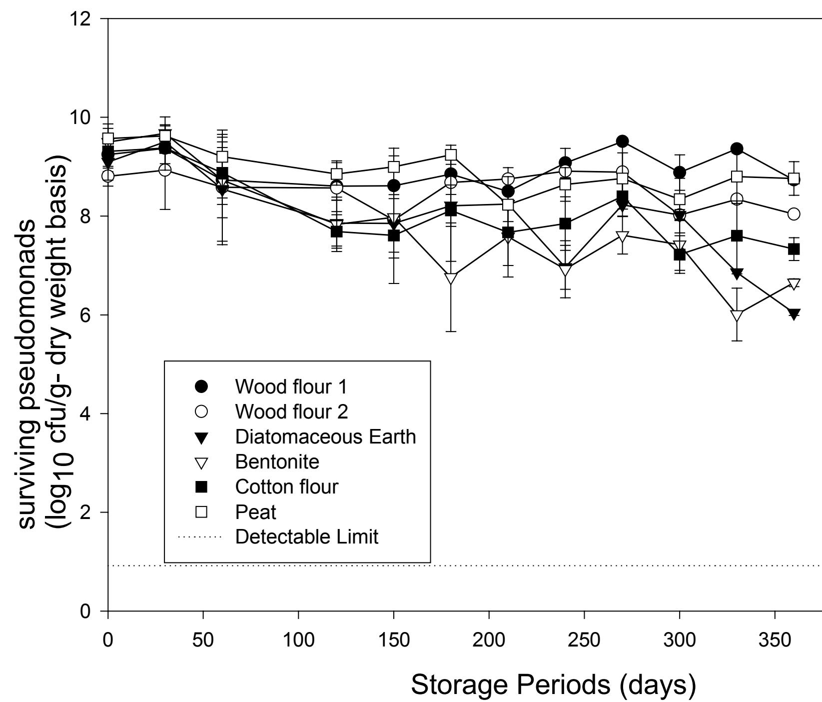

Figure 35. The survival of *Ps. fluorescens* B5 in different wet materials (initial water content ~60 %, storage at 5°C).

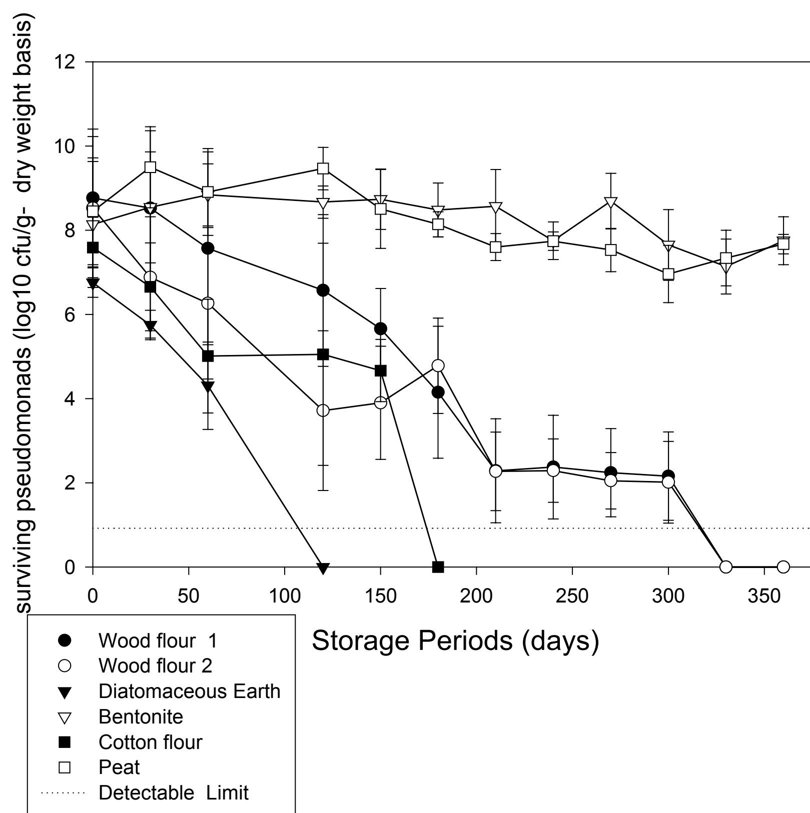

Figure 36. The survival of *Ps. fluorescens* B5 in different dry materials (initial water content ~10 %, storage at 5°C).

3.4.1.3. Survival of *Pseudomonas fluorescens* B5 in different materials

Long time survival of the antagonists in the pelleting materials is one of the most important features of the formulation of Pf B5. Therefore, the survival of Pf B5 with two initial water contents, wet (60%) and dry (10%), at 5°C during storage, was assessed for a period of 12 months Fig. 35 and Fig. 36 show obviously that in all tested materials, survival of Pf B5 in wet materials was superior to survival in dry materials. The survival of B5 in the wet materials was very stable, especially in wood flour type 1 and 2 and peat. The survival rate of cells at the end of observation (12 months) was as high as the initial density with 9 log cfu/ g dry material. Survival rate in the other tested materials was quite high with 7 log cfu/g, 7 log cfu/g and 8 log cfu/g dry material for bentonite, diatomaceous earth and cotton flour respectively.

In contrast to the very stable survival in wet materials, the survival of Pf B5 declined rapidly in dry materials, except for bentonite and peat formulations (Figure 36) with a considerably high cfu of 7.6 log_{10} and cfu of 7 log_{10}, respectively, was found at the end of estimation (12 months). The initial density of Pf B5 was about 8 log_{10}/g dry material. Survival of bacteria decreased rapidly in dry diatomaceous earth and cotton flour and could not be detected after 4 months for the diatomaceous earth and after 6 months for cotton flour formulations. Bacterial survival in both types of wood flour declined drastically up to 2 log_{10} cfu / g after 7 months, and could not be detected after 12 months.

3.4.1.4. Antagonistic activity of *Pseudomonas fluorescens* B5 *in vitro* after storage in different materials

Besides the survival rate, the stability of antagonistic activity during storage in tested materials is a prerequisite for a material that would be used in formulations. The *in vitro* antagonistic activity of Pf B5 was determined at the end of evaluation after 12 months. Except in wet bentonite and wet peat no significant decrease of antagonistic activity *in* until one year (Figure 37). But antagonistic activity of PF B5 in wet bentonite *vitro* was found among the

tested materials which support survival of Pf B5 and wet peat stored under the same conditions declined significantly (p<0.05).

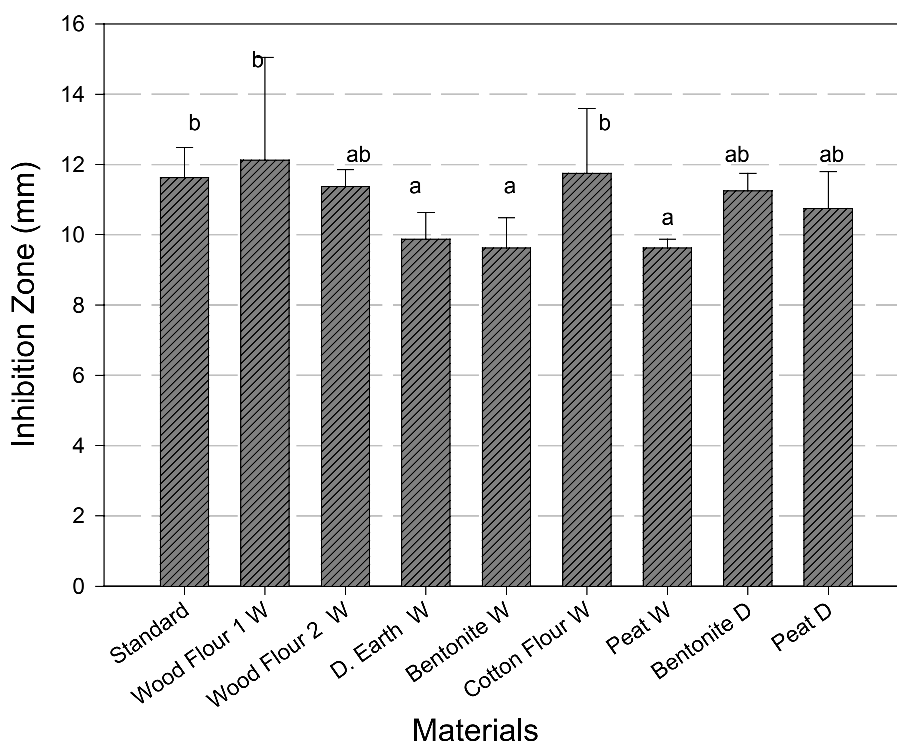

Figure 37. Antagonistic *in vitro* activity of *Ps. fluorescens* B5 against *P. ultimum* in different materials stored for 12 months (W=Wet D=Dry**,** at 5 °C. Dual culture test on TSA, pH 6.5, and evaluated 24 h after fungal plating).

3.4.2. Effect of nitrogen compounds and trace elements as additives in the formulation of *Ps. fluorescens* B5

It is well known that nutrition *i.e.* carbon and nitrogen source and trace elements could have an effect on the production of antifungal metabolites of fluorescent pseudomonads. Their use as pelleting additives in the formulation of *Ps. fluorescens* B5 to support their antagonistic effect should therefore be studied.

3.4.2.1. Effect of an addition of nitrogen compounds and trace elements on the *in vitro* production of antifungal substances by *Ps. fluorescens* B5

The stimulatory effect of trace elements and nitrogen compounds on *in vitro* production of antifungal substances of antagonists was studied.. Among

the trace elements tested, H_3BO_3 (0.2 and 0.05 mM), $MnSO_4$ (0.2 and 0.5mM) and $ZnSO_4$ (0.2 and 0.05 mM) have been proven to enhance the inhibitory effect of culture filtrate of *Ps. fluorescens* B5 against *P. ultimum* (Figure 38). Within the tested concentrations, except for H_3BO_3, all trace elements alone showed an inhibitory effect against *P. ultimum in vitro* (Figure 38). There was no significant increase of the inhibitory effect of the culture filtrate of Pf B5 if one of the five nitrogen materials was added (Figure 39).

Addition of nitrogen sources in certain concentrations *i.e.* urea (0.5 % and 0.1 %), ammonium sulphate (0.5 % and 0.02 %), ammonium nitrate (0.02 %) and tryptone (all tested concentration), even eliminated the inhibitory effect of the culture filtrate produced by Pf B5 *in vitro* (Fig. 39). On the contrary, control media containing urea (0.5%),

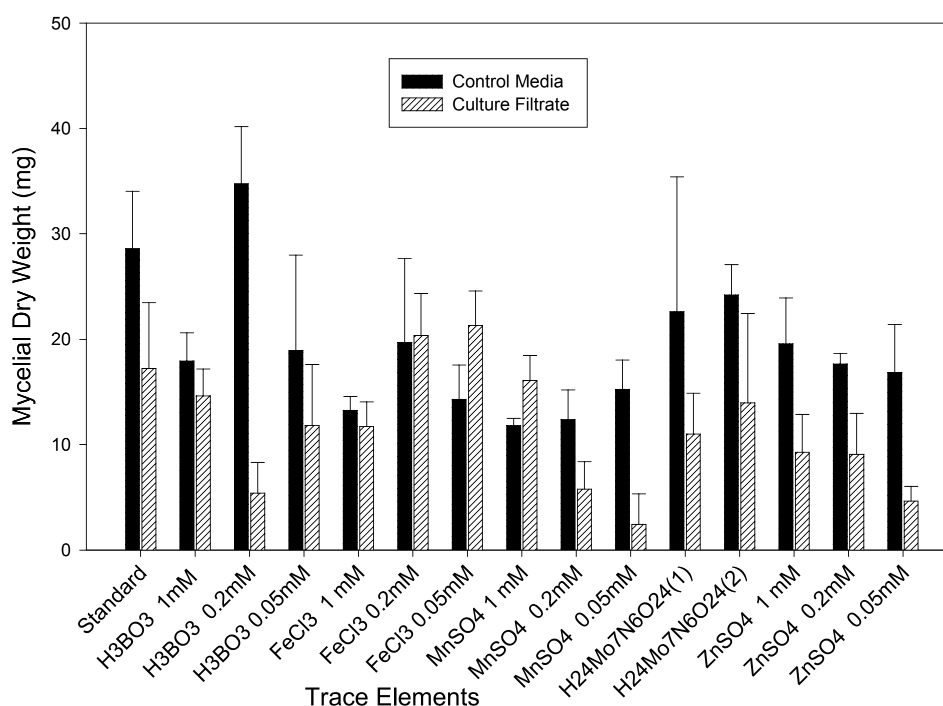

Figure 38. Effect of trace elements on the inhibitory effect of the culture filtrate of *Ps. fluorescens* B5 against *P. ultimum in vitro* (mineral medium, pH=7.0, CF from 96 h old culture). Control media is the mineral medium containing appropriate trace elements to check if tested elements have any direct effect on *P. ultimum.* $H_{24}Mo_7N_6O_{24}$ at concentration 0.2mM (1) and 0.05 mM (2)

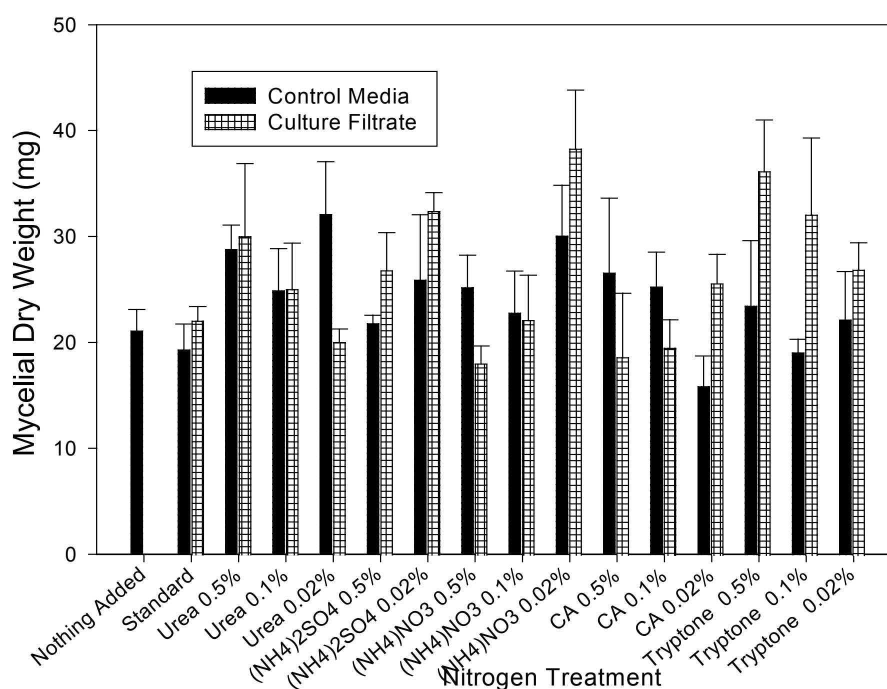

Figure 39. Effect of an amendment of nitrogen sources on the inhibitory effect of the culture filtrate of *Ps. fluorescens* B5 against *P. ultimum in vitro* (Mineral medium, pH 7.0, CF from 96 h-old culture). Mineral medium with appropriate concentrations of nitrogen compounds served as a control to check if tested compounds have any direct effect on *P. ultimum.*

$(NH_4)_2SO_4$ (all tested concentration), NH_4NO_3 (0.02 %) and tryptone (all tested concentration) stimulated the mycelial growth of *P. ultimum*.

3.4.2.2. Effect of an addition of nitrogen compounds and trace elements on the growth of *Ps. fluorescens* B5 *in vitro*

The direct toxicity of tested trace elements and nitrogen compound on *Ps. fluorescens* B5 is also an important factor for the selection of formulation additives. Trace elements and nitrogen sources had also direct effects on the growth of Pf B5. Figure 40 demonstrates that all tested trace elements at the rate of 1 mM inhibited the growth of Pf B5. The inhibitory effect of these tested substances to Pf B5 was strongly reduced in the concentration of 0.2 mM and finally in a concentration of 0.05 mM no inhibitory effect was observed.

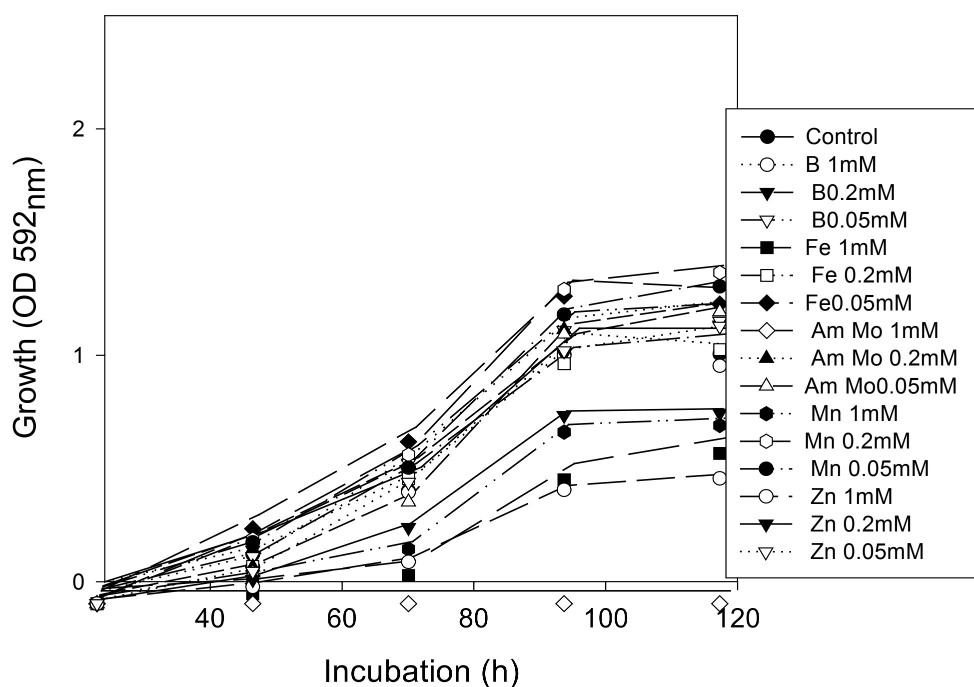

Figure 40. Effect of the addition of trace elements on the growth of *Ps. fluorescens* B5 i*n vitro* (mineral medium, pH 7.0, 25°C, 100 rpm)

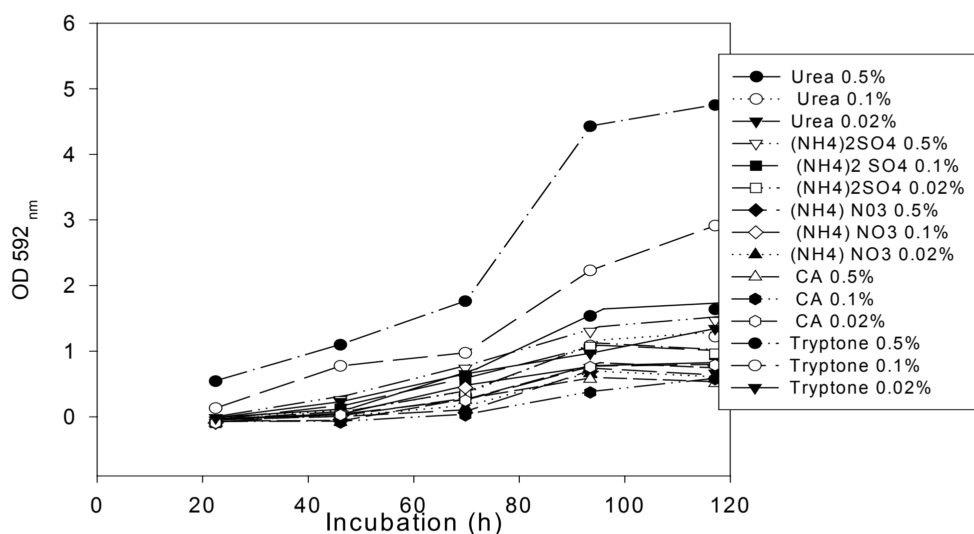

Figure 41. Effect of nitrogen sources on the growth of *Ps. fluorescens* B5 i*n vitro* (Mineral medium, pH=7.0, 25 °C, 100 rpm, 94 h)

Moreover, Figure 41 reveals that none of the nitrogen substances in the tested concentrations was toxic to Pf B5 *in vitro*. On the contrary, they stimulated the growth of Pf B5 and tryptone was the most effective one.

3.4.2.3. Effect of trace elements and nitrogen compounds as formulation additives on the antagonistic activity of *Pseudomonas fluorescens* B5 *ad planta*

The promoting effect of the tested trace elements on antagonistic activity *ad planta* is a determining factor for their selection as formulation additives. The results of the a*d planta* experiments as depicted in Figure 42 and 44, were different to those in the *in vitro* test. Only $MnSO_4$ in the concentration of 0.05 mM and 0.2 mM and 0.05 mM of $ZnSO_4$ enhanced the antagonistic effect of Pf B5 against *P. ultimum.* Although H_3BO_3 (0.2 and 0.05 mM) and 0.2 mM ammonium molybdate increased the inhibitory effect of the culture filtrate of Pf B5 (Figure 38) *in vitro*, these elements had no effect on the antagonistic activity *ad planta* (Figure 42 and 43).

A concentration of 0.05 mM of the two trace elements $MnSO_4$ and $ZnSO_4$ improved the antagonistic activity of *Ps. fluorescens* B5 *ad planta* (Figure 42, 43, and 44). The treatment of the pelleted seed with $MnSO_4$ resulted in an increased activity of PF B5 of ~ 21 %, 16 % and 19 % in the first, second and third experiment, respectively. Without additives, the activity was ~ 50 %, 39 %, and 40 % in the corresponding experiments. $ZnSO_4$ in pelleted sugar beet seeds at the rate of 0.05 mM enhanced the antagonistic activity of Pf B5 in average of ~ 20 % in three experiments. Figure 48 and Figure 49 reveal clearly the enhancement of the biocontrol activity of Pf B5 *ad planta* after dipping pelleted seeds in a solution of these two trace elements. A high concentration of $ZnSO_4$ at the rate of 0.2 mM had a direct antifungal effect against *P. ultimum ad planta* (Figure 42, 43, 44 and 45). Interestingly, there was no longer an antagonistic effect of Pf B5 against *P. ultimum ad planta* if combinations of the two compounds were applied in two different concentrations of 0.05 mM and 0.01 mM (Figure 43).

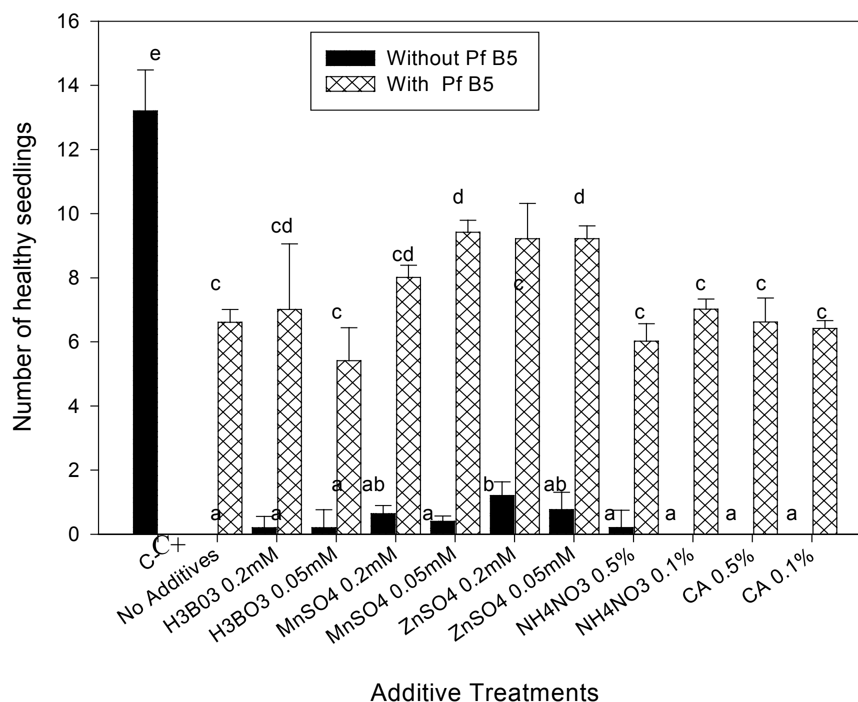

Figure 42. Effect of trace elements and nitrogen compounds in pelleted seeds on the antagonistic activity of *Ps. fluorescens* B5 against damping-off of sugar beet seedlings *ad planta* (EXP I)

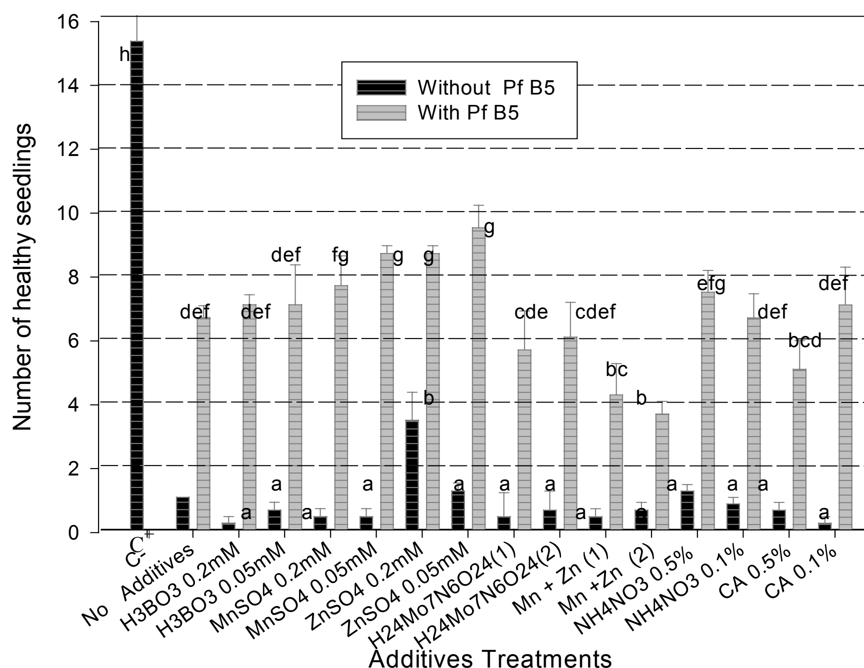

Figure 43. Effect of trace elements and nitrogen compounds in pelleted seeds on the antagonistic activity of *Ps. fluorescens B5* against *P. ultimum* in sugar beet seedling *ad planta* (EXP II). $H_{24}Mo_7N_6O_{24}$ at concentration 0.01mM (1) and 0.05 mM (2) Combination of Mn+Zn with concentration in the mixture 0.05mM (1), and 0.01 mM each (2)

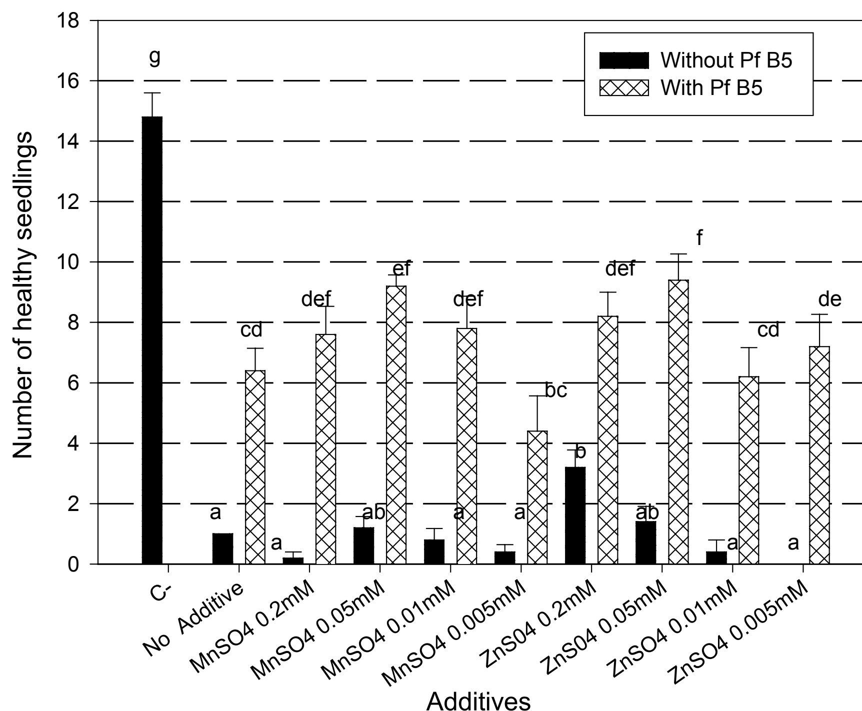

Figure 44. Effect of $MnSO_4$ and $ZnSO_4$ in various concentrations in pelleted seeds on the biological control activity of *Ps. fluorescens* B5 against *P. ultimum* in sugar beet seedlings.

3.4.2.4. **Direct effect of trace elements incorporated into pelleted seeds as formulation additives on emergence and growth of sugar beet seedlings**

It is well known that trace elements have direct effects on plants. The experiment was carried out to investigate the effect on emergence and growth of sugar beet seedlings. Among the tested concentration range and observed variables a toxicity of $MnSO_4$ and $ZnSO_4$ to sugar beet seedlings could no be observed (Figure 46 and 47). The germination rate was not significantly ($p<0.05$) reduced (Figure 46). On the other hand, it is also demonstrated that a combined treatment of $MnSO_4$ and $ZnSO_4$ in a concentration of 0.05 mM each, reduced significantly the germination rate (Figure 46). The application of the two substances increased significantly the total and individual shoot weight of treated sugar beet seedlings.

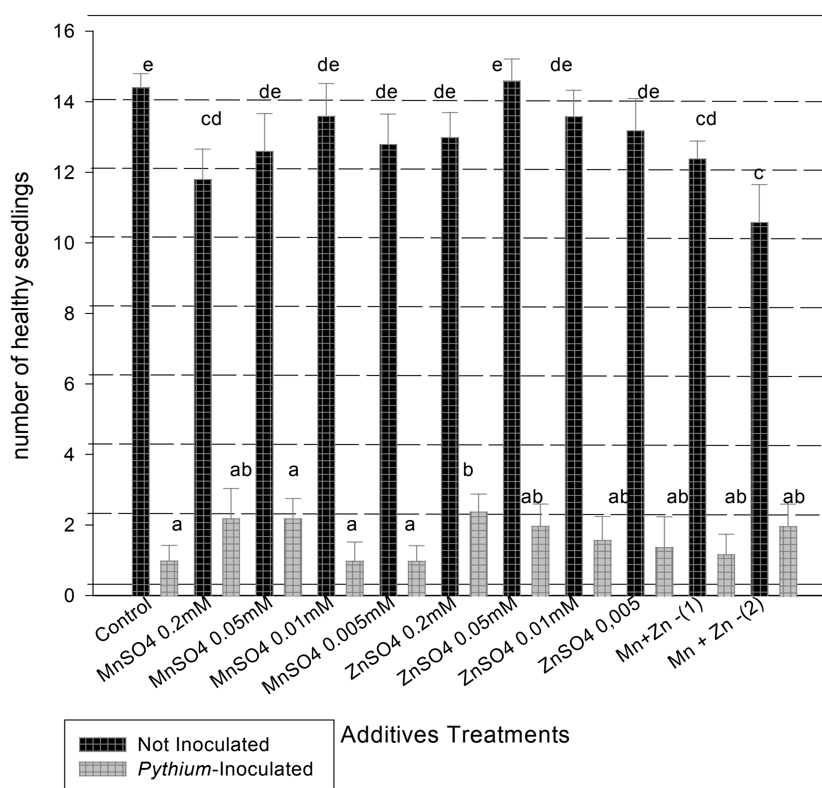

Figure 45. Direct effect of $ZnSO_4$ and $MnSO_4$ in various concentrations in pelleted seeds on damping-off of sugar beet (without Pf B5) Combination of Mn+Zn with concentration in the mixture 0.05mM (1), and 0.01mM each (2)

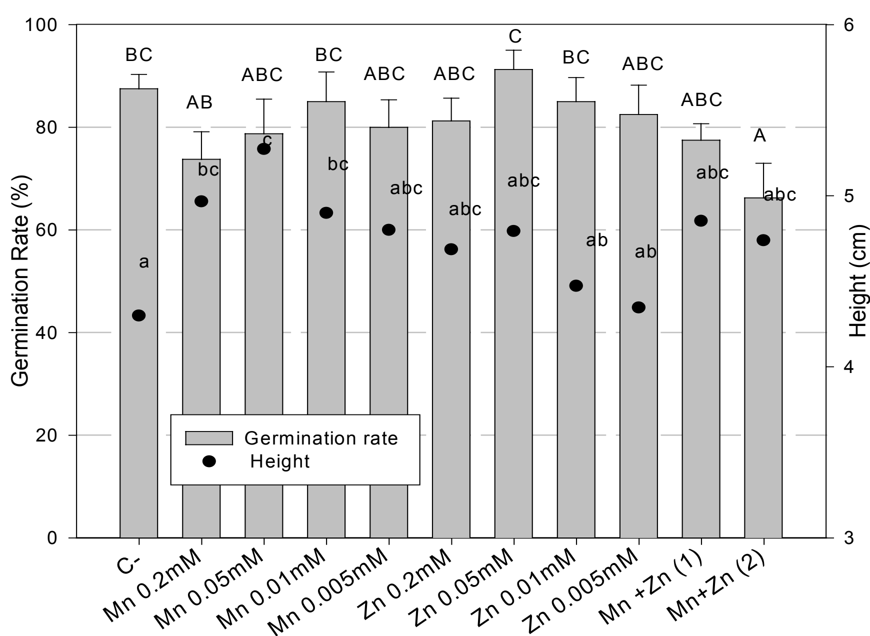

Figure 46. Effect of various concentrations of $MnSO_4$ and $ZnSO_4$ in pelleted seeds on the germination rate and seedling's height of sugar beet (without pathogen and antagonist).

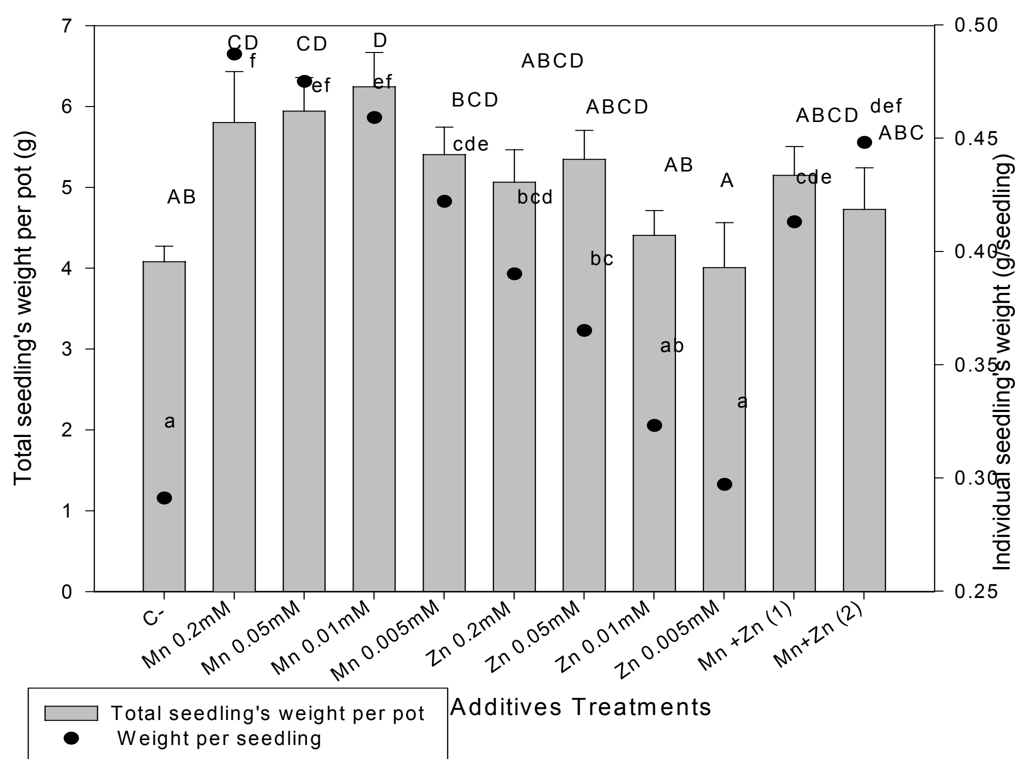

Figure 47. Effect of $MnSO_4$ and $ZnSO_4$ in pelleted seeds on the total and individual seedling's weight

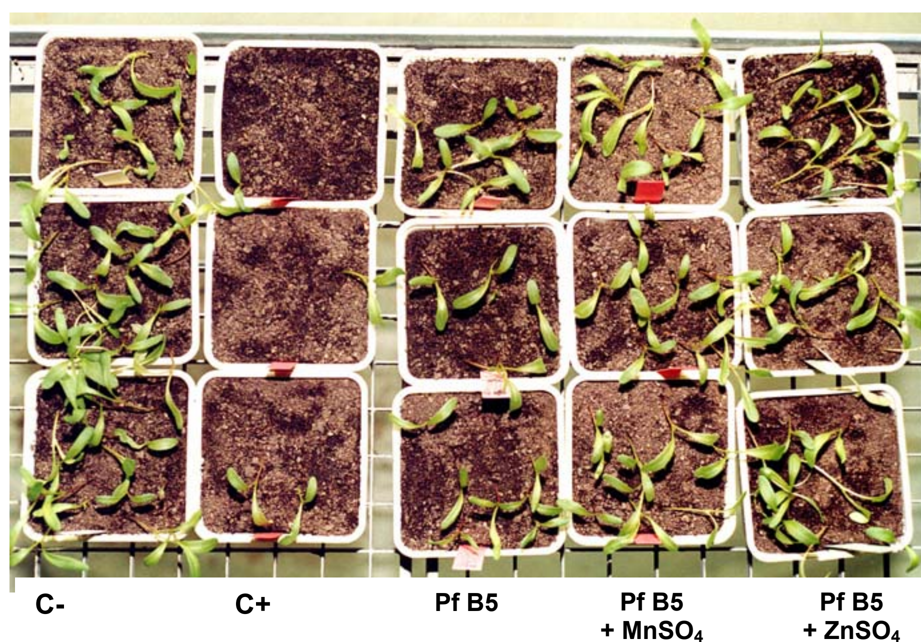

Figure 48. Effect of $MnSO_4$ and $ZnSO_4$ amendments in pelleted seeds in combination with *Ps. fluorescens* B5.

4. DISCUSSIONS

Fluorescent pseudomonads belong to a group of rhizobacteria that was intensively studied in the context of biological control of plant pathogenic fungi. This is due to the fact that *Pseudomonas* spp. has the following biological properties: 1) good root colonization, 2) easy to grow in the laboratory, 3) various modes of action in controlling plant diseases, 4) a relative high growth rate (Weller, 1988; Whipps, 1997; Chin-A- Woeng, 2000).

Most studies on biocontrol of fungal plant diseases by using *Pseudomonas* spp. are focused on finding effective antagonists and modes of action. Fluorescent pseudomonads have various mechanisms in controlling plant disease. The first is antibiosis, proven by reduction of its efficacy *ad planta* of antibiotic negative- producing mutants (Defago and Haas, 1990; Chaterjee *et al.*, 1996; Schulz and Wolf, 2002). *Pseudomonas* spp. is well known to produce a diverse array of antifungal compounds such as phenazines, pyoluteorin, 2,4-diacetylphloroglucinol, pyrrolnitrin, oomycin, hydrogen cyanide, pseudobactin, rhamnolipids (Duffy and Defago, 1999; Chin-A-Woeng *et al.*, 2003; Hwang *et al.*, 2002; Lee *et al.*, 2003). Even though *Pseudomonas* spp. produces hydrolytic enzymes *in vitro* such as chitinase, glucanase (Heupel, 1992), gelatinase, proteinase and arginin dihydrolase (Fakhouri and Buchenauer, 2002). The role of these enzymes in antagonistic activity *ad planta* is not clear. The second mechanism involved is siderophore production by fluorescent pseudomonads, which can compete for ferric iron with pathogen in an iron-limited environment (Whipps, 1987; Defago and Haas, 1990). The third mode of action is induced systemic resistance of host by fluorescent pseudomonads as stated by Ongena *et al*. (1999), Chen *et al*. (1999) and Ramamoorthy *et a*l.(2001). The mechanisms of induced resistance include structural and ultra-structural cell wall modifications of host plants, biochemical/physiological changes in the host plant (Zhang *et al*., 2002), especially the activation of pathogenesis-related-proteins *(*PR protein) such as ß-1,3 glucanases and endochitinases and other chemical defence (Ramamoorthy *et al*., 2002).

The main aim of the present work was to improve antagonistic activity of *Ps. fluorescens* B5 by a multiple approach *i.e*. 1) by applying antifungal metabolite-overproducing mutants 2) by selecting compatible sugar beet varieties, 3) by improving formulation technique through selection of appropriate pelleting materials and the use of formulation additives. In addition, one of the physiological characteristics of *Ps. fluorescens* B5 *i.e.* IAA production and its possible involvement for biological control was also studied.

4.1. The use of antifungal metabolite-overproducing mutants to enhance antagonistic activity of *Ps. fluorescens* B5 against damping-off disease of sugar beet caused by *Pythium ultimum*.

Since antibiosis seems to be a main mode of action of fluorescent pseudomonads in controlling plant diseases (Defago and Haas, 1990; Chatterjee *et al.,* 1997; Chin-A-Woeng, 2000; Walsh *et al.* 2001; Schulz and Wolf, 2002), its antagonistic activity may be improved by applying antibiotic-overproducing mutants. The main aim of the first part of this work was to optimise biological control of damping-off by using antifungal metabolite-overproducing mutants of *Pseudomonas fluorescens* B5.

It could be clearly demonstrated that antagonistic activity of Pf B5 can be improved by applying Tn-5-antifungal metabolites-overproducing mutants, however, only three out of 7 mutants found *in vitro* had also a higher activity up to about 30% against damping-off *ad planta,* than wild type Pf B5 WT. This result is in agreement with foregoing research on other strains of antagonistic fluorescent pseudomonads. For example Maurhofer *et al.* (1992 and 1995) reported that biocontrol efficacy of *P. fluorescens* CHAO against *P. ultimum* in cucumber can be improved by applying pyoluterin-overproducing mutants. Application of 2,4-diacetyl-phloroglucinol-overproducing *P. fluorescens* F113 could increased its antagonistic activity against *P. ultimum* in sugar beet seedlings (Delany *et al.* , 2001). Moreover, Legon *et al.* (2000) stated the enhancement of biocontrol efficacy by *Pseudomonas* biocontrol strains by applying pyrrolnitrin-overproducing mutant.

In the present work, screening of mutants *in vitro* by dual culture technique only reveals the production of total antifungal metabolites, indicated by the size of the inhibition zone, without determining the type of antibiotic. However, a more detailed study of the two antifungal metabolites HCN and IAA showed that the mutants produced higher amounts of these two antifungal substances *in vitro* than the wild type Pf B5 WT. The question whether the main antibiotics known to be produced by *Ps. fluorescens* B5 show a clear correlation with the antagonistic activity has to be studied in more detail

Even though some mutants (No. 010, No. 825, Tn XII) showed significantly higher inhibition *in vitro* (dual culture on TSA), they did not result in higher control level *ad planta* than the wild type. This indicates the poor relationship between antagonistic test *in vitro* and *ad planta* which is also supported by the fact that there is no significant correlation between inhibition zone on TSA or KBA culture with the antagonistic activity *ad planta* (Table 4). Poor correlations between antagonism *in vitro a*nd biocontrol activity *ad planta* were reported in many papers on biocontrol (Smilanick, 1994; Ongena *et al.*, 1999; Folman *et al.,* 2003). The fact indicates multiple factors affecting biocontrol activity *ad planta* of Pf B5.

There is no general pattern of relation between *in vitro* and *ad planta* tests with bacterial antagonists. Some papers stated a strong correlation between inhibition zone *in vitro* and efficacy *ad planta* (Hultberg *et al*., 2000; Zhao, 2001) but others found contrasting results (Ongena *et al.*, 2001; Folman *et al.*, 2003). *In vitro* features of antagonists were also analysed for their relationship with antagonistic activity *ad planta* to determine the main mechanism involved in biocontrol (Nautiyal, 1997;Hultberg *et al.*, 2000,; Schulz and Wolf, 2002; Folman *et al.* 2003,). In addition, studies on the relation between the *in vitro* and *ad planta* activity are very useful for the improvement of screening techniques. For this reason, correlation analysis was performed to examine the relationship between *in vitro* traits and *ad planta* antagonistic activity of mutants. Among the *in vitro* properties of the tested mutants (doubling times in LB, doubling time in KB^+, inhibition zone in

TSA, inhibition zone in KBA^+, mycelial growth reduction in LB-based culture filtrate, mycelial growth reduction in KB+-based culture filtrate, total HCN production, HCN production/10^9 cells, total IAA production, IAA production/10^9 cells, mycelial growth reduction in volatile substances bioassay, and motility), only four characteristics *i.e.* production of HCN, production of HCN/10^9 cells, total production of IAA and mycelial growth reduction in KB+-CF exhibited significant correlations to biocontrol activity of the bacteria *ad planta*. Correlation of HCN production *in vitro* with antagonistic activity of fluorescent pseudomonads *ad planta* was also reported by Hulterg *et al.*(2000), Ellis *et al.* (2000), and Folman *et al.* (2003), although Hultberg (1999) stated the minor role of these traits. A weak but significant correlation of total IAA production *in vitro* ($r=0.47$, $P<0.05$), IAA production/10^9 cells of mutants ($r=0.60$, $P<0.05$) as well as mycelial growth reduction in KB^+-based CF with *ad planta* antagonistic activity was firstly reported by this experiment. Nautiyal *(*1997) reported that isolates of *Ps. fluorescens* that produced high amount of HCN and IAA *in vitro* has relatively high antagonistic activity *ad planta*. Therefore, it can be concluded that total HCN production *in vitro*, HCN production/10^9 cells, total IAA production *in vitro*, IAA production/10^9 cells and mycelial growth reduction in KB^+-based culture filtrate allow a prediction on the biocontrol efficacy of different mutants of Pf B5 *ad planta*.

Total HCN production *in vitro* correlated (0.55, $P<0.05$) significantly to mycelial weight reduction in volatile antifungal metabolites–bioassay. This shows that most of the volatile antifungal substances produced on TSA is HCN. Zhao (2001) reported among tested media *i.e.* TSB and straw extract, *Ps. fluorescens* RII2 produced more HCN *in vitro* on TSB. A further volatile antifungal substance produced by Pf B5 WT *in vitro* is ammonia (Schulz *et al*., 1994), which was not determined in this study.

Colonisation is considered as an important prerequisite of an effective antagonist. Three antifungal metabolites-overproducing mutants had higher control level than Pf B5 WT, *i.e.* No. 311, N 24 and Tn 12 but the ability to colonize the rhizosphere was not significantly different to Pf B5. Moreover,

there was no significant correlation between colonisation and antagonistic activity *ad planta* among the tested mutants. This may be due to that colonisation alone doesn't play a direct role in controlling pathogens, but other mechanisms such as production of non volatile antibiotics and siderophores are much more important.

The first indication of the role of antibiosis in colonisation was revealed by significant correlations between rhizosphere and rhizoplane colonisation and antibiosis-related properties among tested mutants. Rhizosphere colonisation correlated significantly to inhibition zone on TSA (0.72, $p < 0.01$), mycelial growth reduction in CF-LB ($r=0.53$, $p < 0.05$), and total IAA production *in vitro* ($r=0.76$, $p < 0.01$). A significant correlation of mycelial growth reduction in CF-LB ($r=0.75$, $p < 0.05$) and rhizoplane was also found. In contrast, Toyota and Ikeda (1997), who worked with antibiotic-negative mutants, stated little contribution of antibiosis in colonisation of *Pseudomonas fluorescens* MelRC2Rif on tomato and melon. The importance of antibiosis in root colonisation was also confirmed for other rhizobacteria *i.e. Azospirillum brasilense* (van de Broek *et al.*, 1998). More direct evidence using antibiotic-negative mutants of Pf B5 is necessary to determine more exactly the role of antibiosis in colonisation.

Since colonisation is considered to be one of the limiting factors in the success of antagonist application, bacterial properties affecting it have been widely investigated. Some properties related to the colonisation ability of fluorescent pseudomonads are motility, chemotaxis, growth rate, ability to utilize root exometabolites, synthesis of O-antigen lipopolysaccharide, flagella, biosynthesis of vitamine B1 and amino acids (Dekkers *et al.*, 1999; Jiemba and Alexander, 1999; Lugtenberg and Dekkers, 1999; Lugtenberg *et al.*, 2001). Other publications demonstrated that growth rate, chemotaxis, and motility had no siginificant effect on colonisation (Scher *et al.,* 1988; Toyota and Ikeda). The result of the present study is in agreement with the latter, and suggests no significant correlation between growth *in vitro*, motility and colonisation of tested mutants. Toyota and Ikeda (1997) stated that motility of fluorescent pseudomonads is important in movement towards the root, when

fluorescent pseudomonads are applied as soil amendment. The significant correlation between rhizosphere colonisation and motility among tested mutants indicates that motility may play a role in movement in the rhizosphere.

Among four screening techniques *in vitro i.e.* dual culture test on TSA, dual culture test on KB^+A, LB-based culture filtrate (CF-LB) and KB^+-based culture filtrate (CF-KB^+), only CF-KB^+ has a significant correlation ($r=0.64$, $P<0.01$) to biocontrol efficacy *ad planta.* This finding highlights the importance of this technique in *in vitro* screening of fluorescent pseudomonads for biocontrol agent purpose.

In experiments on the direct effect of the antagonist on plant growth *i.e.* without *Pythium* inoculation, slight toxicity of Pf B5 WT and almost all tested mutants except No. 010 was observed, indicated by the reduction of emergence rate of sugar beet seedlings. This effect may be true only for young plants because other growth parameters of sugar beet seedlings such as height and weight were not affected. The toxicity of antifungal substances such as pyoluterin and pyrrolnitrin on seedlings of cotton and tobacco (Maurhofer *et al.*, 1992), HCN on various plants (Cambell, 1985; Blumer and Haas, 2000) may explain this phenomenon. Phytotoxicity of antifungal metabolites produced by fluorescent pseudomonads can be a limiting factor in the application of antifungal metabolites-overproducing mutants as also mentioned by Maurhofer *et al*. (1995).

The experiments using antifungal metabolites-overproducing mutants allow the following conclusions, firstly they may improve biocontrol efficacy of *Ps. fluorescens* B5, secondly they are supporting the hypothesis that antibiosis is the pre-dominant mechanism in biocontrol activity of Pf B5, thirdly they underline the role of antibiotics in colonisation.

4.2. The role of indole-3- acetic acid (IAA) in the biocontrol activity of *Ps. fluorescens* B5 against damping-off and growth promotion of sugar beet seedlings

Previous research on IAA produced by fluorescent pseudomonads is focused on its role as plant growth regulator (Kumari and Srivastava, 1999; Kaur *et al*., 2003). For example, IAA produced by plant growth promoting rhizobacteria (PGPR) is responsible for many variables of growth parameters *i.e.* increasing of shoot weight, root elongation and increasing root biomass (Xie *et al.* 1996; Barazani and Friedman, 1999; Asgar *et al*. 2002; Gamalero *et al*., 2002). In the present work the role of IAA in antagonistic activity of *Ps. fluorescens* B5, and also on plant growth was examined.

Pseudomonas fluorescens B5 produced indole acetic acid (IAA) *in vitro* in considerable amounts. Moreover, some mutants such as No. 029, and No. 311 produced a higher amount of total IAA *in vitro* than wild type Pf B5 WT, however related to cell number No. 29 and N 24 are producing a higher rate of IAA *in vitro* than wild type Pf B5 WT. It is well known that IAA is produced by various antagonists and/or plant growth promoting fluorescent pseudomonads (Hultberg, 1999; Zhao, 2001; Bano and Mussarat, 2002). Among the tested mutants, there was a weak but significant correlation ($r=0.47^*$, $P<0.05$) between total production of IAA *in vitro* and antagonistic activity *ad planta.* On the contrary, no significant correlation could be found between IAA production/10^9 cells and antagonistic activity *ad planta*.

Further experiments showed that the antifungal effect of IAA against *Pythium ultimum in vitro* started at 6.25µg/ml. This antifungal activity of IAA *in vitro* against *P. ultimum* is comparable to that of the antibiotic 2,4-diacetylphloroglucinol. De Souza *et al.* (2003) found an EC_{50} for 2,4-diacetylphloroglucinol against *P. ultimum* of 34.7 µg/ml, even though the reliable value of EC_{50} of IAA on *P. ultimum* can not be calculated due to the lack of a representative number of concentrations tested, the predicted value of IAA on *P. ultimum* is between 75-100 µg/ml.

Futhermore, *ad planta* tests reveal that seed dipping with IAA in concentrations of 12.5 µg/ml and 25 µg/ml can suppress significantly

damping-off of sugar beet seedling caused by *Pythium ultimum.* To our knowledge, this is the first described example for an antifungal effect of IAA against *P. ultimum ad planta.* Till now only limited information on fungicidal activity of IAA is available. *In vitro* experiments revealed that IAA is able to inhibit the growth of *P. ultimum* in vitro (Schulz[1], pers. comm.). Previous research studying antifungal activity of plant growth regulators showed different results. Roco and Perez (2001) reported that IAA, gibberelic acid (GA) and BAP (benzyl amino purin) in the rate of 15, 30, 40 ppm *in vitro* did not affect mycelial growth and spore germination of *Alternaria alternata* and *Trichoderma harzianum*. Furthermore, Al-Masri *et al.* (2002) reported that another plant growth regulator *i.e.* naphthalene acetic acid (NAA), has fungicidal activity against *Sclerotinia minor* infecting mungbean and cucumber, both *in vitro* and *in vivo.* Defago and Haas (1990) reported on two approaches assessing the role of particular metabolites in biocontrol performance of fluorescent pseudomonads. Firstly, by adding known purified metabolites to a pathogen-infested soil, and examining its effect on disease suppression. Secondly, applying *Pseudomonas* mutants that do not produce an extracellular metabolite and comparing disease suppression with its wild type. The technique used in the experiment belongs to the first approach, with the advantages to get results faster and easier. However its disadvantage such as difficulties to test whether Pf B5 produces IAA in the rhizoplane, and not being able to exclude a possible inactivation or adsorbtion by the soil. The result of this experiment is a first indication that IAA production is involved in biocontrol activity of *Ps. fluorescens* B5 *ad planta*.

The effect of IAA on the growth of sugar beet seedling was also examined. Addition of exogenous IAA did not affect the germination rate and seedling’s height. On the contrary, all of the tested concentrations increased seedlings weight.

Observations on the growth parameters of mutants-treated seedlings show that there was no significant correlation between total IAA production *in vitro*, production of IAA/10^9 cells *in vitro* with observed growth variables of

[1] Institut für Zierpflanzenzüchtung , Bornkampsweg 31 Ahrensburg

sugar beet seedlings treated with corresponding mutants. On the contrary, a significant negative correlation exists between total HCN production *in vitro* and germination rate of seedlings treated with mutants. IAA produced by fluorescent pseudomonads in PGPR groups has reported to play an important role in plant growth. This plant growth regulator is involved in root development (Mayak *et al*., 2001) and root elongation (Barazani and Friedman, 1999; Xie *at al.*, 1996). Ashgar *et al*. (2002) stated that total production of IAA *in vitro* of various IAA-producing rhizobacteria correlated significantly with the increasing height, pod number and yield and oil content of corresponding treated *Brassica juncea*. However, Xie *et al*. (1996) reported the loss of root elongating ability of IAA-overproducing mutants of *Ps. aeruginosa* GR 12-2 compared to its wild type. Based on the results of this experiment, there was no correlation between total production of IAA and the growth parameters of seedlings treated with the corresponding mutants. The role of IAA production by various mutants in seedling growth is not clear because other physiological factor *i.e.* HCN production is also involved, indicated by a significant correlation between IAA production/10^9 cells and total HCN production, which may be toxic to various plants species (Schippers *et al.,* 1987; Campbell, 1989; Blumer and Hass, 2002;). Goel *et al.* (2002) reported that co-inoculation of S*ternorhizobium* with HCN-producing strains of antagonistic pseudomonads caused significant lower shoot dry weight and shoot nitrogen of chickpea, in comparison to non HCN-producing strains.

4.3. The role of sugar beet varieties in the antagonistic activity of *Pseudomonas fluorescens* B5 against damping-off disease

The success of biocontrol by fluorescent pseudomonads in the field is influenced by many abiotic and biotic factors. Abiotic factors affecting growth, colonisation and antagonistic activity of fluorescent pseudomonads are soil physical and chemical characteristics such as pH, temperature, water content, the content of macro and microelements and cation exchange capacity (Harris and Adkins, 1999; Ownley *et al.*, 2003; Clays-Josserand *et*

al., 1999; Naseby and Lynch, 1999). Biotic factors influencing the antagonistic activity of microorganism are other soil biota such as bacteria, nematodes and protozoa (Mauerhoffer *et al.*, 1995; Elsherrif and Grossman, 1996; Clays-Josserand *et al.*, 1999; Whipps, 2001), host species (Maurhofer *et al*., 1995; Marschner *et al*., 1999), and host varieties (Leeman *et al.,* 1995; Siddiqui and Shaukat 2003).

Most of biological control researches are focused on the interaction of antagonists and pathogens. On the contrary, the role of the host plant on the biocontrol activity of antagonists attracts little attention (Smith and Goodman, 1999). As a consequence, there are still limited informations on the role of host genotype. On the one hand, some reports show an interaction between antagonistic activity of biocontrol agents and plant genotypes *i.e.* in the system *Pseudomonas fluorescens* - *Fusarium oxsyporum* - tomato as studied by Leeman *et al. (*1995), *Pseudomonas fluorescens* CHAO - *Meloidogyne incognita* - soybean (Siddiqui and Shaukat, 2003), non pathogenic *Fusarium oxxsyporum* - *Fusarium oxysporum* - pea (Hervas et al., 1997), *Pseudomonas chlororapis* -*Fusarium oxysporum* f.sp. *ciceris* - chickpea (Landa *et al*., 1997). In contrast, other publications show the contrary *i.e.* no effect of host genotypes on biocontol activity of antagonists as reported by Hebbar *et al.* (1998), who studied the system *Pseudomonas fluorescens* - *Fusarium moniliforme* – maize.

The results of the work show high a significant interaction (P<0.001) between application of Pf B5 and sugar beet varieties. With regard to the antagonistic activity of *Ps. fluorescens* B5, eight tested varieties can be classified into two groups. The first group is a group with an *ad planta* efficacy above 65 % (V1, V6, V7, V8) and the second with an effect lower than 50 % (V2, V3, V4, V5). It also became apparent that there was no relationship between resistance level and biocontrol activity of Pf B5 on tested varieties. This finding was in agreement with the results of Leeman *et al.* (1995) for tomato, Hebbar *et al.* (1992) for maize and for the *Pseudomonas fluorescens* CHAO - *Meloidogyne incognita* - soybean system (Siddiqui and Shaukat,

2003). Thus, it can be stated that antagonistic activity of Pf B5 WT is dependent on the sugar beet variety.

So far studied the main theory is that the different colonizing ability of antagonists and/ or PGPRs explains the different efficacy on different varieties (Lemanceau *et al.*, 1995; Smith & Goodman, 1999; Simon *et al.,* 2001; Dasti *et al.*, 2000). In the present work, significant different root colonization abilities of Pf B5 among eight tested sugar beet varieties was found. Interestingly, this does not fit with the antagonistic activity on the corresponding varieties. Furthermore, the correlation of colonisation and antagonistic activity was weak (0.31) and not significant. This aspect may be explained by differences among varieties with respect to the regulation of secondary metabolite synthesis. It is supported by the observation that production of antibiotics of *P. fluorescens* CHAO *ad planta* is influenced by host species (Maurhofer *et al.*, 1995) and also found most recently for maize varieties and *P. fluorescens* CHAO (Notz *et al.*, 2001). Differences with respect to chemical and physical properties of roots among sugar beet varieties may influence this effect. It could be that different compositions of sugar beet root exudates are responsible. Further studies on the role of host varieties in the regulation of the synthesis of secondary metabolites such as antibiotics, siderophores, and enzymes of *Ps. fluorescens* B5 and the relation with antagonistic activity are necessary.

Since colonisation is considered as one of important factors in the success of antagonist application, factors affecting it has been widely investigated. Some factors related to the colonisation ability of fluorescent pseudomonads are motility, growth rate, ability to utilize root exometabolites, synthesis of O-antigen lipopolysaccharide, flagella, the biosynthesis of vitamine B1 and amino acids (Dekkers *et al.*, 1999 Lugtenberg *et al*., 1999; Lugtenberg *et al*, 2001). Unfortunately most of intensively investigated factors are related to the antagonistic microorganism, whereas limited information about factors depending on the host plant is available.

Howie *et al.* (1987) described that root colonisation by rhizobacteria involves two phases: phase I is the attachment of bacterial cells on the root

surface, also called adherence and phase II is the multiplication of the bacteria on the root. Adherence is affected by several bacterial characteristics such as the presence of pili (Vesper, 1987), the presence of root-adhesive protein, surface-charge properties (James *et al.*, 1998) and a plant specific glycoprotein termed agglutinin (Anderson *et al.*, 1988). The present study indicates that there was no significant correlation between both types, root adherence and colonisation and also antagonistic activity of Pf B5 on eight tested varieties. This is in agreement with Glandorf *et al.* (1994) who reported no correlation of adherence and colonisation in different tomato varieties. The results suggest that adherence play a minor role in colonisation among different tested varieties.

4.4. Optimisation of biological control efficacy by improving formulation techniques

In biological control, some antagonists which are effective in laboratory and greenhouse fail to provide high efficacy in the field. Besides ecological factors, inappropriate formulation often contributes to such failure. Hence, formulation is a critical step in developing biocontrol methods. Burges and Jones (1998) defined formulation of biocontrol agents as a technique to preserve and deliver organisms (antagonists) to their targets and to improve their activities. Furthermore Jones and Burges (1998) stated that basic functions of formulations are: 1) stabilizing the organisms during production, distribution and storage, 2) facilitating the handling and application of products, 3) protecting the agent from harmful environmental factors at target sites, thereby increasing persistence, 4) enhancing activities of antagonist.

Antagonistic strains of fluorescent pseudomonads can be formulated as *i.e.* aqueous cell suspensions (Fravel *et al.*, 1998), seed coating (Imam Ali *et al*., 2001; Krishnamurti and Gnanamanickam, 1998), powder formulation (Vidhyasekaran *et al.*, 1997), alginate encapsidation (Russo *et al.*, 1996; Bashan and Gonsales, 1999) and seed pellet (Tilcher, 2002; Pedersen *et al.*, 2002).

4.4.1. Testing for Appropriate Pelleting Materials for *Pseudomonas fluorescens* B5

Sugar beet seeds are commonly available in pelleting form. Hence, in previous studies fluorescent pseudomonads were incorporated into sugar beet seed pellet (Heupel, 1992; Tilcher, 2002; Pedersen *et al.*, 2002). However, a normal pelleting process of sugar beet seeds provide adverse effect on *Pseudomonas* mainly due to high temperature (60°C) during drying process. Heupel (1992) stated that the survival of Pf B5 in pelleted seeds depends on the drying technique, which caused a drastic decrease of survival during machine-drying process. She found that only 6.35×10^{1} cfu survived in storage at 4°C up to 8 week under these conditions, whereas in air-dried pills could survive up to 4.18×10^{6} cells/pills. Special pelleting methods as done by Schulz and Wolf (1998) who applied air drying provided high survival of Pf B5 after storage at 5°C for 6 months. Briefly, seed pelleting of sugar beet with Pf B5 can be improved by two approaches, firstly by applying appropriate pelleting materials and secondly by optimising the pelleting process, mainly the drying temperature.

Sugar beet seeds are industrially pelleted with wood flour as the main ingredient. Concerning the compatibility for Pf B5, different pelleting materials *i.e*. two types of wood flour, diatomaceous earth, bentonite, cotton flour, and peat were tested in the present study.

The peculiarities of this experiment were that bacteria are cultivated in TSB amended by tested materials 5% (w/v). The vacuum filtrated, washed and air-dried materials were furthermore stored and evaluated. The experiment with eight tested materials with two initial water contents (60% and 10%) showed that under storage at 5°C, survival of Pf B5 was very stable in all materials under wet conditions. On the contrary, under dry conditions survival of Pf B5 was only sufficient in bentonite (material containing mostly montmorillonite) and peat. The high sensitivity of fluorescent pseudomonads to desiccation can be explained by the fact that they are non spore-forming bacteria compared to the more resistant *Bacillus* species, which are able to survive as spores. The high survival of Pf B5 in wet tested materials makes it

possible to develop these materials as wet carrier in formulation of Pf B5, which has also been reported by Fravel *et al.* (1998) and Warrior *et al*. (2003).

In tested dry materials (10% moisture), only bentonite and peat provided high survival of Pf B5 that was 7.7 log10 cfu for both materials after 12 month of storage. This is quite high compared to the initial density of pseudomonad cells of 8.14 and 8.45 log10 cfu for bentonite and peat respectively. Moreover, the antagonistic activity *in vitro* in the two carriers did not decrease significantly after 12 months of storage. Bentonite was reported as a useful fine clay that can be used as carriers for preserving fluorescent pseudomonads. Dandurrand *et al.* (1994) reported that clay with small particle size such as montmorrilonite, vermicullite and zeolite support higher survival of *Ps. fluorescens* compared to larger particle size such as talc, kaolinite and pyrrophillite. The survival of *Ps. fluorescens* in bentonite in the present experiments was even better than reported by Kurdish *et al.* (1999), in which bentonite with initial moisture of 30% after 12 month storage at 4°C resulted in a decreased survival of *Ps. aureofaciens* of about two fold log10 cfu. The mechanism how bentonite can support long-term survival of bacteria is not yet clearly known. Other publications stated that betonite has positive effect on the activity of beneficial rhizobacteria if it was amended to the soil under field conditions. For instance, bentonite added in the field enhanced the activity of *Ps. fluorescens* against *Fusarium culmorum* in rye (Kurek and Jarozuk-Schisel, 2002). Robert and Cenu (1992) hypothesized the mechanism how different types of clays (such as bentonite with main content montmorrilonite) can provide different protection of microbes under dry condition: 1) clay provides more available water to bacteria upon dessication, 2) related to water retention properties of different clay minerals, clay with higher water retention such as montmorrilonite provided better protection, 3) related to drying rate, clay with slower drying rate protects microbes better than the fast drying type, 4) ‘encapsulation’, bacteria (especially gram negative bacteria) or bacterial colonies are embedded in polysaccharides, which are surrounded by a layer of clay mineral. The ability of bentonite to

absorb and detoxify toxins has been already well known in animal nutrition science (Rosa *et al.,* 2001). Kurdish and Titova (2001) reported that addition of 1 % montmorrilonite into growth media increased the growth and resistance of *Agrobacterium radiobacter* against adverse effects of supra optimal temperatures. Moreover, Hwang and Latte (1997) reported altered metabolic activity of other soil bacteria like *Arthrobacter crystallopoietes* which was grown in media added with clay minerals.

Aside from bentonite, other tested material *i.e.* peat also supported sufficient survival of Pf B5 under dry condition. Peat has been previously reported as a good carrier for rhizobacteria formulations (Fravel *et al.,* 1998). If it was used as material for coating pigeon pea seeds with *Ps. fluorescens*, shelf life of bacteria under room temperature was 60 days (Vidhyasekaran *et al.*, 1997). Georgakopoulos *et al.* (2003) is claiming that *Ps. fluorescens* B5 (the same strain used in this study) is able to survive in peat carrier at ambient temperature for 2 years with a decrease of survival of 1 log10 cfu/g peat. In addition, peat is also reported as the best carrier in formulations of *Rhizobium* (McQuilken *et al.*, 1998)

The ability of the two materials supporting long-term survival of Pf B5 under dry conditions without significant loss of antagonistic activity *in vitro* highlights that bentonite and peat can be furthermore tested as filling materials in seed pelleting process of sugar beet with Pf B5. However, further research is needed to study the survival of Pf B5 in the mixture of bentonite and peat in different ratios.

4.4.2. **Effect of formulation additives on the antagonistic activity of *Pseudomonas fluorescens*** B5.

It is well known that antagonistic activity of fluorescent pseudomonads is affected by nutrients, like carbon and nitrogen sources as well as trace elements. Almost all studies on the effect of nutrients on antagonistic activity of fluorescent pseudomonad were done by their applying as plant fertilizer by soil application. Only Schulz and Wolf (1998) investigated the role of defined nutrients (C and N source) as formulation additives on the efficacy *ad planta* of

Pseudomonas fluorescens B5. The aim of the experiment was to study the effect of nitrogenous compounds and trace elements as formulation additives on the antagonistic activity of Pf B5.

None of the screened nitrogen compounds (urea, ammonium sulphate, ammonium nitrate, tryptone and casamino acids) increased the antifungal activity of Pf B5 *in vitro* and antagonistic activity *ad planta* as well. The effective concentration of these tested nitrogen compounds to enhance the production of antifungal substances and antagonistic activity of fluorescent pseudomonads is unknown, this may explain the phenomena. There was no previous research on the role of nitrogen compounds as formulation additives on the antagonistic activity of fluorescent pseudomonads. The use of nitrogenous compounds as formulation additives is reported for other groups of bacteria, *e.g.* the formulation of *Bacillus thuringiensis* containing organic nitrogen compounds such as D-L tryptophane and L-tyrosine as synergists (Bernhard *et al.,* 1998). Schmidt *et al.* (2001) also reported that the application of peptone (1%) as additive can enhance the efficacy of *Bacillus subtilis* and *Erwinia herbicola* against *Eutypa lata* on grape. The effects of nitrogen fertilizer applications on plant disease in the presence of biocontrol agent were variable. Höfflich *et al.* (2001) reported that the application of mineral nitrogen fertilizers on winter wheat, winter rye, spring barley, and pea did not affect the abundance of indigenous *Pseudomonads* on rhizosphere. In contrast, treatment with nitrogen fertilizers containing mixtures of NO_3^- and NH_4^+ enhanced the capacity of *Pseudomonas fuorescens* strains to promote plant growth and inhibited *Fusarium* growth on rye (Kurek and Jaroszuk-Sciseł, 2003). The present experiment reveals that among the tested nitrogen compounds in the range of 0.05% to 1%, ammonium nitrate and urea promoted the growth of *Pythium ultimum in vitro* but did not enhance disease *ad planta.* An enhancement of the biocontrol activity of Pf B5 by the addition of nitrogen compounds into sugar beet pills could not be demonstrated in the experiment. However, this should not underestimate the role of nitrogen compounds in formulation. A study of Weßendorf (1988) showed that incorporation of nitrate into clay soil at the rate of 0.8 mg/kg improved the survival rate of *Pseudomonas fluorescens* W1

applied to the soil. Further studies on the use of nitrogenous compounds as formulation additives towards the survival rate of *Psudomonas* spp. would be useful.

Screening of various trace elements ($FeCl_3$, H_3BO_3, $CoCl_2$, ammonium molybdate, $MnSO_4$ and $ZnSO_4$) at different concentrations as additives had been done *in vitro* and *ad planta*. The results clearly show that the addition of 0.05 mM $ZnSO_4$ and $MnSO_4$ could enhance the production of antifungal metabolites *in vitro* and applied to sugar beet pellets increased the antagonistic activity of Pf B5 *ad planta* against *Pythium.* The increase *ad planta* was between 13 % - 21 % and 18 - 20 % for $MnSO_4$ and $ZnSO_4$ respectively. To our knowledge, this is the first report on a positive effects of these trace elements applied as formulation additive on the antagonistic activity of *Pseudomonas* spp.. The previous research on the role of trace elements on the production of antibiotics and antagonistic activity of fluorescent pseudomonads, both *in vitro* and *ad planta*, generally is conducted by applying these substances as fertilizer or soil application. For example, the increase of the antagonistic activity of some other biocontrol strains of the fluorescent pseudomonads *in vitro* and *at planta* was mediated by amending zinc as a soil application as reported by Duffy and Defago (1997), Siddiqui *et al.* (2002), Hamid *et al.* (2003) and Ownley *et al.* (2003). The possible explanation of his effect may be an increased production of antifungal metabolites that has also been reported for *Ps. fluorescens* CHAO by Duffy and Defago (1999) and Shaukat *et al.* (2003). A further mechanism is that a $ZnSO_4$-solution can increase the ability of the cell attachment of *Ps. fluorescens* to target fungi by increasing its agglutination as reported by Sritivasta *et al.* (1996) for *Macrophomina phaseolina*. Further research is needed to answer the question whether Zn^{2+} can increase antifungal metabolites production of fluorescent pseudomonads in the rhizosphere.

There are limited informations on the effect of manganese on the production of antibiotics and antagonistic activity of fluorescent pseudomonads. It turned out in the present study that addition of 0.05 mM $MnSO_4$ to sugar beet seed pellet increased the antagonistic activity Pf B5 against *Pythium ultimum*

under controlled conditions. In addition, the stimulation of antifungal substances *in vitro* by amendment of 0.05 mM $MnSO_4$ has also been proven. In contrast, *in vitro* studies of Duffy and Defago (1999) with *Ps. fluorescens* CHAO made it clear that amending $MnCl_2$ into mineral media containing glycerol or glucose as carbon source did not have any effect on the production of antibiotics and siderophores *in vitro.* Antagonistic bioassays *in vitro* using *Pseudomonas aeruginosa* IE-6S+ and *Pseudomonas fluorescens* CHAO against *Macrophomina phaseolina* showed that $MnCl_2$ amendment enhanced the inhibition of *Pseudomonas fluorescens* CHAO but not that of *Pseudomonas aeruginosa* (Shaukat and Siddiqui, 2003). In contrast, experiments under gnotobiotic conditions revealed that manganese fertilization does not affect the antagonistic activity of *Pseudomonas fluorescens* CHAO against take-all disease of wheat (Wüthrich *et al*., 1990). Thus, in contrast to zinc that appeared to have a general effect, manganese seams to be strain specific in case of pseudomonads.

Other ideal features of formulation additives are that they should not favour the pathogen. This is also fulfilled by the two microelements Mn and Zn in the effective concentration of 0.05 mM. On the contrary, the two compounds in the tested range (0.05 mM-0.2mM) alone significantly inhibited the growth of *Pythium in vitro*. Manganese and zinc are already known as fungicidal substances (Anderson, 2002). Moreover, application of manganese as a soil application could induce cowpea resistance against *Rhizoctonia solani* and *Rhizoctonia bataticola* by stimulating the increase of defense enzymes such as polyphenol oxydase (PPO) and peroxidase (PO) and total phenol (Kalim *et al.*, 2003a; Kalim *et al.*, 2003b). However, the fungicidal activity *ad planta* against *P. ultimum* in the present study was only provided by Zn in higher concentration (0.2 mM) and not by Mn.

Other advantages of the two compounds as formulation additives are that $MnSO_4$ and $ZnSO_4$ at effective ratse did not have any detrimental effects on sugar beet seedlings. The two compounds applied as formulation additives did not reduce the emergence rate, but even increased height (Mn), total and individual fresh weight (Mn and Zn). Increasing of growth parameters due to

soil applications in low concentrations of Mn and Zn was reported in sunflower, in which Mn increased the height and Mn and Zn increased the number of leaves (El-Fouly, 2001). Growth inhibition due to Zn fertilizer application such as in *Mentha spicata,* reported by Bekiaroglou and Karataglis (2002) did not occur in the present system. Therefore, the effective rate at which $MnSO_4$ and $ZnSO_4$ enhance the antagonistic activity of Pf B5 against *P. ultimum* is equivalent to 12.33 mg/kg pills and 14.37 mg/kg pills respectively. In these concentrations the two compounds can act also as plant fertilizer.

Although single applications of Mn or Zn as formulation additives had a positive effect but this was no longer true if the two microelements were applied in combination. Surprisingly, the antagonistic activity *ad planta* was even lower. The reason for this phenomenon is not understood yet. One explanation is given by Anderson (2002), who stated that two trace elements *i.e.* Mn and Zn are antagonists in the uptake by plant root system.

Whereas $MnSO_4$ and $ZnSO_4$ had a positive effect on the antagonistic activity of *Ps. fluorescens in vitro* and *ad planta*, H_3BO_3 promoted an inhibitory effect *in vitro* but not *ad planta*. The reason why one of the tested compound like boric acid significantly enhanced the production of antifungal metabolites *in vitro* but not the antagonistic activity *ad planta* is also not known. *Ad planta* tests of antibiotics non-stimulating compounds (ammmonium molybdate, ammonium nitrate and casamino acids) did not provide any enhancement of antagonistic activity of Pf B5. Based on the tested compounds (5 trace elements, 5 nitrogenous compounds), it can be concluded that compounds that stimulate the inhibitory effect of the antagonist Pf B5 *in vitro* should be further investigated *ad planta*, however, the finding also underlines that it is not worthwhile to carry out *ad planta* tests with compounds which do not have any effect *in vitro*.

Even though Hamid *et al.* (2003) reported that ammonium molybdate can stimulate the production of nematicidal compounds *in vitro* and antagonistic activity *ad planta* of *Ps. fluorescens* CHAO against *Meloidogyne javanica*, the similar effects could not be found with *Ps. fluorescens* B5 in the present investigation. Probably, the effect of ammonium molybdate acts strain-specific.

The significance of the two microelements to enhance the antagonistic activity *ad planta*, their effect on the growth of *Pseudomonas fluorescens B*5 *in vitro*, and the effect on host and *Pythium* supports the theory that further research in the field of nutrition should attract more attention in the context of developing biological control methods.

5. OUTLOOK

In recent time a lot of research was focused on the biological control of fungal plant diseases including the use of fluorescent pseudomonads. However, compared to pest control, their introduction into the practice is very limited.

The success in development of biological control programs depend on the three following technical steps: 1) screening of antagonists in *vitro* and *ad planta,* 2) study on the physiological and ecological properties of antagonists in the context of the corresponding agroecosystem, 3) testing under field conditions 4) developing a suitable mass production, formulation and delivery system.

This lack of application of antagonists of plant diseases may be explained by the fact that most research in this field is mainly conducted at universities focused mainly on a more basic knowledge about processes on the molecular level. More practical aspects of biological control of fungal plant diseases such as mass production, formulation and application technique did not get enough attention and resource allocation. To ensure the success of biological control of fungal plant diseases in practical level these aspects should be more elaborated.

There are many efforts that can still be done after a superior antagonist is discovered. The present work using *Pseudomonas fluorescens* B5 as model is supporting the suggestion that there is still a reservoir of different factors or agents for improving biocontrol methods of fungal plant diseases. Innovations in the area of preservation, formulation and application of antagonists may contribute to a better acceptance in the agricultural practice.

6. SUMMARY

A series of experiments was carried out with the aim to optimise biological control of damping-off of sugar beet caused by *Pythium ultimum* by using *Pseudomonas fluorescens* B5 (Pf B5). The research consisted of the four following aspects: **1)** the use of Tn-5 antifungal metabolites-overproducing mutants to improve antagonistic activity of Pf B5, **2)** the role of indole-3-acetic acid (IAA) in biocontrol activity of Pf B5*,* **3)** the significance of plant varieties in biocontrol of *P. ultimum* in sugar beet by using Pf B5, and **4)** improvement of formulation techniques by applying appropriate pelleting materials and additives which can improve the antagonistic activity of Pf B5.

5000 transconjugants were obtained from transposon mutagenesis with *E. coli* Tn5 and Pf $B5^{Str}$. Seven mutants *i.e.* No. 010, No. 029, No. 311, N 24, Tn 12, Tn XII and No. 825 showed a higher antagonistic activity *in vitro* than wild type strain *Ps. fluorescens* B5. The application of the mutants No. 311, N 24 and Tn 12 *ad planta* improved biological control up to 19 %, 8 % and 29 % respectively compared to Pf B5 WT.

Studies on seven Tn5-mutants and on Pf B5 WT and Pf B5 ^{Str} reveal that the antagonistic activity *ad planta* correlated significantly with some physiological features *in vitro i.e.:* total HCN production($r=0.75$, $P < 0.001$), production of HCN/10^9 cells ($r=0.69$, $P < 0.01$), total production of IAA ($r=0.47$, $P < 0.05$), IAA production/10^9 cells ($r=0.60$, $P < 0.05$) and mycelial growth reduction using KB^+-based culture filtrate ($r=0.64$, $P <0.01$). No significant correlation found between antagonistic activity *of* mutants *ad planta* colonisation on rhizosphere and rhizoplane. A significant correlation was found between rhizosphere and rhizoplane colonisation and mycelial growth reduction in LB-based culture filtrates, and inhibition zone on TSA. Motility correlated significantly with the rhizosphere colonization but not with the colonization in the rhizoplane.

The second study of the research has proved a first indication for the importance of indole-3-acetic acid (IAA) in biological control of Pf B5. A significant correlation was found between IAA production *in vitro* and

antagonistic activity *ad planta*. The growth of *P. ultimum* was suppressed *in vitro* using commercially available IAA at the rate of 6.25µg/ml, 12.5µg/ml, 25µg/ml, 50µg/ml, 75 µg/ml 100µg/ml and 200 µg/ml. Moreover, seeds dipping with 12.5 µg/ml and 25 µg/ml IAA before planting suppress damping-off of sugar beet seedlings. On the other hand, seeds dipping with IAA increased fresh weight of sugar beet seedlings.

Eight tested sugar beet varieties treated with Pf B5 showed a different level of disease suppression in standard biocontrol assay in pot test against *P. ultimum*. The level of disease suppression was 74.8%, 58.3%, 47.9%, 44.0%, 51.2%, 80.2%, 72.1%, and 67.8 % for the varieties 300-0132B, 300-0088B, 300-0364B, 300-0483B, 300-0448B, 300-0226B and 300-0493B respectively. Under the same environmental condition and disease pressure, eight-tested sugar beet varieties show different resistance against *P. ultimum*. However, resistance level of sugar beet varieties against tdamping-off did not correlate to the biocontrol performance of Pf B5. Even though the adherence and colonising ability of Pf B5 was significantly different among these varieties, no correlation of these two features with the antagonistic activity was found.

Six different materials *i.e.,* two kinds of wood flour, diatomaceous earth, bentonite, cotton flour and peat were used to screen for appropriate pelleting materials. All tested pelleting materials in wet form (60% initial water content) and cold storage at 5°C supported survival of Pf B5 without significant loss after one year of storage. However, only peat and bentonite were able to support high survival of Pf B5 in the case of using the pelleting material in dry form (10% initial water content), with loss of only 1 log cfu/g material after one year and without significant reduction of antagonistic activity *in vitro*.

Various nitrogen compounds *i.e.* urea, ammonium nitrate, ammonium sulphate, tryptone, and casamino acids and trace elements *i.e.* boric acid, ferric chloride, manganese sulphate, ammonium molybdate, cobalt (II) chloride, copper (II) sulphate and zinc sulphate were tested for their use as formulation additives. None of the tested nitrogen compounds could enhance

the antagonistic activity of Pf B5 *in vitro* and *ad planta.* The production of antifungal metabolites of Pf B5 *in vitro* was improved with the addition of $MnSO_4$, $ZnSO_4$ and H_3BO_3 at the rate of 0.05 mM. Moreover, a treatment of sugar beet seeds with $MnSO_4$ and $ZnSO_4$ at the rate of 0.05 mM increased the biocontrol activity of Pf B5 *ad planta* under controlled conditions, but the two compounds did not favour the pathogen *in vivo* and *ad planta* and also did not have any detrimental effects on sugar beet seedlings, but increased height (Mn^{2+}) and fresh weight of treated seedlings (Mn^{2+} and Zn^{2+}).

7. REFERENCES

Al-Masri, M. I., M. S. Ali-Shtayeh, Y. Elad , A. Sharon, P. Tudzynski, and R. Barakat, 2002. Effect of plant growth regulators on white mould (*Sclerotinia sclerotiorum)* on bean and cucumber. *J. Phytopathology* 150:481–487

Anderson, A. J., P. Habibzadegah-Tari, and C. Tepper. 1988. Molecular studies on the role of root surface agglutinin in adherence and colonization by *Pseudomonas putida*. *App. Environ. Microbiol*. 54:375-380

Anderson, S. 2002. The relationship between nutrients and other elements to plant diseases. *Tree Care Industry*: 25-32

Asghar H. N., Z. A. Zahir, M. Arshad, and A. Khaliq. 2002. Relationship between *in vitro* production of auxins by rhizobacteria and their growth-promoting activities in *Brassica junce*a L. *Biol. Fertil. Soils*. 35:231–237

Asmus, E. and H. Garschagen. 1953. Über die Verwendung von Barbitursäure für die photometrische Bestimmung von Cyanid und Rhodanid. *Z. Analyt. Chem* 138:414-422

Bano, N. and J. Musarrat. 2003. Characterization of a new *Pseudomonas aeruginosa s*train NJ-15 as a potential biocontrol agent. *Current Microbiol*. 46:324–328

Barazani, O. and J. Friedman, 1999. Is IAA the major root growth factor secreted from plant-growth-mediating bacteria? *J. Chem. Ecol.* 25:2397-2406

Bashan, Y., L. E. Gonzalez. 1999. Long-term survival of the plant-growth-promoting bacteria *Azospirillum brasilense* and *Pseudomonas fluorescens* in dry alginate inoculant. *Appl. Microbiol. Biotechnol.* 51:262-266

Bekiaroglou, P. and S. Karataglis. 2002. The efect of lead and zinc on *Mentha spicata* . *J. Agron. & Crop Sci*. 188:201-205

Bernhard, K., P. J. Holloway and H. D. Burges. 1998. A catalogue of formulation additives: function, nomenclature, properties and suppliers. *In*: H. D. Burges *(ed.*). Formulation of Microbial Biopesticides-Beneficial Microorganism, Nematodes and Seed Treatment. Kluwer Academic Publ. Dordrecht. pp. 333-366

Blumer, C. and D. Haas. 2000. Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. *Arch. Microbiol.* 73:170-177

Bollack, J. M. and G. Strotzky (*eds.*). Soil Biochemistry Vol. 6. Marcel Dekker. New York.

Burges, H. D. and K. A. Jones. 1998. Trends in formulation of microorganisms and future research requirements. *In*: H. D. Burges (*ed.*). Formulation of Microbial Biopesticides- beneficial Microorganism, Nematodes and Seed Treatment. Kluwer Academic Publ. Dordrecht. *pp*. 311-332

Burges, H. D. (*ed.*). 1998. Formulation of Microbial Biopesticides- Beneficial Microorganism, Nematodes and Seed Treatment. Kluwer Academic Publ. Dordrecht

Campbell, R. 1989. Biological Control of Microbial Plant Pathogens. Cambridge University Press, Cambridge.

Chatterjee, A. , R. Valasubramanian, W. L. Ma, A. K. Vachhani, S. Gnanamanickam, and A. K. Chatterjee. 1996. Isolation of *ant* mutants of *Pseudomonas fluorescens* strain Pf7-14 altered in antibiotic production, cloning of *ant1* DNA, and evaluation of the role of antibiotic production in the control of blast and sheath blight of rice. *Biol. Control* 7:185–195

Chen,C. R. R. Belanger, N. Benhamou and T. C. Paulitz. 1999. Role of salicylic acid in systemic resistance induced by *Pseudomonas* spp. against *Pythium aphanidermatum* in cucumber roots. *European J. of Plant Pathol.* 105: 477–486

Chin-A-Woeng, T. F. C. 2000. Molecular Basis of Biological Control of Tomato Foot and Root Rot by *Pseudomonas chlororaphis* strain PCL 1391. Dissertation at Leiden University, the Netherlands.

Chin-A-Woeng, T. F. C., G. V. Bloemberg and B. J. J. Lugtenberg. 2003. Phenazines and their role in biocontrol by *Pseudomonas* bacteria. *New Phytologist* 57:503-523

Clays-Josserand, A., J. F. Ghiglione, L. Philippot, P. Lemanceau and R. Lensi. 1999. Effect of soil type and plant species on the fluorescent pseudomonads nitrate dissimilating community. *Plant & Soil* 209: 275–282

Dandurrand, L. M., M. J. Morra, M. H. Chaverra and C. S. Orser. 1994. Survival of *Pseudomonas* spp. in air-dried mineral powders. *Soil Biol. Biochem*. 26:1423-1430

Dasthi, N., B. Prithiviraj, R. K. Hynes, and D. L. Smith. 2000. Root and rhizosphere colonisation of soybean (*Glycine* max L.) by plant-growth-promoting rhizobacteria at low root zone temperatures and short-season conditions. *J. Agron. & Crop Sci.* 185:15-22

Defago, G and D. Haas. 1990. Pseudomonads as antagonist of soilborne plant pathogens: modes of action and genetic analysis. *In*: J-M. Bollack and Strotzky (*eds.*). Soil Biochemistry Vol. 6. Marcel Dekker. New York. pp:249-291

De Oliveira Pinheiro, R., L. H. Boddey, E. K. James, J. I. Sprent, and R.M. Boddey. 2002. Adsorption and anchoring of *Azospirillum* strains to roots of wheat seedlings. *Plant & Soil* 246:151-166

Dekkers, L. C., C. C. Phoelich, and B. J. J. Lugtenberg. 1999. Bacterial traits and genes involved in rhizosphere colonization. *In*: CR. Bell, M Brylinksky and P Jhonson-green (*eds.*). Proc. of 8th International Symposium on Microbial Ecology, Atlantic Canada Soc. For Microbial Ecology, Halifax, Canada.

Delany, I. R., U. F. Walsh, I. Ross, A. M. Fenton, D. M. Corkery and F. O'Gara. 2001. Enhancing the biocontrol efficacy of *Pseudomonas fluorescens* F113 by altering the regulation and production of 2,4-diacetylphloroglucinol. *Plant & Soil* 232:195–205

Desai, S., M. S. Reddy and J. W. Kloepper. 2003. Comprehensive testing of biocontrol agent. *In*: S. Gnanamanickam (*ed.).* Biological Control of Crop Diseases. Marcell-Dekker. New York. *pp*. 387-420

de Souza, J. T., C. Arnould, C. Deulvot, P. Lemanceau, V. Gianinazzi-Pearson, and J. M. Raaijmakers. 2003. Effect of 2,4-diacetyl phloroglucinol on *Pythium*: cellular responses and variation in sensitiviy among propagules and species*. Phytopathology* 93:966-975

Duffy, B. K. and G. Defago, 1997. Zinc improve biocontrol of Fusarium crown and root rot by *Psedomonas fluorescens* and repress the production of pathogen metabolites inhibitory to bacterial antibiotic biosynthesis. *Phytopathol* 87:250-1257

Duffy, B. K. and G. Defago, 1999. Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. *App. Environ. Microbiol* . 65: 2429-2423

El-Fouly, M. M, O. A. Nofal and Z. M. Mobarak. 2001. Efects of soil treatment with iron, manganese and zinc on growth and micronutrient uptake of sunflower plants grown in high-pH soil. *J. Agron. & Crop Sci.* 186: 245-251

Ellis, R. J. , T. M. Timms-Wilson and M. J. Bailey. 2000. Identification of conserved traits in fuorescent pseudomonads with antifungal activity. *Environ. Microbiol*. 2: 274-284

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

Fakhouri, W. and H. Buchenauer. 2002. Characteristics of fluorescent pseudomonad isolates toward controlling of tomato wilt caused by *Fusarium oxysporum* f.sp. *lycopersici*. *J. Plant Dis. & Plant Protection* 110: 143-156

Fravel, D. R., W. J. Connick. J. A. Lewis. 1998. Formulation of microorganisms to control plant diseases. *In*: H.D. Burges (*ed*.). Formulation of Microbial Biopesticides-Beneficial Microorganism, Nematodes and Seed Treatment. Kluwer Academic Publ. Dordrecht. *pp*:187-202.

Friedrich, B., C. Hogrefe and H. G. Schlegel. 1981. Naturally occurring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus. J. Bacteriol*. 147: 198-205

Folman, L. B. , J. Postma, J. A. Van Veen. 2003. Inability to find consistent bacterial biocontrol agents of *Pythium aphanidermatum* in cucumber using screens based on ecophysiological traits. *Microb. Ecol.* 45:72–87

Gamalero, E., M. G. Martinotti, A. Trotta, P. Lemanceau and G. Berta. 2002. Morphogenetic modifications induced by *Pseudomonas ffluorescens* A6RI and *Glomus mosseae* BEG12 in the root system of tomato differ according to plant growth conditions. *New Phytologist* 155:293–300

Georgakopoulos, D. G., P. Fiddaman, C. Leifert and N. E. Malathrakis. 2002. Biological control of cucumber and sugar beet damping-off caused by *Pythium ultimum* with bacterial and fungal antagonists . J. *App. Microbiol*. 92:1078–1086

Glandorf, D. C., M. Van der Sluis, A. J. Anderson, P. A. H. M Bakker and B. Schippers. 1994. Agglutination, adherence, and root colonization by fluorescent pseudomonads. *App. Environ. Microbio*l 60:1726-1723

Glickmann, E. and Y. Dessaux. 1995. A critical examination of specificity of the salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *App. Environ. Microbiol* 61:793-796

Gnanamanickam, S. (*ed.*). 2003. Biological Control of Crop Diseases. Marcell-Dekker. New York.

Goel, A. K., S. S. Sindhu K. R. Dadarwal. 2002. Stimulation of nodulation and plant growth of chickpea (*Cicer arietinum* L .) by *Pseudomonas* spp. antagonistic to fungal pathogens. *Biol. Fertil. Soils* 36:391–396.

Gomez, K. A. and Gomez, A. A. 1984, Statistical Procedures for Agricultural Research. John Wiley and Sons. New York.

Hamid, M., I. A. Siddiqui and S. Shahid Shaukat. 2003. Improvement of *Pseudomonas fluorescens* CHA0 biocontrol activity against root-knot nematode by the addition of ammonium molybdate*. Letters in App. Microbiol.* 36:239–244

Harris, A. R. and P. G. Adkins. 1999. Versatility of fungal and bacterial isolates for biological control of damping-off disease caused by R*hizoctonia solani* and *Pythium* spp. *Biol. Control* 15**:**10–18

Hebbar, K. P, M. H. Martel and T. Heulin, 1998. Suppression of pre and post emergence damping off in corn by *Burkholderia cepacia. European J. of Plant Pathol.* 104:29–36

Hebbar, K. P., A. G. Davey, J. Merrin, P. J. Dart. 1992. Rhizobacteria of maize antagonistic to *Fusarium moniliforme*, a soilborne fungal pathogen: colonisation of rihizosphere and roots. *Soil Biol. Biochem* 24: 989-997

Hervas, A., B. Landa and Rafael M. Jimenez-Dýaz . 1997. Influence of chickpea genotype and *Bacillus* sp. on protection from Fusarium wilt by seed treatment with nonpathogenic *Fusarium oxysporum. European J. of Plant Pathol.* 103: 631–642

Heupel, M. 1992. Biologische Bekämpfung bodenbürtiger Wurzelbranerreger der Zuckerrübe (*Beta vulgaris* L. ssp*. vulgaris* var. *altissima* Doell) durch den Einsatz mikrobieller Antagonisten. Dissertation at University of Göttingen, Germany.

Höfflich, G., M Tauschke, G. Kuhn and J. Rogasik. 1999. Infuence of agricultural crops and fertilization on microbial activity and microorganisms in the rhizosphere *J. Agron. & Crop Sci.* 73:38-43

Howie W.J., R. J. Cook and D. M. 1987. Effects of soil matric potential and cell motility on wheat root colonization by fluorescent pseudomonads suppressive to take-all. *Phytopathology* 77:286–292

Hultberg, M., 1999. Seed Bacterization with *Pseudomonas fluorescens* and Interaction with *Pythium ultimum* on Tomato Soilless System. Doctoral Thesis at Swedish University of Agricultural Science. Alnarp

Hultberg, M. and B. Waechter-Kristensen. 1998. Colonisation of germinating tomato seeeds with the plant growth-promoting rhizobacteria, *Pseudomonas fluorescens* 5.014 and its mutant 5-2/4. *Microbiol. Res.* 153:105-111

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

Hwang, J. W. S. Chilton, and D. M. Bensona,. 2002. Pyrrolnitrin production by *Burkholderia cepacia* and biocontrol of *Rhizoctonia* stem rot of poinsettia. *Biol. Control* 25 :56–63.

Hwang, S. and R.L. Tate. 1997. Interactions of clay minerals with *Arthrobacter crystallopoietes*: starvation, survival and 2-hydroxypyridine catabolism. *Biol. Fertil. Soils* 24:335–340

Imam Ali N., I. Ali Siddiqui, S. Shahid Shaukat and M. J. Zaki. 2001. Survival of *Pseudomonas aeruginosa* in various carriers for the inhibition of root rot-root knot disease complex of mungbean. *Phytopathology Mediterrania* 40:108–112

James, D. W., Jr, T. V. Suslow and K. E. Steinback. 1985. Relationship between rapid, firm adhesion and long term colonisation of roots by bacteria. *App. Environ. Microbiol.* 50 :392-397

James, W. C. 1982. Estimated losses of crops from plant pathogens. *In*: D. Pimentel (*ed.*). CRC Handbook of Pest Management in Agriculture Vol. I Boca Raton Florida, *pp*. 253-245

Jiemba, P. K and M. Alexander. 1999. Possible determinants of rhizosphere competence of bacteria. *Soil Biol. & Biochem*. 31:623-632

Jones, K. A. and H. D. Burges. 1998. Technology of formulation and application. *In*: H. D. Burges (*ed.*). Formulation of Microbial Biopesticides-Beneficial Microorganism, Nematodes and Seed Treatment. Kluwer Academic Publ. Dordrecht. *pp*. 7-30

Kalim , S., Y. P. Luthra and S. K. Gandhi . 2003[a]. Cowpea root rot severity and metabolic changes in relation to manganese application. *J. Phytopathology* 151: 92–97

Kalim , S., Y. P. Luthra and S. K. Gandhi . 2003[b]. Role of zinc and manganese in resistance of cowpea root rot. *J. Plant Disease and Plant Protection* 110: 235-243

Kaur, S., Anil K. Gupta and N. Kaur. 2003. Indole acetic acid mimics the effect of salt stress in relation to enzymes of carbohydrate metabolism in chickpea seedlings. *J. Plant Growth Regul.* 39: 91–98

Kiewnick, S and B. Jacobsen. 1996. Biologische Bekämpfung von *Cercospora beticola* an Zuckerrübe mit Phyllosphere-Bakterien unter Verwendung verschiedener Nährstoffzusätze. *Mittl. Biol. Bundesamt Land-Forstwirtsch*. 321

Koble, W. 1987. 200 Jahre Pflanzenschutz im Zuckerrübenbau. Rheinischer Landwirtschaft-Verlag. Bonn

Krishnamurthy , K. and S. S. Gnanamanickam. 1998. Induction of systemic resistance and salicylic acid accumulation in *Oryza sativa*, L. in the biological suppression of rice blast caused by treatments with *Pseudomonas* spp.. *World J. Microbiol. & Biotechnol.* 14:935-937

Krishnamurthy, K. and S. S. Gnanamanickam. 1998. Biological control by *Pseudomonas fluorescens* Strain Pf7–14: evaluation of a marker gene and formulations. *Biol. Control* 13**:**158–165

Kumari, V and J. S. Srivastava. 1999. Molecular and biochemical aspects of rhizobacterial ecology with emphasis on biological control. *World J. of Microbiol. & Biotechnol*. 15: 535-543

Kurdish, I. K. and L. V. Titova. 2001. Use of high-dispersion materials for culturing and obtaining granular *Agrobacterium radiobacter* preparations. *App. Biochem. & Microbiol. 37:318–321.*

Kurdish, I. K., A. A. Roi., A. D. Garagulya and E. A. Kiprianova. 1999. Survival and antagonistic activity of *Pseudomonas aureofaciens* UKM B-111 stored in fine materials. *Microbiol.* 68:387-393

Kurek, E., and J. Jaroszuk-Sciseł. 2003. Rye (*Secale cereale*) growth promotion by *Pseudomonas florescens* strains and their interactions with *Fusarium culmorum* under various soil conditions. *Biol. Control* 26 :48 –56

Laermann, H.T. 1972. Die Wirkung von Terbutryn und Methbenzthiazuron auf Wintergerste in Abhängigkeit von verschiedenen Bodeneigenschaften und Umweltbedingungen. PhD Thesis, Universität Göttingen.

Landa, B. B., A. Hervas, W. Bettiol, and R. M. Jimenez Dyaz. 1997. Antagonistic activity of bacteria from the chickpea rhizosphere against *Fusarium oxysporum* f.sp. *ciceris.* *Phytoparasitica* 25:305-318

Lee, J. Y. , S. S. Moon and B. K. Hwang . 2003. Isolation and *in vitro* and *in vivo* activity against *Phytophthora capsici* and *Colletotrichum orbiculare* of phenazine-1-carboxylic acid from *Pseudomonas aeruginosa* strain GC-B26. *Pest Manag. Sci.* 59:872–882

Leeman M., J. A. van Pelt, F. M. den Ouden, M. Heinsbroek, P. A. H. M. Bakker and B. Schippers. 1995. Induction of systemic resistance by *Pseudomonas fluorescens* in radish cultivars differ in susceptibility to Fusarium wilt, using a novel bioassay. *European J. Plant Pathol* 101:655-664

Lemanceau, P., T. Corberand, L. Gardan, X Latour, G Laguerre, J.M Boeufgrass and C Alabouvette. 1995. Effect of two plant species, flax (*Linum usitatissianum* L.) and tomato (*Lycopersicon esculentum* Mill), on the diversity of soilborne populations of fluorescens pseudomonads. *App. Environ. Microbiol*. 61:11004-1012

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

Liu L., J. W. Kloepper and S. Tuzun . 1995. Induction of systemic resistance in cucumber by plant growth-promoting rhizobacteria: duration of protection and effect of host resistance on protection and root colonization. *Phytopathology* 85:1064–1068

Lugtenberg, B. J. J. and L. C. Dekkers. 1999. What makes *Pseudomonas* bacteria rhizosphere competent? *Environ. Microbiol*. 1:9–13

Lugtenberg, B. J. J., L. V. Kravchenko and Marco Simons. 1999. Tomato seed and root exudate sugars: composition utilization by Pseudomonas biocontrol strains and role in rhizosphere colonization the mutant cells reached the same population levels. *Environ. Microbiol*. 1:439-446

Lugtenberg, B. J. J., L. Dekkers and G. V. Bloemberg. 2001. Molecular determinat of rhizosphere colonisation by pseudomonads. *Ann. Rev. Phytopathology* 39:461-490

Maass, F. 1996. Untersuchungen zu den Hemmmechanismen von *Pseudomonas fluorescens* B5 gegen den Erreger des Wurzelbrands an der Zuckerrübe (*Beta vulgaris* var. *altissima) Pythium ultimum* unter besonderer Berücksichtigung der Siderophoren. MSc. Thesis. Institut für Pflanzenpathologie und Pflanzenschutz Georg-August Universität Göttingen.

Marschner, P. , J. Gerendas and B. Sattelmacher. 1999. Effect of N concentration and N source on root colonization by *Pseudomonas fluorescens* 2-79RLI. *Plant & Soil* 215:135–141

Maurhofer, M., C. Keel, D. Haas and G. Defago, 1995. Influence of plant species on disease suppression by *Pseudomonas fluorescens* strain CHAO with enhanced antibiotic production. *Plant Pathol*. 44: 40-50

Maurhofer, M., C. Keel, U. Schneider, C. Voisard, D. Haas and G. Defago. 1992. Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHAO on its disease suppresive capacity. *Phytopathology* 82:190-195

Mayak, S., T. Tirosh,and B. R. Glick. 2001. Effect of wild-type and mutant plant growth-promoting rhizobacteria on the rooting of mung bean cuttings. *J.* Plant Growth Regul 18:49–53

McQuilken, M. P., P. Halmer and D. J. Rhodes. 1998. Application of microorganisms to seeds *In*: Burges, H.D. Formulation of Microbial Biopesticides- Beneficial Microorganism, Nematodes and Seed Treatment. Kluwer Academic Publ. Dordrecht. pp. 255-286

Meena, B. T. Marimuthu, P. Vidhyasekaran and R. Velazhahan. 2001. Biological control of root rot of groundnut with antagonistic *Pseudomonas fluorescens* strains*. J. of Plant Dis. and Plant Prot.* 108: 369-381

Naseby, D. C., J. A. Way, N. J. Bainton and J. M. Lynch. 2001. Biocontrol of *Pythium* in the pea rhizosphere by antifungal metabolite producing and non-producing *Pseudomonas* strains. *J. App. Microbiol*. 90: 421-429

Naseby, D. C. and J. M. Lynch. 1999. Effects of *Pseudomonas fluorescens* F113 on ecological functions in the pea rhizosphere are dependent on pH. *Microb. Ecol.* 37:248–256

Nautiyal, C. S. 1997. Selection of chickpea-rhizosphere-competent *Pseudomonas fluorescens* NBRI1303 antagonistic to *Fusarium oxysporum* f. sp. *ciceri, Rhizoctonia bataticola* and *Pythium* sp. *Current Microbiol.* 35: 52–58

Niemann, S. , C. Keel, A. Puhler and W. Selbitschka. 1997. Biocontrol strain *Pseudomonas fluorescens* CHA0 and its genetically modified derivative with enhanced biocontrol capability exert comparable effects on the structure of a *Sinorhizobium meliloti* population in gnotobiotic systems. *Biol. Fertil. Soils* 25:240–244

Notz, R., M. Maurhofer, U Schneider-Keel, B. Duffy, D. Haas and G. Defago. 2001. Biotic factors affecting expression of the 2,4-diacetylphoroglucinol biosynthesis gene ph1a in *Pseudomonas fluorescens b*iocontrol strain CHAO in the rhizosphere. *Phytopathol* 91:873-881

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

Owney, B-H., B. K. Duffy and D. M. Weller. 2003. Identification and manipulation of soil properties to improve the biological control performance of phenazine-producing *Pseudomonas fluorescens*. *Appl. Environ. Microbiol* 69 : 3333-3343

Pedersen, H. C., I. Weiergang, M-M. Pontopiddan, L. Joergensen and A. Svingel. 2002. Prolonged shelf-life of carrier-loaded dehydration sensitive microorganisms. IOBC wprs (International Organization for Biological and Integrated Control of Noxious Animals and Plants West Palearctic Regional Section) Bull. 25:315-318

Pimentel, D. (*ed.*). 1982. CRC Handbook of Pest Management in Agriculture Vol. I Boca Raton. Florida

Ramamoorthy V, R. Viswanathan, T. Raguchander, V. Prakasam and R. Samiyappan. 2001. Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. *Crop Prot.* 20:1-11

Ramamoorthy, V., T. Raguchander and R. Samiyappan. 2002. Induction of defense-related proteins in tomato roots treated with *Pseudomonas fluorescens* Pf1 and *Fusarium oxysporum* f. sp. l*ycopersici. Plant and Soil* 239:55–68

Robert, M. and C. Chenu. 1992. Interactions between soil minerals and microorganisms. *In*: G. Strotzky and J-M. Bollack (*eds.*). Soil Biochemistry Vol. 7 Marcel Dekker. New York. *pp*:307-359.

Roco, A. and L. M. Perez. 2001. *In vitro* biological control activity of *Trichoderma harzianum* on *Alternaria alternata* in the presence of growth regulators. *Plant Biotechnol*. 4:1-6

Rosa, CAR., R. Miazzo, C. Magnoli, M. Salvano, S. M. Chiacchiera, S. Ferrero, M. Saenz, E.C.Q. Carvalho, and A. Dalcero. 2001. Evaluation of the efficacy of bentonite from the south of argentina to ameliorate the toxic effects of aflatoxin in broilers. *Poultry Sci*. 80:139–144

Russo, A., Y. Moenne-Loccoz, S. Fedi, P. Higgins, A. Fenton, D. N. Dowling M. O'Regan F. O'Gara. 1996. Improved delivery of biocontrol *Pseudomonas* and their antifungal metabolites using alginate polymers. *Appl. Microbiol. Biotechnol* . 44:740–745

Scher, F. M., J. W. Kloepper, C. Singleton, I . Zaleska and M. Laliberte. 1988. Colonization of soybean roots by *Pseudomonas* and *Serratia* species: relationship to bacterial motility, chemotaxis and generation time. *Phytopathology* 78:1055-1059

Schippers, B., A. W. Bakker and P. H . A. M. Bakker. 1987. Interaction of deleterious and beneficial rhizosphere microorganisms and their effect on the cropping of potato. *Ann. Review. Phytopathology* 25:339-358

Schmidt, C. S, D. Lorenz, G. A. Wolf and J. Jäger. 2001. Biological control o grapevine dieback fungus *Eutypa lata* II: influence of formulation additives and transposon mutagenesis on the antagonistic activity of *Bacillus subtilis* and *Erwinia herbicola*. *J. Phytopathol* 149:437-445

Schulz, D. and G. A. Wolf. 1998. Der Einfluss definierter Nährstoffgaben in einer Saatgutbehandlung auf die biologische Bekämpfung des Zückerrubernpathogens *Pythium ultimum* durch Pseudomonas fluorescens B5. *Mittl. Biol. Bundesamt Land-Forstwirtsch*. 51

Schulz, D. and G. A. Wolf. 2002. Influence of motility, adhesion and the production of sidrophore and antibiotics of *Ps. fluorescens* B5 and *Ps. corrugata* in rhizosphere colonisation and biocontrol in microcosm. *Mittl. Biol. Bundesamt Land-Forstwirtsch*. 390:394

Schulz, D., M. Heupel, and G. A Wolf. 1994. Untersuchungen zu Mechanismen und Steurungfaktoren der antagobsitischen Wirkung von *Pseudomonas fluorescens* B5. *Mittl. Biol. Bundesamt Land-Forstwirtsch*. 301: 354

Shaukat, S. S. and I. A. Siddiqui. 2003. The influence of mineral and carbon sources on biological control of charcoal rot fungus, *Macrophomina phaseolina* by fluorescent pseudomonads in tomato. *Letters in Appl. Microbiol* 36:392-398

Siddiqui I. A. and S. S. Shaukat. 2002. Resistance against the damping-off fungus *Rhizoctonia solani* systemically induced by the plant-growth-promoting rhizobacteria *Pseudomonas aeruginosa* (IE-6S+) and *P. fuorescens* (CHA0). *J. Phytopathology* 150:500–506

Siddiqui, I. A., S. S. Shaukat and M. Hamid. 2002. Role of zinc in rhizobacteria-mediated suppression of root-infecting fungi and root-knot nematode. J. *Phytopathology.* 150:569-575

Siddiqui, I. A. and S. S. Shaukat. 2003. Plant species, host age and host genotype effects on *Meloidogyne incognita* biocontrol by *Pseudomonas fuorescens* strain CHAO and its genetically modified derivatives. *J. Phytopathology* 151:231–238

Simon, R., U. Priefer and A. Pühler. 1983. Vector plasmids for *in vivo* and *in vitro* manipulation of gram-negative bacteria. *In*: Pühler *(ed.).* Molecular Genetics of Bacteria-Plant Interaction. Springer Verlag, Berlin

Simon, H. M, K. P. Smith, J. A. Dodswordth, B. Guenthner, Jo Handelsman and R. M. Goodman. 2001. Influence of tomato genotype on growth of inoculated and indigenous bacteria in the spermosphere. *App. Envion. Microbiol.* 67: 514-520

Smilanick, J. L. 1994. Strategies for the isolation and testing of biocontrol agents In: C. L. Wilson and M. E. Wiesniewski (*eds.*). Biological Control of Postharvest Diseases Theory and Practices. CRC Press, Florida. *pp.*25.35.

Smith, K. P. and R. M. Goodman. 1999. Host variation for interactions with beneficial plant-associated microbes. *Ann. Rev. Pythopathology* 37:473-491

Srivastava, A . K., S. Gupta and D. K. Arora. 1996. Aglutination response of *Pseudomonas fluorescens* and *Trichoderma harzianum* to *Macrophomina phaseolina* under different growth condition. *Microbiol. Res.* 151:193-200

Strotzky, G. and J-M. Bollack (*eds.*). 1992. Soil Biochemistry Vol. 7 Marcel Dekker. New York.

Tilcher, R. 2002. Integration of antagonistic bacteria in the process of pelleting sugar beet seed- results and problems. IOBC wprs (International Organisaton for Biological and Integrated Control of Noxious Animals and Plants West Palearctic Regional Section) Bull. 25:53-56

Toyota, K. and K. Ikeda. 1997. Relative importance of motility and antibiosis in the rhizoplane competence of a biocontrol agent *Pseudomonas fluorescens* MelRC2Rif. *Biol Fertil Soils* 25:416–420

van de Broek, A., M. Lambrecht and J. Vanderleyden. 1998. Bacterial chemotactic motility is important for the initiation of wheat root colonization by *Azospirillum brasilense. Microbiol.* 144:2599–2606

Vesper, S. J. 1987. Production of pili (fimbriae) by *Pseudomonas fluorescens* and correlation with attachment to corn roots. *App. Environ. Microbiol*. 53:1397-1405

Vidhyasekaran, P., K. Sethuraman, K. Rajappan, and K. Vasumathi. 1997. Powder Formulations of *Pseudomonas fluorescens* to Control Pigeonpea Wilt. *Biol. Control* 8:166–171

Walsh, U. F, J. P. Morsisey and F. O Gara. 2001. Pseudomonas for biocontrol of phytopathogens: from functional genomics to commercial exploitation. *Current Opinion in Biotechnol.* 12:289-295

Warrior, P., K. Krishnamurthy and P. Vasudevan. 2003. Formulation of biological control agents for pests and disease management. In: S. Gnanamanickam (*ed.*). Biological Control of Crop Diseases. Marcell-Dekker. New York. *pp*. 421-442

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

Weßendorf, J. 1988. Einfluss von Anzucht-und Bodenbedingungen auf das Überleben eines *Pseudomonas- fluorescens*-Stammes im Boden. Dissertation at Hohenheim University.

Whipps, J. M. 1997. Development in the biological control of soil-borne plant pathogens. *Adv. in Bot. Res*. 26:1- 99

Whipps, J. M. 2001. Microbial interaction and biocontrol in the rhizosphere. *J. of Exp. Bot. 52:*487-511

Wilson, C.L. and M.E. Wisniewski (*eds*). 1994. Biological Control of Postharvest Disease Theory and Practice. CRC Press. Boca Raton

Wirtschaftliche Vereinigung Zücker- Verein der Zücker Industrie. 2003. Strukturdaten. http://www.zuckerwirtschaft.de/3_1_1_1_3.html. Download at 28.07.03

Wüthrich, B., P. Haldimann and G. Defago. 1990. Effect of pH, nitrogen sources and manganese on suppression of wheat take- all by *Pseudomonas fluorescens* strain CHAO under gnobiotic condition. *In*: C Keel, B Koller and G. Defago. Plant Growth –promoting Rhizobacteria Progress and prospects. The Second International Workshop on PGPR, Interlaken Switzerland 14-19 Oct 1990. *pp.* 343-377

Xie, H., J. J. Pasternak and B. R. Glick. 1996. Isolation and characterization of mutants of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2 t hat overproduce indoleacetic acid. *Current Microbiol.* 32 :67–71

Zhang, S. M. S. Reddy, and J. W. Kloepper. 2002. Development of assays for assessing induced systemic resistance by plant growth-promoting rhizobacteria against blue mold of tobacco. *Biol. Control* 23**:**79–86

Zhang, S., A. L. Moyne, M. S. Reddy and J. W. Kloepper. 2002. The role of salicylic acid in induced systemic resistance elicited by plant growth-promoting rhizobacteria against blue mold of tobacco. *Biol. Control* 25:288–296

Zhao, Q. 2001. Untersuchungen zum Infektionmodus, immunologischen Nachweis und zur biologischen Bekämpfung von *Leptosphaeria maculans* (Desm) Ces. & de Not., dem Erreger der Wurzelhals-und Stängelfäule an Winterraps (*Brassica napus* L.). Cuvellier Verlag. Göttingen.

AUTHOR BIOGRAPHY

Name	: Suryo Wiyono
Place/Birth Date	: Bojonegoro-Indonesia, 12 February 1969
Sex	: Male
Marital Status	: Married with 2 sons
Office Address	: Department of Plant Pests and Diseases, Faculty of Agriculture, Bogor Agricultural University. Jl.Kamper Kampus IPB Darmaga - Bogor 16880 Indonesia, email: suryow@hotmail.com, swiyono2@yahoo.de

Education:

1974-1980	Elementary School. SDN Tinawun Kec Malo- Bojonegoro, East Java Indonesia
1980-1983	Lower Intermediate School. SMPN I Padangan- Bojonegoro, East Java Indonesia
1983-1986	Upper Intermediate School. SMA N Cepu- Blora, Central Java, Indonesia
1986-1991	Sarjana Degree. Bogor Agricultural University, Field of Study: Plant Pests and Diseases, School of Agriculture. Bogor Indonesia
1995-1997	Master Study in Tropical and Subtropical Agriculture, Georg-August University Göttingen Germany
Since Aug 2000	Ph D Program at the Institute of Plant Pathology and Plant Protection Georg-August University Göttingen Germany

Other Courses

Oct 1999-March 2000	German Course, Goethe Institut Jakarta, Indonesia
April-Juli 2000	German Course, Goethe Institut Göttingen, Germany

Professional Experience

1991-1992	Research Assistant and Facilitator of Farmer Participatory Research at FAO-Indonesian National Integrated Pest Management Program
1992-present	Teaching and Scientific Staff of Bogor Agricultural University Indonesia, at the Department of Plant Pest and Diseases, School of Agriculture

Organisation

Member of Nastari (a small farmers-facilitating NGO based in Bogor-Indonesia)
Member of Indonesian Phytopathological Society
Member of Indonesian Microbiological Society