Fates and Impacts of the Genetically Modified Plant Growth-Promoting Bacterium *Pseudomonas fluorescens* SBW25

-Under Controlled and Field Conditions

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Cover: A confocal picture of beneficial *Pseudomonas fluorescens* SBW25 cells tagged with red fluorescent protein and phytopathogenic *Ralstonia solanacearum* 1609 tagged with green fluorescent protein, inhabiting a potato root. (Photo: Jim van Vuurde, Maria Hellman and Lotta Jäderlund)

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Fates and Impacts of the Genetically Modified Plant Growth-Promoting Bacterium *Pseudomonas fluorescens* SBW25 - Under Controlled and Field Conditions

Abstract

Plant growth-promoting bacteria may be used in agriculture to minimize the utilization of chemical pesticides and fertilizers. This thesis studies one plant growth-promoting bacterial strain, *Pseudomonas fluorescens* SBW25, and its interactions with winter wheat, potato and tomato in both field and laboratory conditions. This bacterium was tagged with a novel marker gene cassette containing *gfp* (green fluorescent protein), *luxAB* (bacterial bioluminescence) and *telABkilA* (potassium tellurite oxide resistance) and the resulting strain SBW25::tgl was released in the first field trial with a genetically modified microorganism in Sweden. Bacterial numbers on wheat plants and in soil were determined by selective plating on media containing potassium tellurite oxide. The impact of SBW25::tgl on wheat plant growth was measured using plant length and weight, and possible effects on resident bacterial and fungal microflora was evaluated throughout the 8 months sampling period using terminal restriction fragment length polymorphism (T-RFLP). The GMM was found in high numbers on all plant parts throughout the 8 months period, but only minor impacts were found on native microflora due to bacterial (GMM or wild-type) inoculation.

SBW25 was also evaluated as a biocontrol agent towards the phytopathogenic bacterium *Ralstonia solanacearum* on tomato and potato plants. By using *gfp*-tagged *R. solanacearum* and red fluorescent protein (*rfp*) -tagged SBW25 it was possible to distinguish these two strains on plant surfaces using microscopic techniques. Plate counting, flow cytometry and luminometry were used to monitor the strains on plants. Some biocontrol effect of SBW25 was detected on tomato plants.

The interactions of SBW25 and arbuscular mycorrhizal (AM) fungi *Glomus mosseae* and *G. intraradices* were also studied. Very specific interactions were found, and also synergistic biocontrol effects suggesting that a consortium of microorganisms might be a better choice when applied as plant growth-promoters or biocontrol agents.

Keywords: plant growth-promoting bacteria (PGPB), *Pseudomonas fluorescens* SBW25, marker genes, field trial, arbuscular mycorhizal fungi, *Microdochium nivale*, *Ralstonia solanacearum*, terminal restriction fragment length polymorphism (T-RFLP)

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Till mamma och pappa.

Min släkt är full av hjältar, decennier av slit,
brustna hjärtan, trötta leder, deras stolthet bar mig hit.
Nu är de gömda bakom stjärnor, vid Vintergatans kant,
de är glömda men de talar genom pennan i min hand,
och de bar mig ända hit.

”Elite” - Kent

Svensk sammanfattning

Vissa sorters bakterier kan främja tillväxt hos vissa grödor och dessa bakterier kallas PGPB (plant growth-promoting bacteria). Vissa av dessa hämmar sjukdomsframkallande svampar eller bakterier som kan orsaka stora skördeförluster. Användningen av levande organismer för att bekämpa sjukdomar kallas biologisk kontroll (biokontroll) och detta kan användas för att minska användningen av kemiska bekämpningsmedel i jord- och skogsbruk. Bakterien som den här avhandlingen är baserad på kallas *Pseudomonas fluorescens* SBW25 och den är vanlig i både jord, vatten och på växter.

Vårt mål med det här arbetet var att undersöka hur användning av SBW25 som PGPB påverkade växterna som behandlades, de sjukdomsframkallande organismerna och de naturligt förekommande bakterier och svampar som finns på växterna och som är viktiga för ekosystemets funktion. För att kunna följa SBW25 i den komplexa miljön som en växtplanta representerar så satte vi in så kallade markörgener i bakteriens kromosom. De inmärkta (genetiskt modifierade) SBW25::tgl-bakterierna släpptes ut i ett fältförsök i september 2005. Vetekorn som var naturligt infekterade med en sjukdomsframkallande svamp, *Microdochium nivale* (snömögel), behandlades med bakterielösning innan sådd. Veteplantorna undersöktes sedan med jämna mellanrum med hjälp av markörgenerna efter förekomst av SBW25-celler tills försöket avslutades i maj 2006. På alla delar av de behandlade plantorna fanns ett högt antal SBW25::tgl celler under hela fältförsöksperioden, även efter vintern. Trots detta hittades få och inkonsekventa effekter av bakterien när molekulära metoder (Terminal restriction fragment length polymorphism, T-RFLP) användes för att undersöka förändringar i bakterie- och svampfloran som redan fanns på veteplantorna.

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Jäderlund, L., Hellman, M., Sundh, I., Bailey, M. J. & Jansson, J. K. (2008). Use of a novel nonantibiotic triple marker gene cassette to monitor high survival of *Pseudomonas fluorescens* SBW25 on winter wheat in the field. *FEMS Microbiol Ecol* 63(2), 156-168.

II Jäderlund, L., Ihrmark, K., Hellman, M., Sundh, I., Stenlid, J. & Jansson, J. K. Impact of wild-type and genetically modified *Pseudomonas fluorescens* SBW25 on wheat plant associated bacterial and fungal communities in the field (manuscript).

III Jäderlund, L., Hellman, M., Hörnaeus, K., Che Jian, M., & Jansson, J. K. Monitoring colonization of marker gene-tagged plant pathogen *Ralstonia solanacearum* and plant beneficial *Pseudomonas fluorescens* SBW25 on tomato and potato (manuscript).

IV Jäderlund, L., Arthurson, V., Granhall, U., & Jansson, J. K. Specific interactions between arbuscular mycorrhizal fungi and plant growth-promoting bacteria - as revealed by different combinations (manuscript).

Abbreviations

AM(F)	Arbuscular mycorrhizal (fungi)
BCA	Biocontrol agent
GFP	Green fluorescent protein
GMM	Genetically modified microorganism
MHB	Mycorrhization helper bacteria
PGPB	Plant growth-promoting bacteria
RFP	Red fluorescent protein
TRF	Terminal restriction fragment
T-RFLP	Terminal restriction fragment length polymorphism

No microbe is an island – C. J. Hurst

Introduction

In soil there are a tremendous number of microorganisms, and they are constantly interacting with each other and the surrounding environment in order to survive. One gram of soil may contain 10^{10} microorganisms, and as much as 10^{6} species of bacteria (Gans, Wolinsky & Dunbar, 2005). These microorganisms perform a wide range of functions, such as decomposing organic material, converting dinitrogen to biologically usable ammonia, degrading toxic pollutants and reducing or oxidizing different forms of elements. Soil can be a very harsh habitat and competition for nutrients and space is a soil microbe's every day life. However, many microorganisms are also dependent on each other, for example by performing different steps in nutrient cycling that benefit both populations. Interactions between microorganisms may be of several types such as neutralism, which is often defined as the lack of interactions between microbial populations, or commensalism, an interaction in which one population benefits from another that is not affected. There is also synergism, or mutualism (symbiosis), which is characterized as a process where all populations involved benefit. Of course not all interactions are good for everyone involved. Parasitic and predatory interactions mean that one population benefits at the expense of another. Competition is usually seen as a relationship where one or both of the involved populations are negatively affected, often in their growth rate. Amensalism, also known as antagonism, is the interaction where one population affects the other one negatively, for example by producing toxic compounds (Bottomley, 1999; van Elsas *et al.*, 2007a).

Plant roots may offer a completely different milieu for the microorganisms that inhabit the soil. The region of soil that is influenced by roots is known as the rhizosphere. Microbial numbers and activities are much higher in the rhizosphere soil than the surrounding bulk soil (Smalla *et al.*, 2001), partly due to bioavailable nutrients released by the roots, or root exudates. Also, plant

surfaces are colonized by a large number of microbes and some even live inside of plant cells as endophytes. These plant-associated microorganisms interact with each other and their plant hosts, giving rise to an extremely complex ecosystem that we are only beginning to understand. Plant-microbial interactions may be deleterious, neutral or beneficial to the plant. The intricacy and specificity of these plant-microbe interactions are yet to be discovered, as well as their significance for plant health. Modern agriculture needs new ways of controlling plant pests and increase crop yields without being dependent on chemical pesticides and fertilizers. Biocontrol (the use of living organisms to defeat pathogenic fungi or bacteria) may be a part of this solution. In addition plant beneficial bacteria and fungi, such as mycorrhizal fungi can be used to increase plant uptake of nutrients from the soil (Akköprü & Demir, 2005; Ravnskov *et al.*, 2006). One way of studying the interactions of these microorganisms and their plant hosts and pathogenic competitors is to use fluorescent marker genes (Bloemberg *et al.*, 2004), an example is shown in Figure 1.

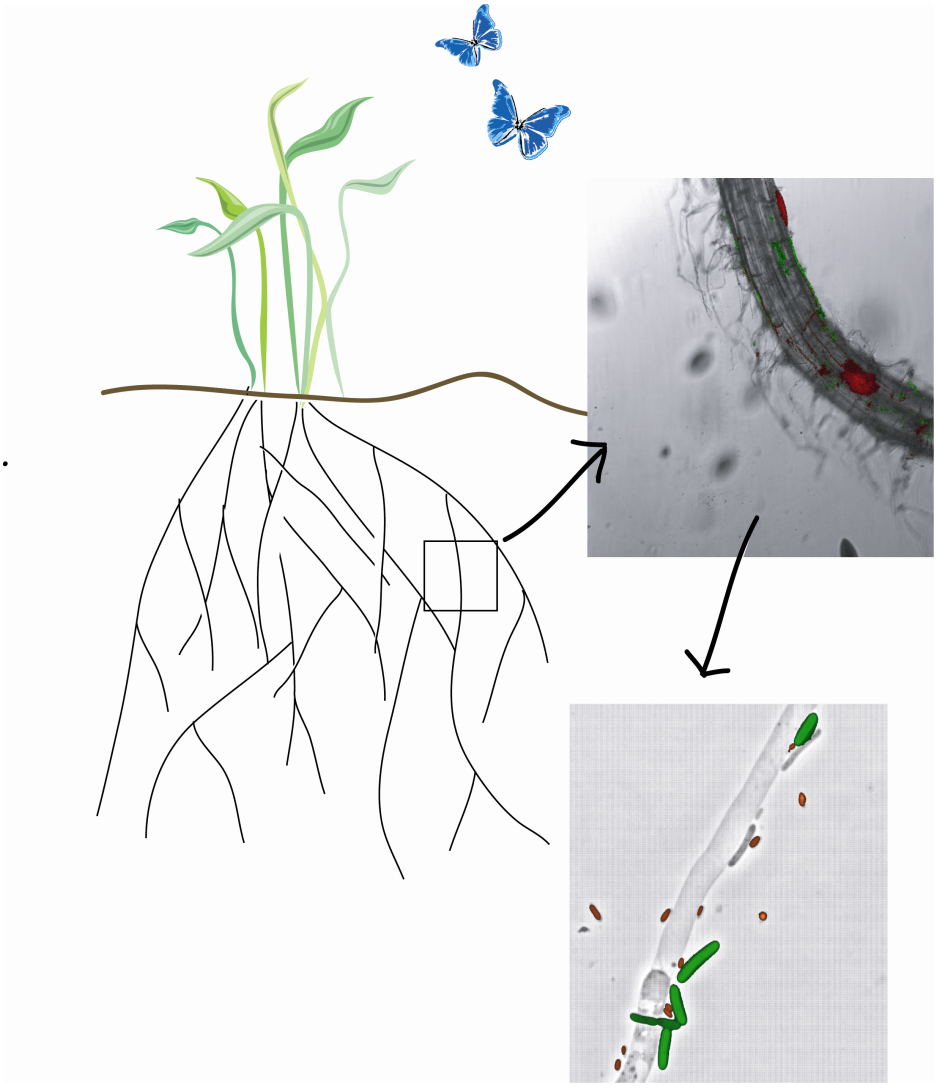

Figure 1. An example on how autofluorescent green and red proteins may be used as marker genes to study bacterial interactions in complex environments. Confocal picture above taken by Jim van Vuurde, Maria Hellman and Lotta Jäderlund. Epifluorescence photograph taken by Lotta Jäderlund.

This thesis aims to elucidate some of the interactions between beneficial and harmful microbes and the plants they colonize. It focuses on the plant growth-promoting bacterium (PGPB) *Pseudomonas fluorescens* SBW25 (Thompson, Ellis & Bailey, 1995) and its survival on plants, beneficial effects, possible negative

effects on resident plant associated fungi and bacteria and the interactions between SBW25 and arbuscular mycorrhizal fungi.

Aims and objectives of this thesis

The main aim of this thesis was to conduct the first field trial of a genetically modified microorganism (GMM) in Sweden and to use *Pseudomonas fluorescens* SBW25, a known PGPB as the model organism for the field study, see Figure 2 for an overview. Some specific objectives were:

· To develop a novel non-antibiotic marker gene cassette for marking of GMMs intended for release into the environment (**Paper I**)

· To determine the survival, activity, plant root colonization and possible dispersal of genetically modified *Ps. fluorescens* SBW25 in a Swedish field trial using a novel non-antibiotic marker gene cassette (**Paper I**)

· To examine the impact of wild-type and GMM strains of *Ps. fluorescens* SBW25 on resident bacterial and fungal populations on wheat seeds, roots and rhizosphere soil (**Paper II**)

· To study potential antagonism of and interaction with *Ralstonia solanacearum* KZR5 (tagged with green fluorescent protein) by *Ps. fluorescens* SBW25 (tagged with red fluorescent protein) on potato and tomato (**Paper III**)

· To determine the interactions of PGPB strains (*Ps. fluorescens* SBW25 and *Paenibacillus brasilensis* PB177) with arbuscular mycorrhizal (AM) fungi (*Glomus mosseae* and *G. intraradices*) and pathogenic fungi (**Paper IV**)

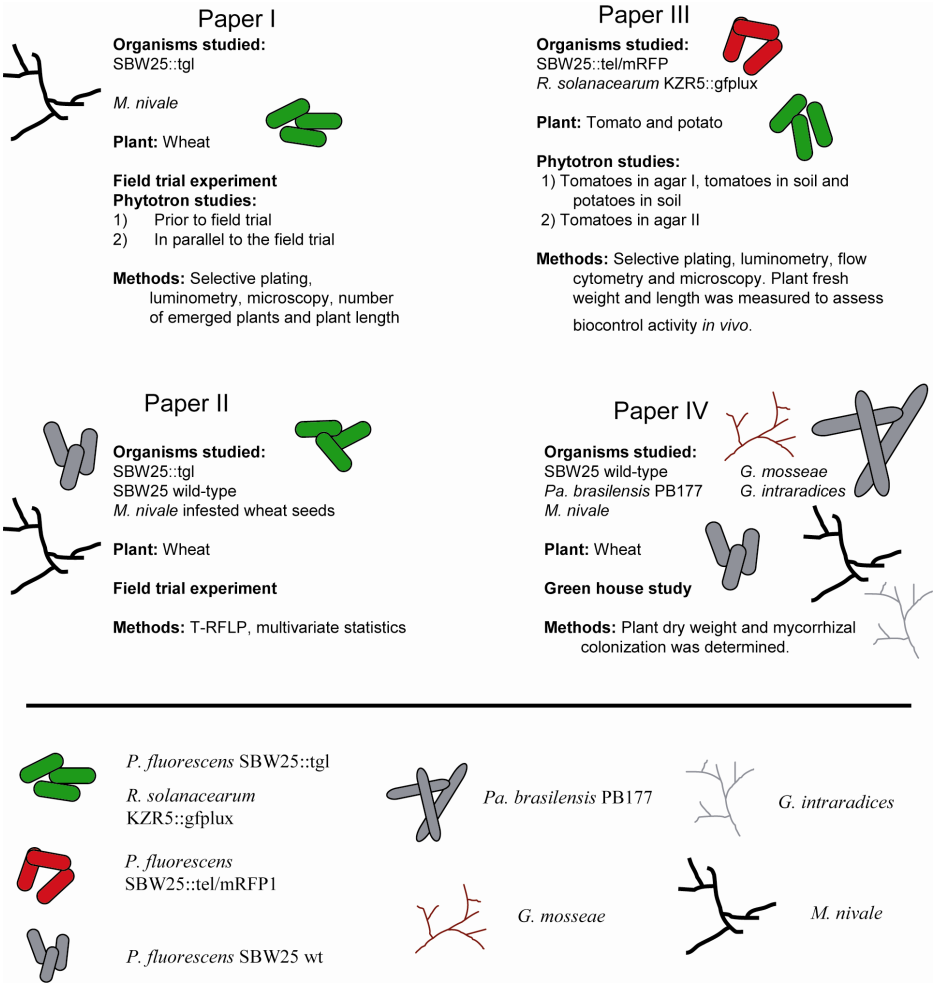

Figure 2. An overview of the methods and microorganisms used in the different papers presented in this thesis.

The soil and plant environments

Soil is a very complex habitat that may seem homogenous to the naked human eye but all soils are extremely heterogeneous at the micro-level, consisting of solid, liquid and gaseous phases (Standing & Killham, 2007). The plant-influenced environment in the soil is very different from the bulk soil in terms of nutrients and thus microbial activity. The term rhizosphere is used to describe the soil nearest to the roots that is influenced by compounds released by the roots and thus having an increased microbial activity. Plant derived substances include exudates (for example amino acids, vitamins, tannins etc),

mucilage (gelatinous substances produced by plant roots), mucigel (gelatinous material at the surface of plant roots), and different lysates from epidermal plant cells. These substances constitute a large part of the bioavailable carbon in the rhizosphere and is therefore of great importance for microbial activity (Hartel, 1999). There are also other factors that influence the rhizosphere environment such as soil moisture, which may affect microbial activity both directly and indirectly. When the water content is too low this will result in decreased nutrient transfer and lower microbial activity, while too high amounts of water will fill micro-pores and result in an anoxic environment. Furthermore, differences in soil texture, temperature and pH will give rise to differences in microbial composition and metabolic activities (Standing & Killham, 2007). When it comes to the soil habitat, it contains high numbers of bacteria (10^8-10^9 g^{-1} of soil) but there are many other organisms that share the soil environment, such as archaea (10^7-10^8 g^{-1} of soil), fungi (10^5-10^6 g^{-1} of soil), viruses (10^{10}-10^{11} g^{-1} of soil) algae (10^3-10^6 g^{-1} of soil) and protozoa (10^3-10^5 g^{-1} of soil) (Hartel, 1999; van Elsas *et al.*, 2007b). The significance of the interactions between these organisms is poorly understood and we need to explore these in order to produce efficient and sustainable agricultural methods.

Plant growth-promoting bacteria and biocontrol

Bacteria may be associated with plants in several ways. Some may inhabit the rhizosphere, taking advantage of root exudates, others may live on root or leaf surfaces and some may colonize intracellular spaces and vascular tissues inside the plant (Preston, 2004). Plant growth-promoting bacteria (PGPB) are specific strains that enhance seed germination and/or plant growth. This term was coined in the 1970's by researchers that noticed the beneficial effects that some bacterial strains had on plants (Kloepper *et al.*, 1980). Common to all PGPB is the ability to competitively colonize plant tissue (Weller, 1988). The molecular mechanisms for plant growth-promotion vary between different bacteria and may include one or several of those discussed below (for an overview see Figure 3).

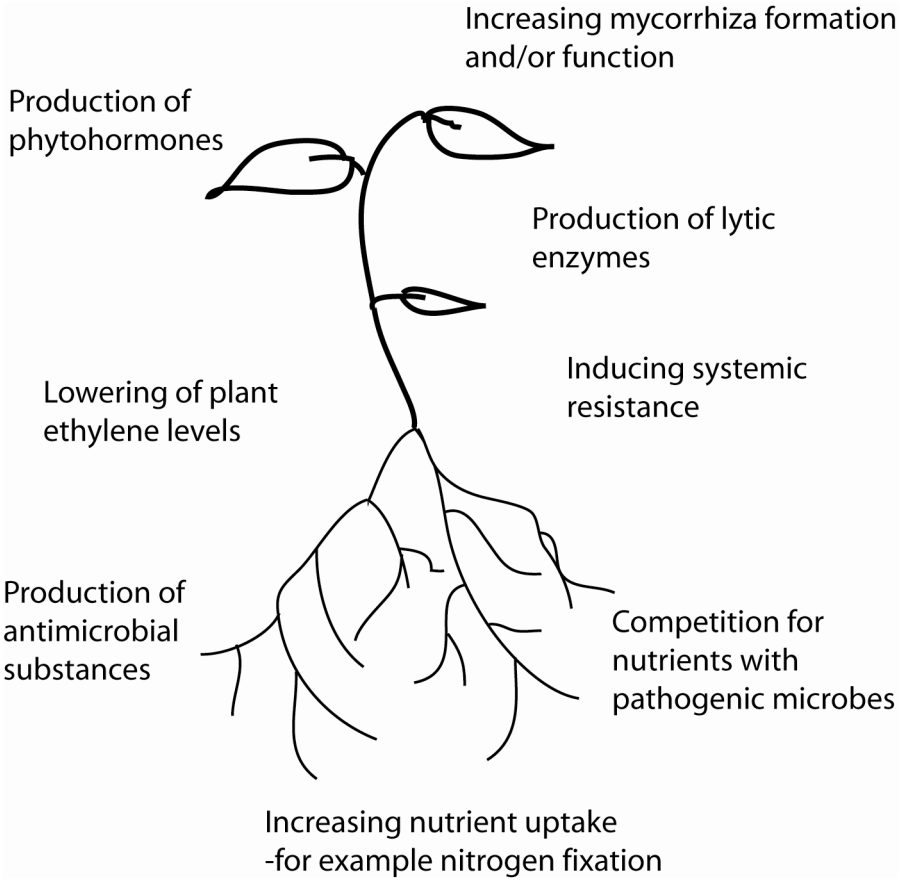

Figure 3. An overview of possible mechansims of plant growth-promoting bacteria (PGPB).

Many rhizobacteria produce phytohormones such as auxins, cytokinins and gibberellins and these substances promote plant growth in a direct way. For example, indole acetic acid (IAA) is an auxin produced by many rhizobacteria. IAA is a regulator of plant genes and the responses from plants to this substance vary from beneficial to harmful primarily depending on the concentration of IAA (Lambrecht *et al.*, 2000). Another regulatory substance used by plants is ethylene, and it is involved in for instance seed germination, root elongation and stress responses of plants to both biotic and abiotic factors (Saleh-Lakha & Glick, 2007). Elevated levels of ethylene will cause fruit to rot and have a negative impact on plant growth. However, some PGPB produce an enzyme called 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) that is able to decrease ethylene levels, thus limiting the negative effects (Glick, 2005). Some PGPB may initiate a phenomenon called induced systemic resistance (ISR), giving a similar response in the plant as if a pathogen had made an attack,

however no symptoms of disease will arise (van Loon, Bakker & Pieterse, 1998). If this response is triggered by plant beneficial bacteria prior to pathogen infection the incidence of disease will be reduced, thus giving higher plant yields. Another mechanism for plant growth promotion is the fact that some PGPB may increase mycorrhiza formation and/or function, and these strains are called mycorrhization helper bacteria (MHB) (Garbaye, 1994). Furthermore, nitrogen-fixing bacteria provide plants with nitrogen by converting the molecular nitrogen to ammonia that can be taken up by plants (Kneip *et al.*, 2007). The most efficient nitrogen-fixing bacteria (for example *Rhizobium*) form host-specific symbioses with leguminous plants. However, plant growth promotion by nitrogen fixation is unlikely to be of large importance unless nitrogen levels are very low due to an energy-intensive mechanism (Saleh-Lakha & Glick, 2007).

One way of indirect plant growth-promotion is via biocontrol of plant pathogens by microorganisms. Biocontrol can function by different mechanisms. For example, some bacteria produce antimicrobial substances to increase their competitive abilities and they could inhibit pathogen growth (antibiosis). One of the most studied groups of antibiotic-producing bacteria is the fluorescent pseudomonads. The first group of antibiotics to be discovered as a biocontrol mechanism were the phenazine derivatives (Chin-A-Woeng, Bloemberg & Lugtenberg, 2002). Other substances that are often produced by *Pseudomonas* spp. are pyoluteorin, 2,4-diacetylphloroglucinol and hydrogen cyanide (Weller, 2007). There are of course other ways of reducing pathogen numbers, and one of those is competition for nutrients such as iron (Handelsman & Stabb, 1996). Iron is one of the most abundant elements in soil but the concentration of biologically available iron (Fe^{2+}) in aerobic soil environments is usually low. Many microorganisms produce low-molecular-weight iron-transporting substances called siderophores, which they use to solubilize iron. This is done by strong complex building of the siderophore with Fe^{3+}, which will then be reduced to Fe^{2+} by a series of mechanisms (Mullen, 1999). One last example of a biocontrol mechanism is the production of lytic enzymes such as chitinases, glucanases and hydrolases (Compant *et al.*, 2005).

In order to get a commercially useful biocontrol agent (BCA) there are many criteria that need to be fulfilled, such as:

- Stable plant colonization of the BCA
- Consistent biocontrol activity
- Acceptable environmental risks
- Easy to culture and mass-produce

- Competitive with chemical agents
- Uncomplicated formulations
- Long shelf life
- Easy to apply and safe to farmers

The efficiency and consistency of biocontrol agents may be increased by the insertion of functional genes, for example genes encoding antimicrobial substances. In a previous work by Timms-Wilson et al (2000) the researchers inserted antibiotic producing genes *phzABCDEFG* into the bacterium *Pseudomonas fluorescens* SBW25, which was not previously known to produce antimicrobial substances. These genes encode the antibiotic compound phenazine-1-carboxylic acid and were originally isolated from *Ps. fluorescens* 2-79 (Mavrodi *et al.*, 1998). The genetically modified SBW25::phz strain was more efficient in reducing damping-off disease in pea than the wild-type strain (Timms-Wilson *et al.*, 2000). Another example of increasing the biocontrol efficacy of *Ps. fluorescens* SBW25 using inserted genes was the study by Bainton et al (2004) where the researchers introduced genes encoding the antibiotic 2,4-diactylphloroglucinol from *Ps. fluorescens* F113 into the chromosome of SBW25. The original F113 strain was known to reduce the number of pathogenic lesions on pea roots, but had the disadvantage of also reducing the number of emerged pea plants. Inserting the antibiotic genes into SBW25 resulted in a strain that had the competitiveness of the SBW25 wild type strain and also the disease reduction property of strain F113, but without the reduction of emerged pea seedlings (Bainton *et al.*, 2004). This example could have significance for the future of novel biocontrol agents.

Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal (AM) fungi are globally widespread and most plant species have some mycorrhizal symbionts. In AM fungi carbon can only be acquired from the plant (Sylvia, 1999). Both intra- and inter-cellular hyphae grow inside the roots and an extraradical mycelial phase extends out into the soil, maximizing the root surface area used for nutrient uptake. AM fungi initially grow between the plant cortical cells but then penetrate the cell wall and develop highly branched arbuscles (tree-shaped structure thought to be used for nutrient transfer between plant and fungus) within the host plant cortical cell. Often other structures are also found in AM fungi, such as vesicles (thin-walled, lipid-filled structures for storage) in intra-cellular spaces, auxiliary cells (function unknown) in the soil and asexual spores.

When a plant root becomes colonized with AM fungi the functions of the root will change, thus changing growth conditions for the other microbial inhabitants on the root or in the rhizosphere (Hodge, 2000). The zone including the soil surrounding plant roots and mycorrhizal hyphae is called the mycorrhizosphere (Rambelli, 1973) (Figure 4) and this is one of the hot spots where many microbial interactions take place. Some bacterial strains even increase the mycorrhiza colonization of plants, and these are referred to as mycorrhization helper bacteria (MHB)(Garbaye, 1994). It has been shown that some MHB and mycorrhiza may together create synergistic effects that will benefit plant health (Ravnskov & Jakobsen, 1999). Bacteria associated with AM fungi have been shown to represent different genus, such as *Bacillus*, *Arthrobacter* and *Pseudomonas* based on culturing methods (Andrade *et al.*, 1997) and uncultured bacteria and *Paenibacillus* using molecular fingerprinting approaches (Artursson, Finlay & Jansson, 2005).

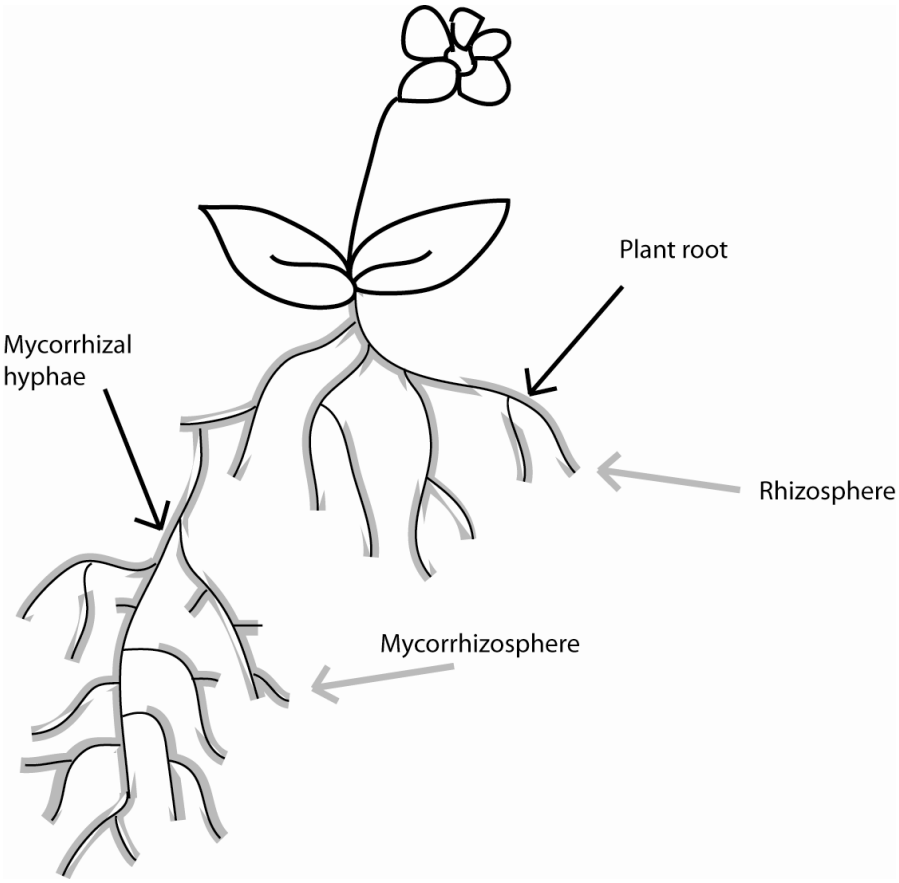

Figure 4. A schematic view of a plant and its mycorrhizal symbiont.

Risk assessment of genetically modified bacteria used as inoculants

Bacteria may be used commercially for environmental applications; for example as inoculants to degrade toxic pollutants and as PGPB or BCAs in agriculture. These bacteria may be tagged with marker genes to facilitate monitoring of the released cells or they may have functional genes inserted in order to increase efficacy. Each GMM requires rigorous risk assessment, for both human and environmental safety. It has been suggested that release of bacterial inoculants (genetically modified or not) may pose a threat to the environment (van Elsas & Migheli, 1999) and this has lead to extensive efforts in evaluation of possible risks before field releases (Gustafsson, 2000). Recently, a scientific and technical guidance on safety evaluation has been proposed (Mensink & Scheepmaker, 2007) which will help in risk assessment of BCAs.

There are several issues that have to be addressed regarding the safety of a GMM for plant growth promoting purposes (van Elsas & Migheli, 1999). Dispersion from the active site and the survival capacity are important parameters to evaluate. If a bacterium is overly competitive it could have a negative effect on other microorganisms, which are required for ecosystem functioning. However, many plant-associated bacteria may be very active on plant roots or in the rhizosphere but vulnerable in the harsh soil surrounding plant parts, which could make them more suitable to use as inoculants since they do not spread to a large extent. In evaluating risks associated with the use and environmental release of GMMs it is important to investigate the impact of the inocula on indigenous microorganism and the wider soil ecology (Glandorf *et al.*, 2001; Heuer & Smalla, 1999; Lilley *et al.*, 2006; Viebahn *et al.*, 2003). Any inoculum has an impact on the environment it is released at some scale. These impacts may be minimized if a bacterium that is already naturally present in large numbers in the environment is used. Therefore it may be of interest to isolate native strains from the specific environment of interest and search for the desired traits among those isolates.

Another concern regards the genetic stability and potential for the horizontal gene transfer of the introduced trait from GMM to indigenous microorganisms (Dröge, Pühler & Selbitschka, 1998). It is important to analyze the effects of the genes to be released in order to minimize the risks. When possible, genes should be introduced into the bacterial chromosome to further limit the potential for transfer, for example by using suicide minitransposons (de Lorenzo *et al.*, 1990).

In Europe it is the European Union Directive 2001/18/EC that regulates the deliberate environmental release of genetically modified microorganisms (http://gmoinfo.jrc.it/; 18-Feb-08). This directive has a wide approach to risk assessment, taking into account both the nature of the parent organism, the

introduced genes and their expected effects on the GMM and any possible negative effects on the environment. This directive defines environmental risk assessment as the evaluation of the GMM release and possible risks to human health and environment safety, whether these risks are direct or indirect, immediate or delayed. Also, an analysis of the "cumulative long-term effects" is to be performed. This analysis should take into consideration the accumulated environmental effects, on for example flora and fauna, soil fertility, biodiversity and soil degradation of organic material. The directive does not apply to GMMs obtained by conjugation, transduction or transformation by mutagenesis, which is considered relatively safe due to conventional use in a number of applications and a long record of safety. The issue of antibiotic resistance genes is to be particularly considered when performing field trials, because of possible spread of antibiotic resistance. Comments by the public are also taken into consideration when giving permission to perform a deliberate release of a GMM. However, this directive states that field trials have to be regarded as a necessary step in the development of new products containing GMMs.

The present study

Methods used to monitor GMM survival, impacts and interactions

Marker genes

Marker genes are genes resulting in some kind of detectable phenotype to the bacterial cells that are tagged. They are commonly used to track specific bacterial strains in complex environments such as soil and plant material (Jansson, 2003) (Figure 5). Marker genes are often introduced using plasmids, and antibiotic resistance genes are often used to select positive clones and later on to select the GMM from indigenous bacteria on agar plates. However, lately the excessive use of antibiotic resistance genes has been questioned since this may increase the antibiotic resistance threat in bacteria (Berger-Bächi & McCallum, 2006; Davison, 2002; Egan & Wellington, 2000). Therefore, we used resistance to the heavy metal oxide potassium tellurite oxide, K_2TeO_3, as selection (Sanchez-Romero, Diaz-Orejas & Lorenzo, 1998) for the GMM released in the field trial described in **Papers I** and **II**. The *telAB/kilA* genes confer resistance to potassium tellurite oxide (K_2TeO_3). When cultivated on agar containing K_2TeO_3 only cells that are resistant are capable of growth and thus this is a selective screening method. Interestingly, before the development of modern antibiotics tellurite was used as a therapeutic agent for leprosy, tuberculosis, dermatitis, cystitis and eye infections (Taylor, 1999).

Another marker gene used in this work is the green fluorescent protein (GFP) that is encoded by the *gfp* gene, originally isolated from the jellyfish *Aequorea victoria* (Chalfie *et al.*, 1994). GFP emits green light at 508 nm upon ultraviolet light illumination at 396 nm and no additional substrates are needed. In this study, a mutant variant of GFP (P11) with the excitation maximum shifted towards the red region of the light spectrum was used because the red-shifted

mutants have been shown to have higher fluorescence output in bacteria, and are more easily distinguished by fluorescence detectors in analytical equipment. A strong constitutive promoter, P*psbA*, which originates from the chloroplasts of pigweed, *Amaranthus hybridus*, was previously coupled to the P11 *gfp* gene to enable constitutive expression (Unge *et al.*, 1999). Since the phenotype of *gfp*-tagged cells is their green fluorescence they can be monitored by the same methods that have been developed for detection of other fluorescently labeled cells. Methods used in this work were epifluorescence microscopy (Figure 5), fluorescence stereomicroscopy, confocal laser scanning microscopy (**Papers I** and **III**) and flow cytometry (**Paper III**).

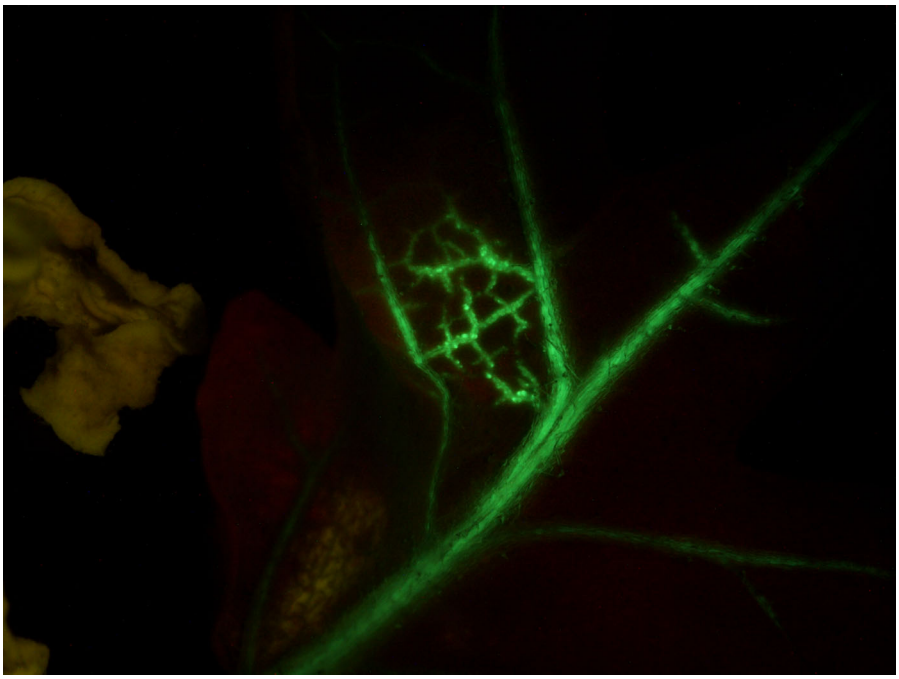

Figure 5. Photograph with an epifluorescence microscope showing GFP-tagged *Ralstonia solanacearum* infecting a tomato leaf. Photo: Lotta Jäderlund

For measuring the metabolic activity of the released GMM cells the *luxAB* genes, encoding bacterial luciferase, were used as an additional marker (Meikle *et al.*, 1994; Unge, *et al.*, 1999) (**Papers I, II** and **III**). The phenotype, bioluminescence, is dependent on the energy reserves of the cells, or $FMNH_2$. Therefore, the *luxAB* genes can be used as a marker for metabolically active cells. However, the bioluminescence reaction requires that *n*-decanal be added as a substrate, in contrast to GFP that does not require any additional substrate.

In order to have an additional marker gene for monitoring two different cell types in the same samples, the *mRPF1* gene (encoding red fluorescent protein, RFP, was also used in some experiments (**Paper III**).

Construction of a non-antibiotic marker gene cassette

A very convenient way of introducing marker genes into the chromosome of Gram-negative bacteria is to use mini-transposons (de Lorenzo, *et al.*, 1990), which are derived from transposons Tn5 or Tn10. The miniTn5 transposon does not carry its own transposition functions and this makes secondary transposition unlikely. In this study variations of the original pUT mini-transposon system (Herrero, Lorenzo & Timmis, 1990) were designed and used for tagging of bacterial strains of interest. The mini-Tn5 vector consists of two different parts, one mobile portion that contains the genes to be inserted, and one portion containing the transposase gene (*tnp*), ampicillin resistance for selection (*bla*), plasmid R6K origin of replication (*ori R6K*) and the RK2 origin of transfer (*oriT*). The delivery system of mini-Tn5 vectors is based on the plasmid R6K, and is dependent on the specific replication protein λ. If the plasmid is inserted into a bacterial strain that does not produce this protein, the mobile part of the vector (containing the marker genes) will integrate into the bacterial chromosome. Thus, these plasmids can only be stably maintained on a plasmid in λpir lysogens or in *E. coli* strains with the λpir gene inserted into their chromosome, otherwise the transposable region will integrate into the host chromosome (Herrero, Lorenzo & Timmis, 1990).

Two different variants of the pUT mini-transposon vector were used for this work; one based on kanamycin resistance (*nptII*) and the other on potassium tellurite oxide resistance (*kilA/TelAB*). The plasmid pUTgfplux, containing genes encoding both GFP and bacterial luciferase, was constructed previously (Unge, *et al.*, 1999). However, this plasmid contains the *nptII* gene, encoding resistance to kanamycin, which is not suitable for field release due to potential spread of antibiotic resistance genes. Therefore it was necessary to create a novel combination of marker genes and pUT vector without antibiotic resistance genes, and for this purpose plasmid pUTtel (Sanchez-Romero, Diaz-Orejas & Lorenzo, 1998) was chosen because it uses tellurite for selection instead of antibiotic resistance. The *gfpluxAB* genes were excised from plasmid pUTgfplux as a *Not*I fragment and ligated into the unique *Not*I-site of pUTtel (**Paper I**). This vector was subsequently used to tag GMM cells released into the field (**Papers I** and **II**). With the help of this novel combination of marker genes we had the possibility to monitor tagged strains on agar plates containing potassium tellurite oxide, to use fluorescence microscopy to visualize GFP fluorescent cells

on plant parts and to measure bioluminescence in order to determine the metabolic activity of the cell population.

In addition, a novel vector containing the tellurite resistance genes as well as the *mRFP1* gene encoding red fluorescence was constructed. The monomeric protein mRFP1 was designed to overcome the problems with slow maturation and tetramerization of the dsRed fluorescent protein (Campbell *et al.*, 2002). The *mRFP1* gene was excised from vector p519mRFP1 kindly provided from Prof. Steven E. Lindow and ligated into the pUTtel vector. The resulting vector was designated pUTtelrfp and used to tag strain *P. fluorescens* SBW25 in **Paper III**.

Terminal restriction fragment length polymorphism (T-RFLP)

An important aspect of the risk assessment of any GMM, and of PGPB in particular, is to evaluate how they interact with and influence other resident microorganisms in the plant/soil ecosystem. In this work terminal restriction fragment-length polymorphism (T-RFLP) (Liu *et al.*, 1997) was used to assess any possible impacts on the bacterial and fungal community structures associated with wheat seeds, roots and rhizosphere soil (**Paper II**). T-RFLP is a PCR based method where one or both primers are fluorescently labeled. The PCR products are cleaved with different restriction enzymes, each organism ideally having different restriction sites, thus giving rise to terminal restriction fragments (TRFs) of different sizes. These TRFs are separated using capillary gel electrophoresis or a polyacrylamide gel. The lengths of the fluorescent TRFs are measured by a DNA sequencer with a fluorescence detector. The relative abundances of the TRFs give a fingerprint of the dominant members of microbial communities in a specific sample. Differences between different samples can then be determined using multivariate statistical methods. In **Paper II** T-RFLP was used with both bacterial and fungal primers to assess the impact of the GMM and wild type strains on the resident microflora in a field trial.

Experimental conditions

Greenhouse

The most common way to study plant-microbe interactions is to use greenhouses where water, nutrients and climate are at least partly controlled by the researchers. When performing a greenhouse trial it is important to compensate for possible spatial differences in the greenhouse, for example variations in temperature or light conditions. Rearranging the pots with suitable differences in time can help to solve this problem. Of course it is also important

to realize that greenhouse experiments lack many of the factors that are present in the field, and may only be used as a compliment or pre-study, and in order to commercialize the products it will be necessary to perform field trials. A study performed by Eller et al. (2005) compared greenhouse and field experiments on the composition of methano- and methylo-trophic bacteria in paddy soil. They found that the diversity of these bacteria in soil samples assessed by DGGE was comparable between the field and the greenhouse but that care should be taken with quantitative measurements such as plate counts (Eller, Krüger & Frenzel, 2005). In this work a greenhouse experiment was conducted in **Paper IV** when examining the interactions of PGPB with AM fungi on healthy or pathogen infested winter wheat.

Phytotron

The Phytotron at the Swedish University of Agricultural Sciences (SLU) contains highly controllable growth chambers where for example temperature, relative humidity and light intensity can be programmed in advance. For this thesis we used the phytotron in **Papers I** and **III**. In **Paper I** we first evaluated the impacts of PGPB inoculants on two cultivars of winter wheat in prior to a field experiment (**Papers I** and **II**). Then we also performed a second phytotron experiment in parallel with the field trial (with one week delay for the phytotron). In this second experiment relative humidity and night/day temperatures as well as light hours for the Uppsala region were applied so as to mimic the field conditions day by day in the best way possible. In **Paper III** the phytotron was also used because of requirements for higher safety measures when working with a genetically modified plant pathogen.

Field trial

Field trials are necessary to provide information, for example, about how climatic factors and soil conditions effect survival of microbial inoculants, such as PGPB strains. Even if good and reproducible results can be obtained in greenhouse trials these may be difficult to repeat under field conditions (Whipps & Gerhardson, 2007). Field trials are the ultimate tests for bacterial inoculants used in agriculture. Randomized block design is used to compensate for possible variations in soil factors in the field. Field trials with genetically modified microorganisms have been performed in several countries. For example, in a study by Bakker et al., (2002) the bacterial strain *Ps. putida* WCS358r was modified with genes encoding the antimicrobial substances phenazine-1-carboxylic acid and 2, 4-diacteylphloroglucinol and repeatedly introduced to wheat seeds in a field trial in 1997, 1998, 1999 and 2000. The impact of this bacterium on the wheat plants was evaluated by measuring plant

weight and effects on the native microflora were assessed using plating techniques and amplified ribosomal DNA restriction analysis (ARDRA). Some metabolic soil activities were also determined, such as substrate induced respiration (SIR) and soil nitrification potential. Only in 2000 bacterial inoculation increased plant yield by approximately 35 %, no plant growth effect was detected in 1997-1999. Some transient changes in fungal and bacterial microfloras were observed, but these shifts did not result in any significant changes in the metabolic activities of the microbial communities (Bakker *et al.*, 2002). Many other studies have also shown that transient changes may occur due to GMM inoculation, however plant species, growth stage and seasonal changes have a larger impact (Castro-Sowinski *et al.*, 2007). The field trial included in this thesis was the first field trial in Sweden with a genetically modified microorganism (*Ps. fluorescens* SBW25::tgl) and was initiated September 8, 2005 and ended May 10, 2006 (**Papers I** and **II**).

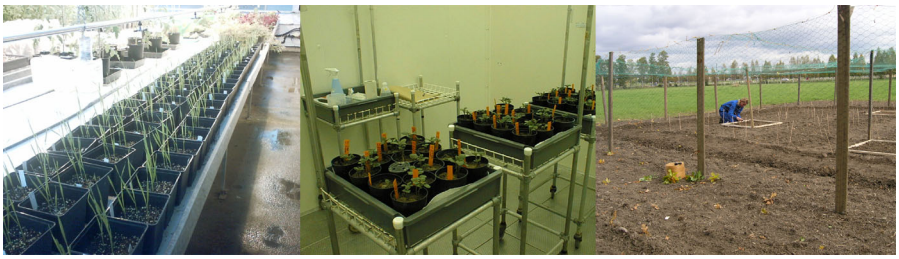

Figure 6. The three experimental conditions used in this thesis; greenhouse, phytotron and field. Photos: Lotta Jäderlund and Maria Hellman.

Pseudomonas fluorescens SBW25

The genus *Pseudomonas* is a very wide and heterogeneous group and it consists of many different species. One of the largest and most heterogeneous is the fluorescent pseudomonad group where *Ps. fluorescens* is a member. *Ps. fluorescens* is a common bacterium in soil, water and on plants (Balows *et al.*, 1991). They are Gram-negative rods, which are motile by polar flagella and belong to risk group 1 (the lowest one) according to European Community classification (2002). The *Ps. fluorescens* SBW25 strain (hereafter referred to as SBW25 in this thesis) is a widely studied plant growth-promoting bacterium, which was previously isolated from the phyllosphere of sugar beet in the UK (Thompson, Ellis & Bailey, 1995). SBW25 is an abundant member of the phytosphere community, a very competent root-colonizer of several plants and considered to be non-pathogenic to humans, animals or plants. It is an excellent colonizer of different plants (both rhizosphere and phyllosphere) such as pea (Naseby *et*

al., 2001), sugar beet (Thompson *et al.*, 1995), wheat (de Leij *et al.*, 1995a; Unge & Jansson, 2001)(**Papers I**, **II** and **IV**), and potato and tomato (**Paper III**). Several studies have addressed the colonization and dissemination ability of SBW25 in order to elucidate some mechanisms responsible for its ecological success (Naseby*, et al.*, 2001; Preston, Bertrand & Rainey, 2001; Rainey, 1999; Rainey & Rainey, 2003; Timms-Wilson*, et al.*, 2000; Unge & Jansson, 2001). Probably the most important factor for spreading of the cells from the inoculated plant part is some form of passive transport, such as traveling with elongating roots and stems (Turnbull *et al.*, 2001). Percolating water and small soil animals are also likely to be a cause of spread of bacteria in soil and on plant surfaces. The importance of motility for SBW25 attachment to sterile wheat roots and survival in non-sterile soil was assessed by Turnbull et al (2001). They found that motile SBW25 survived significantly better than the non-motile strain in soil but there was no significant difference in vertical spread. There was also a significant increase of attachment to sterile wheat roots by motile SBW25. When the strains were inoculated into soil and wheat seeds were planted there was a significantly higher number of motile bacteria on the germinating wheat seed but not on the rest of the plant, suggesting that motile SBW25 may be able to move towards nutrients using chemotaxis (Turnbull*, et al.*, 2001). These results imply that active transport may give an advantage in the early stages of colonization, such as attachment to plant roots. When competing for the same attachment sites the motility could give an advantage, to more quickly get to the site or movement into protective microniches.

In greenhouse trials it has been shown that SBW25 is able to control of phytopathogenic fungi such as *Pythium ultimum* (Ellis, Timms-Wilson & Bailey, 2000; Naseby*, et al.*, 2001), although no antimicrobial substances have yet been identified from this bacterium. Instead, this inhibitory effect has been related to an excellent colonization ability and hence out-competition of other microorganisms. However, in the present study in vitro dual culture agar plate assays have shown that SBW25 inhibits growth of the pathogenic bacterium *Ralstonia solanacearum* (**Paper III**). This implies either that the two microorganisms compete for nutrients (for example iron) or that some unknown substance is produced by SBW25 that inhibits the growth of *R. solanacearum*.

Previous greenhouse experiments have been performed to elucidate the survivability of SBW25 in soil and its colonization pattern on wheat by using a marker gene combination of *gfp* (green fluorescent protein), *luxAB* (bacterial bioluminescence) and *nptII* (kanamycin resistance) (Unge & Jansson, 2001; Unge*, et al.*, 1999). During 30 days incubation in soil the number of SBW25::gfplux cells were stable (at a concentration of 10^9 cells g^{-1} soil

compared to 10^8 cells g^{-1} soil at day 0), although the luciferase activity of the cells decreased, probably due to nutrient limitations. After inoculating spring wheat with *gfp-luxAB*-tagged SBW25, metabolically active cells were detected on seeds, roots and leaves in a greenhouse trial. The highest concentration was found on the seeds, whereas there was considerable variation in cell number and luciferase activity on leaves (Unge & Jansson, 2001).

Recently IVET (in vivo expression technology) was used to study SBW25 gene expression under different environmental conditions. The IVET approach is a promoter-trapping method that uses random integration of a promoterless gene into the bacterial chromosome and then detects mutants that have this gene inserted behind a promoter that is active during some special conditions. Using this method 20 rhizosphere-induced (*rhi*) genes of SBW25 were identified in the rhizosphere of sugar beet seedlings (Rainey, 1999). Fourteen of these genes were involved in nutrient acquisition, secretion and stress response. Seven genes had homology to known *Pseudomonas* genes and one of the *rhi* genes was a component of a type III secretion pathway that was not previously known in non-parasitic bacteria. This type III secretion system (TTSS) was further investigated (Preston, Bertrand & Rainey, 2001) showing that the gene found was part of a 20 kb gene cluster that resembles the type III hypersensitive response and pathogenicity (*hrp*) gene cluster in *Ps. syringae* (also found in the pathogen *R. solanacearum* studied in **Paper III** in this work), and that this TTSS was present in many other plant associated *Pseudomonas* strains. However, SBW25 did not elicit hypersensitive response (HR) in the plants tested (Preston, Bertrand & Rainey, 2001) and mutants still reached high population levels on plant surfaces, and therefore the function of this gene cluster is still unknown in PGPB. A similar study was performed with a different kind of promoter trap (finding the gene *dapB* instead of *panB*) (Gal *et al.*, 2003), thus giving more specificity to the method by the fact that the product of *dapB* is not available for bacteria in the plant rhizosphere; they need to synthesize it themselves. In that study the researchers found 25 *rhi* genes with predicted roles in nutrient acquisition, stress responses and biosynthesis of phytohormones and antibiotics. One specifically interesting gene fusion was to *wss*, an operon encoding an acetylated cellulose polymer. By conducting mutant experiments it was shown that a *wss* deficient mutant had significantly reduced survival levels compared to the wild type in rhizosphere and phyllosphere but not in bulk soil (Gal*, et al.*, 2003). It therefore seems likely that this *wss* operon is needed for ecological performance in the plant environment but not in bulk soil. Another of the *rhi* fusions found was *rhi-74*, showing similarity to plant derived nitrilases. Nitrilases are involved in the production of indole-3-acetic acid, a plant hormone that is commonly found in PGPB.

Another way of studying genes expressed during certain conditions is using shotgun proteomics. This has been applied to SBW25 to elucidate some of the factors that are involved in nutrient starvation and dormancy of cells during stressful conditions (Maraha, 2007). Cells were incubated in rich medium or in medium limited for carbon and nitrogen and these two proteomes were then compared. During starvation conditions, proteins for DNA replication, recombination and repair, cell motility, chemotaxis and secretion/signal transduction systems were upregulated in SBW25 cells. Many of the *rhi* fusions found in the two IVET studies mentioned above were also found by the shotgun proteomics approach, such as the *wss* operon, implying that SBW25 cells may experience starvation not only in the bulk soil but also in the rhizosphere soil (Maraha, 2007). This may seem contradictory to most views of the rhizosphere soil as a nutritional paradise to soil microorganisms (Kennedy, 1998; Rovira, 1965). However, the nutrient status in the rhizosphere will depend on the type of plant, growth stage and location of the bacteria in the rhizosphere soil layer (far from or close to roots) and location along the root (Kragelund, Hosbond & Nybroe, 1997). Both nutrient limitations of phosphate (Kragelund, Hosbond & Nybroe, 1997) and nitrogen (Jensen & Nybroe, 1999) have been detected in rhizosphere environments. The rhizosphere is with certainty one of the most complex microbial environments there is to study.

Other plant beneficial microorganisms studied in this work

Paenibacillus brasilensis PB177

Paenibacillus brasilensis PB177 (PB177) was isolated from maize rhizosphere in Brazil and has been studied because of its abilities to promote plant growth (von der Weid *et al.*, 2002). It has been shown to produce some antimicrobial substances that for example inhibited 5 different fungal isolates (*Diploida macrospora* EM1, *Fusarium moniliforme* Fm2, *F. oxysporum* LMS.1, *Rhizoctonia solani* AG3 and *Verticillium dahliae* Vda-1) in a dual culture agar plate assay and in liquid dual culture experiments (von der Weid *et al.*, 2005). In an earlier study, PB177 was shown to attach to vital AM fungal hyphae to a larger extent than to non-vital AM hyphae for both AM fungi tested (*G. intraradices* MUCL 43194 and *Glomus* sp. MUCL 43205) (Toljander *et al.*, 2006).

PB177 was also studied in combination with arbuscular mycorrhizal fungi to promote growth of wheat plants and inhibit *M. nivale* (**Paper IV**). It was shown to reduce growth of *M. nivale* in dual culture agar plate assays and the number of emerged wheat plants from *M. nivale* infested seeds was slightly increased after 21 days in a phytotron study prior to the field trial (Jäderlund &

Jansson, unpublished data). It was also included in our Swedish field trial due to its interesting properties as a PGPB, however no effects were detected against *M. nivale* in the field.

Arbuscular mycorrhizal fungi

In this thesis the arbuscular mycorrhizal (AM) fungi, *Glomus mosseae* and *G. intraradices*, were used in combination with either bacterial strain SBW25 or PB177 to study their respective combined and individual effects on wheat plants infested with snow mold (*M. nivale*) (**Paper IV**). It has been shown in earlier studies that mycorrhizal interactions have significance for microbial activity and composition in soil. For example *G. intraradices* has been shown to alter microbial activity in the rhizosphere of pea. The nature of these changes were dependent on plant growth stage, AM colonization decreased the rhizosphere respiration before flowering and stimulated respiration during flowering (Wamberg *et al.*, 2003). The culturable bacterial populations associated with *G. intraradices* mycelia were studied by Mansfield-Gise et al (2002). They found that the most frequent bacterial isolates associated with *G. intraradices* in hyposphere and rhizosphere of cucumber plants were *Pseudomonas*, *Arthrobacter* and *Burkholderia* (Mansfeld-Giese, Larsen & Bødker, 2002). These species have also been found in sorghum plant rhizospheres using fatty-acid-methyl-ester analysis for both *G. intraradices* and *G. mosseae* (Andrade*, et al.*, 1997). However, culture dependent methods will only provide a part of the information about bacteria associated with AM fungi. In another study, Bromodeoxyuridine (BrdU) immunocapture and terminal restriction length polymorphism (T-RFLP) were used to detect active bacteria associated with *G. mosseae*. These were found to be mostly uncultured bacteria and *Paenibacillus* (Artursson, Finlay & Jansson, 2005).

Both *G. mosseae* and *G. intraradices* have been used previously to promote plant growth, either singly or in combination with other beneficial microorganisms. For example *G. intraradices* was used in combination with the biocontrol agent *Clonostachys rosea* to promote tomato plant growth (Ravnskov*, et al.*, 2006). In a different study *G. mosseae* was tested together with *Ps. fluorescens* biocontrol agents on leek and tomato with good results (Edwards, Young & Fitter, 1998).

Plant pathogenic microorganisms studied in this work

Ralstonia solanacearum

Ralstonia solanacearum is one of the world's most economically important phytopathogenic bacteria. It causes wilting diseases of more than 200 plant species, belonging to 50 families, including economically important ones like potato, tomato, tobacco and banana (Hayward, 1991). The pathogen was discovered in the last decades of the 19th century when it caused wilting disease in different parts of the world, including Southeast Asia, Southern USA, Japan, Australia and South America. *R. solanacearum* was first described in 1896 as *Bacillus solanacearum* by Erwin F. Smith and transferred by him to the genus *Pseudomonas* in 1914. Recently it has been transferred again, first to *Burkholderia*, then to *Ralstonia*. For a long time, bacterial wilt caused by *R. solanacearum* was regarded mostly as a tropical disease but in the last decades isolates have also been found in colder climates. *R. solanacearum* race 3 (biovar 2) is responsible for the outbreaks of brown rot of potato in temperate climates (van Elsas *et al.*, 2000).

Due to the intricate infection process of *R. solanacearum* this pathogen has an unusually wide host-span, and is also capable of surviving long times in soil and water outside host plants (van Elsas*, et al.*, 2000; Wenneker *et al.*, 1999). The key to this success is a sophisticated regulation system that controls virulence and pathogenicity genes. For example, the *Phc* system consists of five genes used to regulate exopolysaccharide production and cell wall degrading enzymes (Schell, 1996). The *Phc* system is controlled via self-produced signal substances. There is also a Type III secretion pathway, the *hrp* pathogenicity genes, which is used to make fine adjustments to virulence gene expression (Boucher *et al.*, 1987).

Bacterial wilt is still a disease that is difficult to control effectively, in spite of different long-term control strategies. Chemical control of bacterial diseases in infected soils is difficult in developing countries. Prophylactic measures together with resistant cultivars are still the most effective ways to reduce the incidence of the disease (Fortnum & Martin, 1998; French, Anguiz & Aley, 1998; Mariano, Silveira & Michereff, 1998). Alternative control measures such as biocontrol have been investigated with an increased interest in the last decade. Both avirulent mutants of *R. solanacearum* and other antagonistic bacteria, such as *Pseudomonas* spp. have been tested as biocontrol agents towards bacterial wilt (Guo *et al.*, 2004; Trigalet & Trigalet-Demery, 1990). In a study by Schönfeld et al (2003) the effect of compost addition and solarization on the survival of *R. solanacearum* 1609 (biovar 2 race 3) was evaluated. They found that solarization of the soil did not affect the pathogen survival or infectiveness but the compost-

amended soil inhibited *R. solanacearum* survival and gave less number of infected potato plants (Schönfeld *et al.*, 2003). A similar study by Gorissen et al (2004) used pig slurry, solarization or a combination of both and measured number of *R. solanacearum* 1609 and wilted potato plants. Pig slurry addition to infested soil resulted in significantly lower populations of *R. solanacearum* and also fewer wilted plants. The combinatory treatments showed additive effects and gave the best results (Gorissen, van Overbeek & van Elsas, 2004). In the present study the antagonistic effects of SBW25 towards *R. solanacearum* KZR5 (biovar 2 race 3) was investigated in **Paper III**.

Microdochium nivale

The causative agent of pink snow mold, *Microdochium nivale* (Ces. Ex. Sacc.), is a large problem especially in temperate regions, where it causes disease in economically important plants such as winter-sown wheat, rye and barley, turf grasses and conifers. *M. nivale* is very closely related to *Fusarium* spp. and has been transferred several times, from *Gerlachia nivalis* to *Fusarium nivale* and now to *M. nivale*. This mold can be both seed and soil borne and develops under a thick layer of snow, preferably on unfrozen ground (Smith, 1981). *M. nivale* may cause pre- or post-emergence death of seedlings. When disease is severe leaves, shoots and sometimes the whole plant are killed, possibly accompanied by white or pink mycelium giving the disease its name. For the field trial described in **Papers I** and **II**, *M. nivale* infested wheat seeds were used and the same seed batch was used in **Paper IV** when examining the effects of the bacterial strains SBW25 and PB177 together with AM fungi on *M. nivale*.

Colonization ability of SBW25 on different plant species

From earlier studies it is known that *Ps. fluorescens* SBW25 is able to colonize a wide range of different plant species (de Leij, *et al.*, 1995a; Naseby, *et al.*, 2001; Thompson, *et al.*, 1995; Unge & Jansson, 2001). In the present work several different greenhouse and phytotron experiments, together with one field trial have been performed using SBW25 as a seed/tuber coating before planting (see Figure 1 for an overview). Field grown wheat (**Paper I**) was compared with tomatoes grown in agar or soil and potatoes grown in soil (**Paper III**) with respect to the numbers of SBW25 found in plant tissue after 6 weeks (Figure 7). For a more in detail description of the methods used the reader is referred to the respective papers. These experiments give together an extended view of the colonization ability of SBW25 on a variety of plants after 6 weeks (wheat, tomato and potato) (Figure 7). From seed/tuber coating SBW25 readily colonizes both roots and shoots.

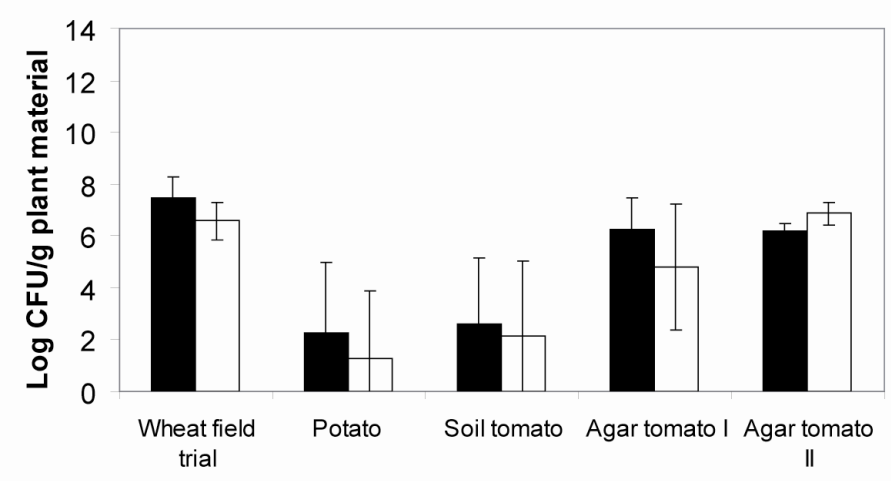

Figure 7. Comparisons between numbers of SBW25 detected on shoots (black bars) and roots (white bars) of different plants after 6 weeks. Inoculum densities: 10^7 cfu ml^{-1} (wheat), 10^8 cfu ml^{-1} (potato, tomato in soil and agar I) and 10^{10} cfu/ ml^{-1} (tomatoes in agar II).

An interesting observation is the difference in numbers of SBW25 colonizing tomatoes in non-sterile soil and sterile agar. Tomatoes grown in soil seem to have a lower number of SBW25 cells than those grown in agar. This could be due to competition from the resident soil microbiota (van Veen, van Overbeek & van Elsas, 1997), or to a slower growth rate of the tomato seedlings in agar. Both wheat plants and tomato plants in agar, showing highest numbers of SBW25, were smaller than potatoes and tomatoes in soil when harvested after 6 weeks.

The phytotron study prior to the Swedish field trial conducted in this thesis (**Papers I** and **II**) tested the influence of different inoculum densities on the final wheat plant number of SBW25. This study revealed that SBW25 established an optimum colonization density with the same metabolic activity on the plants irrespective of inoculum densities: 10^7, 10^8 or 10^9 cfu SBW25 ml^{-1} (Figure 8) (**Paper I**). So, why would there be an optimal colonization capacity on a plant by a specific PGPB? Competition among SBW25 cells could restrict population numbers, since there have to be nutrients and space enough to provide a niche for a bacterial population.

Numbers of plant wounds and cracks are the same irrespective of PGPB inoculum density and these are probably the main attachment sites in early plant colonization by bacteria (Preston, 2004). Quorum sensing mechanisms, signaling systems dependent on bacterial densities, are known to be involved in microbial adaptation to environmental conditions (Whitehead *et al.*, 2001) and have been shown to be control root colonization and biocontrol of strain *Ps.*

fluorescens 2P24 on wheat (Wei & Zhang, 2006). Although no such system has yet been found in strain SBW25 it is likely that some kind of signaling between SBW25 cells, or plant cells and bacteria, is controlling bacterial concentrations in the plant proximity. It has been found that SBW25 when left in unshaken liquid cultures spontaneously forms a wrinkly spreader phenotype (WS) that readily forms a biofilm in the air-liquid interface in order to avoid anoxic conditions. These cells have been found to cooperate by over-producing an adhesive polymer to create this biofilm (Rainey & Rainey, 2003). Thus SBW25 cells are capable of cell–to-cell signaling.

In the present study another experiment with different inoculation doses was performed with tomato plants, giving similar results. In the first agar tomato assay the seeds were soaked in a suspension of SBW25 at 5.3 x 10^8 CFU ml^{-1} and in the second assay a suspension at 1.6 x 10^{10} CFU ml^{-1} was used. Once again, no differences in detected numbers of SBW25 were seen, but the deviations between replicate plants were smaller with the higher inoculum density (Figure 8) (**Paper III**). Both these assays were performed in non-sterile soil, so this colonization was in competition with resident microorganisms.

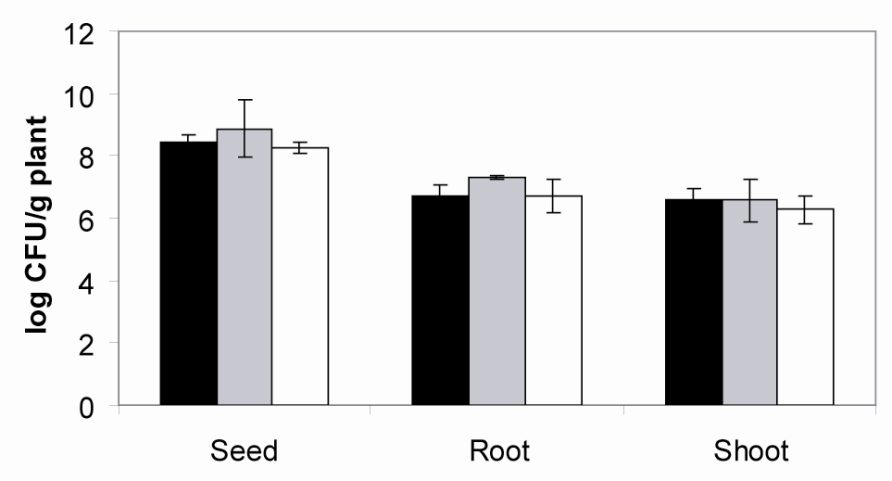

Figure 8. Numbers of SBW25 detected on different plant parts. Wheat seeds were coated with bacterial suspensions of 10^9 (black bars), 10^8 (grey bars) or $10^{6.5}$ (white bars) cfu SBW25/ml.

Survival and impacts of SBW25 on indigenous soil microorganisms in the Swedish field trial

In order for a PGPB or BCA to work properly it is of greatest importance that it is capable of colonizing the plants of interest, reach values high enough at the

desired location and survive during the growing season, or at least long enough to prevent disease. This has been a problem for several candidate PGPB bacteria (Thomashow, 1996). Climatic factors are probably very important since different bacterial strains have different temperature requirements and it is therefore necessary to choose PGPB candidates suitable for each climatic condition. SBW25 has been introduced into field trials both in the UK (de Leij, *et al.*, 1995a; Thompson, *et al.*, 1995) and Sweden (**Paper I**), and these two countries have different climates, especially during the winter season. A genetically modified variant of SBW25 with introduced *lacZY* and *kan*^{r}*-xylE* genes (*Ps. fluorescens* SBW25EeZY-6KX) was evaluated in two separate field trials in the spring of 1993 in the UK. One field trial took place at Wytham Farm in Oxford with sugar beet and another with wheat at Horticulture Research International, Littlehampton. Following sugar beet seed inoculation the GMM colonized the leaves, rhizosphere and root cortex throughout the growing season (270 days post release). The GMM was not detected in soil samples at a depth of 3 cm. Limited dispersal to other plant species was recorded. No GMMs were detected at the roots of any weed species in the field. Transfer of the GMM to guard row sugar beet was limited to outer leaves of the inner guard row of sugar beet in direct physical contact with the inoculated plants. No GMMs were detected in the homogenates of flying insects from traps within the treated plot (Thompson, *et al.*, 1995). In the 1993 wheat field trial vertical and lateral dispersal of the GMM was detected up to 45 cm depth and 200 cm width, however these numbers declined both during wheat growth and after harvest. GMM numbers persisted at levels of 10^6 cfu g^{-1} root during the growing season and for more than 200 days after harvest when the roots were nearly completely decomposed (de Leij, *et al.*, 1995a).

In the present study (initiated in Sep 2005) the genetically modified SBW25::tgl strain was detected at high densities on wheat roots, seeds and shoots throughout an 8 month period, even after winter. There were no major differences between survival in the phytotron and the field for the four first weeks (**Paper I**), even though the numbers of SBW25::tgl cells were significantly higher on shoots in the field than in the phytotron. No dissemination to bulk soil was detected until Nov 2007 (more than 2 years post release) when the GMM could be detected at low levels in bulk soil from all treated plots, guard rows and fallow areas (Jäderlund & Jansson, unpublished data). Numbers of the GMM strain were however less than 1 % of the native *Pseudomonas* population as determined by using an agar medium selective to *Pseudomonas*.

There are clear differences between these field trial studies in the UK and Sweden. The dispersal of the GMM strain to the surrounding environment was

much more prominent in the 1993 wheat trial in the UK than in the other two studies, even if the survival of the strain on plants was highest in the Swedish trial. Soil type may be a part of the explanation for these observed differences as the 1993 wheat trial was performed in a silty loam soil as compared to the 1993 sugar beet and 2005 wheat trials where heavier clay loam soils were used. It is possible that bacteria have easier to spread by water dispersion in sandy compared to clay soils. Another issue could be that the inoculated wheat seeds in 1993 were encapsulated in a 1.25 % guar gel to protect the cells (de Leij, *et al.*, 1995a) and this could have also protected the bacterial cells in the soil environment.

In the 1993 sugar beet trial in the UK the impact of the GMM strain on the culturable pseudomonad community was evaluated as well as community diversity by MIS-FAME (Microbial Identification System – Fatty Acid Methyl Esther analysis), and only temporary effects could be seen (Bailey *et al.*, 1997). In the 1993 wheat experiment culturable bacterial populations were evaluated for disturbances when using SBW25EeZY-6KX as a seed coating and foliar spray, and also here some significant but transient effects were found. The release of the GMM, and the wild-type strain, resulted in significant, but transient changes of some of the culturable microbial community that inhabits the phylloplane and rhizosphere of wheat. No significant effects were detected in bulk soil. Organisms that were fast growing and could not produce resting structures seemed to be the most sensitive to impact by the inoculation, for example fluorescent pseudomonads and yeasts. The alterations caused by the GMM were not significantly different from those caused by the wild-type organism, the changes were mainly small and the inoculated bacteria had no negative effect on plant growth or plant health (de Leij *et al.*, 1995b).

Similar to the field trials in the UK, some small and inconsistent changes were detected in the Swedish field trial (**Paper II**); and none of the ten differences found was significant ($p<0.05$), the others ranging between $0.05<p<0.1$. No effects due to bacterial inoculation were found after 69 days post sowing (Figure 9). These results are in accordance with the English field trials.

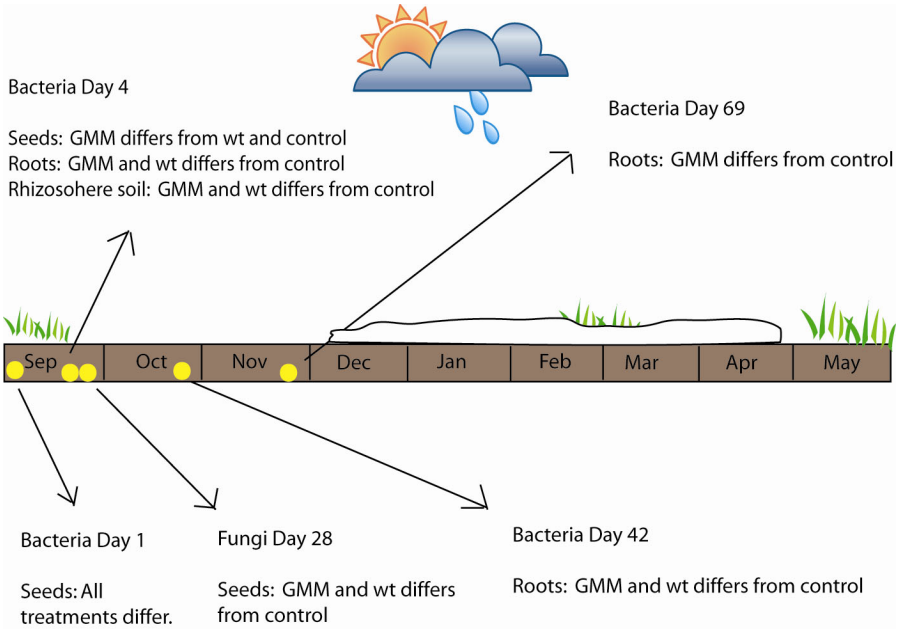

Figure 9. An overview of the detected differences due to treatment using blocked MRPP (p-values <0.10) in the Swedish field trial.

Biocontrol efficency of SBW25

Biocontrol of *R. solanacearum*

Over 80 countries worldwide are affected by bacterial wilt caused by different strains of *R. solanacearum* (Breukers *et al.*, 2005). Many different methods have been applied to control this pathogen in order to develop a low-impact soil management control strategy. Some of the recent attempts are the use of added compost (Schönfeld, *et al.*, 2003) or pig slurry (Gorissen, van Overbeek & van Elsas, 2004) combined with solarization of the soil. Both of these studies showed a reduced survival of *R. solanacearum* in the soil and also a decrease of the infectiveness of the pathogen. Another study used combinations of PGPB (*Bacillus pumilus* and *Ps. putida*), acibenzolar-S-Methyl and a soil amendment consisting of different minerals and organic compounds, also with reduced incidence of disease (Anith & Momol, 2004).

In this thesis, the biocontrol efficacy of SBW25 towards *R. solanacearum* KZR5 was evaluated with tomato and potato as host plants (**Paper III**). SBW25 was shown to inhibit KZR5 growth on agar plates, but results from in vitro tests are often poorly correlated to what will happen *in planta*. However,

in these plant experiments it was shown that SBW25 reduced incidence of disease symptoms on tomatoes by 50 % compared to non-treated control plants (three experiments in total with four replicate plants per treatment). Green house experiments in China with three strains of PGPB showed similar results to those presented in this thesis. One *Serratia* sp., one fluorescent pseudomonad and one *Bacillus* sp. were evaluated as biocontrol agents towards bacterial tomato wilt. Disease was reduced between 63.6 % and 94.1 % by these bacteria (Guo*, et al.*, 2004). Even though infested tomato plants in **Paper III** looked healthy when coinoculated with SBW25 there were latent *R. solanacearum* infections in all infected plants. Latent infections were also noted in the lower parts of stems in a previous study of *R. solanacearum* 1609 (Schönfeld*, et al.*, 2003).

In some cases in **Paper III** the bacterial density of SBW25::telrfp was higher in plants treated with both SBW25 and KZR5, than single inoculation of SBW25, and could be a sign of the SBW25 actively mobilizing as a response to presence of KZR5 cells. It could also be a sign of SBW25 cells taking advantage of the nutrient released by the decaying plants in the presence of KZR5, as shown for an *Acinetobacter* sp. on tomato in presence of a strain of *R. solanacearum* (Kay *et al.*, 2002). However, these findings need further evaluation in larger scale experiments before any conclusion can be drawn. For a more in detail discussion about this, the reader is referred to **Paper III**.

Synergistic interactions between microorganisms to control *M. nivale*

Interactions between PGPB and AM fungi are known to occur, and it has been hypothesized that some of the interactions may be very specific (Aspray *et al.*, 2006; Bending, 2007). **Paper IV** examines the effects of SBW25 and PB177 together with AM fungi *G. mosseae* and *G. intraradices* on wheat plants (infected with *M. nivale* or healthy), and the results from this study is that the interactions are highly specific between these organisms. Both the AM colonization of the wheat roots and the beneficial plant effect are dependent on the microorganisms used, however high AM colonization was not correlated to a higher plant dry weight yield.

When treating *M. nivale* infested wheat seeds, one combination of bacterium and AM-fungus (SBW25 together with *G. intraradices*) actually gave a synergistic effect on shoot and root dry weight, resulting in a shoot weight that was significantly higher than healthy plants. Such synergistic effects have been reported before, for example in a study with *P. fluorescens* D57 on cucumber together with *G. intraradices* and *G. caledonium* it was shown that D57 in dual inoculation with *G. intraradices* gave significantly higher root dry weights than other treatments (Ravnskov & Jakobsen, 1999). These effects could be used

when searching for biocontrol agents or plant growth promoting microbial consortia.

Conclusions – Main findings

· A novel non-antibiotic marker gene vector was constructed and used to monitor a specific bacterial strain in the first field trial with a GMM (SBW25::tgl) in Sweden.

· The GMM strain successfully colonized all plant parts of winter wheat in the field trial at high numbers throughout the 8 months period. They were also metabolically active on all plant parts.

· Only minor and non-consistent impacts, assessed by T-RFLP, of the SBW25::tgl and wt strains were detected on bacterial and fungal resident populations in the field trial.

· Plate assay inhibition of *R solanacearum* KZR5 was detected by SBW25. Some protection was also seen in phytotron studies with tomatoes.

· *G. intraradices*, alone or in combination with SBW25, significantly increased the shoot dry weight of *M. nivale* infested wheat plants. *G. intraradices* + SBW25 even increased shoot dry weight of infested plants compared to healthy ones.

· No correlations could be made between the level of AM colonization and increase in plant dry weight.

Future perspectives

Agriculture needs to find solutions to some of the problems that are connected to environmental health, such as the over-use of chemical pesticides and fertilizers. Plant growth-promoting bacteria can be a part of that solution, together with mycorrhizal fungi and other microorganisms to increase crop yields in a sustainable fashion. This work has focused on the use of one single PGPB, however the most efficient use will probably be the use of a consortium of different microorganisms performing different functions and contributing with specialized traits. This was also shown in this work where the use of SBW25 in combination with AM fungi increased wheat plant shoot weight in comparison to single inoculation with the two microorganisms. More research using consortia of different PGPB working together is needed in order to reveal some of the mechanisms involved in the synergistic interactions, leading to increased plant growth promotion.

Many molecular tools may also be used to investigate the interactions between microorganisms, such as the marker genes used in this thesis. New techniques, and the sequencing of genomes will give new opportunities to focus on important relationships between microbes and their environments.

> We are at the very beginning of time for the human race. It is not unreasonable that we grapple with problems. But there are tens of thousands of years in the future. Our responsibility is to do what we can, learn what we can, improve the solutions, and pass them on.
>
> - Richard Feynman

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Gratitude is the memory of the heart - Jean Baptiste Massieu

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