Tracking the Fate of Biocontrol Microorganisms in the Environment Using Intrinsic SCAR Markers

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Cover: *Pseudomonas brassicacearum* MA250
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Abstract

Biocontrol microorganisms can be used as an alternative to conventional chemical pesticides to control plant diseases. This thesis investigated the fate and behaviour of three microbial model control agents with effect against fungal pathogens in cropping systems, and the spread of one of the model strains in bioaerosols. The bacterium *Pseudomonas brassicacearum* MA250 was monitored in winter wheat systems, in a climate chamber trial and in field conditions. The contribution of strain MA250 to the total microbial load in bioaerosols was also investigated. Two filamentous fungi, *Hypocrea parapilulifera* IMI206039 and *Trichoderma atroviride* IMI206040, were investigated on a golf green and in microcosms with soil sampled from the green and with agricultural soil. To track and enumerate the applied strains, intrinsic strain-specific sequence-characterised amplified region (SCAR) markers were utilised in quantitative real-time PCR. Supplementary methods, such as cultivation-based methods and analysis of the Gram-negative bacterial cell constituent endotoxin, were also included.

In both winter wheat studies, MA250 colonised the roots of healthy seedlings. Withered seedlings that had died due to snow mould infection were not colonised, indicating the importance of a healthy seedling for proliferation of MA250. In the microcosm studies, with the two fungal biocontrol strains, populations of introduced cells behaved differently depending on the soil. On the golf green, the two fungal biocontrol strains were detected only sporadically, but nevertheless maintained their control effect. In the bioaerosol study, the SCAR marker consistently enabled detection of strain MA250, whereas other methods tested did not. The results showed that MA250 significantly contributed to the total microbial load in aerosols after 60 minutes of coating, when MA250 was added to the system. However, after 240 minutes of coating this significant increase was not seen and MA250 could only be detected against the background with use of the SCAR marker.

In conclusion, the use of strain-specific SCAR markers proved a reliable method in quantifying added populations in soils, plants and aerosols. This type of information is valuable for optimising the use of the biocontrol microorganisms and to determine the risks associated with application of the strains.

Keywords: SCAR marker, quantitative real-time PCR, *Pseudomonas brassicacearum, Trichoderma/Hypocrea*

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List of Publications

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

I Johnsson Holmberg, A-I., Melin, P., Levenfors, J.P., Sundh, I. (2009). Development and evaluation of SCAR markers for a *Pseudomonas brassicacearum* strain used in biological control of snow mould. *Biological Control* 48, 181-187.

II Johnsson Holmberg, A-I., Melin, P., Levenfors, J.P., Sundh, I. Determining the fate and behaviour of a seed-applied *Pseudomonas brassicacearum* strain in a winter wheat field trial, by analysis with SCAR markers. (Manuscript).

III Feng, X.M., Johnsson Holmberg, A-I., Sundh, I., Ricard, T., Melin, P. Specific SCAR markers and multiplex real-time PCR for quantification of two *Trichoderma* biocontrol strains in environmental samples. (Submitted)

IV Johnsson Holmberg, A-I., Nilsson, A.I., Melin, P., Hökeberg, M., Madsen, A.M., Sundh, I. Contribution of a biocontrol *Pseudomonas* strain to the total microbial load in bioaerosols during experimental seed inoculation. (Manuscript).

Paper I is reproduced with the permission of the publishers.

My contribution to the papers included in this thesis was as follows:

I Participated in the planning of SCAR marker identification and climate chamber sampling, performed all laboratory work. Analysed the data with co-authors and wrote the majority of the manuscript.

II Planned the project and sampling together with co-authors. Did the majority of sampling in the field trial. Performed all laboratory work and all data analysis. Evaluated the data with the supervisors and wrote the majority of the manuscript.

III Planned the project with co-authors, participated in the field and laboratory work. Contributed to the analysis of the data and to writing the manuscript.

IV Planned the study with co-authors. Planned and performed the aerosol samplings and laboratory work with Nilsson and Madsen. Analysed and evaluated data, with input from the supervisors, and wrote the majority of the manuscript.

Abbreviations

BCA	Biological Control Agent
cfu	Colony forming units
DNA	DeoxyriboNucleic Acid
EFSA	European Food Safety Authority
FDA	The Food and Drug Administration
GRAS	Generally Recognised As Safe
ISO	International Organization for Standardization
ITS	Internal Transcribed Spacer
MBCA	Microbial BioControl Agent
qPCR	Quantitative real-time Polymerase Chain Reaction
QPS	Qualified Presumption of Safety
RAPD	Random Amplified Polymorphic DNA
RNA	RiboNucleic Acid
SCAR	Sequence Characterised Amplified Region

1 Introduction

Today, concern for the environmental state of the earth is widespread and much effort is being devoted to finding ways towards a sustainable future. There is a growing wish among consumers that more regard for sustainability should be taken in the production of food, clothes and other consumables. Besides energy costs the reduced use of xenobiotic chemicals is an important issue as the use of chemicals, for example in agriculture, has a potentially negative impact on the environment in terms of toxicity. In order to find alternative options to chemicals, new methods are needed and eyes have turned to the microbes. Microorganisms have long served humans, most prominently within food production (Jay, 2000), and new uses are being found in more areas, such as agriculture.

The term 'ecology' was coined in 1866 by Häeckel (Atlas & Bartha, 1998) and in the 1960s, when the vast world of microorganisms and their implications in general ecology were recognised, the term microbial ecology began spreading. Microbial ecology refers to the interrelationships between microorganisms and their living and non-living environments. Microbes carry out many crucial biological processes, including the cycling of elements such as nitrogen and carbon, which are essential for all organisms (Atlas & Bartha, 1998).

With the active culturing and intentional use of microorganisms in various applications comes responsibility. Microorganisms in large numbers added to specific environments have the potential to spread to new areas and have effects on resident microbial and eukaryotic populations such as plants and animals. Before a fungus or bacterium can be utilised, for example in biological control, its biology and ecology must be investigated and its safety ensured. Such studies not only address the biocontrol efficiency but also provide insights to the dynamics of the selected microorganism compared with indigenous populations. This thesis describes the current challenges

concerning strain-specific tracking and advances the use of intrinsic DNA markers as a method to address these challenges.

1.1 Aims and outline of this thesis

One of the major fields in which single strains of microorganisms are released in large numbers is biological control. In this thesis three microbial control agents (MBCAs), the bacterium *Pseudomonas brassicacearum* MA250 (hereafter denoted MA250) and the two filamentous fungi *Hypocrea parapilulifera* IMI206039 (anamorph *Trichoderma*; hereafter denoted Tp039) and *Trichoderma atroviride* IMI206040 (hereafter denoted Ta040) were used as model organisms. The two fungi were used together in one formulation.

The aim of the thesis was to investigate the fate and behaviour of the three model organisms in cropping systems and the spread of MA250 in bioaerosols. Such understanding is important in optimising the use of biocontrol organisms and identifying potential risks, but also in learning more about the dynamics of added or introduced populations (Kluepfel, 1993). In order to achieve this, sequence-characterised amplified region (SCAR) markers specific for the biocontrol strains were developed and tested under realistic conditions of use (**Papers I** and **III**). The *Pseudomonas* strain, which has demonstrated biological control effect against *Microdochium* spp. infection, was added to winter wheat seeds and monitored in growing plants in a climate chamber trial (**Paper I**) and a field trial (**Paper II**). The potential impact of aerosol exposure to strain MA250 during experimental seed inoculation was evaluated using the MA250-specific SCAR marker and other primers in quantitative real-time PCR (qPCR), along with cultivation-based methods and endotoxin measurements (**Paper IV**). The two fungal biocontrol strains were monitored in soil microcosms and applied on a golf green, to replace conventional chemical pesticides against *Botrytis cinerea* and *Chondrostereum purpureum* infection. The fungi were then analysed individually using cultivation-based methods and a probe-based multiplex qPCR assay (**Paper III**).

This thesis begins by providing a general background to environmental applications with microorganisms. It then discusses the strengths and weaknesses of SCAR markers in comparison with other available techniques for tracking and quantifying microorganisms released into the environment, with special attention to the genera *Trichoderma* and *Pseudomonas*. The use of strain-specific tracking, and SCAR markers in particular, through qPCR is discussed in more detail. In the experimental studies that form part of this thesis, microorganisms utilised in biological control of plant diseases were

used as model organisms, but the same questions and challenges apply to other types of environmental applications with microbes.

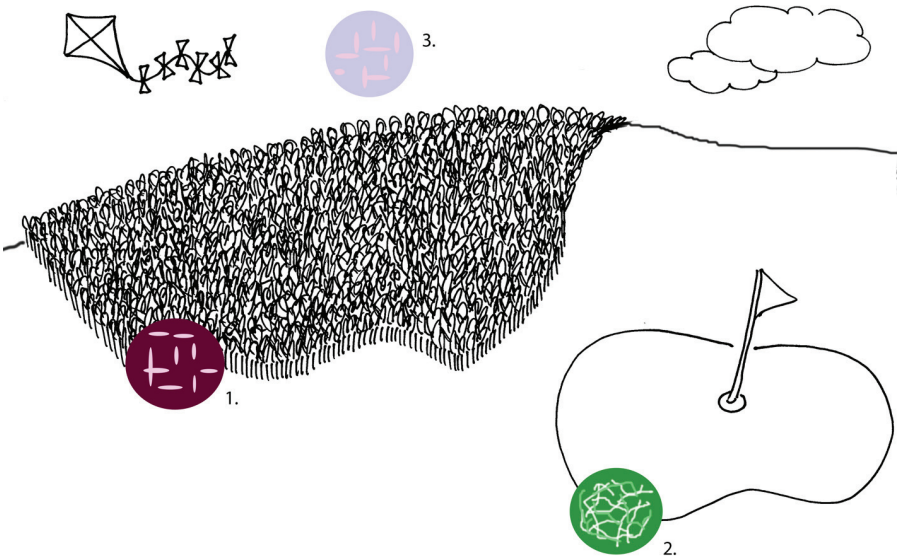

Figure 1. The environments studied in this thesis; *Pseudomonas brassicacearum* MA250 in winter wheat production systems (1), *Hypocrea parapilulifera* Tp039 and *Trichoderma atroviride* Ta040 on golf greens (2) and *Pseudomonas brassicacearum* MA250 in bioaerosols (3).

2 Microbial applications in the environment

Microorganisms are everywhere. They are found in and on our bodies, in our food, in the air and in most environments on earth. The cell numbers of bacteria in arable soils can amount to billions per gram (Øvreås & Torsvik, 1998) with around a thousand-fold fewer fungal cells (Sullivan & Krieger, 2001). The competition for nutrients is often strong, not only among microorganisms but also between them and higher organisms, i.e. eukaryotes. This has driven the need for specialisation with regard to survival and function (Lawrence, 2001).

Because of this species and strains have different characteristics and some have qualities which can be beneficial to humans, who have long tried to domesticate selected microorganisms for various purposes (Jay, 2000). Today, microorganisms are utilised for production or protection in many different environmental application areas (Table 1). Food made with the help of microbes is found in homes all over the world. Other strains are used in agriculture for protection of crops in biological control or enhancement of crop yield in plant growth promotion. In bioremediation, microorganisms are utilised for removal of unwanted compounds (Kuiper et al., 2004; Juhanson et al., 2009).

The production and protection of foodstuffs, and the development of probiotic products using bacteria and fungi, have a long history in the food industry (Jay, 2000). To prevent spoilage of feed, beneficial microorganisms have long been used as protective cultures in the production of silage. There are a number of examples of the use of genetically modified microorganisms in environmental applications (Wilson & Lindow, 1993) but this thesis focuses mainly on unmodified, wild-type isolates with biocontrol activity.

Table 1. *Examples of applications with microorganisms*

Application area	***Purpose***
Food and feed production	Production of fermented foods, cheese, bread, beverages, single cell protein foods and silage
Postharvest protection and biopreservation	Prevention of food spoilage
Crop production	Biological control, plant growth promotion, increased soil fertility
Bioremediation	Decontamination of digestion residues, in soils, waters or wastewaters

2.1 Biological control

Biological control, or biocontrol, is a large environmental microbial application area. In biocontrol, living organisms with antagonistic properties are used to suppress the population density or impact of a specific pest (Eilenberg et al., 2001). This area of research has been intensified the past 40 years (Alabouvette et al., 2009) and this thesis takes as its example microbiological control agents against crop pests. The use of BCAs can be divided into four strategies: classical, inoculation, inundation, and conservation biological control (Eilenberg et al., 2001). For the winter wheat studies with MA250 (**Papers I** and **II**) inoculation biological control was implemented, with the intention of releasing 'a living organism as a biological control agent with the expectation that it will multiply and control the pest for an extended period, but not permanently' (Eilenberg et al., 2001). This definition, where the progeny of the added population is expected to exert the control effect, distinguishes the strategy from inundation biological control, where the added population directly controls the pest. The biocontrol effects of MA250, Tp039 and Ta040 are discussed further in the section 'Soil and cropping systems'.

An efficient biocontrol microorganism should be able to establish a sufficiently large population and carry out the modes of action needed for control effect (Alabouvette et al., 2009). The mechanisms behind biological control of plant diseases by antagonistic microbes are diverse, ranging from parasitism of the pest to induction of a host plant's own resistance. The different interactions between pests and biocontrol organisms have been

reviewed by Whipps (2001) and Alabouvette et al. (2009) respectively, and are only treated briefly here.

The *Pseudomonas* and *Trichoderma* genera have been much studied in the field of biological control. *Trichoderma* strains often have direct parasitic activity, which reduces pest populations (Schuster & Schmoll, 2010). Some *Pseudomonas* biocontrol strains are known for their ability to produce iron-binding molecules, siderophores, which give them an advantage over other strains in an iron-limited environment (Cornelis, 2010). Still other microbial strains release antibiotic substances, such as phenazines or hydrogen cyanide, which may be toxic to other microorganisms but also trigger an immune response in plants (Haas & Défago, 2005). A successful microbial control agent often relies not on one but on several compounds and mechanisms.

Biocontrol agents can be applied for example through seed coating, direct spraying or by delivery with insects. The potential for spread of microbes in bioaerosols is discussed in the section 'Air exposure'. Application of the biocontrol agent to the seed prior to sowing is an established method in the control of soil-borne plant pests (Bennett & Whipps, 2008) and was implemented in **Papers I** and **II**. By selecting this mode of delivery, the biocontrol agent is in place to protect against the pest when the seed germinates. Spray application is utilised in greenhouses but also in biological control of turf grass diseases, allowing spores to reach and colonise the roots (Lo et al., 1997; 1998) and this approach was used in **Paper III**. Some delivery systems utilise pollinators to deliver products, such as BINAB® Vector (Table 2), directly to the flowers of the host plant (Johnson & Stockwell, 1998).

Biological control products currently available on the market are based on approximately 150 species and marketed by around 85 producers worldwide (van Lenteren, 2008). The use of mites and spiders dominates in greenhouses markets (van Lenteren, 2003), while in MBCAs the species *Bacillus thuringiensis* (Bt) has been the most consistent and significant (Whalon & Wingerd, 2003). Table 2 presents a selection of microbial control products targeting fungi (Hansen, 2010).

To register a new product based on a MBCA today, there are several bottlenecks to pass through. Besides safety assessment for registration, further discussed in the section 'On the need to monitor added strains', one challenge is that the efficacy of biological control products is compared with that of chemical pesticides. Chemical pesticides are generally more efficient, can be stored for longer periods of time and are less expensive to produce than biological control products (Harman, 2000). These differences are, however, becoming less of a problem with the improvements made in the

fermentation and formulation of microorganisms, since this progress has led to better efficacy and stability during storage (Kiewnick, 2007).

One strategy in promoting biocontrol agents has been to focus the development of new products to specific niches where biocontrol is regarded a better option compared to chemical options. Chemical pesticides are not always applicable, or there may a wish to reduce such use. Golf courses and parks are examples of such niches for biological control, since they are environments where people spend their leisure time (Harman, 2000). In agriculture, the use of biocontrol agents can allow for control of a pest shortly before harvest, whereas chemical pesticides often have a pre-harvest interval (Kiewnick, 2007). Pests are also less likely to develop resistance against biocontrol agents, than against chemical pesticides, when the control effect is based on several modes of action (Kiewnick, 2007).

Table 2. *A selection of commercially available microbial products against fungal plant diseases.*

Active species	***Product name***	***Distributor***
Bacillus subtilis	Serenade®	BASF SE, Germany
Coniothyrium minitans	Contans®WG	Prophyta Biologischer Pflanzenschutz GmbH
Gliocladium catenulatum	Gliomix®, Prestop®	Verdera Oy, Finland
Pseudomonas chlororaphis	Cedomon®, Cerall®	Lantmännen BioAgri, Sweden
Streptomyces griseoviridis	Mycostop®	Verdera Oy, Finland
Trichoderma atroviride *Hypocrea parapiluliferum*	BINAB® Vector BINAB® TF WP BINAB® Pellets	BINAB Bio-Innovation AB, Sweden

3 On the need to monitor added strains

Knowledge about the fate and behaviour of specific organisms is important for many areas of microbial ecology. Kluepfel (1993) identified four particular areas; population dynamics, microbial dissemination, persistence in the environment and community effects. There are many methods for assessing populations, activity, biomass and collective phylogenetic groups (Parkinson & Coleman, 1991), but fewer for monitoring specific strains in complex samples from the environment. The monitoring of biocontrol strains is also of interest in investigating mechanisms involved in the control effect exerted in biological control or in optimising application strategies for enhanced effect. There are several reasons why for example MBCAs are not used more widely than they are, including slow registration processes, technical difficulties where not enough is known of the intended application environment and the efficiency of the physical product (Cook et al., 1996). Knowledge of the interactions between microorganisms and their environment can facilitate the development of the biocontrol area (Whipps, 2001) and investigating specific strains can assist in obtaining such understanding.

3.1 Determining the fate of an added strain

A strain released into an environment other than the source of isolation, will face a number of opportunities and challenges depending on the strain and the circumstances (Gentry et al., 2004). The ability to persist in a given environment can vary between strains of the same species (Xiang et al., 2010) and added cells may not survive the transfer into the new environment, but quickly decline and vanish. The challenges comprise competition from resident populations for nutrients and niches and grazing

by protozoa or attack by bacteriophages (Gentry et al., 2004). It has been shown that bacteria that have been allowed to establish in sterile soil have greater colonisation ability upon introduction into natural soil than bacteria introduced directly into natural soil (Van Dyke & Prosser, 2000). This finding indicates the impact of stress and exposure to predators, which affect microorganisms that are released into soil (Alabouvette et al., 2009).

A successful biocontrol strain finds protective niches, colonises and spreads to new locations (van Veen et al., 1997; Johansen et al., 2005). To make full use of an added strain in biological control of soil-borne plant diseases, information on the ecology must be obtained. With specific monitoring, the locations and population numbers of a given microorganism can be followed over time, thus providing data on the size, distribution and survival time of the population following inoculation (Whipps, 2001; Alabouvette et al., 2009).

3.2 Non-target effects and safety assessment

Addition of microorganisms to an environment can lead to non-target effects on resident microorganisms, protozoa, nematodes and plants (i.e. organisms other than the target organism), thereby affecting their role in ecological function (Winding et al., 2004). It is generally considered that the non-target effects of microorganisms introduced into soil are small and desist when the introduced population has disappeared or decreased to a critical level (Winding et al., 2004; Johansen et al., 2005). Natural variation in diversity and cell numbers normally arises due to seasonal influence and agricultural practices (Winding et al., 2004; Johansen et al., 2005) as microbial communities are also sensitive to other frequently occurring disturbances, such as mechanical perturbation, desiccation, starvation and freezing/thawing (Kluepfel, 1993; Torsvik et al., 2002).

Studies of the fate and behaviour of specific strains have become more important with the increasing demands for documentation by regulatory agencies evaluating microorganisms for release (Green & Jensen, 1995; Kiewnick, 2007). Many species utilised in environmental applications today have a long documented history of safe use. Some regulatory agencies, such as the European Food Safety Authority (EFSA) and the Food and Drug Administration (FDA), have therefore identified species with a qualified presumption of safety (QPS) or which are generally recognised as safe (GRAS). Microorganisms on these lists usually undergo simplified safety assessment, and the system allows regulatory agencies to focus on those species that may pose the greatest risk (Leuschner et al., 2010).

Safety assessments of particular strains often consist of several parts. A general literature review of the candidate genus at an early stage can reveal potential reasons not to pursue work with a strain. Predictable traits, i.e. those found through relatedness, are of great value (Cook et al., 1996). If the strain passes the initial assessment threshold, laboratory testing is often carried out, followed by studies on the fate and behaviour in the intended target environment. Several specific areas in safety assessment have been identified in which data are lacking and more knowledge is needed (Mensink & Scheepmaker, 2007). Besides modes of action and secretion of toxins or enzymes, field investigations regarding population dynamics, emission and exposure patterns, natural background levels and long-term effects are needed.

The risk with using microorganisms varies with the type of organism and method of application and is a combination of severity of hazard and level of exposure (Cook et al., 1996; Sheppard et al., 2003; Johansen et al., 2005). A complete *risk analysis* has three parts. The *risk assessment* quantifies the risk as a function of the probability of an adverse health effect and the severity of that effect. It also identifies actions and strategies to decrease the level of the risk (Forsythe, 2002). The remaining two are *risk management* and *risk communication*, i.e. the control and prevention of the identified risk and informing others. Collectively, the risk evaluation leads to a defined level where the risk can be accepted.

4 Model microorganisms of the present study

Pseudomonas strains (phylum γ-proteobacteria, family *Pseudomonadaceae*) are commonly found in soils, aquatic systems and many other ecosystems (Achouak et al., 2000). They are often found in association with plants and members of the genus have been used in biological control for a long time (Weller, 2007). Some species such as *P. syringae* (Rico et al., 2011) and *P. corrugata* (Catara, 2007) are pathogenic to plants and a few species, most notably *P. aeruginosa,* are pathogenic to humans and animals.

The species *Pseudomonas brassicacearum* was originally described by Achouak et al. (2000) and the type strain of the species is CFBP DBK11^{T}. It shares general characteristics with *P. thivervalensis* and has been described as a close relative of *P. kilonensis* (Sikorski et al., 2001a). Siderophore typing, i.e. grouping of strains and species by isoelectrophoresis, with regard to the pyoverdine type responsible for iron uptake in fluorescent pseudomonads (Cornelis, 2010), strengthens the identification based on DNA hybridisation (Meyer, 2010).

P. brassicacearum strains have been proposed as putative biocontrol (Ross et al., 2000) and plant growth promoting bacteria (Belimov et al., 2007). Many of the strains currently available were isolated from *Arabidopsis thaliana* or *Brassica napus* plants and different genotypes of *P. brassicacearum* are found on different genotypes of plant species (Fromin et al., 2001). *P. brassicacearum* has been placed in pathogenicity class I (BGChemie, 2005) but strains have been implicated as phytopathogens (Sikorski et al., 2001b). Depending on dosage and environmental circumstances, some strains can have plant growth promoting, neutral and phytopathogenic effects in one single plant cultivar (Belimov et al., 2007).

Pseudomonas brassicacearum strain MA250 (GenBank accession number of the 16S rDNA sequence DQ886486; Fig. 2) was selected as a model organism in the present study, since it has shown potential for biocontrol of fungal diseases in cereal production (Hökeberg et al., 1997; Levenfors et al., 2008). The strain was isolated from the roots of an oilseed rape (*Brassica napus*) plant in a field near Uppsala (Levenfors et al., 2008).

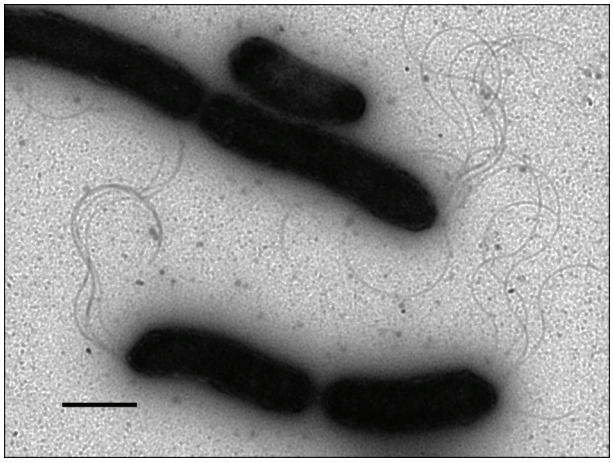

Figure 2. Transmission electron microscopy (TEM) image of air-dried *P. brassicacearum* MA250. The polar flagella are clearly visible. Scale bar = 1 μm. (Photo: P. Wessman)

Strains of the filamentous *Trichoderma* fungi (teleomorph *Hypocrea*, phylum *Ascomycota*, family *Hypocreaceae*), in particular those of the *T. harzianum* species (many reclassified as *T. atroviride*), have a long history in biological control as known antagonists to various plant pathogenic fungi. They also have a place in commercial production of cellulase enzymes (Samuels, 1996). *Trichoderma* are commonly found in soils, and have even been regarded as plant symbionts (Harman, 2000; Alabouvette et al., 2009). Many *Trichoderma* strains produce various types of antibiotics, lytic enzymes and volatile compounds, antagonistic to other fungi (Schuster & Schmoll, 2010). *Trichoderma* fungi have not been reported to parasitise on plants or cause post harvest crop losses, but *T. aggressivum* can cause serious loss in commercial mushroom production (Samuels, 1996; Schuster & Schmoll, 2010). In rare cases, *T. atroviride* has been implicated in the death of immunosuppressed human patients (Samuels, 1996; Schuster & Schmoll, 2010).

The two strains investigated in this study, *Hypocrea parapilulifera* IMI206039 (Tp039; anamorph *Trichoderma parapiluliferum*) and *Trichoderma*

atroviride P. Karst 1892 IMI206040 (Ta040; teleomorph *Hypocrea atroviridis*), are components of several commercial products available through the biocontrol company BINAB Bio-Innovation AB (www.binab.se). The teleomorph *H. parapilulifera*, i.e. the sexual reproductive stage of the fungus, was described by Lu et al. (2004) and the teleomorph *H. atroviridis* was described by Dodd et al. (2003). Teleomorphs are known for many anamorphs of the *Trichoderma* genus (Samuels, 2006). Strain Ta040 was previously classified as *T. harzianum*, but was re-classified after ITS sequencing (Kullnig et al., 2001). Throughout this thesis, the anamorph *Trichoderma* is used to refer to both strains when discussed in general, as the anamorph name is still most widely used in the literature.

There is little detailed information available on the modes of action of these strains, but other members of the genus control fungal pests through mycoparasitism, antibiosis, induction of plant resistance and competition for nutrients and space (Hoitink, 2006; EC 2008a, 2008b). Tp039 and Ta040 have both been shown to parasitise a range of different plant pathogenic fungi (Fig. 3) and are believed to complement each other in terms of pest specificity and biocontrol effect. Other members of the genus are known to produce toxic compounds, such as trichothecenes and peptaibols (EC 2008a, 2008b), but these compounds have not been found in Tp039 or Ta040.

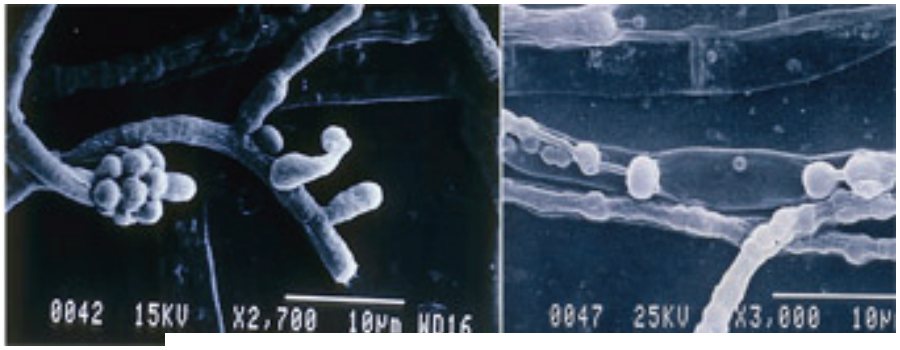

Figure 3. Left image shows a scanning electron microscope (SEM) photo of Ta040 conidiophores parasitising the fungal pest *Poria carbonica*. Right image shows Tp039 hyphae growing on the fungal pest *Antrodia carbonica*. (Photos: BINAB Bio-Innovation AB)

5 Monitoring methods

There are several readily applicable methods available for assessing the structure of whole microbial communities in environmental samples. These include denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) or the analysis of phospholipid fatty acids (PLFA), (Parkinson & Coleman, 1991; van Elsas et al., 1998). These methods give a fingerprint of the microorganisms present, distinguish between the genotypes present and, in the case of T-RFLP and PLFA, allocate identities to broad groups but not to species.

For monitoring of specific groups, species or strains of microorganisms, different approaches ranging from cultivation-based techniques to molecular techniques have been applied, as summarised in the following sections. Several techniques often are used simultaneously to supplement each other (van Elsas et al., 1998).

5.1 Cultivation-based monitoring

The most conventional monitoring technique is plating, where a microorganism is recovered on a solid growth medium. Such methods were implemented in **Papers III** and **IV** of this thesis. Sometimes, a strain naturally carries a trait, such as a rare pigment (Kropp et al., 1996) or resistance to an antibiotic, which makes it easily distinguishable from others within the genus. The latter trait can also be achieved by genetically modifying the strain.

Cultivation-based assays depend on the growth of viable cells, and do not detect cells which are metabolically active but not culturable on plates ('viable but not culturable', VBNC) (Jansson & Prosser, 1997; Pujol et al., 2006). Chapon et al. (2003) detected only 4-13% by plating, compared with PCR-based quantification with a SCAR marker, of the population of a

Pseudomonas strain introduced in wheat. The size of populations can also be underestimated due to competition or antagonism between microorganisms on plates, if rapidly growing microorganisms overgrow microorganisms with slower growth (Jansson & Prosser, 1997; Chapon, 2003). This was seen in **Paper III**, where strain Tp039 could not be enumerated after inoculation into natural soil.

Some selective media can be made specific down to genus level, e.g. for *Pseudomonas*, which can be selected for by using media such as King's B or the derivatives PsF and PsB used in **Paper IV**. The distinction between species and strains based on morphology is often difficult. Pseudomonads, for example, show phenotypic phase variation where the same bacterial clone can give morphologically distinct colony types (Achouak et al., 2000). In addition, even the selected microorganisms can have problems growing on the medium due to the high selective pressure.

Trichoderma colonies on general agar media for fungi are easily recognised, with rapid growth, green conidia and abundant powder (Samuels, 1996). Of the two biocontrol strains investigated in **Paper III**, Tp039 conidia were white and Ta040 conidia green on semi-selective Dichloran Rose Bengal Chloramphenicol (DRBC) agar (Ottow & Glathe, 1968). This medium has previously been used in the tracking and enumeration of *Trichoderma* biocontrol strains (Cordier et al., 2007; Savazzini et al., 2008) and enables detection of fungi with low growth rates. Although culturing is an established method for tracking and quantifying *Trichoderma* strains, the natural background present in environmental samples makes strain-specific identification very difficult (Abbasi, 1999). The problem arises with the distinction between conidia, as these morphological traits are not very variable between species and are difficult to interpret.

5.2 Microscopy-based monitoring

Microscopic detection is a straightforward technique for monitoring microorganisms (Jansson & Prosser, 1997), especially in clean sample matrices such as air. However, this method is less useful in complex sample types such as soils, due to masking by soil particles. The technique does not readily distinguish between species, but allows broad groups to be identified by basis of morphology (Jansson & Prosser, 1997). The use of DNA stains, such as 4',6-diamidino-2-phenylindole (DAPI) or acridine orange, facilitates distinction between cells and other particles in a sample (Jansson & Prosser, 1997).

For more specific detection, fluorescent antibodies targeting epitopes expressed by the microorganism can provide sufficient resolution from genus level (Thornton et al., 2002) down to strain level, provided that the epitope is sufficiently specific and constitutively expressed. Fluorescent in situ hybridisation (FISH) with probes targeting for example 16s rRNA can assist in identification and enumeration of microorganisms on group, genus or species level (Bottari et al., 2006).

5.3 Detection of genetically modified microorganisms

Spontaneous mutants, such as clones which are resistant to rifampicin, are commonly applied in tracking added strains (Natsch et al., 1997; Fischer et al., 2010). One drawback is that such resistance can be too common in a given environment for correct quantification of the introduced cells (Pujol et al., 2006). Many different marker genes can be artificially inserted into bacteria and fungi and one of the more popular is the green fluorescent protein (gfp) system (Chalfie et al., 1994), which does not require the addition of a substrate for detection. Other options for genetic modification are the introduction of one or several genes coding for luminescence, heavy metal resistance (Jäderlund et al., 2008), colour change or antibiotic resistance detected through plating. The introduction of a gene has the additional advantage of offering quantification using real-time PCR, through specific primer binding sites.

Genes inserted in the chromosome yield a more stable expression system compared with plasmid-borne traits. Chromosomal insertion also minimises the risk of transferring the gene to other microbes, such as indigenous populations, and loss of the inserted gene (Errampalli et al., 1999). The introduction of a gene can, however, alter the morphology or ecological fitness of a mutant compared with the wild type (van Elsas et al., 1998; Errampalli et al., 1999). This can affect extrapolation to the wild-type strain, as growth, colonisation ability or even production of metabolites can differ (Lübeck et al., 2002).

5.4 Intrinsic DNA markers

The unmodified genome of a microorganism offers several possibilities for monitoring. Strains which carry a functional gene can be targeted through amplification of the sequence (Mavrodi et al., 2007; Xiang et al., 2010). Other sequences, such as the 16s rDNA in bacteria or the internal transcribed spacer (ITS) and intergenic spacer (IGS) regions in fungi, can be

targets for tracking of specific microorganisms. Primers targeting these regions have been developed for specific identification of genera (Widmer et al., 1998) or species (Atkins et al., 2005) but they rarely offer the specificity necessary for identification of strains.

Sequences where the function is unknown are also utilised for monitoring. There are many approaches to identify these intrinsic markers, which may distinguish between species or even strains within species. One of the most widely used techniques is random amplified polymorphic DNA-PCR (RAPD-PCR) (Welsh & McClelland, 1990; Williams et al., 1990), where a genome is amplified with short, randomly defined decamers, resulting in a fingerprint of the genome. By comparing fingerprints of related strains within a species, PCR products singling out one strain can be identified and sequenced. Other fingerprinting methods include amplified fragment length polymorphism-PCR (AFLP-PCR) (Vos et al., 1995; Olive & Bean, 1999), single strand conformation polymorphism-PCR (SSCP-PCR), repetitive PCR (rep-PCR)(Olive & Bean, 1999) and universally primed PCR (UP-PCR) (Bulat et al., 2000).

SCAR markers are sequence-characterised amplified regions, i.e. any regions in the genome with a known sequence (Paran & Michelmore, 1993). The function of the region does not have to be known, which makes this technique an appealing approach. Regardless of the technique used for identification of a unique sequence, the fragment can be sequenced and new primers designed. If PCR kinetics allow, the original fingerprinting primers may be elongated, but entirely new primers targeting different regions in the SCAR marker are most commonly chosen (**Paper II**). The reason for not using the shorter RAPD primers is the very reason that makes them useful in fingerprinting. Short primers can bind to and amplify target sequences that are not completely complementary, which makes the fingerprinting protocols vulnerable to even small changes in concentration, temperature and time. Longer primers increase the specificity and robustness of the PCR assay, which is needed when the strain is monitored in complex samples, such as soil (Chapon, 2003; Enkerli & Widmer, 2010).

SCAR primers can be made specific for a species, for example *Chondrostereum purpureum* (Becker et al., 1999) and *Botrytis cinerea* (Suarez et al., 2005). Strain-specific SCAR markers have also been developed for several isolates with biological control effect including *Colletotrichum coccodes* 183088 (Dauch et al., 2003), *Pichia anomala* strain K (De Clercq et al., 2003), *Pseudomonas fluorescens* EPS62e (Pujol et al., 2005), *Trichoderma atroviride* T1 (Cordier et al., 2007), *Rhizoctonia solani* AG 1-IB (Grosch et al., 2007), *Pantoea agglomerans* CPA-2 (Nunes et al., 2008), *Trichoderma atroviride*

SC1 (Savazzini et al., 2008), *Pseudomonas brassicacearum* MA250 (**Papers I** and **II**), *Hypocrea parapilulifera* IMI206039 and *Trichoderma atroviride* IMI206040 (**Paper III**).

5.5 Real-time quantitative PCR (qPCR)

In order to quantify populations of the three biocontrol strains, the use of quantitative real-time PCR was implemented in all experimental studies of the present thesis. The concept of amplifying genetic material using primers was first described in the early 1970s (Kleppe et al., 1971). This method was refined to utilise thermal cycling and a thermostable polymerase (Saiki et al., 1985; 1988), an improvement that has led to the prominent position of the technique in biological science today and to a Nobel Prize in chemistry for Kary B. Mullis in 1993.

PCR with continuous recording of product formation allows for more information than conventional end-point PCR. The key principles of real-time PCR are described in detail by Higuchi et al. (1993). The PCR generates copies of a DNA template exponentially (Ginzinger, 2002) and in real-time PCR the amplification of a target site is monitored by detection of fluorescent signal during all amplification cycles (Lee et al., 1993). When the reaction rate slows down, for example due to shortage of reagent, the reaction reaches a plateau phase. Different reactions will reach this plateau at different time points and in end-point PCR, the results from the plateau phase are analysed. In real-time PCR the analysis instead takes place in the exponential phase, which allows extrapolation to the original template concentration. A cycle threshold (Ct), where the signal is significantly higher than the background signal, is determined and the thresholds for each reaction then form the basis for calculations. With the inclusion of a standard curve with known template concentrations, the DNA content of unknown samples can be quantified.

Intercalating binding dyes such as SYBR Green, that bind to double-stranded DNA, are commonly used in qPCR (Morrison et al., 1998). This approach is very useful in amplification with so-called general primers used in **Paper IV**, but can also be used with strain-specific primers as seen in **Papers I** and **II**. In bound form the dye emits fluorescent signal, but in unbound form it does not. The method permits an evaluation of specificity, since the melting point of the resulting product can be determined and compared against the known target template.

For detection of specific targets, e.g. strain-specific detection, probe-based qPCR is more commonly employed (Heid et al., 1996) as the use of a

probe can lead to increased specificity. Hydrolysis probes, such as TaqMan® or molecular beacons, have two dyes that form a donor-acceptor pair where energy from a reporter is transferred to a quencher. During amplification, the quencher and reporter are separated and the reporter emits fluorescent light. The background noise from probe-based qPCR is theoretically smaller than in chemistries binding any double-stranded DNA, due to this specific emission of fluorescent light (Smith & Osborn, 2009).

Probe-based quantification was utilised in **Papers II**, **III** and **IV**. The use of probes enables simultaneous quantification of several markers in one sample (Loncaric et al., 2007) and this approach was used with two SCAR markers in **Paper III**. The quantification of two strains in the same samples allows for the study of the relationship between these strains and can also constitute an economical and time-saving approach (Loncaric et al., 2007).

5.5.1 DNA extraction

The extraction recovery and purity of nucleic acids from environmental samples is critical for the downstream PCR processing (Sharma et al, 2007). Some protocols employ indirect DNA extraction, where cells are first extracted from the sample matrix prior to retrieval of the DNA. Such approaches may lead to lower DNA yield but are often preferred when it is crucial that the DNA is pure and intact, such as in cloning of large inserts (Liles et al., 2008).

Direct DNA extraction, where cells are lysed within the sample matrix, is less time-consuming than indirect extraction and was selected for all experimental studies in this thesis. It is based on cell lysis followed by chemical precipitation and removal of unwanted proteins and RNA, followed by precipitation of the DNA (Bürgmann et al., 2001). Cell lysis can be obtained by mechanical disruption such as bead beating, grinding, freezing/thawing and sonication, or by chemical and enzymatic lysis, e.g. by detergents or lysozymes (Miller et al., 1999).

DNA yields from soil can be low and levels of 10% are not uncommon (Gobbin et al., 2007; **Papers I** and **II**). The extraction of DNA from soil is influenced by soil qualities, with a negative influence from clay or organic matter (Petric et al., 2011), but there are also differences in recovery between biological structures. This is perhaps most pronounced in fungi, where DNA from hyphae is more easily accessed than DNA from spores or conidia (Dodd et al., 2004; Cordier et al., 2007; Savazzini et al., 2008; 2009). If the extracted DNA is intended for quantification, estimation of population size is influenced by the efficiency of extraction of genetic material. If there are large variations in extraction of DNA for example

between structures, or if the yield is much lower than expected, population numbers can be underestimated.

To determine the recovery of DNA from environmental samples, cells or plasmids with a known target sequence can be added to and co-extracted with the DNA already present (**Papers I** and **II**). This can give an approximation of the overall DNA extraction efficiency in a specific soil or matrix. However, it is important to remember that added cells may represent a different extractability compared with resident cells in soil, which are found in a range of morphological and physiological states (Mumy & Findlay, 2004; Sharma et al., 2007).

There has been a debate on whether DNA quality or DNA yield is preferable in the extraction process. Mechanical disruption may cause substantial shearing of DNA, but can result in higher DNA yields (Sharma et al., 2007). Repeated extractions from samples can also lower the variation and increase the yield in extraction between samples and protocols (Bürgmann et al., 2001; Feinstein et al., 2009). Recently, an ISO (International Organization for Standardization) standard for soil extraction was accepted (Petric et al., 2011). The use of a standardised protocol can facilitate comparison of results between laboratories and this protocol favours DNA quality over DNA yield.

5.5.2 PCR inhibition

Another concern in amplification of genetic material is that co-extracted compounds, especially humic substances in samples from soils or sediments, may inhibit PCR (Miller et al., 1999; Opel et al., 2010). High levels of non-target DNA in the PCR can also affect the amplification negatively (Savazzini et al., 2009), along with long PCR products, secondary structures and high content of GC in the DNA (Xiang et al., 2010). In real-time PCR relatively short amplicons are often designed, which can improve the amplification efficiency.

Co-extracted substances inhibiting the PCR may be difficult to remove, but several methods for purification of extracts are available. Gel or column purification with polyvinyl polypyrrolidone (PVPP), Sephadex or polyethylene glycol (PEG) can separate DNA from inhibitors (Miller et al., 1999) but may result in DNA losses. In the PCR itself, addition of proteins (e.g. bovine serum albumin or T4 gene 32 protein) or dilution of DNA extracts can lower the impact of these compounds on amplification (Kreader, 1996). Dilution may be preferable to addition of proteins, since the latter can result in underestimation of the template in the PCR (Sharma

et al., 2007). On the other hand, dilution of DNA extracts also reduces the target numbers for PCR and may lead to problems with detection. One approach to estimate the amplification is to compare quantification of specific genes or markers against 16S rDNA data (or ITS data) amplified from the same samples (as performed in **Paper IV**). These types of controls can provide grounds for comparison between samples and serve as a quality control when many samples are below the detection limit (Gobbin et al., 2007).

The implementation of nested qPCR, where two rounds of amplification are performed, can be of use during low detection conditions (Enkerli & Widmer, 2010; Xiang et al., 2010) but makes quantification more complicated (Haff, 1994).

6 Fate and behaviour of added biocontrol strains

6.1 Development of strain-specific SCAR markers

In the present experimental study, SCAR markers for all three biocontrol strains were developed using an identical approach, which is described in detail in **Papers I** and **III** and summarised in Figure 4.

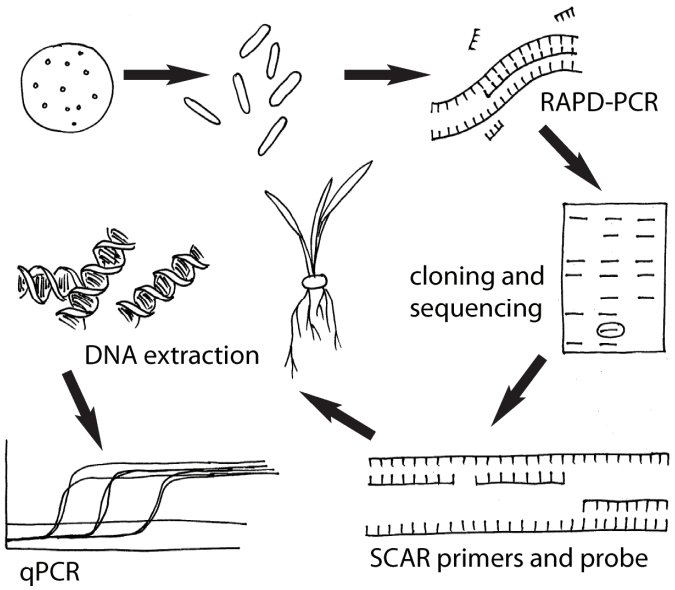

Figure 4. Flowchart diagram of a SCAR marker development process. DNA extracted from pure cultures was subjected to RAPD-PCR, cloning and sequencing to characterise a region unique to the strain. Specific primers and probe for a SCAR region were used to amplify DNA, extracted from environmental samples, in quantitative real-time PCR (qPCR).

The biocontrol strains, along with selected reference strains, were cultured and the genomic DNA was extracted and subjected to RAPD-PCR (**Papers I** and **III**). The resulting PCR products were separated by electrophoresis and unique bands, all candidates for SCAR markers, were excised, cloned and sequenced. New, longer primers based on the sequenced SCAR region were designed and these new primers were tested in qPCR to verify their specificity to the candidate strain. For strains Tp039 and Ta040 a multiplex assay was developed and optimised to avoid cross-amplification due to the combination of primers (**Paper III**).

The primers and probes were applied in qPCR to DNA extracted from environmental samples, such as winter wheat (**Papers I** and **II**), soils and golf greens (**Paper III**) and bioaerosols (**Paper IV**). The original primers developed for strain MA250 were improved further and a probe was added to increase specificity (**Paper II**). In two of the specificity tests (**Papers II** and **III**), signal from reference strains were detected. The signals from these reference strains were, however, near the detection limit of the assays and the strains would have to be present in very large populations to have an impact on the quantification.

6.2 Soil and cropping systems

Monitoring of strains in soils often begins under more controlled conditions in microcosms or in climate chamber trials, rather than in the field. When tracking populations in soils, the first step is often to use potting soil or sterilised field soil in climate chamber assays (Grosch et al., 2007; Felici et al., 2008; **Paper I**). However, it is difficult to fully determine a strain's fate and behaviour using a confined space and a sterile soil. Therefore, such determinations require field trials. The greater need for specificity has been seen in applying SCAR markers for tracking a biocontrol strain in natural soil compared with inoculated sterile soil (Rubio et al., 2005), due to the diversity of resident populations.

6.2.1 *P. brassicacearum* MA250

In the first two experimental studies of this thesis, MA250 cells were inoculated on seeds of winter wheat and tracked with the use of a SCAR marker specific for this strain (**Papers I** and **II**). Experiments were conducted in collaboration with the biocontrol company Lantmännen BioAgri (www.bioagri.se), which offered the opportunity to sample these studies. First, a climate chamber trial with commercial potting soil was sampled, followed by a full-scale field trial (Figure 5; **Paper II**). In both

studies, seeds were treated with either strain MA250 or a chemical control agent. Untreated seeds were used as controls.

Climate chamber study

In the climate chamber study (**Paper I**), the original inoculum was 10^6-10^7 cfu per seed. Roots, shoots and the remaining part of the seed were collected 11 and 27 days after sowing. On day 11, the total MA250 cell level was 10^7 copies per seedling and populations were significantly higher on seeds than on roots ($P < 0.05$). On day 27, the total MA250 cell level was 10^6 copies per seedling and populations on roots were significantly larger ($P < 0.05$) than on day 11. Strain MA250 was found on the seeds and roots at a level of approximately 10^6 cells per treated seedling during the four weeks of the trial. In shoot samples and in all control seedlings, MA250 levels were below the detection limit.

The results showed that strain MA250 colonised the roots, but not the shoots, over the course of weeks after sowing. In contrast, a couple of studies have shown that inoculated *P. fluorescens* strains can spread to and persist in the phyllosphere of the plant and outside the plant (de Leij et al., 1994; Jäderlund et al., 2008). Lack of colonisation of the phyllosphere in our case confirmed findings of a previous study with bacteria inoculated onto wheat seeds (Omer et al., 2004). The colonisation of the above-ground parts of a plant thus seems to be strain-dependent.

Field trial

In the field trial in the present study (**Paper II**), roots and the remaining part of the seed were sampled at days 38 and 221 after sowing. The field located in Tierp, approximately 60 kilometres north of Uppsala, was selected due to its previous history of snow mould and its short crop rotation with winter cereals. The field was sown in September, with the first sampling performed in November and the second in May. The trial was unfortunately terminated after the second sampling, due to severe snow mould infestation that had developed after the first sampling (Figure 5). The year of the field trial was generally very favourable for snow mould in Sweden. From the end of November to April, the field was covered with snow, which favoured this development. Because of the development of snow mould, withered seedlings were sampled in addition to healthy seedlings, after 221 days (Figure 5). The trial was arranged according to a randomised block design with four blocks in order to minimise the effects of natural variation in the field, and three of these four blocks were sampled for

analysis. From each block, three separate seedlings were collected from each treatment (untreated control, chemical control and MA250).

The original inoculation level in the field was approximately 10^9 MA250 cfu per seed. Analysis with the new probe marker showed that the total number of cells per seedling had decreased to 10^4-10^7 cells on day 38 and 10^6-10^7 cells on day 221. In the first sampling, the majority of the cells were found on the seeds with few on the roots. On day 221, however, cells were found on the roots, with levels on most MA250-treated seeds below the detection limit, which indicated root colonisation. Unlike the climate chamber trials, there was a decrease of several log units from sowing to the first sampling, whereas the cell numbers in the climate chamber trial remained stable during the whole trial.

Figure 5. Upper left: Sampled winter wheat field trial in Tierp, Sweden. Upper right: Sampling of plants in May, 2006. Lower left: Effect of snow mould infection. Lower right: Withered and healthy winter wheat seedlings in May, 2006. (Photos: Ingvar Sundh, Jens Levenfors)

A decline in populations of added strains in field trials over a period of weeks or months, as seen in **Paper II**, has also been reported in previous studies (Weller, 2007). This decline in cell numbers can in part be due to an effect of natural succession of populations, where a change in the environment, e.g. maturation of a host plant, affects the composition of the

microbial communities present. Another explanation is the sampling effect, i.e. that some cells remained in the soil at sampling and were never detected. Regardless of the decline, strain MA250 colonised the seedling, as previously demonstrated in wheat field trials with genetically modified seed-applied *Pseudomonas* species (Weller, 1983; de Leij et al., 1995; Kropp et al., 1996; Jäderlund et al., 2008).

The ability to colonise roots has been cited as a critical factor in biological control, with poor colonisation leading to inefficient control of the pest (Fromin et al., 2001; Achouak et al., 2004). Withered seedlings in the present field trial carried high cell numbers on the remaining seeds, but not on the roots (with one exception), indicating that roots had not been colonised prior to withering and that the degrading seed may have created a favourable environment for the MA250 populations. Root exudates have been shown to shape bacterial populations residing in the rhizosphere (Haichar et al., 2008), increasing the activity and survival of microbial communities (Kuiper et al., 2004) and roots of the withered plants failed in this respect. For a wider picture, it is therefore important to sample outside the plant and after the crop growing season in order to determine the potential persistence and fate of an added strain in the longer terms.

Sensitivity, specificity and resident populations

In the qPCR assays in the present study, the technical detection limit was approximately 10^3 plasmid standard copies per reaction. A low detection limit is preferable in the quantification of populations in natural environments, but poor DNA extraction recovery from soils and co-extracted inhibitors may pose problems, as discussed in the sections 'DNA extraction' and 'PCR inhibition'. In practice, this can result in high detection limits, which can be a problem if there are few added cells present. In the two winter wheat trials, the DNA extraction efficiency was estimated to range between 10% and 47%. Similar numbers have been reported by others, but higher recovery rates would be preferable. Regardless of the prospects of removing contaminants in the DNA extracts, the level of inhibition will provide information on the sensitivity of the PCR quantification. A semi-quantitative evaluation can be performed by amplifying an external template in the presence of increasing concentrations of extract (Schneider et al., 2009). In the climate chamber study, inhibition of the qPCR was not seen (**Paper I**). In the field trial, however, co-extracted compounds caused inefficient amplification in the qPCR (**Paper II**).

One potential drawback of PCR for enumeration is the quantification of DNA from dead cells. Nucleic acids are an important part of the soil phosphorous pool (Levy-Booth et al., 2007) and DNA from dead cells is often rapidly degraded and recycled in new genomes by uptake of other organisms. However, in some cases, naked DNA adheres to soil minerals and humic substances. The magnitude of this phenomenon is highly dependent on the properties of the soil, but DNA from genetically modified plants has been shown to persist in field soils for several months (Widmer et al., 1997) to years (Gebhard & Smalla, 1999).

Control wheat plants in the present field study also carried detectable populations with the SCAR marker, although cell numbers in the controls were below the detection limit in the majority of the samples. This detection was possibly due to contamination during sowing, but could also be due to the presence of indigenous populations in the field. The specificity of the SCAR markers developed in **Papers I**, **II** and **III** was evaluated against several reference strains but amplification with the markers in field samples indicated the presence of organisms carrying the target site. The amplified sequence was verified by melting point analysis in the SYBR Green assay and by sequencing of amplicons, showing that it was identical to the SCAR region. Analysis of bulk soil from the field, with the probe SCAR marker, showed no indication of indigenous populations. However, analysis of two other agricultural soils, within a radius of 120 km of Uppsala, indicated the presence of populations carrying the SCAR region at the same levels as were found on control plants in the field trial. The signal obtained with the SCAR marker for control samples from the field trial, raises the possibility that populations of MA250 or closely related strains were present in the field.

One advantage with using SCAR markers, compared to other methods, is that such resident populations in an environment can be quantified. If a reliable SCAR marker with verified specificity for a selected strain is applied, it offers valuable information on potential resident populations in an environment. Such information cannot be obtained using genetically modified strains or the cultivation-based methods available today.

6.2.2 *Trichoderma* strains Tp039 and Ta040

Turf grasses require intensive management (Nelson, 2003) and sports turf is an interesting niche for the implementation of biological control (Harman, 2000). The two biocontrol strains Tp039 and Ta040 were applied to a golf green in St. Arild, southern Sweden, in an attempt to prevent fungal disease.

Two strain-specific markers, combined in a multiplex qPCR assay, were used to track the strains after application. Experiments were performed in microcosms and in the field (**Paper III**).

Microcosm studies

In the microcosms, agricultural soil taken from Ultuna, just outside Uppsala, and soil from the golf green were used with different inoculum concentrations of the formulation, respectively. The agricultural soil was repeatedly autoclaved, and then inoculated with 10^4, 10^6 or 10^8 cfu per gram soil. The microcosms were incubated at 25°C and samples were removed immediately and up to four weeks after inoculation. Analyses were performed using cultivation-based methods and qPCR. The populations had increased by 10- to 1000- fold to 10^7-10^9 copies per gram soil between days one and four. In the highest inoculum, there was a 10- fold additional increase in both strains after two weeks of incubation, but the other populations remained at the established levels throughout the four weeks of the experiment. The higher cell numbers were seen for inoculation with 10^8 cfu per gram soil and there were no differences between the two lowest inocula. These findings confirm results obtained using other *Trichoderma* strains in sterile soil (Cordier et al., 2007). There, initial levels were similar to the inoculum levels but had increased by approximately 1000- fold after three weeks of incubation.

The viable counts for Ta040 correlated well with the qPCR up to 28 days, but for Tp039 the viable counts were lower by 10- to 100- fold than qPCR throughout the experiment. These differences between the methods can be explained by PCR inhibition or incomplete DNA extraction from conidia (Dodd et al., 2004) and other structures (Cordier et al., 2007) or by dormant cells, as discussed in the section 'DNA extraction'.

The golf green soil was autoclaved only once and inoculated with 10^4 or 10^8 cfu per gram soil. The Ta040 populations were after three days found at 10^7 copies per gram soil, regardless of original inoculation numbers, and these levels persisted throughout the nearly two weeks of incubation. This was a 10- fold decrease from the highest inoculum, but a 1000- fold increase from the lowest. Strain Tp039 increased 10- fold in six days from the lowest inoculum, but at the same time decreased 1000- fold from the highest.

The cell levels in the golf green soil determined by qPCR were largely confirmed by cfu for strain Ta040, but not for Tp039, since this strain was overgrown by other fungi on the plates. These findings were different from the sterile agricultural soil, where the inoculated cell numbers of both strains increased in numbers during the experiment, and indicated a difference in

the two soils and that Ta040 established better in this soil than Tp039. A large decline similar to Tp039, in non-sterile soil compared with sterile soil, has also been shown in studies with *Trichoderma harzianum* 1295-22 (Lo et al., 1998).

Golf green application

Strains Tp039 and Ta040 were applied once a month by spraying directly on the golf green in St. Arild, at an inoculum level of 10^3 cfu per gram soil. Samples from the golf green and outside the green were collected before and after application (from April to November). The detection limit was 10^4 qPCR copies per gram soil, and the strains were detected only sporadically shortly after application. The highest concentration of the strains during the trial was 10^5 cells per gram dry soil, but from August to December the strains were not detected at all after application. Grass clippings removed from the green were collected once and contained 10^5-10^7 cells of each strain per gram dry weight.

The efficiency of biological control largely depends on the ability of the inoculant to survive and establish in the target environment. This ability, in turn, can be influenced by the physiological state of the cells and whether they are delivered at a position favourable for growth and colonisation (Alabouvette et al., 2009). Studies have shown that added *Trichoderma* strains can persist in vineyards at high cell levels for up to one year after inoculation (Savazzini et al., 2009). The establishment of released *Trichoderma* populations can depend on the type of soil, possibly due to differences in biological activity (Cordier et al., 2007), as seen in the microcosms in the present study. There, strain Tp039 was outcompeted by Ta040 in the golf green microcosm soil, indicating that Ta040 was better equipped for release in that soil. *Trichoderma* species are generally regarded as highly adaptable, which is an advantage for colonisation of a new habitat (Schuster & Schmoll, 2010).

Other biocontrol *Trichoderma* strains have been reported to establish in soils at various population levels. *T. harzianum* 1295-22, applied to putting greens once every month, was seen to have increased 10- to 100- fold in population levels after five months of treatment, although the added strain was difficult to distinguish from resident populations (Lo et al., 1997). The biocontrol agent *Enterobacter cloacae*, which is used to control dollar spot in grass, was in another study reported to decline rapidly in the course of weeks (Nelson & Craft, 1991). It decreased from high inoculation levels (10^7-10^9 cfu per gram soil) to population levels that would remain

undetected in the present experimental study and remained there for several months.

In the present study, the fungi were applied repeatedly at a concentration where one application would hardly have been detected if the organism did not proliferate at least 10- fold. The control effect on fungal disease on the treated green was observed regardless of detection. It is possible that the *Trichoderma* strains added to the golf greens in the present study had an immediate effect on pathogens, as seen in inundation biocontrol, and then the populations quickly vanished. However, it is also possible that they colonised the turf grass to levels below the detection limit due to low growth rates, which would imply that the approach represents inoculation biocontrol.

6.3 Air exposure

Microbial control agents or other microorganisms can disseminate through bioaerosols, which consist of airborne particles of biological origin (Douwes et al., 2003). Exposure to microbial cells or particles can occur during handling of a biocontrol product, or even after application. This means that bystanders and those directly handling the product can be negatively affected, as constituents of bioaerosols are known to have effects which are sometimes deleterious to human health (Douwes et al., 2003).

The viability of microbes in bioaerosols is of minor importance for these effects, although viable cells that enter the human body have the potential to cause infections (Thorne et al., 2004). Endotoxins, peptidoglycans and $\beta(1\rightarrow3)$-glucans are examples of microbial cell components that can cause respiratory symptoms in humans (Heederik et al., 2000). Besides the clinical implications the components are all, more or less, utilised as markers for the presence of microorganisms (Heederik et al., 2000).

In order to determine the level of risk associated with use of a microorganism, and to manage this risk, it is important to determine the potential exposure associated with specific handling (Madsen, 2006a). Today, there are no international threshold limits for air exposure to microorganisms or their cell components. One reason for this is the lack of dose-response data for specific cells and cell components (Douwes et al., 2003). A major obstacle preventing the collection of such data is the lack of standardised sampling protocols (Madsen, 2006b) or of rapid and specific methods for quantification of exposure (Douwes et al., 2003; Thorne et al., 2004).

Fungal spores and Gram-negative bacteria are commonly found on for example grains (Halstensen et al., 2007) but the addition of MBCAs may increase the total microbial load to harmful levels, compared to those already present. The potential increase in exposure caused by the handling of strain MA250 was assessed in **Paper IV**, by comparing strain-specific MA250 data with the background microbial load (total aerobic bacteria and fungi, Gram-negative bacteria and *Pseudomonas*). Strain MA250 was inoculated by mechanically mixing a cell suspension with wheat seeds and the resulting cell levels were compared with a control treatment using sterile buffer solution. Analyses were also performed using pilot-scale equipment to determine the exposure to MA250 and background populations during seed-coating in this setting. Aerosol samples collected through air-filtration were analysed with cultivation-based methods (using general and selective media), qPCR (with primers targeting different groups) and endotoxin measurements.

Fume hood trial

An experimental seed-coating system, which mimicked small-scale seed coating (Wang et al., 2001), was set up in a fume hood to collect high enough levels of material for analysis. Approximately $5x10^9$ MA250 cells were inoculated onto 50 grams of wheat seeds by mechanical mixing, creating high bioaerosol levels. Air samples were collected at an air-flow rate of two litres per minute for 10, 60 and 240 minutes of seed-coating, after which the contents of the filters were eluted. The suspension was used for DNA extraction with subsequent qPCR analysis, but also for viable counts and determination of the endotoxin content.

The results from the fume hood trials showed that the cell levels of all groups and endotoxin levels analysed significantly increased in the MA250 treatment compared with the control treatment ($P < 0.01$). However, this significant increase in levels was only observed after 60 minutes of sampling and not after 240 minutes ($P > 0.05$). The data obtained from 10 minutes of seed-coating were not sufficient for statistical analysis.

Previous studies have shown that the release pattern of different microbial group is affected by the physiological state of the cells, but also the type of material which they are released from (Swan and Crook, 1998; Madsen et al., 2006c). It is possible that the added cells in the present study never adhered to the grains, but were present in aerosols already before mixing, which would have made them more accessible to sampling. The added MA250 cells may also have been more loosely attached to the seeds and

therefore more easily released than the resident populations, and this can explain the difference between coating times.

The endotoxin measurements constituted the most sensitive method for detection, and significantly reflected the addition of MA250 cells after 60 minutes ($P < 0.05$), but this method only accounts for the presence of Gram-negative bacteria and not other microbial groups (Ławniczek-Wałczyk & Górny, 2010).

The viable count data in the fume hood samples were only sporadically above the detection limit of approximately 10^3 cfu m^{-3}. However, the general trends in cfu levels were 100- to 1000- fold lower than those obtained with qPCR and this probable underestimation has also been shown in previous studies (Fallschissel et al., 2010). The most likely explanation for this poor culturability is that the background populations on the seeds were less metabolically active than the added MA250 cells in seed inoculation. The collection of cells through air-filtering, however, may also cause significant physiological stress and damage, as has been documented for *Pseudomonas* (Buttner & Stetzenbach, 1991; Wang et al., 2001).

Unlike cultivation, qPCR detected cells regardless of their physical condition, which can be important as dead and non-viable cells may also cause negative health effects (Thorne et al., 2004). MA250 was detected in all samples treated with MA250, but only in one control sample, which strengthens the hypothesis that the SCAR marker is specific for strain MA250 (**Papers I** and **II**) and also that the strain was not resident on the wheat seeds. There was no statistically significant difference ($P > 0.05$) in cell levels between the *Pseudomonas* and MA250 populations, indicating that the added strain constituted most of the total *Pseudomonas* population. The qPCR analysis with the strain-specific SCAR marker was also the only method that consistently detected added MA250 against the microbial background, regardless of coating time. Without the use of this marker, the contribution made by MA250 after 240 minutes of seed-coating would not have been quantifiable.

Pilot-scale equipment

The investigation of potentially increased exposure due to seed treatment with MA250 proceeded using pilot-scale equipment for seed inoculation. Here, approximately $5x10^9$ MA250 cells were added to 100 gram of wheat seeds, and seed-coating was performed for 90 seconds. The aerosol sampling began during the mixing and continued up to 240 minutes after seed-coating.

In the cultivation-based and qPCR-based assays, cell levels of all groups investigated were below the detection limit. The endotoxin levels were above the detection limit, but the measured levels were low during seed inoculation using the pilot-scale equipment (<0.1-6 EU m^{-3}). This indicated that the exposure levels to Gram-negative bacteria in this setting was low, which could in turn explain why there were not enough cells or DNA for detection in the two other analyses in the experiment.

7 Conclusions

The main findings of this thesis were:

- RAPD-PCR was successfully implemented for the development of SCAR markers (**Papers I** and **III**).
- *Pseudomonas brassicacearum* MA250 persisted for at least 221 days on below-ground parts of winter wheat in a field trial (**Paper II**), where it colonised the roots of healthy seedlings but not those of withered seedlings.
- *Hypocrea parapilulifera* Tp039 and *Trichoderma atroviride* Ta040 strains were detected in microcosms for several weeks after application (**Paper III**), where they behaved differently in two selected soils. The strains were only sporadically detected in the field, but regardless of this lack of detection, a control effect was reported.
- Strain MA250 caused a significant increase in total levels of Gram-negative and *Pseudomonas* populations in fume hood bioaerosols, compared with the controls, after 60 minutes of sampling but not after 240 minutes (**Paper IV**).
- The use of a strain-specific SCAR marker for enumerating MA250 in bioaerosols (**Paper IV**) enabled study of the impact caused specifically by strain MA250, whereas other methods did not. These findings highlight the need to quantify the resident microbial microflora as well as implementing specific monitoring assays.
- Endotoxin measurements from pilot-scale equipment for seed-coating indicated that exposure levels in this setting to Gram-negative bacteria (including strain MA250) was low (**Paper IV**).

- SCAR markers were valuable tools to determine the fate and behaviour of added populations of specific strains in soil and plant (**Papers I**, **II** and **III**) or aerosol (**Paper IV**) samples, but more work is needed to lower the detection limit.

7.1 Future perspectives

The use of MBCAs and other microorganisms in environmental applications can be facilitated by rapid and reliable methods for evaluating and tracking strains after release.

The markers developed in the present studies are readily available for a range of experiments with the aim of elucidating key mechanisms in their ecology and biology. Regardless of application, the present work demonstrates that the high detection levels in qPCR can be a problem and this must be addressed with regard to data collection and DNA extraction. The use of SCAR markers in exposure studies could assist in establishing relevant exposure limits that take the potential risks associated with specific strains into account.

A SCAR marker also makes evaluations of resident populations possible, as touched upon in **Papers II** and **IV**. Considering the previously established biocontrol effect of MA250, it would be interesting to conduct a larger study on indigenous populations in order to determine whether it is a common inhabitant of agricultural soils or other environments.

The multiplex assay with the SCAR markers for strains Tp039 and Ta040 could be of interest in the investigation of the interaction between the strains, since they are used in one formulation. The biocontrol mechanisms of the two strains are unknown, but they are believed to complement each other with respect to target pests and control mechanisms. Simultaneous quantitative detection of the two strains in the field and under controlled conditions could potentially shed more light on this.

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Those who contemplate the beauty of the earth find reserves of strength that will endure as long as life lasts

Rachel Carson

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