Chapter 8

Summary and General Discussion
Introduction

Measures for preventing and monitoring plant diseases are among the major constituents of pest management in agriculture. Early detection of the phytopathogen can help to predict a disease outbreak and consequently allow the grower to choose a suitable defense strategy. Due to large progress in methods in molecular biology, the monitoring of phytopathogens underwent many improvements during the past two decades. Modern techniques based on DNA amplification can detect negligible amounts of a target organism and can deliver results within a few hours (Lievens and Thomma, 2005). This fast analysis of the disease status gives the time required to define what measure or which pesticide has to be applied to stop or delay disease development.

Since the use of chemical pesticides in agriculture has become a great concern to society, biopesticides have become popular as alternative tools for chemicals to combat plant diseases. Biopreparations, which do not cause a threat to the environment and do not accumulate as toxic residues in end products, are attractive substitutions for chemicals (Cross and Polonenko, 1996; Montesinos, 2003; Lugtenberg and Bloemberg, 2004). Although the advantages of biological control are obvious, there are doubts with respect to the efficacy of biological pesticides (Fravel, 1999). Indeed, strongly varying conditions in the field can influence the expression of many biocontrol traits (Lee and Cooksey, 2000; Tomashow and Weller, 1996; Duffy and Défago, 1997, van Rij et al., 2005) and consequently cause variation in efficacy of biopreparations. Perhaps, that is why many of the over one hundred microbial biocontrol products, which have been marketed (Koch, 2001), are not as successful as chemicals are.

It is generally agreed that biopesticides show a more stable control of diseases in the greenhouse than in the field because under the former circumstances conditions can be controlled better (Paulitz and Belanger, 2006). In fact, the introduction of new substrates such as stonewool created a highly controllable environment, resulting in a higher crop yield. New stonewool is practically free of phytopathogens, which is an advantage. On the other hand, the problem of fungal
infections is still big in stonewool. Fungi can spread through air and water (Brayford, 1996; Postma et al., 2000) or can be introduced by plants. Being practically sterile, new stonewool does not suppress incoming phytopathogens (Postma et al., 2000; Folman et al., 2001). This makes plants growing in stonewool highly vulnerable to infection. On the other hand, used stonewool on which healthy plants have been growing have a buffering microflora which makes life for incoming pathogens difficult (Postma et al., 2000). Since many chemical pesticides are banned for application in greenhouses, the demand for alternatives to control phytopathogens is high. Therefore biopesticides are used more and more as plant protection tools in greenhouses. They are safe and can be effective.

**Bacteria able to control *Folr* in potting soil and stonewool can be selected from rhizosphere samples using new enrichment procedure (Chapter 2 and Chapter 3)**

In theory it is conceivable that super-colonizing bacteria, isolated by the enrichment procedure described in chapter 2 (Fig.1), can act as biocontrol agents using the mechanism “competition for nutrients and niches”, since these can be expected to colonize the rhizosphere better than phytopathogens do (Lugtenberg et al., 2001). In chapters 2 and 3 we describe the isolation, by enrichment for super-colonisers, of bacteria controlling *Folr*, presumably through the mechanism "competition for nutrients and niches". By this method, enhanced colonizers are selected from a mixture of rhizosphere bacteria using the plant root as a selective tool (Chapter 2, Fig. 1; Chapter 3, Fig. 1). This selection method is the first one which allows selection of biocontrol bacteria. So far, antagonistic biocontrol bacteria could only be isolated by much more labour-intensive screening methods. In the case of biocontrol bacteria acting through antagonism, a rather simple pre-screening for antagonistic strains can be is used prior to biocontrol tests. This is why many biocontrol products act through antagonism. However, in the case when induction of systemic resistance is the mechanism, no selection or pre-screening is possible.

Enrichment for competitive tomato root tip colonizers from crude rhizosphere samples, which presumably consists of thousands of different strains, effectively
caused the accumulation of strains with excellent colonizing abilities at the root tip. This could result in the presence of only one or a few strains at the root tip, with a serious risk of siblings (e.g. clones of the same strain). This is what was observed indeed (Chapters 2 and 3). We developed methods for the fast detection of siblings (Chapter 3). Elimination of siblings from our collection before starting the labour-intensive screening tests for competitive colonization and biocontrol ability reduced the amount of work considerably (Chapter 3).

Among the enhanced colonizers were also potentially animal and plant pathogenic bacterial species. This finding confirms results from an earlier report that pathogens have to colonize their targets and that therefore features of pathogenic microorganisms frequently include good colonizing abilities as well (Berg et al., 2005). Indeed, many pathogens can be found in the rhizosphere and they sometimes can control plant diseases (Bano and Musarrat, 2003; Berg et al., 2005; Egamberdiyeva et al., in press). Since application of such microbes should not be allowed in agriculture for reasons of biosafety (Anonymous, 1999), it was important to detect them, and to do this in an early stage to prevent unnecessary work. Identification using 16S rDNA analysis of our isolates, and comparison with known potential pathogens found in the rhizosphere, allowed us to recognize the pathogens and eliminate them from further experiments (Validov et al., 2007).

The ability to produce antibiotics is a disadvantage for registration of a biopesticide product. It appeared that from the thirteen biocontrol strains isolated by enrichment, only three strains produce AFMs (anti-fungal metabolites). Apparently, and as expected, the applied enrichment procedure does not select for antagonistic strains (Chapter 2; Chapter 3).

Enrichment for competitive tomato root tip colonizers is suitable for the isolation of strains controlling TFRR using the mechanism “competition for nutrients and niches” (Kamilova et al., 2005, Chapter 2). The enrichment procedure can be modified for the isolation of bacteria suited to act well in a certain substrate (for example stonewool) and/or for bacteria which can survive certain formulation treatments (Validov et al., 2007, Chapter 3). We believe that the application of the methods described in chapters 2 and 3 will make the isolation of non-pathogenic,
non-antagonistic biocontrol bacteria a routine job. Exploitation of this method can lead to the fast isolation of biocontrol bacteria which originate from the indigenous microflora of the geographical region where they are planned to be applied for plant protection.

**Super colonizers can protect plants using the mechanism “competition for nutrients (and niches)” (Chapters 2 and 4)**

Four mechanisms are known for the biocontrol of fungal phytopathogens: (i) antibiosis, (ii) induction of systemic resistance (ISR), (iii) predation and parasitism and (iv) competition for nutrients and niches (CNN). Many of the described biocontrol agents combine mechanisms to protect plants against infection by a fungus. For example, the traits (i) production of AFMs and (ii) efficient colonization using the trait motility are both important for the biocontrol activity of *P. chlororaphis* strain PCL1391 (Chin-A-Woeng et al., 1998; Chin-A-Woeng et al., 2000) and of *P. fluorescens* strain Q8r1-96 (Mavrodi et al., 2002). *P. chlororaphis* O6 apparently needs its colonization ability to induce full scale systemic resistance in tobacco (Num et al., 2006). In contrast, biocontrol agent *P. fluorescens* strain WCS365, which triggers ISR (Kamilova et al., 2005), is such an aggressive colonizer that colonization of the whole root system is not a requirement for good biocontrol (Dekkers, 1997). *Trichoderma* strains, which control *Fori* using the mechanism predation and parasitism, are good root colonizers as well (Bolwerk et al., 2005).

In these examples an excellent colonizing ability, which is the basis for the mechanism CNN, is considered as a delivery tool, which helps the biocontrol agents to colonize the root niche and to develop sufficient biomass for biocontrol or for reaching the target prey. We showed that bacterial strains *P. fluorescens* PCL1751 (Chapter 2) and *P. putida* PCL1760 (Chapter 4) can control TFRR using their very high colonizing abilities. These strains efficiently colonize the rhizosphere and efficiently consume nutrients. Aggressive consumption of the nutrients by the biocontrol bacteria deprives *Fori* from the carbon sources that it needs for its proliferation (Fig. 4 in chapter 4) and also could also prevent targeted growth of the
fungus towards the plant root, since chemoattractants are consumed by the biocontrol agent.

Interestingly, the biocontrol efficacy in stonewool, in which strains were introduced as a cell suspension in plant nutrient solution, did not depend significantly on the motility trait of the biocontrol agent (Chapter 4). Apparently, conditions of the stonewool and the way the biocontrol strain was introduced provide the required colonization level (Fig. 5 in Chapter 4). Therefore, it may be possible that even strains with reduced motility can control TFRR in stonewool based on the property that they can consume carbon sources in tomato root exudate faster than Forlan (Fig. 4, Chapter 4).

Identification of the mechanism used for biocontrol is required for registration as a product. In the case of antagonistic strains, AFMs can be toxic to humans and useful organisms, or cause allergic reactions (Skrobek et al., 2006). Those non-pathogenic microorganisms, which protect plants using the mechanism CNN, do not have these disadvantages. This facilitates the registration procedure for these strains for application as a product in agriculture.

Abundance of mobile elements and redundancy of fungal genomes provide genetic flexibility for these organisms (Teunissen et al., 2003). This allows fungal phytopathogens to develop resistance to chemical fungicides. Similarly, races of pathogens resistant to a toxic compound produced by a biocontrol agent or fungi avoiding the defense system of the plant, can appear. Moreover, some fungi produce compounds which inhibit production of the secondary metabolites needed by the biocontrol agent to control the pathogens (van Rij et al., 2005) or even metabolize antifungal compounds (Schouten et al., 2004). All these mechanisms decrease the efficacy of biocontrol based on antibiosis. Since root colonization abilities and fitness in the rhizosphere are mediated by perfectly balanced gene activities, fast and substantial improvement of colonization for a phytopathogen is unlikely. Therefore it is difficult to imagine that phytopathogens would become resistant to the action of bacteria using the mechanism CNN. Thus, biopesticide products based on bacteria using CNN may become the ideal effective and safe tools for sustainable biocontrol.
Diversity and monitoring of pathogenic strains of *Fusarium oxysporum* (Chapter 5 and Chapter 6)

*Fusarium oxysporum* (Fox) is a cosmopolitan species represented by both phytopathogenic and nonpathogenic strains (Burgess 1981). Pathogenic strains are divided into more than 150 special forms (formae speciales) which specifically parasitize certain plant species (Baayen et al., 2001). For growers it is important to determine as early as possible which pathogen is present. Since both pathogenic and non-pathogenic strains of Fox are ubiquitous and are able to colonize plants, Fox DNA, detected in and on plant material, is not the ultimate proof for an infection with a pathogen which will cause damage to the plant (Chamber 6). Therefore monitoring tools able to distinguish pathogenic and non-pathogenic Fox are required. For the majority of the formae speciales, genetic determinants for pathogenicity, which could be used for monitoring, are not known. Multi-copy ribosomal gene sequences could be convenient targets for sensitive monitoring of these pathogens, provided that both conserved and non-conserved regions exists in the formae speciales. We compared 33 Fox strains from seven different formae speciales using three criteria: (i) pathogenicity towards tomato and cucumber, (ii) vegetative compatibility and (iii) nucleotide sequence of the intergenic spacer region (IGS). The results obtained show that four out seven formae speciales are heterogeneous. Interestingly, even representatives of the same vegetative compatibility group can greatly differ in the sequence of such a conservative region as IGS is supposed to be. This observed genetic heterogeneity does not support the notion that pathogenic Fox strains of certain forma specialis, and even of some VCGs, descend from a single ancestor (Chapter 5).

All 33 tested strains were able to colonize tomato and cucumber plants endophytically (Chapter 5), but only Fox f. sp. *radicis-lycopersici* (Forl) and Fox f. sp. *radicis-cucumerinum* (Forc) produced foot and root rot on tomato and cucumber plants, respectively. To identify proposed differences between pathogenic and non-pathogenic Fox strains, we followed the colonization of tomato by strains Fox f. sp. *radicis-lycopersici* ZUM2407 (a tomato foot and root rot pathogen), Fox f. sp. *lycopersici* 004 (causing tomato wilt), Fox f. sp. *radicis-cucumerinum* V03-2g (a
cucumber root rot pathogen) and Fox Fo47 (a well known non-pathogenic biocontrol strain). We determined fungal DNA concentrations in tomato plantlets by quantitative PCR (qPCR) with primers complementary to the Intergenic Spacer region (IGS) of these four Fox strains. The found fungal DNA concentration, as determined by qPCR, appeared to be in good agreement with data of the score of visible disease symptoms of tomato foot and root rot obtained 3 weeks after incubation of tomato with Forl ZUM2407. Our results show that targeting of the multicopy ribosomal operon gives highly sensitive qPCR reaction for the detection of Fox DNA. The observed difference in plant colonization between pathogenic and non-pathogenic strains revealed by our results strongly indicates that, when a concentration of Fox DNA in plant material above 50 fg per ng of total plant DNA (e.g. ≥0.005%) is present, this is due to proliferation of a pathogenic Fox (Chapter 6).

**Biocontrol of tomato foot and root rot under industrial conditions (Chapter 7)**

*Pseudomonas putida* strain PCL1760 is an enhanced colonizer isolated using enrichment for competitive tomato root tip colonization (Chapter 3). Acting through the mechanism “competition for nutrients and niches”, strain PCL1760 shows stable TFRR suppression under laboratory conditions (Chapter 3; Chapter 4). *Pseudomonas putida* is a species which has never been reported to have representatives which are pathogenic for animal or for plants. This species is therefore allowed for application in agriculture (Anonymous 1999). *In vitro*, strain PCL1760 does not produce AFMs or enzymes inhibiting the pathogen (Chapter 4). The absence of secreted toxic compounds facilitates its registration as a biocontrol agent. *P. putida* strain PCL1760 perfectly obeys the requirements for biosafety.

To test the efficacy of PCL1760 for plant protection under industrial conditions, an experiment to control TFRR was carried out in a certified greenhouse (PPO, Bleiswijk, The Netherlands). The results of the trial showed that *P. putida* strain PCL1760 controls TFRR in stonewool significantly and decreases the negative
influence of the pathogen on seed germination and on growth of the tomato plants (Chapter 7).

PCR quantification revealed a significantly lower amount of Forl DNA in tomato plant tissue in the variants, in which the biocontrol agent P. putida PCL1760 was added (Fig. 1D, Chapter 7). Mycostop®Biofungicide (Kemira Agro OY, Helsinki, Finland), a biopesticide recommended for application against Fusarium in greenhouses, did not control TFRR.

<table>
<thead>
<tr>
<th>Diseased tomato plants (%)</th>
<th>Forl (Negative control)</th>
<th>Forl + PCL1760</th>
<th>Reduction of TFRR symptoms (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18.7</td>
<td>8.3</td>
<td></td>
<td>55</td>
</tr>
<tr>
<td>29.9</td>
<td>13.7</td>
<td></td>
<td>54</td>
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<tr>
<td>39.8</td>
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<td></td>
<td>56</td>
</tr>
<tr>
<td>53.3</td>
<td>25.9</td>
<td></td>
<td>51</td>
</tr>
<tr>
<td>62.2</td>
<td>10.3</td>
<td></td>
<td>83</td>
</tr>
<tr>
<td>62.4</td>
<td>28.6</td>
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<td>54</td>
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<tr>
<td>62.5*</td>
<td>46*</td>
<td></td>
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</tr>
<tr>
<td>62.6</td>
<td>18.1</td>
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<tr>
<td>72</td>
<td>38.9</td>
<td></td>
<td>45</td>
</tr>
</tbody>
</table>

Differences in disease level among treatments were determined by analysis of variance (ANOVA) and mean comparisons were performed by Fisher’s least-significant-difference test (α = 0.05).

*Only the difference between the variants in this trial was not significant.

TFRR reduction was counted as percentage of disease decrease. The result of the negative control was counted as 100% disease occurrence.

The laboratory conditions we used for biocontrol assays are close to the conditions of the industrial trial: the same stonewool substrate, the same cultivar (Carmello) of tomato plants, and the same pathogen Forl ZUM2407 was used (Chapter 3, Chapter 4). The laboratory conditions we used for biocontrol assays are close to the conditions of the industrial trial: the same stonewool substrate, the
same cultivar (Carmello) of tomato plants, and the same pathogen *F. oxysporum* was used (Chapter 3, Chapter 4). *P. putida* strain PCL1760 controlled the disease reproducibly and efficiently (> 50% reduction of TFRR) in many trials under laboratory conditions (Table 1) under different levels of disease pressure. The results of the trial under industrial conditions show that *P. putida* strain PCL1760 can efficiently control TFRR in the greenhouse (Chapter 7). This makes PCL1760 an attractive candidate for industrial application in stonewool substrate.

**Concluding remarks**

The research described in this thesis shows that the enrichment technique based on competitive root tip colonization allows the isolation of bacteria which can protect plants from TFRR through the mechanism competition for nutrients (and niches) (Chapters 2, 3 and 4). The efficacy of biocontrol of one of these strains, *P. putida* PCL1760, was estimated under industrial conditions using both conventional and molecular techniques (Chapter 7). Quantitative PCR was used also to distinguish between different forms of *Fusarium oxysporum* (*Fox*), which cannot be distinguished using otherwise so far (Chapter 5). It was shown that nonpathogenic *Fox* strains in plant material cannot reach a concentration higher than 50 fg of fungal DNA per ng of total DNA (Chapter 6).

The obtained results are an illustration of an efficient strategy, which can be exploited for the isolation of biocontrol agents for greenhouses and of the application of fast molecular methods for the estimation of their efficacy in plant protection. Moreover, this molecular method a quantitative PCR can be used to monitor pathogenic strains in tomato.