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"Biocontrol of tomato foot and root rot by *Pseudomonas* bacteria in stonewool"
by Shamil Validov

Мне кажется, нет никаких оснований
Гордиться своей судьбой.
Но если б я мог выбирать себя,
Я снова бы стал собой.

*25 к 10, БГ**

посвящается всем кого я люблю:
моим родителям, родным и друзьям**

***** Although I don't see any reason
To be proud of how I dwelt
If my fate was again to be chosen
I would rather become myself

25 to 10, BG

** To people I love:
my parents, relatives and friends

Contents

List of abbreviations

AFM	antifungal metabolites
ARDRA	amplified ribosomal DNA restriction analysis
CFU	colony forming units
CNN	competition for nutrients and niches
Forl	*Fusarium oxysporum* f. sp. *radicis-lycopersici*
Fox	*Fusarium oxysporum*
IAA	indole-3-acetic acid
ISR	induced systemic resistance
(N-)AHL	(*N*-)acyl-L-homoserine lactone
PBS	phosphate buffered saline
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RFLP	restriction fragment length polymorphism
rpm	rotations per minute
RT-PCR	real-time polymerase chain reaction
SAR	systemic acquired resistance
TFRR	tomato foot and root rot
VCG	vegetative compatibility group

Chapter 1

General Introduction

Introduction

Plant diseases have become a permanent threat since human societies started to rely on agriculture as on a major food provider. Back in history, outbreaks of plant diseases resulted in human catastrophes. For example, the Great Potato Famine killed hundreds of thousands of Irish people and forced the emigration to the USA in 1845-1846. Similarly, an epidemic of brown spot rice was the cause of a devastating famine in India in 1943 (Bent 2003). Even nowadays the crop loss due to phytopathogens is still a serious economical problem in agriculture. It is estimated to cause a 15-20 % reduction of the crop yield worldwide (www.apsnet.org). Diseases caused by fungi are the major threat to plants. Out of a million known species, only eight thousand fungi are phytopathogenic (Bent 2003). Fungal pathogens are important not only because they reduce crop yield, but also due to certain compounds they produce during proliferation on/in plants. These compounds, called mycotoxins, are highly poisonous and can adversely affect human and animal health (Pier 1981; Pitt 2000).

1. Pest management

Strategies of pest management were known by humankind in high antiquity. Crop rotation, which breaks life-cycles of soilborne phytopathogens and reduces their build-up, was already mentioned in the Roman literature, and referred to by great civilizations in Africa and Asia. From the medieval time until the 20th century, a three-year rotation was practiced by farmers in Europe: rye or winter wheat, followed by spring oats or barley, and finally letting the soil rest (fallow) during the third stage.

Selection of cultivars which are resistant to certain pathogens happened at farms throughout the history of agriculture. The scientific background for this selection and, the possibility of resistance breeding was discovered in 1905 by the British scientist R.H. Biffen. It was shown that wheat resistance to rust disease was

controlled by a single gene (Biffen 1905). Soon after this, it has been revealed that resistance against many plant diseases is controlled by single genes. Resistance breeding, like many other plant protection methods, has often a temporary nature: it breaks down due to appearance of new strains of the pathogen. Disadvantages of breeding for resistance include a loss of fittness of the plant, and the fact that resistance is not always based on single gene (Robinson 1997).

Another old method is exploitation of solar energy for controlling disease agents in soil and in plant material. This was already used in the ancient India. Crop rotation, resistance breeding and soil solarization are presently still used as simple, effitient and environmentally friendly procedures of plant disease control (Katan et al., 1987).

1.1 Chemical pesticides for crop protection

Crop protection in modern agriculture heavily depends on chemical fungicides. Being extensively used in 1950s -1970s, they seemed to be a final solution against many plant diseases. Disadvantages of chemical pesticides soon became apparent as damage to the environment and a hazard to human health. Moreover, it results in emergence of pesticide-resistant races of the pathogens. Extensive use of pesticides caused a pollution problem in agricultural regions. For example, in 1987 it appeared that surface water in a greenhouse area in The Netherlands must be diluted thirty times before water-flea could survive in it (Working group 1988). Similarly, stable halogen-organic pesticides can be found now in ecosystems far away from the sites where they were applied (Curwin et al., 2005). Due to growing concerns on the negative impact of chemicals, the use of these pesticides is being restricted: more than half of the chemical pesticides used in 1996 were banned in 2003 in the European Union (EU).

The strong reduction of the number of agrochemicals increased the need for alternative plant protection measures. Although genetically engineered pathogen-resistant plants are promising, the European politicians are reluctant or negative

about such products, and genetically modified plants still receive quite a negative public perception.

1.2 Biological control of plant diseases

The use of wild type microbes has become the promising alternative for replacing chemicals or, at least, reducing their use. Over one hundred microbial biocontrol products have been marketed (e.g. Koch, 2001) but their success is variable. This is presumably due to strongly varying conditions in the field since the expression of many biocontrol traits is strongly influenced by biotic (Lee and Cooksey, 2000; Smith et al., 1999) and abiotic (Schnider-Keel et al., 2000; Tomashow and Weller, 1996; Duffy and Défago, 1997, van Rij et al., 2005) conditions. Indeed, it is generally agreed that biocontrol products are more successful under the better controlled greenhouse conditions than in the open field (Paulitz and Bélanger, 2001).

Biosafety of microbial products is a great concern of the society. In fact, many human/animal and plant pathogens can be found among microorganisms which are able to control plant diseases (Bano and Musarrat 2003; Chiarini et al., 2006; Mari et al., 2003). However, the increase of the number of these microorganisms in the environment and even more so their presence in food or forage is highly undesirable. The regulations for microorganisms which can be used as biopesticides vary among countries. According to EU rules, species which have a pathogenic representative cannot be used in agriculture (Anonymous 1998). The United States has less strict requirements. Therefore preparations based on non-pathogenic strains, which belong to species harbouring pathogenic representatives, can be found among commercial biocontrol products (BioFox C, Bio-Save 10, Blue Circle and PSSOL in Table 1).

High efficacy and biosafety are not the only requirements for a biopreparation to be commercialized. Since the majority of biocontrol products must contain live microorganisms (Table 1), methods to preserve them and facilitate their application on the target are important. These methods are known as formulations, e.g.

measures for preservation, delivering to the target and, in some cases, improving the activity of a biocontrol agent. Formulation affects many aspects of a biocontrol organism, including shelf-life and the ability to survive and proliferate in the environment of the target to control the disease. There are many types of formulations, but they all can be divided in seed treatment, wettable powder, liquid and granulation (Jones and Burges 1998). The choice of an appropriate formulation technique depends upon both the biology of the biocontrol organism as well as on peculiarities of the biocontrol process. Fungal strains have been formulated in many different ways (see Table 1) due to robustness and resistance of their spores to drying. Endospore-forming bacteria, such a *Bacillus spp.*, are more suitable for formulation than strains which only exist in the vegetative stage. Dried spores of bacilli can be kept alive for decades at ambient temperature. Spores resist high temperature treatments and can be effectively formulated using spray-drying. Pseudomonads and other Gram-negative bacteria can also be dried; in this case freeze-drying should be applied. These bacteria can be stored as long as bacilli spores as a lyophilizate with no access of oxygen. Freeze-drying is an expensive treatment. Therefore biocontrol products, based on non-sporulating bacteria are frequently marketed as an aqueous suspension of fermenter biomass supplemented by carriers (Table 1).

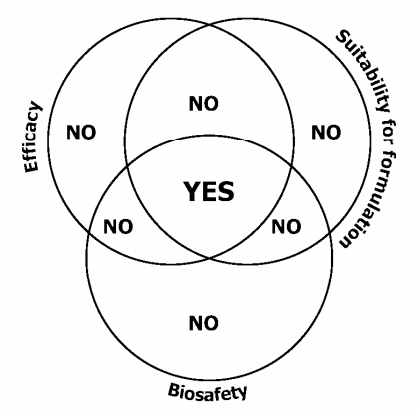

Fig.1 Important criteria for the choice of microorganism meant to be used as a biocontrol preparation

The decision whether a biocontrol strain will be scaled-up and taken in industrial production depends to a great extent on the following characteristics: market size, cost, efficacy, biosafety and the possibility to formulate the biocontrol agent (Fig. 1). Efficacy and suitability for formulation can compensate each other to a certain extent. For example, one of the successful commercial biocontrol products, BlightBan A506 (*Pseudomonas fluorescens*) is supplied as a wettable powder (lyophilizate). Due to its high biocontrol efficacy

against *Erwinia amylovora* (Wilson and Lindow 1993; www.ag.us.nufarm.com), the cost of freeze-drying can be tolerated. An opposite case is a production of biocontrol products based on endo-spore forming bacteria: Kodiak-AT is recommended to apply in combination with chemical fungicides (Jones and Burges 1998), so the biocontrol efficacy of this bacillus is not high. Nevertheless, the low price of the formulation of this strain and the long shelflife of the spores make this commercial product successful (Table 1). Biosafety is a rigorous requirement. Neither high biocontrol efficacy nor ease of formulation are sufficient to allow a pathogenic strain into agricultural practice. Moreover, an application permission of biocontrol products can be revoked, if pathogenisity of their strains have been discovered. An example of it is the fate of biocontrol products based on *Burkholderia cepacia,* which were banned in 2004 after years of successful application (http://www.epa.gov/fedrgstr/EPA-PEST/2004/September/Day-29/p21695.htm).

2. Mechanisms of Biocontrol

The phenomenon of biocontrol by microbes was discovered 70 years ago when studies with suppressive soils were carried out (Baker and Snyder 1965). Some agricultural regions, for instance Salinas Valley (California, USA), the Chateaurenard area (France), the Canary Islands and the Broye Valley (Switzerland) have fields in which agricultural plants do not suffer from the effect of pathogens, although phytopathogenic microorganisms are present in the soil. It was shown that the ability of this kind of soils to suppress pathogens is due to an activity of microorganisms. Elimination of these microorganisms using pasteurization or γ-irradiation makes this soil conducive, i.e. allows the development of the disease (Cook and Rovira, 1976; Scher and Baker, 1980). Moreover suppressivenes can be transferred. If at least 0.1% of a suppressive soil is introduced into a conductive soil, the latter soil can become disease suppressive (Shipton et al., 1973).

An explanation for the way biocontrol microorganisms can inhibit pathogens came from the notion that many soil bacteria can produce antifungal metabolites *in vitro* (Baker and Snyder 1965). Nowadays four mechanisms, which can mediate

biocontrol, are generally recognized: (i) antibiosis, (ii) induction of systemic resistance, (iii) predation and parasitism, and (iv) competition for nutrients and niches.

2.1 Antibiosis

Historically, the antibiosis is the first revealed mechanism of biocontrol and, according to the opinion of some scientists, it is the most efficient one (Haas and Defago 2005). Many rhizosphere bacteria produce secondary metabolites – small organic molecules that inhibit the growth of other microorganisms. The biological role of the production of these compounds is in providing an advantage in colonization of the plant rhizosphere by elimination of competitive microorganisms from the niche (Ligon et al., 2000). Involvement of secondary metabolites in disease suppression was demonstrated by using mutants of *P. fluorescens* CHA0 (Keel et al., 1992) and *P. chlororaphis* PCL1391 (Chin-A-Woeng et al., 1998) impaired in the biosynthesis of 2,4-diacetyl phlorogucinol and phenazine-1-carboxoamide, respectively, which suppressed black root rot of tobacco and foot root rot of tomato, respectively, to a significantly lesser extent than the wild type strains.

2.2. Induction of Systemic Resistance

After interaction with a necrotizing pathogen or with biocontrol bacteria, plants can establish an immunity state that protects them partially or completely from subsequent phytopathogen attacks. Necrotizing pathogens trigger the developing of systemic acquired resistance (SAR) which leads to programmed death of the plant cells near the site of pathogen penetration (van Loon et al., 1998). Proteolytic enzymes and reactive oxygen species that are released from the dying plant cells can kill the pathogens and stop further infection. Salicylic acid accumulates and starts SAR by inducing of production of pathogenesis-related (PR) proteins. Therefore SAR is also called the salicylic acid pathway (Hunt et al., 1996).

Table 1. Examples of commercial biocontrol products for use against soilborne crop diseases [a]

Product biocontrol organism	**Target pathogen**	**Crop**	**Formulation**
BioFox C *Fusarium oxysporum* (non-pathogenic)	*Fusarium oxysporum* *Fusarium molineforme*	Basil, carnation, cyclamen, tomato	Dust or alginate granules
Bio-Fungus *Trichoderma spp.*	*Sclerotinia, Phytophthora, Rhyzoctonia solani, Pythium spp., Fusarium, Verticillum*	Flowers, strawberries, trees, vegetables	Granules, wettable powder, sticks and crumbles
Bio-Save 10 *Pseudomonas syringae*	*Botrytis cinerea, Penicillium spp., Mucor pyroformis, Geotrichum candidum*	Citrus, pome fruit (postharvest disease control)	Wettable powder
BlightBan A506 *Pseudomonas fluorescens*	Frost, *Erwinia amylovora*	Almond, apple, cherry, peach, potato, strawberry,	Wettable powder
Victus *Pseudomonas fluorescens*	*Pseudomonas tolassii*	Mushrooms	Aqueous biomass suspension
Blue Circle *Burkholderia cepacia*	*Fusarium, Pythium*, lesions, spiral, lance, and sting nematodes	Vegetables	Peat carrier or liquid
Ateze *Pseudomonas chlororaphis* 6328	*Pythium, Rhizoctonia, Cylindrocladium, Fusarium*	Pea, ornamentals, cucumber	Powder
Kodiak A-T *Bacillus subtilis*	*Rhizoctonia solani, Fusarium spp., Alternaria spp., Aspergillus spp*	Cotton, legumes	Dry powder (5.5×10^{10} spores/ g) Applied with chemical fungicides
Mycostop *Streptomyces griseoviridis*	*Fusarium spp., Alternaria brassicicola, Phomopsis spp., Botrytis spp., Pythium spp.*	Field, ornamental and vegetable crops	Powder
PSSOL *Pseudomonas solanecearum* (non-pathogenic)	*Pseudomonas solanecearum*	Vegetable	Aqueous biomass suspension
Galltrol-A *Agrobacterium radiobacter*	Crown gall disease, *Agrobacterium tumefaciens*	Fruit, nut and ornamental nursery stock	Petry dishes with pure culture grown on agar (1.2×10^{11} CFU/plate)

a) Jones and Burges 1998, Lugtenberg and Kamilova 2004, http://www.genoeg.net

The plant defence mechanism induced by non-pathogenic bacteria or non-compatible pathogens is known as induced systemic resistance (ISR). ISR was observed in cucumber and tomato against mosaic virus (Raupach et al., 1996), in carnation (Duffy et al., 1983) and in tomato (Kroon, 1990) against *Fusarium oxysporum*, and in *Arabidopsis thaliana* against *P. syringae* pv. *tomato* DC3000 (Zipfel et al., 2004). It was shown that components of bacterial cells such as flagella (Zipfel et al., 2004), lipopolysaccharides (van Loon et al., 1998; Desaki et al., 2006), siderophores (Audenaert et al., 2002), some secondary metabolites such as 2,4 diacetyl phloroglucinol (Iavicoli et al., 2003) and N-acyl-L-homoserine lactone (Schuhegger et al., 2006) and even bacterial cytoplasmic proteins (Kunze et al., 2004) can trigger ISR.

2.3. Predation and parasitism

Some beneficial microorganisms can attack cells of fungal phytopathogens directly by producing lytic enzymes such as chitinases (Carsolio et al., 1994), β(1,3)-glucanases (Lorito et al., 1996), lipases and proteases, which are able to degrade fungal cell wall compounds. This results in destruction of the pathogen, and products of fungal cell degradation can be consumed by the beneficial microorganism. The best known examples of the microorganisms using parasitism and predation as a mechanism are *Trichoderma spp.* (Lorito et al., 1996; Bolwerk, 2005)

2.4. Competition for nutrients and niches

The rhizosphere contains substances such as organic acids, sugars and vitamins, which are exuded from the roots and they are the most important nutrients for the rhizosphere microbes. The mechanism of "competition for nutrients and niches" (CNN) is based on the ability of a biocontrol agent to consume nutrients and to occupy the sites on the roots before the pathogen arrives there. (Lugtenberg and Dekkers, 1999). For the first time CNN was shown to be a sole mechanism for

biocontrol of *Fusarium* wilt of carnation by nonpathogenic *F. oxysporum* strain 618-12. In carnation, treatment with strain 618-12 decreased disease incidence by 80% (Postma and Luttikholt, 1996). Another example, non-pathogenic *F. oxysporum* strain Fo47 suppresses disease only when it is introduced in concentration 10 – 100 higher than the pathogenic *F. oxysporum* f. sp. *radicis-lycopersici* ZUM2407 (Bolwerk et al., 2005). This initial numerical superiority gives Fo47 an advantage to colonize the tomato root faster than the pathogen does and makes this strain capable of disease suppression. Logically, efficient root colonization is an essential characteristic of any biocontrol agent acting through CNN.

So far, CNN is studied for fungus-fungus interaction. One of the strains *F. oxysporum* Fo47 is marketed already in several countries (Paulitz and and Bélanger, 2001).

2.4.1. Exudate consumption

Plants secrete 5% to 21% of all photosynthetically fixed carbon into the rhizosphere as root exudate (Marschner, 1995). Root exudation depends on the substrate in which the plant is growing and it can be altered by microorganisms (Walker et al., 2004). A recent study on root exudation of plants growing on stonewool showed that the root exudates of cucumber, tomato and sweet pepper are similar in composition. Citric, succinic, and malic acids represent the major organic acids, whereas fructose and glucose are the major sugars. Exudation of both organic acids and sugars increases during plant growth. Organic acids represent the major fraction of utilizable carbon, their amounts were considerably higher than those of sugars (Kamilova 2006a).

Efficient consumption of root exudate is an important characteristic of good root colonizers. Mutants of *P. fluorescens* strain WCS365 impaired in organic acid utilisation cannot effectively colonize the plant root (Dekkers 1997; Lugtenberg et al., 2001).

2.4.3. Role of motility and chemotaxis

Another important trait for efficient colonization is motility. It was shown that flagella-less mutants are able to occupy the part of root in the close proximity to the seed (Howie et al., 1987), but they cannot colonize the root tip efficiently (de Weger et al., 1987). Although motility, in relation to root colonization, has been reported to depend on the soil type, the plant and bacterial strains used (Weller and Thomashow, 1994), functional flagella are apparently important for migration of bacteria along the growing root and for reaching the root tip (Lugtenberg et al., 2001).

Plant roots do not produce exudate evenly along their surface. Intracellular junctions are supposed to be the major locations where nutrients are being released from the roots. Another "hot spot" of exudation is the tip of a growing root. Since intracellular junctions are the sites of pathogen penetration into the root tissue (Bolwerk et al., 2005), colonization of these sites as well as of the root tip by beneficial microorganisms is a key event in biocontrol. Bacteria are able to track the exudation sites by chemotaxis. The efficient root colonizer *P. fluorescens* strain WCS365 gives a positive chemotactic response towards tomato root exudates and its major components, such dicarboxylic and tricarboxylic acids, and several amino acids. Strain WCS365 shows no chemotaxis towards exudate sugars (de Weert et al., 2002). Mutants of this strain, which are deficient in sugar utilization, retain their root colonizing ability at the level of wild type strain (Dekkers, 1997). These two observation together with that of the root exudate composition show that (i) sugars are not crucial for *P. fluorescens* strain WCS365 as a carbon source and (ii) chemotaxis drives this excellent colonizer towards several major root exudate compounds. Chemotaxis plays an important role in root colonization: *che*A$^-$ mutants of four *P. fluorescens* strains, which retain their general motility, are impaired in competitive root tip colonization, both in a gnotobiotic system and in non-sterile potting soil (de Weert et al., 2002).

2.4.4 Selection of enhanced root colonizing bacteria

2.4.4.1 Gnotobiotic system to study root colonization

Microorganisms in soil form a complicated network of interactions. Supposedly therefore the soil microflora is quite resistant to introduction of new microorganisms. Non-sterile soil can be used for selection of good colonizers, but fine differences between wild type strain and its mutants cannot be revealed due to background of other microorganisms. Soil also contains nutrients which can influ-

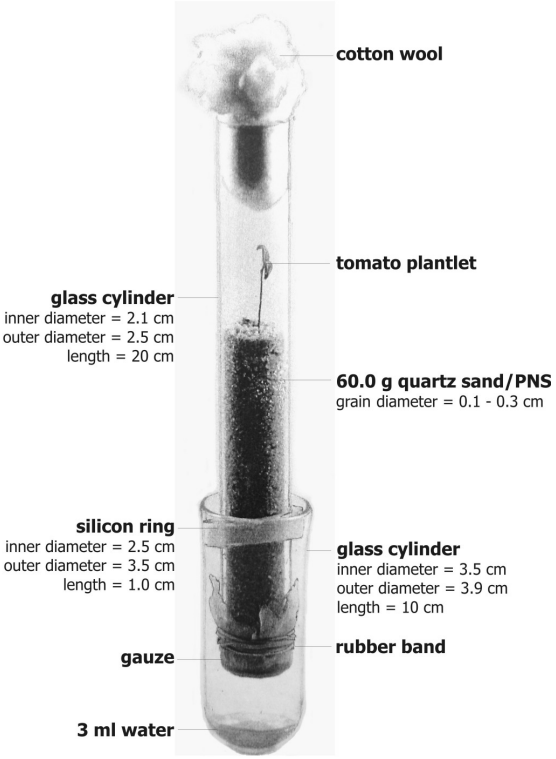

Fig. 2. Gnotobiotic system with growing tomato plant (Simons et al., 1996)

ence colonization of the roots by bacteria. In gnotobiotic system (Fig.2), developed by Simons et al., (1996), bacterized tomato seedlings are planted in a sterile column of quartz sand moistened with plant nutrient solution (PNS, Hoffland et al., 1989). After 7 days of growth in a climate controlled growth chamber, bacteria are isolated from the root. After plating, numbers of bacteria and ratios between wild type and mutant were determined. Using this system a number of traits important for colonization were identified (Dekkers 1997; reviewed by Lugtenberg et al., 2001).

2.4.4.2. Isolation of enhanced root tip colonisers

A criterion for a good root coloniser is that it can efficiently reach the root tip after seed inoculation. An enrichment procedure for rhizoremediating bacteria described by Kuiper et al.(2001a) was developed to isolate efficient naphthalene degrading, root colonising bacteria from soil samples. In this system plant can select good colonizing bacteria. By using this selection procedure, *P. putida* strain PCL1444 was isolated (Kuiper et al., 2001a; Kuiper et al., 2002). By modifying this procedure and subjecting a complete Tn5luxAB mutant bank of WCS365 to this procedure it is possible to select for enhanced competitive root tip colonising mutants. A mutant isolated by using this enrichment appeared to have *mutY* gene disrupted (de Weert et al 2004).

3. *Fusarium oxysporum* as a model pathogen

F. oxysporum (*Fox*) is well represented species among the communities of soilborne fungi, in every soil type worldwide (Burgess 1981). All strains of *Fox* are able to persist on organic matter in soil and to grow in rhizosphere of many plant species (Garret 1970). Many strains of *Fox* are phytopathogenic, they cause rots when penetrating the roots and tracheomycosis, when they invade vascular system of the plant (Fravel et al., 2003). These strains of *Fox* are responsible for yield lost of many economically important crops. *Fox* strains produce variety of mycotoxins,

such diacetoxyscirpenol, HT-2 toxin, deoxynivalenol, trichothecenes, moniliformin, fusarochromanone, fumonisin B1, and wortmannin. These compounds are highly toxic for animals and human. For example, they cause in rats body weight loss, feed refusal, hemorrhage in the stomach and intestines, and, at higher concentrations, death (Mirosha et al., 1989; Abbas et al., 1990).

Being economically important *Fox* species attracts considerable attention of biologists. Genome sequencing project was recently started for this microorganism. A number of scientific teams study pathogenicity and biocontrol of *Fox* strains (Fravel et al., 2003). In our group *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*) strain ZUM2407, a causal agent of tomato foot and root rot, is used to study mechanisms of biocontrol by different microorganisms (Chin-a-Woeng et. al., 1998; Dekker et al., 1999; Bolwerk et al., 2005).

3.1. Taxonomy of *Fusarium oxysporum*

Sexual reproduction has never been observed in *Fox* (Booth 1971). Significant gametic disequilibrium reported among isolates of *Fox* implies that asexual reproduction is an exclusive multiplication strategy in this species (Kistler et al., 1997).

Somatic fusion and heterokaryon formation can occur usually between strains with similar genotypes. This network of strains able to form heterokaryons have been named vegetative compatibility group (VCG).

Fox strains can cause disease to impressive number of plant species. Over than 150 special forms of *Fox* are described (Baayen et al., 2000) as *formae speciales*. Each *forma specialis* includes phytopathogenic strains, which are able to cause disease (wilt or rot) on a unique host or on set of hosts. Since they have the same host, members of a given forma specialis are supposed to be closely related and may have been descended from the common ancestor (Kistler 1997).

However, recent studies revealed ten clonal lineages among strains of *F. oxysporum* f. sp*. cubense* using restriction fragment length polymorphism (RFLP) of anonymous single copy fragment (Koening et al., 1997). These results show that

banana strains of *Fox* f. sp *cubense* could be a closely related to pathogens of another hosts, such as tomato. Considerable genetic diversity within *Fox* f. sp. *cubense* was revealed from the chromosomal polymorphism among the strains random amplified polymorphic DNA and VCGs distribution (O'Donnel et al., 1998). It is generally accepted now that formae speciales which comprise of more than one VCG can have polyphyletic origin (Baayen et al., 2001).

3.2 Monitoring of pathogenic strains of *Fusarium oxysporum*

Detection of pathogen in field, greenhouse or store and monitoring of its development are important parts of pest managements. Classical approach for monitoring of *Fusarium* species is to follow the disease symptoms. The symptoms of different pathogens can be similar, for example, vascular wilt can be caused by *Verticillium dahliae* and *Fox* (Lievens et al., 2003). Therefore this work cannot be carried out without cultivation and identification of the pathogen, which was isolated from lesions.

Many *Fox* strains produce toxins which also can be a target for monitoring (Labuda et al., 2003). This approach is extensively exploited in food and forage production, but it gives more information on the mycotoxins rather than on strains which are producing these compounds.

Microbiological monitoring of filamentous fungi is difficult. Hyphae are continuous structures which are breaking to propagules of different size during the plating. So number of colony forming units (CFU) in the case of fungal material does not reflect real amount of fungal cells (i.e. biomass of the fungus).

Recent progress in molecular biology made possible to follow fungi using specific DNA probes and real-time/quantitative polymerase chain reaction (RT-PCR/qPCR; Schaad and Frederick 2002). Target fragments for qPCR can be obtained using RAPD (Pasquali et al., 2003) or be derived from transposon sequences (Chiocchetti et al., 1999). In addition to estimation of the fungal material amount in the sample, RT-PCR can identify infected plants earlier than symptoms appear (Pasquali et al., 2004).

4. Aims of the thesis

Biological control of phytopathogens gains popularity as an environmentally and end-user friendly approach for crop protection against fungal diseases. It is generally accepted that biocontrol is more reliable under controlled conditions in artificial substrates, such as stonewool, in greenhouses than in open fields. The research, described in this thesis, is aimed at biological control of tomato foot and root rot, caused by *Fusarum oxysporum* f. sp. *radicis-lycopersici* (*Forl*) in stonewool substrates in greenhouses. The aims were the following: (i) To develop an enrichment procedure for the isolation of non-antagonistic bacterial strains which can protect plants against *Forl* (chapter 2); (ii) To use stonewool substrate and isolate and characterize such bacterial strains using this enrichment procedure. One of these isolates is *Pseudomonas putida* PCL1760 (chapter 3); (iii) To unravel the mechanism(s) of action used by *P. putida* strain PCL1760 to control TFRR (chapter 4), (iv) To elucidate the diversity and heterogeneity of *Fusarum oxysporum,* the model phytopatogen used in our biocontrol studies (chapter 5); (v) To quantify *Fusarum oxysporum* biomass in plant tissue as a predictive tool for an ongoing infection (chapter 6) and (vi) To test the efficacy of *P. putida* PCL1760 in the biocontrol of TFRR under industrial conditions in a certified greenhouse under practical conditions using routine and newly developed tools of disease monitoring.

Chapter 2

Enrichment for enhanced competitive plant root tip colonizers selects for a new class of biocontrol bacteria

Kamilova,F., Validov,S., Azarova,T., Mulders,I., and Lugtenberg,B. (2005) Environ. Microbiol. **7:** 1809-1817

Abstract

Our group studies tomato foot and root rot, a plant disease caused by the fungus *Forl* (*Fusarium oxysporum* f. sp. *radicis-lycopersici*). Several bacteria have been described to be able to control the disease, using different mechanisms. Here we describe a method that enables us to select, after application of a crude rhizobacterial mixture on a sterile seedling, those strains that reach the root tip faster than our best tomato root colonizer tested so far, the *Pseudomonas fluorescens* biocontrol strain WCS365. Of the five tested new isolates, four appeared to be able to reduce the number of diseased plants. Analysis of one of these strains, *P. fluorescens* PCL1751, suggests that it controls the disease through the mechanism 'competition for nutrients and niches', a mechanism novel for biocontrol bacteria. Moreover, this is the first report describing a method to enrich for biocontrol strains from a crude mixture of rhizobacteria. Another advantage of the method is that four out of five strains do not produce antifungal metabolites, which is preferential for registration as a commercial product.

Introduction

Many plant diseases are caused by phytopathogenic fungi. In order to decrease the input of agrochemicals in agriculture, biocontrol microbes are used as possible alternatives (Schippers et al., 1987; Lugtenberg and Bloemberg, 2004). We study tomato foot and root rot (TFRR), which is a disease caused by the fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*). This disease cannot efficiently be prevented by chemicals. The isolation of biocontrol bacteria involves a labor intensive screening process that, in the case of antibiosis, can be enhanced by introducing a screening step for strains that produce antifungal metabolites (AFMs) *in vitro*.

To our knowledge, no procedures have been described, which facilitate the selection of biocontrol microbes that act through other mechanisms. Kuiper and colleagues (2001) described a method to select enhanced grass root tip colonizing bacteria. In this method a mixture of rhizosphere bacteria is applied on a sterile seedling. After plant growth in a gnotobiotic system (Simons et al., 1996), those bacteria that have reached the root tip are isolated. These are subsequently used to inoculate a fresh sterile seedling, which again is allowed to grow. After three of these enrichment cycles, excellent competitive root tip colonizers were obtained (Kuiper et al., 2001). In the present paper we used this method to select enhanced tomato and cucumber root tip colonizers. Based on the notion that not only biocontrol fungi but perhaps also biocontrol bacteria exist, which act through the mechanism "competition for niches and nutrients", we screened the selected enhanced root tip colonizers for their ability to control the disease TFRR. The results are described in this paper.

Materials and Methods

Microbial strains and growth conditions

The bacterial strains used are listed in Table 1. All newly isolated strains were routinely cultured in KB (King et al., 1954) at 28°C under vigorous shaking. In some cases the synthetic medium BM (Lugtenberg et al., 1999), supplemented with 1% succinic acid, was used. *Chromobacterium violaceum* was grown in LB medium (Sambrook and Russel, 2001). *Agrobacterium tumefaciens* was grown on yeast mannitol broth (YMB) medium (Smit et al., 1987). Solid growth medium contained 1.8% agar (Difco Laboratories, Detroit, MI, USA). Spontaneous gentamycin-resistant derivatives of PCL1751 were generated by plating 100 ml of overnight culture on KB containing 20 mg of gentamycin per milliliter. After 48 h of growth spontaneous resistant colonies were collected separately and their motility was determined on plates containing 20-fold diluted KB, solidified with 0. 3%agar as described by Dekker and colleagues (1998). Non-motile Gm-resistant derivative was assigned as PCL1752. To analyze competitive growth between wild-type PCL1751 and mutant PCL1752, cells were grown overnight and diluted to a final OD_{620} of 0.1 and subsequently diluted in fresh medium in a 1:1 ratio. After growth overnight, cells were diluted 1000-fold in fresh medium and colony forming units (CFUs) were determined by plating dilutions of samples on KB plates with and without Gm. All fungi used were routinely cultivated on potato-dextrose agar (PDA, Difco Laboratories) or in Czapek-Dox liquid medium (Difco Laboratories) at 28°C under vigorous aeration. Kanamycin (50 mg ml-1), gentamycin (20 mg ml-1) and cycloheximide (100 mg ml-1) were added where applicable.

Isolation of microbes from roots of tomato and cucumber plants and enrichment of enhanced competitive root tip colonizers

Three-month-old tomato and cucumber plants with adhering rhizosphere soil were collected from greenhouses just outside Tashkent, Republic of Uzbekistan. Roots and adhering rhizosphere soil (total 25 g) of each plant species were shaken

vigorously for 2 h in 50 ml of sterile PBS. The samples were diluted, plated on 20-fold diluted solidified Tryptic Soy agar (1/20 TSA; Difco Laboratories) supplemented with cycloheximide (100 mg ml-1), and incubated overnight at 28°C. Subsequently all colonies were scraped together from the plate and suspended in 10 ml PBS. The resulting suspension was used to inoculate germinated sterile seedlings of tomato (cultivar Carmello, Syngenta, Enkhuizen, the Netherlands)and cucumber (cultivar Grendel, Syngenta, Enkhuizen, the Netherlands) for enrichment (Fig. 1) using the gnotobiotic system described by Simons and colleagues (1996). Those rhizobacteria that succeeded to reach the 1-cm-long root tip after growth for 7 days in the gnotobiotic quartz sand moisturized with plant nutrient solution (Hoffland et al., 1989), estimated to contain 1% of the total number of root colonizing bacteria (Simons et al., 1996), were subjected to two more selection cycles to enrich for the best enhanced competitive root tip colonizers (Fig. 1).

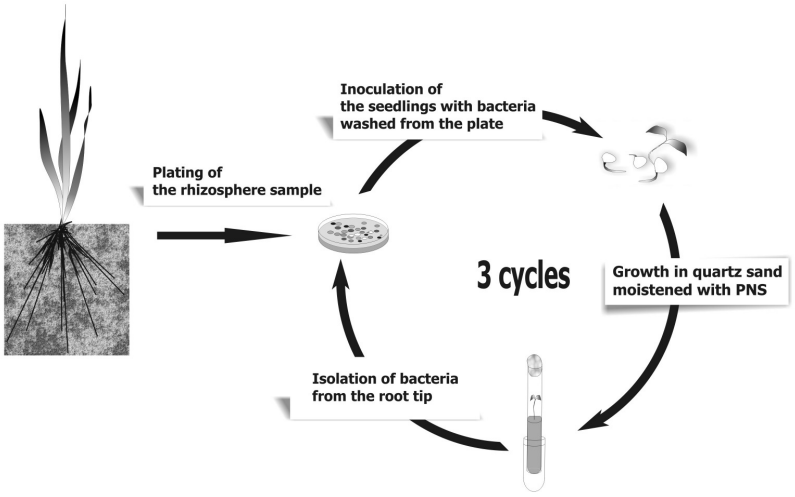

Fig. 1. Selection of enhanced root colonizing bacteria. Microbes were isolated from the rhizospheres of tomato and cucumber plants, grown in diluted TSB and used to inoculate sterile germinated seedlings of the same plant species as the rhizobacteria were derived from. These are tomato cultivar Carmello and cucumber cultivar Grendel. After growth in the gnotobiotic system, those microbes that had reached the 1 cm long root tip were shaken off the root tip, plated on KB agar, judged for colony diversity, and the cells from the combined colonies were used for another enrichment cycle. After a total of three cycles the bacteria from the root tip were plated and selected for competitive root colonization experiments.

Table 1. List of microorganisms used in this study

Strains	Characteristics	Reference or source
Bacteria		
WCS365	*Pseudomonas fluorescens* ; excellent competitive root colonizer; biocontrol strain of tomato foot and root rot	Geels and Schippers, 1983;
PCL1285	Tn5luxAB derivative of WCS365, comparable with wild type in root colonization ability; Km ^{r}	Simons et al.1996;Dekkers et al., 2000; De Weert et.al., 2004
PCL 1391	*P. chlororaphis*; efficient competitive root colonizer; biocontrol strain of tomato foot and root rot which produces phenazine-1-carboximide	Chin-A–Woeng et al. 1998
PCL1751	Wild-type *P. fluorescens*, isolated from Uzbekistan tomato rhizosphere	This study
PCL1752	Spontaneous nonmotile mutant of *P. fluorescens* PCL1751; Gm^{r}	This study
PCL1753	Wild-type *P. fluorescens*, isolated from Uzbekistan tomato rhizosphere	This study
PCL1754	Wild-type *P. putida*, isolated from Uzbekistan cucumber rhizosphere	This study
PCA0067	Wild-type *Pantoae agglomerans*, isolated from Uzbekistan cucumber rhizosphere	This study
PCA0081	Wild-type *Aeromonas hydrophila*, , isolated from Uzbekistan tomato rhizosphere	This study
CV026	*Chromobacterium violaceum* N-AHL reporter strain	Milton et al, 1997
NT1	*Agrobacterium tumefaciens* NT1 N-AHL reporter strain harboring pJM749 containing a lacZ reporter fused to a tra gene of which expression is dependent on TraR	Piper et al., 1993
Fungi		
LBOP17	*Pythium ultimum*; causes damping-off and fruit rot of tomato	IPO-DLO, Wageningen, The Netherlands
R3-11A	*Gaeumannomyces graminis pv.tritici* (Ggt), causes take-all disease of wheat and other cereals.	Raaijmakers and Weller, 1998
ZUM2076	*Botrytis cinera*; causes gray mold of tomato	Novartis Seeds BV, Enkhuizen, The Netherlands
ZUM2372	*Alternaria dauci* isolated from carrot seeds. Pathogen of carrot.	Novartis Seeds BV, Enkhuizen, The Netherlands
ZUM2407	*Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*); causal agent of tomato foot and root rot	IPO-DLO, Wageningen, The Netherlands

Competitive tomato root tip colonization assay

Seeds of tomato were sterilized, allowed to germinate, and the seedlings were inoculated with a 1:1 mixture of two bacterial strains and planted in the gnotobiotic quartz sand system as described by Simons and colleagues (1996). Plants were grown in climate-controlled chambers with 16 h of day light at 24°C during 7 days. To estimate competitive root tip colonization, the root tip (1 cm) with adhering rhizosphere soil and was cut off and shaken vigorously for 15 min in 1.0 ml PBS to remove the bacteria. Dilutions of the bacterial suspensions were plated onto KB and on KB supplemented with Km to determinate the numbers of Km-resistant and Km-sensitive bacteria in the suspension. All colonization experiments were performed in 10-fold. The average number of bacteria and the standard deviation were calculated. The non-parametric Wilcoxon–Mann–Whitney test (Sokal and Rohlf, 1981) for mixed inocula was used to perform statistics.

Preliminary characterization of plant growth promotion traits

To test AFM production *in vitro*, 0.5 ×0.5 cm agar plugs of each fungus were stabbed in the centre of PDA and KB agar plates that were subsequently inoculated with individual bacterial test strains at a distance of 3.0 cm from the fungus. Bacterial strains that caused an inhibition zone of at least2 mm were judged as positive. Hydrogen cyanide was detected using cyanide indicator paper (Castric, 1975), protease on 10% milk agar plates (Brown and Foster, 1970), chitinase on plates containing colloidal chitin (Shimahara and Takiguchi, 1988) and β-glucanase on plates containing lichenan (Sigma, St. Louis, MO, USA) (Walsh et al., 1995). Production of biosurfactant was determined using the drop collapsing assay (Jain *et* al., 1991). Phase variation was judged as described by Van den Broek and colleagues (2003). Motility was tested as described by Dekker and colleagues (1998) on 0. 3% agar.

The production of auxin was determined by a colorimetric method. Briefly, test strains were inoculated in BM/succinate without or with tryptophan (100 mg ml-1) and incubated at 28°C at 150 rpm min^{-1}. After 1, 4 and 8 days of cultivation,

aliquots of bacterial cultures were centrifuged at 13 000 rpm for 10 min. Two milliliters of supernatant fluid was added to a tube with 100 ml 10 mM orthophosphoric acid and 4 ml of Salkowski reagent (Gordon and Weber, 1951). The mixture was incubated at room temperature for 30 min and absorbance of the developed pink color was read at 530 nm. The indole-3-acetic acid (IAA) concentration in the culture was determined by using a calibration curve of pure IAA as a standard. Autoinducers were extracted from supernatant fluids using dichloromethane and the activity of the extracts was analyzed using *Chromobacterium* (Milton et al., 1997) and *Agrobacterium* (Piper et al., 1993) reporter strains as described by Chin-A-Woeng and colleagues (2001).

Isolation of tomato root exudates

Tomato root exudate was isolated as described by Simons and colleagues (1997). Briefly, batches containing 100 sterile seedlings were placed in 100 ml PNS and cultivated in a climate-controlled growth chamber at 24°C, 70% relative humidity and 16 h of daylight. After 14 days, sterility was tested, and root exudate of sterile samples was collected, filtered through 0. 22 mm filters and kept at 4°C until use.

Strain identification

Strains were identified after colony PCR (Williams et al., 1990) for amplification of 16S rDNA. The PCR products were sequenced by ServiceXS (Leiden, the Netherlands) and analyzed for homology using BLAST (Altschul et al., 1997). Amplified ribosomal DNA restriction analysis was performed according to Vaneechoutte and colleagues (1990).

Growth of bacteria in tomato root exudate

Bacteria were pre-grown overnight in 20-fold diluted TSB medium. Cells were spun down, washed three times in PBS, and used for inoculation of tomato root exudate to a final concentration of approximately 10^4 CFU ml-1. The suspension

was incubated at 21°C under aeration at 150 rpm. Growth was measured by dilution plating on KB.

Biocontrol of TFRR using seedling inoculation

Tomato seeds cultivar Carmello were coated with bacteria by dipping the seeds in a mixture of 1% (w/v) methylcellulose (Sigma, St Louis, MO, USA) and 10^9 CFU ml^{-1}bacteria in PBS. *Forl* spores were prepared as described by Chin-A-Woeng and colleagues (1998). Tomato seeds were placed in non-sterile potting soil (Jonkind grond B.V., Aalsmeer, the Netherlands) infested with *Forl* spores (2×10^6 spores kg-1). For each treatment, 96 plants were tested in eight trays of 12 plants each. Plants were grown in a greenhouse at 21–24°C, 70% relative humidity and 16 h daylight. After 15–21 days of growth, plants were removed from the soil, and the plants roots were examined for foot and root rot symptoms as indicated by browning and lesions. Only roots without any disease symptoms were classified as healthy. 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 (a = 0. 05), using SPSS software (SPSS, Chicago, IL, USA). All experiments were performed at least twice. In all biocontrol experiments positive controls consisted of application of *P. fluorescens* WCS365, causing induced systemic resistance (Gerrits and Weisbeek, 1996), and *P. chlororaphis* PCL1391, a phenazine producing TFRR biocontrol strain (Chin-A-Woeng et al*.,* 1998), which requires delivery of this AFM along the root for biocontrol activity (Chin-A-Woeng et al*.,* 2000).

Induction of resistance against TFRR

The root system and hypocoteledons of 3-week-old tomato plants (cv. Moneymaker, purchased from Rijnsburg Zaadhandel, Rijnsburg, the Netherlands) were split and each half of the root was replanted in separate pots, whereas the stem remained intact. After 1 week one part of the root system was inoculated with bacteria (10^9 CFU per plant in 5 ml PBS) or with PBS in control plants. After another week the other part of the root system was challenged by adding to each plant 5 ml PBS containing 10^6 *Forl* spores. Three weeks after challenging, roots were analyzed

for the presence of lesions. Seventeen or 23 plants were grown per treatment. The difference in health conditions (healthy or sick plants) between two different treatments was statistically analyzed using chi-squared goodness-of-fit test (Heath, 1995).

Plant growth promotion

For the evaluation of the effect of bacterial isolates on the growth of tomato and cucumber plants, seeds were coated with bacterial mixtures as described in the biocontrol assay and grown under greenhouse conditions in non-sterile potting soil. Each variant consisted of three replicas with eight seeds per replica. After 3 weeks of growth fresh and dry weight of shoots was determined and analyzed using analysis of variance followed by Fisher's least-significant-difference test (a = 0. 05), using SPSS software (SPSS, Chicago, IL, USA). All experiments were performed at least twice.

Results and discussion

Isolation of enhanced root colonizing bacteria

The mixtures of rhizosphere bacteria from cucumber and tomato plants, cultivated for 12 weeks under greenhouse conditions in soil that had never been treated with fungicides were, respectively, used to inoculate seedlings of the same plants and enhanced competitive root tip colonizers were enriched as described in the *Experimental procedures* section and illustrated in Fig. 1. After each cycle we observed that diversity of colonies in terms of color, size, transparency, etc. somewhat decreased. This observation was true for both types of plants. After the third cycle of enrichment, 16 colonies were randomly chosen to test in competitive tomato root tip colonization assays against PCL1285 (Table 1), a kanamycin-resistant derivative of the best known competitive tomato root tip colonizer *P. fluorescens* WCS365 (Lugtenberg *et al* ., 2001). Seven of the newly isolated strains appeared to be better competitive colonizers than *P. fluorescens* WCS365 or its colonization-proficient Km-resistant derivative PCL1285. The latter strains showed

log 10 [(cfu + 1)/ cm of root tip] root tip colonization values between 4.6 and 5.0 (Table 2). The nine strains derived from the enrichment procedure, which were worse competitive colonizers than PCL1285, showed log 10 [(cfu + 1)/cm of root tip] values between 3.7 and 4.5 (results not shown). Ten rhizosphere isolates, randomly chosen before the enrichment procedure was started, appeared to be 100- to 1000-fold poorer competitive colonizers than the best new isolates: the number of bacteria isolated from the root tip varied from not detectable (the detection limit has a log 10 [(cfu + 1)/cm of root tip] value of 2.7) to a log 10 [(cfu + 1)/cm of root tip] value of 3.7. The seven isolates that showed enhanced competitive root colonizing ability were subjected to the amplified ribosomal DNA restriction analysis (ARDRA) procedure to identify putative siblings. Three out of the seven best colonizers appeared to be siblings (data not shown). The resulting five unique isolates (Table 2) were subjected to further characterization. Note that two of these isolates are derived from cucumber but are able to colonize tomato roots efficiently. The lesson of the ARDRA result is that it is advisable to check for siblings prior to starting the labour-intensive colonization and biocontrol work. Furthermore, we conclude that the used enrichment procedure not only works for the monocot grass but also for the dicot tomato. The enrichment method makes it easy to isolate better competitive tomato root tip colonizers than *P. fluorescens* WCS365, a strain that we considered as the best tomato root colonizer for almost a decade.

Taxonomic characterization of enhanced competitive colonizers

Gram staining showed that all five isolates with enhanced competitive root colonizing ability are Gram-negative. Nucleotide sequencing of amplified 16S rDNA fragments, obtained after colony polymerase chain reaction (PCR), and comparative analysis with the DNA databases, revealed that the isolated enhanced competitive root tip colonizing strains belong to genera *Pseudomonas* , *Aeromonas* and *Pantoae* (Table 1).

Table 2. Competitive tomato root tip colonization ability of the newly isolated strains in competition with WCS365 or PCL1285, a Tn5luxAB derivative of WCS365 [x]

Competing strains	Competitive root tip colonization [lg(cfu+1/cm) root tip][y]	
	Test strain	Reference strain
PCL1751 vs WCS365	5.30 ± 0.37 (a)	4.67 ± 0.58 (b)
PCA0081 vs PCL1285	5.40 ± 0.25 (a)	4.60 ± 0.75 (b)
PCL1753 vs PCL1285	5.70 ± 0.62 (a)	4.70 ± 0.57 (b)
PCA0067 vs PCL1285	5.95 ± 0.35 (a)	4.85 ± 0.72 (b)
PCL1754 vs PCL1285	5.60 ± 0.30 (a)	5.00 ± 0.60 (b)

The 16S rDNA sequences of the new isolates show the following percentages of homology with that of the following strains: strain PCL1751 99% with *P. fluorescens* 62e, strain PCL1753 99% with *P. fluorescens* Q2-87, strain PCL1754 99% with *P. putida* ATCC11250, strain PCA0081 99% with *Aeromonas hydrophila* 45/90 and strain PCA0067 99% with *Pantoea agglomerans* strain JCM1236. The sequences of the five new isolates have been deposited in GenBank under the following accession numbers. *Aeromonas hydrophila* PCA0081, AY900170; *P. agglomerans* PCA0067, AY900169; *P. putida* PCL1754, AY9001168; *P. fluorescens* PCL1751, AY9001171 and *P. fluorescens* PCL1753, AY9001172.

Potential biocontrol traits

The five enhanced competitive colonizers (Table 2) were tested for a number of potential biocontrol traits (Table 3). Only one strain, *P. fluorescens* PCL 1753, is antagonistic towards (four out of five of the tested) phytopathogenic fungi. It also produces autoinducer and hydrogen cyanide (HCN). None of the strains has biosurfactant activity and only two strains secrete at least one of the five tested exoenzymes. Strain PCL1753 is able to induce AHL reporter genes, in both *Chromobacterium violaceum* and *Agrobacterium tumefaciens* (Table 3). In the last

case the signal was weaker. None of these isolates showed colony phase variation (results not shown).

Table 3. Overview of possible plant growth promoting and biocontrol properties of the five newly isolated enhanced tomato root tip colonizers

	Antifungal activity [a]					**Exo-enzyme activity**					**Secondary metabolites**			
Strain	*Forl*	*P.ultimum*	*A.dauchi*	*B.cinera*	*Ggt*	protease	lipase	β-gluconase	cellulase	chitinase	AI[b]	HCN	BS	IAA[c]
PCL1751	-	-	-	-	-	+	-	-	-	-	-	-	-	-
PCA0081	-	-	-	-	-	+	+	-	-	+	-	-	-	10.2 ± 0.6
PCA0067	-	-	-	-	-	-	-	-	-	-	-	-	-	19.8 ± 2.3
PCL1753	-	+	+	+	+	-	-	-	-	-	+	+	-	7.5 ± 1.8
PCL1754	-	-	-	-	-	-	-	-	-	-	-	-	-	n.d.

[a] Antifungal activities were tested as inhibition of growth *in vitro*. Results obtained on PDA and KB were similar.
[b] Abbreviations: AI, autoinducer; BS, biosurfactant; IAA, indole-3-acetic acid; n.d., not determined.
[c] Values represent amounts of IAA in µg/ml in the spent culture medium after 8 days of cultivation of the bacteria in the BM medium supplemented with 1% succinic acid and tryptophan (100 µg/ml).

Auxin production and effect on plant growth

Auxin production was tested in the absence and presence of the auxin precursor tryptophan. All strains reached the stationary phase within 24 h, but no auxin was detectable at that time. It appeared that in 4- and 8-day-old cultures *P. agglomerans* PCA 0067, *A. hydrophila* PCA 0081 and *P. fluorescens* PCL1753 (Table 3), as well as the well known efficient competitive root tip colonizer and biocontrol strain *P. fluorescens* WCS365 (result not shown), produce auxin. Another biocontrol strain, *P. chlororaphis* PCL1391 (not shown) and new isolate *P. fluorescens*

PCL1751 (Table 3), did not produce a detectable amount of auxin. No auxin production was detected in cells grown in the absence of tryptophan.

To test whether the ability to produce auxin has a significant influence on plant growth, tomato seeds were inoculated with the newly selected enhanced colonizing bacteria and grown in potting soil for 21 days. Measurements of fresh and dry weight of tomato shoots (see *Experimental procedures* section for details) showed that inoculation with these bacteria did not cause a significant effect on plant growth (results not shown). We conclude that none of the new isolates promotes tomato in the absence of a pathogen.

Biocontrol of tomato foot and root rot

Of the five enhanced colonizers isolated after three enrichment cycles, the strains *P. fluorescens* PCL1751 and *P. agglomerans* PCA0067 significantly control TFRR and do so to a similar extent as our standard biocontrol strain WCS365 (Table 4). Strains *A. hydrophila* PCA 0081 and *P. fluorescens* PCL1753 showed significant biocontrol of TFRR in one experiment. A reduction of diseased plants was also found in the two other experiments, but the effect was not significant in those cases. *Pseudomonas putida* strain PCL1754 failed to control TFRR in any of the two performed biocontrol experiments. We conclude that four out of the five enhanced colonizers derived from the enrichment procedure have a moderate- to-good biocontrol activity. Interestingly, the two best biocontrol strains *P. fluorescens* PCL1751 and *P. agglomerans* PCA0067 are not antagonistic (Table 3). The results in Tables 2 and 4 with *P. putida* strain PCL1754 show that excellent colonization is not sufficient for biocontrol. Consultation of a classification list of bacteria in safety risk groups (Anonymous, 1998) showed that four of the five isolates fall in risk group 2 and that one of the two consistent new biocontrol strains, *P. fluorescens* PCL1751, is the safest one for application as it falls in risk group 1. Therefore, we continued with this strain.

Table 4. Effect of enhanced competitive tomato root tip colonizers on control of tomato foot root rot

ExP.No	Percentage of diseased plants [x] Test strains					
	None	PCL1751	PCA0081	PCA0067	PCL1753	PCL1754
1	75±15 (a)	51±15(b)		57±8(b)		
2	49±12(a)	27±12(b)	43±15(a)	33±10(b)	40±6(a)	40±11(a)
3	84±8(a)		68±16(b)		64±16(b)	80±10(a)
4	68±9(a)		60±9 (a)		65±15(a)	

[x] The percentage of diseased plants was determined 2-3 weeks after inoculation. Per strain, 96 plants in 8 trays of 12 plants were tested. For statistics, a variance analysis followed by Fisher's least- significant-difference test (α=0.05), using *SPP*S software (*SPP*S Inc., Chicago, Il., USA), was used

Competition for niches and nutrients

PCL1752 was isolated as a spontaneous mutant of *P. fluorescens* PCL1751 impaired in motility when tested on semisolid King's medium B (KB). The mutant was as competitive as the wild type when grown 1:1 in competition with its parent in KB and in tomato root exudate. We conclude that the non-motile mutant has intact housekeeping genes.

In competitive tomato root tip colonization assays, in which sterile seedlings were inoculated with mutant and wild-type cells in equal numbers, followed by plant growth in the gnotobiotic sand system (Simons et al., 1996), the mutant was completely outcompeted by its parental strain both in the middle part of the root and on the root tip. Whereas the wild type reached 5×10^5 cfu per centimetre of root tip, the mutant was not recovered at all from the root tip. In biocontrol experiments in potting soil under greenhouse conditions, the mutant showed no significant biocontrol activity against TFRR, in contrast to the wild type (results not shown). We therefore conclude that competitive colonization, or competition for niches, is required for biocontrol activity of *P. fluorescens* PCL1751.

To test whether the enhanced colonizers can efficiently grow on exudate, the major nutrient source in the rhizosphere, the growth of the five enhanced colonizers was compared with that of five random strains isolated from the starting

material before enrichment. All five enhanced colonizers, as well as *P. fluorescens* WCS365, reached densities of 2×10^7 cfu ml^{-1} within 24 h and remained at that level for the next 48 h. In contrast, the best growing control strain reached a density of 7×10^6; the other control strains reached a maximum value of 3×10^5. The observation that the enhanced colonizers, as well as *P. fluorescens* WCS365 (not shown), grow much better on exudate than random rhizobacteria shows that the enrichment method (Fig. 1) selects for strains that utilize exudates components efficiently for growth. It suggests that competition for nutrients plays a major role in the biocontrol activity of the enhanced colonizers. Therefore this result, combined with the lack of biocontrol by the non-motile mutant, suggests that strain PCL1751 acts through competition for niches and nutrients.

Induction of systemic resistance against TFRR

Strains *P. fluorescens* WCS365 and *P. fluorescens* PCL1751 have in common that they are not antagonistic *in vitro*, do not secrete exo-enzymes, are excellent competitive tomato root tip colonizers, grow to the same high cell density on tomato root exudates and can control TFRR. However, their mechanisms of biocontrol seem to be different: WCS365 causes ISR, at least in *Arabidopsis thaliana* (Gerrits and Weisbeek, 1996) whereas our present results indicate that PCL1751 acts through competition for niches and nutrients. To test whether the two strains really use different mechanisms for their biocontrol action we decided to test, using the split root system described by Kroon (1992), whether the strains can induce resistance towards TFRR in tomato. In this system one of the root parts is treated with a putative biocontrol agent to allow induction of resistance and the other root part is challenged 1 week later with the pathogen.

The results are shown in Table 5. When no bacteria were added to one of the parts of the root system, 70–88% of the plants showed disease symptoms in the part of the root system that had been treated with *Forl*. Strain *P. fluorescens* PCL1751 appeared not to be able to prevent disease in the split root system. In contrast, strain *P. fluorescens* WCS365 showed significant biocontrol. It reduced the number of diseased plants in the tomato split root system to 39–47%. No

introduced *Pseudomonas* bacteria could be recovered from the non-inoculated root part. This result indicates that the induced resistance is systemic. The results of Table 5 therefore show that strain *P. fluorescens* WCS365, in contrast to *P. fluorescens* strain PCL1751, can systemically induce resistance against TFRR. This is the first time that it is shown that *P. fluorescens* WCS365 induces resistance in tomato plants without direct contact with the pathogenic fungus.

Plant growth promotion

For the evaluation of the effect of bacterial isolates on the growth of tomato and cucumber plants, seeds were coated with bacterial mixtures as described in the biocontrol assay and grown under greenhouse conditions in non-sterile potting soil. Each variant consisted of three replicas with eight seeds per replica. After 3 weeks of growth fresh and dry weight of shoots was determined and analysed using analysis of variance followed by Fisher's least-significant-difference test (a = 0.05), using SPSS software (SPSS, Chicago, IL, USA). All experiments were performed at least twice.

Table 5. Induction of systemic resistance against tomato foot and root rot by *P. fluorescens* PCL1751 and *P. fluorescens* WCS365 using a tomato split root system [x]

Microorganism(s) present	Experiment 1		Experiment 2	
	Healthy	Sick	Healthy	Sick
(i) *Forl*	7	16(a)	2	15(a)
(ii) PCL1751 and *Forl*	5	18(a)	5	12(a)
(iii) WCS365 and *Forl*	14	9(b)	9	8(b)

[x] Twenty three (exp.1) or seventeen (exp.2) plants were grown (i) in the presence of *Forl* and the absence of bacteria, (ii) in the presence of *Forl* and *P. fluorescens* PCL1751, and (iii) in the presence of *Forl* and *P. fluorescens* WCS365. Three weeks after addition of *Forl* spores, the diseased plants were scored. Values in one column followed by a different letter are significantly different from each other (P≤0.05), according to the chi-squared goodness-of-fit test (Heath, 1995). For details of inoculation and growth conditions, see Materials and Methods section.

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Chapter 3

Selection of bacteria able to control *Fusarium oxysporum* f.sp. *radicis-lycopersici* in stonewool substrate

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Abstract

Aims: Tomato foot and root rot (TFRR), caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*), is an economically important disease of tomato. The aim of this study was to develop an efficient protocol for the isolation of bacteria which control TFRR based on selection of enhanced competitive root-colonizing bacteria from total rhizosphere soil samples.

Methods and Results: A total of 216 potentially enhanced bacterial strains were isolated from 17 rhizosphere soil samples after applying a procedure to enrich for enhanced root tip colonizers. Amplified Ribosomal DNA Restriction Analysis (ARDRA), in combination with determination of phenotypic traits, was introduced to evaluate the presence of siblings. One hundred and sixteen strains were discarded as siblings. Thirty eight strains were discarded as potential pathogens based on the sequence of their 16S rDNA. Of the remaining strains, 24 performed equally well as, or better than the good root colonizer *Pseudomonas fluorescens* WCS365 in a competitive tomato root tip colonization assay. Finally, these enhanced colonizers were tested for their ability to control TFRR in stonewool, which resulted in seven new biocontrol strains.

Conclusions: The new biocontrol strains, six Gram-negative and one Gram-positive bacteria, were identified as three *Pseudomonas putida* strains and one strain each of *Delftia tsuruhatensis*, *Pseudomonas chlororaphis, Pseudomonas rhodesia* and *Paenibacillus amylolyticus*.

Significance and Impact of Study: We describe a fast method for the isolation of bacteria able to suppress TFRR in stonewool, an industrial plant growth substrate. The procedure minimizes the laborious screens which are a common feature in the isolation of biocontrol strains.

Introduction

Since chemical fungicides are banned more and more frequently and because genetically–engineered fungus-resistant plants are unacceptable in several countries, there is a growing interest in the use of BCAs (biological control agents). Our group uses tomato foot and root rot (TFRR), caused by the fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*), as a model disease to elucidate mechanisms of action used by biocontrol bacteria (Chin-A-Woeng et al., 1998; Bolwerk et al., 2003; Kamilova et al., 2005). The major mechanisms described for disease control by biocontrol bacteria are antagonism (Thomashow and Weller, 1995; Chin-A-Woeng et al., 2003; Haas and Defago, 2005) and induction of systemic resistance in the plant (van Loon et al., 1998; Haas and Defago, 2005; Iavicoli et al., 2003; Kamilova et al., 2005).

Recently, a procedure was developed to select more or less directly for biocontrol bacteria. It appeared that, after application of bacteria from rhizosphere soil to seeds and followed by selection for enhanced root tip colonizers, many of the enhanced colonizers are able to control TFRR. Moreover, the procedure does not select for antagonistic isolates. Production of antibiotics is a disadvantage for registration as a product. For one of the isolates, *P. fluorescens* strain PCL1751, evidence was provided that it acts through the mechanism "competition for nutrients and niches", a mechanism not previously shown for biocontrol bacteria. Considering the selection procedure, this mechanism was expected (Kamilova et al., 2005).

In retrospect, Kamilova et al. (2005) spent a large amount of time on strains which later appeared to be siblings and potential pathogens. This happened because the taxonomic studies on the isolates were carried out in a late stage. In the work described in the present paper we apply the procedure described by Kamilova et al. (2005) with the following modifications and improvements. (i) To isolate strains that not only have biocontrol properties but can also survive drying procedures which are part of most commercial formulation methods, three rhizosphere samples were subjected to freeze or spray drying and subsequently

used for colonization enrichment. (ii) The colonization enrichment experiments were performed on a much larger scale: bacteria from seventeen different rhizosphere samples were used. (iii). To adapt the procedure to commercial practice, stonewool was used as the plant growth substrate instead of the artificial substrate quartz sand. (iv) An ARDRA (amplified ribosomal DNA restriction analysis) method was introduced to identify siblings and potential pathogens at an early stage. (v) The effects of drying and colonization enrichments on the percentages of Gram-negative bacteria, exo-enzyme producing bacteria and pseudomonads were followed.

Materials and Methods

Strains and growth conditions

Bacterial and fungal strains used and their characteristics are listed in Table 1. Bacteria were grown in King's medium B (KB) (King et al., 1954). Twenty-fold diluted tryptic soy broth (1/20 TSA, Difco Laboratories, Detroit, USA) supplemented with 1.5% of agar (Difco) was used for all platings during the enrichment procedure. *Pseudomonas* isolation agar (PIA, Difco) was used to select pseudomonads. Where applicable, kanamycin (Duchefa, Haarlem, The Netherlands) was used at a concentration of 50 μg per ml. Cycloheximide (Sigma, St. Louis, USA), at a final concentration of 100 μg/ml, was used to prevent growth of fungi. Fungal strains were grown on Potato Dextrose Agar (PDA) or Capek-Dox Agar (CDA), both from Difco Laboratories, Detroit, USA.

Collection and processing of rhizosphere samples

The origins of plants used to isolate rhizosphere samples are indicated in Table 2. One gram of plant roots with adhered soil particles, or one gram of stonewool threads, were shaken in 10 ml of phosphate buffered saline (PBS) - 30% glycerol and frozen at -80°C. When needed, samples were defrosted and serial dilutions were plated on 1/20 TSA supplemented with cycloheximide to determine the

number of Colony Forming Units (CFUs). When necessary, the colonies were purified by restreaking on KB agar.

Freeze and spray drying of rhizobacterial samples

Rhizosphere samples STII, STIII and Mix (a mixture of the remaining 15 samples) were plated on 1/20 TSA supplemented with cycloheximide (100 μg/ml) and grown at 28°C for 48 h. Colonies were washed from the plates with PBS and the density of the resulting cell suspensions was adjusted to OD_{620}=0.1. CFUs were determined by plating of serial dilutions of bacterial suspensions on KB agar. To protect cells during freeze- and spray-drying procedures, the bacterial suspensions were amended with skimmed milk powder (Nestlé, Frankfurt, Germany) and sugar beet syrup (Grafschafter Krautfabrik, Meckeheim, Germany) to final concentration of 10% and 1%, respectively.

Freeze drying was performed in an Advantage EL freeze dryer (Virtis, Gardiner, USA). Five ml of cell suspension supplemented with 10% skimmed milk and 1% sugar syrup were placed in 12 ml glass vials and subjected to thermal treatment. The temperature profile of thermal treatment included incubation at 5°C for 30 min, cooling down to 1°C for 30 min, fast freezing of the samples at -40°C and incubation at this temperature for 1 hour. Thermal treatment was followed by primary drying by increasing the temperature stepwise in 90 minutes, -30°C, -10°C, 5°C and finally to 20°C under vacuum changing stepwise from 400 to 300, 200 and 50 mtorr. After drying, the samples were kept in the freeze dryer for an additional 6 hours at 10°C and 10 mtorr. The water content of dried control samples (10% skimmed milk and 1% sugar syrup without bacterial suspension) was determined after both drying procedures using a MA30 moisture analyzer (Sartorius, Göttingen, Germany).

Three samples (20 ml each) were subjected to spray drying in a Mini Spray dryer (Büchi, Flawil, Switzerland) with inlet temperature 150°C, aspirator 12 and flow 600 NL/h. Outlet temperature for all samples was kept in the range of 60-65°C. The resulting powder was collected and sealed in sterile glass vials. Part of the powder

was used for determination of the water content. Numbers of viable cells were determined by plating serial dilutions of this suspension on KB agar. The powder was subsequently dissolved in such a volume of PBS that the original mass over volume ratio was restored.

Phenotypic characterization of strains

Protease secretion was judged after growing the strains on 1/20 TSA amended with 5% skimmed milk. A halo appearing on the first or second day of growth around the inoculated area is indicative for exo-protease activity (Brown and Foster, 1970). Lipase production was tested on 1/20 TSA amended with 2% of Tween 80 (BDH, Poole, UK) according to Howe and Ward (1976). The Gram character was determined using the alkaline test (Bourgault and Lamothe, 1988). A sample of a colony from a KB agar plate was suspended in 50µL of a 3% KOH solution. Indicative for Gram-negative bacteria is the observation that, when a needle is removed from the cell suspension it draws a thread out of it. Hydrogen cyanide was detected using cyanide indicator paper (Castric, 1975). Production of biosurfactants was determined using the drop-collapsing assay (Jain *et al*. 1991). Antifungal activity was determined as described by Kamilova et al. (2005). *P. chlororaphis* PCL1391 and *P. fluorescens* WCS365 were used as positive and negative controls, respectively.

DNA techniques

A nearly full-length portion of the 16S rRNA gene was obtained via PCR amplification with primers 27fm (5′- AGA GTT TGA TCM TGG CTC AG -3′) and 1522R (5′- AAG GAG GTG ATC CAG CCG CA -3′) (Weisburg et al., 1991). To perform Amplified Ribosomal DNA Restriction Analysis (ARDRA), the amplified DNA fragments were digested with *Hha*I, *Hin*fI, *Hpa*II, and *Rsa*I and separated in 2% agarose gel. Ladders GeneRuler 100 plus (Fermentas GmbH, St. Leon-Rot, Germany) was included in order to normalize the banding pattern of 16S rRNA gene restriction fragments. Digitalized images of ARDRA banding pattern were converted,

normalized, combined, and analyzed with Quantity One software (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). A band-matching algorithm (band matching tolerance of 0.75%) was selected to calculate pairwise similarity matrices with the Dice coefficient. Cluster analysis of similarity matrices was performed by the unweighted pair group method using arithmetic averages (Sneath and Snokal, 1973).

To determine the nucleotide sequences, the same primers were used for amplification of the 16S rDNA portion. PCR products were separated in 1% agarose gels, recovered and purified from agarose using QIAGEN kits (QIAGEN Benelux BV, Venlo, The Netherlands). Sequencing was performed by ServiceXS (Leiden, The Nederlands). Sequences were processed with DNAMAN 4.0 software package (Lynnon Biosoft, Quebec, Canada). Similarity searches in GenBank were performed using BLASTN software (Altschul et al. 1990).

Procedure for enrichment of enhanced competitive tomato root tip colonizers

The gnotobiotic glass tube system described by Simons et al. (1996) was used. The tubes were filled with 20 gram of granulate (Grodan BV, Roermond, The Netherlands) moistened with commercial PNS (PPO, Naaldwijk, The Netherlands) and autoclaved. Samples chosen for enrichment were dilution-plated on 1/20 TSA medium supplemented with cycloheximide. After for 48 hours of growth at 28°C, bacteria were washed from crowded plates with PBS. Bacterial suspensions were adjusted to an optical density at 620 nm of 0.1 (OD_{620}=0.1) and used for inoculation of sterile tomato seedlings. The rest of procedure was performed as described previously by Kamilova et al. (2005). After the third cycle of enrichment twelve colonies were randomly chosen from a plate with single colonies, and analyzed for traits.

Competitive tomato root tip colonization assay

This assay is based on the method described by Simons et al. (1996). Strains tested were grown overnight in KB and washed twice in PBS. Cell suspensions were

adjusted to OD_{620}=0.1, for pseudomonads corresponding with a cell density of approximately 10^8 cells/ml, and mixed in equal amounts. To determine the real ratio between the strains, dilutions of 10^{-5} and 10^{-6} were plated on KB agar and the phenotypes of the colonies were determined. Sterile tomato seedlings were dipped into the mixture of cell suspensions of two to four strains and incubated for 10 minutes. Inoculated seedlings were sown in sterile glass tubes with quartz sand and plant nutrient solution (Simons et al. 1996). When groups of 3-4 strains were allowed to compete, three plantlets were tested for each mixture. In the case of competition of one isolate versus WCS365, ten seedlings were used. After incubation for 7 days the 1 cm root tip was removed and the average number of the different bacteria as well as the standard deviation were calculated. The non-parametric Wilcoxon-Mann -Whitney test (Sokal and Rohlf, 1981) for mixed inocula was used to perform statistics.

Biocontrol of Tomato Foot and Root Rot (TFRR)

Biocontrol of TFRR was carried out in stonewool plugs (Grodan BV, Roermond, The Netherlands). The microspore concentration of *Forl* was determined using light microscopy. Plastic trays with 96 stonewool plugs were soaked in 1L of commercial Plant Nutrition Solution (PPO, Naaldwijk, The Netherlands) supplemented with *Forl* (2×10^6 microspores per L) and cells of the strain of interest (10^7 cells per mL). Seeds of tomato var. Carmello were sown in stonewool, covered with 0.5 g of vermiculite, and allowed to germinate in the dark at 23°C for 48h. Once germinated, the seedlings were kept at 21-23°C with 80% relative humidity and 16h of daylight. Biocontrol was scored after 17 days. Dead plants or plants with brown spots or lesions on their feet/roots were considered as diseased. 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), using *SPPS*-software (*SPPS* Inc.,Chicago, IL., USA). All experiments were performed at least twice.

Table 1. Microorganisms and their relevant characteristics.

Strains	Relevant characteristics	Origin, references
Reference bacteria		
Pseudomonas chlororaphis PCL1391	Produces phenazine-1-carboxamide; biocontrol strain of tomato foot and root rot	(Chin-A-Woeng et al., 1998)
Pseudomonas fluorescens WCS365	Excellent competitive root colonizer; biocontrol strain of tomato foot and root rot	(Geels and Schippers 1983; Simons *et al*. 1996)
Pseudomonas fluorescens PCL1285	*Tn5luxAB* derivative of WCS365; comparable with wild type in competitive root colonization ability; Km^r	(de Weert et al. 2004)
Pseudomonas fluorescens PCL1751	Wild-type biocontrol strain, isolated as enhanced competitive root tip colonizer	(Kamilova *et al*., 2005)
Aeromonas hydrophyla PCA0081	Isolated from tomato rhizosphere; risk group 2	(Kamilova *et al*., 2005)
Agrobacterium tumefaciens A334	Plant pathogen; risk group 2	IBL[1]
Burkholderia cepacia JK120	Animal and human pathogen; risk group 2	IPO-DLO[2]
Erwinia carotovora PCA0069	Plant pathogen; risk group 2	IBL
P. aeruginosa PAO1	Opportunistic human pathogen	IBL
P. syringae pv. coronofaciens PCA0074	Plant pathogen; risk group 2	IBL
P. syringae pv. maculicola JK363	Plant pathogen; risk group 2	IPO-DLO
P. syringae pv. syringae JK396	Plant pathogen; risk group 2	IPO-DLO
P. tolaasii NCPPB 1116	Pathogen of cultivated mushrooms; risk group 2	(Munsch et al. 2000)

Table 1 (continued)

Strains	Relevant characteristics	Origin, references
Fungi		
Alternaria alternata ZUM 2372	Isolated from carrot seeds; pathogen of carrot	Novartis Seeds BV[3]
Botrytis cenerea ZUM2076	Causes grey mould of tomato	Novartis Seeds BV
Fusarium oxysporum f. sp. *radicis-lycopersici* ZUM2407	Causes tomato foot and root rot	IPO-DLO
Gaeumannomyces graminis var. *tritici* (Ggt) R3-11A	Causes take-all disease of wheat and other cereals	Novartis Seeds BV
Pythium ultimum LBOP17	Causes of damping-off and fruit rot of tomato	IPO-DLO
Phytophthora cryptogea	Pathogen of tomato and gerbera	IPO-DLO

1 IBL, Leiden, The Netherlands

2 IPO-DLO, Wageningen, The Netherlands

3 Novartis Seeds BV, Enkhuizen, The Netherlands

Results

Analysis of rhizosphere samples

Most samples used as the starting material for the isolation of beneficial rhizobacteria were collected in regions of the Republic of Uzbekistan and in Andalucia, Spain, from greenhouses and fields with a practice of sustainable agriculture (Table 2). Two samples originate from Spanish greenhouses where plants are grown on stonewool and chemicals are used if this is considered necessary for plant protection. These samples represent microbial communities developing in stonewool and contain bacteria adapted to that substrate. Two samples were taken from the Aral See area of Uzbekistan from desert plants growing in soil with a low organic content (Table 2).

The number of colony forming units (CFU's) per gram of rhizosphere soil from greenhouses and the agricultural field varied between 10^6 and 10^8. Similar numbers were found on stonewool threads of greenhouse samples of tomato roots of plants grown on stonewool. The rhizosphere samples taken from the desert plants camel-thorn (*Alhagi pseudalhagi*) and wormwood (*Artemisia maritime*) contained many less bacteria compared to all the other samples used (Table 2).

One hundred and seventy randomly chosen strains representing bacterial communities of all seventeen rhizosphere samples were tested for their Gram character (as evidenced by testing lysis in the presence of 3% KOH), ability to grow on PIA (*Pseudomonas* isolation agar), and their ability to produce lipase and protease. The results are presented in Table 3 (see original sample, under B).

Effect of freeze and spray drying on survival and traits

Samples STII, STIII and Mix (the latter is a mixture of the remaining 15 samples) were subjected to freeze and spray drying to select bacteria resistant to these treatments. The number of CFU's decreased more than 100-fold in all samples due to freeze drying. An even more dramatic drop in CFU's was observed after spray drying: only 0.01% – 0.3% of the cells survived. Comparison of

properties of strains from the original sample with those that had undergone drying (compare B-samples in Table 3) showed that the fraction of Gram-negative bacteria including pseudomonads dropped dramatically during both drying procedures. The same applied for the percentage of producers of lipase and protease.

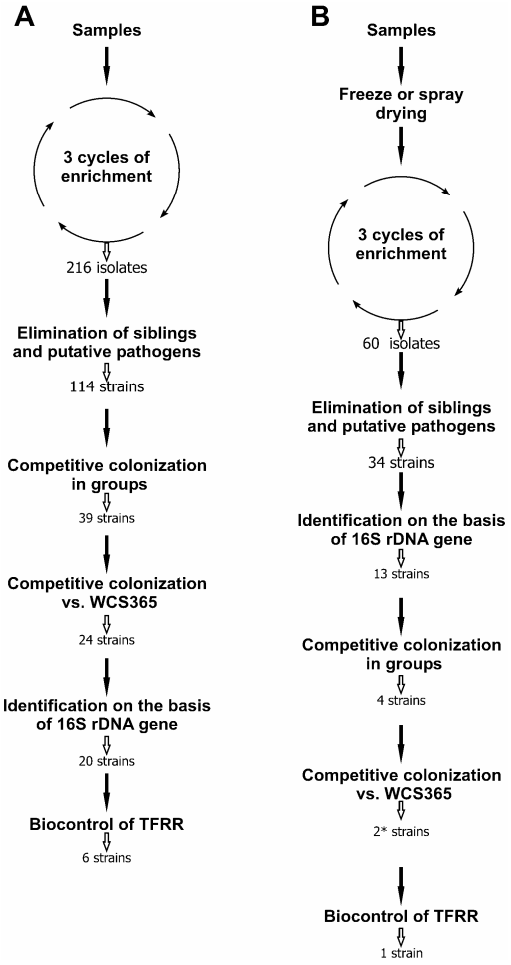

Fig. 1 Strategy for the selection, starting from rhizosphere samples, of enhanced competitive tomato root tip colonizing bacteria (A) and of enhanced competitive root tip colonizing bacteria that also can survive freeze or spray drying (B).

Influence of enrichment for enhanced root tip colonizers on characteristics of strains

Fig.1 illustrates the way various samples were treated. The seventeen original samples from the rhizosphere and the six samples obtained after freeze or spray dryings were treated separately for enrichment in the stonewool gnotobiotic system to select bacteria with the best tomato root tip colonizing activity. The diversity of the morphology of the colonies decreased during the enrichment procedure in all samples (data not shown). Comparison of the characteristics of the samples (Table 2) before (B columns) and after (A columns) colonization enrichment shows that enrichment enhanced the percentages of Gram-negatives, of pseudomonads, and of lipase and protease producers (Table 2).

Table 2. Effect of enrichment for enhanced competitive tomato root tip colonizers on changes of traits of microbial populations [a]

Traits	**Percentage of bacteria with the indicated traits**					
	Original samples		Freeze dried samples		Spray dried samples	
	B	A	B	A	B	A
G-negative bacteria, %	88	98	34	80	2	93
Growth on PIA	18	42	2	25	<1	24
Lipase production	8	11	3	45	<1	30
Protease production	4	28	1	40	<1	30

[a] Original rhizosphere samples and samples that had been subjected to freeze and spray drying procedures were subjected to the enrichment procedure for obtaining enhanced competitive colonizers. This table shows the percentages of bacteria with the indicated traits before (B) and after (A) enrichment for competitive tomato root tip colonization.

Siblings and potential pathogens

Two hundred and seventy six isolates were randomly chosen from the colonies obtained after the third cycle of enrichment (Fig.1). ARDRA was performed on all these isolates to identify siblings that can be present among bacteria originating from the same sample. ARDRA data were compared with those for protease and lipase production, for growth on PIA and for the Gram-character of the strains. The isolates originating from the same sample with indistinguishable phenotypes and patterns of 16S rDNA restriction fragments were considered to be siblings of the same strain. One hundred and sixteen isolates were deleted from the collection as probable siblings.

ARDRA patterns of a set of known pathogenic strains representing human and plant pathogens from risk group 2 (Anonymous, 1998) (Table 1) were compared with the patterns from the remaining 160 isolates to identify potential pathogens among the remaining enhanced colonizers. According to this analysis, twelve isolates were identified as potential pathogens. So, from the 276 isolates, we discarded 116 probable siblings and 12 possible pathogens, which left us with 148 strains for further analysis.

Molecular taxonomy of survivors from freeze and spray drying

After elimination of siblings and potential pathogens, thirty four isolates that survived drying were identified on the basis of the sequence of part of their 16S rDNA. To this end, fragments of 600 to 800 base pairs of 16S rDNA from these isolates were sequenced. BLAST searches in the GenBank database revealed that the strains belong to the following species: *Agrobacterium tumefaciens* (6 strains), *Delftia tsuruhatensis* (6 strains), *Paenibacillus amylolyticus* (5 strains), *Pseudomonas pseudoalcaligenes* (one strain), *Pseudomonas mendocina* (one strain), *Pseudomonas putida* (one strain), *Stenotrophomonas maltophilla* (14 strains). All these species were found in both the freeze and spray dried samples, except *P. pseudoalcaligenes* and *P. mendocina* which were found only in the spray-

dried samples. Strains of *A. tumefaciens, P. mendocina* and *S. maltophilla* belong to risk group 2 (Anonymous, 1998), representing potentially pathogenic bacteria. Therefore, they were excluded from further experiments.

Competitive colonization

Since testing of 127 (114 + 13) (see Fig.1) isolates versus our standard good colonizer *P. fluorescens* WCS365 in competitive colonization assays is very labor-intensive, it was decided to divide the isolates in groups of three or four strains. These groups were chosen in such way that the individual strains could be distinguished based on exo-enzyme production and growth on PIA. The groups were allowed to compete with each other for tomato root tip colonization. This resulted in 43 "winners", each of which was subsequently tested in competition experiments versus *P. fluorescens* WCS365. Those strains which appeared to be equal to, or better colonizers than, *P. fluorescens* WCS365 were retested against the latter strain. Twenty four strains appeared to be reproducibly equal to or better than *P. fluorescens* WCS365 ($P<0.05$). No strain originating from freeze or spray dried samples was equal to or better than *P. fluorescens* WCS365 in colonization of the tomato root tip. Nevertheless two *P. amylolyticus* strains were chosen for biocontrol experiments, since these Gram-positive bacteria are apparently good enough in root colonization to reach the end of the enrichment procedure. Results of competitive colonization for seven strains are given in the Table 3. The number of bacterial cells for strains, which were equal to, or better than *P. fluorescens* WCS365 in root colonization, was around 10^5 cells per 1 cm of root tip, whereas poor colonizers were recovered from the root at approx. 10^3 per 1 cm of root tip.

Molecular taxonomy of enhanced competitive tomato root tip colonizers

The 24 enhanced colonizers were identified on the basis of the nucleotide sequence of their 16S rDNA. BLAST searches in GenBank database revealed that these strains with enhanced colonizing abilities belong to the species *P. putida* (11 strains), *D. tsuruhatensis* (7 strains), *Pantoea agglomerans* (2), *P. chlororaphis* (1),

P. mendocina (1), *Pseudomonas oryzihabitans* (1) and *Pseudomonas rhodesiae* (1). Species affiliation is based on 99% of similarity of 16S rDNA sequence with the corresponding sequences from strains with known taxonomy. Despite our attempt to delete all potential pathogens in an earlier stage, *P. mendocina*, *P. oryzihabitans* and *Pan. agglomerans* species, all belonging to risk group 2 (Anonymous, 1998), were found. We used these strains to extend our data base of potential rhizosphere pathogens.

Table 3. Competitive tomato root tip colonization ability of the newly isolated strains in competition with *P. fluorescens* WCS365 or its kanamycin resistant derivative PCL1285

Competing strains	**Competitive root tip colonization Log10 [(cfu + 1)/cm of root tip]**	
	Test strain	Reference strain
D. tsuruhatensis PCL1755 vs WCS365	5.03±0.74(a)	3.60±0.43(a)
P. amylolyticus PCL1756 vs PCL1285	3.24±1.22(a)	4.31±0.32(b)
P. chlororaphis PCL1757 vs PCL1285	5.09±0.40(a)	4.99±0.47(a)
P. putida PCL1758 vs PCL1285	5.12±0.52(a)	5.13±0.41(a)
P. putida PCL1759 vs PCL1285	5.59±0.33(a)	4.92±0.62(b)
P. putida PCL1760 vs PCL1285	5.52±0.35(a)	4.33±1.25(b)
P. rhodesiae PCL1761 vs PCL1285	5.37±0.40(a)	5.45±0.53(a)

Values in one row followed by a different letter are significantly different from each other ($P \leq 0.05$)

Biocontrol of tomato foot and root rot

Twenty strains originating from the enrichment in the tomato/stonewool system which had not undergone drying were screened for their ability to suppress TFRR in stonewool substrate. Seven strains did not have any influence or even increased the disease severity from 63% in the control to 64 – 75%. Another seven strains decreased the disease pressure slightly but not significantly to 58 – 60%. Six

strains, namely PCL1755, PCL1757, PCL1758, PCL1759, PCL1760 and PCL1761, showed significant biocontrol of TFRR (Fig. 2a). We also tested two *P. amylolyticus* strains, FSTII0 and PCL1756, originating from freeze and spray dried samples, respectively which subsequently had been isolated from the root tip. These Gram-positive strains were outcompeted by *P. fluorescens* WCS 365 in tomato root tip competitive colonization assays. However, the fact, that they had been isolated via the competitive colonization enrichment procedure, indicates that they are among the best colonizers among the survivors of drying procedures. Therefore they were tested for their biocontrol ability. Strain PCL1756 showed biocontrol of TFRR to a level comparable to that of *P. fluorescens* WCS365 (Fig. 2a). All strains that showed significant disease suppression in the first biocontrol experiment were tested again. Despite the fact that the disease pressure was higher in the second experiment (72%) all retested strains showed significant biocontrol (Fig. 2b).

Table 5 gives an overview of the newly isolated biocontrol strains. Their 16S rDNA sequences were deposited in the GenBank database and their accession numbers are shown in Table 4.

Discussion

Recently, Kamilova et al. (2005) described for the first time a method to enrich for biocontrol bacteria, starting from a mixture of rhizosphere bacteria. In the present paper we applied this method on a much larger scale and improved the procedure considerably to make it more efficient. Moreover, we have introduced a variant in which, in addition to enrichment for enhanced colonizers, an additional enrichment step was added to kill those bacteria that could not survive drying procedures which are an essential part of the preparation of many biopesticides.

Table 4. Characteristics of enhanced competitive tomato root tip colonizing biocontrol strains.

Taxonomy [a]	Name	Acc. number in GenBank (16S rDNA)	Production of				Growth		Antagonism towards					
			Protease	Lipase	Biosurfactant	HCN	PIA	37°C	*A. alternata*	*B. cinerea*	*Ggt*	*Forl*	*Ph. cryptogea*	*P. ultimum*
Delftia tsuruhatensis	PCL1755	DQ313378	–	±	–	–	–	–	–	+	–	–	–	+
Paenibacillus amylolyticus	PCL1756	DQ313379	–	–	–	–	–	+	–	–	–	–	–	–
P. chlororaphis	PCL1757	DQ313380	–	+	–	+	+	–	–	–	–	–	+	–
P. putida	PCL1758	DQ313381	–	–	–	–	+	+	–	–	–	–	–	–
P. putida	PCL1759	DQ313382	–	–	+	–	+	+	–	–	–	–	–	–
P. putida	PCL1760	DQ313383	–	–	–	–	+	–	–	–	–	–	–	–
P. rhodesiae	PCL1761	DQ313384	+	–	–	–	+	+	–		+	+	+	–

[a]Based on 16S rDNA sequence comparison

The most useful improvement was the detection of siblings and potential pathogens in an early stage of the procedure. Out of 276 strains 149 strains or 53 percent of the total number fell into this category (Fig.1). In contrast to the original procedure, this improvement saved us from testing these strains in the labor-intensive competitive colonization and biocontrol assays. A further reduction of the work was obtained by testing the potential enhanced colonizers in groups of three or four. We were able to design combinations of distinguishable strains because we had determined the phenotypic properties of all strains in advance (see Table 5). It cannot be excluded, however, that some good strains were lost since we continued with only the "winners" from each group. In contrast to the previous work (Kamilova et al., 2005) we performed the biocontrol tests in stonewool because that is the substrate of choice in modern horticulture.

Enrichment for enhanced colonizers correlated with enrichment for Gram-negative bacteria, pseudomonads and/or lipase- and protease-producers (Table 3).

Drying is part of many procedures for formulation of microbial inoculants (Burges, 1998). In contrast to enrichment for enhanced colonizers, drying selects against Gram-negatives, pseudomonads and lipase- and protease-producers (see Table 3, samples marked B).

The drying procedures that we applied killed most viable rhizobacteria. Apparently these drying procedures are not very suitable for incorporation in a formulation protocol. However, Amiet-Charpentier et al. (1998) and Palmfeldt et al. (2003) have shown that it is possible to formulate non-sporulating bacteria using both freeze and spray drying. It was demonstrated that a methacrylic copolymer carrier, an ethylcellulose and a modified starch product all increase survival of rhizosphere bacteria during spray drying (Amiet-Charpentier et al. 1998; Amiet-Charpentier et al. 1999; Palmfeldt et al. 2003). Some of the enhanced colonizers that survived drying were identified (see Results section). Remarkable is the low percentage of endospore formers. Moreover, this result confirms previous observations in our laboratory (results not shown) which show that *Bacillus* species are poor competitive colonizers. Since good competitive root tip colonization is essential for biocontrol by a biocontrol agent that acts through antagonism (Chin-A-

Woeng et al., 2000), these results suggest that many *Bacillis* biocontrol agents, despite the fact that they produce antibiotics, must also use another mechanism to be good biocontrol agents. Another remarkable observation concerning the strains that survived drying and subsequently underwent colonization enrichment is the under-representation of pseudomonads in comparison with other Gram-negatives such as *S. maltophila*, *Ag. tumefaciens* and *Delftia tsuruhatensis*.

This work resulted in the isolation of seven new TFRR biocontrol agents, including five pseudomonads (Table 5), from seven different rhizosphere samples (Table 2). Six out of these seven strains do not inhibit *Forl* in the plate assay. *P. rhodesiae* PCL1761 was the only antagonist of *Forl* in the plate assay among the biocontrol strains isolated. This confirms the observation of Kamilova et al. (2005) that enrichment for competitive colonizers yields a low percentage of antagonists.
P. putida and *P. chlororaphis* were known previously as biocontrol agents (Bloemberg and Lugtenberg 2001; Chin A-Woeng *et al*. 1998; Pierson, III and Thomashow 1992).

P. rhodesiae was first isolated from natural mineral water (Coroler et al. 1996) and is taxonomically affiliated to the *P. fluorescens* group (Anzai et al. 2000). The representatives of this species were known as degraders of aromatic compounds (Yoon et al. 2002; Kahng et al. 2002) or as isonovalal producers (Fontanille and Larroche 2003), but had not been reported yet as control agents for plant disease.

Seven enhanced colonizers were identified as *D. tsuruhatensis*. *Delftia* is newly classified genus closely related to *Comamonas*. These bacteria were isolated for the first time from active sludge as degraders of terephthalate (Shigematsu et al. 2003). *D. tsuruhatensis* has also been reported as a diazotrophic plant growth promoting rhizobacterium. Strain *D. tsuruhatensis* HR4 was reported to be able to control blast and blight of rice caused by *Xanthamonas oryzae*, *Rhizoctonia solani* and *Pyricularia oryzae* (Han et al. 2005). Our strains of *D. tsuruhatensis* were isolated from the rhizospheres of egg-plant, tomato, sweet pepper and avocado, from both soil and stonewool. This indicates that *D. tsuruhatensis* is widely distributed in the rhizospheres of different plant species.

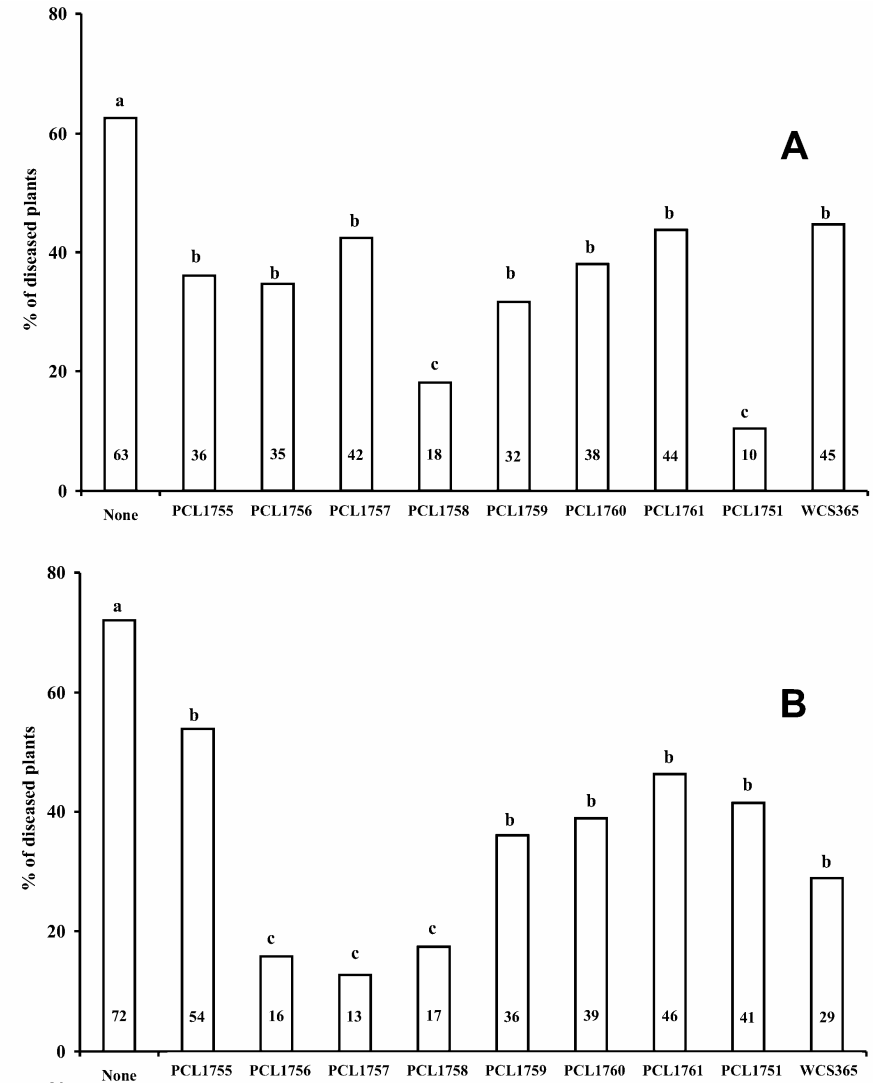

Fig. 2. Biocontrol of Tomato foot and root rot. Seeds were bacterized with bacteria except the negative control (None). Seeds treated with the known biocontrol strains *P. fluorescens* WCS365 and PCL1751 were used as positive controls. The other seeds were treated with bacteria isolated via the enrichment procedure based on competitive tomato root tip colonization. Percentages of diseased plants, as scored 2-3 weeks after inoculation, are indicated inside the bars. Per strain 96 plants in eight trays were tested. Statistically different values are indicated with different letters. A and B represent different experiments.

Of the seven new biocontrol agents, *P. amylolyticus* is the only strain that was isolated after both drying and colonization enrichment. It is an endospore-former. Although it was a good colonizer among the survivors of drying, it loses colonization in competition with *P. fluorescens* WCS365. No antagonistic activity was detected (Table 4). *P. amyloliticus* was reported previously as an endophyte of coffee cherries (Sakiyama et al. 2001) and of entomopathogenic nematodes (Enright et al. 2003). The closely related species *Paenibacillus alvey* can protect *Arabidopsis* against leaf pathogen by triggering systemic resistance of the plant (Tjamos et al. 2005).

Considering the procedure used to isolate these seven new biocontrol strains, we suggest that their mechanism of action likely is, or includes, competition for nutrients and niches. Evidence for this mechanism has been obtained for the first strain isolated using this procedure, *P. fluorescens* PCL1751 (Kamilova et al., 2005).

Acknowledgements

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We thank Prof. Dr. A. Durston for correcting the English.

Chapter 4

Pseudomonas putida strain PCL1760 controls tomato foot and root rot in stonewool using the mechanism "competition for nutrients and niches"

Shamil Validov, Faina Kamilova and Ben Lugtenberg

Abstract

In a previous study, we showed that *Pseudomonas putida* strain PCL1760, can control tomato foot and root rot (TFRR), caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici (Forl),* in stonewool substrate. Since the strain was selected using enrichment for competitive root tip colonizers in stonewool, we hypothesized that biocontrol was based on the mechanism "competition for nutrients and niches". Indeed, in the present study we could show that all other known biocontrol mechanisms are unlikely. To test our hypothesis further, mutants impaired in the uptake of a group of major exudate nutrients, dicarboxylic acids (strain PCL1762; *dctA*) and in motility (strain PCL1764; *flhA*) were constructed. It appeared that mutant PCL1762, but surprisingly not mutant PCL1764, was statistically significant impaired in suppressing TFRR. In contrast to the parental strain and the motility mutant, the *dctA* mutant PCL1762 was not able to stop proliferation of *Forl* in sterile tomato root exudate, the only carbon source available in stonewool. This result indicates that, as expected, competition for nutrients plays a role in the observed disease suppression by the wild type strain. As expected from previous studies, both mutants appeared to be impaired in colonization of tomato roots in a gnotobiotic sand system. However, under biocontrol conditions in stonewool substrate, to which the bacterial cells are added as a cell suspension in plant nutrition solution, both mutants colonized all root parts to a similar extent as the parental strain did. These results indicate that motility does not play an important role, if any, in the biocontrol of TFRR in stonewool by *P. putida* PCL1760. Furthermore, a very high affinity of cells of strain *P. putida* PCL1760 and its derivatives for stonewool was observed. We conclude that the ability to compete for nutrients is crucial for biocontrol of TFRR by *P. putida* PCL1760 in stonewool and that flagellar motility does not play a crucial role because the density of bacteria needed for biocontrol is provided the way of introduction of the biocontrol agent into stonewool.

Introduction

Diseases caused by phytopathogenic fungi are the major threat for agriculture in both field and greenhouse. The introduction of the artificial substrate stonewool created highly controllable conditions for plant cultivation, leading to increased crop production in greenhouses. In new stonewool the young plants start to grow under practically sterile conditions without pathogens. The disadvantage is that they are vulnerable to attack by pathogens. Since many chemical pesticides are banned, protection of plants by beneficial microorganisms against diseases, known as biological control, are environmentally and end-consumer friendly tools in modern agriculture. Practical interest for biological pesticides stimulated research on the mode of action of biocontrol agents (Haas and Defago, 2005). Established mechanisms of biocontrol are (i) antibiosis, (ii) induction of systemic resistance (ISR) in the plant, and (iii) predation and parasitism. The mechanism (iv) competition for nutrients and niches has been proposed, but especially for bacteria, strong experimental support of this mechanism is lacking.

Biocontrol agents acting via antibiosis produce toxic compounds, which can kill the pathogen or, at least, slow down its growth (Haas and Defago, 2005). This allows the biocontrol agent to prevent proliferation of the pathogen and to occupy more space on the root (Ligon et al., 2000). In a number of cases the pivotal role of toxic compounds for biocontrol activity of beneficial microorganisms was confirmed by mutant studies and the isolation and sometimes analysis of the responsible compounds from the rhizosphere (Thomashow et al., 1990; Keel et al., 1992; Chin-A-Woeng et al., 1998; Cazorla et al., 2006; Romero et al., 2007; Weller et al., 2007).

Some beneficial bacteria are able to induce plant defence. After being challenged by such bacteria, plants develop a defence reaction faster upon contact with phytopathogens (van Loon et al., 1998). ISR was observed in plants from different families against various phytopathogens such as mosaic virus (Raupach et al., 1996), *Fusarium oxysporum* (Duijff et al., 1998) and *P. syringae* pv. *tomato* DC3000 (Zipfel et al., 2004).

Fungal phytopathogens can be directly attacked by beneficial microorganisms producing lytic enzymes such as protease, chitinase and β (1,3)-glucanase (Carsolio et al., 1994; Harman et al., 2004; Bolwerk et al., 2005). Products resulting from the destruction of the pathogen can be utilised by the attacking microorganism (Lorito et al., 1996). *Trichoderma spp.* strains are the best known example of such microbes which act through the mechanism of predation and parasitism (Harman et al., 2004; Bolwerk et al., 2005).

Until recently, no bacteria were known to act through the mechanism "Competition for nutrients and niches". Kamilova et al. (2005) used an enrichment procedure in an attempt to isolate such strains. Many of such selected enhanced colonizers were able to control TFRR of plants growing in potting soil. The enrichment procedure was improved and applied to stonewool, resulting in a collection of bacteria which are able to control TFRR in stonewool substrate (Validov et al., 2007).

Effective growth in root exudate is typical for efficient root colonizing bacterial strains: enhanced colonizers as well as the good colonizer *P. fluorescens* WCS365 (Lugtenberg et al., 2001) reach 10-100 times higher cell densities in tomato root exudate than random bacteria isolated from the starting material used for enrichment (Kamilova et al., 2005). In the rhizosphere of tomato grown in stonewool with plant nutrient solution, the only carbon source available is root exudate, in which organic acids (especially citric, succinic and malic acids) are the major components (Kamilova et al., 2006a). Competitive growth in exudate allows the good colonizer *P. fluorescens* strain WCS365 to restrict multiplication of *Forl* (Kamilova et al., 2006b).

Rhizoplane microorganisms do not colonize their niche, the plant root, evenly. Microcolonies of *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 are observed mostly on junctions between plant epidermal cells (Chin-A-Woeng et al., 1997; Bloemberg et al., 2000). Also the pathogen *Forl* ZUM2407 colonizes preferentially the junctions between the epidermal cells (Lagopodi et al., 2002). These results were interpreted as evidence that junctions between the plant cells are the sites where root exudate is released (Lagopodi et al., 2002). It has been shown that

motility is a major mechanism which allows the biocontrol agent to reach sites on the root surface which are rich in root exudate (de Weger et al., 1987; Lugtenberg et al., 2001). Recently the role of motility in biocontrol was defined more precisely and it was shown that chemotaxis of the biocontrol bacterium towards certain root exudate compounds is the crucial trait (de Weert et al., 2002).

In the present paper we address the mechanism of biocontrol of TFRR used by the enhanced competitive root colonizer *P. putida* PCL1760 in stonewool substrate. We focused on the hypothesis that biocontrol is based on competition for nutrients and niches.

Materials and Methods

Strains and growth conditions

The characteristics of bacterial and fungal strains used in this study are listed in Table 1. Bacteria were grown in LB (Difco Laboratories, Detroit, MI, USA). When needed, kanamycin (Km) and tetracyclin (Tc), both from Duchefa (Haarlem, The Netherlands), were added to final concentrations of 50 and 40 μg/ml, respectively. *Pseudomonas* Isolation Agar (PIA; Difco Laboratories), supplemented with Km, was used for selection of transconjugants of *P. putida* PCL1760 and for the isolation of Km resistant mutants from stonewool at the end of the biocontrol experiment. *Forl* strain ZUM2407 was grown on Potato Dextrose Agar (PDA) or Capek-Dox Agar (CDA), both from Difco Laboratories. To produce microspores, PDA pieces with hyphae of *Forl* ZUM2407 were placed in Czapek-Dox broth and incubated under vigorous aeration at 28°C for 72 hours. The fungal culture was filtered through cheese cloth to remove agar pieces and mycelium. The microspore concentration in the filtrate was determined using light microscopy.

For all experiments in which bacterial cell suspensions were needed, wild type strain PCL1760 and its mutants were grown overnight in LB under vigorous aeration at 28°C and washed twice in phosphate buffered saline (PBS), pH 7.0 (Sambrook and Russell 2001). Cell suspensions were adjusted to OD620=0.1, which corresponds with approximately 10^8 CFUs/ml.

Construction of PCL1760 mutant strains

The Polymerase Chain Reaction (PCR) was carried out with *Taq* polymerase (Fermentas GmbH, St. Leon-Rot, Germany). Primers were synthesized by Isogen Life Science (IJsselstein, The Netherlands). Restriction enzymes and T4 DNA-ligase were purchased from Fermentas GmbH. These enzymes were used according to the manufacturer's recommendations. The plasmids and primers used in this study are listed in Tables 1 and 2, respectively. For mutagenesis of *dctA* and *flhA* genes, internal fragments of these two genes were amplified with primers complementary to conserved regions of the *dctA* and *flhA* genes of *P. aeruginosa* PAO1 (Acc.No. NC002516), *P. fluorescens* Pf5 (NC004129), *P. syringae* pv. *tomato* DC3000 (NC004633), and *P. putida* KT2440 (NC002947). *Eco*RI sites were introduced in 5' ends of primers (Table 2). Nucleotide sequences of the amplified fragments were determined by ServiceXS (Leiden, The Nederlands) and were subsequently processed with Vector NTI 10.1.1. software package (Invitrogene Corp., Breda, The Netherlands). Similarity searches in GenBank were performed using BLASTN (Altschul et al.,1990; http://www.ncbi.nlm.nih.gov/blast/). The obtained PCR products were digested with *Eco*RI and subsequently separated in a gel containing 1% agarose in 0.5×TBE buffer (Sambrook and Russell 2001). Bands of the expected sizes were cut out from the gel and purified using the QIAGen gel purification kit (QIAGEN Benelux BV, Venlo, The Netherlands). Purified fragments were cloned into the *Eco*RI site of pME5285, resulting in pMP7701 and pMP7702 (Table 1).

Table 1. Microorganisms and their relevant characteristics.

Strains, plasmids	Relevant characteristics	Origin, references
Wild type bacterial strains		
Pseudomonas fluorescens WCS365	Excellent competitive root colonizer; biocontrol strain of tomato foot and root rot	Simons et al. 1996; Dekkers, 1997
Delftia tsuruhatensis PCL1755	Biocontrol strain, isolated as enhanced competitive root tip colonizer	Validov et al. 2007
Paenibacillus amylolyticus PCL1756	Biocontrol strain, isolated after spray drying	Validov et al. 2007
Pseudomonas chlororaphis PCL1757	Biocontrol strain, isolated as enhanced competitive root tip colonizer	Validov et al. 2007
Pseudomonas putida PCL1758	Biocontrol strain, isolated as enhanced competitive root tip colonizer	Validov et al. 2007
Pseudomonas putida PCL1759	Biocontrol strain, isolated as enhanced competitive root tip colonizer	Validov et al. 2007
Pseudomonas putida PCL1760	Biocontrol strain, isolated as enhanced competitive root tip colonizer	Validov et al. 2007
Pseudomonas rhodesiae PCL1761	Biocontrol strain, isolated as enhanced competitive root tip colonizer	Validov et al. 2007
Bacterial Mutants		
PCL1762	Derivative of PCL1760 impaired in dicarboxylic acid uptake, *dctA*::pMP7701, Km^R	This study
PCL1764	Non-motile derivative of PCL1760, *flhA*::pMP7702, Km^R	This study
Fungal strain		
Fusarium oxysporum f. sp. *radicis-lycopersici* ZUM2407	Causes tomato foot and root rot	IPO-DLO, Wageningen, The Netherlands

Table 1 (*continued*)

Plasmids		
pMP5285	Suicide plasmid for *Pseudomonas spp.*; used for homologous recombination, Km^R	Kuiper et al.,(2001)
pRK2013	Helper plasmid for transconjugative transfer	Ditta et al.,(1980)
pMP7701	pMP5285 with inserted in *Eco*RI site the 750 bp fragment of *dctA* gene amplified from PCL1760 chromosome	This study
pMP7702	pMP5285 with inserted in *Eco*RI site the 1050 bp fragment of *flhA* gene amplified from PCL1760 chromosome	This study

Table 2. Oligonucleotides used.

Primers	Oligonucleotide Sequence (5′ – 3′)	Target Regions
oMP1120[1]	TTTTTGAATTCCAGCAGTACCAGCAGCAGGGTGAT	*dctA*[2]
oMP1121	AAAAAGAATTCTTCTCGGTGCTGTTCGGCTTCG	*dctA*[2]
oMP1195	ATGACGACACGTCAGCCGCTGTAC	5′-end of *dctA*[2]
oMP1191	TTTTTGAATTCGTGATCCAGGCCTTCGGTGA	flh*A*[2]
oMP1192	AAAAAGAATTCAAACCGTCACCGATGGTCAGCA	flh*A*[2]
oMP1193	ATGGATCGCACTCAGTTAATCAGCAACG	5′-end of *flhA*[2]
oMP686	TTAAGTTTATTCTTATCAATATAGG	pMP5285[3]
oMP1197	GTCAGGGCAA	Operon G19; Clerc et al. (1998)

[1] Introduced *Eco*RI sites are underlined
[2] *dctA* and *flhA* regions are homologous to those of *P. putida* KT2440
[3] The primer is annealing close to the MCS of plasmid pMP5285 (Table 1)

These vectors were introduced into strain PCL1760 for single homologous recombination by triparental mating using helper plasmid pRK2013 (Ditta et al., 1980). Transconjugant clones of PCL1760 were selected on PIA supplemented with Km.

The obtained mutants were verified by amplification of the flanking regions from the genomic DNA of mutants using primers oMP686 and oMP1195 for the *dctA* gene and primers oMP686 and oMP1193 for the *flhA* gene (see Table 2). Nucleotide sequences of the PCR fragments were determined by ServiceXS and compared using BLASTN.

Loss of motility of the *flhA* mutant was checked on 1/20 KB medium containing 0.3% agar (Difco Laboratories). To this end, clones of interest were spot-inoculated in the middle of a Petri dish. PCL1760 was used as a positive control. Clones which could not spread from the spot of inoculation were considered as non-motile.

For the *dct*A mutant, inability to grow on succinate and malate was tested on M9 medium (Sambrook and Russell 2001) to which these dicarboxylic acids were added as the sole carbon source. The mutants were also checked in competition with wild type strain PCL1760 for their relative growth rate in LB broth. For this purpose overnight cultures of PCL1760 and its mutants were prepared as described in the section "Strains and growth conditions" and mixed in a ratio 1:1. The mixtures were inoculated in LB to a final concentration 10^4 CFUs/ml. The mixtures were grown for 24h and subsequently reinoculated in fresh LB broth to a final concentration of 10^4 CFUs/ml. In total this procedure was carried out three times. The actual ratio of wild type PCL1760 and its mutant was determined by plating of serial dilutions on LB agar with and without Km in the beginning of the test and after every 24h of incubation.

Competitive growth of bacteria and Forl in tomato root exudate

Sterile tomato root exudate obtained as described by Kamilova et al., (2006a) was used for cultivation and co-cultivation of bacterial and fungal strains. Cultures of PCL1760 and its mutants were prepared as described in the

section "*Strains and growth conditions*". Bacterial cultures and fungal spores were introduced in 2.0 ml tomato root exudate to a final concentration of 10^5 cells/ml. Each variant was carried out in triplicate. Inoculated and uninoculated control samples were incubated at 28°C under vigorous aeration (190 rpm) for 72 hours. To determine the numbers of bacterial cells and fungal propagules in tomato root exudate, serial dilutions were plated after 0, 24, 48 and 72 hours on LB agar and CDA. The latter medium was amended with Tc to prevent bacterial growth. Colonies were counted after 24 – 48 hours of growth at 28°C.

Tomato root colonization assay in quartz sand

Cell suspensions for inoculation of tomato seedlings were prepared as described in the section "*Strains and growth conditions*". Sterile tomato seedlings were dipped in the mixture of cells and incubated for 10 minutes. Inoculated seedlings were sown in sterile glass tubes containing quartz sand and plant nutrient solution (Simons et al., 1996). After incubation for 7 days, one cm pieces of base, middle part and tip were isolated and shaken in PBS. Dilutions of bacteria released from every part of the root were plated separately on solid LB agar. For each strain five plants were used.

To compare colonizing abilities, cells of wild type *P. putida* strain PCL1760 and its mutants were mixed in a ratio 1:1. Ten sterile seedlings of tomato were inoculated with the mixtures. After seven days of growth, bacteria colonizing the root tip of tomato plantlets were recovered as described above. Dilutions of bacteria released from every single root tip were plated separately on solid LB and on LB agar supplemented with Km. The experiment was performed twice. The average numbers of the various bacteria as well as the standard deviation were calculated. The non-parametric Wilcoxon-Mann-Whitney test (Sokal and Rohlf, 1981) for mixed inocula was used to perform statistics.

Tomato root colonization assay in stonewool

Sterile tomato seeds were sown in stonewool moistened with sterile Plant Nutrition Solution (PNS; PPO, Naaldwijk, The Netherlands) containing 10^7 cell/ml of the bacterium of interest. Plants were harvested after seven days of

growth and bacteria were recovered from one cm pieces of the base, the middle part and the tip of the root. The numbers of cells was determined by plating of serial dilutions of the bacteria washed off the root.

Attachment of P. putida strain PCL1760 to stonewool

To determine the numbers of bacterial cells that attach to stonewool, six ml of cell suspension in commercial PNS with a cell density of 10^7 cell/ml was applied per stonewool plug (Grodan BV, Roermond, The Netherlands). This volume was used because six ml is the maximum amount which can be held by a single plug. Plugs saturated with cell suspension of *P. putida* strain PCL1760 were incubated for one hour at room temperature and the aqueous phase was pressed out and collected. Plugs were washed twice with 6 ml of PNS and the flow-through liquid was collected. The cell concentration in the combined aqueous phases was determined using plating of serial dilutions on LB agar. Numbers of attached cells were calculated as the difference between numbers of introduced bacteria and numbers of bacteria in flow-through PNS.

Biocontrol of Tomato Foot and Root Rot

Biocontrol of TFRR was carried out in stonewool plugs. Spores of *Forl* were obtained and counted as described in the section "*Strains and growth conditions*". Plastic trays with 96 stonewool plugs were soaked in 1L of PNS supplemented with *Forl* ZUM2407 microspores (2×10^6 spores per L PNS) and cells of the strain of interest (10^7 cells per mL). Seeds of tomato var. Carmello were sown in stonewool, covered with 0.5 g of vermiculite, and allowed to germinate in the dark at 23°C for 72h. Once germinated, the seedlings were kept at 21-23°C, 80% relative humidity and 16h of daylight. Biocontrol was scored after 17-21 days. Dead plants or plants with brown spots or lesions on their feet/roots were considered as diseased. 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), using *SPP*S-software (*SPP*S Inc., Chicago, IL, USA). All experiments were performed at least twice.

To determine colonization by bacteria, the roots from eight tomato plants were removed from the stonewool plugs and placed in 5 ml of sterile PBS. The roots were shaken in PBS for 1 h at 4°C. For each variant of bacterial inoculation 40 plants were analyzed. The stonewool (500 mg) from the plugs in which tomato plantlets had grown was sampled and homogenized in FastPrep FP220A (MP Biochemicals, Amsterdam, The Netherlands) with 0.5 ml of PBS at top speed for 30 s. Five samples were taken from the each variant. Serial dilutions of washes from the roots and the stonewool were plated on LB and PIA. The latter medium was supplemented with Km if the strain used for inoculation was resistant to this antibiotic.

Random Amplified Polymorphic DNA (RAPD)

To distinguish the introduced strains from other bacteria isolated from stonewool and plant roots at the end of a biocontrol experiment, 30 colonies were picked up from PIA plates and compared with *P. putida* strain PCL1760 using the RAPD method. To generate RAPD profiles, fresh bacterial colonies were picked from the plate and resuspended in 1% NaCl. The optical density at 620 nm of the suspension was adjusted to 0,1. Cells were lysed by incubation of the suspensions at 100°C for 5 min. The suspensions were spun at 14.000g for 5 min. Five µl of cleared cell lysate were used to carry out a PCR reaction with the primer oMP1197 (Operon G19) as described in Clerc et al. (1998). The DNA fragments were separated on a 2% agarose gel. Ladders GeneRuler 100 plus (Fermentas GmbH, St. Leon-Rot, Germany) was included in order to normalize the banding pattern of the RAPD profiles. Digitalized images of the RAPD patterns were converted, normalized, combined, and analyzed with Quantity One software (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). A band-matching algorithm (band matching tolerance of 0.75%) was selected to calculate pairwise similarity matrices with the Dice coefficient. Cluster analysis of similarity matrices was performed by the unweighted pair group method using arithmetic averages (Sneath and Snokal, 1973). Cells of the closely related *P. putida* strain PCL1758 was used as the negative control in

comparison of profiles. Colonies clustered together with PCL1760 were considered as colonies of introduced strain. Uninoculated control (with *Forl* only) scored negatively.

Tomato seed germination

The percentage of germinated seeds was determined by counting the number of tomato plantlets in biocontrol experiments 2.5-3 weeks after sowing. All plugs without tomato plantlet were examined for the presence of the seed. If the seed was present it was considered as failed in germination. In total 384 seeds were analyzed for each variant (negative control and all strains tested).

Induction of systemic resistance against TFRR (Kamilova et al.*, 2005)*

Thirty plants were grown per treatment. The root system and hypocoteledons of 3-week-old tomato plants of cv. Moneymaker (Rijnsburg Zaadhandel, Rijnsburg, The Netherlands) were split in two. Each half of the root was replanted in a separate pot, keeping the stem intact. After one week, one part of the root system was inoculated with bacteria (10^9 cfu per plant in 5 ml PBS) or with sterile PBS for control plants. One week later, the other part of the root system was challenged by adding 5 ml of PBS containing 10^6 *Forl* spores to the pot. Three weeks after challenging, the roots were analyzed for the presence of lesions. Plants inoculated with *P. fluorescens* strain WCS365 were used as positive control (Kamilova et al., 2005). To score the disease level, the following indexing system was used: healthy plants were given a value of 0, plants with one lesion on the root were given a value of 1, plants with two, three and four lesions were given a value of 2, 3 and 4, respectively. The value 5 was given to the plants with five or more root lesions. The difference in disease index between two different treatments was statistically analyzed using chi-squared goodness-of-fit test (Heath, 1995).

Results and Discussion

Criteria of plant health and human safety used for the selection of the strain to be investigated

Biocontrol agents, meant for application as products in agriculture, must meet many criteria. Bacterization should result in efficient and reproducible biocontrol without side effects such as inhibition of seed germination. Moreover, the active agent should not belong to a bacterial species that is related to potential or real pathogens (Anonymous, 1998). Also, the inability to grow at 37°C would eliminate the risk for human heath. Based on these criteria the following evaluation was made for the seven enhanced colonizing biocontrol strains PCL1755 through PCL1761 isolated by Validov et al. (2007) (Table 1).

Biocontrol efficacy of the seven strains was determined four times. Controls included uninoculated seeds as well as seeds inoculated with the good colonizer *P. fluorescens* WCS365 that previously has shown consistent biocontrol in both potting soil and stonewool. The average biocontrol results of these four experiments are depicted in Fig. 1. All seven new strains showed significant biocontrol ability. *Pn. amylolyticus* strain PCL1756 suppressed TFRR significantly in only two out of four experiments. The two *P. putida* strains PCL1758 and PCL1759 showed biocontrol in all four experiments, but the level of disease suppression varied strongly. Moreover, these strains can grow at 37°C. *D. tsuruhatensis* strain PCL1755 decreases tomato seed germination from 95% in the negative control to 89% (significant difference, $P<0.05$; data not shown). The remaining three strains isolated from stonewool enrichment, *P. chlororaphis* PCL1757, *P. putida* PCL1760 and *P. rhodesiae* PCL1761, showed high disease suppression (around 50%) in all four independent experiments. In conclusion, out of the seven biocontrol strains tested, *P. chlororaphis* PCL1757, *P. putida* PCL1760 and *P. rhodesiae* PCL1761, are suitable for application when all used criteria of biocontrol, germination and biosafety are applied.

Criteria used to exclude other mechanisms in the selection of the strain to be investigated

From the point of view of testing the mechanism "competition for nutrients and niches" of the strain to be investigated, it would be convenient when the strain does not use other mechanisms. Since PCL1757 produces HCN and phenazine-1-carboxamide, antibiosis may play a role. Similarly, since PCL1761 produces protease and can inhibit *Forl* ZUM 2407 *in vitro*, this strain might control TFRR via the mechanisms antibiosis or "predation and parasitism".

P. putida PCL1760 does not inhibit fungal strains *in vitro*, and does not produce N-acyl homoserine lactones, which makes antibiosis unlikely. Similarly, it is unlikely that predation and parasitism is used by *P. putida* PCL1760, since

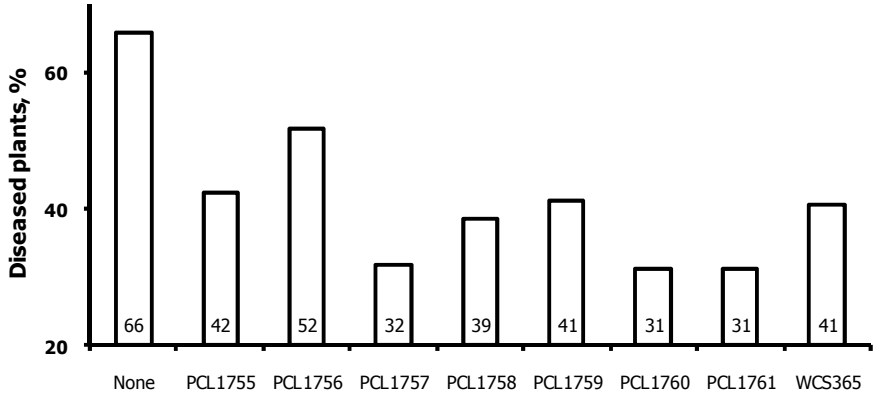

Fig.1. Biocontrol of TFRR by enhanced colonizers (Validov et al., 2007) under stonewool conditions. The score results of four independent experiments were joined and average values of biocontrol were plotted. Statistical analysis was performed on joined sets of plants. All strains show significant difference with negative control (None), where only spores of *Forl* ZUM2407 were added. Treatment with *P. fluorescens* WCS365 was used as a positive control.

this strain produces no protease, glucanase or chitinase (Validov et al., 2007).

Depletion of iron ions can limit growth of deleterious and pathogenic rhizosphere bacteria and fungi, thereby protecting plants from diseases (Buyer and Sikora, 1990; Thomashow and Weller, 1990; Lemanceau et al., 1992). Like many other pseudomonads, *P. putida* PCL1760 can produce Fe^{3+} scavenging siderophores under iron limitation and may inhibit pathogens by limitation of

this ion in alkaline soil. However, the plant nutrient solution which is used for growing tomato plants in stonewool contains as much as 50 mM of Fe^{3+}. Therefore it is unlikely that siderophore production by *P. putida* PCL1760 plays a role in the biocontrol activity of this strain in stonewool.

To test induction of systemic resistance as a possible mechanism of biocontrol by *P. putida* PCL1760, we used a tomato split-root system. *P. fluorescens* strain WCS365, known to trigger ISR (Kamilova et al., 2005), was used as a positive control in our tests. Treatment of one part of the tomato root with strain WCS365 significantly decreased the disease index from 4,5 in the negative control to 3,3 ($P<0.05$). Strain PCL1760 also caused a decrease in the disease index (3,5) but the difference with the control was not statistically significant. In the second experiment *P. fluorescens* WCS365 again reduced the disease index significantly from 2,9 in the negative control to 2,3 ($P<0.05$) whereas *P. putida* strain PCL1760 did not cause a significant resistance against TFRR. In conclusion, in two independent experiments, in which the disease pressure was quite different, *P. fluorescens* strain WCS365 showed a statistically significant reduction of the disease index. In contrast, *P. putida* strain PCL1760 demonstrated no significant reduction of the disease on tomato roots in either experiment.

We conclude that, since the mechanisms antibiosis, predation and parasitism, depletion of iron and ISR can practically be excluded to be involved in the biocontrol of TFRR under stonewool conditions by *P. putida* strain PCL1760, the strain is likely to act mainly or completely through competition for nutrients and niches. The observation that the strain appeared to be susceptible to kanamycin, tetracyclin, streptomycin and gentamycin is helpful for mutant studies required to test further mechanistic aspects.

Rationale for studying mechanistic aspects of "competition for nutrients and niches"

The mechanism competition for nutrients and niches is supposed to be firstly based on the ability of the biocontrol agent to utilize nutrients from the

rhizosphere for its own multiplication, thereby out competing the pathogen. Secondly, the biocontrol agent is assumed to colonize the strategically important exudation sites on the root surface faster than the pathogen. Obviously, there is an interrelationship between these two abilities. Colonization of exudation sites is required for efficient nutrient uptake. Nutrient uptake is necessary for cell multiplication and for providing energy to travel along the roots towards exudation sites.

The composition of root exudates of tomato, cucumber and sweet pepper grown in stonewool is quite similar and contain as major carbon sources organic acids and sugars (Kamilova et al., 2006-a). Organic acids represent the major fraction of utilizable carbon. Citric acid is the major organic acid, whereas dicarboxylic acids such as succinic and malic acid constitute approximately 30% of all organic acids in root exudates of tomato grown in stonewool (Kamilova et al., 2006-a). Uptake of dicarboxylic acids in many γ-proteobacteria is carried out by a specific transporter protein, which is encoded by the *dct*A gene. A *dct*A-deficient mutant of *P. chlororaphis* O6 does not grow on minimal medium containing dicarboxylic acids as the sole carbon source, but it grows on citrate, glucose, fructose, sucrose and inositol as fast as wild type strain (Nam et al., 2003). We decided to construct a *dctA* mutant of the biocontrol strain *P. putida* 1760 to test whether its expected inability to use part of the root exudate nutrients would result in a decreased ability to compete with *For1* in isolated tomato root exudate and in a decrease of biocontrol efficacy.

Competition for niches requires effective root colonization. Of the established traits involved in colonization, motility of the strain is perhaps most crucial (Lugtenberg et al., 2001). Later investigations showed that in fact chemotaxis towards the exudate compounds malic acid and citric acid (de Weert et al., 2002) is the basis for the requirement of motility for competitive colonization. To render *P. putida* strain PCL1760 non-motile we decided to disrupt its *flhA* gene. FlhA protein is part of the motor transport apparatus, which carries out translocation of all external compounds of the flagellum across the inner membrane and indirectly derepresses the genes coding for

structural elements of the flagellum (Berg, 2003). A non-motile mutant was repeatedly proven to be impaired in competitive root colonization in a gnotobiotic test system as well as in potting soil (Lugtenberg et al., 2001). If this is also the case for the *flhA* mutant of PCL1760, the causal relationship between competition for niches and biocontrol efficiency will be tested.

Construction and characterization of a mutant impaired in dicarboxylic acid uptake

An internal 753 bp fragment of the *dctA* gene was amplified using oMP1120 and oMP1121 (Table 2). Nucleotide sequencing of the obtained fragment showed 99% of homology with the sequence of the *dctA* gene from *P. putida* KT2440. The fragment was cloned in plasmid pMP5285 and the resulting construct was designated as pMP7701. Mutants impaired in growth on succinate as the sole carbon source were obtained by disruption of the *dct*A gene. For this purpose pMP7701 was transferred to *P. putida* strain PCL1760. Km resistant clones were tested for growth on M9 media containing the dicarboxylic acid succinate or the tricarboxylic acid citrate as the sole carbon source. None of the 20 checked clones was able to grow on M9/succinate, but all grew well on M9/citrate. To test their environmental fitness, the mutants were tested for competitive growth against their parental strain in LB medium. A mutant, which was equal in the growth rate to parental strain *P. putida* PCL1760 in LB broth, was designated as PCL1762. Correct placement of the pMP7701 plasmid (see Table 1) in the chromosome of PCL1762 was confirmed by amplification of the adjacent region using the primer pairs oMP686 and oMP1195 (Table 2). Blast searches showed that the nucleotide sequence of the obtained PCR fragment shares 99% homology with the 5' end of the *dctA* gene from *P. putida* KT2440. Mutant PCL1762 is unable to grow on minimal media with succinate or malate as the sole carbon source. In both performed biocontrol trials (Fig.2) mutant PCL1762 was less efficient than parental strain *P. putida* PCL1760.

When single strains were tested in colonization in quartz sand after seed inoculation, it appeared that the *dct*A mutant PCL1762 was impaired in colonization of the middle and lower root parts (Fig. 3).

Growth experiments in tomato root exudate, of which the organic acid fraction contains 30% dicarboxylic acids (Kamilova et al., 2006-a), showed that mutant PCL1762, which is impaired in the uptake of dicarboxylic acids, grew

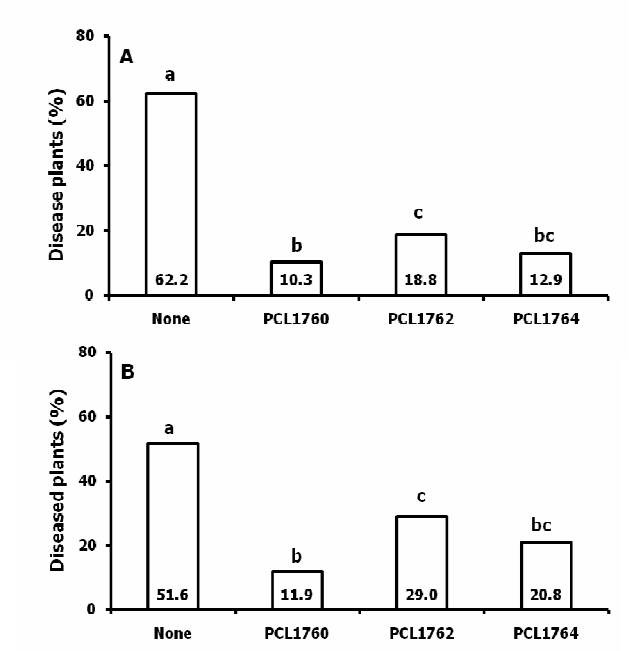

Fig.2. Biocontrol of TFRR in stonewool by *P. putida* PCL1760 and its mutants PCL1762 (*dctA*) and PCL1764 (*flhA*). Panels A and B represent two independent trials. Statistically different variants are marked with different letters.

slower than its parental strain and reached a lower final cell density (5×10^7 CFUs/ml) after 48 h of incubation, whereas the wild type strain PCL1760 reached its highest density (2×10^8 CFUs/ml) already after 24 h (Fig.4A). These growth defects of the mutant in root exudate explain its root colonization defect in quartz sand (Fig. 3).

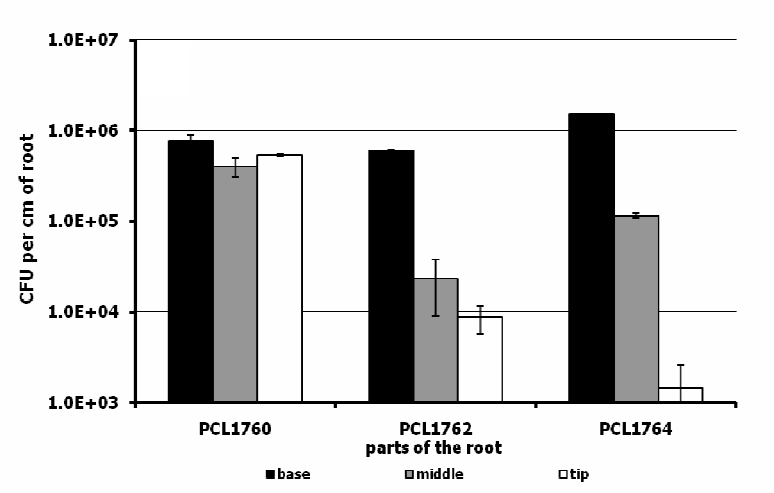

Fig. 3. Colonization of the different parts of tomato by single strain of *P. putida* strain PCL1760 its mutants PCL1762 (*dctA)* and PCL 1764 (*flhA*) in the gnotobiotic sand – PNS system.

Summarizing, the *dctA* mutant is less efficient in biocontrol of TFRR in stonewool than its parental strain (Fig.2). It is less able than its parental strain to inhibit growth of *Forl* in tomato root exudate (Fig. 4), and it colonizes the root as well as the parental strain under biocontrol conditions in stonewool (Fig.5). We conclude that the biocontrol ability of wild type strain PCL1760 is due to competition with *Forl* for exudate nutrients.

Motility mutant of P. putida strain PCL1760 and its characteristics

Conserved regions were used to develop the primer pair for amplification of an internal fragment of the *flh*A gene (Table 2). The nucleotide sequence of the fragment, obtained by PCR with oMP1191 and oMP1192 and chromosomal DNA of strain PCL1760, shares 99% similarity with *flh*A gene of *P. putida* KT2440. The internal fragment of *flh*A gene was cloned in pMP5285. The resulting construct was designated pMP7702 and was transferred to *P. putida* strain PCL1760. Twenty Km resistant clones of PCL1760 resulting from the transconjugation experiment were tested for motility. Ten non-motile mutants were checked for competitive growth with parental strain *P. putida* PCL1760.

Mutants which were growing as good as the parental strain were tested for integration of the pMP7702 plasmid into the chromosome of *P. putida* strain PCL1760 by amplification and sequencing of the flanking regions. The nucleotide sequence of the amplified fragment appeared to be 99% similar to the 5'-end of *flh*A gene of *P. putida* KT2440. One of the non-motile mutants with confirmed presence of pMP7702 inside the *flh*A gene was designated as PCL1764.

In both performed biocontrol trials mutant PCL1764 controlled TFRR to a level not significantly different from that performed by the parental strain (Fig 2).

When single strains were tested for root colonization in quartz sand after seed inoculation, it appeared that the mutant colonized the middle root part and the root tip worse than the wild type (Fig. 3), as expected for a non-motile mutant (Lugtenberg et al., 2001).

When tested for growth rate and maximum growth level in tomato root exudate, PCL1764 grew (Fig. 4A) and stopped proliferation of *Forl* ZUM2407 (Fig. 4B) like the wild type strain PCL1760 did.

Kamilova et al. (2005) described that a non-motile mutant of biocontrol strain *P. fluorescens* PCL 1751, a strain isolated in a similar way as PCL1760, and therefore expected to control TFRR by competition for niches and nutrients, was impaired in root colonization in sand as well as in biocontrol of TFRR in potting soil (Kamilova et al., 2005). We observed the same colonization defect for *P. putida* mutant PCL1764, which however, was not impaired in biocontrol of TFRR (Fig.2). To explain the different results, we tested colonization of the root system in stonewool. It appeared that during biocontrol in stonewool the flhA mutant colonizes the tomato root as good as its parental strain (Fig.5A).

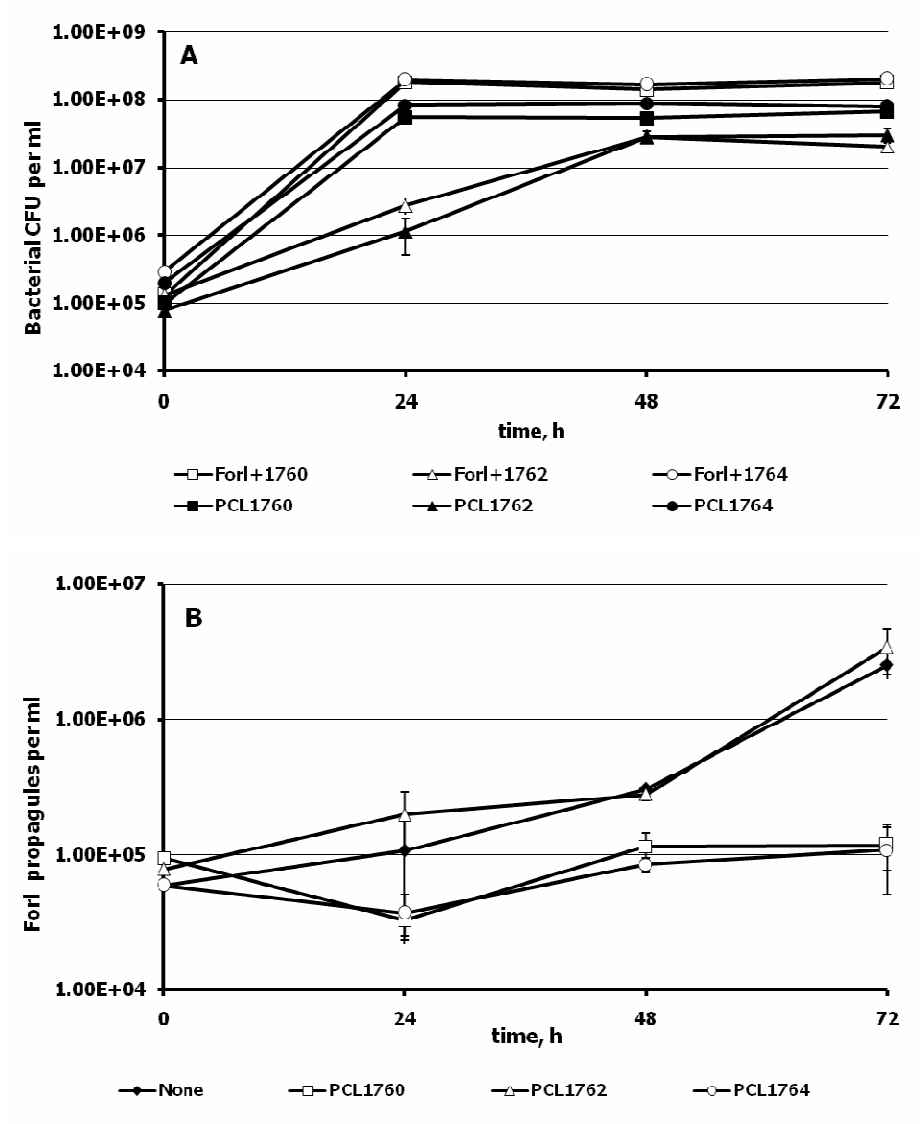

Fig.4. Competitive growth of *ForI* ZUM2407 against *P. putida* PCL1760 and its mutants PCL1762 (*dctA)* and PCL1764 (*flhA)* in tomato root exudate. Panel A represents the growth of the bacterial strains in the absence (closed symbols) or presence (open symbols) of *ForI* ZUM2407. Panel B shows numbers of *ForI* ZUM2407 propagules in the absence (closed symbol) and presence (open symbols) of PCL1760 and its mutants PCL1762 and PCL1764.

This explains the good biocontrol in stonewool by the non-motile mutant (Fig.2). Furthermore, since PCL1760 and its derivatives appeared to colonize the stonewool to levels of at least 10^5 CFUs per plug (Fig. 5B), it is likely that during biocontrol in stonewool, to which the bacteria were added at the beginning of the experiment, bacteria firmly attach to the stonewool. This was confirmed by applying cell suspensions in a concentration of 10^7 CFU/ml to stonewool. It appeared that 85 – 90% of the cells from the introduced suspension of parental strain PCL1760 and its mutants PCL1762 and PCL1764 remain attached to stonewool. Consequently, it is likely that during biocontrol in stonewool the growing root continuously picks up bacteria from the stonewool, making flagellar motility relatively unimportant in this substrate.

Summarizing, the *flhA* mutant is not significantly impaired in biocontrol of TFRR in stonewool (Fig.2), is as competitive as the parental strain for exudate nutrients (Fig. 4), and colonizes the root as well as the parental strain under biocontrol conditions in stonewool (Fig.5). We conclude that competition of *P. putida* PCL1760 with *Forl* for the root niche/space by flagellar motility does not play an important role in stonewool substrate. This is different from potting soil in which flagellar motility of strain *P. fluorescens* PCL1751 was shown to be important for a strain which likely acts by the same mechanism (Kamilova et al., 2005). We conclude that flagellar motility plays different roles during biocontrol in the substrates stonewool and potting soil. The explanation probably is that in potting soil the bacteria are applied on seeds whereas in stonewool experiments the biocontrol bacteria are distributed as a suspension over the stonewool plug and subsequently stick firmly to stonewool from which they are easily colonizing the passing growing root. A very interesting observation was made when also the culturable associated microflora was quantified. At the end of the biocontrol experiment, three weeks after inoculation, the parental strain appeared to be present on the root in higher numbers than the culturable associated microflora (Fig. 5A). In contrast, the associated microflora was more dominant in stonewool. From these results we conclude that during germination and young seedling growth, growth stages in which the plant is most vulnerable for

pathogen attack, the enhanced colonizing biocontrol bacterium *P. putida* PCL1760 is by far the most dominant strain on the root and therefore is in an excellent position to control TFRR.

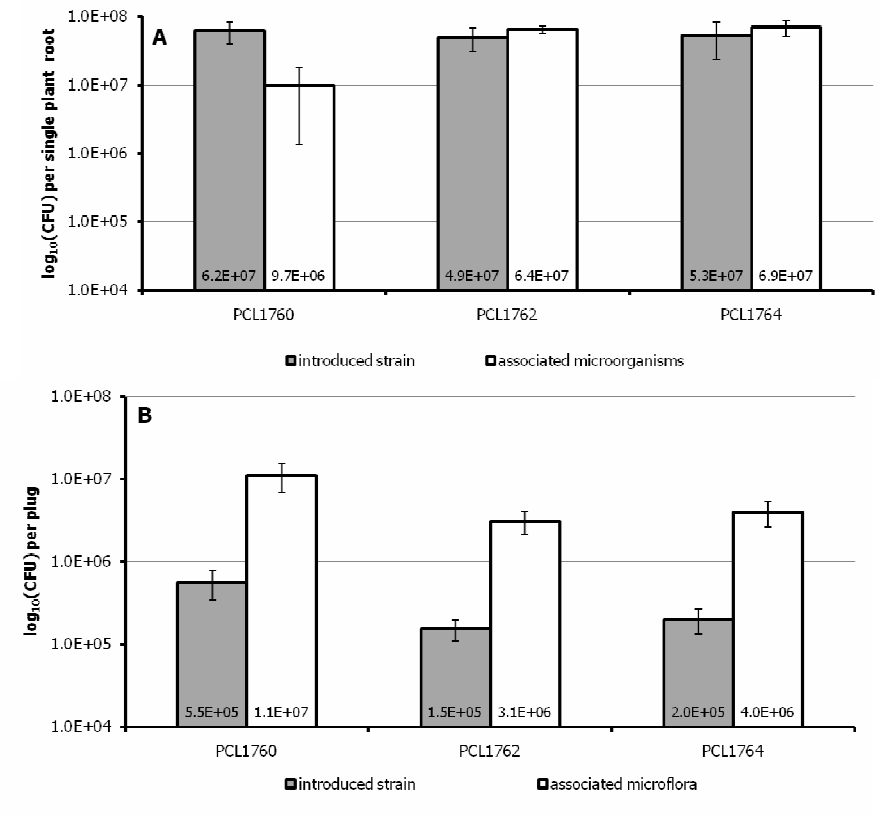

Fig. 5. Colonization of the tomato root (Panel A) and of the stonewool substrate (Panel B) by *P. putida* strainPCL1760 and its mutants PCL1762 (*dctA*) and PCL1764 (*flhA*) at the end of a biocontrol experiment.

General conclusions

In present study we have shown that, in stonewool, *P. putida* strain PCL1760 uses the mechanism of competition for nutrients to control tomato foot and root rot caused by *Forl* ZUM2407. Although flagellar motility was shown previously several times to play a crucial role in competition for the root niche in the biocontrol of TFRR in potting soil (Kamilova et al., 2005), we

observed that this trait hardly plays a role in biocontrol in stonewool substrate (Fig.2). This difference is likely caused by the different methods used to inoculate the plants. In contrast to potting soil, in which bacterized seeds were sown, the cells of the biocontrol bacterium were added as a suspension to stonewool to which they were efficiently bound. This supposedly enabled even the nonmotile mutant to colonize the root efficiently, thereby circumventing the need for flagellar motility.

Acknowledgements

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Chapter 5

Heterogeneity of phytopathogenic strains of *Fusarium oxysporum*

Shamil Z. Validov, Juan Juan Wang, Shufan Qi, Marjan de Boer, Ben Lugtenberg and *Faina Kamilova*

Abstract

Thirty three *Fusarium oxysporum* (*Fox*) strains from seven different formae speciales were taxonomically characterized using three criteria: (i) pathogenicity, (ii) vegetative compatibility and (iii) nucleotide sequence of the intergenic spacer region (IGS). Based on the comparison of mitochondrial small subunit rDNA (mt SSU rDNA) sequences, which have been used to confirm species affiliation of the studied fusaria, we concluded that strain Na10, which is pathogenic to narcissus, does not belong to *Fox.* All strains were able colonize tomato and cucumber plants endophytically, but only *Fox* f. sp. *radicis-lycopersici* (*Forl*) and *Fox* f. sp. *radicis-cucumerinum* (*Forc*) produced the foot and root rot on tomato and cucumber plants, respectively. No vegetative compatibility was observed when strains of different formae speciales were tested against each other. *Forl* strains C63F, C142 and RL2309 formed heterokaryons with tester strains from more than one vegetative compatibility group (VCG). Comparison of IGS sequences revealed heterogeneity of the majority of the formae speciales. *Fox* f. sp. *lycopersici* (*Fol*) strains 004 and 007 appeared to be part of the largest clade of *Forl. Forl* strain PD 95/1187 is more related to cucumber pathogens than to the other *Forl* strains. These results support the notion of polyphyletic origin of the formae speciales. Several strains representing the same VCG of *Forl* were nested in different clades of the phylogenetic dendrogram built on the comparison of IGS sequences. The observed genetic heterogeneity of VCGs does not support the notion of the clonal evolution of vegetatively compatible strains of *Fox*.

Introduction

Fusarium oxysporum is an important pathogen of many agricultural and ornamental crops (Nelson, 1981). Representatives of *Fox* are known to infect an extraordinary large range of host plants, causing wilt, root rot and crown rot diseases. For example, *Fusarium* wilt and *Fusarium* foot and root rot of tomato caused by *Fol* and *Forl*, respectively, are probably economically the most significant plant diseases and have been reported in at least 32 countries (Jones et al., 1991). These tomato diseases occur both in the greenhouse and in the field and cause significant crop losses. Root and stem rot, caused by *Fusarium oxysporum* f. sp. *radicis-cucumerinum* (*Forc*), a disease of cucumber, was recorded in many countries, including The Netherlands, to cause significant yield losses in greenhouses (Vakalounakis and Fragkiadakis, 2003; Vakalounakis et al., 2004). *Fox* also causes wilt disease on carnation and bulbrot of several flowers such as tulip, lily, daffodil and hyacinth (Baayen et al., 1998; Postma and Luttikholt, 1996; Hanks, 1996), which results in huge losses in horticulture (Moore et al., 1979).

Being economically important, *Fox* has received considerable attention from plant pathologists (Edel et al., 2001). Since no sexual cycle has been observed for *Fox,* it was proposed to divide *Fox* into formae speciales which reflect disease host specificity and symptoms that appear on infected plants (Smith et al., 1988). Currently, over 150 host-specific forms are known for this species (Baayen et al., 2001). Strains of certain forma specialis have no morphological features in common which clearly distinguish them from the strains of other host-specific forms. Therefore, identification of *Fox* strains has traditionally involved pathogenicity testing with sets of different host plants appropriate for the forma specialis in question (Summerell et al., 2003). Many formae speciales can be further subdivided into races on the basis of virulence on different host cultivars (Gordon and Martyn, 1997). The formae speciales are suitable for classification of phytopathogens, but this concept cannot be applied to non-pathogenic or multi-host *Fox* strains.

Vegetative compatibility is another method to classify *Fox.* It has been proven to be a good complementation to the morphological and phytopathological approaches

(Elias et al., 1992; Elias et al., 1993; Marlatt et al., 1996; Appel and Gordon, 1996). A vegetative compatibility test divides a special form into VCGs. This test is typically used to characterize pathogenic populations in asexual fungi. Strains from the same VCG can form stable heterokaryons after anastamosis (Correll et al., 1987). In many fungi, multiple vegetative incompatibility loci (*vic*) mediate vegetative compatibility. Strains are vegetatively compatible when the alleles at each of their *vic* loci are identical. According to the clonal theory, which describes the evolution of asexually reproducing organisms, compatibility of the *Fox* strains is evidence for their close relationship. Several studies have shown that affiliation to VCGs is a good indicator of the evolutionary lineage of *Fol* (Elias et al., 1992; Marlatt et al., 1996) and other formae speciales of *Fox* (Appel and Gordon, 1996; Koenig, 1997). Due to the high frequency of mutation rendering *Fox* strains unable to utilize nitrate, the VCG test is widely employed for the classification of *Fox* isolates. Self-incompatibility of some strains and heavy dependence on tester strain collections should be mentioned as disadvantages of this method.

In the past years, a strong increase in the use of molecular methods has emerged in fungal diagnostics. It provides a possible answer to the problems which are encountered with the routine identification methods mentioned previously. To investigate a possible correlation between pathotype and genotype, numerous genetic diversity studies have been conducted using fingerprinting techniques (Elias et al., 1993; Woo et al., 1996; Koening et al., 1997; Nelson et al., 1997; Vakalounaki et al., 1999). Use of these methods has shown that formae speciales with two or more VCGs are of polyphyletic origin (O'Donnell et al., 1998[a]).

One of the most robust and informative techniques used in fungal diagnosis is nucleotide sequencing of orthologous genes, in which DNA sequence variation has been used to judge the relationship between strains or to design species-specific primers and probes (Donaldson et al., 1995; Taylor et al., 1999). The sequences of mt SSU rDNA, translation elongation factor IF-1α, Internal Transcribed Spacer region (ITS) and Intergenic Spacer Region (IGS) have been successfully used in

recent years for the identification and systematics of *Fox* species (Lee and Taylor, 1992; Baayen et al., 2001; Hong et al., 2000).

In this paper we compare the results of pathogenicity and vegetative compatibility tests with data on mtSSU rDNA and IGS sequence analyses to clarify the relationship between 33 *Fox* strains. These strains originate from both greenhouses and open soil and represent seven different formae speciales. Comparing the different methods to identify and classify these strains provides insight in the reliability of these methods.

Materials and Methods

Strains and growth conditions

Fungal strains used and their characteristics are listed in Table 1. *Fusarium* strains were maintained on Potato Dextrose Agar (PDA, Difco Laboratories, Detroit, MI, USA). Czapek-Dox Agar (CDA, Difco Laboratories, Detroit, MI, USA) was used for complementation tests of *nit* mutants and Czapek-Dox Broth (CDB, Difco Laboratories, Detroit, MI, USA) was employed to obtain fungal spores for phytopathogenicity test. Mineral medium (MM) was prepared according to Correll et al., (1987), but without nitrogen source, and when a nitrogen source was needed, supplemented with hypoxanthine (Sigma, St. Louis, MO, USA), sodium nitrate, sodium nitrite or ammonium sulfate, the latter three compounds were purchased from ICN Biomedicals BV, Zoetemeer, The Netherlands.

Phytopathogenicity test on cucumber and tomato

Pathogenicity tests were carried out in stonewool (Grodan BV, Roermond, The Netherlands) for both tomato (cultivar Carmello) and cucumber (cultivar Symbal) both provided byDr. B. Kroon (Syngenta, Enkhuizen, The Netherlands). Spores of *Fox* strains were obtained on CDB as described by Chin-A-Woeng et al., (1998). In the case of tomato plant infection plastic trays with 48 stonewool plugs were soaked in 0.5 L of commercial Plant Nutrition Solution (PNS, PPO, Naaldwijk,

Table1 *Fusarium* strains and their characteristics

Phytopathogens	VCG number	Host plant	Accession Numbers of mt SSU rDNA/IGS	Source, reference
F. oxysporum* f. sp. *radicis-lycopersici				
ZUM 2407	0091	Tomato	EF437292/EF437260	Syngenta[A]
RL2037	0096	Tomato	EF437293/EF437261	SyngentaA
RL2039	0094	Tomato	EF437294/EF437262	SyngentaA
MDI031216059	0094	Tomato	EF437295/EF437263	GACB
MDI040126018	ND*	Tomato	EF437296/EF437264	GACB
PD 20006163	ND	Tomato	EF437297/EF437265	PDC
PD 87/192	0091	Tomato	EF437298/EF437266	PDC
PD 87/193	0091	Tomato	EF437299/EF437267	PDC
PD 87/245	ND	Tomato	EF437300/EF437268	PDC
PD 95/1187	0094	Tomato	EF437301/EF437269	PDC
C142	0090	Tomato	EF437302/EF437270	UVAD
C560,	SI	Tomato	EF437303/EF437271	UVAD
C63F	0090	Tomato	EF437304/EF437272	UVAD
VCG tester strains of *Forl*, (mutant genotype)				
*Forl*102, nitM	0090 I	Tomato		FPFS[E]
*Forl*104, nitM	0090 III	Tomato		FPFS[E]
*Forl*106, nitM	0091 I	Tomato		FPFS[E]
*Forl*111, nitM	0091 II	Tomato		FPFS[E]
*Forl*120, nitM	0092	Tomato		FPFS[E]
*Forl*129, nitM	0093	Tomato		FPFS[E]
*Forl*122, nitM	0094 I	Tomato		FPFS[E]
*Forl*124, nitM	0094 II	Tomato		FPFS[E]
*Forl*125, nitM	0094 III	Tomato		FPFS[E]
*Forl*126, nitM	0094 IV	Tomato		FPFS[E]
*Forl*128, nitM	0094	Tomato		FPFS[E]
*Forl*130, nitM	0096	Tomato		FPFS[E]
*Forl*100, nit1	0098	Tomato		FPFS[E]
*Forl*101, nitM	0099	Tomato		FPFS[E]

F.oxysporum* f. sp. *lycopersici				
Fol 004		Tomato	EF437305/EF437289	UVA[D]
Fol 007		Tomato	EF437306/EF437290	UVA[D]
F. oxysporum f. sp. ***radicis-cucumerinum***				
Fukk 04/01	0260	Cucumber	EF437307/EF437273	PPO, Naaldwijk[F]
Fukk 04/02	0260	Cucumber	EF437308/EF437274	PPO, Naaldwijk[F]
Fukk 04/03	0260	Cucumber	EF437309/EF437275	PPO, Naaldwijk[F]
Fukk 05/01	0260	Cucumber	EF437310/EF437276	PPO, Naaldwijk[F]
Fukk 05/02	0260	Cucumber	EF437311/EF437277	PPO, Naaldwijk[F]
Fukk 05/03	0260	Cucumber	EF437312/EF437278	PPO, Naaldwijk[F]
V03-2g	0261	Cucumber	EF437313/EF437279	ARIAM[G]
F. oxysporum* f. sp. *lilii				
Fol 3	ND	Lily	EF437314/EF437280	PPO, Lisse[H]
Fol 101	ND	Lily	EF437315/EF437281	PPO, Lisse[H]
F. oxysporum* f. sp. *gladioli				
IR1	ND	Iris	EF437316/EF437282	PPO, Lisse[H]
IR19	ND	Iris	EF437317/EF437283	PPO, Lisse[H]
F. oxysporum* f. sp. *narcissi				
Na5	ND	Narcissus	EF437318/EF437284	PPO, Lisse[H]
F. oxysporum* f. sp. *tulipae				
Tu11	0230	Tulip	EF437320/EF437286	PPO, Lisse[H]
Tu58	0230	Tulip	EF437321/EF437287	PPO, Lisse[H]
Tu67	0230	Tulip	EF437322/EF437291	PPO, Lisse[H]
Fusarium sp.				
Na10	ND	Narcissus	EF437319/EF437285	PPO, Lisse[H]
Fusarium hostae* f. sp. *hyacinthi				
Hy9	ND	Hyacinth	EF437323/EF437288	PPO, Lisse[H]

[A] Syngenta (The Nederlands)

[B] Groen Agro Control (The Nederlands);

[C] Plantenziektekundige Dienst(The Nederlands);

[D] Universiteit van Amsterdam (The Nederlands);

[E] Flore Pathogène Faune du Sol, Inst. National de la Recherche Agronomique Dijon, France;

[G] All-Russian Institute of Agricultural Microbiology (Russia);

[F,H] Praktijkonderzoek Plant & Omgeving (The Nederlands)

ND not determined; SI self incompatible

The Netherlands) supplemented with *Fox* to a final concentration of 2×10^6 microspores per L. For cucumber plants, stonewool blocks were moistened with 0.5 L of PNS with the same spore concentration as in the tomato test.

Plants were grown for 2.5 – 3 weeks, harvested and scored. Dead plants or plants with brown (orange in the case of *Forc* V03-2g) spots or lesions on their feet/roots were considered as diseased. 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 ($\alpha = 0.05$), using *SPPS*-software (*SPPS* Inc., Chicago, IL, USA). All experiments were performed at least twice.

Phytopathogenicity test on flowerbulbs

The different *Fusarium spp.* strains have been tested in several bioassays. Isolates from narcissus have been tested two consecutive years in the field. Narcissus bulbs were planted in artificially infested soil (10^4 cfu/ g soil). Thirty bulbs were planted in the soil per treatment and experiments were carried out with 3 replicates. After 9 months the bulbs were harvested and stored for 3 months so disease symptoms were fully developed.

Tulip isolates have been tested in a bioassay in the greenhouse on "Monte Carlo", "Viking", "White Dream" and "Prominence" tulip cultivars. Five cm artificially infested soil (10^5 cfu/g soil) was put on top of non infested potting soil in a 3 L plastic pot. The pots were stored at 18°C in the greenhouse under plastic before 5 tulip bulbs per pot were planted in the soil. Per treatment 3 pots were planted. During 4 weeks crop stand was scored and after 4 weeks the bulbs were harvested and scored for bulb rot.

Vegetative compatibility test

Nitrate non-utilizing mutants of *Fox* strains were generated on PDA supplemented with 1.5% of $KClO_3$. Fast growing sectors without aerial mycelium were isolated and tested for impaired growth on CDA. The phenotype of the mutants was determined by growth on MM supplemented with hypoxanthine (0.2

g/L), $NaNO_2$ (0.5 g/L) or $(NH_4)_2SO_4$ (0.72 g/L) as the nitrogen source. The mutants which did not grow in medium with hypoxanthine or $NaNO_2$, added as a sole nitrogen source, were designated as *nit*M or *nit*3, respectively. The rest of mutants were designated as *nit*1 mutants.

Compatibility was checked on CDA. Self-compatibility was tested by pairing *nit*M and *nit*1 mutants from the same strain.

DNA techniques

Fungal strains were grown on sterile filter paper placed on PDA agar. Filter paper with fungal hyphae was collected and ground in liquid nitrogen. DNA was isolated from pulverized fungal biomass using the Nucleon Phytopure kit (Amersham Biosciences GmbH, Freiburg, Germany).

The mitochondrial small subunit RNA (mt ssuRNA) gene fragment was amplified using primers MS1 and MS2 (Zeng et al., 2003) and sequenced by ServiceXS (Leiden, The Netherlands) with the same primers. The IGS fragment of rDNA was amplified using the primers CNL12 and CNS1 (Cai et al., 2003). The nucleotide sequences of the PCR fragments were determined by ServiceXS (Leiden, The Netherlands) using primers CNL12 and U49:65 (Cai et al., 2003). The obtained chromatograms were normalized, assembled and exported to FASTA format using Vector NTI 10.1.1. software (Invitrogen Corporation, Breda, The Netherlands).

Sequence alignment was performed using the CLUSTALW 1.8 program (Thompson et al., 1994). For construction of the consensus tree, bootstrapping was performed with 1,000 data sets and unweighted pair group and visualized with Njplot free software (designed by Gouy M, http://pbil.univ-lyon1.fr/software/njplot.html).

Results

mt SSU rDNA fragment comparison

Comparison of mt SSU rDNA nucleotide sequences of *Fox* strains (see Table 1) resulted in a strongly supported (99%) ingroup, comprising of two subclades (Fig.

1). No subclade with bootstrapping higher then 10% was formed inside the big *Fox* subclade. The three strains Fol3, Fol101 and IR19, were nested as a separate small subclade (75% of bootstrap) in the proximity of the rest of the *Fox* strains. BLAST searches reveal that mtSSU rDNA fragments of Fol101 and IR19 strains share 99% of homology with *Fox* f. sp. *melonis* strains TX388 (Acc.N. DQ831943).

Fusarium sp. strain Na10 was clustered with *F. bulbicola* strain NRRL 13618 and with *F. circinata* NRRL 29945, although the distances between these three strains are large.

Pathogenicity test

Pathogenicity of all 33 *Fox* strains was tested on tomato, cucumber and flower bulbs. Inoculation of tomato and cucumber seeds with the *Fox* strains showed that all of them were able to colonize plants endophytically. However, only *Forl* and *Forc* strains caused foot and root rot on tomato and cucumber, respectively. The most aggressive among *Forl*s are strains C63F, 2037, PD 95/1187, MDI040126018 and PD20006163. Severity of the disease, caused by these strains, ranged from 79% to 87% and was significantly different from that caused by the other *Forl*s (60%). Small lesions were observed on the roots when non-*Forl* strains were used to infect tomato plantlets. In comparison with the extensive foot and root rot lesions caused by *Forl* strains, the ones caused by non-compatible pathogens were much smaller and did not lead to plant death. Virulence of *Forc* strains varied between 56 and 63% with no significant difference between the seven strains tested. When cucumber plantlets had been infected with spores of the representatives of *Forl*, *F. oxysporum* f. sp. *tulipae* (*Fot*), *F. oxysporum* f. sp. *gladioli* (*Fog*) and *F. oxysporum* f. sp. *narcissi* (*Fon*), no damage to plants was observed.

In field experiments *Fon* strain Na 5 (isolated from bulb rot) and Na 10 (isolated from the diseased skin of bulbs) were both able to cause disease. The main symptoms caused by Na5 were severe bulbrot (50 % of the bulbs) and skin disease. Na10 mainly caused skin disease (75%) and only slightly bulb rot. Compared to other isolates Na 5 is relatively virulent concerning bulb rot whereas Na10 can cause relatively severe skin disease.

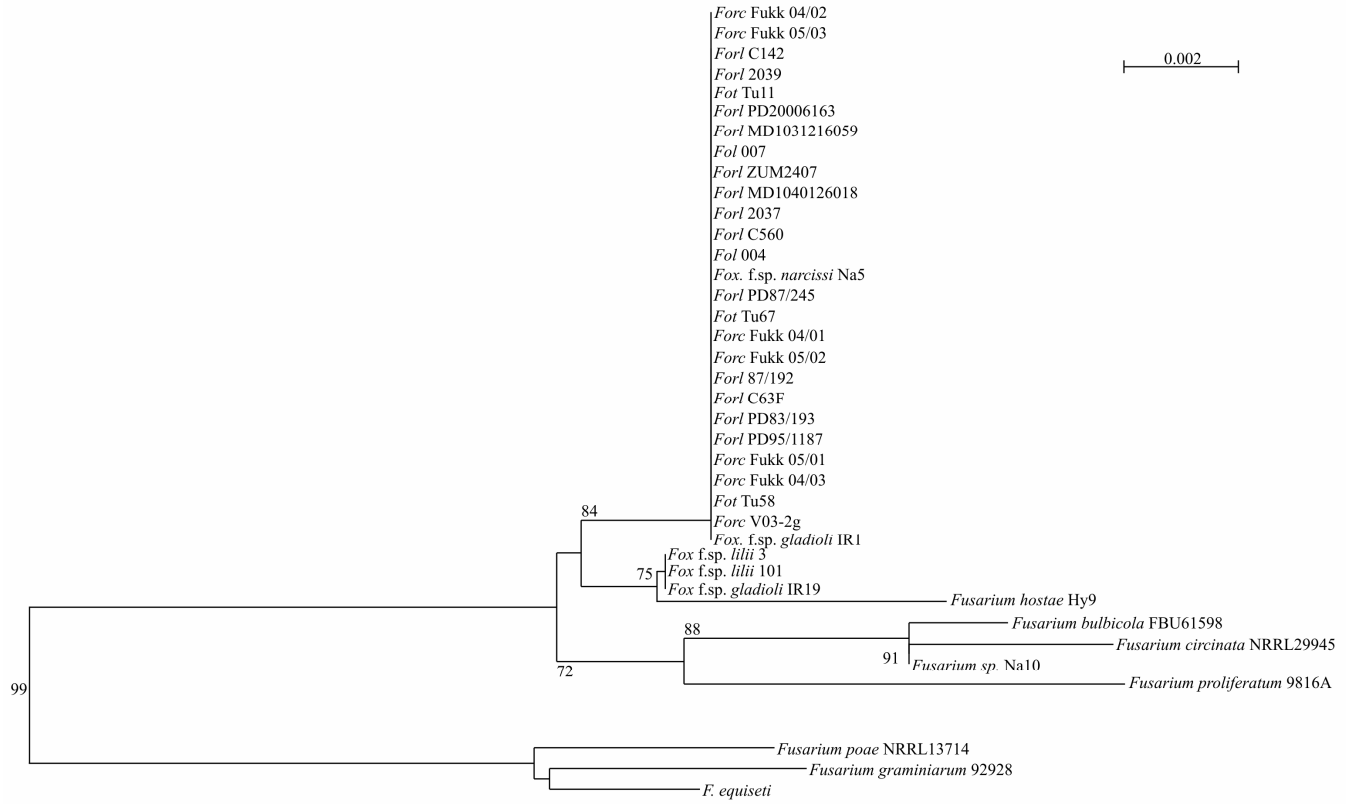

Fig. 1 Phylogenetic tree based on the comparison of the mt SSU rDNA sequence of *Fox* strains from seven different formae speciales. Strain *Fusarium equiseti* was used as the outgroup. The numbers at the nodes represent bootstrap support >50% from 1,000 replications.

Tulip isolates were tested on 4 different tulip cultivars in a greenhouse experiment. Strain Tu58 was able to cause severe bulb rot in the cultivars "White Dream" and "Prominence" that are very susceptible to *Fusarium* bulb rot (100 % diseased bulbs). In the less susceptible cultivars "Monte Carlo" and "Viking" Tu58 caused much less severe bulb rot (up to 43% diseased bulbs). Tu67 was the most virulent strain and caused 100% severely rotted bulbs in all cultivars.

Vegetative compatibility test

The *Fox* strains were tested for vegetative compatibility according to Puhalla et al., (1985). No complementation was observed between strains of different formae speciales. *Forc* strains are represented by two VCGs . All *Forc* strains originating belong to the same VCG (Fig 2). *Forl* strains were assigned with VCGs 0090, VCG 0091, VCG 0092, VCG 0094 and VCG 0096. Strains *Forl* C142 and *Forl* C63F showed heterokaryon formation with tester strains from VCG 0090 and VCG 0092, although aerial hyphae appeared earlier with the former tester strain. Multiple complementation was also shown for *Forl* 2309. This strain could form heterokaryons with several testers, with the strength decreasing from VCG 0094, via VCG 0092 to VCG 0090. All tulip strains are vegetatively compatible with each other. The strains from other formae speciales did not show compatibility.

IGS sequences

IGS nucleotide sequences of *Fusarium* strains were determined and the results were used to build a dendrogram (Fig. 2). *Fusarium hostae* strain Hy9 was used as an outstanding control. *Fusarium sp.* strain Na10 is nested with 99% of bootstrap in the immediate proximity of *F. bulbicola* NRRL 13618. All *Fox* strains form a strongly supported clade (100% of bootstrapping). Three strains, *Fog* IR19, *Fox* f. sp. *lilii* 3 and *Fox* f. sp. *lilii* 101, are nested in a separate small subclade. The major *Fox* subclade is not homogeneous and contains five subclades supported with high bootstrap values (79 – 100%). Subclade 1 includes seven *Forl* strains. *Fol* strains 007 and 004 are nested inside this subclade at some distance from the *Forl* strains.

The second subclade is related to the first one and comprises two *Forl* strains. These two subclades are most related among the *Forl* strains studied, but their separation is highly supported by bootstrapping. The third subclade is represented by the causal agents of tulip bulb rot. This subclade is joining the first two subclades, although the bootstrap of this connection is of low value. Strains *Fon.* Na5 and two *Forl*s, C63F and MDI040126018, with identical IGS sequences, form the fourth subclade. The fifth subclade is comprised of cucumber pathogens and the single *Forl* strain PD95/1187 (81% bootstrap). *Forc* strains form a highly supported ingroup inside the fifth subclade. Strains *Fog* IR1 and *Forl* PD 87/245 are not included in any of the mentioned subclades. The latter strain is most distantly related to the other strains in the biggest *Fox* subclade (Fig. 2).

Discussion

We studied 33 phytopathogenic strains of *Fusarium oxysporum* isolated from greenhouses and fields. These strains represent seven host-specific forms and cause tomato foot and root rot, cucumber root rot, tomato vascular wilt and root or bulb rot of tulips, iris, lily or daffodil (Table 1).

To avoid comparison of *Fox* strains with non-*Fox* ones, mtSSU rDNA sequence analysis was used to clarify the species affiliation of the studied strains. Twenty eight strains from *Forl*, *Forc*, *Fol*, *Fog, Fot* and *Fon* were not distinguishable from each other at the level of mtSSU rDNA sequence. Strains *Fox* f. sp *lilii* 3, 101 and *Fox* f. sp *gladioli* IR19 formed separate clade. Nevertheless, these three strains might belong to *Fox,* because the genetic distance between these two clades is short in comparison to those for closest species from *Fusarium* genus (Fig. 1.). The difference between mtSSU rDNA sequences of strains from these two clades of *Fox* is due to nucleotide substitutions, whereas the divergence between species is caused by insertions/deletions. It was shown that the mitochondrial (mt) rRNA genes evolve more rapidly than nuclear rRNA genes. Due to variable sequence regions even closely related taxa can be distinguished (Zeng et al., 2003). Comparison of fragments of mtSSU rDNA genes allows us to conclude that Na10 belongs to another species than *Fox* (Fig. 1). It should be noted that earlier work

based on RFLP analyses of the ITS region using ITS 1-4 primers and *Hinf*1 digestion affiliated Na 10 to *Fox* (M. de Boer and S. Breeuwsma, *unpublished data*). This shows that ITS – RFLP is not always sufficient in identifying *Fusarium* species and that mtSSU rDNA sequence analysis probably is a more reliable method for the identification of *Fusarium* species.

Pathogenicity of *Fox* strains was tested on tomato and cucumber in a stonewool substrate system, which is nowadays widely used in greenhouses. Being practically sterile in the beginning of plant cultivation, stonewool is a more convenient substrate for the experimental purposes than potting soil, which contains many indigenous microorganisms able to compete with the pathogenic fungus and causing deviation of results. Testing of *Fox* strains confirmed their affiliation to formae speciales they previously were assigned with. All strains were able to colonize tomato and cucumber and were found in the endophytic stage in the shoots and roots of the plantlets. All strains of *Fox* are able to grow in the rhizosphere of many plant species (Garret 1970). Several compounds of the plant root exudates, such as organic acids and sugars, can be utilised by *Fox* (Kamilova et al., 2006). It allows *Fox* to colonize the rhizosphere of non-host plants. Endophytic colonization was shown for both pathogenic and non-pathogenic *Fox* (Olivain and Alabouvette, 1999; Rowe, 1980). Infection by non-compatible phytopathogenic *Fox* strains does not lead to lesion formation on cucumber plants, whereas colonization of tomato plantlets by non-compatible *Fox* strains results in the formation of small lesions. It indicates that phytopathogenic *Fox* strains can damage some noncompatible plant species, but to a much lesser extent than strains of homologous forma specialis. Similarly, the non-pathogenic strain Fo5a4 is able to reach the cortex but, in contrast to *Fol*32, is unable to get into the xylem due to barriers formed in the hypodermis (Olivain and Alabouvette, 1999).

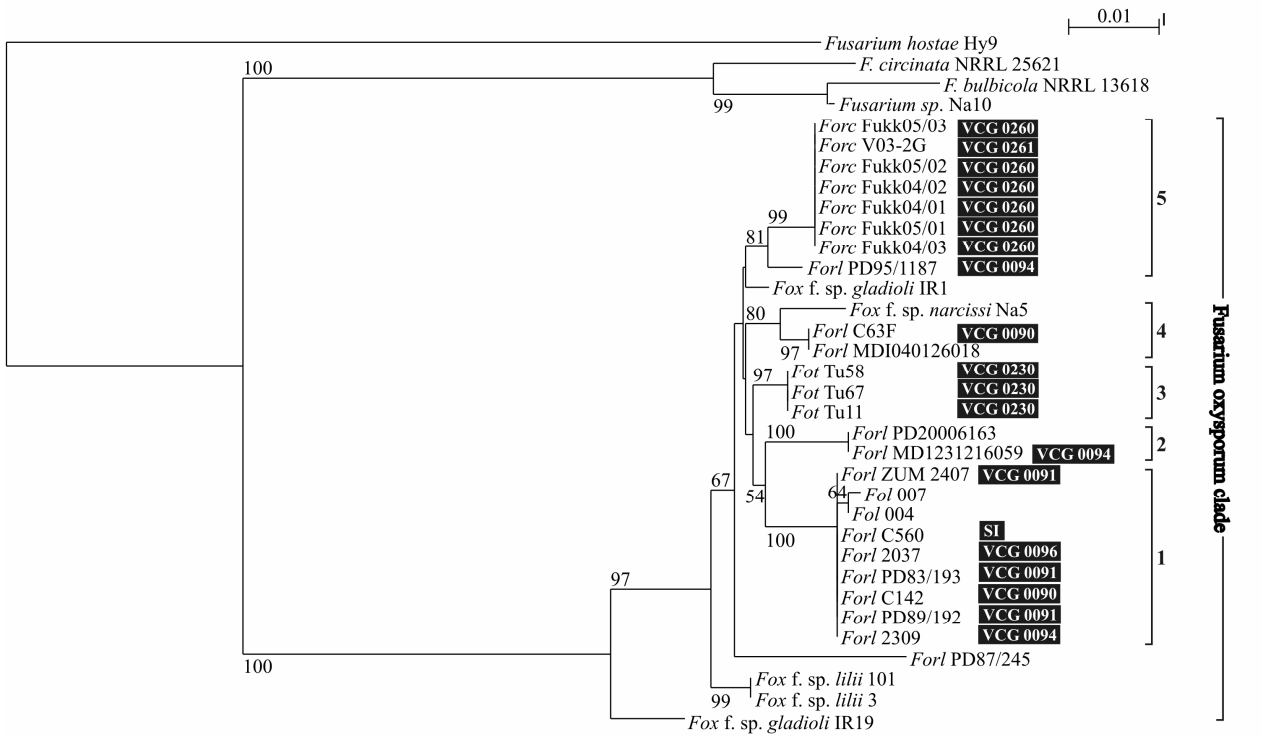

Fig. 2. Phylogenetic relationship of *Fox* strains built on the basis of IGS sequence alignment. The numbers at the nodes represent bootstrap support >50% from 1,000 replications. Strain *Fusarium hostae* f. sp. *hyacinthi* Hy9 (Baayen et al., 2001) was used as an outgroup. VCG are given in black boxes next to the corresponding strain. SI – self incompatibility

Similar effect was observed in the bioassay with tulip isolates. Using tulip cultivars with different susceptibility, it was shown that *Fot* strain Tu11 (Baayen et al., 1998) and Tu67 cause severe bulb rot even in resistant cultivars, whereas Tu58 shows a smaller percentage of disease pressure and only slight bulb rot (M. de Boer and S. Breeuwsma; *unpublished data*).

The vegetative compatibility test allowed us to assign *Forl* strains to the five VCGs. Three strains C63F (VCG 0090), C142 (VCG 0090) and RL2309 (VCG 0094) were compatible with the tester strain from VCG 0092. VCGs of *Fox* for thirty-eight formae speciales was listed in Katan (1999) and previously discussed in the article of Kistler et al., (1998). Multiple compatibility was described for *Forl* strains from VCG 0090 (Katan et al., 1991). Moreover, there is a possibility that *Forl* strains of VCG 0094 can be compatible with strains *Fog* VCG 0034 (Kim et al., 1997; Yoo et al., 1995).

The data on comparison of IGS sequences shows that pathogenicity to certain plant species can evolve from quite a range of different *Fox* genotypes. The dendrograms in Fig. 2 represent the relationship between *Fox* strains. Three formae speciales, *Forc, Fot* and *Fox* f.sp *lilii,* are comprised of strains with a monophyletic origin. *Forl* strains are distributed among 5 clades. *Fol* strains 007 and 004 form a subclade within subclade 1 of *Forl*. *Fol* and *Forl* are pathogenic to the same host plant. They can clearly be distinguished as formae speciales by symptoms: representatives of *Fol* cause tomato wilt by blocking the vascular system of the infected plant (Charest et al., 1984; Rep et al., 2004; Attitalla et al., 2004), whereas *Forls* cause foot and root rot and cannot invade the plant's vessels (Rowe 1980). According to the results of the IGS comparison, *Fol* strains are closer to the seven strains of the clade 1 than to the six other *Forls* which are nested in the other subclades. It was shown that *Fol* is comprised of several lineages (Cai et al., 2003). Our results demonstrate that some of these lineages can be closely related to representatives of *Forl*. IGS sequences in the pairs of *Forl* strains MDI031216059/PD 20006163 and C63F/MDI040126018 are identical and these strains form two separate subclades. Strain PD 95/1187 is close to cucumber pathogens and PD87/245 is most remote to any other tomato pathogen tested in

this study. This divergence of IGS sequences among representatives of *Forl* shows how different the genetic background of this forma specialis can be. It may explain the occurrence of *Forl* strains which are pathogenic to hosts outside of the Solanaceae (Rowe 1980). *Forl* is not the only host-specific form with a multiple origin. It has been shown that *Fox.* f. sp. *cubense* or of *Fox.* f. sp. *melonis* are comprised of strains which have polyphyletic evolutionary origin (Kistler, 1997; Jacobson and Gordon, 1990). Comparison of combined datasets including EF-1α and mtSSU rDNA genes gave a dendrogram in which representatives of *Fox.* f. sp. *cubense* were divided between the clades, indicating polyphyletic origin of host-specific forms of *Fox* (O'Donnel et al., 1998).

Pathogenicity test revealed that five *Forl* strains, C63F, 2037, PD 95/1187, MDI040126018 and PD20006163, are significantly more virulent than the rest of *Forl*s. These five most aggressive strains form four subclades (1, 2, 4 and 5) together with less virulent *Forl*s (Fig.2). Similarly, the tulip isolates nested in third subclade show different levels of virulence. This indicates that virulence is not a property of a certain phylogenetic group of *Forl* (Fig.2). Variation of the aggressiveness to the host, among *Fox* strains, could be a result of fungal genome rearrangements generated by mobile elements (Teunissen et al., 2003). A high frequency of transposition could be a cause of the fast evolution for these asexual species.

Remarkably, strains from the same VCG are distributed among more than one clade of *Forl* (Fig.2). Strains RL2309, MDI031216059 and PD 95/1187 nested in different subclades, but they are compatible with the tester strain of VCG 0094. Vegetative compatibility is a routine tool to characterize strains of *Fox* (Kistler et al., 1998). It is generally agreed that VCGs of *Fox* represent a lineage with common evolutionary origin (Cai et al., 2003). Our results show that there are some exceptions: the two strains C63F and C142, both from VCG 0090, have pronounced differences in their IGS regions. In contrast, strains representing VCG 0090, 0091, 0094 and 0096 are nested together in clade 1 of *Forl* due to high similarity of their IGS sequences. Furthermore, strains *Fox* f. sp. *lilii* 101 and 3 that cause different disease symptoms (101 causes stem rot and 3causes bulb rot) and belong to

different VCG and RAPD-PCR groups (M. de Boer and S. Breeuwsma; *unpublished data*) are placed together in one IGS clade. The discrepancies between vegetative compatibility test and IGS sequences comparison might be explained by (i) peculiarity of *vic* loci behavior in *Fox* (little is known on vegetative compatibility genes of *Fox*) or (ii) sexual processes that might occur between strains of *Fox* under natural conditions (Arie et al, 2000; Kerenyi et al., 2004).

Taking into consideration the heterogeneity of the strains revealed in this study and the diversity of the strains from *Fox* f. sp. *cubense*, *Fox* f. sp. *melonis* and *Fox* f. sp. *lycopersici* reported previously, the possibility to design primers or probes, which could be based on the multi-copy ribosomal gene sequences and could discriminate between different host specific forms of *Fox*, is quite low. The polyphyletic origin of *Forl* strains and the close relationship between the strains from different formae speciales may indicate that pathogenicity to a certain plant is not a result of a long-term co-evolution of *Fox* lineages with the host, but could be the result of relatively fast changes caused by gene exchange or rearrangement of genetic material. The specific loci responsible for pathogenicity might be more proper tools for monitoring of phytopathogenic forms of *Fox* strains.

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Chapter 6

Monitoring of pathogenic and nonpathogenic *Fusarium oxysporum* strains during tomato plant infection

Shamil Z. Validov, Faina D. Kamilova and Ben J.J. Lugtenberg

Abstract

Monitoring of pathogenic strains of *Fusarium oxysporum* (*Fox*), which cause wilt and rots on agricultural and ornamental plants, is important for predicting the disease outbreaks. Since both pathogenic and non-pathogenic strains of *Fox* are ubiquitous and are able to colonize plants, *Fox* DNA, detected in plant material, is not the ultimate proof for an ongoing infection which would cause damage to the plant. 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) and *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. Two weeks after tomato seedling inoculation by these *Fox* strains the DNA concentration of *Forl* ZUM2407 was 5 times higher than that of the non-compatible pathogen *Forc* V03-2g and 10 times higher than that of Fo47. In three week old plantlets the concentration of *Forl* ZUM2407 DNA was at least 10 times higher than those of the other strains. The fungal DNA concentration, as determined by qPCR, appeared to be in good agreement with data of the score of visible symptoms of tomato foot and root rot obtained 3 weeks after incubation of tomato with *Forl* ZUM2407. Strain *Fol* 004, causing wilt on tomato by blocking plant vessels, was developing much slower in the plantlets. This is probably reflecting the usual way of infection of this special form, namely infiltration into the plant through the wounds.Our results show that targeting of the multicopy ribosomal operon gives highly sensitive qPCR reaction for the detection of *Fox* DNA. Since formae speciales of *Fox* cannot be distinguished by comparison of ribosomal operons, detection of *Fox* DNA is not evidence of plant infection by the compatible pathogen. Nevertheless, the observed difference in plant colonization between pathogenic and non-pathogenic strains revealed by our present results strongly suggests that a concentration of *Fox* DNA in plant material above a certain threshold level is due to proliferation of pathogenic *Fox*.

Introduction

Fox is a well-known pathogen of agricultural or ornamental crops (Nelson, 1981). Phytopathogenic strains of *Fox* are responsible for yield loss of many economically important crops worldwide. For example, *Fusarium* wilt and foot and root rot of tomato caused by *Fusarium oxysporum* f.sp *lycopersici* (*Fol*) and *Fusarium oxysporum* f.sp *radicis-lycopersici* (*Forl*), respectively, have been reported in at least 32 countries (Jones, 1991). These tomato diseases occur both in the greenhouse and in the field and cause significant crop losses (Hahn 2002, Cai *et al*, 2003). Root and stem rot of cucumber, caused by *Fusarium oxysporum* f. sp. *radicis-cucumerinum* (*Forc*) was also recorded in many countries to cause significant yield losses in greenhouses (Vakalounakis *et al*., 2004).Besides yield decreases the *Fox* produces toxins which can accumulate in the end products and therefore can be dangerous for human and animal health (Pitt 2000).

Fusarium oxysporum (*Fox*) is a cosmopolitan species whose representatives, both phytopathogenic and non-pathogenic, can survive as saprophytes in soil (Burgess 1981). Due to their ability to utilize a large variety of nutrients, both pathogenic and non-pathogenic *Fox* strains can colonize rhizosphere of various plants and, moreover, transit to endophytic stage (Garret 1970). Some non-pathogenic strains of *Fox* have been shown to control tomato foot and root rot caused by *Forl* (Olivain and Alabouvette, 1999; Bolwerk *et al*., 2005). Moreover "Bio*Fox* C" – a product based on a non-pathogenic strain of *Fox* is used for the protection of basil, carnation, cyclamen and tomato against pathogenic *Fox* and *Fusarium molineformae* (Jones and Burges 1998).

Monitoring of plant pathogens is crucial for disease management. Early detection, identification and quantification of the infestation level can help to choose appropriate defense measures. Monitoring of a phytopathogenic microorganism can be done indirectly by following of disease symptoms appearing on the plants or by analyzing volatiles excreted during pathogen multiplication (Prithiviraj *et al*., 2004). Direct approaches, such a dilution plating of infested plant or soil on selective media (Vujanovic *et al*., 2002), detection of fungal spores in plant material (Hahn 2002), immunological and molecular detection of the causal

agent of the disease, give more precise information on the pathogens (Paulitz 2000).

Development of real-time PCR (RT-PCR) has provided a powerful tool for pathogen monitoring. RT-PCR techniques give a high sensitivity for the detection of fungal strains (Zhang *et al.*, 2005; Pasquali et al., 2006). It allows the detection of the pathogen earlier than symptoms of the disease appear on the plants (Pasquali et al., 2004). Real-time PCR also can give semi-quantification of fungal pathogens such as *Fusarium oxysporum*, *Fusarium solani*, *Pythium ultimum* and *Rhizoctonia solani* in a single assay (Lievens *et al.*, 2005).

Since plants can be colonized by pathogenic and nonpathogenic *Fox* strains, detection of *Fox in planta* is not necessarily evidence of pathogen attack. It was shown that the patterns of tomato root penetration by pathogenic and non-pathogenic *Fox* are quite similar and the differences are mainly quantitative (Olivain and Alabouvette, 1999). In the case of non-pathogenic *Fox,* flax plants appeared to be able to stop invasion of the fungus by building barriers in the cortex whereas pathogenic strains appeared to avoid the defense system of the host plant (Olivain *et al.*, 2003). These two observations, done by using microscopical methods, show that, due to the reaction of the plant, the nonpathogenic strain Fo47 is restricted in multiplication in tomato and flax.

If differences in proliferation of *Fox* strain *in planta* exist, it should be possible to detect them by quantitative PCR. To test this idea we compared colonization of tomato plants by different *Fox* strains: (i) the tomato foot and root rot pathogen *Forl* ZUM2407 strains, (ii) the tomato vascular wilt pathogen *Fol* 004, (iii) the cucumber root rot pathogen *Forc* V03-2g and (iv) the non-pathogenic biocontrol strain *Fox* Fo47 using qPCR. The results are reported in this paper.

Materials and Methods

Strains and growth conditions

Strains used in this study are listed in Table 1. Strains were kept frozen at -80°C. When needed cultures of the strains were plated on Czapec-Dox agar (CDA, Difco Laboratories, Detroit, MI, USA) and grown at 28°C for 5 – 10 days. To avoid

bacterial contamination CDA was amended with kanamycin (Duchefa, Haarlem, The Netherlands) and tetracyclin (Duchefa, Haarlem, The Netherlands) in final concentrations of 50 and 40 µg per ml, respectively.

Table 1. Relevant characteristics of *Fusarium oxysporum* (*Fox*) strains used in this study

Strain	Host	Acc. N. of the IGS region	Reference/Source
Fox f. sp. *radicis-lycopersici* ZUM2407	tomato	EF437260	Syngenta, The Netherlands
Fox f. sp. *lycopersici* 004	tomato	EF437289	UVA, The Netherlands*
Fox f. sp. *radicis-cucumerinum* V03-2g	cucumber	EF437279	ARIAM, Russia**
Fox Fo47	none	EF437222	Olivian and Alabouvette, 1999

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** All-Russian Institute of Agricultural Microbiology, Russia

Plant inoculation with Fox strains and scoring of the disease symptoms

To obtain spores, 1-liter Erlenmeyer flasks containing 200 ml of Czapec-Dox broth (CDB, Difco Laboratories, Detroit, MI, USA) were inoculated with one-third of a 10-day-old CDA petri dish culture of the *Fox* strains. The cultures were grown at 28°C for 72 hours under aeration (110 rpm). Spores were separated from the mycelium and the agar pieces by filtering of the cultures through miracloth (Omnilabo International BV, Breda, The Netherlands).The microspore concentration was determined using light microscopy. The filtrate was adjusted to concentrations of 10^7, 10^6, 10^5 or 10^4 spores/L of Plant Nutrient Solution (PNS; PPO, Naaldwijk, The Netherlands).

Tomato (*Lycopersicon esculentum* Mill.) seeds (cv. Carmello) were sown in stonewool plugs (Grodan BV, Roermond, The Netherlands). Plastic trays with the stonewool plugs were soaked in PNS supplemented with *Fox*. Seeds were allowed to germinate for 3 days in the dark at 23-27°C and subsequently plants were grown in a greenhouse at 21–24°C, 70% relative humidity and 16 h daylight.

For each treatment, 144 plants were tested. Forty eight plants were harvested after 1, 2 and 3 weeks and the disease level was scored using six replicates of 8 plants each. To score the disease, plants were removed from the stonewool,

washed, and the plant roots were examined for foot and root rot symptoms as indicated by browning and lesions. Only roots without any disease symptoms were classified as healthy. The disease score, in this case, was calculated as a percentage of plants with a lesion. Alternatively, the disease level was scored by indexing the disease severity: healthy plants were given a value of 0, plants with small lesions (<2 mm) were given a value of 1, plants with developed lesions – 2, plants with deadly lesions (rotten foot, vast root rot) a value of 3. The value for dead plants was 4. The disease index (DI) was calculated using following formula:

$$DI=(n0\times0+n1\times1+n2\times2+n3\times3+n4\times4)/(n0+n1+n2+n3+n4),$$

In which n0, n1, n2, n3 and n4 are number of plants with indexes 0, 1, 2, 3 and 4 respectively.

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 (a = 0.05), using SPSS software (SPSS, Chicago, IL, USA). The experiment was performed twice.

Sample collection and DNA isolation

Replicates of 8 plants (whole plant) each were pulverized in liquid nitrogen. One gram of ground plant material was mixed with 1 ml of extraction buffer consisting of 2% hexadecatrimethylammonium bromide (CTAB; Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), 100 mM Tris-HCl (pH 8.0), 1.4M NaCl and 20 mM EDTA (MP Biochemicals, Amsterdam, The Netherlands). The mixture was incubated at 55°C for 20 min and then centrifuged at 14.500 rpm. The supernatant was extracted with one volume of chloroform. The upper phase was transferred to a new Eppendorf tube and the DNA was precipitated by adding 0,6 volume of isopropanol followed by centrifugation. The pellets were dissolved in 500 µl of TE buffer (pH 8.0). To remove RNA from the preparations, RNAse (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) was added at a final concentration 2 µg/ml. DNA preparations were incubated at 60°C for 30 min and subjected to phenol-chloroform extraction. DNA was precipitated by adding 50 µl of 3M sodium acetate and 350 µl of isopropanol followed by centrifugation at 14.500

rpm for 5 min. DNA pellets were washed twice with 70% ethanol and dried. DNA was dissolved in 50 µl of µQ water. The DNA concentration in the preparations was adjusted to 5 ng/µl.

To isolate DNA, the biomass of the *Fox* strains was ground in liquid nitrogen and further isolation was performed as described for the isolation of DNA from tomato plants.

Quantitative PCR reaction

Primers OMP1049 (5'-TGCGATTTGGACGAGATATGTG-3') and OMP1050 (5'-ATTTGCCTACCCTGTACCTACC-3') for quantitative PCR reaction were designed using Beacon Designer 5.0 (Bio-Rad Laboratories BV, Veenendaal, The Netherlands) on the basis of the IGS sequence of *Forl* ZUM2407 strain. Real-time PCR was performed in Chromo4™ Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories BV, Veenendaal, The Netherlands) with the following thermal profile: initial DNA denaturation and polymerase activation at 95°C for 10 min, followed by 40 cycles each containing denaturation and annealing steps at 95°C and at 58°C, respectively, both for 15 sec. Amplification cycles were followed by a melting curve built from 50°C to 90°C, with measurements made every 0.2°C. The PCR mixture was prepared using qPCR Core kit for SYBR® Green I No ROX (Eurogentec, Seraing, Belgium) according the recommendations of the manufacturer (see ref. number RT-0000-06, at www.eurogentec.com). A standard curve for quantification was generated by plotting the log of the concentrations (from 5 ng to 28 fg) of total DNA isolated from *Forl* strain ZUM2407 in the presence of 5 ng of tomato plant DNA.

Results

Infection of tomato with Fox strains from different formae speciale

Tomato seeds were inoculated with strains *Forl* ZUM2407, *Fol* 004, *Forc* V03-2g and non-pathogenic *Fox* Fo47 in concentrations of 10^5 spores per liter of PNS. No difference in rate and level of germination was observed between untreated seeds and seeds inoculated with any of these four *Fox* strains. Tomato plants harvested one week after sowing had no symptoms of TFRR. Two and three week old tomato plants sometimes had brownish lesion and some plants were dead. The results of the score in which no differentiation of lesion heaviness was taken into account are shown in Fig.2A. No statistical difference in disease level between two-week old plants was observed using the undifferentiated lesion score. Most dead or heavily damaged tomato plantlets were observed in the variant treated with *Forl* ZUM2407. Tomato plants which were inoculated by *Fol* 004, *Forc* V03-2g and *Fox* Fo47 strains had mainly light lesions. This is illustrated by the indexed score results which are plotted in Fig.2B. The disease levels as determined by indexing of the lesions already show statistical differences for two week old plants. The disease level was significantly higher in three week old tomato plants with *Forl* ZUM2407. Stain *Fol* 004 did not cause any wilting, which is the typical disease symptom for this *forma specialis*.

Quantification of fungal DNA in planta

Equal efficacy of templates for DNA fragment amplification is important for comparison of different strains. DNA isolated from *Forl* ZUM2407, *Fol* 004, *Forc* V03-2g and *Fox* Fo47 strains was used to check the efficiency of the fragment amplification with primers OMP1049 and OMP1050. No significant difference was observed when the DNA dilutions ranged from 10 ng to 1 fg from each individual strain were compared using qPCR.

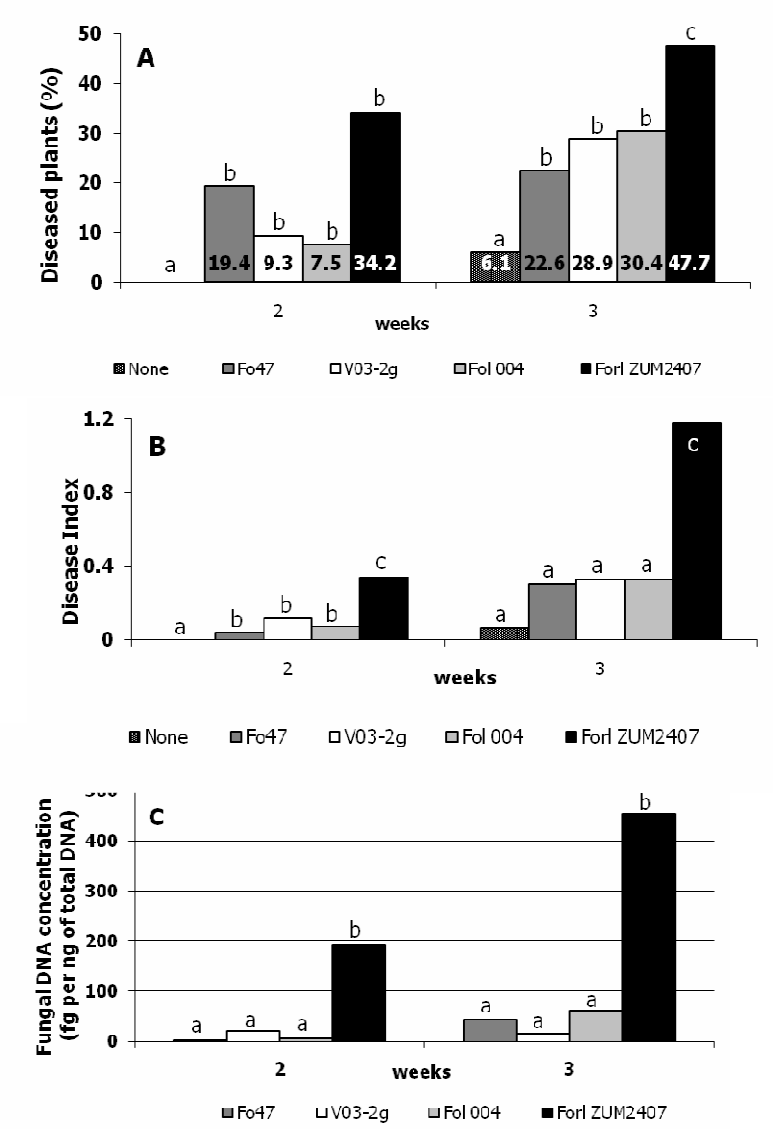

Fig. 1. Quantification of tomato infection by four *Fox* strains based on the results of two independent experiments. (A) results with no differentiation of lesion heaviness; (B) disease index score; (C) comparison of fungal DNA concentration in tomato plantlets. For statistics, a variance analysis followed by Fisher's least-significant-difference test (a = 0.05), was used. Statistically different values are labeled with different letters (a,b and c)

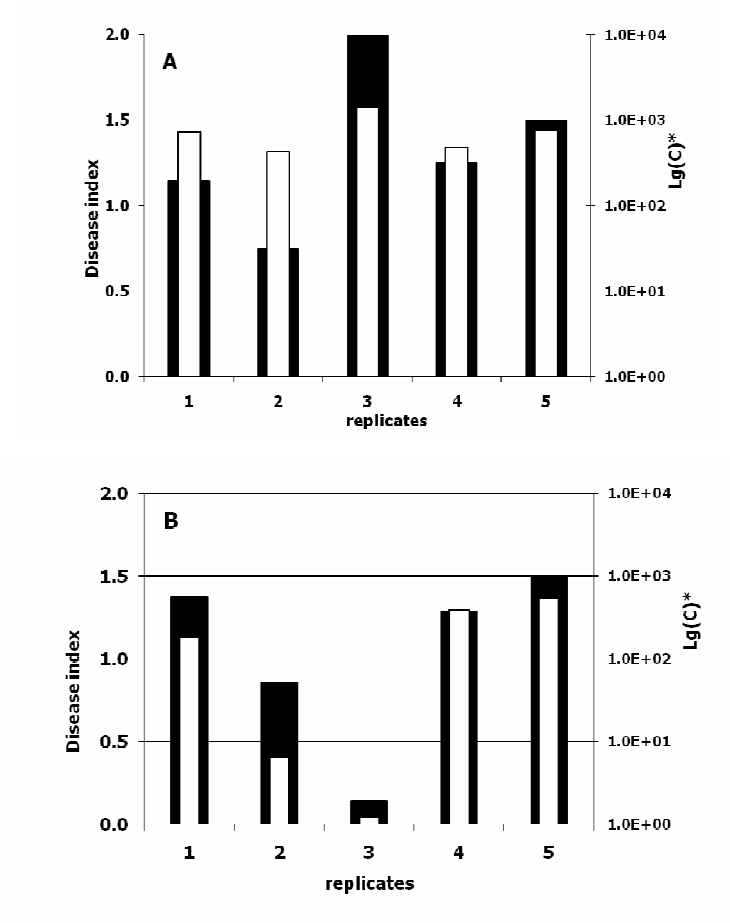

Fig. 2. Comparison of disease index and fungal DNA concentration
A and B represent results of two independent experiments on tomato infection with *Forl* ZUM2407. Plants have been scored in 3 weeks after the seed inoculation with fungal spores. Black bars correspond to disease index (left Y-axis), white ones show fungal DNA concentration in logarithmic scale (right Y-axis). Lg(C), C is fungal DNA concentration in fg per 1 ng of total DNA

To follow proliferation of *Fox* strains total DNA was isolated from harvested and scored groups (replicates) of tomato plants and used for qPCR. Comparisons of the indexed disease score and DNA quantification are shown on Fig. 2. In two independent trials DNA concentration shows the same trend as the disease index does. Results of the quantification show that the concentration of *Forl* ZUM2407 DNA is 5-10 times higher than those observed for the other *Fox* strains (Fig. 1C.).

Statistical analysis revealed difference in fungal DNA concentration between *Forl* ZUM2407 and the other *Fox* strains studied, starting from week 2 (Fig 1C).
The dynamics of fungal DNA concentration in plant material is given for all four strains in Figure 3. In both independent experiments, the DNA concentration of strains *Fol* 004, *Forc* V03-2g and Fo47 did not exceed 100 fg per 1 ng of total DNA. In the second week of growth, the DNA concentration of *Forl* ZUM2407 in tomato plants was 5 and 10 times higher, respectively, in first and second experiment, than those of strains *Fol* 004, *Forc* V03-2g and Fo47.

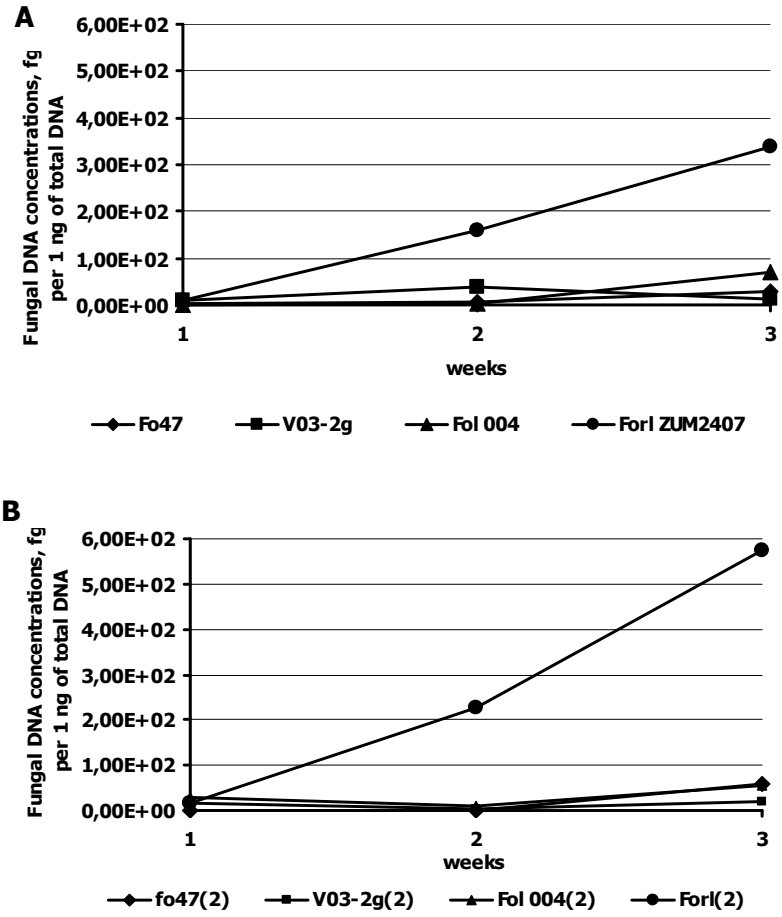

Fig. 3. Changes in fungal DNA concentrations during growth of inoculated tomato plants. A and B represent two independent experiments.

Discussion

We used IGS region as a target for qPCR. Ribosomal operon, in which the space between 18S and 28S rRNA genes is designated as IGS region, is presented in 200 copies per haploid genome of *Fox*, which gives high sensitivity to qPCR reaction. It was possible to detect 20 fg of DNA from the four *Fox* strains in 1 ng of tomato plant DNA, using primer pair OMP1049 – OMP1050 which amplify 150-bp fragment within the IGS. Similar results were obtained when IGS region was used for detection of *Fox* f. sp. *vasinfectum* in cotton seedlings (Abd-Elsalam et al., 2006). Lower detection level of 100 fg of pure fungal DNA reported in this article might be explained by the bigger size of the fragment for amplification (438-bp).

Fungal DNA concentrations determined by qPCR were in a good agreement with the results of indexed disease score. Replicates containing more plants with heavy lesions were given high disease index and they showed higher DNA concentration of *Forl* strain ZUM2407 (Fig.2). Similar correlation between biomass of *Alternaria brassicola* and *Botrytis cinerea* were obtained when disease progression of the pathogens on *Arabidopsis thaliana* was quantified (Brouwer *et al.*, 2003).

Results of score with no differentiation of lesion heaviness give no statistical difference between *Fox* strain on the second week (Fig. 1A). In contrast, disease index shows statistical difference between *Forl* ZUM2407 and the other three *Fox* strains, since *Fox* Fo47, *Forc* V03-2g and *Fol* 004 strains were producing only small lesions on tomato plantlets (Fig. 1B). Fungal DNA concentration determined in plants inoculated with different *Fox* strains correlated with disease index score and showed statistical difference between *Forl* strain ZUM2407 and the other three *Fox* strains (Fig. 1C).

Colonization of tomato plants by four *Fox* strain was followed by fungal DNA concentration in isolated DNA of plant. Concentration of *Forl* strain ZUM2407 DNA was increasing from week 1 to week 3, whereas concentration of DNA from *Fox* Fo47, *Forc* V03-2g and *Fol* 004 strains did not change dramatically and did not exceed 100 fg per ng of total DNA (Fig. 3).

Although *Fol* strain 004 is a vascular wilt pathogen of tomato, it showed neither disease symptoms nor DNA increase in our experiments. The passive behavior of this strain can be explained by long incubation period needed (more than four weeks) for the development of tomato wilt. It has been also shown that infection by the representatives of *Fol* progresses faster if plants has been wounded (Rep). In our experimental settings tomato plantlets were not damaged and had been harvested in three weeks after infection.

PCR-based methods give high sensitivity of detection; they also can facilitate analysis and reduce the time of detection procedure (Lievens *et al*., 2005). Choice of the target DNA fragment is a pivotal for the monitoring. Both types of targets: fragments specific to a certain group of *Fox* strains (supposedly *forma specialis*) and orthologous sequences (ribosolmal operon, tubulin gene etc.) were used for detection of the fungal strains in various substrate.

For example, anonymous fragments generated with RAPD methods were used for detection of *Fox* pathogenic to Paris daisy and basil (Pasquali et al*.,* 2004; Pasquali et al*.,* 2006). The target fragments used for amplification gave high sensitivity to detection of specific pathogens of Paris daisy and basil. Authors do not discuss whether all *Fox* which are pathogenic to these crops can be detected using primers specific for these anonymous fragments. Many of formae speciales such a *Fox*. f. sp. *cubense*, *Fox*. f. sp. *melonis, Forl* comprise of strains which have polyphyletic evolutionary origin (Kistler, 1997; Jacobson and Gordon, 1990; Validov et al., chapter 4). It means that unrelated *Fox* strains can be pathogenic to the same plant species, and consequently, some of these strains can miss an anonymous fragment, which is the target for qPCR. Vice versa, revealing of DNA fragments in the samples, except of genes which are involved in host-plant infection (Rep et al., 2004), cannot guarantee fully that detected *Fox* is a specific pathogen of the given plant.

Orthologuous sequences cannot be used for distinguishing of heterogenic *formae speciales* or for discrimination of pathogenic *Fox* strains from non-pathogenic ones. On another hand, multicopy orthologuous sequences are

convenient targets for qPCR due to wide range of strains which can be detected and high sensitivity of the reaction.

According Olivian and Alabouvette (1999), difference in colonization of tomato by pathogenic and non-pathogenic strains of *Fox* is mainly quantitative. This difference, perhaps, can be explained by observation made during linen root colonization by pathogenic and non-pathogenic strains of *Fox.* Strain *Fox* Fo47 trigger formation of barriers, which apparently stop further invasion by the fungus, whereas pathogenic strain avoids plant defense system (Olivian et al. 2003). In this scenario non-pathogenic strains are doomed to stay outside of the roots and compete for scant nutrients from root exudates with rhizosphere microorganisms. In opposite, pathogenic strains proliferate on abundant nutrients, being in plant cortex.

Our results show that non-pathogenic *Fox* strain Fo47 and non-compatible pathogen *Forc* V03-2g, as well as, *Fol* strain 004 could not exceed 40 fg per ng of total DNA neither on week two nor on week three. This concentration might be the level of colonization by non-pathogenic strain or by non-compatible pathogen *Fox,* and this colonization level apparently is not dangerous for tomato plant health.

Exploitation of conservative multicopy region, such an IGS fragment, gives highly sensitive detection of *Fox* strains, in the same time, quantification of the *Fox* DNA in plants material might distinguish progressing of specific pathogen from root colonization by non-pathogenic *Fox* strains.

Acknowledgements

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Chapter 7

Biocontrol of tomato foot and root rot in stonewool by *Pseudomonas putida* strain PCL1760 in a certified greenhouse under industrial conditions

Shamil Z. Validov, Faina Kamilova and Ben J.J. Lugtenberg

Abstract

Fusarium oxysporum f. sp. *radicis-lycopersici* (*Forl*) causes tomato foot and root rot (TFRR). The bacterium *P. putida* strain PCL1760 is able to control the disease when it is added as a suspension in the plant nutrient solution (PNS) to the stonewool substrate. Here we report the results of a trial performed in a certified greenhouse under industrial conditions. From an analysis 31 days after sowing, it appeared that the addition of *Forl* spores to the PNS not only caused disease symptoms but also decreased seed germination as well as plant fresh weight. Addition of cells of *P. putida* strain PCL1760 together with *Forl* spores significantly decreased the disease severity and also had a positive effect on seed germination and fresh weight. qPCR showed that the presence of PCL1760 also decreased the amount of *Forl* DNA in the plant. Re-application of the biocontrol bacterium one week after the first application did not improve the results. Application of Mycostop®Biofungicide, a registered biocontrol product claimed to control damping off, root and stem rot, and wilt caused by *Fusarium* of greenhouse ornamentals and vegetables such as cucumber, tomatoes and peppers (www.acornorganic.org) as a positive control, did not decrease the disease severity.

Introduction

Diseases caused by phytopathogenic *Fusarium oxysporum* strains decrease crop yields of vegetable plants such as cucumber, sweet pepper and tomato (Brayford, 1996). The introduction of stonewool substrate provides the grower with a highly manageable and controllable system for plant cultivation. New stonewool is practically free from (both beneficial and pathogenic) microbes. The absence of pathogens is a clear advantage but the absence of buffering microbes is a disadvantage (Moulin et al., 1994; Postma et al., 2000). Seedlings growing in stonewool are very vulnerable for pathogens entering the substrate (Postma et al., 2000).

Previously it has been shown that microflora, developed in stonewool, can suppress diseases (Postma et al., 2000; Folman et al., 2001). We have isolated bacteria which efficiently utilize root exudate nutrients and efficiently colonize both plant root and stonewool. Under laboratory conditions such bacteria were shown to control tomato foot and root rot (TFRR) caused by *Forl* ZUM2407 of plants growing in potting soil (Kamilova et al., 2005) and in stonewool (Validov et al., 2007).

In the present paper we report the results of an efficacy trial of the control of TFRR by *P. putida* strain PCL1760 in a certified greenhouse under practical plant growth conditions.

Materials and Methods

Strains and growth conditions

Forl is the causal agent of tomato foot and root rot (Brayford, 1996). *Forl* strain ZUM2407 (Chin-A-Woeng et al., 1998) was maintained on Czapec-Dox agar (CDA, Difco Laboratories, Detroit, MI, USA). To grow *Forl*, mycelium was placed on CDA and incubated at 28°C for 5 – 10 days. To avoid bacterial contamination, CDA was supplemented with kanamycin and tetracyclin, both purchased from Duchefa (Haarlem, The Netherlands), to final concentrations of 50 and 40 µg per ml, respectively. To generate microspores, PDA pieces with mycelium of *Forl* ZUM2407 were placed in Czapek-Dox broth and incubated under vigorous aeration at 28°C for 72 hours. The fungal culture was filtered through cheese cloth to remove agar pieces and mycelium. The microspore concentration in the filtrate was determined using light microscopy.

Biocontrol agent *P. putida* strain PCL1760, isolated from the avocado rhizosphere (Validov et al., 2007), was maintained on *Pseudomonas* Isolation Agar (PIA; Difco Laboratories). When a liquid culture was needed, PCL1760 was grown in KB (King et al., 1954) at 28°C under vigorous aeration for 15 – 18 hours.

Mycostop®Biofungicide was added to PNS to a final concentration o,35 g/L PNS (2 g per 1000 tomato plants) as recommended by the manufacturer (Kemira Agro OY, Helsinki, Finland) simultaneously with microspores of *Forl*.

Generation of an environmentally fit rifampicin resistant mutant of P. putida PCL1760

To obtain spontaneous rifampicin resistant mutants, an concentrated overnight culture (approx. 10^{11} cells/ml) of *P. putida* PCL1760 was plated on PIA supplemented with rifampicin (Duchefa, Haarlem, The Netherlands) to a final concentration of 100 µg/ml. Approximately 100 rifampicin resistant colonies were scraped off plates and suspended in PBS (phosphate-buffered saline; Sambrook and Russel, 2001). Tomato seeds were inoculated by immersion in this suspension for ten minutes. After growth of the seedlings for seven days, the root tip was cut off,

the bacteria were washed off the root tip, and serial dilutions were plated. Ten single colonies were tested for competitive tomato root tip colonization against the wild type strain PCL1760 (Simons et al., 1996). One mutant, strain PCL1765, which was equal to the parental strain in competitive colonization of the tomato root tip after seed inoculation, and therefore environmentally fit, was used in further experiments.

Biocontrol of Tomato Foot and Root Rot

Seeds of tomato (*Lycopersicon esculentum* Mill.) var. Carmello were sown in stonewool plugs in styropor trays (Grodan BV, Roermond, The Netherlands) and each plug was covered with 0.5 g of vermiculite. The trays were soaked in PNS (PPO, Bleiswijk, The Netherlands) with or without fungal spores and with or without *P. putida* PCL1760 cells as shown in Table 1. To allow seed germination, the trays were incubated in the dark at 25-30°C for 72h. After germination, trays with tomato plantlets were transferred to the greenhouse at 20-24°C, 80% relative humidity and 16 h of daylight. After 17 days of growth, plugs with plants were transferred to stonewool blocks (Grodan BV, Roermond, The Netherlands). After transplanting, blocks were placed on tables and watered automatically for five minutes every day with PNS without added microbes. Humidity, daylight duration and temperature of tomato cultivation were the same as indicated previously.

Biocontrol was scored after 31 days. Tomato roots were removed from stonewool blocks and analyzed. The disease level was scored by indexing the disease severity: healthy plants were given a value of 0, plants with small lesions (<2 mm) were given a value of 1, plants with developed lesions – 2. The value for dead plants was 3. The disease index (DI) was calculated using following formula:

$$DI = \frac{n0 \times 0 + n1 \times 1 + n2 \times 2 + n3 \times 3}{n0 + n1 + n2 + n3}$$

In which n0, n1, n2 and n3 are the number of plants with indexes of 0, 1, 2 and 3, respectively. Differences in disease index among treatments were determined by analysis of variance (ANOVA) and mean comparisons were performed by Fisher's

least-significant difference test (α= 0.05), using SPSS software (SPSS, Chicago, IL, USA).

Table 1. Scheme of the biocontrol experiment in stonewool

Treatments	**Microbes added**	
	***Forl* ZUM2407**	***P. putida* PCL1760***
None (Positive control)	No	No
Forl (Negative control)	10^7 spores per L PNS	No
PCL1760	10^7 spores per L PNS	10^{10} CFU per L
PCL1760 (2×)**	10^7 spores per L PNS	2×10^{10} CFU per L (*Total*)

* *P. putida* PCL1760 was added as a mixture with its Rif^R mutant PCL1765 in a ratio of 9:1.
** Another mixture of strains PCL1760 and PCL1765 was added one week after the first introduction of the strains.

The stonewool (500 mg) from the plugs, in which tomato plantlets had grown, was sampled and homogenized in FastPrep FP220A (MP Biochemicals, Amsterdam, The Netherlands) with 0.5 ml of PBS (Sambrook and Russel, 2001) at top speed for 30 s. Five samples were taken from each variant. Serial dilutions of washes from the roots and the stonewool were plated on LB and on PIA supplemented with rifampicin 100 µg/ml. Circulating PNS from the irrigation system used for watering the stonewool blocks with growing tomatoes was sampled and plated directly on PIA with and without rifampicin at a final concentration of 100 µg/ml. Colonies grown on selective media were tested for their PCL1760 identity using RAPD as described in chapter 4.

The fresh weight of the above stonewool parts of the tomato plants was determined as follows: groups of eight to ten plants were weighed and the average fresh weight of the plants was calculated. Differences in fresh weight among treatments were evaluated by analysis of variance (ANOVA) and mean comparisons were performed by Fisher's least-significant difference test (α= 0.05), using SPSS software.

Sample collection and DNA isolation

Replicates of 6 plants each were pulverized in liquid nitrogen. One gram of ground plant material was mixed with 1 ml of extraction buffer consisting of 2% hexadecatrimethylammonium bromide (CTAB; Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), 100 mM Tris-HCl (pH 8.0), 1.4M NaCl and 20 mM EDTA (MP Biochemicals, Amsterdam, The Netherlands). The mixture was incubated at 60°C for 20 min and then centrifuged at 14.500 rpm for 5 min. The supernatant fluid was extracted with one volume of chloroform. The aqueous upper phase was transferred to a new Eppendorf tube and the DNA was precipitated by adding 0,6 volume of isopropanol and collected by centrifugation. The pellets were dissolved in 100 µl of TE buffer pH 8.0 (Sambrook and Russel, 2001). To remove RNA from the preparations, RNAse (Sigma-Aldrich Chemie BV) was added to a final concentration of 2 µg/ml. DNA preparations were incubated at 60°C for 30 min and mixed with five volumes of PB buffer and applied on columns of PCR-purification kits (QIAGEN Benelux BV, Venlo, The Netherlands). Subsequently, DNA purification was carried out as described in the instructions supplied with the QIAGEN PCR purification kit. DNA was eluted from the column with 50 µl of EB buffer of the same purification kit. The DNA concentration in the preparations was adjusted to 5 ng/µl.

To isolate DNA, the biomass of the *Fusarium oxysporum* (*Fox*) strains was ground in liquid nitrogen and further isolation was performed as described for the isolation of DNA from tomato plants.

Quantitative PCR reaction

Primers OMP1049 (5'-TGCGATTTGGACGAGATATGTG-3') and OMP1050 (5'-ATTTGCCTACCCTGTACCTACC-3') for quantitative PCR reaction were designed using Beacon Designer 5.0 (Bio-Rad Laboratories BV, Veenendaal, The Netherlands) on the basis of the IGS sequence of *Forl* ZUM2407 strain. Real-time PCR was performed in Chromo4^{TM} Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories BV, Veenendaal, The Netherlands) with the following thermal profile: initial DNA denaturation and polymerase activation at 95°C for 10 min, followed by 40 cycles each containing denaturation and annealing steps at 95°C and at 58°C,

respectively, both for 15 sec. Amplification cycles were followed by a melting curve built from 50°C to 90°C, with measurements performed every 0.2°C. The PCR mixture was prepared using the qPCR Core kit for SYBR® Green I No ROX (Eurogentec, Seraing, Belgium) according the recommendations of the manufacturer (see ref. number RT-0000-06, at www.eurogentec.com). A standard curve for quantification was generated by plotting the log of the concentrations (from 5 ng to 28 fg) of total DNA isolated from *Forl* strain ZUM2407 in the presence of 5 ng of tomato plant DNA.

Results

Effects of biocontrol strain P. putida PCL1760

The percentage of germinated seeds was scored 17 days after seed sowing. In the positive control, in the absence of added microbes, 95% of the seeds had germinated. The presence of *Forl* decreased this value to 57% but the addition of both *Forl* and *P. putida* PCL1760 resulted in a value of 76% (Fig. 1 A).

The average fresh weight per plant of the above stonewool parts of the tomato plants was scored 31 days after sowing the seeds. The presence of *Forl* decreased the fresh weight significantly, from 58.8 g in the positive control to 36.4 g (Fig. 1.B). The additional presence of *P. putida* PCL1760 increased the fresh weight to 50.0 g.

The disease severity was scored 31 days after sowing the seeds. The variants, in which *P. putida* strain PCL1760 was added showed significant biocontrol: the disease index decreased from 1.5 in negative control to 0.8 (Fig. 1.C).

When DNA of tomato plants was used as the template for quantitative PCR (qPCR) with primers specific to the IGS region of *Fusarium oxysporum*, no *Forl* DNA was detected in the positive control (Fig. 1D). In the positive control, the melting curves of the PCR products consist of a few peaks (Fig 2A), which are different from the peak of the IGS fragment amplified from the negative control, which contains only *Forl* DNA (Fig 2B).

qPCR semi-quantification showed that addition of biocontrol strain *P. putida* PCL1760 decreased the concentration of *Forl* DNA in plant material approximately 5-fold in comparison with the amount found in the negative control.

Also the numbers of biocontrol strain PCL1760 cells were determined in circulating PNS as well as in stonewool. None of the 30 colonies, obtained after recirculating PNS was plated on PIA, was identified as *P. putida* strain PCL1760, when RAPD profiles of these colonies and were compared with those of PCL1760. Rif^R mutants of *P. putida* PCL1760 were found in the growth substrate in a concentration of 3.1×10^5 per g of dry stonewool.

Effects of additional application of the biocontrol strain P. putida PCL1760

In the variant, in which PCL1760 was added twice, the seed germination was 74% (Fig.1A). Additional introduction of the biocontrol strain did not increase the average fresh weight of the tomato plants (Fig.1B). The disease index was significantly decreased from 1.5 in negative control to 1.2. (Fig. 1C).

The concentration of fungal DNA *in planta,* determined by using qPCR, was 0.2 pg per ng of total DNA (Fig.1D).

Effects of application of the biocontrol product Mycostop® Biofungicide

Mycostop®Biofungicide is a commercial product from Kemira Agro Oy, Helsinki, Finland. It is claimed to control damping off, root and stem rot, and wilt caused by *Fusarium* of greenhouse ornamentals and vegetables such as cucumber, tomatoes and peppers (www.acornorganic.org). In our experiment Mycostop®Biofungicide slightly increased seed germination from 57% in the negative control (with *Forl* alone) to 62% (Fig. 1A). The average fresh weight of tomato plants increased from 36.4 g in the negative control to 46.0 g (Fig. 1B). Addition of Mycostop®Biofungicide did not decrease the disease level *Forl* (Fig. 1C).

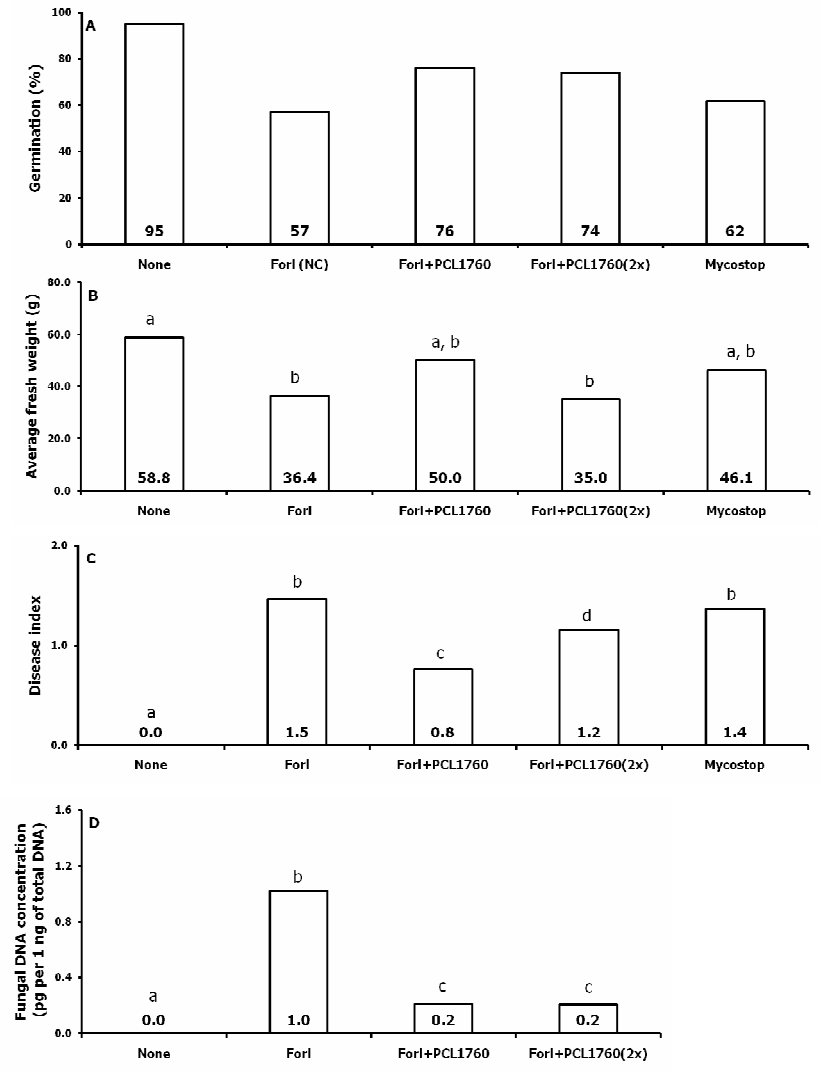

Fig. 1. Influence of biocontrol strains on tomato development: germination of tomato seeds (Panel A), average fresh weight of the above stonewool part of tomato plants (Panel B), disease severity (Panel C) and fungal DNA concentration *in planta* (D). Microconidia of *Forl* ZUM2407 and cells of *P. putida* PCL1760, its rif-resistant mutant PCL1765 and Mycostop®Biofungicide were added to PNS simultaneously. For *Forl*+PCL1760(×2) the bacterial strain was added again one week after the first introduction (Table 1). Different letters above the bars on the graphs indicate significantly different results ($p<0.05$). The fungal DNA concentration was not determined for the plants treated with Mycostop®Biofungicide.

Discussion

We have shown that under practical horticultural conditions in stonewool substrate in a greenhouse, *Forl* decreases seed germination of tomato cv. Carmello, decreases the fresh weight of the plants and causes severe disease symptoms (Fig. 1). Under the same conditions, *P. putida* strain PCL1760, which has been reported to control TFRR in the laboratory (chapters 3 and 4), when added together with *Forl* spores, decreases the disease severity of the plants significantly. Moreover, it alleviates the negative effects of *Forl* on the germination of tomato seeds (Fig. 1A) and on the fresh weight of the plants (Fig. 1B). This beneficial effect of *P. putida* strain PCL1760 is accompanied by a significant decrease of the amount of *Forl* DNA in plant material (Fig. 1D) It is likely that the decrease of *Forl* biomass is caused by competition of *P. putida* strain PCL1760 cells with the fungus for exudate nutrients (chapter 4). Furthermore, inhibition of fungal spore germination in exudate by *P. putida* strain PCL1760, which was proven for biocontrol bacterium *P. fluorescens* WCS365 (Kamilova et al., 2006), may contribute to this beneficial effect.

Recirculating PNS and stonewool were tested for the presence of the biocontrol strain. *P. putida* strain PCL1760 was not found in PNS which was recirculating in the

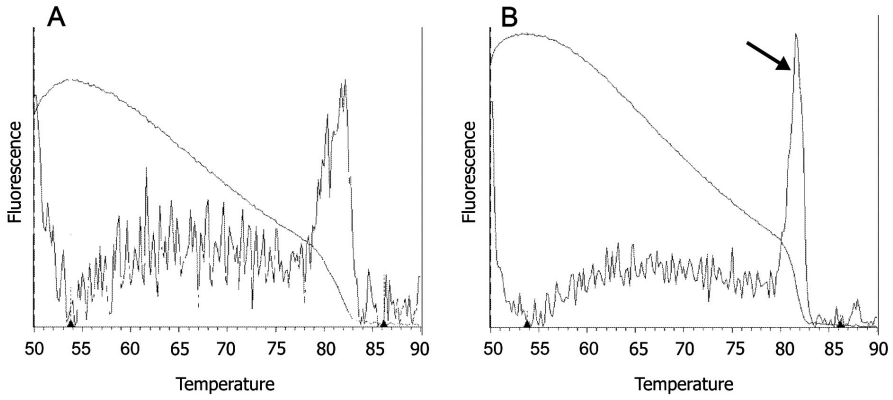

Fig. 2. Melting curves of fragments amplified from the template of tomato DNA used as isolated from the plants from positive control (Panel A) and negative control (Panel B). The black arrow points the peak which indicates denaturation of the IGS fragment from *Forl* strain ZUM2407

stonewool block watering system. Strain PCL1765, which is a Rif^R mutant of *P. putida* PCL1760, was recovered from stonewool in a concentration of 3.1×10^5. Taking the original ratio of Rif^S/Rif^R into consideration, the total concentration of *P. putida* strain PCL1760 must be 10 times higher. These results are in the good agreement with ones obtained under laboratory conditions: during the biocontrol PCL1760 was colonizing stonewool in a concentration of approx. 10^6 cells per gram of dry substrate (Chapter 4). Apparently, cells of the biocontrol agent *P. putida* strain PCL1760 stay on the plant roots (chapter 4) and on the stonewool surrounding them, and did not reach the PNS circulating in the watering system. This result indicates that by far most cells of the biocontrol strain stay close to the place where they should exert their beneficial action and also that the risk of dissimination of the biocontrol strain to the environment is low.

Additional application of *P. putida* strain PCL1760 cells had no advantage over single application (Fig. 1). In spite of the difference in fresh weight of the tomato plants and in disease levels found in variants in which PCL1760 was applied once or twice, the *Forl* DNA concentration in tomato plants was nearly the same (Fig.1C).

The commercial product Mycostop®Biofungicide, which is based on the bacterium *Streptomyces griseoviridis*, and is supposed to control TFRR, was not effective (Fig.1).

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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 trough 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 *Forl* in potting soil and stonewool can be selected from rhizoshere 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 *Forl,* 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 *Forl* 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 *Forl* 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 *Forl* can (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 develope 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 rhizoshpere 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 1. Efficacy of biocontrol of TFRR by *P. putida* PCL1760 in trials under laboratory conditions.

Diseased tomato plants (%)		**Reduction of TFRR**
***Forl* (Negative control)**	***Forl* + PCL1760**	**symptoms (%)**
18.7	8.3	55
29.9	13.7	54
39,8	17	56
53.3	25.9	51
62.2	10.3	83
62.4	28.6	54
62.5*	46*	26
62.6	18.1	71
72	38.9	45

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 ($\alpha = 0.05$).

*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 (Chapter3, 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 *Forl* ZUM2407 was used (Chapter3, 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 *Fusarum 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.

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Nederlandse samenvatting

Samenvatting

Inleiding en doelstelling

De grootste bedreiging voor de productie van planten is infectie met ziekteverwekkende schimmels. Veel schimmels kunnen worden bestreden met chemische pesticiden. Politiek en publieke opinie hebben zich tegen deze bestrijdingswijze gekeerd.

Recent is een aantal microbiologische producten ontwikkeld om infectie met schimmels te voorkomen of de infectie te bestrijden. Dit is een vorm van biologische controle. In ons laboratorium richten we ons op biocontrole-bacteriën die actief zijn tegen schimmels die het wortelstelsel aanvallen. Meer precies, we gebruiken als model de ziekte tomaten-voet-en-wortelrot (in het Engels tomato foot and root rot, TFRR), die wordt veroorzaakt door de bodemschimmel *Fusarium oxysporum* f. sp. *radicis-lycopersoci* (*Forl)*. We proberen de mechanismen te ontrafelen die bacteriën gebruiken om *Forl* te bestrijden. Bovendien proberen we nieuwe biocontrole-bacterien te isoleren.

Mechanismen van biocontrole

Toen dit door STW gesteunde onderzoek begon waren vooral de volgende mechanismen bekend.

1. Antibiose. Hierbij produceert de bacterie een antibioticum dat actief is tegen de schimmel. Voorwaarde voor effectieve bestrijding is dat de bacterie het wortelstelsel van de plant goed koloniseert. In dat geval wordt het antibioticum overal op de wortel geproduceerd en is de bescherming tegen de schimmel succesvol.

2. Inductie van Systemische Resistentie (ISR). Sommige bacteriën produceren stoffen die de plant aanzetten tot een snellere reactie op infectie met schimmels.

3. Predatie en parasitisme. Hierbij maakt de biologische bestrijder enzymen die de celwand van de pathogene schimmel afbreken. Het best bekende voorbeeld is

de biocontrole-schimmel *Trichoderma*. Bij bacteriën is dit mechanisme voor bepaalde *Serratia* stammen voorgesteld.

Hoe worden biocontrole bacteriën geisoleerd?

Het vinden van biocontrole-microben is een arbeidsintensieve zaak. Het minst moeilijk is nog het vinden van stammen die werken via antibiose. Zulke bacteriën kunnen op een plaat met vast groeimedium worden getest op de productie van stoffen die de groei van de pathogene schimmel, die op dezelfde plaat wordt aangebracht, remmen. Sommige van de positieve bacteriestammen blijken ook te werken als ze op zaad of plant worden aangebracht. Op deze wijze is een aantal biocontrole-stammen geisoleerd, vooral *Pseudomonas* en *Bacillus* soorten. Een voorbeeld is de in ons laboratorium geisoleerde bacterie *Pseudomonas chlororaphis* PCL1391, die actief is tegen TFRR. Een groot nadeel bij toepassing van antibioticum-producerende bacteriën is echter dat het bij de registratie als product moeilijk is om bacteriën, die antibiotica in het milieu uitscheiden, voor toepassing goedgekeurd te krijgen.

Men zou verwachten dat, via eenzelfde voor-screening als werd gebruikt bij bacteriën die werken via de productie van antibiotica, bacteriën te isoleren zijn die werken via predatie en parasitisme. Dit is echter nooit gerapporteerd in de vakliteratuur.

Het vinden van stammen die werken via ISR kan alleen gedaan worden via het testen van grote aantallen bacterien in een biocontrole test. Aangezien de kansen laag zijn en de test arbeids-intensief, is dit een vrijwel onmogelijke taak. In ons laboratorium is lang gewerkt met de stam *Pseudomonas fluorescens* WCS365, die het wortelstelsel van veel planten voortreffelijk koloniseert. De stam functioneerde dan ook als model-stam om het mechanisme van kolonisatie te bestuderen. Tot onze grote verbazing bleek echter dat de stam niet werkte via het tot dan toe hypothetische mechanisme "competitie voor voedingsstoffen en plekken op de wortel" (in het Engels "competition for nutrients and niches; CNN"), maar via ISR. Geluk komt ook in de wetenschap voor. De stam werkt zeer goed tegen TFRR.

Bestaat het mechanisme "Competitie voor Nutrienten en Niches" (CNN)?

Tijdens het bestuderen van wortelkolonisatie was in ons lab in de jaren negentig van de vorige eeuw een systeem ontwikkeld om het competitief koloniserend vermogen van bacteriën ten opzichte van elkaar te meten. In ons STW project LBI.5884 werd besloten daarmee de hypothese te testen dat het mechanisme "competitie voor voedingsstoffen en plekken op de wortel" bestaat. Voor dit mechanisme was nooit een overtuigend bewijs geleverd. Een mengsel van (honderd)duizenden wortelbacteriën werd door ons in een simpele test aan de competitie om de wortel onderworpen en de beste koloniseerders werden op biocontrole van TFRR getest. Het resultaat was zeer positief: een groot deel van de geteste bacteriën bleek actief te zijn tegen TFRR. De meeste van deze bacteriën produceren geen antibiotica wat voordelig is in geval van registratie als product. (Hoofdstukken 2 en 3).

In eerste instantie werd biocontrole van TFRR getest in grond, een klassiek substraat voor de productie van tomaat (hoofdstuk 2). Moderne, hoog-productieve tomatenproductie vindt echte plaats op steenwol-substraat. Zo worden in Nederland alle tomaten op steenwol gekweekt en in Spanje 40%. Daarom werd de door ons ontwikkelde techniek ook op steenwol toegepast, eveneens met succes (hoofdstuk 3). We zijn er dus als eerste in geslaagd biocontrole bacteriën op te hopen.

Als "proof of priciple "werd getest of onze veronderstelling, dat de isolaten werken via "competitie voor voedingsstoffen en plekken op de wortel", klopt. Dit leek het geval te zijn voor stam *P. fluorescens* PCL1751 als tomaat werd gekweekt in grond (hoofdstuk 2). Het werkingsmechanisme werd in groter detail bestudeerd voor de stam *P. putida* PCL1760 voor tomatenproductie op steenwol (hoofdstuk 4). Competitie voor nutrienten als mechanisme werd duidelijk bewezen: de biocontrole-stam consumeerde het enige beschikbare voedsel, tomaten wortel-exudaat (dit is een soort zweet dat de wortel uitscheidt), veel efficiënter dan *Forl*: de bacterie verhinderde zelfs de groei van *Forl* compleet. Een bewegingsloze mutant, die de wortel (de niche) niet kan koloniseren, vertoonde geen biocontrole in grond.

Daarmee is bewezen dat competitie voor de wortel-niche een essentieele rol speelt in de biocontrole van TFRR door stam PCL1760.

Tot onze verbazing konden we in steenwol niet bewijzen dat competitie voor een plaats op de wortel een rol speelde in de controle van TFRR. Daartoe werd dezelfde bewegingsloze mutant gebruikt. In steenwol bleek dat deze mutant significante bescherming gaf. Toen we controleerden of de bacterie de wortel koloniseerde, hetgeen niet werd verwacht voor een bewegingsloze mutant, bleek dat de mutant de wortel net zo effectief als de ouder had gekoloniseerd. Als mogelijke verklaring werd bedacht dat steenwol de biocontrole bacterie vasthoudt waardoor de in steenwol groeiende wortel continu in contact met de bacterie blijft. Uit onze testen bleek vervolgens dat toevoegen van onze bacterie aan steenwol, zoals voor biocontrole wordt gedaan, ertoe leidt dat de steenwol meer dan 95% van de bacteriën bindt. Het lijkt er dus op dat steenwol sterk bijdraagt aan wortelkolonisatie en dat in steenwol beweeglijkheid van de bacterie niet zo belangrijk is. Wortelkolonisatie zal waarschijnlijk ook in steenwol essentieel zijn voor biocontrole maar de bewegingsloze mutant is geen goed hulpmiddel om dat te testen.

Diversiteit van Fusarium stammen en monitoring van infectie met pathogenen

Fusarium komt erg algemeen voor, niet alleen als ziekteverwekker maar ook als ongevaarlijke of zelfs als voor de mens gunstige schimmel. In dit onderzoek werden 33 stammen bestudeerd afkomstig van diverse planten en al of niet als ziekteverwekker geisoleerd. Om infectie met een pathogene *Forl* snel te kunnen detecteren werd in eerste instantie getracht een DNA fragment te vinden dat uniek is voor alle tomaten-pathogenen en dat niet voorkomt in andere pathogenen en ook niet in niet-pathogenen. Verwacht werd dat we zo'n fragment konden vinden in de zgn. intergenic spacer region (ISR). Dit bleek echter niet het geval. Wel werd een *Fox* –specifiek fragment gevonden (hoofdstuk 5). Daarmee werd gekeken of misschien de hoeveelheid *Fox* DNA in de tomatenplant een maat voor infectie met tomaatpathogenen kon zijn. Tot onze verrassing werd gevonden dat alle 33

teststammen zowel tomaat als komkommer binnendringen. Tomatenpathogenen bleken echter te onderscheiden van de andere stammen omdat de laatsten al vroeg na infectie van de plant stoppen met hun groei in de plant. We concludeerden dat, zodra de hoeveelheid *Fox* DNA in de plant groter is dan 50 fg per ng totaal planten DNA (d.w.z. meer dan 0.005%), gesproken kan worden van infectie met een tomatenpathogeen. Zo kan dus infectie van tomaat met een tomatenpathogeen toch snel worden ontdekt (hoofdstuk 6).

Kan Pseudomonas TFRR ook onder praktijkomstandigheden bestrijden?

Hoewel we uitgebreid hebben laten zien dat ons isolaat *P. putida* PLC1760 in een laboratoriumkas prima TFRR kan bestrijden (significant succes in zeven van de acht uitgevoerde tests) , zijn praktijkmensen pas te overtuigen als dit ook onder praktijkomstandigheden, en uitgevoerd door derden, het geval is. Daartoe werd door het gecertificeerde, dus onafhankelijke, instituut PPO in Bleiswijk een proef uitgevoerd waarbij tomaten van het cultivar Carmello in steenwol werden besmet met sporen van de ziekteverwekker *Fusarium oxysporum* f. sp. *radicis-lycopersici* ZUM2407. Om effectiviteit van biocontrole te testen werd een deel van de planten tevens beent met onze *P. putida* PCL1760 of van het commercieel verkrijgbare biofungicide Mycostop®Biofungicide, dat wordt aanbevolen voor toepassing tegen o.a. *Fusarium* in kassen. Eenendertig dagen na infectie bleek onze *Pseudomonas* de ziekte significant en overtuigend te hebben beperkt terwijl het commercieel verkrijgbare product niet bleek te werken. Uit deze resultaten kunnen we concluderen dat *P. putida* PL1760 een aantrekkelijke kandidaat is voor de industrieele toepassing tegen *Fusarium* in kassen waarin tomaat wordt gekweekt. Gezien het werkingsmechanisme van de stam, CNN, is het waarschijnlijk dat PCL 1760 ook tegen andere wortelziekten van tomaat kan worden ingezet. Bovendien, aangezien de exudaatsamenstelling van bv. komkommer en paprika sterk op die van tomaat lijkt, is het waarschijnlijk dat PCL 1760 ook wortelpathogenen van andere groentegewassen kan bestrijden.

Краткое изложение работы:

Биологический контроль корневой гнили томатов бактериями рода *Pseudomonas* в исскуственном субстрате (каменном волокне)

Summary and General Discussion (in Russian)

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Актуальность проблемы.

Заболевания растений являются одним из факторов серьезно ограничивающих продуктивность сельского хозяйства во всем мире. Ежегодный ущерб, причиняемый фитопатогенными микроорганизмами, значительная часть которых представлена паразитическими грибами, составляет, по разным оценкам, от 15 до 20% общей продуктивности мирового растениеводства. Ранняя диагностика заболеваний растений, например, методами на основе полимеразной цепной реакции (ПЦР), позволяет обнаружить следовые количества патогенных микроорганизмов в максимально сжатые сроки (Lievens and Thomma, 2005), выбрать и своевременно применить меры защиты, что снижает экономические риски сельского хозяйства связанные с заболеваниями растений.

В настоящее время химические пестициды являются наиболее востребованным инструментом в защите растений. Токсичность этих агрохимикатов для окружающей среды, персонала сельскохозяйственных (с/х) предприятий и конечного потребителя с/х продукции общеизвестна и вызывает серьезную озабоченность в обществе. Введенные Европейским Союзом ограничения на использование химических пестицидов в тепличных хозяйствах стимулировали поиск альтернативных методов борьбы с заболеваниями растений. Одним из таких методов является применение биологических препаратов, которые в отличие от химических пестицидов безвредны для окружающей среды и потребителя (Cross and Polonenko, 1996; Montesinos, 2003; Lugtenberg and Bloemberg, 2004). Не смотря на значительный перечень зарегистрированных биопрепаратов (Koch, 2001) и очевидные преимущества, они пока не находят широкого применения в с/х. Причина такого пренебрежения, коренится не только в сложных и дорогих процедурах сертификации биопестицидов, но и в нестабильности их защитного эффекта в полевых условиях (Fravel, 1999). Варьирующие условия окружающей среды могут оказывать значительное влияние на защитные свойства микроорганизмов (Lee and Cooksey, 2000; Tomashow and Weller, 1996; Duffy and Défago, 1997, van Rij et al., 2005), что сказывается на эффективности биопрепаратов.

Условия культивирования растений в тепличных хозяйствах отличает большая постоянность, что предполагает также стабильный защитный эффект от применения биопестицидов (Paulitz and Belanger, 2006). Использование каменного волокна (КВ), в качестве субстрата для роста, повысило продуктивность растений и избавило от таких

почвенных вредителей как фитопатогенные нематоды. Поражение растений фитопатогеными грибами в КВ возможна, поскольку споры этих микроорганизмов могут передаваться по воздуху, вноситься вместе с растительным материалом и затем распространятся от растения к растению циркулирующим в системе питательным раствором (Brayford, 1996; Postma et al., 2000). Было установлено, что присутствие непатогенных микроорганизмов в КВ защищает растения от поражения фитопатогенным грибом *Pythium afanidermatum* (Postma et al., 2000). Инокуляция КВ тщательно подобранными микроорганизмами в начале культивации, очевидно, может защитить растения от патогенов. С учетом ограниченного набора химических пестицидов разрешенных к применению в тепличных хозяйствах, биопестициды становятся привлекательным альтернативным методом защиты растений в высокотехнологичных и контролируемых условиях искусственных субстратов в теплицах.

Цель и задачи исследования.

Исследования, описанные в данной работе, направлены на создание средств биологического контроля корневой гнили томатов, вызванной патогенным грибом *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*) в условиях КВ в теплице. В соответствии с поставленной целью были определены следующие задачи:

1. Разработка метода накопительной культуры для выделения бактерий, которые могут защитить растения от фузариозной корневой гнили (биоконтрольные бактерии; Глава 2)

2. Выделение биоконтрольных бактерий с использованием этого нового метода, адаптированного к условиям КВ, и характеризация этих бактерий (Глава 3).

3. Исследование механизма контроля корневой гнили томатов используемого штаммом *P. putida* PCL1760 – наилучшим биоконтрольным агентом, выделенным с помошью метода накопительной культуры (Глава 4),

4. Изучение разнообразия *Fusarium oxysporum* – модельного фитопатогена, использованного при изучении биоконтроля фузариозной корневой гнили томатов (Глава 5)

5. Количественное определение биомассы *Fusarium oxysporum* в растительных тканях для обнаружения инфекции томатов данным патогеном (Глава 6)

6. Испытание защитных (биоконтрольных) свойств штамма *P. putida* PCL1760 по отношению к фузариозной гнили томатов в промышленный условиях в сертифицированной теплице. Оценка эффективности биологической защиты (биоконтроля) с помощью рутинных и молекулярных методов.

Настоящая работа является частью проекта (LBI.5884) «Селекция микроорганизмов, оптимально приспособленных для контроля заболеваний растений, вызванных паразитическими грибами в условиях исскуственного субстрата – каменного волокна» поддержанного Службой Технических Наук, Отделения Прикладных Наук Голландской организации Научных Исследований и Технологических Программ Минестерства Экономики Королевства Нидерланды.

Научная новизна

Результаты, полученные в данной работе, являются оригинальными и несомненно имеют теоретическую ценность. В ходе работы выделены бактерии, подавляющие корневую гниль томатов за счет конкуренции с фитопатогеном за пространство и питательные вещества в ризосфере. До настоящего времени, этот механизм биоконтроля описывался только как теоретически возможный, причем защитный потенциал этого механизма в реальных условиях подвергался сомнению.

Предполагается, что вегетативные группы совместимости *Fusarium oxysporum* состоят из штаммов происходящих от одного и того же клона (клональная теория эволюции), поскольку половой процесс для этого вида грибов не описан. Изучение разнообразия фитопатогенных штаммов *Fusarium oxysporum* выявило гетерогенность внутри вегетативных групп совместимости, что отвергает клональную эволюцию специальных форм и групп вегетативной совместимости этого патогена.

Практическая значимость работы

Предложенный нами метод мониторинга фитопатогенных штаммов *Fusarium oxysporum,* вызывающих корневые гнили растений, является на сегодняшний день единственным способом, пригодным для различения фитопатогенных и сапрофитных штаммов для конкретного вида растений. Метод мониторинга, разработанный и

примененный в данном исследовании, может быть использован для фитосанитарного контроля томатов (растений) без дополнительных модификаций.

Предложенный метод накопительных культур и последующие стадии селекции позволяют выделять бактерии пригодные для защиты растений из образцов ризосферы для нужд тепличных хозяйств в сжатые сроки.

Изолированный в ходе настоящей работы штамм *P. putida* PCL1760 является активным средством защиты растений , эффективность которого доказана в промышленных условиях в сертифицированном тепличном хозяйстве.

Апробация работы.

Материалы диссертации были представлены на следующих конференциях:

1. Second International Symposium on Tomato Diseases 8-12 October, 2007, Kusadası, Turkey
2. IOBCwprs Working Group "Multitrophic Interactions in Soil" June 24-27, 2007, Dijon, France,
3. 2nd FEMS Congress of European Microbiologists, July 4-8 2006 Madrid, Spain
4. 7th PGPR workshop, 28 May – 2 June 2006, Nordwijkerhout, Netherlands
5. 10th International Congress on *Pseudomonas*, August 27-31, 2005, Marseille, France
6. IOBCwprs Working Group "Multitrophic Interactions in Soil" June 5-7, 2005, Wageningen, Netherlands

Публикации.

По материалам диссертации опубликовано 3 статьи и 7 тезисов.

Структура и объем диссертации.

Диссертация состоит из общего введения, включающего обзор литературы, из шести экспериментальных глав, общего обсуждения, выводов, и списка литературы, изложенных на английском языке; краткого изложения диссертационной работы на голландском языке и краткого изложения данной работы на русском языке. К диссертации также прилагаются биография автора (англ. яз) и перечень его публикаций. Диссертация изложена на 188 страницах машинописного текста, содержит 17 рисунков и 17 таблиц, нумерация которых возобновляется в каждой главе. Список литературы включает 235 источников.

Результаты исследования и их обсуждение

Бактерии, защищающие растения от *Forl* в почве и каменной вате, могут быть изолированы из образцов ризосферы методом накопительной культуры, основанной на конкурентной колонизации корня растений (Главы 2 и 3)

Бактерии, способные защитить растения от фитопатогенов (агенты биоконтроля), могут быть отобраны из образцов ризосферы методом накопительной культуры, основанном на конкурентной колонизации корня растений. Этот метод позволяет селектировать бактерии, которые агрессивно колонизируют корневую систему растения. Теоретически эти бактерии могут осуществлять защиту растений (биоконтроль) за счет конкуренции с патогенными микроорганизмами за питательные вещества и пространство в ризосфере (Lugtenberg et al., 2001). Во второй и третьей главах описывается выделение бактерий, подавляющих развитие корневой гнили томатов, вызванной *Forl*, предположительно посредством механизма конкуренции за питательные вещества и пространство в ризосфере (конкуренция за экологические ниши). Эти бактерии были *селектированы* из образцов ризосферы с помощью метода накопительной культуры, где собственно само растение отбирает наиболее активных колонизаторов корневой системы. Возможность подобного отбора отличает микроорганизмы, контролирующих фитопатогены посредством механизма конкуренции за экологические ниши от биоконтрольных агентов, использующих другие механизмы для защиты растений. Например, для выделения антагонистов возможен пре-скрининг выделенных микроорганизмов *in vitro*, но никаких процедур, облегчающих поиск микроорганизмов, инициирующих системную устойчивость растений, пока не существует.

В процессе работы накопительной культуры, изначальное разнообразие микроорганизмов в ризосфере экспериментальных растений изменяется таким образом, что бактерии, колонизующие корень наиболее активно, становятся преобладающей частью микробного сообщества. Бактериальные изоляты, отобранные в конце накопительной культуры, с большой вероятностью являются клонами одних и тех же штаммов, что и наблюдалось в наших экспериментах (Главы 2 и 3). Мы разработали

метод выявления клонов среди изолированных штаммов, благодаря чему объем работ по тестированию изолятов был значительно сокращен. (Validov et al., 2007= Глава 3).

Среди выделяемых активных колонизаторов были обнаружены штаммы, принадлежащие к потенциально патогенным видам. Эти результаты согласуются с сообщениями, в которых указывается, что многие патогены являются также хорошими корневыми колонизаторами (Berg et al., 2005). Многие патогены животных и человека обнаружены в ризосфере растений, где они могут контролировать заболевания растений (Bano and Musarrat, 2003; Berg et al., 2005; Egamberdiyeva et al., в печати). Поскольку применение этих микроорганизмов в сельском хозяйстве не допустимо (Anonymous, 1999), патогены должны быть распознаны и как можно раньше изъяты из экспериментов. Мы идентифицировали потенциально патогенные штаммы на основе сравнения последовательностей 16S рДНК и не использовали их в последующих экспериментах (Validov et al., 2007).

При регистрации биопестицида, антибиотики, производимые агентом биоконтроля, являются скорее недостатком, поскольку требуют дополнительных тестов на токсичность для человека и животных. Из 13 штаммов выделенных с использованием разработанного нами метода накопительной культуры, основанной на конкурентной колонизации корня растений, только три синтезировали антигрибные метаболиты. Это показывает, что предложенный нами метод не способствует выделению антагонистических штаммов (Главы 2 и 3).

Данный метод накопительной культуры может быть модифицирован в зависимости от условий, в которых будет применен агент биоконтроля (Validov et al., 2007). Использование метода накопительной культуры описанного в главах 2 и 3 позволяет выделять непатогенные, неантагонистические биоконтрольные бактерии из образцов ризосферы растений, взятых из географических точек, где впоследствии планируется применение биопрепаратов, созданных на основе этих бактерий.

Активные колонизаторы могут защищать растения за счет механизма конкуренции с фитопатогеном за экологическую нишу.

В настоящее время описаны четыре механизма биоконтроля заболеваний вызванных грибными фитопатогенами: (1) антибиозис, (2) индукция системной устойчивости растения, (3) хищничество/паразитизм и (4) конкуренция за экологические ниши. Многие известные агенты биоконтроля используют несколько механизмов для защиты

растения от грибного патогена. Например: и продукция антибиотиков и эффективная колонизация важны для контроля грибных заболеваний растений штаммами *P. chlororaphis* PCL1391 (Chin-A-Woeng et al., 1998; Chin-A-Woeng et al., 2000) и *P. fluorescens* strain Q8r1-96 (Mavrodi et al., 2002). Колонизирующая активность штамма *P. chlororaphis* O6 важна для развития полноценного системного ответа растения (Num et al., 2006). Штаммы *Trichoderma ssp.*, которые контролируют *Forl* посредством хищничества/паразитизма, являются также превосходными колонизаторами (Bolwerk et al., 2005).

В приведенных примерах высокая колонизационная способность, которая является основой механизма конкуренции за экологические ниши, рассматривается как средство, которое помогает агенту биоконтроля заселить ризосферу/ризоплану и достичь в этой нише численности необходимой для эффективного контакта с фитопатогеном или для стимуляции системной устойчивости растения. Псевдомонады *P. fluorescens* PCL1751 (Глава 2) и *P. putida* PCL1760 (Глава 4) контролируют фузариозную корневую гниль томатов благодаря своим экстраординарным колонизирующим способностям. Эти штаммы быстро заселяют ризосферу томатов и эффективно используют питательные вещества из корневых экссудатов. Активное потребление питательных веществ лишает *Forl* источников углерода, которые нужны этому грибу для роста (Рис. 4.4), и возможно дезориентирует направленный рост гиф фитопатогена, поскольку хемоаттрактанты (органические кислоты и т.д.) потребляются агентом биоконтроля.

При культивировании растений в каменной вате, в которую биоконтрольные агенты вносили в качестве суспензии клеток в питательном растворе для растений, подвижность штамма не играла значительную роль в контроле заболевания томатов корневой фузариозной гнилью (Глава 4). Было показано, что способ внесения агента биоконтроля в КВ обеспечивает достаточный уровень колонизации как субстрата, так и корня томата (Глава 4, Рис. 5). Вероятно поэтому, быстрое потребление питательных веществ было достаточным условием для снижения заболеваемости растений в условиях КВ (Глава 4).

Определение механизма действия необходимо для регистрации биоконтрольного штамма и его дальнейшего коммерческого применения. В случае антагонистических штаммов, антигрибные метаболиты могут быть токсичны для человека и животных, или быть причиной аллергических реакций (Skrobek et al., 2006). Непатогенные бактерии, защищающие растения посредством механизма конкуренции за экологические ниши,

лишены этого недостатка, что, несомненно, облегчит регистрацию биопрепаратов на основе таких микроорганизмов.

Генетическая изменчивость грибов, в том числе и фитопатогенных, обусловлена обилием мобильных элементов и избыточностью их геномов (Teunissen et al., 2003). Это объясняет быстрое возникновение рас фитопатогенных грибов, устойчивых к химическим фунгицидам. Сходным образом может возникнуть устойчивость к токсичным веществам, синтезируемым агентами биоконтроля. Кроме того, некоторые штаммы фитопатогенных грибов выделяют вещества, которые подавляют продукцию вторичных метаболитов у бактериальных штаммов – агентов биоконтроля (van Rij et al., 2005), или даже метаболизируют антигрибные вещества (Schouten et al., 2004). Все эти защитные механизмы фитопатогенных грибов снижают эффективность биоконтроля, основанного на механизме антибиозиса.

Агент биоконтроля, использующий механизм конкуренции за экологические ниши, является суперколонизатором корней. Поскольку колонизационные способности штаммов и их приспособленность к условиям ризосферы являются результатом доведенного до совершенства балланса в экспрессии генов, трудно представить, что случайные единичные изменения в грибном геноме могут породить штамм, который превосходит в колонизационных способностях биоконтрольный штамм – суперколонизатор. Таким образом, биопестициды основанные на бактериальных штаммах, использующих механизм конкуренции за экологические ниши, могут стать идеальным с точки зрения эффективности и безопасности инструментом стабильного биоконтроля.

Гетерогенность и мониторинг фитопатогенных штаммов *Fusarium oxysporum* (Главы 5 и 6)

Фитопатогенные и сапрофитные штаммы *Fusarium oxysporum* (*Fox*) встречаются повсеместно, практически в любом типе почв (Burgess 1981). Среди патогенных штаммов различают 150 специальных форм (*formae speciales*), представители которых специфично паразитируют на определенном виде растений (Baayen et al., 2001). Диагностика инфекций очень важна в условиях промышленного выращивания растений. Поскольку патогенные и непатогенные формы *Fox* могут присутствовать в ризосфере, определение ДНК этого микроорганизма в растительном материале не означает, что растение подверглось атаке патогенным штаммом *Fox* и вскоре будет им

повреждено (Глава 6). Для большинства *formae speciales* генетические детерминанты патогенности к тому или иному растению неизвестны. Мультикопийные регионы, такие как рибосомальные гены, могут служить удобной мишенью для разработки праймеров и, соответственно, высокочувствительного мониторинга этого патогена, если между *formae speciales* существуют генетические различия в этих регионах. Мы сравнили 33 штамма *Fox* из семи разных *formae speciales* используя следующие критерии: (1) патогенность по отношению к томатам (*Lycopersicon esculentum*), огурцам (*Cucumis sativum*) и лилейным декоративным культурам (Глава 5); (2) вегетативная совместимость; (3) нуклеотидная последовательность межгенной области между 28S и 18S рРНК генами (IGS). Было показано, что четыре из семи специальных форм гетерогенны, т.е. содержат штаммы значительно отличающиеся по последовательности IGS. Более того, даже представители одной и той же группы несовместимости могут сильно различаться по последовательностям IGS. Наблюдаемая гетерогенность противоречит догме происхождения групп вегетативной совместимости и уж тем более специальных форм от единого предкового штамма (Глава 5).

Все 33 исследованных штамма были способны колонизировать растения томатов эндофитно (Глава 5). Тем не менее только заражение *Fox* f. sp. *radicis-lycopersici* (*Forl*) приводило к развитию корневой гнили на опытных растениях. Для того, чтобы выявить различия между патогенным и непатогенным штаммом, мы исследовали колонизацию томатов штаммами *Fox* f. sp. *radicis-lycopersici* ZUM2407 (корневая гниль томатов), *Fox* f. sp. *lycopersici* 004 (фузариозное увядание томатов), *Fox* f. sp. *radicis-cucumerinum* V03-2g (корневая гниль огурца) и *Fox* Fo47 (непатогенный биоконтрольный штамм). Концентрация ДНК фузариев в растениях томатов определялась методом количественной ПЦР (qPCR); фрагмент IGS был выбран в качестве мишени для амплификации. Результаты молекулярного мониторинга грибов соответствовали результатам визуального определения симптомов болезни через три недели после заражения штаммом *Forl* ZUM2407. Наши результаты свидетельствуют, что использование фрагментов рибосомального оперона в качестве мишеней для qPCR позволяет осуществлять высокочувствительную детекцию ДНК *Fox* в растении. Выявленные различия показывают, что можно говорить о заражении фитопатогенным штаммом специфичным к данному растению, при обнаружении ДНК *Fox* в концентрации выше 50 fg в нанограмме растительной ДНК (≥0.005%).

Биоконтроль фузариозной корневой гнили томатов в промышленных условиях (Глава 7)

Штамм *Pseudomonas putida* PCL1760, являющийся превосходным колонизатором, был выделен методом накопительной культуры, основанной на конкурентной колонизации корня томатов (Глава 3). Используя механизм конкуренции за экологические ниши в ризосфере, данный штамм демонстрирует стабильное подавление фузариозной корневой гнили томатов (Главы 3 и 4). *Pseudomonas putida* является видом, в котором не выявлено патогенных штаммов. Поэтому для штаммов этого вида псевдомонад нет ограничений для использования в сельском хозяйстве (Anonymous 1999). В условиях *in vitro*, данный штамм не производит антигрибные метаболиты или экзоферменты ингибирующие *Forl* (Глава 4). Отсутствие выделяемых токсических веществ облегчает регистрацию штаммов для использования в сельском хозяйстве. Таким образом штамм *P. putida* PCL1760 соответствует требованиям биологической безопасности.

Таблица 1. Эффективность биоконтроля фузариозной корневой гнили томатов штаммом *P. putida* PCL1760 в лабораторных экспериментах

Растения с симптомами корневой гнили(%)		**Снижение уровня заболеваемости (%)**
Forl **(Негативный контроль)**	***Forl*** **+ PCL1760**	
18.7	8.3	55
29.9	13.7	54
39.8	17	56
53.3	25.9	51
62.2	10.3	83
62.4	28.6	54
62.5*	46*	26
62.6	18.1	71
72	38.9	45

Различия в уровнях заболеваемости между обработками определялись с помощью анализа вариаций (ANOVA), и сравнение средних проводилось с помощью теста Фишера по наименее значимым различиям ($\alpha = 0.05$).
*Только в этом эксперименте различие между вариантами не было значимым.
Снижение уровня заболеваемости корневой гнилью определялось как процент уменьшения количества больных растений. Результат негативного контроля считался за 100% проявление заболеваемости.

Промышленные испытания штамма PCL1760 были проведены на базе сертифицированных теплиц (PPO, Bleiswijk, The Netherlands). Результаты этих испытаний показали, что штамм *P. putida* PCL1760 подавляет фузариозную корневую гниль томатов в условиях исскуственного субстрата - каменной ваты, и снижает негативное влияние фитопатогена на прорастание семян и рост растений томата (Глава 7).

С помощью qPCR было показано, что растения выращенные в присутствии агента биоконтроля *P. putida* PCL1760, содержат в 5 раз меньше ДНК *Forl* ZUM2407 (Глава 7). Следует отметить, что Mycostop®Biofungicide (Kemira Agro OY, Helsinki, Finland), которой рекомендован этой компанией как средство борьбы с корневыми гнилями томатов, не подавлял развитие этой болезни.

Лабораторные условия, которые были использованы в наших экспериментах, были близки к промышленным условиям теплиц: те же субстрат, сорт томатов, питательный раствор для растений и фитопатоген (Главы 3 и 4). Штам *P. putida* PCL1760 демонстрировал высокий (>50% подавление болезни) и стабильный биоконтроль фузариозной гнили томатов во многих экспериментах с разным уровнем заболеваемости растений (Таблица 1). Результаты промышленного испытания (Глава 7) подтверждают полученные ранее лабораторные результаты, что позволяет предложить штамм *P. putida* PCL1760 для создания биологического препарата использования в теплицах с искусственным субстратом в качестве надежного и безопасного средства защиты растений.

Выводы

1. Метод накопительной культуры, основанный на конкурентной колонизации корня растения, способствует выделению штаммов с высокими колонизирующими способностями. Существенная доля выделенных таким образом штаммов способна снижать заболеваемость растений, вызванную *Forl*.

2. Штаммы *P. fluorescens* PCL1751 и *P. putida* PCL1760 являются первыми выделенными и изученными бактериями ингибирующими фитопатоген исключительно посредством механизма конкуренции за экологические ниши.

3. Специальные формы, а также группы вегетативной совместимости у вида *Fusarium oxysporum* могут состоять из генетически неродственных штаммов.

4. Инфекцию растения фитопатогенным штаммом и колонизацию непатогенным штаммом *Fusarium oxysporum* можно различить по концентрации ДНК гриба в растительном материале.

5. Штамм *P. putida* PCL1760 является безопасным агентом биоконтроля, эффективность которого доказана в промышленных условиях.

Curriculum vitae

Shamil Zavdatovich Validov was born on the 5th of March 1971 in Kazan, Republic Tatarstan, Russian Federation. He attended high school No 111. In 1988 he entered the Kazan State University. In 1993 he received his Master degree in genetics. In the same year he joined the Laboratory of Plasmid Biology at the Institute of Biochemistry and Physiology of Microorganisms of the Russian Academy of Sciencies in Biology Centre of Excellence in Puschino near Moscow. In 2001, within the framework of a NWO-sponsored cooperation between The Netherlands and Russian Federation (NWO-3 Project N. 047.007.020 "Enhancing Effectiveness of Bacterial Inoculants for Sustainable Agriculture"), he visited the Dept. of Molecular Microbiology at the Institute of Biology of Leiden University. Under the supervision of Dr. G.V. Bloemberg he studied the influence of the genes of polyaromatic hydrocarbon degradation pathways on the synthesis of secondary metabolites of plant growth promoting *Pseudomonas* strains. In 2002 he visited the Dept. of Plant Pathology, Laboratory of Dr. L. Thomashow, Washington State University, Pullman, WA, USA on a Short Term Scholarship. As a visitor scientist he studied the distribution of bacteriocin production among soil *Pseudomonas* strains, active plant disease control agents and the impact of bacteriocins on competition between bacteria in the rhizosphere. From September 1st 2003 through December 31st 2007 he was working on his PhD thesis "Biocontrol of tomato foot and root rot by *Pseudomonas* bacteria in stonewool" at Leiden University, Institute of Biology, Dept. of Molecular Microbiology under the supervision of Prof. Dr. E.J.J. Lugtenberg and Dr. F. Kamilova.

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