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ORIGINAL ARTICLE

Biosurfactants are involved in the biological control of *Verticillium* microsclerotia by *Pseudomonas* spp.

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biological control, biosurfactants, competition, phenazines, *Pseudomonas, Verticillium dahliae, Verticillium longisporum*.

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Abstract

Aims: To examine the effect of previously described bacterial antagonists on the viability of *Verticillium* microsclerotia *in vitro* and to elucidate the possible modes of action of bacterial strains in the suppression of *Verticillium* microsclerotia viability.

Methods and Results: A microplate assay was developed to test the suppressive effect of well-defined *Pseudomonas* spp. on the viability of *Verticillium* microsclerotia *in vitro*. Experiments using phenazine- and biosurfactant-deficient mutants indicated that biosurfactants and phenazine-1-carboxylic acid play a role in the suppression of microsclerotia viability by *Pseudomonas* spp. In addition, microsclerotia colonization tests revealed that *Pseudomonas* spp. are able to colonize the surface of the microsclerotia, but not the inner matrix. Growth response curves showed that the population levels of *Pseudomonas* spp. increased when they were in the vicinity of *Verticillium* microsclerotia, indicating that *Pseudomonas* spp. may utilize nutrients from the microsclerotia for their growth.

Conclusions: *Pseudomonas* spp. seem to be good candidates for *Verticilllium* microsclerotia biocontrol. Biosurfactant production is one of the main mechanisms involved in their mode of action.

Significance and Impact of the Study: This line of work may contribute to a better understanding of biological control agents and their working mechanisms.

Introduction

Verticillium wilt is an important disease responsible for dramatic yield losses in many crops all over the world (Pegg and Brady 2002). *Verticillium dahliae* and *Verticillium longisporum* produce melanized resting structures, microsclerotia, which can survive for more than a decade in soil. Microsclerotia are stimulated to germinate by root exudates. The infectious hyphae that emerge from the microsclerotia penetrate the roots of the host plant (Schnathorst 1981). Because the microsclerotia are the most important structures of the pathogen for survival and in causing initial infections, the microsclerotia are regarded as one of the direct targets of biological control of *Verticillium* (Tjamos 2000).

In the past, studies on biological control of *Verticillium* microsclerotia have typically focused on the use of the fungus *Talaromyces flavus*. This fungal antagonist kills individual *Verticillium* microsclerotia *in vitro*, because of the production of glucose oxidase that converts glucose to hydrogen peroxide (Fravel *et al.* 1987; Kim *et al.* 1988; Fahima *et al.* 1992). In recent years, bacterial antagonists, e.g. *Pseudomonas* spp., have also been identified as potential biological control agents for *Verticillium* wilt (e.g. Leben *et al.* 1987; Berg *et al.* 2001; Mercado-Blanco *et al.* 2004). These previous studies have specifically targeted the suppression of the hyphal growth of *Verticillium*. However, to our knowledge, no research has ever looked at the direct effect of these bacterial antagonists on the viability of individual *Verticillium* microsclerotia *in vitro*. As mentioned, a better control of microsclerotia is

important, as they are the key structures responsible for survival and infection by *Verticillium*.

A common mechanism underlying the direct antagonistic activity towards soil pathogens by *Pseudomonas* is the production of antifungal compounds. These antifungal compounds include the secretion of antibiotics and siderophores (Dwivedi and Johri 2003), biosurfactants (de Souza *et al.* 2003; Raaijmakers *et al.* 2006) and lytic enzymes (Velazhahan *et al.* 1999).

In this article, we investigate the role of phenazines and biosurfactants in the *Pseudomonas*-mediated reduction of *Verticillium* microsclerotia viability. Phenazines are a large family of heterocyclic nitrogen-containing pigments with broad-spectrum antibiotic activity. It is assumed that they diffuse across or insert into the membrane of plant pathogens and act as a reducing agent, resulting in the generation of toxic superoxide radicals and hydrogen peroxide (Chin-A-Woeng *et al.* 2003). Some of the best-known phenazines involved in biological control are phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN) and pyocyanin (Chin-A-Woeng *et al.* 2003). For example, *Pseudomonas chlororaphis* PCL1391 produces PCN, which is responsible for the biological control of *Fusarium oxysporum* (Chin-A-Woeng *et al.* 1998, 2001). *Pseudomonas aeruginosa* PNA1 produces PCA and PCN, which are involved in the control of *Pythium* spp. (Anjaiah *et al.* 1998; Tambong and Höfte 2001). *Ps. fluorescens* is able to suppress the take-all disease on wheat by the production of PCA (Thomashow and Weller 1988). Biosurfactants are surface-active compounds produced by a variety of microorganisms. The mode of action of biosurfactants in biological control involves the formation of channels in the cell wall and perturbations of the cell surface of the pathogen (Raaijmakers *et al.* 2006). The best-known biosurfactants in biological control are cyclic lipopeptides (Raaijmakers *et al.* 2006) and rhamnolipids (Stanghellini and Miller 1997). For example, *Pseudomonas* CMR12a produces a cyclic lipopeptide responsible for the control of *Pythium* and *Phytophtora* spp. (Perneel 2006), while *Ps. aeruginosa* PNA1 produces rhamnolipids (Perneel 2006).

Apart from the production of antifungal compounds, a second important mechanism by which bacterial antagonists may control soil pathogens, is that they may compete with the pathogen for nutrients, as both the pathogen and the bacterium can have similar carbon source nutrient utilization profiles (Ellis *et al.* 1999). For example, Jana *et al.* (2000) found that loss of nutrients from the sclerotia of *Macrophomina phaseolina* incubated with isolates of *Ps. fluorescens* was directly correlated with germination suppression.

A first aim of the present study was to examine the effect of previously described bacterial antagonists on the viability of *Verticillium* microsclerotia *in vitro*. More specifically, four *Pseudomonas* spp., including *Ps. chlororaphis* PCL1391, *Pseudomonas* CMR12a, *Ps. aeruginosa* PNA1 and 7NSK2; were tested for their ability to suppress *Verticillium* microsclerotia viability. These bacterial strains have previously shown biological control activity towards several soil pathogens (e.g. Buysens *et al.* 1996; Anjaiah *et al.* 1998; Chin-A-Woeng *et al.* 1998; Tambong and Höfte 2001).

The second aim of the present study was to elucidate the possible modes of action of bacterial strains in the suppression of *Verticillium* microsclerotia viability. Two hypotheses were investigated. Initially it was investigated whether the secretion of phenazines and biosurfactants might be involved in the biological control ability of the bacterial strains. This strategy was inspired by previous findings, demonstrating that phenazines and/or biosurfactants are involved in the biological control activity of other plant pathogens by these bacterial strains (Anjaiah *et al.* 1998; Chin-A-Woeng *et al.* 1998; Tambong and Höfte 2001). Second, we investigated whether the bacterial antagonists might colonize *Verticillium* microsclerotia and utilize nutrients from *Verticillium* microsclerotia for their growth, as described above.

Material and methods

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All the bacteria listed in Table 1 have been previously described, except for *Pseudomonas* CMR12a-PhzH (see below) and *Ps. aeruginosa* PNA1-PhzH (see below).

King's medium B (KB) (King *et al.* 1954) was used routinely to culture *Pseudomonas* spp. strains at 37°C (*Pseudomonas* CMR12a and *Ps. chlororaphis* PCL1391) or at 37°C (*Ps. aeruginosa* PNA1 and 7NSK2). *Escherichia coli* strains were grown in Luria-Bertani medium at 37°C (Sambrook *et al.* 1989). Gentamycin, tetracyclin and kanamycin antibiotics were used at the following concentrations; for *E. coli* 25 μg ml^{-1} and for *Pseudomonas* spp. 100 μg ml^{-1}.

Construction of *Pseudomonas phzH* mutants

Plasmid preparation, ligation, cloning and transformation were performed according to standard methods (Sambrook *et al.* 1989). *Taq* DNA Polymerase and Q-solution were purchased from Qiagen (Venlo, the Netherlands) and polymerase chain reaction (PCR) was carried out as recommended by the manufacturer. The primers used are listed in Table 1.

Table 1 Bacterial strains, primers and plasmids used in this study

Strain, primer or plasmid	Description	Medium	Reference or source
Strains			
Pseudomonas chlororaphis			
PCL1391	Wild type, PCA$^-$, PCN$^+$, BS$^+$	PPA	Chin-A-Woeng *et al.* (1998)
PCL1121	*phzH* mutant of PCL1391, obtained by gene replacement, Tc^R, PCA$^+$, PCN$^-$, BS$^+$	PPA	Chin-A-Woeng *et al.* (2001)
PCL1119	*phzB* mutant of PCL1391, obtained by insertion of a promoterless Tn5*luxAB*, Tc^R, PCA$^-$, PCN$^-$, BS$^+$	PPA	Chin-A-Woeng *et al.* (1998)
Pseudomonas			
CMR12a	Wild type, PCA$^-$, PCN$^+$, BS$^+$	KB	Perneel (2006)
CMR12a-7·97	*panC* mutant of CMR12a, obtained by miniTn5*Pho*A3 mutagenesis, Gm^R, PCA$^-$, PCN$^+$, BS$^-$	KB + Gm	Perneel (2006)
CMR12a-PhzH	*phzH* mutant of CMR12a, obtained by gene replacement using plasmid pJK100-PhzHUp-PhzHDown, Km^R, PCA$^+$, PCN$^-$, BS$^+$	KB + Km	This study
Pseudomonas aeruginosa			
PNA1	Wild type, PCA$^+$, PCN$^+$, BS$^+$	KB	Anjaiah *et al.* (1998)
PNA1-Rhl	*rhlB* mutant of PNA1, obtained by gene replacement, Gm^R, PCA$^+$, PCN$^+$, BS$^-$	KB + Gm	Perneel (2006)
PNA1-PhzH	*phzH* mutant of PNA1, obtained by gene replacement using plasmid pKOG-phzH, Gm^R, PCA$^+$, PCN$^-$, BS$^+$	KB + Gm	This study
Pseudomonas aeruginosa			
7NSK2	Wild type, PCA$^-$, PYO$^+$, BS$^+$	PPA	Iswandi *et al.* (1987)
7NSK2-PhzM	*phzM* mutant of 7NSK2, obtained by gene replacement, Gm^R, PCA$^+$, PYO$^-$, BS$^+$	PPA	De Vleesschauwer *et al.* (2006)
Escherichia coli WM3064	Genetically modified *E. coli* strain carrying the *pir* gene for propagation of plasmids with the R6 Kγ origin of replication	LB	Denef *et al.* (2006)
Escherichia coli DH5α	General-purpose *E. coli* host strain used for the transformation and propagation of plasmids	LB	Hanahan (1983)
Escherichia coli HB101	General-purpose *E. coli* host strain containing the helper plasmid	LB	Boyer and Roulland-Dossoix (1969)
Primers*			
12aPhzH-F1	gtgAGATCTaaccagctggccgacat (*Bgl*II)	–	This study
12aPhzH-R1	gtgGAATTCcaacgcgtcgtggttgta (*Eco*RI)	–	This study
12aPhzH-F2	gtgGGGCCCcgcaggagattctcaatc (*Apa*I)	–	This study
12aPhzH-R2	gtgGAGCTCccaggataccggtcgatt (*Sac*I)	–	This study
PNA1PhzH-F	gtgTCTAGAgtgcagccgatgtcctatc (*Xba*I)	–	This study
PNA1PhzH-R	gtgGGGCCCcaggtgcagcgaggtatc (*Apa*I)	–	This study
Plasmids			
pBluescript II KS/SK (+)	General purpose cloning vector Amp^R	–	Fermentas
pJK100	Allelic-exchange vector Km^R Tc^R	–	Denef *et al.* (2006)
pKnockout-G	Mobilizable suicide vector Gm^R Amp^R	–	Windgassen *et al.* (2000)
pBlue-PhzHUp	pBluescript with a *Bgl*II-*Eco*RI 592 bp *phzH*-upstream fragment of CMR12a Amp^R	–	This study
pBlue-PhzHDown	pBluescript with a *Apa*I-*Sac*I 539 bp *phzH*-downstream fragment of CMR12a Amp^R	–	This study
pJK100-PhzHUp	pJK100 with a *Bgl*II-*Eco*RI 592 bp *phzH*-upstream fragment of CMR12a Km^R Tc^R	–	This study
pJK100-PhzHUp-PhzHDown	pJK100-PhzHUp with a *Apa*I-*Sac*I 539 bp *phzH*-downstream fragment of CMR12a Km^R Tc^R	–	This study
pKOG-phzH	pKnockout-G with a 921 bp *Xba*I-*Apa*I bp *phzH* fragment of PNA1 Gm^R Amp^R	–	This study
pRK2013	Conjugation helper plasmid *ori* ColE1 *tra*$^+$ *mob*$^+$ Km^R	–	Figurski and Helinski (1979)

*Capital letters in primer sequences indicate 5′ extensions containing restriction enzyme recognition sites (mentioned in brackets) used for vector construction.

LB, Luria Bertani medium (Sambrook *et al.* 1989); PPA, Pseudomonas P agar (Difco); KB, King's B medium (King *et al.* 1954); Gm, gentamycin; Km, kanamycin; Tc, tetracyclin; Amp, ampycilin; Gm^R, Km^R, Tc^R, Amp^R, resistant to gentamycin, kanamycin, tetracyclin and ampicillin, respectively; PCA, phenazine-1-carboxylic acid; PCN, phenazine-1-carboxamide; BS, biosurfactants; PYO, pyocyanin.

CMR12a-PhzH is a *phzH* mutant of CMR12a, generated by the use of the allelic exchange vector pJK100. Plasmid pJK100 (3854 bp) is a suicide vector carrying a tetracyclin resistance element, an R6 Kγ origin of replication, the RP4 origin of transfer and MCS-*lox*P-Km^R-*lox*P-MCS (Marx and Lidstrom 2002; Denef *et al.* 2006). Plasmids with the R6 Kγ origin of replication were propagated in a genetically modified *E. coli* strain carrying the *pir* gene (WM3064; Denef *et al.* 2006). The regions immediately flanking the *phzH* gene (592 bp *phzH*-upstream and 539 bp *phzH*-downstream) were amplified by PCR and inserted into pJK100 (intermediate and final constructs in Table 1) as described previously (Marx and Lidstrom 2002). A *ΔphzH::kan* mutant of CMR12a was generated by introducing pJK100-PhzHUp-PhzHDown by triparental conjugation from *E. coli* WM3064 with the aid of *E. coli* HB101 (pRK2013) (Boyer and Roulland-Dossoix 1969). Kanamycin-resistant transconjugants were screened for tetracyclin sensitivity, to confirm the double-crossover event.

PNA1-PhzH is a *phzH* mutant of PNA1, generated by the insertion of the vector pKnockout-G, a mobilizable suicide vector (6 kb) with a gentamycin resistance cassette (Windgassen *et al.* 2000). An internal fragment (921 bp) of the *phzH* gene of *Ps. aeruginosa* PNA1 was amplified by PCR with primers designed in frame with the *phzH* gene (PNA1PhzH-F/R). The PCR fragment was cloned into pKnockout-G, resulting in pKOG-phzH (Table 1). This plasmid was used as a suicide vector in a *Ps. aeruginosa* PNA1 background to obtain a *phzH* mutant of strain PNA1 by single homologous recombination.

CMR12a-PhzH and PNA1-PhzH transconjugants were screened for the loss of PCN-production by means of thin-layer chromatography (TLC). The performance of the constructed phzH mutants when compared with the wild type has been checked thoroughly. More specifically, the production of biosurfactants was assessed using the drop collapse technique as described by Jain *et al.* (1991). HCN was detected by cyanide-indicator paper according to Bakker and Schippers (1987). Protease activity was detected as clearing zones on LB-medium amended with 2% skim milk powder (Sacherer *et al.* 1994). Swarming phenotype and fluorescence were checked on KB agar plates (Kinscherf and Willis 1999).

Inoculum preparation

Each strain and its derivatives were grown on a specific medium (Table 1), as previous research showed that these media stimulate the production of the secondary metabolites under study. More specifically, Pseudomonas P Agar (PPA) is a phosphate-limiting medium and phosphate limitation stimulates the production of phenazines by *Pseudomonas* spp. (Frank and Demoss 1959). Furthermore, we showed that KB medium seems to stimulate biosurfactants production by *Pseudomonas* spp. (data not shown). For inoculum preparation, all strains were grown at 28°C for 24 h on the respective medium, except for the *Ps. aeruginosa* strains, which were grown at 37°C. After incubation, the bacteria were scraped from the medium and suspended in sterile physiological solution. Bacterial concentration in the suspension was determined spectrophotometrically by measuring absorbance at 620 nm.

Verticillium isolate and microsclerotia preparation

The cauliflower isolate O1 of *V. longisporum* was used throughout this study. This isolate was randomly chosen out of five European *Verticillium* strains from cauliflower previously described (Debode *et al.* 2005). The isolate was routinely grown on potato dextrose agar plates at 24°C and conserved in a Microbank^{TM} (Pro-lab Diagnostics, ON, Canada) at −80°C. Dry, individual microsclerotia were produced as described by Hawke and Lazarovits (1994). During this procedure, the microsclerotia are grown in semisolid medium, harvested by wet sieving (to remove medium, spores and mycelium), mixed with sand (to dry without clumping) and sorted into size classes by dry sieving. Subsequently, microsclerotia with a size of 75 μm or more were retained and suspended in sterile water containing 0·08% water agar to obtain a homogeneous suspension. To determine the concentration, the number of microsclerotia in the suspension was determined in small subsamples (15 μl) using a dissecting microscope.

Microsclerotia germination assay

To test the effect of bacterial antagonists on the viability of microsclerotia, a microplate assay was developed (Fig. 1). Nylon mesh filters (pore size 41 μm, diameter 25 mm, Millipore, Billerica, MA, USA) were placed in wells of a 96-well microplate. Subsequently, 20 μl of the microsclerotia preparation was embedded in the filters, resulting in about 50 microsclerotia per filter. In addition, 180 μl of the bacterial suspension was added to the wells (2×10^7 and 2×10^9 CFU ml^{-1}). Sterile physiological solution (180 μl) served as control treatment. A total volume of 200 μl per well was used, because volumes exceeding 200 μl per well may cause limited oxygen diffusion (Broekaert *et al.* 1990). The microplate was incubated for 2 days at 24°C. After incubation, the filters were retrieved from the microplate and placed on sterile filter paper under sterile conditions. After 15 minutes, the fil-

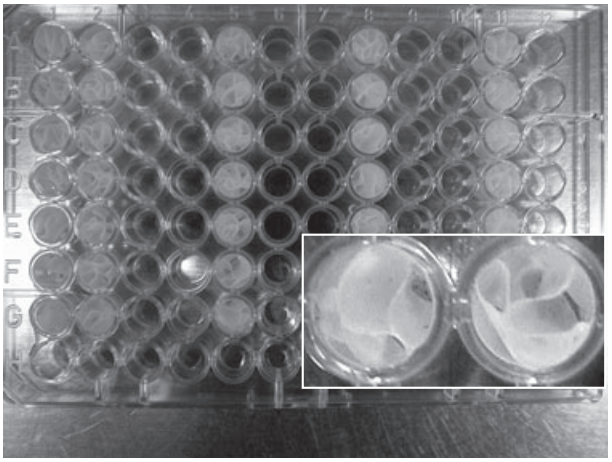


Figure 1 Microplate assay: nylon mesh filters placed in wells of a 96-well microplate.

ters were dry and placed on soil-pectate-tergitol agar (SPTA) plates containing 50 mg l^{-1} each of chloramphenicol, tetracyclin and streptomycin sulfate (Hawke and Lazarovits 1994). Ten days after incubation of the plates, the filters were examined under a dissecting microscope both for germination percentage and formation of secondary microsclerotia. There were five replications per treatment and each experiment was repeated once.

In a first step, the four wild–type bacteria listed in Table 1 were screened for their ability to suppress the viability of *Verticillium* microsclerotia *in vitro*. In a second step, several mutants deficient in phenazines or biosurfactants production were tested to elucidate the involvement of phenazines and biosurfactants in the *Pseudomonas*-mediated reduction of *Verticillium* microsclerotia.

Microsclerotia colonization assay

To determine the ability of *Ps. chlororaphis* PCL1391, *Pseudomonas* CMR12a, *Ps. aeruginosa* PNA1 and 7NSK2 to colonize the surface and the inner matrix of *Verticillium* microsclerotia, 20 μl of a microsclerotia suspension containing about 200 microsclerotia was added to each well of a microplate. In addition, 180 μl of a bacterial suspension was added in a concentration of 2×10^7 CFU ml^{-1}. Sterile physiological solution (180 μl) served as control treatment. The microsclerotia were incubated for 2 days at 25°C. To determine the surface and inner colonization after incubation, the microsclerotia were washed two times with sterile distilled water and ground in 200 μl physiological solution using an autoclaved pestle. To determine the inner matrix colonization alone, the microsclerotia were surface-disinfested in 0·5% NaOCl for 2 min, washed two times in sterile distilled water and ground in 200 μl physiological solution using an autoclaved pestle. This disinfection procedure should be robust enough to kill the bacteria on the surface of the microsclerotia (Melouk 1992). The bacteria in the ground microsclerotia solutions were enumerated by serial dilutions. Each experiment consisted of two replicates per treatment and each experiment was repeated once.

Growth response of bacteria in vicinity of *Verticillium* microsclerotia

To determine the growth response of *Ps. chlororaphis* PCL1391, *Pseudomonas* CMR12a, *Ps. aeruginosa* PNA1 and 7NSK2 in vicinity of *Verticillium* microsclerotia, 20 μl containing a concentration rage of 0–500 microsclerotia were put in each well of a microplate. Subsequently, 180 μl of a bacterial suspension containing 2×10^1, 2×10^3, 2×10^5, 2×10^7 and 2×10^9 CFU ml^{-1} was added to each well. Sterile physiological solution (180 μl) served as control treatment. The microplate was incubated for 2 days. The concentration of bacteria after incubation was determined by serial dilutions. Each experiment consisted of two replicates per treatment and each experiment was repeated once.

Data analysis

The percentage of *Verticillium* microsclerotia germination was expressed relative to the control. Univariate repeated measurements were used and multiple comparisons of the means were performed using *post hoc* Tukey test ($P = 0{\cdot}05$).

Results

Effect of wild-type bacteria on the viability of *Verticillium* microsclerotia

The effect of the four wild-type bacteria listed in Table 1 on the viability of *Verticillium* microsclerotia was evaluated with regard to (i) microsclerotia germination and (ii) formation of secondary microsclerotia. Figure 2 shows the effect of the wild-type bacteria on microsclerotia germination. When 2×10^7 CFU ml^{-1} bacteria were added in the microtiter assay, only *Ps. chlororaphis* PCL1391 was effective in reducing microsclerotia germination. When 2×10^9 CFU ml^{-1} bacteria were added, all bacteria were able to suppress the germination of the *Verticillium* microsclerotia. In addition, only a few of the *Pseudomonas*-treated microsclerotia formed secondary microsclerotia, whereas all colonies arising from the control treatment formed secondary microsclerotia (data not shown).

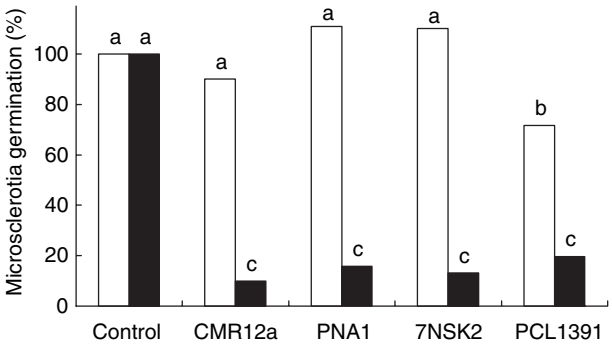


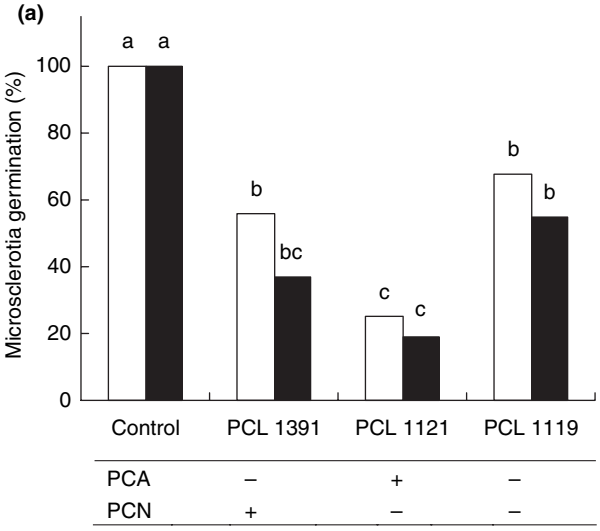
Figure 2 Percentage of germinated *Verticillium* microsclerotia exposed to 2×10^7 CFU ml^{-1} (open bars) and 2×10^9 CFU ml^{-1} (solid bars) of four different wild-type *Pseudomonas* spp. Bars indicated with the same letter are not significantly different after data analysis with a *post hoc* Tukey test ($P < 0{\cdot}05$).

Characterization of the phzH mutants

TLC plates showed that PNA1-PhzH and CMR12a-PhzH are impaired in PCN production and produce PCA instead. In addition, both mutants were not altered in biosurfactants production, HCN production, protease activity, swarming phenotype, fluorescence ability and viability.

Involvement of phenazines and biosurfactants

Pseudomonas chlororaphis PCL1391 has been shown to be the best candidate for *Verticillium* microsclerotia suppression (see Fig. 2). The involvement of the phenazine PCN in *Ps. chlororaphis* PCL1391-mediated reduction of *Verticillium* microsclerotia viability was investigated by testing two mutants of PCL1391: PCL1121 and PCL1119. A seven-gene operon (*phzABCDEFG*) is responsible for the synthesis of phenazine-1-carboxylic acid (PCA), and *phzH* encodes for an asparagine synthetase like enzyme, which is responsible for the conversion of PCA to phenazine-1-carboxamide (PCN). A *phzB* mutant (such as PCL1119) is phenazine-deficient and a *phzH* mutant (such as PCL1121) produces PCA instead of PCN (Chin-A-Woeng *et al.* 1998, 2001; Mavrodi *et al.* 2001). PCL1119 was as effective in the inhibition of microsclerotia germination as the wild type, whereas PCL1121 was more effective when compared with the wild type (Fig. 3a). In addition, PCL1121 was also more effective in the suppression of the formation of secondary microsclerotia when compared with the wild type. None of the germinated microsclerotia treated with PCL1121 were able to form secondary microsclerotia, whereas all the germinated microsclerotia in the control treatment formed secondary microsclerotia. A few of the germinated microsclerotia treated with PCL1391 and PCL1119 were able to form secondary microsclerotia (Fig. 3b).



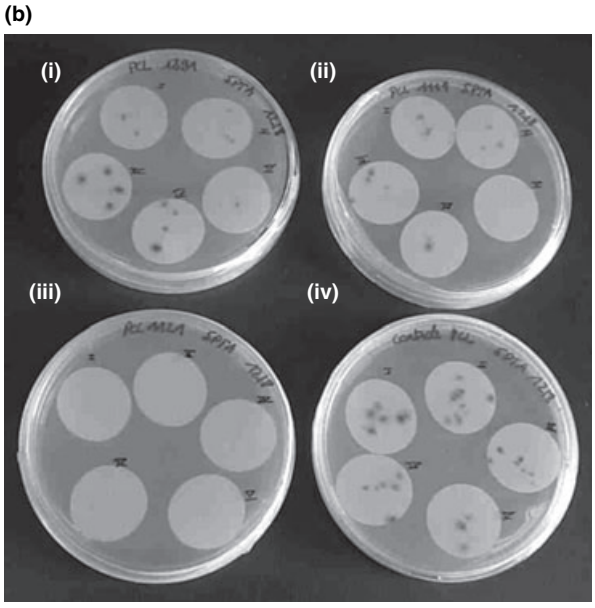


Figure 3 (a) Percentage of germinated *Verticillium* microsclerotia exposed to 2×10^7 CFU ml^{-1} (open bars) and 2×10^9 CFU ml^{-1} (solid bars) of the wild-type bacterium, *Pseudomonas chlororaphis* PCL1391 and his mutants PCL1121 and PCL1119. Bars indicated with the same letter are not significantly different after data analysis with a *post hoc* Tukey test ($P < 0{\cdot}05$). PCN, phenazine-1-carboxamide; PCA, phenazine-1-carboxylic acid. (b). Effect of 2×10^9 CFU ml^{-1} of *Ps. chlororaphis* PCL1391, PCL1119 and PCL1121 on the formation of secondary microsclerotia when compared with the control treatment. (i) PCL1391, wild type (ii) PCL1119, PCA and PCN deficient mutant (iii) PCL1121, PCA$^+$ mutant (iv) control treatment.

The role of the phenazine pyocyanin produced by *Ps. aeruginosa* 7NSK2 was investigated by testing the effect of *Ps. aeruginosa* 7NSK2-PhzM on the viability of *Verticillium* microsclerotia. The pyocyanin-negative mutant 7NSK2-PhzM was constructed by gene replacement of the

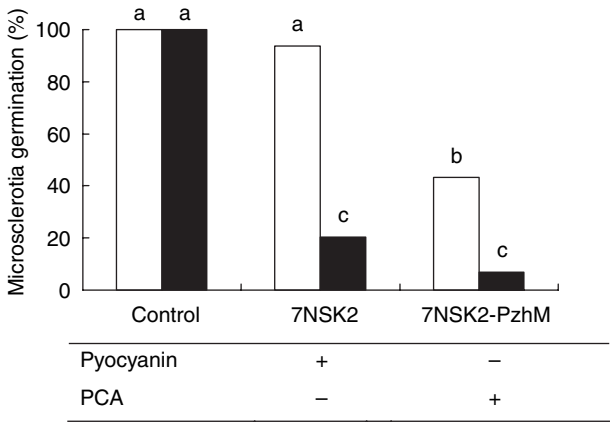


Figure 4 Percentage of germinated *Verticillium* microsclerotia exposed to 2×10^7 CFU ml^{-1} (open bars) and 2×10^9 CFU ml^{-1} (solid bars) of the wild-type bacterium, *Pseudomonas aeruginosa* 7NSK2 and its mutant 7NSK-PhzM. Bars indicated with the same letter are not significantly different after data analysis with a *post hoc* Tukey test ($P < 0{\cdot}05$). PCA, phenazine-1-carboxylic acid.

phzM gene (De Vleesschauwer *et al.* 2006), encoding an *O*-methyl transferase, which is necessary for the conversion of PCA to the pyocyanin precursor 5-methylphenazine-1-carboxylic acid betaine (Mavrodi *et al.* 2001). As can be seen in Fig. 4, *Ps. aeruginosa* 7NSK2-PhzM was more effective in inhibition of the *Verticillium* microsclerotia germination when compared with the wild type (Fig. 4). In addition, none of the germinated microsclerotia treated with 7NSK2-PhzM were able to form secondary microsclerotia, whereas a few microsclerotia treated with the wild type were able to form secondary microsclerotia. All the germinated microsclerotia in the control treatment were able to form secondary microsclerotia (data not shown).

To determine the involvement of biosurfactants (rhamnolipids) and phenazines (PCA and PCN) produced by *Ps. aeruginosa* PNA1 in the reduction of *Verticillium* microsclerotia viability, the effect of two mutants, PNA1-PhzH and PNA1-Rhl, on the viability of *Verticillium* microsclerotia was compared with the wild type. Similar to PCL1121, PNA1-PhzH is impaired in PCN production because of a *phzH* mutation and produces PCA (TLC – data not shown) and PNA1-Rhl is impaired in biosurfactants (rhamnolipid) production (Perneel 2006). PNA1-PhzH was as effective as the wild-type treatment, whereas PNA1-Rhl was no longer able to reduce microsclerotia germination (Fig. 5a). All the germinated microsclerotia in the control treatment were able to form secondary microsclerotia. None of the germinated microsclerotia treated with PNA1 or PNA1-PhzH were able to form secondary microsclerotia. For the microsclerotia treated with the biosurfactants mutant, PNA1-Rhl, a few of the germinated microsclerotia formed secondary microsclerotia, indicating that this mutant had an inhibitory effect on the viability of *Verticillium* microsclerotia (Fig. 5b).

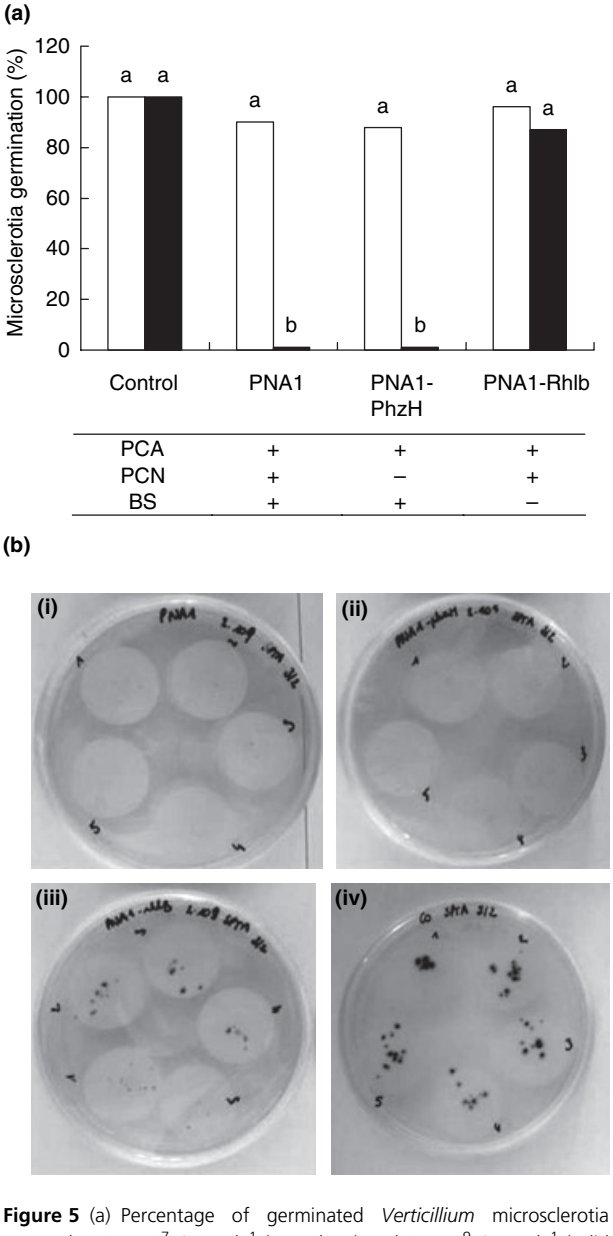


Figure 5 (a) Percentage of germinated *Verticillium* microsclerotia exposed to 2×10^7 CFU ml^{-1} (open bars) and 2×10^9 CFU ml^{-1} (solid bars) of the wild-type bacterium, *Pseudomonas aeruginosa* PNA1 and its mutants PNA1-PhzH and PNA1-Rhl. Bars indicated with the same letter are not significantly different after data analysis with a *post hoc* Tukey test ($P < 0{\cdot}05$). PCN, phenazine-1-carboxamide; PCA, phenazine-1-carboxylic acid; BS, biosurfactants. (b) Effect of 2×10^9 CFU ml^{-1} of *Ps. aeruginosa* PNA1, PNA1-PhzH and PNA1-Rhl on the formation of secondary microsclerotia when compared with the control treatment. (i) PNA1, wild type (ii) PNA1-PhzH, PCA$^+$ mutant (iii) PNA1-Rhl, biosurfactant deficient mutant (iv) control treatment.

The effect of CMR12a-7·97 and CMR12a-PhzH was tested on the viability of *Verticillium* microsclerotia when compared with the effect of the wild type. CMR12a-7·97 is impaired in the *panC* gene, coding for pantoate-β-alanine ligase and responsible for the production of pantothenate, a precursor of an important cofactor of cyclic lipopeptide synthetases (Perneel 2006). As such, CMR12a-7·97 is impaired in biosurfactant production. Many transconjugants had to be screened to find a *phzH* mutant in CMR12a, as the frequency of double homologous recombination with pJK100-phzHUp-phzHDown is probably very low. Whereas CMR12a produces PCA and PCN, its *phzH* mutant CMR12a-PhzH accumulates PCA (TLC – data not shown). CMR12a-7·97 was less effective when compared with the wild type, but still had an inhibitory effect on the microsclerotia germination. The effect of CMR12a-PhzH on the viability of *Verticillium* microsclerotia was not significantly different from the wild-type treatment (Fig. 6). A few of the germinated microsclerotia treated with CMR12a-7·97 were able to form secondary microsclerotia, whereas none of the germinated microsclerotia treated with CMR12a and CMR12a-PhzH were able to form secondary microsclerotia. All of the germinated microsclerotia in the control treatment were able to form secondary microsclerotia (data not shown).

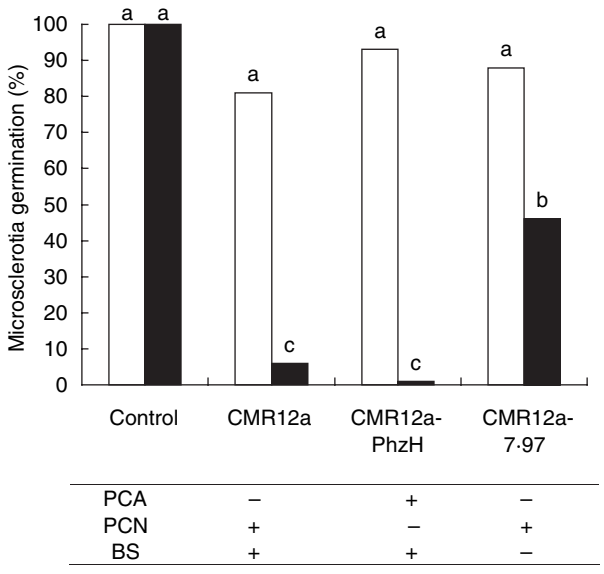


Figure 6 Percentage of germinated *Verticillium* microsclerotia exposed to 2×10^7 CFU ml^{-1} (open bars) and 2×10^9 CFU ml^{-1} (solid bars) of the wild-type bacterium, *Pseudomonas* CMR12a and its mutants CMR12a-PhzH and CMR12a-7·97. Bars indicated with the same letter are not significantly different after data analysis with a *post hoc* Tukey test ($P < 0·05$). PCN, phenazine-1-carboxamide; PCA, phenazine-1-carboxylic acid; BS, biosurfactants.

Microsclerotia colonization assay

To determine the ability of the bacteria to colonize the surface and the inner matrix of the microsclerotia, microsclerotia were treated with 2×10^7 CFU ml^{-1} bacteria and incubated for 2 days. After incubation, the microsclerotia were washed to determine the surface colonization and disinfected to determine the inner matrix colonization.

The bacterial population on the surface of the microsclerotia was $3·9\ (\pm 3·2) \times 10^4$ CFU ml^{-1} for *Ps. chlororaphis* PCL1391, $4·1\ (\pm 1·9) \times 10^4$ CFU ml^{-1} for *Ps. aeruginosa* 7NSK2, $5·2\ (\pm 1·3) \times 10^4$ CFU ml^{-1} for *Ps. aeruginosa* PNA1 and $4·8(\pm 2·8) \times 10^4$ CFU ml^{-1} for *Pseudomonas* CMR12a (values between brackets indicate standard deviations). There was no significant difference in surface colonizing ability between the tested *Pseudomonas* strains.

When we looked at the inner matrix colonization ability of the strains, no bacteria could be detected in the disinfected microsclerotia (detection limit was 100 CFU ml^{-1}), indicating that the tested *Pseudomonas* strains are not able to colonize the inner matrix of the microsclerotia.

Growth response of bacteria in vicinity of *Verticillium* microsclerotia

To determine the growth response of *Ps. chlororaphis* PCL1391 and *Ps. aeruginosa* 7NSK2 bacteria in the vicinity of *Verticillium* microsclerotia, various concentrations of bacteria were incubated for 2 days in a solution containing increasing amounts of *Verticillium* microsclerotia per 200 μl. When *Ps. chlororaphis* PCL1391 and *Ps. aeruginosa* 7NSK2 were incubated with 0 or 5 microsclerotia per 200 μl, populations after 2 days of incubation were similar to the initial bacterial population added. When *Ps. chlororaphis* PCL1391 and *Ps. aeruginosa* 7NSK2 were incubated at low concentrations (2×10^1, 2×10^3 and 2×10^5 CFU ml^{-1}) with 25 or more microsclerotia per 200 μl, a clear increase in the population was observed. When a high bacterial concentration (2×10^7 and 2×10^9 CFU ml^{-1}) was initially added, there was no additional growth after 2 days of incubation (Fig. 7a,b).

Similar results were obtained for *Ps. aeruginosa* PNA1 and *Pseudomonas* CMR12a (data not shown).

Discussion

Effect of wild-type bacteria on the viability of *Verticillium* microsclerotia

Contrary to the common assumption that melanized structures are resistant to microbial attack (Bell and

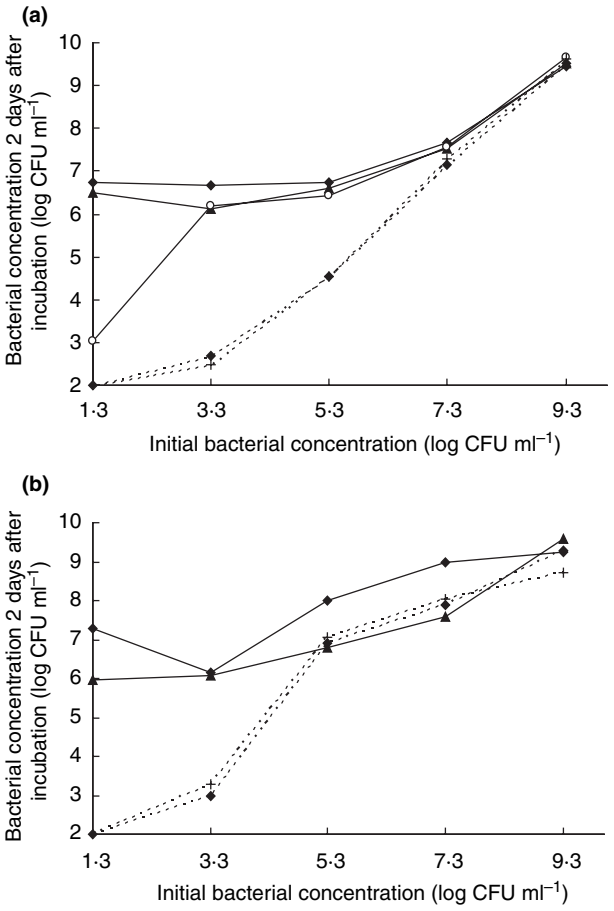


Figure 7 (a) *Pseudomonas chlororaphis* PCL1391 concentration 2 days after incubation in vicinity of 0 (--+--), 5 (--◆--), 25 (—○—), 50 (—▲—) and 200 (—◆—) microsclerotia. Detection limit was 100 CFU ml^{-1}. (b) *Pseudomonas aeruginosa* 7NSK2 concentration 2 days after incubation in vicinity of 0 (--+--), 5 (--◆--), 50 (—▲—) and 500 (—▲—) microsclerotia. Detection limit was 100 CFU ml^{-1}.

Wheeler 1986), the present study showed that *Pseudomonas* spp. can suppress the viability of *Verticillium* microsclerotia *in vitro*.

Reduction in *Verticillium* microsclerotia germination and formation of secondary microsclerotia may result in a lower *Verticillium* infection pressure in the field and in a reduced survival of *Verticillium* microsclerotia in soil (Coley-Smith and Cooke 1971). Thus, this study suggests that *Pseudomonas* spp. may be promising biological agents to control *Verticillium* wilt and they deserve closer attention in future field studies.

Involvement of biosurfactants and phenazines

It should be noted that the majority of the tested *Pseudomonas* spp. were only effective at high cell densities (2×10^9 CFU ml^{-1}, see Fig. 2). Based on this finding, we hypothesized that secondary metabolites, such as phenazines and biosurfactants might be involved in their mode of action, as secondary metabolites are mainly produced by bacteria at high cell densities (Haas *et al.* 2000). Another reason that can be put forward to underpin this hypothesis is that filter-sterilized culture supernatants of several of the tested *Pseudomonas* spp. showed antifungal activity against *Verticillium* microsclerotia (Debode *et al.* 2004).

Two different groups of secondary metabolites were investigated. A first group of secondary metabolites that were investigated were the biosurfactants. Biosurfactants are surface-active compounds capable of affecting the cell surface of plant pathogenic fungi (Raaijmakers *et al.* 2006). They can act on lipids and are able to form pores in the membrane layer (Kim *et al.* 2004). Given that the storage compounds present in *Verticillium* microsclerotia are lipids (Gordee and Porter 1961), we hypothesized that biosurfactants may act on these lipids by their permeabilizing activity, resulting in a loss of storage compounds that are necessary for *Verticillium* microsclerotia germination. The present study shows that the biosurfactant deficient mutants of *Pseudomonas* CMR12a and *Ps. aeruginosa* PNA1 were less effective in the suppression of the viability of the *Verticillium* microsclerotia when compared with the wild type, indicating that the former hypothesis may be supported. In addition, all the wild-type *Pseudomonas* strains that were tested in the present study were effective and they all produce biosurfactants (data not shown). A third indication that biosurfactants might be involved in the biological control of the tested wild-type *Pseudomonas* strains is that they were all effective at a cell density of 2×10^9 CFU ml^{-1} (Fig. 2). This cell density seems to be necessary to obtain the Critical Micelle Concentration (CMC) (de Souza J.T. *et al.*, personal communication). The CMC is the minimum amount of surfactants required to cause a decrease in surface tension (de Souza *et al.* 2003; Raaijmakers *et al.* 2006). Consequently, this amount of biosurfactants may be needed to cause a suppressive effect on *Verticillium* microsclerotia viability. Finally, it should be noted that biosurfactant production does not fully account for *Verticillium* microsclerotia suppression and other mechanisms are most likely involved in the biological control of *Verticillium* microsclerotia. More specifically, the present study reveals that the biosurfactant mutant of *Ps. aeruginosa* PNA1 had a residual effect on the formation of secondary microsclerotia. In addition, the biosurfactant mutant of *Pseudomonas* CMR12a had a residual effect both on *Verticillium* germination and the formation of secondary microsclerotia. These residual effects may be attributed to the production of PCA by those strains (see below).

A second group of metabolites that were investigated were the phenazines. Phenazines are able to diffuse across or insert into the membrane of plant pathogens and act as a reducing agent, resulting in the generation of superoxide radicals and hydrogen peroxide (Chin-A-Woeng *et al.* 2003). The main findings of the present study are that PCN and pyocyanin are not responsible for the biocontrol activity of tested *Pseudomonas* spp., whereas PCA seems to have a suppressive effect on *Verticillium* microsclerotia. PCA is a well-known metabolite involved in the biological control of several soilborne pathogens by *Pseudomonas* spp. (e.g. Mazzola *et al.* 1995). PCA is the first phenazine compound formed by those strains, and can be modified into PCN or pyocyanin by the aid of the *phzH* or the *phzM* gene (Chin-A-Woeng *et al.* 2001; De Vleesschauwer *et al.* 2006). In this study, PCA involvement was demonstrated by the fact that mutants of *Ps. chlororaphis* PCL1391 and *Ps. aeruginosa* 7NSK2 overproducing PCA (PCL1121 and 7NSK2-PhzM, respectively) were more effective in the inhibition of microsclerotia germination and formation of secondary microsclerotia when compared with the wild-type treatment. In addition, the biosurfactant mutants of *Ps. aeruginosa* PNA1 and *Pseudomonas* CMR12a (PNA1-Rl and CMR12a-7·97, respectively) both had a residual effect on the formation of secondary microsclerotia and this may be attributed to the production of PCA by those two strains. There was no additional impact of the mutants of *Ps. aeruginosa* PNA1 and *Pseudomonas* CMR12a overproducing PCA (PNA1-PhzH and CMR12a-PhzH, respectively) on the viability of the *Verticillium* microsclerotia. This may be attributed to the fact that the two wild-type strains were already highly effective in the inhibition of *Verticillium* microsclerotia viability and no additional effect of the PCA production could be obtained. Up to now, we were not able to construct mutants of *Ps. aeruginosa* PNA1 and *Pseudomonas* CMR12a that are completely PCA deficient.

The actual phenazine and biosurfactants concentrations in the microplate assay have not been measured. These data would show whether the antagonistic effects were truly related to the metabolites addressed by the gene knock-out, and whether effects which only occurred at 10^9 CFU ml^{-1} and not at lower concentrations were related to threshold concentrations of the metabolites produced by the *Pseudomonas* spp.

Microsclerotia colonization and competition for nutrients

Apart from secondary metabolites, a second possible underlying mechanism that may be involved in the *Pseudomonas*-mediated reduction of *Verticillium* microsclerotia viability is the ability of *Pseudomonas* spp. to colonize microsclerotia and utilize nutrients from the microsclerotia for their own growth. If this is the case, microsclerotia germination will be most likely inhibited, as these nutrients are normally used by the microsclerotia themselves for their germination (Willetts and Bullock 1992). However, it can also be hypothesized that *Pseudomonas* spp. might utilize the nutrients that are released by the microsclerotia. The release of nutrients by sclerotia in so-called 'exudation droplets' has been suggested to play an important role in maintaining the physiological balance within sclerotia (Willetts and Bullock 1992). Therefore, it can be hypothesized that when *Pseudomonas* spp. utilize the nutrients released in the exudation droplets, it follows that the physiological balance of the microsclerotia gets disturbed and the germination of the microsclerotia is inhibited.

The present study shows that *Pseudomonas* spp. might colonize the surface of the microsclerotia, but not the inner matrix. The adhesion of bacteria on the surface of the pathogen is an important feature in antagonistic interactions (Jana *et al.* 2000) and our results support that this mechanism may play a role in the biocontrol activity of the tested *Pseudomonas* strains. However, the maximum *Pseudomonas* carrier capacity of the microsclerotia was approx. 5×10^4 CFU ml^{-1} or approx. 50 *Pseudomonas* colonies per microsclerotium and this bacterial density is insufficient to kill *Verticillium* microsclerotia *in vitro* (see Fig. 2). Thus, the adhesion of *Pseudomonas* spp. on the surface of *Verticillium* microsclerotia cannot be the main mechanism involved.

In addition, we also found that the number of *Pseudomonas* bacteria increased when they were in the vicinity of a sufficient amount of *Verticillium* microsclerotia. This result indicates that *Pseudomonas* spp. might utilize nutrients that are present in the microsclerotia for their growth. However, the *Pseudomonas* spp. were only able to grow until they reached a concentration of approx. 1×10^7 CFU ml^{-1} (Fig. 7) and this bacterial density is insufficient to kill the *Verticillium* microsclerotia *in vitro* (Fig. 2). Thus, the growth of *Pseudomonas* spp. in vicinity of *Verticillium* microsclerotia cannot be the main mechanism involved in the *Pseudomonas*-mediated reduction of *Verticillium* microsclerotia viability.

Interferences between modes of actions

As until now no single mode of action could be demonstrated, we believe that there might be interference between the modes of actions. For example, biosurfactants and phenazines might act synergistically in the biological control of plant pathogens (Perneel 2006). In this hypothesis, biosurfactants migrate to the surface of the microsclerotia and form a thin film. Biosurfactants

function as such as carrier molecules for phenazines, enhancing the possibility of encountering the target cell and concentrating the antibiotics at the place of action (Perneel 2006). Besides this, the permeabilizing activity of biosurfactants on the cell wall and membrane can enhance the efficacy of phenazines by facilitating their access into the cell (Perneel 2006). Our results support this hypothesis as all tested wild-type *Pseudomonas* strains produce both phenazines and biosurfactants. To further investigate this hypothesis, mutants that are deficient in both phenazine- and biosurfactants-production, need to be constructed and tested for their ability to suppress the viability of *Verticillium* microsclerotia.

A second interference may be that the biosurfactants produced by the *Pseudomonas* spp. may facilitate the adhesion of the *Pseudomonas* spp. on the surface of the pathogen (Ron and Rosenberg 2001).

Biological relevance and practical implications

This study was carried out under *in vitro* conditions and more research is needed to see whether these results can be extrapolated to field conditions.

First, it needs to be clarified which *Pseudomonas* densities can possibly be achieved when *Pseudomonas* spp. are incorporated in soil. In this respect, preliminary experiments have been conducted in which the tested *Pseudomonas* strains were incorporated in soil naturally infested with *Verticillium* microsclerotia. Results showed that 14 days after incubation, the population density of the *Pseudomonas* strains was from 1×10^5 to 1×10^6 CFU g^{-1} soil (Debode 2005). However, these experiments were carried out at room temperature (24°C) and this is far above the temperature regime in a field soil. Therefore, further research is needed to examine what would happen at lower temperature (16°C/12°C).

Second, it needs to be examined whether the tested *Pseudomonas* strains have a significant biocontrol effect under field conditions, as research in the past has shown that biocontrol agents are often unsuccessful in field experiments (Mazzola 2004). Preliminary results showed that the combined incorporation of the tested *Pseudomonas* spp. with ryegrass or lignin in soil was more effective in reducing the viability of the *Verticillium* microsclerotia than the application of the *Pseudomonas* spp. alone (Debode 2005). These results indicate that in field conditions, a combined treatment may be more successful in controlling *Verticillium* than the application of *Pseudomonas* spp. alone.

Conclusions

In summary, this study demonstrates the potential of some previously described *Pseudomonas* bacteria as effective biocontrol agents against *Verticillium* microsclerotia. The efficacy of these bacteria when introduced into field soil (either alone or in combination), is a scope for future research.

The exact modes of action of the tested *Pseudomonas* strains in the suppression of *Verticillium* microsclerotia could not be elucidated in the present study. However, important conclusions can be drawn. First, biosurfactants are the main mechanism involved in the biological control of *Verticillium* microsclerotia by *Pseudomonas* CMR12a and *Ps. aeruginosa* PNA1. Second, PCA seems to have an inhibitory effect on *Verticillium* microsclerotia viability. Third, *Pseudomonas* spp. can colonize the microsclerotia surface and utilize nutrients from the microsclerotia for their growth. Future research should further explore these modes of action and possible interferences among them should be studied.

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