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Suppression of fusarium wilt of radish by co-inoculation of fluorescent *Pseudomonas* spp. and root-colonizing fungi

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Abstract

In an earlier study, treatment of radish seed with the bacterium *Pseudomonas fluorescens* WCS374 suppressed fusarium wilt of radish (*Fusarium oxysporum* f. sp. *raphani*) in a commercial greenhouse [Leeman *et al.*, 1991b, 1995a]. In this greenhouse, the areas with fusarium wilt were localized or expanded very slowly, possibly due to disease suppressiveness of the soil. To study this phenomenon, fungi were isolated from radish roots collected from the greenhouse soil. Roots grown from seed treated with WCS374 were more abundantly colonized by fungi than were roots from nonbacterized plants. Among these were several species known for their antagonistic potential. Three of these fungi, *Acremonium rutilum*, *Fusarium oxysporum* and *Verticillium lecanii*, were evaluated further and found to suppress fusarium wilt of radish in a pot bioassay. In an induced resistance bioassay on rockwool, *F. oxysporum* and *V. lecanii* suppressed the disease by the apparent induction of systemic disease resistance. In pot bioassays with the *Pseudomonas* spp. strains, the pseudobactin-minus mutant 358PSB$^-$ did not suppress fusarium wilt, whereas its wild type strain (WCS358) suppressed disease presumably by siderophore-mediated competition for iron. The wild type strains of WCS374 and WCS417, as well as their pseudobactin-minus mutants 374PSB$^-$ and 417PSB$^-$ suppressed fusarium wilt. The latter is best explained by the fact that these strains are able to induce systemic resistance in radish, which operates as an additional mode of action. Co-inoculation in pot bioassays, of *A. rutilum*, *F. oxysporum* or *V. lecanii* with the *Pseudomonas* spp. WCS358, WCS374 or WCS417, or their pseudobactin-minus mutants, significantly suppressed disease (except for *A. rutilum*/417PSB$^-$ and all combinations with 358PSB$^-$), compared with the control treatment, if the microorganisms were applied in inoculum densities which were ineffective in suppressing disease as separate inocula. If one or both of the microorganism(s) of each combination were applied as separate inocula in a density which suppressed disease, no additional suppression of disease was observed by the combination. The advantage of the co-inoculation is that combined populations significantly suppressed disease even when their individual population density was too low to do so. This may provide more consistent biological control. The co-inoculation effect obtained in the pot bioassays suggests that co-operation of *P. fluorescens* WCS374 and indigenous antagonists could have been involved in the suppression of fusarium wilt of radish in the commercial greenhouse trials.

Abbreviations: CFU = colony forming units; KB = King's B; PGPR = plant growth-promoting rhizobacteria; CQ = colonization quotient.

Introduction

The Châteaurenard soil in France and the Salinas Valley soil in California are known for their natural suppressiveness to fusarium wilt diseases [Alabouvette, 1986; Kloepper *et al.*, 1980; Louvet *et al.*, 1981, Scher and Baker, 1980]. In the French soil, strains of nonpathogenic *Fusarium oxysporum*, were considered

to be responsible for the soil suppressiveness [Alabouvette, 1986; Louvet *et al.*, 1981], whereas fluorescent pseudomonads were thought to be the organisms responsible for soil suppressiveness in the Salinas Valley soil [Kloepper *et al.*, 1980; Scher and Baker, 1980, 1982]. These root-colonizing pseudomonads, which are beneficial to plant growth, were termed plant growth-promoting rhizobacteria (PGPR) [Kloepper and Schroth, 1978]. When the PGPR were introduced into a disease conducive soil, fusarium wilt disease was significantly suppressed [Alabouvette, 1986; Kloepper *et al.*, 1980; Paulitz *et al.*, 1987]. However, infestation of a disease conducive soil with a disease-suppressing microorganism does not reach the level of suppression observed in the naturally suppressive soils, and the positive effects are often inconsistent [Weller, 1988]. Improvement of suppression of fusarium wilt of radish and take-all of wheat was demonstrated by combinations of *Pseudomonas* spp. [Raaijmakers, 1994; Weller and Cook, 1983]. By combining fluorescent pseudomonads and nonpathogenic fusaria, the suppression of fusarium wilts was more efficient and more consistent than by separate inoculation of the disease-suppressing organisms [Lemanceau and Alabouvette, 1991; Lemanceau *et al.*, 1992, 1993; Park *et al.*, 1988]. It is now of consensus, that natural disease suppressiveness of soils is based on a concerted action of several disease-suppressing microorganisms and modes of disease suppression [Lemanceau and Alabouvette, 1993; Schippers, 1992].

The accepted modes of disease suppression of the disease-suppressing microorganisms are 1) competition for substrate (e.g. carbon, nitrogen and ferric iron), 2) niche exclusion, 3) antibiosis and 4) induction of resistance [Couteaudier and Alabouvette, 1990; Lemanceau and Alabouvette, 1993; Schippers, 1992; Weller, 1988]. Lemanceau *et al.* [1992, 1993] have demonstrated that suppression of fusarium wilt of carnation by co-inoculation of *Pseudomonas putida* WCS358 and nonpathogenic *Fusarium oxysporum* Fo47 was based on increased sensitivity of the pathogen to carbon competition with the antagonistic Fo47, in the presence of the fluorescent siderophore of WCS358.

Suppression of fusarium wilt by fluorescent pseudomonads, through fluorescent siderophore (called pyoverdine or pseudobactin) mediated competition for ferric iron, was first reported by Scher and Baker [1982]. Growth-promoting effects on radish by fluorescent pseudomonads were first published by Kloepper and Schroth [1978] and later by Geels *et al.* [1985]. Seed bacterization of radish and of seed tubers of potato with *Pseudomonas fluorescens* WCS374 resulted in significant plant growth-promotion in high-frequency radish and high-frequency potato-cropping soil, respectively [Geels and Schippers, 1983b; Geels *et al.*, 1985]. In these studies, siderophore-mediated iron deprivation of deleterious microorganisms was considered the mode of action for the observed growth-promotion.

Recent studies have demonstrated that WCS374 and WCS417 induce systemic resistance in radish against fusarium wilt [Leeman *et al.*, 1995b]. Their lipopolysaccharides were demonstrated to be involved in the induction of resistance [Leeman *et al.*, 1995c]. The PGPR strain WCS374 significantly suppressed fusarium wilt of radish, caused by *Fusarium oxysporum* f. sp. *raphani* (syn. *F. o.* f. sp. *conglutinans*), in commercial greenhouse trials [Leeman *et al.*, 1991b, 1995a]. In this greenhouse, the disease reoccurs annually at the same locations. These expand very slowly and suggest that the soil surrounding the localized areas with wilting plants is disease suppressive.

The objective of this study was to investigate if the suppression of fusarium wilt of radish in the localized areas in the commercial greenhouse by bacterization with strain WCS374 [Leeman *et al.*, 1991b, 1995a], is due to strain WCS374 alone, or could possibly have operated in concert with antagonistic elements of the indigenous root-colonizing saprophytic mycoflora. Saprophytic fungi therefore were isolated from radish roots obtained from the greenhouse soil [Leeman *et al.*, 1991b]. Their fusarium wilt suppressive capacity, separately, as well as co-inoculated with selected disease-suppressing *Pseudomonas* strains, was studied in pot bioassays and evaluated with respect to their modes of disease suppression by e.g. pseudobactin siderophores and induction of systemic disease resistance. The significance of co-inoculation for commercial application is discussed.

Materials and methods

Radish cultivar

The radish (*Raphanus sativus* L.) cultivar Saxa*Nova (moderately resistant to fusarium wilt, seed size 2.50–2.75 mm) (S&G Seeds B.V., Enkhuizen, the Netherlands) was used in all experiments.

Microbial cultures and inocula

The wilt pathogen of radish, *Fusarium oxysporum* Schlecht. f. sp. *raphani* Kendrick and Snyder (formerly called *Fusarium oxysporum* Schlecht. f. sp. *conglutinans* [(Wollenw.) Snyder & Hansen] race 2 Armstrong & Armstrong) (WCS600), was obtained from infected radishes from a commercial greenhouse, by isolation on Komada's agar [Komada, 1975] modified by Gams and Van Laar [1982]. The fungal culture was maintained on modified Komada's agar.

The pathogen was cultured in aerated 2% malt extract broth. After seven days of incubation at 22 °C, washed microconidia were mixed with gamma sterilized peat (Agrifutur s.r.l., Alfianello, Italy). The inoculum was incubated four days at 22 °C, and stored at 6 °C. The number of colony-forming units (CFU) in the peat before being used in the bioassays, was determined by dilution plating on modified Komada's agar.

An inventory was made of the radish root-colonizing fungi in the commercial greenhouse soil, in the spring of 1991. In this greenhouse, biological control of fusarium wilt of radish by *Pseudomonas fluorescens* WCS374 was obtained in a number of successive crops of radish, during 1989-92 [Leeman *et al.*, 1991b; 1995a]. Roots were sampled in 4 × 3 fixed plots (each 0.5 m^2) belonging to the localized areas with fusarium wilt disease (summer periods of 1989-90) or the surrounding areas without fusarium wilt incidence, both in combination with the film-coating (polyvinyl acetate) control treatment as well as with the treatment with *P. fluorescens* WCS374 in the film-coating. Per fixed plot, eight root parts of 5 cm length, starting 1 cm below the radish, were rinsed in tap water for 10 min to remove soil particles. Roots were transferred into bottles (8 cm × 3 cm diam.) and vigorously shaken in sterile water with glass beads (1 g, 1 mm diam.) for 3 min. Afterwards each root was dried on filter paper, cut into four parts of 3–5 mm length and placed on carboxymethylcellulose agar supplemented with 50 ppm aureomycin [Gams *et al.*, 1987]. After incubation for 4 days at 21 °C, fungi growing from the root parts were isolated and cultured on malt agar (2% malt extract + 1.5% agar, MA), oatmeal agar (3% oatmeal extract + 1.5% agar), carrot-potato agar (0.5 % carrot root extract + 0.5% potato tuber extract + 0.8% agar) or soil agar (filtered garden soil extract (taken from the garden of the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands) 1:1= soil:water + 0.8% agar). Fungi were identified when the colonies were sporulating and were maintained on MA. Three relatively abundant species were selected out of the collection (Table 1): *Acremonium rutilum* Gams, *Fusarium oxysporum* Schlecht. and *Verticillium lecanii* (Zimm.) Viegas. The *Fusarium* isolate caused no disease symptoms on radish (hereafter termed nonpathogenic *F. oxysporum*). They were screened for their disease suppressive properties against fusarium wilt of radish in the pot bioassay.

Each species was cultured in 2% malt extract. The washed microconidia of the nonpathogenic *F. oxysporum* were mixed through peat. Cultures of the two other fungi were homogenized with an ultra-thurrax, washed and also mixed through peat. After four days incubation at 21 °C, the inocula were stored at 6 °C. The number of CFU in the peat was determined by dilution-plating on MA, before being used in the pot bioassay.

Pseudomonas putida WCS358 and *P. fluorescens* WCS374 were originally isolated from potato rhizosphere [Geels and Schippers, 1983a, 1983b] and *P. fluorescens* WCS417 was isolated from wheat rhizosphere in field soil suppressing the take-all disease in wheat caused by *Gaeumannomyces graminis* var. *tritici* [Lamers *et al.*, 1988]. Plant growth-promoting traits of WCS358 (siderophore mediated competition for iron), WCS374 and WCS417 (siderophore mediated competition for iron, induction of systemic disease resistance) have been described in a number of papers [Bakker *et al.*, 1986, 1987; Duijff *et al.*, 1993; Geels and Schippers, 1983b; Geels *et al.*, 1985, 1986; Lamers *et al.*, 1988; Leeman *et al.*, 1991b, 1995a, 1995b, 1995c; Van Peer *et al.*, 1990, 1991; Van Peer and Schippers, 1989, 1992]. Pseudobactin-minus mutants JM218 of *P. putida* WCS358 (designated 358PSB$^-$) [Marugg *et al.*, 1985], JM374 of *P. fluorescens* WCS374 (designated 374PSB$^-$) [Weisbeek *et al.*, 1986] and S680 of *P. fluorescens* WCS417 (designated 417PSB$^-$) [Duijff *et al.*, 1993] were used to study the role of siderophores in the disease-suppressing effect. These mutants are nonfluorescent, Tn*5* insertion mutants, that do not produce a functional pseudobactin.

Bacteria were cultured on King's B (KB) agar [King *et al.*, 1954] at 27 °C. Bacterial suspensions were prepared by scraping one day old cultures from KB agar plates in 0.01 M $MgSO_4$. These suspensions were diluted and mixed with gamma sterilized peat (Agrifutur s.r.l., Alfianello, Italy). The number of CFU in the bacterized peat was determined by dilution-plating on KB agar.

Table 1. Potentially antagonistic fungi isolated from radish roots in the commercial greenhouse

Species	Potential antagonist of:
Acremonium rutilum Gams	fungi
Arthrobotrys oligospora Fr.	nematodes
Dactylella haptotyla (Drechsler) de Hoog & van Oorschot	nematodes
Emericellopsis terricola van Beyma	fungi
Fusarium oxysporum Schlecht.*	fungi
Paecilomyces farinosus (Holm:Fr.) Brown & Smith	insects
Septofusidium herbarum (Brown & Smith) Samson	fungi
Trichoderma harzianum Rifai	fungi
Trichoderma viride Pers.:Fr.	fungi
Verticillium lamellicola (Smith) Gams	fungi
Verticillium lecanii (Zimm.) Viegas	fungi, insects
Verticillium leptobactrum Gams	fungi, insects
Verticillium psalliotae Treschow	fungi, insects

* a nonpathogenic strain on radish

Pot bioassay

The peat inocula containing either the pathogen, fungal antagonist, or bacterium, were mixed with river sand to a final inoculum density of 10^3 CFU pathogen, $5 \times 10^3-10^4$ CFU fungal antagonist, and $5 \times 10^5-10^6$ bacteria g^{-1} peat/sand mixture, respectively. The final peat/sand mixtures always contained 1% peat, by adding sterile peat where necessary.

The inoculated peat/sand mixtures were transferred into pots (500 g pot^{-1}) containing a layer of hydro culture granules (100 ml) at the bottom. Per pot six radish seeds were sown. Each treatment was replicated ten times. Experiments were repeated at least three times.

Plants were maintained in the greenhouse at temperatures of 22 °C at night, 24–30 °C during the day, at a relative humidity of approximately 70% and with supplemental Son-t lighting for 16 h day^{-1}. The first week the plants received water, and in the remaining 2 weeks they received a modified Hoagland's [Hoagland and Arnon, 1938] nutrient solution containing 10 μM Fe-EDDHA (5% ferric iron of which 80% is bound as Fe-ethylenediamine di (o-hydroxyphenylacetic acid)) (Ciba-Geigy) [Leeman *et al.*, 1995b], on top of the pot. Three weeks after sowing, plants were harvested and the percentage of plants with fusarium wilt symptoms (browning and/or blackening of the xylem tissue in the roots and radish, and yellowing or browning of leaves) was recorded.

Induced systemic resistance rockwool bioassay

Radish seeds were sown in sand and after 5 days transferred to rockwool growth cubes (Rockwool/Grodan B.V., Roermond, The Netherlands). In this bioassay, the disease-suppressing fungi or strain WCS374 were inoculated on the root tips (0.2 g peat $root^{-1}$ containing 10^7 CFU fungi or WCS374 g^{-1}) and the pathogen inoculated on the root base (0.2 g peat $root^{-1}$ containing 10^6 CFU g^{-1}), as described by Leeman *et al.* [1995b]. Each treatment was replicated twelve times, each replication consisted of three plants. Three weeks after inoculation of the roots with the pathogen, the plants were harvested and the percentage of plants with fusarium wilt symptoms was recorded. Experiments were repeated at least three times.

Microbial root colonization

At harvest time, the internal colonization of the plants in the induced resistance bioassay by the inoculated saprophytic fungi (and pathogen) was checked by incubating (7 days, 22 °C) sections of externally disinfected roots and radishes on 2% malt agar. Disinfection of the plant parts was accomplished by dipping intact parts in 96% ethanol, followed by burning the alcohol. The plants used to determine the extent of internal colonization by the saprophytic fungi were not inoculated with the radish pathogen. In this way the pathogen did not interfere with the detection of saprophytic fungal populations. External colonization of the roots at the base (zone of pathogen inoculation) by the saprophytic fungi was determined by plating (malt agar and

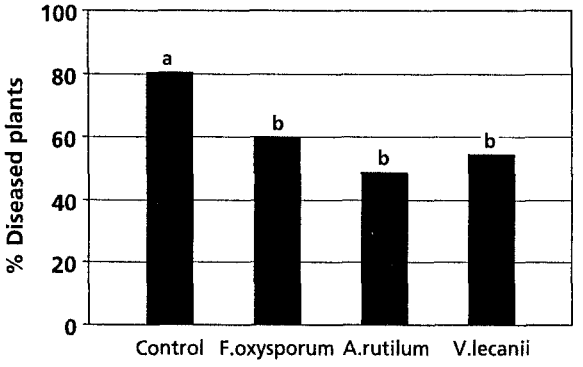

Fig. 1. Suppression of fusarium wilt of radish in a pot bioassay. *Fusarium oxysporum*, *Acremonium rutilum* or *Verticillium lecanii*, before sowing, were mixed through river sand as peat inocula (5 × 10^4 CFU g^{-1} peat/sand mixture). The pathogen was mixed through the sand as a peat inoculum (10^3 CFU g^{-1}). (Bars with the same letter are not significantly different at P≤ 0.05, Fisher's least-significant-difference test)

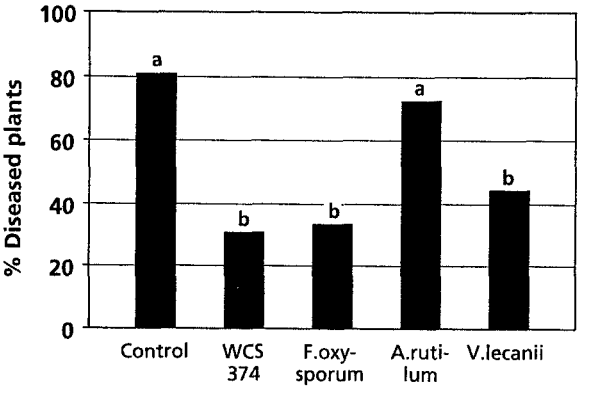

Fig. 2. Suppression of fusarium wilt of radish in the induced systemic resistance rockwool bioassay. Five day old radish seedlings were treated on their root tips with sterile peat or peat biological control inocula containing *Pseudomonas fluorescens* WCS374, *Fusarium oxysporum*, *Acremonium rutilum* or *Verticillium lecanii*, while the pathogen was delivered in peat on the root base two days after the biocontrol organisms. The pathogen and the biocontrol agents stayed spatially separated throughout the experiment. (Bars with the same letter are not significantly different at P≤ 0.05, Fisher's least-significant-difference test)

Komada's agar) root washings, which were obtained from plants not inoculated with the pathogen.

The root colonization by the introduced *Pseudomonas* strains in the induced resistance bioassay was determined in root macerates, by using the immunofluorescence colony-staining method [Van Vuurde and Roozen, 1990], modified by Leeman *et al.* [1991a, 1995b].

Data analysis

Data on diseased plants were analyzed for significance after arcsine square root transformations using analysis of variance followed by Fisher's least-significant-difference test (α= 0.05), using SAS-software (SAS Institute, Cary, NC, USA). Fungal root colonization data from the commercial greenhouse were analyzed by using the Kruskal-Wallis test (α= 0.05) followed by Wilcoxon's two sample rank sum test, because of non-normal distributions and nonhomogeneous variances after transformations.

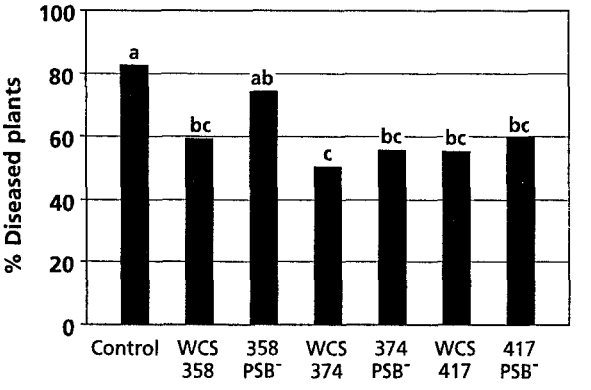

Fig. 3. Suppression of fusarium wilt of radish in a pot bioassay. *Pseudomonas putida* WCS358, *P. fluorescens* WCS374 or WCS417, or the respective pseudobactin mutants 358PSB⁻, 374PSB⁻ and 417PSB⁻, before sowing, were mixed through river sand as peat inocula (5 × 10^6 CFU g^{-1} peat/sand mixture). The pathogen was mixed through the sand as a peat inoculum (10^3 CFU g^{-1}). (Bars with the same letter are not significantly different at P≤ 0.05, Fisher's least-significant-difference test)

Results

Root colonization by soil fungi in a commercial greenhouse

In the spring of 1991, 51 fungal species were isolated from the roots of radish in the commercial greenhouse. Based on literature [Domsch *et al.*, 1980], 13 species were considered potential antagonists of fungi, nematodes or insects (Table 1).

The total fungal colonization quotient (CQ = total number of CFU of fungi isolated/total number of root parts) was significantly (P≤ 0.05) higher on the roots of the crop of radish from the WCS374 treatment (CQ= 1.23 or 1.08) compared with the film-coating control treatment (CQ= 0.77 or 0.70) (Table 2). No difference in CQs was observed between plots where fusarium wilt of radish occurred in 1989–90 and plots where the

Table 2. Root colonization quotients of fungi present on the roots of radish grown in a commercial greenhouse in the spring of 1991

Sampling plot characteristics	Colonization quotient[a]	
	Total fungi	Potentially antagonistic fungi
No fusarium wilt, no WCS374 applied	0.77 a[b]	0.40 a[b]
With fusarium wilt, no WCS374 applied	0.70 a	0.35 a
No fusarium wilt, WCS374 applied	1.23 b	0.59 a
With fusarium wilt, WCS374 applied	1.08 b	0.35 a

[a] number of fungi isolated/total number of root parts

[b] per column, means followed by the same letter are not significantly different at $P \leq 0.05$, Kruskal-Wallis test, followed by Wilcoxon's rank sum test

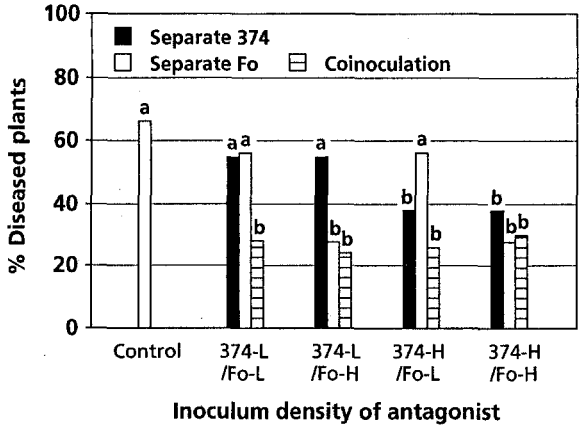

Fig. 4. Suppression of fusarium wilt of radish in a pot bioassay. *Pseudomonas fluorescens* WCS374 or *Fusarium oxysporum*, before sowing, were mixed through river sand as separate- or co-inocula in peat (374-L[ow]: 5×10^5, 374-H[igh]: 5×10^6, Fo-L: 5×10^3, Fo-H: 5×10^4 CFU g^{-1} peat/sand mixture). The pathogen was mixed through the sand as a peat inoculum (10^3 CFU g^{-1}). (Bars with the same letter are not significantly different at $P \leq 0.05$, Fisher's least-significant-difference test)

disease did not occur. In 1991, fusarium wilt of radish was almost completely absent; however *F. oxysporum* f. sp. *raphani* was present at low levels in the plots with a history of fusarium wilt, both in the absence and presence of WCS374. No significant difference occurred between the antagonistic fungi root colonization quotients of the WCS374 treatment (CQ= 0.59 or 0.35) and the film-coating control treatment (CQ= 0.40 or 0.35).

Suppression of fusarium wilt of radish by saprophytic fungi or Pseudomonas *spp.*

Three potentially antagonistic fungi were screened for their disease-suppressing properties against fusarium wilt of radish using the pot bioassay. The saprophytic fungi *Acremonium rutilum*, *Fusarium oxysporum* and *Verticillium lecanii*, significantly suppressed disease when applied at an inoculum density of 5×10^4 CFU g^{-1} sand (Fig. 1). In the induced resistance bioassay on rockwool, disease was suppressed by *F. oxysporum* and *V. lecanii*, but not by *A. rutilum* (Fig. 2). Neither the bacterium, nor the disease-suppressing fungi colonized the plants internally. Similarly, the pathogen could be isolated from inoculated plants. No evidence was found for external root colonization by the disease-suppressing bacterium and fungi at the root base (zone of pathogen inoculation).

Of the *Pseudomonas* spp. tested, strains WCS358, WCS374, 374PSB$^-$, WCS417, and 417PSB$^-$ significantly suppressed fusarium wilt in the pot bioassay, at inoculum densities of 5×10^6 CFU g^{-1} sand (Fig. 3). Only the pseudobactin-minus mutant strain 358PSB$^-$ was not able to suppress disease. *P. fluorescens* WCS374 induced systemic resistance in the rockwool bioassay (Fig. 2).

Suppression of fusarium wilt of radish by co-inoculation of saprophytic fungi and Pseudomonas *spp.*

The combination of *F. oxysporum* and WCS374 only reduced disease significantly compared with separate inoculation of the organisms, if both were applied at densities which were not individually able to reduce disease significantly (Fig. 4). The combination *F. oxysporum* and WCS358 or WCS417 gave similar results (data not shown). The efficacy of the organisms in suppressing disease could be changed by applying different inoculum densities ([Low] 5×10^3 or [High] 5×10^4 CFU *F. oxysporum* g^{-1} soil, and [Low] 5×10^5 or [High] 5×10^6 CFU WCS374 g^{-1} soil). To study co-inoculation effects in the pot bioassay, the disease-suppressing microorganisms were applied at

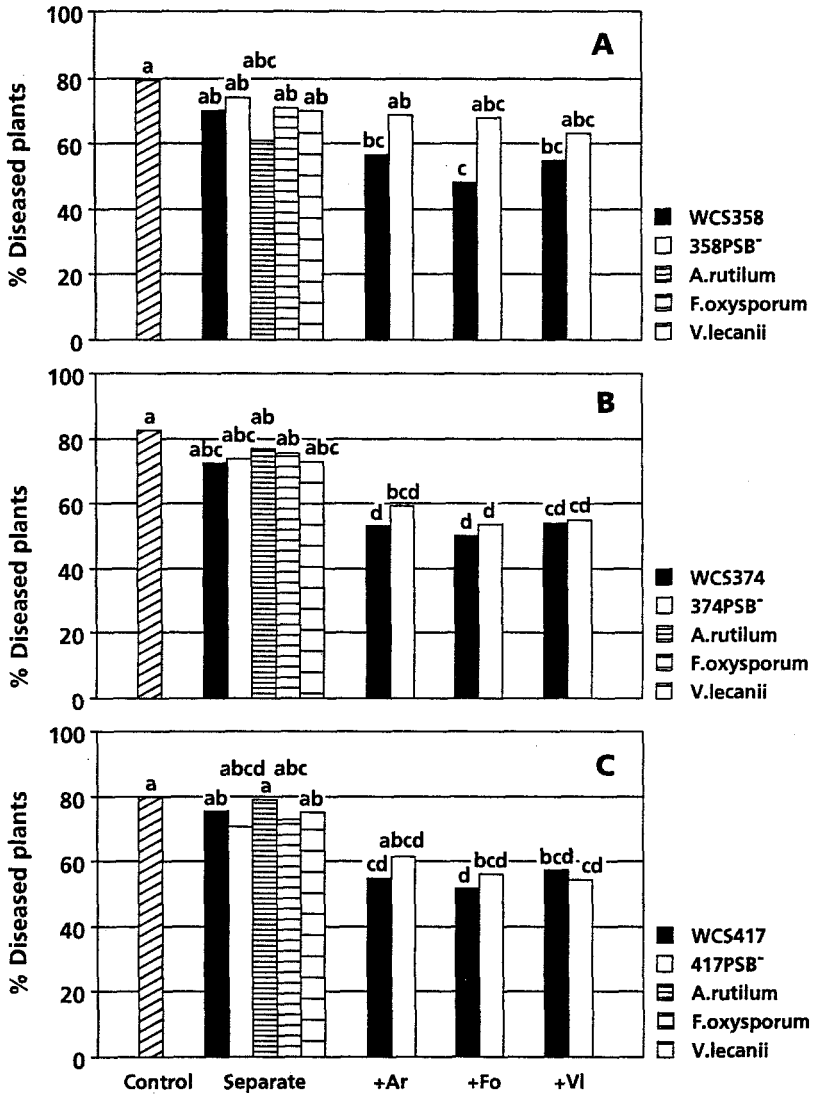

Fig. 5. Suppression of fusarium wilt of radish in a pot bioassay. (A) *Pseudomonas putida* WCS358, (B) *P. fluorescens* WCS374, (C) *P. fluorescens* WCS417, their respective pseudobactin mutants 358PSB$^-$, 374PSB$^-$, or 417PSB$^-$, or *Acremonium rutilum*, *Fusarium oxysporum*, or *Verticillium lecanii*, before sowing, were mixed through river sand as separate- or co-inocula in peat (bacteria: 5×10^5, fungi 5×10^3 g^{-1} peat/sand mixture). The pathogen was mixed through the sand as a peat inoculum (10^3 CFU g^{-1}). (Bars with the same letter are not significantly different at $P \leq 0.05$, Fisher's least-significant-difference test.)

inoculation densities at which they were not individually able to significantly suppress disease. In this way significant suppression of disease was observed by co-inoculation of strains WCS358, WCS374, 374PSB$^-$, WCS417, or 417PSB$^-$ together with *F. oxysporum*, *A. rutilum* (except in combination with 417PSB$^-$ where there was only a tendency) or *V. lecanii*, compared with the control treatment (Fig. 5). The combinations with 358PSB$^-$ did not show a significant disease suppression. Compared with the separate inoculation of the respective bacteria and fungi, a significant reduction of disease only was observed for the combinations 358+Fo, 374+Fo, 374PSB$^-$+Fo, 417+Fo, 374+Ar, and 417+Ar.

Discussion

Root-colonizing fungi antagonistic to *F. oxysporum* f. sp. *raphani*, causing agent of fusarium wilt of radish

were isolated from the soil of a commercial radish production greenhouse. Fusarium wilt spreads very slowly in this greenhouse and it is possible that these fungi are involved. They may also co-operate with *P. fluorescens* WCS374, in the disease suppression obtained by radish seed treatment with this strain [Leeman *et al.* 1991b, 1995a]. After metham-sodium fumigation of the soil in this greenhouse in August of 1990, strain WCS374 was applied as a radish seed treatment beginning in September of 1990 and continuing until the end of the trials in 1992. In that period, seed bacterization significantly reduced fusarium wilt, in 8 out of 9 of the successive crops of radish.

In the spring of 1991, no differences were detected in colonization of the roots by potentially antagonistic fungi, between plots with and without a history of fusarium wilt of radish (Table 2). On plants treated with strain WCS374, however, significantly more root-colonizing fungi were found per root part, compared with the control treatment. Recolonization by saprophytic fungi after soil fumigation, apparently had been strongly favoured by strain WCS374. A relationship of application of plant growth-promoting rhizobacteria and a change in composition of the root microflora also has been demonstrated by Kloepper and Schroth [1981]. A replacement of endophytic pseudomonads by *P. fluorescens* WCS417 was reported by Van Peer and Schippers [1989].

The differential effect of WCS374 on the recolonization of roots by the saprophytic mycoflora and the fusarium wilt pathogen (present at low levels, both in the absence and presence of the bacterium), may be due to the lower sensitivity of saprophytes, compared with the pathogen, to antagonism by WCS374. A higher sensitivity to competition for iron with *P. putida* WCS358, of the fusarium wilt pathogen *F. oxysporum* f. sp. *dianthi* of carnation compared with the antagonistic nonpathogenic *F. oxysporum* Fo47 was reported by Lemanceau *et al.* [1992, 1993]. The siderophore-mediated competition for iron made the pathogen more sensitive to carbon competition with Fo47, resulting in an increased suppression of wilt in carnation by co-inoculation of Fo47 with WCS358, compared with individual inoculations [Lemanceau *et al.*, 1992, 1993].

The saprophytic, antagonistic fungi *A. rutilum*, *F. oxysporum* and *V. lecanii*, frequently isolated from radish roots from greenhouse soil, significantly suppressed fusarium wilt of radish in pot bioassays (Fig. 1). The mode of action of disease suppression by *F. oxysporum* and *V. lecanii* could be induced systemic resistance, since both fungi suppressed disease when inoculated on the root at spatially separate locations from the pathogen (Fig. 2). In these experiments, none of the isolates was found in or on the root at the zone of inoculation of the pathogen, indicating that the organisms remained spatially separated from the pathogen. Induction of systemic resistance by nonpathogenic or avirulent strains of *F. oxysporum* was earlier demonstrated against fusarium wilt of watermelon and cucumber [Biles and Martyn, 1989; Mandeel and Baker, 1991]. Postma and Rattink [1992] showed suppression of fusarium wilt of carnation by a nonpathogenic *F. oxysporum*. However, in their system the antagonistic fungus was isolated from the plant interior and they did not obtain evidence for systemic induced resistance. Another mode of action of *A. rutilum*, *F. oxysporum* and of *V. lecanii* may be competition for nutrients with the pathogen as was demonstrated for carbon with nonpathogenic fusaria [Couteaudier and Alabouvette, 1990; Lemanceau *et al.*, 1993].

The suppression of fusarium wilt of radish by *P. putida* WCS358 most probably was siderophore-mediated, because the wild type strain suppressed disease, while the pseudobactin-minus mutant 358PSB$^-$ did not (Fig. 3). This is in accordance with other reports [Bakker *et al.*, 1986, 1987; Duijff *et al.*, 1993; Raaijmakers, 1994]. For *P. fluorescens* strains WCS374 and WCS417 there was no difference in disease suppression between wild type strains and pseudobactin-minus mutants. This can be explained by the fact that these strains and their mutants induced systemic resistance in radish against fusarium wilt [Leeman, 1994; Leeman *et al.*, 1995b]. This does not necessarily imply that pseudobactins of these strains were not a mechanism of disease suppression. That mechanism may have been masked by induced systemic resistance.

A significant increase in suppression of disease by co-inoculation of WCS374 (or WCS358, or WCS417) and the antagonistic saprophytic *F. oxysporum*, compared with their effects when inoculated separately, could only be demonstrated if the bacterium and the fungus were applied at densities that did not suppress disease when applied on their own (Fig. 4). This possibly is best explained by the observation that the disease can not be reduced below a certain level. When this level of maximum disease reduction is already realized by one of the biocontrol organisms, the combination simply can not further reduce disease. The 'co-inoculation' effect in the fusarium wilt of radish system differs from the additional disease suppres-

sion obtained by co-inoculation of *P. putida* WCS358 and the nonpathogenic *F. oxysporum* Fo47, even when the fungus alone already reduced disease significantly compared with the control [Lemanceau *et al.*, 1992]. The suppression of disease by Fo47 in the fusarium wilt of carnation model of Lemanceau et al. [1992], however, was much less than the level of reduction of disease by *F. oxysporum* obtained in the radish model.

Significant disease suppression was observed for the co-inoculation of the nonpathogenic *F. oxysporum* with one of each of the three wild type *Pseudomonas* strains or the pseudobactin-minus mutant 374PSB$^-$, compared with ineffective individual inoculations (Fig. 5). Two other combinations also significantly suppressed disease: *A. rutilum* with WCS374 or WCS417, compared with ineffective individual inoculations.

If compared with the control treatment, significant disease suppression was observed for 14 out of the 18 possible co-inoculation combinations, when the microorganisms were applied in inoculum densities which were ineffective as separate inocula. All combinations of 358PSB$^-$ with fungi tested, and the combination 417PSB$^-$ with *A. rutilum*, never (tested 6 times) showed significant disease suppression, compared with the control treatment. However, the latter combination showed a strong tendency of disease reduction in all tests. Since the pseudobactin-minus mutant 358PSB$^-$ is unable to suppress disease on its own, it is concluded that the 'co-inoculation effect' of the microorganisms used in these experiments can only be expected when the organisms applied have the potential to suppress disease, when inoculated on their own. The combinations of the pseudobactin-minus mutant 358PSB$^-$ with the fungi did not significantly suppress disease. The absence of pseudobactin-mediated competition for iron probably was the reason for this lack of suppression of disease. The involvement of pseudobactin 358 of strain WCS358 in co-inoculation experiments was earlier demonstrated in bioassays, in which the suppression of fusarium wilt of carnation by *P. putida* WCS358 and nonpathogenic *F. oxysporum* strain Fo47 was based on increased sensitivity of the pathogen for carbon competition with the antagonist Fo47 in the presence of the pseudobactin of WCS358 [Lemanceau *et al.*, 1992, 1993]. The observation that *A. rutilum*/WCS417 reduced disease significantly and the combination with the 417PSB$^-$ did not, may be explained by assuming that pseudobactin-mediated competition for iron operated as an additional mechanism of disease suppression. The fact that the combination *A. rutilum*/417PSB$^-$ did not significantly reduce disease and the combination *A. rutilum*/374PSB$^-$ did, is best explained by the observation in the induced systemic resistance rockwool bioassay, that WCS374 and derivatives reduce disease to a lower level than WCS417 and its derivatives do [Leeman, 1994].

An advantage of the co-inoculation is that the combined populations still significantly suppress disease even when their population density may be too low to do so on their own. A combination of disease-suppressing organisms may have the advantage of different traits (pseudobactin-mediated competition for iron, induced systemic resistance, and others) being involved in suppression of disease. If one of the organisms or disease suppressive traits is not active, e.g. due to unfavourable environmental conditions, the combination still can be. This may provide more consistent biological control.

It is conceivable that 'co-operation' between the indigenous antagonistic fungi and *P. fluorescens* WCS374 contributes to the wilt suppression obtained after introduction of WCS374, in the slowly expanding localized areas of the fusarium wilt of radish in the commercial greenhouse [Leeman *et al.*, 1991b; 1995a]. Combinations of biological control agents also have been reported that did not result in improved suppression of disease compared with the separate inoculants [Dandurand and Knudsen, 1993; Hubbard *et al.*, 1983; Miller and May, 1991; Sneh *et al.*, 1984]. These results may be ascribed to incompatibility of the co-inoculants [Baker, 1990; Bakker *et al.*, 1993; Raaijmakers, 1994], e.g., one of the combined *Pseudomonas* strains could be outcompeted for iron by the other [Raaijmakers *et al.*, 1995].

In conclusion, significant suppression of disease by co-inoculation was obtained in bioassays and therefore may also have been involved in the suppression of fusarium wilt of radish in the commercial greenhouse trials where *P. fluorescens* WCS374 was applied as a seed treatment. The success of biological control of soil-borne diseases by application of single disease-suppressing agents may not only depend on the level and quality of antagonism of the applied agent, but also on that already present in the soil. Co-inoculation needs further exploitation to contribute to a more consistent microbial biological control of plant pathogens.

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References

Alabouvette C (1986) Fusarium wilt suppressive soils from the Chateaurenard region: review of a 10 year study. Agronomie 6: 273–284

Baker R (1990) An overview of current and future strategies and models for biological control. In: Hornby D (ed), Biological Control of Soil-Borne Plant Pathogens (pp. 375–389) C.A.B. International, Wallingford

Bakker PAHM, Lamers JG, Bakker AW, Marugg JD, Weisbeek PJ and Schippers B (1986) The role of siderophores in potato tuber yield increase by *Pseudomonas putida* in a short rotation of potato. Neth J Plant Pathol 92: 249–256

Bakker PAHM, Bakker AW, Marugg JD, Weisbeek PJ and Schippers B (1987) Bioassay for studying the role of siderophores in potato growth stimulation by *Pseudomonas* spp. in short potato rotations. Soil Biol Biochem 19: 451–457

Bakker PAHM, Raaijmakers JM and Schippers B (1993) Role of iron in the suppression of bacterial plant pathogens by fluorescent pseudomonads. In: Barton LL and Hemming BC (edS) Iron Chelation in Plants and Soil Microorganisms (pp. 269–278) Academic Press Inc., San Diego

Biles CL and Martyn RD (1989) Local and systemic resistance induced in watermelons by formae speciales of *Fusarium oxysporum*. Phytopathology 79: 856–860

Couteaudier Y and Alabouvette C (1990) Quantitative comparison of *Fusarium oxysporum* competitiveness in relation to carbon utilization. FEMS Microbiol Ecol 74: 261–268

Dandurand LM and Knudsen GR (1993) Influence of *Pseudomonas fluorescens* on hyphal growth and biocontrol activity of *Trichoderma harzianum* in the spermosphere and rhizosphere of pea. Phytopathology 83: 265–270

Domsch KH, Gams W and Anderson TH (1980) Compendium of Soil Fungi. Academic Press, London

Duijff BJ, Meijer JW, Bakker PAHM and Schippers B (1993) Siderophore-mediated competition for iron and induced resistance in the suppression of fusarium wilt of carnation by fluorescent *Pseudomonas* spp. Neth J Plant Pathol 99: 277–289

Gams W and Laar W van (1982) The use of solacol (validamycine) as a growth retardant in the isolation of soil fungi. Neth J Plant Pathol 88: 39–45

Gams W, Aa HA van der, Plaats-Niterink AJ van der, Samson RA and Stalpers JA (1987) CBS Course of Mycology. Centraalbureau voor Schimmelcultures, Baarn, the Netherlands

Geels FP and Schippers B (1983a) Selection of antagonistic fluorescent *Pseudomonas* spp. and their root colonization and persistence following treatment of seed potatoes. J Phytopathol 108: 193–206

Geels FP and Schippers B (1983b) Reduction of yield depressions in high frequency potato cropping soil after seed tuber treatments with antagonistic fluorescent *Pseudomonas* spp. J Phytopathol 108: 207–214

Geels FP, Schmidt EDL and Schippers B (1985) The use of 8-hydroxyquinoline for the isolation and prequalification of plant growth-stimulating rhizosphere pseudomonads. Biol Fert Soils 1: 167–173

Geels FP, Lamers JG, Hoekstra O and Schippers B (1986) Potato plant response to seed tuber bacterization in the field in various rotations. Neth J Plant Pathol 92: 257–272

Hoagland DR and Arnon DI (1938) The water culture method for growing plants without soil. Calif Agric Exp Stn Bull 347: 36–39

Hubbard JP, Harman GE and Hadar Y (1983) Effect of soilborne *Pseudomonas* spp. on the biological control agent, *Trichoderma hamatum*, on pea seeds. Phytopathology 73: 655–659

King EO, Ward MK and Raney DE (1954) Two simple media for the demonstration of pyocyanin and fluorescin. J Lab Clin Med. 44: 301–307

Kloepper JW and Schroth MN (1978) Plant growth promoting rhizobacteria on radishes. Proceedings of the Fourth International Conference on Plant Pathogenic Bacteria, Angers, France 2: 879–882

Kloepper JW and Schroth MN (1981) Relationship of *in vitro* antibiosis of plant growth-promoting rhizobacteria to plant growth and the displacement of root microflora. Phytopathology 71: 1020–1024

Kloepper JW, Leong J, Teintze M and Schroth M (1980) *Pseudomonas* siderophores: a mechanism explaining disease-suppressive soils. Curr Microbiol 4: 317–320

Komada H (1975) Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soils. Rev Plant Prot Res 8: 114–125

Lamers JG, Schippers B and Geels FP (1988) Soil-borne diseases of wheat in the Netherlands and results of seed bacterization with pseudomonads against *Gaeumannomyces graminis* var. *tritici*. In: Jorna ML and Slootmaker LAJ (eds) Cereal Breeding Related to Integrated Cereal Production (pp. 134–139) Pudoc, Wageningen

Leeman M (1994) Suppression of fusarium wilt of radish by fluorescent *Pseudomonas* spp. – Induction of disease resistance, co-inoculation with fungi and commercial application. Ph.D. thesis, Utrecht University, Utrecht

Leeman M, Raaijmakers JM, Bakker PAHM and Schippers B (1991a) Immunofluorescence colony staining for monitoring pseudomonads introduced into soil. In: Beemster ABR, Bollen GJ, Gerlagh M, Ruissen MT, Schippers B and Tempel A (eds) Biotic Interactions and Soil-Borne Diseases (pp. 374–380) Elsevier, Amsterdam

Leeman M, Scheffer RJ, Pelt JA van, Bakker PAHM and Schippers B (1991b) Control of fusarium wilt of radish by *Pseudomonas fluorescens* WCS374, in greenhouse trials. In: Keel C, Koller B and Défago G (eds) Plant Growth-Promoting Rhizobacteria: Progress and Prospects, West Palaearctic Reg Sec Bull Vol 14/8 (pp. 34–38) Int Org Biol Integrated Control Noxious Anim Plants

Leeman M, Pelt JA van, Hendrickx MJ, Scheffer RJ, Bakker PAHM and Schippers B (1995a) Biocontrol of fusarium wilt of radish in commercial greenhouse trials by seed treatment with *Pseudomonas fluorescens* WCS374. Phytopathology: in press

Leeman M, Pelt JA van, Ouden FM den, Heinsbroek M, Bakker PAHM and Schippers B (1995b) Induction of systemic resistance by *Pseudomonas fluorescens* in radish cultivars differing in

susceptibility to fusarium wilt, using a novel bioassay. Europ J Plant Pathol: in press

Leeman M, Pelt JA van, Ouden FM den, Heinsbroek M, Bakker PAHM and Schippers B (1995c) Induction of systemic resistance against fusarium wilt of radish by lipopolysaccharides of *Pseudomonas fluorescens*. Phytopathology: in press

Lemanceau P and Alabouvette C (1991) Biological control of fusarium diseases by fluorescent *Pseudomonas* and non-pathogenic *Fusarium* Crop Prot. 10: 279–286

Lemanceau P and Alabouvette C (1993) Suppression of fusarium wilts by fluorescent pseudomonads: mechanisms and applications. Biocontrol Sc Techn 3: 219–234

Lemanceau P, Bakker PAHM, Kogel WJ de, Alabouvette C and Schippers B (1992) Effect of pseudobactin 358 production by *Pseudomonas putida* WCS358 on suppression of fusarium wilt of carnations by nonpathogenic *Fusarium oxysporum* Fo47. Appl Environ Microbiol 58: 2978–2982

Lemanceau P, Bakker PAHM, Kogel WJ de, Alabouvette C and Schippers B (1993) Antagonistic effect of nonpathogenic *Fusarium oxysporum* Fo47 and pseudobactin 358 upon pathogenic *Fusarium oxysporum* f. sp. *dianthi*. Appl Environ Microbiol 59: 74–82

Louvet J, Alabouvette C and Rouxel F (1981) Microbiological suppressiveness of some soils to fusarium wilts. In: Nelson PE, Toussoun TA and Cook RJ (eds) Fusarium: Diseases, Biology and Taxonomy (pp. 261–275) Pennsylvania State University Press

Mandeel Q and Baker R (1991) Mechanisms involved in biological control of fusarium wilt of cucumber with strains of nonpathogenic *Fusarium oxysporum*. Phytopathology 81: 462–469

Marugg JD, Spanje M van, Hoekstra WPM, Schippers B and Weisbeek PJ (1985) Isolation and analysis of genes involved in siderophore biosynthesis in plant growth-stimulating *Pseudomonas putida* WCS358. J Bacteriol 164: 563–570

Miller RH and May S (1991) Legume inoculation: successes and failures. In: Keister DL and Cregan PB (eds) The Rhizosphere and Plant Growth (pp. 123–135) Kluwer Academic Publishers, Dordrecht

Park CS, Paulitz TC and Baker R (1988) Biocontrol of fusarium wilt of cucumber resulting from interactions between *Pseudomonas putida* and non-pathogenic isolates of *Fusarium oxysporum*. Phytopathology 78: 190–194

Paulitz TC, Park CS and Baker R (1987) Biological control of fusarium wilt of cucumber with non pathogenic isolates of *Fusarium oxysporum*. Can J Microbiol 33: 349–353

Postma J and Rattink H (1992) Biological control of fusarium wilt of carnation with a nonpathogenic isolate of *Fusarium oxysporum*. Can J Bot 70: 1199–1205

Raaijmakers JM (1994) Microbial interactions in the rhizosphere – Root colonization by *Pseudomonas* spp. and suppression of fusarium wilt. Ph.D. thesis, Utrecht University, Utrecht

Raaijmakers JM, Sluis I van der, Koster M, Bakker PAHM, Weisbeek PJ and Schippers B (1995) Utilization of heterologous siderophores and rhizosphere competence of fluorescent *Pseudomonas* spp. Can J Microbiol: 41: 126–135

Scher FM and Baker R (1980) Mechanism of biological control in a fusarium-suppressive soil. Phytopathology 70: 412–417

Scher FM and Baker R (1982) Effect of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to fusarium wilt pathogens. Phytopathology 72: 1567–1573

Schippers B (1992) Prospects for management of natural suppressiveness to control soilborne pathogens. In: Tjamos EC, Papavizas GC and Cook RJ (eds) Biological Control of Plant Diseases, Progress and Challenges for the Future, NATO ASI Series, Series A: Life Sciences. Vol. 230 (pp. 21–34) Plenum Press, New York and London

Sneh B, Dupler M, Elad Y and Baker R (1984) Chlamydospore germination of *Fusarium oxysporum* f.sp. *cucumerinum* as affected by fluorescent and lytic bacteria from Fusarium-suppressive soil. Phytopathology 74: 1115–1124

Van Peer R and Schippers B (1989) Plant growth responses to bacterization and rhizosphere microbial development in hydroponic cultures. Can J Microbiol 35: 456–463

Van Peer R and Schippers B (1992) Lipopolysaccharides of plant growth-promoting *Pseudomonas* sp. strain WCS417r induce resistance in carnation to fusarium wilt. Neth J Plant Pathol 98: 129–139

Van Peer R, Kuik AJ van, Rattink H and Schippers B (1990) Control of fusarium wilt of carnation grown on rockwool by *Pseudomonas* sp. strain WCS417r and by FeEDDHA. Neth J Plant Pathol 96: 119–132

Van Peer R, Niemann GJ and Schippers B (1991) Induced resistance and phytoalexin accumulation in biological control of fusarium wilt of carnation by *Pseudomonas fluorescens* strain WCS417r. Phytopathology 81: 728–734

Van Vuurde JWL and Roozen NJM (1990) Comparison of immunofluorescence colony-staining in media, selective isolation on pectate medium, ELISA and immunofluorescence cell staining for detection of *Erwinia carotovora* subsp. *atroseptica* and *E. chrysanthemi* in cattle manure slurry. Neth J Plant Pathol 96: 75–89

Weisbeek PJ, Hofstad GAJM van der, Schippers B and Marugg JD (1986) Genetic analysis of the iron-uptake system of two plant growth-promoting *Pseudomonas* strains. In: Swinburne TR (ed) Iron, Siderophores and Plant Diseases (pp. 299–313) Plenum Press, New York

Weller DM (1988) Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu Rev Phytopathol 26: 379–407

Weller DM and Cook RJ (1983) Suppression of take-all of wheat by seed treatments with fluorescent pseudomonads. Phytopathology 73: 463–469