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Combining fluorescent *Pseudomonas* spp. strains to enhance suppression of fusarium wilt of radish

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Abstract

Fusarium wilt diseases, caused by the fungus *Fusarium oxysporum*, lead to significant yield losses of crops. One strategy to control fusarium wilt is the use of antagonistic, root-colonizing *Pseudomonas* spp. It has been demonstrated that different strains of these bacteria suppress disease by different mechanisms. Therefore, application of a mixture of these biocontrol strains, and thus of several suppressive mechanisms, may represent a viable control strategy. A prerequisite for biocontrol by combinations of biocontrol agents can be the compatibility of the co-inoculated micro-organisms. Hence, compatibility between several *Pseudomonas* spp. strains, that have the ability to suppress fusarium wilt of radish, was tested *in vitro* on KB agar plates. Growth of *P. fluorescens* strain RS111 was strongly inhibited by *Pseudomonas* spp. strains RE8, RS13, RS56 and RS158, whereas a mutant of strain RS111 (RS111-a) was insensitive to inhibition by these strains. Strains RS111 and RS111-a only slightly inhibited some other strains. Suppression of fusarium wilt of radish in a potting soil bioassay by the incompatible combination of RE8 and RS111 was comparable to the effects of the single strains. However, disease suppression by the compatible combination of RE8 and RS111-a was significantly better as compared to the single strains. In contrast, the incompatible combination of RS56 with RS111 resulted in enhanced disease suppression as compared to the single strains. Increased disease suppression by combinations of RS13 or RS158 with RS111 or RS111-a was not observed. This indicates that specific interactions between biocontrol strains influence disease suppression by combinations of these strains.

Introduction

Fusarium wilt diseases, caused by the fungus *Fusarium oxysporum*, lead to significant yield losses of horticultural and agricultural crops. The pathogen infects the roots and colonizes the vascular tissue, leading to wilting and finally death of the plant (Peterson and Pound, 1960). Possibilities to manage fusarium wilt, e.g. by using fungicides or resistant cultivars, are limited. Therefore, other strategies to control this disease, such as biological control, are being developed.

Worldwide, disease-suppressive soils, including those suppressive to fusarium wilt, have been described (Alabouvette, 1986; Kloepper et al., 1980; Scher and Baker, 1980). The suppressiveness of these soils is of microbial origin (Schippers, 1992; Weller, 1988). Especially fluorescent *Pseudomonas* bacteria and non-pathogenic strains of *Fusarium oxysporum*, isolated from these soils, have the ability to reduce fusarium wilt, and have been studied for their potential as biological control agents. Mechanisms demonstrated to be involved in suppression of fusarium wilt by these antagonistic microorganisms are: competition for substrate (Couteaudier and Alabouvette, 1990), siderophore-mediated competition for iron (Duijff et al., 1994; Raaijmakers et al., 1995a), and induction of systemic resistance in the host (Leeman et al., 1995, 1996; Pieterse et al., 1996; Van Peer et al., 1991). Inoculation of disease conducive soils with a single strain of a biological control agent only rarely leads to a

level of suppression as observed in naturally disease-suppressive soils, and positive effects with single inoculants are often inconsistent (Schippers, 1992; Weller, 1988). Hence, it is postulated that in suppressive soils a concerted action of several microorganisms and mechanisms is responsible for the highly consistent disease suppressiveness (Alabouvette, 1986; Lemanceau and Alabouvette, 1991; Schippers, 1992). Consequently, application of a mixture of biocontrol agents is likely to more closely mimic the natural situation and may, therefore, represent a more viable control strategy.

Pierson and Weller (1994) showed that combinations of several fluorescent pseudomonads have the potential for greater biocontrol activity against take-all of wheat as compared to the same strains applied individually. Duffy et al. (1996) demonstrated that combinations of a strain of the biocontrol fungus *Trichoderma koningii* with different *Pseudomonas* spp. isolates provided greater suppression of take-all disease than either the fungus or the bacterium alone. Lemanceau et al. (1992, 1993) described increased suppression of fusarium wilt of carnation by combining *P. putida* WCS358 with non-pathogenic *Fusarium oxysporum* Fo47. The enhanced disease suppression by this combination is due to siderophore-mediated competition for iron by WCS358, which makes the pathogenic *F. oxysporum* strain more sensitive to competition for glucose by the non-pathogenic strain Fo47. Furthermore, Leeman et al. (1996) showed that combining strains of non-pathogenic *Verticillium lecanii*, *Acremonium rutilum* or *Fusarium oxysporum* with the fluorescent *Pseudomonas* spp. strains WCS358, WCS374 or WCS417 resulted in significantly better suppression of fusarium wilt of radish compared to the single organisms.

There are also reports stating that combinations of biological control agents do not result in improved suppression of disease as compared to the separate inoculants (Miller and May, 1991; Sneh et al., 1984). For example, combining a *Trichoderma harzianum* strain with a *Pseudomonas fluorescens* strain, both able to suppress root rot of pea caused by *Aphanomyces euteiches* f. sp. *pisi*, did not result in better disease suppression (Dandurand and Knudsen, 1993). Positive and negative interactions between introduced biocontrol microorganisms or between an introduced biocontrol agent and the indigenous microflora can influence their performance in the rhizosphere. For example, two groups of microorganisms that occupy the same ecological niche and have the same nutritional requirements, are bound to compete for nutrients (Bakker et al., 1988; Fukui et al., 1994; Janisiewicz and Bors, 1995; Raaijmakers et al., 1995b). Raaijmakers et al. (1995b) demonstrated that siderophore-mediated competition for iron between the two biocontrol agents *P. putida* WCS358 and *P. fluorescens* WCS374 decreased colonization of radish roots by the latter strain. Hubbard et al. (1983) described negative effects of endemic *Pseudomonas* spp. strains on the biocontrol agent *Trichoderma hamatum*. They suggested that these negative effects were caused by effective competition for iron by the *Pseudomonas* spp. strains, because addition of iron suppressed growth inhibition of *T. hamatum* by *Pseudomonas* strains *in vitro*. Moreover, *T. hamatum* suppressed *Pythium* seed rot of pea only when iron was added to naturally infested soil. Another negative interaction between two populations of biocontrol microorganisms can be due to detrimental effects of secondary metabolites produced by one organism on the growth of the other (Mew et al., 1994). Thus, an important prerequisite for the desired effectiveness of strains appears to be compatibility of the co-inoculated microorganisms (Baker, 1990; Li and Alexander, 1988; Raaijmakers et al., 1995b).

The objective of this study was to determine whether specific interactions between *Pseudomonas* spp. strains influence disease suppression by combinations of these strains. Interactions between several disease-suppressive *Pseudomonas* strains were first studied *in vitro*. Subsequently, suppression of fusarium wilt of radish by the single strains and their combinations was investigated to determine in how far interactions *in vitro* between the strains have predictive value for disease suppression by combinations of these strains *in vivo.*

Materials and methods

Microbial cultures

From collections of fluorescent *Pseudomonas* spp. isolates, recovered from the root tissue or rhizosphere of radish (P.A.H.M. Bakker and C. Remkes, unpublished) or tomato (Van Peer et al., 1990), 24 strains that were effective in reducing fusarium wilt of radish in potting soil bioassays, were selected. Based on *in vitro* interactions between the strains on KB agar plates (see: *in vitro* interactions between *Pseudomonas* strains), a number of strains were selected for further experiments (Table 1). The strains were stored at $-80\,^{\circ}$C in 50% glycerol. The pathogen used was

Table 1. Pseudomonas spp. strains selected for study

Strain	Origin	Reference
P. putida WCS358	potato rhizosphere	Geels and Schippers, 1983
P. putida RE8	radish root tissue	Bakker et al., in preparation
P. putida RS13	radish rhizosphere	„
P. putida RS56	radish rhizosphere	„
P. fluorescens RS158	radish rhizosphere	„
P. fluorescens RS111	tomato rhizosphere	Van Peer et al., 1990
P. fluorescens RS111-a		This study
P. fluorescens En401	tomato root tissue	Van Peer et al., 1990

Fusarium oxysporum Schlecht f.sp. *raphani* Kendrick & Snyder strain WCS600 (Leeman et al., 1995a).

Typing of strains by gel electrophoresis of LPS

In this study a spontaneous mutant of RS111 (RS111-a) was isolated. To identify this mutant it's lipopolysaccharides (LPS) pattern was compared to the LPS patterns of the parental strain and strain RE8. Cells were grown in KB medium (King et al., 1954) for 24 h at 28 °C. Cell envelopes containing LPS were obtained by differential centrifugation after disruption of the cells by ultrasonic treatment. Cell envelope samples were solubilized in standard sample mixture (Lugtenberg et al., 1975) and treated with proteinase K to degrade proteins, for the analysis of LPS (De Weger et al., 1987). These samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained with silver reagent (Silver Stain Kit; Bio-Rad).

In vitro *interactions between* Pseudomonas *strains*

Bacterial suspensions were made by suspending cells cultured on KB agar plates in 10 mM $MgSO_4$. The density of the bacterial suspensions was measured spectrophotometrically at 660 nm and adjusted to 10^7 colony forming units (cfu)/ml. The *Pseudomonas* strains were spot-inoculated on KB agar plates by pipetting 2 droplets of 5 µl of the bacterial suspension on a plate. The spot-inoculated plates were incubated at 27 °C for 48 h. Subsequently, a suspension of the target strain (10^7 cfu/ml) was atomized over the spot-inoculated plates. After an additional incubation period of 24 h at 27 °C zones of growth inhibition of the target strain around the spot-inoculated strains were measured. To determine the influence of iron-regulated metabolites, such as siderophores, the same experiment was performed on KB supplemented with 100 µM $FeCl_3$. Inhibition is expressed as the diameter of the inhibition zone divided by the diameter of the spot-inoculated colony.

Suppression of fusarium wilt

Effects of single strains and their combinations on fusarium wilt in radish were tested in a potting soil bioassay (Leeman et al., 1996; Raaijmakers et al., 1995a). The pathogen was cultured in aerated 2% malt extract (DIFCO) medium at 22 °C. After 14 days of growth, cultures were filtered through glass wool to remove mycelial mats. Microconidia were harvested by centrifugation (8000 rpm, 20 min), resuspended in 10 mM $MgSO_4$, and mixed through a potting soil/sand mixture to a final concentration of 3.75×10^5 cfu/g mixture. This *F. oxysporum*-infested soil was incubated for 3–5 days at 20 °C. Bacteria were grown for 24 h at 27 °C on KB agar plates, and suspensions were prepared in sterile 10 mM $MgSO_4$. The bacteria were introduced in an autoclaved (2×20 min with a 24 h interval) potting soil/sand mixture (Raaijmakers et al., 1995a) to a final density of approximately 7×10^6 cfu/g mixture. For the assay the fusarium-infested soil, bacterized soil, additional autoclaved soil and non-autoclaved river sand were mixed to final densities of 10^4 *F. oxysporum* conidia/g soil and 10^6 cfu/g soil for the bacteria. When combinations of *Pseudomonas* strains were tested, each strain was added at a final density of 10^6 cfu/g. Per treatment 9 pots (11 cm high, 14 cm diameter) were filled with 750 g of the soil/sand mixture, in which ten radish seeds (*Raphanus sativus* L.; cultivar Saxa 2*Nova, S&G Seeds B.V. Enkhuizen) were sown. The plants were grown in a climatized greenhouse at 20 °C

with a photoperiod of 16 hrs. After approximately 21 days the percentage of diseased plants per pot was scored on the basis of both external wilting and internal browning symptoms (Leeman et al., 1996). All experiments were repeated once.

Data analysis

Results of the two bioassays were pooled after establishing that there was no significant interaction at $p = 0.05$ between experiments and treatments, and variances were homogeneous. The pooled data were analyzed for significance using analysis of variance (anova) followed by Fisher's least-significant-difference test ($p \leq 0.05$), using SAS-software (SAS Institute, Cary, NC, USA).

Results

In vitro *antagonism*

A total of 24 strains of fluorescent *Pseudomonas* spp., that were demonstrated to suppress fusarium wilt of radish in a potting soil bioassay (P.A.H.M. Bakker, C. Remkes and P. Vogel, unpubl.), were tested pairwise in all possible combinations to establish their interactions *in vitro* (results not shown). Several types of interactions were observed: strains did not inhibit each other, only one strain was inhibited by the other, or both strains inhibited each other's growth. Some isolates were inhibited in growth by the majority of the other strains, whereas others were rarely inhibited by any of the strains. The observed growth inhibition was sometimes strong, resulting in a clear zone surrounding the spotted colony (Figure 1A). Other spot-inoculated colonies inhibited growth of the target strain only slightly, resulting in a zone of reduced growth around the spotted colony (Figure 1B), or did not inhibit growth at all (Figure 1C). In these *in vitro* interactions different mechanisms were involved since in 33% of the interactions addition of 100 μM $FeCl_3$ to the medium interfered with the antagonism. This indicates that iron-regulated metabolites were involved in some of the interactions. Other interactions may have resulted from competition for substrates or production of antibiotics.

To further examine the *in vitro* interactions, four *Pseudomonas* spp. strains were selected, based on their differential interactions *in vitro* (Table 2). Growth of the target strain RE8 was slightly inhibited by the spot-inoculated strains WCS358, RS111 and En401. When $FeCl_3$ was added inhibition by WCS358 and En401 disappeared, while the slight growth inhibition by RS111 was maintained. Strain En401 was hardly inhibited only on plates without Fe. En401 itself slightly inhibited growth of RE8 and RS111 based on iron-regulated compounds. WCS358 was not inhibited by any of the other strains, whereas WCS358 itself reduced growth of RE8 and En401 and inhibited growth of RS111. This growth inhibition was also caused by iron-regulated compounds, because addition of iron repressed growth inhibition in all cases. Also RE8 inhibited growth of

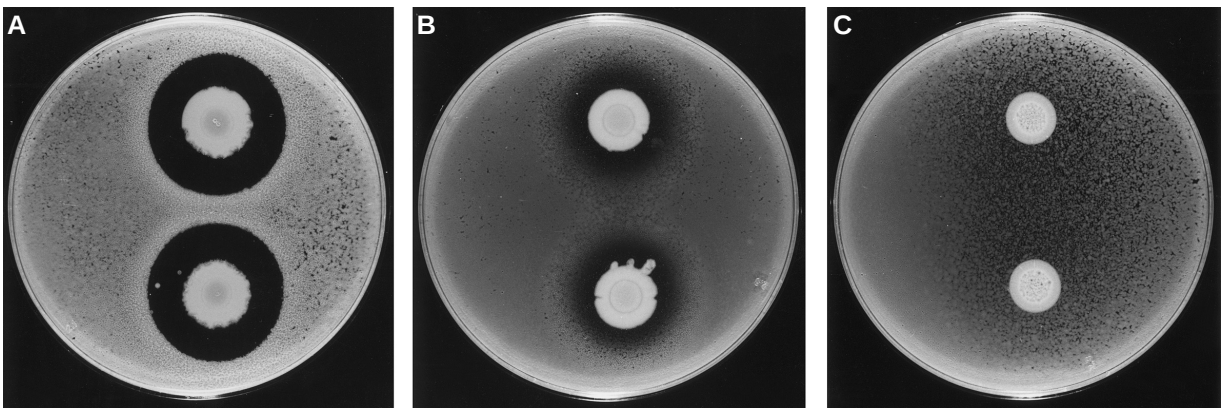

Figure 1. In vitro antagonism between fluorescent *Pseudomonas* spp. strains. The strains were spot-inoculated by pipetting 2 droplets of 5 μl of a suspension (10^7 cfu/ml) on KB agar plates. After 48 h of growth at 27 °C a suspension of the target strain (10^7 cfu/ml) was atomized over the spot-inoculated plates. After an additional 24 h several interactions were observed: A; the atomized strain was inhibited strongly by the spot-inoculated strain resulting in a clear zone of growth inhibition, B; the sprayed strain was slightly inhibited resulting in a zone of reduced growth or C; the sprayed strain was not inhibited by the spot-inoculated strain.

Table 2. In vitro interactions between fluorescent *Pseudomonas* spp. strains RE8, WCS358, RS111 and En401 on KB agar plates and KB agar plates containing 100 μM $FeCl_3$. +; clear zone of growth inhibition (see Figure 1A), ±; zone of reduced growth (see Figure 1B), —; no zone of growth inhibition (see Figure 1C)

KB					KB +100 μM $FeCl_3$			
Spot	RE8	WC358	RS111	En401	RE8	WC358	RS111	En401
Spray								
RE8	—	±2.2[x]	±2.0	±1.9	—	—	±2.3	—
WCS358	—	—	—	—	—	—	—	—
RS111	+2.4	+2.1	—	±1.5	+1.3	±[y]	—	—
En401	±1.8	±2.7	—	—	—	—	—	—

[x] total diameter/colony diameter; when this value = 1 there is no growth inhibition by the spot-inoculated colony. [y] no clear measurement of inhibition zone possible.

Table 3. In vitro antagonism of four *Pseudomonas* spp. strains against *P. fluorescens* strains RS111 and RS111-a on KB agar plates and on KB agar plates containing 100 μM $FeCl_3$. +; clear zone of growth inhibition (see Figure 1A), ±; zone of reduced growth (see Figure 1B), —; no zone of growth inhibition (see Figure 1C)

KB					KB + 100 μM $FeCl_3$			
Spot	RE8	RS13	RS56	RS158	RE8	RS13	RS56	RS158
Spray								
RS111	+2.4[x]	+2.5	+2.7	+2.5	+1.3	+1.2	+1.2	+1.3
RS111-a	±1.7	±1.7	±1.8	±1.7	—	—	—	—

[x] total diameter/colony diameter; when this value = 1 there is no growth inhibition by the spot-inoculated colony.

RS111. When iron was added to the medium the size of the inhibition zone was reduced. The apparent sensitivity of the growth of RS111 to inhibition by the spotted strains was further examined by testing all other 23 strains for their ability to inhibit RS111 on KB and KB+Fe. Only three strains, *P. putida* RS13 and RS56 and *P. fluorescens* RS158, inhibited growth of RS111, indicating that this interaction is fairly specific (Table 3).

A mutant of RS111 (RS111-a) was isolated from the inhibition zone surrounding the spot-inoculated strain RE8. A colony that appeared in the inhibition zone was isolated and crude cell wall extracts were prepared to compare the outer membrane LPS pattern on SDS-PAGE gels to those of the wildtype strains RS111 and RE8. The LPS pattern of RS111-a was identical to the pattern of RS111, and clearly different from that of RE8. The sensitivity of mutant RS111-a to growth inhibition by strains RE8, RS13, RS56 and RS158 was examined on KB and KB+Fe agar plates. In contrast to the wildtype RS111, mutant RS111-a was only slightly inhibited in growth by these four isolates on plates without added Fe (Table 3). When $FeCl_3$ (100 μM) was added, mutant RS111-a was not inhibited in growth whereas the wildtype RS111 was (Table 3).

Based on the observed *in vitro* interactions (Tables 2 and 3), compatible and incompatible combinations of strains (e.g. compatible: RS111+En401 and incompatible: RE8+RS111, Table 2) were chosen. Incompatible and compatible combinations were also obtained by combining strain RS111 or mutant RS111-a with strains RE8, RS13, RS56 and RS158 (e.g. incompatible: RS13 and RS111 and compatible: RS13 and RS111-a). These *in vitro* compatible and incompatible combinations were tested for suppression of fusarium wilt of radish in a potting soil bioassay to study the relationship between *in vitro* interactions between strains and disease suppression by combinations of these strains *in vivo*.

In vivo *suppression of fusarium wilt*

Combinations of the four strains RE8, WCS358, RS111 and En401 were tested for their disease suppression

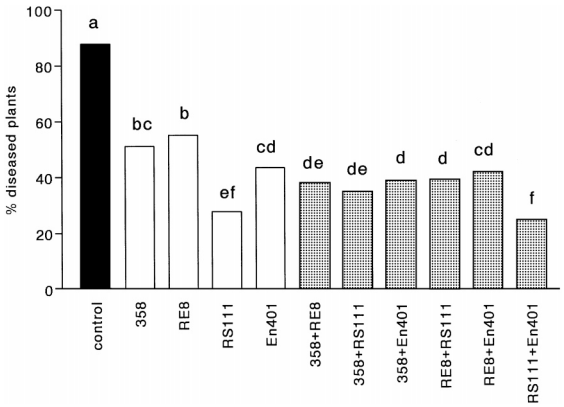

Figure 2. Suppression of fusarium wilt of radish in a pot bioassay. Before sowing, soil was bacterized with fluorescent *Pseudomonas* spp. strains WCS358, RE8, RS111 and En401. Strains were mixed through soil to a final concentration of 10^7 cfu/g soil (in single and combination treatments). The pathogen, *Fusarium oxysporum* f.sp. *raphani*, was mixed through soil to a final concentration of 10^4 conidia/g soil. Disease was scored 18 days after sowing. Bars indicated with the same letter are not significantly different at $p \leq 0.05$, analysis of variance followed by Fisher's least-significant-difference test.

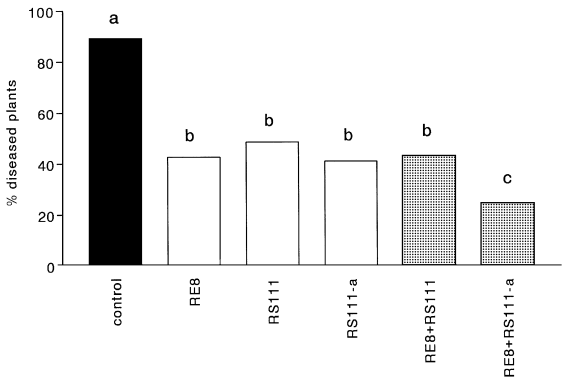

Figure 3. Suppression of fusarium wilt of radish in a pot bioassay. Before sowing, soil was bacterized with fluorescent *Pseudomonas* spp. strains RE8, RS111, RS111-a and combinations. Strains were mixed through soil to a final concentration of 10^6 cfu/g soil for single treatments and $2 * 10^6$ cfu/g soil for the combinations. The pathogen was mixed through soil to a final concentration of 10^4 conidia/g soil. After mixing bacterized soil, fusarium inoculated soil and sand radish seeds were sown. Disease was scored 21 days after sowing. Bars indicated with the same letter are not significantly different at $p \leq 0.05$, analysis of variance followed by Fisher's least-significant-difference test.

in vivo. All bacterial treatments, including the combinations, resulted in a significantly lower percentage of diseased plants as compared to the non-bacterized control treatment (Figure 2). Strain RS111 was most effective in disease suppression when comparing the single inoculants. Combining strains RE8 and WCS358 resulted in a significantly better disease suppression compared to the single strains (Figure 2). The combinations of En401 with RE8 or WCS358 resulted in disease suppression comparable to that obtained by En401 alone. Disease suppression by the combination of the strains RS111 and En401 was comparable to that by the single strain RS111. On the other hand, combinations of RS111 with RE8 or with WCS358 resulted in less disease suppression than RS111 alone (Figure 2). Only the combination RE8+RS111 resulted in a significantly lesser disease suppression than RS111 alone and a significantly better disease suppression than RE8 alone. This agrees with the strong antagonism between the strains *in vitro* (Table 2).

Whether this negative effect of RE8 on disease suppression by RS111 in the combination was correlated with the strong *in vitro* inhibition of RS111 by RE8, was further investigated by conducting bioassays with RE8, RS111 and mutant RS111-a. RS111-a was as effective as RS111 in suppressing disease (Figure 3). Disease suppression by RS111 was comparable to that by RE8, and the combination of strains RE8 and RS111 resulted in the same percentage diseased plants as compared to the single strains. However, the percentage diseased plants in the combination of RE8 and RS111-a was significantly lower compared to the single strains and to the combination of RE8 and RS111 (Figure 3). These results suggest that disease suppression by the combinations of RE8 and RS111, and RE8 and RS111-a reflect *in vitro* interactions observed between these strains.

Strains RS13, RS56 and RS158 showed the same differential inhibition of RS111 and RS111-a as strain RE8. To further elucidate the importance of this *in vitro* (in)compatibility in disease suppression, combinations of RS13, RS56 or RS158 and RS111 or RS111-a were studied in the radish-fusarium bioassay. Disease suppression by the single strain RS13 (Figure 4) and strain RS158 (Figure 5) was comparable to that by strains RS111 or RS111-a alone. In none of the combinations enhanced disease suppression was found, indicating that the combinations of RS111 or RS111-a with RS13 or RS158 did not increase the effectiveness of disease suppression. In contrast, when RS56 was combined with RS111, the percentage diseased plants was significantly lower as compared to the single

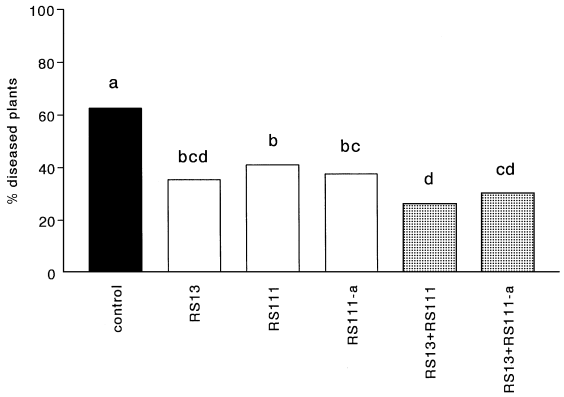

Figure 4. Suppression of fusarium wilt of radish in a pot bioassay. Before sowing, soil was bacterized with fluorescent *Pseudomonas* spp. strains RS13, RS111, RS111-a and combinations. Strains were mixed through soil to a final concentration of 10^6 cfu/g soil for single treatments and $2 * 10^6$ cfu/g soil for the combinations. The pathogen was mixed through soil to a final concentration of 10^4 conidia/g soil. After mixing bacterized soil, fusarium inoculated soil and sand radish seeds were sown. Disease was scored 21 days after sowing. Bars indicated with the same letter are not significantly different at $p \leq 0.05$, analysis of variance followed by Fisher's least-significant-difference test.

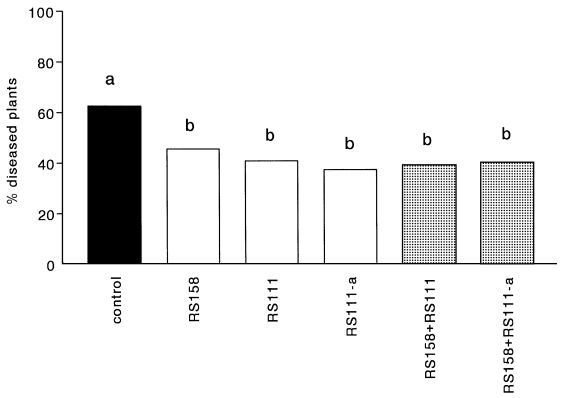

Figure 5. Suppression of fusarium wilt of radish in a pot bioassay. Before sowing, soil was bacterized with fluorescent *Pseudomonas* spp. strains RS158, RS111, RS111-a and combinations. Strains were mixed through soil to a final concentration of 10^6 cfu/g soil for single treatments and $2 * 10^6$ cfu/g soil for the combinations. The pathogen was mixed through soil to a final concentration of 10^4 conidia/g soil. After mixing bacterized soil, fusarium inoculated soil and sand radish seeds were sown. Disease was scored 21 days after sowing. Bars indicated with the same letter are not significantly different at $p \leq 0.05$, analysis of variance followed by Fisher's least-significant-difference test.

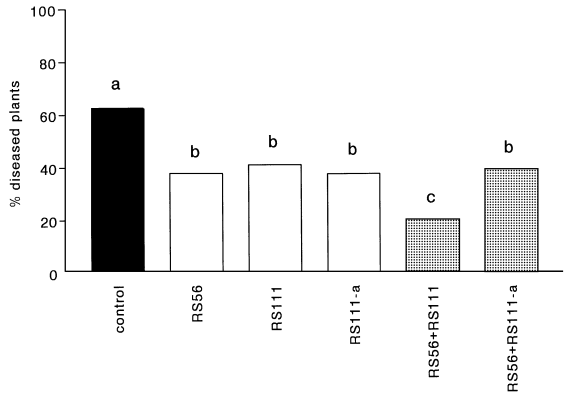

Figure 6. Suppression of fusarium wilt of radish in a pot bioassay. Before sowing, soil was bacterized with fluorescent *Pseudomonas* spp. strains RS56, RS111, RS111-a and combinations. Strains were mixed through soil to a final concentration of 10^6 cfu/g soil for single treatments and $2 * 10^6$ cfu/g soil for the combinations. The pathogen was mixed through soil to a final concentration of 10^4 conidia/g soil. After mixing bacterized soil, fusarium inoculated soil and sand radish seeds were sown. Disease was scored 21 days after sowing. Bars indicated with the same letter are not significantly different at $p \leq 0.05$, analysis of variance followed by Fisher's least-significant-difference test.

inoculants. However, this effect did not occur in the combination of RS56 with RS111-a (Figure 6). Clearly, RS56 combined favourably with RS111, but not with RS111-a.

Discussion

Increasing the genetic diversity of biological control systems through the use of mixtures of microorganisms may result in treatments that persist longer in the rhizosphere and utilize a wider array of biocontrol mechanisms (e.g. induction of systemic resistance, production of antibiotics and competition for nutrients) under a broader range of environmental conditions (Pierson and Weller, 1994). However, introducing combinations of biocontrol microorganisms does not always result in a better and more consistent disease suppression, as was demonstrated by Dandurand and Knudsen (1993). Numerous biotic and abiotic factors are likely to contribute to this inconsistent performance of biocontrol microorganisms (Weller, 1988). Inadequate colonization of the rhizosphere, limited tolerance to changes in environmental conditions and fluctuating production or activity of antifungal metabolites are among the most important factors (Duffy et al., 1996;

Pierson and Weller, 1994). Antagonism between the indigenous microbial population and a biocontrol agent or between biocontrol agents applied in a mixture can also influence the performance of a biocontrol agent in the rhizosphere. For example, competition for limited carbon sources or iron in the soil can influence the outcome of root colonization (Kragelund and Nybroe, 1996; Raaijmakers et al., 1995b) and consequently disease suppression. It has been demonstrated that a positive relationship exists between population size of the biocontrol strain on roots and disease suppression (Bull et al., 1991; Johnson, 1994; Montesinos and Bonaterra, 1996; Raaijmakers et al., 1995a; Smith et al., 1997). The rhizosphere population density of the biocontrol agent has to reach a threshold level before suppression of disease occurs. It is possible that in combinations of biocontrol agents this threshold level is not reached by one or both agents, due to negative interactions between the agents, and enhanced disease suppression cannot be expected. Therefore, several authors have suggested that combinations of introduced biocontrol agents have to be compatible in order to establish a better and more consistent disease suppression (Baker, 1990; Janisiewicz and Bors, 1995; Janisiewicz, 1996; Raaijmakers et al., 1995b). This compatibility can be tested *in vitro*, but whether this has predictive value for interactions *in vivo* remains to be seen. It may be envisaged that treatment with *in vitro* incompatible combinations can result in enhanced disease suppression. For instance, the coinoculated strains may not influence each other *in vivo* e.g. by spatial separation on the roots. Or the production of the *in vitro* inhibiting secondary compounds might take place in the stationary phase and not play a role in early stages of colonization (Fukui *et al*., 1994; Duffy et al., 1996).

When *in vitro* antagonism (Table 2) is compared to *in vivo* disease suppression (Figure 2), it appears that the *in vitro* test has some predictive value for the disease suppression by combinations of pseudomonads. This especially accounts for *P. fluorescens* strain RS111, which is strongly inhibited *in vitro* by *P. putida* strain RE8. The percentage of diseased plants in the combination of RE8 with RS111 was intermediate between the single strain treatments. The disease-suppressive effect of RS111 was not reduced by En401. The latter strain hardly inhibited growth of RS111 *in vitro*. Since the combination of RS111 and RE8 resulted in a disease suppression significantly lower compared to RS111 alone, but significantly better compared to RE8 alone, it seemed that the disease suppressive activity of RS111 was diminished by the presence of RE8. It is possible that the growth inhibition of RS111 by RE8 occurring *in vitro* also occurs in the rhizosphere, resulting in reduced disease suppression. This hypothesis was further investigated using a mutant of RS111 (RS111-a) that was compatible with RE8 *in vitro*. In this case disease suppression by RE8, RS111 and the incompatible combination of RE8 with RS111 was similar. The compatible combination of RE8 and mutant RS111-a resulted in a better disease suppression as compared to the single strains (Figure 3). Thus, the better disease suppression by the combination of RE8 and RS111 is consistent with their *in vitro* compatibility. At present, the rhizosphere population densities of strains RE8, RS111, RS111-a and the combinations are being investigated.

In contrast to the combination of RE8 with RS111-a, combinations of strains RS13 or RS158 with RS111-a, that are also compatible *in vitro* (Table 3), did not result in a better disease suppression (Figures 4 and 5). An explanation for these results can be that both strains in the combination suppress disease by the same mechanism, and thus better disease suppression by the compatible combinations of RS13 or RS158 with RS111-a is not to be expected. In contrast, although strains RS56 and RS111 are incompatible *in vitro*, combining these strains resulted in a better disease suppression as compared to the single strains and to the compatible combination of RS56 and RS111-a (Table 3, Figure 6). Such lack of correlation between *in vitro* and *in vivo* antagonism was also demonstrated by Pierson and Weller (1994). They suggested that incompatibility results in earlier and greater competition, and therefore in earlier and more consistent expression of traits involved in competition and disease control, particularly antibiotic production. Also for the combination WCS358+RE8 disease suppression was better as compared to the single strains, despite the slight *in vitro* inhibition between these strains (Table 2, Figure 2). Hence, enhanced disease suppression by combinations of biocontrol agents may rely on *in vitro* compatibility, but this does certainly not apply to all combinations. Further characterization of mutant RS111-a, insensitive to growth inhibition by other strains, can increase our knowledge of interactions between biocontrol strains. These interactions can influence (biocontrol) activity as well as population densities of strains. To examine how the strains influence each other in the rhizosphere, the population dynamics of the strains RE8, RS56, RS111, RS111-a and their combinations will be investigated.

Another condition to achieve a better disease suppression by a combination of biocontrol agents, is that these agents should suppress disease by different disease-suppressive mechanisms. This was observed for suppression of *Phytophthora cinnamoni* causing root rot in *Banksia grandis*. Combining a cellulase-producing *Micromonospora carbonacea* and an antibiotic-producing *Streptomyces violascens* resulted in an enhanced disease suppression (El-Tarabily et al., 1996). Suppression of fusarium wilt of carnation was enhanced by combining non-pathogenic *Fusarium oxysporum* Fo47, which acts through competition for carbohydrates, with *P. putida* strain WCS358 which suppresses disease by iron competition (Lemanceau et al., 1992, 1993). The enhanced disease suppression by the combination of strains WCS358 and RE8, the combination of RE8 and RS111-a, and the combination of RS56 and RS111 could be the result of a combination of different disease-suppressive mechanisms. Strain WCS358 suppresses fusarium wilt of radish by effectively competing for iron (Raaijmakers et al., 1995a). The disease-suppressive mechanisms of the other strains are being investigated.

In conclusion, these results indicate that specific interactions between biocontrol strains can influence disease suppression by the combination of these strains. It is necessary, therefore, to further investigate microbial interactions that enhance or detract from biocontrol (Handelsman, 1996) in order to understand and predict the performance of (mixtures of) biocontrol agents.

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