In vitro compatibility between fluorescent *Pseudomonas* spp. strains can increase effectiveness of Fusarium wilt control by combinations of these strains

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Abstract

In vitro interactions between Fusarium wilt suppressing *Pseudomonas* spp. strains RE8 and RS111 were investigated. Growth of strain RS111 on SSM agar medium was strongly inhibited by strain RE8, whereas RE8 was not inhibited by RS111. From the inhibition zone, produced by RE8, a mutant of strain RS111 (RS111-a) was isolated, that was no longer inhibited by RE8. When applied together in soil, the incompatible combination of RE8 plus RS111 did not result in a better suppression of Fusarium wilt of radish as compared to the single strains. However, the compatible combination RE8 plus RS111-a did result in a better disease suppression as compared to the single strains and to the incompatible combination. Thus, better performance in biocontrol by the combination of strains RE8 and RS111 appears to be dependent on in vitro compatibility between the strains.

Introduction

Wilt diseases caused by *Fusarium oxysporum* can lead to significant yield losses of horticultural and agricultural crops. Possibilities to manage Fusarium wilt, such as with fungicides, are limited. Therefore, other strategies to control this disease, including biological control, are being developed. Microorganisms, and especially fluorescent pseudomonads and non-pathogenic strains of *F. oxysporum* isolated from Fusarium wilt suppressive soils, have the ability to reduce Fusarium wilt. Mechanisms demonstrated to be involved in suppression of this disease include (i) competition for substrate, (ii) siderophore-mediated competition for iron, and (iii) induction of systemic resistance. It is postulated that in disease-suppressive soils a concerted action of several disease-suppressing microorganisms and mechanisms is responsible for the highly consistent disease suppressiveness (Alabouvette 1986, Lemanceau and Alabouvette 1991, Schippers 1992). Application of a mixture of different biocontrol agents may more closely mimic the natural situation and be a more viable control strategy (Duffy et al. 1996). Several authors have shown that combining disease-suppressive microorganisms results in improved biocontrol (Duffy et al. 1996, Leeman et al. 1996, Lemanceau et al. 1992, Pierson and Weller 1994).

It has been postulated that an important prerequisite for successful co-inoculation of strains is the compatibility of the co-inoculated microorganisms (Baker 1990, Li and Alexander 1988, Raaijmakers et al. 1995). Thus, incompatibility of the co-inoculants may explain the reports of combinations of biological control agents that do not result in improved suppression of disease as compared to the separate inoculants (Dansdorand and Knudsen 1993, Hubbard et al. 1983, Sneh et al. 1984).

The objective of this study was to determine whether interactions between *Pseudomonas* spp. strains influence disease suppression by combinations of these strains. Interactions
between disease-suppressive *Pseudomonas* strains RE8 and RS111 were first studied in vitro. Subsequently, suppression of Fusarium wilt of radish by the single strains and combinations of strains was investigated to determine in how far interactions in vitro are predictive of disease suppression by combinations of these *Pseudomonas* strains in vivo.

**Materials and Methods**

*Pseudomonas putida* strain RE8 was isolated from radish root tissue. *Pseudomonas fluorescens* strain RS111 was isolated the rhizosphere of tomato (Van Peer et al. 1990). RS111-a, a mutant of RS111, was isolated from the inhibition zone surrounding a colony of strain RE8. This mutant was not inhibited in vitro by strain RE8. The pathogen used was *F. oxysporum* Schlecht, *fascitrum* Kendrick, & Snyder, strain WCS600 (Leeman et al. 1996). Compatibility between *P. fluorescens* strains RS111 or RS111-a and *P. putida* strain RE8 was tested in vitro on standard succinate medium (SSM) agar plates (Meyer et al. 1978) without or with FeCl₃ (200 mM). Test strains were spot-inoculated. After 48 h of incubation at 27°C a suspension of the target strain (10⁷ CFU/ml) was atomized over the spot-inoculated plates. Zones of growth inhibition of the overlay strains around the spot-inoculated strains were scored after an additional incubation period of 24 h at 27°C.

To study the relation between in vitro compatibility and disease suppression, single strains and their combinations were tested for suppression of Fusarium wilt of radish in a potting mix composed of potting soil and river sand. The pathogen was cultured in a shaking culture of 2% malt extract (Difco, Detroit, MI) at 22°C for 14 days. Inoculum of *F. oxysporum* was produced by mixing microconidia into a non-autoclaved potting mix to give a final concentration of 3.75 x 10³ microconidia/g mix. The infested potting mix was incubated for 3 to 5 days at 20°C. Bacteria were grown for 24 h at 27°C on King's medium B (KB) agar plates (King et al. 1954) and scraped into water. Bacterial suspensions were mixed into twice autoclaved potting mix to give a approximately 7 x 10⁶ CFU/g mix. For the bioassay, potting mix infested with *Fusarium* and bacteria were added to noninoculated mix (soil but not sand was autoclaved) to give 10⁸ *Fusarium* microconidia/g and 10⁶ CFU bacteria/ml (or 2 x 10⁸ CFU/g for the bacterial combinations. Treatments consisted of nine pots filled with approximately 750 g potting mix. Ten radish seeds (*Raphanus sativus* L.; cultivar Saxa 2*Nova*, S&G Seeds B.V. Enkhuizen) were planted per pot. Plants were grown in the greenhouse at 20°C with a 16 h photoperiod. After approximately 21 days, the percentage of diseased plants was scored (Leeman et al. 1996). Results of two bioassays were pooled after establishing that there was no significant interaction (*P* = 0.05) between experiments and treatments, and that variances were homogeneous. The pooled data were analyzed for significance with ANOVA followed by Fisher's LSD test (*P* = 0.05) using SAS software (SAS Institute, Cary, NC).

**Results**

*Pseudomonas fluorescens* strain RS111 was strongly inhibited in vitro by strain RE8 (Table 1) on SSM agar plates without or with FeCl₃, whereas, strain RE8 was not inhibited by RS111 (results not shown). Mutant RS111-a was less sensitive to inhibition by RE8 on plates without Fe and was not inhibited on plates with Fe (Table 1). Thus, an incompatible (RE8 plus RS111) and a compatible (RE8 plus RS111-a) combination of *Pseudomonas* spp. strains was established. Disease suppression 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 (Table 2).

Table 1. In vitro antagonism of *Pseudomonas putida* strain RE8 against *P. fluorescens* strain RS111 and mutant RS111-a on SSM agar plates without or with FeCl₃ (200 mM).

<table>
<thead>
<tr>
<th>Overlay strain</th>
<th>SSM</th>
<th>SSM + Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS111</td>
<td>+5.2</td>
<td>+2.2</td>
</tr>
<tr>
<td>RS111-a</td>
<td>±3.3</td>
<td>-</td>
</tr>
</tbody>
</table>

* '−' indicates no growth inhibition of the overlay strain around the spot-inoculated colony. ‘±’ indicates slight growth inhibition, ‘+’ indicates strong growth inhibition. Values represent the diameter of inhibition zone/colony diameter of RE8.

Table 2. Effect of bacteria used alone and in combination on Fusarium wilt of radish.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Diseased plants⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>62.2 a</td>
</tr>
<tr>
<td>RE8</td>
<td>38.2 b</td>
</tr>
<tr>
<td>RS111</td>
<td>40.9 b</td>
</tr>
<tr>
<td>RS111-1</td>
<td>37.4 b</td>
</tr>
<tr>
<td>RE8 plus RS111</td>
<td>39.4 b</td>
</tr>
<tr>
<td>RE8 plus RS111-a</td>
<td>28.3 c</td>
</tr>
</tbody>
</table>

⁷ Mix was bacterized with fluorescent *Pseudomonas* spp. strains RE8, RS111, RS111-a (10⁶ CFU/g potting mix) or combinations of RE8 with RS111 or RS111-a (10⁶ CFU/g potting mix each). The pathogen was mixed through the potting mix to a final concentration of 10⁴ microcomidial/g mix. Percentage of diseased plants was scored 21 days after sowing.

⁸ Means followed by the same letter are not significantly different (P ≤ 0.05) according to Fisher’s LSD test.

Discussion
The introduction of biocontrol microorganisms or combinations of these organisms does not always result in a significant and consistent disease suppression. Numerous biotic and abiotic factors are likely to contribute to this variable 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). Several authors have suggested that combinations of introduced biocontrol agents can act more reliably but strains have to be compatible in order to establish a better and more consistent disease suppression (Baker 1990, Janisiewicz 1996, Janisiewicz and Bors 1995, Raaijmakers et al. 1995).

To achieve a better disease suppression by the combination of *P. putida* strain RE8 and *P. fluorescens* strain RS111, in vitro compatibility seems to be a prerequisite. A probable
explanation for these observations is that interactions between the strains influence root colonization. When RS111 is mixed through soil together with RE8, RS111 is probably inhibited in growth and not able to sufficiently colonize the roots. Mutant RS111-a is not inhibited by RE8 and is apparently not impaired in the colonization of the roots in the presence of RE8. Hence, under these conditions, both RE8 and RS111-a can fully express their disease-suppressive properties resulting in an enhanced disease suppression. These results indicate that interactions between biocontrol strains can influence the disease suppression by the combination of these strains. Therefore, it is essential to investigate microbial interactions that enhance or detract from biocontrol (Handelsman and Stabb 1996) to understand and predict the performance of biocontrol agents and strain combinations.

Currently the population dynamics of the strains RE8, RS111, RS111-a and their combinations are under investigation using the immunofluorescence colony staining technique, as modified by Leeman et al. (1995). The disease-suppressive mechanisms of the strains are being elucidated. Disease suppression by combinations of RS111 or RS111-a with several other *Pseudomonas* spp. strains that exhibit similar in vitro interactions is currently being investigated.

**Acknowledgments**

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**Literature**


