BIOLOGICAL CONTROL OF FUSARIUM WILT OF RADISH BY COMBINATIONS OF FLUORESCENT PSEUDOMONAS SPP. STRAINS

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Abstract

In this study the hypothesis that compatibility between *Pseudomonas* spp. strains is important to improve biological control by combinations of the strains is tested. Four strains of fluorescent pseudomonads that have the ability to suppress fusarium wilt of radish were tested for their interactions *in vitro*. Some strains were compatible, that is they do not inhibit each other, whereas other combinations were not compatible *in vitro*. To study the relation between interactions *in vitro* and interactions *in vivo*, single strains and combinations of the strains were tested for suppression of fusarium wilt of radish. All bacterial treatments significantly reduced the disease. Treatment with *Pseudomonas fluorescens* strain RS 111 reduced the disease significantly better than the other strains. In combinations with *Pseudomonas fluorescens* strains RE 8 or WCS 358, this disease-suppressive effect of RS 111 was reduced. *In vitro*, strains RE 8 and WCS 358 strongly inhibited RS 111. The combination of RS 111 and *Pseudomonas fluorescens* strain En 401 resulted in the same disease suppression compared to that by RS 111 alone. *In vitro*, RS 111 was not inhibited by En 401. The reduced disease-suppressive effect of RS 111 in combination with WCS 358 or RE 8 might be due to reduced root colonization by RS 111 in the combinations. Combining RE 8 and WCS 358 resulted in a significantly better disease suppression compared to the single strains. However, *in vitro* growth of RE 8 was slightly inhibited by WCS 358. In this case effects of WCS 358 on population densities of RE 8 might be smaller compared to effects of either RE 8 or WCS 358 on RS 111. Thus, in *vitro* interactions between *Pseudomonas* strains to some extend can predict disease suppressive effects by combinations of strains. Population dynamic studies will be conducted to further test this hypothesis.

Introduction

Fusarium wilt diseases cause considerable damage to horticultural and agricultural crops. The fungal pathogen, *Fusarium oxysporum*, infects the roots and colonizes the vascular tissue, leading to wilting of the plant and finally death (Peterson and Pound, 1960). Methods to control the disease are not always reliable. Steam desinfection is expensive and the created microbial vacuum can sometimes lead to devastating disease development. Soil fumigation has negative effects on the environment and will probably be banned in the near future. Therefore other strategies to control the disease, like biological control, have to be developed.

Worldwide, several fusarium wilt-suppressive soils have been described (Alabouvette, 1986; Kloepper *et al.*, 1980; Scher and Baker, 1980). This suppressiveness is of microbial origin (Schippers et al., 1992; Weller, 1988). Especially fluorescent pseudomonads and non-pathogenic strains of *Fusarium oxysporum*, isolated from these soils, have the ability to reduce fusarium wilt. Mechanisms demonstrated to be involved in suppression of fusarium wilt are: competition for substrate, siderophore-mediated competition for iron, and induction of disease resistance (Leeman, 1995; Lemanceau et al, 1992, 1993). Inoculation of a conducive soil with a single strain of a biological control microorganism never reaches the level of suppression observed in naturally suppressive soils, and the positive effects are often inconsistent (Schippers, 1992; Weller, 1988). In

suppressive soils a concerted action of several disease-suppressing microorganisms and mechanisms is postulated to be responsible for the highly consistent disease suppressiveness (Alabouvette, 1986; Lemanceau and Alabouvette, 1991; Schippers, 1992). Therefore, applications of combinations of micro-organisms were tested for their disease controlling abilities. Indeed, in some cases combinations resulted in improved disease control (Lemanceau *et al.*, 1992, 1993; Leeman *et al.*, 1995; Park *et al.*, 1988; Pierson *et al.*, 1994). An important prerequisite for a successful co-inoculation of strains appears to be the compatibility of the co-inoculated microorganisms (Baker, 1990; Li and Alexander, 1988; Raaijmakers, 1994).

In the present study the relation between interactions *in vitro* and disease suppression *in vivo* was investigated for several combinations of strains of fluorescent pseudomonads.

Materials and methods

Microbial cultures and inocula

Pseudomonas putida WCS 358 and RE 8 were isolated from potato rhizosphere and radish root tissue , respectively (Geels *et al.*, 1983a, 1983b; P.A.H.M. Bakker and C. Remkes, unpublished). *Pseudomonas fluorescens* RS 111 and En 401 were isolated from, respectively, the rhizosphere and root tissue of tomato (Van Peer et al, 1990). These four strains significantly reduce fusarium wilt of radish in potting soil bioassays (P.A.H.M. Bakker, C. Remkes and L.C. van Loon, unpublished). The strains were maintained at -80°C in glycerol.

Bacteria were grown for 48 h at 27°C on King's medium B (KB) agar plates (King *et al.*, 1954), and suspensions were prepared in sterile 10 mM $MgSO_4$. The pathogen used was *Fusarium oxysporum* Schlecht f.sp. *raphani* Kendrick & Snyder. It was cultured in aerated 2% malt extract (DIFCO) at 22°C. After 7 days of growth at room temperature, cultures were filtered through glass wool to remove mycelial mats. The microconidia were harvested by centrifugation (8000 rpm, 20 min) and resuspended in 10 mM $MgSO_4$.

In vitro antagonism between Pseudomonas strains

The *Pseudomonas strains* were spot-inoculated on KB agar plates. To test growth inhibition by the spot-inoculated strain, a suspension of the target strain (10^7 cfu/ml) was atomized over the spotinoculated plates after 24 h of incubation at 27°C. Zones of growth inhibition around the spot-inoculated strains were scored after an additional incubation of 24 and 48 h at 27°C. The experiment was designed such that all possible combinations of the 4 strains were tested.

In vivo suppression of Fusarium wilt by (combinations of) pseudomonads

Disease suppression by the single strains and their combinations was tested in a potting soil bioassay (Raaijmakers, 1994; Leeman, 1995). The pathogen was mixed in a potting soil/sand mixture (non autoclaved) to a final concentration of 3.5×10^5 cfu/g mixture. This *Fusarium oxysporum*-infested soil was incubated for 3 days at 20°C. The bacteria were introduced in an autoclaved (2 x 20 min with a 24 h interval) potting soil/sand mixture to a final density of approximately 7×10^7 cfu/g mixture. For the potting soil bioassay fusarium-infested soil, bacterized soil, additional autoclaved soil and non-autoclaved river sand were mixed in a 1:5:9:22.5 ratio. Final densities of *Fusarium oxysporum* and bacteria in this mixture were 10^4 cfu/g and 10^7 cfu/g respectively. Per treatment 9 pots (11 cm high, 14 cm diameter) were filled with this mixture and ten radish seeds (*Raphanus sativus* L.; cultivar Saxa*Nova, S&G Seeds B.V. Enkhuizen) were sown. The plants were grown in

the greenhouse at 20 °C at a photoperiod of 16 hrs. After 19 days fusarium wilt symptoms were scored externally, as well as internally by making cross sections of the root and tuber and examining these for discoloration of the vascular tissues (Leeman, 1995; Raaijmakers, 1994).

Results

In vitro antagonism

The interactions between *Pseudomonas* spp. strains RE 8, WCS 358, RS 111 and En 401 on KB agar plates are summarized in table 1.

Sprayed / Spot	RE 8	WCS 358	RS 111	En 401
RE 8	-	-	++	-
WCS 358	+	-	++	-
RS 111	+	-	-	-
En 401	+	-	-	-

Table 1 . *In vitro* interactions between fluorescent *Pseudomonas spp.* strains RE 8, WCS 358, RS 111 and En 401 on KB agar plates. - ; no inhibition, + ; slight inhibition, ++ ; strong inhibition. The columns show inhibition of growth of the sprayed strains and the rows inhibition by the spot-inoculated strains.

Growth of strain RE 8 was slightly inhibited by WCS 358, RS 111 and En 401. RE 8 itself strongly inhibited growth of RS 111. WCS 358 was not inhibited by any of the other strains, whereas WCS 358 itself inhibited the growth of RE 8 slightly and that of RS 111 strongly. The growth of En 401 was not inhibited by the other strains and En 401 slightly inhibited growth of RE 8. RS 111 was strongly inhibited by strains RE 8 and WCS 358 whereas RS 111 itself slightly inhibited growth of RE 8.

Thus, the combinations of RS 111 and En 401, and WCS 358 and En 401 are considered to be compatible and the combinations of RS 111 and WCS 358 or RE 8, and of RE 8 and WCS 358 or En 401 are considered to be incompatible.

Suppression of fusarium wilt

All bacterial treatments, including the combinations, resulted in a significantly lower percentage of diseased plants compared to the non-bacterized control treatment. Strain RS 111 reduced disease significantly better than any of the other strains. The combination of the strains RS 111 and En 401 did not result in a better disease suppression than the single strain RS 111. The combinations of RS 111 with RE 8 or with WCS 358 resulted in an intermediate disease suppression compared to the disease suppression by each of these strains on their own. The combinations of En 401 with RE 8 or WCS 358 also did not result in a better disease suppression compared to En 401 alone. One combination, RE 8 and WCS 358, did result in significantly higher disease suppression compared to the disease suppression by the single strains (table 2).

Treatment	Percentage diseased plants
Control	87.8 A[1]
WCS 358	51.1 BC
RE 8	55.1 B
RS 111	27.8 EF
En 401	43.5 CD
WCS 358 + RE 8	37.8 DE
WCS 358 + RS 111	34.9 DE
WCS 358 + En 401	38.8 D
RE 8 + RS 111	39.1 D
RE 8 + En 401	41.8 CD
RS 111 + En 401	24.6 F

Table 2 : Percentage of fusarium wilted plants in a potting soil bioassay. Treatments consist out of bacterization of soil with fluorescent *Pseudomonas* spp. strains WCS 358, RE 8, RS 111 and En 401 and their combinations. The strains were mixed through soil as inocula in soil to a final concentration of 10^7 cfu/g soil (in the single and combination treatments). The pathogen was mixed through soil as inoculum in soil to a final concentration of 10^4 cfu/g soil. Plants were score 18 days after sowing. [1] Means followed by the same letter are not significantly different at $P \leq 0.05$, analysis of variance followed by Fisher's least-significant-difference test.

Discussion

When *in vitro* antagonism is compared to the results of *in vivo* disease suppression, it appears that the *in vitro* test has some predictive value for the disease suppression by combinations of pseudomonads. In these experiments this especially accounts for RS 111, which is strongly inhibited *in vitro* by RE 8 and by WCS 358. The percentage of diseased plants in the combination RE 8 + RS 111 was significantly intermediate compared to the single strain treatments. In the combination RS 111 + WCS 358 the percentage of diseased plants was also intermediate compared to the single strain treatments. The disease-suppressive effect of RS 111 was not reduced by En 401. The latter strain also did not inhibit growth of RS 111 *in vitro*. However, the combination of RE 8 with WCS 358 did give a significant reduction of the percentage diseased plants compared to the single treatments although RE 8 was *in vitro* slightly inhibited by WCS 358. It is possible that WCS 358 has a different effect on population densities of strains RS 111 and RE 8. This will be investigated by studying the population dynamics of the different (combinations of) strains.

In the combination of RE 8 and WCS 358 the reduction of the percentage of diseased plants may be due to synergistic effects resulting from different disease-suppressive actions. It has been demonstrated that WCS 358 suppresses disease by competition for iron (Raaijmakers *et al*, 1995). For RE 8 the disease-suppressive mechanism(s) are under investigation.

It is possible that non-compatibility (like in the combination of RE 8 and WCS 358) results in earlier and greater competition among introduced bacteria in the rhizosphere and, therefore, earlier and more consistent expression of traits involved in competition and disease control (Pierson and Weller, 1994). However, this does not seem to apply to the combinations of RE 8 and WCS 358 with RS 111. Unraveling the mechanisms involved in disease suppression by and non-compatibility of combinations, will offer us tools to improve biological control of soil-borne diseases.

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