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Implication of systemic induced resistance in the suppression of fusarium wilt of tomato by *Pseudomonas fluorescens* WCS417r and by nonpathogenic *Fusarium oxysporum* Fo47*

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Abstract

Fluorescent pseudomonads and nonpathogenic *Fusarium oxysporum* have been shown to suppress fusarium wilts. This suppression has been related to both microbial antagonism and induced resistance.

The aim of the present study was to assess the relative importance of systemic induced resistance in the suppression of fusarium wilt of tomato in commercial-like conditions by a reference strain of each type of microorganism (*P. fluorescens* WCS417r and nonpathogenic *F. oxysporum* Fo47). The spatial separation of the pathogen and the biocontrol strains excluded any possible microbial antagonism and implicated the involvement of the systemic induced resistance; whereas the absence of any separation between these microorganisms allowed the expression of both mechanisms. Since systemic induced resistance has often been associated with the synthesis of PR-proteins, their accumulation in tomato plants inoculated with WCS417r or with Fo47 was determined.

The analysis of the results indicates that the suppression of fusarium wilt by *P. fluorescens* WCS417r was ascribed to systemic induced resistance without any detection of the PR-proteins tested (PR-1 and chitinases). In contrast, the suppression achieved by nonpathogenic *F. oxysporum* Fo47 appeared to be mainly ascribed to microbial antagonism but also to a lesser extent to systemic induced resistance. This induced resistance could be related to the accumulation of PR-1 and chitinases.

The possible relationship between the ability of Fo47 to suppress fusarium wilt more efficiently than WCS417r and its ability to show both mechanisms is discussed.

Introduction

Natural suppressiveness to fusarium wilts has been ascribed to two types of microorganisms: fluorescent pseudomonads and nonpathogenic *Fusarium oxysporum* (Alabouvette and Lemanceau, 1996). Since then, different studies have underlined their possible use to control fusarium wilts (Alabouvette et al., 1993). However, biological control is often inconsistent (Weller, 1988; Schippers, 1992). In order to overcome this limitation, a better knowledge of their modes of action is required.

Microbial suppression of fusarium wilts results from microbial antagonism during the saprophytic growth of the pathogen and from induced resistance of the host-plant during the parasitic growth of the pathogen (Lemanceau and Alabouvette, 1993). Although many studies have investigated the role of each mode of action, to our knowledge none of them has addressed their relative importance. Furthermore, since most experiments have been performed on young plants, conclusions made in such experimental conditions may not be valid in older plants.

The aim of the present work was to assess, in tomato crops, the implication of systemic induced resistance in the suppression of fusarium wilt by a reference strain

*Dedicated to the memory of Ben Duijff.

of fluorescent pseudomonad (*P. fluorescens* WCS417r, Lamers et al., 1988) and of nonpathogenic *F. oxysporum* (Fo47, Alabouvette et al., 1987). Since systemic induction of resistance has often been associated with the accumulation of PR-proteins *in planta* (Van Loon, 1997), the induction of their synthesis as a consequence of the tomato inoculation with either WCS417r or Fo47 was also evaluated.

Materials and methods

Microorganisms and culture conditions

Nonpathogenic *Fusarium oxysporum* Fo47 was previously isolated from the Châteaurenard (France) soil that naturally suppresses fusarium wilts; the efficacy of Fo47 in reducing the severity of fusarium wilts has been demonstrated several times (Alabouvette et al., 1993). Fol32 is a virulent strain of *Fusarium oxysporum* f. sp. *lycopersici* race 2. *F. oxysporum* Fo47 and Fol32 are single-spore isolates; they are cryopreserved by freezing conidial suspensions at $-80\,^{\circ}$C in 50% glycerol. *F. oxysporum* strains were grown in malt extract liquid medium ($10\,g\,l^{-1}$) at 23 °C on a rotative shaker (150 rpm). After 7 days of growth, cultures were filtered through glass wool to remove mycelial mats. Microconidia left in the filtrate were pelleted by centrifugation ($5000 \times g$, 20 min) and rinsed twice in 0.01 M $MgSO_4$. The conidial densities of the suspensions were determined by direct observation on a haemocytometer and adjusted by dilution.

Pseudomonas fluorescens WCS417r was kindly provided by Prof. LC Van Loon and Dr. PAHM Bakker (University of Utrecht, The Netherlands). This bacterial strain is a spontaneous mutant resistant to rifampicin of the strain WCS417 isolated from wheat rhizosphere (Lamers et al., 1988). Its ability to suppress fusarium wilts has previously been demonstrated (Van Peer et al., 1991; Duijff et al., 1995; Leeman et al., 1995). Cultures of bacterial cells, grown in Luria broth medium (Miller, 1972), were stored at $-80\,^{\circ}$C in 50% glycerol. Bacterial inoculants were produced in King's B medium agar plates (King et al., 1954) at 25 °C for 48 h. Bacteria were scraped from the medium and suspended in 0.1 M $MgSO_4$, pelleted by centrifugation ($5000 \times g$, 20 min) and rinsed twice in 0.01 M $MgSO_4$. The bacterial densities of the suspensions were determined by direct observation on a haemocytometer and adjusted by dilution.

Host-plant and growing conditions

Seeds of tomato (*Lycopersicon esculentum* Mill.) cultivar Monalbo (susceptible to all races of *F. oxysporum* f. sp. *lycopersici*) were sterilized in a NaClO solution (1.5%, v/v) for 20 min, washed 3 times with sterile distilled water and placed on a sterile filter at 25 °C for 72 h to germinate. Depending on the experiments, the pregerminated seeds were either sown in 9 ml rock wool plugs (Rockwool/grodan BV, The Netherlands) (bioassays for disease suppression) or in glass flasks, containing 300 ml of sterile rock wool flocks, sealed with screw caps and autoclaved (1.5 l; 3 seeds per flask) (bioassays for protein extractions). Rock wool, a growing substrate used for soilless culture, is made by melting rock (most commonly a form of basalt) at a temperature of 1600 °C and spinning into fibres (Vaughan, 1990). According to the experiments, the seedlings were either planted in large cubes (365 or 500 ml), to enable their growth for a long period in the biocontrol experiments, or not for the plants used for the protein extractions.

Plants were grown either in a glasshouse (bioassays for disease suppression) or in a sterile cabinet (bioassays for protein extractions) with a photoperiod of 16 h at 25 °C during the light period and at 22 °C during the dark period. Plants were watered with a standard nutrient solution for tomato (TNS) resulting from a 500-fold dilution of a commercial nutrient stock solution ('Hydrokani AO', Hydro Agri, France) to which $Ca(NO_3)_2$ (0.65 mM) and FeEDDHA (10 μM) (Sequestrene 'Fe100SG', CIBA-GEIGY, Switzerland) were added. All through the plant growth, the pH and the electrical conductivity of the TNS were kept in the rock wool at the following values 5.7 ± 0.2 and $3.3 \pm 0.5\,mS\,cm^{-2}$, respectively. The TNS was filter sterilized prior watering the plants grown in gnotobiotic conditions for the protein extractions.

Bioassays for disease suppression and induced resistance

After 3 weeks growth, half of the seedlings were split in two parts with a scalpel from the hypocotyl down to the root system. Each half of the root systems was planted in separate 365 ml rock wool cubes. This so-called split root system, performed in order to physically separate the antagonist and the pathogen, has been previously described (Liu et al., 1995; Olivain et al., 1995; Fuchs et al., 1997). The non-split seedlings were planted in

500 ml rock wool cubes. Two days later, one side of each split plant was watered with 20 ml of either a bacterial suspension of WCS417r, or of a conidial suspension of Fo47 or of 0.01 M $MgSO_4$. In the same way, the non-split plants were watered with 27 ml of either a bacterial suspension of WCS417r, or of a conidial suspension of Fo47 or of 0.01 M $MgSO_4$. The final densities of WCS417r and of Fo47 were 2×10^7 cells and 1×10^6 conidia per ml of rock wool, respectively. Three days after the inoculation of the biocontrol strains, the non-treated side of the split plants and the non-split plants were watered with 20 and 27 ml, respectively, of a conidial suspension of Fol32 resulting in a final density of 1×10^4 conidia per ml rock wool.

The percentage of plants with fusarium wilt symptoms (disease incidence) was scored every week. The experiments were ended 11 weeks after inoculation with Fol32.

In order to exclude any direct contact between the antagonists and the pathogen in the split plants, the absence of WCS417r and of Fo47 on the root side infested with the pathogen, and in the stem was checked during microbial analyses performed at the end of the experiment as described below.

Samples of root pieces (0.3 g) were vigorously shaken in glass tubes containing 5 ml of 0.01 M $MgSO_4$ and 1 g of glass beads (0.18 mm diameter) for 30 s. The presence on the rhizoplane of the bacterial strain WCS417r was evaluated after plating (Spiral System, Interscience, France) the rhizoplane suspensions on modified King's B agar medium (Geels and Schippers, 1983) supplemented with rifampicin ($150\,mg\,l^{-1}$, KB^+rif). The presence of Fo47 on the rhizoplane was evaluated after plating 100 µl of the rhizoplane suspensions on Komada agar (Komada, 1975). Three Petri dishes were plated per suspension-dilution.

The stems were surface-disinfected (flamed after dipping into a 95% ethanol solution) and cut into sections at 1, 15 and 25 cm above the split of the stem. These sections were placed on KB^+rif and Komada agar plates. After incubation for 72 h at 27 °C, the plates were checked for bacterial and fungal growth.

The absence of any contamination by WCS417r and by Fo47 was assessed after 48 and 72 h of incubation at 27 °C, respectively. The colonies of *F. oxysporum* strain Fo47 were discriminated from Fol32 ones on the basis of their different morphology.

Protein extraction and assays

Twenty-four-day-old plants, grown in gnotobiotic conditions, were watered with either 20 ml of a bacterial suspension of WCS417r, or a conidial suspension of Fo47 or of 0.01 M $MgSO_4$. The final densities of WCS417r and of Fo47 were 2×10^7 cells and 1×10^6 conidia per ml of rock wool, respectively. Six days after the microbial inoculation, plants were harvested for protein extraction. Roots, stems and leaves were collected, blotter-dried and weighed. The plant materials were ground with a pestle in an ice-chilled mortar in liquid nitrogen. The resulting powder was suspended in the following extraction buffer (pH 2.8) (1 ml per g of fresh tissue): Na_2HPO_4 (32 mM), citrate (84 mM), ascorbic acid ($1\,g\,l^{-1}$) containing 1% mercaptoethanol. The homogenates were centrifuged ($12000 \times g$, 30 min) at 4 °C and the supernatant fractions were kept frozen at −20 °C until used for PR-protein quantification. In order to check the efficacy of the extraction procedure, the protein concentration of the supernatants was determined according to Bradford (1976) by using the Bio-Rad protein assay (Bio-Rad laboratories, Richmond, CA, USA) (data not shown). The amount of chitinase and PR-1 proteins was quantified by ACP-ELISA according to the procedure described by Eparvier and Alabouvette (1994). Antisera against tobacco PR-1 (Van Loon et al., 1987) and against a 26 kD chitinase (Joosten and De Witt, 1988; Benhamou et al., 1990) were used as heterologous and homologous antisera for detection of PR-1 and chitinase homologs in tomato, respectively. The antisera were kindly provided by Prof. PJGM De Witt (Wageningen Agricultural University, The Netherlands). One hundred microliters of protein extracts were used for coating. Absorbance values were recorded at 405 nm one hour after substrate addition. Each sample, corresponding to a replicate of each experimental treatment, was ELISA assayed three times.

Experimental design and statistical analysis

The experimental design for scoring disease was a randomized complete block with six replications of six plants per treatment. The percentage of diseased plants was submitted to angular transformation before statistical analysis. Data on PR-protein quantification resulted from ELISA assays made on 6 replicates of 3 pooled plants per experimental treatment. Transformed values of disease incidence and non-transformed values of absorbance were analyzed by analysis of variance and Fisher's least significant test (Sokal and Rohlf, 1981).

Microbial enumerations were performed on six replicates of two plants per treatment.

Both types of experiment (disease suppression and PR quantification) were repeated at least twice with similar results. Results shown in Tables 1 and 2, and in Figure 1 were obtained from pooled data from two replicates of each type of experiment.

Results

Suppression of fusarium wilt and systemic induced resistance

The effect of the biocontrol agents WCS417r and Fo47 on kinetics of disease incidence in non-split and in split plants are shown in Figure 1A and B, respectively. In non-split plants, the disease incidence in the infested control increased with time and reached a plateau 10 weeks after infestation. Both WCS417r and Fo47 delayed the manifestation of the disease and reduced the disease incidence. Reduction of disease incidence by WCS417r and Fo47 were found to be significant when analysed at the end of the experiment. However, the biocontrol achieved by Fo47 was significantly more effective than the one achieved by WCS417r (Figure 1A). In split plants, the apparition of the disease in the infested control was delayed compared to the corresponding control in non-split plants. At the end of the experiment, both antagonists reduced the disease incidence, however this reduction was only significant with WCS417r (Figure 1B).

Splitting the seedlings in two parts lead to a reduction of the disease incidence in the infested control (66.6%) compared to that of the non-split plants (91.7%). This difference was found to be statistically non-significant (data not shown). However, in order to compare the efficacy of the biocontrol strains in the two experimental conditions (non-split and split plants), the disease incidence was expressed as a percentage of the corresponding infested control (Table 1). When the antagonist and the pathogen were applied together (non-split plants), both WCS417r and Fo47 reduced significantly the disease incidence. However, the disease suppression was significantly more effective with Fo47 than with WCS417r. In contrast, when the antagonists and the pathogen were spatially separated (split plants), only WCS417r reduced significantly the disease incidence.

In the split plants, neither Fo47 nor WCS417r could be isolated from the side infested by the pathogen. Similarly, none of the antagonists could be isolated from the tomato stem tissue (data not shown). These results indicate that antagonists and pathogen remained spatially separated all through the experiment.

Table 1. Effect of *P. fluorescens* WCS417r and of *F. oxysporum* Fo47 on fusarium wilt incidence in non-split and split plants of tomato 77 days after infestation with *F. oxysporum* f. sp. *lycopersici* Fol32. Relative disease incidence is expressed as the percentage of the disease incidence of the corresponding infested control: 91.7% and 66.6% for the non-split and split plants, respectively

	Relative disease incidence	
	Non-split plants	Split plants
Infested control	100c	100b
WCS417r	69.8b	58.3a
Fo47	42.3a	78.5ab

Within a same column, means designated with the same letter are not significantly different ($P = 0.05$).

PR-proteins accumulation

Using indirect ACP-ELISA, the accumulation of PR-proteins was assessed in roots, stems and leaves 6 days after inoculation of the biocontrol strains.

Table 2. Quantification by ACP-ELISA of PR-proteins in tomato inoculated with *P. fluorescens* WCS417r or nonpathogenic *F. oxysporum* Fo47. PR-protein concentrations are given as absorbance values (405 nm) per ml of extraction buffer

	Chitinases			PR-1		
	Roots	Stems	Leaves	Roots	Stems	Leaves
Control	2.55a	2.38a	6.0a	1.08a	0.17a	0.40a
Fo47	8.56b	4.20a	14.2b	1.05a	1.25b	1.61b
WCS417r	1.13a	3.42a	6.7a	0.67a	0.50ab	0.56a

Within a same column, means designated with the same letter are not significantly different ($P = 0.05$).

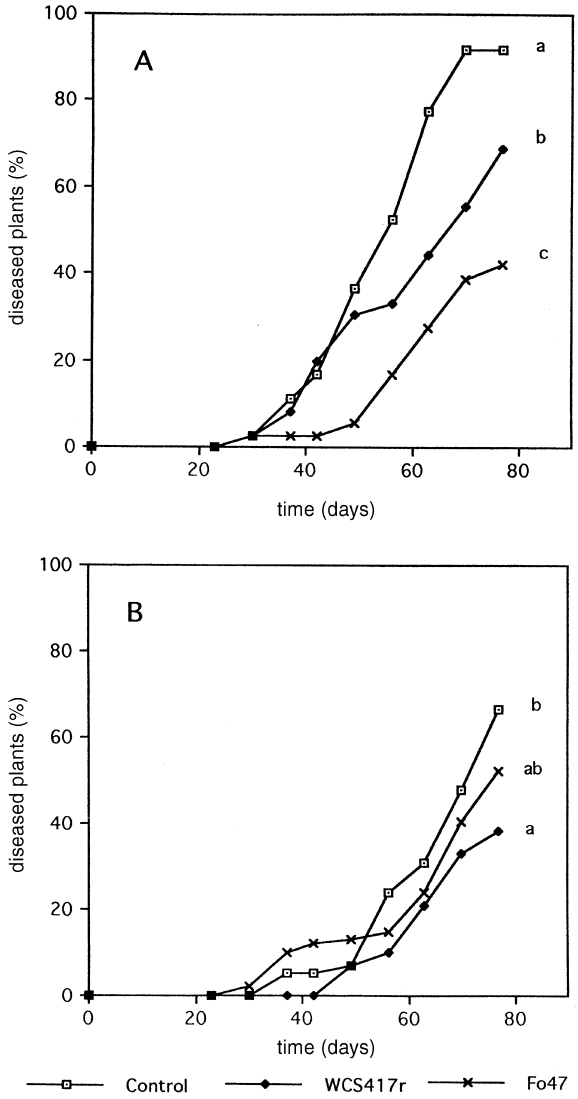

Figure 1. Effect of *P. fluorescens* WCS417r and of *F. oxysporum* Fo47 on the kinetics of the disease incidence of fusarium wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici* Fol32 in non-split (A) and in split plants (B). Means of disease incidence recorded on day 77 and designated with the same letter are not significantly different ($P = 0.05$).

In all the plant parts tested, inoculation with *P. fluorescens* WCS417r did not lead to any detectable accumulation of PR-proteins compared to the uninoculated control (Table 2).

In contrast, accumulation of proteins cross-reacting with the antiserum against tomato chitinases was observed in the roots and leaves of the plants inoculated with nonpathogenic *F. oxysporum* Fo47. In these plants, the quantity of chitinase homologs was much larger in leaves than in roots. Chitinases were found constitutively present in the leaves of the control plants. An accumulation of PR-1 proteins was also recorded in the stems and leaves of the plants inoculated with Fo47 compared to the control. Similar magnitudes of PR-1 accumulation were measured in both the stems and leaves of Fo47 inoculated plants.

Discussion

The aim of the present study was to explore the relative importance of systemic induced resistance in the suppression of fusarium wilt of tomato. Such an evaluation was made possible by comparing the decrease in disease incidence after inoculation of the biocontrol strains in non-split plants and in split ones. Splitting the root system in two parts lead to a non-significant reduction of the disease incidence in the control compared to the non-split plants. Such a reduction was previously recorded by Olivain et al. (1995) but not by Mandeel and Baker (1991).

In non-split plants, the disease suppression could be ascribed to both microbial antagonism and induced resistance in the host-plant. Cultivation of these plants was performed in condition close to the commercial ones and was carried out for a long period of time (11 weeks). In these conditions, the nonpathogenic *F. oxysporum* strain Fo47 was shown to suppress more effectively fusarium wilt of tomato than *P. fluorescens* strain WCS417r.

In split plants, the absence of any direct contact between the pathogen and the biocontrol strains prevented any microbial antagonism. The disease suppression recorded with split plants could then only be ascribed to a systemic induced resistance of the host-plant. Since the efficacy of the biocontrol achieved by *P. fluorescens* WCS417r was similar in split and non-split plants, the implication of microbial antagonism in the disease suppression appears to be limited compared to the systemic induced resistance. These results are in agreement with previous studies. Antagonism against pathogenic *F. oxysporum* by fluorescent pseudomonads has been mainly associated with siderophore mediated iron competition (Lemanceau and Alabouvette, 1993). Due to the low pH of the nutrient solution, the iron competition performed by *P. fluorescens* WCS417r in soilless culture was previously assumed to be reduced (Duijff et al., 1995). Systemic induced resistance by *P. fluorescens* WCS417r was already established in

three plant species: *Arabidopsis*, carnation and radish (Van Peer et al., 1991; Djuiff et al., 1995; Leeman et al., 1995; Hoffland et al., 1996; Pieterse et al., 1996). Our results describe the ability of *P. fluorescens* WCS417r to induce systemic resistance against fusarium wilt in a new plant species (tomato). The non-specificity of the host-plant in which *P. fluorescens* WCS417r is able to induce systemic resistance makes it different from the others bacterial strains which are usually only able to induce resistance in a specific plant species (Van Loon, 1997). The induction of resistance in tomato by *P. fluorescens* WCS417r could be associated with its ability to colonize the root tissue of this host-plant. Indeed, a mutant of this bacterial strain affected in the O-antigenic side chain of its outer membrane lipopolysaccharides showed both a reduced endophytic root colonization (Duijff et al., 1997) and a lower ability to induce systemic resistance (Van Peer et al., 1991; Leeman et al., 1995).

Nonpathogenic *F. oxysporum* Fo47 reduced the incidence of fusarium wilt in split plants although not significantly. Such a reduction confirms the ability of Fo47, previously described, to induce systemic resistance in tomato (Olivain et al., 1995; Fuchs et al., 1997). The ability of nonpathogenic *F. oxysporum* and of pathogenic *F. oxysporum* belonging to another *forma speciales* than the pathogen to induce resistance has been shown several times (Tamietti and Matta, 1991; Olivain et al., 1995; Fuchs et al., 1997). However, the efficacy of the induced resistance varies according to the fungal biocontrol strain and the strain Fo47 was shown to induce resistance less efficiently than another nonpathogenic *F. oxysporum* strain (Olivain et al., 1995). This relative low efficacy of the strain Fo47 to induce systemic resistance in tomato is in agreement with the results of the present study in which the disease suppression in split plants was not significant. In contrast, the nonpathogenic *F. oxysporum* Fo47 reduced significantly the disease incidence in non-split plants. The more effective biocontrol achieved by Fo47 in non-split plants than in split ones indicates that another mechanism (microbial antagonism) additional to systemic induced resistance is implicated. Competition for carbon has previously been demonstrated to be involved in the microbial antagonism performed by nonpathogenic *F. oxysporum* specially by Fo47 against pathogenic *F. oxysporum* (Couteaudier and Alabouvette, 1990; Lemanceau et al., 1993). In the present study, this mode of action is likely to contribute to the disease reduction by Fo47 and the combination of induced resistance and competition for carbon resulted in an efficient disease suppression by Fo47 in non-split plants.

The spatial separation between the biocontrol strains used to induce resistance and the challenging pathogen in the split root system led to the conclusion that the reduction of the disease incidence by the inducing microorganisms was plant mediated (Hoffland et al., 1996). Because of their potential antifungal activity, most of the research dealing with the plant biochemical responses upon induction have focused on the synthesis of pathogenesis-related proteins and classic systemic acquired resistance (SAR) has been found associated with their accumulation (Van Loon, 1997). Recently, it has been demonstrated that *P. fluorescens* strain WCS417r induced systemic resistance against fusarium wilt in radish and *Arabidopsis* without inducing PR-proteins accumulation in either the two plant species (Hoffland et al., 1995, 1996; Pieterse et al., 1996). The authors suggested that the protection achieved upon induction with this bacterial strain was the same as with classic SAR, but mediated via a partly different pathway. In our study, we demonstrated that PR proteins accumulation is not a prerequisite for the expression of induced systemic resistance by strain *P. fluorescens* WCS417r in tomato. In contrast, Fo47 was found to induce the synthesis of chitinase homologs in tomato roots and leaves. This result is in agreement with previous studies showing an increase in glycosidase activities in tomato upon inoculation with nonpathogenic strains of *F. oxysporum* (Matta, 1989; Fuchs et al., 1997). PR-1 homologs also accumulated in stems and leaves of Fo47-treated tomato plants. In our study, the ability of Fo47 and WCS417r to induce resistance of the host plant and to induce PR proteins accumulation was evaluated in two different types of experiment. To ascribe the accumulation of PR proteins to the presence of a specific microbial strain, experiments were conducted on young plants grown in gnotobiotic conditions, in growth chamber. Whereas, implication of induced resistance in disease suppression was evaluated on older plants, split and grown in greenhouse. These differences do not allow to draw any definitive conclusion on a possible relation between PR protein accumulation and induced resistance. Nevertheless, our data, showing that (i) Fo47 both induces resistance of the host plant and accumulation of PR proteins, (ii) WCS417r induces resistance of the host plant but does not induce any accumulation of PR proteins, suggest that Fo47 may act as an

inducer of resistance through a classic SAR-like mechanism whereas WCS417r may act through a different mechanism.

In conclusion, our results indicate that non-pathogenic *F. oxysporum* Fo47, which expresses both mechanisms systemic induction of resistance and microbial antagonism, suppresses more efficiently fusarium wilt of tomato in commercial-like conditions than *P. fluorescens* WCS417r which mainly induces systemic resistance. This conclusion is supported by previous data showing an increased efficacy and consistency of the biological control by combining modes of actions expressed by one or two biocontrol strains (Alabouvette and Lemanceau, 1998).

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