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Suppression of *Rhizoctonia* root-rot of tomato by *Glomus mossae* BEG12 and *Pseudomonas fluorescens* A6RI is associated with their effect on the pathogen growth and on the root morphogenesis

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Abstract

Rhizoctonia solani root-rot is a major soilborne disease causing growth and yield depression. The ability of *Glomus mosseae* BEG12 and *Pseudomonas fluorescens* A6RI to suppress this soilborne disease in tomato was assessed by comparing the shoot and root growth of plants infested with *R. solani* 1556 when protected or not by these beneficial strains. The epiphytic and parasitic growth of the pathogenic *R. solani* 1556 was compared in the presence and absence of the biocontrol agents by microscopical observations allowing the quantification of roots with hyphae appressed to epidermal cells (epiphytic growth) and of roots with intraradical infection (parasitic growth). The root architecture of the tomato plants under the different experimental conditions was further characterized by measuring total root length, mean root diameter, number of root tips and by calculating degree of root branching. *G. mosseae* BEG12 and *P. fluorescens* A6RI fully overcame the growth depression caused by *R. solani* 1556. This disease suppression was associated with a significant decrease of the epiphytic and parasitic growth of the pathogen together with an increase of root length and of the number of root tips of inoculated tomato plants. The combined effects of *G. mosseae* BEG12 and *P. fluorescens* A6RI on pathogen growth and on root morphogenesis are suggested to be involved in the efficient disease suppression.

Introduction

Rhizoctonia solani negatively affects the growth and health of many plant species. Populations belonging to this fungal species are known to cause damping-off and root-rot. Root-rot induces reduction of plant growth and may even lead to plant death, in any case significant yield losses in field conditions (Moulin et al., 1994; Sneh et al., 1996, Steinberg et al., 2004) and for re-planting disease in orchard (Mazzola, 1997) are associated with this major class of soilborne disease. Research has been performed over the last years to identify potential biocontrol agents to reduce severity of root-rot caused by *Rhizoctonia* and also by *Phytophthora* and *Pythium* (Moulin et al., 1996; Sneh et al., 1996; Paulitz and Belanger, 2001; Folman et al., 2003a, b).

Among potential biocontrol agents, specific attention has been given to fluorescent pseudomonads and AM fungi (Whipps, 1997; Pozo et al., 2002a; Sharma and Johri, 2002). Disease suppression of soilborne diseases by fluorescent pseudomonads and AM fungi has mostly been ascribed to reduction of pathogen saprophytic growth leading to decreased root infections

(microbial antagonism) (Cook et al., 1995; Vigo et al., 2000) and/or to reduction of the pathogen parasitic growth resulting from stimulation of host-plant defence reactions (Van Loon et al., 1998; Pozo et al., 2002b).

Beside microbial antagonism and elicitation of defence reactions of the host-plant, specific strains of AM fungi and fluorescent pseudomonads were recently shown to affect the root architecture of inoculated plants (Berta et al., 2002; Gamalero et al., 2002). Among them, the model strains *Glomus mosseae* BEG12 and *Pseudomonas fluorescens* A6RI were shown to increase root length and surface and also number of root tips of tomato plants; the expression of these beneficial effects being affected by the soil fertility (Gamalero et al., 2002). Although modifications of root architecture induced by microbial inoculants are likely to affect plant susceptibility to root-rot (Berta et al., 2002), little attention has been given to their real consequences on plant health.

The aim of the present study was first to assess the efficiency of the model strains *G. mosseae* BEG12 and *P. fluorescens* A6RI in suppressing root-rot of tomato due to *R. solani*, and second to determine the effect of BEG12 and A6RI on the epiphytic and parasitic growth of *R. solani* and on the root morphogenesis when infested or not.

Materials and methods

Microorganisms and microbial inoculants

Rhizoctonia solani Kühn strain 1556 isolated from tomato (kindly provided by S. L. Woo, University of Naples "Federico II" culture collection, Italy) was grown on potato dextrose broth at 150 rpm, 25 °C in light for 5 days, and the mycelium was vacuum harvested, weighed, and homogenized in distilled water.

Mycorrhizal inoculum, obtained from Biorize (Dijon, France), was prepared by mixing leek roots, infected with *Glomus mosseae* (Nicol and Gerd) Gerdemann and Trappe strain BEG 12, and quartz sand.

A rifampicin resistant derivative (A6RI) (Gamalero et al., 2002) of *Pseudomonas fluorescens* A6 (Lemanceau and Samson, 1983) was used. Bacteria cultures, grown in Luria-Bertani broth medium (Miller, 1972) supplemented with rifampicin (100 μg ml^{-1}), were stored at −80 °C in 50% glycerol. *P. fluorescens* A6RI inoculant was produced on KB agar plates (King et al., 1954) at 28 °C for 48 h. Bacteria were scraped from the medium and suspended in 0.1 M $MgSO_4 \cdot 7H20$, pelletted by centrifugation (5000 rpm, 20 min), washed twice and suspended in 0.1 M $MgSO_4 \cdot 7H_2O$. The bacterial density of the suspension was determined using a calibration curve assessed by turbidity ($\lambda = 600$ nm) and adjusted to 10^7 colony forming units per ml (cfu ml^{-1}).

Treatments and plant growth conditions

A mixture of 0.3–0.7 mm coarse grade quartz sand was sterilized at 200 °C for 2 h and distributed in 600 ml containers. Tomato seeds (*Lycopersicon esculentum* cv. Early Mech), kindly provided by Petoseed (Peto Italiana, Parma, Italy), were surface sterilized by gently shaking in a 5% NaClO solution for 3 min, and rinsed six times for 5 min and four times for 20 min in sterile demineralised water. The seeds were pre-germinated on moist sterile filter paper at 24 °C for 3 days. One pre-germinated seed was sown in each container.

Inoculation of *G. mosseae* BEG12 was performed by incorporating 10% (v/v) of the inoculum-quartz sand mix into the culture substrate. Bacterization was performed before sowing by dipping the germinated seeds for 20 min in a suspension (10^7 cfu ml^{-1}) of *P. fluorescens* A6RI. Infestation of the culture substrate with *R. solani* 1556 was obtained by spreading 1 g of pathogenic mycelium at the surface of each container which was covered with a 5 cm sand layer. Plants were harvested 7, 14, 21 and 28 days after sowing for analysis.

Five plants were cultivated per experimental condition, including uninoculated control plants, and per sampling date. Plants were grown in a chamber (16/8 h light/dark photoperiod, 24/20 °C light/dark thermoperiod, 150 μE m^{-2} s^{-1} light irradiance, 60% relative humidity) and watered to saturation with a modified Long Ashton nutrient solution containing 32 μM phosphate three times per week (Trotta et al., 1996).

Quantification of root infection by R. solani *1556, and of root colonization by* G. mosseae *BEG12 and* P. fluorescens *A6RI*

Whole root systems were aseptically cut. Sand loosely adhering to roots was removed by gently washing the roots with sterile water. The pathogenic fungus was revealed by microscopic observations on whole squashed roots stained with cotton blue. According to these observations, roots not colonized by the pathogenic fungus, roots with hyphae appressed to epidermal cells (epiphytic growth) and roots with intraradical infection (parasitic growth) were differentiated. And percentages of roots with epiphytic and parasitic colonisation by *R. solani* 1556 were expressed as: length of colonized roots/total root length × 100.

The degree of AM colonization was evaluated by determining the total frequency of mycorrhization (Trouvelot et al., 1986), after random sampling 30 pieces of root 1 cm long per experimental condition.

To measure bacterial densities on roots, roots were weighed, placed in 0.1 M $MgSO_4 \cdot 7H_2O$ buffer and vortexed for 15 min. Suspensions obtained were serially diluted and plated on solid KB supplemented with rifampicin (100 μg ml^{-1}). After incubation for 48 h at 25 °C, the number of cfu were determined and expressed per gram of fresh root weight.

Quantification of plant growth and root morphogenesis characterization

Parameters measured to quantify plant growth were fresh root and shoot weight. Variables used to characterize root architecture were total root length, mean root diameter, number of root tips and root branching degree, represented by root tip number divided by total root length. For this purpose, whole root systems were put in a transparent container and digitised by using a Desk Scan II scanner (Régent Instrument Inc., Quebec, Canada) connected to a Power Macintosh 4400/200 computer (Appel Computer Inc., Cupertino, CA, USA). Digitised root images were analyzed by MacRhizo V 3.9 software (Régent Instrument Inc., Quebec, Canada) and total root length, mean root diameter, number of root tips and root system branching were evaluated.

Statistical analysis

Since populations of bacteria approximate a log normal distribution (Loper et al., 1985), bacterial density values were logarithmically transformed before analysis. Non-transformed and transformed values were submitted to ANOVA and then to Fisher's least significant test ($P < 0.05$) using a Statview statistics package (SAS Institute Inc., Cary, NC, USA). Five plants were cultivated per experimental condition. All the experiments were performed three times, and the results shown represent one representative experiment.

Results

Effect of G. mosseae *BEG12 and* P. fluorescens *A6RI on the rate of infection of tomato roots by* R. solani *1556*

Many hyphae were first observed on epidermal cells on which they developed appressoria (Figure 1a), then they appeared to penetrate the root cortex in 21 day-old plants (Figure 1b), and finally invaded the root vessels in 28 day-old plants (Figures 1c, d). The percentage of roots colonized by *R. solani* 1556 increased with time (data not shown). So, only quantifications of roots colonized by *R. solani* 1556 in 28 day-old plants are reported. The percentage of roots with external hyphae appressed to epidermal cells recorded in the presence of *G. mosseae* BEG12 and *P. fluorescens* A6RI was decreased by 62.3% and 69.4%, respectively, compared to the infested control (Table 1). The percentage of roots showing intraradical root infections recorded in the presence of *G. mosseae* BEG12 and of *P. fluorescens* A6RI was even more sharply reduced (86.8%, 92.3%, respectively) (Table 1). The pathogenic *R. solani* 1556 only reached the central cylinder when inoculated alone (Figures 1c, d).

Effect of R. solani *1556 on the degree of mycorrhization of tomato roots by* G. mosseae *BEG12 and on the density of* P. fluorescens *A6RI*

The density of *P. fluorescens* A6RI in the rhizosphere and the total frequency of mycorrhization were significantly reduced in plants infested with *R. solani* 1556 compared to uninfested plants.

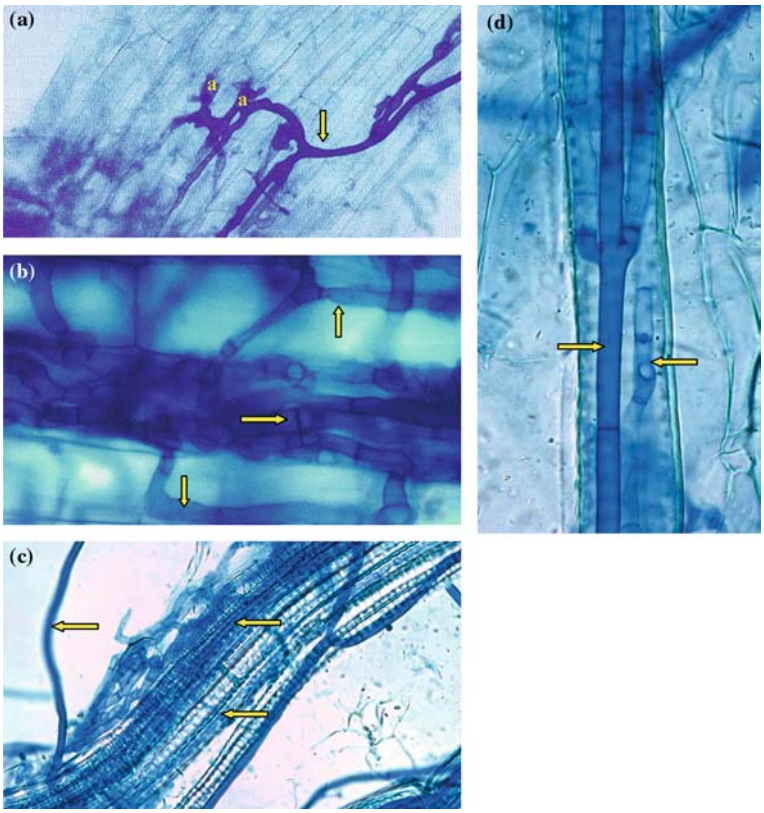

Figure 1. Micrographs of tomato roots infected with *Rhizoctonia solani* 1556, squashed and stained with cotton blue. (a) External hyphae and appressoria [a] on epidermal cells (236×), (b) Root cortex cells highly infected with fungal hyphae (591×), (c) Central cylinder of a root in which many fungal hyphae are running along the tracheids (300×), (d) A tracheid completely invaded by fungal hyphae (700×). Arrows indicate the pathogen hyphae.

Table 1. Effect of *Glomus mosseae* BEG12 and of *Pseudomonas fluorescens* A6RI on the percentage of roots showing appressed hyphae *R. solani* 1556 to epidermal cells, and intraradical infection by *Rhizoctonia solani* 1556 in 28 day-old tomato

	Roots showing external hyphae appressed to epidermal cells (%)	Roots showing intraradical infection (%)
1556	20.56 a	14.5 a
BEG12 + 1556	7.76 b	1.92 b
A6RI + 1556	6.30 b	1.12 b

These reductions were recorded only in 28 day-old plants for bacterial density, and in both 21 and 28 day-old plants for degree of mycorrhization. In 28 day-old plants, bacterial density (log cfu g^{-1}) significantly decreased from 5.34 in uninfested plants down to 4.50 in infested plants; and the total frequency of mycorrhization was significantly decreased from 25.60 down to 22.32.

Effect of G. mosseae *BEG12 and* P. fluorescens *A6RI on growth of tomato plants infested or not with* R. solani *1556*

R. solani 1556 reduced significantly shoot weight compared to the uninfested control (40.2%). The root weight was also reduced (37.2%) although not significantly (Figure 2a, b).

G. mosseae BEG12, but not *P. fluorescens* A6RI, promoted plant growth compared to the uninfested control, this promotion being significant only for the shoot weight. Neither BEG12 nor A6RI modified the fresh root/shoot weight ratio in uninfested plants (Figure 2c).

Both BEG12 and A6RI suppressed the deleterious effect induced by *R. solani* 1556. In the presence of *R. solani* 1556, *G. mosseae* BEG12 induced

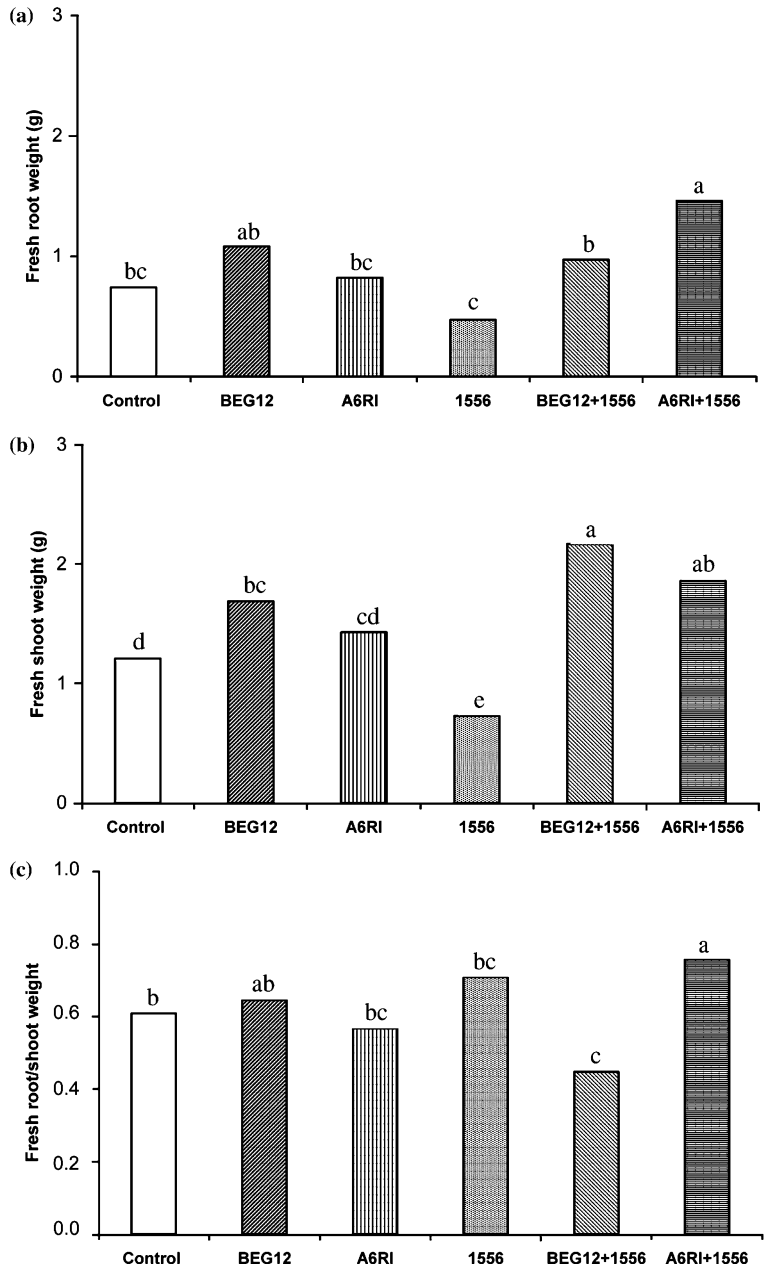

Figure 2. Fresh root weight (a), shoot weight (b), and fresh root/shoot weight ratio (c) of 28 day-old tomato infested with *Rhizoctonia solani* 1556 or not (control), and inoculated or not with either *Glomus mosseae* BEG12 or *Pseudomonas fluorescens* A6RI.

a significant increase of shoot weight compared to both infested and uninfested control (198%, 78.6%, respectively), but induced a significant increase of root weight (107.3%) only when compared to the infested control (Figure 2b, a). In the presence of the pathogen, *P. fluorescens* A6RI allowed a growth of root and shoot significantly higher than the infested control (213.7%, 155.7%, respectively), and also significantly higher than the uninfested control (97%, 53%, respectively) (Figure 2a, b). Therefore, the impact of the biocontrol agents on the fresh root/shoot weight ratio in the presence of the pathogenic fungus differed according to the beneficial strains, this ratio being

Table 2. Effect of *Glomus mosseae* BEG12 and of *Pseudomonas fluorescens* A6RI on root morphogenetic parameters (total root length, mean root diameter, number of root tips and root branching degree) in 28 day-old tomato infested with *Rhizoctonia solani* 1556 or not (control)

	Total root length (cm)	Mean root diameter (mm)	Number of root tips	Root branching degree
Control	517.69 ab	0.44 bc	616.60 c	1.19 c
BEG12	500.42 b	0.44 bc	917.60 b	1.84 ab
A6RI	502.97 ab	0.38 c	723.00 bc	1.35 c
1556	264.99 c	0.52 a	480.00 d	1.80 b
BEG12 + 1556	482.89 b	0.50 ab	915.00 b	1.88 ab
A6RI + 1556	664.70 a	0.43 bc	1376.60 a	2.26 a

significantly higher in plants inoculated with A6RI than with BEG12 (Figure 2c).

Effects of G. mosseae *BEG12 and* P. fluorescens *A6RI on root morphogenesis of tomato plant infested with* R. solani *1556*

Root morphogenesis was characterized by measuring total root length, mean root diameter, number of root tips, and by calculating root branching degree. The corresponding values, measured or calculated in 28 day-old plants, are shown in Table 2.

In the infested control, the total root length was reduced by 48.8% compared to the uninfested control, whereas the number of root tips was only reduced by 22.15%, in such way that the root branching was significantly increased compared to the uninfested control (51.3%). The mean root diameter of *R. solani* infested plants was significantly higher than that of all other treatments except the treatment BEG12 + 1556.

Compared to the uninfested control, *G. mosseae* BEG12 increased significantly the number of root tips but did not affect the total root length, and consequently root branching was significantly increased. *Pseudomonas fluorescens* A6RI did not affect significantly any of the root morphogenetic parameters compared to the uninfested control.

In the presence of *R. solani* 1556, *G. mosseae* BEG12 increased the number of root tips compared to both the infested and uninfested controls (90.6%, 48.4%, respectively), but only increased significantly total root length compared to the infested control and root branching degree compared to the uninfested control; *P. fluorescens* A6RI increased dramatically the number of root tips compared to both the infested and uninfested controls (186.6%, 55.2%, respectively), and restored the root length to a level not significantly different from the uninfested control. Therefore the bacterial strain induced a significantly higher degree of root branching compared to the infested and uninfested controls (Table 2).

Altogether these modifications lead to clear differences between the root systems of the experimental treatments as shown in Figure 3. The most reduced root system despite its high degree of branching was found in plants infested with *R. solani* 1556. The most extensive and branched root system was that of plant inoculated together with *P. fluorescens* A6RI and *R. solani* 1556.

Discussion

The two beneficial microorganisms *G. mosseae* BEG12 and *P. fluorescens* A6RI were tested for their ability to suppress root-rot of tomato caused by *R. solani* 1556. This pathogenic strain significantly reduced plant growth as measured by fresh root and shoot weight, and also affected root architecture. Plants infested with *R. solani* 1556 showed thicker roots in comparison to all the uninfested controls (uninoculated, A6RI, BEG12), in which root diameter did not differ. The largest root diameter of infested plants was not related to an increased size of whole plant which on the contrary was reduced. This result is therefore in disagreement with the general statement that plants regulate the size of their organs in relation to their total size (Barlow, 1994). The increased root diameter could rather be ascribed to a hormonal imbalance that would also account for the scarcity of short adventitious roots and the emergence of many short laterals, leading to a more

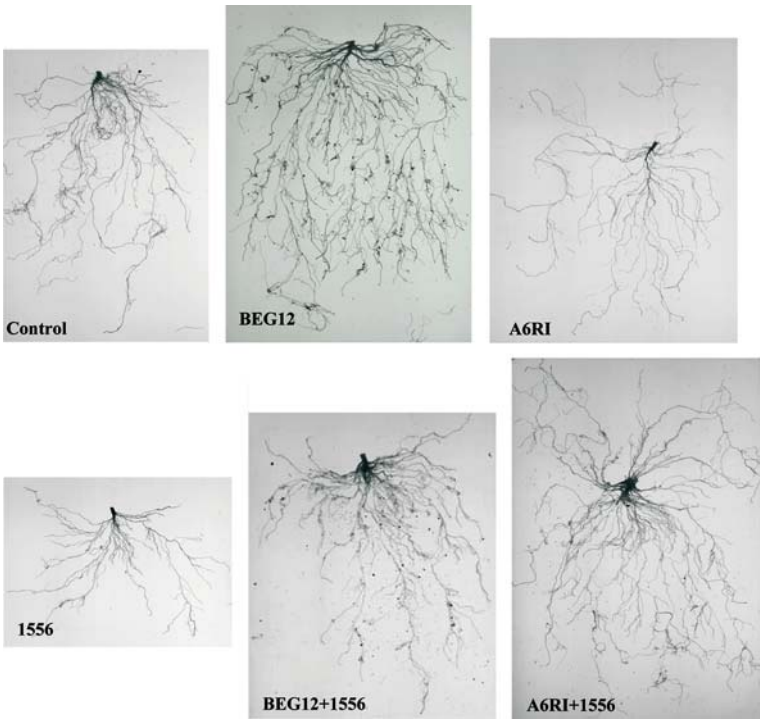

Figure 3. Root systems of 28-day old tomato, infested with *Rhizoctonia solani* 1556 or not (control), and inoculated or not with either *Glomus mosseae* BEG12 or *Pseudomonas fluorescens* A6RI.

branched root system, as previously reported for tomato roots infected with *Phytophthora nicotianae* var. *parasitica* (Trotta et al., 1996). Since the total root length of the infested plants was significantly reduced, the total soil volume explored is expected to be lower than that explored by the root system of healthy plants. This difference would account for the reduced growth of the diseased plants as measured by the plant weight.

In the absence of pathogen, controversial data are reported in the literature about the beneficial effect of AM fungi on the growth of tomato (Trotta et al., 1996; Gamalero et al., 2002). In the present study, the number of root tips of plants inoculated with *G. mosseae* BEG12 was significantly increased but not the total root length. Root branching was then significantly increased and plant growth promoted as indicated by the increased shoot weight. However, root weight was not enhanced. In agreement with Gamalero et al. (2002), *P. fluorescens* A6RI neither promoted plant growth nor modified root architecture of tomato in the low fertility conditions used in the present study.

G. mosseae BEG12 and *P. fluorescens* A6RI fully overcame the reduced growth induced by the pathogenic fungus. The growth of the infested plants inoculated with BEG12 or A6RI was even significantly higher than that of the uninfested control. This was recorded despite the lower colonization of the beneficial strains in the presence than in the absence of the pathogen. A lower colonization of the biocontrol agent *P. fluorescens* 2–79 in the wheat rhizosphere when inoculated in the presence of *Pythium* was also previously recorded by Mazzola and Cook (1991). After having suggested that this lower colonization may involve cellular communication, using reporter bacterial mutants Fedi et al. (1997) have showed that a strain of *P. ultimum* could repress the expression of genes in the biocontrol *P. fluorescens* F113 resulting in altered ecological fitness. In our study, the lower colonization of BEG12 and A6RI combined with their stronger beneficial effects in infested plants suggest that their activity was different in the presence and in the absence of the pathogen. Possible signalisation between the pathogenic and beneficial strains might account for these differences.

Altogether, these observations stressed the ability of *G. mosseae* BEG12 and *P. fluorescens* A6RI to suppress root-rot of tomato induced by *R. solani*. However, their ability to promote growth of infested plants differed according to the beneficial strains. Promotion of root growth was greater in infested plants when inoculated with *P. fluorescens* A6RI than with *G. mosseae* BEG12, therefore the fresh weight root/shoot ratio was significantly higher in plant inoculated with A6RI than in plants inoculated with BEG12. This observation is in agreement with those made previously (Berta et al., 2002; Gamalero et al., 2002), indicating that *G. mosseae* mostly promoted shoot growth. The fact that the fresh weight root/shoot ratio was significantly lower in plants inoculated with BEG12 than in plants inoculated with A6RI suggests that, in contrast to bacterized plants, the net benefit of mycorrhizal association largely exceeded its net cost, as already stressed by Fitter (1991).

Microscopic observations of the pathogen growth at the root surface indicated that in the presence of *G. mosseae* BEG12 and *P. fluorescens* A6RI, the epiphytic growth of *R. solani* 1556 was significantly reduced, suggesting the occurrence of microbial antagonism. Antagonistic activities against *R. solani* fungi by fluorescent pseudomonads have been described several times in the literature (Homma, 1996). The number of infections by the root-rot pathogen *Phytophthora parasitica* was also shown to be reduced when protected by AM fungi (Cordier et al. 1996; Vigo et al., 2000). Microscopic observations of the root tissues further indicated that in the presence of *G. mosseae* BEG12 and *P. fluorescens* A6RI, the parasitic growth of *R. solani* 1556 was significantly reduced as stressed by the decreased frequency of intraradical infections and by the fact that the pathogen never reached the central cylinder, suggesting a possible induced resistance of the host-plant by BEG12 and A6RI. Our observations are in agreement with those made by Benhamou et al. (1994) showing that in mycorrhizal roots the growth of the pathogen was usually restricted to the epidermis and cortical tissues, whereas in non-mycorrhizal roots the pathogen reached a higher level of development, infecting the vascular stele.

Glomus mosseae BEG12 and *P. fluorescens* A6RI increased the total root length and the number of root tips. These parameters being significantly increased, the deleterious impact of root necroses is expected to be a lot less important in inoculated than in non-inoculated plants. However, since root-rot pathogens usually infect immediately behind the root tip, the higher number of root tips in inoculated plants could have lead to an increase of their susceptibility to root-rot, as indicated by Norman et al. (1996) who showed an increased rate of infections by root-rot pathogen in more branched root systems. But, AM fungi also had a positive effect on the size of root apices which then appear to be less susceptible to pathogen infections (Fusconi et al., 1999). This observation could account for the disease suppression achieved by *G. mosseae* BEG12 recorded in the present study despite its positive effect on the number of root tips. *P. fluorescens* A6RI, shown to increase the number of root apices, would also be expected to contribute to increase the susceptibility of the plant to root-rot. However, Gamalero et al. (2004) have recently shown that this bacterial strain intensively colonizes apices in the first days of emergence, corresponding to the period during which the apices are the most active, and then the bacterial strain is likely to suppress the pathogen when the apices are the most susceptible to root infection. After these early days, since tomato roots have determinate growth, their apices become parenchymatized when getting older (Berta et al., 1993) and consequently become less susceptible to pathogen infections.

Altogether, our observations indicate that root-rot suppression by BEG12 or A6RI was associated with (i) reduced epiphytic and parasitic growth of the pathogenic *R. solani* and with (ii) modifications of the root architecture. These observations would suggest that the addition of these effects might account for the efficient disease suppression recorded by *G. mosseae* BEG12 and *P. fluorescens* A6RI. Further studies are underway to support this hypothesis and to assess the possible additive effect of the combined inoculation of *G. mosseae* BEG12 plus *P. fluorescens* A6RI in the root-rot suppression.

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