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Biocontrol of Fusarium crown and root rot of tomato and growth-promoting effect of bacteria isolated from recycled substrates of soilless crops

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Summary. Fusarium crown and root rot (FCRR) of tomato caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* is a serious problem in agriculture today. Biological control of this disease may be an effective option to deal with this problem. Antagonistic bacteria isolated from suppressive substrates or soils were tested against FCRR in the glasshouse in a sandy soil mixture infested with the pathogen and compared with other experimental or commercial FCRR biocontrol agents. The antagonistic bacteria were applied twice before artificial infection with the pathogen. *Pseudomonas* sp. strain FC-24B and *P. putida* FC-8B provided the best and most effective suppression in all trials, reducing vascular discoloration significantly from 65 to 77% and from 52 to 100% respectively. These strains also reduced root rot by 66% and 70% respectively. *Pseudomonas* sp. FC-24B also substantially increased plant growth, when the pathogen was absent. *Pseudomonas* sp. FC-9B, *Achromobacter xylosoxydans* MM1, *Streptomyces griseoviridis* strain K61 and the commercial formulation *Trichoderma harzianum* ICC012 + *T. viride* ICC080 also gave promising results in terms of disease control.

Key words: *Pseudomonas* sp., suppressive soil, biological control, *Solanum lycopersicum*.

Introduction

Fusarium crown and root rot (FCRR) of tomato, caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* is an important soilborne disease, that limits the yield of glasshouse-, field- and soilless-grown tomato crops in many countries. Yield losses due to FCRR range from 20 to 60% (Rowe and Farley, 1981; Jarwis, 1988; Mihuta-Grimm *et al*., 1990). The disease was first detected in Japan (Yamamoto *et al*., 1974) and was subsequently identified in many other areas, including Europe, USA, Canada and Israel. In Italy, it was first reported by Tamietti and Lento (1986).

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Management of FCRR is difficult because the pathogen has an airborne dissemination (Katan *et al*., 1997; Rowe *et al*., 1977), is spread with symptomless transplants (Hartman and Fletcher, 1991), and because of the soilless media used (Brammall and McKeown, 1989). Although host resistance has been identified and incorporated into many commercial cultivars, serious outbreaks of the disease are still occurring. Since FCRR resistance can be overcome (Elmhirst, 1997) and since susceptible cultivars are preferred because they have higher yields, it is necessary to identify alternative control methods. Chemical control of FCRR with fumigants (methyl bromide in the past, metham-sodium more recently) or with fungicides (benzimidazoles) has sometimes provided adequate control in the field, but rapid disease spread can occur again if the disinfested soil is re-contaminated by the pathogen (Rowe *et al*., 1977).

The relatively poor effectiveness of chemical control and the lack of resistance of some commercially important tomato cultivars have stimulated a search for biological control agents effective against this pathogen. Several studies have been carried out in order to find and evaluate biocontrol agents. Although biological control is not always satisfactory, some bacterial and fungal antagonists have proved effective. Among others, selected strains of *Pseudomonas fluorescens* and *P. putida* suppressed FCRR both in soil and soilless crops (Kamilova *et al*., 2009). The endophytic strain 63-28 of *P. fluorescens* induced resistance to FCRR in tomato plants (Piga *et al*., 1997). Biocontrol of FCRR by *P. fluorescens* was improved by zinc (Duffy and Défago, 1997). Two isolates of *Bacillus megaterium* and *Burkholderia cepacia*, used alone or in combination with carbendazim, reduced FCRR tomato (Omar *et al*., 2006). Attempts to biologically control FCRR using fungal antagonists have relied on saprophytic *Fusarium oxysporum* (Alabouvette and Olivain, 2002), *Trichoderma harzianum* (Ozbay *et al*., 2001) and binucleate *Rhizoctonia solani* (Muslin *et al*., 2003). A combination of a plant growth-promoting strain of *Fusarium equiseti* with biodegradable pots was recently suggested as a means to control FCRR (Horinouchi *et al*., 2008).

A recent development in biological control involves the understanding and exploitation of mechanisms of suppression occurring in soilless systems. Rockwool and perlite substrates have a natural capacity to suppress FCRR in soilless-grown tomatoes (Minuto *et al*., 2007; Clematis *et al*., 2009a). This suppressiveness is due to several bacteria and fungi in these substrates, mostly from the genera *Pseudomonas*, *Trichoderma* and *Fusarium* (Clematis *et al*., 2009b, Srinivasan *et al*., 2009).

The aim of this work was to compare the biocontrol effectiveness of bacteria isolated from suppressive substrates or soils, with other experimental or commercial biocontrol agents of FCRR on tomato, and to evaluate the effect of these bacteria on plant growth.

Materials and methods

Experimental plan, plant material and substrates

Five glasshouse trials were carried out in the period February 2007–June 2009, at the Centro di Competenza per l'Innovazione in campo agro-ambientale, AGROINNOVA, located at Grugliasco, Italy, of the University of Torino. Trials 1, 2 and 3 examined the efficacy of several micro-organisms against FCRR and trials 4 and 5 examined the growth-promoting activity of the test microrganisms in the absence of the pathogen.

Tomato seeds (cv. Cuore di bue) were sown in 60-plug trays filled with Brill Type 5 substrate (Brill Substrate GmbH & Co. KG, Georgsdorf, Germany) composed of a mix of blond peat:black peat, 15:85 (v:v), with a fine structure for nursery production, pH 5.5–6.0, and steam disinfested at 80°C for 30 min. The substrate was fertilized with 1100 g m^{-3} of NPK (nitrogen, 150 mg L^{-1}; phosphorus, P_2O_5, 170 mg L^{-1}; potassium, K_2O, 170 mg L^{-1}) and traces of molybdenum. The same substrate and fertilisers were used in all trials.

Glasshouse temperatures were 18–23°C for all trials. Fourteen-day-old tomato seedlings were transplanted to 30-L pots (52×42×26 cm), filled with 25 L of a mix of sand: perlite: peat: vermiculite, 55:12.5:12.5:20 (v:v:v), and steam disinfested at 80°C for 30 min. Each treatment had three pots, each containing 12 plants. Six of these plants were harvested and assessed 48–52 days after transplanting, and the remaining six plants 65–67 days after transplanting.

Biocontrol agents and application

Six bacterial isolates previously identified by 16S rDNA sequencing (Srinivasan *et al*., 2009) were used in the trials and compared with commercial and experimental biofungicides. The bacteria *Pseudomonas putida* FC-6B, *Pseudomonas* sp. FC-7B, *P. putida* FC-8B, *Pseudomonas* sp. FC-9B and *Pseudomonas* sp. FC-24B were isolated from recycled rockwool substrates of soilless crops grown at Albenga (northern Italy) (Clematis *et al*., 2009b), while *Achromobacter xylosoxydans* MM1 was isolated from Fusarium-suppressive soils in Liguria (northern Italy) (Moretti *et al*., 2008). The commercial formulations used were *Trichoderma harzianum* strain ICC012+*T. viride* strain ICC080 (Remedier WP, Isagro Ricerca s.r.l., Milano, Italy) and *Streptomyces griseoviridis* strain K61 (Mycostop®, Verdera OY, Helsinki, Finland).

The bacterial strains were maintained on Luria Bertani (LB) slants (Miller, 1987). Fresh bacte-

rial suspensions were prepared by inoculating a loopful of bacterial cells into 25 mL LB medium in 100-mL Erlenmeyer flasks, and incubating on a rotary shaker (Vibramax 100, Heidolph Italia Srl. instruments, Milan, Italy) at 600 rpm in a growth chamber for 7 h at 20°C±1. Bacterial concentrations (CFU ml^{-1}) were determined by the serial dilution method. To develop a standard curve for bacterial growth, the OD_{600} (optical density) values correlating with the CFU mL^{-1} were determined at the same time. Thereafter, the culture concentrations of bacteria in CFU ml^{-1} were measured by the corresponding OD_{600} value.

In trials 1 to 3, bacteria were applied to the substrate at a concentration of 10^7 CFU mL^{-1} substrate. The two commercial formulations were applied at 0.25 (Remedier) and 0.02 g L^{-1} substrate (Mycostop) according to the manufacturer's instructions. All biocontrol agents were applied twice before inoculation with the pathogen: 14 days before transplanting, by homogenizing the antagonist with the substrate at the time of seeding in the 60-plug trays; and again, 10 days before transplanting, by applying it to the sandy soil used to grow the seedlings in the 30-L pots. The 30-L pots containing sandy soil were maintained under greenhouse conditions (18–23°C) and watered daily during the 10 days before transplantation. Since the survival of bacteria after their application to the soil was not monitored, any losses in viability in the 10 days preceding seedling transplant were taken to be caused by inherent differences among the strains. The pots were maintained in the same condition as the 60-plug trays. Fourteen days after sowing, the antagonist-treated tomato seedlings were transplanted to the antagonist-treated sandy soil.

Pathogen strain and inoculation

Fusarium oxysporum f. sp. *radicis-lycopersici* Protector 1, a highly virulent strain of FCRR, was applied to the sandy mixture using chlamydospores formulated in talc. The pathogen was grown on liquid casein hydrolysate medium (Locke and Colhoun, 1974) and maintained on a rotary shaker at 200 rpm for 10 days at 28°C. The culture was centrifuged at 8,000 *g* for 20 min at 4°C. The pellet was thoroughly mixed with a double volume of dry talc powder (1:2 w:w) and kept for 10 days at 25°C. The number of chlamydospores per gram of talc was assessed by the serial dilution plating method on potato dextrose agar (PDA) containing 25 mg L^{-1} of streptomycin sulphate. To achieve optimal symptom development, the pathogen was added to the 30-L pots filled with sandy soil (a mix of substrate containing sand) to achieve a final concentration of 10^4 CFU mL^{-1} (chlamydospores dispersed in talc per volume of soil), 10 days after the second treatment with the biocontrol agents. These conditions were established by a preliminary trial carried out to evaluate the best method for inoculating the soil with the pathogen. A pathogen-only control and non-pathogen control were included in each trial. Another two trials (4 and 5) were also set up to evaluate the plant growth-promoting ability of the micro-organisms in the absence of the pathogen, otherwise conditions were the same as in the trials 1–3.

Disease assessment

After transplanting, plants were monitored weekly and the status of FCRR was recorded. Trials 1–3 were assessed at 48–52 and at 65–67 days after transplanting. FCRR was scored on the basis of the presence/absence of brown lesions on the shoot/root transition zone. For the evaluation, each plant was cut at the basal stem or crown and the severity of vascular discoloration (VD) was calculated using the formula: VD= $\sum$(No. plants × x) / total of plants recorded, where 'x' is scored on a 5 point scale in which: 0, healthy (no vascular discoloration); 1, ≤25% discoloration of the vascular tissue area; 2, >25 to 50%; 3, >50 to 75%; and 4, >75 to 100%. Root rot (RR) was measured by evaluating the discoloration of the total root system caused by FCRR, which was rated on a scale from 0 to 100%.

Evaluation of plant growth-promoting activity

In all trials, plant height was measured two weeks after transplanting. Fresh shoot weight and root weight were measured twice by cutting the first half of the plants grown in each pot at 48–52 days, and the second half at 65–67 days after transplanting (trials 1–3). The same parameters were measured at the end of trials 4 and 5, where the pathogen was absent.

Statistical analysis

A randomised block design was used in all trials, with three replicates per treatment, which

included a pathogen-only control and a non-pathogen control. The VD data were analysed by univariate ANOVA with Duncan's multiple range test (P<0.05) using SPSS software 17.0. The data relating to root system discolouration were analysed with the non-parametric Kruskal-Wallis test.

Results

Since there were significant differences in VD between treatments and trials, the results of each trial are presented for each evaluation carried out (Figure 1). In contrast, for the root rot data (trials 1–3), significant differences occurred only between

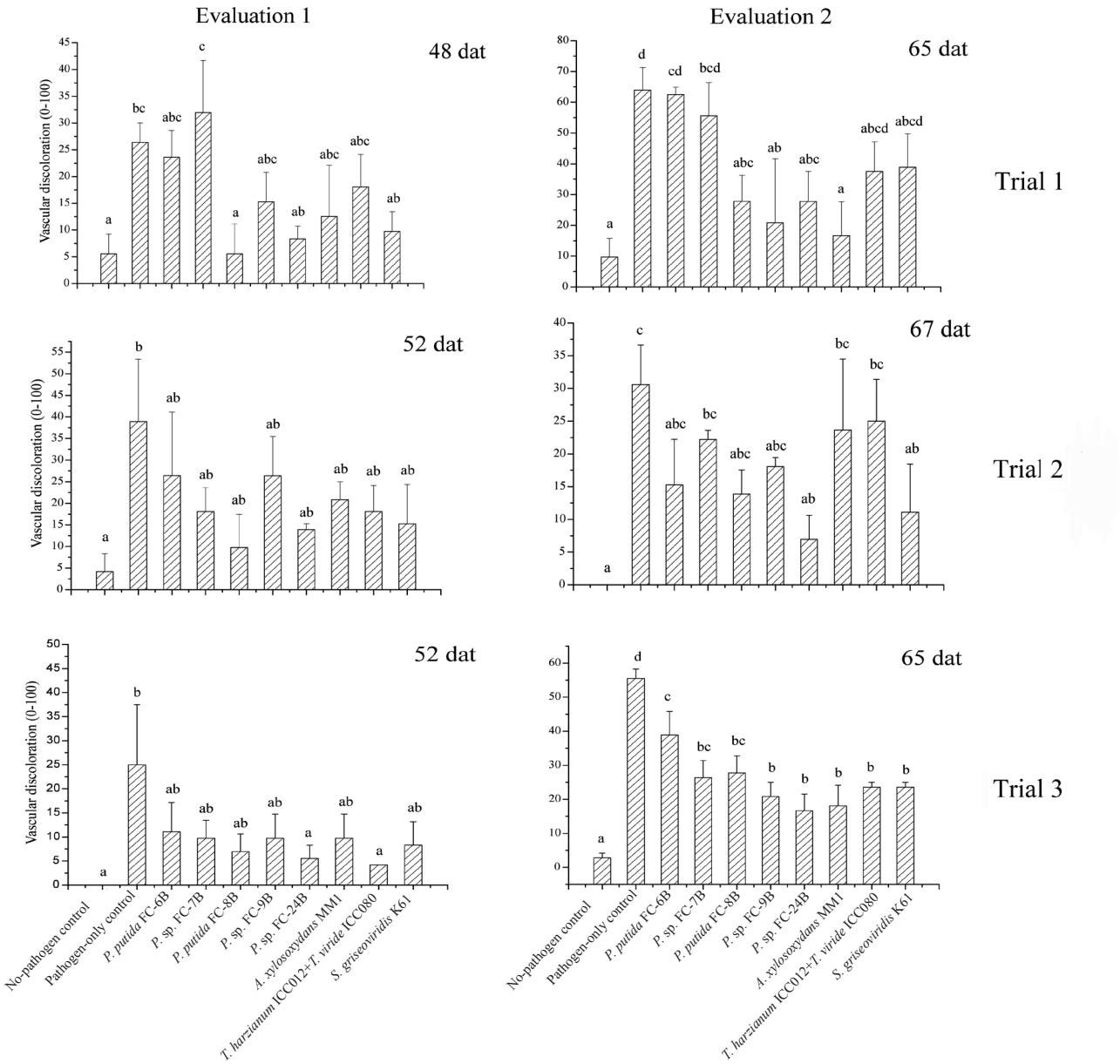

Figure 1. Biocontrol effects of various agents against *Fusarium oxysporum* f. sp. *radicis-lycopersici* on tomato in three glasshouse trials evaluated at different days after transplanting (dat), expressed as percent of vascular discoloration (VD). Vertical bars represent the standard error of three replicates from the mean at each date. Means designated by the same letter are not significantly different (P<0.05) according to Duncan's test.

treatments, thereby allowing data from different trials to be combined (Figure 2). Based on the same type of analysis as above, plant height and fresh shoot weight in the two trials carried out in the absence of the pathogen (trials 4 and 5) are shown individually (Figure 3), whereas the fresh root weights were combined (Figure 4).

In trials 1, 2 and 3, artificial infestation of the substrate with the pathogen produced a normal disease incidence (VD) in the control plots, ranging from 25 to 39% in the first evaluation, and from 31 to 64% by the end of the trial (Figure 1); the root rot mean value was 37% (Figure 2). At the second evaluation in trial 2, the VD decreased slightly with all treatments as compared with trials 1 and 3. *Pseudomonas* sp. FC-24B and *P. putida* FC-8B caused the largest reduction of vascular discolouration in most trials and periods of evaluation (Figure 1). Similar trends were seen in the reduction of root rot with these two strains (Figure 2). *Pseudomonas* sp. FC-9B, *A. xylosoxydans* MM1 and the commercial formulation *Trichoderma harzianum* ICC012+*T. viride* ICC080 also gave promising control with a >50% reduction in disease incidence. *P. putida* FC-6B and *Pseudomonas* sp. FC-7B generally were less satisfactory, reducing disease incidence by less than 50%, as disease mitigation failed (Figures 1 and 2).

In the presence of the pathogen (trials 1-3), there were no significant differences among the treatments for plant height, fresh shoot weight and fresh root weight (data not shown).

In the absence of the pathogen (trials 4 and 5), *Pseudomonas* sp. FC-24B and *A. xylosoxydans* MM1 substantially improved plant height, shoot and root weight. Improvements with *Pseudomonas* sp. FC-24B were 30, 26 and 33% for these growth variables respectively, and improvements with *A. xylosoxydans* MM1 31, 33 and 28% respectively. *P. putida* FC-6B and by *S. griseoviridis* also promoted plant growth (Figures 3 and 4).

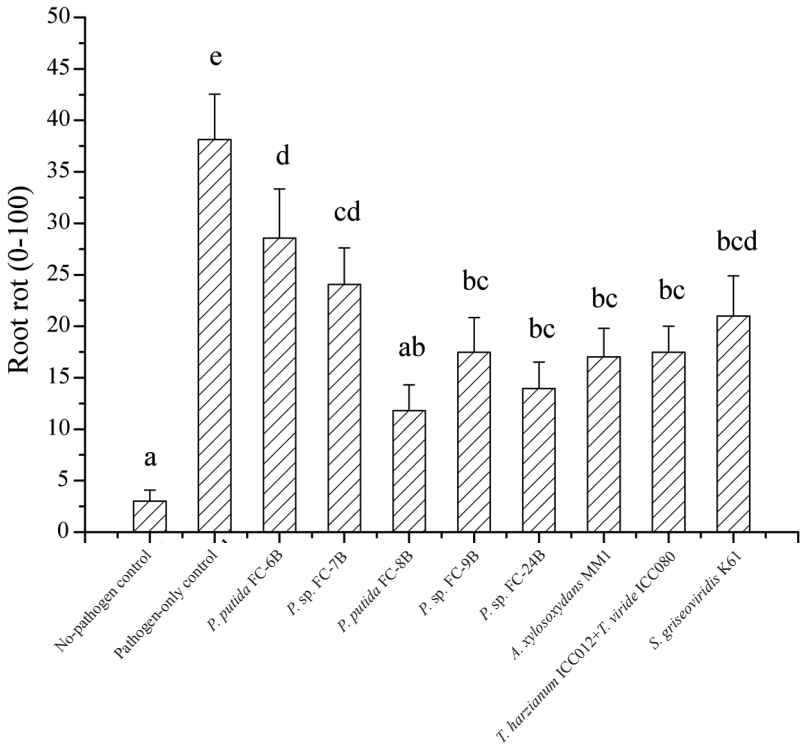

Figure 2. Biocontrol effects of various agents against *Fusarium oxysporum* f. sp. *radicis-lycopersici* on tomato in three glasshouse trials evaluated at different days after transplanting (dat), expressed as percent of root rot (RR). Vertical bars represent the standard error (trials 1, 2 and 3). Means designated by the same letter are not significantly different (P<0.05) according to Duncan's test.

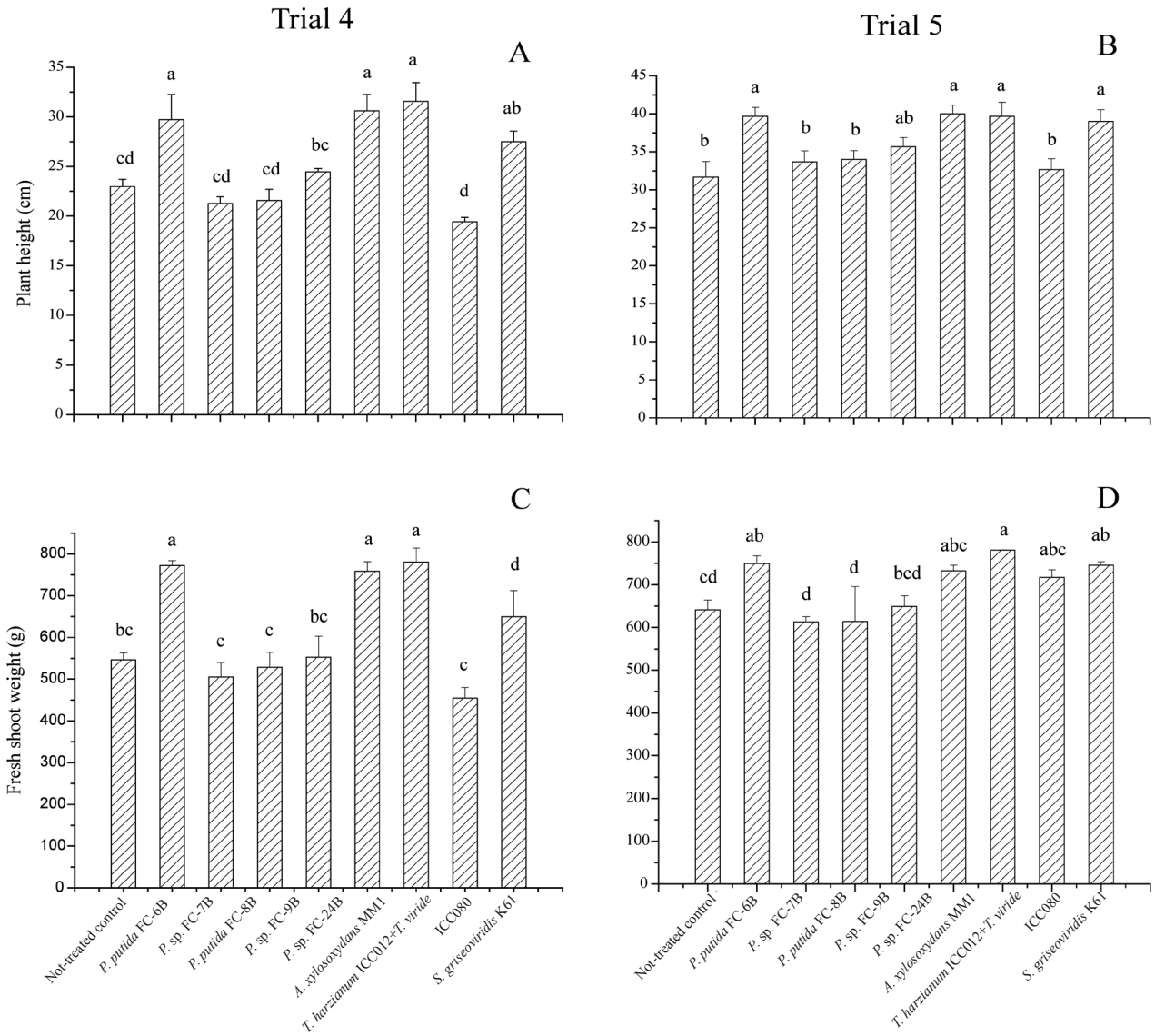

Figure 3. Plant growth promoting activity of biocontrol agents on tomato in the absence of the pathogen 52 days after transplanting, expressed as plant height (cm) and fresh shoot weight (g). Vertical bars represent the standard error of three replicates in A and C (trial 4), and B and D (trial 5). Means designated by the same letter are not significantly different (P<0.05) according to Duncan's test.

Discussion

Fusarium wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici* is a serious soil-borne disease of tomato worldwide that is difficult to control by traditional means, thus making the development of alternatives a high priority (Lemanceau *et al.*, 1992).

Fluorescent pseudomonads have been used to control root diseases caused by fungal pathogens: isolates of *P. fluorescens*, *P. putida, P. aeruginosa* and *P. aureofaciens* suppress soil-borne pathogens through rhizosphere competition (Elad and Chet, 1987), antibiosis (Pierson and Thomashow, 1992) and iron chelation by production of siderophores

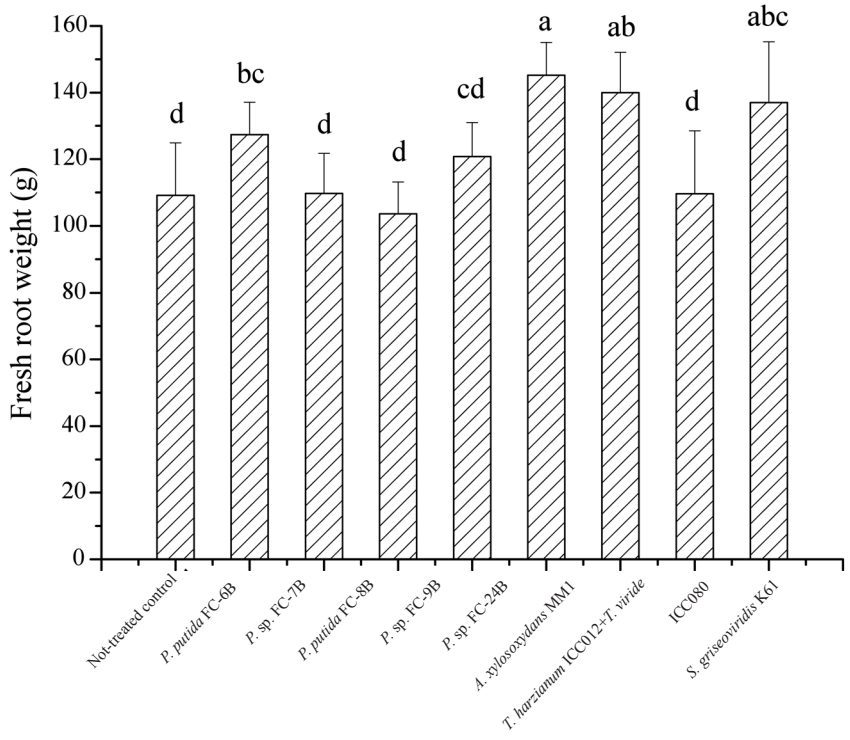

Figure 4. Root growth promoting activity of biocontrol agents on tomato in the absence of the pathogen 52 days after transplanting. Vertical bars represent the standard error of 6 replicates (two trials, 4 and 5). Means designated by the same letter are not significantly different ($P<0.05$) according to Duncan's test.

(Lemanceau *et al*., 1992). *P. fluorescens* WCS365 and *P. putida* PCL1760 suppressed tomato foot and root rot in stonewool (Kamilova *et al*., 2006; Validov *et al*., 2009).

In the present study, some strains of *Pseudomonas* sp. were very effective against FCRR at different disease pressures (trials 1–3). At the second evaluation in trial 2, the VD decreased slightly in all treatments compared with the other two trials, which may have resulted from an increase in temperature (maximum temperature 26°C) between the first and second evaluation. These characteristics of *Pseudomonas* strains are well known and documented (Kamilova *et al*., 2006; Validov *et al*., 2009). Of special interest in this study is the fact that the effective *Pseudomonas* strains were isolated from re-used rockwool soilless systems (Minuto *et al*., 2007; Clematis *et al*., 2009b). The indigenous microflora residing in re-used rockwool has been reported to reduce FCRR and damping-off caused by *Pythium aphanidermatum* in soilless systems (Postma *et al*., 2005; Minuto *et al*., 2007). Microbes from such an origin should be well adapted to the particular niche of this study (sandy soil) and so should be especially effective against FCRR, which is a disease typical of soilless crops. These bacteria can be used as disease control agents for both Fusarium wilt (Srinivasan *et al*., 2009) and FCRR on tomato. Their broad-spectrum disease suppression capacity and environmental adaptability should facilitate their commercial exploitation. The disease suppression and plant growth-promoting activity found in this study may be related to the fact that the biocontrol agents were applied in two steps.

Disease control and/or plant growth-promoting activity has previously been attributed to a com-

mon characteristic of *Pseudomonas* sp., its endophytic nature (Misaghi and Donndelinger, 1990). The two-step method of applying the bacteria at sowing and at transplanting, most likely gave the biocontrol agents an opportunity to survive in these particular niches and colonise them. The findings suggest that the antagonists survived in the rhizosphere and interacted with the pathogen by competing at the same infection site on and inside the root surface of the host (rhizosphere competition) (Nemec *et al*., 1996). Endophytic bacteria and their plant hosts can coevolve in a manner that leads to intimate and effective means of communication and cooperation in suppressing vascular diseases (Nejad and Johnson, 2000). Obviously, other mechanisms of disease biocontrol by *Pseudomonas* spp., such as secreting antibiotics, inducing systemic resistance in the host, or suppressing the pathogen through a number of regulatory mechanisms (Haas and Dèfago, 2005), could also have occurred. Further studies are needed to investigate the main biocontrol mechanisms of the bacterial strains reported here.

Interestingly, *P. putida* FC-8B gave the best root rot control, but it did not significantly promote plant growth when the pathogen was absent. In addition, this strain did not significantly increase plant height, fresh shoot weight and fresh root weight when the pathogen was present. This suggests that there is no explicit association between the plant growth-promoting activity and the disease suppression capacity of the *Pseudomonas* spp. strains tested in the study. However, in other studies, endophytic bacteria have shown both plant growth-promoting activity and disease control capacity (Haas and Dèfago, 2005). Future work should examine the effect of combined treatments of disease suppressive strains and growth promoting strains.

In conclusion, tomato plants grown in sandy soil previously inoculated with potentially endophytic *Pseudomonas* spp. has reduced FCRR disease symptoms and/or enhanced plant growth. Under controlled conditions, *Pseudomonas* sp. FC-24B showed the greatest capacity for disease-suppression and growth-promotion. Further work with this strain is in progress to elucidate its mechanisms of action on tomato under greenhouse conditions. The study shows that effective *Pseudomonas* spp. strains can be obtained from re-used substrates in soilless crops, thus providing a new source of biocontrol agents, adapted to new cultivation methods.

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