Introduction

The fungus *Fusarium oxysporum* Schlecht f.sp. *radicis-lycopersici* Jarvis and Shoemaker (FORL) causes tomato crown and root rot (TCRR, synonym: tomato foot and root rot (TFRR) in tomato (*Lycopersicon esculentum* Mill.), which is a serious problem for field and greenhouse production (Jarvis, 1981) causing significant losses. The disease has been reported in at least 32 countries (Jones et al. 1981). Chemical pesticides do not efficiently suppress TCRR. Hence, alternative measures such as the application of biocontrol agents for disease management are required. At present, an increasing number of commercial products based on plant growth-promoting rhizobacteria (PGPR) to control root pathogens is becoming available worldwide. Many of them contain strains of *Pseudomonas spp., Bacillus spp.*, etc., but practical applications are still limited.

Biological control of soilborne pathogens to improve productivity of the plants has been reported in several pathosystems including cucumber, hot pepper, tobacco and tomato by single strains or mixture of PGPR stimulating plant defense responses to pathogen infection (Anith et al., 2004; Jetiyanon et al., 2003; Jetiyanon and Kloepper, 2002; Kloepper et al., 2004; Murphy et al., 2003; Murphy et al., 2000; Raupach and Kloepper, 1998; Ryu et al., 2004). The use of combinations of multiple antagonistic organisms also may improve disease control over the use of single organisms. Application of multiple organisms simultaneously may also enhance the level, efficacy and consistency of biocontrol measures by providing multiple mechanisms of action, a more stable rhizosphere community, effectiveness over a wider range of environmental conditions and a reduced risk for the development of resistances. In particular, combinations of arbuscular mycorrhizal fungi (AMF) and PGPR may provide protection at different times or under different conditions, and occupy different or complementary niches. But, only few studies investigated the synergistic effect of beneficial microorganisms such as *Pseudomonas, Bacillus* and AMF to improve plant growth and resistance of plants against soilborne diseases particularly in replant disease soil infected by FORL.

It is hypothesized that improved mycorrhization by single or combined application of *Pseudomonas* sp. "Proradix®" (DSMZ 13134) and *Bacillus amyloliquefaciens* FZB42 can improve nutrient acquisition, healthy growth of tomato plants and suppress *Fusarium* crown and root rot disease caused by *Fusarium oxysporum* Schlecht f.sp. *radicis-lycopersici* Jarvis and Shoemaker (FORL). Furthermore, single or dual inoculation combined with AMF will lead to synergistic effects on the healthy growth of tomato plants in replant disease soils caused by FORL. This research was conducted as a first step toward the development of effective biological control systems as an alternative strategy for the management of TCRR.

Materials and methods

Plant, microbial inoculums and experimental set-up.

For surface disinfection, Tomato seeds (*Lycopersicon esculentum* Mill. Money Maker variety) were first shaken in a 70% ethanol solution for 1 min, then in a 1,5% Sodium hypochloride (NaOCl) solution for 3 min and finally washed with tap water. Seeds with and without 0.25 ml *Pseudomonas* sp. "Proradix®" (DSMZ 13134, Sourcon Padena, Tübingen-Germany)

dressing (4.5 x 10^{10} cfu l^{-1} sterile distilled water), with and without *Bacillus amyloliquefaciens* FZB42 (RhizoVital® 42 TB, ABiTEP, Berlin, Germany) coating (5 – 15 gr kg^{-1} seed), and with and without AMF-inoculum (approx. 8000 propagules kg^{-1} substrate of *Glomus intraradices* strain 510, Mycotek Biotechnik Maßholder & Poehling GbR, Hannover, Germany) were sown in pots containing 50 g loamy sandy soil/sand mixture (3:1/by volume). The clay loamy soil used in this study was collected from Brazil and the characteristics of soil are as follows; pH ($CaCl_2$) 6.2 , P 1.3 , K 17 , Mg 31 100 g^{-1} soil, Mn 118 , Zn 1.9 , B 0.22 and Fe 45 mg kg^{-1} soil (soil pH and plant available nutrients analyzed according to VLUDFA (2007). Three weeks after sowing, the seedlings of tomato were transplanted to pots containing 2 kg replant disease soil. To increase the FORL-infection potential of the soil, FORL infected tomato plants had been grown in the soil before for three and a half months. Severely disease affected plants were then cut in 5 mm pieces and incorporated into the replant disease soil (250 g plant material kg^{-1} replant disease soil) and mixed. Before planting, the soil was fertilized with 100 mg N kg^{-1}, 50 mg P kg^{-1}, 150 mg K kg^{-1}, 50 mg Mg kg^{-1}, 0.06 mg Fe kg^{-1}. The pots were arranged in a completely randomized design in a soil-heating-cooling system in the greenhouse to provide optimal soil temperature conditions (19°C) for FORL. The experimental growth phase ended six weeks after transplanting.

Plant harvest and nutrient concentration analysis, root colonization by arbuscular mycorrhizal fungi (AMF) and Disease severity assessment.

At harvest, shoots and roots were separated, roots were thoroughly washed and blotted, and fresh weight was determined. For analysis of nutrient concentrations, harvested samples (shoots) were washed and gently pressed between tissue papers to remove adhering water, after which fresh weights were determined. The plant samples were dried at a temperature of 65°C for 3 days. Root and shoot dry weights were determined. Mineral elements were determined by atomic absorption spectrophotometry (Mn and Zn) and photo-spectrophotometry (P) after wet digestion. Assessment of AMF root colonization was based on Koske and Gemma (1989) and Kormanik and McGraw (1984). The disease severity was estimated according to the method described by Hibar et al., (2006). The disease index percentage was determined using the equation described by Song et al. (2004).

Experimental design and statistical analysis

The experimental design was a completely randomized design pattern consisting of eight treatments, i.e; CONTROL (No microbial inoculation), *P.*sp. "Proradix®" (PR), *B. amyloliquefaciens* FZB42 (BA), PR+BA, Arbuscular mycorrhizal fungi (AMF), PR+AMF, BA+AMF and PR+BA+AMF. Tukey tests at a significant level of $P<0.05$ were conducted on with transformed data after one-way ANOVA to identify significant differences between the treatments. The results in tables and figures are given as means. All statistical analyses were performed using Sigma Stat version 2.03 statistical software (SPSS Inc. Chicago. IL. USA).#

Results

Mycorrhiza infection could not be detected in the control treatment and in the treatments with *P*.sp. ”Proradix®” and *B. amyloliquefaciens* FZB42 inoculation (Fig. 1). Inoculation with AMF induced mycorrhiza infection and this was enhanced in combination *P*.sp. ”Proradix®” and *B. amyloliquefaciens* FZB42 inoculation. Combined inoculation with both PGPRs did not lead to an additional increase of mycorrhiza infection. Single inoculation with *P*.sp. ”Proradix®”, *B. amyloliquefaciens* FZB42 or AMF similarly increased root and shoot dry weight (Figs. 2 and 3), increased shoot nutrient concentrations (Tab. 1) and decreased the disease index (Fig. 4). Simultaneous inoculation with both PGPRs further increased P shoot concentrations but did not have an additional significant effect on any of the other measure variables. Combined inoculation of AMF with one of the PGPRs led to a further significant increase of the dry weights and of some nutrient concentrations (P and Zn). However, this further increase did not exceed the sum of the single effects. The observed additional decrease of the disease index was not significant. The combination of AMF inoculation with both PGPRs did not lead to additional effects.

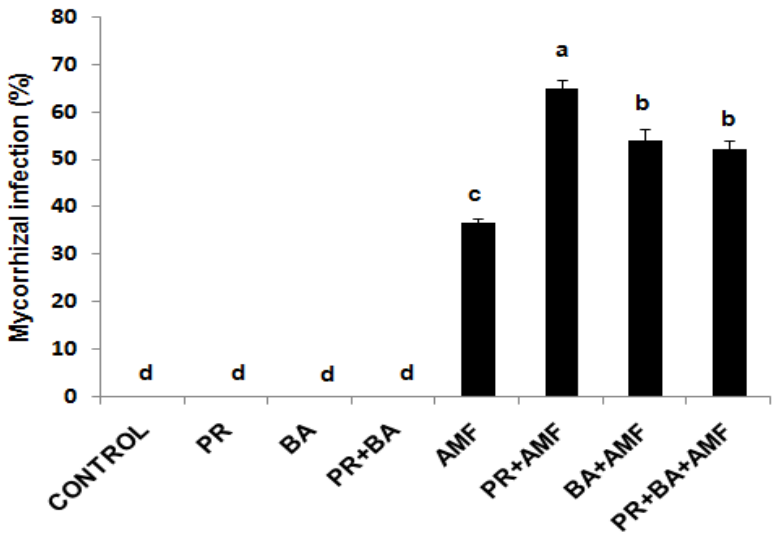

FIGURE 1. Percentage of roots infected by AMF 9 weeks after planting with *Pseudomonas* sp. ”Proradix®” (PR), *Bacillus amyloliquefaciens* FZB42 (BA) and arbuscular mycorrhizal fungi (AMF) on replant disease soil infected by FORL. Letters above the bars indicate significant differences between the treatments (Tukey, $p<0.05$).

Treatments	P (mg g^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})
Control	0,953 d	40,398 b	23,799 b
PR	1,552 c	78,293 a	26,550 b
BA	1,552 c	89,637 a	35,821 ab
PR+BA	1,914 b	70,158 a	32,579 ab
AMF	1,896 bc	80,256 a	32,813 ab
PR + AMF	2,421 a	84,108 a	48,307 a
BA + AMF	2,068 ab	91,793 a	40,572 ab
PR + BA + AMF	1,912 bc	99,570 a	33,533 ab

TABLE 1. Concentration of the total P, Mn and Zn in the shoots of tomato plants 9 weeks after planting and inoculation with *Pseudomonas* sp. "Proradix®" (PR), *Bacillus amyloliquefaciens* FZB42 (BA) and arbuscular mycorrhizal fungi (AMF). Different letters indicate significant differences between the treatments within the same column (Tukey, $p<0.05$).

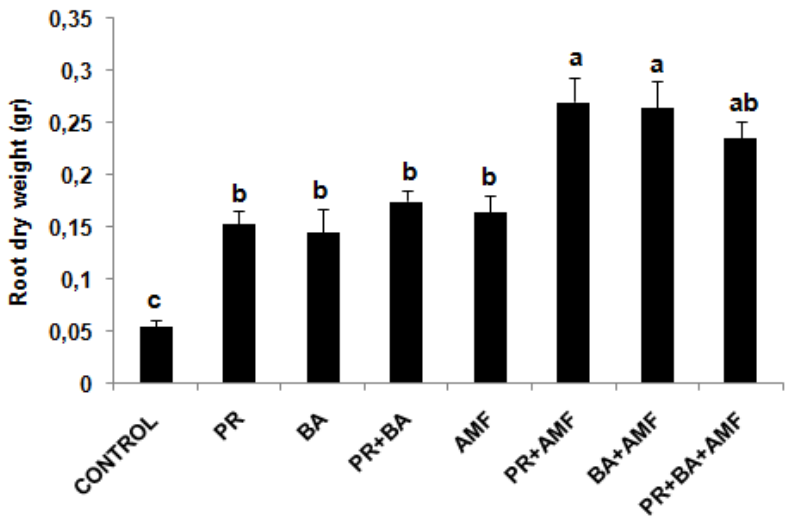

FIGURE 2. Root dry weight of tomato plants 9 weeks after planting with *Pseudomonas* sp. "Proradix®" (PR), *Bacillus amyloliquefaciens* FZB42 (BA) and arbuscular mycorrhizal fungi (AMF) inoculation on replant disease soil infected by FORL. Different letters above the bars indicate significant differences between the treatments (Tukey, $p<0.05$).

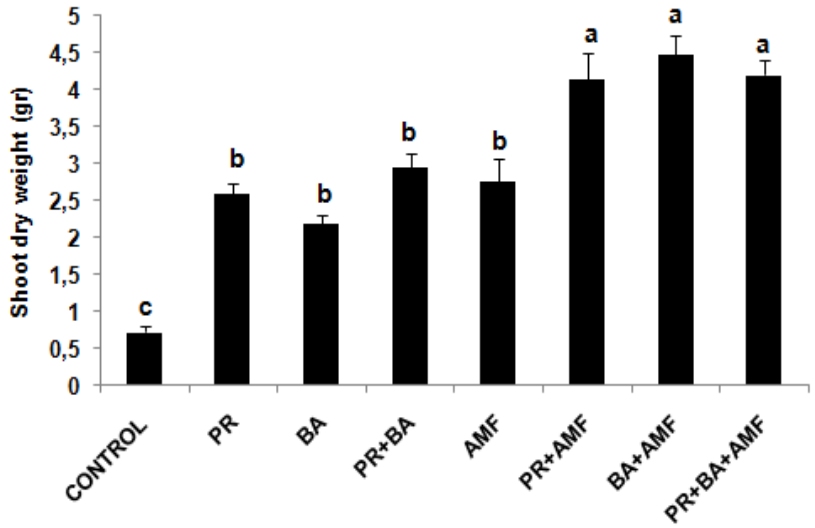

FIGURE 3. Shoot dry weight of tomato plants 9 weeks after planting with *Pseudomonas* sp. "Proradix®" (PR), *Bacillus amyloliquefaciens* FZB42 (BA) and arbuscular mycorrhizal fungi (AMF) inoculation on replant disease soil infected by FORL. Different letter above the bars indicate significant differences between the treatments (Tukey, $p<0.05$).

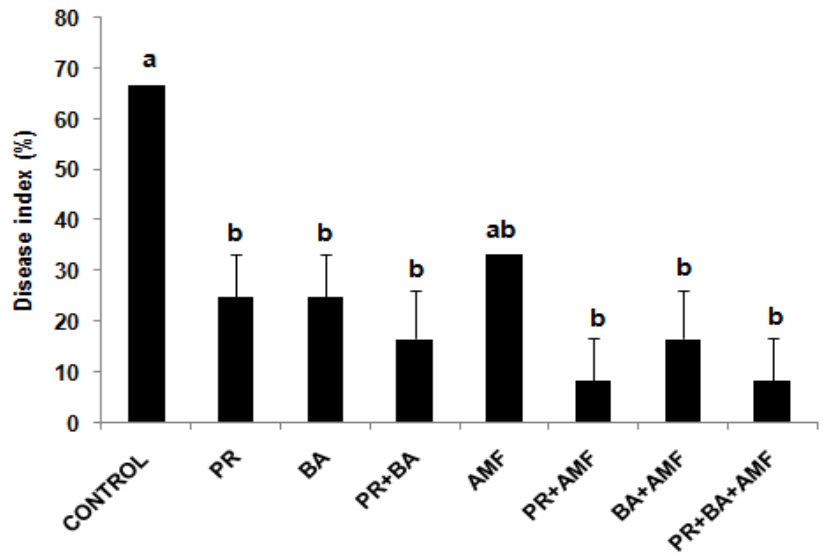

FIGURE 4. Disease index of tomato plants 9 weeks after planting with *Pseudomonas* sp. "Proradix®" (PR), *Bacillus amyloliquefaciens* FZB42 (BA) and arbuscular mycorrhizal fungi (AMF) inoculation on replant disease soil infected by FORL. Different letter above the bars indicate significant differences between the treatments (Tukey, $p<0.05$).

Discussion

The results presented here demonstrate that inoculation with PGPRs and AMF can induced significant disease suppression after FORL infection in a soil where AMF is not (sufficiently) present or suppressed by other factors, e.g. FORL.

The efficiency could be improved by combining PGPRs with AMF. Synergistic effects on plant growth under several conditions when PGPR and AMF are coinoculated are reported (Vivas et al., 2003; 2006; Artursson et al., 2006). In our study, however, the effects of combined inocculation with two PGPRs or with PGPRs and AMF never exceeded the sum of the effects of single inocculation with PGPRs and AMF. This indicates additive effects of PGPRs and AMF rather than clear synergistic effects.

Roots of tomato after application of both bacteria strains were not only healthier but also showed a significantly higher colonization by AMF *Glomus intraradices*, indicating that AMF infection in the soils was suppressed directly by pathogens or indirectly as consequence of poor root development. Azcón (1987) and Linderman (1997) reported that unidentified PGPR have a strong stimulatory effect on the growth of AMF and increased mycelia growth of *G. mosseae* spores. *P. fluorescens* 92rk, alone or co-inoculated with *P. fluorescent* P190r, increased mycorrhizal colonization of tomato roots by *G. mosseae* BEG12 (Gamalero et al., 2004). Similarly to the results obtained by Marulanda-Aguirre et al. (2008), where *Bacillus megaterium* inoculated with *G. intraradices* showed the highest percentage of mycorrhizal root length of *Lactuca sativa* plants compared to the single inoculation of *G. intraradices*. These results suggest that PGPR and AMF might be co-inoculated, at least in soils with a low AMF status, to optimize the formation and function of the mycorrhizal symbiosis**.**

Both PGPR and AMF inoculation treatments directly and indirectly improved the nutrients acquisition and allocation to the shoots of tomato plants. The concentrations of P, Mn and Zn in tomato shoots were higher after inoculation with *P*. sp. ”Proradix®” and *B. amyloliquefaciens* FZB42 when compared to the untreated control. The ability of *P. fluorescens* and AMF to promote plant growth by improved nutrient acquisition and suppression of soilborne pathogens is well documented (Smith and Goodman, 1999., Barea et al, 2002., Gamalero et al., 2003, Yusran et al., 2009). Both functions may promote plant growth but by different mechanisms. AMF facilitated mineral and water uptake, and increased the defense against soilborne pathogens (Filion et al., 1999; Smith et al., 2001; Marulanda-Aguirre et al., 2003; 2008). PGPRs induced the release of plant growth regulators (Koch et al., 1998). Siddique (2006) reported that *Pseudomonas* spp. can synthesize certain enzymes that can modulate plant hormone levels, might limit the available iron via siderophore production and can also kill pathogens by production of certain antibiotics. Our study confirmed that, *B. amyloliquefaciens* FZB42 can act as a PGPR, as described by De Freitas et al. (1997), Kokalis-Burelle et al. (2002), Kishore et al. (2005) and Marulanda-Aguirre et al. (2008). Phae et al. (1992) reported that *B. subtilis* NB22 significantly reduce the occurrence of crown and root rot disease of tomato.

Another aspect in the present study was to test if the mixtures of different bacteria species improve the control against FORL compared to one bacterium species alone. Our results did not confirm Pierson and Weller (1994) and Schisler et al. (1997) who proposed a strategy to increase the efficacy and the consistency of disease control by mixed application of antagonistic microorganisms with different modes of actions. Cordier et al. (2000) stated that dual or multiple inoculations of beneficial microorganisms can be neutral, positive or negative depending on the inoculants used. However, our study showed that combined application of two PGPRs improved tomato growth and suppressed FORL to the same extent as single application or further increases P shoot concentrations. Our results are in agreement with studies by Raupach et al. (1998), Pierson and Weller (1994) and Duffy et al. (1995), all of which demonstrated that certain mixtures of PGPR were significantly suppressive to cucumber pathogens and take-all disease. Different mechanisms of action for different PGPR strains may explain why combinations of *P.* sp. "Proradix®" and *Bacillus amyloliquefaciens* FZB42 suppress disease similar to inoculation with single strains. Sung and Chung (1997) demonstrated that chitinase-producing *Streptomyces* spp. and *B. cereus* isolates used in combination with antibiotic-producing *P. fluorescens* and *Burkholderia* (*Pseudomonas*) *cepacia* isolates had a synergistic effect on the suppression of rice sheath blight and Szczech and Dyśko (2008) who reported that among tested bacterial inoculations, only mixture of the bacteria B125 and PT42 tended to affect positively the growth of the plants and to reduce the density of *Fusarium* spp. in the rhizosphere of tomato plants. These results indicate that the consistency of biocontrol agents in suppression of soilborne pathogens influenced by many factors, i.e. bacterial strains, soilborne pathogen species, species of plant, etc.

Conclusion

The hypotheses that improved mycorrhization by single or combined inoculation with *Pseudomonas* sp. "Proradix®" (DSMZ 13134) and *Bacillus amyloliquefaciens* FZB42 can enhance nutrient acquisition, healthy growth of tomato plants and suppress *Fusarium* crown and root rot disease caused by *Fusarium oxysporum* Schlecht f.sp. *radicis-lycopersici* Jarvis and Shoemaker (FORL) was confirmed here. Effects of combined inoculation were additive rather than clearly synergistic. The results obtained suggest an important role of rhizosphere interactions for the expression of bio-control mechanisms by inoculation with effective *Pseudomonas* and *Bacillus* strains independent of simple antagonistic effects. The commercial biological control agents may be effective reducing Fusarium crown and root rot such as TCRR and that further evaluation of these is justified. Further studies on the use of PGPR and their synergistic effect with AMF may consider the optimal stage of plant development for non-pathogen colonization and different methods for infesting plants under field conditions.

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Pseudomonas sp. "Proradix®" inoculums, Rhizovital®, ABiTEP, Berlin (Germany) for providing *Bacillus amyloliquefaciens* FZB42 inoculums and Mycotek, Biotechnik, Maßholder & Poehling GbR, Hannover (Germany) for providing arbuscular mycorrhiza fungi (AMF) *Glomus intraradices* strain 510 inoculums.

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