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ORIGINAL ARTICLE

Biological control of Fusarium wilt on common beans by in-furrow application of *Trichoderma harzianum*

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Abstract Two field trials were conducted during the summer and winter seasons of 2010 to evaluate the effect of biocontrol agents against Fusarium wilt of common bean. Treatments included six *Trichoderma harzianum* isolates applied in planting furrows at 1.2×10^{12} conidia/ha, and one seed treatment with carboxin + thiram. Non-treated and infested plots served as control. Three *T. harzianum* isolates, CEN287, CEN290 and CEN316, reduced Fusarium wilt incidence and disease severity in the summer trial; incidence averaged 43.9 and 82.4 % in effective treatments and infested control plots, respectively. During the winter season, four isolates, CEN290, 1306, CEN316 and CEN287, reduced wilt incidence by 22.4, 26.7, 41.2 and 51.3 %, respectively, compared to the infested control (92.8 %) and fungicide-treated seeds (85.8 %). In general, ranking of treatments were similar whether incidence or severity was evaluated. However, CEN287 and CEN316 were ranked as the most effective isolates in both seasons. Crop yield-related variables were not affected by the treatments. *Fusarium oxysporum* population in soil was positively associated with disease incidence and severity, and negatively associated with grain number per pod and 100-grain mass in the summer experiment. In the winter trial, increasing densities of *Trichoderma* spp. in soil were correlated with increased 100-grain mass and number of grains per pod. The results highlight the value of in furrow applications of biocontrol agents for managing Fusarium wilt of common bean.

Keywords *Phaseolus vulgaris* L · Antagonist delivery · Antagonist survival · Rhizocompetence · Soilborne plant pathogen · Vascular wilt

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Introduction

Fusarium wilt (*Fusarium oxysporum* f. sp. *phaseoli* J.B. Kendr. & W.C. Snyder) is one of the most important diseases of common bean worldwide (Schwartz et al. 2005). Root infections cause yellowing and wilt in plants especially at the blooming and pod-filling stages; the wilt is irreversible and the plants may eventually die prematurely. The disease is favored by mild temperatures and high soil moisture and can lead to yield reductions of 80 % in susceptible common bean cultivars (Sartorato and Rava 1994; Salgado et al. 1996). Infested seeds and agricultural machinery are the main means of dissemination of the pathogen. In infested soils, diseased plants first appear in small patches, which tend to increase in the following cropping seasons. In the absence of effective disease management, the infested area gets larger and may expand throughout the entire field (Abawi and Pastor-Corrales 1990).

The pathogen survives as a saprophyte in soil or in plant debris in the form of chlamydospores, or even in roots of some non-host plants (Dhingra and Coelho-Neto 2001; Schwartz et al. 2005; Toledo-Souza et al. 2012). Successful disease management of susceptible cultivars is hardly achieved and there are few effective practices recommended for this disease (Hall and Nasser 1996), including host plant resistance

(Alves-Santos et al. 2002). Chemical fungicides are ineffective and not recommended for vascular wilts (except for seed treatment) since they do not prevent further root infection and phloem colonization by the pathogen.

In a biocontrol strategy, rhizosphere colonization using beneficial microorganisms has been shown to protect different crops against wilt pathogens such as soybean, banana, cotton, chickpea (Khan et al. 2004; Thangavelu et al. 2004; Alabouvette et al. 2009; John et al. 2010; Pereg and McMillan 2015). However, data on the efficacy of antagonists against Fusarium wilt of common bean is lacking.

Seed treatment with *Trichoderma* species may protect seedlings against Fusarium wilt in common bean (Carvalho et al. 2014; Guimarães et al. 2014), but results based on field data are limited. *Trichoderma* antagonists combine different desired traits such as hyperparasitism, competition and antibiosis and survival on different soils, especially in the rhizosphere (Harman et al. 2004; Mohamed and Haggag 2006; John et al. 2010). Their identification requires a thorough selection and testing under disease-conducive weather.

Successful management of Fusarium wilt leading to yield loss reduction in commercial fields using competitive isolates of *Trichoderma* has been reported (Shali et al. 2010). Particularly in Brazil, the adoption of *Trichoderma*-based biofungicides has increased over the years due to their proven benefits in integrated management of white mold (*Sclerotinia sclerotiorum*) on different crops (Morandi and Bettiol 2009; Görgen et al. 2009; Geraldine et al. 2013; Aguiar et al. 2014). Nevertheless, there is an urgent need to test the usefulness of biocontrol for managing soilborne diseases such as root rots and vascular wilts. Beyond selection of competitive isolates, the delivery of antagonists at the target site is a critical step in a biocontrol program (Glare et al. 2012). Among the different methods of antagonist release, in-furrow application is a low-cost and promising alternative (Teixeira et al. 2012). Therefore, our study aimed to evaluate the efficacy of six *Trichoderma harzianum* isolates applied via in-furrow method to suppress Fusarium wilt and reduce *F. oxysporum* population in soil.

Materials and methods

Origin of *T. harzianum* and *F. oxysporum* f. sp. *phaseoli* isolates

The effects of six *T. harzianum* isolates and a seed treatment with a commercial fungicide mixture were compared to infested non-treated plots. A non-infested and non-treated plot served as negative control. Isolates CEN287, CEN288, CEN289, CEN290 and CEN316 were previously selected for rhizocompetence, production of volatile metabolites and hyperparasitism (Carvalho et al. 2011, 2014) and recovered from the culture collection of biocontrol agents of Embrapa Genetic Resources and Biotechnology (Brasília, Federal District, Brazil). The commercial isolate *T. harzianum* '1306' (Itaforte Bioprodutos, Itapetininga, Brazil), registered in Brazil for the biocontrol of common bean root rots was also tested.

The experimental units consisted of field plots (1 m^2) infested with an aggressive *F. oxysporum* f. sp. *phaseoli* race 2 isolate (Fop 46) used as standard in breeding for disease resistance (Cândida et al. 2009). This isolate is deposited in the collection of fungal and functional microorganisms from Embrapa Arroz e Feijão (Santo Antônio de Goiás, Goiás State, Brazil). All fungal isolates used in this work were stored in liquid nitrogen and sterilized filter paper at −20 °C, respectively, and recovered on Potato-Dextrose-Agar (PDA) before application.

Growth of *F. oxysporum* f. sp. *phaseoli* and *T. harzianum*

Soil infestation was accomplished with two different methods to provide sufficient inoculum for disease development. In the first, macro and microconidia were produced by transferring four PDA disks with Fop 46 mycelia to 250 mL Erlenmeyers with 50 mL of Tochinai media (10 g Bacteriological Peptone; 0.5 g KH_2PO_4; 2.5 g $MgSO_4.7H_2O$; 20 g Maltose; 1000 mL distilled water). Flasks were kept in an orbital shaker at 120 rpm and room temperature during 4 days. In the second method, chlamydospores and conidia of Fop 46 isolate were produced on 9:1 sand:cornmeal medium, after 20 days of growth at 25±2 °C (Nene and Haware 1980).

Six 5 mm agar plugs of *T. harzianum* isolates were removed from 7-day-old colonies and transferred to 250 mL flasks containing 15 g of autoclaved parboiled rice, previously moistened at 60 %. Flasks were incubated at 25 °C and 12 h photoperiod. After 6 days, conidia were harvested by shaking colonized grains with autoclaved distilled water, and filtered through sterile gauze.

Field experiments

Field trials were conducted in the summer and winter of 2010 at the experimental area of Embrapa Arroz e Feijão in a field previously cropped with common bean. For both trials, plots were infested with a suspension of macroconidia and microconidia adjusted to 1.3×10^6 colony-forming units (CFU) /mL using a Neubauer chamber. This suspension was used in consecutive soil infestations with *F. oxysporum* f. sp. *phaseoli* (Sivan and Chet 1986), using a 3.8 L manual compression sprayer, made on 14 and 27 Nov 2009, 12 and 19 Dec 2009, 8 and 22 Jan 2010, 22 Apr 2010 and 20 May 2010. The last soil infestation was made in furrows simultaneously with the sowing of common bean susceptible cultivar BRS Valente (19 Dec 2009 and 22 Apr 2010). Each plot consisted

of two planting furrows of approximate 0.1 m depth, spaced 0.5 m, which received 15 seeds of susceptible cultivar BRS Valente per linear meter.

Thereafter, planting furrows were sprayed with *T. harzianum* isolates (1.2×10^{12} conidia/ha) using a handheld sprayer of 1.0 L. Immediately after spraying, furrows were covered with a 2–3 cm soil layer and sprinkle irrigated. Finally, to ensure *F. oxysporum* f. sp. *phaseoli* density in soil, an extra infestation was made 20 days after sowing (DAS) at V2 phenological stage, by careful addition to the bean lines chlamydospores and conidia obtained in sand:cornmeal, after adjustment to 1.5×10^{6} CFUs/mL.

Soil was prepared with a hoe and kept free of weeds throughout the experiment. Fertilization consisted of NPK (5-30-15 at 400 kg/ha) with nitrogen topdressing of 60 kg/ha at the V3 stage. Other cultural practices followed regional recommendations (Paula Júnior et al. 2008). The summer trial was rain fed with sprinkle irrigation used only when necessary to avoid water stress. The winter trial was irrigated daily, since the season is typically dry in central-western Brazil. Treatments were assigned to the same plots in the summer and winter trials.

The trials were conducted in a randomized block design with four replications. Both trials had control treatments with in-furrow spraying of sterilized water. Carboxin + thiram (300 mL/100 kg of seeds, containing 200 g/L of carboxin; 200 g/L of thiram) is commonly used by farmers of the country to manage soilborne diseases of common bean and so it was included as a treatment. Since we aimed to test whether *Trichoderma*-based treatments reduce Fusarium wilt as an eco-friendly approach, further in-furrow treatments with chemicals were not considered for testing. A negative control was included, that is, inoculated with neither the pathogen nor the antagonist.

Quantification of *F. oxysporum* and *Trichoderma* from rhizosphere soil

Composite samples with approximately 200 g of rhizospheric soil were collected at 0–10 cm depth to estimate initial and final populations of both *F. oxysporum* and *Trichoderma* spp. The same procedure was used to quantify population density of *F. oxysporum* during the crop cycle. Soil samples were carefully homogenized and stored at 4 °C, before laboratory procedures. Such procedures involved quantification of overall *F. oxysporum* and *Trichoderma* spp. soil populations in semi-selective media. Even though such media do not track specific *Trichoderma* strains or *Fusarium formae specialis*, they are useful to compare treatments according to the inoculum density of soil fungi (Bakarat and Al-Masri 2009; Zachow et al. 2009; Martínez-Medina et al. 2010; Zhou and Wu 2012).

Inoculum density of *F. oxysporum* during the course of the experiments was assessed by 1:100 serial dilution of soil samples in sterilized water and spread of 1 mL aliquots on Komada semi-selective media (20.0 g D-Galactose; 2.0 g L-Asparagine; 1.0 g KH_2PO_4; 0.5 g KCl; 0.5 g $MgSO_4.7H_2O$; 10 mg Fe_3Na EDTA; 18.0 g agar; 750 mg PCNB; 1.0 g $Na_2B_4O_7.10H_2O$; 1000 mL distilled water). With the same dilution procedure, density of *Trichoderma* spp. was estimated in TSM (0.12 g KH_2PO_4; 0.26 g $MgSO_4.7H2O$; 0.26 g KNO_3; 1.0 g $CaCl_2.2H_2O$; 1.0 g $Ca(NO_3)_2$; 0.05 g citric acid; 1.0 ml igepal; 2.0 g saccharose; 18.0 g agar; 0.0025 g vinclozolin; 1000 ml distilled water). *Trichoderma* spp. and *F. oxysporum* were identified according to colony morphology and morphological traits, and their population density was expressed in CFUs/g .

Disease assessment

Fusarium wilt incidence on common beans was assessed in all plants at 64–67 DAS (R7 phenological stage), always in the morning, based on the proportion of plants with typical disease symptoms. Further, wilt severity was estimated with an ordinal scale ranging from 1 to 9 (Pastor-Corrales and Abawi 1987), as follows: 1 – no visible symptoms; 3 – one to three leaves, representing no more than 10 % of total foliage with wilt and chlorosis; 5 and 7 – plants respectively with approximately 25 and 50 % of wilting and chlorotic leaves; and 9 - dead or severely infected plants with wilt, chlorosis, necrosis and/or premature defoliation. Disease scores were further used to calculate a disease severity index (DSI), according to McKinney (1923): (DSI)=[S (score in the scale × frequency) / (total number of units × maximum score in the scale)]×100.

Common bean yield

The crop was harvested at R9 stage in 14 Mar (summer trial) and 16 Jul (winter trial) 2010 and grain yield from all manually harvested plants from each plot was expressed in kg/ha. The number of seeds per pod and 100-grain mass were assessed in 10 plants selected at random from each plot.

Statistical analysis

Data of all variables were checked for normality (Shapiro-Wilk test) and further subjected to analysis of variance (Anova). Data on yield in the summer trial, wilt incidence in winter trial and pathogen population density in both trials were transformed to $\sqrt{x}+0.5$ in order to normalize data and stabilize variances prior to Anova. Data on the progress curve of *F. oxysporum* in soil was modeled using linear regression. The Dunnet test ($P\leq0.05$) was used to compare mean of each treatment with the control mean. Pearson's

correlation coefficient was calculated for all pairs of variables assessed in the trials. All statistical procedures were performed with R software (R Development Core Team, Vienna, Austria).

Results

Increasing levels of *F. oxysporum* were achieved by the sequential soil infestation and growth of a susceptible cultivar (BRS Valente), thus providing a disease-conducive environment. Endemic populations of *F. oxysporum* averaged around 50 CFUs/g (Fig. 1) prior to first infestation, and reached a maximum of nearly 300 CFUs/g. A second order linear model (r^2=0.96, $P \leq 0.01$) was fitted to data on population density of *F. oxysporum* in the check plots. In contrast, mean initial population of *Trichoderma* spp. was close to 10 CFUs, and therefore the differences in biocontrol efficacy were attributed to treatment effects.

All plots showed yellowed and wilted plants at the R7 stage in both trials, with varying disease incidence, even in the non-infested plots. The successful soil infestation led to 57.5 and 26.1 %, on average, of Fusarium wilt incidence and severity index, respectively, in the summer trial (Fig. 2). Disease levels increased 66.3 and 29.7 % on average for disease incidence and severity in the winter trial. In the Fop 46-infested control plots, wilt incidence was 2.97 and 5.83 times higher than non-infested plots, respectively in the summer and winter trials.

CEN287, CEN290 and CEN316 significantly reduced wilt incidence in the summer trial compared to the infested and non-treated control, while CEN288, CEN289, 1306 and carboxin + thiram did not differ from the control. In this season, infested plots averaged 82.4 % wilt incidence, with 36.8 mean disease severity. *Trichoderma*-based treatments reduced incidence and severity by 47.1 and 20.7 % (CEN287), 47.1 and 22.7 % (CEN290), 37.5 and 20.4 % (CEN316), respectively. The non-infested and non-treated control plot averaged 27.7 and 17.5 % of incidence and severity, respectively.

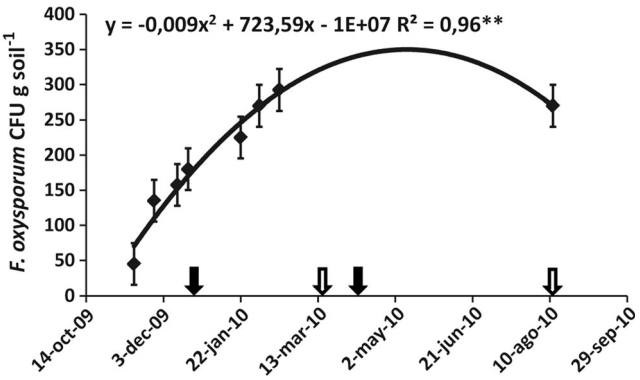

Fig. 1 Progress of *Fusarium oxysporum* colony forming units (CFUs)/g soil on control microplots infested with *Fusarium oxysporum* f. sp. *phaseoli* without any treatments. Black and white arrows indicate respectively planting and harvest dates of common bean 'BRS Valente', in two consecutive experiments. The adjusted model represents the buildup of *F. oxysporum* after standard soil infestation procedures in both trials: with a 1.3×10^6 macro and micro conidia /mL suspension of *F. oxysporum* f. sp. *phaseoli* at November 14, 27, December 12, 19 (2009) and January 8, 22 April 22 and May 20 (2010). Complementary soil infestation was done with a suspension of conidia and chlamydospores adjusted to 1.5×10^6 CFUs /mL (SC) on January 8 and May 20, 2010. **Significant according to F test ($P \leq 0.01$)

In the winter trial, with increased inoculum levels, all treatments showed high disease intensity levels despite the 15.9 % of symptomatic plants in non-infested and non-treated plot (Fig. 2). CEN290, 1306, CEN287 and CEN316 reduced disease incidence by 22.4, 26.7, 45.0 and 55.0 %, respectively, compared to the infested plot. Furthermore, CEN287 and CEN316 also achieved severity similar to the negative control (correspondingly 24.3, 20.9 and 14.6 %), lower than others treatments, where severity ranged from 28.3 to 40.5 %. Moreover, other *Trichoderma* treatments did not differ from infested control. In both trials, means of wilt incidence and DSI did not differed between fungicide-treated and the infested control.

Common bean mean yield ranged from 2941 to 3664 kg ha^{-1} and 3336 to 3948 kg ha^{-1}, respectively in the summer and winter trial, without differences among treatments. Also, there were no differences concerning yield components, in both trials. The pathogen/antagonist ratio was not affected by growing season and data from both trials were combined for analysis (Fig. 3). Isolates CEN 288 and CEN290 were ineffective to reduce Fusarium wilt. With the exception of these two isolates, for both trials, the highest population density of *Trichoderma* spp. corresponded to the lowest *F. oxysporum* density, assessed in treatments at crop harvest.

Regarding the *F. oxysporum* × *Trichoderma* spp. relationship, treatments were apparently clustered in four different groups: 1) enduring population of *Trichoderma* spp. with higher reduction of *F. oxysporum* on CEN287 treatment, corresponding to the best biocontrol results of Fusarium wilt; 2) CEN289, CEN316 and 1306 isolates forming an intermediate group, with recovered populations of *Trichoderma* spp. between 40 and 80 CFUs; 3) infested control with the pathogen; and 4) chemical seed treatment, with the smallest population of *Trichoderma* spp. and the highest of *F. oxysporum.* The final population of *F. oxysporum* in the Fop 46-infested plots treated with *T. harzianum* CEN287 (62 CFUs) was at levels similar to the initial density of *F. oxysporum* determined prior to the establishment of the trials.

Correlation analysis showed distinct associations among disease variables, fungal populations and yield components (Table 1). *F. oxysporum* density in soil was positively correlated to Fusarium wilt incidence and severity in the summer 2009/2010 (ρ=0.78 and ρ=0.66, respectively), but negatively associated to 100-grain mass (ρ=−0.71) and number of grains per pod (ρ=−0.69) in the same trial. In 2010 winter, 100-grain

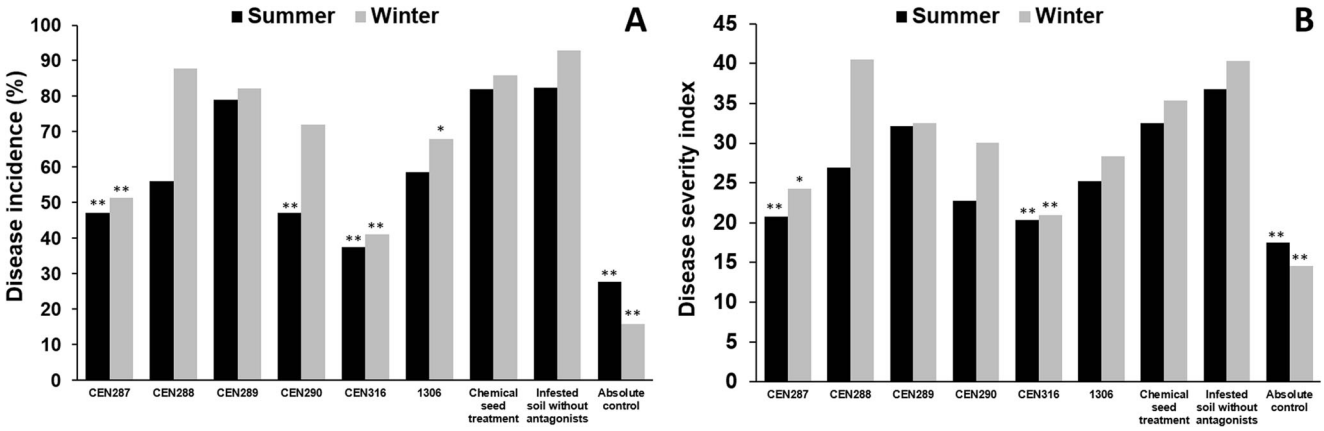

Fig. 2 Effect of *Trichoderma harzianum* isolates on Fusarium wilt incidence (**a**) and disease severity index (**b**), assessed in field microplots cropped with common bean 'BRS Valente', in the summer 2009/2010 and winter 2010 season. * and ** are significant at 5 and 1 %, according to the Dunnet test, in comparison to non-treated infested microplots

mass and number of grains per pod were positively correlated to *Trichoderma* spp. density in soil, respectively with ρ=0.67 ($P \leq 0.05$) and ρ=0.97 ($P \leq 0.01$). Also in the winter trial, the number of pods per plant was negatively correlated to DSI (ρ=0.74, $P \leq 0.05$).

Discussion

Successive sowing of annual crops in our study increased density of *Fusarium* population in soil at levels similar to previous reports of increase of *F. solani* f. sp. *phaseoli* population (up to 400 propagules/g of soil) after the first growing of common bean (Hall and Phillips 1992). Although the plating methods used here did not allow to differentiate Fop 46 isolate from endemic *F. oxysporum* isolates, the inoculum density of

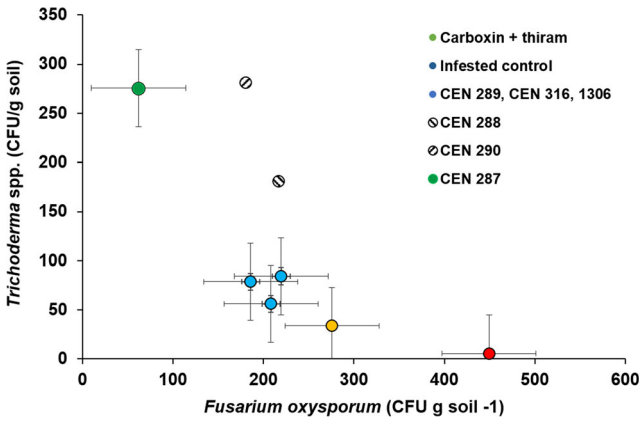

Fig. 3 Scatter plot of *Fusarium oxysporum* density in relation to *Trichoderma* spp. density on experimental microplots after in-furrow treatment with *Trichoderma harzianum* isolates, in contrast to seeds treated with carboxin + thiram and control plots. Fungal populations were assessed at harvest of common bean 'BRS Valente', in a joint analysis with results from 2009/2010 (summer) and 2010 (winter) season

wilt-booster isolates on infested control was sufficient to achieve disease incidence at levels well above the non-infested plots. With the natural increase of *F. oxysporum* f. sp. *phaseoli* in infected plants and survival in soil, the soil infestation during winter trial was probably ineffective, as *Fusarium* populations almost reached a plateau in the second bean-growing season.

The pod-filling (R7) is the growth stage when treatments aiming to reduced inoculum density and vascular wilt progress are evaluated (Toledo-Souza et al. 2012). In the case of biocontrol treatments, antagonist survival until such advanced stage of crop development is a quite needed trait. Despite the nearly 300 CFUs/g of *F. oxysporum* during the onset of the winter trial, and the former Fusarium wilt records, the disease development followed the same patterns in this season (Fig. 2), probably favored by temperatures around 20 °C and high soil moisture.

Correlation analysis supported biocontrol results, and the relevance in reducing the pathogen inoculum density to improve disease control and increase yields. CEN287 and CEN316 were the most effective isolates to reduce wilt incidence and DSI in the two trials. Among the six *T. harzianum* isolates, only these two have shown hyperparasitic capacity against *F. oxysporum* f. sp. *phaseoli* (Carvalho et al. 2014). Their higher effectiveness relates to the fact that biocontrol does not rely solely on plant-microorganism interactions, but also on the ecological adaptability required to survive on soil and protect infection sites (Alabouvette et al. 2009; Longa et al. 2009). Therefore, our findings suggest that only *T. harzianum* CEN287 fulfilled the requirements for plant protection and antagonist survival in soil, in comparison to other treatments.

Although differences up 723 kg/ha in yield were found among treatments, there was no significant difference corroborating previous findings (Hoyos-Carvajal et al. 2009; Vinale

Table 1 Pearson's correlation coefficients for all pairs of variables - Fusarium wilt incidence (INC), disease severity index (DSI), number of pods per plant (NP), 100-grain mass (GM), crop yield (YLD), number of grains per pod (NGP), density (units/g soil) of *Fusarium oxysporum* (Fox) and *Trichoderma* spp. (Tricho) – evaluated in two experiments conducted in the summer (shaded lower left panel) and winter (non-shaded upper right panel) of 2010 year. Numbers in bold represent significance at 5 % probability

	INC	SEV	NP	GM	YLD	NGP	*Foxy*	*Tricho*
INC		**0.98**	**-0.63**	-0.24	-0.26	-0.01	0.58-	-0.01
SEV	**0.95**		**-0.74**	-0.27	-0.43	-0.08	0.53	-0.08
NP	-0.30	-0.16		0.56	**0.79**	0.47	-0.51	0.52
GM	-0.34	-0.21	0.57		0.23	0.49	-0.60	**0.67**
YLD	-0.30	-0.16	**0.99**	0.57		0.37	-0.06	0.36
NGP	-0.27	-0.10	**0.90**	0.56	**0.89**		-0.24	**0.97**
Foxy	**0.78**	**0.66**	-0.59	**-0.71**	-0.58	**-0.69**		-0.36
Tricho	-0.32	-0.22	0.59	-0.11	0.58	0.52	-0.27	

et al. 2009). Previous studies reported significantly greater yield return for crops at stressed rather than non-stressed environments (Harman et al. 2004).

The final population of *Trichoderma* spp. in plots treated with CEN287 was seven times higher than the infested control plots, and corresponded to the lowest population density of *F. oxysporum*. These results are also in accordance with Mohamed and Haggag (2006), who reported reductions in *Fusarium oxysporum* f. sp. *lycopersici* levels under greenhouse and field soil using seeds treated with *T. harzianum* and respective reductions in tomato wilt incidence.

In summary, we demonstrated the effectiveness of biocontrol of Fusarium wilt of common bean with *T. harzianum*. The in-furrow application of biocontrol treatments provided satisfactory control using CEN287 and CEN316 isolates at levels above seed treatment with fungicides. These findings corroborate results by Prasad et al. (2002) and Bora et al. (2004), and suggest this strategy as a feasible alternative for managing Fusarium wilt. Other practices could be integrated into a biocontrol program aiming to improve crop health and providing new standards of disease management where other techniques are inefficient.

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