

Tropical Plant Pathology



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CARVALHO, Daniel D. C. et al. Biological control of *Fusarium oxysporum* f. sp. phaseoli by *Trichoderma harzianum* and its use for common bean seed treatment. **Tropical Plant Pathology**, Brasília, v. 39, n. 5, p. 384-391, out. 2014. DOI: <http://dx.doi.org/10.1590/S1982-56762014000500005>. Disponível em: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1982-56762014000500005&lng=en&nrm=iso. Acesso em: 30 out. 2019.



Biological control of *Fusarium oxysporum* f. sp. *phaseoli* by *Trichoderma harzianum* and its use for common bean seed treatment

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ABSTRACT

Biological control of seed-borne pathogens has shown to enhance germination and physiological quality of seeds. The objectives of this study were to evaluate the *in vitro* antagonistic effect of five *Trichoderma harzianum* isolates (CEN287, CEN288, CEN289, CEN290, and CEN316) against *Fusarium oxysporum* f. sp. *phaseoli* (Foxy) and test its potential use in seed treatment. Initially, pathogen and antagonists were grown in paired cultures at 25°C, from which samples were assessed using scanning electron microscopy (SEM). Then, clean or Foxy-infected seeds were treated with conidial suspension of the antagonists. Percent of Foxy-infected seeds and normal seedlings were evaluated at seven and nine days of incubation, respectively. All but one *Trichoderma* isolate (CEN290) inhibited Foxy mycelial growth. SEM analysis revealed that only one *Trichoderma* isolate (CEN287) showed parasitic interaction with Foxy. Two isolates (CEN287 and CEN316) significantly reduced the Foxy incidence and enhanced seed germination, though less effective than the fungicide mixture (carboxin + thiram). A principal component analysis indicated the importance of volatile metabolites in reducing Foxy incidence on common bean seeds. CEN287 *Trichoderma harzianum* isolate formed a single group due to its increase in germination rate of Foxy-infected seeds.

Key words: *Phaseolus vulgaris*, seed pathology, soil-borne disease

INTRODUCTION

Infected seeds are considered the main inoculum source and mean of dispersal of *Fusarium oxysporum* f. sp. *phaseoli* J.B. Kendr. & W.C. Snyder. This fungus causes the Fusarium wilt disease, and is among the most damaging pathogens of common bean (*Phaseolus vulgaris* L.) in Brazil (Carvalho et al., 2011a). Following its introduction into an area, Fusarium wilt may appear in small, isolated foci and, after several seasons, spread throughout the entire area (Abawi & Pastor-Corrales, 1990). The disease is difficult to control and there are a few effective management strategies (Hall & Nasser, 1996). Fungicides are not recommended for the management of vascular wilts (except for seed treatment), since they may not prevent plant infection and subsequent phloem colonization. Biological control with *Trichoderma* species has contributed to reduce Fusarium wilt incidence mainly when applied on seeds (Carvalho et al., 2011a; Carvalho et al., 2011b).

Competition, parasitism and antibiosis are likely mechanisms involved in the antagonistic activities of *Trichoderma* spp. (Alabouvette et al., 2009). These fungi

can establish on the seed surface, and colonize and reproduce in the rhizosphere (Carvalho et al., 2011a). In recent years, biofungicides have been developed and commercialized worldwide (Shali et al., 2010). Due to the negative aspects involved in the use of agrochemicals such as contamination of the environment with toxic residues that may affect humans and animals (Carvalho et al., 2007), the use of biofungicides in seed treatments has been expanded to many crops (Corrêa et al., 2008), which is true especially in Brazil where *Trichoderma* spp. is the most used microorganism in bioformulations (Carvalho et al., 2011a).

Systematic screening and selection of potential biocontrol agents should be performed as a first step prior to their use for treating seeds. However, few are the reports of the biological control of Fusarium wilt on various crops, particularly of the efficiency of *Trichoderma* spp. for controlling *F. oxysporum* f. sp. *phaseoli* on common bean, as well as its potential for seed treatment. Therefore, our study aimed to evaluate the *in vitro* antagonistic effect of five selected isolates of *T. harzianum* (Carvalho et al., 2011a) against *F. oxysporum* f. sp. *phaseoli* and to test its potential for disease control based on their application on seeds of common bean.

MATERIALS AND METHODS

Fungal isolates

The five *T. harzianum* isolates (CEN287, CEN288, CEN289, CEN290, and CEN316) used in this work was obtained from the Biological Control of Plant Pathogens Collection of Embrapa Recursos Genéticos e Biotecnologia (Brasília, DF, Brazil) and were collected at the Brazilian Cerrado region. The isolate Fop 46 of *F. oxysporum* f. sp. *phaseoli* was obtained from the collection of Embrapa Arroz e Feijão (Santo Antônio de Goiás, GO, Brazil). Cultures stored in liquid nitrogen were recovered on Potato Dextrose Agar (PDA).

Plate confrontation assays

The *Trichoderma* spp. isolates were evaluated for antifungal activity against *F. oxysporum* f. sp. *phaseoli*, *in vitro*, according to Dennis & Webster (1971). Mycelial plugs (5 mm diameter) of the pathogen, isolate C-25-02, were positioned on one half portion of the PDA plates near the agar edge, and allowed to pre-establish for three days. Then, a 5 mm mycelial plug of the antagonist was positioned on the other half portion of the plate. Controls containing only *F. oxysporum* f. sp. *phaseoli* plugs on both half portions of the PDA plates were also set up. After five days at 25°C and 12 h photoperiod, the radial growth of *F. oxysporum* f. sp. *phaseoli* colonies were measured and, at nine days of simultaneous growth, the tested *T. harzianum* isolates were classified for their level of antagonism according to the scale of Bell et al. (1982). The experiment was conducted twice, each with four replicates.

Scanning electron microscopy (SEM)

Agar plug samples (5 mm) from the confrontation zone (five day paired cultures) were removed and fixed in a modified Karnovsky solution (2.5% glutaraldehyde, 2% paraformaldehyde in 0.05 M sodium cacodilate buffer at pH 7.2 containing 0.001 M CaCl₂) at 4°C for 24 h. This was followed by four washings using the same buffer for 30 min and post-fixation for 1 h at 4°C in 1% osmium tetroxide in 0.05 M sodium cacodilate buffer at pH 7.2. Samples were then washed three times using distilled water, and dehydrated in an ethanol gradient (10, 20, 30, 50, 70, 80, 90, 95 and 100%). The specimens were dried with carbon dioxide in a critical point dryer (Elmitech K850), mounted on aluminum stubs with double-sided tape and coated by vacuum evaporation in an Elmitech k550 gold evaporator. Samples were visualized in a ZEISS DSM962 scanning electron microscope, including all replicates from the plate confrontation assays.

Action of volatile and non-volatile metabolites of *T. harzianum* on *F. oxysporum* f. sp. *phaseoli*

For volatile metabolites (VM), the bottom of a Petri dish containing fresh culture of the antagonist inoculated on PDA and a similar Petri dish bottom containing three-

day cultures of *F. oxysporum* f. sp. *phaseoli* (C-25-02) were joined. A transparent plastic film was used to seal the two plates facing each other, with the pathogen culture at the upper dish. After five days at 25°C and 12 h photoperiod, the radial growth of the *F. oxysporum* f. sp. *phaseoli* colonies was measured. The average values of percent inhibition were obtained in respect to the control (without *Trichoderma*).

To test the action of non-volatile metabolites (NVM), *Trichoderma* isolates were grown in potato dextrose broth for five days at 25°C in the dark and 250 rpm shaking. Culture supernatant was filtered using a 22 nm Millipore syringe filter and added to melted PDA (2.8% agar) at a ratio of 25% (Carvalho et al., 2011b). Then, mycelial plugs (5 mm diameter) of *F. oxysporum* f. sp. *phaseoli* (C-25-02) colonies were placed in the center of each Petri dish containing the medium supplemented with antagonist cultural filtrate. Controls consisted of PDA plates, in which sterile water replaced the cultural filtrate. Colonies were incubated at 25°C and in a 12 h photoperiod until the control plates were fully colonized, when radial growth of the pathogen was measured. The VM and NVM experiments were carried out twice with four replicates and radial growth inhibition values, compared to controls, were converted to percentages.

Spore production of *T. harzianum*

Seven-days-old agar plugs (5 mm) of the antagonists were transferred to 250 mL flasks (six plugs per flask), containing parboiled rice (15g per flask) that had been previously moistened (60% p v⁻¹) and autoclaved (121°C for 40 min). Flasks were kept at 25°C with a 12 h photoperiod. After six days, spores were collected by flushing with distilled water and filtered through sterile gauze. The conidial concentration was measured using a Neubauer chamber.

Infection of seeds with *F. oxysporum* f. sp. *phaseoli*

The water restriction technique was used to obtain seeds contaminated with *F. oxysporum* f. sp. *phaseoli* (isolate Fop 46), using PDA medium + mannitol at -1.0 MPa. After cultivation of the pathogen (25°C and 12 h photoperiod), clean common bean seeds cv. 'BRS Valente' (56g) were placed on top of the colonies and incubated for five days at 25°C.

Detection of *F. oxysporum* f. sp. *phaseoli* on seeds treated with *T. harzianum*

This experiment was conducted with the five *T. harzianum* isolates and Trichodermil® SC, a commercial isolate of the same fungal species (Itaforte Bioprodutos, Itapetinga, SP, Brazil). The common beans seeds cv. 'BRS Valente' contaminated by *F. oxysporum* f. sp. *phaseoli* (Fop 46) were treated with 2 mL 100g⁻¹ antagonist suspension (2.5 x 10⁸ conidia mL⁻¹) and were distributed onto layers of germitest paper (44.0 x 34.0 cm) moistened

RESULTS

with distilled water, according to the RAS (2009). Rolls of germitest paper containing the seeds were kept in a growth chamber at 20°C, 98% relative humidity in the dark. After seven days, seeds were observed for the presence of aerial mycelium of *F. oxysporum* f. sp. *phaseoli* around the infected seeds and seedlings. Typical monophialides, microconidia and macroconidia of *F. oxysporum*, were also observed using a Zeiss Stemi DV4 stereoscopic microscope (Leslie & Summerell, 2006; RAS, 2009). To confirm the fungus species, semi-permanent microscope slides were prepared with the biological material for examination under an optical microscope (Nikon Eclipse 55i). The experiment was performed twice, using four replicates of 50 seeds, in a randomized block design. Seeds treated with Carboxin + Thiram (300 mL 100 kg⁻¹ of seeds, containing 200 g L⁻¹ of Carboxim; 200 g L⁻¹ of Thiram) and untreated seeds were used as positive and negative controls, respectively.

Effect of *T. harzianum* on common bean seed germination

This test was conducted twice, with the same treatments and replicates mentioned above. Briefly, the prepared germitest rolls were kept in a germinator at 25°C, for nine days. Evaluations were made in terms of normal seedlings number, absence or presence of necrosis and pathogen signals, abnormal seedlings, and dead seeds, according to the RAS (2009).

Statistical analysis

The data were subjected to analysis of variance (ANOVA) and to the Scott-Knott test ($P \leq 0.05$). Regression analysis was performed for all variables, using SISVAR software (Ferreira, 2011). Also, principal component analysis (PCA) was used to analyze the effect of all variables of the confrontation plate assay, volatile and non-volatile metabolites, incidence of *F. oxysporum* on seeds, percentage of normal seedlings from seeds inoculated and non-inoculated with *F. oxysporum* f. sp. *phaseoli*. The PCA analysis was conducted using the FactoMineR package of R (R Development Core Team, Vienna, Austria).

Results showed that all *T. harzianum* isolates inhibited mycelial growth of the pathogen. Mean scores for antagonism were similar among the *T. harzianum* isolates (Table 1); all antagonists colonized around 70% of the surface of the medium at the 9th day of growth.

The microscopic examinations of the confrontation zones showed antagonistic interactions between *T. harzianum* isolate CEN287 and *F. oxysporum*, evidenced by hyphal encoiling (Figures 1A and 1B) (Benhamou & Chet, 1993). Emergence of conidiogenous cells with conidia of the antagonist along the hyphae of the pathogen was also observed (Figures 1C and 1D).

All but the CEN290 isolate showed antifungal action in the VM assay (Table 2). The percentages of inhibition ranged from 23 and 40%, but differences among *T. harzianum* inhibiting the pathogen were not significant ($P \leq 0.05$). In the NVM assay, CEN316 and CEN289 showed significantly higher growth inhibition (26 and 20%, respectively) than the other antagonists and the control. VM from CEN287 and CEN288 were more inhibiting to the pathogen growth than their NVM.

In the seed assay, germination was reduced in the non-treated seeds inoculated with *Fusarium oxysporum* f. sp. *phaseoli* (Table 3). Seeds treated with the Carboxin + Thiram mixture showed 66% reduction in the incidence of *F. oxysporum* f. sp. *phaseoli*. Although none of the biological treatments was as efficacious as the fungicide mixture, CEN287 and CEN316 differed significantly from the negative control and the other isolates, reducing the incidence of disease by 40 and 31%, respectively (Table 3). As to germination (% of normal seedlings) CEN287 performed similar to Carboxin + Thiram. The CEN287 isolate colonized 'BRS Valente' seedlings that developed from the Fop-infected seeds (Figure 2). The effect of the pathogen on seeds was also demonstrated by a linear negative relationship between the incidence (%) of *F. oxysporum* f. sp. *phaseoli* on seeds and the emergence of normal seedlings from contaminated seeds ($y = -0.2471x + 61.3299$; $R^2 = 64.87\%$; $P \leq 0.01$).

TABLE 1 - *In vitro* antagonism (confrontation plate assay) of *Trichoderma harzianum* against *Fusarium oxysporum* f. sp. *phaseoli*.

Isolate of <i>T. harzianum</i>	Mycelial growth inhibition of <i>F. oxysporum</i> (%) at the 5th day ^(a)	<i>T. harzianum</i> antagonism class by the 9th day ^(a, b)
CEN287	51.8	2.0
CEN288	54.4	2.0
CEN289	54.1	2.0
CEN290	53.3	2.0
CEN316	54.1	2.3
Coefficient of variation (%)	2.44	10.22

^(a)Not significant according to analysis of variance ($P \leq 0.05$).

^(b)Antagonism according to Bell et al. (1982): Class 1: *Trichoderma* completely colonizes the pathogen and covers the whole surface of the medium; Class 2: *Trichoderma* colonizes 2/3 of the surface of the medium; Class 3: *Trichoderma* colonizes 1/2 of the surface of the medium; Class 4: *Trichoderma* colonizes 1/3 of the surface of the medium; Class 5: the pathogen completely colonizes the antagonist and covers the whole surface of the medium.

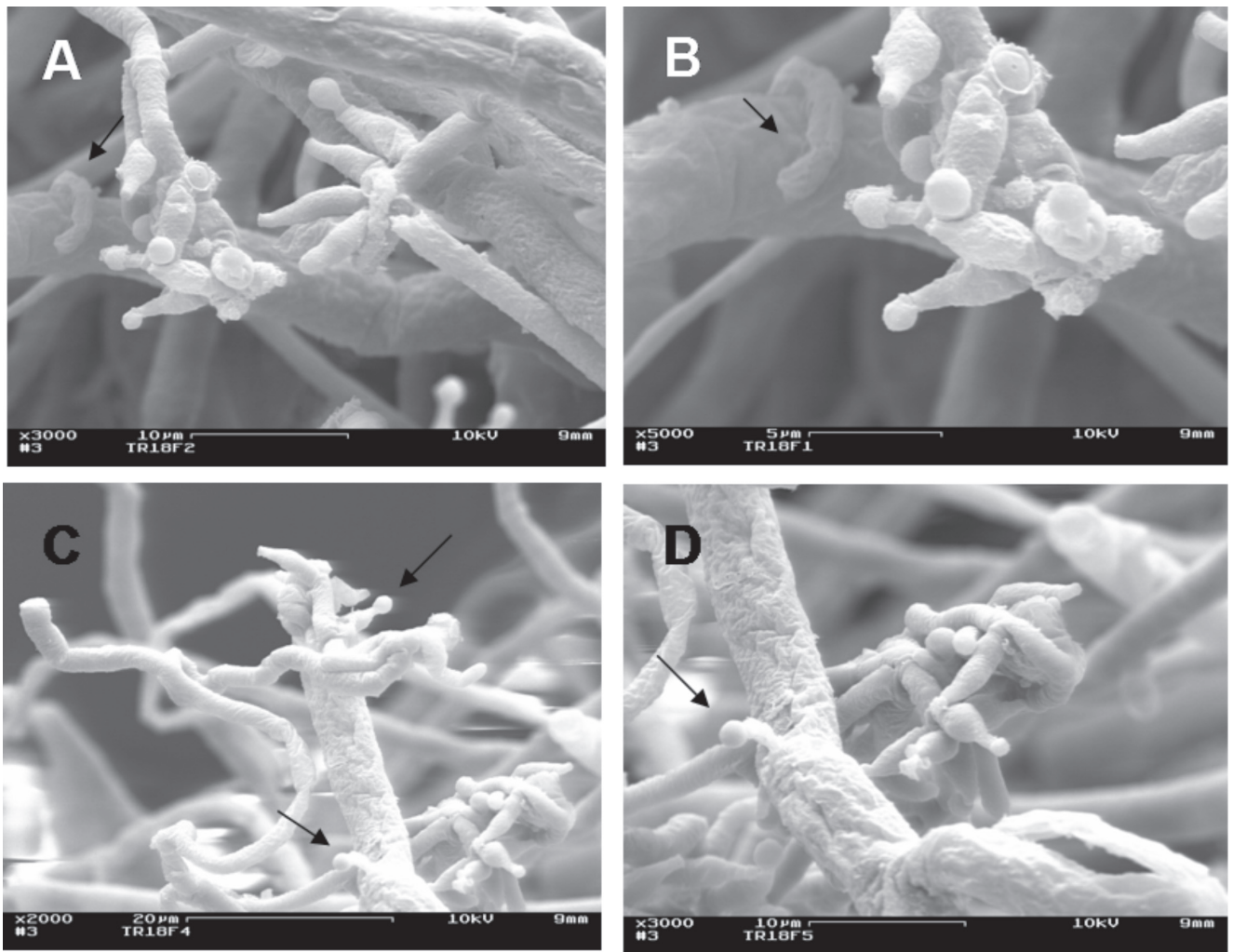


FIGURE 1 - Scanning electron micrographs showing interactions between *Trichoderma harzianum* (CEN287) and *Fusarium oxysporum* f. sp. *phaseoli* (C-25-02): **A**. Arrow showing the pathogen encircled by the antagonist; **B**. encircling in greater detail; **C**, **D**. conidiogenous cell with conidia of *Trichoderma* emerging from hyphae of *F. oxysporum*.

The multivariate analysis showed that 76.09% of the variance was explained by the first two components (54.22% and 21.87%) (Figure 3). Eigenvalues of the first and second components were 3.25 and 1.31, respectively. Among the variables, *Fusarium oxysporum* incidence (%) was the only that showed influence on variance in PCA; 0.866 was the correlation of this variable in the biocontrol of *F. oxysporum* ($P \leq 0.05$).

Mycelial growth inhibition of *F. oxysporum* (%) at the 5th day in the confrontation plate assay was negatively correlated with mycelial growth of *F. oxysporum* in the presence of *T. harzianum* non-volatile metabolites (NVM) (Figure 3). Similarly, percentage of normal seedlings from seeds inoculated with *F. oxysporum* (inoculated seeds) was negatively correlated with *F. oxysporum* incidence (Foxy incidence). On the contrary, *F. oxysporum* incidence was positively correlated with VM.

DISCUSSION

Several can be the modes of action that inhibit the growth of *F. oxysporum* f. sp. *phaseoli* by *T. harzianum* isolates, such as competition for nutrients and space, mycoparasitism and antibiosis (Alabouvette et al., 2009). In the NVM tests, CEN316 and CEN289 inhibited pathogen growth by 26 and 20%, respectively, and also performed similarly in the VM test. Antibiosis, whether by VM or NVM, plays an important role in biological control, which can act together with nutrient competition and with mycoparasitism, leading to higher levels of antagonism (Lorito et al., 1994). Notably, greater inhibition of the pathogen growth was observed in the VM tests than in the NVM tests, though this warrants further investigation in order to determine the influence of inhibitor dosage and stability.

TABLE 2 - Inhibition of growth of *Fusarium oxysporum* f. sp. *phaseoli* by volatile (VM) and non-volatile (NVM) metabolites of *Trichoderma harzianum*.

Isolate of <i>T. harzianum</i>	Mycelial growth (% of control) of <i>F. oxysporum</i> in the presence of <i>T. harzianum</i> metabolites ^(a)	
	VM(% ^(b))	NVM (% ^(b))
CEN287	64.6 aA	96.4 bB
CEN288	60.6 aA	89.9 bB
CEN289	73.2 aA	80.1 aA
CEN290	100.0 bA	95.2 bA
CEN316	77.6 aA	74.2 aA
Control	100.0 bA	100.0 bA
Mean	75.2 A	87.2 B
Coefficient of variation (%)	19.84	11.90

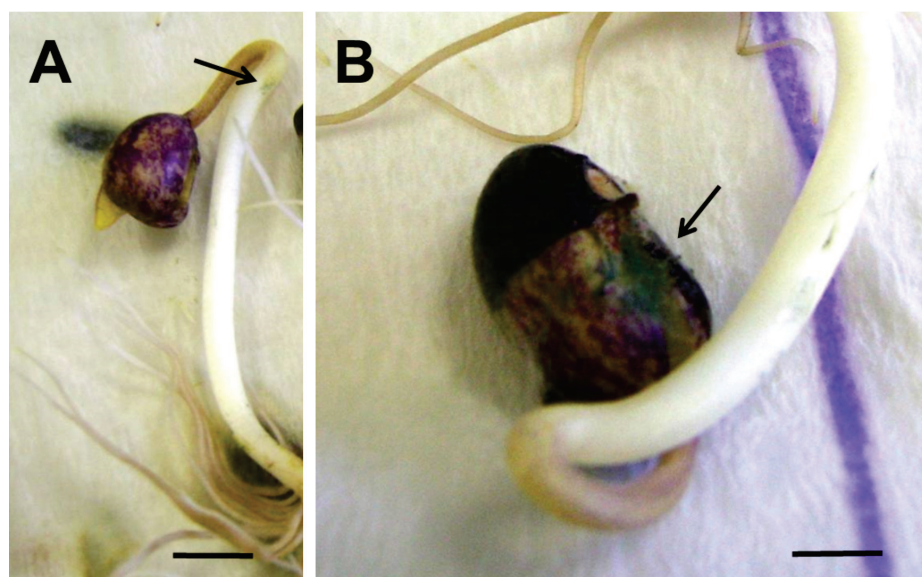
^(a)Means followed by the same letters, lowercase in the columns and uppercase in the rows, do not differ significantly, according to the Scott-Knott test ($P \leq 0.05$).

^(b)Values compared to the Control, obtained from 5 and 9 day colonies for VM and NVM, respectively.

TABLE 3 - Effect of treatments (*Trichoderma harzianum* isolates from this study, a commercial *Trichoderma harzianum* isolate and fungicide) on the incidence of *Fusarium oxysporum* f. sp. *phaseoli* on seeds, on the germination of contaminated seeds, and on seedling growth from healthy seeds of variety BRS Valente.

Treatment	<i>Fusarium oxysporum</i> f. sp. <i>phaseoli</i> incidence (% ^(a))	Germination (% of normal seedlings) ^(a)	
		Inoculated with <i>F. oxysporum</i> f. sp. <i>phaseoli</i>	Uninoculated seeds
CEN287	56.0 b	51.5 aA	92.5 B
CEN288	89.5 d	43.5 bA	94.0 B
CEN289	74.0 c	42.0 bA	92.5 B
CEN290	82.0 c	35.0 bA	94.0 B
CEN316	63.5 b	43.5 bA	93.0 B
Commercial isolate	74.5 c	40.5 bA	90.0 B
Carboxin+Thiram	31.5 a	54.0 aA	91.0 B
Control	92.0 d	41.5 bA	93.5 B
Mean	-	43.9 A	92.6 B
Coefficient of variation (%)	8.22	13.81	4.24

^(a)Means followed by equal letters, lowercase in the columns and uppercase in the rows, do not differ according to Scott-Knott test ($P \leq 0.05$).

**FIGURE 2** - *Trichoderma harzianum* (CEN287) colonizing common bean cv. 'BRS Valente' seedlings from seeds previously contaminated with *Fusarium oxysporum* f. sp. *phaseoli* (Fop 46): **A**, arrow shows hypocotyl colonized by the antagonist; **B**, cotyledons extensively colonized by the antagonist after its application on the seeds (Bars to figures **2A** and **2B** corresponding to 7.5 mm and 4.0 mm, respectively).

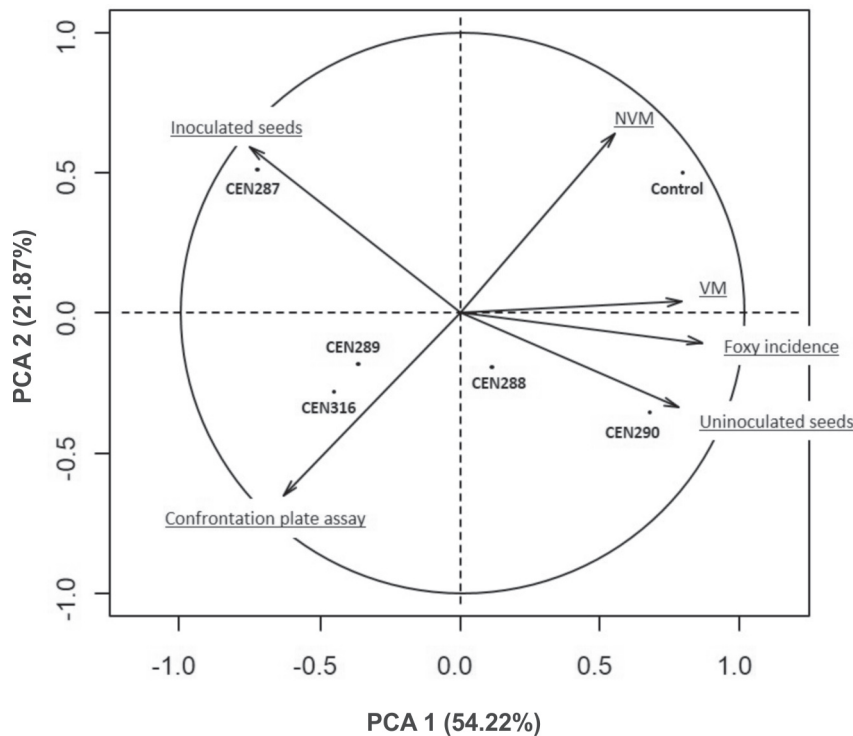


FIGURE 3 - Principal component analysis (PCA) of the following traits. Confrontation plate assay: in vitro antagonism at the 5th day shown in the Table 1; VM: volatile metabolites; NVM: non-volatile metabolites; Foxy incidence: *Fusarium oxysporum* incidence; Inoculated seeds: percentage of normal seedlings from seeds inoculated with *F. oxysporum* and; Uninoculated seeds: percentage of normal seedlings from seeds non-inoculated with *F. oxysporum*, investigated for biological control of *Fusarium oxysporum* f. sp. *phaseoli* by *Trichoderma harzianum*, according to the first two main components.

The multivariate data analysis with PCA showed that VM explained most of variation in *F. oxysporum* f. sp. *phaseoli* incidence in common bean seeds. Agüero et al. (2008) reported antibiosis as the main mechanism involved in the control of *Aspergillus flavus* in maize seeds. Exposure of *A. flavus* to volatile metabolites produced by *T. harzianum* reduced 31.7% of pathogen biomass in seeds (Agüero et al., 2008). There is no report of NVM as a key mechanism in the biological control of seedborne pathogens. Furthermore, it is worth mentioning that VM has advantages over NVM, since VM have greater capacity for soil diffusion, when soluble in water (Mangenot & Dien, 1979; Lobo Junior & Abreu, 2000).

Confrontation plate assay is frequently used for screening of biological control agents (Carvalho et al., 2011b; Lopes et al., 2012). PCA analysis indicated that NVM were negatively correlated with confrontation plate assay. In the confrontation plate assay, hyperparasitism was observed microscopically as well as the NVM effects and nutrient competition. The isolates CEN289 and CEN316 grouped together in the multivariate analysis, highlighting their similarity, differing from CEN287, which more efficiently increased the percentage of seed germination.

VM are often considered a key mechanism in the biological control of seed-borne pathogens by fungal antagonists (Agüero et al., 2008; Lopes et al., 2012). However, the most efficient among our *Trichoderma* strains, CEN287, behaved similarly to the other strains regarding VM. Thus, the combination of mycoparasitism, antibiosis,

and fast coverage of the seed surface (data not shown) seemed to be associated with the greater antagonistic efficiency of CEN287 compared to the other isolates. These mechanisms may also contribute to the escape mechanism as reported by Mertz et al. (2009), wherein pathogens may be limited to the seed coat, preventing transmission during emergence and the detachment of cotyledons, resulting in healthy seedlings.

The interactions observed in the SEM analysis can be considered as typical of hyperparasitism (Agrios, 2005). Similarly, Louzada et al. (2009) observed hyphae of *F. solani* encircled by *Trichoderma* in SEM images. As reported by Zeilinger & Omann (2007), during mycoparasitism, lectines from the cell wall of the pathogen can induce the association of antagonist hyphae with the pathogen and, consequently, hyphae of the pathogen may be colonized. Furthermore, intrahyphal colonization can also occur (Benhamou & Chet, 1993), as also observed in our study. Descriptions of encircled hyphae are common in many papers on mycoparasitism, but intrahyphal colonization is not commonly reported for antagonistic interactions of *Trichoderma* and other fungi.

As suggested in a previous study (Carvalho et al., 2011a), seed treatments with 2 mL of conidial suspension at 2.5×10^8 conidia mL⁻¹ of *T. harzianum* per 100g of seeds did not cause any adverse effects on the germination of common bean seeds. The fungus *F. oxysporum* f. sp. *phaseoli* reduced 55% of normal seedlings of common bean, thus efficient biological control of *F. oxysporum* f. sp. *phaseoli* on seeds,

even at relatively low rates, can reduce the introduction and dissemination of the pathogen into pathogen-free areas, preventing yield losses. Harman et al. (2004) reported efficient dissemination of the antagonist on corn seeds treated with *T. harzianum*, which resulted in seedling roots colonized by the antagonist. This characteristic is among the key features for selecting potential biocontrol agents for the control of seed-borne diseases. Khan et al. (2004) inoculated *T. harzianum* on chickpea seeds for the control of chickpea wilt caused by *F. oxysporum* f. sp. *ciceris* and observed a 60% decrease in the wilt incidence 45 days after sowing; the use of chemical control (carbendazim fungicide at 2 g kg⁻¹ seeds) in that study reduced only 40% of the wilt incidence. Furthermore, a drastic reduction in pathogen cfu's in the rhizosphere was also obtained by treatment with *Trichoderma* (Khan et al., 2004). Similarly, in the present study, strain CEN287 significantly reduced the incidence of *F. oxysporum* f. sp. *phaseoli* on seeds. The top-ranked *T. harzianum* isolate, CEN287, incorporated at least three attributes (hyperparasitism capacity, VM and NVM metabolites production and hypocotyl colonization), all contributing to its greater efficacy in the biocontrol of bean seeds infected with *F. oxysporum* f. sp. *phaseoli* compared to what is commercially available.

Similarly to the chemical treatment, *T. harzianum* (CEN287) was able to significantly reduce incidence of *F. oxysporum* f. sp. *phaseoli* on seeds of common bean 'BRS Valente' and promoted vigorous plant growth. Excepting CEN290, all *Trichoderma* isolates in this study were capable of showing antibiosis against the pathogen (VM or NVM). Nevertheless, strain CEN287 was more efficient than the other strains in the seed treatment assays; it was able to establish on, and colonize the surface of common bean seeds, as well as hypocotyls. The multivariate analysis suggested the importance of volatile metabolites in reducing *F. oxysporum* incidence on common bean seeds, which should be further explored.

ACKNOWLEDGEMENTS

The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Fundação de Apoio à Pesquisa do Distrito Federal (FAP-DF) for the financial support.

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TPP-2013-0220

Submitted: 17 December 2013

Revisions requested: 10 March 2014

Accepted: 6 May 2014

Section Editor: Marciel J. Stadnik