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Antagonist activity of *Trichoderma harzianum* against *Sclerotinia sclerotiorum* from common bean

Daniel Diego Costa Carvalho[1], Paulo Henrique Pereira Costa Muniz[1], Thiago Alves Santos de Oliveira[2], Irene Martins[3], Sueli Corrêa Marques de Mello[3]

[1]Universidade Estadual de Goiás, Câmpus Ipameri. Rodovia GO 330, km 241, Anel Viário, Setor Universitário, 75780-000, Ipameri, Goiás, Brasil.
[2]Universidade Estadual de Feira de Santana, Feira de Santana, Bahia, Brasil.
[3]Embrapa Recursos Genéticos e Biotecnologia, Brasília, Distrito Federal, Brasil.

E-mail autor correspondente: daniel.carvalho@ueg.br
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Abstract: A key point in the development of new biopesticides is the discovery of strains with greater activity or more adapted to the environmental conditions under which such products are used. Thus, there is need to maintain a continuous flow of characterization and assessment of biocontrol agents. Therefore, five Brazilian *Trichoderma harzianum* isolates were evaluated in vitro against *Sclerotinia sclerotiorum*, the causal agent of the white mold of common bean. Initially, the isolates grown with the pathogen by paired culture and antibiosis assays. Light microscopy studies showed mycoparasitism by isolate CEN316 on *S. sclerotiorum* hyphae. Besides, reductions in the mycelial growth of *S. sclerotiorum* were verified with non-volatile metabolites of *T. harzianum*, wherein CEN287 and CEN316 inhibited the pathogen growth in 95% and 90% respectively. In vitro results, credit such isolates as potential holders in the development of soil inhabitant pathogens biological control program.

Keywords: biological control; antibiosis; white mold; *Phaseolus vulgaris.*

Atividade antagonista de *Trichoderma harzianum* contra *Sclerotinia Sclerotiorum* de feijão comum

Resumo: Um ponto-chave no desenvolvimento de novos biopesticidas é a descoberta de cepas com maior atividade ou mais adaptadas às condições ambientais sob as quais esses produtos são utilizados. Assim, é necessário manter um fluxo contínuo de caracterização e avaliação de agentes de controle biológico. Portanto, cinco isolados brasileiros de *Trichoderma harzianum* foram avaliados in vitro contra *Sclerotinia sclerotiorum*, agente causal do mofo branco do feijoeiro. Inicialmente, os isolados foram cultivados com o patógeno por meio de ensaios de cultura pareada e antibiose. Estudos de microscopia de luz mostraram micoparasitismo do isolado CEN316 sobre hifas de *S. sclerotiorum*. Além disso, as reduções no crescimento micelial de *S. sclerotiorum* foram verificadas com metabolitos não voláteis de *T. harzianum*, em que CEN287 e CEN316 inibiram o crescimento do patógeno em 95% e 90%, respectivamente. Resultados in vitro, creditam tais isolados como detentores de grande potencial para o desenvolvimento do programa de controle biológico de patógenos habitantes do solo.

Palavras-chave: controle biológico, antibiose, mofo branco, *Phaseolus vulgaris.*

Introduction

White mold, caused by *Sclerotinia sclerotiorum*, has been responsible for major losses in agriculture. Plants susceptible to this soil pathogen cover 408 species in 278 genera and 75 families (TOLEDO-SOUZA et al., 2008). Chemical fungicides have not been unable to suppress the pathogen. Furthermore, these products are harmful to health, lead to the reduction of beneficial microbial flora of the soil, besides increasing the costs of production (FAHMI et al., 2012). Therefore, biological control has been seen in a growing interest worldwide, especially in the case of diseases caused by soilborne plant pathogen. The advantages of this control method are more evident in protected crops, not only due to the high value of these plants, but also with the possibility of manipulation of environmental parameters, which may significantly alter the effectiveness of the agents used. However, large crops such as beans, currently has concentrated high demand for biofungicides for control of diseases that has proven to be difficult to control.

Thus, it is no surprise that *Trichoderma* species are dominant components of fungal soil microbiota. Given the economic importance of this fungus, nowadays research aiming at the selection and maintenance of promising strains for the development of biofungicides and inoculants in culture collection has been stimulated. Brazilian soils, especially the Cerrado biome, are promising source of microbial genetic resources and are habitats for numerous biotypes of *Trichoderma* (GERALDINE et al., 2013). Microbial cultures support the development of biological control programs, as they offer stocks of isolates samples and opportunity to generate economically important information about the use of these microorganisms (LOUZADA et al., 2009).

Isolates of *Trichoderma* with efficient antagonistic capacity against *S. sclerotiorum* is a promising alternative strategy to pesticides for the white mold management since several commercial products (biopesticides, biofertilizers and soil amendments) in basis of this fungus has been used as part of environmental friendly protocols to protect crop plants against pathogens and to increase yields (MAHMOUD et al., 2015). Biocontrol of diseases promoted by *Trichoderma* comprises a complex process that can occur by different mechanisms, either separately or together, such as antibiosis (volatile and non-volatile metabolites), competition for nutrients, and mycoparasitism (HOWELL, 2003). The objective of this study was to investigate the antagonistic effect of five *T. harzianum* isolates against *S. sclerotiorum* under in vitro conditions, in order to provide information for the future use of these isolates maintained in culture collection in a white mold biological control program.

Materials and Methods

Fungal isolates

The five isolates (CEN287, CEN288, CEN289, CEN290, and CEN316) of *T. harzianum* used in this work belong to the Biological Control of Plant Pathogens Collection of Embrapa Recursos Genéticos e Biotecnologia (Brasília, Distrito Federal, Brazil). The isolates were identified on bases of morphological characteristics (SAMUELS et al., 2014) and sequencing of ITS-1 and ITS-4 regions of rDNA. Cultures stored in liquid nitrogen were recovered on potato dextrose agar (PDA). The pathogen *S. sclerotiorum* (isolate C-54-01) was isolated from naturally infected common bean plants and maintained in the same medium.

Dual culture assay for antagonism of Trichoderma harzianum against Sclerotinia sclerotiorum

For this assay, there was a pre-establishment of the pathogen, which was placed two days before antagonists. Mycelial plugs (5 mm) taken from pathogen and antagonist cultures were positioned diametrically opposite each other on Petri dishes containing solidified PDA medium, and incubated at 25°C and 12 h photoperiod. The radial growth of the pathogen was measured after seven days of simultaneous growth. The same plates were used to look for mycoparasitism signs, under a light microscope at 400x magnification.

Activity of volatile metabolites (VM) of Trichoderma Harzianum

Petri dishes containing PDA medium were inoculated with mycelial plugs (5 mm) of C-54-01 and *T. harzianum* isolates. Plates containing the antagonist (recently inoculated) and plates containing two-day-old colonies of the pathogen were fitted to each other and, sealed with transparent plastic film. The Petri dishes were kept at 25°C and 12 h photoperiod, with pathogen occupying the superior base (CARVALHO et al., 2014). Control plates were *S. sclerotiorum* without isolates of *T. harzianum*. Colonies of *S. sclerotiorum* were measured at the time of the full colonization of control Petri dishes (four days). The average values (in percentages) of radial mycelial growth were obtained in comparison with the control.

Activity of non-volatile (NVM) and thermostable metabolites (TNVM) of Trichoderma harzianum

Each *T. harzianum* isolate was grown in 250 mL of potato-dextrose-broth (PDB) in 500 mL Erlenmeyer flasks at 250 rpm in an orbital shaker (Lab line Instruments, Inc., 60160) at 25°C in the dark, for five days. Cultures were filtered through Whatman nº1 filter paper (Schleicher & Schuell GmbH, Dassel, Germany) and afterwards in 0.45 µm Millipore syringes. Aliquots (5 mL) of it were added to 20 mL of PDA and the mixture was poured into Petri dishes (90 mm). Mycelium plugs (5 mm) of C-54-01 were placed in the center of each Petri dish, which were kept at 25°C and 12 h photoperiod for five days. The radial growth of the pathogen was measured and used to calculate the inhibition of mycelial growth (%), when compared with the control. To determine the thermal stability of metabolites (TNVM), filtrates of the five *T. harzianum* isolates were obtained as described above. Forty-five milliliters of the cultural filtrate were autoclaved at 121°C for 21 min and were incorporated into 135 mL of flux PDA, which was distributed in Petri dishes. A mycelium plug (5 mm) of C-54-01 was placed in the center of each Petri dish, which was kept at 25°C and 12 h photoperiod (GUIMARÃES et al., 2016). The treatments were evaluated as described above. All *in vitro* assays had four replicates per treatment.

Statistical analysis

The results were submitted to ANOVA, the Scott-Knott test ($P \leq 0.05$) and regression analysis to determine correlation among all variables were carried out using the SISVAR 5.3 software (FERREIRA, 2011).

Results and Discussion

Dual culture assay for antagonism of Trichoderma harzianum against Sclerotinia sclerotiorum

T. harzianum isolates showed rapid growth, so that after three days, hyphae intermingled with that of *S. sclerotiorum*, and in seven days, the

mycelium covered the entire surface of the culture medium. There was no difference among the isolates in terms of mycelial growth (Table 1).

Table 1. *In vitro* antagonism (dual culture) of *Trichoderma harzianum* against *Sclerotinia sclerotiorum*(1)

Isolate of *T. harzianum*	Dual culture Colonies of *S. sclerotiorum* - diameter (mm) on the 7th day
CEN287	36.6
CEN288	39.6
CEN289	35.0
CEN290	35.0
CEN316	40.3
Coefficient of variation (%)	8.65

(1)Not significant according to analysis of variance (P≤0.05).

Parasitic capacity on *S. sclerotiorum* was clearly observed at least with *T. harzianum* CEN316. This parasitism was confirmed in several portions on hyphae of *S. sclerotiorum* from light microscopy images, where the hyphal growth of *T. harzianum* occurred with subsequent coiling the hyphae of *S. sclerotiorum* (Figure 1A). Hyphae of CEN316 were also observed extending along the hyphae of the pathogen (Figure 1B).

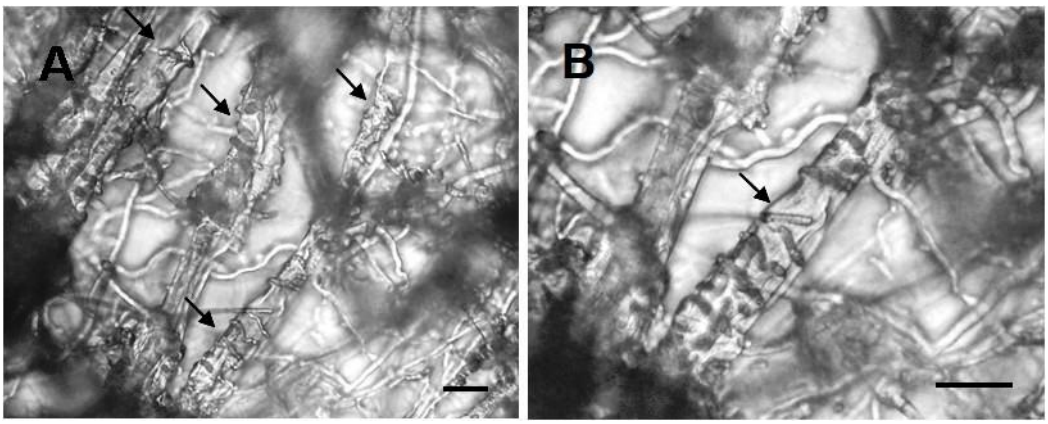

Figure 1. Light micrographs of interactions between *Trichoderma harzianum* (CEN316) and *Sclerotinia sclerotiorum* (C-54-01). (A) Arrows show CEN316 developing on the pathogen hyphae; (B) Hypha of *S. sclerotiorum* colonized by the antagonist, in larger amplification. (Bars: 32 and 30 µm to the figures 1A and 1B, respectively).

Activity of volatile (VM), non-volatile (NVM) and thermostable metabolites (TNVM) of Trichoderma harzianum

Summary of analysis of variance (ANOVA) is presented in the Table 2. Thus, concerning volatile metabolites (VM), except CEN290, all isolates of *T. harzianum* presented antifungal effects and did not differ from each other (Table 3). The average percentage of inhibition was between 35% and 62%. Further reduction in mycelial growth of *S. sclerotiorum* was observed with non-volatile metabolites (NVM) of *T. harzianum* CEN287 and CEN316, for which the mean values of percent inhibition were 95% and 90%

respectively. After autoclaving, the metabolites produced by CEN287, CEN288 and CEN289 inhibited in 45%, 50% and 37% the growth of *S. sclerotiorum*, respectively.

The growth reduction of *S. sclerotiorum* in dual cultures can be attributed to competition for space and nutrients present in the culture medium (VINALE et al., 2008). However, the main biocontrol mechanisms used by the fungus of the genus *Trichoderma* when confronted with the pathogenic fungi, are mycoparasitism (HOWELL, 2003; LOUZADA et al., 2009; CARVALHO et al., 2014) and antibiosis (GERALDINE et al., 2013), which were also explored in the present study. No correlation was observed between VM and NVM in this work. According to Dennis e Webster (1971), high production of NVM is not correlated to the high production of volatile inhibitors. Although there was an increase in the growth of *S. sclerotiorum*, under the effect of TNVM when compared to VM and NVM (Table 3), founded thermal stability indicates that active substances produced by CEN288 can remain stable, regardless of the environmental temperature. Besides, active fraction present in NVM of CEN287 and CEN316 seems to have decomposed in inactive substances after autoclaving metabolites, while the opposite seems to have occurred for CEN289. Such events are in accordance with Carvalho et al. (2007), who postulated that biological active fractions present in metabolites produced by microorganisms can to decompose to another one when subjected to temperature increases. Nevertheless, antibiosis is an important biocontrol mechanism, since the rate of antibiotics secretion is correspondent to the percentage of biocontrol, and after being purified, the isolated antibiotic has the same capacity of biocontrol (Benítez et al. 2004). According to Vinale et al. (2008), the specificity to exercise antibiosis occurs owing to the production of metabolites belonging to a variety of chemical compounds, which can suggest different action mechanisms. These evaluations are important, since the capacity to produce toxic metabolites with fungicide or a fungistatic effect can vary among species and among isolates from the same species (CARVALHO et al., 2014).

Table 2. Summary of analysis of variance (ANOVA): Source of variation (FV), liberty degree (GL), sum of squares (SQ), mean square (QM) of *Trichoderma harzianum* on *Sclerotinia sclerotiorum.*

Source of variation (FV)	GL	SQ	QM	Fc	Pr>Fc
Isolate of *T. harzianum* (T)	4	2739.44	6959.86	58.64	0.0000
Metabolite (M)	2	260.38	1030.19	8.68	0.0007
T x M	8	17590.44	2198.80	18.52	0.0000
Error	42	4984.12	118.66		
CV (%)	17.78				

CV(%) - Coefficient of variation.

Table 3. Inhibitor effect of volatile (VM), non-volatile (NVM) and autoclaved metabolites (TNVM) of *Trichoderma harzianum* on *Sclerotinia sclerotiorum*

Isolate of *T. harzianum*	Colonies of *S. sclerotiorum* under effect of *T. harzianum* metabolites - Growth (%)[1, 2]		
	VM	NVM	TNVM
CEN287	55.8 aB	4.7 aA	54.9 aB
CEN288	52.1 aA	60.5 bA	50.0 aA
CEN289	65.9 aA	99.8 cB	63.9 aA
CEN290	85.8 bA	99.4 cB	100.0 cB
CEN316	38.2 aB	10.7 aA	76.7 bC
Control[3]	100.0 bA	100.0 cA	100.0 cA
Mean	66.32 A	62.65 A	74.27 B
Coefficient of variation (%)	20.67	12.15	11.08

[1]Means followed by equal letters, lowercase in the columns and uppercase in the rows, do not differ according to the Scott - Knott test (P≤0.05).
[2]Values compared to the control, obtained from colonies with 4, 5, and 5 days of growth for VM, NVM and TNVM, respectively.
[3]VM: without antagonist; NVM: addition of 5 mL of sterile distilled water at flux BDA; TNVM: BDA without adding *T. harzianum* metabolites.

Although Moretini e Melo (2007) and Louzada et al. (2009) have used scanning electron microscopy to verify in vitro interactions between biocontrol agents and *S. sclerotiorum*, images were verified in the present work, exhibiting mycoparasitism interactions, which can to be obtained from a simpler technique: light microscopy. To exemplify light microscopy as an adequate technique for such studies, it can cite the work of Rodríguez et al. (2006), that employing light microscopy to verify mycelium of *S. sclerotiorum* in contact with *Fusarium oxysporum* (non-pathogenic isolate). As consequence of the parasitism exerted by *F. oxysporum*, mycelium of *S. sclerotiorum* alterations included increased branching, reduced length of branches, granulation, retraction of the plasmalemma and collapse of cytoplasm. Thus, interactions verified between CEN316 and *S. sclerotiorum* (Figure 1) can to be considered as hyperparasitism, which is defined as a complex process, compounded by several events, including host recognition, which is attacked by the antagonist hyphae, and subsequently penetration (AGRIOS, 2005; VINALE et al., 2008; CARVALHO et al., 2014). During this process, the fungus *Trichoderma* can also secrete enzymes or even produce secondary metabolites with antifungal properties, when grown in a culture medium (BENÍTEZ et al., 2004).

A logical sequence aiming to select antagonists for the biological control of diseases proceeds through several steps, and theoretically from in vitro (screening in Petri dishes) to in vivo (field experiments) (KÖHL et al., 2011). Although no relationship between in vitro inhibition tests and performance in the field has been reported Fravel (2005), CEN287 and CEN316 proved to be effective for management of Fusarium wilt of common bean under field conditions (CARVALHO et al., 2015a) and in the control of seedborne fungi (CARVALHO et al., 2011). In addition, in field trials, isolates CEN287 and CEN316 also proved to be effective biological control agents of white mold of common bean, in which they reduced the severity of the disease in 96 and 80%, respectively, compared to the untreated bean

(CARVALHO et al., 2015b). Due to these isolates to be effective strains for the control of soil inhabitant pathogens, CEN287 and CEN316 reduced the incidence of common bean fusarium wilt by 51.3 and 41.2% in field experiments when compared to the control, respectively (CARVALHO et al., 2015a).

This isolates present high capacity for spore production and, they must be considered in the development of soil inhabitant pathogens biological control program.

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