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Cell wall-degrading enzymes and parasitism of sclerotia are key factors on field biocontrol of white mold by *Trichoderma* spp.

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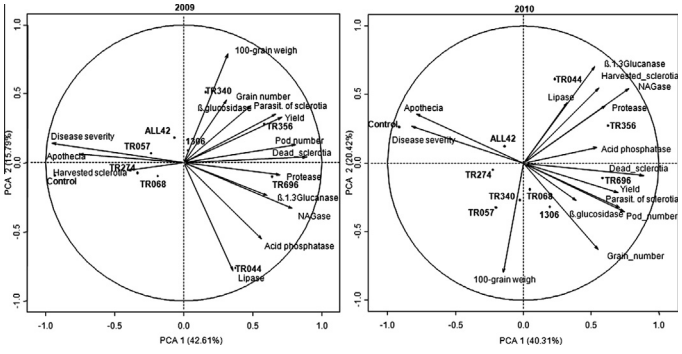
HIGHLIGHTS

- *Trichoderma* spp. isolates were investigated for field biocontrol of white mold.
- Field and laboratory results were submitted to principal component analysis.
- Consistent results were found in 2009 and 2010.
- Cell wall degrading enzymes and parasitism are key components of field biocontrol.
- NAGase and β-1,3-glucanase may be used as markers for selection of new isolates.

GRAPHICAL ABSTRACT



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ABSTRACT

Field outcomes of 10 *Trichoderma* spp. isolates against white mold (*Sclerotinia sclerotiorum*) on common beans were matched to laboratory results, to identify the causes of variance related to biocontrol effectiveness. Laboratory assays estimated sclerotia parasitism and production of the cell wall-degrading enzymes (CWDEs) lipase, NAGase, β-1,3-glucanase, β-glucosidase and protease. Field trials were carried out in 2009 and 2010 under a randomized block design and sprinkling irrigation, where 2×10^{12} spores mL^{-1} of each antagonist were applied to the plots at the early R5 stage. The density of *S. sclerotiorum* apothecia m^{-2} and disease severity were assessed, respectively at R7 and R8 stages, with yield and its components also estimated for each year. Field results were analyzed jointly by the Tukey–Kramer multiple comparison test, and all variables from both field and laboratory experiments were subjected to principal component analysis (PCA). In both years, isolates TR696 and TR356 of *Trichoderma asperellum* were effective in reducing apothecia density and disease severity. Biocontrol increased the number of pods per plant and yields up to 40% when compared to controls, even under higher disease pressure in 2010. PCA demonstrated in 2009 and 2010 that apothecia density, disease severity, NAGase, β-1,3-glucanase and number of pods were the main sources of the first component of variance. Such results suggest that the CWDEs NAGase and β-1,3-glucanase and sclerotia parasitism are key components of *Trichoderma*

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spp. action in biocontrol of *S. sclerotiorum* in the field, and may be used as markers to hasten the selection of new, promising isolates.

1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is the causative agent of white mold on more than 400 plant species (Boland and Hall, 1994). The disease is a main cause of yield losses of common beans (*Phaseolus vulgaris* L.) in all major producing regions of the world (Ramasubramaniam et al., 2008), due to the lack of resistant varieties and survival of the pathogen in soil as sclerotia over several years. Sclerotia germination in soil is a key event in disease onset as it triggers the growth of apothecia, considered the main source of the initial inoculum (ascospores) that infects plants after blooming (Adams and Ayers, 1979; Purdy, 1979; Sun and Yang, 2000).

The degradation of sclerotia by fungal and bacterial antagonists is one of the most effective ways to decrease the burden of *S. sclerotiorum* sclerotia in soil. Many antagonistic species have been described as effective mycoparasites of *S. sclerotiorum* sclerotia, such as *Coniothyrium minitans* and *Ulocladium atrum* (Gerlagh et al., 2004; Huang et al., 2000; Jones et al., 2011). Various mechanisms may be involved in the pathogen's biocontrol (*e.g.*, antibiosis by *Pseudomonas* spp.) and may affect sclerotia numbers in soil or inhibit the germination of ascospores (Fernando et al., 2007). Among many reports of antagonists against *S. sclerotiorum*, the genus *Trichoderma* (Persoon) includes several species that have been extensively studied in the biocontrol of soilborne pathogens. In general, it is accepted that higher frequencies of species such as *Trichoderma harzianum* and *Trichoderma asperellum* correlate negatively with the viability of *S. sclerotiorum* sclerotia in soil (Ferraz et al., 2011), but there are as yet no reports of soil that is suppressive to this pathogen based solely on endemic populations of *Trichoderma* spp.

The sclerotia consists principally of a melanin rind and an inner component composed of carbohydrates (mainly β-1,3-glucans) and proteins (Le Tourneau, 1979). Sclerotinia pathogens stimulate *Trichoderma* spp. to produce cell wall-degrading enzymes (CWDEs) including β-1,3-glucanase, chitinases and proteases (Vázquez-Garcidueñas et al., 1998), and these CWDEs hydrolyze the cell wall of the sclerotia, which then decay and die in the soil. (Kubicek et al., 2001; Verma et al., 2007). Hence, it may be expected that antagonists with increased secretion of extracellular enzymes such as chitinase, β-1,3-glucanases and proteases should be responsible for more a pronounced decline in the *S. sclerotiorum* inoculum levels in soil (Woo et al., 2006).

Numerous studies have demonstrated the efficiency of *Trichoderma* spp. in controlling *S. sclerotiorum* on different crops (Abdullah et al., 2008; Carvalho et al., 2011). However, there are a limited number of reports linking CWDEs and hyperparasitism results achieved under controlled conditions with the field performance of *Trichoderma* spp. isolates. As such, it is not yet clear that better CWDE producers perform well in soil, showing marked decreases in initial inoculum and disease severity, and improvement in yields. Such a demonstration could also identify and emphasize the main factors involved in effective control of white mold in the field, and fine-tune the selection process of antagonists.

An appropriate statistical approach such as principal components analysis (PCA) may be suitable to summarize and identify correlations among qualities related to biocontrol, since it reduces a large number of observed variables to a smaller number of factors (Tabachnick and Fidell, 2007). This multivariate approach could describe a set of antagonists on the basis of their most relevant variables that correlate to the first dimensions of the PCA. Hence, PCA can help in understanding the role in the field of laboratory-assessed characteristics such as CWDE production and sclerotia parasitism by *Trichoderma* spp., in the effective control of white mold.

Therefore, the main objective of this paper was to test, under field conditions, 10 isolates of *Trichoderma* spp., in the biocontrol of white mold on common beans. The second objective was to employ a multivariate statistical approach to gathering both laboratory and field results, and identify the variables that best explain the efficiency of *Trichoderma* spp. isolates in white mold biocontrol in the field.

2. Material and methods

2.1. Field trials

The field experiments were conducted from July to October in 2009 and 2010 at Palmital Farm, an experimental area of Embrapa Rice and Beans, located at Goianira, in the Brazilian State of Goiás (coordinates: 16°26'04, 18"S, 49°24'06, 80"W; 735 m). The soil was classified as clayey, with pH 6.6 and 20 g dm^{-3} of organic matter. Soil management began with conventional tillage in an area of 1000 m^2. The crop of common bean cv. Pérola was established in both years with 0.50 m between rows and a population of 240,000 seeds ha^{-1}, supported by 400 kg ha^{-1} of NPK 5–25–15 as recommended based on previous soil analysis. Soil inoculation with *S. sclerotiorum* was performed annually with a homogenous spread of sclerotia mixed with soybean residues from a seed-cleaning unit, with the aid of a manual fertilizer dispenser. The distribution of sclerotia in the experimental area was carried out soon after crop sowing with an average of 145 sclerotia per m^2 in 2009 and 2010, in accordance with Huang et al. (2000). The re-inoculation of soil in 2010 provided a uniform distribution of inoculum and avoided patches that would impact negatively on treatment effects and data analysis.

The experimental area had no crop history before 2009, and was used formerly as a pasture. Before planting the experimental crop, grasses were killed with glyphosate (4 L ha^{-1}) with straw incorporated into the soil by means of a plough. After harvesting in October 2009, the experimental area was planted with soybeans. In the following year, crop residues and weeds were killed as in the first trial, with the 2010 experiment following recommendations for no-tillage cropping.

A sprinkling irrigation system provided optimal conditions for both crop and white mold development (Gerlagh et al., 1999; Weiss et al., 1980). Irrigation was carried out every three days, after 16:00, with a water supply of 15 mm m^{-2}. The experimental design consisted of randomized complete blocks with four replications. Each plot was composed of seven lines of common bean of 4 m in length, totaling 12 m^2. Plots were separated from each other by 1 m borders also planted with common beans.

The treatments consisted of application of 10 *Trichoderma* spp. lines plus a negative control. Eight of the *Trichoderma* spp. lines were from the Embrapa Rice and Beans collection of multifunctional microorganisms, labeled as: TR274 and TR068 (*T. harzianum*), TR356, TR696, TR022 and TR044 (*T. asperellum*), TR057 and 451/2 (*Trichoderma* sp.). The isolate series was completed by ALL-42 (*T. harzianum*) from the Federal University of Goiás culture collection and *T. harzianum* 1306 (ESALQ/USP), component of a biofungicide called Trichodermil. Isolates described at species level

were previously identified according to their ITS and TEF1 sequences, obtained with DYEnamic™ ET Terminator Cycle Sequencing Kit from GE Healthcare, according to the manufacturer's instructions, and electrophoresed using the ABI Prism 3100 (Applied Biosystems). Sequence analysis of the ITS amplicons to species identification was performed using the TrichOKEY 2.0 (Druzhinina et al., 2005) and TrichoBLAST (Kopchinskiy et al., 2005) tools available online at http://www.isth.info/. Their sequences were deposited in GenBank and correspond to the accession numbers KC993072 to KC993077 (TR022, TR696, TR044, TR068, TR274 and TR057), HQ857122 (ALL-42) and HQ857128 (TR356).

The isolates were cultured in 250 ml Erlenmeyer flasks containing previously autoclaved (121 °C, 40 min.) parboiled rice. The vials were kept in a BOD incubator at 25 °C with a 12-h photoperiod for seven days, to stimulate profuse sporulation. Subsequently, the colonized rice was carefully washed with autoclaved distilled water to obtain a suspension of conidia. All suspensions obtained were adjusted to a concentration of 2×10^8 conidia ml^{-1} using a hemacytometer.

The application of the isolates was performed at the beginning of pre-blooming (R5) of common beans in a proportion equivalent to 2×10^{12} conidia ha^{-1} with the aid of a CO_2-pressurized sprayer. Control plots were treated with distilled water. The sprayer consisted of a bar with four full cone nozzles, spaced 50 cm apart, connected to a CO_2 cylinder giving a spray volume of 400 L ha^{-1} at 2.6 bar. Immediately after treatment application, the experimental area was irrigated with water to a depth of 5 mm, so that the conidia of *Trichoderma* spp. retained in the crop canopy could reach the ground.

Inoculum density was estimated early in the R7 stage (beginning of pod filling) soon after apothecia were formed. Apothecia density was estimated as the average number in two individual 1.0 m^2 random samples within each plot. At the R8 stage (complete pod filling) the severity of white mold was assessed through a disease scale adapted from Napoleão et al. (2005). The disease scale consisted of points from 1 to 7, with #1 corresponding to the absence of disease symptoms; #2 from 1% to 5% of the plot area with white mold symptoms (ACS), #3 from 6% to 20% of ACS, #4 from 21% 50% ACS, #5 from 51% to 70% ACS, #6 from 71% to 90% ACS, and #7 from 91% to 100% of diseased area. Disease assessments were made by means of careful opening of 1.0 m^2 of the crop canopy in the two central rows of each plot. Two evaluators individually scored the plots, and disease severity was defined by the arithmetic mean of these assessments.

At the ripening stage (R9), the two central lines of 1.5 m within each plot were manually harvested and stored in burlap bags. After plant threshing and separation of residues, yield and its components (total number of pods, number of seeds per pod and average weight of 100 grains) were estimated, after adjustment of grain moisture to 13%. The number of pods and the number of seeds per pod were assessed from random samples of five common bean plants and 20 pods, respectively. The number of new sclerotia m^{-2} in all treatments was estimated after manual separation from plant residues, using a 5 mm pore-width sieve.

2.2. Enzyme production and enzymatic assays

For hydrolytic enzyme production, discs (3 mm diameter) from *Trichoderma* strains previously grown on malt extract, yeast extract and glucose (MYG) medium were inoculated into TLE medium ($CaCl_2.2H_2O$, 0.3 g L^{-1}; KH_2PO_4, 2.0 g L^{-1}; $(NH_4)_2SO_4$, 1.4 g L^{-1}; $MgSO_4.7H_2O$, 0.3 g L^{-1}; Urea, 0.3 g L^{-1}; Peptone, 1.0 g L^{-1}; trace elements solution (Fe^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}), 0.1%). Lyophilized cell wall of *S. sclerotiorum* '1370' (0.5%) obtained after maceration with mortar and pestle was used as a carbon and nitrogen source. The cultures were grown in triplicate, in 125 ml conical flasks with constant shaking (120 rpm) at 28 °C. After 48 h, the mycelium was harvested by filtration through Whatman No. 1 filter paper, and the culture filtrate was used as a source of enzymes. The following enzymatic essays were carried out with three sub-samples of 10 ml each. Only those with standard deviation below 20% were accepted.

The N-β-acetylglucosaminidase (NAGase), acid phosphatase and β-glucosidase activities were determined using ρnp-derived substrates: ρ-nitrophenyl-β-N-acetylglucosamine (ρ-NPNAG) (5 mM), ρ-nitrophenyl-phosphate (ρ-NPP) (5 mM) and ρ-nitrophenyl-β-D-glucopyranoside (ρ-NPGluc) (5 mM). The reactions were carried out in microplate assay format. The assay mixtures contained 10 μl of enzyme solution, 40 μl of ρnp-derived solution and 100 μl of buffer. The molarity and the pHs of the buffers were: 50 mM sodium acetate buffer, pH 4.8, 5.5 and 6.0 for acid phosphatase, β-glucosidase and NAGase, respectively. After incubation of the mixture at 37 °C for 15 min, the reaction was stopped by the addition of 100 μl of NaOH (0.1 M). The amount of ρ-nitrophenol (ρ-NP) was determined spectrophotometrically at A_{405}. One unit of enzyme activity was defined as the amount of enzyme necessary to release 1 μmol de ρ-NP per minute.

The lipase activity was determined using ρ-nitrophenyl-palmitate (ρ-NPPa) (Sigma®) as a substrate at a concentration of 5 mM in acetonitrile (Jain et al., 2005). The assay mixtures contained 100 μl of enzyme solution, 20 μl of ρ-NPPa solution and 100 μl of 0.1 M sodium phosphate buffer (pH 7.0) containing 0.27 M NaCl and 0.9% v/v Triton X-100. After incubation of the mixture at 37 °C for 30 min the reaction was transferred to a domestic microwave oven and irradiated for 30 s on medium low power to stop the reaction. The enzyme activities were calculated as above.

The β-1,3-glucanase activity was determined using laminarin (Sigma®) as a substrate at a concentration of 0.75% w/v in sodium acetate buffer (50 mM, pH 5.0) (Ramada et al., 2010). The assay mixtures contained 10 μl of enzyme solution and 20 μl of laminarin solution. After incubation of the mixture at 50 °C for 10 min, 100 μl of 3,5-dinitrosalicylic acid (DNS) were added and the reaction was incubated at 95 °C for 5 min. The amount of reducing sugar was determined spectrophotometrically at A_{540}. One unit of enzyme activity was defined as the amount of enzyme necessary to release 1 μmol of reducing sugar per minute.

The protease activity was determined using azocasein (Sigma®) as a substrate at a concentration of 0.25% w/v in phosphate/citrate buffer (50 mM) pH 5.0 (Cabral et al., 2004). The assay mixtures contained 20 μl of enzyme solution, 40 μl of azocasein solution and 40 μl of the respective buffers. After incubation of the mixture at 37 °C for 30 min, 100 μl of trichloroacetic acid (TCA) 10% w/v were added and the samples incubated at 4 °C for 10 min. The samples were centrifuged at 2500 rpm for 30 min and 100 μl of supernatant were transferred to another microplate containing 100 μl of NaOH (1 M), and examined at 450 nm. One unit of enzyme activity was defined as the amount of enzyme necessary to elevate 1 unit of absorbance per minute.

The protein concentrations of samples were determined by the Bradford assay (Bradford, 1976) using bovine serum albumin (BSA-Sigma) as a standard.

2.3. Parasitism tests

For in-laboratory parasitism tests, sclerotia of *S. sclerotiorum* were produced in autoclaved 250 mL Erlenmeyer flasks containing 100 'Pérola' common bean grains each (Garcia et al., 2012). Each autoclaved flask received three 5-mm PDA disks with *S. sclerotiorum* '1370' mycelia, taken from the edge of a seven-day old colony. The flasks were incubated at 20 °C and manually shaken briefly at every two or three days. After 30 days, sclerotia were removed

from the flasks, air-dried for 24 h and transferred to the surface of 250 g autoclaved soil samples with moisture adjusted to 100% of field capacity, uniformly spread on 11 × 11 × 3.5 cm acrylic transparent boxes. Each box received 20 sclerotia randomly distributed.

To evaluate the 10 *Trichoderma* spp. isolates, the pathogen's sclerotia were sprayed in their respective boxes with 10 ml suspensions at a concentration of 2.4×10^6 conidia ml^{-1}. After random distribution of the boxes at 20 °C ± 2 °C and continuous light for 15 days, the sclerotia were removed, surface disinfested with ethanol (70%) and sodium hypochlorite (2%), and rinsed three times in distilled autoclaved water. Subsequently, the resistant structures were placed on disinfected carrot disks and arranged in 90-mm Petri dishes according to Hoes and Huang (1975). After seven days of incubation at 20° ± 2 °C, the sclerotia were evaluated for viability and parasitism by *Trichoderma* spp. This experiment was repeated twice.

2.4. Statistical analysis

2.4.1. Univariate analysis

Univariate statistical analysis was carried out with the SAS 9.1 statistical package (SAS Institute, Cary, NC, USA) GLM procedure to verify treatment effects. The analysis of variance ($\alpha = 0.05$) was supported by the Shapiro–Wilk test for the normality of residuals, Leneve test for homogeneity of variance, and the Durbin–Watson test for independency of residues and to check for outliers. To compare the 2009 and 2010 results, a joint analysis was performed (GLM procedure) with a mixed model, where treatments were considered as a fixed effect. To meet the assumptions of residuals normality and the homogeneity of variance, the number of apothecia m^{-2} was adjusted after determining its square root plus 0.5. The number of new sclerotia m^{-2} was also adjusted after square root transformation. The data were analyzed jointly with Tukey–Kramer multiple comparisons $\alpha = 0.05$. Linear regressions were estimated by PROC REG. A heat map with all CWDE outcomes was constructed by MeV software 4.8.1 (Saeed et al., 2003).

2.4.2. Multivariate analysis

Principal components analysis (PCA) gathered all field and laboratory variables. The PCA was performed using the Rcmdr and FactoMineR packages from the 'R' software 2.15 (R Development Core Team, Vienna, Austria). To assess the multivariate effect of field variables (apothecia, severity, number of pods, level of grain productivity, sclerotia harvested) with enzymatic (total Proteins, NAGase, acid phosphatase, β-glucosidase, lipase, β-1,3-glucanase) and parasitism (dead sclerotia and *Trichoderma* spp. colonies parasitizing sclerotia) traits, and the results stability in 2009 and 2010, PCA was performed separately for each year.

3. Results

3.1. Field trials

White mold infections were successfully established at varying severity levels in both years of study. The average densities of apothecia in the control plots were 9.5 m^{-2} and 109 m^{-2}, respectively in 2009 and 2010, providing a conducive environment for white mold. Despite the amount of sclerotia distributed being equivalent in both years, the apothecia density in 2010 was on average 13.7 times greater than in 2009 (Table 1).

The number of apothecia m^{-2} in the plots was affected by the treatments in the two years. Joint analysis showed a significant interaction between this variable and year of trial. There was a drastic reduction in apothecia density in plots treated with the TR696 isolate of *T. asperellum* in both years, even under the higher inoculum pressure recorded in 2010. In 2009, the isolate ALL-42 of *T. harzianum* did not decrease the apothecia density. In 2010 the isolates TR022, TR356 as TR696, all *T. asperellum*, showed superior efficiency in comparison with isolate 1306 (*T. harzianum*), which did not differ from the control (Table 1).

Disease severity in 2010 was on average 90% higher than in 2009 ($p < 0.05$). In 2009, most of the isolate treatments differed from the control, and TR696, TR356 and TR044 (*T. asperellum*), TR340 (*Trichoderma* sp.) and 1306 *T. harzianum* decreased white mold severity at average values of 62.5%, 47.0%, 56.2% 43.7% and 43.7%, respectively. In 2010, treatments were ranked in two distinct groups, where isolates TR696 and TR022 of *T. asperellum* stood out from the control with a 51.4% reduction in disease severity. In the second group, isolates TR356 of *T. asperellum* and '1306' of *T. harzianum* also differed from the control. However, they performed equally to the other isolates, providing reductions of 45.7% and 41.4% in white mold severity, respectively.

Despite their better performance under a higher disease pressure in 2010, most isolates kept the same rank in reducing disease severity as in the previous year. Moreover, isolates TR696 and TR356 of *T. asperellum* were the only two that were effective in reducing the severity and apothecia numbers in two consecutive years. Despite the fact that the '1306' strain of *T. harzianum* was effective in reducing disease severity in the two years, it did not differ from controls in reducing apothecia.

Linear regression models for disease severity and apothecia density were adjusted in different ways, according to the quantity of apothecia in soil. In 2009, the model explained only 23.9% ($p \leqslant 0.005$) of disease severity (Fig. 1). A better fit for this association was observed in 2010, when 49.1% ($p \leqslant 0.001$) of white mold severity was explained in terms of the number of apothecia formed in soil. These results endorse the connection between apothecia and high severity of white mold in common bean crops. Further, simple linear models also showed the impacts of disease severity on grain yield (Fig. 1). The amount of apothecia m^{-2} was also negatively linked to 100-grain weight and grain yield in 2009 (data not shown), suggesting depreciation of the harvest (grain) as an impact of the disease.

The number of pods per plant was affected by the application of *Trichoderma* spp. in 2009 and 2010. Isolates 1306, TR696 and TR356 increased the number of pods per plant by 89.4%, 81.3% and 82.5%, respectively in 2009, when compared to the control (Table 1). The white mold severity in 2010 also affected the average number of pods per plant, which was reduced drastically in untreated plots. In the same year, plots treated with isolates TR356, TR696 and TR022, all *T. asperellum*, differ from the control with increases of 110.1%, 106.5% and 97.4%, respectively, in the number of pods per plant. In contrast, there were no differences between the isolate treatments and the control in terms the number of grains per pod and 100-grain weight, in 2009 and 2010.

Common bean yield in untreated plots was reduced, causing significant losses in 2009 and, markedly, in 2010 (Table 1). On average, yield losses on untreated plots were 445 kg ha^{-1}, close to the 524 kg ha^{-1} reported by Ramasubramaniam et al. (2008). However, in 2009 only isolate TR356 of *T. asperellum* differed from the control with a 40% gain in productivity. This same isolate provided once again a proportional increase in yield (44.2%) in 2010, though it did not differ from the other treatments. A more pronounced effect of Trichoderma treatments on yield was observed in 2010 under higher disease pressure, with an increase of 70.3% and 59.9% in productivity resulting from treatments with isolates TR022 and 1306, respectively (Table 1).

The *T. asperellum* isolates TR696 and TR356 had a marked positive performance on the majority of variables assessed in 2009 and 2010. However, they did not differ from the control in terms of common bean yield. The TR696 isolate was efficient in reducing

Table 1
Effect of *Trichoderma* spp. on *Sclerotinia sclerotiorum* apothecia number m^{-2}, white mold severity, 'Pérola' common bean yield (kg ha^{-1}) and yield components (number of pods, number of seeds per pod, 100-grain weight), and amount of new *S. Sclerotiorum* sclerotia m^{-2} obtained from plant residues, in 2009 and 2010.

Treatment	Apothecia		Disease severity[a]		Number of pods		Grains pod^{-1}		100-grain weight (g)		Grain yield (kg ha^{-1})		New sclerotia m^{-2}	
	2009													
TR022	7.0	abc	2.75	ab	17.0	abc	5.8	NS	25.2	NS	2036.9	b	19.3	ab
TR696	3.0	c	1.50	b	22.4	ab	5.5		27.2		2346.4	ab	14.0	ab
TR356	4.5	bc	2.12	b	22.5	ab	5.4		28.7		2662.2	a	20.0	ab
TR044	6.5	bc	1.75	b	18.6	abc	5.2		25.7		2287.9	ab	23.8	ab
TR068	10.5	abc	2.75	ab	17.4	abc	5.2		27.4		1956.1	b	24.8	ab
TR057	4.5	bc	2.87	ab	18.4	abc	5.1		27.7		2310.8	ab	36.2	ab
TR340	9.5	abc	2.25	b	17.0	abc	5.6		28.3		2456.3	ab	21.5	ab
TR274	15.0	ab	2.75	ab	14.8	Bc	5.6		26.1		1929.1	b	44.7	a
ALL42	18.5	a	2.75	ab	19.1	abc	5.8		26.8		2371.4	ab	27.5	ab
1306	9.5	abc	2.25	b	23.4	a	5.5		27.1		1928.4	b	13.2	b
Control	19	a	4.00	a	12.3	c	5.2		26.6		1901.6	b	21.5	ab
	2010													
TR022	39.0	c	2.12	c	19.5	a	4.7	NS	29.0	NS	3424.5	a	48.2	NS
TR696	47.0	c	2.12	c	20.4	a	5.0		28.2		2666.9	abc	33.7	
TR356	42.0	c	2.37	bc	20.8	a	4.9		25.5		2905.0	abc	48.3	
TR044	74.0	abc	3.12	abc	15.1	ab	4.4		26.3		2752.8	abc	40.3	
TR068	65.2	abc	2.87	abc	13.5	ab	5.1		28.7		2951.4	abc	35.6	
TR057	75.0	abc	2.50	bc	19.0	ab	4.8		28.8		2658.6	abc	10.3	
TR340	82.5	abc	3.75	ab	17.6	ab	5.0		28.2		2282.5	bc	27.3	
TR274	107.0	ab	3.50	abc	18.2	ab	4.7		27.8		2468.6	bc	17.3	
ALL42	49.5	bc	3.62	abc	13.6	ab	5.0		27.7		2270.1	bc	34.0	
1306	50.5	abc	2.56	bc	18.5	ab	4.9		27.4		3205.4	ab	29.2	
Control	109.0	a	4.37	a	9.9	b	4.2		26.6		2010.5	c	26.2	

Means followed by same letters in the column do not differ statistically according to Tukey test ($P \leq 0.0\,5$).
NS = not significant ($P \geq 0.0\,5$).
[a] Disease severity was assessed according to a 1–7 scale, with 1 corresponding to all healthy plants and 7 to 91–100% of area covered with symptoms (adapted from Napoleão et al., 2005).

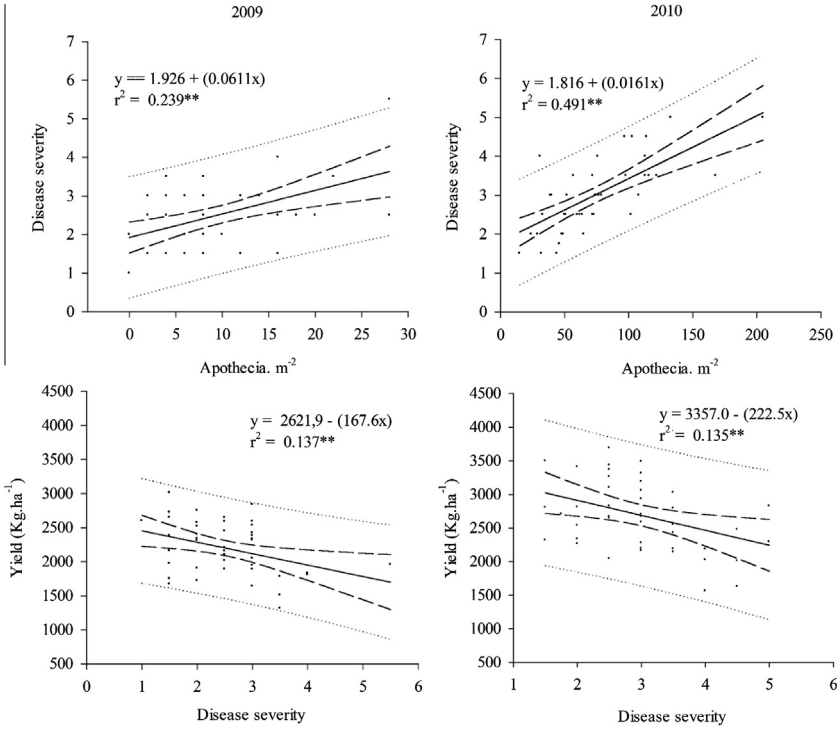

Fig. 1. Linear regression models adjusted for white mold severity, *Sclerotinia sclerotiorum* apothecia density and 'Pérola' common bean yield. **Significance at $\alpha = 0.01$. Disease severity was estimated after back transformation and interpolation of scores attributed by a modified scale from Napoleão et al. (2005), where "1" means no symptoms in the field plots, and 7 corresponds to 91–100% of plant surface in plots with white mold symptoms. CI and PI correspond to confidence intervals and predicted intervals after model adjustment at 95% confidence.

apothecia and disease severity and boosted the number of pods per plant. Moreover, plots treated with isolated TR696 in 2009 and 2010 showed, respectively average yields that were 23.3% and 31.7% higher than the lowest productivity values for each year (control). Likewise, isolate TR356 treatment showed a gain of 40.0% in 2009 and of 44.3% in 2010. Such results with antagonists are comparable to those from del Río et al. (2004), in which chemical control of white mold increased productivity by between 26% and 33%. According to their consistent and convincing performances, the results with isolates TR696 and TR356 were

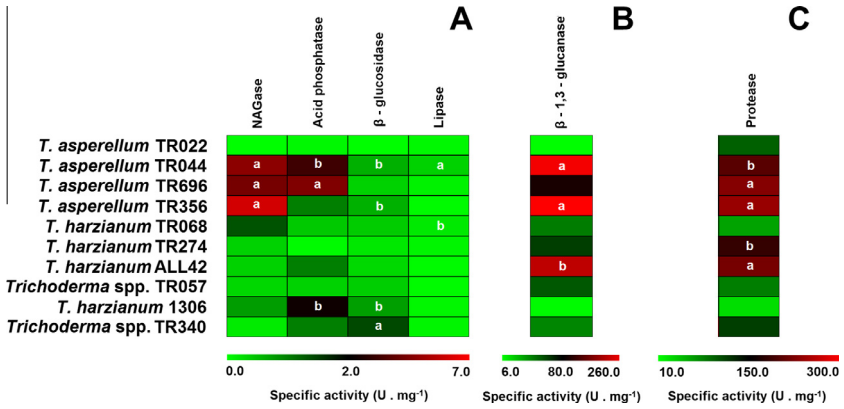

Fig. 2. Heat maps for *Trichoderma* spp. cell wall degrading enzymes, from isolates grown on *S. sclerotiorum* cell wall. Specific activities of enzymes that have ρnp-derivatives as substrates: NAGase, acid phosphatase, β-glucosidase and lipase (A), β-1,3-glucanase (B) and proteases (C). All the experiments were performed in triplicate and the results show the mean with error less than 10%. Similar letters do not differ by Scott Knott test ($\alpha = 0.05$). a = highest activity; b = high activity; other outcomes do not shown relevant activities.

considered worthy of further studies including the development of commercial biological fungicides.

3.2. CWDE analysis

All strains of *Trichoderma* secreted all of the analyzed cell wall-degrading enzymes (CWDEs) after exposure to *S. sclerotiorum* cell wall in different proportions (Fig. 2) and, without exception, all results reported below were obtained at $p \leqslant 0.0001$. The highest NAGase specific activities were exhibited by three *T. asperellum* isolates, identified as TR356 (6.15 U mg^{-1}), TR044 (4.765 U mg^{-1}) and TR696 (4.355 U mg^{-1}). The highest acid phosphatase specific activity was exhibited by *T. asperellum* TR696 (4.535 U mg^{-1}), followed by *T. asperellum* TR044 (3.235 U mg^{-1}) and *T. harzianum* 1306 (2.315 U mg^{-1}). The highest β-glucosidase specific activity was shown by *Trichoderma* sp. TR340 (1.425 U mg^{-1}), followed by *T. harzianum* 1306 (0.75 U mg^{-1}) and finally by *T. asperellum* isolates TR044 (0.615 U mg^{-1}) and TR356 (0.605 U mg^{-1}). The highest lipase specific activity was exhibited by *T. asperellum* TR044 (0.35 U mg^{-1}), followed by *T. harzianum* TR068 (0.17 U mg^{-1}).

For β-1,3-glucanase the highest specific activities were exhibited by *T. asperellum* TR356 (270.385 U mg^{-1}), *T. asperellum* TR044 (254.35 U mg^{-1}) and *T. harzianum* ALL-42 (216.16 U mg^{-1}). The highest acid proteolytic specific activity was exhibited by *Trichoderma* sp. TR340 (234.075 U mg^{-1}), followed by *T. asperellum* TR044 (221.25 U mg^{-1}). The highest neutral proteolytic specific activity was exhibited by *T. harzianum* ALL-42 (291.67 U mg^{-1}), followed by *Trichoderma* sp. TR340 (260.74 U mg^{-1}). The highest basic proteolytic specific activity was exhibited by *T. asperellum* TR356 (240.4 U mg^{-1}), followed by *T. asperellum* TR696 (229.19 U mg^{-1}).

3.3. Principal component analysis (PCA)

The 2009 PCA analysis revealed that the first three components accounted for 42.61%, 15.79% and 14.06% of the variance, totaling 72.46% (Fig. 3A). In 2010, 71.02% of the variance was explained

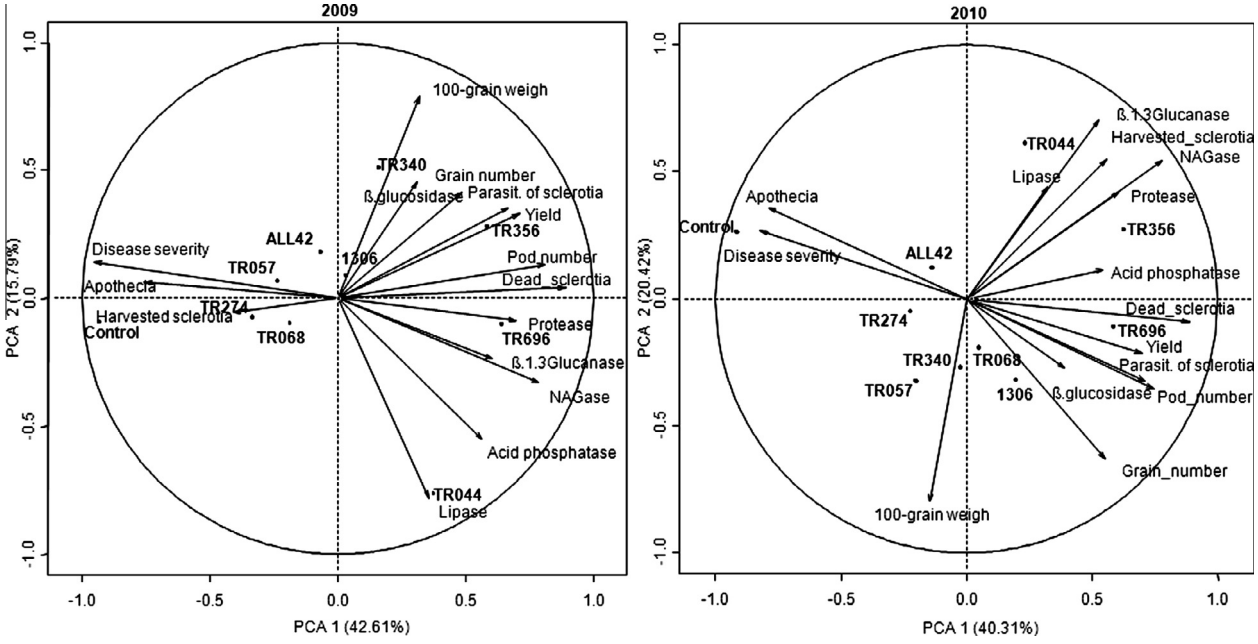

Fig. 3. PCA biplot with field and laboratory traits investigated for biocontrol of white mold (*Sclerotinia sclerotiorum*) of common bean by *Trichoderma* spp. in 2009 (A) and 2010 (B), according to the first two main components.

Table 2
Correlation matrix of field and laboratory variables related to biocontrol of white mold (*Sclerotinia sclerotiorum*) by *Trichoderma* spp. and dimensions of principal component analysis of 2009 and 2010 with significance of $p < 0.05$.

Variables	2009			2010		
	PCA1	PCA2	PCA3	PCA1	PCA2	PCA3
Apothecia	−0.753	ns	ns	−0.788	ns	ns
Disease severity	−0.952	ns	ns	−0.820	ns	ns
Common bean yield	0.710	ns	ns	0.695	ns	ns
Grain number	ns	ns	ns	ns	ns	ns
100-grain weigh	ns	0.790	ns	ns	−0.79	ns
Harvested sclerotia	ns	ns	0.671	ns	ns	ns
Pod number	0.807	ns	ns	0.742	ns	ns
β-1.3-glucanase	ns	ns	0.642	ns	0.703	ns
Lipase	ns	−0.779	ns	ns	ns	ns
NAGase	0.786	ns	ns	0.779	ns	ns
Dead sclerotia	0.891	ns	ns	0.884	ns	ns
Protease	0.697	ns	0.665	ns	ns	ns
Parasitism of sclerotia	0.668	ns	ns	0.710	ns	ns
Eigenvalue	6.392	2.368	2.109	6.046	3.062	1.545
Explained Variance (%)	42.61	15.78	14.06	40.30	20.41	10.30
Cumulative variance	42.61	58.40	72.46	40.30	60.72	71.02

Dim = dimension. ns = not significant at $p < 0.05$.

by the first three components (40.30%, 20.41% and 10.30%), as shown in Fig. 3B. We consider that the first two main components provided enough information to support the main results presented here, and the discussion will be restricted to these. Concerning field variables, disease severity and number of pods per plant accounted for much of the variance in the data. Among the CWDEs, NAGase and β-1,3-glucanase showed a clear influence on variance. However, the proteases only influenced the variance in 2009. Furthermore, dead sclerotia also showed a strong association with the first principal component (Table 2).

Apothecia density was negatively correlated with NAGase and β-1,3-glucanase. Such a correlation indicates that NAGase and β-1,3-glucanase enzymes play a key role in reducing the number of apothecia and the chain of events in the field that account for disease severity, underlining the importance of these CWDEs in the control of white mold. The parasitism of sclerotia in the laboratory indicated by both proportion of dead sclerotia and *Trichoderma* spp. sporulation on sclerotia in the laboratory assays also showed a strong correspondence with the decrease in disease severity observed in the field in 2009 when compared with 2010 (Fig. 3B). β-Glucosidase by its turn was positively correlated to Trichoderma parasitism on sclerotia, in contrast to *S. sclerotiorum* apothecia and white mold severity.

4. Discussion

The results introduce new possibilities for biological control of white mold on common beans, show the feasibility of isolate selection by laboratory procedures (Lopes et al., 2012; Monteiro et al., 2010), and identify the relevant traits that are involved in biological control in the field. The experimental conditions provided enough disease pressure to achieve epidemic levels of disease in 2009 and 2010, representative of inoculum density and disease pressure reported in commercial crops attacked by white mold (Görgen et al., 2010). The significant increase in apothecia density and disease severity in 2010 was attributed to the field management strategy, designed on purpose to maintain or increase disease levels in the second trial. Thus, soybean planting followed by implementation of a no-tillage approach was a successful strategy to achieve high disease pressure and verify the consistency of results from the previous year. These facts, added to proper soil temperature and moisture favor an increase in carpogenic germination of sclerotia (Sun and Yang, 2000). It is also possible that an improvement in soil fertility in 2010 positively influenced crop development which, in turn, enhanced the conducive background for sclerotia germination, produced higher leaf area available for infection and gave higher average yields (Blad et al., 1978; Vieira et al., 2012).

Both in 2009 and in 2010, the treatments with *Trichoderma* spp. showed differences in grain yield compared to the control. Such results concerning biological control of *S. sclerotiorum* are not always observed in the field, as reported by del Río et al. (2007). These authors attributed the lack of differences in yield to low disease incidence and severity of white mold. Additionally, there are several other reports regarding antagonists that efficiently reduce incidence and severity of white mold without impacts on crop yield (Zeng et al., 2012; Carvalho et al., 2011; Fernando et al., 2007). Here, we believe that the combination of planting conditions, methods and data analysis demonstrate the merits of isolates TR696 and TR356 in breaking through this threshold, and encourage in a modest but consistent way the adoption of biocontrol of *S. sclerotiorum* in the field.

Higher disease pressure recorded in 2010 affected the efficiency of several isolates of *Trichoderma* spp., despite it almost did not affect the results of PCA. Similarly, Zeng et al. (2012) also observed that biological control agents were more efficient at higher disease pressure. Apart from isolates TR696 and TR356, which showed stable results in both years, isolates TR022 and 1306 were the only two with higher efficiency under increased severity of white mold. Furthermore, environmental factors such as solar radiation, humidity, and temperature and soil microbial community have their own influence on antagonists, making it difficult to reduce to only a few factors the causes of variation seen between the two field essays (Bae and Knudsen, 2005; Eastburn and Butler, 1991; Naar and Kecskés, 1998).

Several studies have shown that isolates of *Trichoderma* spp. can significantly increase the growth of different plant species (Fontenelle et al., 2011; John et al., 2010; Yedidia et al., 2000), and this has been attributed to phosphate solubilizing enzymes, indoleacetic acid and other metabolites by which Trichoderma promotes plant growth (Harman et al., 2004; Tasaki et al., 2006; Hoyos-Carvajal et al., 2009; Leitão et al., 2010). According to Fig. 2, we infer that isolates 1306, TR696 and TR044 are plant growth promoters and that they increased productivity of bean plants, despite their different capacities to destroy sclerotia in soil.

The association between disease severity and number of apothecia, especially in 2010, also endorses the importance of

reducing the sclerotia burden for the successful management of white mold (Görgen et al., 2010). In this context, efficient antagonists may be considered a valuable tool for disease management according to their effectiveness as observed in the short term.

The effects of biocontrol on *S. sclerotiorum* reproduction (*i.e.*, number of new sclerotia formed after plant infection) were unclear. Despite the significant reduction in disease severity with some treatments, the number of sclerotia in plant residues was in general inversely proportional to white mold severity. In spite of reports that biocontrol of white mold can reduce the number of sclerotia after common bean infection (Huang et al., 2000), we theorize that disease cycle was accelerated in non-protected plots, with earlier attack of plants, higher disease severity and anticipated fall of new sclerotia. Hence, there would be a higher proportion of sclerotia lying on the ground, which was not assessed by this study.

We believe that the multivariate data analysis with PCA evidence in an unprecedented way the importance of NAGase and β-1,3-glucanase in reducing apothecia density, with consequent reduction in disease severity. Trichoderma isolates with antagonistic potential have been mainly characterized by their ability to secrete CWDEs such as chitinases, glucanases and proteases that hydrolyse the cell walls of pathogens (López-Mondéjar et al., 2011; Seidl, 2008; Verma et al., 2007). For the most part, these results were achieved under *in vitro* conditions and do not correlate with the efficiency of *Trichoderma* spp. in the presence of *S. sclerotiorum* hosts.

The chitinolytic system, especially genes encoding NAGase, chitinase, protease and β-glucanase, is considered as essential to the mycoparasitism process (Kubicek et al., 2001; López-Mondéjar et al., 2011). In the present study, we demonstrate the importance of good producers of NAGase and β-1,3-glucanase in the control of *S. sclerotiorum* under field conditions, and recommend their use as biochemical markers for the selection of new *Trichoderma* spp. isolates against *S. sclerotiorum*, in addition to other requirements for effective biological control. In particular, NAGase was the most highly correlated CWDE in terms of higher variance in PCA, emphasizing its relevance for white mold biocontrol. Beyond the high production of NAGase and β-1,3-glucanase, parasitism and death of sclerotia under controlled conditions was another trait linked to control of white mold under field conditions. The top-ranked *T. asperellum* isolates TR696 and TR356 incorporate these three attributes and are an example of the versatility available within the Trichoderma genus, and its potential for successful biological control. These results do not diminish in any way the role of other metabolites or traits not analyzed here, but the same multivariate approach is suggested for future studies.

5. Conclusions

Brazilian isolates of *T. asperellum* TR696 and TR356 are efficient in controlling white mold under field conditions with consistent results shown over the two years of the study.

Principal component analysis offers the appropriate approach to indicate the importance of laboratory-assessed traits for the performance of biocontrol of white mold under field conditions.

The high production of NAGase and β-1,3-glucanase should be used as biochemical markers in screening for new isolates of *Trichoderma* spp. potentially effective against *S. sclerotiorum*.

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