



New isolates of *Trichoderma* antagonistic to *Sclerotinia sclerotiorum*

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Abstract: Forty-nine isolates of *Trichoderma* from the Brazilian Midwest were evaluated for their antagonistic activity *in vitro* against *Sclerotinia sclerotiorum* (causal agent of white mold), which were then identified based on their nuclear ribosomal ITS sequences. Paired culture tests showed that all isolates exhibited some antagonism, with a maximum of 77% mycelial inhibition and complete inhibition of sclerotia production. Two isolates were found to be the most promising biocontrol agents, considering both antagonistic parameters (CEN1253 - *T. koningiopsis* and CEN1265 - *T. brevicompactum*). Five different species were identified: *T. harzianum* (23), *T. spirale* (9), *T. koningiopsis* (8), *T. brevicompactum* (7) and *T. asperellum* (2). These isolates are stored in the Embrapa Fungi Collection for Biological Control and the information obtained in the experiments will be incorporated into the database of biological assets within the genetic resources information system (Allele) and be made available for further studies.

Keywords: Microbial genetic resources, molecular identification, biocontrol, white mold.

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Resumo: Quarenta e nove isolados de *Trichoderma* obtidos no centro-oeste do Brasil foram avaliados quanto a sua atividade antagonística *in vitro* contra *Sclerotinia sclerotiorum* (agente causal do mofo branco) e identificados com base nas sequências ITS do DNA ribossômico nuclear. Os testes de cultivo pareado mostram que todos os isolados exibiram algum antagonismo, com um máximo de 77% de inibição micelial e inibição total da produção de escleródios. Dois isolados se destacaram como os mais promissores, considerando ambos os parâmetros avaliados (CEN1253 - *T. koningiopsis* e CEN1265 - *T. brevicompactum*). Cinco espécies diferentes foram identificadas: *T. harzianum* (23), *T. spirale* (9), *T. koningiopsis* (8), *T. brevicompactum* (7) and *T. asperellum* (2). Estes isolados estão armazenados na Coleção de Fungos para Controle Biológico da Embrapa e as informações obtidas nos experimentos serão incorporadas na base de dados de ativos biológicos, no sistema de informações de recursos genéticos, e disponibilizados para estudos futuros.

Palavras-chave: Recursos genéticos microbianos, identificação molecular, biocontrole, mofo branco.

Introduction

Sclerotinia sclerotiorum Lib. de Bary (*Ss*) is the causal agent of white mold, a disease that affects many hosts such as legumes, sunflowers, canola, most vegetables, and others. This pathogen is widespread in Brazil and the fungus can cause serious losses, mainly due to its contamination or infestation of seeds (Heffer Link & Johnson 2007). Furthermore, the pathogen survives for long periods using specialized resistant structures, called sclerotia (Bianchini et al. 2005). For soil pathogens, chemical control rarely leads to satisfactory results. Furthermore, chemical pesticides pose health risks, lead to the reduction of beneficial soil microflora and increase production costs (Fahmi et al. 2012). In this context, biological control of plant diseases using antagonists has already shown good results in Brazil, as in other countries with more tradition in this practice, such as USA, Canada, Australia and France (Campanhola & Bettiol 2003). Particularly in the areas of organic production, there is high demand for biological control agents, because they have a low impact on the environment, leave no toxic residues in food and are fully compatible with other alternative control measures. However, one of the keys to success in the use of biofungicides is that they must be developed from strains with high activity against the pathogens in question and be adapted to the environmental conditions under which they will operate.

It is necessary to maintain a continuous flow of collection, isolation, evaluation and characterization of biocontrol agents to improve and maintain their efficiency (Lopes et al. 2013). Species of *Trichoderma* Pers. are widely used in the biological control of plant pathogens, but commercial formulations have only become available recently. The lack of properly registered formulations has been a serious obstacle in the use of bioproducts in Brazil (Machado et al. 2012). According to the Brazilian Ministry of Agriculture Livestock and Food Supply (MAPA) database (Agrofit 2016), there are five commercial products recommended for *Ss* control, formulated with either *T. asperellum* and *T. harzianum*. In order to select isolates for new formulations, some studies have reported the effectiveness of different species of *Trichoderma* in control of *Ss*, showing both inhibition of mycelial growth (Amin et al. 2010, Samuel et al. 2010, Matroudi et al. 2009) and the parasitism of sclerotia (Abdullah et al. 2008).

The current taxonomy of *Trichoderma* is based mainly on molecular analysis (Druzhinina et al. 2010, Kullning et al. 2000), since morphological identification of the anamorph forms are unreliable (Druzhinina et al. 2006). With the development of molecular phylogeny, the genus *Trichoderma* has been grouped into taxonomic sections (Bissett 1984); based on phylogenetic clades (Druzhinina et al. 2010). As an extension of this work, Druzhinina and colleagues (2005) developed a DNA barcode for molecular identification of *Trichoderma* species, called *TrichOKEY*. Given the importance of biological control within the sustainable management of agriculture and environmental protection, the current investigation was undertaken to prospect for *Trichoderma* isolates with potential for inhibition of *Ss* in soil of the Federal District, Brazil and to identify them using nrRNA gene ITS sequences.

Material and Methods

The samples were obtained from soils from organic farming systems and native vegetation, from Rural Center Rajadinha (Planaltina, Federal District). The region is part of the Brazilian central highlands, with

an altitude of around 954 m. The main soil type that predominates in the region is red latosol. The collected soils were extracted from fractions of rhizoplane, rhizosphere and roots, up to 5 cm from the following plants: maize (*Zea mays* L.); eggplant (*Solanum melongena* L.); okra (*Abelmoschus esculentus* L. Moench); tomato (*Solanum lycopersicum* L.); squash (*Cucurbita* sp.); cassava (*Manihot esculenta* Crantz); cabbage (*Brassica oleracea* var. *capitata* L.); ornamental fern (*Pleopeltis* sp.); kale (*Brassica oleracea* L. var. *acephala* DC.); *Cestrum* sp. and *Miconia* sp. (Table 2). Pure cultures of the antagonist, originating from monosporic cultures, were tested against *Ss* strain CEN1157 in paired culture experiments, according to Dennis & Webster (1971). The plates were incubated aerobically for seven days at 25 °C, in a photoperiod of 12 hours.

The confrontation tests were performed in triplicate and the control was a disc of culture medium with *Ss* in the center of plate. Radial mycelial growth of the pathogen (cm) was measured with the aid of a millimeter ruler and used for calculating inhibition of mycelial growth rate (Menten et al. 1976) using the equation: $MGI = [(D_{test} - D_{trat} / D_{trat}) * 100]$; where D_{test} is the diameter of the radial mycelial growth of *Ss* in the control treatment without *Trichoderma*, D_{trat} is the diameter of the radial mycelial growth of *Ss* in treatment with *Trichoderma*. The number of sclerotia produced by *Ss* was also counted in the confrontation cultures zones.

Comparison of means was performed using the Scott-Knott clustering test. The model residues were subjected to Box-Cox transformation when they had normal variance. Box-Cox consisted of transforming the data according to expression: $Y' = (Y\lambda - 1) / \lambda$, where Y is the response variable under investigation and λ is a parameter to be estimated. Analyses were performed in the statistical program R (<http://www.r-project.org/>). The significance level for all analyses was 5%.

Genomic DNA was extracted using a phenol-chloroform method (Raeder & Broda 1985) from *Trichoderma* isolates grown in potato dextrose broth in a shaking incubator at 150 rpm for five days. The gDNA was quantified by comparison to a high molecular weight DNA Mass Ladder (Invitrogen). The nuclear ribosomal ITS locus (rDNA; ITS1-5.8S-ITS2) was amplified by PCR using the primers ITS1 5'-TTCCGTAGGTGAACCTGCGG-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3' (White et al. 1990). The PCR reaction volume was 25 µL and contained: 5 ng template DNA; 1 X PCR buffer; 0.2 mM dNTPs; 1.5 mM MgCl₂; 0.4 µM each primer; 1 U Taq DNA polymerase. PCR cycling consisted of an initial 96 °C for 5 min, then 30 cycles 94 °C for 45 s, 60 °C for 45 s and 72 °C for 1 min with a final extension at 72 °C for 10 min. PCR products were visualized using 1% agarose gels and the products subjected to Sanger chain termination sequencing (both strands) using the amplification primers (Macrogen Inc.; Seoul, South Korea).

Contigs were assembled using DNA BASER (<http://www.dnabaser.com/index.html>). BLAST (Basic Local Alignment Search Tool) searches of the NCBI (National Center for Biotechnology Information) Genbank nucleotide database and *TrichOKEY* 2.0 (<http://www.isth.info/>) were used to verify the ITS sequences. Experimentally derived sequences and related reference ITS sequences obtained from Genbank were aligned using MAFFT with its G-INSI option (Katoh & Standley 2013). A phylogenetic hypothesis was calculated using MrBayes (v3.2.5; Ronquist & Huelsenbeck 2003). The Bayesian analysis was conducted using default program settings except that reversible-jump

MCMC (Huelsenbeck et al. 2004) was used to optimize base substitution model parameters within the general time reversible (GTR) framework. MCMC sampling was run for 2 million generations, which allowed the standard deviation of split frequencies to fall to 0.006853, strongly indicating stable convergence. The analysis was repeated to confirm the result obtained. The first 25% of trees were discarded as *burn-in* prior to calculation of the 50% majority rule consensus phylogram. The ITS sequence from *T. longibrachiatum* (Table 1) was used as outgroup for tree rooting.

Table 1. Reference ITS sequences used for identification.

GenBank accession number	Species	Reference
GU198318.1	<i>T. asperellum</i>	Samuels et al. (2010)
EU330949.1	<i>T. brevicompactum</i>	Degenkolb et al. (2008)
AY857253.1	<i>T. harzianum</i>	Druzhinina et al. (2005)
DQ313142.1	<i>T. koningiopsis</i>	Samuels et al. (2006)
EU401576.1	<i>T. longibrachiatum</i>	Druzhinina et al. (2008)
AY857246.1	<i>T. spirale</i>	Druzhinina et al. (2005)

Results

In the paired culture experiments, we observed inhibition of mycelial growth of *Ss* ranging between 28, and 77% (Table 2).

In the statistical analysis of MGI, we observed the formation of five distinct groups (horizontal bars; Figure 1) at the 5% significance level in the Scott-Knott test, as shown (Figure 1). The data were transformed (Box-Cox, $\lambda = 0.42$) and the model was able to explain 91.69% of the total variability in the data (SQResidues= 33.14, SQTreat= 109.98, $R^2=69%$, $F_{48,98} = 6.775$, p-value= 7.8e-16).

Production of *Ss* sclerotia was also reduced in the confrontation cultures by the action of *Trichoderma* isolates (Figure 2). At the 5% level of significance, the ScottKnott test showed that the isolates formed two separate groups. The data were transformed (Box-Cox, $\lambda = 0.55$) and the model was able to explain 66% of the total variability in the data (SQResidues= 222.6, SQTreat= 660.3, $R^2=66%$, $F_{37,76} = 6.092$, p-value < 1.61e-11). The isolates CEN1253, CEN1314, CEN1313, CEN1257 and CEN1304 all caused complete inhibition of sclerotia production (Table 2). The first group of 25 isolates had less inhibition of sclerotia production, ranging between 27 and 10 sclerotia, while in the second group, between eight and two sclerotia were produced.

In the phylogenetic analysis of ITS sequences, rooted with *T. longibrachiatum*, we observed five major clades (Figure 3). The first 23 isolates grouped with the *T. harzianum* reference with a posterior probability of one, although some variability within the clade was present. The second clade contained nine isolates clustering with *T. spirale*, again with a posterior probability of one. The third group comprised into two related clades, one containing eight isolates and *T. koningiopsis*, and the other two isolates clustering with *T. asperellum*. Finally, the fifth clade contained seven isolates and *T. brevicompactum*.

Table 2. List of the *Trichoderma* isolates, results of the *in vitro* mycelial growth inhibition test (MGI) percentage and number of sclerotia (NS) produced by *Sclerotinia sclerotiorum*.

Embrapa Culture collection number	Origin of isolates		Observations	
	Plant	Niche	MGI(%)	NS
CEN1242	Maize	Soil	48	8
CEN1243	Eggplant	Soil	57	23
CEN1244	Okra	Rhizoplane	54	22
CEN1245	Tomato	Rhizosphere	53	27
CEN1247	Squash	Rhizosphere	50	6
CEN1248	Squash	Rhizoplane	51	18
CEN1249	Tomato	Rhizoplane	62	2
CEN1250	Tomato	Soil	63	10
CEN1251	Maize	Soil	53	14
CEN1252	Maize	Soil	50	16
CEN1253	Cassava	Soil	77	0
CEN1254	Tomato	Rhizoplane	60	14
CEN1256	Tomato	Rhizoplane	60	19
CEN1257	Tomato	Soil	59	0
CEN1258	Cassava	Soil	58	14
CEN1259	Cassava	Soil	46	1
CEN1260	Cassava	Rhizosphere	54	17
CEN1261	Cabbage	Rhizosphere	53	10
CEN1262	Eggplant	Rhizosphere	55	15
CEN1263	Eggplant	Rhizosphere	53	13
CEN1264	Eggplant	Rhizosphere	71	4

Continued Table 2.

Embrapa Culture collection number	Origin of isolates		MGI(%)	Observations	
	Plant	Niche		NS	
CEN1265	Tomato	Rhizoplane	70		1
CEN1266	Ornamental fern	Rhizosphere	48		10
CEN1267	Maize	Rhizosphere	44		12
CEN1268	Maize	Rhizoplane	51		6
CEN1269	<i>Miconia</i> sp.	Soil	28		18
CEN1270	<i>Miconia</i> sp.	Soil	53		26
CEN1271	Cassava	Rhizosphere	62		3
CEN1272	<i>Cestrum</i> sp.	Soil	36		19
CEN1274	Kale	Soil	48		11
CEN1298	Tomato	Rhizoplane	63		2
CEN1299	Kale	Solo	65		11
CEN1302	Tomato	Soil	58		6
CEN1303	Eggplant	Soil	61		18
CEN1304	Squash	Rhizoplane	58		0
CEN1306	Tomato	Rhizoplane	59		1
CEN1311	Cassava	Soil	54		2
CEN1313	Cabbage	Rhizosphere	62		0
CEN1314	Eggplant	Rhizosphere	66		0
CEN1319	Ornamental fern	Rhizosphere	69		7
CEN1326	Kale	Rhizoplane	59		3
CEN1333	Cassava	Rhizoplane	56		4
CEN1337	<i>Miconia</i> sp.	Rhizoplane	52		7
CEN1339	<i>Miconia</i> sp.	Soil	60		7
CEN1345	Squash	Soil	50		6
CEN1346	Cassava	Soil	51		17
CEN1347	Squash	Rhizoplane	65		9
CEN1348	Squash	Soil	63		10
Control treatment	-	-	-		30

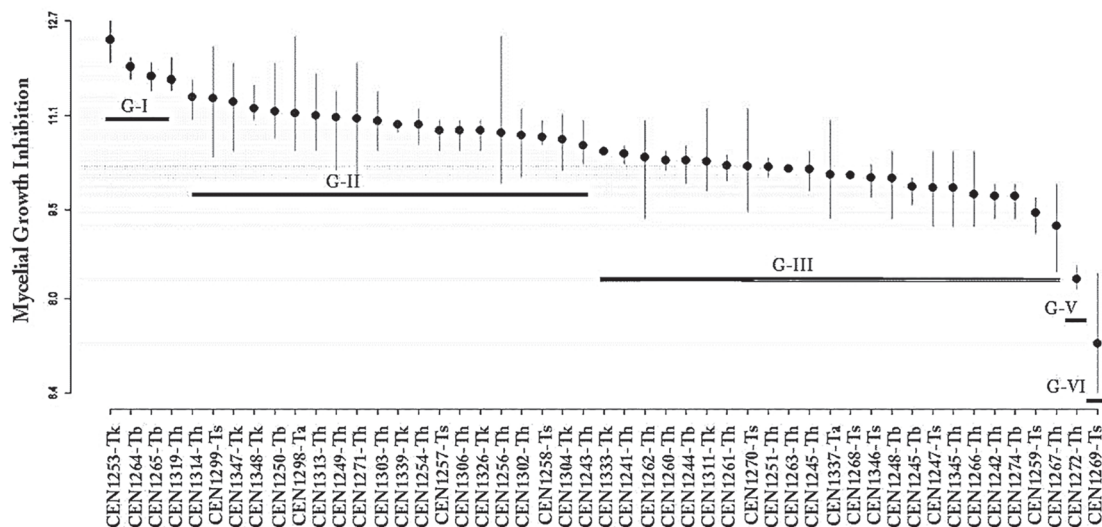


Figure 1. Average inhibition of mycelial growth of *Sclerotinia sclerotiorum* by *Trichoderma* spp., according to the Scott-Knott test.

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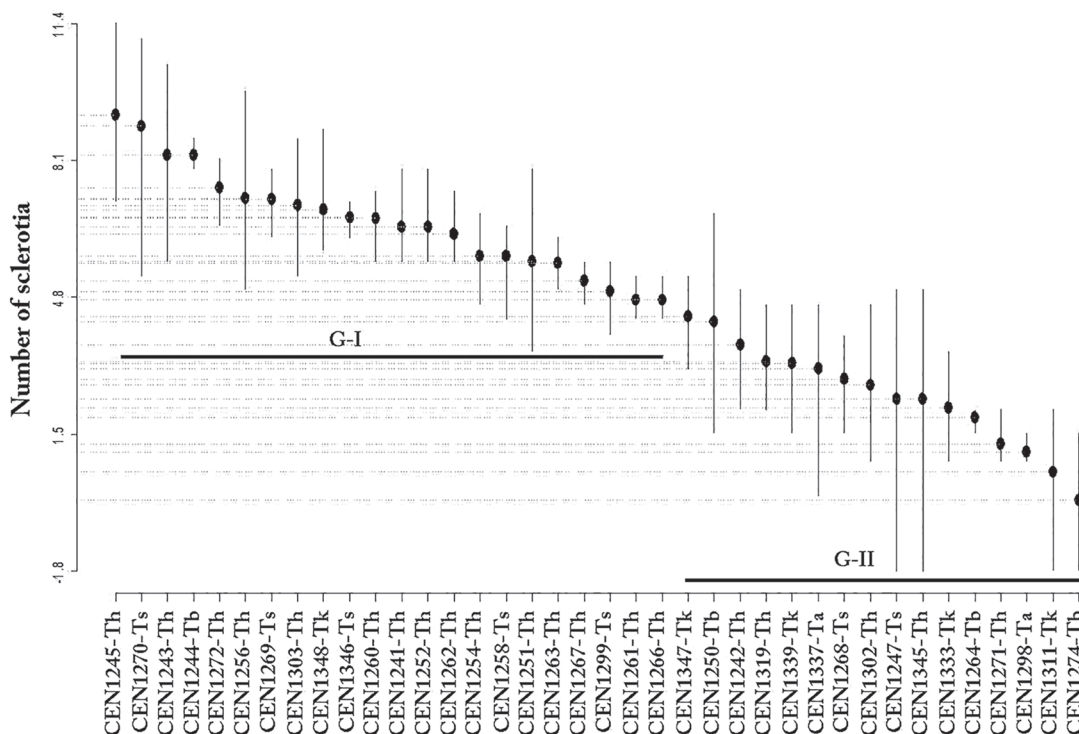


Figure 2. Mean production of *Ss* sclerotia in the presence of *Trichoderma*, according to the Scott-Knott test.

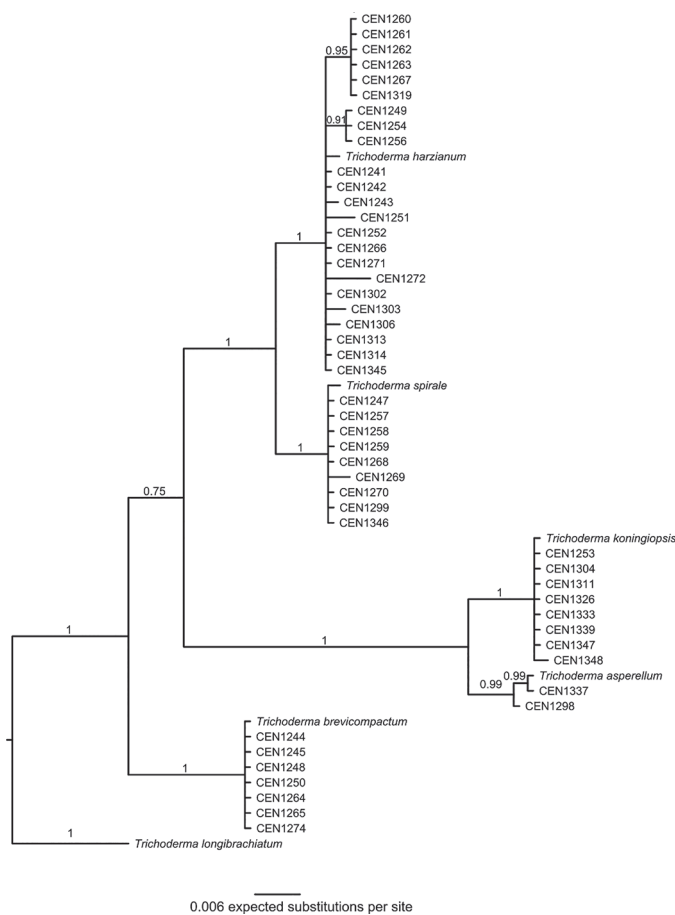


Figure 3. Bayesian phylogenetic tree based on ITS sequences from 49 *Trichoderma* isolates.

Discussion

In the present work, isolates with greater inhibitory potential were found to belong to the species *T. koningiopsis*, *T. brevicompactum* and *T. harzianum*. Experiments carried out by Amin et al. (2010) showed that *T. viride* was most inhibitory to *S. sclerotiorum* (66%), while two isolates of *T. harzianum* exhibited MGI values of 56.57% and 38.12% respectively. Matroudi et al. (2009) used an *Ss* isolate from canola, to evaluate antagonistic *Trichoderma* spp., reporting variation between isolates. However, *T. atroviride* was the most efficient species and reduced growth between 85-93% compared to *T. harzianum* that presented an MGI of 70-80%. Likewise, there was no correlation between the source niche of the antagonist and its inhibitory potential *in vitro*. Here, the most efficient isolates were obtained from both the rhizosphere and rhizoplane as well from soil samples.

The ability to reduce the production of sclerotia is a valuable quality for the selection of *Trichoderma* isolates for biocontrol in view of the importance of the resistant structure on the survival of the pathogen in soil (Bianchini et al. 2005). Some isolates from this study (*T. koningiopsis*, *T. harzianum* and *T. spirale*) completely inhibited the production of *Ss* sclerotia. In the same sense, Abdullah et al. (2008) observed inhibition of the formation of *Ss* sclerotia by *T. harzianum*, that decreased from 31.66 (treatment control) to 12.07 and 18.12 sclerotia. On the other hand, the most inhibitory isolate from the work reported by Amin et al. (2010) was *T. viride*.

Following the classification of Druzhinina et al. (2010), we observed some ITS sequence variation among the isolates clustering with *T. harzianum*. The formation of subgroups within *T. harzianum* is recognized, since it is regarded as a species complex (Kullnig et al. 2000). The second clade included *T. spirale* and these first two

species have phylogenetic affinity (Druzhinina & Kubicek 2005), belonging to *Trichoderma* Section *Pachybasium* B. The third group (*T. koningiopsis* and *T. asperellum*) with related clades are classified in Section *Pachybasium* A. The fifth and final clade contained *T. brevicompactum* are placed in Section *Lutea* (<http://www.isth.info/biodiversity/>). Although the ITS sequences showed multiple hits in BLASTn for each query, these were clarified and confirmed by the *TrichOKEY* program which corroborates the phylogenetic analysis. The advantage of this online tool is that it was developed by taxonomists skilled in *Trichoderma/Hyphocrea*, where the database is restricted to correctly identified sequences (Druzhinina et al. 2005).

A majority of the isolates were identified as *T. harzianum*, followed by *T. spirale*, *T. koningiopsis*, *T. brevicompactum* and *T. asperellum*, respectively. This distribution was expected, since it is known that *T. harzianum* is a global species, colonizing the most diverse substrates and ecological niches with a broad geographic distribution (Kubicek et al. 2008).

This study permitted the selection of isolates with good antagonistic potential for *S. sclerotiorum* and two of them: CEN1253 and CEN1265, identified as *T. koningiopsis* and *T. brevicompactum*, respectively, were considered to be promising for both biocontrol parameters (inhibition of mycelial growth and reduction of sclerotia). These fungi are stored in the Embrapa Fungi Collection for Biological Control and the information obtained in the experiments will be incorporated into the database of biological assets and genetic resources system (Allele, <http://alelomicro.cenargen.embrapa.br/>) for further studies required for the process of development of commercial products and procedures established by the Brazilian Ministry of Agriculture Livestock and Food Supply, until they are available for use by farmers.

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