Original Research Article

Characterization of novel Brazilians rhizosphere soil *Trichoderma* to select effective biocontrol agents against *Sclerotinia sclerotiorum* on beans

ABSTRACT

Trichoderma spp. are fungus considered one of the most attractive for biological control due to its different mechanisms action against plant pathogens and action to promote plant growth and productivity. The molecular characterization of this group of antagonists is of great importance for conducting studies in the area of biodiversity and for identifying and selecting the possibility of their use in biological control. Objective of this work was to identify 29 *Trichoderma* isolates based on molecular profiles and phylogenetic analysis sequence the rRNA gene ITS1-5-8S-ITS2, as well as to select the isolates that presented the highest levels of antagonism against *S. sclerotiorum* and that promoted growth in common bean using experiments in vitro and in a greenhouse. Among the sequences obtained, a total of five different *Trichoderma* species were identified: *T. asperellum*, *T. harzianum*, *T. koningiopsis*, *T. brevicompactum* and *T. tomentosum*. The Trichoderma isolates used showed a variation in the mycelial inhibition of the pathogen by non-volatile metabolites between 84.11% to 100%. Regarding the dry weight of the plants treated with *Trichoderma* and the pathogen, it was observed that ten *Trichoderma* isolates promoted plant growth in relation to the control with a variation of 2.6% to 34%. *T. asperellum* CEN201 proved to be the best candidate to participate in a broader selection that would include field tests for the biological control of *S. sclerotiorum* and growth promotion in common bean, as in the laboratory and at home of vegetation showed great success in inhibiting the pathogen and promoting plant growth.

Keywords: Biological control, mycelial inhibition, phylogenetic analysis, white mold

1. INTRODUCTION

Beans are one of the most important crops based on harvested areas and global production of this legume [1]. Brazil is the world's largest producer of common bean (*Phaseolus vulgaris*) with an annual production of 3,250,000 t and an average seed yield of 1,024 kg/ha [2]. Although this crop is susceptible to several diseases, especially in large cultivated areas, few of them are as harmful as white mold, caused by the soil fungus *Sclerotinia sclerotiorum* (Lib.) de Bary (1884) [1,3].

This pathogen is necrotrophic, capable of infecting more than 400 plant species, a fact that makes it an economic threat to world agriculture [4]. White mold epidemics are reported worldwide with estimated losses of 13% to 80%, depending on year and geographic region [5, 6].

In beans, white mold can affect the entire shoot, causing initially small, watery lesions that quickly increase in size. As the disease progresses, the affected parts lose color, becoming yellowish and then brown, producing soft tissue rot, followed by the white cottony mycelium characteristic of the fungus [7].

Sclerotinia sclerotiorum can produce survival structures by clumping hyphae with a high melanin content in the outer layer, called sclerotia. These structures are generally rounded, elongated or irregular in shape of different sizes which, depending on the host, range from a few millimeters to a few centimeters [8,9,10].

Modern agriculture is dependent on a number of synthetic fungicides that significantly contribute to increasing the economic efficiency of crop production to meet the food needs of the rapidly growing global population [11,12]. However, the unbalanced application of agrochemicals leads to environmental degradation and poses numerous challenges to agriculture, ecosystems and soil health [13].

Dozens of fungi and bacteria are widely described as acting in the biocontrol of *S. sclerotiorum* and promoting plant growth, including species of the genus *Trichoderma* Persoon (1794), a fungus considered one of the most attractive for biological control due to its different mechanisms action against plant pathogens and action to promote plant growth and productivity [14,15,16]. Several commercial *Trichoderma* based products are available in most of the world's best-developed large arable areas [17], so its use has become a viable and sustainable resource for reducing the use of agrochemicals in agriculture.

While correct species identification is important in the selection and validation of biocontrol agents, the assessment of infraspecific variation is also important to protect commercial strains and understand the genetic resources available in natural populations [18]. Regarding the identification and taxonomy of *Trichoderma*, until the 1990s, it was entirely based on morphological characteristics observed under optical microscopy and most *Trichoderma* isolates cited in the literature were misidentified due to the difficulty in interspecific differentiation of reproductive structures [19]. The phylogenetic species concept, based on concordance of genealogies of multiple genes, revolutionized fungal taxonomy and exposed weaknesses in traditional identification based only on morphology [20].

After the 1990s, when molecular techniques began to be routinely employed, confirmation or correction of previously designated species occurred. In the past decade, a virtual database (Gen Bank) was established, where nucleotide sequences of most known species are deposited. In addition to this database, a website dedicated to the comparison of nucleotide sequences unique to *Trichoderma* species and their teleomorphs of the genus Hypocrea was created [21].

Given the above, the objective of this work was to identify 29 *Trichoderma* isolates based on molecular profiles and phylogenetic analysis, as well as to select the isolates that presented the highest levels of antagonism against *S. sclerotiorum* and that promoted growth in common bean using experiments *in vitro* and in a greenhouse.

UNDER PEER REVIEW

2. MATERIAL AND METHODS

2.1 Microorganisms and culture conditions

Twenty nine *Trichoderma* strains (Table 01) and one of *Sclerotinia sclerotiorum* (CEN217) were supplied by the Fungal Collection for Biological Control of Plant Pathogens and Weeds of the Brazilian Agricultural Research Corporation (Embrapa), Brazil.

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Table 1. A. rDNA-based molecular identification of *Trichoderma* strains from different Brazilian geographical origins and plant rhizospheres.

Strain code	Geographical origin / substrate	ITS1-5.8S-ITS2	Genbank accession number
CEN162	Distrito Federal / rhizosphere soil from rice	*T. asperellum*	KC561056
CEN201	Mato Grosso / rhizosphere from native soil	*T. asperellum*	KC561057
CEN209	Distrito Federal / rhizosphere from native soil	*T. koningiopsis*	KC561058
CEN210	Distrito Federal / rhizosphere from native soil	*T. koningiopsis*	KC561059
CEN234	Distrito Federal / rhizosphere soil from cotton	*T. harzianum*	KC561060
CEN250	Distrito Federal / rhizosphere soil from cotton	*T. harzianum*	KC561061
CEN252	Distrito Federal / rhizosphere soil from cotton	*T. tomentosum*	KC561062
CEN284	Distrito Federal / rhizosphere soil from cotton	*T. harzianum*	KC561063
CEN287	Distrito Federal / rhizosphere soil from cotton	*T. harzianum*	KC561064
CEN289	Distrito Federal / rhizosphere soil from cotton	*T. harzianum*	KC561065
CEN503	Pernambuco / rhizosphere soil from guava	*T. asperellum*	KC561066
CEN504	Pernambuco / rhizosphere soil from guava	*T. asperellum*	KC561067
CEN509	Pernambuco / rhizosphere soil from guava	*T. brevicompactum*	KC561068
CEN510	Pernambuco / rhizosphere soil from guava	*T. harzianum*	KC561069
CEN511	Pernambuco / rhizosphere soil from guava	*T. brevicompactum*	KC561070
CEN512	Pernambuco / rhizosphere soil from guava	*T. asperellum*	KC561071
CEN514	Pernambuco / rhizosphere soil from guava	*T. asperellum*	KC561072
CEN518	Pernambuco / rhizosphere soil from guava	*T. asperellum*	KC561073
CEN519	Pernambuco / rhizosphere soil from guava	*T. asperellum*	KC561074
CEN520	Pernambuco / rhizosphere soil from guava	*T. asperellum*	KC561075
CEN522	Pernambuco / rhizosphere soil from guava	*T. brevicompactum*	KC561076
CEN698	Distrito Federal / rhizosphere soil from strawberry	*T. koningiopsis*	KC561077
CEN747	Distrito Federal / rhizosphere soil from strawberry	*T. asperellum*	KC561078
CEN761	Distrito Federal / rhizosphere soil from strawberry	*T. koningiopsis*	KC561079
CEN776	Distrito Federal / rhizosphere soil from strawberry	*T. koningiopsis*	KC561080
CEN786	Distrito Federal / rhizosphere soil from strawberry	*T. asperellum*	KC561081
CEN847	Distrito Federal / rhizosphere soil from soybean	*T. asperellum*	KC561082
CEN854	Rio Grande do Sul / native soil	*T. harzianum*	KC561083
CEN865	Rio Grande do Sul / native soil	*T. asperellum*	KC561084

UNDER PEER REVIEW

2.2 Genomic DNA extraction, PCR amplification and sequencing

The *Trichoderma* strains were grown on potato dextrose agar (PDA, Acumedia, Michigan, USA) and the genomic DNA was obtained according to [22]. The rRNA gene ITS1-5.8S-ITS2 regions were amplified with universal primers ITS1 (5´-CCG TAG GTG AAC CTG CGG-3´) and ITS4 (5´-TCC TCC GCT TAT TGA TAT GC-3´) according to [23]. PCR

reactions were performed in 25 µL containing: genomic DNA (5–25 ng), each primer (0.4 mM), dNTPs (0.2 mM, GE Healthcare, Connecticut, USA), $MgCl_2$ (1.5 mM, Healthcare, Connecticut, USA), Taq polymerase (2.0 U, Life Technologies, Ca, USA) and reaction buffer (10 µL, Invitrogen). Amplification was performed as described by [24] with an initial denaturation for 5 min at 94 ºC, 30 cycles of 30 s at 94 ºC, 30 s at 55 ºC, 1 min at 72 ºC, 10 min at 72 ºC, and finally cooling to 4 ºC. Purified PCR products were sequenced at Sanger Macrogen INC, South Korea, using the same primers described for PCR reactions.

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2.3 Analysis of sequence data

DNA sequences were aligned using Clustal X v2.0.12. The terminal regions with poor alignment were manually removed using BioEdit v7.0.5.3, and the remaining portions were aligned together. Sequence analysis of the rRNA gene ITS1-5-8S-ITS2 amplicons was performed using the TrichOKEY 2.0 and TrichoBLAST tools available online at http://www.isth.info/. The sequences were deposited in GenBank.

2.4 Phylogenetic analysis

The rRNA gene ITS1-5.8S-ITS2 sequences were analyzed by maximum parsimony (MP) and Bayesian phylogenetic Inference (BI) for taxonomic characterization and establishment of phylogenetic relationships among the *Trichoderma* strains. Sequences were aligned using the program MUSCLE - Multiple Sequence Alignment v3.5 [25] and the alignment manually corrected using BioEdit Sequence Alignment Editor v7.0.8.0, 1997-2007 [26]. Gaps present in the original alignment were preserved and coded separately using the program FastGap v1.2 [27]. MP analysis was performed using PAUP* program v4.0b10, 2001 [28]. The heuristic search consisted of 1,000 replicates using the random addition of taxa (Stepwise Addition). The exchange was carried out by the algorithm branches TBR (Tree Bisection and Reconnection) and the robustness of the tree topologies was evaluated by bootstrap analysis with 1,000 replicates. Indels were treated as newstate. Bayesian approach was performed using the program MrBayes v3.1.2 [29]. The nucleotide substitution model more appropriate for alignment of the rDNA model was the GTR (General Time Reversible). The algorithm Monte Carlo Markov Chain (MCMC) was started from a random tree and six processed Markov chains for 1,000,000 generations, with samples collected every 100 generations. In the analysis, 25% were discarded of initial samples (burn-in) and the remainder used to determine the distribution of the posterior probability values. The indels were excluded from analysis. MP and IB trees were edited with Fig Tree - Tree figure drawing tool v1.3.1 [30] and Adobe Illustrator Cs5 v15.0.0.0 programs. The *Trichoderma longibrachiatum* CEN1067 was used as outgroup for rooting the tree.

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2.5 Dual culture

To evaluate the antagonism of twenty nine *Trichoderma* strains against *Sclerotinia sclerotiorum* (CEN217), 5-mm diameter discs of PDA medium were taken from the edge of actively growing colonies of fresh fungal cultures, and placed on the surface of a fresh PDA plate at a spacing of 4 cm. The plates were incubated in a BOD at 25 °C, with a 12-h photoperiod. The evaluation was performed when the pathogen completely covered the control plate without *Trichoderma*. The evaluation of antagonism was carried out according to the classification proposed by [31]: grade 1, *Trichoderma* grows on the pathogen and occupies the entire surface of the medium; grade 1.5, *Trichoderma* grows on 87.5% of the surface of the medium; grade 2, *Trichoderma* grows on 66.6% of the surface of the medium; grade 2.5, *Trichoderma* grows on 62.5% of the surface of the medium; grade 3, *Trichoderma* occupies 50.0% of the surface of the medium; grade 3.5, *Trichoderma* grows on 37.5% of the surface of the medium; grade 4, *Trichoderma* grows on 33.3% of the surface of the

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UNDER PEER REVIEW

medium; and grade 5, *Trichoderma* does not grow and the pathogen occupies the entire surface of the medium. The experiment was replicated three times for each *Trichoderma* strains and conduced two times.

2.6 Non volatiles metabolites

To evaluate the *Trichoderma* no volatile metabolite action againt *S. sclerotiorum*, from 7 days old of each *Trichoderma* cultures, 5cm^2 dics of mycelial agar plugs were removed with a cork borer and inoculated in 50 ml sterilized potato dextrose broth (PDB) in 100 ml flasks and incubated at 25 ± 2ºC under orbital shaking at 150 rpm for seven days. Thereafter, the cultures were filtered through whatman N° 1 filter paper and filtered through a 0.45-µm membrane filter. To determine the effect of *Trichoderma* culture filtrate on *S. sclerotiorum* mycelia growth. Sterile PDA medium was distributed into tubes and the fungi filtrate were added to reach final fungi filtrate concentrations of 25% (v/v). The mixtures were individually distributed in Petri dishes (9 cm Ø) and kept in UV light for solidification for 20 minutes. Five mm diameter of 7 days old *S. sclerotiorum* culture was inoculated at the center of Petri dishes. The plates were there after incubated at 25± 2 °C for seven days under alternate cycles of 12 h light and 12 h darkness. Each test was repeated three times, and the efficiency of the filtrates was measured in terms of percentage of *S. sclerotiorum* mycelial growth inhibition calculated using the following formula: mycelial growth inhibition (%) = (dc-dt/dc) ×100 Where dc = mean diameter of control (untreated) and dt = mean diameter of treated mycelium. The experiment was replicated three times for each *Trichoderma* strains and conduced two times.

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2.7 Sporulation in solid substrate fermetation

To determine the sporulation of *Trichoderma* isolates, 1000 mL flasks were filled with 100 g of parboiled rice with 60% distilled water (V/V), sealed (gauze and cotton plug) and then autoclaved at 121 ºC for 25 minutes. After autoclaving, under aseptic conditions, 4 ml of *Trichoderma* conidia suspension (10^8 conidia/mL) were transferred onto the parboiled rice. The plates were thereafter incubated at 25± 2 °C for seven days under alternate cycles of 12 h light and 12 h darkness. To determine the mean sporulation of each of the 29 *Trichoderma* isolates, each erlemeyer was shaken by hand and one gram was collected from each repetition of the treatments to then count each sample in a newbauer chamber. The trataments with each *Trichoderma* strain was repeated three times and experiment repeted twice.

2.8 Greenhouse experiments

The experiments to verify plant growth promotion and white mold action in common bean plants (cultivar “carioca”) were carried out in a greenhouse with temperature variation from 17.2 °C to 21.78 °C and humidity with variation from 85.8% to 88.0%. The treatments were elaborated with 29 *Trichoderma* isolates and the pathogen and as follows: *Trichoderma* + Pathogen; Pathogen and absolute witness (no fungi).

The experiment was set up in polypropylene trays with 72 cells (120 cm^3/cell) containing substrate based on pine bark and vermiculite (Plantmax®). After filling the cells with substrate, 1g of the pathogen was transferred to each corresponding cell and, 48 hours later, 10 ml of the spore suspension was inoculated ($1.0x10^6$ spores ml^{-1}). Plants previously germinated for three days in the laboratory were planted individually in each cell of the tray. The treatments used for the experiment were elaborated in the following three ways: *Trichoderma* + Pathogen; only the pathogen is an absolute witness (no fungi). Dry weight and disease intensity caused by the pathogen were evaluated 21 days after planting.

UNDER PEER REVIEW

The experiment was conducted with four replications per treatment in a completely randomized design and repeated twice. Results were subjected to analysis of variance and to the Scott- Knott test at 5% probability, using the Sisvar software [32].

3. RESULTS AND DISCUSSION

3.1 Identification of *Trichoderma* isolates

The *Trichoderma* strains were identified to species level based upon Blast and Trichokey comparisons with ITS1-5.8S-ITS2 rRNA gene sequence data for known species within the genus *Trichoderma* (Table 1). Among the sequences obtained, a total of five different *Trichoderma* species were identified: *T. asperellum* Samuels, (13), *T. harzianum* Rifai (7), *T. koningiopsis* Samuels (5), *T. brevicompactum* Kraus, (3) and *T. tomentosum* Bisset (1).

Table 1. B. rDNA-based molecular identification of *Trichoderma* strains from different Brazilian geographical origins and plant rhizospheres.

Straisns	Geographical origen/substrate	Identification	Accession number
CEN162	Distrito Federal/rhizosphere soil from rice	*T. asperellum*	KC561056
CEN201	Mato Grosso / rhizosphere soil from native vegetation	*T. asperellum*	KC561057
CEN209	Distrito Federal / rhizosphere soil from native vegetation	*T. koningiopsis*	KC561058
CEN210	Distrito Federal / rhizosphere soil from *copaiba*	*T. koningiopsis*	KC561059
CEN234	Distrito Federal / rhizosphere soil from cotton	*T. harzianum*	KC561060
CEN250	Distrito Federal / rhizosphere soil from cotton	*T. harzianum*	KC561061
CEN252	Distrito Federal / rhizosphere soil from cotton	*T. tomentosum*	KC561062
CEN284	Distrito Federal / rhizosphere soil from cotton	*T. harzianum*	KC561063
CEN287	Distrito Federal / rhizosphere soil from cotton	*T. harzianum*	KC561064
CEN289	Distrito Federal / rhizosphere soil from cotton	*T. harzianum*	KC561065
CEN503	Pernambuco / rhizosphere soil from guava	*T. asperellum*	KC561066
CEN504	Pernambuco / rhizosphere soil from guava	*T. asperellum*	KC561067
CEN509	Pernambuco / rhizosphere soil from guava	*T. brevicompactum*	KC561068
CEN510	Pernambuco / rhizosphere soil from guava	*T. harzianum*	KC561069
CEN511	Pernambuco / rhizosphere soil from guava	*T. brevicompactum*	KC561070
CEN512	Pernambuco / rhizosphere soil from guava	*T. asperellum*	KC561071
CEN514	Pernambuco / rhizosphere soil from guava	*T. asperellum*	KC561072
CEN518	Pernambuco / rhizosphere soil from guava	*T. asperellum*	KC561073
CEN519	Pernambuco / rhizosphere soil from guava	*T. asperellum*	KC561074
CEN520	Pernambuco/rhizosphere soil from guava	*T. asperellum*	KC561075
CEN522	Pernambuco / rhizosphere soil from guava	*T. brevicompactum*	KC561076
CEN698	Distrito Federal / rhizosphere soil from strawberry	*T.koningiopsis*	KC561077
CEN747	Distrito Federal / rhizosphere soil from strawberry	*T. asperellum*	KC561078
CEN761	Distrito Federal / rhizosphere soil from strawberry	*T. koningiopsis*	KC561079
CEN776	Distrito Federal / rhizosphere soil from strawberry	*T. koningiopsis*	KC561080

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UNDER PEER REVIEW

CEN786	Distrito Federal / rhizosphere soil from strawberry	*T. asperellum*	KC561081
CEN847	Distrito Federal / rhizosphere soil from soybean	*T.asperellum*	KC561082
CEN854	Rio Grande do Sul/rhizosphere soil from native vegetation	*T. harzianum*	KC561083
CEN865	Rio Grande do Sul/rhizosphere soil from native vegetation	*T. asperellum*	KC561084

Phylogenetic trees generated from analysis of ITS1-5.8S-ITS2 rRNA gene sequences (Figure 1) using Maximum Parsimony and Bayesian analyses exhibited similar topologies with four well-defined groups with branches strongly supported by posterior probability higher than 0.9 and bootstrap value higher than 80%. These groups corresponded to Hamatum, Viride, Brevicompactum and Harzianum clades, each of which contained a reference sequence for the species. The Hamatum clade was composed of 13 strains of *T. asperellum*, six of which (CEN503, CEN504, CEN512, CEN514, CEN519 and CEN520) forming a monophyletic group, supported with posterior probability of 0.95 and a bootstrap value of 92%. These strains were all collected from guava rhizosphere soil samples in Pernambuco State (Brazil). The Viride clade consisted of five strains of *T. koningiopsis* and infer that CEN776 and CEN761 strains, collected in strawberry crop in Distrito Federal (Brazil), show high similarity. The CEN698 strain, collected in the same geographic origin/substrate, was shown to be phylogenetically distinct to the two other strains. Two monophyletic clades within the Harzianum clade were observed which were very well defined and strongly supported with posterior probability higher than 0.80 and bootstrap value higher than 75%: one containing seven *T. harzianum* strains and the other with the *T. tomentosum* strain. This is informative data that show good separation of the *T. harzianum* and *T. tomentosum* strains.

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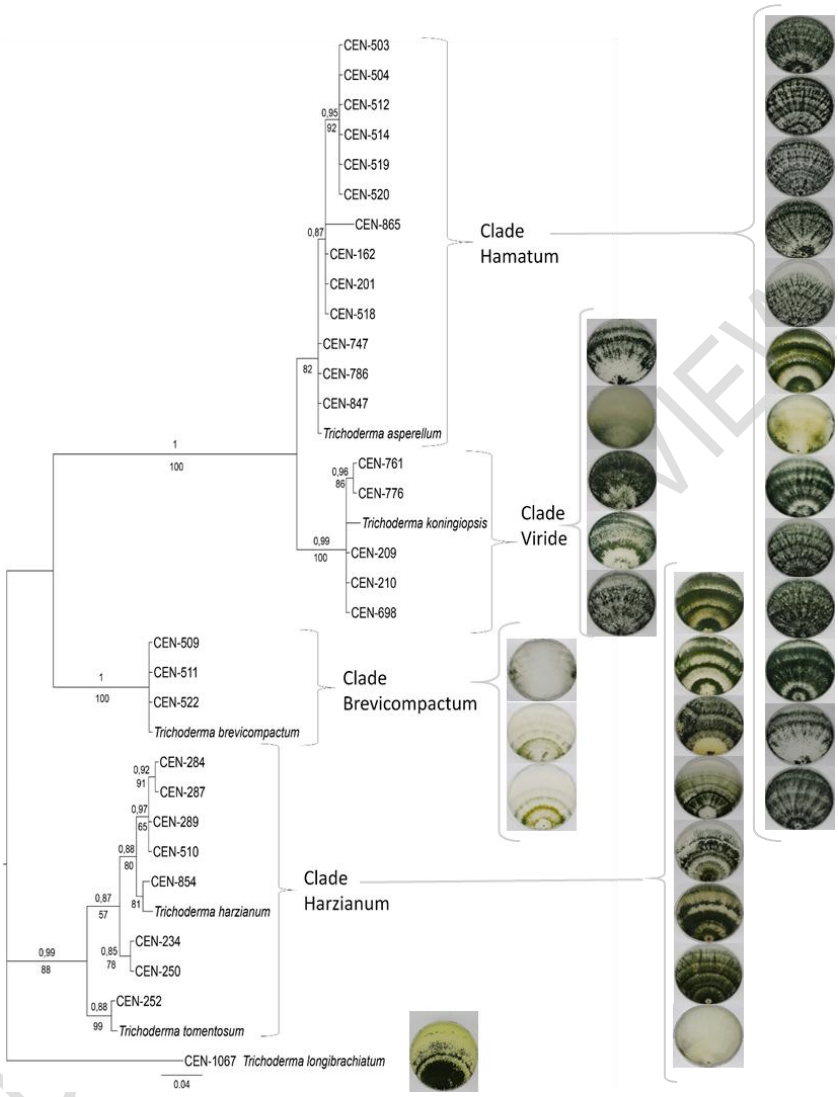

Figure 01 - Phylogenetic tree based on ITS1-5.8S-ITS2 rRNA gene of 29 *Trichoderma* strains. The tree topology was inferred from Bayesian analysis. Numbers above branches indicate Bayesian posterior probabilities (value > 0.9) and numbers below branches indicate bootstrap values (value > 50%) for 1,000 replicates derived from maximum parsimony analysis. The strains *T. asperellum* (JN004182), *T. brevicompactum* (JQ040334), *T. harzianum* (JN179079), *T. koningiopsis* (HQ857120) and *T. tomentosum* (HQ857116) are external reference standard strains obtained from GenBank. *Trichoderma longibrachiatum* CEN1067 was used as outgroup. The images aligned in each clade are the colonies of the respective *Trichoderma* isolates cultivated in PDA medium for 7 days at 25 °C in a 12h photoperiod.

3.2 *Selection of Trichoderma isolates in vitro against Sclerotinia sclerotiorum*

The selection of 29 *Trichoderma* isolates was based on experiments that proved the pathogen's biological control capacity in a laboratory and greenhouse environment.

Regarding the laboratory experiments, the *Trichoderma* isolates used showed a variation in the mycelial inhibition of the pathogen by non-volatile metabolites (Table 02) between 84.11% (CEN511) to 100% (CEN201). Isolates CEN201, CEN503, CEN504, CEN509, CEN512, CEN514, CEN518, CEN519, CEN520, CEN698 and CEN786 showed the highest mycelial inhibition rates against *S. sclerotiorum*, ranging from 90% to 100%.

In the pairing of cultures (Table 02), according to the scale by [31], only grades 01 and 02 were observed. Trichoderma isolates considered as grade 01 were: CEN162, 201, 504, 511, 512, 514, 518, 519, 520.522 and 747. The other isolates were considered as with grade 02.

Analyzing the sporulation of *Trichoderma* isolates in parboiled rice (Table 02), a discrepancy was observed between the production of antagonist spores ranging from $4.35x10^{6}$ spores mL^{-1} (CEN210) to $2.20x10^{8}$ spores mL^{-1} (CEN511). Four isolates produced spores in the house of 106 (CEN209, CEN210, CEN776, CEN786), nineteen isolates in the house of 10^{7} (CEN201, CEN252, CEN284, CEN287, CEN503, CEN504, CEN509, CEN510, CEN514, CEN518, CEN519, CEN520, CEN522, CEN698, CEN747, CEN761, CEN847, CEN854, CEN865) and six isolates in the house of 10^{8} (CEN162, CEN234, CEN250, CEN289, CEN511, CEN512).

Table 2 - Antagonism, growth inhibition of *S. sclerotiorum* and sporulation by *Trichoderma* strains.

Strains Code	Species	Inibition by V.M.%	Dual culture	Conidia mL^{-1}
CEN162	*T. asperellum*	88,89 d	1	1,25E+08 d
CEN201	*T. asperellum*	100,00 a	1	8,65E+07 f
CEN209	*T. koningiopsis*	86,11 f	2	7,75E+06 m
CEN210	*T. koningiopsis*	88,33 e	2	4,35E+06 m
CEN234	*T. harzianum*	88,00 e	2	1,25E+08 d
CEN250	*T. harzianum*	85,78 f	2	1,65E+08 b
CEN252	*T. tomentosum*	87,78 e	2	1,80E+07 m
CEN284	*T.harzianum*	85,78 f	2	9,65E+07 e
CEN287	*T. harzianum*	86,11 f	2	1,35E+07 l
CEN289	*T. harzianum*	88,89 d	2	1,45E+08 c
CEN503	*T. asperellum*	92,22 b	1	8,55E+07 f
CEN504	*T. asperellum*	92,22 b	1	2,80E+07 j
CEN509	*T. brevicompactum*	90,00 d	2	1,45E+07 l
CEN510	*T. harzianum*	87,22 e	2	1,80E+07 l
CEN511	*T. brevicompactum*	84,11 g	1	2,35E+08 a
CEN512	*T. asperellum*	91,56 b	1	1,25E+08 d
CEN514	*T. asperellum*	92,22 b	1	2,55E+07 j
CEN518	*T. asperellum*	92,22 b	1	7,80E+07 f

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UNDER PEER REVIEW

CEN519	*T. asperellum*	92,22 b	1	8,55E+07 f
CEN520	*T. asperellum*	89,78 d	1	9,65E+07 e
CEN522	*T. brevicompactum*	88,56 d	1	3,45E+07 i
CEN698	*T. asperellum*	92,22 b	2	8,75E+07 f
CEN747	*T. asperellum*	88,00 e	1	6,65E+07 g
CEN761	*T. asperellum*	88,56 d	2	7,65E+07 f
CEN776	*T. koningiopsis*	85,00 g	2	8,75E+06 m
CEN786	*T. asperellum*	90,56 c	2	8,65E+06 m
CEN847	*T. asperellum*	87,89 e	2	5,25E+07 h
CEN854	*T. harzianum*	87,89 e	2	3,75E+07 i
CEN865	*T. koningiopsis*	87,89 e	2	1,60E+07 l
CEN217	Testemunha	0,0 h	**	**
C.V.	**	5,71	**	8,91

3.2 Growth promotion and pathogen control in common bean with *Trichoderma* in a greenhouse

Regarding the dry weight of the plants (Figure 2) treated with *Trichoderma* and the pathogen, it was observed that ten *Trichoderma* isolates promoted plant growth in relation to the control with a variation of 2.6% (CEN510) to 34% (CEN201). Of these isolates, nine are of the species *T. asperellum* and one of *T. harzianum*. Isolates CEN201 and CEN162 promoted the greatest development of plants, followed by isolates CEN786, CEN512, CEN503, CEN518, CEN519, CEN854, CEN847 and CEN510. The rest of the 19 isolates tested showed a decrease in plant growth in relation to the control, which ranged from -0.6% (CEN209) to -67.3% (CEN284).

Regarding the control of the pathogen in bean plants under greenhouse conditions, there was an absence of symptoms and signs in treatments that showed an increase in dry weight (Figure 2). In treatments where the dry weight was negative, strong symptoms and signs of the pathogen were observed. Plants treated with isolates CEN209, CEN250 and CEN504 had lower dry weight compared to the control, which ranged from -0.6% (CEN209) to -4.5% (CEN504), however, they did not show symptoms or signs of the pathogen.

When analyzing the correlations between the results of the laboratory experiments and the results in the greenhouse (data not shown), a positive correlation was observed between the tests of "non-volatile metabolites" x "Dry weight of plants in a greenhouse" ($r = 0.45$; $p = 0.34$).

UNDER PEER REVIEW

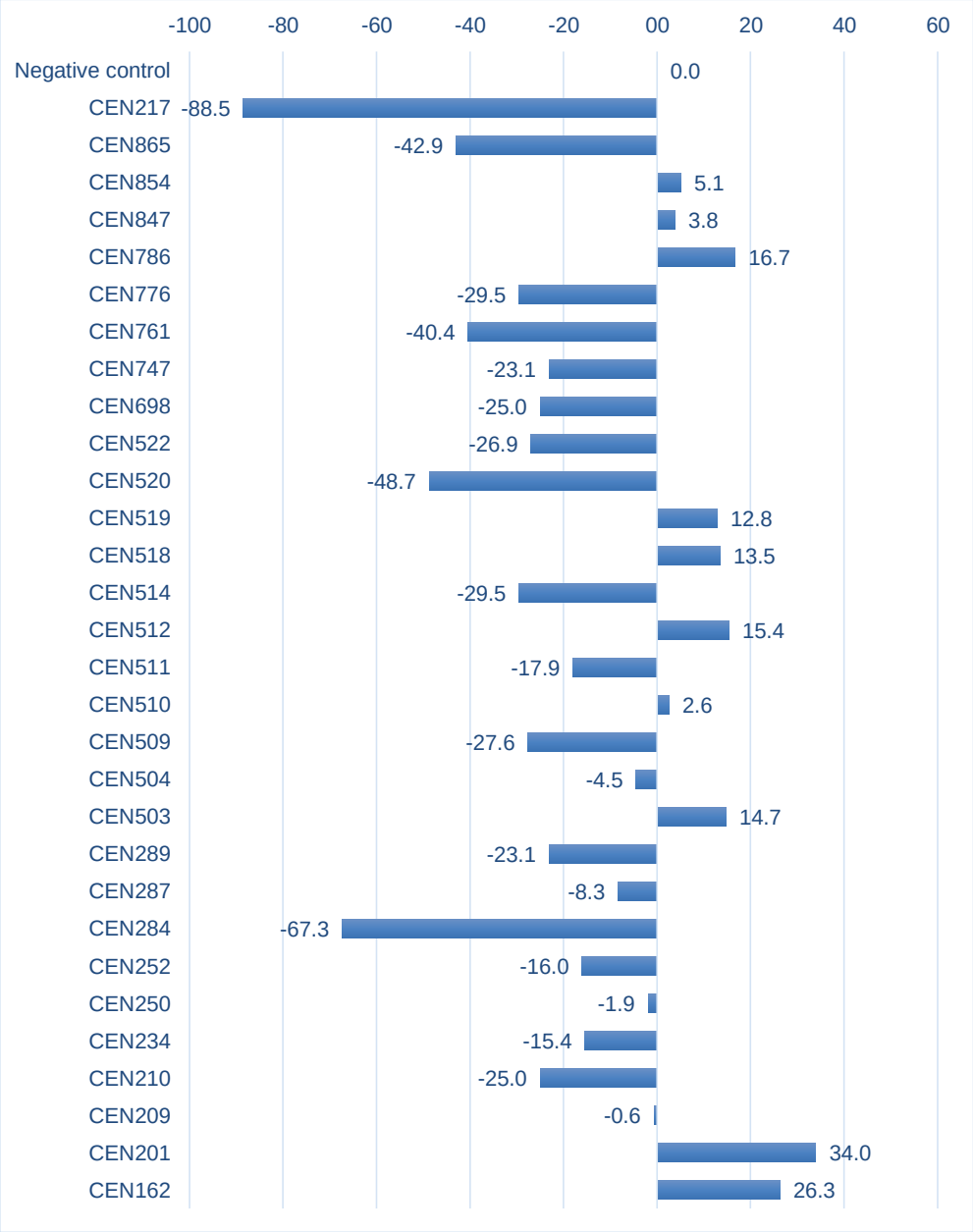

Figure 2. Relative efficiency (%) of dry weight evaluated at 16 days after planting of bean inoculated with *Trichoderma* spp. Coefficient of variation: 10.29

Fungi of the *Trichoderma* genus can act by different mechanisms against phytopathogens and as plant growth promoters. However, it is difficult to be precise about which mechanisms and to what extent they use them to benefit plants and reduce the population of phytopathogenic fungi. These parameters depend on the physical-chemical conditions of the environment. For this reason, the action of *Trichoderma* isolates can be unpredictable [33].

It is important to understand the genetic diversity of *Trichoderma* species, their biocontrol mechanisms and reproductive potential in order to be more successful in applying the best *Trichoderma* isolates in the pathositema in which the problem is observed [33,34]. This genetic diversity was revealed in the *Trichoderma* isolates in this work, according to the information extracted from the ITS1-5.8S-ITS2 rRNA gene, the formation of four different clades after analysis and a clear separation between the groups of five *Trichoderma* was observed species among the 29 sequences obtained.

Thirteen strains of *T. asperellum*, one strain of *T. tomentosum, sevem* strains of *T. harzianum,* five strains of *T. koningiopsis* and tree strains of *T. brevicompactum* were identified on the basis of similarity to the representative identified species. The molecular taxonomy of the *Trichoderma* strains is in agreement with the delineations of [35,36], where species were grouped into respective clades, Hamatum, Viride, Brevicompactum and Harzianum, and were the same as the groupings in Figure 1.

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[37] also obtained results that corroborate this work, the authors assembled a phylogenetic tree using the ITS1-5.8S-ITS2 rRNA gene from 13 *Trichoderma* isolates and revealed similar groupings for the *T. asperellum* group (Hamatum clade), *T. harzianum* (Harzianum clade) and *T. longibrachiatum.* [38] obtained equivalent results when six different species were grouped using the ITS1-5.8S-ITS2 DNA nuclear ribosomal gene, however, the group showed ambiguous identifications for *T. asperellum* and *T. brevicompactum*, unlike the situation in Figure 1 where the difference between the *Trichoderma* species is clear.

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As it is essential to have the correct identification of the bioagent species, knowing the mechanisms of microbial biological control is essential for the selection of *Trichoderma* isolates. Its abilities to inhibit or reduce the mycelial growth of phytopathogens by direct parasitism, antibiosis, competition for space, nutrients or gases are mechanisms that naturally fungi of the genus *Trichoderma* have, although with different intensities depending on biological targets, climate, soil, matter available organic and *Trichoderma* isolate itself [39,40,41].

In this work, it was observed that all obtained from *Trichoderma* dissipated high rate of mycelial inhibition by paired culture and by volatile metabolites against pathogen. One of the most prominent results was CEN201 (*T. asperellum*) with grade 1 on the scale [31] and did not generate mycelial growth of the pathogen when in contact with its non-volatile metabolite. This result is in compliance with [42], noting that the isolate T-aloe (*T. harzianum*) inhibited mycelial growth by pairing cultures against *S. sclerotiorum* by 56.3% (equivalent percentage between grade 2 and 3 on the scale by [31] and 48% inhibition by non-volatile metabolites[43] obtained similar results in the pairing of cultures of two results of *Trichoderma asperelloides* (T25 and T42) against nine demanded of *S. sclerotiorum*, with the isolate T25 scores lower than the isolate T42 in the scale of [31].

Still on the CEN201 isolate, it was observed that it reached a good sporulation in parboiled rice, but it was not the highest among the *Trichoderma* isolates tested. The isolate that reached the highest sporulation was CEN511 (*T. brevicompactum*), 63.19% higher than

UNDER PEER REVIEW

CEN201, and also reached the highest mycelial inhibition index by pairing and non-volatile metabolite against the pathogen (84.11%). The great difference between these two isolates, in addition to the species, was the behavior in the greenhouse, the isolate CEN201 totally controlled the pathogen and promoted the growth of plants by 26%, whereas the isolate CEN511 did not prove to be a good agent for biocontrol of the pathogen, allowing the expression of signs and symptoms of *S. sclerotiorum* in common bean plants.

In the selection of *Trichoderma* for biological control of phytopathogens and promotion of plant growth, it is very important that the microorganism has a high sporulation rate in the chosen culture medium, because in large-scale commercial production, high spore productivity directly influences the reduction the costs of the entire production chain of commercial biological products [44]. Furthermore, the high spore production significantly affects the formulation, shelf life and dose of the product applied in the field [45], generating a product of higher quality and lower price for the farmer. From a biological point of view, the large production of spores can influence disease management and increase the agronomic aspects of the plant, given that one of the main biocontrol mechanisms used by *Trichoderma* is competition for space and nutrients, however, the best isolate for pathogen control was CEN201, which sporulated 26% less than the best isolate in the sporulation item (CEN511). Therefore, sporulation cannot always serve as parameters to indicate that a *Trichoderma* isolate is better or worse for disease control. This was demonstrated by [46] who evaluated the potential for volatile metabolites of *Trichoderma* spp. to inhibit the mycelial growth of *S. sclerotiorum*. These authors demonstrated that the species that sporulated the most was the one that least inhibited the growth of the pathogen's mycelium and inferred that the pathogen's mycelial inhibition seems to be more related to the qualitative profile of the volatile metabolites emitted by *Trichoderma* spp.

Another isolate that stood out in laboratory and greenhouse tests was CEN162 (*T. asperellum*), this isolate reached a high level of mycelial inhibition by non-volatile metabolites (88.9 %) and a maximum score on the scale of [31] against the pathogen and greater sporulation in parboiled rice than CEN201. The great difference between these two isolates was the promotion of bean growth in a greenhouse, CEN162 promoted a 26.3% increase in dry mass of the bean plant, 7.69% more than isolate CEN201.

Several authors use greenhouse trials to analyze disease control and plant growth promotion. These works are highly relevant, as they indicate that microorganisms can be more successful in the field than those tested only in the laboratory. [47] used 19 *Trichoderma* isolates and selected the best 04 according to laboratory tests. Of these four isolates, one isolate showed better plant growth promotion and the other showed the highest rate of inhibition of *Fusarium* wilt in tomato. [48] selected 29 *Trichoderma* isolates in laboratory and greenhouse. The results showed that an isolate UFT201 (*T. asperelloiodes*) was the most efficient (both in laboratory tests and in a greenhouse) for the promotion of growth in Beans Caupi. [49] used the *T. asperelloides* isolate UFT201, mentioned above, to verify the growth promotion in soybean under field conditions in the state of Tocantins, Brazil. The authors concluded that its use promoted greater development of soybean plants and significantly increased grain yield, and showed that the selection of *Trichoderma* isolates in laboratory and greenhouse had positive results in the field.

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The ability of microorganisms to promote plant growth has also been related to the emission of volatile and non-volatile metabolites near plant roots. As for the correlation between the experiments in this work, there was only a positive correlation, although low, between the tests of “Volatile metabolites” vs. “Dry weight of plants in a greenhouse”. This correlation makes a lot of sense, as *Trichoderma* metabolites may have small molecules acting as secondary metabolites in plant growth promotion and against phytopathogens.

UNDER PEER REVIEW

Studies have already shown that several volatile metabolites produced by microorganisms have also been reported in vegetable volatile metabolite profiles [50,51]. This reinforces the theory that metabolites emitted by *Trichoderma* spp. and other microorganisms act as mediators of their biochemical relationships with plant roots and, consequently, can trigger responses capable of stimulating plant growth [52,53,54], favoring the synthesis of plant hormones such as auxins and ethylene [55].

4. CONCLUSION

According to the results of this work, *T. asperellum* CEN201 proved to be the best candidate to participate in a broader selection that would include field tests for the biological control of *S. sclerotiorum* and growth promotion in common bean, as in the laboratory and at home of vegetation showed great success in inhibiting the pathogen and promoting plant growth.

UNDER PEER REVIEW

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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UNDER PEER REVIEW