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Research Article

Antagonism and molecular identification of *Trichoderma* isolated from rhizosphere of medicinal plants

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ABSTRACT: *Trichoderma* is the most studied and used fungal agent in biological disease control worldwide. Its prospection is a necessary routine, in order to select more effective and specific strains for the different existing agro pathosystems. This work reports the *in vitro* antagonism (Mycelial Growth Inhibition - MGI) of five *Trichoderma* isolates, obtained from rhizospheric and organic soil of medicinal plants cultivated in Brazil, to five different phytopathogenic fungi and their molecular identification based on actin (*act*), calmaldulin (*cal*), rDNA gene (ITS) and translation elongation factor 1-α (*tef1*-α). Regarding the fungus *Macrophomina phaseolina*, the MGI varied between 63.33 and 67.03%; for *Fusarium verticillioides* between 67.20 and 85.92%; *Phaeocytostroma sacchari* between 84.00 and 92.90%; in the case of *Sclerotinia sclerotiorum,* the inhibition was total (100%), and for *Sclerotium rolfsii,* the antagonism was between 62.03 and 79.07%. According to the molecular phylogeny performed, concatenated analysis of the genetic markers revealed that the five antagonist fungi belong to the *Trichoderma afroharzianum* species. It is concluded that the *T. afroharzianum* isolates evaluated showed good levels of *in vitro* control of the plant pathogenic fungi in question and will be studied via *in vivo* tests and in plant growth promotion.

KEY WORDS: Antagonistic fungi, biological plant disease control, dual culture, organic cultivation, phytopathogenic fungi, phylogeny

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INTRODUCTION

Fungi are among the most diverse groups of microorganisms, occupying different niches and environmental functions on the planet. Molecular studies, some independent of laboratory cultivation, evidence this diversity and reveal new taxonomic groups existing in the Fungi Kingdom (Wu *et al.,* 2019).

Despite their great environmental, agricultural and industrial importance, a high number of fungal species are pathogenic to plants or affect their post-harvest by-products. Therefore, phytopathogenic fungi can drastically reduce the yield and quality of agricultural products (Peng *et al.,* 2021). Some of these plant diseases are limiting, difficult to control and occur in a complex or multiple way, demanding more and more efficient or, at least, satisfactory management measures (Coque *et al.,* 2020).

Among the available measures for Integrated plant Diseases Management (IDM), biological control is considered an important tool and, in some cases, indispensable. The introduction of Biological Control Agents (BCAs) alters the interaction between plants, pathogens and the environment, triggering a cascade of biological and physical events that not only influence the phytopathogen, but also affect plant health and the ecological functions they exert (He *et al.,* 2021). Thereby, some BCAs interact with plants, inducing latent mechanisms of resistance and act through competition for nutrients or other pathways that modulate the growth conditions of the phytopathogen; antagonists act by direct hyperparasitism and antibiosis (directly interfering with the pathogen). Understanding the mode of action of antagonists is essential to achieve optimal control of plant diseases. Screening studies or selection of BCAs in bioassays has the advantage that those with multiple modes of action and their combinations can be detected (Köhl *et al.,* 2019).

Concerning plant disease BCAs, fungi of the genus *Trichoderma* stand out regarding their wide use in agriculture. These fungi are found in a wide variety of ecosystems, often in forest or agricultural soils (Zin and Badaluddin, 2019; Silva *et al.,* 2020).

Its multifunctionality makes *Trichoderma* the most used BCA in the biological control of plant diseases. The selection and study of such antagonists is a laborious and constant process, involving laboratory techniques such as paired cultivation, which has been carried out for decades and was initiated by Dennis and Webster (1971). These steps are essential because they reveal the mode(s) of action of the antagonists. In addition, a key point in the development of new biopesticides is the discovery of fungal strains with greater activity and more adapted to the agro pathosystems in which these products will be used (Marques *et al.,* 2016; Carvalho *et al.,* 2019).

Screening of the antagonist potential of *Trichoderma* isolates has been demonstrated in scientific works for several plant pathogenic fungi, such as *Macrophomina phaseolina* (El-Benawy *et al.,* 2020, Choudhary *et al.,* 2021, Martínez-Salgado *et al.,* 2021, Paul *et al.,* 2021); *Fusarium* spp. (Pellan *et al.,* 2020; Kumar *et al.,* 2021; Yassin *et al.,* 2021); *Sclerotium rolfsii* (Kotasthane *et al.,* 2014; Kamel *et al.,* 2020; Blanco *et al.,* 2021) and *Sclerotinia sclerotiorum* (Marques *et al.,* 2016; Amaral *et al.,* 2018; Sumida *et al.,* 2018; Carvalho *et al.,* 2019).

In Brazil, some commercial products of biological origin are already available for IDM and are formulated with species such as *T. afroharzianum, T. asperellum, T. atroviride, T. asperelloides, T. harzianum, T. koningiopsis, T. stromaticum* and *T. zelobreve* (Agrofit, 2022). Therefore, the present study aimed to evaluate the antagonism of *Trichoderma* isolates to five phytopathogenic fungi and perform their molecular identification.

MATERIAL AND METHODS

Isolation of the fungal pathogen

Trichoderma isolates were obtained by means of the methodology of serial dilutions of rhizospheric soils in Martin's semi-selective medium (Martin, 1950). They were obtained from previous studies carried out at UPIS Faculdades Integradas, Departamento de Agronomia. These samples come from an organic medicinal garden located near Brasília, in Planaltina-DF, Brazil (15.58ºS, 47.73ºW), a region constituted by the cerrado biome, and which were collected during the month of March 2021. According to the Köppen classification, the municipality has an AW tropical seasonal mega thermal savanna climate (Cardoso *et al.,* 2014). To purify the antagonist colonies, monosporic cultures were performed. After purification, a working collection of fungi was kept in cryovials with 10% glycerol for future tests, and they are described in Table 1, along with other references used.

Concerning phytopathogenic fungi, they belong to the collection of the Núcleo de Pesquisa em Fitopatologia – NPF, Universidade Federal de Goiás, Goiás, Bazil, namely: *Macrophomina phaseolina* (Tassi) Goid (Common bean - *Phaseolus vulgaris* L.), *Fusarium verticillioides* (Sacc.) Nirenberg (Sugarcane – *Saccharum officinarum* L.), *Phaeocytostroma sacchari* (Ellis & Everh.) Sutton (Sugarcane), *Sclerotinia sclerotiorum* (Lib.) DeBary (Common bean), *Sclerotium rolfsii* Sacc. (host not known) and *Rhizoctonia solani* Kühn (host not known).

Antagonism by paired cultures

The antagonism of the *Trichoderma* isolates to the phytopathogenic fungi was evaluated by the technique of paired cultures, where the Petri dishes, containing Potato-Dextrose-Agar (PDA) culture medium, received the PDA discs (5 mm ∅) on diametrically opposite sides with mycelium of the respective antagonists and plant pathogens, according to Dennis and Webster (1971). The plates were incubated aerobically at 25 °C, in a 12 h photoperiod in B.O.D. (Biochemical Oxygen Demand).

Mycelial growth inhibition index

The radial mycelial growth of the pathogens in centimeters (cm) was measured with the aid of a millimeter ruler and the data used to calculate the mycelial growth inhibition index (Menten *et al.,* 1976) using the equation: $MGI = [(D_{control} - D_{treat}/D_{control}) * 100]$; where test $D_{control}$ is the diameter of the radial mycelial growth with the phytopathogens in the control treatment (without *Trichoderma*), and D_{treat} is the diameter of the radial mycelial growth of the phytopathogenic fungi in the treatment confronted with *Trichoderma*.

Experimental design and statistical analysis

For each *Trichoderma* isolate (treatment), three replications were performed (Petri dishes), distributed in a completely randomized design, in a factorial scheme 5 (*Trichoderma* isolates) x 5 (Phytopathogenic fungi). Assays were performed twice.

The radial mycelial growth data (in cm) of the bioassay obtained were submitted to ANOVA and application of the

Table 1. Description of *Trichoderma* isolates identified in rhizospheric soils of medicinal plants in organic cultivation in Brazil and Genbank accession number references of partial sequences of *act*, *cal*, ITS and *tef1*-α used in the phylogenetic analysis

Species	Strain	Location	Source	*act*	*cal*	ITS	*tef1-α*
T. afroharzianum	Tricho 1	Brazil	Lemongrass	OM918745	ON128708	OM654041	Na
T. afroharzianum	Tricho 2	Brazil	Lemongrass	OM918746	ON128709	OM654042	OM885991
T. afroharzianum	Tricho 3	Brazil	Citronella	OM918747	ON128710	OM654043	OM885992
T. afroharzianum	Tricho 4	Brazil	Citronella	OM918748	ON128711	OM654044	OM885993
T. afroharzianum	Tricho 5	Brazil	Citronella	OM918749	ON128712	OM654045	OM885994
T. afroharzianum	CBS466.94	Poland	Na	Na	Na	KP009262	KP008851
T. afroharzianum	CEN1410	Brazil	Onion	MK696756	MK696702	MK714890	MK696648
T. afroharzianum	CEN1414	Brazil	Onion	MK696760	MK696706	MK714894	MK696652
T. pyramidale	CBS:135574	Italy	Olive	Na	Na	KJ665699	Na
T. lentiforme	CEN1415	Brazil	Onion	MK696761	MK696707	MK714895	MK696653
T. guizhouense	GJS 06-100	Cameroon	Soil	FJ442506	FJ442343	FJ442276	FJ463289
T. camerunense	CBS:138272	Cameroon	Soil	FJ442537	AF442875	NR_137300	AF348107
T. endophyticum	GJS 08-184	Argentina	Jatay palm	MH371415	MH371400	Na	MH371384
T. endophyticum	GJS 08-188	Argentina	Jatay palm	MH371416	MH371416	Na	MH371386
T. afarasin	DIS 314F	Cameroon	Wood	FJ442468	FJ442259.1	FJ442259	FJ463400
T. rifaii	DIS 337F	Ecuador	Cacao	FJ442471	FJ442315	FJ442621	FJ463321
T. simmonsii	TR05	Hungary	Grapevine	Na	Na	OK560828	OK655889
T. harzianum	CBS 226.95	UK	Soil	FJ577684	FJ442567	MH874152	AY605833
T. atrobrunneum	GJS 90-254	Na*	Na	FJ442525	AF442886	Na	Na
T. longibrachiatum	CEN1399	Brazil	Garlic	MK696741	MK696687	MK714875	MK696633

*Na: Not available

mean comparison test (Scott-Knott at 5% probability), using SISVAR 5.6 software (Ferreira, 2014).

Molecular identification of *Trichoderma* isolates

DNA was extracted using the CTAB method (Doyle and Doyle, 1987). DNA sequences of actin (*act*); calmodulin (*cal*); internal transcribed spacers rDNA regions (ITS 1 – 5.8S rRNA – ITS2 = ITS), and translation elongation factor 1-α (*tef1-α*) were used in the phylogenetic analyses. The primers used were for *act*, act512F and act783R (Carbone and Kohn, 1999); for *cal*, cal-228, cal-737 (Carbone and Kohn, 1999); for ITS, ITS5 and ITS4 (White *et al.,* 1990); and for *tef1*-α, EF1 and EF2 (O'Donnell *et al.,* 2009).

After amplification, 10 µL of each PCR product was submitted to 1.5% agarose gel electrophoresis, precast with GelRed in TBE buffer at 80 volts and visualized in an UV transilluminator (Boiteux, 1999). PCR products were purified and sequenced by Macrogen Inc., Korea (http://www.macrogen.com). The sequences obtained were analyzed with BioEdit (Hall 1999) and compared with the National Center for Biotechnology Information (NCBI) GenBank using Basic Local Alignment Search Tool (BLAST).

The sequences of the isolates from this study were aligned with the additional sequences using MEGA v. 6 software (Tamura *et al.,* 2013) employing the MUSCLE®

algorithm (Edgar 2004). The alignments were checked, and manual adjustments were made, when necessary. Gaps were treated as missing data. The best nucleotide substitution model for each gene/region was determined using the Akaike Information Criterion (AIC) implemented in MrMODELTEST 2.3 (Posada and Buckley, 2004).

Bayesian Inference (BI) analyses employing the Markov Chain Monte Carlo (MCMC) method were performed with the selected sequences, first with each region separately, then with the combined dataset. The phylogenetic analysis of each region and of the concatenated dataset was conducted in MrBayes on XSEDE v.3.2.6 (Ronquist *et al.,* 2012) at the CIPRES Science Gateway (Miller *et al.,* 2010). Four MCMC chains were run simultaneously, starting from random trees for 10,000,000 generations. Trees were sampled every 1,000 generations, resulting in 10,001 trees. The first 2,500 trees were discarded as the burn-in phase of each analysis. The Posterior Probabilities (PP) (Rannala and Yang, 1996) were determined from a majority-rule consensus tree that was generated from the remaining trees. The trees were visualized in FigTree v. 1.3.1 (Rambaut 2009), exported and edited using the graphics programs. *Trichoderma longibrachiatum* CEN1399 was used as outgroup.

RESULTS

Mycelial growth inhibition

As regards the bioassays, it was possible to observe that all *Trichoderma* isolates exhibited *in vitro* antagonistic effect to all the phytopathogenic fungi tested (Table 2, Fig. 1). For the fungus *M. phaseolina*, the MGI varied between 63.33 and 67.03%; for *F. verticilioides* between 67.20 and 85.92%; *P. sacchari* between 84.00 and 92.90%; in the case of *S. sclerotiorum* the inhibition was total (100%), and for *S. rolfsii* the antagonism ranged between 62.03 and 79.07%.

Statistical analysis

Statistical analysis of radial mycelial growth of fungi (cm) showed that all isolates differed significantly from the control (Fig. 2). As for the plant pathogenic fungi *M. phaseolina* and *F. verticillioides*, there was no difference in antagonism among the *Trichoderma* isolates evaluated. On the other hand, for *P. sacchari* in the treatment with isolate *Tricho* 2, slightly higher growth was observed than with the others (1.43 cm), but still differing significantly from the control. The same was observed for the fungus *S. rolfsii*, although in this case one isolate (*Tricho* 3) stood out with the lowest mycelial growth (1.88 cm), differing significantly from the others and from the control. For *S. sclerotiorium*, there was no significant difference between the antagonist isolates, since all of them completely inhibited the growth of this fungus.

Molecular identification of *Trichoderma*

In reference to phylogenetic analyses carried out, it was observed that the ITS and *act* markers individually were not sufficiently informative, as they did not allow the grouping of our isolates with the reference sequences used (Table 1). However, *cal* and *tef1-α* allowed better resolution, and although not all isolates clustered in the same clade, *Tricho 2* clustered with the *T. endophyticum* reference sequences (data not shown). The isolates in this work were identified as *Trichoderma afroharzianum* because they form a clade with high posterior probability support (PP=1) close to the *T. afroharzianum* species (Fig. 3).

Table 2. Bioassay result, MGI (%)*, in paired culture between *Trichoderma* isolates and five phytopathogenic fungi

***Trichoderma* isolates**	**Plant pathogenic fungi inhibition**				
	Macrophomina phaseolina	***Fusarium verticillioides***	***Phaeocytostroma sacchari***	***Sclerotinia sclerotiorum***	***Sclerotium rolfsii***
Tricho 1	64.07	82.50	89.60	100.00	65.18
Tricho 2	66.29	67.20	84.00	100.00	62.03
Tricho 3	63.33	74.80	88.80	100.00	79.07
Tricho 4	67.03	74.40	92.90	100.00	67.22
Tricho 5	66.11	85.92	89.00	100.00	64.63

* Mycelial Growth Inhibition Index

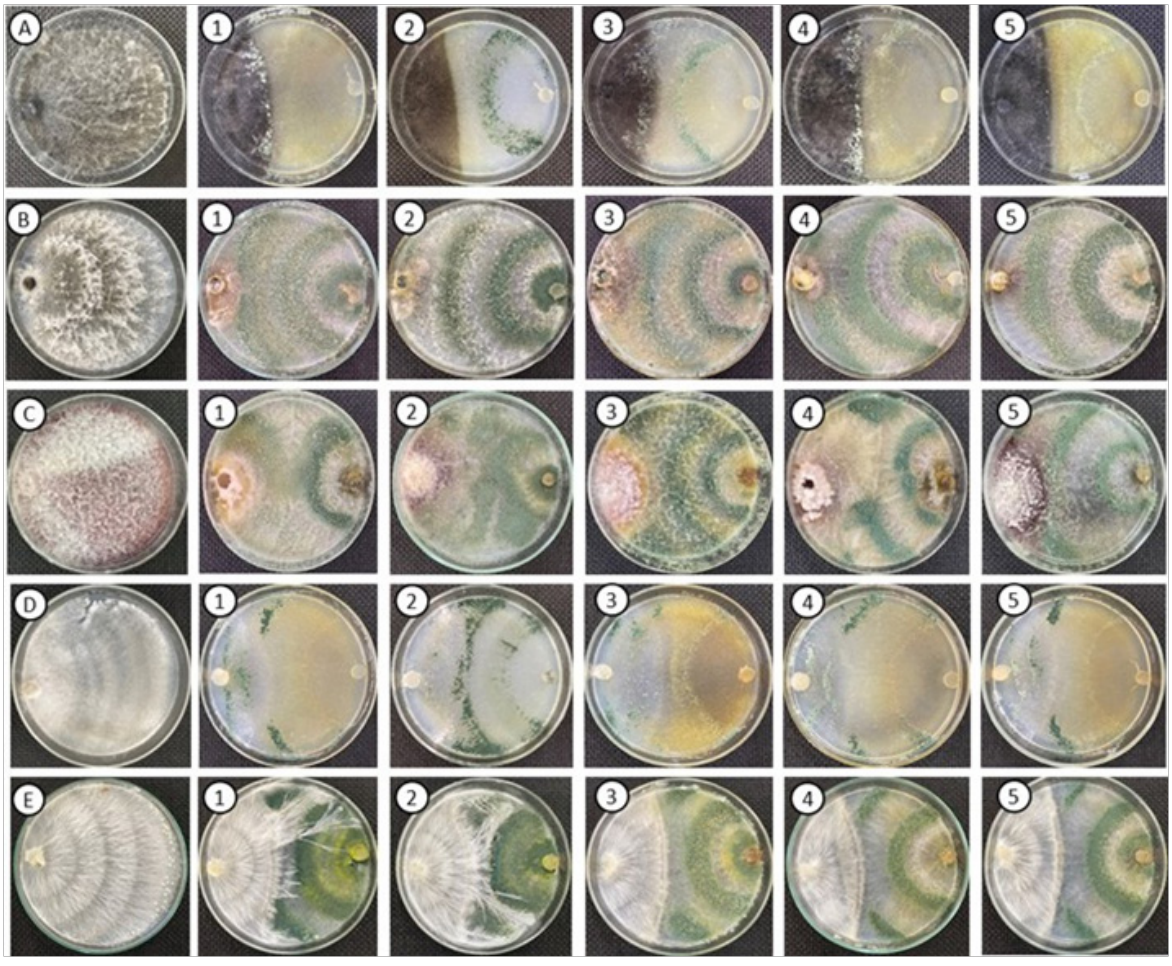

Fig. 1. Paired culture bioassay between *Trichoderma* isolates, of organic origin, and five phytopathogenic fungi where in line A there is *Macrophomina phaseolina*, line B *Phaeocytostroma sacchari*, line C *Fusarium verticillioides*, line D *Sclerotinia sclerotiorum* and line E *Sclerotium rolfsii*; columns 1, 2, 3, 4 and 5 correspond to treatments with *Trichoderma* isolates 1, 2, 3, 4 and 5, respectively.

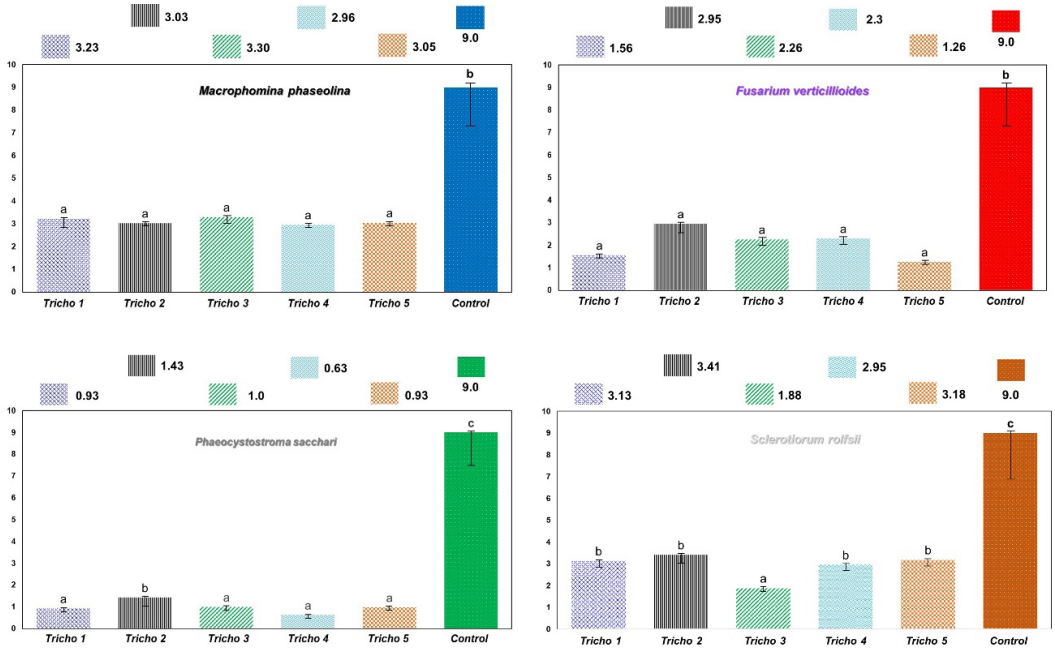

Fig. 2. Statistical analysis of radial mycelial growth, in cm, from paired culture bioassays between *Trichoderma* isolates and four phytopathogenic fungi. Means followed by the same letter do not differ significantly by the Scott-Knott test ($p>0.05$)

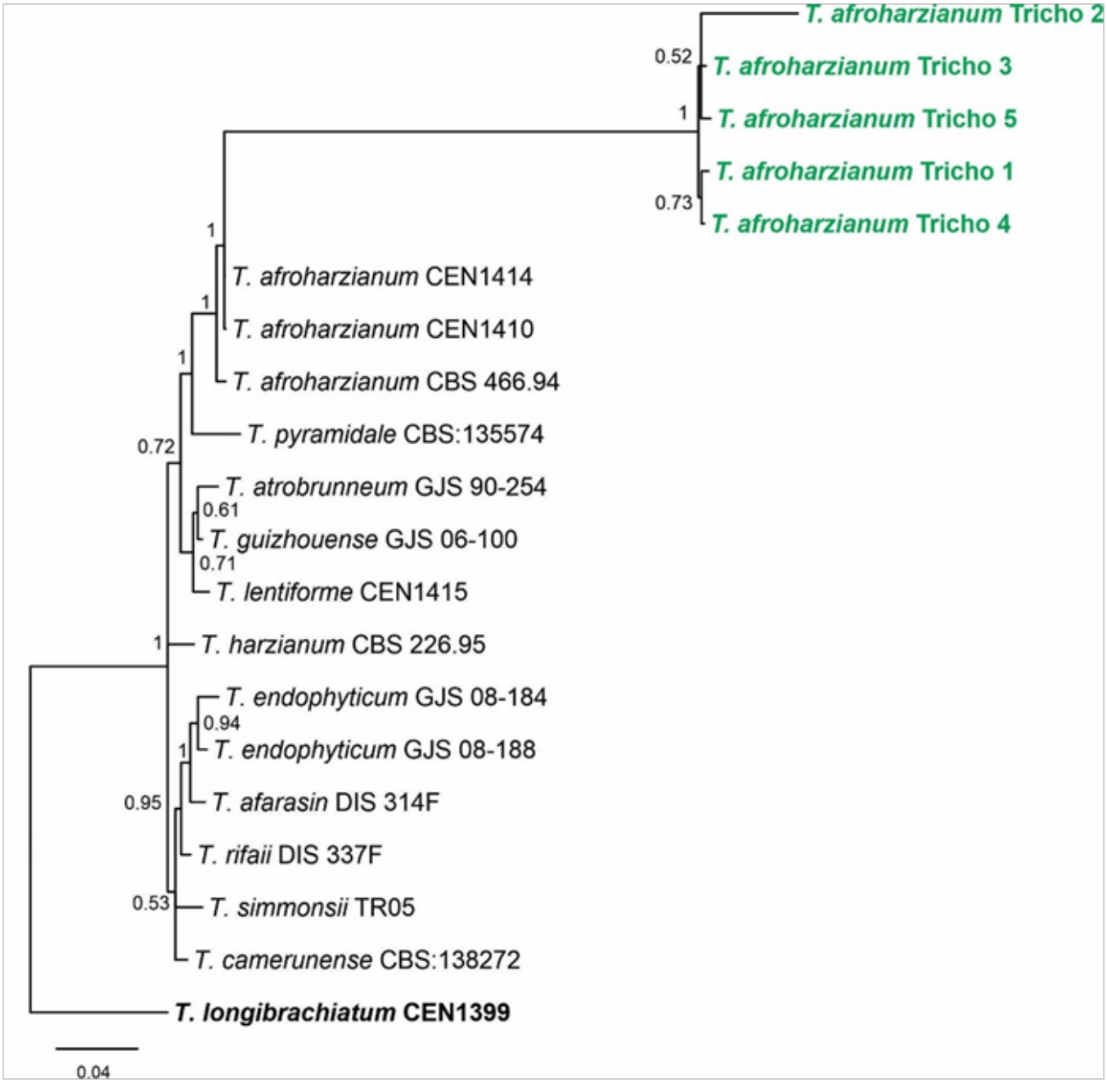

Fig. 3. Multilocus phylogenetic tree as inferred from Bayesian analysis based on the combined sequences of the *act*, *cal*, *ITS* and *tef1*-α. The Bayesian posterior probabilities are indicated next to the nodes. The tree was rooted with *Trichoderma longibrachiatum* CEN1399 (bold). The species in this study are highlighted in green

Discussion

As mentioned above, there was no difference between the antagonist isolates evaluated in reducing the mycelial growth of *M. phaseolina*, the causal agent of charcoal rot. Similar mean inhibition was observed by Choudhary *et al.* (2021), where *T. harzianum*, which restricted the growth of the fungus by 67.59% in relation to the control, stood out. Martínez-Salgado *et al.* (2021) reported the highest percentage of growth inhibition of this phytopathogen (71.11%) by *T. koningipsis*. According to studies by Paul *et al.* (2021), the antagonism exhibited by *T. harzianum* ranged between 75.01 and 78.22%. A slightly higher inhibition rate (72.42%) than in the present study was observed by El-Benawy *et al.,* (2020) by the *T. atroviride* isolate T22.

With respect to the phytopathogenic fungus *F. verticillioides*, one of the causal agents of red rot in sugarcane, no variation in antagonism was observed among the evaluated isolates. According to Kumar *et al.* (2021), the greatest inhibition of the growth of this phytopathogen was demonstrated in treatment with the species *T. harzianum* and *T. viride*, with 64.0 and 70.0%, respectively. The work by Yassin *et al.* (2021) showed an inhibition rate similar to the present study, where *T. viride* inhibited 70.46% of its growth, while *T. harzianum* exhibited a rate of 60.64%. Also, in paired culture and not very different from what has already been described, *T. asperellum* showed the ability to inhibit the growth of *F. verticillioides* by 72% (Pellan *et al.,* 2020). According to Bounaka *et al.* (2021), *T. afroharzianum* inhibited the growth of *F. culmorum* by 80.68% (crown rot of wheat).

Searches were performed in indexed journals, but no studies were found in the literature on the antagonism of species of *Trichoderma* to *P. sacchari* or its synonyms *Phaeocytosporella sacchari* G.L. Stout and *Pleocyta sacchari* Petr. & Syd., which shows that this work is a pioneer in describing the excellent *in vitro* antagonistic potential of a biological control agent to this sugarcane pathogen. Studies such as these are important, as stem rot has been considered an emerging plant disease (Carabez *et al.,* 2014).

The plant-pathogenic soilborne fungus *S. rolfsii* was inhibited at rates above 62% in the present study. A lower level of *in vitro* antagonism (57%) was observed by Blanco *et al.* (2021) when this pathogen was confronted with *T. asperellum*. Analyzing different species of *Trichoderma*, Kamel *et al.* (2020) reported that *T. koningii* was the one that stood out in inhibiting the growth of this fungus, followed by *T. harzianum*. Corroborating the present work, good levels of control (up to 81%) for the phytopathogenic fungus were reported by Kotasthane *et al.* (2014) for several species of the antagonist such as *T. harzianum, T. virens, T. viride, T. aureoviride*, among others.

The antagonistic capacity of the *Trichoderma* isolates evaluated in the present study was absolute, that is, there was total inhibition of the mycelial growth of *S. sclerotiorum*. Marques *et al.* (2016) reported inhibition ranging between 28 and 77% for different species of antagonists evaluated. According to Sumida *et al.* (2018), *T. asperelloides* isolates exhibited good levels of antagonism to this phytopathogenic fungus. Amaral *et al.,* (2018) reported median inhibition (49-64%) of this phytopathogen by *T. afroharzianum* isolates. Inhibition analogous to that observed here was reported by Carvalho *et al.* (2019), where two *T. harzianum* isolates suppressed 95% and 90% of the mycelial growth of the pathogen in question.

Here the ITS and *act* molecular markers were not able to group the *Trichoderma* isolates with the reference isolates. In contrast, only *tef1-α* and *cal* did not group into a single clade, one of the five isolates. Modern fungal taxonomy has evolved to determine global lists of recognized species. Although species delimitation is subjective, identification is expected to be accurate. With regard to the low resolution of the ITS region, Cai and Druzhinina (2021) argue that this region has insufficient polymorphism and cannot distinguish sister species. As for *tef1-α* sequences, the same authors report that 99% of the species can be identified by this DNA barcoding. According to Inglis *et al.* (2020), *cal* was also one of the most informative characters, after *tef1* and *rpb2*, and *act* the least resolutive, corroborating the results of the present work.

The species *T. afroharzianum* was described by Chaverri *et al.* (2015), when they were reviewing and reclassifying isolates from the *T. harzianum* species complex (Clade Harzianum). According to these authors, this species has worldwide distribution, occurring mainly in soils. Thus, the species is also part of the formulation of several commercial products for biological control of diseases (previously considered *T. harzianum*) and had already been studied and reported in Brazil, for example by Amaral *et al.* (2018) and Inglis *et al.,* (2020). In addition, *T. afroharzianum* has was also observed to be associated with ant colonies (Montoya *et al.,* 2016) and with ear rot in maize (Pfordt *et al.,* 2020). This double, or perhaps multiple, faceted nature of the *Trichoderma* genus has already been discussed by Kredics *et al.* (2018).

CONCLUSION

It is concluded that the *Trichoderma* isolates evaluated showed good levels of *in vitro* control of the plant pathogenic fungi tested, with emphasis on *Phaeocytostroma sacchari,* up

to 92.9% and *S. sclerotiorum*, which was totally inhibited by the antagonist isolates and will be studied via *in vivo* tests and in plant growth promotion. Multilocus phylogenetic analysis revealed that the isolates belong to the species *T. afroharzianum.*

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