



## Molecular identification of isolates of *Trichoderma* spp as biocontroller of *Fusarium falciforme*, causal agent of root rot of table manioc (*Manihot esculenta* Crantz) var. rosinha in the State of Alagoas/Brazil

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### ABSTRACT

Root rot caused by pathogens limits the production of cassava (*Manihot esculenta* Crantz), a crop of high interest for the Northeast region of Brazil. The difficulty in management has led to the search for alternatives, among which, the biocontrol with *Trichoderma* spp, has been showing significant results. Due to the importance of the cassava crop and the existing phytosanitary problems, this work aimed to identify, through molecular tools, the isolates of *Trichoderma* spp. that obtained the best performance in inhibiting the development of *F. falciforme*. *Trichoderma* isolates were selected by the method of confrontation and production of metabolites. Subsequently, the DNA was extracted and the sequencing of the ITS region was performed for phylogenetic analysis. Twelve *Trichoderma* isolates were obtained, among which, five (T5; T6; T10; T13; T15) produced bioactive substances that significantly inhibited the development of *F. falciforme*. The T5; T6 and T15 were submitted to a phylogenetic study based on the ITS region of the DNA because they presented better results. The results of the phylogenetic analysis based on the gene sequences of the ITS region of the DNA, revealed with 100% reliability that the isolates are of the genus *Trichoderma*, being T5 of the species *T. citrinoviride* with 99% of similarity in the sequence; T6 is *T. harzianum* with 95% and T15 is *T. orientale* with 95%. The isolates *T. citrinoviride* and *T. orientale* are the first reports of these species acting as biocontrollers of phytopathogens in cassava plantation areas in the state of Alagoas.

**Keywords:** Molecular biology; Biological control; Antagonism.

## 1 INTRODUCTION

There are several phytopathogens that cause rot in the cassava root (*Manihot esculenta* Crantz) and limit its production. For this reason research to minimize deleterious actions has been occupying an increasing level. Research focused on cassava culture and management has been intensified every day in an attempt to seek viable alternatives that reduce the damage caused by losses caused by pests and diseases, and biocontrol becomes one of the main weapons in due to its ability to control and minimize the use of pesticides and their residues that end up causing soil contamination and reduction of the microbiota (Silva et al., 2020a, 2020b).

Antibiosis, the production of bioactive substances and excretion of enzymes are capable of preventing the development or resulting in complete lysis and dissolution of the cellular structure, regardless of physical contact. These metabolites, even in low concentration, interfere in biological activities, in the reduction or paralysis of growth, sporulation and germination of spores, as well as distortions in hypha and endolysis (Otsubo & Lorenzi, 2004; Santos et al., 2005).

*Trichoderma* is a fungus widely used in biocontrol due to its antagonistic potential and is present in suppressive soils. This microorganism belongs to the phylum Ascomycota which holds several classes, among them are Euscomycetes and Pyrenomycetes of the order Hypocreales, family Hypocreaceae and genus *Hypocrea* (teleomorph) (Melo, 1991).

There are several advantages of *Trichoderma* spp. among them are the disharmonious relationships established by the antibiosis, withpetition, decomposition of organic matter, colonization of the rhizosphere in which a competition is established between soil phytopathogen microorganisms, and in plants can induce resistance (Harman, 2000, 2006; Harman et al., 2004).

Several studies have been published proving the biocontrolling activity of *Trichoderma* spp., among them are the works of (Melo et al., 1998) who worked with *Rhizoctonia solani* Kühn, *Sclerotium rolfsii* Sacc., *Sclerotinia sclerotiorum* (Lib.) de Bary, *Fusarium* spp. (Azevedo et al., 2021) and *Pythium* spp. obtaining satisfactory results using the biocontroller.

Marques et al., (2014); Rollán et al., (1999) and Rondón et al., (2007) comment that among numerous species of *Trichoderma* spp. that they worked, the species that presented the highest rate of hyperparasitism and antagonism to phytopathogens to *S. rolfsii*, *S. minor* and *S. sclerotiorum*, *R. solani* and *Pyricularia grisea* Sacc was *T. Hifai harzianum*.

Silva et al., (2020b) in their studies obtained a satisfactory result where all the isolates used obtained a satisfactory rate of antibiosis and hyperparasitism (*in vitro*) with *Trichoderma* spp. isolates, collected from soils of areas producing cassava in the state of Alagoas. Dos Santos (2010) reports that his work using *S. rolfsii* confronted with *T. harzianum*, was able to antagonize and hyperparasite *in vitro*, the hyphae of phytopathogens in addition to competing for nutrients and space. Comtroller *S. lignicola*, today *Neoscytalidium* sp, with *Trichoderma* spp., Silva et al., (2013) observed that the biocontroller isolate was promising because it was perceived that there was an overlap of hyphae in the phytopathogen colony. Semelhantes results were obtained in the work of Silva et al., (2020b) where *Neoscytalidium* sp from cassava plantation areas were confronted with *Trichoderma* isolates. The pathogen proved to be very competitive by space and nutrient due to its speed of growth and colonization that exceeded that of the biocontroller, but was noticed the action of *Trichoderma* spp, hyperparasitizing the phytopathogens ( unpublished data).

In relation to antibiosis and competition for food, *Trichoderma* spp. has as its acteristic carthe rapid colonization due to its speed of growth and efficiency in assimilating the nutrients of the medium, ensuring its biological cycle (Barbosa & Meza, 2009; Hobbs, 1992).

For Ethur, (2006), among *Trichoderma* spp.'s strategies the most efficient is competition, because from it is established the dispute for space, nutrients and later antibiosis and hyperparasitism will occur.

From the process of antibiotic production by the biocontroller, antibiosis is more active and even if it is in minimal concentrations, they can cause metabolic and physiological interferences paralyzing the growth, reduction and inhibition of spore production, hydrolysis degradation and hyphae distortion (Bettiol & Wagner Bettiol, 1991; Carvalho Filho, 2013; Claydon et al., 1987; Days, 2011; Souza, 2013).

According to Amorim et al., (2016) several bioactive substances are synthesized by *Trichoderma* spp. such as gliotoxin, viridine and trichodermin, which interferes in the development of phytopathogens. Ethur's (2006) study concluded that gliotoxin was able to interfere with *pythium ultimum* physiology by inhibiting mycelial growth and germination of sporangias.

The successor of a biocontroller is due to the ability to produce enzymatic bioactives capable of hydrolysing the cell wall that is composed of lipid, proteins, carbohydrates, chitin and cellulose. Thus, the biocontroller needs to synthesize lipases, proteases, chitinases, cellulase, glucanases, etc. These enzymes need to be produced in the quantities necessary to interrupt the biological process of phytopathogens and ensure the penetration, antibiosis and hyperparasitism of the biocontroller (Bomfim et al., 2010; Harman et al., 2004; Melo, 1991; Silva, et al., 2020a, 2020b; Souza, 2013).

Another important mechanism for the success of biocontrollers such as *Trichoderma* spp., is mycoparasitism that has as its strategy to survive inside or on the phytopathogen and to remove the nutrients necessary for its development. This type of antibiosis uses enzymes for penetration and hydrolysis causing the death of the phytopathogen or other biocontroller that is at a nutritional or enzymatic disadvantage, leading to a cannibalism action that is the disharmonic relationship between individuals of the same species where the weakest serves as food for the strongest. (Bomfim et al., 2010; Harman et al., 2004; Silva, et al., 2020a, 2020b; Zucchi, 2010).

Among the enzymes produced by *Trichoderma* spp hydrolysing chitin,  $\beta$ -1,3-glucanase is the one that most interacts in mycoparasitism. This is due to most phytopathogens having a cell wall rich in chitin (Bauermeister et al., 2010; Giese, 2010; Marcello, 2008).

The most efficient form of mycoparasitism is necrotrophic, because this type of biocontrol antagonizes fungi that have resistance structures such as sclerotia, chlamydospores and microsteria (Medeiros et al., 2021; Melo et al., 1998). The genus *Trichoderma* spp. an excellent biocontroller is shown, due to the characteristics presented, such as : enzymatic activity capable of hydrolysing hyphae and resistance structures, biochemically detecting the structures of phytopathogens, curling, strangling and penetrating via a pressium, and nourishing the host, thereby reducing the phytopathogen population, besides being easily spread, surviving unfavorable environments and not offering environmental impact (Azevedo et al., 2021; Melo Et. al., 1998; Spiegel & Chet, 1998; Vinale et al., 2008).

Due to the importance of cassava culture and existing phytosanitary problems, this study aimed to identify through molecular tools the isolates of *Trichoderma* spp. that obtained the best performance in inhibiting the development of *F. sickle cell*.

## 2 METHODOLOGY

The studies were conducted in the Laboratory of Phytopathology and Molecular Phytopathology, of the Academic Unit Center of Agrarian Sciences (CECA), of the Federal University of Alagoas (UFAL) - Delza Gitaí Campus, in Rio Largo, State of Alagoas.

### 2.1 DNA EXTRACTION FROM *TRICHODERMA* SPP ISOLATES

The isolates used in this experiment were previously selected in a previous study, according to (Silva et al., 2020a, 2020b).

Mycelium discs of *Trichoderma* spp isolates were transferred to Erlenmeyers with 30 mL of sucrose-extrat yeast-asparagine liquid medium (Alfenas et al., 2007). After five days, the mycelium was removed and the excesses of the medium washed with sterilized distilled water. For extraction, 200mg of the mycelium of each isolate were transferred to mortar and refrigerated at  $-80^{\circ}\text{C}$  for five minutes. Then, mycelium was macerated in the presence of the extraction buffer, according to the CTAB protocol (*Cationic Hexadecyl Trimethyl Ammonium Bromide*), according to (Doyle & Doyle, 1987).

The crushed mycelium of the isolates were transferred to two micro-centrifuge tubes with a capacity of 1.5 mL. Then, 1 mL of extraction buffer was added (CTAB 4%, NaCl 1.4M, EDTA 20 mM, Tris-HCl 100 mM, PVP 1%), 4  $\mu\text{L}$  of  $\beta$ -mercaptoethanol, and then the tubes were kept in a bath at  $65^{\circ}\text{C}$  for 30 minutes. Subsequently, the samples were centrifuged at 12,000 rpm for 15 minutes. The supernatant was transferred to a new tube, where 600  $\mu\text{L}$  of CIA was added (chloroform: 24:1 isoamyl alcohol). After centrifugation, the aqueous phase was transferred to a new tube, adding 400  $\mu\text{L}$  of absolute ethanol.

The precipitated DNA was washed with 70% ethanol and dried at room temperature and then resuspended with 40  $\mu\text{L}$  of ET (Tris-EDTA; Tris-HCl 10 mM, EDTA 1mM) + RNase (10  $\mu\text{g}/\text{mL}$ ). DNA quality was visually estimated in 0.8% agarose gel.

### 2.2 DNAMPLIFICATION AND SEQUENCING OF PCR PRODUCTS

The isolates obtained were amplified with the gene encoding for the ITS-rDNA region (Table 1).

Table 1. Description of the primer used to identify fungal genera.

Gene	Primer	Sequence (5'-3')	References
ITS	ITS1	TCCGTAGGTGAACCTGCGG	White et al., 1990
	ITS4	TCCTCCGCTTATTGATATGC	

Source: Authors (2022).

PCR reactions were prepared with 10X buffer, MgCl<sub>2</sub> 50mM, DNTP's 10mM, 10µM of each oligonucleotide, 1U of *Taq* DNA Polymerase and 1µL of diluted DNA (1:20). The final volume of the reactions was adjusted to 60µL with autoclavada Milli-Q water. PCR reactions occurred in applied biosystems thermocycler (*2720 Thermal Cycler*) under the following conditions for the ITS region: initial denaturation of 95°C for 2 min and 38 cycles of 95°C for 1 min, 55°C for 30s, 72°C for 45s and a final cycle of 10 min for 72°C. Then, the PCR product was sent, for purification and sequencing with the same *primers* used in amplification, to Macrogen Inc. (Seoul, South Korea).

### 2.3 SEQUENCING AND PHYLOGENETIC ANALYSES

Sequencing was performed by Macrogen Inc. (Seoul, South Korea) directly from purified PCR products and later, compared with other sequences deposited in *Genbank* ([www.ncbi.nlm.nih.gov/genbank](http://www.ncbi.nlm.nih.gov/genbank)), using the BlastN (*Nucleotide Basic Local Alignment Search Tool*) program, for the preliminary identification of the fungus species with highest percentage of identity. They were also compared with sequences in the Q-Bank Fungi database (<http://www.q-bank.eu/fungi/>) to determine the fungal genera with which they shared the highest sequence identity.

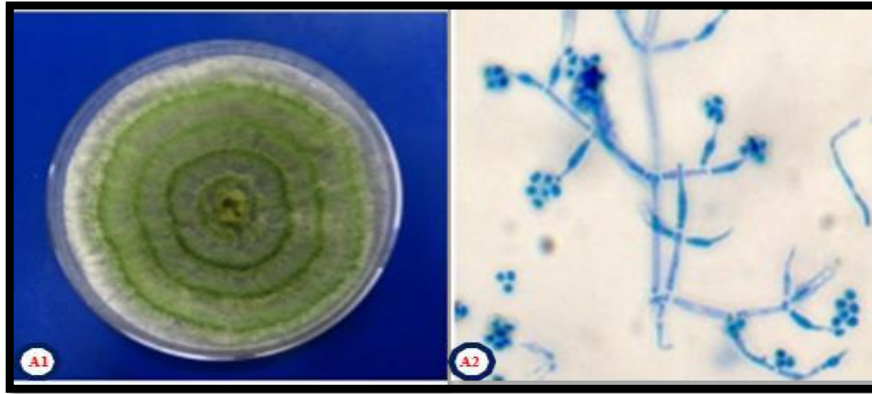
Multiple alignments of nucleotide sequences were prepared using the MUSCLE algorithm (Edgar, 2004) and adjusted manually in the MEGA6 package (Tamura et al., 2013). Bayesian Inference was performed on the CIPRES web portal (Miller et al., 2010) using MrBayes v. 3.2.3 (Ronquist et al., 2012). The best nucleotide replacement model was determined using MrModeltest 2.3 (Posada & Buckley, 2004) according to the *Akaike Information Criterion* (AIC). Analyses were rotated for each dataset for 10 million generations using four chains and showing each 1,000 generations, for a total of 10,000 trees. The first 2,500 trees were discarded as a *burn-in phase*. Later probabilities (Rannala & Yang, 1996) were determined from a *majority-rule consensus tree generated* with the remaining 7,500 trees. The trees were viewed and edited in figtree v. 1.4 ([ztree.bio.ed.ac.uk/software/figtree](http://ztree.bio.ed.ac.uk/software/figtree)) and Inkscape (<https://inkscape.org/pt/>).

## 3 RESULTS AND DISCUSSION

### 3.1 MOLECULAR IDENTIFICATION OF *TRICHODERMA* SPP.

In a previous experiment, according to Silva (2016) and Silva et al (2020ab), twelve *isolates of Trichoderma* spp. were obtained, which presented whitish mycelial growth that quickly originated hyaline conidiophores, aggregated and very branched, not verticillated, simple lyalides or in groups, corroborating with (Micffhere et al., 1993). The conidia found were unicellular, ovoid, with smooth or rough walls produced in small terminal clusters, green or hyaline spreading rapidly on the colony surface, measuring 3.8-4.0 x 3.1-3.7 µm, characteristic of *Trichoderma* species s (Figure 1).

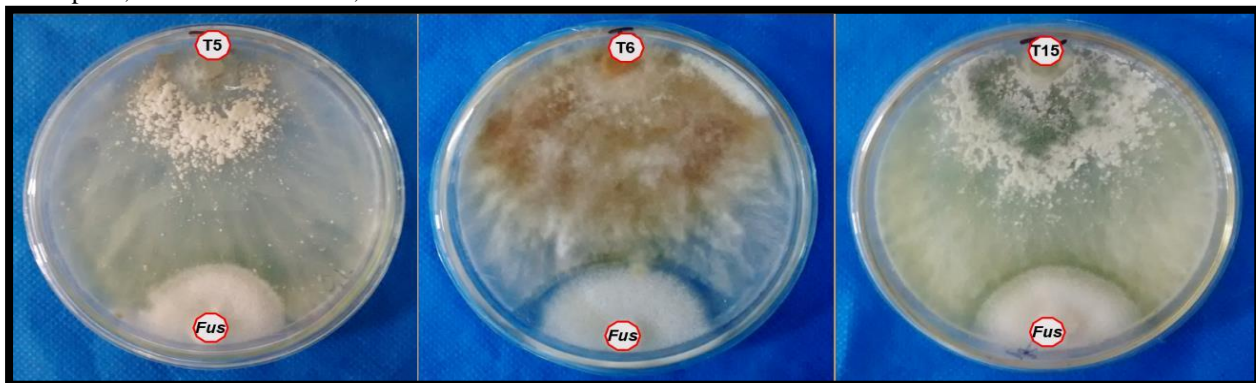
Figure 1. Colony aspect (A1) and reproductive structures of T6 isolate of *Trichoderma* spp. (A2).



Source: Authors (2022).

From the results obtained by the adopted scale, it was verified that at seven days, the 15 isolates of *Trichoderma* spp. already caused some degree of inhibition in the mycelial growth of the phytopathogen. After 14 days it was verified that the isolates (T1; T2; T3; T5; T6; T9 and T15) obtained high biocontrol activity (grades 1) and isolates (T4; T8; T10; T12 and T14) presented good performance (note 2). The isolates T7 and T13 were the ones that obtained the lowest performance in their biocontrol activity (Figure 2).

Figure 2. Confrontation between *trichoderma* spp isolates in the upper position and *F. sickle cell*. in the lower ca position of the plate, with the isolates T5, T6 and T15.



Source: Authors (2022).

The PCR amplification product with primers ITS1 and ITS4 resulted in a fragment of approximately 550 base pairs (pb). Phylogenetic analysis based on gene sequences of the ITS region of the DNA of the isolate, using the "maximum Likelihood Tree" method based on 1000 bootstrap replicas.

The results are acceptable for completion at the species level, because the similarity value found was 99%. Menezes et al., (2010) and O'Donnell et al., (2000); concluded in their work that sequencing of the ITS region was an efficient method for identifying *Trichoderma* spp.

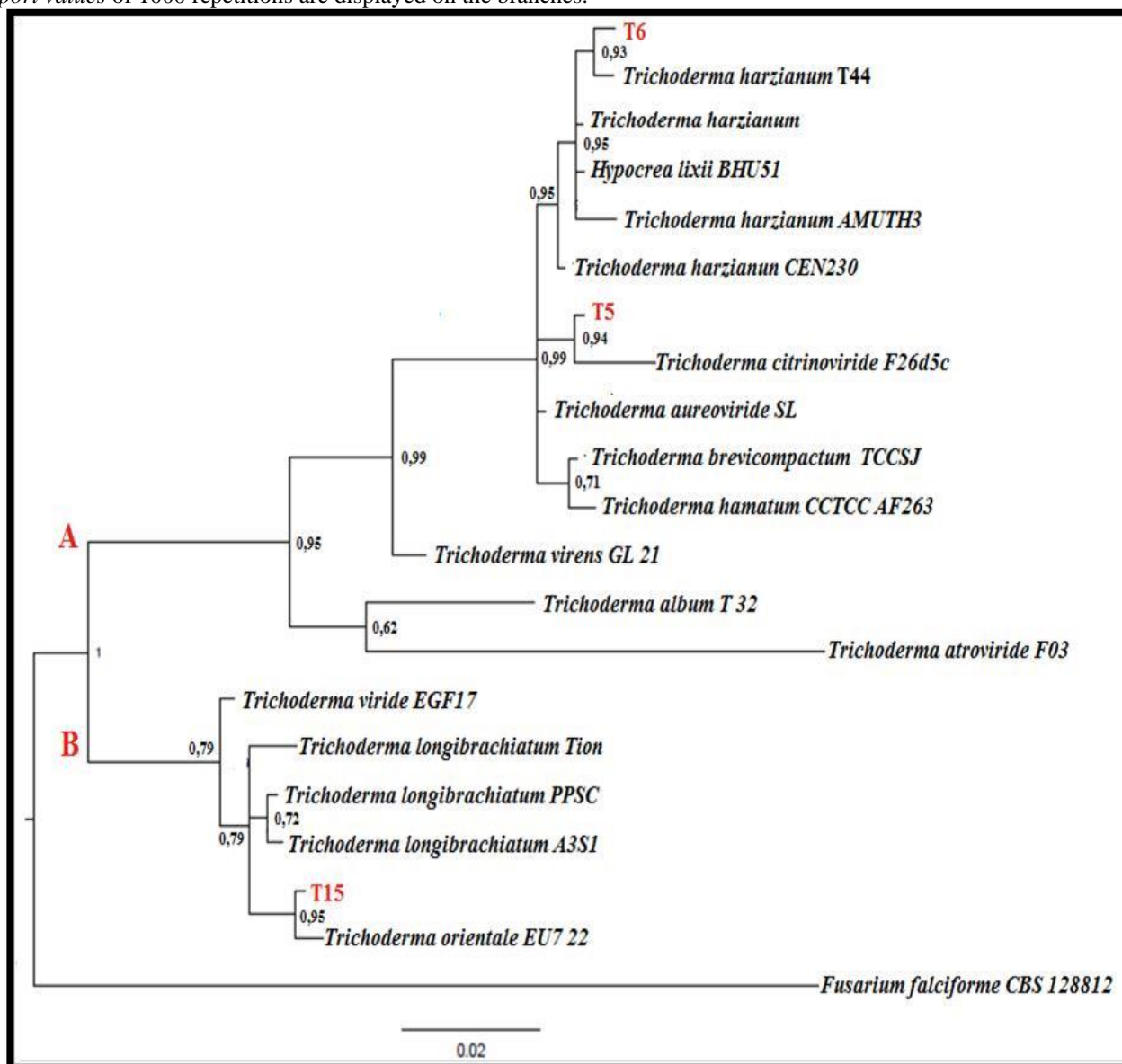
The isolates of *Trichoderma* T5, T6 and T15 showed the best score on the Bell et al. scale (1982), higher production of secondary metabolites, as well as those with the best inhibition rate of *F. falciforme* (Silva, 2016; Silva et al., 2022), were submitted to DNA extraction and sequencing for molecular identification and phylogenetic analysis. The phylogenetic tree generated from the sequences of the ITS1 and ITS5 genes

of rDNA presented two large Clades with 100% support. These sides were classified as A, which presented the highest number of branches and also grouped the isolates T5 and T6 into B, where the isolate T15 was grouped (Figure 3).

Observing the tree we can verify that T6 was grouped in clade A and presented 99% support where are sequences of *Trichoderma harzianum* and *Hypocrea lixii* its teleomorphic form (sexual), with 95% similarity, thus confirming that the isolate in which it is *Trichoderma harzianum*.

The T5 isolate also showed 99% support, equal to T6, now identified as *T. harzianum* and its similarity occurred as *Trichoderma citrinoviride* with 94% support, thus stating that the isolate is of the same species. More recently, the teleomorphic forms *Hypocrea lixii*, *H. atroviridis*, *T. harzianum* and *T. atroviride* were discovered and proven by DNA analysis (Chaverri et al., 2003; Dodd et al., 2003).

**Figure 3.** Phylogenetic tree based on the ITS region of *Trichoderma* spp. DNA, constructed using the 'maximum Likelihood Tree' method. The isolate CBS 128812 used as an "outgroup". The scale bar represents a change of 0.02, and bootstrap support values of 1000 repetitions are displayed on the branches.



Source: Authors (2022).

The Isolate T15 was grouped in clade B, where it presented 100% origin support, but its similarity in the branch in which it was grouped was lower. However, the similarity presented was 95% with *Trichoderma orientale*. Due to the information generated we can suggest the species of this isolate as the same in which it was grouped in the phylogenetic tree. A similar study was carried out by Carvalho Filho (2013), who, when analyzing the same genomic region, reports that ITS is sufficient to identify *Trichoderma* spp isolates comparing sequences deposited in ncbi *genebank*. (Corabi-Adell, 2005), studying phylogeny, using the same region, also corroborates the results found in this study, by identifying the isolates of *Trichoderma* spp taking as support the transcribed region. (Reyes-Figueroa et al., 2016) also managed to identify at species level their isolates of *Trichoderma* spp, only with this region and with tree support with lower score than those found in this study. Menezes et al. (2010) concluded that sequencing of the ITS region was an efficient method for identifying and separating isolates from *Trichoderma* spp. Lima-Luz (2012) corroborates with the authors mentioned above in concluding the efficiency in the identification of *Trichoderma* spp isolates from agricultural soils in northern Maranhão.

Samuels et al., (2004) in their study did not identify some isolates at the species level, due to the fact that this region is very conserved. For this reason the isolates that were not identified had another genomic region sequenced (EF1 — *Translation elongation*) and concatenated with the transcript to identify the species of these isolates. Jaklitsch and Voglmayr, (2015) report that the Region RPB1 and RPB2 are also regions that have a high number of loci and that it also gives great support for molecular identification of *Trichoderma* spp isolates and their teleomorphic form.

Sánchez and Rebolledo, (2010) comment that research focused on molecular biology with *Trichoderma* is still very scarce and that from the study conducted by him with the ITS region, four new species were described. The phylogenetic trees generated showed that the isolates were correctly identified, since the groups resulted in branches containing the individuals belonging to their respective sections and species, with their high degree of similarity, support in the clades and branches.

#### 4 CONCLUSION

Phylogenetic analysis based on gene sequences of the ITS region of DNA revealed with 100% reliability that the isolates are of the genus *Trichoderma*, being T5 of the species *T. citrinoviride* with 99% similarity in the sequence; T6 of the species of *T. harzianum* with 95% and T15 of the species *T. orientale* with 95%.

The isolates *T. citrinoviride* and *T. orientale* are the first reports of these species acting as biocontrollers of phytopathogens in cassava plantation areas in the state of Alagoas.

Other studies need to be carried out in order to identify new species of *Trichoderma* spp as a biocontrol alternative of soil-inhabitant phytopathogens that cause significant losses in cassava crop in the State of Alagoas.



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