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Article

Characterization of Novel Native Mycoparasitic *Trichoderma* Isolates from Mangrove Sediments and its Potential Biocontrol against *Fusarium* spp.

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Abstract: Native strains of *Trichoderma*, isolated from mangrove sediments of PE, Brazil were determining their morphological and molecular characterization, and were investigated to assess of their biocontrol potential over the phytopathogenic *Fusarium* strains isolated from Caatinga soil, PE, Brazil. The *Trichoderma* strains were characterized by polyphasic approach, which combined their morphological characteristics, macro- and microculture results, growth evaluation by Tukey test, with significance of 5%. The DNA was extracted and the product was amplified with primers ITS 1 and 2, and sequenced. *Trichoderma* strains were compatible morphologically with the description of the genus. The molecular identification of *Trichoderma*, sequences of 500 bp were amplified, deposited in GenBank and used for phylogenetic analysis. The growth rate analysis showed rate of 0.1207 cm^{h-1} to *Trichoderma* strains and *Fusarium* spp. lower growth rate (0.031 cm^{h-1}) was observed. The antibiosis tests showed the best antagonistic level of effectiveness to *T. asperellum* UCP 0149 against *F.* solani UCP 1395(82.2%) and *F.* solani UCP 1075(70.0%), followed of *T. asperellum* UCP 0319 against *F.* solani UCP1083 (73.4%), and *T. asperellum* UCP 0168 against *F.* solani UCP1098 (71.5%), respectively. The data obtained could serve as the basis for developing several biotechnological processes of safe use.

Keywords: Filamentous fungi, Bioactive substances, Antibiosis, Phytopathogenic.

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1. Introduction

Several studies on species of microorganisms isolated in marine environments have demonstrated degradation and high tolerance to a wide variety of pollutants including pesticides, polyaromatic hydrocarbons and heavy metals as well as to stress conditions of radiation, acidity, alkalinity and temperature. This remarkable potential means they can be used as agents of environmental bioremediation [1-4].

Fungi of the genus *Trichoderma* have been isolated from marine environments rich in lignocellulosic materials. Its great capacity for competition and adaptation in these environments is due to its ability to synthesize biomolecules such as enzymes and volatile and non-volatile antimicrobial metabolites such as antibiotics and hydrolytic enzymes which are of great economic

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and industrial interest. These fungi have also aroused research interest worldwide because of their role in hyperparasitism and the competition for space, oxygen and nutrients [5-7].

Assays using microorganisms, such as those of the *Trichoderma* species, which produce biomolecules that can control phytopathogens, have been extensively developed, especially in biological and agricultural areas. This has resulted in formulations of products that are regulated and marketed for agricultural use [8-11]. Among these, *T. harzianum* is one of the most researched biocontrol species, followed by *T. viri*, *T. koningii*, *T. hamatum* and *T. pseudokoningii* [12].

Trichoderma spp. have been used as biocontrol agents against soil-borne pathogens such as *Fusarium*, *Pythium* and *Rhizoctonia* spp. in French beans among other crops as described Kariuki et al.[13]. *Trichoderma* spp. is also being used in the management of soil-borne diseases in French beans particularly for the export market. It is hypothesized that *Trichoderma* spp. acts by antibiotic production, mycoparasitism, production of cell walldegrading enzymes and competition for nutrients or space to achieve biocontrol of the pathogens [14,15].

However, this ability to produce fungitoxic substances can vary between species and among isolates of the same species [16]. Some strains can produce antimicrobial metabolites while others act as promoters of plant development. Therefore, it is important to identify these microorganisms [17-23].

The taxonomic confirmation of species of the genus *Trichoderma*, based only on morphological markers, can be considered limited and of low accuracy, due to the plasticity of its characteristics [24]. Therefore, molecular techniques must be combined with adopting a variety of parameters in order to identify species correctly [25]. This enables phylogenetic comparisons to be made, based on target sequences, thus determining the precise relationships between *Trichoderma* spp. [26].

The pathogenic action of fungi inhabitants of the soil, which can cause diseases in the root system or even the aerial part of plants, is a factor that limits agricultural production. Currently, pest and disease control measures are carried out on a large scale using agrochemicals [27]. However, using such chemicals is associated with several problems. For example, they are expensive to produce and such costs are recovered by including them in the price of final products. Moreover, their use can result in the emergence of resistant pathogens, environmental contamination and ill-effects on human health, besides which they can harm other living beings [28].

Biological control stands out as an alternative that leads to more sustainable agricultural development, besides which it contributes to conserving the environment. Futhermore, it has become increasingly widespread because it is relatively simple, clean and inexpensive. *In vitro* tests are the basis for selecting and assessing the potential and feasibility of biocontroller microorganisms that can prompt the growth or development of phytopathogenic agents [29,30].

The objectives of the presente study were identification of a novel *Trichoderma* spp. strains, isolated from mangrove sediments (Pernambuco, Brazil) by morphological and molecular characterization and tested the antibiosis potential and its efficacy against the pathogen *Fusarium* spp. isolated from Caatinga soils(Pernambuco, Brazil) .

2. Results

2.1. Identification of microorganisms

Thirteen strains of *Trichoderma* (UCP 149, UCP 168, UCP 217, UCP 230, UCP 236, UCP 258, UCP 319, UCP 367, UCP 376, UCP 432, UCP 529, UCP 314 and UCP 476) and nine of *Fusarium* (UCP 1396, UCP 1395, UCP 1083, UCP 1073, UCP 1084, UCP 1074 , UCP 1075, UCP 1096 and UCP 1098) were identified, in accordance with the standard characteristics of each species, and maintained in periodic cultivation in PDA medium. They were deposited in the Collection of Cultures of the Nucleus of Research in Environmental Sciences and Biotechnology (NPCIAMB) - Catholic University of Pernambuco (UCP).

2.2. Morphological characterization

Strains of *Trichoderma* spp. obtained were morphologically identified at the genus level, all of which were morphologically compatible with the description of the genus. Their growth in the culture medium was rapid and showed concentric halos and a floccose or compact surface that looked like tufts. The mycelium, initially of a white color, acquired green shades, due to the abundant production of conidia. The changes in shade, which ranged from yellow to brown, may be associated with the synthesis and diffusion of different metabolites in the culture medium. Microscopically, abundant sporulation of smooth or rough-appearance conidia was observed, originating from branched and irregularly verticillated conidiophores, and presented conidiogenic (phialides) cells, which generally were ampiliform or fusiform, and arranged in clusters (Figure 1).

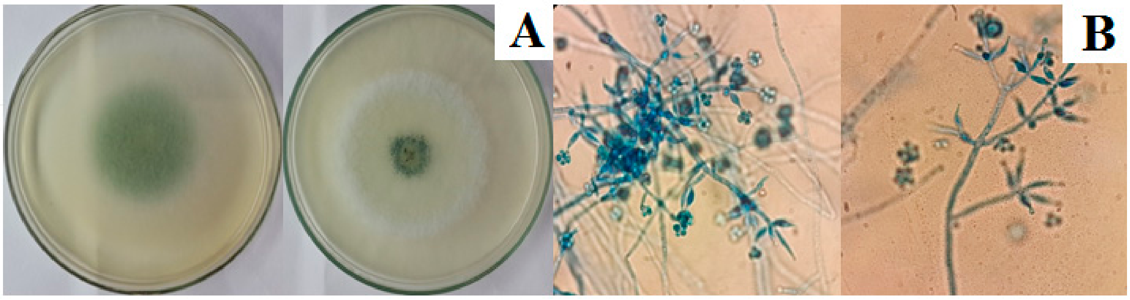

Figure 1. Macroscopic morphology of the growth pattern of the colony in PDA (A) and microscopic arrangements of hyphae, conidiophores and conidia (B) of *Trichoderma* spp.

2.3. Extraction and amplification of genomic DNA

The thirteen samples of *Trichoderma* spp. were identified at the molecular level. The ITS primers amplified sequences of about 500 bp, as seen on the agarose gel in Figure 2.

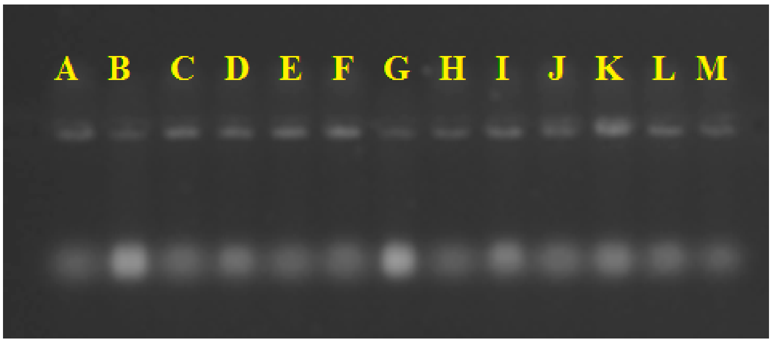

Figure 2. Running on 1.5% agarose gel with PCR products obtained from the ITS region primers of *Trichoderma* isolates (from left to right, the thirteen isolates are: UCP 0149, UCP 0168, UCP 0217, UCP 0230, UCP 0236, UCP 0258, UCP 0319, UCP 0367, UCP 0376, UCP 0432, UCP 0529, UCP 0314 and UCP 0476).

2.4. Analysis of the products obtained by PCR

The ITS1, 5.8S and ITS2 regions of the samples of the genus *Trichoderma* were sequenced and edited which generated sequences of 543 to 587 bp. These sequences were then subjected to a BLAST search and deposited in the NCBI Genbank. The deposit access codes of the sequences are awaited (Table 1).

Table 1. *Trichoderma* spp. used in this research study: host, place and accession number of the genetic sequences of the ITS region.

Taxon	Collection number	Host	Place	Number NCBI/ ITS
Trichoderma asperellum	CBS 433.97 *	soil	USA	AY380912
T. asperellum	IBSD T39	Mangrove sediments	India	JX518901
T. asperellum	UCP 0149	Mangrove sediments	Brazil	MF974884
T. asperellum	UCP 0168	Mangrove sediments	Brazil	MF974875
T. asperellum	UCP 0217	Mangrove sediments	Brazil	MF974876
T. asperellum	UCP 0236	Mangrove sediments	Brazil	MF974877
T. asperellum	UCP 0319	Mangrove sediments	Brazil	MF974878
T. asperellum	UCP 0367	Mangrove sediments	Brazil	MF974879
T. asperellum	UCP 0376	Mangrove sediments	Brazil	MF974880
T. asperellum	UCP 0432	Mangrove sediments	Brazil	MF974881
T. asperellum	UCP 0314	Mangrove sediments	Brazil	MF974883
T. asperellum	UCP 0476	Mangrove sediments	Brazil	MF974882
T. brunneoviride[2]	CBS121130*	-	Germany	EU518659
T. longibrachiatum	CBS 816.68 *	-	USA	EU401556
T. longibrachiatum	NRRL 54514	*Gloeophyllum trabeum*	USA	HQ882796
T. longibrachiatum	UCP 0529	Mangrove sediments	Brazil	MF974874
T. harzianum	CBS 226.95 *	Soil	UK	AJ222720
T. harzianum	CBS 227.95	Soil	UK	AJ222721
T. harzianum	UCP 0230	Mangrove sediments	Brazil	MF974886
T. harzianum	UCP 0258	Mangrove sediments	Brazil	MF974885

[1] Taxon in bold were found in this study. ITS: Internal transcribed spacer (ITS1-5.8S-ITS2); [2]Outgroup isolate; [*] *Type* species culture; *BOT* – A. M. Ismail, Plant Pathology Research Institute, Giza, Egypt; *CBS* – Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; *IBSD* - Institute of Bioresources and Sustainable Development, Manipur, India; *NRRL* – Agricultural Research Service Culture Collection, Peoria, USA.; UCP – Universidade Católica de Pernambuco.

2.5. Molecular identification and phylogenetic analysis

All sequences were used for the formation of the phylogenetic tree, along with sequences of species types. The aligned sequences presented 602 characters, of which 68 were informative, 94 were variable and 506 were constant. The maximum likelihood analysis generated a consistent topology tree, in which three groups corresponding to the genus *Trichoderma* were observed, namely: *T. harzianum*, *T. longibrachiatum* and *T. asperellum*, which showed ramifications among the individuals of the other clades, as verified in Figure 3.

The isolates UCP 0230 and UCP 0258 were grouped in the *T. harzianum* section (85% bootstrap support) while the England isolate type formed a clade with bootstrap values of 85%. When related to each other, they formed a clade with bootstrap values of 99% (Figure 3). In the T. *longibrachiatum* section, the England isolate formed a clade with bootstrap values of 100% and included the isolate UCP 0529 with the isolate type of the United States of America. The *T. asperellum* section comprised the remaining isolates in two subclades with 100% bootstrap values, where UCP 376, UCP 0314 and UCP 0432 were grouped with the India rate and UCP 0476, UCP 0319, UCP 0236, UCP 0168, UCP

0367, UCP 0149, UCP 0217 with the US type rate. However, the clade formed a subgroup with bootstrap values of 90% when compared to the isolate from the *T. longibrachiatum* section. This indicates a probable speciation event between these two species, thus forming a consistent grouping within the section (Figure 3). Then the sequences were searched in the BLASTN, arising from which only published results, with high score (1000) and "e-value" of zero were used (Supplementary Material).

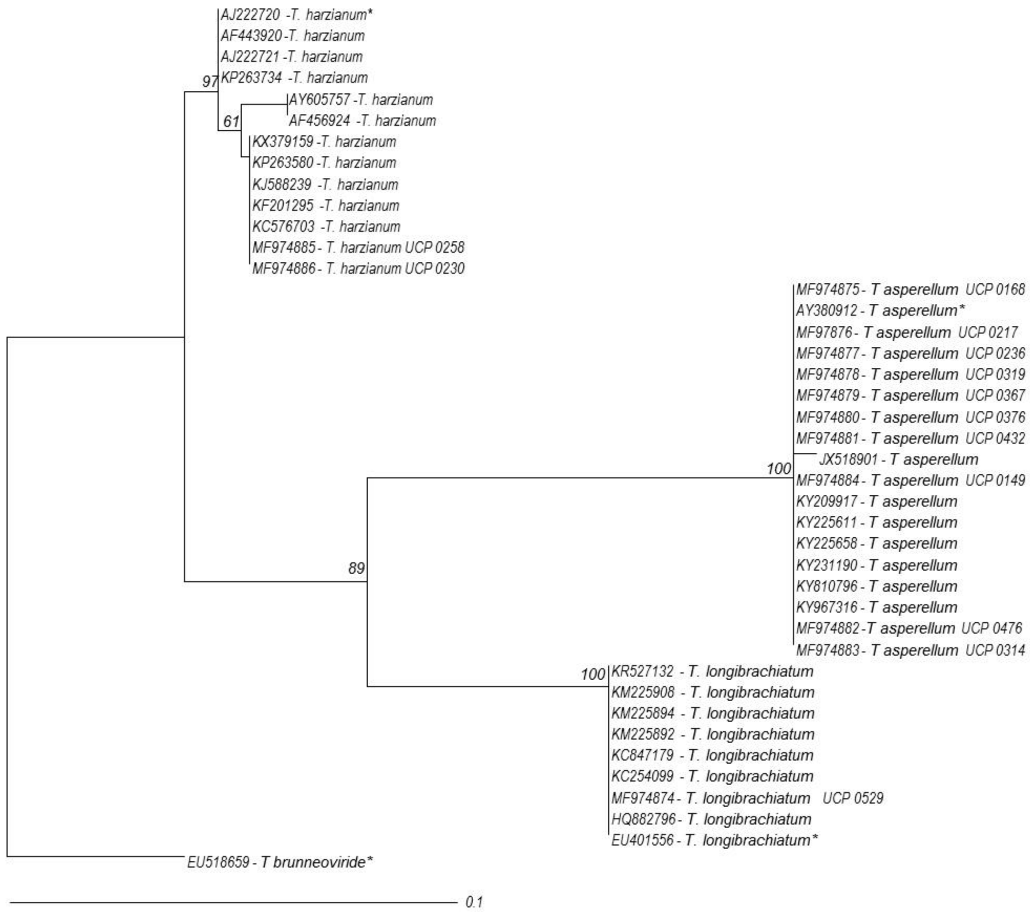

Figure 3. Maximum likelihood tree based on 602 bp aligned with partial sequences of the Internal Transcribed spacer - ITS (ITS1, 5.8 S and ITS4), with 19 taxa belonging to the *Trichoderma* species, compatible with the isolates examined in this study. Bootstrap values above 70% (indicating the ML/MP ratios) are shown near the nodes. Isolates from this study were written in bold in the highlighted branch; *Trichoderma brunnroviride*, isolate CBS121130, was used as outgroup, and taxa typed with NCBI access code followed by (*) represents the type of species.

2.6. Evaluation of mycelial growth rate of Trichoderma and Fusarium strains

All *Trichoderma* strains showed a similar *in vitro* growth pattern, with an average growth rate of 0.1207 cm $^{h-1}$. However, *Fusarium* spp. growth rates showed the media of 0.031 cm $^{h-1}$. This can be considered significant when compared to the mean deviation of 0.0146, which represents a variation of approximately half of this average. The growth rates of *Trichoderma* spp. (Table 2) were approximately four times greater than the evaluation of *Fusarium* strains average growth rate in 72 h of (Table 3).

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Table 2. *In vitro* mycelial growth interval pattern of *Trichoderma* strains

***Trichoderma* strains**	**DG(cm)**		**MGR (cm h-1)**
	24h	**72h**	
Trichoderma asperellum UCP 0149	1.9	4.7	0.11
T. asperellum UCP 0168	2.0	5.2	0.13
T. asperellum UCP 0217	2.2	5.2	0.12
T. harzianum UCP 0230	1.5	4.0	0.10
T. asperellum UCP 0236	2.0	5.0	0.12
T. harzianum UCP 0258	2.0	5.0	0.12
T. asperellum UCP 0319	1.7	4.8	0.12
T. asperellum UCP 0367	1.3	5.0	0.15
T. asperellum UCP 0376	1.3	4.5	0.12
T. asperellum UCP 0432	2.0	5.0	0.12
T. longibrachiatum UCP 0529	1.2	4.3	0.12
T. asperellum UCP 0314	1.5	4.5	0.12
T. asperellum UCP 0476	2.0	5.0	0.12
			0.1207 ± 0.0059[1]

[DG] In vitro Colony Diameter Growth (cm); [MGR] Mycelial Growth Rate (cm $^{h-1}$); [1] Mean values of mycelial growth rate ± mean deviation values.

Table 3. *In vitro* mycelial growth interval pattern of *Fusarium* strains

***Fusarium* strain**	**DG(cm)**		**MGR(cm $^{h-1}$)**
	24 h	**48 h**	
F. oxysporium UCP 1396	1.1	2.4	0.05
F. solani UCP 1395	0.8	1.3	0.02
F. solani UCP 1083	0.7	1.1	0.01
F.oxysporum UCP 1073	0.7	2.1	0.05
F. solani UCP 1084	0.8	1.9	0.04
F. solani UCP 1074	0.7	1.3	0.02
F.solani UCP 1075	0.6	1.3	0.02
F. solani UCP 1096	0.6	1.1	0.02
F. solani UCP 1098	0.7	2.0	0.05
			0.031 ± 0.0146[1]

[DC] In vitro mycelial growth diameter (cm); [TC] Mycelial growth rate (cm h-1);
[1] Mean values of mycelial growth rate ± mean deviation values.

2.7. Antibiosis phenomenon of Trichoderma against Fusarium strains

The results of the pairing tests showed that all strains of *Trichoderma* paired with strains of *Fusarium* presented antagonistic action, thereby directly affecting the development pattern of the colonies. That is, *Trichoderma* colonies were able to grow on the pathogens, not only hindering their mycelial development by nutrient and space, but effectively demonstrating the ability to interact with the pathogen [30]. The Figure 4 showed reduced the growth of *Fusarium* and showed direct action by the identified strains as *T. asperellum* UCP 0168 against to *F. solani* UCP 1084 and *F. solani* UCP 1074 (Fig. 4A). However, the *T. asperellum* UCP 0319 strain showed two behavior when paired

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with some *Fusarium* isolates (Figures 4B, C), respectively. The analysis indicated halo formation in figure 4B against to *F. solani* UCP 1395 and *F. solani* UCP 1096, as well as to strains *F. solani* UCP 1084 and *F. solani* UCP 1074 (Fig. 4A). In the analysis of variance, in a factorial arrangement by Tukey's test of 5% probability, the percentages of inhibition of the *Fusarium* pathogenic strains were significantly to different strains of *Trichoderma* ($p \leq 0.01$).

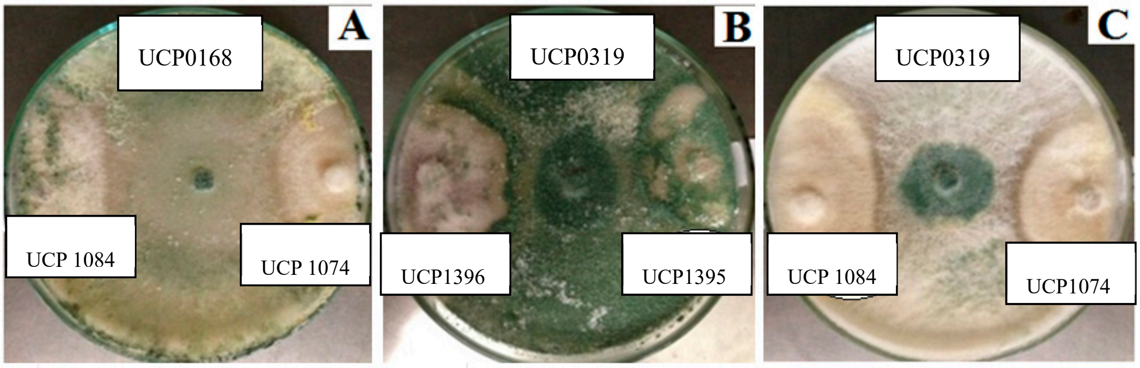

Figure 4. *In vitro* efficacy of antagonistic effect of *Trichoderma asperellum* UCP 0168 against *Fusarium* strains UCP 1084 and UCP 1074 (**A**); *T. asperellum* UCP 0319 against the effect against to *Fusarium* UCP 1396 and UCP1395 (**B**), and *T. asperellum* UCP 0319 against *Fusarium* strains UCP 1084 and UCP 1047 (**C**)

Among the isolates, strains of *T. asperellum* were significantly different from *T. harzianum* and *T. longibrachiatum*, with a percentage of inhibition equal to or greater than 50% in most antagonistic associations, especially UCP0149, UCP 0168, UCP 0319 and UCP0367, with regard to promoting greater inhibition in at least two distinct *Fusarium* isolates (Table 4). For *T. asperellum*, UCP 0149 showed a higher percentage inhibition when coupled with UCP 1395 (82.2% inhibition) (Figure 5B) as this was 70% inhibition with UCP 1075 . *T. asperellum* UCP 0168 showed its highest percentages of inhibition when paired with UCP 1396, which reduced colony growth by 57.2% (Figure 5A) and with UCP 1098, with a percent inhibition of 71.5% (Figure 5I). *T. asperellum* UCP 0432 promoted inhibitions of 62.5% and 63.15% in UCP 1074 and UCP 1096, respectively (Figure 5D and 5H). The same range of inhibition was observed in UCP 314, with inhibitory action of 63.2% on UCP 1096 (Figure 5H). *T. asperellum* UCP 0319 was the best antagonist, acting directly on four different isolates of *Fusarium* sp. and showed a percentage of inhibition of 73.4% for UCP 1083 (Figure 5C), 50% for UCP 1073 (Figure 5D), 55% for UCP 1084 (Figure 4E) and 62.5% for UCP 1096 (Figure 5H). The highest percentage of inhibition, when compared to the other treatments, was observed in *T. asperellum* UCP 0476, namely, 57.2% when paired with *F. solani* UCP 1084 (Figure 5E), and *T. asperellum* UCP 0367 in 57.2% when paired with *F. solani* UCP 1396 (Figure 5A) and 61.4% when paired with *F. solani* UCP 1074 (Figure 5F).

2.8 Level of Effectiveness of Trichoderma strains agaist Fusarium

The mycelia growth of both the dual culture and control plates were measured at intervals of 24 hours beginning from the 72nd hours of incubation (Table 4). It was observed that *Trichoderma* strains grew faster than the phytopathogenic *Fusarium* strains in the dual culture plates. The control plates also grew faster than their respective dual cultures (Figures 4). Minimum inhibition concentration (MIC) according to Sangoyomi [54] and described by Okigbo and Emeka[55]shows that *Trichoderma* strains were selected by the level of effectiveness in controlling some strains of *Fusarium* indicated in the Table 4. The results showed the effective phenomenon of antibiosis in *T. asperellum* UCP 0149, *T. asperellum* UCP 0168, *T.*

asperellum UCP 0432, *T. asperellum* UCP 0314, *T. asperellum* UCP 0319, *T. asperellum* UCP 0476 and *T. asperellum* UCP 0367 and varies significantly ($P \leq 0.05$) across treatments. However, the all nine strains *F. solani* UCP 1395, *F. solani* UCP 1075, *F. oxysporium* UCP 1396, *F. solani* UCP 1098, *F. solani* UCP 1074, *F. solani* UCP 1096, *F. solani* UCP 1074, *F. solani* UCP 1083 and *F. oxysporium* UCP 1073 showed inhibition response to *Trichoderma* strains used, respectively.

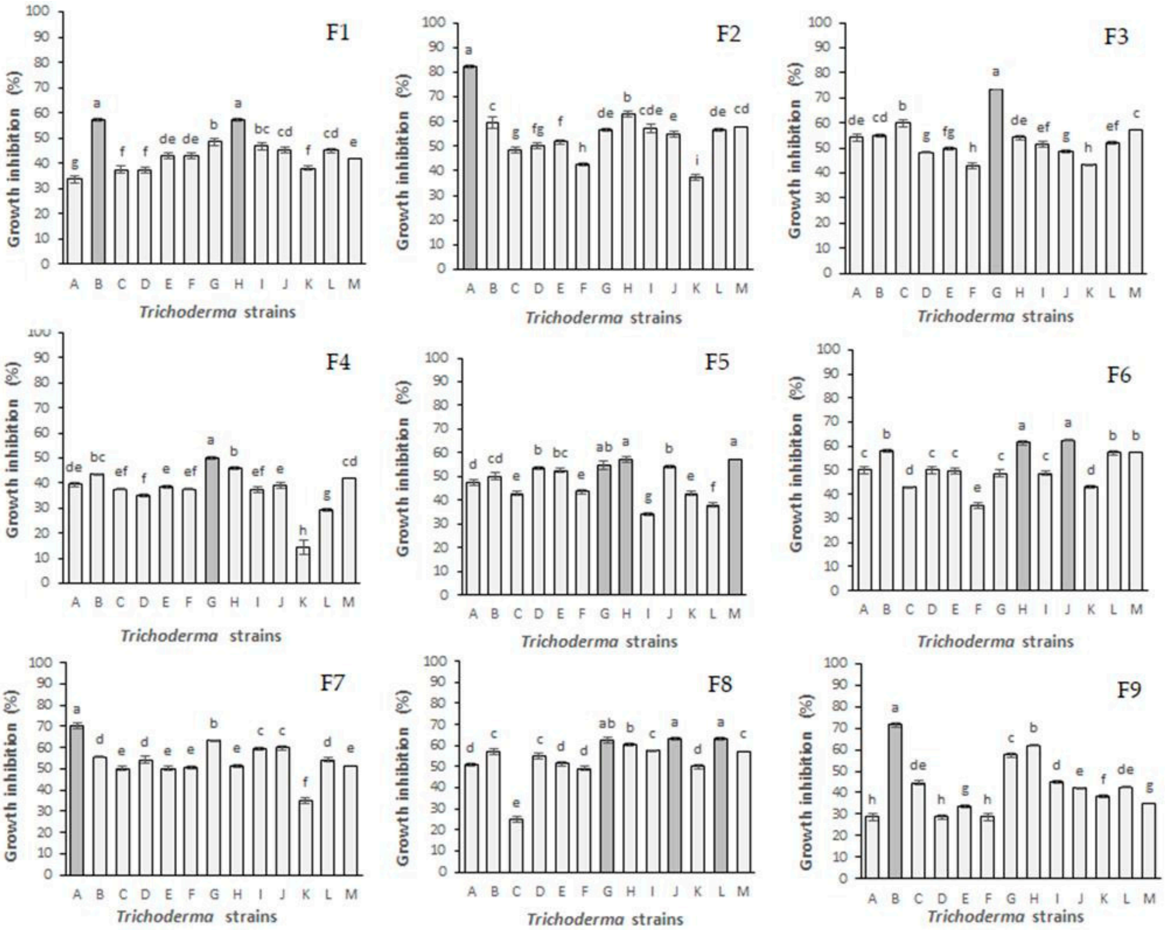

Figure 5 . Percentage Growth Inhibitions (PGI) of Fusarium strains: *Fusarium oxysporium* UCP 1396(F1); *F.solani* UCP1395(F2); *F. solani* UCP1083 (F3); *F. oxysporum* UCP1073 (F4); *F. solani* UCP1084 (F5); *F. solani* UCP1074 (F6); *F. solani* UCP1075 (F7); *F. solani* UCP1096 (F8); and *F. solani* UCP1098 (F9) under cultivation of nine isolates of *Trichoderma* strains identified in the horizontal axis by capital letters: *T. asperellum* UCP0149 (A); *T. asperellum* UCP0168 (B); *T. asperellum* UCP0217 (C); *T. harzianum* UCP0230 (D); *T.asperellum* UCP 0258 (E); *T. asperellum* UCP 0235 (F); *T. asperellum* UCP 0319 (G) ; *T. asperellum* UCP 0367 (H) ; *T. asperellum* UCP 0376 (I); *T. asperellum* UCP 0432 (J); *T. longibrachiatum* UCP 0529 (K); *T. asperellum* UCP 0314 (L) and *T. asperellum* UCP 0476 (M). Growth inhibition averages, represented by the same lower case letters above the bars, do not differ significantly from each other by the Tukey test at 5% probability.

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Table 4. *In vitro* efficacy of antagonistic effect and level of effectiveness of selected *Trichoderma* strains against to *Fusarium* strains

***Trichoderma* paired with *Fusarium* strains**				
Trichoderma	***Fusarium***	***Growth Inhibition (PGI)**	**MIC (%)**	****Level of Effectiveness**
T. asperellum UCP 0149	*F. solani* UCP 1395	82.20[a]	>50-<100	effective
T. asperellum UCP 0149	*F. solani* UCP 1075	70.00[a]	>50-<100	effective
T. asperellum UCP 0168	*F. oxysporium* UCP 1396	57.20[a]	>50-<100	effective
T. asperellum UCP 0168	*F. solani* UCP 1098	71.50[a]	>50-<100	effective
T. asperellum UCP 0432	*F. solani* UCP 1074	62.50[a]	>50-<100	effective
T. asperellum UCP 0432	*F. solani* UCP 1096	63.15[a]	>50-<100	effective
T. asperellum UCP 0314	*F. solani* UCP 1096	63.20[a]	>50-<100	effective
T. asperellum UCP 0319	*F. solani* UCP 1083	73.40[a]	>50-<100	effective
T. asperellum UCP 0319	*F. oxysporium* UCP 1073	50.00[a]	>50-<100	effective
T. asperellum UCP 0319	*F. solani* UCP 1084	55.00[a]	>50-<100	effective
T. asperellum UCP 0319	*F. solani* UCP 1096	62.50[a]	>50-<100	effective
T. asperellum UCP 0476	*F. solani* UCP 1084	57.20[a]	>50-<100	effective
T. asperellum UCP 0367	*F. solani* UCP 1074	61.40[a]	>50-<100	effective

*Bell et al. [56] **Sangoyomi [54] and described by Okigbo and Emeka[55]

3. Discussion

Trichoderma are an alternative to chemical pesticides that can be more reliable and ecologically safety as well as economically sustainable. All the *Trichoderma* strains isolated from mangrove sediments reduced the mycelial growth of pathogenic fungi *Fusarium* isolated from caatinga soil. *Trichoderma* species clearly exhibited varying levels of growth rate and antagonism towards *Fusarium* species described by literature. Some of them are economically important because of their production of enzymes and antibiotics and their action as biocontrol agents against a variety of plant pathogens and the specificity of biocontrol agents against the pathogens was also evident [30]. *Trichoderma* spp. has been recognized as a source of various cell wall degrading enzymes and secondary metabolites [31,32].

Results of the finding with the novel *Trichoderma* strains isolated from mangrove sediments revealed that phylogenetic clusters formed in the maximum likelihood tree, where reliable support values are verified in each group and the taxonomic inferences given to the individuals according to their respective grouping sections are assumed to be adequate. In our studies *T. harzianum* strains formed a consistent cluster, supported by a bootstrap value of 85%. However, the subdivision observed in this clade, which separates UCP 0230/ UCP 0258 isolates from CBS 226.95 and CBS 227.95 (Figure 3), may be related to early speciation phenomena, or even genetic variabilities present in this species. For Druzhinina et al. [33], *T. harzianum* is one of the species with the greatest variability, and is currently considered a complex species. In this sense, Neuhof et al. [34] described the presence of peptaibols characterized as linear a-aminoisobutyrate-containing peptides produced by the genus *Hypocrea*/*Trichoderma* (*Hypocrea* and *Trichoderma* are the teleomorph and anamorph forms, respectively for the same taxon); however, the teleomorph stage are known to occur in nature.

The species chosen as an external group was *T. brunneoviride*, because, although it is within the genus, has a nucleotide sequence that diverges in the members of the other clades and occupies a basal position that differs from the other clusters [35,36].

However, *T. brunneoviride* can be chosen as an external group, despite belonging to the same genus of the other species, since it has a distinct nucleotide sequence in relation to the members of other clades and occupies a basal position that differs from the other clusters [26,37].

The antagonism by the novel strains of *T. asperellum* UCP 0149, *T. asperellum* UCP 0168, *T.asperellum* UCP 0432, *T. asperellum* UCP 0314, *T. asperellum* UCP 0319, *T. asperellum* UCP 0476 and *T. asperellum* UCP 0367 performed better in inhibiting the growth of all strains of *F. solani* UCP 1395, *F. solani* UCP 1075, *F. oxysporium* UCP 1396, *F. solani* UCP 1098, *F. solani* UCP 1074, *F. solani* UCP 1096, *F. solani* UCP 1074, *F. solani* UCP 1083 and *F. oxysporium* UCP 1073, respectively.

According to Waghunde et al. [38], *Trichoderma* species grow faster because they use their food source more efficiently. In our study, *Trichoderma* strains not only had higher growth rates in relation to *Fusarium* strains. (Supplementary Material) but also significantly exceeded significantly exceed the values of about 0.33 mm/h which Moretto et al. [39] indicated. This mechanism of accelerated growth makes it easier for *Trichoderma* to assume some control over pathogenic fungi, not only due to its competing for occupied space, but mainly due to nutritional competition.

A biocontrol agent may excrete one or more metabolites which can retard or inhibit the growth of pathogens in the surrounding area of such a compound. This phenomenon is called antibibiosis and *Trichoderma* species can also suppress fungi by means of parasitism, competition and antibiosis which can directly influence both plant growth and the response to disease [40,41]. In the present study, it is inferred that the inhibitory actions of the *Trichoderma* strains studied are possibly linked to the action of antibiosis, hyperparasitism and competition.

According to Bosah et al. [42], the paired culture test is extremely important in the area of biological control of phytopathogens, since a good performance in this test indicates that the antagonist agent is effective at enabling the rapid growth of the pathogen, thus extending the potential of the agent to prompt hyperparasitism and, especially, competition for nutrients and space. According to Howell [43], *Trichoderma* species have the ability to suppress the growth of various fungi in solid culture media.

Strains of *T. asperellum* UCP 0149, *T. asperellum* UCP 0168 and *T. asperellum* UCP 0319 were shown to be the best antagonist effect when submitted to cultures matched with all *Fusarium* strains. However, a similar inhibition capacity was observed corresponding to >50-<100 to strains *T. asperellum* UCP 0314, *T. asperellum* UCP 0432, *T. asperellum* UCP 0367and *T. asperellum* UCP 0476 on the growth of

Fusarium strains on observing the zone of inhibition between the colonies of both fungi or the overlap of *Trichoderma* mycelium on the colony of *Fusarium* strains. (Figure 4). Observations of this nature are used to evaluate the antagonistic potential of *Trichoderma* spp. and the results obtained prove that the strains studied have the potential to suppress the growth of *Fusarium* species [44-45].

Trichoderma species detect and localize the mycelium of susceptible fungi, and grow in their direction, as they respond to the chemical stimuli produced by the host fungus [5]. In addition, competition is one of the main characteristics of *Trichoderma* isolates, due to their high mycelial growth rate [42].

The findings showed that *T. asperellum* strains have inhibitory effects on the mycelia growth of *Fusarium* strains in all the treatments in the dual culture. The results also revealed that the mechanism of action by *T. asperellum* strains were by competition with *Fusarium* for nutrients and space, as well as mycoparasitism over the pathogen and probably secretion of antibiosis. The rapid growth of *Trichoderma* and competition for nutrient and space by the antagonist inhibited the growth of the pathogen all strains of *Fusarium* (Tables 2, 3 and 4).

The overall mean inhibition observed was greater than 50%, and the best results were tied to values above 70% which showed that each *Trichoderma* species has different abilities to inhibit *Fusarium* strains (Table 4) [5,42,46].

T. asperellum stand out not only because they are the isolates of the highest incidence in the mangrove sediment in Pernambuco, but also because they are the best inhibitory agents on *Fusarium* spp. Different results were observed by Taribuka et al. [47], when selecting strains of *Trichoderma* that are potentially antagonistic to *F. oxysporum*. The highest percentages of growth inhibition were provided by *T. gamsii* (60.61%), followed by *T. harzianum*.psr-1 (59.08%), *T. harzianum*.swn-1 (55.80%), *T. koningiopsis* (55.58%), *T. harzianum*.swn-2 (54.05%) and *T. asperellum* (49.67%). These results differ from those found in this study, which presented the lowest values of inhibition.

4. Materials and Methods

4.1. Microorganisms and culture conditions

Nine *Fusarium* spp. and thirteen *Trichoderma* strains were kindly released to the researchers from the UCP (Universidade Católica de Pernambuco) Culture Collection, which is registered in the WFCC (World Federation for Culture Collection. The strains of *Fusarium* were isolated from Caatinga soil in Serra Talhada, Pernambuco, Brazil; and *Trichoderma* spp. were obtained from mangrove sediments of Rio Formoso, Pernambuco, Brazil, and maintained on Sabouraud dextrose agar at 5^0C.

4.2. Morphological characterization

Morphological identification was undertaken by using the classification keys of Gams and Bisset [48], Rifai [49] and Samuels et al. [50]. The percentage of growth and morphological characteristics were analyzed in PDA. Microscopic characters were analyzed in accordance with the morphology, size and disposition of the conidia and the phialides, using slides prepared by the microculture in EMA (Himedia) technique. The material was then dyed with cotton blue so that it could be visualized.

4.3. Extraction and amplification of genomic DNA

Thirteen samples of *Trichoderma* spp. and *Fusarium* spp. were selected for species-level identification and phylogenetic analysis. Genomic DNA was extracted from 7-10 day old mycelial growth by the method adapted from Murray and Thompson [51]. Internal transcribed spacer (ITS) sequences 1 and 2, including the 5.8S interval, and the 1-α Elongation Factor 1-α (TEF1-α) were amplified using the primers ITS 1 (TCC GTA GGT GAA CCT GCG G) and ITS 4 (TCC TCC GCT TAT TGA TAT GC), for ITS [52]. Each 25 µl of the polymerase chain reaction (PCR) mix included: 13.85 µl ultrapure water, 1 µl template DNA, 1.5 µl of each primer (10 µM, synthesized by

Invitrogen-Carlsbad, CA), 2.5 µl of dNTP mix and 4.63 µl of Taq DNA polymerase mix (0.05 µl -1 Taq DNA polymerase, reaction buffer, 4 mM MgCl 2, Thermo Scientific, Waltham, USA). PCR reactions were performed in a SimpliAmp™ Thermal Cycler (applied biosystems) at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min (denaturation), 57°C for 1 min (for ITS) or 55°C for 1 min (for TEF1-α) (annealing), 72°C for 1 min (elongation), and 72°C for 10 min (final extension).

4.4. Analysis of the products obtained by PCR

To verify the efficiency of the PCR reaction, 3 µL of the substances obtained were stained with 3 µL of SYBR® Green dye (Thermo Scientific, Waltham, USA) and analyzed by 1.5% agorose electrophoresis in 0.5x TBE buffer (Tris-borate-EDTA 100 mM Tris base and 2.0 mM EDTA solution pH 8.0) and 50 mM boric acid. Electrophoresis occurred at 75v for a period of 40 min. After the run, the gels were developed and visualized under an ultraviolet transilluminator to check the amplification and purity and were then photographed for documentation purposes [53]. The amplicons were purified and sequenced by Macrogen Inc., Korea (http: //www.127 macrogen.com). The nucleotide sequences obtained were checked and edited using Staden Package 2.0 software packages. Subsequently, the consensus sequences, obtained in this study, were compared with each other using the Mega BLAST tool and deposited in the GenBank database (http://www.ncbi.nlm.nih.gov).

4.5. Molecular identification and phylogeny

The nucleotide sequences obtained were checked and edited with the Staden Package 2.0 software packages. Subsequently, the consensus sequences, obtained in this study, were compared with each other using the Mega BLAST tool and deposited in the GenBank database (http://www.ncbi.nlm.nih.gov). Nineteen taxa belonging to the *Trichoderma* species, compatible with the isolates of this study were used to construct of the phylogenetic tree, with *Trichoderma brunnroviride*, isolate CBS121130, used as outgroup and taxa written with NCBI access code and followed by (*) representing the type species. The statistical method used was "Neighbor-Joining" (NJ) with the Jukes and Cantor model to 1000 replicates [54].

4.6. Determination of growth rates

In order to evaluate growth rates, each isolate of *Trichoderma* strains (UCP 0149, UCP0168, UCP 0217, UCP 0230, UCP 0236, UCP0258, UCP 0319, UCP 0367, UCP 0376, UCP 0432, UCP 0529, UCP 0314 and UCP 0476) and *Fusarium* strains (UCP 1396 , UCP 1395, UCP 1083, UCP 1073, UCP 1084, UCP 1074 , UCP 1075, UCP 1096 and UCP 1098) was cultured individually. Disks (0.6 cm in diameter) of fungal structures were deposited in the center of a Petri dish containing PDA. These dishes were incubated at 26 ± 2°C, with a 12-h photoperiod. After 24 and 48 h of incubation, the diameters (cm h^{-1}) of colonies were measured in two perpendicular directions, and growth rates were determined according to the equation: $G_R = (G_2 - G_1) / (T_2 - T_1)$; in which: G_R = growth rate; G_1 = growth after 24 h; G_2 = growth after 48 h; T_1 = 24 h; T_2 = 48 h [36].

4.7. Percentage inhibition of Fusarium growth

To analyze the antagonistic action of the isolates of *Trichoderma* spp. on the samples of Fusarium spp., the techniques used were direct confrontation in vitro or culture pairing in Petri dishes. Following Moretto et al. [39], for each *Fusarium* sp., discs (6 mm diameter) containing fungal structures were deposited at one end of the Petri dish containing PDA medium (approximately 1 cm from the end of the plate). After 72 h, a disk of *Trichoderma* spp, with five days of growth, was deposited 3.5 cm away from the colony of the possible phytopathogen. The design was entirely randomized with four replicates. Control was represented by the pathogen without the presence of the antagonist. The plates were maintained at 26°C with a 12 h photoperiod for six days and the growth of the *Fusarium* colony was checked.

In accordance with the methodology of Camporota [55], the percentage of colonization (%C) of each antagonist isolate was calculated using the formula: %C = (DT /DE) x 100, where DT is the distance between colonies after mycelial growth stabilizes and DE is initial distance between the two mycelial discs. The inhibition index of *Fusarium* in relation to *Trichoderma* was determined by the relation I = 100 - %C. In addition to the %C values, each *Fusarium* isolate was classified as per the degree of antagonism (G), according to a scale of notes by Bell et al. [53], shown in Table 4.
The percent growth inhibition was determined as a guide in selecting the minimum inhibition concentration (MIC) that will be effective in controlling the rot-causing fungus for the three treatments. Antagonist was also rated for inhibitory effects using a scale established by Sangoyomi [54] and described by Okigbo and Emeka[55], as: ≤ 0% inhibition (not effective); >0-20% inhibition (slightly effective); >20-50% inhibition (moderately effective); >50-<100% inhibition (effective) and 100% inhibition (highly effective).

4.8. Statistical analysis

Differences of the antagonistic action of *Trichoderma* isolates over *Fusarium* were determined by factorial design in ANOVA and the means were compared by Tukey test at 5% significance using the ASSISTAT ® program.

Table 4. Classification of the degree of antagonism (G), according to the scale of Bell et al. [56].

Colonization pattern	Degree of antagonism (G)
Biocontrol agent grows completely over the pathogen, covering the entire surface of the culture medium	1
Biocontrol agent grows to at least about 2/3 of the surface of the culture medium	2
Biocontrol agent and pathogen colonize approximately half the surface of the culture medium (more than 1/3 and less than 2/3) and neither appears to dominate the other	3
Pathogen colonizes at least 2/3 of the surface of the culture medium and exhibits resistance to the biocontrol agent	4
Pathogen grows completely on the biocontrol agent and occupies the entire surface of the culture medium	5

5. Conclusions

The finding has revealed that the novel *Trichoderma* strains revealed wide variability of fungi living in ecosystem niche in mangrove sediment from Brazil. It is also important to have greater knowledge of the ecology of these species and their responses to environmental or anthropogenic disturbances which may interfere with the equilibrium of these ecosystems. We studied a wide morphological and defined phylogenetic lineages based in the morphological characters. The results suggested that *T. asperellum*, *T. harzianum* and *T. longibrachiatum* fungal strains which exhibit heterogeneity in genome structure, DNA sequence and similarity of ITS1 and 2 sequences in most taxa. *Trichoderma* strains showed capacity of inhibiting mycelia growth of seven strains of *Fusarium solani* and two strains of *F. oxysporium* all of them isolated from caatinga soil of Brazil. However, only three selected strains identified as *Trichoderma asperellum* showed the best antagonist results in order to achieve highest level of effectiveness and the possibility of eco-friendly application and low cost as biological agent against phytopathogenic *Fusarium* strains, and as well as target specific when is compared with synthetic fungicides. Besides, the studies those filamentous fungi which have a good potential for antagonistic interaction, can both aid the conduct of biotechnological processes, and improvements in environmental conditions of plant health.

Acknowledgments: This work was financially supported by CNPq (National Council for Scientific and Technological Development) Process No. 311373/2014-3, FACEPE (Foundation for the Support of Science and Technology of the State of Pernambuco) Process No. APQ-0291-2.12/15, and Coordination Unit for the Improvement of Higher Level Education Personnel (CAPES). The authors are grateful to the Nucleus of Research in Environmental Sciences and Biotechnology, Catholic University of Pernambuco, Brazil, for the use of laboratories.

Author Contributions: Patrícia Rego Barros Filizola and Galba Maria Campos Takaki conceived and designed the experiments; Patrícia Rego Barros Filizola performed the experiments; Adriana Ferreira de Souza, Iwanne Lima Coelho and Delson Laranjeira analyzed the data; Galba Maria Campos Takaki contributed reagents/materials/analysis tools; Patrícia Rego Barros Filizola, Marcos Antônio Cavalcante Luna and Galba Maria Campos Takaki wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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