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Article

Antagonistic activity of biocontrol agent *Trichoderma* spp. against *Fusarium* sp., the causal agent of *Ananas comosus* fruitlet rot

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ABSTRACT

Pineapple (*Ananas comosus*) is a significant crop, with an annual production exceeding 25 million tons. However, fusariosis can severely impact its cultivation, a fungal disease that causes fruitlet rot and results in substantial yield losses. To decrease dependency on chemical control methods, biocontrol agents (BCAs) present a promising alternative. Among these, *Trichoderma* species are noteworthy due to their diverse antagonistic mechanisms. The efficacy of each mechanism can be assessed through fungal confrontation assays. This study aimed to isolate, identify, and evaluate *in-vitro* nine *Trichoderma* spp. strains as potential BCAs against *Fusarium* sp. associated with pineapple fruitlet rot. The antagonistic fungi were isolated from rhizosphere soils in both open-field and greenhouse pineapple farms in Misiones province, Argentina. Identification of the fungi required both morphologic and genetic data. In the *in-vitro* assays, the capabilities for direct competition for substratum, production of metabolites, and mycoparasitism were evaluated. The results indicated that isolates *T. harzianum* TC7, *T. harzianum* TC9, *T. asperellum* TU3, and *T. asperellum* TU4 had statistically superior inhibitory effects against *Fusarium* sp. These isolates can be potentially used in formulating natural fungicides to reduce pineapple fruitlet rot caused by *Fusarium*, promoting sustainable production practices.

Keywords: pineapple, confrontation, mycoparasitism, metabolites, ITS region

INTRODUCTION

The excessive or inadequate use of pesticides in tropical and subtropical crops can result in economic, social and environmental harm[1]. Furthermore, pesticide-mediated control of microorganisms is often inefficient, as these organisms may develop resistance". In tropical and subtropical regions, higher frequencies of pesticide applications are required to manage microorganism-related diseases, such as fungal infections caused by *Fusarium*[2]. Fusariosis in both open-field and greenhouse pineapple farms is particularly problematic, as it is associated with fruitlet rot, leading to severe yield losses and significant economic costs [3–5].
Concerns regarding chemical-based control have driven the development of alternative approaches[6], such as integrated crop management (ICM) strategies[7]. A key component of ICM is biocontrol agents (BCAs), also

known as biopesticides[8]. These can be applied on farms as natural antagonists of pests. The use of BCAs is considered environmentally friendly and harmless to human health[9].

Most investigations concerning BCAs have focused on *Trichoderma* species due to their multiple antagonistic mechanisms, which act synergistically to control plant diseases. *Trichoderma* species exhibit high growth and reproductive rates, survival across various environmental conditions, nutrient consumption and competition efficiency, and ability to act as necrotrophs against other fungi.

In practice, morphological classification must be complemented with molecular data, for which multiple deoxyribonucleic acid (DNA) loci analysis is required. In particular, the Internal Transcribed Spacer region (ITS), within ribosomal DNA serves as a reference locus for identifying *Trichoderma* species[10].

As an initial step in evaluating a *Trichoderma* isolate as a BCA, qualitative in-vitro assays are necessary. These trials are predictive tools to determine mycelial growth inhibition against specific pathogens. It is possible to separately analyze the antagonistic mechanisms of direct competition for substrate, mycoparasitism, and the production of fungistatic compounds, known as metabolites[11,12]. As the final steps in this evaluation, it is necessary to investigate the most promising isolates in planta assays under controlled or field conditions and develop mass production techniques for the biopesticide.

This work aimed to isolate, identify, and evaluate *in-vitro* the potential of native *Trichoderma* isolates as BCAs against *Fusarium* sp., associated with pineapple fruitlet rot.

MATERIALS AND METHODS

Isolation of rhizosphere *Trichoderma* strains

Rhizospheric soil samples were collected from 9 pineapple farms in Argentina, and two farming systems were considered: open-field and greenhouse cultivation (see Table 1). 100g of soil was collected from each farm, mixed, and homogenized manually. Fungal colonies were obtained using serial dilution and cultured on *Trichoderma* Selective Medium (TSM)[13,14] at 28±2ºC for 7 days. *Trichoderma* colonies were individually subcultured on Potato Dextrose Agar (PDA) at 28±2ºC for 7 days.

Isolate name	Type of farming system	Region	Town
TU1	Open-field cultivation	Southwest	Posadas
TU2	Open-field cultivation	South	Apostoles
TU3	Open-field cultivation	West	Puerto Rico
TU4	Open-field cultivation	Northwest	Libertad
TU5	Open-field cultivation	Southwest	Posadas
TU6	Open-field cultivation	Centre	Obera
TC7	Greenhouse cultivation	Northwest	Wanda
TC8	Greenhouse cultivation	Centre	Campo Ramón
TC9	Greenhouse cultivation	South	Apostoles

Table 1. Information on the *Trichoderma* isolates, including the type of farming system (open-field or greenhouse cultivation), region, and town of the pineapple farms

Morphological identification of the *Trichoderma* isolates

Monosporic colonies were obtained for further proposals. These were cultivated in PDA and Malt Extract Agar at 28±2°C, in darkness, for 15 days. Regular observations of macroscopic characteristics, including color, shape, pigment production or liberation, and growth rate, were recorded. Microscopic examination involves the identification of vegetative and reproductive structures according to [15] using an optical microscope (Olympus, CX23).

DNA extraction, amplification, and sequence analysis

Genomic DNA was extracted according to [16]. The ITS1-5.8S-ITS2 region was amplified using the polymerase chain reaction (PCR) technique according to [17]. Macrogen Inc. (Seoul, Korea) standard sequencing services purified and sequenced PCR products. The quality of DNA sequencing was verified using Chromas Lite v2.1, and sequences were manually trimmed using BioEdit v7.2[18]. Subsequently, the sequences underwent an essential local alignment search tool for nucleotides (BLASTn) analysis against the National Centre for Biotechnology Information (NCBI) database. Multiple sequence alignment was performed using AliView v1.28[19] with the ClustalW method.

Phylogenetic analysis of ITS1-5.8S-ITS2 region

Phylogenetic inference was conducted using the Bayesian method. All characters were treated as equally weighted, with gaps considered missing data. The results from the BLASTn submission were downloaded, verified and selected for phylogenetic reconstruction (sequences are listed in Table 2). The outgroup in the inference was *Clonostachys* spp., with *C. rosea* CBS 154.27 and *C. solani* CBS 183.30 (NCBI database Reference Sequence, accession numbers: NR_165993 and NR_163540, respectively).

The find-best-fit-model function was performed in Mega v7[20] to identify the most informative phylogenetic model. With the resulting model, the *Maximum Likelihood* method with a bootstrap of 1000 repetitions was applied. The tree was visualized and edited with the same software.

Taxonomic identification	Accession number	Isolate name	Source/ publication
***T. asperellum* species-complex**	ON877392	TU1	This work
	ON877396	TU2	
	ON877391	TU3	
	ON877395	TU4	
	ON877393	TU5	
	ON877394	TU6	
	EU280109	CIB T05	[30]
	EU280110	CIB T113	
	NR130668	CBS 433.97	NCBI Reference Sequence
	MK210562	T2	[52]
	MK210429	T3	
	MK210428	T4	
	MK211208	T16	
	MK210235	T20	
	MK209012	T21	
	MH825714	IIRRCK 1	[53]
	MZ323879	RMCK01	[54]
***T. harzianum* species-complex**	ON877397	TC7	This work
	ON877399	TC8	
	ON877398	TC9	
	EU280091	DAOM 234005	[30]
	EU280092	DAOM 233963	
	EU280077	CIB T44	
	EU280103	CIB T99	
	EU280079	CIB T02	
	EU280075	CIB T131	
	EU280078	CIB T136	
	EF568084	MITS2507	NCBI Reference Sequence
	KC254097	MITS2506	
	NR131264	CBS 110080	

	NR137301	CBS 130755	
	NR137305	CBS 130746	
	NR137297	CBS 130431	
	NR137300	CBS 138272	
	NR137298	CBS 548.92	
	NR137304	CBS 124620	
***Clonostachys* spp.**	NR165993	CBS 154.27	NCBI Reference Sequence
	NR163540	CBS 183.30	

Table 2. The taxonomic identification, isolate name, accession number in the NCBI database, and publication or reference are listed for each sequence used in the phylogenetic reconstruction of the ITS1-5.8S-ITS2 region of the native *Trichoderma* isolates and sequences from NCBI database

Antagonism potential of rhizosphere *Trichoderma, in-vitro*

In-vitro assays were made in Petri dishes with PDA at 28±2°C in darkness for 10 days. The pathogenic *Fusarium* sp. strain used in these assays was obtained from the culture collection of the Laboratory of Mycology "Dra. Martha G. Mevdeveff" from the "Facultad de Ciencias Exactas, Químicas y Naturales, UNaM". This strain was isolated on a diseased pineapple plant from Apostoles City, Misiones province. Negative controls consisted of identical assays but without *Trichoderma* being inoculated.

Mycoparasitic activity determination

The mycoparasitic activity was confirmed by directly observing cellular interactions between the *Trichoderma* and *Fusarium* isolates. The interactions between the colonies were investigated using the microculture method [21] with slight modifications [22], and the colonies were cultivated in 0.1ml of PDA for 5 days. During observation with an optical microscope, the mycelium was stained with lactophenol-cotton-blue to enhance visualization and examined using 40X and 100X magnifications.

Estimation of the growth inhibition of *Fusarium* sp. by *Trichoderma*

The antagonistic activity was estimated as %I=(C-T/C)x100, where %I is the percentage of mycelial growth inhibition and T and C are the mean *Fusarium* colonies radius in the treatments (with *Trichoderma*) and the controls (without *Trichoderma*), respectively. Three biological replicates were performed for each treatment.

Direct confrontation (DC)

%I was measured in the DC experiments according to [23]. In this case, 5mm discs of *Trichoderma* and *Fusarium* were deposited oppositely at 5mm of the edge of a single Petri dish. We also used the antagonism scale (from 1 to 5) proposed by [24].

Indirect confrontation by diffusible (ID) and volatile (IV) metabolites

This study evaluated two types of indirect confrontation assays: diffusible metabolites (ID) and volatile metabolites (IV).

The ID production experiments were carried out as per [11]. 5mm discs of *Trichoderma* were plated on sterile PDA plates, and the medium surface was covered with a layer of cellulose paper. *Trichoderma* strains were grown for 2 days. Afterward, the *Trichoderma* colonies were carefully removed, and a 5mm disc of *Fusarium* was plated on the medium.

The IV production experiments followed the protocol outlined in [12]. 5mm discs of *Trichoderma* and *Fusarium* were plated on separate PDA dishes. The plates were overlaid, with *Trichoderma* at the bottom and *Fusarium* at the top. The junction was sealed with parafilm. The technique was modified to visualize Fusarium sp. colonies exposed to Trichoderma volatiles as per [25].

Statistical analysis of the growth inhibition

The antagonistic activities were compared with one-way ANOVA to find the statistically significant inhibitions. Tukey's test was implemented for the pairwise comparisons. Data analysis and visualization were performed using GraphPad Prism v8[26].

RESULTS

Isolation of rhizosphere *Trichoderma* strains

Nine fungal isolates with the classic *Trichoderma* characters were obtained from the rhizosphere soils of pineapple plants from farms located in Misiones province. Six isolates were obtained from open-field farms and named TU1, TU2, TU3, TU4, TU5, and TU6. The remaining three isolates were obtained from greenhouse farms and named TC7, TC8, and TC9. All fungal isolates were maintained and stored in PDA at 4ºC.

Morphological identification of the *Trichoderma* isolates

Colonies of *Trichoderma* spp. were initially white for the first 48-72 hours (early mycelium), later turning dark or light green (late mycelium), with scarce aerial mycelia. No diffusible pigment was observed when *Trichoderma* isolates were cultured alone. Conidial production was observed after 4 days of incubation, with denser conidia in the center of the colonies. Older colonies formed 1-3 concentric rings. Conidiophores were symmetrical, with a uniform central axis from which paired secondary axes emerged. Phialides were slender, hooked, and flask-shaped. Conidia were dark green and globose, appearing after 48-72 hours. Chlamydospores were green and globose, appearing after 5-7 days. We identified these isolates as members of the genus Trichoderma based on morphological characteristics.
During the late mycelium state, the growth rate and sporulation pattern were slightly different between *Trichoderma* isolates depending on the type of farming system (open-field or greenhouse cultivation) from which they were collected.

Phylogenetic study of the ITS1-5.8S-ITS2 region

The two main groups formed consisted of sequences from the *T. harzianum* and *T. asperellum* species complexes (see Fig. 1). These groups had significant statistical support, with bootstrap values higher than 70. The sequences of isolates TU1, TU2, TU3, TU4, TU5, and TU6 grouped with *T. asperellum* species-complex sequences from NCBI. The sequences of the isolates TC7, TC8, and TC9 grouped with *T. harzianum* species-complex.

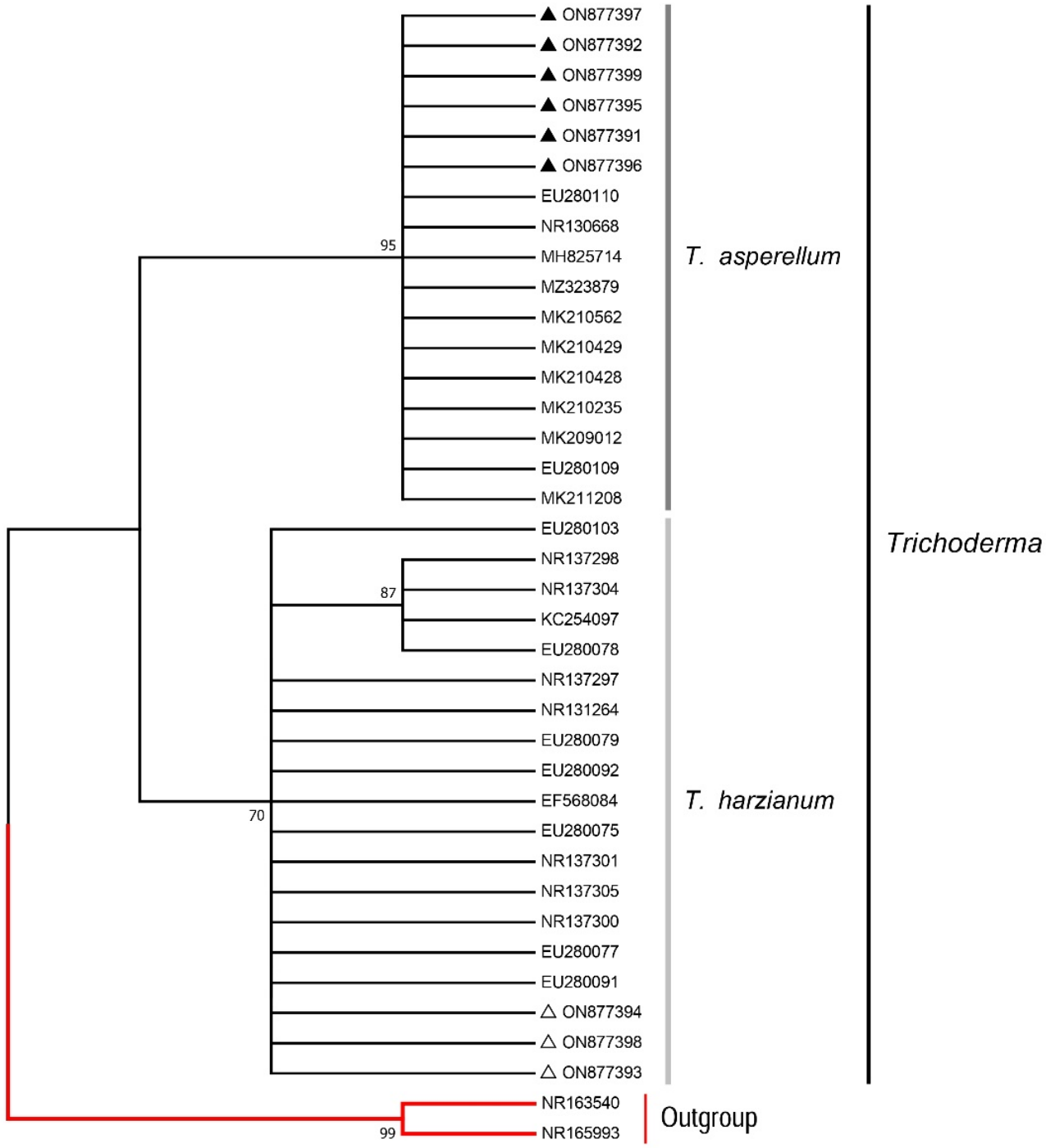

Figure 1. Phylogenetic reconstruction of the ITS1-5.8S-ITS2 region. The tree was constructed using the *Maximum Likelihood* method with the Jukes-Cantor model, and bootstrap support values were generated from 1000 replicates. Only bootstrap supports equal to or higher than 70 are shown. The isolates from this work are indicated by black triangles (▲) for open-field cultivation and white triangles (△) for greenhouse cultivation. The outgroup (*Clonostachys* spp.) is colored in red. The vertical bars indicate sequences from the same *Trichoderma* species complex. These groups are supported by bootstrap values of 70 for the *T. harzianum* species-complex isolates and 95 for the *T. asperellum* species-complex isolates.

Mycoparasitic activity determination

The mycoparasitic capability of all the *Trichoderma* isolates was confirmed by observing the microscopic interaction of the hyphae. These interactions included cellular adhesion, coiling, and penetration toward *Fusarium* sp. (Fig. 2).

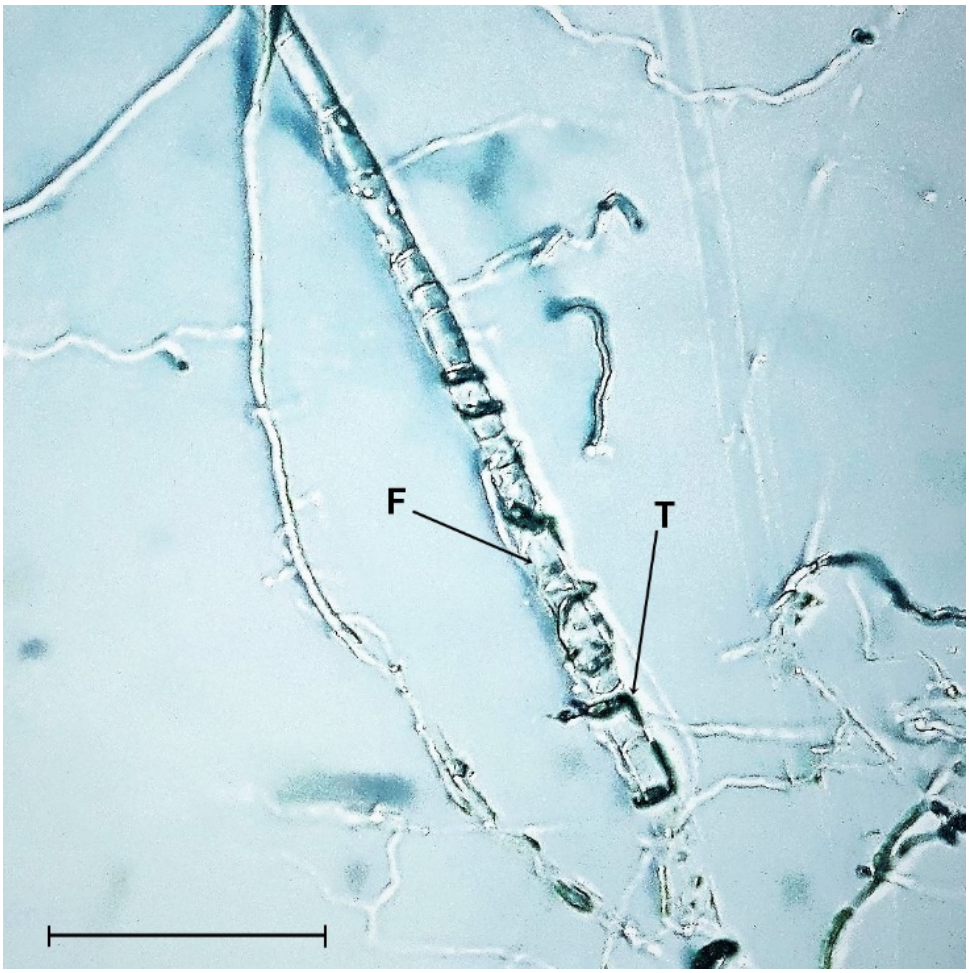

Figure 2. Microphotography of a microculture assay between the isolate *T. asperellum* TU2 (T) and *Fusarium* sp. (F), associated with pineapple fruitlet rot. The image shows multiple coiling of *Trichoderma* towards the *Fusarium* hyphae. Scale: 20um.

Estimation of the growth inhibition of *Fusarium sp.* by *Trichoderma*

The antagonistic strains exhibited a remarkable inhibitory effect against the *Fusarium* isolate after 10 days of cultivation. The percentage of mycelial growth inhibition (%I) varied among the antagonistic treatments tested. In other words, the inhibition effect against *Fusarium* sp. varied among the different assays involving *Trichoderma* isolates (see Table 3). The average colony radius for the *Fusarium* sp. isolates, without *Trichoderma*, was 39±2,4mm across three biological replicates after 10 days of cultivation.

Isolate name	DV	ID	IV
TU1	60.7 ± 1.2	53.3 ± 1.8	49.7 ± 0.9
TU2	66.3 ± 1.4	52.3 ± 0.9	39.0 ± 1.2
TU3	51.7 ± 0.5	57.7 ± 0.9	42.8 ± 0.7
TU4	53.7 ± 0.7	56.7 ± 1.3	45.3 ± 1.6
TU5	61.7 ± 0.5	48.0 ± 1.4	46.3 ± 1.2
TU6	58.3 ± 0.4	43.4 ± 0.7	43.3 ± 0.7
TC7	69.4 ± 0.7	48.0 ± 0.6	51.3 ± 1.2
TC8	63.0 ± 0.6	48.7 ± 0.9	56.3 ± 0.8
TC9	66.8 ± 1.4	43.8 ± 1.4	60.3 ± 0.7

Table 3. PICP values for nine *Trichoderma* isolates against *Fusarium* sp. in dual culture (DC) and diffusible (IV) and volatile (IV) metabolites assays. Values are shown as mean ± standard deviation. Absolute values for the Fusarium colonies are detailed in Table S1.

Direct confrontation (DC)

The T. harzianum isolates showed the highest antagonistic activity in the DC cultures. In this case, the inhibition ranged from 63 to 69.3%. The inhibition obtained by the *T asperellum* isolates ranged from 51.7 to 66.3%.

The *Trichoderma* isolates showed high scores on Bell's scale, with *T. asperellum* and *T. harzianum* isolates scoring 1 and 2, respectively. At the end of the 10th day, they exhibited moderate to high overgrowth on the *Fusarium* sp. colonies.

In all the DC plates tested, the contact zone between the fungi exhibited a curved shape, with the concavity orientated towards the *Fusarium* isolate. The *T. asperellum* isolates produced inhibition halos (3-5mm wide) between both colonies, indicating the presence of diffusible metabolite production. The *T. harzianum* isolates emanated a profuse coconut-like smell, a classical indicator of volatile metabolite production in *Trichoderma* species[27–29].

Indirect confrontation by diffusible (ID) and volatile (IV) metabolites

In ID production assays, *T. asperellum* isolates showed the highest antagonistic activity. In this case, inhibition ranged from 43.3 to 57.6%. In contrast, inhibition for *T. harzianum* isolates was weaker for this assay and ranged from 43.7 to 48.7%.

The T. harzianum isolates obtained the strongest inhibitions against Fusarium sp in IV cultures. The %I values ranged from 51.3 to 60.3%. The inhibition for *T. asperellum* isolates was lower and ranged from 39 to 49.7%.

Remarkably, in the IV assays, the *Trichoderma* colonies had a yellow coloration after 10 days of cultivation, and the *Fusarium* isolate did not produce any pigments in the presence of the volatile metabolites produced by *Trichoderma* spp., so the late mycelia of the colonies remained white (Fig. 3).

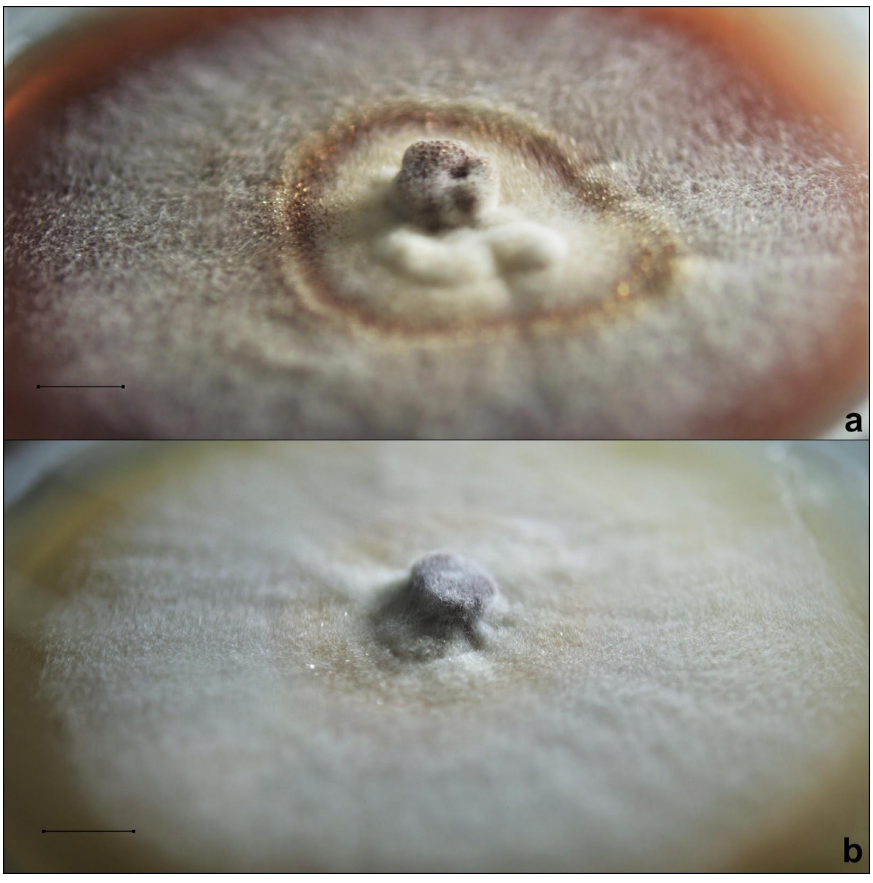

Figure 3. Illustrative picture of macro-morphological changes of the *Fusarium* colonies in controls and treatments within the IV assays. In (a), the *Fusarium* colony exhibits the typical red pigment produced during cultivation on potato dextrose agar. In (b), the *Fusarium* colony is exposed to the *Trichoderma* volatile compounds and does not produce any observable pigments, resulting in a white appearance after 10 days of cultivation. Scale: (a, b): 5mm

Statistical analysis of the growth inhibition

The one-way ANOVA test revealed statistically significant differences in the inhibition values among some of the *Trichoderma* isolates for each type of the antagonism mechanisms tested (see Fig. 4). Particularly, in the DC experiments ($F= 133.3$, $df= 26$, $P <0.0001$), *T. harzianum* TC7 was statistically superior to other isolates (Tukey, $P<0.05$). In the ID assays ($F= 59.2$, $df= 26$, $P <0.0001$), *T. asperellum* TU3 and *T. asperellum* TU4 had a statistically different inhibitory effect on *Fusarium* sp. (Tukey, $P<0.05$) but not between each other (Tukey, $P\geq0.5$). In the IV assays ($F= 147.3$, $df= 26$, $P <0.0001$), *T. harzianum* TC9 produced a statistically higher inhibition than any other isolate (Tukey, $P<0.05$).

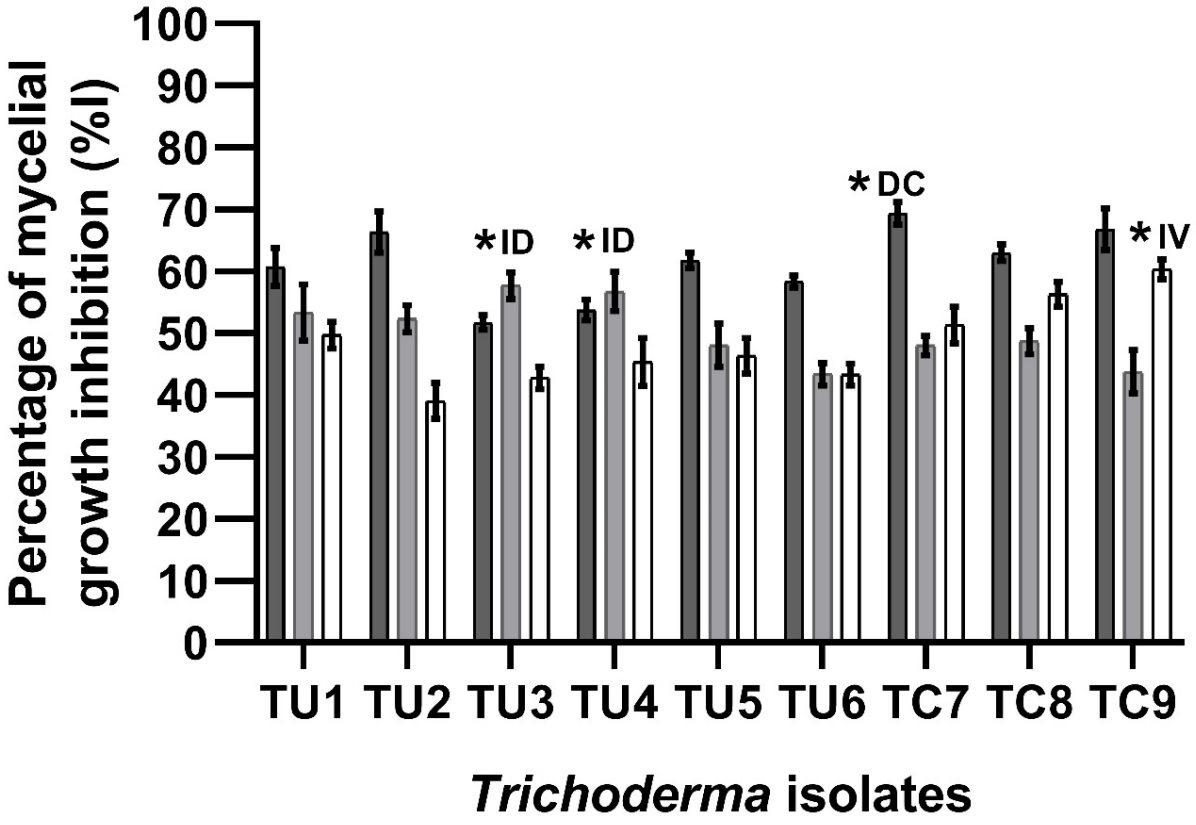

Figure 4. The values of %I (vertical axis) of the nine *Trichoderma* isolates (horizontal axis) against *Fusarium* sp., associated with pineapple fruitlet rot, in DC (dark bars), ID (grey bars), and IV (white bars) assays. For each %I value, the 95% confidence intervals are shown. The asterisks (*) indicate statistically higher inhibitions (Tukey, P<0,05) for the dual culture (*DC), diffusible metabolites (*ID), and volatile metabolites (*IV) assays.

DISCUSSION

Isolation of rhizosphere *Trichoderma* strains

The reports suggest that *Trichoderma*[30] and *Fusarium*[5] are prevalent fungal genera in pineapple farms. Specifically, *T. harzianum* and *T. asperellum* are frequently isolated in tropical and subtropical environments[31], which comprise the primary geographic zones for pineapple cultivation.

Morphological and molecular identification of the *Trichoderma* isolates

In the present assay, two groups were delineated based on *Trichoderma* morphological characters. Each group presented the typical morphotypes reported for the *T. asperellum* species-complex[32] and the *T. harzianum* species-complex isolates[33]. However, it should be noted that these morphotypes serve as broad descriptors.

On average, the analysis of the ITS1-5.8S-ITS2 region leads to an accurate taxonomic fungal species identification, particularly within the ITS1 and the ITS2 introns[34]. The formation of phylogenetically significant subgroups within specific species is standard, as observed in [35] and [17], where the *T. harzianum* isolates were grouped in two or more "clades." Similarly, in the experiments by [32,] the *T. asperellum* isolates formed various subgroups. The two Trichoderma morphotypes in this assay corresponded to distinct ITS1-5.8S-ITS2 phylogenetic groups. The sequences of the *T. harzianum* species-complex isolates formed subgroups with statistically significant bootstrap values.

However, taxonomic identification at the species level in *Trichoderma* necessitates the utilization of additional DNA markers[36]. For example, the Translational Elongation Factor 1α (TEF1α)[37] and the RNA Polymerase II Second Largest Subunit (RPB2)[38] have been used to distinguish species within the *T. asperellum*[39] and *T. harzianum* complexes[40].

Mycoparasitic activity determination

The ability to parasitize other fungi is widespread among species of the genus *Trichoderma*. *T. asperellum* and *T. harzianum* are no exceptions, as their mycoparasitic capacity has been validated against numerous phytopathogens[35].

In this research, the ability of native *T. asperellum* and *T. harzianum* isolates to mycoparasitize *Fusarium* sp. associated with pineapple fruitlet rot was demonstrated. These results are similar to those obtained by [41], where most *Trichoderma* isolates exhibited the capacity to mycoparasitic *Fusarium* spp., showing a range of antagonist-pathogen cellular interactions, including adhesion, coiling, and penetration of host hyphae.

Direct confrontation (DC)

The autochthonous *T. asperellum* and *T. harzianum* isolates inhibited the mycelial growth of *Fusarium* sp. by at least 50 and 60%, with maximum %I values of 66.3% (TU2) and 69.3% (TC7), respectively. These %I values are similar to those previously reported by [7] for *T. asperellum* [33,42] for *T. harzianum*, with most isolates exhibiting medium to high antagonistic activity in dual cultures. Nevertheless, %I values documented in the literature range from 20% to 70% for T. asperellum and 30% to 90% for *T. harzianum*. Consistent with the findings in this study, the highest dual culture %I values for *T. harzianum* isolates were also reported by [42].

The highest %I values were primarily obtained in the DC tests, compared to other assays. This is logical, considering that under these conditions, multiple antagonistic mechanisms could be present simultaneously, such as producing diffusible and volatile metabolites, competition for the substrate, and mycoparasitism, etc[43].

Indirect confrontation by diffusible (ID) and volatile metabolites (IV)

The production of diffusible metabolites has been demonstrated in several *Trichoderma* species, including *T. asperellum*[44] and *T. harzianum*[45]. In the present work, the native *T. asperellum* and *T. harzianum* isolates produced diffusible substances with fungistatic action against *Fusarium* sp. *in-vitro*. This was especially remarkable for the *T. asperellum* isolates, which exhibited %I values greater than 50%. Moreover, the presence of inhibition halos during the dual cultures may be attributed to these isolates' high production of diffusible compounds.

Similar to the previous case, the ability to produce volatile metabolites has been demonstrated for *T. asperellum*[46] and *T. harzianum*[47]. The %I values obtained by the *Trichoderma* isolates in this assay suggest the production of volatile compounds capable of inhibiting *Fusarium* sp. mycelial growth. This was particularly notable for *T. harzianum* isolates, which achieved %I values greater than 50% in this assay. Remarkably, these fungi emitted an intense coconut-like smell during cultivation with *Fusarium* spp., a scent previously associated with a *Trichoderma* volatile antibiotic[12,27–29].

Utilization of *Trichoderma* spp. as BCAs in pineapple farms

Our study provides preliminary insights into the presence of *T. harzianum* and *T. asperellum* species complexes and their mechanism for controlling *Fusarium* sp., the causal agent of pineapple fruitlet rot. In the present work, the antagonistic effects of the *Trichoderma* isolates varied within isolates from the same species complex. The interactions of fungal isolates are considered strain-specific [48,49]. In this context, the interactions of novel *Trichoderma* isolates against multiple pathogenic *Fusarium* strains associated with pineapple fruitlet rot could improve our understanding of the behavior of BCAs under different conditions.
While promising *Trichoderma* isolates were identified and evaluated in this work, further research on the formulations and applications of these BCAs for field treatments is necessary[50]. The effectiveness of *Trichoderma* isolates in protecting pineapple plants from *Fusarium* infection should be evaluated *in planta* to represent a more realistic scenario[51]. Future research in these areas will enhance the development of robust biocontrol strategies using *Trichoderma* species against *Fusarium*-induced fruitlet rot in pineapples under both open-field and greenhouse cultivation in Misiones farms using ICM strategies.

CONCLUSIONS

This work has studied the biocontrol mechanisms of Trichoderma strains belonging to different species groups. Furthermore, the *Trichoderma* isolates from healthy pineapple rhizosphere soil displayed robust antagonistic activity against *Fusarium* sp. These results showed that native *Trichoderma* spp. could reduce the mycelial growth of *Fusarium* sp., associated with pineapple fruitlet rot.

This is the first report on the isolation, identification, and characterization of antagonistic *Trichoderma* species from rhizosphere soil in open-field and greenhouse-cultivated pineapple farms in Misiones, Argentina. The antagonistic activity of *T. asperellum* and *T. harzianum* species complexes highlights the potential of using these BCAs to formulate natural and highly effective fungicides. Different formulation and application methods must be studied to gain further insight into the utility of *Trichoderma* as BCA. These isolates might be integrated with other management strategies to reduce pineapple fruitlet rot caused by *Fusarium* under a sustainable production practice.

Supplementary Materials: Table S1

Isolate name	DC			ID			IV		
TU1	15.5	14.8	15.7	17.9	17.7	19	19.4	19.4	20
TU2	12.6	13.7	13.2	18.2	18.9	18.7	24.3	23.5	23.6
TU3	18.7	18.8	19	16.2	16.8	16.6	22	22.4	22.6
TU4	17.8	18.3	18.2	16.3	17.2	17.1	22	21.2	20.8
TU5	14.8	14.9	15.1	20.8	20.3	19.7	21.1	21.3	20.5
TU6	16.2	16.4	16.2	22.2	22.3	21.8	22.3	21.8	22.2
TC7	11.6	12.2	12.1	20.5	20.4	20	19.5	18.5	19
TC8	14.2	14.4	14.7	20.1	20.3	19.7	17.4	16.7	17.1
TC9	13.4	12.4	13.2	21.9	22.5	21.4	15.5	15.7	15.2

Table S1. Values of the radius (mm) of the *Fusarium* sp. colonies during *in vitro* antagonism assays against nine *Trichoderma* isolates.

Author Contributions: LMM made the taxonomic identification, the *in-vitro* characterization of the fungal isolates, the analysis of the data, and the writing of the manuscript. AEA made a significant contribution in the manuscript's interpretation and writing. MCV designed and conducted the experiments and isolated the fungal material. All authors read and approved the final manuscript.

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