Article

Identification of endophytic fungi of *Ageratina pichinchensis* with antagonistic activity against phytopathogens of agricultural importance

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Abstract

Ageratina pichinchensis is a medicinal plant, endemic to Mexico, known as *Axihuitl*. The extracts of the leaves show antifungal activity against dermatophytic fungi, but there are no studies of the identification of endophytic fungi. The objective was to identify endophytic fungi of *A. pichinchensis* with potential as biological control agents of phytopathogens. Fifty-five morphospecies of endophytic fungi that belong to the phylum Ascomycota were isolated from the leaves of *A. pichinchensis*. Molecular identification based on the analysis of the sequences of internal transcribed spacers (ITSs) amplified by PCR showed that six of the most frequent fungi correspond to *Remotididymella anthropophila* and *Diaporthe caatingaensis* and to the genera *Diaporthe*, *Phomopsis* and *Fusarium*. In multiple antagonism assays, seven morphospecies showed strong antagonistic activity against the pathogens *Fusarium oxysporum*, *F. proliferatum* and *Stemphylium vesicarium*. Two endophytic fungi belong to *Alternaria alternata*, another to *Trichoderma longibrachiatum* and two others are from the genera *Alternaria* and *Phomopsis*. While *Nigrospora oryzae* was the only most frequent endophyte and with antagonistic activity against the three pathogens. In dual culture assays, endophytes with strong antagonistic activity inhibited the mycelial growth of *F. oxysporum* and *F. proliferatum* by 37 to 80%, but in the poisoned food assay, *T. longibrachiatum* inhibited the mycelial growth of the two pathogens by 79% and 66%, respectively. For the first time, *R. anthropophila* as an endophytic fungus, as well as the identification and antagonistic activity of endophytic fungi of *A. pichinchensis*, are reported.

Keywords: *Fusarium*, *Nigrospora*, *Remotididymella*, *Trichoderma*, biocontrol.

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Introduction

Endophytic microorganisms are those that colonize plant tissues, but without causing visible symptoms of the disease (Hardoim *et al*., 2015). In medicinal plant extracts, antimicrobial activity is related to the proportion of endophytes (Egamberdieva *et al*., 2017). Therefore, endophytic fungi of medicinal plants could be used as biocontrol agents of phytopathogens.

The medicinal plant *Ageratina pichinchensis* (Kunth) R.M. King & H. Rob (formerly called *Eupatorium aschembornianum* S. Schauer) is endemic to Mexico and is a wild perennial herb that grows in forested areas in 28 of the 32 Mexican states (Rzedowski and Rzedowski, 2001). In the state of Morelos, it is known as *Axihuitl* and grows in the protected natural area of the Chichinautzin Biological Corridor. It is a plant that is used in traditional medicine to treat gastric ulcers, skin infections, wounds and tumors (Avilés and Suárez, 1994). Leaf extracts have antifungal activity against the dermatophytic fungi *Candida albicans* and *Aspergillus niger* (Ríos *et al*., 2003). However, studies of the identification of endophytic fungi of *A. pichinchensis* with antagonistic activity against phytopathogens are scarce.

Fungi of the genera *Fusarium* and *Stemphylium* cause disease in various crops. *Fusarium oxysporum* causes vascular wilt or root rot in crops such as alfalfa, beans, cotton, lettuce, onion, peas, pepper, potato, soybeans, spinach, and tomato (Munkvold, 2017). *F. proliferatum* is a component of the ear and stem rot complex in corn, asparagus, bananas, date palms, figs, mangoes, pines, sorghum and onions (Munkvold, 2003). *Stemphylium vesicarium* is the causative agent of the leaf blight disease in onion and garlic (Rao and Pavgi, 1975; Zapata-Sarmiento *et al*., 2020) and also affects asparagus, broad beans and rice (Sheikh *et al*., 2015; Graf *et al*., 2016; Foster *et al*., 2019). Therefore, the objective of this study was the identification of endophytic fungi of *A. pichinchensis* with potential for the biological control of phytopathogens of the genera *Fusarium* and *Stemphylium*.

Materials and methods

Collection of plant material

Ageratina pichinchensis plants were collected in October 2019 in the Chichinautzin Biological Corridor, Morelos, Mexico, geographical coordinates 18° 59’ 26.4” north latitude 99° 17’ 09.2” west longitude. A specimen was deposited in the HUMO Herbarium of the Autonomous University of the State of Morelos (Voucher 3571), and the plants were identified by trained personnel from the same institution. The plants were about 1 m tall and were in the flowering stage. In total, 40 leaves without symptoms of disease were collected from 20 plants, two leaves per each plant.

Isolation of endophytic fungi and their classification into morphospecies

Endophytic fungi were isolated and classified into morphospecies according to Arnold *et al*. (2001). Five fragments of 5 mm^2 were cut from the leaves and the surface of the fragments was disinfected with ethanol (70%) for 2 min, with sodium hypochlorite (0.52%) for 2 min and two washes with sterile distilled water.

To evaluate the efficacy of disinfection, an impression from each fragment was obtained in potato, dextrose and agar culture medium (PDA, Bioxon) in Petri dishes, which were incubated for eight days. The absence of mycelial growth indicated that the disinfection method was effective in eliminating epiphytic fungi. At the same time, the five fragments were dried and placed in Petri dishes with PDA culture medium. The Petri dishes were incubated at 27 ±2 °C with a photoperiod of 12 h light: 12 h darkness until observing the growth of the hyphae. The tips of the hyphae were subcultured to obtain pure colonies in Petri dishes with PDA.

Fungi were classified into morphospecies according to the following morphological characteristics: spore production, aerial mycelium, colony color, culture medium color, surface texture, and edge characteristics. The relative frequency (RF) of each morphospecies was calculated according to Photita *et al*. (2001) and the following formula: $RF=\left(\frac{\text{Number of fragments colonized by the fungus}}{\text{Total fragments}}\right)\times 100$.

Multiple antagonism assay

Previously, isolates of *F. oxysporum*, *F. proliferatum* were obtained from onion bulbs and the isolate of *S. vesicarium* was obtained from onion leaves. Prior to carrying out the assays, pathogenic fungi such as endophytes were cultured in the PDA medium (Bioxon), at 27 ±2 °C with a photoperiod of 12 h light:12 h darkness for seven days.

The antagonistic activity of each morphospecies against the three phytopathogens was evaluated in a multiple antagonism assay described by Sánchez-Fernández *et al*. (2015). Three repetitions were made for each endophyte in multiple confrontation and the controls. The results were analyzed in triplicate by means of cluster analysis with the 'fastcluster' package of Rstudio version 3.4.2. Antagonistic activity was classified according to the modified scale of Yuen *et al*. (1999) as: a) strong, the endophytic fungus inhibits the growth of pathogens and grows to and surrounds the pathogen; b) weak, the endophytic fungus and the pathogen grow and their hyphae intermix and do not reduce their growth; c) mutual, the endophytic fungus and the pathogen grow to contact and stop growing; and d) null, the pathogen grows to the endophyte, surrounds it and inhibits its growth.

Molecular identification of endophytic fungi with the highest antagonistic activity

Endophytic fungi that were found with a RF greater than 5% and that in the multiple antagonism assay showed strong antagonistic activity were cultured in PDA medium (Difco) for 7 days. The mycelium was collected, frozen and pulverized in a mortar with liquid N_2. For DNA purification, the Dneasy Plant Mini Kit (Quiagen, Germany) was used. The ITS regions of the fungi were amplified using the primers ITS 1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' CTGTTGGTTTCTTTTCCTCCGC 3') designed by White *et al*. (1990). The amplification conditions were those reported by Zapata-Sarmiento *et al*. (2020).

The sequencing was performed by the company Macrogen Inc Services (Seoul, Korea). The consensus sequence was obtained with the BioEdit Program (version 7.0.5) and the sequences were deposited in GenBank with the Blast program of the National Center for Biotechnology Information database. Based on the results of the Blast analysis, an identity ≥ 98 to 100% and a coverage ≥ 80% with other sequences were considered to assign a name to a species. Sequences that did not meet these criteria were assigned the name at the genus level.

Dual culture assay

Endophytic fungi with strong antagonistic activity were selected to evaluate their antagonistic activity in dual culture assays against *F. oxysporum* and *F. proliferatum* according to Zapata-Sarmiento *et al*. (2020). Six repetitions were made for each endophytic fungus in dual culture with each pathogen and the controls. Every 24 h photographs of the cultures were taken, and the images were analyzed using the ImageJ program (version 1.8) to calculate the area (cm^2) of mycelial growth of the pathogen.

The percentage of mycelial growth inhibition (MGI) was calculated using the equation: MGI= (C-T) × 100 ÷ C. Where: C corresponds to the area of mycelial growth of the pathogen in the control; and T to the area of mycelial growth in the dual culture.

The data were analyzed by means of an analysis of variance (Anova) and the comparison of means using the Tukey test in Rstudio (version 1.2.1335) with the Agricolae library. The type of interaction between the endophytic fungus and the phytopathogen was recorded after 15 days of incubation. According to Bertrand *et al*. (2013), interactions were classified into: remote inhibition, zone of lines, contact inhibition and overgrowth.

Assay of antagonistic activity of non-volatile metabolites

The antagonistic activity of the cell-free filtrates of the endophytic fungi *Trichoderma longibrachiatum*-EA54 and *Nigrospora oryzae*-EA51 against *F. oxysporum* and *F. proliferatum* was evaluated by means of the poisoned culture technique according to Schmitz (1930). *T. longibrachiatum* and *N. oryzae* were grown in Petri dishes with PDA medium (Difco TM) for 7 days. With the culture of *T. longibrachiatum*, a suspension of spores was prepared at a concentration of $1x10^7$ spores ml^{-1}. In Erlenmeyer flasks (250 ml) with 50 ml of potato dextrose broth (PDB, Difco TM), 1.5 ml of the spore suspension was added. Because *N. oryzae* in PDA culture medium lacks reproductive structures, the flasks were inoculated with five blocks of culture medium with mycelium of 0.5 cm in diameter. Three Erlenmeyer flasks were prepared from each fungus.

The liquid cultures were incubated in a stirrer at 150 rpm and at 27 ±2 °C with a photoperiod of 12 h light: 12 h darkness. After four days, the culture broth was collected and centrifuged at 4 500 rpm for 10 min; the supernatant was filtered through 0.45 µm and then 0.22 µm membranes (GVWP, Millipore) to obtain the cell-free filtrate that was used to prepare the culture medium according to Zapata-Sarmiento *et al*. (2020). Six Petri dishes were prepared for each pathogen with each cell-free filtrate and the respective controls. Every 24 hours photographs were taken, and the images were analyzed with the ImageJ program (version 1.8) to calculate the area (cm^2) of mycelial growth of the pathogen. The percentage of mycelial growth inhibition (MGI) was calculated using the equation: MGI= (C-T) × 100 ÷ C.

Results and discussion

Classification and frequency of morphospecies of endophytic fungi

From the leaves of *A. pichinchensis*, 100 isolates of endophytic fungi were obtained. Based on morphological characteristics, the isolates were classified into 55 morphospecies. The isolate EA38 was the most frequent morphospecies with a relative frequency (RF) of 20%; followed by the

isolates EA37, EA39 and EA40 with an RF of 15% and then the isolates EA30, EA42 and EA51 with an RF of 10%. The remaining isolates had a RF of 5%. Morphospecies is a functional taxonomic term useful for classifying endophytic fungi, which are very diverse in plants that grow in tropical environments. This classification is also useful for identifying endophytes that lack reproductive structures when cultured *in vitro* (Fröhlich and Hyde, 1999; Arnold *et al*., 2001). For these reasons, it was decided to use this classification for endophytic fungi of *A. pichinchensis*.

Antagonistic activity of morphospecies of endophytic fungi in multiple antagonism bioassays

The cluster analysis grouped the 55 morphospecies of endophytic fungi according to their antagonistic activity against *S. vesicarium*, *F. proliferatum* and *F. oxysporum*. Seven morphospecies (EA26, EA51, EA28, EA10, EA53, EA55 and EA54) showed strong antagonistic activity against the three pathogens. While the mycelial growth of *S. vesicarium* was strongly inhibited by 12 endophytic fungi and weakly inhibited by six. Mutual inhibition of mycelial growth with the three pathogens was observed with the isolates EA2 and EA37, but the isolate EA48 was the only one that showed mutual inhibition with *S. vesicarium* (Figure 1).

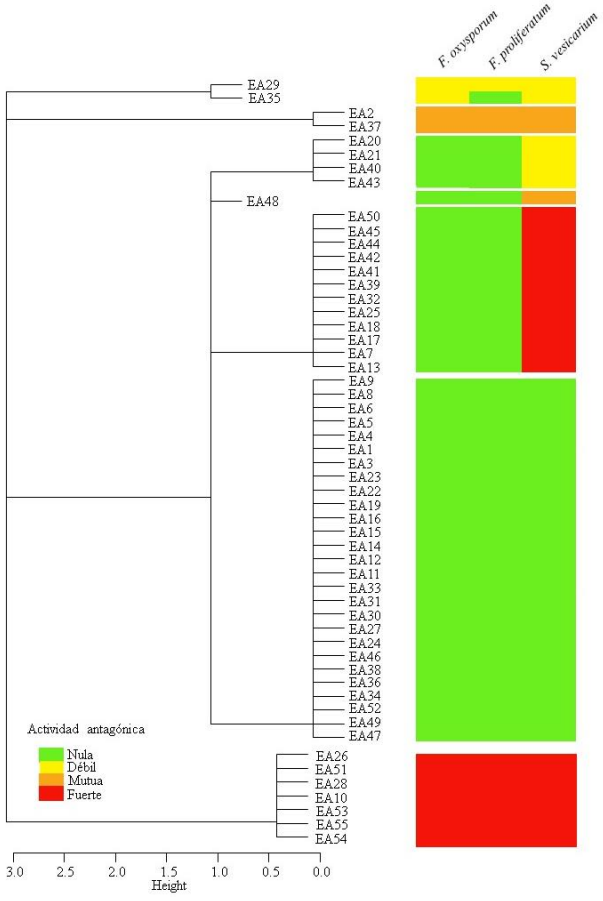

Figure 1. Cluster analysis of the antagonistic activity (strong, mutual, weak or null) of the endophytic fungi of *Ageratina pichinchensis* against *Fusarium oxysporum*, *Fusarium proliferatum* and *Stemphylium vesicarium*.

Figure 2 shows the interactions observed between the seven morphospecies of endophytic fungi classified with strong antagonistic activity against the pathogens. The morphospecies of the isolates EA10, EA28 and EA54 grew on the mycelium of the pathogens. While EA26, EA51, EA53 grew around the pathogens and the morphospecies EA55 grew on *S. vesicarium* and only grew around the two species of *Fusarium*.

Figure 2. Interactions observed in the multiple antagonism assay of the seven morphospecies of the endophytic fungi of *Ageratina pichinchensis* classified with strong antagonistic activity against the pathogens: a) *Fusarium oxysporum*; b) *Fusarium proliferatum*; and c) *Stemphylium vesicarium*.

Of the 55 morphospecies of endophytic fungi that were isolated from *A. pichinchensis*, 12 of them showed antagonistic activity against one pathogen and seven against the three pathogens. Similarly, the isolation of morphospecies of endophytic fungi of medicinal plants with antagonistic activity against pathogenic fungi is reported. In *Etlingera elatior* (ginger), the isolation of six morphospecies of endophytic fungi with antagonistic activity against *Fusarium oxysporum*, *Ganoderma boninense* and *Rigidoporus lignosus* is reported (Lutfia *et al*., 2020) and in *Aloe dhufarensisi*, two morphospecies of endophytic fungi with antagonistic activity against *Fusarium* sp. and *Cladosporium* sp. were isolated (Al-Rashdi *et al*., 2020).

Based on the results of the multiple antagonism assay, the seven morphospecies of endophytic fungi with strong antagonistic activity were selected for their identification at the molecular level and to perform the assays of antagonistic activity in dual culture and poisoned food culture.

Identification of endophytic fungi with strong antagonistic activity against pathogens

Table 1 shows the identification at the molecular level of the 12 isolates of endophytic fungi that were found with a RF greater than 5% and that also showed a strong antagonistic activity. The sequences of the isolates EA30 and EA40 met the criteria of identity (≥ 98%) and coverage (> 80%) corresponding to sequences of *Remotididymella anthropophila* and *Diaporthe caatingaensis*, respectively. The sequence of the isolate EA37 also met the criterion of identity and coverage, but it is with a sequence from the gene bank of an unidentified species of the genus *Phomopsis*. For the isolates EA38 and EA39, the identity value was less than 98%, so they were placed in the genus *Diaporthe*.

Table 1. Molecular identification of endophytic fungi of *Ageratina pichinchensis* with a relative frequency (RF) greater than 5% and that showed a strong antagonistic activity against the pathogens *Fusarium oxysporum*, *Fusarium proliferatum* and *Stemphylum vesicarium*.

Morphospecies		Species	No. of access	Identity (%)	Coverage (%)
With RF greater than 5%	EA30	*Remotididymella anthropophila*	MT150607	99.1	99
	EA37	*Phomopsis* sp.	MT150610	99	100
	EA38	*Diaporthe* sp.	MT150611	97.8	91
	EA39	*Diaporthe* sp.	MT150612	97.1	100
	EA40	*Diaporthe caatingaensis*	MT150613	98.7	98
	EA42	*Fusarium* sp.	MT362619	92.2	96
With RF greater than 5%, antagonists	EA51	*Nigrospora oryzae*	MT150620	99.8	97
Antagonists	EA10	*Alternaria alternata*	MT107053	99.4	100
	EA26	*Alternaria* sp.	MT107054	99.8	100
	EA28	*Alternaria alternata*	MT150606	99.5	100
	EA54	*Trichoderma longibrachiatum*	MT150622	99	96
	EA55	*Phomopsis* sp.	MT150623	99.5	99

In the case of the isolate EA42, the identity was 92.2% and therefore, it was also only placed in the genus *Fusarium*. The isolate EA51 with an RF greater than 5% and with antagonistic activity against all pathogens showed an identity of 99.8% with *Nigrospora oryzae* sequences. The isolates of the fungi EA10 and EA28 with antagonistic activity showed an identity greater than 99% with sequences of *Alternaria alternata* species. The sequence of the isolate EA26 showed an identity of 99.8% with an unidentified species of the genus *Alternaria* and the sequences of the isolates EA54 and EA55 showed an identity of 99 and 99.5% with sequences of *Trichoderma longibrachiatum* and *Phomopsis* sp., respectively. Finally, the isolate EA53 was the only one that was not identified at the molecular level, but in the PDA medium it did not develop reproductive structures, it had aerial mycelium, the colony showed a dusty texture, with irregular edge, with rings and white.

Endophytic fungi belong to the orders Pleosporales, Trichophaeriales, Diapothales and Hypocreales. According to the molecular identification and relative frequency data, the genus *Phomopsis* (anamorph of *Diaporthe*) was the most frequent, followed by *Fusarium* sp., *N. oryzae* and *R. anthropophila*. Some of the species of endophytic fungi that were identified in *A. pichinchensis* are also reported in other species of plants of the genus *Ageratina*. The most abundant endophytic fungi in *A. adenophora* belong to the genus *Phomopsis* (Mei *et al*., 2014), while *P. magnolia* and *N. oryzae* are also reported as endophytes of *A. altissima* (Christian *et al*., 2016). Fungi of the genus *Phomopsis* are the endophytes that are most frequently isolated in tropical plant species (Murali *et al*., 2006).

The fungus *N. oryzae* is an endophyte with a cosmopolitan distribution and a wide range of hosts, Wang *et al*. (2017). The fungus *R. anthropophila* has not been reported as an endophyte in other plants; but this fungus belongs to the family Didymellaceae, which includes other species of fungi reported as endophytes and phytopathogens (Wang *et al*., 2017). Similar to our results, fungi of the genera *Alternaria* and *Diaporthe* are reported as endophytes of the medicinal plant *Ocimum sanctum* Linn., and also show antagonistic activity against *F. oxysporum* (Chowdhary and Kaushik, 2015). However, there are no reports of the antagonistic activity of *Alternaria* sp. and *Phomopsis* sp. against *F. proliferatum* and *S. vesicarium*. With respect to *N. oryzae*, it is reported to be an endophyte of *Gossypium arboreum* (cotton) with antagonistic activity against *F. solani* (Hiremani *et al*., 2020), but there are no reports of the antagonistic activity of *Nigrospora* against other species of *Fusarium* and *S. vesicarium*.

Antagonistic activity of endophytic fungi in dual culture assays

In dual culture, the seven isolates of selected endophytic fungi inhibited the mycelial growth of *F. oxysporum* and *F. proliferatum* from 37 to 80%. The isolates of *T. longibrachiatum* and *N. oryzae* showed the greatest antagonistic activity since they inhibited the growth of the two *Fusarium* species by more than 79% (Table 2).

Table 2. **Antagonistic activity of endophytic fungi of *Ageratina pichinchensis* against *Fusarium oxysporum* and *Fusarium proliferatum* in dual culture assays.**

	Fusarium oxysporum		*Fusarium proliferatum*	
	MGI (%)	MG (cm^2)	MGI (%)	MG (cm^2)
Control	0	51 ±1.4 a	0	58.8 ±3 a
T. longibrachiatum EA54	80	9.8 ±1.4 e	80	11.2 ±1.8 e
Nigrosora oryzae EA51	79	10.6 ±1 e	83	9.7 ±1.5 e
EA53	56	22.1 ±1.1 d	53	27.7 ±1.2 d
Phomopsis sp. EA55	48	26.2 ±1.5 c	49	30.1 ±0.4 cd
A. alternata EA10	46	27.3 ±2.5 c	44	32.9 ±1.7 bc
A. alternata EA28	42	29.4 ±2.2 bc	40	35.3 ±2.1 b
Alternaria sp. EA26	37	31.9 ±3 b	42	34.3 ±1.2 bc

Each value corresponds to the mean ± standard deviation (n= 5). Values in the same column followed by different letters differ significantly according to Tukey's HSD test ($p< 0.05$). MGI= mycelial growth inhibition; MG= mycelial growth.

In relation to *T. longibrachiatum*, it is reported to inhibit the growth of *F. oxysporum* from 27.2 to 68.7% (Sundaramoorthy and Balabaskar, 2013; Abdelrahman *et al*., 2016; Zhang *et al*., 2018). In this study, the isolate of *T. longibrachiatum* was found to inhibit the growth of *F. oxysporum* by up to 80%. In contrast, there are no reports of the antagonistic activity of *T. longibrachiatum* against *F. proliferatum*. But other *Trichoderma* species such as *T. harzianum* and *T. gamsii* inhibit the growth of *F. proliferatum* by 80% (Mondani *et al*., 2021), similar to what was reported in this study.

In the case of *N. oryzae*, studies of inhibition of the growth of fungi of the genus *Fusarium* are scarce. The percentage of growth inhibition of *F. oxysporum* and *F. proliferatum* found in this study is higher than that reported (43.06%) against *F. solani* (Hiremani *et al*., 2020). While *N. oryzae* is an endophyte of *Tylophora indica* that shows no antagonistic activity against *F. oxysporum* (Kumar *et al*., 2010). For *F. proliferatum*, there are no studies of the antagonistic activity of *N. oryzae*. Figure 3 shows the types of interaction between endophytic fungi and the pathogens *Fusarium oxysporum* and *Fusarium proliferatum*.

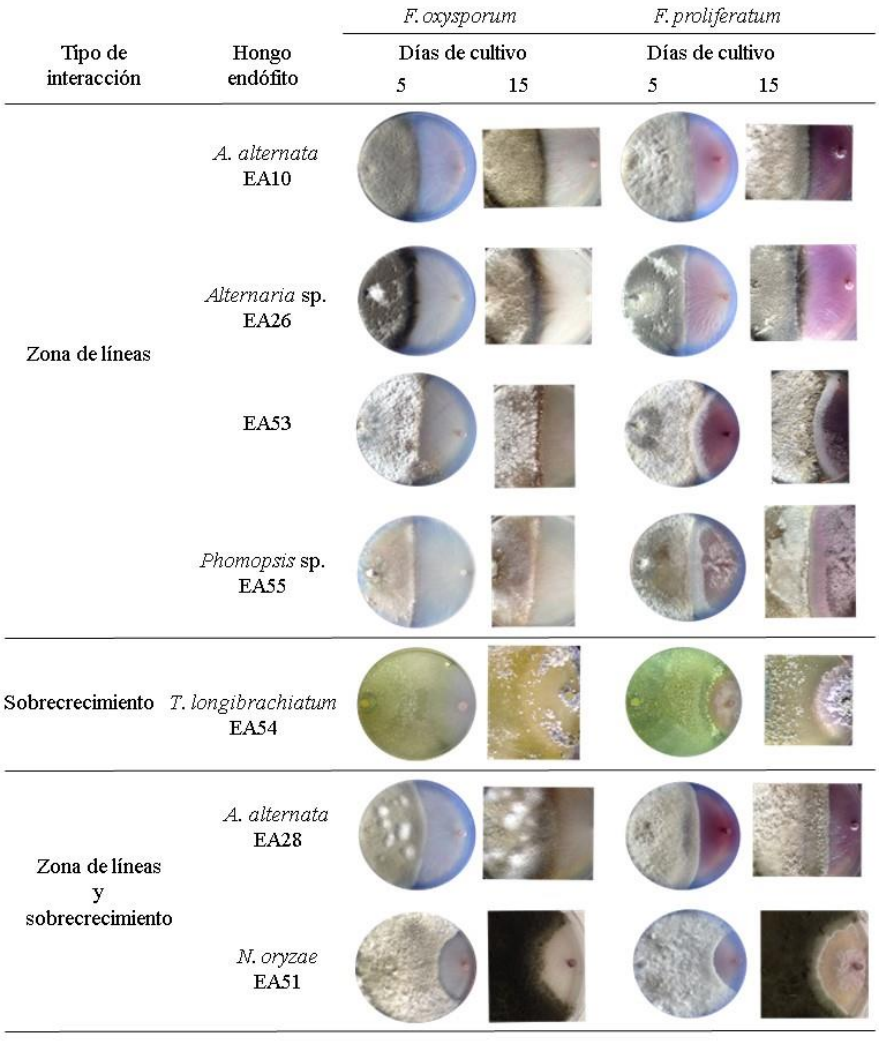

Figure 3. Types of interaction between endophytic fungi of *Ageratina pichinchensis* and the phytopathogens *Fusarium oxysporum* and *Fusarium proliferatum* in dual culture assays.

In the interaction of *F. oxysporum* and *F. proliferatum* with *A. alternata* EA10, *Alternaria* sp. EA26, *Phomopsis* sp. EA55 and EA53 (unidentified), the formation of a zone of lines was observed. The type of interaction between *A. alternata* EA28 and the two pathogens was different depending on the *Fusarium* species. With *F. oxysporum*, a zone of lines was observed, while with *F. proliferatum*, an overgrowth of the endophyte on the mycelium of the pathogen was observed. In contrast, *N. oryzae* grew on the mycelium of *F. oxysporum* but developed a zone of lines with *F. proliferatum*. *Trichoderma longibrachiatum* grew on both pathogens and also sporulated on them.

The presence of a zone of lines indicates that the mechanism of antagonistic activity is antibiosis (Bertrand *et al*., 2013), so it is suggested that *A. alternata* EA10, *Alternaria* sp. EA26, *Phomopsis* sp. EA55 and the isolate EA53 produce antibiotics against *Fusarium*.

In the 'overgrowth' interaction, it may involve, in addition to antibiosis, a competition for nutrients and space (Bertrand *et al*., 2013). Therefore, the results in dual culture and of the type of interaction indicate that the antagonistic activity of *T. longibrachiatum* with the two species of *Fusarium* is antibiosis and competition. However, the results on the type of interaction of the fungi *N. oryzae* and *A. alternata* EA28 against the two species of *Fusarium* indicate that the mechanisms of interaction depend on the species of the pathogen. Based on the results of the dual culture assay and the types of interaction, *T. longibrachiatum* and *N. oryzae* were selected to perform the poisoned food assays.

Antagonistic activity of non-volatile metabolites of *Trichoderma* sp. and *N. oryzae* against *F. oxysporum* and *F. proliferatum*

Cell-free filtrates of *N. oryzae* did not inhibit the growth of *F. oxysporum* and *F. proliferatum*. However, *Trichoderma* sp. inhibited the mycelial growth of both pathogens; *F. proliferatum* was inhibited by 66.5% and *F. oxysporum* by 79.5% (Table 3).

Table 3. Antifungal activity of filtrates of liquid cultures of *Trichoderma* sp. and *Nigrospora oryzae* against *Fusarium oxysporum* and *Fusarium proliferatum*.

	Fusarium oxysporum		*Fusarium proliferatum*	
	MG (cm^2)	MGI (%)	MG (cm^2)	MGI (%)
Control	40.37 ±1.2 a	0	28.78 ±1.1 a	0
Trichoderma sp.	8.27 ±0.7 b	79.5	9.63 ±0.6 b	66.4
N. oryzae	39.37 ±1 a	0	27.87 ±1.5 a	0

Each value corresponds to the mean ± standard deviation (n= 5). Values in the same column followed by different letters differ significantly according to Tukey's HSD test ($p< 0.05$). MGI= mycelial growth inhibition; MG= mycelial growth.

The poisoned food technique confirmed that the antagonistic activity of *N. oryzae* was not due to the production of antibiotics and that *N. oryzae* inhibits the growth of the two *Fusarium* species by competition of space and nutrients. On the contrary, the results with *T. longibrachiatum* indicate that it is an endophytic fungus that inhibited the growth of the two species of *Fusarium* by the production of compounds with antibiotic activity.

Similarly, other authors report the antagonistic activity of *Trichoderma* against strains of *F. oxysporum*, but studies on the antagonistic activity of *T. longibrachiatum* against *F. proliferatum* are scarce. Based on our results, growth inhibition by compounds produced by *T. longibrachiatum* was greater against *F. oxysporum* than against *F. proliferatum*. Future studies could focus on characterizing the non-volatile metabolites produced by *Trichoderma longibrachiatum* and evaluating the effectiveness against the two pathogens.

Strains of *T. longibrachiatum* have been isolated from soil of the rhizosphere of a forest site (Zhang *et al*., 2018), from desert soil in Egypt (Abdelrahman *et al*., 2016) and from the rhizosphere of *Solanum lycopersicum* L. (tomato) (Sundaramoorthy and Balabaskar, 2013). But studies on the antagonistic activity of *Trichoderma* isolated from leaves and aerial parts of medicinal plants are scarce. Sarsaiya *et al*. (2020) reported that *T. longibrachiatum* isolated from stem segments of *Dendrobium nobile* produces dendrobine, a compound similar to that produced by the host plant and that shows antibacterial activity. Likewise, *T. longibrachiatum* isolated from the root of *Suaeda glauca*, a sea plant, produces sesquiterpenes and cyclodepsipeptides with antagonistic activity against soil pathogens (Du *et al*., 2020). These studies show the potential use of *Trichoderma* strains isolated from medicinal plants for agricultural purposes. Future studies will be aimed at identifying and characterizing metabolites produced by *Trichoderma longibrachiatum* isolated from *A. pichinchensis* leaves with antifungal activity against pathogens.

Conclusions

The most frequent endophytic fungi of *A. pichinchensis* belong to the phylum Ascomycota and include *Remotididymella anthropophila* and *Diaporthe caatingaensis*, and others that belong to the genera *Diaporthe, Phomopsis* and *Fusarium*. The endophytic fungi with antagonistic activity were *Alternaria alternata* and *Trichoderma longibrachiatum* and others that belong to the genera *Alternaria* and *Phomopsis*. The only frequent endophytic fungus that showed antagonistic activity is *N. oryzae*, which together with *T. longibrachiatum* stand out for their antagonistic activity against *F. oxysporum* and *F. proliferatum*. But they differ in their mechanism of antagonistic activity, in *T. longibrachiatum* it is due to the production of compounds with antibiotic activity, while the activity of *N. oryzae* is due to the competition for space and nutrients. This is the first report of *R. anthropophila* as an endophytic fungus and of the identification and antagonistic activity of endophytic fungi from *A. pichinchensis* leaves.

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