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Fungal Endophytes in Species of *Paspalum* and *Bromus*, Occurrence and *In Vitro* Antagonistic Activity Assessment

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Research Article

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ABSTRACT

The aims of this work were to study the occurrence of fungi associated to *Paspalum dilatatum, P. notatum, Bromus biebersteinii* and *B. catharticus* and to evaluate their potential as antagonistic agents against fungal pathogens. The genera *Acremonium, Alternaria, Aspergillus, Curvularia, Fusarium, Penicillium* and *Stemphylium* were the most representative isolated fungi. The isolates of the genus *Acremonium* showed antagonistic activity against different putative pathogenic species tested in dual cultures. The results suggest that the interactions and mechanisms of antagonism of the same strain vary according to the putative pathogenic strains to which they are faced to. The results encourage further studies to elucidate if the strains isolated in this study are good candidates for biological control of latent pathogens in pastures. To accomplish this, future studies focused on the analysis of the secondary metabolites produced by the antagonists in the culture medium, as well as assays *in planta* will be conducted.

INTRODUCTION

Fungal endophytes may occur in most plant species. The term endophyte, first proposed by de Bary [1], refers to organisms that colonize internally plant tissues and live in them in an asymptomatic way, for all or at least a significant part of their life cycle [2]. It is a unique cost-benefit plant-microbe association defined by location, not function, where none of the interacting partners is discernibly harmed, and the individual benefits depend on both organisms. Endophytic fungi are a very diverse polyphyletic group of microorganisms that can thrive asymptomatically in aboveground as well as belowground plant tissues [3].

Endophytic fungi form complex relationships with host plants; they utilize nutrients and energy from the plant, while plants may benefit from bioactive metabolites that endophytes produce [3,4]. These metabolites may be able to inhibit or suppress a broad range of organisms, providing protection to plants against nematodes, herbivorous insects, bacterial and fungal pathogens [5]. In this regards, there is detailed literature providing information on pathogens suppression by endophytes [3,6-8]. Other revisions report endophytes that have been used in seed treatment, substantially limiting the negative effects of biotic and abiotic stress in plants [9,10]. Particularly in pastures, where the establishment of forage species is affected by multiple biotic and abiotic factors, the potential benefit of this partnership opens an attractive perspective for biotechnological application, such as the biological control.

Biological control was defined by Baker [11] as the decrease of the inoculum or the activity of pathogenic species by the action of one or more microbial organisms (control agents) including the host plant and excluding human. Biological control mechanisms include the production of antibiotics and antifungal enzymes, competition for nutrients and induction of own host plant defenses [12]. Taking advantage of naturally occurring antagonistic interactions to control pests is an ecologically relevant alternative to chemical-based pest control [13]. In the case of vertically transmitted endophytes, it may also be a cost-effective strategy as the agent can be self-replicating, so that continual applications are not necessary [14].

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Paspalum dilatatum (Poir) and *P. notatum* (Flüggé) are summer-grasses and *Bromus biebersteinii* (Roem and Schult) and *B. catharticus* are winter-grasses widespread in Argentina. They are important forage grasses, especially as regards high productivity, resistance to both, drought and water excess, and vigorous re-growth ability [15]. The isolation of endophytic fungi associated with these species is a good strategy to identify agents potentially useful for biological control in native plants. This work was addressed to: i) determine the occurrence of fungal species in *P. dilatatum, P. notatum, B. biebersteinii and B. catharticus*, and ii) evaluate *in vitro* antagonistic effects against putative fungal pathogens of the same forage species, in order to assess their potential as biological control agents.

MATERIALS AND METHODS

Plant Sampling and Isolation of Fungi

Plant samples of *P. dilatatum, P. notatum, B. biebersteinii and B. catharticus* were collected in Zavalla (32°43´S; 60°55´W) and Funes (32°55′S; 60°49′W), Santa Fe, Argentina. Sampling was done taking into account a separation distance of at least 2 m between plants, in order to avoid taking plants of the same genotype [16]. At each sampling site, whole plants with (color change, wilting or necrosis) or without symptoms of disease were collected for the isolation of fungi.

Stem and leaves sections were selected at random, stained with lactophenol blue and methylene blue and then observed by a Zeiss (Germany) KF2 binocular microscope (40x and 100x). The presence of fungi was confirmed by the appearance of mycelia in the intercellular space of the different tissues [17,18]. Before starting fungal isolation from plant organs, a triple sterilization was carried out, immersing leaf and stem portions (1-3 cm) in alcohol (90% v v^{-1}) 1 min, sodium hypochlorite (50% m v^{-1}) 9 min and alcohol (70% v v^{-1}) 1 min. Finally, three washes with sterile water were performed and the samples were dried with sterile filter paper under a laminar flow cabinet. Fragments (3-5 mm) of leaves and stems were placed in 9 cm Petri dishes (6 to 8 each) with potato dextrose agar (PDA) and 3 drops of lactic acid in order to control bacterial growth. Two biological replicates for each tissue from the same plant were obtained. All the material was incubated in a growth chamber at 28°C in darkness for 20 days. Pure cultures of fungi were obtained, taking portions of 5 mm and inoculating the fungus individually in Petri dishes with fresh PDA. Fungal occurrence was taken as positive when it was detected and/or isolated in at least one sample of each plant. Fungal isolates were maintained at 4°C, and re-streaked every three months. Dry micelia were stored at -20°C for long term preservation.

Taxonomical Identification

Morphological characterization

Macro and microscopic morphological data of the fungal isolates were recorded. Fungal structures were stained with lactophenol blue and methylene blue and then observed by a Zeiss (Germany) KF2 binocular microscope (40x and 100x) based on the technique reported by Arenas [19]. Briefly, the adhesive side of a tape is pressed against the fungal colony, and glued to a glass slide with a drop of the corresponding dye. The distribution of intact fungal structures in microculture was also observed [20]. To this end, a portion of sterile PDA (1 cm × 1 cm) was deposited directly on a slide in which the fungus was spread. The slide was placed in a Petri dish with filter paper imbibed in sterile water, and incubated at 28°C in darkness during 7 days.

The shape and size of spores, hyphae, sporangia, and presence or absence of septa was recorded. Fungal isolates were grouped into morphotypes and identified following taxonomic specific keys [2,21-27].

Molecular characterization

The endophytic fungi were incubated in PD medium with no antibiotics during ten days at 28°C with agitation. Then the cultures were centrifuged, the supernatants were discarded and the mycelia were stored at -20°C until DNA extraction. The DNA extraction was carried out using the protocols published by Möller *et al.* and Allen *et al.* [28,29]. Briefly, dry mycelia (60-75 mg) were grinded in a mortar and pestle with liquid nitrogen. Then, the mycelia were disaggregated in 2 consecutive incubations, the first one with SDS (2% m v^{-1}) and proteinase K (50 µg) at 60°C for 60 min followed by the addition of CTAB and an incubation at 65°C for 10 min. The samples were incubated on ice with a mixture of chloroform: isoamyl alcohol (24:1) for 60 min and then centrifuged at 10000 rpm for 10 min at 4°C. The aqueous phases were transferred to new tubes and were incubated with 5 µl RNAase (1000 µg mL^{-1}) at 37°C for 1 h. Then, another chloroform:isoamyl alcohol extraction was performed and the DNA from the upper phase was precipitated with isopropanol 70% (v v^{-1}). Finally, the DNA was re-suspended in 50 µL of buffer TE (10:1) and checked by electrophoresis in 1.5% (m v^{-1}) agarose gels.

PCR, Sequencing and Phylogenetic Analyses

Genomic DNA (2-10 ng) was used as template in PCR reactions using the primers *ITS1* and *ITS4* to amplify ITS (ITS1-5.8S-ITS2) region, and primers *983* and *2218R* to amplify the *elongation factor 1 alpha* (*TEF1*) gene **(Table 1)** [30-32]. The following thermocycler conditions were used: initial denaturing at 94°C for 3 min; 40 cycles of denaturing at 94°C for 30 s, annealing for 30 s; extension at 72°C for 2 min; and a final extension step of 72°C for 10 min. The annealing temperatures were 55°C *ITS1-ITS4* and 59°C for *983-2218R*. The amplification products were evaluated in agarose gels 1.5% (m v^{-1}) and the samples showing single bands were sequenced.

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Table 1. Primer sequences used for PCR analysis.

Primer name	Sequence (5´-3´)[a]	Annealing temperature (°C)
ITS1	TCCGTAGGTGAACCTGCGG	64.5
ITS4	TCCTCCGCTTATTGATATGC	58.4
983	GCYCCYGGHCAYCGTGAYTTYAT	66
2218R	ATGACACCRACRGCRACRGTYTG	65.4

[a]R (A or G), Y (C or T) and H (A or C or T)

Each sequence fragment was subjected to an individual BLAST search (Basic Local Alignment Search Tool of the NCBI) to verify its identity. Also, the newly obtained sequences were aligned with fungal sequences from GenBank using the PHYLOGENY platform with default parameters in operation [33]. This tool includes MUSCLE for multiple alignment, GBLOCKS for alignment curation, PHYML algorithm to estimate maximum-likelihood in phylogenetic analysis and TREEDYN for tree rendering [33-36].

Antagonistic Activity

Potential antagonists were tested by a dual confrontation approach. Antagonistic activity was determined by evaluating the simultaneous development of the pathogen and the potential antagonist (endophyte) and the interactions between them [37]. Both fungi, pathogen and potential antagonist, were spread at the same time in Petri dishes of 10 cm diameter with PDA at 2 cm from the edge, and at the maximum distance between them. Three replicates of each dual culture were performed. The diameter of each fungal colony at 7 and 15 days after inoculation was measured. The presence or absence of inhibition halo (zone between the two strains where no mycelium growth occurs and the pathogen growth stops towards the antagonist) was also registered. The percentage of growth inhibition (PGI) was calculated using the formula of Skidmore and Dickinson [38]:

PGI (%) = KR-R1/KR × 100

Where KR is the radius of the pathogen from the midpoint of inoculation to the edge of the colony in the control culture (without confronting antagonist), and R1 is the radius of the pathogen from the midpoint of inoculation to the edge of the colony in the direction towards the antagonist colony in dual cultures. The PGI were classified as follows: 0 = no growth inhibition of the pathogen; 1=1-25% growth inhibition of the pathogen (very low); 2=26-50% inhibition of growth of the pathogen (low); 3=51-75% (moderate) and 4=76-100% inhibition of growth of the pathogen (high). The zone of inhibition, defined as the distance in millimeters between the pathogenic fungus and fungal antagonist after 14 days of growth in dual cultures, was also measured [39].

Pathogenic fungi isolated from diseased plants of *P. notatum*, *P. dilatatum*, *B. biebersteinii* and *B. catharticus* used as inocula in the dual cultures were: *Alternaria alternata (Fr.) Keissl* (046), *Alternaria* sp.1 (035), *Curvularia clavata (B.L.) Jain* (048), *C. pallescens Boedijn* (044) and *Fusarium* sp.1 (049).

Data Analysis

The number of endophyte species isolated from plants of the genera *Paspalum* and *Bromus* were recorded and the percentages of endophyte infection were calculated by sampling area and by plant species.

The PGI of the pathogenic fungi tested was transformed by the square root of the arc-sine to fit homocedasticy and a two-way ANOVA was performed. Normality was tested by Shapiro-Wilks test. Multiple comparisons between medias were made among pathogens within the same antagonist with multiple range Tukey's test (α=0.05). All statistical analyzes were done using the statistical program INFOSTAT [40].

RESULTS

Fungi Isolated from Species of *Paspalum* and *Bromus*

Seventy-two fungal strains were isolated from four grass species collected, the vast majority of which were *ascomycete* fungi (60 isolates). The main taxa isolated belong to the genera *Acremonium, Alternaria, Aspergillus*, *Curvularia, Fusarium, Penicillium* and *Stemphylium*. Additionally, 12 unidentified fungal strains were isolated **(Figure 1)**. Regarding the isolation of endophytic fungi from the grass species analyzed, a greater number of endophytes were found associated with *B. catharticus* collected in Funes, which showed 55% of infection over the total of plants sampled **(Table 2)**. On the other hand, *P. notatum* contained the lowest number of fungi (three isolates), two belonging to the genus *Acremonium* and one to *Stemphylium* **(Table 2)**. Several endophytic fungal isolates were found in both sampling sites, such as *Acremonium* sp.1, 4, 5, 6 and 8, *Aspergillus* sp.1, *Fusarium* sp.2 and *Pencillium* sp.4 **(Table 2)**.

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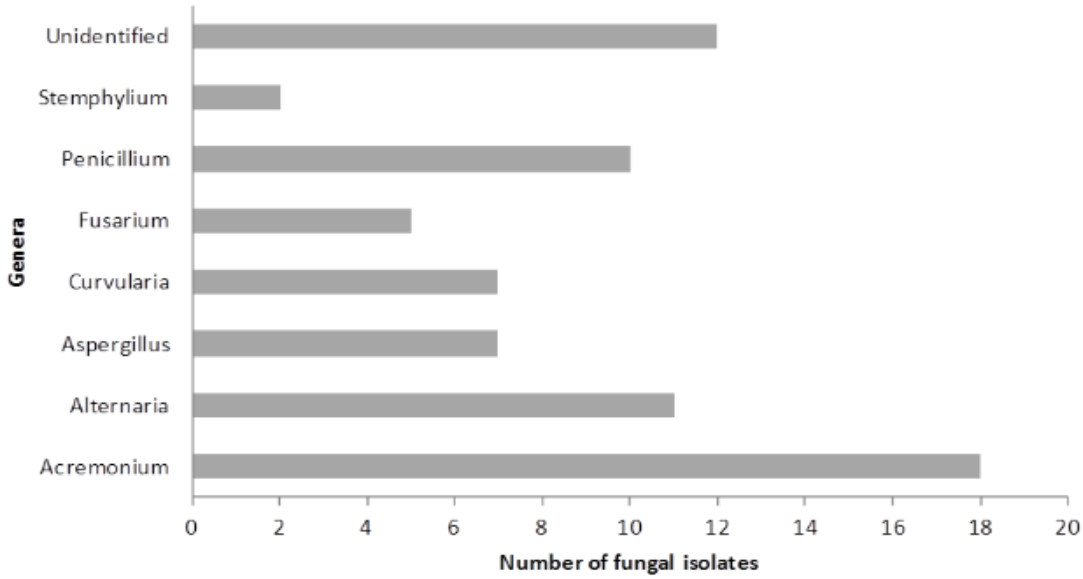

Figure 1. Number and diversity of fungal isolates.

As regards the pathogens, *A. alternata* was isolated only from *P. dilatatum*, while the other isolates of the genus *Alternaria* were all isolated from *B. catharticus*. All the fungi belonging to the genus *Curvularia* were isolated from *P. dilatatum*, except for *Curvularia* sp.1 (034) which was obtained from *B. biebersteinii* **(Table 2)**. Several isolates identified as *Fusarium* sp.2 were obtained from *B. catharticus, B. bierbersteinii and P. dilatatum* in both sampling areas. Meanwhile, *Fusarium* sp.1 was isolated from *B. catharticus*. The isolates classified as *Aspergillus* were found in the four plant species and in both sampling sites **(Table 2)**. Although *Penicillium* and *Stemphylium* are considered pathogens of several plant species [41,42], they were not considered pathogens in this study since they were obtained from plants without symptoms of disease.

A morphological characterization of the 72 isolates was carried out. Microscopically, 18 of the 72 isolates showed septate hyphae giving rise to thin, tapered, mostly lateral phialides produced singly or in small groups. Unicellular conidia were produced in mucoid heads or unconnected chains and the hyphae were usually hyaline. Macroscopically, colony reverse showed variation in the intensity of the yellow or orange pigmentation among isolates. Considering all the characteristics mentioned above, these 18 isolates were all classified as *Acremonium spp.* **(Table 2)** [43].

Table 2. Morphological characterized fungal isolates listed by sampling zone and by host grass species.

Fungal Name[a]	Sampling zone 1 (Zavalla)	Sampling zone 2 (Funes)	Sampling date	Plant tissue	Host grass species	Host plant phenotype
Acremonium sp.1 (030)	X		05-03-2012	stem	*Paspalum notatum*	Healthy
Acremonium sp.1 (032)	X		05-03-2012	stem	*Bromus biebersteinii*	Healthy
Acremonium sp.1 (057)		X	14-09-2012	stem	*Bromus catharticus*	Healthy
Acremonium sp.2 aff. *killiense* (064)	X		05-03-2012	leaf	*Paspalum dilatatum*	Healthy
Acremonium sp.3 (033)		X	14-09-2012	seeds	*Bromus catharticus*	Healthy
Acremonium sp.4 (040)	X		05-03-2012	stem	*Paspalum dilatatum*	Healthy
Acremonium sp.4 (051)		X	14-09-2012	seeds	*Bromus catharticus*	Healthy
Acremonium sp.5 (053)	X		05-03-2012	leaf	*Bromus biebersteinii*	Healthy
Acremonium sp.5 (056)	X		14-09-2012	leaf	*Bromus catharticus*	Healthy
Acremonium sp.5 (058)		X	14-09-2012	seeds	*Bromus catharticus*	Healthy
Acremonium sp.6 (054)	X		05-03-2012	leaf	*Paspalum dilatatum*	Healthy
Acremonium sp.6 (072)	X		03-12-2012	stem	*Paspalum dilatatum*	Healthy
Acremonium sp.6 (073)		X	14-09-2012	seeds	*Bromus catharticus*	Healthy
Acremonium sp.7 (050, 055)		X	14-09-2012	seeds	*Bromus catharticus*	Healthy
Acremonium sp.7 (074)		X	14-09-2012	seeds	*Bromus catharticus*	Healthy
Acremonium strictum (039)	X		13-04-2012	stem	*Paspalum notatum*	Healthy
Acremonium strictum (052)		X	14-09-2012	seeds	*Bromus catharticus*	Healthy
Alternaria alternata (046)	X		05-03-2012	leaf	*Paspalum dilatatum*	Symptoms of disease
Alternaria raphani (001)		X	14-09-2012	leaf	*Bromus catharticus*	Healthy
Alternaria brassicola (010)		X	14-09-2012	leaf	*Bromus catharticus*	Healthy

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Alternaria brassicola (019)		X	14-09-2012	leaf	*Bromus catharticus*	Healthy
Alternaria sp.1 (013, 014, 031, 035)		X	14-09-2012	leaf	*Bromus catharticus*	Symptoms of disease
Alternaria sp.2 aff. *cheiranthi* (018)		X	14-09-2012	leaf	*Bromus catharticus*	Healthy
Alternaria sp.3 (027, 067)		X	14-09-2012	leaf	*Bromus catharticus*	Healthy
Aspergillus niger (024)	X		05-03-2012	leaf	*Paspalum dilatatum*	Symptoms of disease
Aspergillus niger (063)	X		13-04-2012	leaf	*Bromus biebersteinii*	Symptoms of disease
Aspergillus sp.1 (015, 021)	X		05-03-2012	leaf	*Paspalum dilatatum*	Symptoms of disease
Aspergillus sp.1 (061)		X	14-09-2012	stem	*Bromus catharticus*	Symptoms of disease
Aspergillus sp.2 aff. *flavus* (060)	X		05-03-2012	leaf	*Paspalum notatum*	Healthy
Aspergillus sp.3 (070)		X	14-09-2012	stem	*Bromus catharticus*	Healthy
Curvularia sp.1 (029)	X		05-03-2012	leaf	*Paspalum dilatatum*	Healthy
Curvularia sp.1 (034)	X		13-04-2012	leaf	*Bromus biebersteinii*	Healthy
Curvularia pallescens (037, 041, 044)	X		05-03-2012	leaf	*Paspalum dilatatum*	Symptoms of disease
Curvularia sp.2 aff. *eragrostidis* (042)	X		05-03-2012	leaf	*Paspalum dilatatum*	Healthy
Curvularia clavata (048)	X		05-03-2012	leaf	*Paspalum dilatatum*	Symptoms of disease
Fusarium sp.1 (043, 049)		X	14-09-2012	leaf	*Bromus catharticus*	Symptoms of disease
Fusarium sp.2 (023)	X		05-03-2012	leaf	*Paspalum dilatatum*	Symptoms of disease
Fusarium sp.2 (059)	X		13-04-2012	leaf	*Bromus biebersteinii*	Symptoms of disease
Fusarium sp.2 (062)		X	14-09-2012	leaf	*Bromus catharticus*	Symptoms of disease
Penicillium sp.1 (002)		X	14-09-2012	leaf	*Bromus catharticus*	Healthy
Penicillium sp.2 (003)		X	14-09-2012	leaf	*Bromus catharticus*	Healthy
Penicillium sp.2 (065)		X	14-09-2012	stem	*Bromus catharticus*	Healthy
Penicillium sp.3 (005, 007)		X	14-09-2012	stem	*Bromus catharticus*	Healthy
Penicillium sp.4 (008, 011)	X		03-12-2012	leaf	*Paspalum dilatatum*	Healthy
Penicillium sp.4 (016, 068, 069)		X	14-09-2012	stem	*Bromus catharticus*	Healthy
Stemphylium sp.1 (012)	X		05-03-2012	stem	*Paspalum dilatatum*	Healthy
Stemphylium sp.1 (028)	X		03-12-2012	stem	*Paspalum notatum*	Healthy
Mycelia sterilia -not identified (004)		X	14-09-2012	seeds	*Bromus catharticus*	Healthy
Mycelia sterilia -not identified (006)		X	14-09-2012	seeds	*Bromus catharticus*	Healthy
Mycelia sterilia -not identified (017)		X	14-09-2012	leaf	*Bromus catharticus*	Healthy
Mycelia sterilia -not identified (020)		X	14-09-2012	seeds	*Bromus catharticus*	Healthy
Mycelia sterilia -not identified (025)		X	14-09-2012	leaf	*Bromus catharticus*	Healthy
Mycelia sterilia -not identified (026)		X	14-09-2012	stem	*Bromus catharticus*	Healthy
Mycelia sterilia -not identified (036)	X		03-12-2012	leaf	*Paspalum dilatatum*	Healthy
Mycelia sterilia -not identified (038)	X		03-12-2012	leaf	*Paspalum dilatatum*	Symptoms of disease
Mycelia sterilia -not identified (045)	X		05-03-2012	leaf	*Paspalum dilatatum*	Healthy
Mycelia sterilia -not identified (047)	X		05-03-2012	leaf	*Paspalum dilatatum*	Healthy
Mycelia sterilia -not identified (066)	X		05-03-2012	leaf	*Paspalum dilatatum*	Healthy
Mycelia sterilia -not identified (071)		X	14-09-2012	leaf	*Bromus catharticus*	Healthy

[a]Numbers represent isolate code

Additionally, ten isolates grew fast forming colonies in shades of green, sometimes white, mostly consisting of a dense felt of conidiophores. Microscopically, chains of single-celled conidia (ameroconidia) were observed in basipetal succession from a specialized conidiogenous cell called a phialide. Phialides could be observed singly or in groups, a pattern typical of *Penicillium* [25]. Finally, two isolates (012 and 028) grew fast forming velvety to cottony colonies in colors gray, brown, or brownish-black and black reverse. Microscopically, they presented septate hyphae, conidiophores, and conidia. The hyphae were pale brown to brown in color. Conidiophores were dematiaceous (simple or branched), with a number of vesicular swellings or nodes. These isolates where classified as *Stemphylium* [44].

Phylogenetic Analyses

To confirm the taxonomic position of these fungi, phylogenetic analyses were performed. The rRNA ITS (ITS1-5.8S-ITS2) and *elongation factor 1 alpha* (*TEF1*) sequences obtained from eight of the *Acremonium* isolates and GenBank (www.ncbi.nlm.nih. gov/) sequences belonging to 22 genera (*Acremonium, Calonectria, Claviceps, Cordyceps, Cylindrium, Dussiella, Echinodothis, Emericellopsis, Fusarium, Gliomastix, Hypocrea, Hypocrella, Ilyonectria, Metacordyceps, Metarhizium, Microcera, Myrothecium, Mycoarachis, Moelleriella, Sarocladium, Simplicillium and Trichodema)* were compared. The PHYLOGENY platform allowed the analysis of the data in a simple and robust way, using maximum-likelihood (ML) tree construction, which is commonly recognized as one of the most accurate approaches in molecular phylogenetics [33].

The topology of the tree constructed using ITS sequences showed 7 of the 8 isolates as being related to *Simplicillium* **(Figure 2A)**. *Simplicillium lanosoniveum* and *S. obclavatum* are considered synonyms of *Acremonium lanosoniveum* and *A.*

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obclavatum, respectively (www.mycobank.org). On the other hand, isolate 40 showed no clustering pattern to the rest of the groups included in this tree. Moreover, the tree constructed using *TEF1* sequences allowed to confirmed the classification of three isolates (058, 064 and 073) as *Simplicillium* **(Figure 2B)**. The other 5 isolates were not included in this analysis because the PCR reactions rendered no amplification products using *TEF1*-specific primers.

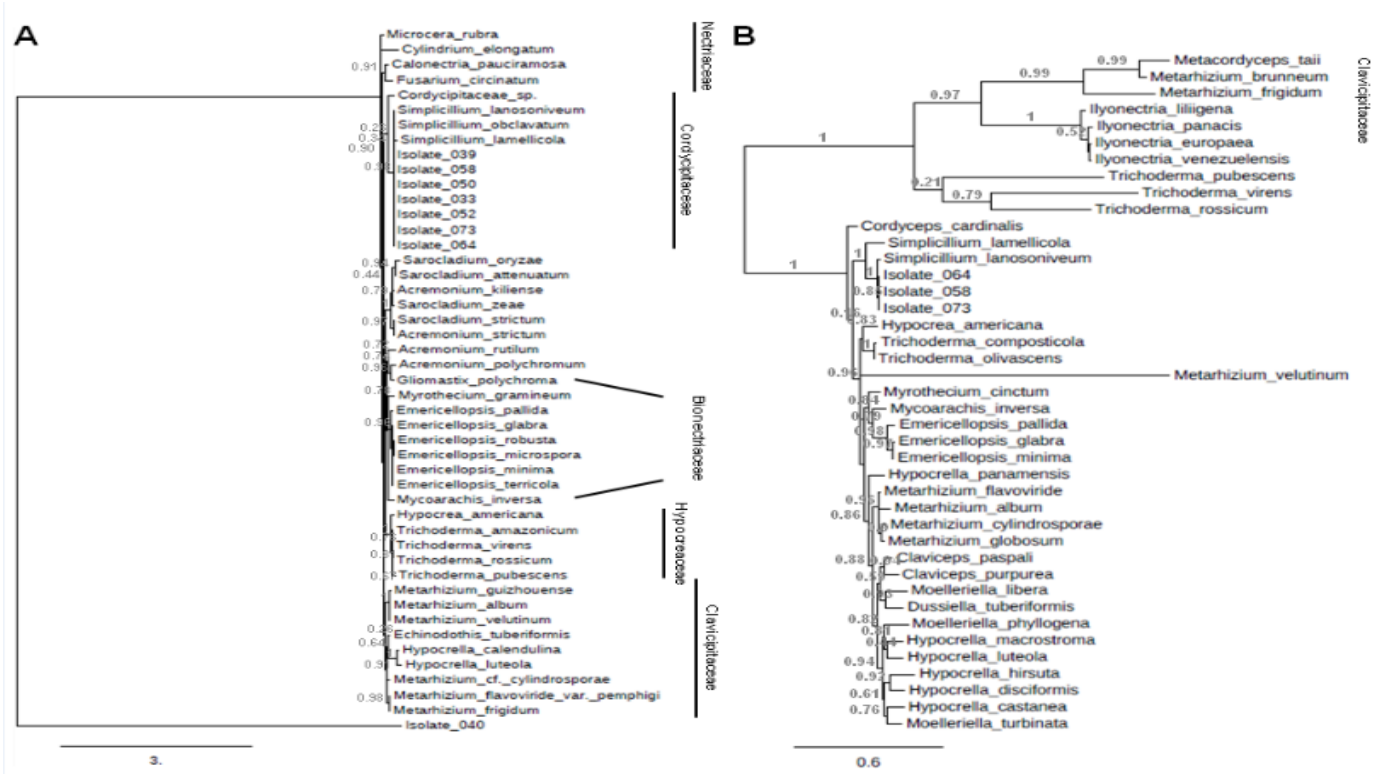

Figure 2. Phylogenetic analyses. (A) Tree obtained from the ITS (ITS1–5.8S–ITS2) sequence alignment of 8 isolates and 30 sequences from the National Center for Biotechnology Information (NCBI), (B) Tree obtained from the *TEF1* sequence alignment of 3 isolates and 38 sequences
from NCBI.

Antagonistic Activity of Fungal Endophytes

The percentage of growth inhibition (PGI) and pathogen growth inhibition category (GIC) are presented in **Table 3**. Endophytes of the genus *Acremonium* showed different antagonistic activity against the pathogenic species tested. Isolate 033 had the highest antagonistic activity against *A. alternata* **(Figure 3A)** and *Alternaria* sp., with a PGI of 91.42% and 91.35%, respectively, and both with a GIC of 4 **(Table 3)**. Isolates 040 and 052 **(Figure 3B)** also had a GIC of 4 (with PGI of 77.14% and 76.18%, respectively) for *A. alternata*, whereas isolates 050 and 064 showed the lowest percentage of inhibition (with PGI of 44.76% and 39.04%, respectively) and a GIC of 2. Antagonistic effect of isolate 064 against *Alternaria* sp. had the lowest value of PGI (4.93%) and a GIC of 1 **(Table 3)**.

For the pathogenic species *C. clavata*, the greatest antagonistic effect in the dual culture was obtained against isolate 050 with a PGI of 63.20%, followed by isolate 033, with a PGI of 55.43% **(Figure 3C)**. In the other dual confrontation against *C. clavata*, the percent of inhibition was similar (44.43-49.86%), with a GIC of 2 **(Table 3)**. For *C. pallescens*, the highest PGI (83.80%) in the dual culture was obtained versus isolate 050 **(Figure 3D)**, followed by a PGI of 76.18% for isolates 033 and 073. The lowest PGI (28.56%) was evidenced in the dual confrontation with isolate 064. Finally, *Fusarium* sp. was the pathogenic species that showed the lowest inhibition in the dual confrontation, with GIC values ranging between 1 and 2. The highest PGI (35.13%) was obtained with isolate 064.

In general terms, isolate 064 showed the lowest inhibition against the pathogenic species tested, except for *Fusarium*. On the other hand, isolate 033 exhibited the highest values of inhibition **(Table 3)**. As regards the zones of inhibition observed in the dual cultures, isolate 040 versus *A. alternata* showed a strong inhibition zone (15-18 mm) **(Table 4)**. Similarly, the inhibition zone against *Alternaria* sp. was of 17-20 mm **(Table 4 and Figure 3E)**. Inhibition zones from moderate to weak were observed in the dual cultures of isolate 050 against *C. pallescens* **(Table 4)**, and no inhibitory halo was evidenced **(Figure 3D)**. In the dual cultures against *Fusarium* sp., no inhibition halo was observed after 14 days of incubation with any of the isolates tested as antagonistic **(Table 4)**.

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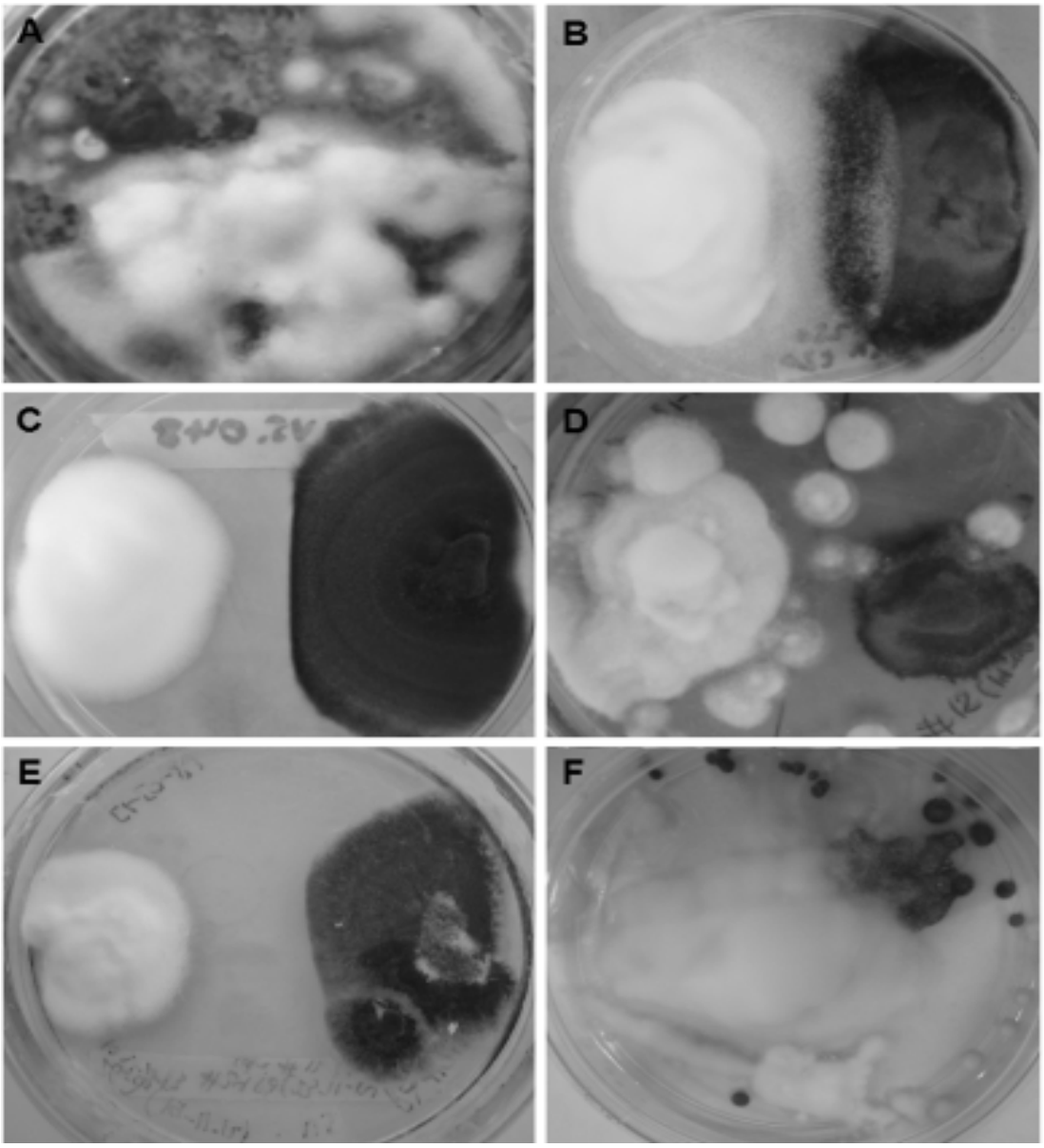

Figure 3. Dual cultures grown for 3 weeks at 28ºC. (A) Colony of *Acremonium* sp.3 (033) covering completely the colony of *A. alternata* (046) 14 days after inoculation, (B) Dual culture between *Acremonium* sp.8 (052) and *A. alternata* (046), (C) Inhibition zone of *Acremonium* sp.3 (033) against *C. clavata* (048), (D) Colonies of *Acremonium* sp.7 (050) growing over a colony of *C. pallescens* (044), (E) Inhibition zone of *Acremonium*

sp.4 (040) against *Alternaria* sp.1 (035), (F) Growth of *Acremonium* sp.6 (073) over the colony of *A. alternata* (046).

Table 3. Antagonistic activity of fungal endophytes.

Antagonists	Pathogen									
	***A. alternata* (046)**		***Alternaria* sp.1 (035)**		***C. clavata* (048)**		***C. pallescens* (044)**		***Fusarium* sp.1 (049)**	
	PGI[1]	**GIC[2]**	**PGI[1]**	**GIC[2]**	**PGI[1]**	**GIC[2]**	**PGI[1]**	**GIC[2]**	**PGI[1]**	**GIC[2]**
Acremonium sp.3 (033)	91.4[a]	4	91.3[a]	4	55.4[b]	3	76.1[c]	4	6.3[d]	1
Acremonium sp.4 (040)	77.1[a]	4	86.4[a]	4	48.6[b]	2	50.4[b]	2	12.6[c]	1
Acremonium sp.7 (050)	44.7[a]	2	23.4[b]	1	63.2[c]	3	83.8[d]	4	17.1[b]	1
A. strictum (052)	76.1[a]	4	34.5[b]	2	44.4[b]	2	36.1[b]	2	9.9[c]	1
Acremonium sp.2 aff. *kiliense* (064)	39.0[ac]	2	4.9[b]	1	46.6[a]	2	28.5[c]	2	35.1a[c]	2
Acremonium sp.6 (073)	71.4[a]	3	70.2[a]	3	49.8[b]	2	76.1[a]	4	11.7[c]	1

Note: [1]Data presented correspond to the mean PGI (percentage of growth inhibition) values calculated using Skidmore and Dickinson´s formula (1976). [2]GIC (growth inhibition category) of pathogen were as follows: 0= no inhibition; 1= very weak inhibition; 2= weak inhibition; 3= moderate inhibition; 4= strong inhibition. Different letters within the same row correspond to significant differences among pathogens (α = 0.05, SMD = 13.0%, Tukey's test)

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Table 4. Inhibiton zones (IZ)[a] of the fungal antagonists.

Antagonists	Pathogens				
	A. alternata (046)	*Alternaria* sp.1 (035)	*C. clavata* (048)	*C. pallescens* (044)	*Fusarium* sp.1 (049)
Acremonium sp.3 (033)	+	-	+++	+++	-
Acremonnium sp.4 (040)	++++	++++	+	+	-
Acremonium sp.7 (050)	+++	++	+	-	-
Acremonium sp.8 (052)	+	++	++	+	-
Acremonium sp.2 aff. *killiense* (064)	+	-	+	+	-
Acremonium sp.6 (073)	+++	+++	++	++	-

[a]IZ: No inhibition zone= -; very weak= +, 0-5 mm; weak= ++, 5-10 mm; moderate= +++, 10-15 mm; strong= ++++, 15-20 mm; very strong= +++++, > 2 mm.

DISCUSSION

Dominant endophyte taxa commonly found in grasslands differ depending on the region under study. The results obtained by Stone *et al.* [45-49] showed that the dominant endophyte taxa found in temperate grasses places are *Alternaria, Acremonium, Cladosporium, Penicillium, Epicoccum* and *Aureobasidium* spp. These studies suggest that endophytic species of temperate and tropical grasses appear to have low host specificity, since they can be found in many grass species. Endophyte mycobiota associated with a particular pasture usually consists of a relatively large number of fungal species [49]. Plant anatomical structure, leaf size and chemical components of grass species are important factors to consider that may affect diversity of fungi found in them [49,50].

In this work, the highest percentage of endophyte infection (55%) was observed in plants of *B. catharticus* collected from Funes. This may be due in part to differences in crop management and the characteristics of the plant species present in both sampling areas. Sampling area in Funes belonged to natural grassland between horticultural crops under organic management, while in Zavalla it was a seed implanted pasture under conventional management. Since seeds generally have a prior antifungal treatment, the transmission of endophytes in this case would occur by the spores that persist in the soil which colonize the hosts [51]. On the other hand, *P. dilatatum* species from Funes area showed 33% of endophyte infection.

In the present study the morphological analysis detected the genus *Acremonium* associated to cosmopolitan temperate grass species of the genera *Paspalum* and *Bromus*. This finding would contribute to the list of endophytes from the order *Hypocreales* in temperate areas, since to our knowledge, there are no reports related to the isolation of *Acremonium* species associated with these forage species. However, some of these isolates were classified as *Simplicillium* based on molecular analyses. Species belonging to this genus have been isolated from soil, plant tissues, fungi, nematodes and human nails [52]. Most species of this genus do not appear to be plant pathogens and some of them have been reported to be mycoparasites. Particularly, *S. lamellicola* BCP effectively suppressed various plant diseases caused by pathogenic fungi, including *B. cinerea*, and was commercialized as the microbial fungicide Acre [53]. It is worth noting that this strain, which was isolated from the mycelia of *B. cinerea*, was previously known as *Acremonium strictum* BCP [54]. Interestingly, two of the fungal isolates described in this work (039 and 052) were classified as *A. strictum*.

In the dual cultures of *Acremonium* sp. against *Alternaria* sp. and *A. alternata*, the largest antagonistic effect was observed with *Acremonium* sp.3 (033), though a similar result was obtained with *Acremonium* sp.4 (040). The percent of inhibition of both antagonistic strains of *Acremonium* allowed their classification in the highest category for inhibiting pathogen mycelial growth (GIC) **(Table 3)**. However, it was interesting to observe the inhibition zones in the dual cultures against the pathogenic strains of *Alternaria* sp. and *A. alternata*, since both antagonistic strains showed a different behavior when faced to these pathogens. A strong inhibition zone was observed in the dual culture with *Acremonium* sp.4 (040) with both pathogens, while there was a very weak zone of inhibition with *Acremonium* sp.3 (033**) (Table 4)**. These results suggest that the mechanism of action of both antagonistic strains may differ despite they presented a similar antagonistic effect. *Mycoparasitism* and production of secondary metabolites or hydrolytic enzymes are both feasible mechanisms operating in the antagonic action. In the case of *Acremonium* sp.3 (033) *mycoparasitism* seems to be involved in the antagonism [55], since this strain grew on *Alternaria* sp. and *A. alternata*, covering the entire medium in the Petri dish with its mycelium, and thus humpering the pathogenic strains growth **(Figure 3A)**. For that observed in dual culture with *Acremonium* sp.4 (040), production of secondary metabolites by the strain, which would constrain the development of the pathogenic fungus, seems to be the more feasible mechanism of antagonism **(Figure 3E)**. Competition for nutrients could be also possible [56,57].

Acremonium sp.7 (050) exhibited increased antagonistic activity against *C. pallescens* when compared to strains of *Alternaria* **(Table 3)**. In the dual culture with *C. pallescens*, the mechanism of antagonism of *Acremonium* sp.7 (050) seems to be *mycoparasitism*, since *C. pallescens* spores were surrounded by large number of spores of the antagonist, which were larger and some of them were hampering the germ pores (data not shown). *Acremonium* sp.7 (050) hyphae invading pathogen fungal structures were also observed. In the dual cultures with *Curvularia* sp. a moderate inhibition zone was observed, suggesting that the production of secondary metabolites could be the mechanism of the antagonism **(Table 4 and Figure 3D)**.

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These results suggest that the interactions and mechanisms of antagonism of the same antagonistic strain vary according to the pathogenic strains to which they are faced to in the dual cultures. Moreover, the analysis of variance showed that there is an interaction between the antagonistic and the pathogens, indicating that the inhibition of the pathogens depends on the antagonistic species. From the above mentioned, we hypothesized that pathogenic strains could modulate or influence in the behavior of the antagonist and vice versa. The similar values of PGI showed by the same antagonistic strain against different pathogenic strains, though to the differences observed between the zones of inhibition, appear to support this hypothesis **(Figure 3 and Table 3)**.

The antagonistic strains tested showed no activity against *Fusarium* sp.1 **(Tables 3 and 4)**. It also had a higher rate of growth in the dual culture and no presence of inhibitory halo was observed (data not shown). *Acremonium* sp.2 aff. *killiense* (064) which evidenced the lowest percentages of inhibition against *Alternaria* and *Curvularia* was the only strain capable of exerting a weak antagonistic effect on the growth of *Fusarium* sp.1 **(Table 3)**.

CONCLUSION

It is important to note that *in vitro* results are not necessarily indicative of what occurs in the plant. However, the *in vitro* study of antagonists-pathogens is a practical approach in order to identify good candidates for use in biological control. The *in vitro* studies also allow understanding the possible mechanisms by which the candidate strains reduce the damage caused by pathogens. The finding of endophytes with antagonist effects could be translated to useful applications to improve the performance of plants, as an alternative to the use of chemicals to control pests.

Finally, based on the results obtained in this study, we propose that the isolates of the *Acremonium* endophyte are good candidates for biological control of latent pathogenic species in pastures as *A. alternata, Alternaria* sp*., C. clavata* and *C. pallescens*. However, since the antibiotic effect varied in a species-specific way, further research will be necessary to complement these results. Specifically, studies focused on the analysis of the secondary metabolites produced by the antagonists in the culture medium, as well as studies *in planta*, will contribute to elucidate the antagonistic effect of the strains tested.

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The authors declare that they have no conflict of interest.

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