Fungal endophytes of *Panicum maximum* and *Pennisetum purpureum*: isolation, identification, and determination of antifungal potential

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ABSTRACT

This study aimed to isolate and identify endophytic fungi from the forage grass *P. maximum* and evaluate their ability to inhibit the growth of plant pathogenic fungi. One sample from *P. purpureum* grass was also included. Surface disinfected stem fragments were used for endophytic fungal isolation. One hundred and twenty-six endophytic fungi were isolated, of which 118 were from *P. maximum* and eight from *P. purpureum*. Morphological characteristics and internal transcribed spacer (ITS) and 18S (NS) sequence comparisons identified most isolated endophytic fungi as belonging to the phylum Ascomycota, with *Sarocladium* being the dominant genus. The isolates were subjected to *in vitro* antagonism tests against pathogenic fungi, and 31 endophytic fungi inhibited the growth of *Bipolaris maydis*, *Penicillium expansum*, and *Sclerotinia minor*. The results expand our knowledge of the diversity of endophytes associated with tropical grasses and suggest that they may represent new sources of antifungal metabolites for biocontrol and biotechnological purposes.

Key Words: antagonism; grasses; molecular identification; plant pathogens; rDNA

INTRODUCTION

In Brazil, forage grasses are the main source of animal feed for dairy and beef cattle (Lima et al., 2010). *P. maximum* and *P. purpureum* are two grass species originating from tropical Africa (Ndemah et al., 2000, Dinardo et al., 2003, Braz et al., 2006). These are the main forage grasses used for pasture formation in Central and South America, since they have high nutritional value and high dry matter production potential (Dias and Alves, 2008, Lédo et al., 2008, Lima et al., 2010, Queiróz et al., 2014). *Panicum maximum* is well diffused among livestock farmers, being considered one of the most productive in the Brazilian livestock industry (Jank et al., 2008; 2011). *Pennisetum*

purpureum is important as forage for silage because of its high growth rate and biomass production per area, with great efforts, therefore, being devoted to determining its palatability and nutritional values as an alternative forage crop ([Wang et al., 2002](#)).

Plants are constantly involved in interactions with a wide range of microbial populations. Endophytic fungi inhabit plant organs at some stage in their life cycle and colonize internal tissues of plants without causing apparent damage to their host ([Petrini, 1991](#)). Endophytes constitute a valuable source of bioactive secondary metabolites ([Naik et al., 2009](#)), which may protect their host against pathogen, insect, or animal attacks, or even have direct or indirect effects on plant growth ([Porras-Alfaro and Bayman, 2011](#)). There have been very few studies on warm season grasses and the endophytes associated with them ([Ghimire et al., 2011](#)). In this study, we surveyed the endophytic fungi naturally occurring in stems of *P. maximum* and *P. purpureum,* collected from three different locations in Brazil, and evaluated their ability to inhibit the growth of plant pathogenic fungi. To test such possible outcome, antagonism bioassays (*in vitro* tests with two or more organisms placed against each other in contact or not) were set. The inhibition, in the context of this study, is the ability of one organism to partially or totally diminish the growth of the other.

MATERIAL AND METHODS

Plant samples of eleven cultivars of *P. maximum* and one of *P. purpureum* were collected in August and September 2012, and January 2013. Sampling sites included three experimental fields located in the Brazilian states of Mato Grosso do Sul and Minas Gerais ([Table 1](#)). Healthy tillers were collected from each plant at 10-15 cm above the soil and transported to a laboratory in plastic bags for fungal isolation.

Table 1 Origin of samples of *P. maximum* and *P. purpureum* cultivars

Location	Sample/Cultivar
Embrapa Beef Cattle,	*P. maximum* cv. Mombaça
Campo Grande - MS	*P. maximum* cv. Tanzânia
	P. maximum cv. Massai
	P. maximum cv. Aruana
	P. maximum cv. Gatton
	P. maximum cv. BRS Zuri
	P. maximum cv. Milênio
	P. maximum cv. Colonião
	P. maximum cv. Tobiatã
Universidade Federal de Lavras	*P. maximum* cv. Mombaça
(UFLA)/Lavras - MG	*P. maximum* cv. Vencedor
	P. maximum cv. Tanzânia
	P. maximum cv. Colonião
	P. maximum cv. Makueni
	P. maximum cv. Massai
Embrapa Dairy Cattle,	*P. maximum* cv. Tanzânia
Juiz de Fora - MG	*P. maximum* cv. Mombaça
	P. purpureum cv. Mott

Tillers were washed under running tap water and cut into 5-7-cm fragments. Surface disinfection was done by treating the stem fragments thrice with sterile water for 1 min, 96% ethanol for 3 min, 5% sodium hypochlorite for 3 min, and sterile water three times for 1 min each. Approximately 100 µL of the last water wash was plated on PDA and incubated, as a sterilization control, followed by drying

on sterile filter paper. Disinfected tissues were cut into 0.5-cm fragments and plated on PDA plates containing cefotaxime (250 µg.mL^{-1}). Following an incubation at 25 °C, plates were checked regularly and fungal colonies emerging from the margins of sectioned tissues were subcultured onto PDA. Purified isolates were stored long-term in sterile microtubes containing sterile water, and kept at 4 °C.

Initial grouping of fungal isolates into morphotypes, and their identification to the genus level were based on colony appearance, mycelium color, and structures of conidiomata, conidiophore, and conidia (size, color, shape, ornamentation, etc.). Cultivation of non-sporulating cultures on malt-extract-agar (MEA) was done to promote sporulation ([Guo et al., 1998]).

Sequencing of the internal transcribed spacer (ITS) and part of the 18S (NS) region of the rDNA was carried out for all isolates to support morphological identification and to identify non-sporulating fungi. Genomic DNA from pure cultures was extracted from the mycelial mat using a Mobio UltraClean$^{®}$ Microbial kit. Amplifications of ITS and 18S (NS) were carried out in 30 µL reactions containing 15 µL Qiagen *Taq* PCR Master Mix kit, 12 µL of H$_2$O, 1 µL of each primer (10 pmol), and 1 µL of genomic DNA at 10 ng/µL. The internal transcribed spacer was amplified using primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC), with the reaction conditions as: 2 min at 95 °C, followed by 35 cycles of 1 min denaturation at 95 °C, 1 min of primer annealing at 50 °C, 1 min of extension at 72 °C, with a final elongation of 7 min at 72 °C. The NS was amplified using NS1 (GTAGTCATATGCTTGTCTC) and NS6 (GCATCACAGACCTGTTATTGCCTC) primers, and the reaction conditions were as follows: 1 min for initial denaturation at 94 °C, followed by 35 cycles of 35 s denaturation at 94 °C, 50 s of primer annealing at 55 °C, 2 min of extension at 72 °C, with a final elongation of 6 min at 72 °C. Amplifications were performed in a Programmable Thermal Controller-100, (MJ Research, Inc) thermocycler.

The PCR products were purified and sequenced by Macrogen. Consensus sequences were assembled using SeqAssem software and compared against the GenBank database through BLAST searches using the Mega 6 software ([Tamura et al., 2013]). The closest hit sequences were checked for their authenticity and used as references for molecular identification of endophytic isolates.

Isolates of seven plant pathogenic fungi, namely *B. maydis, Aspergillus ochraceus, P. expansum, S. minor, Fusarium verticillioides, Pyricularia oryzae*, and *Colletotrichum graminicola*, were used in the antagonism bioassays.

The endophytic fungi and plant pathogens were cultivated for seven days at 25 °C on PDA. Mycelial disks (5 mm) of each endophyte were transferred to one side of a PDA plate. After seven days of incubation, 5 mm mycelial disks of plant pathogens were inoculated onto opposite sides of the plates containing the endophytes. Control plates contained the pathogens inoculated without endophytes. After seven days of paired incubation at 25 °C, the percentage of growth inhibition of the pathogens was calculated in relation to the control ([Fávaro et al., 2012]). Tests were performed in triplicate.

The bioassay was also performed on Petri dishes divided into two partitions to verify if those endophytic fungi that inhibited the growth of phytopathogenic fungi in the first test were producing bioactive volatile compounds instead of, or in addition to, compounds secreted in the culture medium ([Strobel et al., 2011]).

RESULTS

We isolated 118 fungi from 11 *P. maximum* cultivars (17 samples) and eight isolates from *P. purpureum* (one sample). From samples collected in Campo Grande, 53 isolates were obtained, with 52 obtained from Lavras and 21 from Juiz de Fora.

Most isolates belonged to the Ascomycota, with only two members of the Basidiomycota (*Meira* sp. and *Sporisorium* sp.) being isolated from *P. maximum*. The most frequent species was *Sarocladium* sp. (65 isolates), followed by *Ramichloridium* sp. (13) (Figure 1). Seventeen other taxa were isolated in frequencies varying from one to five isolates (Figure 1). Four isolates could only be identified to the order level, Capnodiales and Hypocreales, while one ascomycete isolate was only identified to the phylum level. Five species were identified among the eight endophytes isolated from *P. purpureum*, *Sarocladium* sp. (four isolates), *Acremonium* sp., *Cladosporium* sp., *Paraconiothyrium* sp., and *Sporisorium* sp. (one isolate from each taxon).
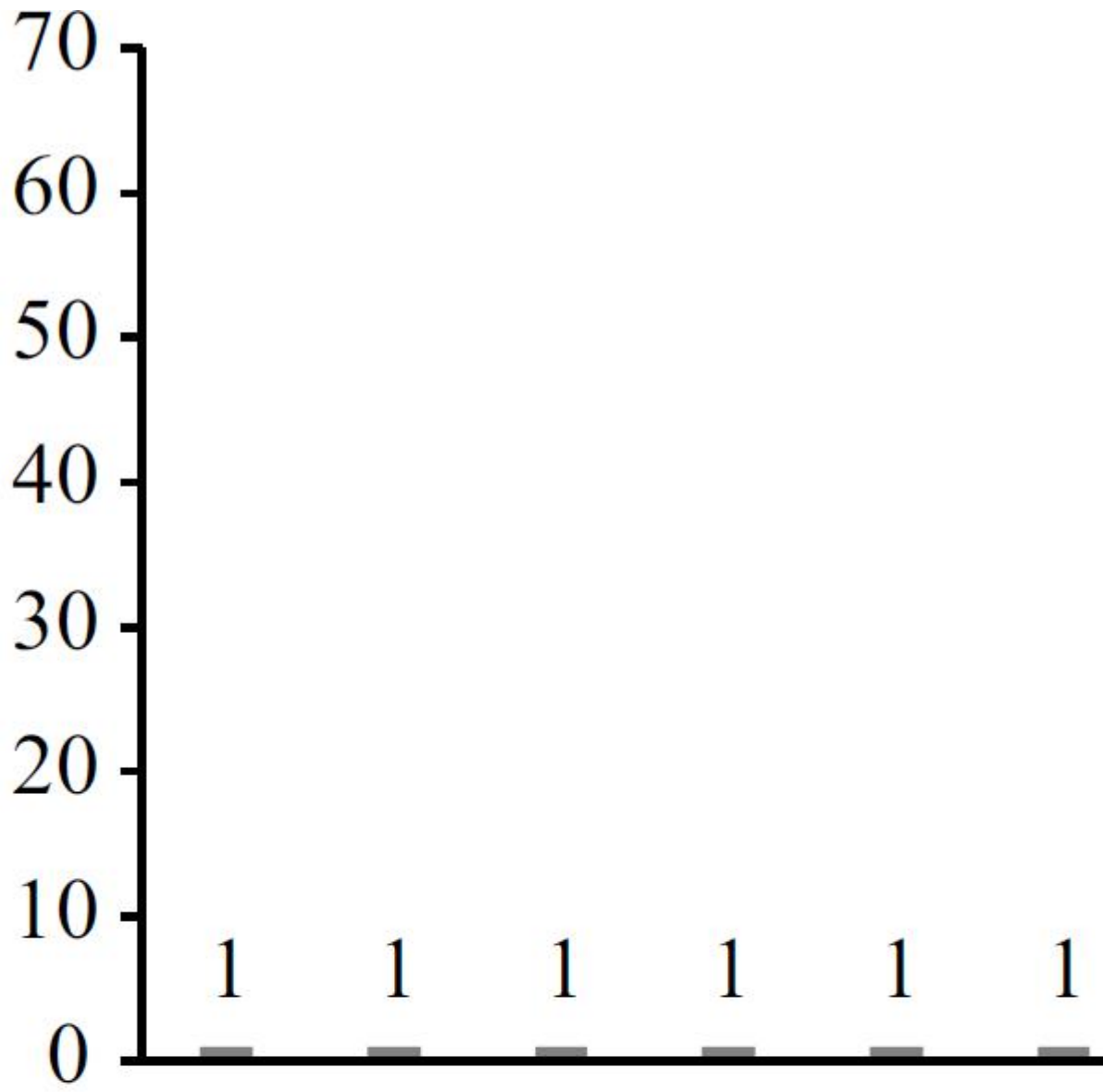
Figure 1 Number of isolates of endophytic fungi from *P. maximum.*

Thirty endophytic isolates from *P. maximum* and one from *P. purpureum* reduced the growth of at least one of the three of pathogenic fungi included in the antagonism bioassay, *B. maydis*, *P. expansum*, and *S. minor* ([Table 2](#)). The remaining tested plant pathogens were not inhibited by the endophytic fungi.

Table 2 Antagonism test of *P. maximum* and *P. purpureum* endophytes against some phytopathogens

Pathogenic fungi	Identification code	GenBank Number (ITS)	Endophytic fungi	Growth reduction (%)
B. maydis	CG-TZ01	-	*Amniculicola parva*	22.41
	LA-VC01	KP968424	*Sarocladium* sp.	27.59
	CG-TT01	KP968452	*Sarocladium strictum*	41.38
	LA-MB01	KP968387	*Sarocladium* sp.	44.82
	LA-MB02	KP968434	*Sarocladium terricola*	55.55
	CG-ML01	-	Unidentified Hypocreales	60.87
	CG-TT02	KP968468	*S. strictum*	60.87
P. expansum	CG-ML02	KP968444	*Ochroconis* sp.	29.41
S. minor	CG-ML03	-	*Ramichloridium* sp.	14.28
	JF-MB01	KP968376	*Sarocladium* sp.	16.66
	LA-MB03	KP968369	*Sarocladium* sp.	19.04
	JF-MT01	KP968390	*Sarocladium* sp.[1]	28.57
	CG-TB01	-	*Ramichloridium* sp.	30.36
	CG-TZ01	-	*Amniculicola parva*	33.77
	CG-ML04	KP968440	*Ochroconis* sp.	38.96
	LA-MB01	KP968387	*Sarocladium* sp.	41.56
	CG-ML01	-	Unidentified Hypocreales	42.86
	CG-ML05	KP968423	*Ramichloridium* sp.	43.21
	CG-ML06	KP968427	*Ramichloridium* sp.	46.75
	CG-ML07	KP968443	*Ramichloridium* sp.	46.75
	CG-ML08	KP968457	*Sarocladium* sp.	48.21
	LA-TZ01	KP968406	*Sarocladium* sp.	50.62
	LA-MB04	KP968385	*Sarocladium* sp.	53.25
	LA-MB05	KP968374	*Sarocladium* sp.	53.25
	CG-ML09	KP968476	*Ochroconis* sp.	53.57
	CG-TT03	KP968455	*Sarocladium* sp.	55.36
	LA-MB06	KP968375	*Sarocladium* sp.	57.14
	JF-TZ01	KP968389	*Sarocladium* sp.	59.74
	CG-ML10	KP968460	*Ramichloridium* sp.	64.29
	LA-VC01	KP968424	*Sarocladium* sp.	67.53
	CG-TT04	-	*Sarocladium* sp.	67.86
	CG-TT05	KP968454	*Sarocladium* sp.	67.86
	CG-TB02	KP968463	*Sarocladium* sp.	75.00
	CG-TT06	KP968451	*Sarocladium* sp.	80.36
	CG-TT07	KP968456	*Sarocladium* sp.	80.36

[1]Isolate from *Pennisetum purpureum.*

LA - Lavras, JF - Juiz de Fora, CG - Campo Grande; MB - Mombaça; VC - Vencedor; TZ - Tanzânia; TT - T65; ML - Milênio, TB - Tobiatã, MT - Mott.

Seven endophytic fungi inhibited the growth of *B. maydis*, including five isolates of *Sarocladium*, with values ranging from 22.41 to 60.87% of inhibition ([Table 2](#)). Twenty-seven out of the 31 tested isolates showed some antagonistic activity *in vitro* and inhibited the growth of *S. minor*. These isolates belonged to five different genera, with *Sarocladium* as the most common, reflecting the dominance of this

genus in our survey. Growth reduction of *S. minor* ranged from 14.28 to 80.36% ([Table 2](#)).

One isolate of *Ochroconis* sp. from *P. maximum* (Milênio cultivar) inhibited the growth of *P. expansum* by 29.41%. Two other *Ochroconis* isolates were active against *S. minor* ([Table 2](#)).

DISCUSSION

The profile of fungal endophytes from *P. maximum* and *P. purpureum* followed the common patterns found in studies of tropical endophytes, in which only few taxa are dominant, and most are rare. Another common finding is the predominance of ascomycetes over basidiomycetes ([Rodriguez et al., 2009](#), [Marquéz et al., 2012](#)).

Isolates of *Sarocladium* dominated the endophytic fungal assemblages in our present investigation. This genus was established in 1975 ([Gams and Hawksworth, 1975](#)); however, it was only recently delimited as a monophyletic group and shown to contain several species formerly classified in the morphogenus *Acremonium* ([Summerbell et al., 2011](#), [Giraldo et al., 2015](#)). These findings highlight the common association of *Sarocladium* species with plants in the Poaceae family, including the rice sheet rot pathogen *Sarocladium oryzae* and the putative plant-protective endophytes *Sarocladium zeae* (formerly *Acremonium zeae*) from maize ([Wicklow et al., 2008](#)) and *Sarocladium implicatum* (formerly *Acremonium implicatum*) from *Brachiaria brizantha* ([Kelemu et al., 2001](#)). Two *Sarocladium* species were recently described from collections of grass endophytes, *Sarocladium spinificis* from *Spinifex littoreus*, a grass found on the Taiwanese coast ([Yeh and Kirschner, 2014](#)), and *Sarocladium brachiariae*, from *B. brizantha* grass, collected in China ([Liu et al., 2017](#)).

Reflecting the dominance of *Sarocladium* sp. among the isolated endophytes, most isolates capable of inhibiting the growth of *B. maydis* and *S. minor* belong to this genus. Chemical characterization of the isolates was not performed in this study, but the production of bioactive secondary metabolites in culture medium is highly likely. Dozens of metabolites are known from *Sarocladium/Acremonium*, including the antibiotic helvolic acid and antifungal cerulenin, produced by *S. oryzae* ([Bills et al., 2004](#)), and antimicrobial pyrrocidines A and B, produced by *S. zeae* ([Wicklow et al., 2005](#)). The putative protective action of *S. zeae* on maize ears against *F. verticillioides* and *Aspergillus flavus* was assigned to pyrrocidines, while a crude organic extract of a strain of *S. oryzae*, enriched in cerulenin content, was capable of reducing the severity of rice blast disease, caused by *Pyricularia oryzae* ([Côrtes et al., 2014](#)).

Thirteen endophytic isolates were assigned to *Ramichloridium* sp., making it the second most abundant taxon isolated in this study. *Ramichloridium* is a heterogeneous group of fungi including species with different lifestyles, such as saprobes and plant and human pathogens ([Arzanlou et al., 2007](#)). Several reports of endophytic *Ramichloridium* species are known mainly from dicotyledonous hosts, including Australian pine (*Pinus nigra*) ([Jurc et al., 1996](#)), Camellia (*Camellia oleifera*) ([Zhou et al., 2013](#)), and Artemisia (*Artemisia annua*) ([Yuan et al., 2011](#)).

CONCLUSIONS

To our knowledge, this is the first work with endophytes in *P. maximum* and *P. purpureum* grasses in Brazil, and contributes to the knowledge of fungal species associated with forage grasses in the country. Our results demonstrate the antifungal activities of *Sarocladium* and *Ramichloridium* isolates, the most common endophytes retrieved in our survey. This work will be expanded with additional *in vitro* and *in vivo* tests and by searching for compounds that are biosynthesized by selected fungal endophytes with potentially useful applications in biotechnology.

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