DOI: http://dx.doi.org/10.1590/1678-992X-2018-0210

ISSN 1678-992X

SCIENTIA AGRICOLA

Research Article

Endophytic fungi from *Brachiaria grasses* in Brazil and preliminary screening of *Sclerotinia sclerotiorum* antagonists

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Edited by: Fernando Dini Andreote

Received June 27, 2018
Accepted October 29, 2018

ABSTRACT: Fungal endophytes of *Brachiaria*, a nonhost of *Sclerotinia sclerotiorum*, may harbor species with antagonistic effects against this plant pathogen. The objective of this work was to investigate the diversity of endophytic fungi associated with different *Brachiaria* species and hybrids and evaluate their potential to inhibit the plant pathogen *S. sclerotiorum*. Stem samples from 39 *Brachiaria* spp. plants were collected in pasture fields and experimental areas of three states of Brazil resulting in 74 endophytes isolated. Twenty-eight species were identified by sequences of the Internal Transcribed Spacer (ITS) and 18S rDNA regions. *Paraconiothyrium* sp. was the most abundant endophyte, accounting for 24 % (14 isolates) of total, and it was isolated from *B. ruziziensis*, *B. decumbens*, *B. humidicola*, and *B. brizantha*. *Phoma sorghina* was the second most abundant taxon, followed by *Sarocladium strictum*, and *Plenodomus* sp. *In vitro* analyses showed that *Paraconiothyrium* sp., *Sarocladium kiliense*, *Acremonium curvulum*, *Setophoma terrestris*, *Dissoconium* sp., and *Cladosporium flabelliforme* exhibited antagonistic activity against *S. sclerotiorum*, with percentages of growth inhibition ranging from 25 to 60 ($p < 0.05$). *Paraconiothyrium* sp. BBXE1 (60 %), BBPB4.1 (60 %), BCMT4.1 (54 %), and *S. kiliense* (54 %) showed the highest values of Antagonism Percentages (AP). Therefore, fungi with inhibitory activity against *S. sclerotiorum* such as *Paraconiothyrium* sp. are naturally endophytic in *Brachiaria* grasses.

Keywords: Coniothyrium-like fungi, *Urochloa*, tropical forage grasses, antagonism, white mold

Introduction

Grass pastures are widely distributed in different regions of Brazil, and the success of their establishment depends on the use of robust forage species. *Brachiaria* (syn. *Urochloa*) species are among the most important forages for cattle feeding in the country, reflecting their adequate adaptation to different conditions (Valle et al., 2009).

The beneficial association of some agricultural grass species from temperate regions with vertically transmitted Clavicipitaceous fungal endophytes is a well stablished phenomenon (Saikkonen et al., 2006). This mutualism may protect plants against the growth of herbivore insects through the balance between antagonistic signaling pathways and increased availability of nutrients (Saikkonen et al., 2006, 2013). This type of symbiosis is not commonly observed in tropical grasses (Sánchez Márquez et al., 2012). Nevertheless, a large diversity of horizontally transmitted endophytic fungi in Poaceae species used for cattle feeding in tropical regions deserves further investigation (Rodrigues and Dias-Filho, 1996).

Grasses can be used for mulching or rotation with dicots to reduce disease inoculum, since they do not share the same range of pathogens (Gasparotto et al., 1982; Görgen et al., 2009, 2010). The white mold is one of these diseases, caused by *Sclerotinia sclerotiorum*. This pathogen has a wide host range, but it mainly affects dicotyledonous hosts, including important crops such as soybean, bean and cotton, causing water-soaked lesions that expand rapidly killing a large portion of the aerial plant tissues, which commonly become covered by the white mycelium of the pathogen (Bolton et al., 2006).

Management of white mold in soybean crop may include rotation/mulching with nonhost Poaceae and the combination with biological control, using for example *Trichoderma harzianum* (Görgen et al., 2009, 2010), a fungus reported as endophytic in *Brachiaria* grasses (Rodrigues and Dias-Filho, 1996; Kago et al., 2016). Thus, a plausible hypothesis is that fungal endophytes of *Brachiaria*, a nonhost of *S. sclerotiorum* (Görgen et al., 2009), may harbor species with antagonistic effects against this plant pathogen. Therefore, we investigated the diversity of fungal endophytes associated with several *Brachiaria* species in Brazil and evaluated *in vitro* antagonism of the isolates against *S. sclerotiorum*.

Materials and Methods

Sampling *Brachiaria* plants

Thirty-nine disease-free plants of different species and hybrids of *Brachiaria* were sampled: *B. ruziziensis*; *B. decumbens* cv. Basilisk; *B. mutica* cv. Angola; hybrid cv. Mulato I and cv. Mulato II; *B. humidicola* common, cv. Llanero and cv. Tupi; *B. brizantha* cv. Piatã, cv. Xaraés, and cv. Marandu, in which 11 were collected from pasture fields and 28 from experimental plots. The samples were collected during the rainy and dry seasons from Aug 2012 to Oct 2013 in the following locations: five at the Universidade Federal de Lavras (Lavras-MG, Bra-

zil); seven at the Embrapa Amazônia Oriental (Belém-PA, Brazil); eight at the Embrapa Gado de Corte (Campo Grande-MS, Brazil); 19 at the Embrapa Gado de Leite (Juiz de Fora-MG, Brazil) (Figure 1). Plants in vegetative stage were harvested at 10 cm above the ground using pruning shears, placed in plastic bags and refrigerated for approximately 48 h until isolation (6 to 10 Apr). A cool box was used to transport samples to the laboratory.

Isolation of endophytic fungi from *Brachiaria* plants

Defoliated stem samples were washed under tap water, cut into 10 cm fragments and placed in 50 mL sterile Falcon tubes. Surface disinfestation was done by successive washes with sterile distilled water (1 min), 96 % ethanol (2 min), sterile distilled water (1 min), 5 % sodium hypochlorite (2 min), and a final wash three times in sterile distilled water (1 min). Stem pieces were dried on sterile filter paper and cut into smaller pieces (0.5 cm) using a sterile scalpel. Fifteen stem pieces per plant sample were seeded onto Petri dishes containing PDA medium (potato infusion-dextrose-agar) amended with cefotaxime (0.25 g L^{-1}) incubated at 25 °C. To assess the efficiency of disinfestation, aliquots of final rinse water of 100 µL were similarly seeded. Samples were examined daily and the endophytic fungi were individually transferred to new plates containing PDA/cefotaxime plates for purification. Isolates were stored with the Castellani's method (Castellani, 1939) at 4 °C in sterile tubes containing 1 mL of sterile distilled water.

ITS and 18S rDNA sequencing and molecular identification

The molecular identification of fungal isolates was performed using DNA sequences of ITS and 18S rDNA (White et al., 1990). Mycelia were scraped from colonies on PDA using a sterile toothpick and total DNA was extracted using the Microbial DNA Isolation Kit (MO BIO). Amplification reactions were performed in 30 µL reaction volumes containing 15 µL of kit, 12 µL of H_2O, 10 pmol of each forward and reverse primer, and 1 ng of DNA. For ITS amplification, primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′- TCCTCCGCTTATTGATATGC-3′) were used, and reaction conditions were: 95 °C for 2 min, followed by 35 cycles at 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. To amplify the 18S region, primers NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and NS6 (5′-GCATCACAGACCTGTTATTGCCTC-3′) were used. Reactions were performed in a thermocycler at 94 °C for 1 min, followed by 35 cycles at 94 °C for 35 s, 55 °C for 50 s, and 72 °C for 2 min, with a final extension at 72 °C for 6 min.

The amplification products were purified and sent to Macrogen (Seoul, South Korea) for Sanger sequencing. The sequences were edited using the SeqAssem 07/2008 software and then compared with sequences of reference strains deposited in international culture collections available in the GenBank database (National Center for Biotechnology Information - NCBI) (https://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST) and identification was performed according to the identities found.

Antifungal activity of isolated endophytic fungi against *Sclerotinia sclerotiorum*

The evaluation of antifungal activity was adapted from Kelemu et al. (2001). The pathogenic strain UFLA44 of *S. sclerotiorum* used in this work was collected from common bean field cultivar Ouro Vermelho by Abreu and Souza (2015) in the municipally of Coimbra, state of Minas Gerais, Brazil. A small mycelial

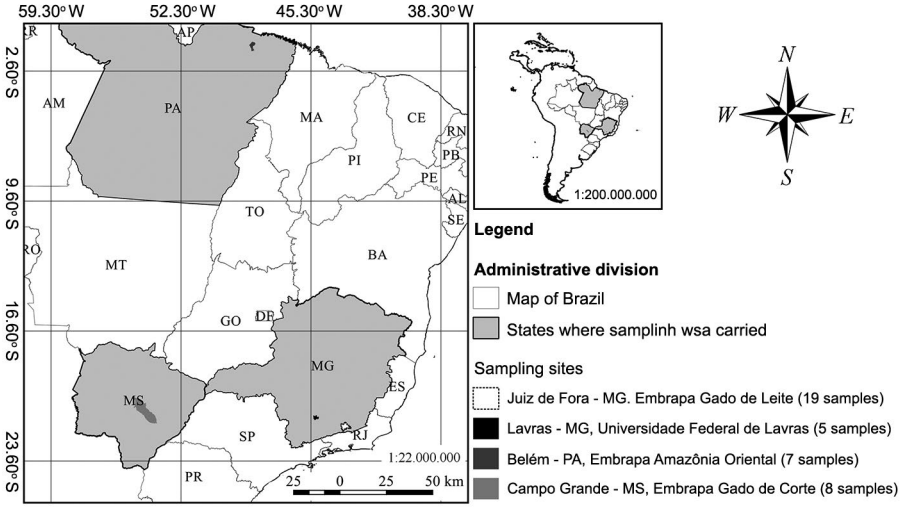

Figure 1 – Brazilian map showing the states and sites where plants were sampled. The map was created on the QGIS software version 2.18 using the Geographic Coordinate Reference System and Datum SIRGAS2000 from data of Instituto Brasileiro de Geografia e Estatística (IBGE).

fragment of the endophyte was placed on one side of a Petri dish (9 cm diameter) containing PDA and incubated for 7 d at 25 °C. Next, 5 mm colony fragment of *S. sclerotiorum* UFLA44 obtained from sclerotia germination was placed on the opposite side the plate containing the endophyte (Figures 4A, B), where the phytopathogen was inoculated after the endophytic fungi because of its higher growth rate. The plates were incubated at 25 °C for additional 7 d and, at the end, the pathogen radial growth was the measured variable. This experiment was conducted in a completely randomized design with 225 experimental units: 74 endophytic fungi; phytopathogen alone, corresponding to the control (Figure 4C); with three replicates.

The Antagonism Percentage (AP) of j-esim replicate (n = 3) and i-esim treatment was calculated according to the equation: AP_{ij} = (DM – dm) / DM x 100, in which DM (cm) = diameter average of the *S. sclerotiorum* colony in the absence antagonist endophytic fungus and dm (cm) = diameter average of the *S. sclerotiorum* colony in dual culture with the antagonist endophytic fungus. Data were statistically evaluated by analysis of variance (ANOVA) and the means were compared using the Tukey test at 5 % probability level in SISVAR statistical software (version 5.6). The standard error of the mean was calculated and presented with the respective data.

Endophytes that exhibited antagonism against *S. sclerotiorum* were used in a second experiment to verify whether the observed inhibition resulted from the production of volatile or nonvolatile compounds by the endophytic fungi in the culture medium. The same methodology described in the first experiment was adopted, however, a bipartite Petri dish that prevented contact between the colonies was used.

Results and Discussion

We isolated 74 endophytic fungi from 39 stem samples of *Brachiaria* spp. collected in three states of Brazil (Figure 2). The 28 samples from experimental plots yielded 48 isolates, while 26 isolates were recovered from 11 samples collected in pasture fields, an overall recovery rate of two isolates per sample.

Twenty-eight species of the endophytic fungi were identified by ITS and 18S rDNA sequences comparison, most belonging to Phylum Ascomycota (Figure 3A). *Paraconiothyrium* sp. was the most abundant endophyte (Figure 3B), accounting for 24 % (14 isolates) of total and isolated from all samples, except for one sample (hybrid cv. Mulato II and *B. mutica*). *Phoma sorghina* (11 isolates) was the second most abundant taxon, followed by *Sarocladium strictum*, and *Plenodomus* sp., both taxa represented by seven isolates. These four species accounted for approximately two-thirds of all isolates (68 %), but corresponded to 32 % of all species recovered.

Paraconiothyrium, *Phoma*, and *Plenodomus* are *Pleosporales* genera belonging to different families of the *Dothideomycetes* class. Taxonomy of this group coniothyrium-like fungi is quite complex and subjected to periodical updates, mainly to accommodate the wealth of DNA sequence data and phylogenetic reassessments of ex-type and other reference strains preserved in culture collections (Verkley et al., 2014; Ariyawansa et al., 2015; Chen et al., 2015). On the other hand, the ITS marker is generally reliable for strain typing and identifications to genus level among this group of fungi (Verkley et al., 2014). Species of *Phoma*-like fungi are common endophytes of grasses, such as the temperate species *Dactylis glomerata* and *Holcus lanatus* (Sánchez Márquez et al., 2007, 2010). Two putative *Phoma* species were reported

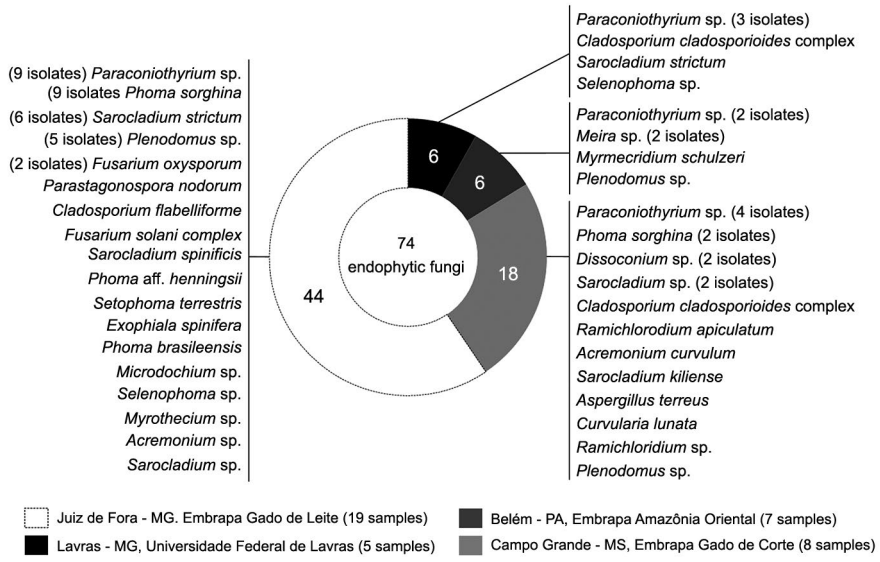

Figure 2 – Endophytic fungi isolated from different species of *Brachiaria* for each sampling site in Brazil.

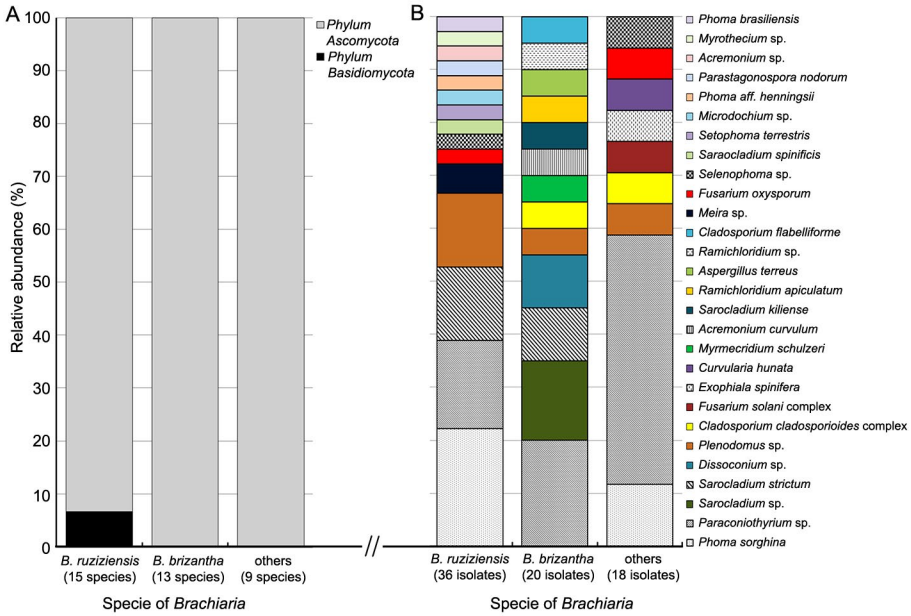

Figure 3 – Relative abundance at (A) phylum and (B) species level of endophytic fungi isolated from different species of *Brachiaria*.

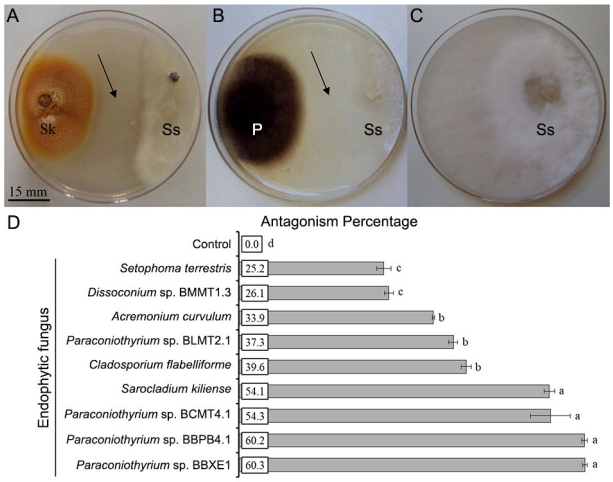

Figure 4 – (A) *In vitro* antagonism of *Sarocladium kiliense* (Sk) and (B) *Paraconiothyrium* sp. BBPB4.1 (P) in relation to *Sclerotinia sclerotiorum* (Ss) in dual cultures. (C) Growth of Ss after 7 days in the absence of antagonist. (D) Antagonism percentage values exhibited by selected endophytic fungi against Ss ($p < 0.05$). Means followed by same letter do not differ by the Tukey test at 5 % level (n = 3) and bars represent the standard error of the mean. Arrows in (A) and (B) show the inhibition zones.

as endophytes of *Brachiaria* in a preliminary inventory conducted in Brazil (Rodrigues and Dias-Filho, 1996). *P. sorghina*, the second most abundant species and isolated from *B. ruziziensis*, *B. decumbens*, and *B. humidicola*, was also one of the most frequently isolated endophyte in perennial grasses *Hyparrhenia hirta* and *Bothriochloa macra* in Australia (White and Backhouse, 2007). Species of *Paraconiothyrium* were the dominant root endophytes of native grasses inhabiting semiarid grasslands in New Mexico (Khidir et al., 2010). We isolated endophytes from the lower part of plant stems and the root system of *Brachiaria* and the soil are possibly the source of the abundant *Paraconiothyrium* taxon, since this is a genus of common soil-borne fungi (Domsch et al., 2007).

Twelve isolates were identified as *Sarocladium* species, the third most common genus of *Brachiaria* endophytes. *B. brizantha* yielded *Sarocladium* sp., *S. kiliense* and *S. strictum*. From *B. ruziziensis*, *S. spinificis* and *S. strictum* were isolated. There is a high diversity of *Sarocladium* in grasses, mostly endophytes and some phytopathogens (e.g. *S. oryzae*). This diversity is shown by the description of two new species, *Sarocladium spinificis*, endophytic to coastal grass *Spinifex littoreus* in Taiwan, and *S. brachiariae*, recently described in *B. brizantha* China (Yeh and Kirschner, 2014; Liu et al., 2017). *S. implicatum* (formerly known as *Acremonium implicatum*) was identified as a seed-transmitted endophyte of *Brachiaria* species, where it may play a role in protecting plants against fungal pathogens, such as *Drechslera* spp., which causes leaf spots (Kelemu et al., 2001). Seedborne *S. implicatum* can colonize other plant parts after germination and provide fitness advantages to the host (Kago et al., 2016).

Two isolates of *Meira* sp. from *B. ruziziensis* were the sole representatives of Phylum Basidiomycota in this study. Species of this genus were described as acaropathogenic and can be found as endophyte of many plant species (Rush and Aime, 2013). Moreover, a recent study reported the isolation of *Meira* sp. as an endophyte of the temperate grass *Ammophila arenaria* (Sánchez Márquez et al., 2012).

In vitro analyses showed that the endophytic fungi *Paraconiothyrium* sp. (isolates BLMT2.1, BCMT4.1, BBPB4.1, and BBXE1), *Sarocladium kiliense*, *Acremonium curvulum*, *Setophoma terrestris*, *Dissoconium* sp. (isolate BMMT1.3) and *Cladosporium flabelliforme* exhibited antagonistic activity against *S. sclerotiorum* ($p < 0.05$), with percentages of growth inhibition ranging from 25 to 60 (Figure 4D). *Paraconiothyrium* sp. BBXE1, BBPB4.1, BCMT4.1, and *S. kiliense* showed the highest values of Antagonism Percentages.

The growth inhibition of *S. sclerotiorum* is not likely caused by the synthesis of volatile molecules, because no inhibition was observed when these fungi were grown in bipartite plates. *Coniothyrium minitans* is a known mycoparasite capable of controlling plant diseases caused by fungal pathogens, including *S. sclerotiorum* (Whipps et al., 2008), reducing survival of sclerotia and production of apothecia (Zeng et al., 2012). Almeida et al. (2014) reported the isolation of graminin B, a compound with antibiotic activity obtained from fermentation of broths of species *P. hawaiensis*. Three isolates of *Paraconiothyrium* sp. showed the highest inhibition rates in our bioassays (BBXE1, BBPB4.1, and BCMT4.1), suggesting that other *Paraconiothyrium* species can be used in the biocontrol of white mold and that antibiosis is another mode of action of them.

One endophytic isolate of *S. kiliense* reduced the growth of *S. sclerotiorum* by more than a half through the excretion of metabolites in the medium and formation of inhibition halo (Figure 4A). The bioactivity of secondary metabolites produced by *Sarocladium* species is well documented in the case of *S. oryzae*, a producer of the phytotoxic helvolic acid and the antifungal compound cerulenin (Hittalmani et al., 2016). Secondary metabolites produced by an endophytic isolate of *S. implicatum* from *Brachiaria* inhibited the growth of *Rhizoctonia solani*, causal agent of foliar blight in *Brachiaria*, and *Pyricularia oryzae*, causal agent of rice blast (Kelemu et al., 2001). Considering that *in vitro* antagonism does not always predict efficacy, *in vivo* studies are needed to confirm these considerations since they were based only on *in vitro* test.

In the management of white mold caused by *S. sclerotiorum*, one of the recommendations is crop rotation of the host-plant with *Brachiaria* spp. This strategy can be potentialized by the application of *Trichoderma harzianum* over grass litter prior to the next sowing of the host plant, increasing eradication effects on the pathogen initial inoculum (Görgen et al., 2009). Although no *Trichoderma* sp. was recovered from *Brachiaria* sp., abundant *Paraconiothyrium* spp. may offer a reliable disease control. *Trichoderma* spp. has limited performance under cooler temperatures (Paula Junior et al., 2012), the most favorable condition for *S. sclerotiorum* ascospore release and white mold epidemic outbreak (Bolton et al., 2006) as well as for *C. minitans* sclerotia parasitism (Whipps et al., 2008).

Once *Brachiaria* sp. harbors endophytic fungi, such as *Paraconiothyrium* sp. with inhibitory activity against *S. sclerotiorum*, rotation with *Brachiaria* sp. enriched with the antagonistic endophytic fungus may offer a package composed of two disease management tools by planting *Brachiaria* sp. This prospect should be explored in future *in vivo* studies with active application of endophytes to *Brachiaria* seeds or to the field. The bioactive compounds produced by these endophytic fungi also deserve further investigation, since they may be used in the control of plant diseases or other fields, such as medicine and industry.

Conclusions

Brachiaria spp. from different experimental plots and pasture fields in Brazil yielded 28 taxa of stem-associated endophytic fungi. Two-thirds of all isolates belong to four of the most common species, *Paraconiothyrium* sp., *Phoma sorghina*, *Plenodomus* sp., and *Sarocladium strictum*.

Four isolates of the most common endophytic fungus *Paraconiothyrium* sp. (BLMT2.1, BCMT4.1, BBPB4.1, and BBXE1) in addition to *Sarocladium kiliense*, *Acremonium curvulum*, *Setophoma terrestris*, *Dissoconium* sp. (isolate BMMT1.3), and *Cladosporium flabelliforme* exhibited *in vitro* antifungal activity against *S. sclerotiorum*. Tests with *Brachiaria* sp. enriched with the antagonistic endophytic fungus and the investigation of bioactive compounds produced should be explored in future studies.

Acknowledgements

To the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), for the scholarships granted to the first author; to the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), for the financial support to this study (CAG-APQ-02100-13); to the researchers of the Universidade Federal de Lavras, Embrapa Gado de Corte, Embrapa Gado de Leite, and Embrapa Amazônia Oriental, for the providing the samples used in this work.

Author's Contributions

Conceptualization: Gama, D.S.; Medeiros, F.H.V.; Cardoso, P.G. Data acquisition: Gama, D.S.; Cardoso, P.G. Data analysis: Gama, D.S.; Santos, Í.A.F.M. Design of methodology: Gama, D.S.; Medeiros, F.H.V.; Cardoso, P.G. Writing and editing: Gama, D.S.; Santos, Í.A.F.M.; Abreu, L.M.; Medeiros, F.H.V.; Duarte, W.F; Cardoso, P.G.

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