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Antagonism of saprobe fungi from semiarid areas of the Northeast of Brazil against *Sclerotinia sclerotiorum* and biocontrol of soybean white mold

Antagonismo de fungos sapróbios do semi-árido do Nordeste brasileiro contra *Sclerotinia sclerotiorum* e biocontrole do mofo branco da soja

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Highlights:
Myrothecium sp., *Volutella minima, Phialomyces macrosporus* and *Dictyosporium tetraseriale* were selected based on their antagonistic activity against *Sclerotinia sclerotiorum.*
Myrothecium sp. isolate 2 completely suppressed sclerotia formation and inhibited ascospore germination by over 95%.
AUDPC of white mold reduced in 55.8%, 79.7%, and 83.2% in soybean treated with *Myrothecium* sp. isolate 2, *P. macrosporus,* and *V. minima*, respectively.

Abstract

The antagonistic activity of 25 saprobe fungi from semiarid areas of the Northeast of Brazil was evaluated against *Sclerotinia sclerotiorum* (Lib.) de Bary (Helotiales: Sclerotiniaceae). Four fungi [*Myrothecium* sp. Tode (Hypocreales: Stachybotryaceae) isolate 2, *Volutella minima* Höhn. (Hypocreales: Nectriaceae), *Phialomyces macrosporus* P.C. Misra & P.H.B. Talbot (Pezizomycotina) and *Dictyosporium tetraseriale* Goh, Yanna & K.D. Hyde (Pleosporales: Dictyosporiaceae)] were selected and further tested their ability to inhibit mycelial growth, sclerotia formation and ascospore germination of *S. sclerotiorum* and to control white mold on soybean plants. *V. minima* and *P. macrosporus* filtrates at 50% effectively suppressed mycelial growth and *Myrothecium* sp. isolate 2 completely suppressed sclerotia formation and inhibited ascospore germination by over 95%, the same result as commercial fungicide fluazinam. Soybean plants pre-treated with *Myrothecium* sp. isolate 2, *P. macrosporus,* and *V. minima* and inoculated with *S. sclerotiorum* showed a reduction of 55.8%, 79.7%, and 83.2% of area under disease progress curve (AUDPC) of white mold, respectively, in relation to water. Collectively, these results underline the antagonistic activity of *V. minima, P. macrosporus*, and *Myrothecium* sp. isolate 2 against *S. sclerotiorum* and their potential as biocontrol agents of soybean white mold.
Key words: Biological control. *Glycine max.* Saprobic fungi. Sclerotinia stem rot.

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Resumo

A atividade antagônica de 25 fungos sapróbios de região do semi-árido do Nordeste brasileiro foi avaliada contra *Sclerotinia sclerotiorum* (Lib.) de Bary (Helotiales: Sclerotiniaceae). Quatro fungos [*Myrothecium* sp. Tode (Hipocreales: Stachybotryaceae) isolate 2, *Volutella minima* Höhn. (Hipocreales: Nectriaceae), *Phialomyces macrosporus* P.C. Misra & P.H.B. Talbot (Pezizomycotina) e *Dictyosporium tetraseriale* Goh, Yanna & K.D. Hyde (Pleosporales: Dictyosporiaceae)] foram selecionados para avaliar sua capacidade de inibir o crescimento micelial, a formação de esclerócios e a germinação de ascósporos de *S. sclerotiorum* e sua eficiência de controle do mofo branco em plantas de soja. Os filtrados de *V. minima* e *P. macrosporus* a 50% de concentração suprimiram efetivamente o crescimento micelial de *S. sclerotiorum*. *Myrothecium* sp. isolado 2 suprimiu completamente a formação de escleródios e inibiu a germinação de ascósporos em mais de 95%, o mesmo resultado que o fungicida comercial fluazinam. Plantas de soja pré-tratadas com *Myrothecium* sp. s isolado 2, *P. macrosporus* e *V. minima* e inoculados com *S. sclerotiorum* apresentaram redução de 55,8%, 79,7% e 83,2% da área abaixo da curva de progresso da doença (AACPD) mofo branco, respectivamente, em relação ao controle com água. Coletivamente, os resultados obtidos *in vitro* e em plantas de soja inoculadas, indicam a atividade antagônica de *V. minima, P. macrosporus* e *Myrothecium* sp. isolado 2 contra *S. sclerotiorum* e seu potencial como agentes de biocontrole do mofo branco da soja.

Palavras-chave: Controle biológico. Fungos sapróbios. *Glycine max*. Podridão de Sclerotinia.

Introduction

In 2018/19 Brazil was the second largest producer of soybean [*Glycine max* L. Merrill (Fabales: Fabaceae)] worldwide, led by the United States and followed by Argentina and China, with a total production of 117 million metric tons (United States Department of Agriculture, 2020). Despite the high productivity, more than 40 diseases can infect soybean crops in Brazil (A. M. R. Almeida et al., 2005).

White mold of soybean or Sclerotinia stem rot is caused by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary (Helotiales: Sclerotiniaceae) and can occur in all Brazilian regions with mild climatic conditions (southern region and *cerrado* highlands above 800 m altitude). For every increase of 10% in white mold severity, yield losses in soybean were estimated at 170 to 330 Kg ha^{-1} (Chun, Kao, Lockwood, & Isleib, 1987; Hoffman et al., 1998; Danielson, Nelson, & Helms, 2004).

Sclerotinia sclerotiorum is considered a polyphagous soil fungus, that infects more than 400 species of plant (Boland & Hall, 1994). This fungus forms resistance structures (sclerotia) that can survive for long periods in the soil. Temperature conditions for sclerotia germination are affected by light intensity and moisture level. At light intensities of 80 to 90 mol $m^{-2}s^{-1}$, the optimal temperature varied from 12 to 18 °C, independent of moisture level, while at light intensity of 120 to 130 mol m^{-2} s^{-1}, the optimal temperature was 20 °C when the soil moisture was high (Sun & Yang, 2000). Sclerotia germinate by forming small, cup-shaped, stalked apothecia where ascospores are formed, or by forming mycelia. Ascospores released from apothecia are carried by the wind and are responsible for plant infections (Dhingra, Mendonça, & Macedo, 2009). Soybean plants are more susceptible to infection from blossom (R1 stage) to seed (R5 stage) (Danielson et al., 2004). Control of soybean white mold is complex due to the long susceptibility stage of the host, lack of genetic resistance and the difficulty of synchronizing fungicide sprayings with ascospore release (Morton & Hall, 1989; Bardin & Huang, 2001). Therefore, the utilization of alternative methods, such as biological control, becomes necessary.

Microorganisms reported to colonize sclerotia or with antagonistic properties against *Sclerotinia* spp. include bacteria, mainly in the genus *Bacillus* (Zeng,Wang, Kirk, & Hao, 2012; Kamal, Lindbeck, Savocchia, & Ash, 2015; Vinodkumar, Nakkeeran, Renukadevi, & Malathi, 2017). Nevertheless, much research has been focused on species of antagonistic or mycoparasitic fungi, including *Clonostachys rosea* (Preuss) Mussat (Hypocreales: Bionectriaceae) (Rabeendran, Jones, Moot, & Stewart, 2006), *Dictyosporium elegans* (Corda) (Pleosporales: Dictyosporiaceae) (McCredie & Sivasithamparam, 1985), and several species of *Gliocladium* and *Trichoderma* (Geraldine et al., 2013; Zhang et al., 2016; Elias, Domingues, Moura, Harakava, & Patricio, 2016; Sumida et al., 2018), *Paraphaeosphaeria minitans* (Campb.) Verkley, Göker, Stielow (Pleosporales: Didymosphaeriaceae) (formerly *Coniothyrium minitans*) (Whipps, Sreenivasaprasad, Muthumeenakshi, Rogers, & Challen, 2008; Zeng et al., 2012; Nicot et al., 2019), and *Paecilomyces lilacinus* (Thom) Samson (Eurotiales: Aspergillaceae) (Yang, Abdelnabby, & Xiao, 2015).

Fungal biodiversity has been prospected in semiarid areas and various saprobe fungi were identified in the litter of the Caatinga forest in Northeast Brazil (Barbosa & Gusmão, 2011; D. A. C. Almeida, Izabel, & Gusmão, 2011; Leão-Ferreira, Pascholati, Gusmão, & Castañeda Ruiz, 2013; Santa Izabel & Gusmão, 2018). Some of these saprobes were being used as potential biological control agents and resistance inducers (Resende, Milagres, Rezende, Aucique-Perez, & Rodrigues, 2015; Barros, Fonseca, Balbi-Peña, Pascholati, & Peitl, 2015; Rodríguez et al., 2016; Peitl et al., 2017; Ribeiro et al., 2018; Botrel et al., 2018).

In this context, the main goal of the present study was to select saprobe fungi from semiarid areas of Northeast Brazil that could control white mold of soybean, which was investigated at *in vitro* and *in vivo* experiments.

Materials and Methods

Saprobe fungi and Sclerotinia sclerotiorum isolates

Saprobe fungi were isolated from the litter of the Caatinga forest (semiarid) in Northeast Brazil and are deposited in the "Coleção de Culturas de Microrganismos da Bahia (CCMB)" at the "Universidade Estadual de Feira de Santana," Bahia State, Brazil (Ministry of the Environment MMA/ SISGEN code AB513B8). Species were identified by comparing their reproductive structures with the descriptions in the specialized literature (D. A. C. Almeida et al., 2011). The 25 isolates used in this work (Table 1) were cultured on potato-dextrose-agar (PDA) (200 g L^{-1} potato infusion, 20 g L^{-1} dextrose, 15 g L^{-1} agar, pH 5.6 ± 0.2) plates at 25 ± 2 °C and 12/12 h photoperiod. Sclerotia of *S. sclerotiorum,* were isolated from naturally infected soybean plants from Londrina, Paraná State, Brazil. Sclerotia were collected and superficially disinfected by immersion into a 70% alcohol solution for three min, then by immersion into a 0.2% sodium hypochlorite solution for three min and rinsed three times with distilled and sterilized water. Sclerotia were dried for 12 h in a laminar flow chamber and then transferred to PDA plates and incubated at 20°C in the dark for 4 days. After germination, mycelia were inoculated on PDA plates and incubated under the same conditions described above.

Saprobe fungi filtrates

Two 5 mm diameter mycelial disks from the edge of the saprobe fungi colonies were transferred to Erlenmeyer flasks containing 100 mL of potato-dextrose (PD) culture broth (200 g L^{-1} potato infusion, 20 g L^{-1} dextrose, pH 5.6 ± 0.2). Cultures were incubated at 25 ± 2 ºC and 12/12 h photoperiod on an orbital shaker (100 rpm) for 10 days. Afterward, cultures were transferred at 5 ± 2 ºC for 48 h. The supernatant was filtrated, centrifuged twice at 5000 rpm for 15 min each for fungal cell removal, and stored at 5 °C.

Screening of saprobe fungi for its toxicity against Sclerotinia sclerotiorum

Filtrates from 25 saprobe fungi cultures were incorporated at 5% and 50% (v/v) into PDA culture medium at 50 °C, according to the methodology used by Sarma, Ameer Basha, Singh and Singh (2007). Five mm diameter mycelial disks from 3-day-old cultures of *S. sclerotiorum* grown on PDA, were transferred to the center of PDA plates with the incorporated filtrates. As a control, *S. sclerotiorum* was transferred to PDA plates without fungal filtrates. Plates were incubated at 20 ± 2 ºC and 12/12 h photoperiod. Colony size was assessed daily by two orthogonal measurements of the diameter of the fungal colony until the control treatment reached the entire plate. Daily diameter measurements were used to determine the mycelial growth rate (MGR) (Oliveira, 1991):

$$MGR = \sum_{i=1}^{n} \left(\frac{y_i - y_{i-1}}{n} \right)$$

Where, y is the diameter (cm) at each measurement at the i^{th} observation and n is the number of days after inoculation.

After 21 days, the number and weight of sclerotia formed on the plates were assessed.

Effect of saprobe fungal filtrates on Sclerotinia sclerotiorum mycelial growth and sclerotia formation

Based on the previous screening, *Myrothecium* sp. Tode (Hypocreales: Stachybotryaceae) isolate 2, *Dictyosporium tetraseriale* Goh, Yanna & K.D. Hyde (Pleosporales: Dictyosporiaceae), *Volutella minima* Höhn. (Hypocreales: Nectriaceae) and *Phialomyces macrosporus* P.C. Misra & P.H.B. Talbot (Pezizomycotina) filtrates were incorporated at 5% and 50% (v/v) in PDA medium at 50 °C. Five mm diameter mycelial disks from 3-day-old cultures of *S. sclerotiorum* grown on PDA, were transferred to the center of PDA plates with the incorporated filtrates and incubated at 20 ± 2 ºC and 12/12 h photoperiod for 21 days. As a control, *S. sclerotiorum* was transferred on PDA plates without fungal filtrates.

Mycelial growth rate, number, and weight of sclerotia were determined as described above.

Effect of saprobe fungal filtrates on the germination of Sclerotinia sclerotiorum ascospores

Fungal filtrates were used at a concentration of 50% (v/v) and commercial fungicide Fluazinam at 0.5% in sterile water. As a control treatment, sterile water was used.

For ascospore production, sclerotia were placed in clear polystyrene boxes (11 × 11 × 3.5 cm) with moistened autoclaved soil (eutrophic red latosol) and incubated at 20 ± 2 ºC and 12/12 h photoperiod for 45 days. Apothecia were cut and homogenized with mortar in 10 ml of sterilized distilled water (Tolêdo-Souza & Costa, 2007).

Aliquots of 50 µL of fungal filtrates were placed in ELISA plate cells with 50 µL of the ascospore suspension (2×10^5 ascospores mL^{-1}). Samples were homogenized and then transferred to water-agar plates (15 g L^{-1} of agar). Plates were incubated at 20 ± 2 ºC in continuous light for 6 h. Lactophenol-cotton blue was used to stop the germination and to stain the structures. Ascospores were considered germinated when the length of their germ tube was equal to or greater than their diameter. One hundred ascospores per plate (replication) were assessed.

Effect of volatiles from saprobe fungi on Sclerotinia sclerotiorum mycelial growth and sclerotia formation

The assay was performed in two-section polystyrene Petri plates. Saprobe fungi were cultivated on PDA on one side of the plate and incubated at 25 ± 2 ºC and 12/12 h photoperiod for 7 days. Afterward, 5 mm diameter mycelial disks of *S. sclerotiorum* grown on PDA, were transferred to the opposite section of the plate. In the case of *P.*

macrosporus, S. sclerotiorum was transferred only 3 days after because this fungus has faster growth than the others. Plates were kept at 25 ± 2 ºC and 12/12 h photoperiod until *S. sclerotiorum* occupied the entire section of the control plates (without saprobe). Colony diameters were measured daily to obtain the MGR. The number and weight of sclerotia were assessed 21 days after inoculation.

Effect of volatiles from saprobe fungi on the germination of Sclerotinia sclerotiorum ascospores

Saprobe fungi were cultivated as described before. Water-agar was poured in the opposite section of the Petri plates, where a 50 µL-aliquot of ascospore suspension (2×10^5 ascospores/mL) was transferred. Plates were incubated at 25°C in continuous light for 6 h. Germination was stopped by lactophenol-cotton blue addition. Two hundred ascospores per plate (replication) were assessed.

Control of white mold of soybean under greenhouse conditions

Soybean seeds (cv. BMX Potencia RR) were sown in one-liter pots containing soil (eutrophic red latosol) and sand (1:1 v/v). Saprobe fungi were cultivated in 100 mL PD broth (200 g L^{-1} potato infusion, 20 g L^{-1} dextrose, pH 5.6 ± 0.2) and incubated at 25 ± 2 ºC and 12/12 h photoperiod for 10 days. Afterwards, 100 mL of distilled and sterilized water was added to the cultures and homogenized. These homogenates were sprayed on soybean plants at V4 stage (four fully expanded trifoliate leaves). As a negative control, soybean plants were sprayed with distilled and sterilized water and, as a positive control, plants were sprayed with commercial inducer acibenzolar-S-methyl (ASM) (30 g ha^{-1}).

Three days after saprobe fungi, ASM or water sprays, soybean plants were inoculated with *S. sclerotiorum* according to the method of Kull et al. (2003), with modifications. For inoculation, a 200 µL micropipette tip was used to remove mycelial disks from 3-day-old colonies of *S. sclerotiorum* grown on PDA (20 ± 2 ºC and 12/12 h photoperiod). These tips containing mycelia were placed on the cut stems of the soybean plants. The stems were previously cut 0.5 cm above the last fully expanded leaf insertion (4^{th} trifoliate leaf) with sterilized scissors. After inoculation, soybean plants were kept at 20 ± 2 ºC and 12/12 h photoperiod in a growth chamber. Lesions lengths were measured weekly, from the cut end of the stem to the end of the lesion, for 21 days. The AUDPC was calculated according to the formula by Shaner and Finney (1977) and normalized by dividing the AUDPC value by the total time (number of days from the first occurrence of the disease to the end of the observation period) (Fry, 1978).

Experimental design and data analysis

The *in vitro* experiments were arranged in a completely randomized design with five replications. The *in vivo* experiment was arranged in a randomized complete block design with five replications and each experimental unit corresponded to a plastic pot with a single soybean plant. The experiments were repeated.

Data were submitted to analysis of variance at 0.05 level of significance. When treatment effects were significant, means were compared by the Scott–Knott test ($p \leq 0.05$) in the screening test and by Tukey test ($p \leq 0.05$) in tests with the four selected saprobes.

Results

Screening of saprobe fungi

Curvularia eragrostidis (Henn.) Meyer (Pleosporales: Pleosporaceae), *Gonytrichum macrocladum* (Sacc.) Hughes (Chaetosphaeriales: Chaetosphaeriaceae), *Myrothecium* sp. isolate 2, *Phialomyces macrosporus, Stachybotrys globosa* Misra, Srivast (Hypocreales: Stachybotryaceae) and *Volutella minima* filtrates at 5% (v/v), reduced the MGR of *S. sclerotiorum*. *Phialomyces macrosporus* showed the lowest rate (0.67 cm/day), which was

statistically different from the control treatment and from the other saprobe filtrates (Table 1). At the concentration of 50%, *P. macrosporus* and *V. minima* filtrates completely inhibited the mycelial growth of *S. sclerotiorum*. At the same concentration, *Beltraniella portoricensis* (Stevens) Piroz., Patil (Amphisphaeriales: Amphisphaeriaceae), *Clonostachys rosea, Dictyosporium tetraseriale, Myrothecium* sp. Isolate 2, and *Sarcopodium circinatum* Ehrenb. (Pezizomycotina) filtrates reduced the mycelial growth in about 76%.

Sclerotia number and weight exhibited greater variation than mycelial growth (Table 1). *Stachybotrys chartarum* (Ehrenb.) Hughes (Hypocreales: Stachybotryaceae) filtrates, completely inhibited sclerotia formation at both concentrations. At 50%, *B. portoricensis, Dictyochaeta heteroderae* (Morgan-Jones) Carris, Glawe, (Chaetosphaeriales: Chaetosphaeriaceae) and *Myrothecium* sp. isolate 2 filtrates also completely inhibited sclerotia formation. Because *P. macrosporus* formed so few sclerotia, their weight was almost zero.

Table 1
Mycelial growth rate (MGR), number and weight of sclerotia of *Sclerotinia sclerotiorum* in potato dextrose agar culture medium amended with saprobe fungal filtrates at 5% and 50% (v/v)

Treatments	MGR[ab] (cm/day)		Sclerotia[a]			
			Number		Weight (g)	
	5%	50%	5%	50%	5%	50%
Control treatment	2.45 ± 0.04a	2.46 ± 0.03a	26.4 ± 4,27a	21.6 ± 2.98b	0.25 ± 0.009a	0.30 ± 0.023b
Beltrania copaifera	2.42 ± 0.26a	2.62 ± 0.03a	13.0 ± 1.92a	3.40 ± 0.51d	0.13 ± 0.020b	0.08 ± 0.008d
Beltrania rhombica	2.52 ± 0.01a	2.44 ± 0.06a	9.80 ± 3.10b	8.20 ± 1.50d	0.31 ± 0.162a	0.21 ± 0.034c
Beltraniella portoricensis	2.41 ± 0.04a	0.77 ± 0.08d	6.60 ± 1.83c	0.00 ± 0.00d	0.18 ±0.012a	0.00 ± 0.000e
Chloridium virescens var. *virescens*	2.46 ± 0.03a	1.24 ± 0.08c	12.0 ± 2.19b	6.00 ± 2.51d	0.13 ± 0.028b	0.06 ± 0.008d
Clonostachys rosea	2.56 ± 0.06a	0.47 ± 0.10d	10.8 ± 3.07b	18.4 ± 1.29c	0.12 ± 0.024b	0.17 ± 0.015c
Curvularia eragrostidis	1.87 ± 0.11b	2.32 ± 0.10b	5.00 ± 1.30c	2.60 ± 2.14d	0.08 ± 0.021b	0.02 ± 0.009e
Curvularia inaequalis	2.42 ±0.04a	2.20 ± 0.04b	14.4 ± 2.14b	14.4 ± 0.68c	0.14 ± 0.012b	0.13 ± 0.011d
Dictyochaeta heteroderae	2.60 ± 0.11a	1.97 ± 0.09b	10.6 ± 3.31b	0.00 ± 0.00d	0.13 ± 0.027b	0.00 ± 0.000e
Dictyosporium tetraseriale	2.47 ± 0.13a	0.52 ± 0.09d	4.60 ± 0.68c	3.60 ± 0.81d	0.08 ± 0.011b	0.05 ± 0.005d
Gonytrichum chlamydosporium	2.31 ± 0.08a	1.37 ±0.35c	7.20 ± 3.38c	3.00 ± 1.26d	0.03 ± 0.014b	0.03 ± 0.006e
Gonytrichum macrocladum	2.05 ± 2.05b	1.80 ± 0.23b	10.2 ± 2.78b	3.25 ± 1.49d	0.08 ± 0.018b	0.05 ± 0.017d
Lappodechium lageniforme	2.77 ± 0.06a	2.69 ± 0.13a	9.80 ± 1.83b	26.8 ± 3.85a	0.20 ± 0.033a	0.41 ± 0.043a
Memnoniella echinata	2.58 ± 0.05a	2.64 ± 0.07a	15.4 ± 2.56b	20.0 ± 5.10b	0.13 ± 0.011b	0.13 ± 0.032d
Myrothecium sp. isolate 1	2.55 ± 0.04a	2.62 ± 0.03a	12.6 ± 1.21b	17.8 ± 4.76c	0.15 ± 0.019a	0.26 ± 0.006d
Myrothecium sp. isolate 2	1.85 ± 0.12b	0.41 ± 0.41d	10.6 ± 2.92b	0.00 ± 0.00d	0.22 ± 0.054a	0.00 ±0.000e
Periconia hispidula	2.73 ± 0.07a	2.18 ± 0.31b	5.40 ± 1.81c	2.80 ± 0.37d	0.06 ± 0.011b	0.06 ±0.009d
Phialomyces macrosporus	0.67 ± 0.17c	0.00 ± 0.00e	4.20 ± 1.11c	0.40 ± 0.40d	0.09 ± 0.022b	0.00 ± 0.000e
Pithomyces chartarum	2.35 ± 0.09a	1.07 ± 0.10c	9.80 ± 2.60b	6.60 ± 0.68d	0.10 ± 0.019b	0.07 ± 0.009d
Pseudobotrytis terrestris	2.34 ± 0.01a	1.49 ± 0.08c	8.00 ± 2.17c	3.20 ± 1.02d	0.17 ± 0.054a	0.07 ± 0.006d
Sarcopodium circinatum	2.51 ± 0.03a	0.73 ± 0.02d	10.4 ± 3.67b	12.4 ± 2.38c	0.17 ± 0.020a	0.07 ± 0.041d
Stachybotrys chartarum	2.48 ± 0.03a	2.66 ± 0.03a	0.00 ±0.00c	0.00 ± 0.00d	0.00 ± 0.000b	0.00 ± 0.000e
Stachybotrys globosa	2.20 ± 0.28b	2.27 ± 0.40b	11.8 ± 3.32b	14.2 ± 2.65c	0.21 ± 0.053a	0.40 ± 0.103a
Stachylidium bicolor	2.53 ± 0.03a	1.99 ± 0.07b	13.4 ± 1.36b	2.00 ± 1.26d	0.18 ± 0.031a	0.04 ± 0.025e
Volutella minima	2.12 ± 0.44b	0.00 ± 0.00e	3.80 ± 1.39c	6.00 ± 1.58d	0.08 ± 0.015b	0.08 ± 0.014d
Zygosporium echinosporum	2.32 ± 0.04a	2.22 ± 0.03b	10.2 ±0.58b	6.40 ± 1.29d	0.22 ± 0.004a	0.18 ± 0.023c

[a]Mean ± SE (n = 5 replicates) within each column followed by the same letter are not significantly different (P<0.05) according to Scott-Knott test.
[b]MGR = (current colony diameter - colony diameter of the previous day)/(number of days after inoculation).

Table 2
Mycelial growth rate (MGR), number and weight of sclerotia of *Sclerotinia sclerotiorum* cultured in potato dextrose agar culture medium amended with saprobe fungal filtrates at 5% and 50% (v/v)

Treatments	MGR (cm/day)[c]		Sclerotia			
			Number		Weight (g)	
	5%	50%	5%	50%	5%	50%
Control treatment[a]	2.5 ± 0.08a	2.4 ± 0.08a	26.4 ± 6.08a	21.6 ± 5.25a	0.25 ± 0.07a	0.30 ± 0.09a
Dictyosporium tetraseriale	2.4 ± 0.08a	0.5 ± 0.02b	5.0 ± 1.15b	3.6 ± 0.87b	0.08 ± 0.02b	0.05 ± 0.01b
Myrothecium sp. isolate 2	1.8 ± 0.06a	0.4 ± 0.01b	10.6 ± 2.44b	0.0 ± 0.00b	0.22 ± 0.06a	0.0 ± 0.00b
Phialomyces macrosporus	0.7 ± 0.02b	0.0 ± 0.00c	3.7 ± 0.85b	0.5 ± 0.12b	0.05 ± 0.01b	0.01 ± 0.00b
Volutella minima	2.1 ± 0.07a	0.0 ± 0.00c	3.8 ± 0.88b	6.0 ± 1.46b	0.08 ± 0.02b	0.08 ± 0.02b
Control treatment[b]	2.4 ± 0.08a	2.4 ± 0.09a	28.7 ± 6.47a	19.8 ± 5.06a	0.30 ± 0.08a	0.30 ± 0.08a
Dictyosporium tetraseriale	2.5 ± 0.08a	0.8 ± 0.03b	5.0 ± 1.13b	2.3 ± 0.59b	0.09 ± 0.02b	0.05 ± 0.01b
Myrothecium sp. isolate 2	1.9 ± 0.06a	0.4 ± 0.01b	12.3 ± 2.77b	0.0 ± 0.00b	0.25 ± 0.07a	0.0 ± 0.00b
Phialomyces macrosporus	0.7 ± 0.02b	0.0 ± 0.00c	3.7 ± 0.83b	0.5 ± 0.13b	0.05 ± 0.01b	0.01 ± 0.00b
Volutella minima	2.1 ± 0.07a	0.0 ± 0.00c	2.9 ± 0.65b	5.4 ± 1.38b	0.08 ± 0.02b	0.08 ± 0.02b

[a]First experiment, mean ± SE (n = 5 replicates) within each column followed by the same letter are not significantly different (P<0.05) according to Tukey test.
[b]Second experiment, mean ± SE (n = 5 replicates) within each column followed by the same letter are not significantly different (P<0.05) according to Tukey test.
[c]MGR = (current colony diameter - colony diameter of the previous day)/(number of days after inoculation).

Effect of saprobe filtrates on mycelial growth, sclerotia formation and germination of ascospores of Sclerotinia sclerotiorum

When saprobe filtrates were assessed at 5%, only *P. macrosporus* reduced MGR in both experiments (Table 2). Regarding sclerotia formation, it was observed that all treatments were different from the control, reducing sclerotia number in both experiments. Considering the effect on sclerotia weight, only *Myrothecium* sp. isolate 2 filtrates did not reduce this variable in both experiments.

When fungal filtrates were assessed at 50%, all treatments reduced *S. sclerotiorum* MGR. *Myrothecium* sp. isolate 2 and *D. tetraseriale* filtrates showed MGR means of 0.4 and 0.65 cm/day, respectively. *Volutella minima* and *P. macrosporus* filtrates completely inhibited *S. sclerotiorum* mycelial growth in both experiments. All treatments reduced the number and weight of sclerotia formed. *Myrothecium* sp. isolate 2 filtrates completely inhibited the formation of these structures.

Treatments with fluazinam and *Myrothecium* sp. isolate 2 reduced the ascospore germination (Table 3) in about 90.7% and 97%, respectively (average from both experiments). *D. tetraseriale* and *V. minima* filtrates reduced ascospore germination in about 23.9% and 30.8%, respectively (average from both experiments). Treatment with *P. macrosporus* filtrate did not significantly reduce ascospore germination.

Table 3
Effect of saprobe filtrates and fluazinam on the germination of ascospores of *Sclerotinia sclerotiorum*

Treatments	Germination (%)	Germination inhibition (%)
Control treatment[a]	92.8 ± 2.66 a[1]	-
Dictyosporium tetraseriale	70.2 ± 2.01 b	24.4
Fluazinam	8.80 ± 0.25 c	90.5
Myrothecium sp. isolate 2	2.60 ± 0.07 c	97.2
Phialomyces macrosporus	89.0 ± 2.55 a	4.10
Volutella minima	65.2 ± 1.87 b	29.7
Control treatment[b]	94.2 ± 2.86 a	-
Dictyosporium tetraseriale	72.3 ± 2.20 b	23.3
Fluazinam	8.70 ± 0.26 c	90.8
Myrothecium sp. isolate 2	3.10 ± 0.09 c	96.7
Phialomyces macrosporus	89.3 ± 2.72 a	5.20
Volutella minima	64.2 ± 1.95 b	31.9

[a]First experiment, mean ± SE (n = 5 replicates) within each column followed by the same letter are not significantly different (P<0.05) according to Tukey test.
[b]Second experiment, mean ± SE (n = 5 replicates) within each column followed by the same letter are not significantly different (P<0.05) according to Tukey test.

Effect of volatiles from saprobe fungi on mycelial growth, sclerotia formation and ascospore germination of Sclerotinia sclerotiorum

Only *P. macrosporus* volatiles inhibited the mycelial growth of *S. sclerotiorum* in about 60%, MGR in approximately 54% (Table 4) and ascospore germination in approximately 30% (Table 5). Regarding sclerotia number, treatments with *D. tetraseriale, P. macrosporus*, and *V. minima* volatiles reduced sclerotia formation. Sclerotia weight was not affected by fungal volatiles.

Control of soybean white mold under greenhouse conditions

Lesion length of white mold at 7 days after inoculation (DAI) in soybean plants treated with ASM and *D. tetraseriale* was not different from that in untreated and inoculated plants (Table 6). Plants treated with *Myrothecium* sp. isolate 2 and *P. macrosporus* showed shorter lesions (0.8 cm) than ASM and water treated plants, but longer than *Volutella minima* treated plants, wherein lesion length was 0.5 cm.

Table 4
Effect of volatiles from saprobe fungi on colony diameter, mycelial growth rate (MGR), number and total weight of sclerotia of *Sclerotinia sclerotiorum*

Treatments	Colony diameter at 3rd day (cm)	MGR (cm/day)[c]	Number of sclerotia	Total weight of sclerotia (g)
Control treatment[a]	8.1 ± 0.46 a	2.6 ± 0.15 a	15.4 ± 3.48 a	0.18 ± 0.05 [n.s]
Dictyosporium tetraseriale	7.5 ± 0.42 a	2.5 ± 0.14 a	10.6 ± 2.39 b	0.14 ± 0.04
Myrothecium sp. isolate 2	7.7 ± 0.43 a	2.6 ± 015 a	12.4 ± 2.80 a	0.12 ± 0.03
Phialomyces macrosporus	3.1 ± 0.17 b	1.1 ± 0.06 b	8.8 ± 1.99 b	0.08 ± 0.02
Volutella minima	8.0 ± 0.45 a	2.6 ± 0.15 a	8.2 ± 1.85 b	0.1 3 ± 0.03
Control treatment[b]	7.9 ± 0.45 a	2.5 ± 0.14 a	15.4 ± 3.55 a	0.19 ± 0.05 [n.s]
Dictyosporium tetraseriale	7.8 ± 0.44 a	2.6 ± 0.14 a	11.2 ± 2.58 b	0.12 ± 0.03
Myrothecium sp. isolate 2	7.9 ± 0.45 a	2.6 ± 0.14 a	12.3 ± 2.83 a	0.12 ± 0.03
Phialomyces macrosporus	3.2 ± 0.18 b	1.2 ± 0.07 b	9.1 ± 2.10 b	0.07 ± 0.02
Volutella minima	8.0 ± 0.45 a	2.6 ± 0.14 a	8.0 ± 1.84 b	0.13 ± 0.03

[a]First experiment, mean ± SE (n = 5 replicates) within each column followed by the same letter are not significantly different (P<0.05) according to Tukey test. Not significant (ns).
[b]Second experiment, mean ± SE (n = 5 replicates) within each column followed by the same letter are not significantly different (P<0.05) according to Tukey test. Not significant (ns).
[c]MGR = (current colony diameter - colony diameter of the previous day)/(number of days after inoculation).

Table 5
Effect of volatiles from saprobe fungi on the germination of ascospores of *Sclerotinia sclerotiorum*

Treatment	Germination (%)	Germination inhibition (%)
Control treatment[a]	96.0 ± 3.48 a	-
Dictyosporium tetraseriale	92.6 ± 3.35 a	3.50
Myrothecium sp. isolate 2	84.6 ± 3.06 a	11.9
Phialomyces macrosporus	67.6 ± 2.45 b	29.6
Volutella minima	85.6 ± 3.10 a	10.8
Control treatment[b]	96.8 ± 3.72 a	-
Dictyosporium tetraseriale	92.6 ± 3.56 a	3.50
Myrothecium sp. isolate 2	84.9 ± 3.27 a	11.9
Phialomyces macrosporus	67.8 ± 2.61 b	29.6
Volutella minima	86.5 ± 3.33 a	10.8

[a]First experiment, mean ± SE (n = 5 replicates) within each column followed by the same letter are not significantly different (P<0.05) according to Tukey test.
[b]Second experiment, mean ± SE (n = 5 replicates) within each column followed by the same letter are not significantly different (P<0.05) according to Tukey test.

Table 6
White mold lesion length at 7, 14 and 21 days after inoculation (DAI) and area under the disease progress curve (AUDPC) in soybean plants treated with water, acibenzolar-S-methyl (ASM) or saprobe fungi and inoculated with *Sclerotinia sclerotiorum*, under greenhouse conditions. Londrina-PR. Brazil. 2014

Treatments	Lesion length (cm)			AUDPC[b]	% reduction of AUDPC
	7 DAI	14 DAI	21 DAI		
Control (water)[a]	5.3 ± 0.08 a	7.4 ± 0.09 a	10.0 ± 0.08 a	66.1 ± 0.47a	-
ASM	5.3 ± 0.08 a	6.9 ± 0.08 b	9.5 ± 0.08 b	63.8 ± 0.46 b	3.47
Dictyosporium tetraseriale	5.3 ± 0.08 a	7.1 ± 0.08 b	9.8 ± 0.08ab	64.9 ± 0.46 ab	1.82
Myrothecium sp. isolate 2	0.8 ± 0.01b	6.1 ± 0.07c	9.7 ± 0.08ab	29.2 ± 0.21 c	55.8
Phialomyces macrosporus	0.8 ± 0.01b	2.1 ± 0.02 d	3.2 ± 0.03c	13.4 ± 0.10 d	79.7
Volutella minima	0.5 ± 0.01 c	1.9 ± 0.02 d	3.1 ± 0.02c	11.1 ± 0.08 e	83.2

[a]Mean ± SE (n = 5 replicates) within each column followed by the same letter are not significantly different (P<0.05) according to Tukey test.

[b] $\sum_{i=1}^{n} = \frac{[(X_i + X_{i+1})/2][t_{i+1} - t_i]}{(T_n - T_1)}$ where X_i and X_{i+1} are two consecutive severity measurements performed at times t_i and t_{i+1}. respectively.

At 14 DAI all treatments showed lesion lengths lower than the negative control. Plants treated with ASM and *D. tetraseriale* exhibited lesion lengths of 6.9 and 7.1 cm, respectively, both shorter than the negative control, but longer than *Myrothecium* sp. isolate 2 treated plants (6.1 cm). Plants treated with *P. macrosporus* and *V. minima* showed lesion length of 2.1 and 1.9 cm, respectively, the shortest lesions recorded.

At 21 DAI, plants treated with *D. tetraseriale* and *Myrothecium* sp. showed lesion lengths not statistically different from the negative control and ASM treatment. Plants treated with *P. macrosporus* and *V. minima* with lesions of 3.2 and 3.1 cm, respectively, showed the highest reduction of white mold severity.

Considering the area under the disease progress curve (AUDPC), the treatment with *D. tetraseriale* was the only one that was not statistically different from negative control. Plants treated with ASM showed shorter lesions from those of the control and the same length of the lesions of *D. tetraseriale* treated plants. Plants treated with *V. minima* showed the greatest reduction of disease progress, followed by plants treated with *P. macrosporus* and *Myrothecium* sp. isolate 2.

Discussion

Most saprobe filtrates showed antagonism against *Sclerotinia sclerotiorum*. Of the 25 saprobe fungi assessed, 72% (18) reduced the MGR and 92% (22) reduced the number of sclerotia of *S. sclerotiorum* when tested at 50% concentration. Four saprobes completely inhibited the formation of sclerotia.

Abdullah, Ali and Suleman (2008) observed that *Bacillus amyloliquefaciens* filtrates (50% v/v) inhibited about 55% of the mycelial growth of *S. sclerotiorum* and reduced in 50% the formation of sclerotia. Ávila et al. (2005) observed that two isolates of *Trichoderma* completely inhibited the colony growth of *Sclerotium rolfsii*. An *in vitro* study performed by Zancan, Machado, Sousa and Matos (2012), assessed the effect of fungicides and a biological product based on *Trichoderma harzianum* on the formation of sclerotia by *S. sclerotiorum.* Their results showed that methyl thiophanate and fluazinam completely inhibited sclerotia formation at concentrations above 100 ppm and the biological control agent completely inhibited sclerotia formation at all tested concentrations. Figueirêdo et al. (2010) observed 37.04% and 32.94% reduction in severity of white mold of common bean using

an isolate of *T. harzianum* and methyl thiophanate fungicide, respectively. Isolates of *T. asperellum* reduced apothecia density of *S. sclerotiorum* and white mold severity in the common bean, increasing the number of pods per plant and bean production by more than 40% (Geraldine et al., 2013). The T-aloe isolate of *T. harzianum* exhibited biocontrol potential of *S. sclerotiorum*, assessed by *in vitro* inhibition of mycelial growth, possible mycoparasitism, increase of activity of peroxidase, superoxide dismutase and catalase, reduction of the injury caused by *S. sclerotiorum* on soybean leaf cell membrane, as well as promotion of soybean plant growth and increase of chlorophyll and total phenol content. Also, the defense-related genes *PR1*, *PR2*, and *PR3* were expressed in the leaves of T-aloe-treated plants (Zhang et al., 2016).

Barros et al. (2015) performed a study to select fungal isolates with antagonistic effects on *S. sclerotiorum* and observed that *Myrothecium* sp. filtrate showed the greater inhibition potential in a dual culture assay. When soybean plants were treated with this fungus and inoculated 3 days after with *S. sclerotiorum*, the lesion length of white mold was reduced by 70% at 21 days after inoculation compared with inoculated plants without fungal filtrate treatment. Zheng et al. (2011), searched among 105 fungal isolates for new biocontrol agents for *Verticillium dahliae* Kleb. (Glomerellales: Plectosphaerellaceae) in cotton and observed that one belonging to the genus *Myrothecium* showed effectiveness of 33.21% under greenhouse conditions.

The reduction on ascospore germination by *Myrothecium* sp. isolate 2 was the same as the chemical control fluazinam. *Sclerotinia sclerotiorum* develops apothecia from sclerotia followed by production of ascospores, which can be disseminated at short distances (Abawi & Grogan, 1979) and the infection in soybean plant in the field is primarily through ascospores infecting flower petals (Grau & Hartman, 2015). Therefore, products that reduce ascospore germination are important in controlling white mold in field conditions, as these structures are the only source of airborne inoculum of this disease. Sumida et al. (2015) verified under field conditions that procymidone and fluazinam fungicides (combined with benzalkonium chloride or alone), reduced the incidence of *Sclerotinia* stem rot in about 74%.

The inhibitive effect of volatile compounds from *P. macrosporus* on the mycelial growth and ascospore germination of *S. sclerotiorum* assessed in our experiments was reported too on the mycelial growth and sporulation of *Colletotrichum gloeosporioides* (Penz.) Penz., Sacc. (Glomerellales: Glomerellaceae) (Rodríguez et al., 2016) and on the bacterial pathogen *Pseudomonas syringae* pv. *garcae* (Botrel, 2013).

Organic volatile compounds (OVCs) are substances of low polarity and molecular weight that can easily transpose membranes and be released in the atmosphere and soil (Pichersky, Noel, & Dudareva, 2006). Many microorganisms produce a mixture of OVCs that may be toxic to beneficial and phytopathogenic fungi (Morath, Hung, & Bennet, 2012; Kottb, Gigolashvili, Großkinsky & Piechulla, 2015) by reducing mycelial growth, sporulation and germination (Li et al., 2012; Morath et al., 2012). Due to their efficient spread through soil porosity, organic volatile compounds could be an alternative strategy to control soil inhabitant fungi (Dudareva, Negre, Nagegowda, & Orlova, 2006).

Despite the aggressive inoculation methodology used in the experiment, soybean plants treated preventively with *V. minima, P. macrosporus* and *Myrothecium* sp. isolate 2 filtrates exhibited significantly lower disease severity during the 21 days of evaluation. Our results of white mold control with ASM suggest that this commercial chemical inducer does not efficiently control this disease in soybean plants and confirmed previous results on the same pathosystem (Barros et al., 2015).

The direct antagonistic activity of *P. macrosporus* filtrates and volatiles against *S. sclerotiorum* verified in our *in vitro* experiments could be complemented by a resistance induction mechanism already found in other pathosystems. *Phialomyces macrosporus* is reported in literature as a potential resistance inducer in coffee plants against *Pseudomonas syringae* pv. *garcae* (Botrel et al., 2018) and *Colletotrichum gloeosporioides* (Rodríguez et al., 2016) and in eucalyptus plants against *Puccinia psidii* Winter (Pucciniales: Pucciniaceae) (Pierozzi, 2013).

The *in vivo* experiment was carried out with inoculation of soybean plants with *S. sclerotiorum* mycelium, and therefore, reflects the ability of the tested biocontrol agents to inhibit mycelial growth. In this context, *P. macrosporus* and *V. minima* filtrates have a higher inhibitory effect. The high efficacy of *Myrothecium* sp. isolate 2 to inhibit ascospore germination was not assessed in our *in vivo* experiment due to the inoculation method used. New experiments should be performed with inoculation of plants with ascospore suspension to test the potential of *Myrothecium* sp. isolate 2 in controlling white mold derived from ascospore infection.

Collectively, our results underline the efficacy of *Myrothecium* sp. isolate 2, *P. macrosporus* and *V. minima* as biocontrol agents of soybean white mold. Further studies are needed to determine if there is a resistance induction mechanism being activated in soybean plants when the filtrates are sprayed prior to pathogen inoculation. Finally, studies are essential to determine how viable the use of these saprobe fungi is under field conditions, including aspects such as the optimal saprobe formulation, timing, and number of saprobe sprays.

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