

Full Length Research Paper

Evaluation of biocontrol properties of *Streptomyces* spp. isolates against phytopathogenic fungi *Colletotrichum gloeosporioides* and *Microcyclus ulei*

Diana Marcela Vinchira Villarraga^{1*}, María Elizabeth Méndez Tibambre¹, Ibonne Aydee García Romero¹, Zulma Rocío Suarez-Moreno² and Nubia Moreno-Sarmiento¹

¹Universidad Nacional de Colombia, Sede Bogotá, Instituto de Biotecnología, Carrera 30 No. 45-03, Edificio Manuel Ancizar, Bogotá D.C, Colombia.

²Investigación y Desarrollo. Empresa Colombiana de Productos Veterinarios VECOL S.A. Avenida El Dorado No.82-93 Bogotá D.C, Colombia.

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South American Leaf Blight (SALB) of the rubber tree, caused by *Microcyclus ulei* and foliar anthracnose caused by *Colletotrichum gloeosporioides*, are diseases that adversely affect rubber cultivation in America. Both diseases have a significant economic impact on this agricultural subsector. The aim of the present study was to evaluate the potential as biological control agents of three *Streptomyces* species strains, namely A20, 7.1 and 5.1, against *M. ulei* and *C. gloeosporioides*. The results of analysis of variance (ANOVA) and Tukey post-hoc test of the *in vitro* antifungal activity assays evidenced the potential of the three *Streptomyces* strains to inhibit *C. gloeosporioides* growth through the production of diffusible (A20 and 5.1) and volatile compounds (7.1). Furthermore, other results indicated that strain 5.1 had a high biocontrol activity against *C. gloeosporioides*, and thus such strain was selected for further evaluations as a possible biocontrol agent against *M. ulei*. *In vitro* assays suggested that active compounds produced by 5.1 inhibited *M. ulei* growth by interfering with conidia germ tube and stroma formation. Bioassay-guided fractionation with organic solvents of 5.1 fermentation broths, suggested that antifungal compounds produced by this strain were nonionic compounds of medium-polarity. Currently, studies are ongoing to elucidate the chemical structure of these antifungal compounds. These approaches aim to generate a biological control agent to provide the Colombian rubber subsector with a preventive measure for controlling *M. ulei* and *C. gloeosporioides*.

Key words: Biological control, foliar anthracnose, rubber tree, South American Leaf Blight (SALB), *Streptomyces* species.

INTRODUCTION

Hevea brasiliensis is a native plant of the Amazon basin, member of the Euphorbiaceae family used for rubber

production (natural rubber). Approximately, 10 million tons/year of natural rubber are produced from this tree,

*Corresponding author. E-mail: dmvinchirav@unal.edu.co. Tel: + (57)13165000/16972 -16970.

93% of the production comes from South East Asian countries such as Thailand, Malaysia, and Indonesia, 4.5% from Africa and only 2.5% from Latin American countries (Berthelot et al., 2014; Rivano et al., 2015). Even though, Colombia is not a natural rubber exporter, it possesses an ample natural rubber cultivation tradition located in the departments of Caquetá, Putumayo, Guaviare and Córdoba and more recently Santander and Meta with a cultivation area of 44,100 ha (Castellanos et al., 2009; Confederación Cauchera Colombiana, 2015). The difference observed in productivity between Asian and Latin American countries is generated in great part by the presence of an endemic disease known as the South American Leaf Blight (SALB) of the rubber tree caused by the Ascomycete *Microcyclus ulei* fungus (Gasparotto and Pereira, 2012). This phytopathogen infects mature fruits, stems, and young leaves (stage B) of the *Hevea* genus reducing the plant's growth, causing premature leaf falling, with reduction of its photosynthetic area. Moreover, it causes death of susceptible greenhouse clones and garden clones (Chee and Holliday, 1986; García et al., 2007; Gasparotto and Pereira, 2012). In addition to SALB, foliar anthracnose caused by *Colletotrichum gloeosporioides* is the second disease of fungal origin limiting *H. brasiliensis* natural rubber production (Guyot et al., 2005; Castro, 2011; Gasparotto and Pereira, 2012). This disease has generated an important impact on Colombian and Brazilian crops, with increasing incidence (Furtado and Trindade, 2005; García et al., 2007; Castro, 2011).

Despite the efficiency obtained from traditional chemical treatments for *M. ulei* and *C. gloeosporioides* in *H. brasiliensis*, high costs and environmental impact from airplane spraying has promoted the search for new strategies of biological control. For most cases, these microbial agents are capable of self-sustained growth after an initial inoculation, with mid- and long-term pathogen suppression; and less biological impact compared with traditional chemical control (Quimby et al., 2002; Palaniyandi et al., 2013; Yuliar et al., 2015). To generate this effect, biocontrol agents can use one or more mechanisms including nutrient competition, niche exclusion (competitive exclusion), signal interference of quorum sensing (Quorum quenching), parasitism, diffusible or volatile secondary metabolite production with antimicrobial activity (antibiosis), and induced systemic resistance in plants (Bloemberg and Lugtenberg, 2001; Hibbing et al., 2010).

Among the microorganisms evaluated as potential biocontrol agents, members of the *Streptomyces* genus stand-out. These gram-positive bacteria are characterized by mycelial growth similar to that of fungi. They are commonly isolated from terrestrial (soil, rhizosphere, and endosphere of plants) or marine environments, as free-living microorganisms or in

association with other organisms (Coombs and Franco, 2003; Cao et al., 2004; Kinkel et al., 2012). Furthermore,

they possess a diverse secondary metabolism that allows them to produce a great array of metabolites with antibacterial, antifungal, and antiviral activity. In addition, they are bioinsecticides, antitumoral and immune suppressors, among others (Omura et al., 2001; Hopwood, 2007; Kaur et al., 2014). In agriculture, numerous *Streptomyces* species have demonstrated capability of controlling diverse fungal phytopathogens of great agroindustrial impact (Samac and Kinkel, 2001; Taechowisan et al., 2003; Tian et al., 2004; Khamna et al., 2009; Zarandi et al., 2009; Gopalakrishnan et al., 2011; Li et al., 2012; Kanini et al., 2013). However, relatively few biological inoculants have been developed for crop use, mainly based on the poor association between the efficiency determined in the laboratory compared to the one observed in the greenhouse or the field (Bonaldi et al., 2015).

For the present study, three strains namely A20, 5.1 and 7.1, previously isolated by the Bioprocess and Bioprospecting Research Group from the National University of Colombia, were selected with the aim to verify its potential to act as biological control agents against *C. gloeosporioides* and *M. ulei* and to perform a preliminary characterization of the compound(s) associated with this activity. Suarez-Moreno et al. (2016, in press), taxonomically classified all three isolates within the *Streptomyces* genus, based on their biochemical profiles, colony macro- and microscopic characterization and sequencing of the rRNA 16S gene by Supplementary Tables 1 and 2. Within the initial characterization of these three strains, isolated from symptomatic rice, carnation and yam plants, their high potentials for bacterial and fungal phytopathogen control were determined. For these reasons, this work screened all three isolates aiming to determine their potential to control rubber fungal pathogens, looking forward to develop biological inoculants for natural-rubber farmers, as an approach to integral control for SALB and foliar anthracnose.

MATERIALS AND METHODS

Isolation, characterization and isolate growth

M. ulei and *C. gloeosporioides* isolation and identification

M. ulei and *C. gloeosporioides* isolates were obtained from affected foliage with SALB and anthracnose symptoms respectively from Corpoica's clonal garden - La Libertad Section, located in Villavicencio in the Mavalle S.A. plantation (Department of Meta, Colombia). For *M. ulei* isolation, pure *in vitro* cultures were obtained in M4 growth media (Junqueira et al., 1984) and identified by conidial asexual morphology reported for *Fusicladium heveae* (Anamorphic form of *M. ulei*, currently known as *Pseudocercospora ulei*) (Schubert et al., 2003; Hora et al., 2014). On the other hand, *C. gloeosporioides* isolation was performed by direct seeding on PDA of anthracnose symptomatic foliage that was previously

treated with hypochlorite (5%) and ethanol (70%) (Agostini et al., 1992; Pinzón, 2014). Obtained monospore cultures were morphologically characterized by macro- and microscopic description of the pathogen including aspect and color of the

mycelium, form, size, segmentation and conidia sporulation according to previous reports in the literature (Gunnell and Gubler, 1992; Barnet and Hunter, 1998; Pérez et al., 2003). For both isolates, internal transcribed spacer (ITS) analysis was performed using ITS1/ITS4 universal primers (White et al., 1990). Obtained amplicons were sequenced in duplicate, assembled and analyzed by BLASTN against GenBank data base.

Streptomyces spp. strains

Streptomyces spp. A20, 5.1 and 7.1 strains were isolated from rice rhizosphere soil from La Pilar (Venadillo) and El Puente (Armero) farms in the Department of Tolima (Colombia) and were identified in previous works of the research group (Suarez-Moreno et al., 2016, in press).

For antifungal activity evaluations, each *Streptomyces* strain was grown in ISP3 solid medium (Oat Meal 20.0 g.L⁻¹, Agar 18.0 g.L⁻¹, FeSO₄.7H₂O 0.001 mg.L⁻¹, MnCl₂.4H₂O 0.001 mg.L⁻¹, ZnSO₄.7H₂O 0.001 mg.L⁻¹, final pH: 7.3±0.2) or M3.7 liquid medium (5 g.L⁻¹ glucose, 5 g.L⁻¹ yeast extract, 2 g.L⁻¹ CaCO₃, 2 g.L⁻¹ tryptose, 10 g.L⁻¹ starch 2 g.L⁻¹ SO₄(NH₄)₂, 2 g.L⁻¹ NaCl, 1 mg.L⁻¹ FeSO₄ at pH 7.2 ± 0.2) depending on the assay performed. In solid cultures, each strain was incubated for 5 days at 30°C, whereas liquid cultures were incubated for 72 h at 30°C with constant agitation (150 rpm), and were used for the antifungal assays as described in the following (Shirling and Gottlieb, 1966).

Effect of *Streptomyces* spp. isolates on *C. gloeosporioides* mycelia growth

Streptomyces spp. A20, 5.1 and 7.1 strains were evaluated by dual culture plate assay to verify their ability to inhibit growth of *C. gloeosporioides* isolates C1, C2 and C3. Each *Streptomyces* strain was seeded on PDA in a straight line at 3 cm from the Petri dish periphery. 5 mm disc containing phytopathogen mycelia from each fungus (previously obtained from a 5 days-old solid PDA culture) was placed in the center of the petri dish. Media without *Streptomyces* was used as negative control. All media were incubated for eight days at 25°C and examined to verify inhibition areas between *C. gloeosporioides* and *Streptomyces* spp. A20, 5.1 or 7.1, respectively (Yuan and Crawford, 1995).

Production of diffusible and volatile compounds with antifungal activity

To evaluate whether the observed antifungal effect was due to diffusible or volatile compounds production, an agar well diffusion test and a volatile compound production assay was performed following CLSI 2011 guide, as well as recommendations suggested by Arrebola et al. (2010), respectively.

For agar well diffusion tests, 200 µl of a conidial suspension (10⁵ conidia.mL⁻¹) of *C. gloeosporioides* isolates C1, C2 or C3 was massively seeded in Petri dishes with 25 ml PDA. Wells of 7 mm in diameter were opened at a distance of 4 mm from the edges of the Petri dish. Each well was inoculated with 100 µl of A20, 5.1 or 7.1 liquid spent supernatants from liquid cultures obtained from each *Streptomyces* isolate, as described previously. 10 µl Clotrimazole (100 µg µl⁻¹) and 100 µl M3.7 of sterile media were used as positive and negative controls, respectively (CLSI, 2011). Plates were incubated for 48 h at 25°C, and inhibition halo diameters were recorded in triplicates for each evaluated sample (Equation 1).

Assays were performed three times using three biological replicates for each *Streptomyces* culture.

$$\text{Inhibition diameter} = \text{Total inhibition diameter} - \text{Well diameter} \quad (1)$$

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To test if antifungal activity of the bacterial strains was due to the production of volatile compounds, a double-dish chamber assay was carried out. Briefly, bases of two Petri dishes containing 25 ml of PDA were used. For the first Petri dish, *Streptomyces* spp. isolates A20, 5.1 and 7.1 were seeded. In the second one, a 5 mm agar disc containing *C. gloeosporioides* C1, C2 or C3 mycelium was seeded. Both plates were then confronted and sealed with Parafilm™ aiming to obtain a chamber with a shared atmosphere without direct contact between both microorganisms. As a negative control, one experiment was set without *Streptomyces* in the first Petri dish (Arrebola et al., 2010). After eight days of incubation at 25°C, radial growth of C1, C2 and C3 were measured and compared to the negative control. Inhibition percentage was calculated using Equation 2 as described by Taechowisan et al. (2012).

$$\text{Inhibition (\%)} = \frac{[(GDU-GDT) \times 100]}{GDU} \quad (2)$$

where GDU refers to the growth diameter in untreated control and GDT corresponds to the growth diameter in treatments.

Effect of *Streptomyces* A20 and 5.1 filtered extracts on *C. gloeosporioides* mycelial growth and conidial germination

To assess the effect of *Streptomyces* A20 and 5.1 filtered extracts on *C. gloeosporioides* mycelial growth, methods suggested by Anthony et al. (2004) were used. Briefly, *Streptomyces* A20 and 5.1 crude extracts were obtained by centrifuging at 6000 rpm for 10 min 500 ml from three independent liquid fermentation cultures of each strain in M3.7 medium. The obtained supernatant was filtered through 0.22 µm nitrocellulose membrane and subsequently used to supplement 25 ml of PDA. Increasing volumes of filtered spent-supernatants were used in order to obtain a medium with concentrations of 6, 4 or 2% (v/v) of extract per petri dish. Later, 7 mm discs from C1, C2 and C3 *C. gloeosporioides* isolates were placed in the center of each dish, and incubated at 25°C to evaluate the radial growth of each isolate every 24 h until day 17 post-inoculation. *C. gloeosporioides* growth on each treatment was compared to a negative control seeded on PDA without extract, and the growth inhibition percentage was obtained 17 days after treatment from Equation 2 (Anthony et al., 2004; Taechowisan et al., 2012).

Based on the results obtained from this assay, supernatant effect on conidia germination process of the three *C. gloeosporioides* isolates was further evaluated for strain *Streptomyces* 5.1. For this purpose, conidia germination counts were measured with treated and no-treated conidia by using lyophilized crude extracts from *Streptomyces* 5.1. To this end, 100 µl of a conidial suspension (1×10⁵ conidia.mL⁻¹) was supplemented with previously lyophilized 5.1 crude extract (obtained as described previously) and seeded onto PDA previously divided into 1 cm² squares. Four different concentrations of crude extract were evaluated (10, 25, 50 and 100 mg ml⁻¹). Conidia germination count was performed after 10 h incubation at 25°C. Percentage of germination inhibition was calculated in relation to the total number of conidia and the present germinated conidia in the sample as defined by Equation 3 (Palaniyandi et al., 2011).

$$\% \text{ Germination} = \left(\frac{\text{No. of germinated conidia} \times 100}{\text{No. of total conidia}} \right) \quad (3)$$

Streptomyces* 5.1 antifungal activity evaluation against *M. ulei

To determine the effects of active compounds produced by *Streptomyces* 5.1 on *M. ulei* pathogenic isolates growth, two antifungal assays were performed evaluating the percentage of

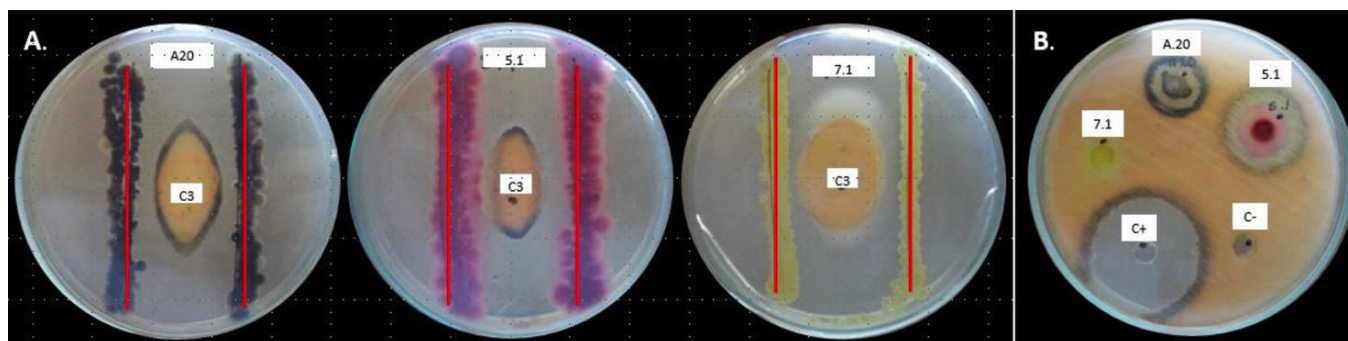


Figure 1. *C. gloeosporioides* C3 mycelial growth inhibition assay by: A. dual confrontation and B. well diffusion. C+: Clotrimazole 10 mg ml⁻¹; Media M3.7 C-: Media without inoculation.

germinated *M. ulei* conidia in suspension (1.65×10^5 conidia ml⁻¹) supplemented with four different concentrations of lyophilized *Streptomyces* 5.1 supernatant (10, 25, 50 and 100 mg ml⁻¹). 300 μ l of saline solution were added to *M. ulei* conidia suspension as negative control. Conidia germination was determined as described for *C. gloeosporioides* assays (Equation 3). Moreover, the effect of 5.1 extracts on *M. ulei* stroma formation was evaluated by seeding 100 μ l of treated conidia suspension on PDA with 5.1 supernatants. Stroma formation count on solid media was performed after 15 day incubation at 25°C, reporting growth as CFU ml⁻¹ (Rocha et al., 2011).

Preliminary characterization of *Streptomyces* 5.1 produced active metabolites

In order to isolate and characterize antifungal compounds produced by *Streptomyces* 5.1, three independent liquid fermentations were carried out in 1 L of M3.7 medium. Culture media was inoculated and grown at 30°C, under constant agitation at 150 rpm. Each culture was then centrifuged for 10 min at 5000 rpm, and the obtained supernatants were filter sterilized by a 0.22 μ m membrane. Spent supernatants were analyzed by sequential fractionation with dichloromethane and butanol (ratio solvent-supernatant 2:1 and 1:1 for each solvent, respectively) in a continuous liquid-liquid extraction system. The aqueous and organic extracts obtained were separated by decantation and dried through lyophilization or in a vacuum rotary evaporator (Labconco® Kansas City, MO USA), respectively.

All extracts were subsequently evaluated for *C. gloeosporioides* (C3) antifungal activity by using the agar well diffusion method described previously. Aqueous extracts were dissolved in distilled water to evaluate concentrations 10, 20, 50 and 100 mg ml⁻¹. Likewise, obtained organic extracts at 0.5, 0.8 and 1 mg ml⁻¹ were dissolved in DMSO (8% v/v). One liter of M3.7 sterile media was subjected to the same extraction process and evaluated under the same conditions as 5.1 samples in order to be used as negative control. As mentioned, this methodology was performed with three 5.1 biological replicates and the negative control, respectively.

Butanolic extracts obtained from isolate 5.1 which maintained antifungal activity against *C. gloeosporioides* were analyzed by Liquid chromatography-mass spectrometry (LC/MS) and Matrix-assisted Laser Desorption and Ionization Time of Flight mass spectrometry (MALDI-TOF). To this end, 2 mg of butanolic extract previously dissolved in ethanol (90% v/v) was injected into the

HPLC VWR-LaChrom coupled to an Amazon x mass spectrometry (Bruker Daltonics, Bremen Germany) at the Universidad Industrial de Santander (Santander, Colombia). Chromatography was run in an x Terra® RP18 5 μ m (4.6 x 250 mm) column with a nitrile acetate gradient with 0.075% formic acid-H₂O as mobile phase. Data were collected and analyzed using the Compass Data analysis (Bruker Daltonics®) and Mzmine 2.14.2® programs.

For MALDI TOF, an Autoflex (Bruker Daltonics, Bremen, Germany) mass spectrometry with a positive ion reflection mode was used. Identification and spec allocation were carried out automatically using a Flexanalysis software version 2.2 (Bruker Daltonics) and Mzmine. All m/z (mass to charge ratio) obtained by both methodologies were compared to those reported in the Streptome DB database (Lucas et al., 2013) and Antimarin Database in order to find compounds previously reported with the m/z ratios found in this study (Blunt et al., 2007).

Statistical analysis

All assays of the present study (either qualitative or quantitative) were performed in triplicate. To test for normal distributions for the quantitative data a Shapiro-Wilk test was carried-out. Additionally, it was evaluated if all replicas for each assay presented similar tendencies. Tukey test for each data group was performed to find atypical data. Analysis of variance (ANOVA) was used to evaluate the effect of different treatments on phytopathogen fungal growth. Significant differences among means were compared with a Tukey post-hoc test ($p = 0.05$). GraphPad Prism® (GraphPad Software, Inc.® 2012) was used for all analyses.

RESULTS

Streptomyces A20, 5.1 and 7.1 were capable of reducing different *C. gloeosporioides* isolates mycelial growth

The potential of *Streptomyces* A20, 5.1 and 7.1 to inhibit the growth of *C. gloeosporioides* isolates was initially determined by a confrontation dual culture test. In this assay, it was evidenced that *Streptomyces* A20 and 5.1 significantly reduced *C. gloeosporioides* mycelial growth

(Figure 1). Furthermore, agar well diffusion assays suggested that A20 and 5.1 antifungal activity could be generated by a diffusible antifungal metabolite production

(Figure 1B). It was determined that *Streptomyces* 5.1, presented significant differences compared with strain

Table 1. Diffusible compound production with antifungal activity by *Streptomyces* strains A20, 5.1 and 7.1.

Treatment	Inhibition halo (mm)		
	<i>C. gloeosporioides</i> C1	<i>C. gloeosporioides</i> C2	<i>C. gloeosporioides</i> C3
<i>Streptomyces</i> A20	11.33 ± 1.22 ^a	9.66 ± 1.0 ^a	10.89 ± 0.92 ^a
<i>Streptomyces</i> 5.1	17.22 ± 0.97 ^b	12.22 ± 1.56 ^b	14.22 ± 1.48 ^b
<i>Streptomyces</i> 7.1	0 ^c	0 ^c	0 ^c
Clotrimazole	34.78 ± 1.4 ^d	27.44 ± 2.06 ^d	29.78 ± 1.64 ^d
M3.7 media	0 ^c	0 ^c	0 ^c

Data shown represents mean in mm ± SD of three biological replicates per treatment. Different letters in the same column represent significant differences among treatments ($p < 0.05$).

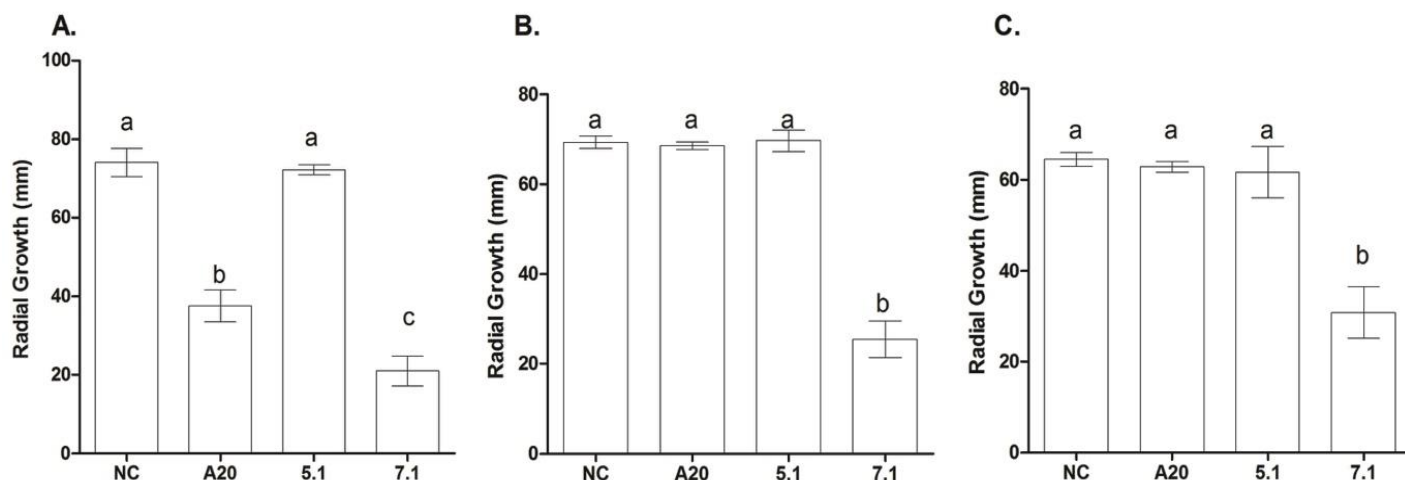


Figure 2. *C. gloeosporioides* C1, C2, and C3 mycelial growth inhibition by *Streptomyces* A20, 5.1 and 7.1 through volatile compound production. A. *C. gloeosporioides* C1, B. C2 and C. C3. Data illustrate mean (mm) ± SD of three biological replicates per treatment. Different letters indicate significant differences among treatments ($p < 0.05$).

A20 in regards to *C. gloeosporioides* mycelial growth inhibition. However, it was not comparable with the positive control (Table 1). Otherwise, strain 7.1 did not demonstrate any antifungal activity mediated by diffusible compounds. Thus to determine if *Streptomyces* 7.1 presented another means of control, it was decided to evaluate active volatile compounds against the three *C. gloeosporioides* isolates.

Through the double-dish chamber assay, it was identified that only 7.1 strain was capable to reduce *C. gloeosporioides* mycelial growth through the production of volatile compounds (Figure 2), generating growth inhibition percentages of 71.62, 63.28 and 52.27% against C1, C2 and C3, respectively. In contrast, A20 was only capable of inhibiting the growth of isolate C1 with an inhibition percentage of 49.24%. These results indicated that *Streptomyces* A20 and 5.1 characteristic

antifungal activity were mediated by the production of antifungal diffusible compounds, whereas for 7.1 such effect was the result of volatile nature compounds.

Streptomyces* 5.1 diffusible compounds had a fungistatic effect on *C. gloeosporioides

Considering the aforementioned results, it was desirable to confirm the efficiency and stability of the diffusible compounds produced by strain A20 and 5.1 based on its ability to reduce *C. gloeosporioides* mycelial growth through time. It was observed that diffusible compounds produced by 5.1, retarded mycelial growth for all three *C. gloeosporioides* isolates generating a maximum growth of 61.11, 51.45 and 38.71% for isolates C1, C2 and C3, respectively at the sixth day of incubation (6% treatment) (Figure 3A to C). An inverse correlation was observed between the pathogen growth and the concentration of

the compounds produced by 5.1 against *C. gloeosporioides* C3, being 6% concentration of the most active between the treatments. Interestingly, there were 146 Afr. J. Microbiol. Res.

no statistical differences within 4 and 6% treatments in the antagonistic assays against C1 and C2, which could be possibly associated with susceptibility differences

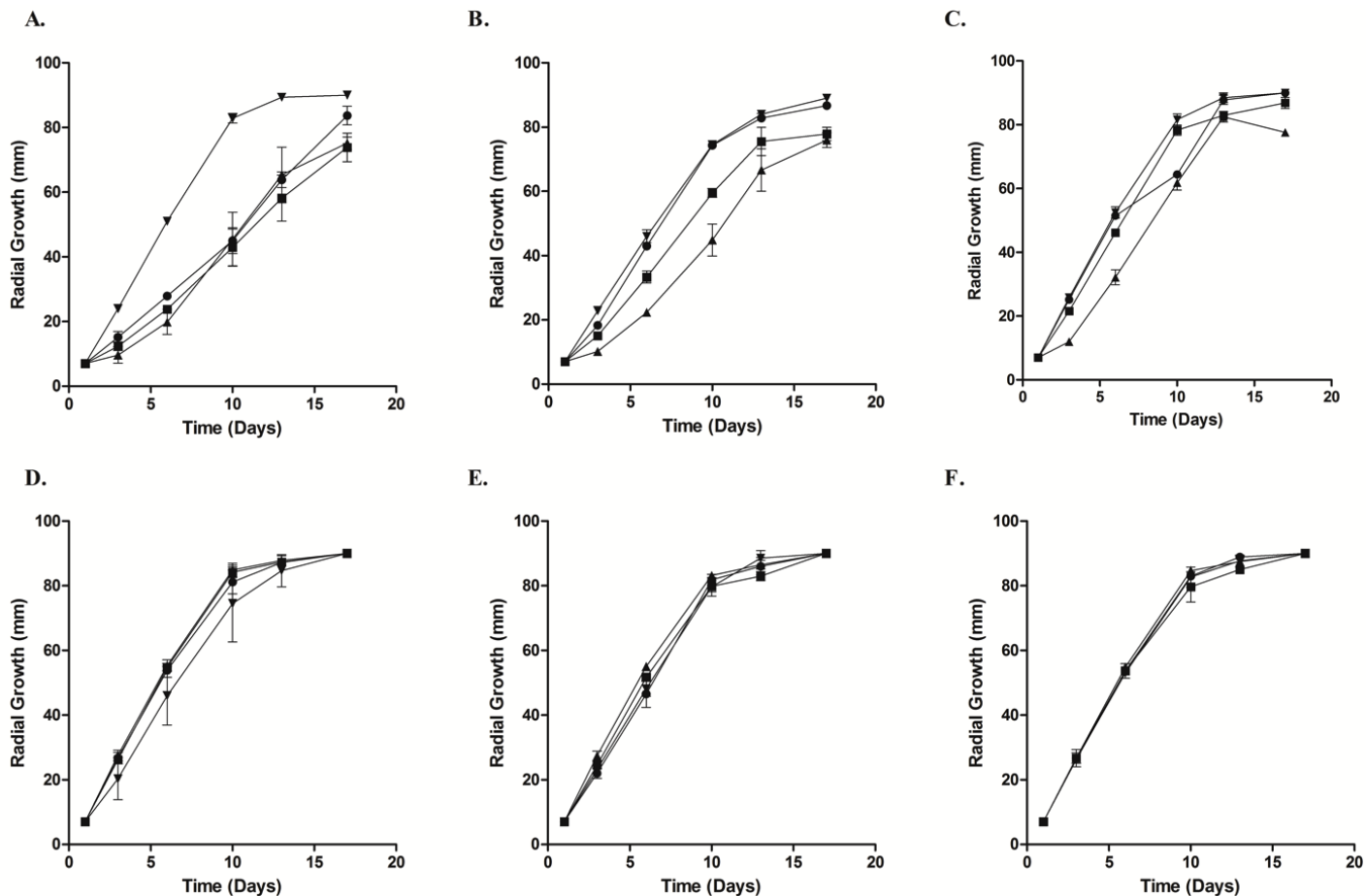


Figure 3. Effect of 5.1 and A20 produced compounds on *Colletotrichum gloeosporioides* C1, C2 and C3 mycelial growth. In the figure, sections A-C corresponds to 5.1 treatments and D-F to A20 treatments. A and D are the assays against to C1, B and E against to C2 and C and F against to C3, respectively. Data presented as mean (mm) \pm Standard Deviation (SD) of six biological replicas for each treatment (2%v/v \bullet , 4%v/v \blacksquare , 6%v/v \blacktriangle , Negative control \blacktriangledown).

Table 2. Growth inhibition percentage of *C. gloeosporioides* isolates treated with A20 and 5.1 crude extracts.

Isolate	Streptomyces 5,1			Streptomyces A20		
	2%	4%	6%	2%	4%	6%
<i>C. gloeosporioides</i> C1	7.037 \pm 3.195 (a, \square)	17.96 \pm 4.94 (b, \square)	16.48 \pm 2.15 (b, \square)	0 (c, \square)	0 (c, \square)	0 (c, \square)
<i>C. gloeosporioides</i> C2	2.622 \pm 0.91 (a, \dagger)	12.55 \pm 2.29 (b, \dagger)	14.61 \pm 2.56 (b, \square)	0 (c, \square)	0 (c, \square)	0 (c, \square)
<i>C. gloeosporioides</i> C3	0 (a, \dagger)	3.519 \pm 1.91 (b, \square)	13.7 \pm 0.90 (c, \square)	0 (a, \square)	0 (a, \square)	0 (a, \square)

Data shown represents mean percentage \pm SD of three biological replicates per treatment at seventeen day of incubation. Different letters in the same row represent significant differences among treatments ($p < 0.05$). Different symbols in the same column represent significant differences among treatments ($p < 0.05$).

among the three isolates of *C. gloeosporioides* to the secondary metabolites produced by 5.1.

After ten days of incubation, the inhibitory effect for all 5.1 treatments was reduced to such extent that at the end

of the evaluation (Day 17), only the 6% treatment presented inhibition percentages greater than 10% against the three *C. gloeosporioides* isolates (16.48, 14.61 and 13.7% for C1, C2 and C3 respectively) (Table

2 and Figure 3A to C). These results suggest that the effect generated by *Streptomyces* 5.1 active compounds is fungistatic and its stability after a single application has

a maximum of 10 days (last measurement where the inhibitory percentage was above 30%).

On the other hand, treatments performed with three Villarraga et al. 147

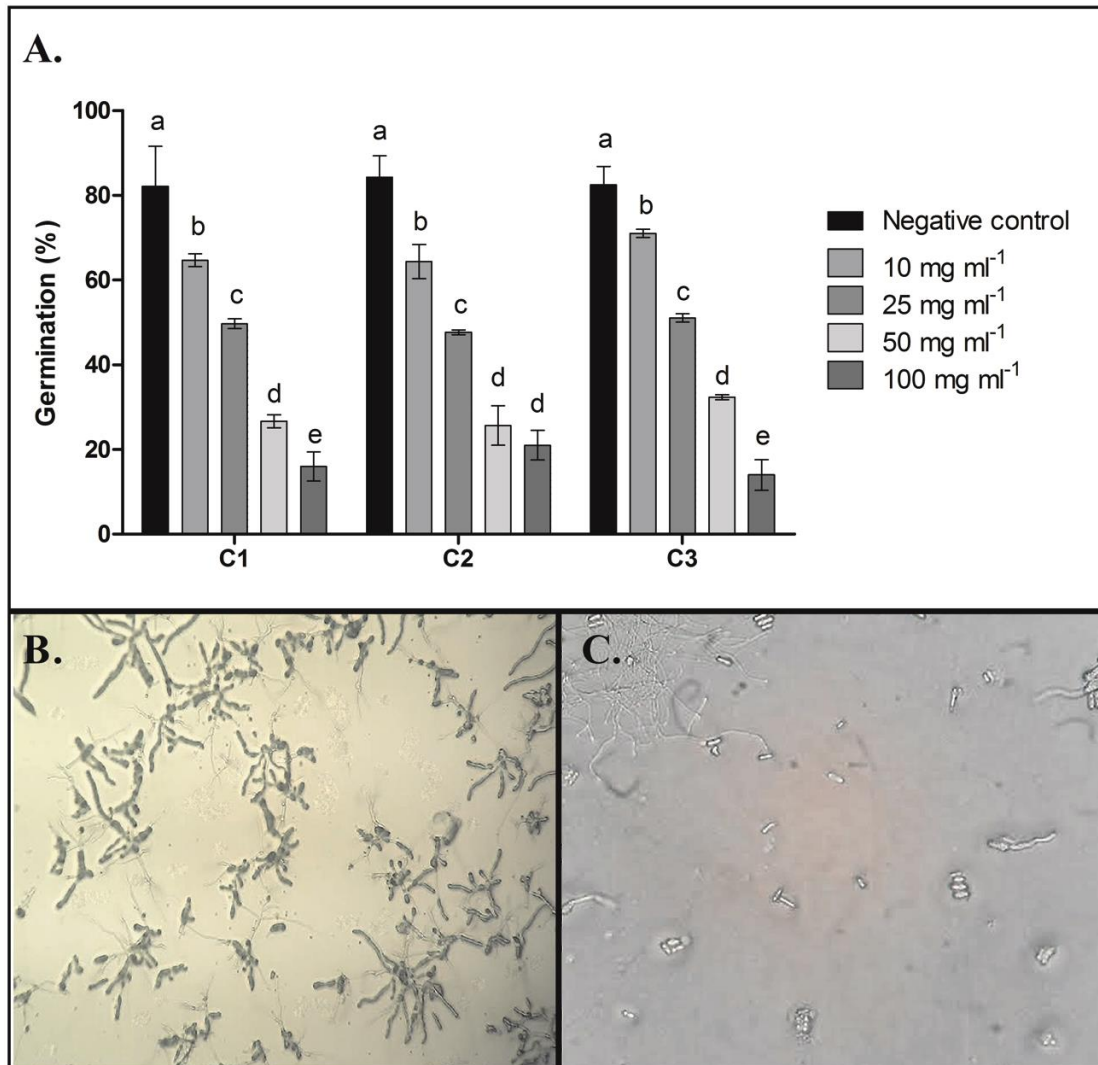


Figure 4. *Streptomyces* 5.1 active compound effect on conidia germination of three *C. gloeosporioides* isolates. A. Germination percentage of *C. gloeosporioides* isolates: C1, C2 and C3 conidia suspension with different concentrations of *Streptomyces* 5.1 lyophilized supernatant. Data presented are mean of germination (%) \pm SD of six biological replicas per treatment. B. Light microscopy of non-treated conidia of *C. gloeosporioides* C3. C. Light microscopy of 100 mg ml⁻¹ treatment conidia of *C. gloeosporioides* C3.

concentrations of the lyophilized crude extract from *Streptomyces* A20 showed no deleterious effect on the growth of the three *Colletotrichum* isolates at the end of the incubation time (Table 2 and Figure 3D to F). Suggesting that the active metabolite produced by A20 either is unstable and was degraded during the test or, it is not produced under the fermentation conditions evaluated at the concentration sufficient to inhibit phytopathogen growth. Due to these results, this isolate was excluded for the following analysis.

Treatment of *C. gloeosporioides* C1, C2 and C3 conidia with different concentrations of 5.1 lyophilized crude extracts, allowed to determine that the antifungal compounds produced by this strain are able to inhibit the phytopathogen's conidia germination (Figure 4A). As it was observed for mycelial development, the inhibitory effect was inversely proportional to the concentration of the lyophilized extract, being 100 mg ml⁻¹ of the most efficient treatment with an inhibition percentage of 79.5%.

In these experiments, light microscopy evidenced that conidia presented the characteristic swelling of the first

stage of germination, in particular for 50 and 100 mg·ml⁻¹ treatments, however, in these treatments conidia were unable to form the germ tube (Figure 4B and C). This result suggests that active compound(s) produced by 148 Afr. J. Microbiol. Res.

Streptomyces 5.1 possibly interfere with the cell wall membrane formation in *C. gloeosporioides*, which could impair the formation of germ tubes. Nevertheless, this

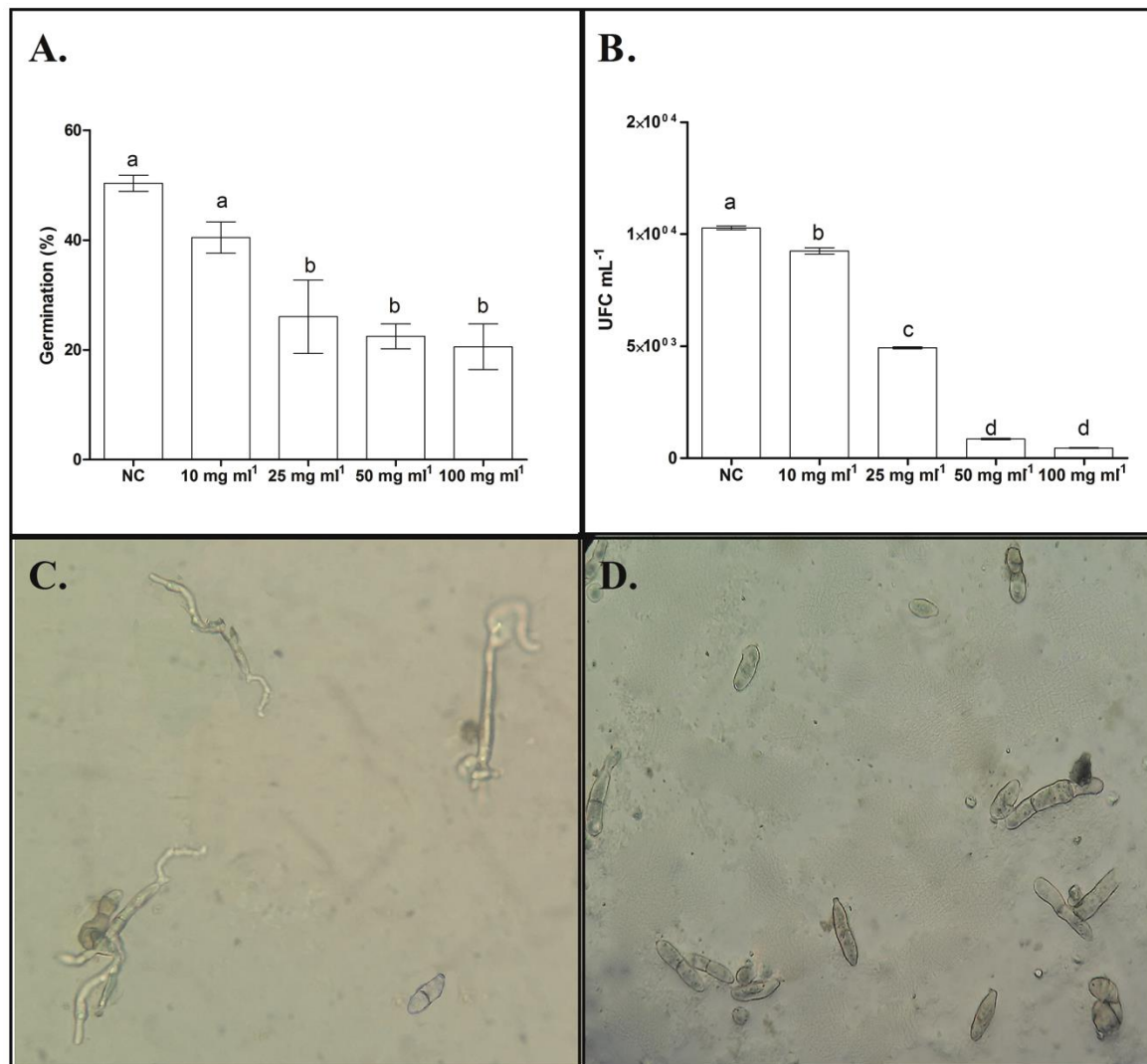


Figure 5. *Streptomyces* 5.1 supernatant effect on *M. ulei* conidia germination and stroma formation. A. Germination percentage for conidia suspension. B. *M. ulei* stroma/ml count treated with different concentrations of lyophilized *Streptomyces* 5.1 supernatant. Data in % represent mean ± SD of three biological replicas/treatment. Different letters depict significant differences among treatments p < 0.05. C. Light microscopy of non-treated *M. ulei* conidia with the characteristic bipolar germination tube form. D. Light microscopy of *M. ulei* conidia treated with 100 mg ml⁻¹ of 5.1 supernatant. In the figure, NC corresponds to Negative control.

hypothesis must be confirmed through cytochemical and microscopic complementary studies. In addition, it had to be established if treated conidia remained viable (fungistatic effect) or were incapable of forming new vegetative mycelium, once they lost contact with the antifungal compound (fungicidal effect).

Streptomyces* 5.1 active compound production against *M. ulei

To determine *Streptomyces* 5.1 biocontrolling potential against the phytopathogenic fungus *M. ulei*, two approaches were carried-out. First, compound capacity to inhibit *M. ulei* conidia germination process present in lyophilized supernatant from 5.1 cultures was determined. As can be observed from Figure 5, *M. ulei* conidia suspension treatment at different 5.1 lyophilized supernatant concentrations significantly reduced germination percentage at 25, 50 and 100 mg·ml⁻¹. On

the contrary, treatment with 10 mg·ml⁻¹ did not present any significant difference compared to negative control (Figure 5A).

Furthermore, it was evidenced that for all 5.1 supernatant treatments (25, 50 or 100 mg·ml⁻¹) conidial

germ tube formation was not completely inhibited. Nonetheless, germ tubes did not present typical *M. ulei* characteristics, where germ tube formation develops at

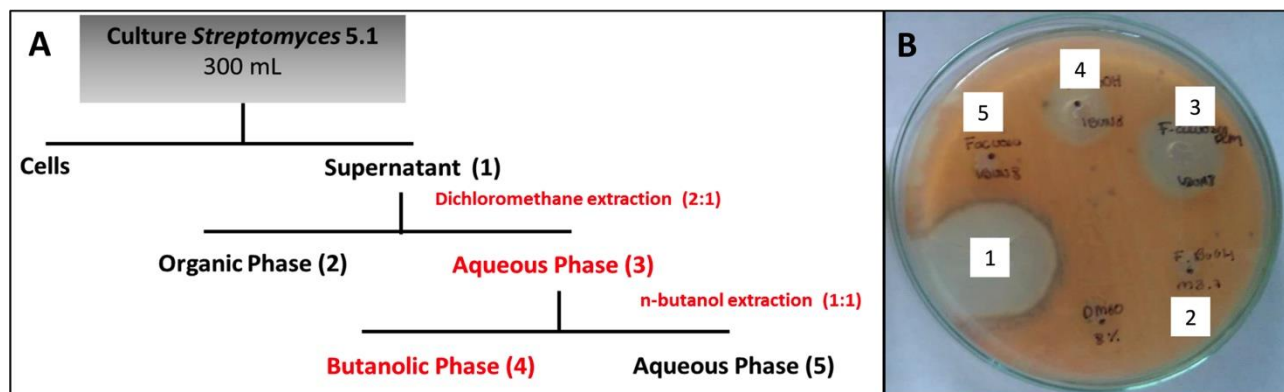


Figure 6. Bioassay guided fractionation of *Streptomyces* 5.1 active compounds. A. Flow diagram with solvents of different polarity. B. Antifungal assay against *C. gloeosporioides* C3 for fractions obtained from strain 5.1. Numbers in the figure of the right correspond to the ones presented in Figure 6A. As a solvent control DMSO (8% v/v) was used.

both poles of the conidia (Figure 5C). For the treated conidia, germination occurred only at one pole of the conidia, and it was not possible to observe the typical division of the germ tube (Figure 5D).

To determine if the *M. ulei* deficient conidia (classified as germinated conidia during germination count) had a subsequent effect on stroma formation (*M. ulei* vegetative growing stage), 100 µl of conidia suspension treated with phytopathogen were seeded in PDA to evaluate mycelia growth of the fungus after being exposed to compounds produced by *Streptomyces* 5.1. It was observed that all treatments with different supernatant concentrations lead to a significant reduction in the number of formed stroma (Figure 5B). This result suggested that conidia with deformed germination were incapable of generating a vegetative mycelium, thus reducing the number of stromas formed per dish. It can therefore be inferred that compounds produced by strain 5.1 have a fungicidal activity against *M. ulei*.

***Streptomyces* 5.1 produces different compounds with medium polarity of non-ionic nature**

To preliminarily determine the nature of active compounds produced by *Streptomyces* 5.1, bioguided fractionation of *Streptomyces* 5.1 supernatant was performed with solvents of different polarity (Figure 6A), followed by antifungal activity evaluation of all obtained fractions against *C. gloeosporioides* isolate C3. Our results indicated that, antifungal compounds produced by strain 5.1 were retained in the aqueous phase of the first fractioning when dichloromethane was used as a solvent.

Subsequent treatment of this aqueous phase using butanol as a solvent favored organic phase (butanolic) compound extraction (Figure 6B). The solubility profile identified for *Streptomyces* 5.1 antifungal compounds suggested that active compounds are characterized by being non-ionic, with medium polarity.

Analysis of *Streptomyces* 5.1 butanolic fractions through LC/MS and MALDI TOF evidenced the presence of six peaks with m/z values of 252,25, 309,17, 427,24, 610,06, 610,39 and 778,28. These peaks were not observed in blank media (Figure 7). Therefore, it was assumed that they were produced by *Streptomyces* 5.1. Out of these peaks, the major peak 610,39 was recognized by both techniques, thus becoming the most interesting for structure elucidation.

Search of compounds produced by different *Streptomyces* spp. presenting an antifungal activity or any antimicrobial activity, in addition to similar m/z values to those reported in this study were not found, which suggests that these compounds have not been described yet. Therefore, it is necessary to continue their purification and structural elucidation by means of complementary techniques to those utilized in the present study.

DISCUSSION

To date, Integrated system crop management of natural rubber (*H. brasiliensis*), which is a crop of agricultural importance, constitutes a main focus in its production chain. Within these systems, management with phytopathogens has a major role given its direct effect on

the crop's productivity. *C. gloeosporioides* and *M. ulei* fungi are known phytopathogens affecting *H. brasiliensis*, traditionally managed with chemicals (Castro, 2011; Berthelot et al., 2014). However, high costs, environmental impact of these practices and emergence

of strains resistant to fungicides has generated a need to search for new alternatives for microorganism handling; for instance, the development of biological inoculants with antifungal capabilities (Fravel, 1998; Compant et al., 2005). In this sense, this present work evaluated the

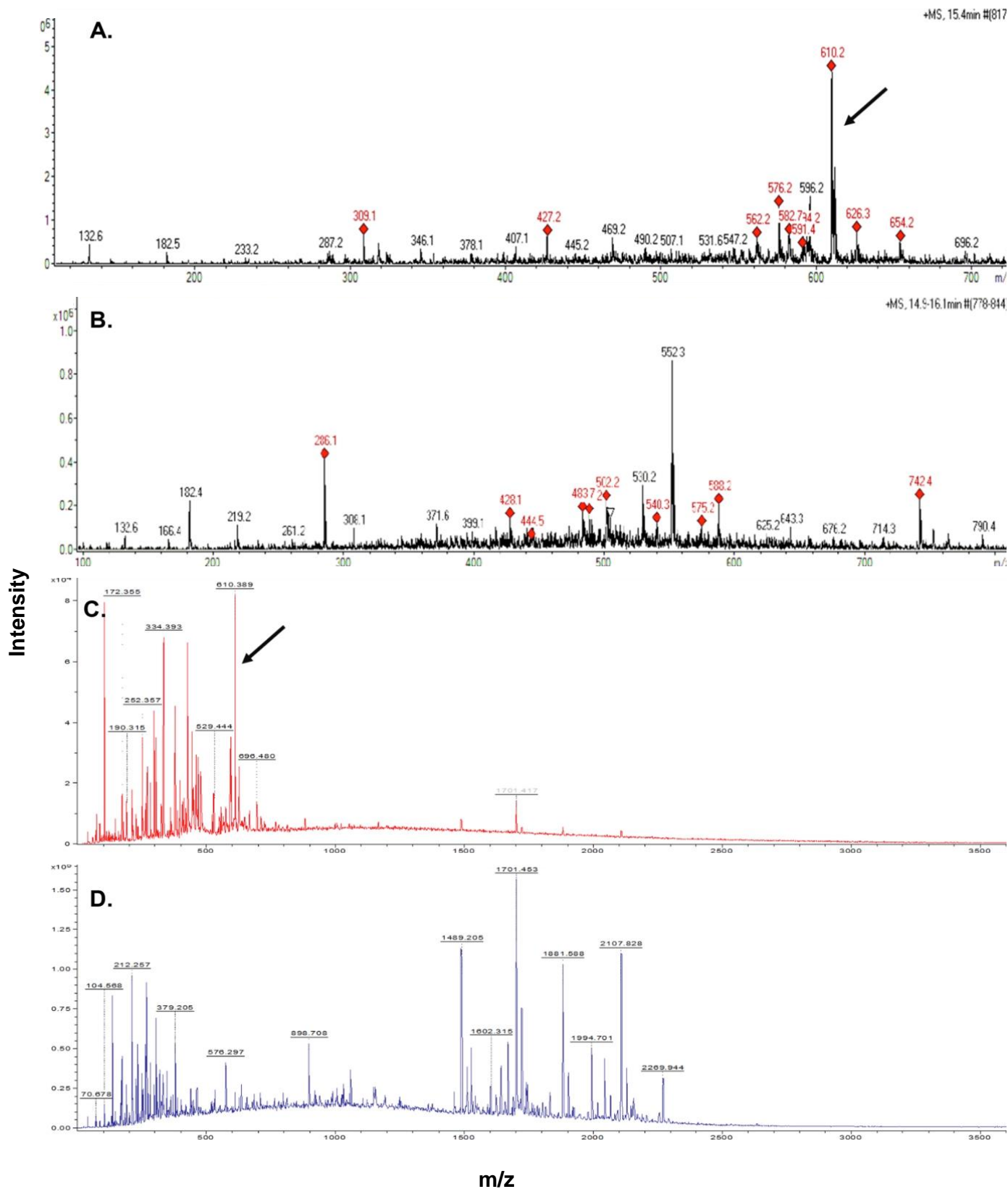


Figure 7. Mass spectrometry of *Streptomyces* 5.1 supernatant butanol fractions obtained by LC-MS and MALDI TOF. Figures A and C, correspond to the Spectrum obtained by MALDI-TOF and LC/MS of 5.1 butanol fraction, and B and D correspond to the media. Highlighted is the main peak m/z 610 present in *Streptomyces* 5.1 butanol fraction recognized by both chromatographic techniques.

possibility of three *Streptomyces* strains previously characterized for their potential to control *Burkholderia glumae* and *Pseudomonas fuscovaginae* phytopathogens in rice cultivations, as biocontrol agents of natural rubber

(*H. brasiliensis*) crops for the pathogens *M. ulei* and *C. gloeosporioides*.

Streptomyces genus is recognized for its ample secondary metabolism, which allows them to produce diverse bioactive compounds of interest for biological control including antibiotics, lytic enzymes (chitinases and glucanases, proteases, among others) and/or siderophores (Qin et al., 2011). An initial evaluation of three *Streptomyces* strains against three *C. gloeosporioides* isolates evidenced their potential to inhibit mycelial growth of the phytopathogenic agent (Figure 1 and Table 1). Further assays permitted to establish that *Streptomyces* 7.1 was able to reduce *C. gloeosporioides* mycelial growth by producing volatile compounds with an inhibition percentage ranging from 52 to 71% (Figure 2). Antifungal activity by *Streptomyces* genus volatile compound production for phytopathogen control has been previously evaluated by Wan et al. (2008), who evidenced *Streptomyces platensis* F1 capacity to inhibit *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Botrytis cinerea* growth, and to reduce the incidence and severity of diseases caused by these pathogens in foliage tissues of rice plants, turnips, and strawberry fruits, respectively, under controlled atmosphere conditions (Wan et al., 2008).

Likewise, Li et al. (2010, 2012) developed similar studies where *Streptomyces globisporus* JK-1 capability to inhibit *B. cinerea* and *Penicillium italicum* growth in tomato plants (*Lycopersicon esculentum*) and Shatang mandarin fruit (*Citrus microcarpa*) was verified, with promising results for growth control of these pathogens through *in vivo* assays under shared atmosphere conditions (Li et al., 2010, 2012). Nonetheless, use of these types of metabolites is recommended for controlling diseases in environments that favour the presence of a microatmospheres, where a higher concentration of a volatile compound is achieved, as it is the case for control of pathogens in soil, management of foliage diseases under controlled conditions in greenhouse and use of post-crop fruit storage containers (Wan et al., 2008; Li et al., 2012). For these reason, application of microorganisms able to produce volatile compounds for management of foliage phytopathogens in *H. brasiliensis* under field cultivation conditions would not be recommended; due to possible reduction in metabolite efficiency by compound dilution effect, since there would not be a controlled atmosphere environment. Therefore, strain 7.1 was removed for posterior assays in the present study, even though its potential as a biocontrol agent should be studied in future research aimed for controlling post-crop disease control.

On the other hand, *Streptomyces* A20 and 5.1 were capable of inhibiting *C. gloeosporioides* growth by

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producing extracellular diffusible metabolites, as suggested by the antifungal activity retained in liquid culture filtered supernatants from both microorganisms (Figure 1 and Table 1). Prapagdee et al. (2008) reported

similar results to those obtained in our study. They described *C. gloeosporioides* 50% radial growth inhibition using filtered extract of *Streptomyces hygrosopicus* during its exponential growth. Furthermore, Shahbazi et al. (2014) demonstrated antifungal activity of *Streptomyces* strains P8 and P42 isolated from chili pepper (*Capsicum annum* L. Kulai) rhizosphere soils evaluated against *C. acutatum*, *C. capsici* and *C. gloeosporioides* phytopathogens. Antifungal activity was due to compounds present in supernatants from liquid culture of *Streptomyces* strains (Shahbazi et al., 2014). In both studies, lysis of the phytopathogen's hyphae cell wall by chitinase was evidenced.

Results obtained in the present study indicate that strain 5.1 had the highest antifungal activity against *C. gloeosporioides* and *M. ulei*, which was statistically significant compared to results obtained with *Streptomyces* A20 (Table 1), being the first report (as far as we know) that describe the antifungal activity of a *Streptomyces* isolate against *M. ulei*. Furthermore, results obtained from the bioassay guided fractionation suggested that enzymatic lytic activity was not produced, since antifungal activity remained without alterations after supernatant fractionation with two different organic solvents of different polarity (dichloromethane and butanol). Since, these two solvents have the capability to degrade enzymes, it can be suggested that *Streptomyces* antifungal compounds can be classified as a non-enzymatic metabolite with antifungal activity. Additionally, analysis of 5.1 filtered supernatants suggested that active compounds of this microorganism suppress *C. gloeosporioides* and *M. ulei* conidia germination process, as well as stroma formation for the later one, with a percentage inhibition of 79.5, 40.88 and 95.56%.

Light microscopy analysis, demonstrated that contact between a *Streptomyces* extract (25 to 100 mg·ml⁻¹) and *C. gloeosporioides* or *M. ulei* conidia, reduced conidia development and germ tube elongation for each pathogen, disrupting mycelia development. This type of activity could interrupt the penetration process of both phytopathogenic fungus in rubber leaves inhibiting or delaying the infection in the plants, as it has been previously reported for others *Streptomyces* species evaluated for biological control of *C. gloeosporioides*, *Fusarium oxysporum* f. sp. *lycopersici*, *Verticillium albo-atrum* and *Alternaria solani*. An inversely proportional association was observed between conidia percentage germination and extract concentration, containing the active compound evaluated (El-Abyad et al., 1993; Palaniyandi et al., 2011), as it was observed in the present study (Figures 4 and 6).

However, mechanism of action against the phytopathogens for antifungal compounds isolated from

Streptomyces 5.1 is still un-known. To elucidate such activity, first of all it is necessary to establish the type of metabolite produced. The present study evidenced *Streptomyces* 5.1 produced at least two non-ionic

compounds. Analysis through LC/MS and MALDI TOF of 5.1 fermentation butanol extractions revealed six unique strain peaks; with the highest peak at a molecular mass of 610.2 m/z. Data obtained from these experiments did not correlate with compounds reported in StreptomeDB or Antimarin Database, therefore, these compounds could be assumed as new. However, it is necessary to perform a discrimination process among the six peaks to determine which of them is responsible for the observed antifungal activity. Moreover, complementary assays must be carried out to reveal the compounds and verify the new molecule hypothesis.

Last, despite our results it is necessary to establish additional *in vitro* and *in vivo* experiments focused on (i) evaluating *Streptomyces* 5.1 and *C. gloeosporioides* and *M. ulei* population behavioral dynamics in association with rubber plants (*H. brasiliensis*), taking into account the plant's defense response to *Streptomyces* 5.1. inoculation, (ii) comparisons between incidence and severity of the disease treated with 5.1, and (iii) determining its efficiency with respect to the traditional chemical control.

This study evidenced that *Streptomyces* 5.1 strain competence to produce extracellular metabolites with antifungal activity to inhibit mycelial growth in addition to impede *C. gloeosporioides* and *M. ulei* germination process under *in vitro* conditions. Obtained results demonstrated *Streptomyces* 5.1 potential to be utilized as a biological control agent destined for rubber plant (*H. brasiliensis*) protection against foliar anthracnose and SALB. To the best of our knowledge this is the first report establishing *Streptomyces* genus for important phytopathogen fungi control of the natural rubber.

Conflict of Interests

The authors have not declared any conflict of interests.

Abbreviations

DMSO, Dimethyl sulfoxide; **ISP**, International *Streptomyces* Project; **ITS**, internal transcribed spacer; **LC-MS**, liquid chromatography–mass spectrometry; **Mbp**, megabase pairs; **PDA**, potato dextrose agar; **ppm**, parts per million; **SALB**, South American Leaf Blight.

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Supplementary Table 1. Biochemical characterization of *Streptomyces* A20, 5.1 and 7.1.

Strain	Catalase	Oxidase	Glucose	Sucrose	Fructose	Rhamnose	Arabinose	Inositol	Xylose	Mannose
A20	+	-	+	-	-	-	-	-	-	-
7.1	+	-	+	+	+	-	-	-	-	-
5.1	+	-	+	-	-	-	-	+	-	-

Single colonies were characterized by their colony morphology in ISP2, ISP3, ISP4, Nutrient agar and Mueller Hinton Agar. Catalase, oxidase and their carbon source utilization were tested, as suggested for *Streptomyces*-like bacteria, as described previously (Shirling and Gottlieb, 1968; Goodfellow, 2012).

Supplementary Table 2. 16S rRNA phylogenetic analyses.

Strain	<i>Streptomyces</i> species	Similarity (%)
A20	<i>S. racemochromogenes</i>	99.93
	<i>S. polychromogenes</i>	99.86
	<i>S. flavotricini</i>	99.66
5.1	<i>S. angustmycinicus</i>	98.77
	<i>S. abikoensis</i>	98.69
	<i>S. sioyaensis</i>	98.64
7.1	<i>S. corchorusii</i>	99.86
	<i>S. canarius</i>	99.79
	<i>S. olivaceoviridis</i>	99.73

PCR amplification, sequencing and analysis of the entire 16S rRNA locus was performed as described by Wang et al. (2013). The cleaned PCR products were directly sequenced using universal primers 27F, 500F, 818R and 1492R by Macrogen (Korea). Closely related sequences were obtained from EZTaxon (Kim et al., 2012) and are listed. 16S rRNA sequencing results showed that our strains display high similarity to those of the *Streptomyces* species, locating strains A20, and 7.1 a closely related to *S. racemochromogenes*, and *S. corchorusii*, whereas strain 5.1 was related to the *S. sioyaensis* clade, respectively.