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Secondary metabolites produced by *Microbacterium* sp. LGMB471 with antifungal activity against the phytopathogen *Phyllosticta citricarpa*

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Abstract

The citrus black spot (CBS), caused by *Phyllosticta citricarpa*, is one of the most important citrus diseases in subtropical regions of Africa, Asia, Oceania, and the Americas, and fruits with CBS lesions are still subject to quarantine regulations in the European Union. Despite the high application of fungicides, the disease remains present in the citrus crops of Central and South America. In order to find alternatives to help control CBS and reduce the use of fungicides, we explored the antifungal potential of endophytic actinomycetes isolated from the Brazilian medicinal plant *Vochysia divergens* found in the Pantanal biome. Two different culture media and temperatures were selected to identify the most efficient conditions for the production of active secondary metabolites. The metabolites produced by strain *Microbacterium* sp. LGMB471 cultured in SG medium at 36 °C considerably inhibited the development of *P. citricarpa*. Three isoflavones and five diketopiperazines were identified, and the compounds 7-*O*- β -D-glucosyl-genistein and 7-*O*- β -D-glucosyl-daizone showed high activity against *P. citricarpa*, with the MIC of 33 μ g/mL and inhibited the production of asexual spores of *P. citricarpa* on leaves and citrus fruits. Compounds that inhibit conidia formation may be a promising alternative to reduce the use of fungicides in the control of CBS lesions, especially in regions where sexual reproduction does not occur, as in the USA. Our data suggest the use of *Microbacterium* sp. LGMB471 or its metabolites as an ecological alternative to be used in association with the fungicides for the control of CBS disease.

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Introduction

The ascomycete *Phyllosticta citricarpa* (teleomorph: *Guignardia citricarpa*) is the causal agent of citrus black spot (CBS) disease (Glienke et al. 2011). The CBS was first reported in 1895 in Australia, and in 1940 was found in Brazil (Kotzé 1981). The disease has worldwide occurrence, reported in Africa, Oceania, South America (Cartens et al. 2017), and the USA (Schubert et al. 2012; Er et al. 2014). Although the fungus has recently been reported in Europe (Guarnaccia et al. 2017), the disease is not yet present, and citrus fruits with CBS lesions are subject to quarantine regulations in the European Union (EU 2015). The regulations restrict market access for countries with the presence of CBS disease and reduce the availability of citrus fruits to consumers in the off-season in Europe (Agostini et al. 2006).

The control of CBS is entirely dependent on the application of fungicides, and the fruits need to be sprayed four to five times during the infection period, which lasts 4 to 5 months after fruiting (Silva Jr. et al. 2016). These chemical control methods reduce the disease outbreak but significantly increase the price of production, up to half of the total operational costs (Maggione 1998; Silva Jr. et al. 2016). Fungicides have also been associated with negative environmental impacts (Fialho et al. 2016). Initially, CBS disease was controlled using benomyl; however, in Southern Africa, 80% of fungal isolates are resistant to this treatment, and resistant strains have been spread across almost all major citrus producing areas (Schutte et al. 2003).

In addition, carbendazim, one of the few effective fungicides for the control of *P. citricarpa*, was banned in the USA (U.S. EPA 2012) after detection of the chemical in imported citrus juice. To further increase the problem of CBS control, symptomless fruit may develop symptoms during transport (Er et al. 2013, 2014). Alternatives for post-harvest control involve the use of benzimidazoles; however, these compounds are effective in delaying, but not eliminating, CBS expression after harvest (Carvalho et al. 2014). According to Silva Jr. et al. (2016), currently, the only chemicals used in the control of CBS in the state of Sao Paulo, Brazil, are strobilurins (quinone outside inhibitors, QoI) and copper-based fungicides.

The concern with the control of CBS disease stimulated the search for alternative methods for the control of the fungus *P. citricarpa*. As a strategy, our group has been investigating endophytes of medicinal plants for the CBS biological control, which have resulted in the description of new species with biological potential (Savi et al. 2015; Hokama et al. 2016; Santos et al. 2016; Peña et al. 2016; Tonial et al. 2017). In addition, endophytic strains isolated of citrus leaves have also been investigated (Mariduena-Zavala et al. 2014; Er et al. 2014). The use of microorganisms and/or their secondary metabolites in a biological control scenario can reduce fungicide applications and consequently decrease the costs of citrus production and environmental impacts caused by current treatments (Fialho et al. 2016; Santos et al. 2016). In this context, we explored a pre-established collection of actinomycetes (Gos et al. 2017) for the production of secondary metabolites to control the *Phyllosticta citricarpa* the causal agent of citrus black spot disease.

Materials and methods

Biological source

The strains utilized in this study were isolated as endophytes from the medicinal plant *Vochysia divergens*, collected in the Pantanal sul-mato-grossense wetland region of Brazil and identified by 16S rRNA phylogenetic analysis (Gos et al. 2017). *P. citricarpa* strain LGMF06 was isolated from citrus black spot lesions. The endophytes and phytopathogen are stored in the biological culture collection of the Laboratory of Genetics of Microorganisms (LabGeM) from Federal University of Paraná (<http://www.labgem.ufpr.br/>).

Selection of culture conditions

Isolates were inoculated in 50 mL of SG medium (Shaaban et al. 2011), incubated for 3 days at 36 °C and 180 rpm. Subsequently, 1 mL from the pre-culture was inoculated in media SG and R5A (100 mL) (Fernández et al. 1998) and incubated for 10 days at two different temperatures, 28 °C and 36 °C, both at 180 rpm. The culture was filtered off on Whatman # 4, and the water fraction was extracted with EtOAc (3 × 100 mL). The combined organics were evaporated in vacuo at 40 °C and resuspended in methanol at 10 mg/mL.

In vitro tests with crude extracts

On PDA plates, 100 µL of the extract was spread with a Drigalski spatula, and discs (6 mm) of the *Phyllosticta citricarpa* colonies were deposited in the center of the plate. Plates were incubated at 28 °C for 21 days. The growth zone was measured and compared with controls Derosal® (1.0 mg/mL) and methanol (Savi et al. 2015; Hokama et al. 2016). The analysis was performed in triplicate. The strain that produced metabolites with highest activity was selected for large-scale fermentation. The compounds produced were identified and their biological activity assessed by MIC, growth inhibition, and inhibition of pycnidia formation in *Citrus sinensis* leaves and fruits.

Large-scale fermentation, extraction, isolation, and compound identification

A large-scale fermentation (8 L) of strain LGMB471 was performed using SG culture medium at 36 °C, 210 rpm, for 10 days. The culture was filtered off on Whatman # 4, and the supernatant was mixed with 5% (w/v) XAD-16 resin and stirred overnight, followed by filtration, extraction with methanol, and then the combined organics were evaporated in vacuo at 40 °C to yield 722 mg of yellowish-brown XAD extract. The XAD extract was subjected to Sephadex LH-20 (MeOH; 1 × 20 cm) gel filtration, to yield fractions 1–4, 182.5 mg, 296.3 mg, 84 mg, and 10.5 mg, respectively. The fractions were purified by HPLC to yield compounds 1–8 in pure forms (Fig. 1).

Ultraviolet-visible (UV-Vis) spectra were taken directly from analytical HPLC-PAD runs and show relative intensities. NMR spectra were measured using a Varian (Palo Alto, CA) Vnmr 400 (¹H, 400 MHz; ¹³C, 100 MHz) spectrometer, where δ values were referenced to respective solvent signals [CDCl₃, δ H 7.24 ppm, δ C 77.23 ppm]. High-resolution electrospray ionization (HRESI) mass spectra were recorded on AB SCIEX Triple TOF® 5600 system. HPLC-MS analyses were accomplished using a Waters (Milford, MA) 2695 LC module (Waters Symmetry Anal C₁₈, 4.6 × 250 mm, 5 µm; solvent A, H₂O/0.1% formic

acid; solvent B, CH₃CN/0.1% formic acid; flow rate, 0.5 mL min⁻¹; 0–4 min, 10% B; 4–22 min, 10–100% B; 22–27 min, 100% B; 27–29 min, 100–10% B; 29–30 min, 10% B). HPLC analyses were performed on an Agilent 1260 system equipped with a photodiode array detector (PAD) and a Phenomenex C₁₈ column (4.6 × 250 mm, 5 µm; Phenomenex, Torrance, CA). Semi-preparative HPLC was accomplished using Phenomenex (Torrance, CA) C₁₈ column (10 × 250 mm, 5 µm) on a Varian (Palo Alto, CA) ProStar Model 210 equipped with a photodiode diode array detector and a gradient elution profile (solvent A, H₂O; solvent B, CH₃CN; flow rate, 5.0 mL min⁻¹; 0–2 min, 10% B; 2–34 min, 10–100% B; 34–36 min, 100% B; 36–42 min, 100–10% B; 42–45 min, 15% B). All solvents used were of ACS grade and purchased from the Pharmco-AAPER (Brookfield, CT). Size exclusion chromatography was performed on Sephadex LH-20 (25–100 µm; GE Healthcare, Piscataway, NJ). All other reagents used were reagent grade and purchased from Sigma-Aldrich (Saint Louis, MO).

Minimum inhibitory concentration of isolated compounds

The MIC was determined using a 96-well plate, 90 µL of malt extract broth was added to each well, plus 15 µL of the compounds and crude extract and 10 µL of a conidial suspension of *P. citricarpa* with 6×10^5 conidia/mL (Tonial et al. 2017). The plate was incubated for 14 days at 28 °C. The absence of fungal growth in the well was considered positive result. The assay was performed in triplicate and methanol was used as solvent control. A serial dilution of the extract was performed to MIC determination. The concentrations evaluated were 1.3 mg/mL, 225 µg/mL, 33 µg/mL, 5 µg/mL, 0.75 µg/mL, 0.11 µg/mL, 0.017 µg/mL, 0.0025 µg/mL, and 0.00038 µg/mL.

Antifungal activity against *Phyllosticta citricarpa* in Petri dish

One hundred microliters of compounds **1** and **2** diluted in a final concentration of 10 mg/mL was spread over the surface of PDA medium (48 × 12 mm plates), using the Drigalski spatula, and one mycelial disc of the phytopathogen was inoculated in the center of the plates. As the positive control, the fungicide Derosal ® (1.0 mg/mL) was used. As the negative control, only the phytopathogen was used. Plates were incubated in BOD at 28 °C for 21 days. The growth inhibition was assessed comparing the diameter of the colony in the presence of treatment and controls.

Inhibition of *Phyllosticta citricarpa* pycnidia development in citrus leaves

Antagonistic activity of compounds produced by *Microbacterium* sp. LGMB491 was evaluated in order to determine their potential to inhibit the development of *P. citricarpa* pycnidia in the surface of autoclaved (20 min; 120 °C; 1 atm) *Citrus sinensis* leaves. In Petri dishes with water agar medium, one citrus leaf fragment (Ø 10 mm) was deposited containing 100 µg of the compound to be evaluated. Four discs of 2-mm-thick *P. citricarpa* LGMF06 mycelia were inoculated close to each leaf fragment. The Petri dishes were sealed and maintained at 28 °C, with 12-h photoperiod for 21 days. After this period, the pycnidia of *P. citricarpa* formed above the leaves were counted under a stereoscopic microscope (Santos et al. 2016).

Inhibition of *Phyllosticta citricarpa* development in citrus fruits

For this analysis, detached *Citrus sinensis* fruits were used. The mycelium of *P. citricarpa* was introduced in the fruit using a wound with cutting drill. To evaluate the inhibition activity against the pathogen, 100 µg of each compound was added to the wounds. The wound was sealed with tape and maintained in light chamber at 28 °C in continuous light. The qualitative activity was evaluated after 21 days of incubation, comparing the development of lesions in the treatments with the compounds with the negative control, only the phytopathogen, and the positive control with 100 µg of the fungicide Derosal.

Results

Culturing conditions

The two culture media and temperatures analyzed were able to stimulate the production of active secondary metabolites by six out of ten evaluated strains that were able to inhibit the *Phyllosticta citricarpa* mycelial growth (Table 1). Cultivation of strain *Microbacterium* sp. LGMB471 resulted in the production of high activity metabolites in all the conditions evaluated (more than 80% of inhibition); however, metabolites produced in SG medium at 36 °C almost completely inhibited the phytopathogen development (Table 1). In addition, the extract of *Microbacterium* sp. LGMB471 cultured in SG medium at 36 °C showed the minimal inhibitory concentration and minimal fungicidal concentration of 225 µg/mL.

Secondary metabolite identification

Scale-up fermentation of strain *Microbacterium* sp. LGMB471 (10 L) using SG medium, followed by extraction afforded 722 mg of crude extract. Fractionation, isolation, and purification of the obtained extract using various chromatographic techniques resulted in compounds **1–8** in pure forms (Fig. 1). Based on HPLC/UV, ESIMS, and NMR data (Supplementary information, Fig. S1–26), and by comparison with literature, the secondary metabolites were identified as 7-*O*-β-D-glucosyl-genistem (**1**) (Fedoreyev et al. 2008), 7-*O*-β-D-glucosyl-daizidin (**2**) (Fedoreyev et al. 2008), 4',7-dihydroxyisoflavanone (**3**) (Fedoreyev et al. 2008; Shaaban et al. 2012), cyclo-(L-Pro-L-Val) (**4**) (Oliveira et al. 2009), cyclo-(L-Pro-L-Leu) (**5**) (Yan et al. 2004), cyclo-(L-Pro-L-Phe) (**6**) (Barrow and Sun 1994), cyclo-(L-Val-L-Phe) (**7**) (Pickenhagen et al. 1975), and cyclo-(L-Leu-L-Phe) (**8**) (Perez-Victoria et al. 2012) (Fig. 2).

Inhibition of *Phyllosticta citricarpa* development

Among the eight compounds isolated from the strain LGMB471, compounds 7-*O*-β-D-glucosyl-genistein (**1**) and 7-*O*-β-D-glucosyl-daizidin (**2**) demonstrated considerable activity against *P. citricarpa* (MIC values of 33 µg/mL), compounds **3** and **4** had moderate activity (MIC of 225 µg/mL), and compounds **5** and **6** demonstrate low activity (MIC of 1.5 mg/mL) (Table 2). Compounds **7** and **8** showed no activity. In view of the considerable activity demonstrated by compounds **1** and **2** in the MIC analysis, they were selected for the next evaluation. 7-*O*-β-D-glucosyl-genistein (**1**) and 7-*O*-β-D-glucosyl-daizidin (**2**) also significantly inhibited the *P. citricarpa* mycelial growth in Petri dish containing PDA media, presenting 57 and 63% of inhibition, respectively (Table 2 and Fig. 3a–d).

We also evaluated the ability of compounds **1** and **2** to inhibit the formation of *P. citricarpa* pycnidia in citrus leaves, mechanism associated with CBS dispersion and symptoms development, and both compounds were able to inhibit more than 50% of *P. citricarpa* pycnidia production in leaves (Table 2). In the analysis of CBS symptoms induction using detached *Citrus sinensis* fruits, 100 µg of 7*O*-β-D-glucosyl-genistein (**1**) and 7*O*-β-D-glucosyl-daizone (**2**) repressed the development of necrosis characteristic of citrus black spot disease (Fig. 3e–h).

Discussion

The use of microorganisms and their metabolites may represent an alternative to decrease the impacts of fungicide applications in the field. As result, several registrations of commercial biocontrol products for control leaf and fruit diseases have been deposited (Junaid et al. 2013). Specially, endophytes have been demonstrated to possess the ability to survive in high diverse plants, thereby helping to reduce of herbivory, to control phytopathogens, to induce systemic resistance, and to promote plant growth (Gao et al. 2010; Farrar et al. 2014). In this way, in the present work, we assessed the potential of an actinomycetes collection (Gos et al. 2017) in the control of the phytopathogen *P. citricarpa*.

The optimization of culture conditions showed that, besides the strain analyzed, the culture media and temperature also drastically influenced the biological activity of the extracts (Table 1). For example, the strains LGMB487 and LGMB466 of *Actinomyces* sp. only inhibit more than 50% of *P. citricarpa* growth when cultured in R5A at 28 °C. The strain *Microbacterium* sp. LGMB471 presented considerable activity in all conditions evaluated; however, cultivation using SG medium at 36 °C almost completely inhibited the phytopathogen development. The genus *Microbacterium* has been reported as a producer of several metabolites with biological activity (Kamil et al. 2014); however, there are no reports of activity against *P. citricarpa*. In this context, we reasoned that it would be valuable to determine the metabolic profile of *Microbacterium* sp. LGMB471 and to define the compounds responsible for the biological activity against *P. citricarpa*.

Three isoflavones (**1–3**) and five diketopiperazines (**4–8**) were isolated from the strain LGMB471. Actinomycetes, bacteria, and fungi were already described as producers of isoflavones (Hazato et al. 1979; Chang et al. 2007). This class of compounds has been associated with antifungal and antibacterial activity against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, and vancomycin-resistant *Enterococcus faecalis* (Azzouzi et al. 2014; Yu et al. 2016). The diketopiperazines are commonly isolated from several microorganisms (Kalinovskaya et al. 2017), with activities described as antitumor, antiviral, antifungal, and antibacterial (Martins and Carvalho 2007). In gram-negative bacteria, cyclic dipeptides have been shown to influence quorum sensing (Ryan and Dow 2008) and play a potential role in plant and animal cell communication (Prasad 1995).

In view of the antimicrobial potential of the isolated compounds, the MIC of all compounds was evaluated. Based on this evaluation, the compounds 7*O*-β-D-glucosyl-genistein (**1**) and 7*O*-β-D-glucosyl-daizone (**2**) were selected for the next analysis, since they showed considerable antifungal activity against *P. citricarpa* (MIC values of 33 µg/mL) (Table 2).

These compounds inhibited 57 (1) and 63% (2) mycelial growth of the fungus in plates and inhibited more than 50% of pycnidia production in leaves and citrus fruits (Table 2). These data are of great importance, since the breakdown in the development of asexual spores is fundamental for the interruption of the CBS disease cycle. Pycnidia are responsible for aggravating the disease within the plant and adjacent areas in the countries where the disease is established (Perryman et al. 2014), and are the only ones responsible for the spread of the disease in the American orchards, since only a single MAT type cloning population is present in this region and sexual reproduction does not occur (Wang et al. 2016).

Using detached fruits, 7*O*- β -D-glucosyl-genistein (1) and 7*O*- β -D-glucosyl-daizone (2) compounds repressed the development of CBS necrosis, suggesting that these compounds or the strain *Microbacterium* sp. LGMF471 can be an ecofriendly alternative to reduce the use of fungicide in citrus orchards for the control of CBS disease. In addition, some authors have suggested that isoflavones may increase plant defense against phytopathogens (Cheng et al. 2015) and may act as phytoalexins in the defense mechanisms (Lee et al. 2013).

In this study, we explored the biotechnological potential of a pre-established culture collection of actinomycetes for the control of the *P. citricarpa* phytopathogen and demonstrated that 7*O*- β -D-glucosyl-genistein (1) and 7*O*- β -D-glucosyl-daizone (2) produced by the strain *Microbacterium* sp. LGMB471 showed considerable activity against *P. citricarpa*, inhibiting the formation of asexual spores and the development of lesions of the CBS disease and detached fruits. In general, our data suggest the use of *Microbacterium* sp. LGMB471 or its metabolites as an ecological alternative to be used in association with the fungicides for the control of CBS disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Work-up scheme of the metabolites produced by the strain *Microbacterium* sp. LGMB471

Fig. 2.

Compounds produced by the strain *Microbacterium* sp. LGMB471 cultivated in SG medium at 36 °C

Fig. 3.

Antifungal activity of compounds 7-*O*-β-D-glucosyl-daidsen and 7-*O*-β-D-glucosyl-genistein against the phytopathogen *Phyllosticta citricarpa* in culture media and *Citrus sinensis* detached fruits. **a, e** Positive control: phytopathogen *P. citricarpa*. **b, f** Treatment with 1 mg and 100 μg of compound 7-*O*-β-D-glucosyl-daidsen, respectively. **c, g** Treatment with 1 mg and 100 μg of compound 7-*O*-β-D-glucosyl-genistein, respectively **d, h** Control with fungicide derosal

Mycelial growth of *Phyllosticta citricarpa* (cm) in the presence of endophytic actinomycetes extracts produced in two culture media (SG and R5A) and two temperatures (28 °C and 36 °C)

Table 1

Strain	Culture conditions for metabolites production			
	SG medium		R5A medium	
	28 °C	36 °C	28 °C	36 °C
<i>Microbispota</i> sp. LGMB461	2.4 ± 0.1	3.2 ± 0.1	2.1 ± 0.1	3.3 ± 0.1
<i>Microbispota</i> sp. LGMB465	3.6 ± 0.2	2.7 ± 0.1	2.6 ± 0.1	2.9 ± 0.2
<i>Actinomadura</i> sp. LGMB466	3.5 ± 0.2	3.1 ± 0.1	3.7 ± 0.2	3.6 ± 0.0
<i>Microbacterium</i> sp. LGMB471	<i>1.1 ± 0.2</i>	<i>0.8 ± 0.1</i>	<i>1.2 ± 0.3</i>	<i>1.0 ± 0.1</i>
<i>Williamsia</i> sp. LGMB479	2.0 ± 0.1	2.0 ± 0.2	2.1 ± 0.2	1.8 ± 0.1
<i>Sphaerisporangium</i> sp. LGMB482	2.9 ± 0.1	3.5 ± 0.1	2.6 ± 0.3	2.4 ± 0.0
<i>Streptomyces</i> sp. LGMB483	2.6 ± 0.1	2.8 ± 0.1	<i>1.6 ± 0.1</i>	3.2 ± 0.3
<i>Micrococcus</i> sp. LGMB485	<i>1.7 ± 0.1</i>	<i>1.7 ± 0.2</i>	<i>1.7 ± 0.1</i>	2.3 ± 0.1
<i>Actinomadura</i> sp. LGMB487	<i>1.6 ± 0.2</i>	2.8 ± 0.1	2.8 ± 0.2	<i>1.4 ± 0.1</i>
<i>Aeromicrobium</i> sp. LGMB491	2.8 ± 0.1	3.0 ± 0.2	<i>1.7 ± 0.2</i>	3.3 ± 0.0

Mean (±SD). *P. citricarpa*, positive control; derosal, 0.0; negative control, 4.2 ± 0.2. Italic values represent inhibition higher than 50%

Table 2

Minimum inhibitory concentrations (in µg/mL), growth rate (in cm), and number of pycnidia produced in leaves by *Phyllosticta citricarpa* in the presence of compounds isolated from *Microbactericum* sp. LGMB471

Compounds	1	2	3	4	5	6	7	8	Derosal	Control
MIC	33	33	225	225	1500	1500	–	–	NE	No inhibition
Growth	1.3 ± 0.2	1.6 ± 0.4	NE	NE	NE	NE	NE	NE	0	3.7 ± 0.3
Pycnidia	62 ± 8	71 ± 10	NE	NE	NE	NE	NE	NE	0	150 ± 15

–, no inhibition; NE, not evaluated. For the growth rate, the concentration used was 50 µg for the compounds and 250 µg for the control Derosal