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Investigation of mycoviruses in endophytic and phytopathogenic strains of *Colletotrichum* from different hosts

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ABSTRACT. Fungi belonging to the *Colletotrichum* genus can be categorized as endophytic or phytopathogenic. These fungi can be infected by viruses, termed mycoviruses, which are known to promote hypovirulence in infected fungi. However, there are few studies that have described mycoviral infections of endophytes. The production of secondary metabolites by endophytes with antimicrobial potential in inhibiting numerous pathogens has gained increasing attention. The aim of the current study was to investigate the presence of mycoviruses in endophytic and phytopathogenic fungi of the *Colletotrichum* genus, as well as to analyze the antimicrobial activity of crude extracts obtained from these samples. To detect the presence of mycoviruses in the samples, dsRNA was extracted, treated with enzymes, and analyzed following electrophoresis in agarose gel. Furthermore, isometric mycoviral particles were observed by transmission electron microscopy. Serial microdilution methodology was used to test crude extracts of *Colletotrichum* spp for antibacterial activity

against *Escherichia coli* and *Staphylococcus aureus*, and antifungal activity against *Fusarium solani*. The results of the molecular and microscopic analyses indicated that a phytopathogenic strain presented infection by mycovirus. The antibacterial activity analysis revealed that the minimum inhibitory concentrations and minimum bactericidal concentrations were low for the fungal extracts of the two endophytes, indicating that these extracts were effective antibacterial agents. However, their antifungal activity against *F. solani* was not statistically different compared to that of the negative control.

Key words: Mycovirus; *Colletotrichum*; Crude extracts; Antimicrobial activity; Endophyte; Phytopathogen

INTRODUCTION

Viruses are infectious agents that can infect almost all living organisms, including fungi. Viruses that infect fungi are termed mycoviruses, most of which have double-stranded RNA (dsRNA) genomes that are packed into non-enveloped isometric particles (Refos et al., 2013).

Mycoviruses have no overt effect on their fungal hosts, although some species can cause changes in the physiology of phytopathogenic fungi, leading to hypovirulence. Hypovirulence is defined as reduced pigmentation, asexual sporulation, loss of fertility, and/or reduced growth rate (van de Sande et al., 2010), leading to a reduction in virulence (Kanematsu et al., 2010), and therefore hypovirulence-associated mycoviruses have described as biological control agents for phytopathogenic fungi (Peever et al., 2000; Ghabrial and Suzuki, 2009; van de Sande et al., 2010). To date, few studies have reported on the presence of mycoviruses in endophytic fungi, the presence of which may be linked with plant adaptation to extreme environments, conferring heat tolerance to plant hosts that contain the endophytes (Márquez et al., 2007; Herrero et al., 2009; Asencio et al., 2013). Different molecular approaches are used for detecting the presence of and identification of mycoviruses. Specifically, the use of transmission electron microscopy (TEM) contributes to the visualization and thus confirmation of mycoviral particles.

Colletotrichum spp have been screened for the presence of mycovirus (Figueirêdo et al., 2012; Lima et al., 2012; Bezerra, 2015). This genus is considered the most widely distributed, and inhabits plants in the endophytic form as well as phytopathogenically. When this fungus acts as an endophyte, it does not cause damage, receives nutrients and plant protection from its host, and in exchange may help the plant under stress conditions through the production of chemical compounds (Petrini, 1991; Azevedo et al., 2000). In contrast, when *Colletotrichum* acts as a phytopathogen, it causes necrotic lesions, leaf and fruit anthracnose (Freitas, 2014).

Interactions between endophytes and their hosts have emerged as a promising alternative for the production of a vast number of secondary metabolites and enzymes (Firáková et al., 2007; Corrêa et al., 2014). A single endophyte may be able to produce a large variety of bioactive metabolites (Ramasamy et al., 2010), and these metabolites may have antimicrobial potential with efficient inhibitory actions against certain pathogens, thereby enabling the procurement of biotechnological products with low financial and environmental cost.

The aim of the current study was investigate the presence of mycoviruses in endophytic and phytopathogenic strains of the *Colletotrichum* genus, as well as to analyze the antimicrobial activity of crude extracts obtained from these samples.

MATERIAL AND METHODS

Biological material

Samples of endophytic fungi of the *Colletotrichum* genus were provided by the Microbial Biotechnology Laboratory at Universidade Estadual de Maringá, Paraná, Brazil, and phytopathogenic strains were provided by Embrapa Grape & Wine of Bento Gonçalves, Rio Grande do Sul, Brazil (Table 1).

Table 1. Fungal strains along with their fungal plant interactions (endophyte/phytopathogen) and host plants.

Isolate	Interaction with plant	Host
Colletotrichum sp 1	Phytopathogenic	Grape
Colletotrichum sp CNPUV 378	Phytopathogenic	Grape
Colletotrichum sp 29b	Endophytic	Coffee
Colletotrichum sp 64	Endophytic	Coffee
Colletotrichum sp 60	Endophytic	Coffee
Colletotrichum sp 27	Endophytic	Coffee
Colletotrichum sp 65	Endophytic	Coffee
Colletotrichum sp 153	Endophytic	Coffee
Colletotrichum sp 163	Endophytic	Coffee
Colletotrichum sp 118	Endophytic	Coffee
Colletotrichum sp 137b	Endophytic	Coffee
Colletotrichum sp 137a	Endophytic	Coffee
Colletotrichum sp 136a	Endophytic	Coffee
Colletotrichum sp 173b1	Endophytic	Coffee
Colletotrichum sp 174	Endophytic	Coffee
Colletotrichum sp 176	Endophytic	Coffee
Colletotrichum sp 155	Endophytic	Coffee
Colletotrichum sp 189	Endophytic	Coffee
Colletotrichum sp 11	Endophytic	Coffee
Colletotrichum sp 47	Endophytic	Coffee
Colletotrichum sp 202a	Endophytic	Coffee
Colletotrichum sp 149	Endophytic	Coffee
Colletotrichum sp 165b3	Endophytic	Coffee
Colletotrichum sp 167	Endophytic	Coffee
Colletotrichum sp 110	Endophytic	Coffee
Colletotrichum sp 61	Endophytic	Coffee

Extraction of total nucleic acids of *Colletrotrichum* spp

Fungal strains were grown for seven days on Petri dishes containing potato dextrose agar (PDA) medium (Smith and Onions 1983) at 28°C. Next, the mycelia were removed from the dishes and ground to a fine powder with liquid nitrogen. Total nucleic acids were extracted as described by Raeder and Broda (1985).

Extraction of dsRNA

dsRNA was extracted using methods described by Morris and Dodds (1979) with modifications. Cellulose columns (CF-11, Whatman International Ltd., Kent, England) for chromatography were set up in disposable 10-mL syringes, with 2 g microgranular cellulose. The columns were buffered with successive washes with 1X STE buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 0.5 mM EDTA) containing 16% (v/v) ethanol to remove DNA and single-stranded RNA. The putative dsRNA that remained in the column was eluted with STE without ethanol, and precipitated with the addition of 0.1 volume of 3 M sodium acetate and 20 mL ice cold absolute

ethanol. The precipitate was collected by centrifugation at 10,000 *g* for 25 min. The supernatant was discarded, and the precipitate was washed with 70% ethanol and centrifuged. The supernatant was discarded, and the pellet was dried and resuspended in diethylpyrocarbonate water. The dsRNA was analyzed following 1.5% agarose gel electrophoresis.

The samples were treated with DNase RQ1 (1 U/µL) and nuclease S1 (89 U/µL) (Promega, Madison, WI, USA). DNase RQ1 digestion was performed at 37°C for 30 min, followed by incubation at 65°C for 10 min to inactivate the enzyme. Nuclease S1 digestion was performed at 37°C for 30 min. The efficiency of treatments was analyzed by agarose gel electrophoresis.

TEM

TEM was used as described by Figueirêdo et al. (2012). Briefly, hyphae and spores of fungi grown in PDA medium were carefully removed with a glass slide, transferred to a mortar containing 0.2 mL 0.05 M cacodylate buffer, pH 7.2, and macerated with pestle. Small drops of this material were placed on a parafilm sheet, on top of which carbon-coated 300 mesh grids were floated for 20 min. The grids were removed, and excess liquid was absorbed with a piece of filter paper. The grids were washed with drops of distilled water, and then floated on a droplet of 1% aqueous uranyl acetate for 15 min. After removal of excess liquid, the grids were dried and examined with a Zeiss EM 900 TEM with digital image recording.

Crude fungal extract isolation for analysis of antimicrobial activity

The extraction of metabolites was performed according to methods described by Rukachaisirikul et al. (2008) with modifications. Two endophytic strains (29b and 64) and one phytopathogenic strain (2) were randomly selected to obtain crude extracts. Pure cultures of fungi were re-cultured on Petri dishes with PDA at 28°C for seven days to obtain young colonies. Three mycelia fragments (5 mm^2) of each fungus were inoculated into 2 L Erlenmeyer flasks containing 1 L potato dextrose broth and incubated at 28°C for 30 days under stationary conditions. The broth cultures were filtered and centrifuged at 5300 *g* for 15 min to separate the culture broth and mycelia. All filtrates were extracted three times with equal volumes of ethyl acetate in a separatory funnel, where the separation of phases occurred by polarity difference after strong agitation. The acetate phase was separated, dried with anhydrous sodium sulfate, and then concentrated in a rotary evaporator at 38°C. The extract residues were resuspended in 10 mL absolute methanol and stored at 4°C.

Antibacterial activity analysis of the crude fungal extracts

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were evaluated for the crude extracts obtained from two fungal strains (29b and 64) against the bacteria *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) from the collection at Universidade Estadual de Maringá Microbiology Laboratory. For these evaluations, a serial microdilution method was used on 96-well microplates as recommended by the CLSI M7-A9 (2012). The concentrations of the extracts ranged from 2.7 to 87.5 µg/mL. Tetracycline was used as positive control for both bacteria at a concentration ranging from 0.48 to 62.5 µg/mL. Bacterial microcultivation in culture medium without the evaluated crude extracts served as a negative control. All tests were performed in triplicate. Mueller Hinton medium (100 µL) was added to each

well of the microplates. The crude extracts were diluted in 5% dimethylsulfoxide, and then 100 μL diluted crude extracts was added into the first well to start the serial microdilution at a ratio of 1:2. At the end of microdilution, 5 μL of the previously prepared bacterial inoculum was added, with a subsequent 1:10 dilution, resulting in a final concentration of inoculum in each well of 10^5 UFC/mL. The plates were then incubated for 24 h at 35°C. For evaluation of the MBC, a microcultivation in 10 μL Mueller Hinton agar was performed for 24 h at 35°C after the initial microplate incubation period. The MBC was defined as the lowest concentration of extract that was able to kill all bacteria after the treatment period. After microcultivation, the colonies were counted using the negative control as the baseline parameter, as the number of colonies is related to the total reduction in bacterial load, which was calculated as bacterial load reduction = log (CFU/mL).

Antifungal activity analysis of the crude fungal extract

The crude extracts obtained from the 29b and 64 fungal isolates were tested against the phytopathogenic fungus *Fusarium solani*, which was provided by the Microorganisms IGSALQ collection from the Laboratory João Lucio de Azevedo, ESALQ, Universidade de São Paulo, Brazil (www.cria.org.br/cgee/junho/docs/cadastro_colecoes.xls). Mycelial fragments from the phytopathogenic colony were inoculated onto Petri dishes with PDA medium 4 cm away from filter paper disks (5 mm). The crude extracts (10 μL) of the endophytic strains were applied to the filter paper at a concentration of 700 μg/mL. Benlate fungicide (700 μg/mL) served as the positive control, and methanol (MeOH) was used as the solvent control. The inhibition rate was evaluated with the ImageJ software, in which the mycelial growth area of the phytopathogen with the crude extracts was compared with that of controls. The index of inhibition percent was calculated as Im% = (1-TM / CM) x 100, where Im% = inhibition index in percentage of growth, TM = mean of the area for treatments in cm^2, and CM = mean of the area for controls in cm^2.

Statistical analysis

All statistical analyses were performed with the Sisvar 5.4 software. The Scott Knott test was used by which $P < 0.05$ was considered statistically significant.

RESULTS

Detection and visualization of mycovirus

Among the *Colletrotrichum* isolates analyzed, the following nine samples presented nucleic acid bands that suggested the possible presence of mycovirus from total nucleic acid extractions: 29b, 47, 61, 136a, 149, 153, 173b1, 174, and CNPUV 378 (data not shown). Based on these results, dsRNA isolation was performed on these nine isolates, and the dsRNA was further subjected to enzyme treatments with DNase RQ1 and nuclease S1. The electrophoretic analysis of the enzyme cleaved dsRNA revealed bands potentially associated with mycovirus only in *Colletotrichum* sp CNPUV 378.

For CNPUV 378, we observed two fragments with sizes corresponding to approximately 1500 and 2500 base pairs (bp) following agarose gel electrophoresis (Figure 1). The samples subjected to dsRNA extraction were then analyzed by TEM. The microscopy results revealed the presence of isometric virus particles measuring between 25 and 50 nm in diameter (Figure 2), with

and without genetic material, in CNPUV 378, which was in agreement with the data obtained by molecular analysis.

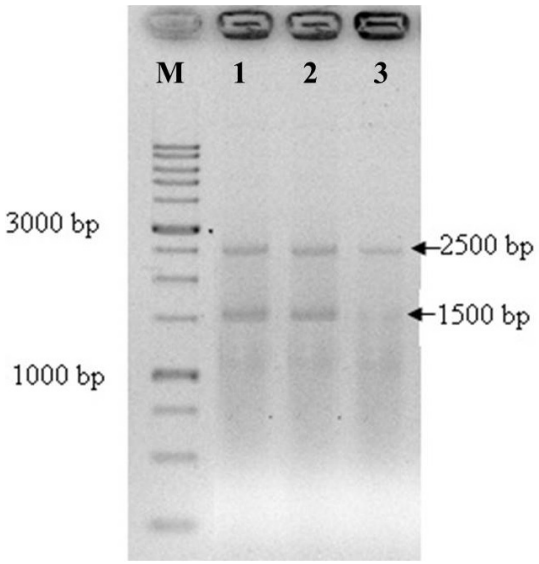

Figure 1. Bands of double-stranded RNA from *Colletotrichum* isolates visualized following agarose gel electrophoresis. *Lane M* = 1-kb DNA ladder; *lane 1* = dsRNA extracted from phytopathogenic isolate CNPUV 378; *lanes 2* and *3* = treatment of the dsRNA from lane 1 with the DNAse RQ1 and nuclease S1 enzymes, respectively.

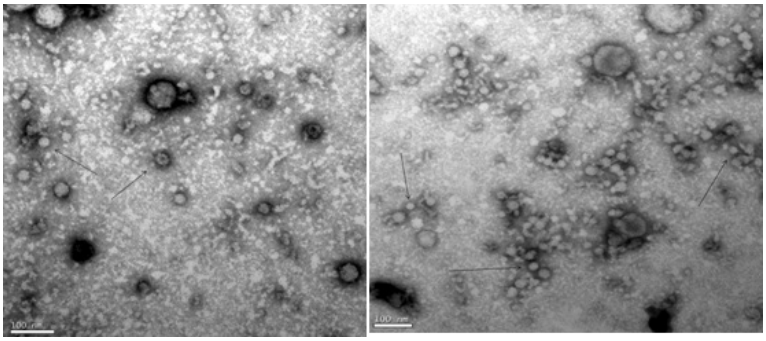

Figure 2. Transmission electron microscopy image of isometric mycovirus particles with diameters between 25 and 50 nm from the *Colletotrichum* phytopathogenic strain. Bar = 100 nm.

Antibacterial activity of the crude extracts

The results of the antibacterial activity assessment of the of crude fungal extracts, including the MICs and MBCs of the endophytic/phytopathogenic fungi are presented in Table 2.

The crude extract of the *Colletotrichum* sp 64 endophytic strain against *E. coli* exhibited the same MIC and MBC bacteriostatic and bactericidal activity at a concentration of 10.94 μg/mL, with bacterial load reduction of 10. The MIC and MBC of the *Colletotrichum* sp 64 extract against *S. aureus* was 2.7 and 5.5 μg/mL, respectively, with a bacterial load reduction of 4.4.

E. coli and *S. aureus* treated with the crude extract of the *Colletotrichum* sp 29b endophytic strain revealed the same MBC able to remove all bacterial cells, which was 43.7 µg/mL. However, the MIC value for the *Colletotrichum* sp 29b extract was better against *S. aureus* (5.5 µg/mL) than that against *E. coli* (21.9 µg/mL) with bacterial load reductions against *S. aureus* and *E. coli* of 4.7 and 6.5, respectively. Comparatively, *Colletotrichum* sp CNPUV 378 extract had MIC and MBC values against *E. coli* of 43.7 and 87.5 µg/mL, respectively, with a bacterial load reduction of 6. The extract of this phytopathogenic fungus had MIC and MBC values against *S. aureus* of 21.9 and 43.7 µg/mL, respectively, with a bacterial load reduction of 4. Together, these results indicate that compared to the other extracts, the crude extract of *Colletotrichum* sp 64 was the most efficient antibacterial agent against both *E. coli* and *S. aureus*.

Table 2. Antibacterial activity of crude extracts from *Colletotrichum* sp endophytic strains (29b and 64), from *Colletotrichum gloeosporioides* phytopathogenic strain (CNPUV 378), and tetracycline against of *Escherichia coli* and *Staphylococcus aureus*.

Antibacterial treatment	*E. coli*			*S. aureus*		
	MIC[1]	MBC[1]	Bacterial load reduction[2]	MIC	MBC	Reduction
Colletotrichum sp 64	10.94	10.94	10	2.7	5.5	4.4
Colletotrichum sp 29b	21.9	43.7	6.5	5.5	43.7	4.7
Colletotrichum sp CNPUV 378	43.7	87.5	6	21.9	43.7	4
Tetracycline	1.95	62.5	*	0.48	7.71	*

[1]Concentration in µg/mL. [2]Bacterial load reduction = log (CFU/mL). *Not calculated.

Antifungal activity of the crude extracts

The antifungal activity analysis revealed that the activity against *F. solani* of the crude extracts of *Colletotrichum* spp 64 and 29b did not differ statistically from that of the negative control treatment with methanol, indicating that these extracts weren't effective antifungal agents (Table 3). Although the extracts tested herein did not work effectively against *F. solani*, there are many reports showing that endophyte extracts may inhibit various pathogenic microorganisms (Assakura et al., 2009; Ramos et al., 2010; Orlandelli et al., 2012; Rhoden et al., 2012; Bezerra, 2015). Therefore, these extracts may be effective in the inhibition other phytopathogens.

Table 3. Antifungal activity of the crude extracts from *Colletotrichum* spp against *Fusarium solani*.

Treatment	Mycelial growth area (cm^2)[1]	Im%
Colletotrichum sp 29b	38.01[b]	7.52
Colletotrichum sp 64	40.42[b]	1.65
Positive control	33.82[a]	17.71
Negative control	41.10[b]	0

[1]Mean mycelial growth area in cm^2; [a]value was significantly different from the negative control by the Scott Knott test ($P < 0.05$); [b]values were not statistically different by the Scott Knott test ($P > 0.05$).

DISCUSSION

The molecular and microscopic analyses suggest that the mycovirus detected herein may belong to the family Partitiviridae, due to the number and size of fragments visualized on the agarose gel, and the particle size observed by TEM. Partitiviruses possess two essential dsRNA genome segments ranging from 1300 to 2500 bp in length with one long open reading frame on one of the RNA strands. Partitivirus particles are isometric, with diameters of different strains ranging from 25 to 40 nm as determined by negative stain electron microscopy (Nibert et al., 2014).

In recent years, the number of studies regarding mycoviruses in phytopathogenic fungi has increased considerably (Figueirêdo et al., 2012; Zhong et al., 2014; Özkan and Coutts, 2015; Shang et al., 2015). The frequency of mycovirus strains in *Colletotrichum* from different plant hosts as detected by mycoviral dsRNA herein was low (1/26; 3.85%). Similarly, in a study of different strains of endophytic fungi from the *Colletotrichum* genus, Lima et al. (2012) detected dsRNA in only three isolates from a total of thirty-nine (7.7%) analyzed.

In the molecular analysis of endophytic fungi, we could not confirm the absence of mycovirus in the majority of samples analyzed. This is due to the methodology used in this study, where we sought to detect dsRNA because the majority of mycoviruses have dsRNA genomes, although it is possible that some mycoviruses present in endophytic fungi may contain single-stranded RNA or DNA genomes. Moreover, the absence of visualization of viral particles with microscopy also does not necessarily signify the absence of mycovirus in endophytic fungal samples. As described by Nuss (2005), this may be because various mycoviruses do not encode proteins that form capsids, and rather exist as nude molecules of dsRNA. Thus, uranyl acetate will fail to stain the viral protein, making it impossible to visualize virus particles. In this case, the detection mycoviruses may be achieved by metagenomic analysis (Al Rwahnih et al., 2011).

Some dsRNA mycoviruses are characterized as beneficial, because they can be used as biological control agents of certain fungi that cause diseases in cultivated plants. Since these viruses can be transmitted via hyphal anastomosis, infected fungi can naturally transmit the virus to healthy fungi, which can cause hypovirulence. Dalzoto et al. (2006) analyzed the hypovirulence associated with mycoviral dsRNA in *Beauveria bassiana*. It was demonstrated that dsRNA in *B. bassiana* strains was responsible for hypovirulence effects against *Euschistus heros*, when compared with isogenic dsRNA-free strains. This study indicated that hypovirulence may be an important tool in agriculture as a biocontrol.

Zhong et al. (2014) observed spherical virus particles of approximately 40 nm in diameter via TEM, and moreover, after nucleic acid extraction from a mycelial preparation of a *Colletotrichum acutatum* HNZJ001 isolated from an anthracnose lesion on immature pepper fruit, two dsRNA bands were observed following gel electrophoresis. Genome comparison and phylogenetic analysis indicated that the virus was of the family Partitiviridae. These results suggested the presence of a novel two-segment dsRNA virus termed *Colletotrichum acutatum partitivirus* 1. Figueirêdo et al. (2012) reported the detection of a mycovirus in a strain of *Colletotrichum gloeosporioides* causing anthracnose in cashew trees. TEM revealed the presence of isometric particles (30-35 nm in diameter), confirming that the infection of this *C. gloeosporioides* strain was by a mycovirus. More recently, mycovirus research has begun to utilize new technology (Pearson et al., 2009), including next generation sequencing and bioinformatics, which has made it more feasible to use whole genome or metagenomic approaches to screen fungi for the presence of potential viruses (Mokili et al., 2012).

The results of the antimicrobial activity analyses stand out in the present study, which highlight the potential of using the extracts of endophytes as alternatives for biocontrol (Orlandelli et al., 2012; Rhoden et al., 2012). For example, as a bactericidal agent, the crude extract of *Colletotrichum* sp 64 was effective in inhibition and elimination of *E. coli* and *S. aureus*, with a bacterial load reduction of 10.0 for *E. coli* and 4.4 for *S. aureus*. This data provides strong evidence that the metabolites of this fungus are a potential source of new antibiotics. Similarly, Arivudainambi et al. (2011) isolated the endophytic fungus *C. gloeosporioides* from the medicinal plant *Vitex negundo* L., and its extracts were screened for their antibacterial activity against methicillin-, penicillin-, and vancomycin-resistant clinical strains of *S. aureus*. The results showed that the antimicrobial metabolites of the endophytic fungus in combination with antibiotics were able to

substantially decrease the MIC of antibiotics against a diverse group of bacteria containing genetic elements responsible for drug resistance. Specifically, the extract showed effective antibacterial activity against *S. aureus* strain 9, with a MIC of 31.25 μg/mL.

Souza et al. (2004), isolated *Colletotrichum* sp, *Guignardia* sp, *Aspergillus niger*, *Phomopsis* sp, and *Xylaria* sp endophytic fungi from *Palicourea longiflora* and *Strychnos cogen*, which are toxic plants from the Amazon. The authors then tested these fungi against pathogenic bacteria, and found that two *Colletotrichum* sp strains had antibacterial activity against *S. aureus, Bacillus* sp and *B. subtilis.*

Silva (2013), isolated endophytic fungi from healthy leaves of *Schinus terebinthifolius* Raddi (mastic-red), and evaluated fungal extracts for their antimicrobial activity against pathogenic microorganisms. Particularly, the antimicrobial activity was evaluated, and it was found that the MIC and MBC against *S. aureus* were 125 and 250 μg/mL, respectively, and against *E. coli*, the MIC and MBC were 500 and 1000 μg/mL, respectively.

Ramos et al. (2010), obtained crude extracts from *Papulaspora immersa* and *Arthrinium state* endophytic fungi isolated from the roots of *Smallanthus sonchifolius* (yacón), and investigated the ability of these extracts to inhibit *S. aureus*, *Kocuria rhizophila*, *Pseudomonas aeruginosa*, and *E. coli*. The MIC of the extract of *P. immersa* against *P. aeruginosa* was 90 μg/mL, and the extracts of *A. state* from *A. montagnei* Sacc. against ATCC bacteria had the best result against *P. aeruginosa* (MIC = 160 μg/mL).

Meng et al. (2015), isolated and identified five new compounds from the culture extract of *Penicillium brocae* MA-231, an endophytic fungus isolated from the fresh tissue of the marine mangrove plant *Avicennia marina*. All of these compounds were examined for antimicrobial activities against *S. aureus* and *Gaeumannomyces graminis*. The screening results showed that the MIC values ranged from 0.25 to 32.0 μg/mL and 0.25 to 64 μg/mL, respectively, against the tested strains.

Assakura et al. (2009) obtained metabolite extracts of an endophytic fungus isolated from *Trichilia elegans*, which were tested against *Colletotrichum* sp and *Moniliophthora perniciosa* phytopathogens. The results showed that against *Colletotrichum* sp, there was no significant inhibition by the secondary metabolites of the endophyte.

In the present study, the presence of mycovirus particles was detected molecularly and by TEM in a *Colletotrichum* grape phytopathogenic strain, but was not observed in the isolates from *Colletotrichum* coffee endophytic strains. The antibacterial activity analysis revealed that low concentrations of secondary metabolites from extracts of fungi of the genus *Colletotrichum* are effective for use against pathogenic bacteria. However, the extracts tested herein were not significantly effective against a representative phytopathogen.

Conflicts of interest

The authors declare no conflict of interest.

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