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Secondary metabolites produced by endophytic fungi: novel antifungal activity of fumiquinone B

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ABSTRACT. Fungi are present in the most diverse environments including the interior of plant tissues, living as endophytes without causing apparent damage. These endophytes are producers of secondary metabolites, also known as natural products, such as fungicides. Here, we evaluated the ethyl acetate fractions obtained from endophytic fungi isolated from plants in the genus *Begonia*. The fractions were submitted to inhibitory test against the plant pathogens *Diaporthe phaseolorum* and *Colletotrichum gloeosporioides*. From the 88 ethyl acetate fractions evaluated, 14.7 % inhibited *C. gloeosporioides* and 11.3 % inhibited *D. phaseolorum*. One fungal isolate displaying an active fraction was selected for chemical investigation. The fungus identified as *Neopestalotiopsis* sp., produced a compound that was active against *D. phaseolorum,* with a MIC of 312 µg mL^{-1} (1,695.3 µM). The compound was identified by mass spectrometry and ^{1}H NMR as the known compound fumiquinone B. The results highlight that the endophytes are capable of producing compounds that may be used to control plant pathogens. The compound fumiquinone B is reported for the first time as an antifungal agent against *D. phaseolorum,* a relevant plant pathogen worldwide. This is also the first report of the production of fumiquinone B by the genus *Neopestalotiopsis*.

Keywords: 2,6-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione; *Neopestalotiopsis* sp.; *Diaporthe phaseolorum*.

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Introduction

Endophytic organisms are those that live inside host plants without causing apparent injury, since they are in harmonic environmental conditions (Higginbotham et al., 2013; Ikram, Ali, Jan, Guljan, & Khan, 2019). According to the plant species, its age, geographic location and the environmental conditions where the plants live, there is a significant variation in the diversity of endophytic fungi (Bandara et al., 2006; Arnold, 2007; Song, Otkur, Zhang, & Tang, 2007). It is known that the greatest biodiversity of endophytic fungi is present in the leaves of tropical plants, which may contain species not yet found in other biomes (Arnold & Lutzoni, 2007; Arora et al., 2019) and which can produce several new compounds (Li et al., 2018). During the evolution, endophytic microorganisms have developed a number of characteristics and functions to survive in their specific ecological niches (Kusari, Hertweck, & Spiteller, 2012). The endophytes are known as a rich source of biomolecules, as alkaloids, terpenoids, flavonoids and steroids, with structural diversity and biotechnological applicability (Nair & Padmavathy, 2014; Li et al., 2018).

Due to the potential of endophytes to assist their hosts in the protection against pathogens, it is interesting to study these fungi aiming for bioactive substances. Moreover, new fungicides are required for the control of diseases that attack the important crops (Leadbeater, 2015), e. g. anthracnose and the pod and stem blight, diseases that cause great damages in crops worldwide. The pod and stem blight are caused by fungi in the genus *Diaporthe* Nitschke (=*Phomopsis* (Sacc.) Bubák). At least four *Diaporthe* species occur in soybeans and are responsible for serious diseases and yield losses (Pereira, Pereira, & Fraga, 2000; Santos, Vrandecic, Cosic, Duvnjak, & Phillips, 2011). The fungus *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. (=*Phomopsis phaseoli* (Desm.) Sacc.) causes seed decay (Hepperly & Sinclair, 1978; Pereira, Pereira, & Fraga.,

2000), pod and stem blight (Pedersen & Grau, 2010) and stem canker in soybean (*Glycine max* (L.) Merrill), leading to considerable crop production losses worldwide (Udayanga, Castlebury, Rossman, Chukeatirote, & Hyde, 2015).

The fungus *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. is one of the plant pathogens responsible for anthracnose, a disease that attacks various crops, such as avocado, strawberry, eggplant, coffee, cashew, citrus, etc. (Freeman, Katan, & Shabi, 1998). In view of the relevance of these diseases, it is important to seek new alternatives that may act in its control, such as fungicides (Leadbeater, 2015). The use of synthetic fungicides is progressively restricted because of harmful effects on the environment and human health, and the emergence of highly resistant fungal strains. Therefore, there is a great demand for new natural fungicides (Ribera & Zuñiga, 2012). Due to the potential of endophytes to protect their hosts against pathogens, the search in these environments becomes promising.

Here, we worked with several endophytic fungi from plants in the genus *Begonia* Plumier found in the Atlantic Rain Forest (Correia, Lira, Assis, & Rodrigues, 2018), to assess their potential to produce active extracts against plant pathogens and identify the compounds.

Material and methods

General procedures

The ^{1}H nuclear magnetic resonance (NMR) was recorded on a Bruker 14.1 Tesla, AVANCE III model spectrometer (operating at 600.23 MHz for hydrogen frequency) with cryoprobe™. The chemical shifts are given on a δ (ppm) scale. High-resolution mass spectrometry (UPLC-qTOF) was acquired on a UPLC Xevo G2-XS Q-TOF (Waters, Milford, USA), quadrupole time-of-flight mass spectrometry operating in electrospray positive mode in the following conditions: Column Acquity UPLC BEH C_{18} (2.1 x 100 mm, 1.7 µm, Waters, Milford, USA). The chromatographic conditions were a gradient of H_2O/ACN (98:2, v v^{-1}, 9 min. 100% ACN) during 10 min., flow rate 0.5 mL $min.^{-1}$. The parameters for the mass spectrometry detection were: MS^E continuum, m z^{-1} 100-1200 Da range and ramp collision energy of 30V. Data acquisition was performed using the software MassLynx

High-performance liquid chromatography (HPLC) was conducted using an Agilent 1100 Series UV/Vis with a quaternary pump, coupled with a UV detector MWD (Multiple Wavelength Detector), using a reversed phase column C_{18} (4.60 x 250 mm, 5 µm; Phenomenex Kinetex), with a mobile phase in gradient of H_2O/ACN (90:10, v v^{-1}, 30 min. 100% ACN), flow rate 1 mL $min.^{-1}$ and λ = 220 nm. HPLC-grade solvents were utilized (LiChrosolv, Merck, Germany). Solid-phase extraction was carried out using silica gel, cyano and C_{18} cartridges of different dimensions (Phenomenex, USA and Supelco, Sigma-Aldrich, Germany) and P.A grade solvents (Synth, Brazil). Silica gel_{254} (Macherey-Nagel) was used for TLC. Spots were detected under UV light (254 and 365 nm).

Optical absorbances were measured on a TECAN reader, model SUNRISE, operated by Magellan v.7.1 software, at 620 nm.

Biological material

The endophytic fungi used in this study (n= 88) were isolated from *Begonia fischeri* Schrank (HRCB 64226), *B. venosa* Skan ex Hook (HRCB 64227) and *B. olsoniae* L.B. Sm. & B.G. Schub (HRCB 64229) and identified by morphological and molecular techniques in a previous study (Correia, Lira, Assis, & Rodrigues, 2018).

Cultivation, extraction and isolation

For the screening of extract with anti-fungal activity, each fungal isolate was cultured on malt extract agar 2% medium (20 g L^{-1} of malt extract - Kasvi and 15 g L^{-1} of agar - Acumedia) at 28°C for 7 days. Then, three mycelium fragments (7 mm^2 in diameter) were transferred to Erlenmeyer flasks with malt extract 2% broth, pH 6.0, and incubated at 28°C under agitation (150 rpm) for seven days (150 mL batch in 250 mL Erlenmeyer flasks for each fungal strain). The mycelium was separated from the supernatant using vacuum filtration and the liquid was extracted three times with EtOAc. Evaporation of the solvent in vacuum gave the ethyl acetate extracts, which were tested against the fungi *C. gloeosporioides* and *D. phaseolorum* in the paper disk assay.

The selected fungal strain was cultured under the same conditions (28 x 250 mL Erlenmeyer flasks, each containing 150 mL of malt medium, pH 6.0). The flask cultures were incubated at 28°C on a rotary shaker at 150 rpm for 9 days. The liquid cultured medium was extracted with EtOAc three times, and the organic solvent was evaporated to dryness under vacuum to afford 1.0 g of the ethyl acetate extract.

The ethyl acetate extract was separated on a Sep-pak cyan column (10 g) using a gradient elution of CH_2Cl_2-MeOH (100: 0 to 0: 100), resulting in 5 fractions (1-5). Fraction 1 (877 mg) was separated on Sep-pak silica gel column (10 g) with a gradient of hexane- CH_2Cl_2-MeOH, yielding 5 fractions (1A-1E). Fraction 1B (60 mg) was fractionated on a C_{18} reverse phase Sep-pak column (1 g) using a H_2O-MeOH gradient, yielding 3 fractions (1B1-1B3). Fraction 1B3 (8.3 mg) was purified by HPLC with a H_2O-ACN gradient to give 1.2 mg of active compound 1B3P1 (1).

Fumiquinone B (1). Purple crystals, UV λ_{max} (MeOH) nm (ε) 226 (2,400), 269 (3,700), 292 (3,200); ^{1}H NMR ($DMSO_{d6}$, 600.23 MHz) δ 1.74 (s, 3H, CH_3-5), 3.72 (s, 3H, OCH_3-3); HRESIMS $[M+H]^+$ *m/z* 185.0438 (calc. for $C_8H_9O_5^+$ 185.0450); HRESIMS $[2M+H]^+$ m z^{-1} 367.0654 (calc. for $C_{16}H_{15}O_{10}^+$ 367.0665).

Antifungal assays

Paper disk assay

The paper disk assay (Heatley, 1944) was used to determine antifungal activity during the screening and the separation, and purification procedures. The fractions were applied to 6 mm diameter sterile paper disks, Filter Macherey Nagel, MN 640 m, 7 cm (1 mg for AcOEt extract, 0.5 mg for fractions and 0.2 mg for pure compounds) and placed on the edge of Petri dishes with 2% malt medium. An inoculum of the pathogen (a 7 mm diameter mycelium fragment removed from a fresh culture) was added to the opposite border of the Petri dish. Areas of inhibition of mycelial growth around the paper disks were measured after incubation for 5 days at 28°C. Inhibition percentages were calculated in comparison with the control containing only the pathogen using the Image J program (Image Processing and Analysis in Java). This experiment was conducted with one replicate. The fungi *C. gloeosporioides* (deposit number CMAA 1688) and *D. phaseolorum* (deposit number CMAA 1695) used in this study were isolated from guarana plant and soybean, respectively, obtained and identified by Prof. Dr. João Lúcio de Azevedo, from the Laboratory of Genetics of Microorganisms, Department of Genetics, ESALQ-USP.

Microdilution assay

The microdilution assay was performed on a 96-well microplate according to the guidelines of the National Committee on Clinical and Laboratory Standards (Clinical and Laboratory Standards Institute [CLSI], 2008) with modifications. For the assay, the pure compound was diluted in DMSO and 2% malt medium to final concentrations of 2500; 1250; 625; 312; 156; 78.1; 39; 19.5; 7.7; 4.8 and 2.4 μg mL^{-1} in each well. A suspension of inoculum of *D. phaseolorum* (10^5 spores mL^{-1}, 60 μL) was added to each well, totalizing a final volume in the well of 100 μL. As negative control, wells containing medium, inoculum and DMSO were evaluated, but without the compound. As a positive control, the commercial fungicide Score was used, whose active principle is difenoconazole. The microplate was incubated in B.O.D. at 28°C and analyzed in a microplate reader at 620 nm at 12 hours intervals for 120 hours.

The minimum inhibitory concentration (MIC) of the compound was determined spectrophotometrically after the above-mentioned incubation periods, according to the (CLSI, 2008) guidelines, with modifications. For both the isolated compound and the commercial fungicide, MIC was determined as the lowest concentration showing absence of growth or equal to the initial growth as compared to growth (up to 12 hours) in the well free of compounds. The commercial fungicide used was the Score (Syngenta, Switzerland).

Results

The bioassays against the plant pathogens *D. phaseolorum* and *C. gloeosporioides* were carried out with the ethyl acetate extracts obtained from 88 endophytic fungi, being 36 from *B. fischeri*, 24 from *B. olsoniae* and 28 from *B. venosa*. From the 88 fractions evaluated, 14.7% were active against *C. gloeosporioides*, 11.3% against *D. phaseolorum* and 6.81% inhibited both plant pathogens, varying in inhibition indexes. In relation to the host plants, 27.7% of the selected endophytes from *B. fischeri* inhibited the growth of at least one of the plant pathogens evaluated, against 50% of *B. olsoniae* endophytes and 7.14% of *B. venosa*

(*D. phaseolorum* inhibition only). The 24 active fractions were reevaluated in a new bioassay (in triplicate). The results are shown in Table 1.

Table 1. Inhibitory activity of 24 endophytic fungi isolated from *Begonia fischeri*, *B. olsoniae* and *B. venosa* against the plant pathogens *Diaporthe phaseolorum* and *Colletotrichum gloeosporioides*

Fungi ID	Fungi identification	Host	*D. phaseolorum*	*C. gloeosporioides*
am 11	*Colletotrichum* sp1	*B. olsoniae*	-	+
am 222	*Colletotrichum* sp1	*B. olsoniae*	-	+
am 229	*Colletotrichum* sp1	*B. olsoniae*	-	+
am 37	*Colletotrichum* sp1	*B. olsoniae*	-	++
am 273	*Curvularia* sp1	*B. fischeri*	+++	-
am 193	*Curvularia* sp2	*B. fischeri*	+++	-
am 02	*Diaporthe* sp1	*B. fischeri*	++	-
am 114	*Diaporthe* sp1	*B. olsoniae*	-	++
am 117	*Diaporthe* sp1	*B. fischeri*	++	+++
am 122	*Diaporthe* sp1	*B. olsoniae*	+	+
am 111	*Diaporthe* sp2	*B. olsoniae*	++	-
am 120	*Diaporthe* sp3	*B. olsoniae*	++	-
am 82	*Diaporthe* sp5	*B. olsoniae*	+++	-
am 173	*Epicoccum nigrum*	*B. fischeri*	+	+++
am 78	*Mucor bainieri*	*B. olsoniae*	+	+
am 110	*Neopestalotiopsis* sp.	*B. fischeri*	-	++
am 29	*Neopestalotiopsis* sp.	*B. fischeri*	**+++**	**-**
am 95	*Neopestalotiopsis* sp.	*B. fischeri*	++	-
am 58	*Trichoderma* sp.	*B. olsoniae*	++	+
am 166	*Xylariales*	*B. olsoniae*	-	+
am 80	*Xylaria* sp2	*B. fischeri*	++	++
am 145	*Xylariales*	*B. fischeri*	-	++
b145	Non-identified ascomycete	*B. venosa*	+++	-
b147	*Colletotrichum* sp1	*B. venosa*	++	-

Inhibition scores (-) no inhibition of the plant pathogen. (+) Plant pathogen got to the paper disk. Very small inhibition halo. (++) Plant pathogen did not reach the paper disk. Small inhibition halo. (+++) Plant pathogen did not reach the paper disk. High inhibition halo.

The strain AM29, identified as *Neopestalotiopsis* sp. (deposited under accession CRM 1920 at CRM-UNESP, Microbial Resource Center (WDCM 1043), was selected by its activity against *D. phaseolorum* for a chemical investigation of its secondary metabolites.

After culturing the *Neopestalotiopsis* sp. AM29 in liquid medium, filtration and partitioning with ethyl acetate, the AcOEt extract (1.0 g) was obtained. This extract showed 23% activity against the mycelial growth of *D. phaseolorum* and was fractioned in a biomonitoring way until it obtained the pure active compound 1B3P1 (1.2 mg), with 58% of activity against *D. phaseolorum*. The molecular mass of the pure compound was searched in the Dictionary of Natural Products database and identified by spectroscopic analysis (MS and NMR) by comparison with Hayashi et al. (2007). With the data obtained in the ^{1}H NMR spectrum, it was possible to correlate the hydrogen shifts (δ_H) of 1B3P1 (1) with the compound fumiquinone B (2,6-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione, CAS 1011259-29-2), a quinone derivative, Figure 1 (Hayashi et al., 2007).

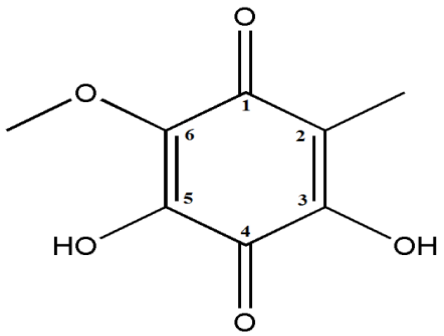

Figure 1. Structure of fumiquinone B ($C_8H_8O_5$) produced by *Neopestalotiopsis* sp., endophytic from *Begonia fischeri*.

The isolated compound fumiquinone B was tested against spores of *D. phaseolorum* using the microdilution assay (Figure 2).

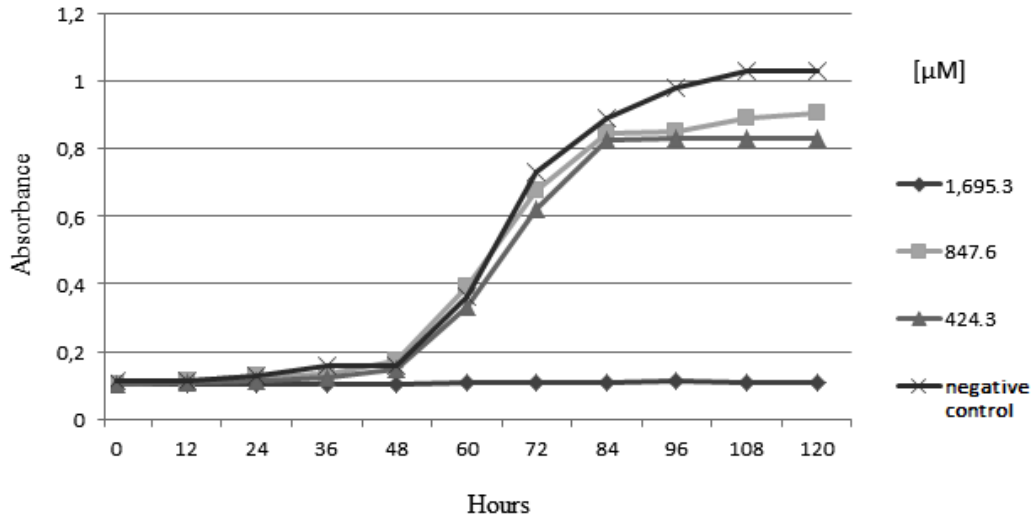

Figure 2. Data obtained in microdilution assay of the isolated compound fumiquinone B against spores of *Diaporthe phaseolorum*

The assay was carried out after 120h of incubation. The compound fumiquinone B was active against spores of *D. phaseolorum*, with a MIC of 312 µg mL^{-1} (1,695.3 µM). The MIC of the commercial fungicide difenoconazole (positive control, not shown) was 2.4 µg mL^{-1} (5.9 µM).

Discussion

Probably the ethyl acetate extracts from the endophytic fungi that showed inhibition against the evaluated plant pathogens have different compounds, since the best inhibitors for *D. phaseolorum* were not the same for *C. gloeosporioides*. Regarding the inhibition of the former, the extracts obtained from the endophytes *Cuvularia* sp1, *Curvularia* sp2, *Diaporthe* sp5 and *Neopestalotiopsis* sp. presented the best results. For *C. gloeosporioides*, the extracts of *Diaporthe* sp1 and *Epicoccum nigrum* presented the higher inhibition scores.

In the literature, there are few studies with secondary metabolites from *Neopestalotipsis* species. This is due to the fact that the genus *Neopestalotiopsis* was recently described by Maharachchikumbura, Hyde, Groenewald, Xu, and Crous (2014). Initially, De Notaris, in 1839, described the genus *Pestalotia* De Not, which was divided by Steyaert, in 1955 in *Pestalotia*, *Pestalotiopsis* Steyaert and *Truncatella* Steyaert. Until the 1990s, the taxonomy of *Pestalotiopsis* and related genera was based on conidial characteristics, mainly pigmentation. However, this method of identification was controversial, due to the variation of other morphological characteristics, such as colony color, texture and shape (Hu, Jeewon, Zhou, Zhou, & Hyde, 2007; Solarte, Muñoz, Maharachchikumbura, & Álvarez, 2018). Then, by morphological and DNA analyzes (ITS, β-tubulin and 1-alpha elongation factor) the genus *Pestalotiopsis* was segregated into two new genera: *Neopestalotiopsis* Maharachch., K.D. Hyde & Crous and *Pseudopestalotiopsis* Maharachch., K.D. Hyde & Crous. Eleven new species were introduced in *Neopestalotiopsis*, twenty-four in *Pestalotiopsis* and two in *Pseudopestalotiopsis* (Maharachchikumbura et al., 2014). Due to this recent review of the genus, most of the available information is on the genus *Pestalotiopsis*, which is commonly isolated as endophyte (Wei et al., 2007; Figueiredo et al., 2007). It has been isolated from the families *Podocarpaceae*, *Theaceae* and *Taxaceae* (Wei et al., 2007), of the species *Rhizophora apiculata* DC., *Cocos nucifera* L., *Taxus wallichiana* Zucc., *Camellia sasanqua* Thunb., *Fragraea bodenii* Wernh., *Cordemoya integrifolia* (Willd.) Baill. (Wei & Tong, 2003), *Maytenus ilicifolia* Mart. Ex Reissek (Figueiredo et al., 2007), *R. mucronata* Lam. (Zhou et al., 2017), among others. This fungus is known for the production of compounds with medicinal applications (Tejesvi, Kini, Prakash, Subbiah, & Shetty, 2008), as antioxidant, antihypertensive, antimicrobial (Tejesvi et al., 2008; Zhao et al., 2015) and antifungal activity (Strobel et al., 2002); agricultural application, as anti-oomycete activity (Li & Strobel, 2001); and antifungal against *Fusarium oxysporum* Schltdl., *F. fujikuroi* Nirenberg (= *F. verticillioides* (Sacc.) Nirenberg), *Macrophomina phaseolina* (Tassi) Goid., *Epicoccum sorghinum* (Sacc.) Aveskamp, Gruyter & Verkley (= *Phoma sorghina* (Sacc.) Boerema, Dorenb. & Kesteren) and *Sclerotinia sclerotiorum* (Lib.) de Bary (Tejesvi, Kini, Prakash, Subbiah, & Shetty, 2007); in addition to producing

compounds with industrial applications (Xu, Ebada, & Proksch 2010; Maharachchikumbura, Guo, Chukeatirote, Bahkali, & Hyde, 2011).

Quinones are a group of natural compounds found in fungi, bacteria and plants (Monks & Jones, 2002). They are generally colored and semi-volatile, belonging to the class of polycyclic aromatic oxygenated hydrocarbons (HPAO). The structure of the quinones presents two carbonyl groups in an unsaturated ring of six carbon atoms, and they are classified as benzoquinones, naphthoquinones, anthraquinones or phenanthraquinones, according to the type of aromatic system (Sousa, Lopes, & Andrade, 2016).

Quinones are synthesized essentially via the polyketide pathway or the shikimate pathway, biosynthetic pathways absent in animals (Thompson, 1971). The biological reactivity of quinones in biological systems can be attributed to the fact that they are oxidizing and electrophilic. In relation to toxicity, this is influenced by the chemical structure, mainly by substitution effects (Monks & Jones, 2002). They have a role in photosynthesis, like electron transporters. Several quinones are vitamins or have antioxidant activity. Quinone derivatives are common in biologically active molecules. Many of the drugs clinically approved or still in clinical trials against cancer are quinone-related compounds (El-Najjar et al., 2011). Quinones, such as 1,4-naphthoquinone, 1,2-naphthoquinone, 1,4-benzoquinone, and anthraquinone have moderate antifungal activity to *Colletotrichum* spp. (Meazza, Dayan, & Wedge, 2003).

The quinone fumiquinone B has been isolated from the fungi *Aspergillus fumigatus* Fresen. (Hayashi et al., 2007) and *Xylaria feejeensis* (Berk.) Fr. (García-Mendez, Macías-Ruvalcaba, Lappe-Oliveiras, Hernández-Ortega, & Macías-Rubalcava, 2016), and is known for its nematicidal activity against *Bursaphelenchus xylophilus* Steiner & Buhrer, 1934 (Hayashi et al., 2007) and for its phytotoxic properties (García-Mendez et al., 2016).

Although the MIC of fumiquinone B against *D. phaseolorum* is higher than the one of the commercial fungicide difenoconazole, the isolated compound may present a different mode of action, as it pertains to the quinones class, and difenoconazole belongs to the group of triazoles that act on the cell membrane of fungi (Odds, Brown, & Gow, 2003).

Here, we emphasize the ability of the endophytes in producing compounds that may be used to control plant pathogens such as *C. gloeosporioides* and *D. phaseolorum*. The *endophyte Neopestalotiopsis* sp. AM29 produced fumiquinone B, which showed activity against the latter pathogen. This is the first report of the production of the compound fumiquinone B by fungi in the genus *Neopestalotiopsis*, as well as it is the first report of its activity against *D. phaseolorum*. Further studies need to be performed to evaluate the toxicity of the compound, additionally *in vivo* tests are also necessary.

Eletronic supplementary material

The mass spectra (positive and negative mode), high-resolution mass spectrum (positive mode) and ^{1}H NMR of the compound fumiquinone B in $DMSO_{d6}$ were shown in the supplementary material (Figures S1, 2, 3, 4 and 5).

Conclusion

Our study showed that endophytic fungi from *Begonia* plant species produce antifungal compounds. One of these metabolites, fumiquinone B, found in a isolate of *Neopestalotiopsis*, inhibited the *in vitro* growth of plant pathogens.

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Supplementary material

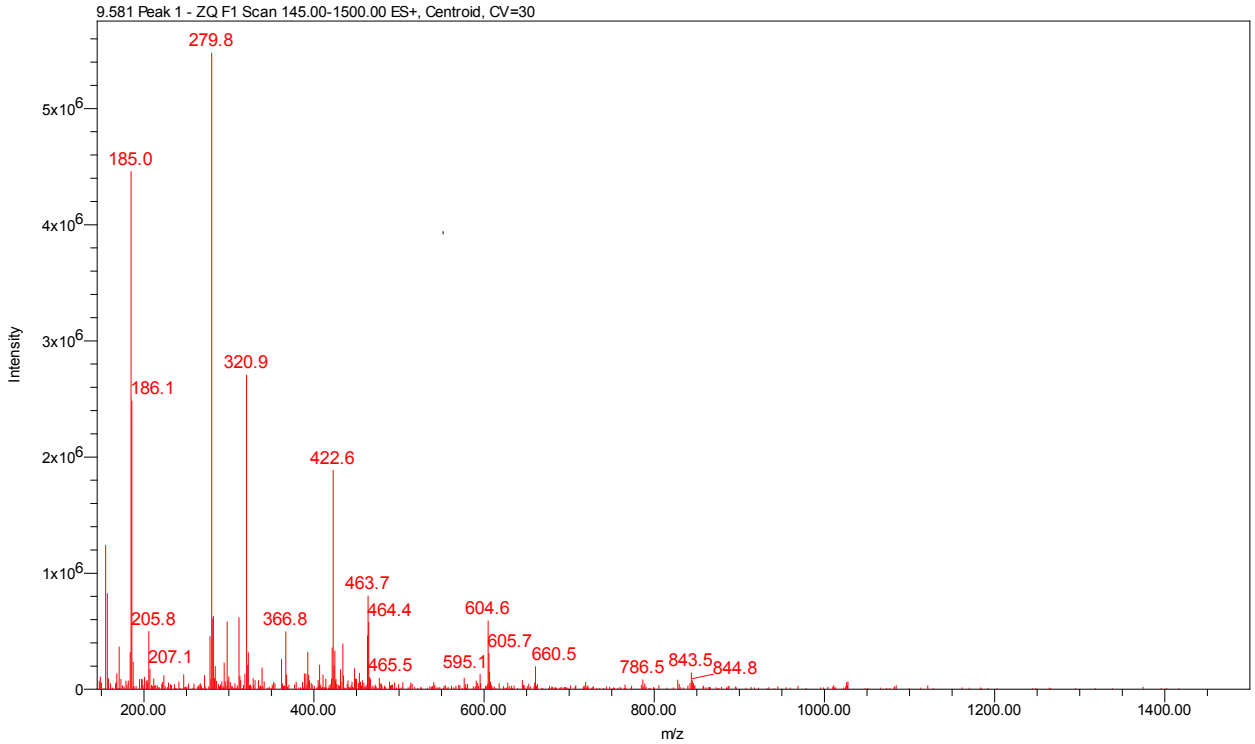

Figure S1. Mass spectrum (positive mode) of the compound fumiquinone B.

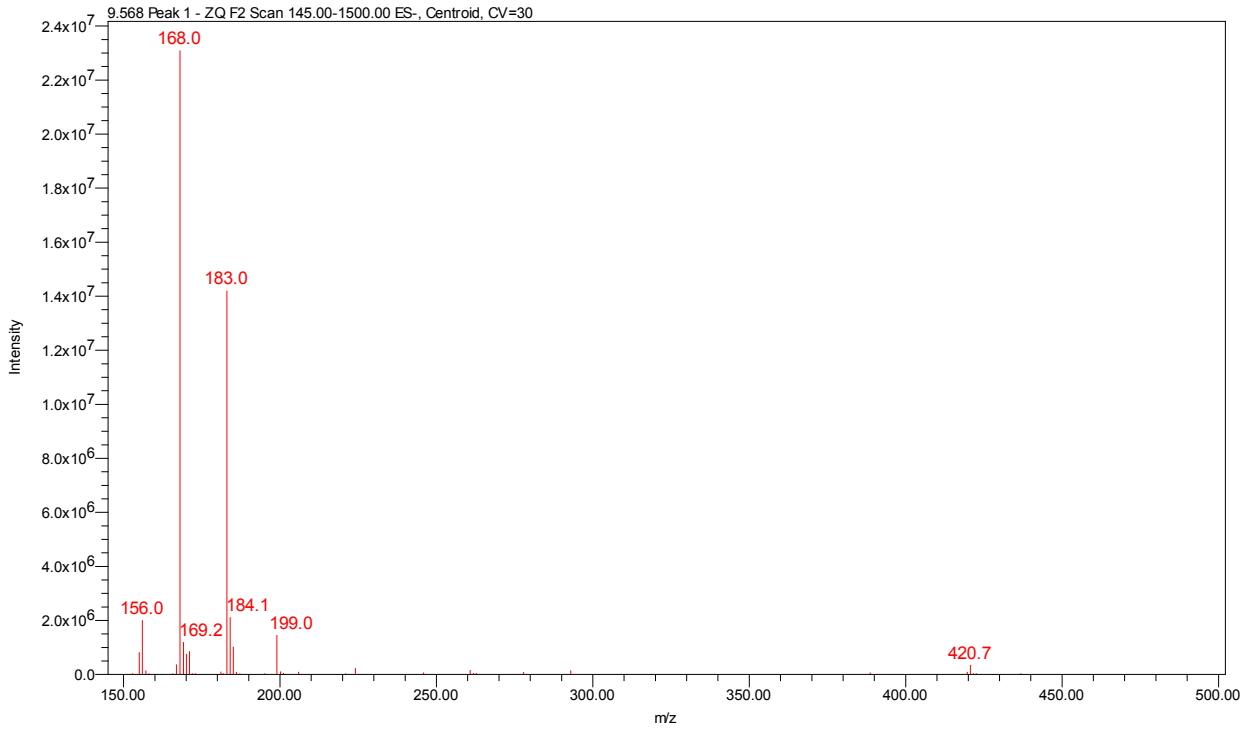

Figure S2. Mass spectrum (negative mode) of the compound fumiquinone B.

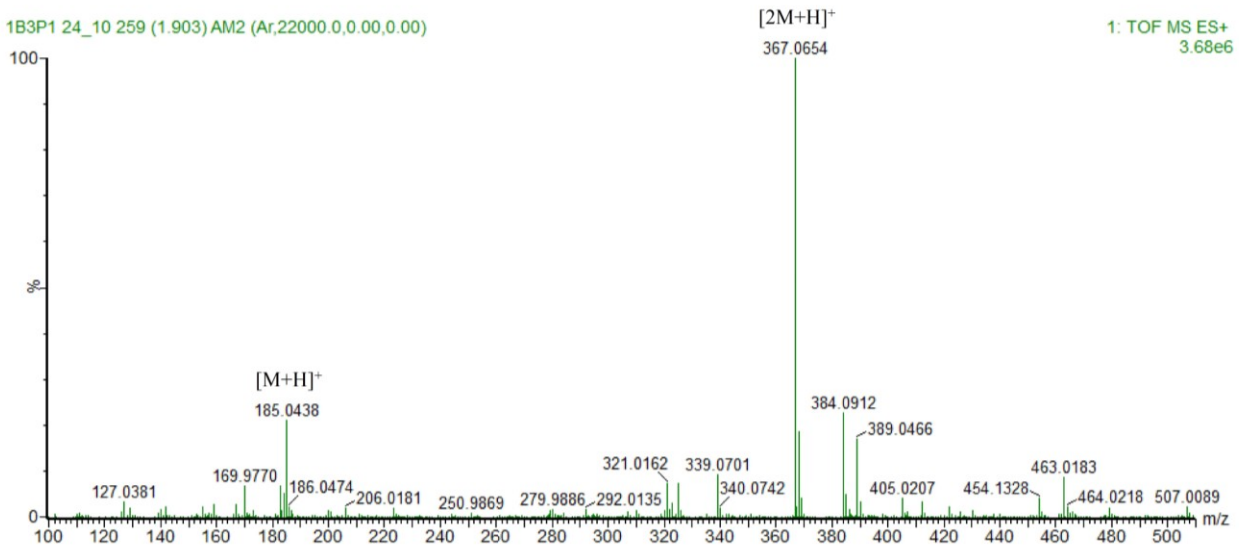

Figure S3. High-resolution mass spectrum of the compound fumiquinone B.

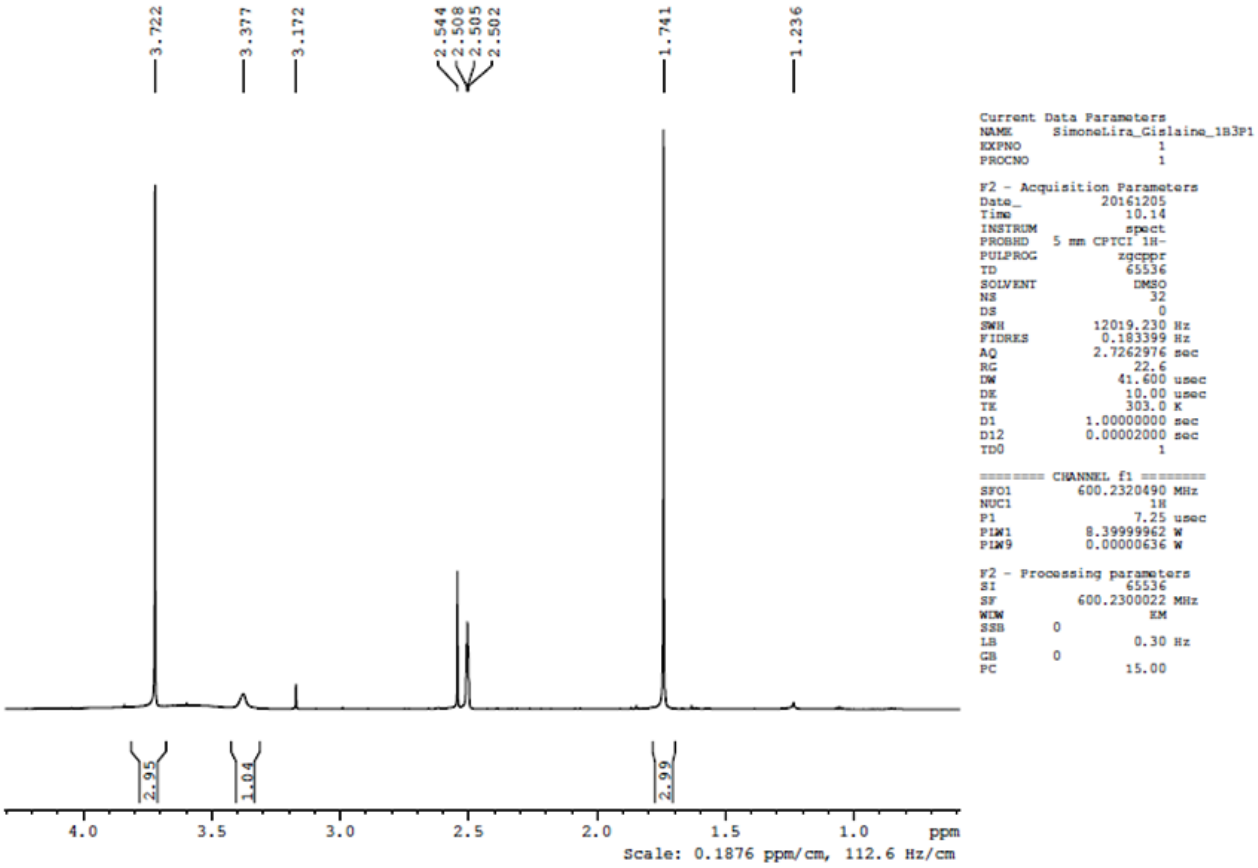

Figure S4. NMR of the compound fumiquinone B in $DMSO_{d6}$.

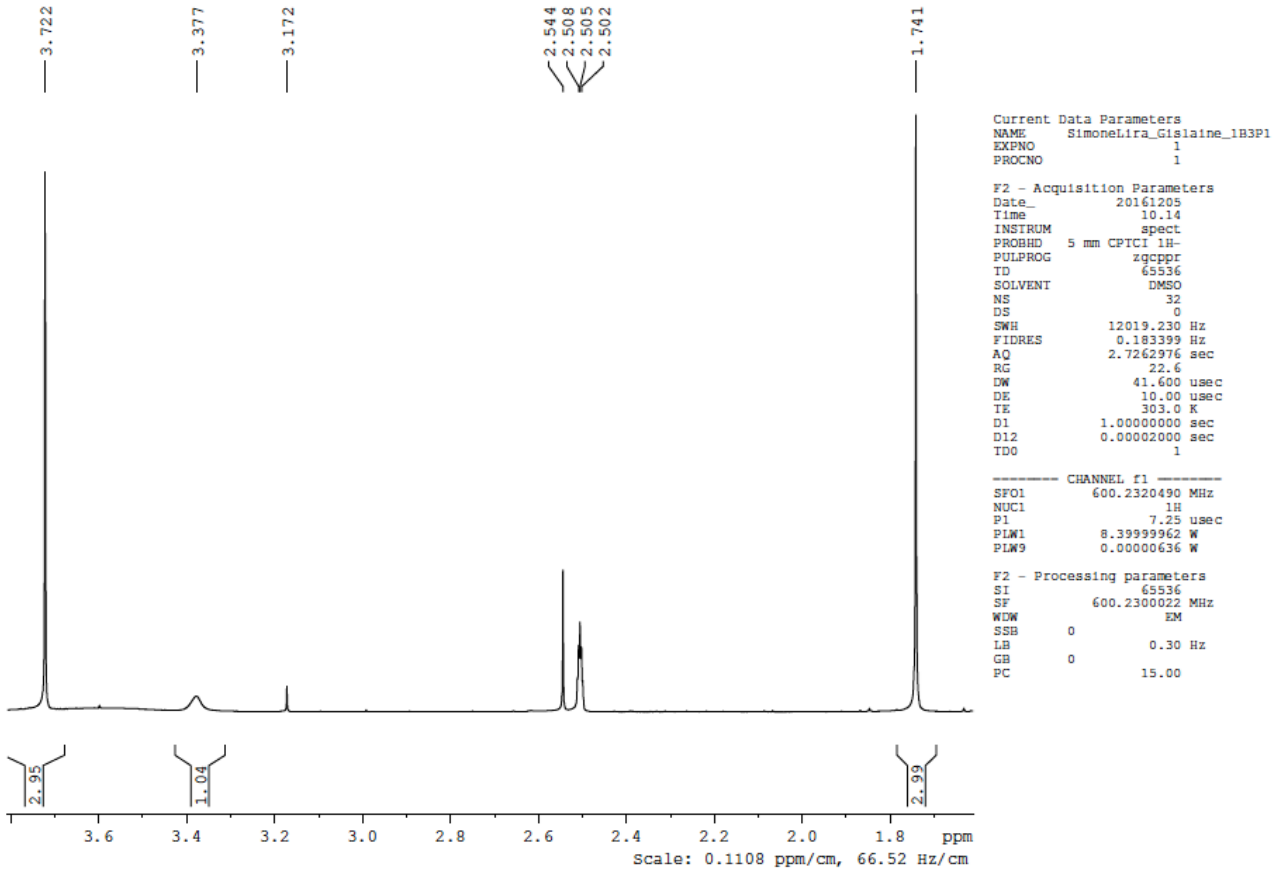

Figure S5. NMR of the compound fumiquinone B in $DMSO_{d6}$ (with enlarged region).