

Article

Secondary Metabolites Produced by *Macrophomina phaseolina* Isolated from *Eucalyptus globulus*

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Abstract: In the course of investigations on the role of secondary metabolites in plant-microbe interactions, the production of secondary metabolites by *Macrophomina phaseolina* isolates from *Eucalyptus globulus*, was studied. This fungus is responsible for several plant diseases which affect crop productivity and industry. Although secondary metabolites may play a role in disease development, there are very few reports on *M. phaseolina* metabolomics and, as far as we know, isolates from eucalypts have not been investigated for secondary metabolites production. In the present paper, metabolites typical of fungi, from the family Botryosphaeriaceae, were identified for the first time as products of *M. phaseolina*. Furthermore, the isolate under examination was grown in the presence and absence of host stem tissue, and metabolite profiles were compared. Five products are reported for the first time in this species and azelaic acid was exclusively produced in the presence of eucalypt stem. Finally, phytotoxicity and cytotoxicity tests of culture filtrates and crude organic extracts were also performed. Key Contribution: Lipophilic metabolites produced by *M. phaseolina* might play a role in the plant-fungus interactions responsible for serious diseases of *E. globulus*.

Keywords: plant-microbe interaction; secondary metabolites; *Eucalyptus globulus*; *Macrophomina phaseolina*; plant diseases; (3R,4S)-botryodiplodin

1. Introduction

The genus *Eucalyptus* belongs to the family Myrtaceae which contains approximately 800 species. Most *Eucalyptus* species are endemic to Australia and they are planted as an important source of fiber and essential oils, many of which have ecological functions as well as medicinal and industrial uses [1–4]. The remarkable adaptability of eucalypts coupled with their fast growth and wood properties has driven their rapid adoption for plantation forestry, making eucalypts the most widely planted hardwood forest trees in the world [2,5].

Due to various diseases, planting of some *Eucalyptus* species was impossible or limited in specific areas and it significantly affected the industry. The most serious diseases of the *Eucalyptus* species are caused by fungi, in particular the ones caused by *Cryphonectria cubensis* and various species of *Mycosphaerella* and Botryosphaeriaceae (e.g., *Botryosphaeria dothidea*, *Diplodia corticola*, *Neofusicoccum eucalyptorum*, *Neofusicoccum kwambonambiense*, *N. parvum*) [6–8].

Many fungi from the family Botryosphaeriaceae are well-known opportunistic pathogens of a broad number of plant hosts [9–11], being able to switch, with unfavorable environmental conditions from asymptomatic endophytes, to virulent pathogens. In fact, species of Botryosphaeriaceae are generally endophytes on eucalypts, but can also cause severe canker and dieback diseases which have negative impacts in both native and introduced plantations [12,13]. Among them, *M. phaseolina* (Tassi) Goid. is responsible for serious damages of eucalypt plantations [14,15] and other economically important crops, such as soybean, sunflower, sesame, melon and strawberry [16–20]. In fact, *M. phaseolina* attacks a wide range of hosts in more than 500 cultivated and wild plant species [21]. The diseases caused by this fungus are well-documented, but few reports on secondary metabolites produced by *M. phaseolina* are present in the literature, although they might play a role in charcoal rot and other plant diseases. So far, *M. phaseolina* has been reported as a producer of only some metabolites, such as asperlin, isoasperlin, phomalactone, phaseolinic acid, phomenon and phaseolinone [22,23].

The aim of this study was to investigate, for the first time, the *in vitro* secondary metabolite production of an isolate of *M. phaseolina* from *Eucalyptus globulus*. The effect of eucalypt tissues on the secondary metabolite profile of *M. phaseolina*, grown in liquid medium, was also evaluated. Furthermore, the phytotoxic and cytotoxic activities of the culture filtrates and crude ethyl acetate extracts were also tested.

2. Materials and Methods

2.1. General Experimental Procedures

Optical rotations of pure metabolites were measured in CHCl_3 or in MeOH on a Jasco polarimeter (Tokyo, Japan). ^1H NMR spectra were recorded at 400 MHz in deuterated chloroform (CDCl_3) or in deuterated methanol (CD_3OD), on Bruker spectrometers (Karlsruhe, Germany) and the same solvents were used as internal standards. Analytical and preparative TLC were performed on silica gel plates (Kieselgel 60, F254, 0.25 mm; Merck, Darmstadt, Germany). The spots were visualized by exposure to UV radiation (253 nm), or by first spraying with 10% H_2SO_4 in methanol, followed by heating at 110 °C for 10 min. Chromatography columns (CC) were carried out on silica gel (Merck, Kieselgel 60, 0.063–0.200 mm).

GC-MS measurements were performed using an Agilent 6850 GC equipped with an HP-5ms capillary column (5% phenyl methyl poly siloxane stationary phase) and the Agilent 5973 Inert MS detector (used in the scan mode). Helium was employed as the carrier gas, at a flow rate of 1 mL min^{-1} . The injector temperature was 250 °C and the temperature ramp raised the column temperature from 70 °C to 280 °C: 70 °C for 1 min; 10 °C min^{-1} until reaching 170 °C; and 30 °C min^{-1} until reaching 280 °C. Then, it was held at 280 °C for 5 min. The electron impact (EI) ion source was operated at 70 eV and at 200 °C. The quadrupole mass filter was kept at 250 °C and was programmed to scan the range 45–550 m/z at a frequency of 3.9 Hz. GC-MS data were acquired on crude extracts after trimethylsilylation according to Guida et al. [24].

2.2. Fungal Isolate and Growth Conditions

M. phaseolina PE35 used in this study was originally isolated from *E. globulus* in Portugal. Cultures were maintained at room temperature on potato dextrose agar (PDA) medium (Merck, Darmstadt, Germany). Before inoculations, each isolate was cultured on PDA at 25 °C for 7 days, in the dark. Mycelium was scraped from PDA plates and suspended in 5 mL of sterilized ultra-pure water. This mycelial suspension was added to 1 L erlenmeyers, containing 250 mL of potato dextrose broth (PDB) (pH 5.6), and incubated for 7 days with and without eucalypt stem (host) in the dark at 25 °C. *E. globulus* stems were sterilized twice by autoclaving and then dried at 50 °C. As a control, sterilized *E. globulus* stems were added to PDB and incubated for 7 days in the dark at 25 °C. Culture filtrates were obtained by filtering the culture through sterile 0.45 μm cellulose acetate membranes in a vacuum system.

2.3. Extraction and Purification of Fungal Metabolites

Culture filtrates (PE35 = 1 L, PE35_{Host} = 1 L, HOST = 250 mL) were extracted 3 times with EtOAc. Each organic phase was dried with Na₂SO₄, and evaporated under reduced pressure, originating a crude extract (CE) as brown oil residue (PE35CE = 90.2 mg, PE35CE_{Host} = 111.6 mg, HOSTCE = 7.7 mg). PE35 crude extracts were purified by silica gel chromatography (CC and/or TLC), producing the identified metabolites.

PE35CE was fractionated by CC on silica gel (40 cm × 1.5 cm ID) eluted with CHCl₃/*i*-PrOH (9:1, *v/v*) originating 10 homogeneous fractions and the last fraction was eluted with methanol (A 0.7 mg, B 1.3 mg, C 11.0 mg, D 7.1 mg, E 1.1 mg, F 9.3 mg, G 1.9 mg, H 6.7 mg, I 6.8, L 15.0 mg). The residue from fraction B, an amorphous solid, was identified as (*R*)-mellein (*R*_f 0.67 on TLC on silica gel eluted with CHCl₃). The residue of fraction C was purified by preparative TLC on silica gel eluted with a mixture of *n*-hexane/EtOAc (1:1, *v/v*), originating an amorphous solid identified as *cis*-(3*R*,4*R*)-4-hydroxymellein (4.5 mg *R*_f 0.46 on TLC on silica gel eluted with *n*-hexane/EtOAc (1:1, *v/v*)). Fraction F, after purification by CC on silica gel (20 cm × 1.0 cm) eluted with *n*-hexane/EtOAc (1:1, *v/v*), produced yellow oil identified as (3*R*,4*S*)-botryodiplodin (7.2 mg *R*_f 0.24 on TLC on silica gel eluted with *n*-hexane/EtOAc (1:1, *v/v*)).

PE35CE_{Host} was fractionated by CC on silica gel (40 cm × 1.5 cm ID) eluted with CHCl₃/*i*-PrOH (9:1, *v/v*), originating 9 homogeneous fractions and the last fraction was eluted with methanol (A 1.0 mg, B 2.0 mg, C 5.7 mg, D 11.6 mg, E 7.6 mg, F 14.8 mg, G 3.7 mg, H 7.1 mg, I 20.0 mg). The residue from fraction B, an amorphous solid, was identified as (*R*)-mellein. The residue of fraction E was purified by preparative TLC on silica gel eluted with a mixture of *n*-hexane/EtOAc (1:1, *v/v*), originating an amorphous solid identified as *cis*-(3*R*,4*R*)-4-hydroxymellein (3.2 mg). Fraction F, after purification by CC on silica gel (20 cm × 1.0 cm ID) eluted with *n*-hexane/EtOAc (1:1, *v/v*), produced yellow oil identified as (3*R*,4*S*)-botryodiplodin (10.5 mg).

2.4. Phytotoxicity Assay

Phytotoxicity was performed both on tomato cuttings and by a leaf puncture assay. Culture filtrates were tested on tomato cuttings using the following dilutions (*v/v*): 1:4, 1:2, and without dilution. Tomato stems of rootless plants (2 weeks old) were dipped in a vial with 2 mL of culture filtrate. After 24 h of incubation at room temperature, the solution was replaced by 2 mL of sterile distilled water for an additional 48 h. Sterile distilled water and PDB were used as controls. Using a 0–4 scale (0 = no symptoms; 1 = slight withering; 2 = intermediate withering; 3 = severe withering; 4 = full withering), symptoms were recorded and converted to a percentage to evaluate phytotoxic activity. All assays were conducted in triplicate.

Phytotoxic activity of crude extracts was assessed by leaf puncture assay. Each tomato plant leaf was punctured with a sterile needle, and a droplet (20 µL) of extract dissolved in 4% of MeOH was applied to the adaxial surface on the leaves. Several dilutions of extract solutions were tested: 1, 0.5, 0.25, 0.1, 0.01 mg mL⁻¹. The leaves were kept in a moist chamber to prevent the droplets from drying and were observed daily. The visual symptoms were recorded after 10 days of incubation. The lesion diameter was expressed in cm. All assays were carried out in triplicate.

2.5. Cytotoxicity Assay

The cytotoxicity tests were conducted as previously reported with slight modifications [25,26]. Two cell lines were grown and maintained according to Ammerman et al. [27]: A Vero cell line (ECACC 88020401, African green monkey kidney cells, GMK clone) and a 3T3 cell line (DSMZ – ACC 173). The microtiter plates were incubated at 37 °C in 5% CO₂ for 24 h. Vero and 3T3 cells were treated, for 20 h, with culture filtrates or crude extracts (1:1 in DMEM—Dulbecco's modified eagle medium). Three dilutions (*i.e.*, 1:2, 1:4, 1:8) of culture filtrates were tested. Six solutions of crude extracts were prepared in 4% MeOH in phosphate buffered saline (PBS), diluted in PBS at 1 — 0.01 mg mL⁻¹ and

tested. PBS and 4% MeOH were used as controls. After the incubation period, the medium was removed by aspiration, and 50 μL of DMEM with 10% resazurin (0.1 mg mL^{-1} in PBS) was added to each well to assess cell viability. The microtiter plates were incubated at 37°C in 5% CO_2 for 3 h. The absorbance was read at wavelengths 570 and 600 nm in a microtiter plate spectrophotometer (Biotek Synergy). PBS was used as the control.

2.6. Statistical Analysis

Two-way analysis of variance (ANOVA), followed by a Tukey multiple comparison test, was used to determine the statistical significance of cytotoxicity between each dilution/concentration and control of each crude extract or culture filtrate obtained from different sources (PE35; PE35_{Host}; host) ($p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$). All the analyses were performed with GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Data are shown as the average of three independent replicates of each condition.

3. Results

3.1. Identification and Distribution of Metabolites

Metabolites from PDB (potato dextrose broth) incubated with eucalypt stems, and from the culture filtrates of *M. phaseolina* PE35 grown with and without host tissue were extracted with ethyl acetate (EtOAc) as described in the methods section (called "HOSTCE, PE35CE_{Host} and PE35CE" from this point forward). Crude extracts were treated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) before GC-MS analysis because secondary metabolites may not have suitable gas chromatographic properties [28]. All metabolites were identified comparing their EI mass spectra at 70 eV with the ones present in the NIST 14 mass spectral library [29] and the custom GC-MS library described by Félix et al. [30,31].

Total ion chromatograms (TICs) of PE35CE_{Host} and PE35CE are shown in Figure 1a,b.

M. phaseolina PE35, under the growth conditions studied (in presence and absence of host tissue), produces (3*R*,4*S*)-botryodiplodin, succinic acid, tyrosol, (*R*)-mellein, and cis-(3*R*,4*R*)-4-hydroxymellein. Azelaic acid was exclusively produced when *M. phaseolina* PE35 was grown in the presence of eucalypt stems. The definitive proof of the metabolites' identity of metabolites (Figure 2) and the stereostructure elucidation of some compounds (i.e., (3*R*,4*S*)-botryodiplodin, cis-(3*R*,4*R*)-4-hydroxymellein and (*R*)-mellein) were obtained after fractionation by column chromatography of the crude extracts and the comparison of the ^1H NMR spectra and optical rotation of pure compounds with those reported in the literature [31].

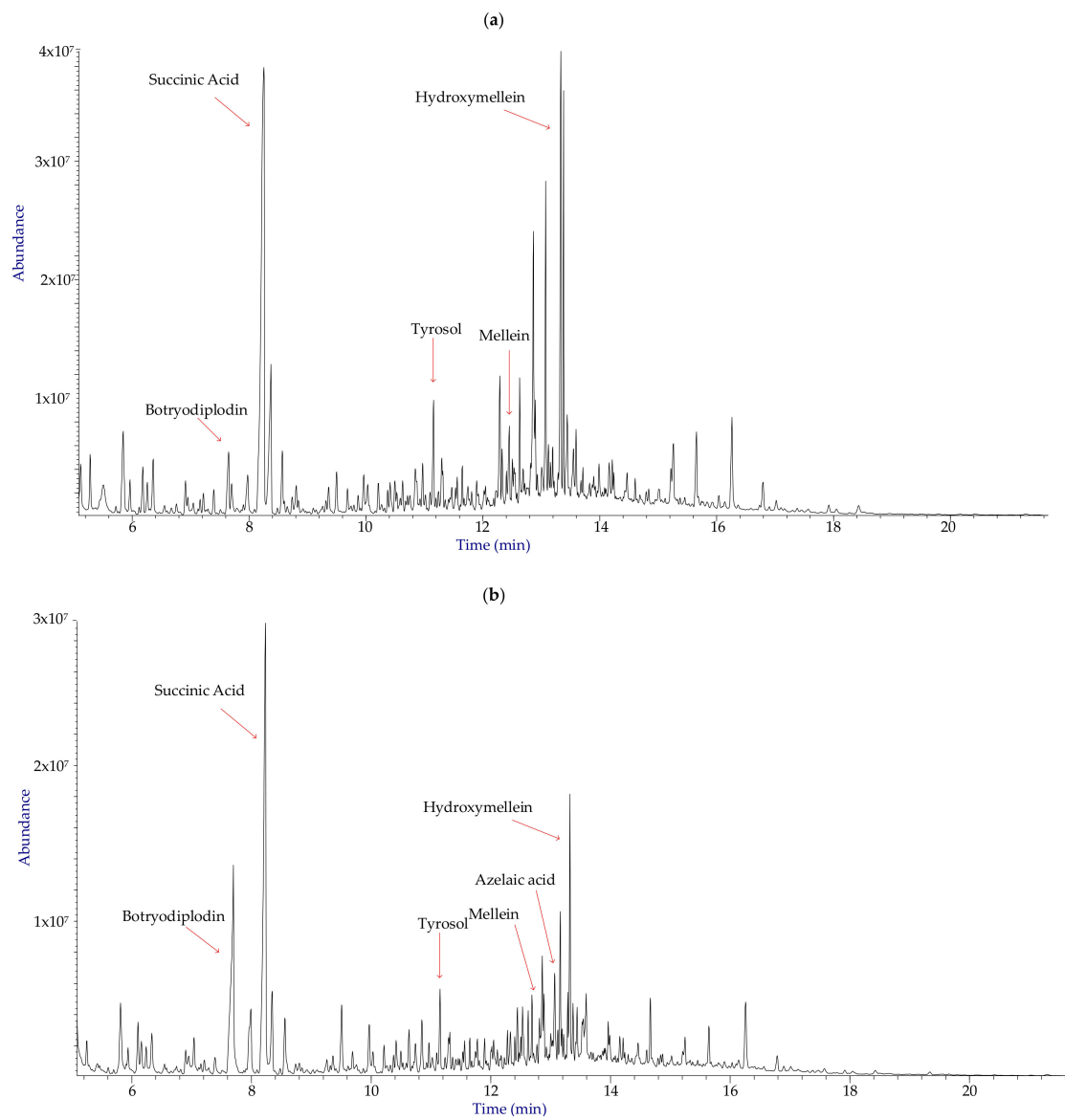


Figure 1. Annotated total ion chromatograms (TICs) acquired by processing samples from crude extracts. (a) Crude extract of *Macrophomina phaseolina* PE35 grown in potato dextrose broth (PDB) (PE35CE), (b) Crude extract of *M. phaseolina* PE35 grown in the presence of *Eucalyptus globulus* (PE35CE_{Host}).

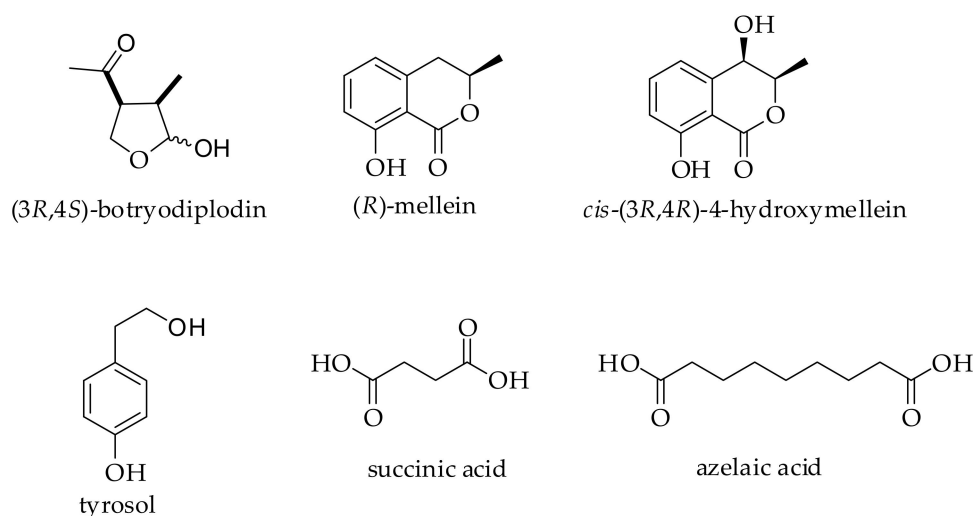


Figure 2. Secondary metabolites identified in crude extract of *M. phaseolina* PE35 grown in absence (PE35CE) and in presence of *E. globulus* (PE35CE_{host}). Azelaic acid was exclusively produced by *M. phaseolina* PE35 grown in the presence of eucalypt stems.

3.2. Phytotoxicity and Cytotoxicity Assays

Phytotoxicity of culture filtrates was assessed on tomato plant cuttings. Under both conditions (PE35 in the presence and in absence of host), *M. phaseolina* PE35 was able to induce symptoms (depigmentation and severe necrosis) on tomato plants (Figure 3). Control culture filtrate (i.e., host) showed the same effect of PDB (no visible symptoms). Comparing the results obtained from phytotoxicity tests of culture filtrates of *M. phaseolina* PE35 grown with and without host, no differences in toxicity were visible on tomato plants cuttings (Figure 3). Phytotoxicity (presence of symptoms) on tomato cuttings was significant only in the use of pure (undiluted) filtrate.

Dilution (v/v)	Phytotoxic activity (%)		
	PE35	PE35 _{Host}	Host
No dilution	75.00 ****	58.33 ****	16.67
1:2	33.33	25.00 **	8.33
1:4	8.33	8.33 **	0

Figure 3. Phytotoxicity (%) of culture filtrates of *M. phaseolina* PE35 grown with (PE35_{Host}) and without (PE35) host tissue (eucalyptus). Two controls were used: No diluted PDB (16.67% of phytotoxic activity) and water (no phytotoxic activity). Statistical analysis was performed using two-way ANOVA followed by a Tukey multiple comparison test. Asterisks correspond to data with statistical difference (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) towards the control.

Phytotoxicity of the crude extracts was tested on tomato leaves (Figure 4). The extracts obtained from *M. phaseolina* grown in the presence of the host seems to be more phytotoxic than the ones corresponding to growth without host. The crude extract of the control (i.e., HOSTCE) induced no phytotoxic symptoms (Figure 4).

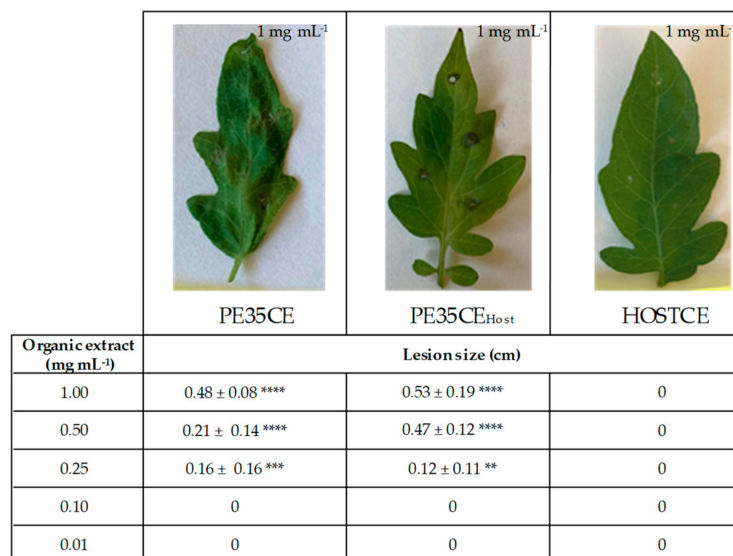


Figure 4. Phytotoxicity of crude extracts of filtrates of *M. phaseolina* PE35 grown in presence (PE35CE_{Host}) and absence host tissue (PE35CE) and only host tissue (HOSTCE). Statistical analysis was performed using two-way ANOVA followed by a Tukey multiple comparison test. Asterisks correspond to data with statistical difference (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) towards the control.

Since *M. phaseolina* was described as a human pathogenic opportunist [32], the toxic effect on mammalian cells was also evaluated. When the culture filtrate was assayed, it was possible to observe significant cytotoxicity on 3T3 and Vero cell lines (Figure 5).

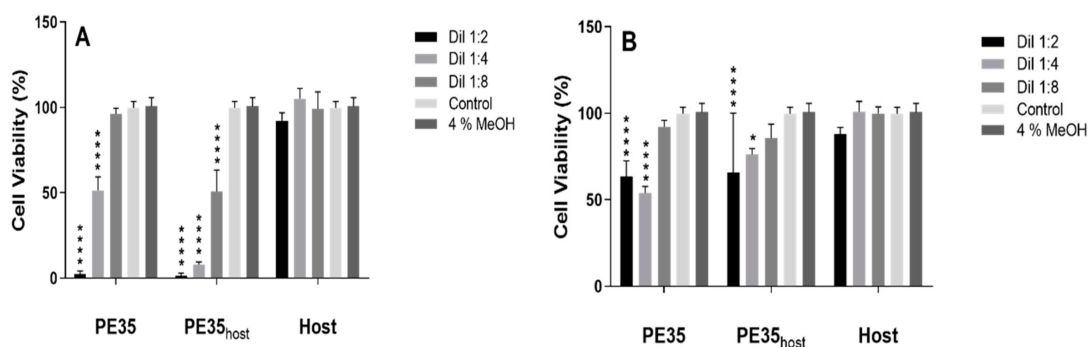


Figure 5. Cytotoxicity of culture filtrates of *M. phaseolina* PE35 grown with (PE35_{Host}) and without (PE35) host tissue (*Eucalyptus*) in 3T3 cells (A) and Vero cells (B). Phosphate buffered saline (PBS) and 4% MeOH were used as the control. Statistical analysis was performed using two-way ANOVA followed by a Tukey multiple comparison test. Asterisks correspond to data with statistical difference (**** $p < 0.0001$, * $p < 0.05$) towards the control.

Cytotoxicity data are in accordance with phytotoxicity: Both *M. phaseolina* extracts (PE35CE and PE35CE_{host}) induced similar phytotoxic symptoms and cell viability. Generally, both crude extracts induced higher cytotoxicity at the higher concentrations tested (Figure 6).

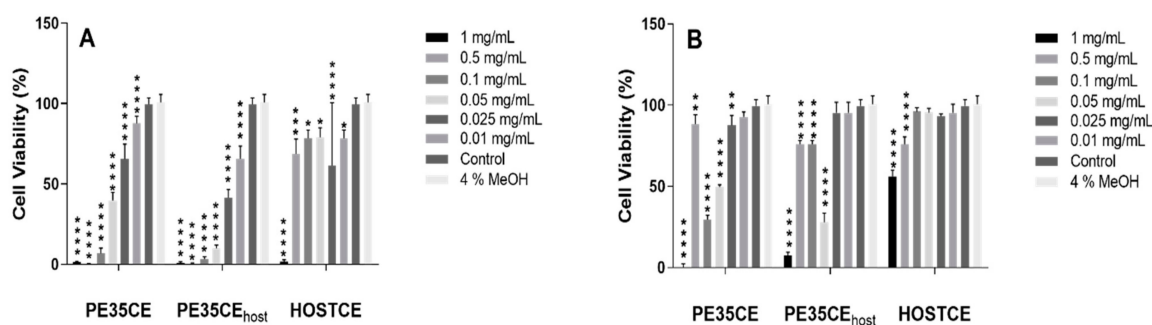


Figure 6. Cytotoxicity of crude extracts of filtrates of *M. phaseolina* PE35 grown in the presence (PE35CE_{Host}) and absence of the host tissue (PE35CE) and only the host tissue (HOSTCE) in 3T3 (A) cells and Vero cells (B). Phosphate buffered saline (PBS) and 4% MeOH were used as the control. Statistical analysis was performed using two-way ANOVA followed by a Tukey multiple comparison test. Asterisks correspond to data with statistical difference towards the control (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

4. Discussion

Macrophomina phaseolina, the causal agent of several plant diseases such as charcoal rot, is a threat for eucalypt crops, resulting in economic losses due to the negative effects on plant physiology [14,15]. Several clinical reports have also established this fungus as an opportunist human pathogen that causes infection in immunocompromised individuals [21,32–36].

Secondary metabolites produced by *M. phaseolina* may be correlated with disease outbreak [37], but there are very few studies on the production of compounds by this fungus [22,23,38,39]. We report, for the first time, the identification of secondary metabolites produced by a *M. phaseolina* isolate from *E. globulus*. Furthermore, the effect of eucalypt tissue on the secondary metabolites production was evaluated. Because *M. phaseolina* might be considered significant to both plant and human health [21], the culture filtrates and the crude extracts produced under both growth conditions (with and without host tissue) were evaluated for toxicity on tomato plants and on mammalian cells. The crude extracts toxicity seems to be slightly increased when the fungus was grown in presence of the host tissue.

Six metabolites, belonging to diverse classes of natural compounds were identified in the crude extracts of *M. phaseolina*. Azelaic acid was the only compound identified to be exclusively produced by *M. phaseolina* PE35 grown in the presence of the host tissue. This dicarboxylic acid was recently detected in cultures of *Neofusicoccum vitifusiforme*, a fungus frequently associated to Botryosphaeria dieback of grapevine [40]. Azelaic acid has antimicrobial and anti-inflammatory properties finding application in the treatment of skin diseases. Furthermore, its activity against several tumor cells has also been documented [41].

Among the identified metabolites, (3*R*,4*S*)-botryodiplodin, *cis*-(3*R*,4*R*)-hydroxymellein and (*R*)-mellein and tyrosol are typical metabolites produced by fungi from the family Botryosphaeriaceae [42–44], but only botryodiplodin was already known as a product of *M. phaseolina*. In 2007, Ramezani et al. identified the mycotoxin (3*R*,4*S*)-botryodiplodin in the culture of *M. phaseolina*, isolated from soybean [38]. Subsequently, (3*R*,4*S*)-botryodiplodin was detected in many cultures of *M. phaseolina* isolated from several plants (e.g., melon, strawberry, soybean), and also as *vivotoxin* in studies on plant tissues naturally infected with charcoal rot disease [39].

Although it has been proposed that (3*R*,4*S*)-botryodiplodin may be involved in the fungal infection of plants, its role in the pathogenicity remains to be clarified. Certainly, the (3*R*,4*S*)-botryodiplodin stereostructure significantly influences the biological activities. In fact, (3*R*,4*S*)-botryodiplodin is reported as a non-phytotoxic compound [44], but its epimer turns out to be phytotoxic and

the cytotoxic effect of 3-*epi*-botryodiplodin on mammalian cells was shown to be higher than (3*R*,4*S*)-botryodiplodin [31].

Melleins are also toxic metabolites produced by fungi and could be responsible for the toxic effects of culture filtrates and crude extracts of *M. phaseolina* PE35 [30,31,45].

The metabolite (3*R*,4*S*)-botryodiplodin present in crude extracts of *M. phaseolina* is responsible for high toxicity against mammalian cells (i.e., Vero and 3T3 cell lines), consistent with the opportunistic nature of *M. phaseolina* towards the human host. In fact, it was tested for cytotoxicity on Vero and 3T3 cell lines in our previous work [31]. In general, 3T3 cells seem to be more sensitive than Vero cells when exposed to crude extracts of *M. phaseolina* PE35.

5. Conclusions

M. phaseolina is frequently associated with plant diseases and with significant economic impact on some crops (e.g., soybean, sunflower, sesame, melon) [17–20]. The present paper describes the identification of six secondary metabolites produced by *M. phaseolina* PE35 (isolated from *E. globulus*) in liquid medium. The identification of metabolites present in extracts of this isolate was carried out mainly with the support of a custom GC-MS library, but the stereostructure determinations were obtained after isolation and NMR and optical rotation analysis of metabolites. This strategy allowed the comparison of metabolite profiles obtained at different experimental conditions (i.e., presence of eucalypt stem in the liquid medium). The presence of host tissue during *M. phaseolina* PE35 growth induced the production of azelaic acid. The metabolomic results correspond with phytotoxicity and cytotoxicity data collected for culture filtrates and crude extracts of *M. phaseolina* PE35.

This work enriches the existing literature with new data on secondary metabolites by *M. phaseolina*. Our findings might be useful in understanding the ecology and host-pathogen relationships, the mechanisms of action of secondary metabolites and their possible application for the screening of diseases.

Author Contributions: Conceptualization, A.C.E., A.S.D., A.A. (Artur Alves) and A.A. (Anna Andolfi); methodology, A.C.E., A.S.D., A.A. (Artur Alves) and A.A. (Anna Andolfi); validation, A.C.E., A.S.D., A.A. (Artur Alves) and A.A. (Anna Andolfi); formal analysis, C.F. and F.L.; investigation, M.M.S., D.N., F.S., C.F., F.L., V.F. and A.A. (Anna Andolfi); data curation, C.F., F.L., M.M.S., V.F.; writing—original draft preparation, M.M.S., D.N., F.S., C.F., F.L., V.F.; writing—review and editing, A.C.E., A.S.D., A.A. (Artur Alves) and A.A. (Anna Andolfi); supervision, A.C.E., A.S.D., A.A. (Artur Alves) and A.A. (Anna Andolfi); project administration, A.C.E., A.S.D., A.A. (Artur Alves) and A.A. (Anna Andolfi); funding acquisition, A.A. (Artur Alves) and A.A. (Anna Andolfi). All authors have read and agreed to the published version of the manuscript.

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