Soybean leaf infusion culture media and phytotoxic metabolite production in *Macrophomina phaseolina**

Medio con infusión foliar de soja y producción de metabolitos fitotóxicos en *Macrophomina phaseolina*

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Abstract

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Introduction. The charcoal rot fungus, *Macrophomina phaseolina*, is a ubiquitous necrotrophic phytopathogen that infecting soybean and other plant species. Despite its significant impact on crops, limited progress has been made in understanding the factors that influence phytotoxic molecule secretion by this phytopathogen. **Objective.** To evaluate the effect of soybean leaf infusion in the culture medium on the differential secretion of phytotoxic molecules of *M. phaseolina*. **Materials and methods.** The study was conducted between 2016 and 2023 at the Departamento de Química Biológica, Universidad Nacional de Asunción, Paraguay. Two fungal isolates were cultured *in vitro* using potato dextrose broth (PDB) and Czapek-Dox broth media, with or without soybean leaf infusion. Phytotoxic activity of secreted molecules was a using soybean leaf discs. The crude organic extract from the cultures was separated using chromatographic techniques, and purified metabolites were characterized by UHPLC-PDA/MS, HRMS (APGC), HRMSESI, 1HNMR and 13CNMR. **Results.** Molecules secreted by *M. phaseolina* FCQ11 cultured in infusion-enriched PDB induced the highest percentage of necrosis. Under these conditions, three differentially secreted metabolites were isolated and identified: (R)-mellein, (3R,4R)-hydroxymellein, and (-)-botryodiplodin. **Conclusions.** Soybean leaf infusion presence in *M. phaseolina* growth media stimulates phytotoxic metabolite production and alters the profile of secreted metabolites.

Keywords: fungal phytopathogen, secondary metabolites, bioactive molecules, charcoal rot.

Resumen

Introducción. El hongo de la pudrición carbonosa *Macrophomina phaseolina* es un fitopatógeno necrotrófico ubicuo que infecta a la soja y a otras especies de plantas. Aunque este hongo causa pérdidas significativas en los cultivos, se han logrado pocos avances en la comprensión de los factores que influyen en la secreción de moléculas fitotóxicas. **Objetivo.** Evaluar el efecto de la infusión de hojas de soja en el medio de cultivo sobre la secreción diferencial de moléculas fitotóxicas de *M. phaseolina*. **Materiales y métodos.** El estudio se realizó entre 2016 y 2023 en el Departamento de Química Biológica de la Universidad Nacional de Asunción, Paraguay. Dos aislados del hongo se cultivaron in vitro utilizando medios de caldo papa dextrosa y el medio Czapek-Dox, con o sin suplementación de infusión de hojas de soja. Se evaluó la actividad fitotóxica de las moléculas secretadas utilizando discos de hojas de soja. El extracto orgánico crudo de los cultivos se separó por medio de técnicas cromatográficas, y los metabolitos purificados se caracterizaron mediante UHPLC-PDA/MS, HRMS (APGC), HRMSESI, 1HNMR y 13CNMR. **Resultados.** Las moléculas secretadas por el hongo *M. phaseolina* FCQ11 cultivado en medio PDB enriquecido con infusión indujeron el mayor porcentaje de necrosis. Bajo estas condiciones, se aislaron e identificaron tres metabolitos secretados de forma diferencial: (R)-meleína, (3R,4R)-hidroximeleína y (-)-botriodiplodina. **Conclusiones.** La presencia de la infusión de hojas de soja en el medio de crecimiento de *M. phaseolina* estimula la producción de metabolitos fitotóxicos, alterando el perfil de los metabolitos secretados.

Palabras clave: hongo fitopatógeno, metabolitos secundarios, moléculas bioactivas, podredumbre carbonosa.

Introduction

The charcoal rot fungus *Macrophomina phaseolina* is a necrotrophic phytopathogen with a wide host range, including economically significant crops such as soybean (*Glycine max* L.) (Deshmukh & Tiwari, 2021), corn (*Zea mays* L.) (Saleh et al., 2010), and beans (*Phaseolus vulgaris*) (Marcenaro & Valkonen, 2016). It causes charcoal rot disease, which affects plants at all growth stages and is promoted by high temperatures and low soil moisture (Chilakala et al., 2022; Kaur et al., 2012; Khasin et al., 2021). Numerous factors contribute to the development of this pathology (Slavov, 2005), including phytotoxins, which facilitate the penetration, invasion, and colonization of the host (Kaur et al., 2012).

Several phytotoxins produced by *M. phaseolina* have been identified, including phaseolinone (Bhattacharya et al., 1987; Dhar et al., 1982), botryodiplodin (Ramezani et al., 2007; Salvatore et al., 2020), phaseocyclopentenones A and B, and Guignardone A (Masi et al., 2021). Other metabolites that may be related to the virulence of this pathogen, including phaseolinic acid (Mahato et al., 1987), macrophominol (Trigos et al., 1995), and mellein (Khambhati et al., 2023; Salvatore et al., 2020), have also been reported. However, many of the expected molecules from genome sequencing information are still undescribed, leaving a significant gap in understanding the role of these compounds in the pathogenesis of M. phaseolina (Islam et al., 2012).

Previous studies have shown that enriching the culture medium with host tissue can increase the secretion of phytotoxic molecules in phytopathogenic fungi such as *Phytophthora capsici* (Flores-Giubi et al., 2013) and, in the case of *M. phaseolina*, supplementing in vitro culture media with host plant infusions can stimulate conidia production (Nouri et al., 2020), alter the profile of secreted proteins (Pineda-Fretez et al., 2023) or modify the metabolite profile (Salvatore et al., 2020). Given the lack of reports on *in vitro* culture conditions that promote phytotoxin production in the soybean–*M. phaseolina* pathosystem and the fungus's multifactorial pathogenicity, the objective of this research was to evaluate the effect of soybean leaf infusion in the culture medium on the differential secretion of phytotoxic molecules of *M. phaseolina*.

Materials and methods

General experimental procedures

The study was conducted from 2016 to 2023 at the Departamento de Química Biológica, Universidad Nacional de Asunción, Paraguay. NMR spectra were recorded on an Agilent 500 MHz NMR spectrometer using $CDCl_3$ as the solvent (Eurisotop, Saint-Aubin, France). Chemical shifts are expressed in ppm (δ) and referenced to the solvent (δ_H 7.25, δ_C 77.0). Optical rotations were measured using an Anton PAAR 5300 polarimeter. Thin-layer chromatography (TLC) was performed on Merck Kieselgel Å F_{254} plates with a 0.25 mm layer thickness. Column chromatography was conducted on silica gel 60 (60–200 μm, VWR). For the ultra-high performance liquid chromatography (UHPLC) analysis, samples were injected into a Waters, Acquity™ UHPLC system coupled to a photodiode array (PDA) detector and a tandem mass spectrometer (Xevo TQD) operating in positive ion electrospray ionization mode (UHPLC/MS/ESI+).

The compounds [1] and [2] were analyzed using gas chromatography-atmospheric pressure chemical ionization coupled to high-resolution mass spectrometry (GC-APGC-HRMS) on an Agilent 7890 GC separation system, coupled with a quadrupole time-of-flight tandem mass spectrometer (Xevo-G2-S QTOF, Waters, Manchester, U.K.) equipped with an APGC source. Compound [3] was analyzed by high-resolution mass spectrometry with electrospray ionization (HRMSESI), into an ultra-high performance liquid chromatograph, coupled to a quadrupole time-of-flight tandem mass spectrometer (Xevo-G2-S QTOF,

Waters, Manchester, U.K.) equipped with an ESI source. Data acquisition and processing for compounds [1-3] were performed with MassLynx™ 4.1 software (Waters, Manchester, U. K.) in positive ion mode.

Microorganisms

The fungal isolates *M. phaseolina* FCQ11 and FCQ39 were isolated from naturally infected soybean plants at Universidad Nacional de Asunción, San Lorenzo (Departamento Central), and Edelira (Departamento de Itapúa), Paraguay, respectively. Molecular identification was performed by Sanger sequencing of the translation elongation factor 1 alpha (*tef1-α*) and beta-tubulin (*β-tub*) genes at Macrogen Korea (Seoul, Republic of Korea). Sequences were deposited in GenBank under the following accession numbers: ON866534 (FCQ11-*tef1-α*), ON959212 (FCQ11-*β-tub*), ON866535 (FCQ39-*tef1-α*), and ON959213 (FCQ39-*β-tub*).

Culture conditions and fungal crude organic extracts preparation

M. phaseolina was grown and maintained on potato-dextrose-agar (PDA, Liofilchem™) in a microbiological incubator at 30 °C in the dark. Mycelium plugs (5 mm in diameter) were preserved in an 80 % glycerol aqueous solution at −20 °C. Stored mycelium plugs were re-inoculated on PDA culture medium and incubated at 30 °C, until the fungal mycelium covered the entire Petri dish surface (4-5 days).

Three fresh mycelium discs from actively growing margins were transferred to Roux bottles containing 200 mL of potato dextrose broth (PDB, Liofilchem™) or Czapek-Dox (CZP, Liofilchem™), both with soybean leaf infusion (PDB INF, CZP INF) or without soybean leaf infusion (PDB, CZP). The infusion was prepared by boiling 20 g of young soybean leaves (V3-V4 stage) in 1 L of distilled water, followed by filtration through gauze. The resulting infusion was then used to replace the water in the supplemented media.

After the fungal mycelium covered the entire culture surface (5-6 days), it was separated from the culture media by vacuum filtration using gauze. The filtrate obtained was extracted with ethyl acetate (HPLC grade), and the organic fraction was dried with anhydrous sodium sulfate and evaporated under reduced pressure using a rotary evaporator to yield crude organic extracts. The result was expressed as milligrams of organic extract per liter of culture medium (mg/L).

Phytotoxic bioassay

To evaluate the phytotoxic activity of crude organic extracts, bioassays were performed using 1.8 cm susceptible soybean cultivar Nidera A5009 (*Glycine max*, V3-V4 stage) leaf discs. Discs were placed on Petri dishes, and 50, 100, and 200 μg of each extract dissolved in acetonitrile were tested; 20 μL of 8 % (v/v) phosphoric acid (H_3PO_4) solution was used as a positive control and the solvent as a negative control (Moreau et al., 1982). The leaf discs were incubated in darkness at 30 °C for 24 hours. Following incubation, the presence of necrosis symptoms was observed. Total leaf area and leaf necrotic (damage) area of each disc were manually measured using ImageJ software (free license), and then the percentage of necrosis was then calculated (Schneider et al., 2012).

Isolation and identification of *M. phaseolina* metabolites

M. phaseolina FCQ11 grown in PDB INF was selected to perform the isolation of the molecules present in the crude organic extract and its subsequent identification. The crude organic extract was fractioned by column chromatography using silica gel as the stationary phase and a gradient of hexane and ethyl acetate with increasing polarity as the mobile phase. Fractions were collected in numbered test tubes, monitored by thin-layer chromatography (TLC), and grouped according to their retention factor (Rf) similarities. The metabolites were purified and identified by nuclear magnetic resonance (NMR) spectroscopy.

Compound 1 (*R*)-mellein

3,4-dihydro-8-hydroxy-3-methylisocoumarin. The organic extraction yield was 4.6 mg/100 mg of crude extract of *M. phaseolina* FCQ11 grown in PDB INF. The NMR spectroscopic data of compound 1 were consistent with the literature (Li et al., 2021). HRMS (APGC$^+$) calcd for $C_{10}H_{11}O_3$ $[M+H]^+$ 179.0708,

found 179.0728; calcd for $C_{10}H_9O_2$ $[M-H_2O+H]^+$ 161.0603, found 161.0626; calcd for $C_9H_{11}O_2$ $[M-CO+H]^+$ 151.0759, found 151.0783; calcd for C_9H_9O $[M-CO-H_2O+H]^+$ 133.0653, found 133.0684.

Compound 2 (3R,4R)-hydroxymellein

(3R,4R)-4,8-dihydroxy-3-methylisochroman-1-one. The organic extraction yield was 7.2 mg/100 mg of crude extract of *M. phaseolina* FCQ11 grown in PDB INF. The NMR spectroscopic data of compound 2 were consistent with the literature (Djoukeng et al., 2009). HRMS (APGC+) calcd for $C_{10}H_{11}O_4$ $[M+H]^+$ 195.0657, found 195.0685; calcd for $C_{10}H_9O_3$ $[M-H_2O+H]^+$ 177.0552, found 177.0583; calcd for $C_9H_9O_2$ $[M-H_2O-CO+H]^+$ 149.0603, found 149.0627.

Compound 3 (-)-botryodiplodin

1-((3S,4R)-5-hydroxy-4-methyltetrahydrofuran-3-yl)ethan-1-one. The organic extraction yield was 9 mg/100 mg of crude extract of *M. phaseolina* FCQ11 grown in PDB INF. The optical rotation and NMR spectroscopic data of compound 3 matched the literature (Ramezani et al., 2007). HRMSESI m/z 167.0686 $[M+Na]^+$ (calcd for $C_7H_{12}O_3Na$, 167.0684), 145.0868 $[M+H]^+$ (calcd for $C_7H_{13}O_3$, 145.0865), 127.0762 $[M+H-H_2O]^+$ (calcd for $C_7H_{11}O_2$, 127.0759).

Results

Total secondary metabolites secreted by *M. phaseolina* isolates

The production of secondary metabolites by *M. phaseolina* isolates FCQ11 and FCQ39 under different culture conditions was evaluated (Figures 1A and 1B for isolates FCQ11 and FCQ39, respectively). No differences were observed in the total production of secondary metabolites from *M. phaseolina* FCQ11 across the evaluated culture media. For *M. phaseolina* FCQ39, a lower production of secondary metabolites was detected in Czapek-Dox (CZP) medium without soybean leaf infusion (32.7 mg/L).

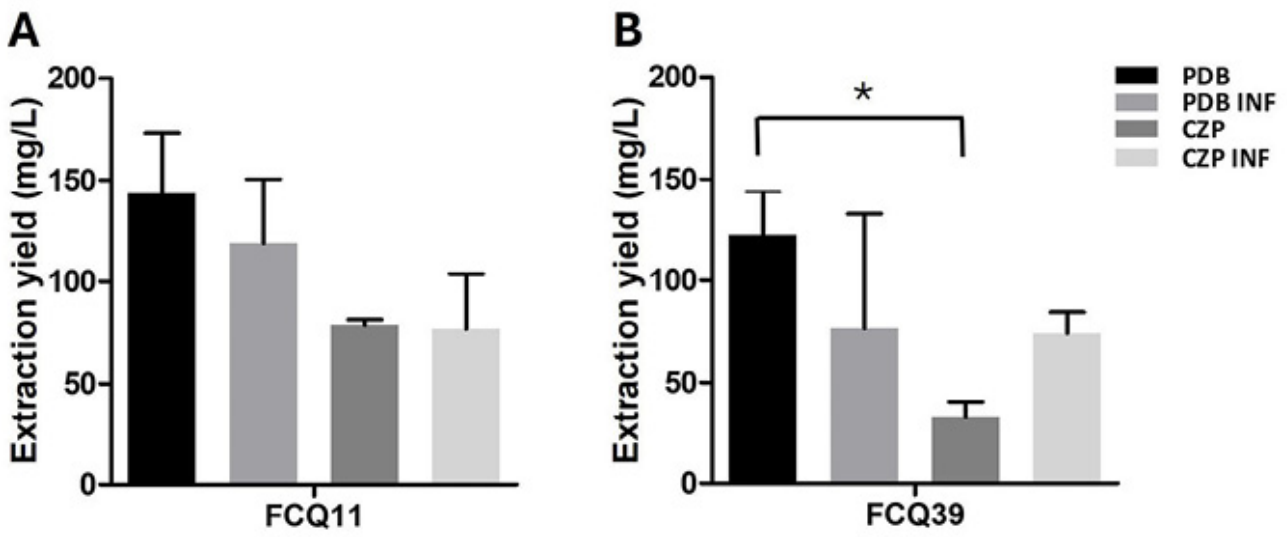

Figure 1

Secondary metabolites extraction yield from *Macrophomina phaseolina* FCQ11 (A) and FCQ39 (B). Departamento de Química Biológica, Facultad de Ciencias Químicas. Universidad Nacional de Asunción, Paraguay. 2016.

Mean organic extract yields (mg/L) was quantified from cultures grown in PDB and CZP media, with or without soybean leaf infusion (PDB INF and CZP INF, respectively). FCQ11 yields: 143.0mg/L (PDB), 118.6mg/L (PDB INF), 78.6mg/L (CZP), and 76.8mg/L (CZP INF). FCQ39 yields: 121.9mg/L (PDB), 76.6mg/L (PDB INF), 32.7mg/L (CZP), and 74.0mg/L (CZP INF). *p < 0.05 (ANOVA; Tukey).

Figura 1. Rendimiento de extracción de metabolitos secundarios de *Macrophomina phaseolina* FCQ11 (A) y FCQ39 (B). Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Asunción, Paraguay. 2016.

El rendimiento promedio de los extractos orgánicos (mg/L) se determinó a partir de cultivos en medios PDB y CZP, con o sin infusión de hojas de soja (PDB INF y CZP INF, respectivamente). Para FCQ11, los rendimientos fueron 143,0 mg/L (PDB), 118,6 mg/L (PDB INF), 78,6 mg/L (CZP) y 76,8 mg/L (CZP INF). Para FCQ39, los rendimientos fueron 121,9 mg/L (PDB), 76,6 mg/L (PDB INF), 32,7 mg/L (CZP) y 74,0 mg/L (CZP INF). *p < 0,05 (ANOVA; Tukey).

Phytotoxicity of metabolites secreted by *M. phaseolina*

The bioassay on soybean leaf discs revealed clear symptoms of tissue damage and visible necrosis, confirming that both *M. phaseolina* isolates produce phytotoxic compounds capable of inducing cell death in soybean tissues. Representative images and results of the bioassay are shown in Figure 2A. For both isolates, a 100 % necrotic effect was observed with 100 and 200 μg of crude organic extract from all media tested. However, with a smaller amount of crude organic extract (50 μg), statistically significant differences were found, with the crude organic extract of *M. phaseolina* FCQ11 grown in PDB INF medium showing the highest foliar damage (Figure 2B). This extract was chosen for metabolites isolation and purification.

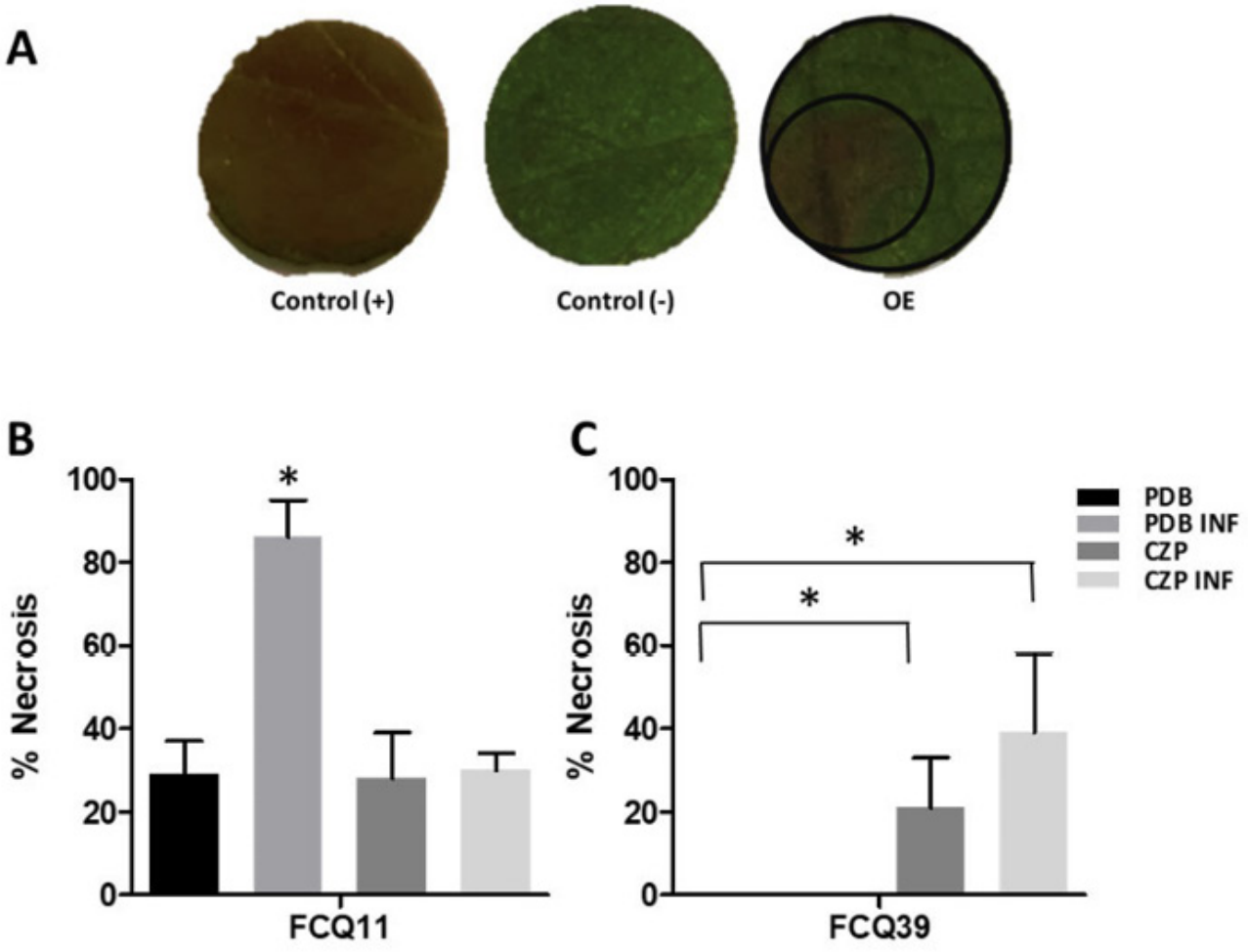

Figure 2

Phytotoxicity bioassay results. A. Representative images showing phytotoxic activity of metabolites secreted by *Macrophomina phaseolina* FCQ11 on soy leaves discs. Control (+): H_3PO_4 solution as positive control; Control (−),]: acetonitrile as negative control; OE: crude organic extract from PDB INF tested at 50 µg. Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Asunción, Paraguay. 2016.

The outer circle demarcates the total leaf area while the inner circle indicates the necrotic region; results are expressed as percentage of leaf area with signs of necrosis,m analyzed using ImageJ software. B, C. Necrosis percentage induced by secreted molecules from *M. phaseolina* FCQ11 and FCQ39 (B and C, respectively). Crude organic extracts of PDB and PDB-INF from *M. phaseolina* FCQ39 showed no phytotoxic activity. For each assessment, crude organic extracts were obtained from *M. phaseolina* filtrates grown in potato dextrose broth (PDB), PDB with soy leaves media infusion (PDB INF), Czapek-Dox (CZP), and Czapek-Dox with soy leaves media infusion (CZP INF), evaluated at 50 µg. *$p < 0.05$ (ANOVA; Tukey).

Figura 2. Bioensayo de fitotoxicidad. A. Imágenes representativas de la actividad fitotóxica de los metabolitos secretados por *Macrophomina phaseolina* FCQ11 sobre discos de hojas de soja. Control (+), control positivo solución H3PO4; Control (−), control negativo acetonitrilo; OE, extracto orgánico crudo de PDB INF evaluado a 50 µg. Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Asunción, Paraguay. 2016.

El círculo mayor indica el área foliar total y el círculo menor el área de necrosis detectada; los resultados se expresaron como porcentaje del área foliar con signos de necrosis. El análisis se realizó con el programa informático ImageJ. B, C. Porcentaje de necrosis inducida por moléculas secretadas de *M. phaseolina* FCQ11 y FCQ39 (B y C, respectivamente). Los extractos orgánicos crudos de PDB y PDB INF de *M. phaseolina* FCQ39 no mostraron actividad fitotóxica. En cada caso, el extracto orgánico crudo se obtuvo del filtrado de *M. phaseolina* cultivado en medios caldo papa dextrosa (PDB), PDB con infusión de hojas de soja (PDB INF), Czapek-Dox (CZP), Czapek-Dox con infusión de hojas de soja (CZP INF), evaluado a 50 µg. *$p < 0,05$ (ANOVA; Tukey).

Isolation and identification of metabolites secreted by *M. phaseolina*

Ten fractions were obtained from the organic extract of *M. phaseolina* FCQ11. Among them, the metabolites were identified, isolated, and later subjected to structural characterization. Three main metabolites as phytotoxic molecules secreted by *M. phaseolina* were detected and isolated: (R)-mellein [1], (3R,4R)-hydroxymellein [2], and (-)-botryodiplodin [3] (Figure 3).

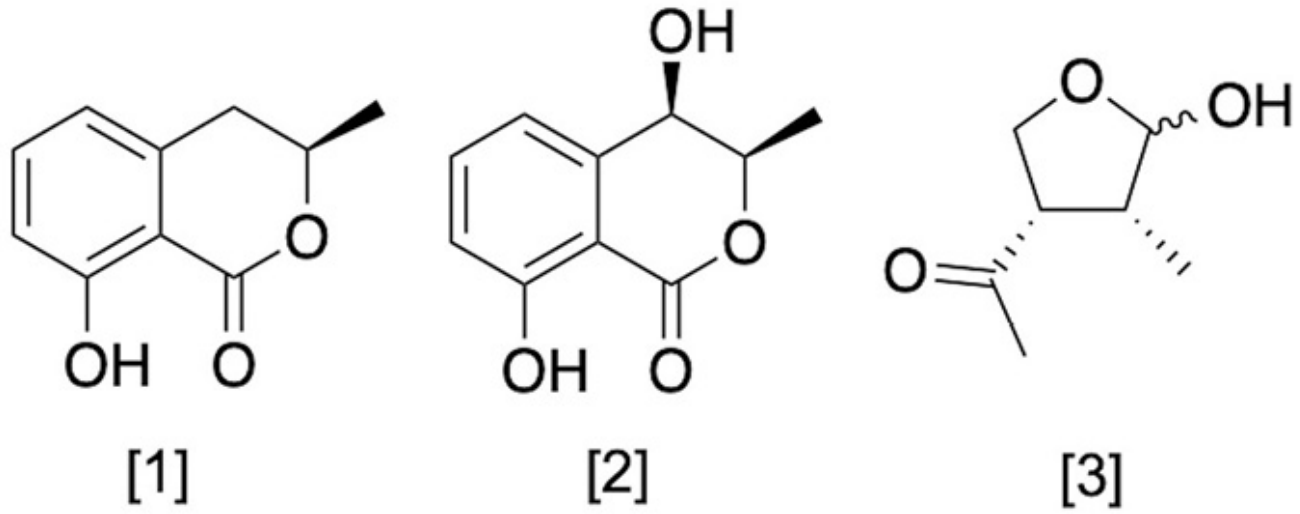

Figure 3

Metabolites structure secreted by *Macrophomina phaseolina*. Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Asunción, Paraguay. 2023.

Figura 3. Estructura de los metabolitos secretados por *Macrophomina phaseolina*. Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Asunción, Paraguay. 2023.

The optical rotation and nuclear magnetic resonance (NMR) spectroscopic data of the pure compounds (R)-mellein [1] and (3R,4R)-hydroxymellein [2] matched those previously described in the literature. A third metabolite [3] was isolated and analyzed by UHPLC/PDA. Despite its low UV absorbance, high-resolution mass spectrometry confirmed the molecular weight of the metabolite with a retention time of 0.48 min at 145.0868 $[M+H]^+$, which was consistent with the data published in the literature for (-)-botryodiplodin.

The chromatographic profiles of the crude organic extracts from *M. phaseolina* FCQ11 grown in PDB and PDB INF revealed distinct patterns of secreted metabolites (Figure 4). The major metabolites identified were (R)-mellein and (3R,4R)-hydroxymellein, with retention times of 2.03 and 1.08 min, respectively. The metabolite (-)-botryodiplodin was detected as a minority peak at 0.48 min. All metabolites were identified by mass spectrometry by comparison with UHPLC-MS analysis.

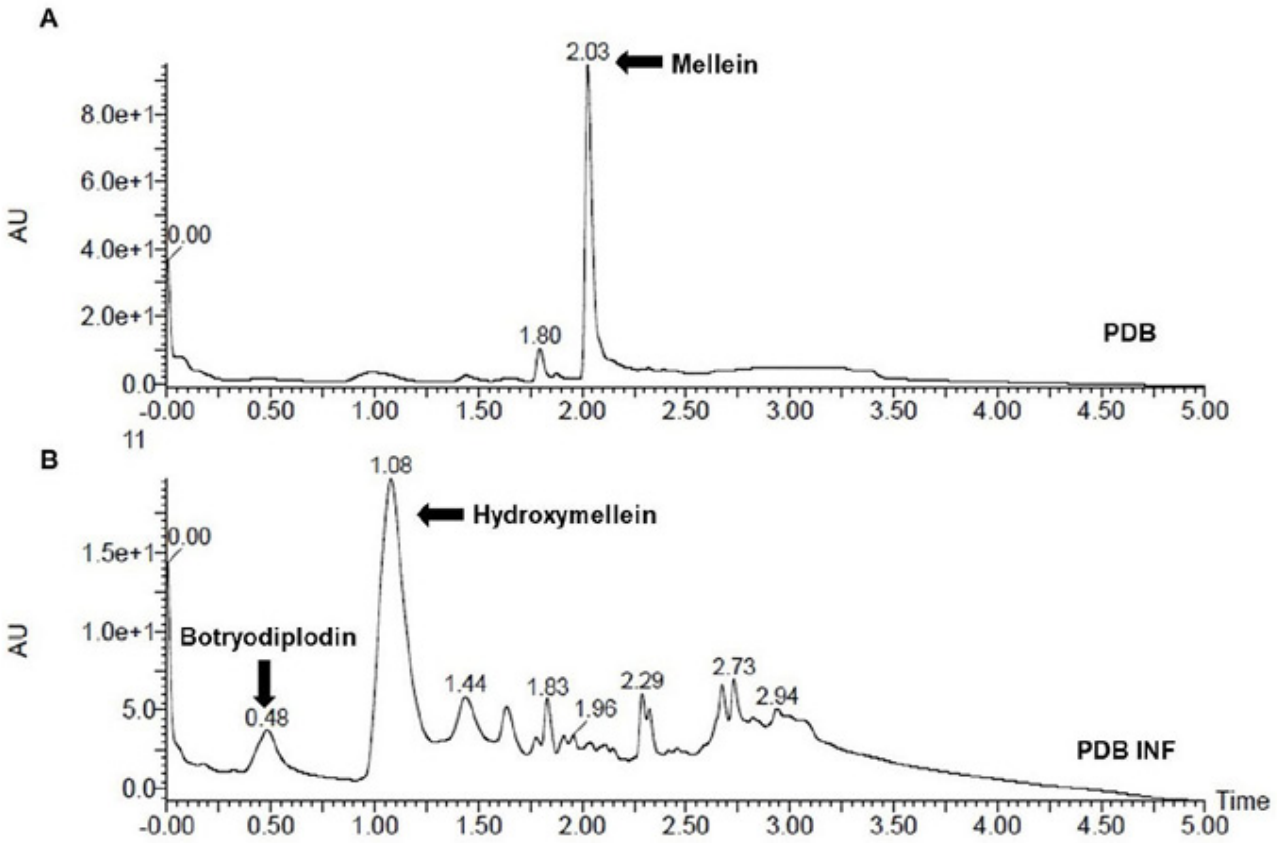

Figure 4

Chromatographic fingerprint by ultra-high efficacy liquid chromatography with diode array detection (UHPLC-PDA) of secondary metabolites secreted by *Macrophomina phaseolina* FCQ11. A. Fungus grew in potato dextrose broth without soybean leaf infusion (PDB). B. Fungus grew in potato dextrose broth with soybean leaf infusion (PDB INF). Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Asunción, Paraguay. 2016.

Figura 4. Cromatograma obtenido por cromatografía líquida de ultra alta eficacia con detección por arreglo de diodos (UHPLC-PDA) de metabolitos secundarios secretados por *Macrophomina phaseolina* FCQ11. A. El hongo fue cultivado en caldo papa dextrosa sin infusión de hojas de soja (PDB). B. El hongo fue cultivado en caldo papa dextrosa con infusión de hojas de soja (PDB INF). Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Asunción, Paraguay. 2016.

The chromatographic analysis showed that the addition of soybean leaf infusion to the PDB culture medium of *M. phaseolina* FCQ11 altered the chemical composition of the medium. When *M. phaseolina* FCQ11 was grown only in PDB, the secretion of (R)-mellein was observed (Figure 4A). However, when a soybean leaf infusion was added, the secretion of (3R,4R)-hydroxymellein and (-)-botryodiplodin was detected, along with other molecules in a lower proportion (Figure 4B).

Discussion

M. phaseolina, the causal agent of charcoal rot, infects soybean plants at any crop growth stage, although post-flowering is the most common (Mishra & Kumari, 2021). As soybean is a key agricultural crop, infections caused by this phytopathogenic fungus highlight the importance of understanding the molecular mechanisms underlying its pathogenicity. This soil-borne pathogen has a high genomic potential for secreting low

molecular weight molecules that could be involved in the infection process of its host plants (Islam et al., 2012).

Research has focused on detecting molecules related to pathogenesis, revealing significant differences in metabolic profiles when the fungus is grown under pathogenesis-simulating conditions. For example, in *Phytophthora capsici*, habanero pepper leaf infusion was used to supplement the medium, enhancing the understanding of its pathogenic behavior (Flores-Giubi et al., 2013). The fungus *M. phaseolina* showed significant changes in metabolite profiles when grown in media supplemented with *Eucalyptus globulus* stem tissues (Salvatore et al., 2020). Supplementation with pistachio leaf extract was shown to affect conidia production of *M. phaseolina* (Nouri et al., 2020). More recently, soybean leaf infusion was found to induce significant alterations in the fungal secretome of *M. phaseolina* (Pineda-Fretez et al., 2023).

The metabolites (R)-mellein [1] and (3R,4R)-hydroxymellein [2], secreted by *M. phaseolina*, were identified through spectroscopic techniques. The data obtained were consistent with previously published data from NMR, optical rotation, and mass spectrometry (Djoukeng et al., 2009; Li et al., 2021). Spectrometric analysis revealed characteristic fragmentation patterns of protonated molecular ions, which were in agreement with the assigned structures (Félix et al., 2019; Khambhati et al., 2023).

Mellein, the main chemical structure from the 3,4-dihydroisocoumarins group, is widely found in different fungi in both R or S form at C-3, with the R form being more common (Braca et al., 2012). The first report of (R)-mellein was isolated from *Aspergillus melleus* (Nishikawa, 1933). It has been described as a phytotoxic compound, causing symptoms such as wilting, chlorosis, and necrosis in pine seedlings, tomato cuttings, grapevines, and others (Abou-Mansour et al., 2015; Cabras et al., 2006; Saeed, 2016). A complete inhibition of wheat seed (*Triticum aestivum*) germination has been observed, and it has also been seen in vine seedlings. In *in vitro* assays, (R)-mellein strongly suppresses the expression of plant defense genes and can accumulate in plants in its native chemical form (Chooi et al., 2015; Trotel-Aziz et al., 2019).

The metabolite (3R,4R)-hydroxymellein, a derivative of (R)-mellein, has several structural isomers (Cabras et al., 2006). It is also considered a phytotoxic metabolite in different fungi, and it is believed that it can generate synergy or addition with other secreted metabolites, thus affecting susceptible plants or even acting alone. However, its phytotoxicity depends on multiple factors, such as the host species and assay conditions used to evaluate it (Abou-Mansour et al., 2015; Cabras et al., 2006), or the conditions to which the fungus is exposed (Pan et al., 2019; Ramírez-Suero et al., 2014; Trotel-Aziz et al., 2019).

Botryodiplodin, a phytotoxin initially described in *Penicillium roqueforti* (Moreau et al., 1982; Nielsen et al., 2006; Renauld et al., 1985) and *P. stipitatum* (Fuska et al., 1974; 1988), has also been reported in *M. phaseolina*. It is believed that this fungus utilizes (-)-botryodiplodin during root infection to induce necrosis in root tissue, facilitating the entry of fungal hyphae into the plant (Abbas et al., 2019; Islam et al., 2012; Ramezani et al., 2007). The metabolites identified in this study could be part of the strategy used by this fungus in the process of interaction with the host, making their analysis crucial for understanding the fungus-host interaction mechanisms (Reveglia et al., 2020).

Although these metabolites have been previously documented, the specific conditions for in vitro secretion by *M. phaseolina* have not been described. The addition of soybean leaf infusion to the culture medium was the key to inducing the production of these metabolites. In addition to the three molecules detected, other secreted molecules, albeit in smaller amounts (Figure 4B), may also have been responsible for the high phytotoxicity observed in the secreted metabolites of strain FCQ11 in the medium enriched with soybean leaf infusion. Further research is underway to identify the molecules and clarify their specific roles.

Conclusions

The inclusion of soybean leaf infusion in the growth medium of *M. phaseolina* promoted the production of phytotoxic metabolites in a potato dextrose broth medium supplemented with soybean leaf infusion (PDB

INF), and under this condition, the profile of secreted metabolites changed. Three metabolites —(R)-mellein [1], (3R,4R)-hydroxymellein [2], and (-)-botryodiplodin [3]— were identified, and these could be related to the symptoms developed in susceptible soybean crops. The findings of this study provide new insights into the mechanisms by which this necrotrophic fungus infects susceptible hosts.

Interests conflict

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Soybean leaf infusion culture media and phytotoxic metabolite production in *Macrophomina phaseolina
Medio con infusión foliar de soja y producción de metabolitos fitotóxicos en *Macrophomina phaseolina***

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