Nematicidal and insecticidal activity of ethyl acetate extracts from culture filtrates of *Arabidopsis thaliana* fungal endophytes

Díaz-González, Sandra[1,2]; Andrés, María Fe[1]; González-Sanz, Carlos[2]; Sacristán, Soledad[2,3] and González-Coloma, Azucena[1]

[1]Instituto de Ciencias Agrarias, CSIC, Serrano, 115, Madrid, 28006, Spain

[2]Centro de Biotecnología y Genómica de Plantas (CBGP, UPM-INIA/CSIC), Universidad Politécnica de Madrid (UPM) - Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA/CSIC) Pozuelo de Alarcón (Madrid) Spain

[3]Departamento de Biotecnología-Biología Vegetal, Escuela Técnica Superior de Ingeniería Agronómica, Alimentaria y de Biosistemas, Universidad Politécnica de Madrid (UPM) Madrid, Spain

Abstract

Endophytic fungi, which live inside plant tissues without causing symptoms of disease, produce a variety of bioactive secondary metabolites. These metabolites have applications in biopesticide development, providing eco-friendly alternatives to conventional plant protection products (PPPs). This study explores the nematicidal and insecticidal properties of ethyl acetate (EtOAc) extracts from endophytic fungi isolated from wild populations of *Arabidopsis thaliana* in central Spain. The effects of these extracts against the plant-parasitic nematode *Meloidogyne javanica*, as well as two common plant pest insects (*Myzus persicae* and *Spodoptera littoralis*) were studied. Our results showed that 9 out of the 13 extracts exhibited significant nematicidal and/or insecticidal activity, indicating their biopesticide potential. Chemical analysis through GC-MS and LC-MS revealed that the active extracts contained a great variety of mostly extract-exclusive secondary metabolites, indicating a rich chemical diversity that was independent of the activity of the extract or origin of the fungal isolate. Our work highlights the importance of exploration into the metabolites produced by fungal endophytes, particularly those isolated from model plants like *A. thaliana*. This research lays the groundwork for future studies aimed at harnessing of endophytic fungi as a source of biopesticide products, contributing to sustainable agricultural practices and improved crop protection methods.

Keywords: fungal endophytes, secondary metabolites, biopesticide, insecticide, nematicide, *Myzus persicae*, *Spodoptera littoralis*, *Meloidogyne javanica*, *Arabidopsis thaliana*, extracts.

1. Introduction

Plant protection products (PPPs) are key agricultural inputs to ensure plant health, crop productivity and food security, since they protect crops from pests and pathogens. However, the inappropriate uses of harmful chemical PPPs can have a negative impact on ecosystems biodiversity and plant, animal and human health. Regulation is nowadays focused on the sustainable and safe production of crops, what has led to a gradual withdrawal of harmful pesticides. In the last decades, alternative approaches to plant protection have been encouraged, such as the use of biopesticides, which tend to pose fewer risks than conventional PPPs (Chandler et al., 2011). Biopesticides are PPPs derived from naturally occurring compounds or agents obtained from biological sources like animals, plants, and microorganisms (Kumar et al., 2021). Among those, fungi are important microbial sources of biologically active secondary metabolites and thus are valuable tools to develop novel microbial-based biopesticides (Macías-Rubalcava and Sánchez-Fernández, 2017; Hashem et al., 2023). Fungal endophytes, in particular, have been recognized as abundant sources of bioactive compounds, with terpenoids and polyketides being the most frequently isolated (Mousa and Raizada, 2013). The potential of

these metabolites for medicinal and agricultural applications has also been emphasized, highlighting the need for further research in this field (Ratnaweera and de Silva, 2017).

Fungal endophytes are a highly diverse group of fungi that naturally colonize internal plant tissues without causing disease symptoms for at least part of their life cycle (Hardoim et al., 2015; Lugtenberg et al., 2016; Yan et al., 2019). Protection against pests and herbivores is one of the multiple benefits that these microorganisms can naturally provide to the host plant. Fungal endophytes support the plant in chemical defense by producing bioactive secondary metabolites that directly inhibit the action of microbial pathogens, insects and animals (Kusari et al., 2012). A well-known example is the endophyte *Neotyphodium* sp. which protects plants against herbivores by the production of alkaloids (Schardl et al., 2013). Bioprospection of endophytic fungal cultures isolated from different plant species has yielded a wide variety of promising bioactive compounds against nematodes and insect pests (Andrés et al., 2017). For example, 4-hydroxybenzoic acid, indole-3-acetic acid (IAA) and gibepyrone D produced by the fungal endophyte *Fusarium oxysporum* strain 162 isolated from tomato plants showed high antagonistic effect against the root-knot nematode *Meloidogyne incognita* (Bogner et al., 2017). In another study, free fatty acids (oleic, linoleic, palmitic and stearic) present in an extract from the endophytic fungus *Trichoderma* sp. EFI 671, isolated from *Laurus* sp., showed strong antifeedant effects against the aphid *Myzus persicae* (Kaushik et al., 2020). Recent reports on biocidal compounds from fungal endophytes include dioxolanones from *Phyllosticta* sp. (YCC4) isolated from *Persea indica* with antifeedant (against *M. persicae)* and nematicidal (against *M. javanica*) effects (Díaz et al., 2024), aphid antifeedant stempholones from *Stemphylium* sp. (Diaz et al., 2024) or acaricidal mellein from *Aspergillus* sp. (Reyes Castillo et al., 2024), both isolated from *Bethencourtia palmensis.* Although the plant species *Arabidopsis thaliana* is one of the best studied model organisms in plant research (Koornneef and Meinke, 2010), the knowledge about secondary metabolites produced by endophytic fungi isolated from it and their potential to produce bioactive compounds against plant pests is still limited.

In the present work, EtOAc extracts from endophytic fungi of wild *A. thaliana* plants from the central Iberian Peninsula have been screened for their nematicidal activity against the plant parasitic nematode *M. javanica* and their antifeedant activity against the plant insect pests *M. persicae* and *S. littoralis.* The diverse nematicidal and insecticidal effects observed among the EtOAc extracts emphasize the rich chemical diversity within these endophytes. Our study provides a foundation for exploring endophytes as biotechnological sources of biopesticides, opening new doors for eco-friendly and effective pest management solutions.

2. Materials and methods

2.1 Fungal strains

The 13 fungal isolates used in this work (Table 1) were isolated in 2010 and 2011 from surface sterilized *A. thaliana* plants from different wild populations of the central Iberian Peninsula described in García et al. (2013).

The identification of each isolate was conducted by sequencing the internal transcribed spacer (ITS) region with the primer pairs ITS1/ITS4 (Bokulich and Mills, 2013). Total fungal genomic DNA was extracted from mycelial fragments scraped from fresh fungal culture plates with CTAB method (Doyle and Doyle, 1987). The PCR conditions were set as follows: 2 min at 94°C; 30 s at 94°C, 30 s at 52°C and 1 min at 72°C for 35 cycles; and a final cycle of extension of 10 min at 72°C. Species was assigned based on the best hit of a blast search against Mycobank Database (https://www.mycobank.org/). ITS sequences were aligned with MAFFT v7.525 (Multiple

Alignment Fast Fourier Transform)(Katoh and Standley, 2013) and trimmed with trimmAl v2.0 (Capella-Gutiérrez et al., 2009). Phylogenetic tree was calculated with FastTree v2.1.11 (Price et al., 2010) and plotted with iTOL v6 (Letunic and Bork, 2024).

Table 1. Description of fungal endophytic isolates used for the screening.

Isolate ID	Species	Year of isolation	Population	Tissue of isolation
10006	*Penicillium solitum*	2010	Polán	root
10010	*Cladosporium cladosporioides*	2010	Polán	silique
10013	*Didymella macrostoma*	2010	Polán	rosette
10027	*Fusarium citricola*	2010	Polán	rosette
10033	*Alternaria sp.*	2010	Polán	rosette
10034	*Alternaria terricola*	2010	Polán	rosette
10050	*Fusarium spartum*	2010	Menasalbas	root
10062	*Penicillium sp.*	2010	Menasalbas	silique
10070	*Alternaria alternata*	2010	Menasalbas	flower stalk
110013	*Pleiochaeta setosa*	2011	Polán	root
110040	*Dothiora maculans*	2011	Menasalbas	rosette
110145	*Penicillium sp.*	2011	Menasalbas	rosette
110180	*Acremonium sclerotigenum*	2011	Rascafría	rosette

2.2. Culture conditions and liquid-liquid extraction

The fungal isolates were restored in potato-dextrose-agar plates (PDA, Difco™) and incubated at room temperature for seven days. Restored colonies were transferred to a new PDA plate and incubated under the same conditions for additional seven days.

The fungal isolates were grown in Malt-Peptone liquid medium (10 g/L Malt Extract, Merck, 2.5 g/L Mycological Peptone, Oxoid™). For each fungal isolate, six 100 mL flasks with a volume of 50 mL of medium each (300 mL in total), were inoculated at a final concentration of 10^4 spores/mL of medium. The flasks were incubated in darkness, at 25ºC and 120 rpm for 21 days. After incubation, the mycelium was removed from the culture by filtration through a double layer of filter paper with the help of an extraction pump.

Each culture filtrate was liquid-liquid extracted with EtOAc three times. The EtOAc fraction was dried at 40ºC in a rotary evaporator and stored at 4ºC.

2.3. Nematicidal activity

2.3.1. Nematicidal Bioassay

The population of *M. javanica* was obtained from Instituto de Ciencias Agrarias, CSIC in Madrid, Spain as described by Moo-Koh et al. (2022). The inoculum (second-stage juveniles, J2) of *M. javanica* were adjusted to a final concentration of 100 J2 nematodes/ 95 µL of distilled water solution.

EtOAc extracts (5 µL) dissolved in DMSO:0.6%-Tween 20 or DMSO:0.6%-Tween 20 for controls, were transferred to each well containing nematode suspension (95 µL), with a final concentration of 1 µg/µL. Four replicates per treatment were included in these experiments.

Plates were sealed with parafilm to prevent evaporation and maintained in a growth chamber in darkness, at 25 ± 1 ºC.

Dead J2s were counted after 72 h using a binocular microscope and mortality rate was calculated (%M) and corrected with Schneider-Orelli's formula (Schneider-Orelli, 1947). Extracts with a %M higher than 90% were considered active and selected for further assays at lower doses (0.5 µg/µL, 0.25 µg/µL and 0.125 µg/µL) and at earlier time points (48 h). LC50 and LC90 were calculated by a Probit analysis with the software Statgraphics 19 (Statgraphics Technologies, Inc.).

$$M\ (\%) = \frac{M - M_{Control}}{100 - M_{control}} x100$$

2.3.2. Egg hatching inhibition assay

Egg hatching assay was performed with active EtOAc extracts from isolates 10034, 10070 and 110040. Four replicates of three egg masses each (a total of 12 egg masses) were tested for each treatment. The three egg masses of each replicate were placed in a well of a 24-well plate and exposed to a total volume of 400 µL of the extracts diluted in DMSO + 0.6% Tween at the concentration of 1 µg/µL or DMSO+0.6 % Tween for controls. Plates were sealed with parafilm to prevent evaporation and maintained in a growth chamber in darkness, at 25 ± 1 ºC and 70% relative humidity. After five days (day 0) the number of juveniles hatched out of the egg masses was recorded and the egg masses were transferred to a new plate containing 400 µl of water per well. This process was repeated each 7 days until the end of the experiment. The number of J2 hatched out of the egg masses was recorded at days 0, 7, 14, 21 and 28.

2.4. Insecticidal activity

2.4.1. Maintenance of *M. persicae* and *S. littoralis* colonies

M. persicae and *S. littoralis* were reared on bell pepper (*Capsicum annuum* L.) plants and artificial diet (Poitout and Bues, 1970), respectively. Host plants together with insect colonies were maintained at 21 ± 2ºC, 60-70% relative humidity, and 16 h light: 8 h dark in a growth chamber.

2.4.2. Bioassay of Settling Inhibition of the aphid *M. persicae*

Pepper leaf disks of 2 cm^2 were cut into two even pieces (1 cm^2 each). The two leaf sections were set on water-agar (1%) coating the bottom of a ventilated plastic box (3x3x1.5 cm). In each box, one leaf section was treated with 10 µL of the fungal EtOAc extract and the other with 10 µL of the solvent (ethanol), as control. After solvent evaporation, 10 apterous aphids (24-48 h old) were placed in each plastic box. A total of 20 replicates (boxes) per extract were included in this experiment. The percentage of aphids that settled on each leaf section was recorded after 24 h (at the environmental conditions described above), as described by Gonzalez-Coloma et al. (2000). Settling inhibition index was calculated at an initial concentration of 100 µg/cm^2, using the following equation:

$$SI\ (\%) = 1 - \left({}^{T}/_{C}\right) X\ 100$$

Where:

T = proportion of aphids on the treated section.

C = proportion of aphids on the control section.

Extracts with a SI index higher than 70% were considered active and selected for further assays at lower doses (50 µg/cm^2, 25 µg/cm^2). EC50 was calculated using a logarithmic regression model with the software Statgraphics 19 (Statgraphics Technologies, Inc.).

2.4.3. Bioassay of Feeding inhibition of *S. littoralis*

Four pepper leaf disks (1 cm^2) were placed at equal distances on a water-agar (1%) petri dish (9 cm diameter). Two leaf disks were treated with 10 µL of the fungal EtOAc extract and the other two with 10 µL of the solvent (ethanol), as control. After solvent evaporation, two newly molted *S. littoralis* L6 larvae were allowed to feed on the leaf disks at room temperature, until the consumption of either the treated or control disks reached 75%. A total of six replicates (petri dishes) per extract were included in this experiment.

Non-consumed leaf disk area was measured on their digitalized images with the software Image J version 1.53k (Schneider et al., 2012). Feeding inhibition index was calculated at an initial concentration of 100 µg/cm^2, using the following equation:

$$FI\ (\%) = 1 - \left({}^{T}/_{C}\right) X\ 100$$

Where:

T = non-consumed areas of treated leaf disks.

C = non-consumed areas of control leaf disks.

Extracts with a FI index higher than 70% were considered active and selected for further assays at lower doses (50 µg/cm^2, 25 µg/cm^2). EC50 was calculated using a logarithmic regression model with the software Statgraphics 19 (Statgraphics Technologies, Inc.).

2.5. Chromatography

2.5.1. Gas Chromatography – Mass Spectrometry

The DCM fractions of the fungal EtOAc extracts were analyzed by gas chromatography mass spectrometry (GC-MS) using a Shimadzu GC-2010 gas chromatograph coupled to a Shimadzu GCMS-QP2010 Ultra mass detector (electron ionization, 70 eV). Sample injections (1 µl) were carried out by an AOC-20i and equipped with a 30 m × 0.25 mm i.d. capillary column (0.25 µm film thickness) Teknokroma TRB-5 (95%) Dimetil- (5%) diphenylpolisiloxane. Working conditions were as follows: split ratio (20:1), injector temperature 300ºC, temperature of the transfer line connected to the mass spectrometer 250 ºC, initial column temperature 110 ºC, then heated to 290 ºC at 7 ºC/min and a Full Scan was used (m/z 35-450). Electron ionization mass spectra and retention data were used to assess the identity of compounds by comparing them with those found in the Wiley 229 and NIST 17 Mass Spectral Database. All extracts (4 µg/µl) were dissolved in 100% DCM for injection.

2.5.2. Liquid Chromatography – Mass Spectrometry

The fungal EtOAc extracts were analyzed by liquid chromatography coupled to mass spectrometry (LC-MS) in a Shimadzu apparatus equipped with LC- 20AD pump and a CTO-10AS VP column oven coupled to a mass spectrometer with triple quadrupole as analyzer (LCMS-8040), with an electrospray ionization source (ESI). An ACE 3 C18 column (150 mm × 4.6 mm, 3 µm particle size) with an ACE3 C18 analytical pre-column was used for the separation. The compounds were eluted with Methanol (HPLC-MS grade) with 0.1% acetic acid (MeOH): MiliQ

water with 0.1% acetic acid. The solvent gradient started at 38% B reaching 100% in 45 min and 100% during 10 min and then 38% B for 7 min at before the next injection, with a flow rate of 0.5 mL/min. The nitrogen flow (drying gas for solvent evaporation) was 15 L/min. The potential for the electrospray capillary was + 4.50 kV and a Full Scan was used in positive mode (m/z 110–850) used the Q3 quadrupole with a potential of 1.98 kV and a capillary temperature of 250ºC. The heat block temperature was 400ºC. The stock solutions of the extracts were injected at 0.5 mg/ml with 5 µl injection through an automatic injector (SIL-20A XR). All extracts (0.5 µg/µl) were dissolved in 100% MeOH for injection.

2.5.3. Hierarchical clustering

Hierarchical clustering of EtOAc extracts based on GC-MS and LC-MS profiles were calculated based on squared euclidean distance and the complete linkage method with the software Statgraphics 19 (Statgraphics Technologies, Inc.).

3. Results

3.3. Nematicidal activity

A total of five EtOAc extracts out of the 13 tested showed activity (>90% M at 72 h) against *M. javanica* (Tables 2 and 3). Extract 10034 (*A. terricola*) exhibited the earliest effect causing a mortality rate of 77% at 48 h, while 10033 (*Alternaria* sp.) and 110040 (*D. maculans*) showed the highest effect at 72 h with a 100% of mortality. The most effective extract in terms of lethal dose was 10070 (*A. alternata*) with LC_{50} of 0.05 mg and LC_{90} of 0.4 mg. Extract 10034 showed the lowest LC50 with 0.04 mg, however LC90 was the second highest.

Table 2. Screening for activity of fungal EtOAc extracts against the plant parasitic nematode *M. javanica* at 1 µg/µL concentration and insect pests *M. persicae* and *S. littoralis* at 100 µg/cm² concentration.

Isolate/Extract ID	Species	*M. javanica* (%M)[1]	*M. persicae* (%SI)[2]	*S. littoralis* (%FI)[3]
10006	***P. solitum***	23.2 ± 2.3	**93.6 ± 2.2**	**72.0 ± 7.9**
10010	*C. cladosporioides*	56.6 ± 3.2	59.2 ± 11	51.5 ± 10.0
10013	***D. macrostoma***	25.1 ± 5.2	68.8 ± 5.3	**81.6 ± 16.4**
10027	***F. citricola***	15.5 ± 4.3	**80.0 ± 7.8**	31.4 ± 13.3
10033	***Alternaria* sp.**	**100 ± 0.0**	63.6 ± 6.2	19.8 ± 6.5
10034	***A. terricola***	**90.2 ± 2.0**	52.8 ± 9.6	29.6 ± 16.0
10050	***F. spartum***	3.4 ± 2.1	**70.1 ± 6.4**	17.3 ± 7.8
10062	*Penicillium sp.*	1.1 ± 0.9	57.9 ± 7.7	42.2 ± 12.5
10070	***A. alternata***	**96.7 ± 0.9**	**80.6 ± 8.1**	**99.4 ± 0.4**
110013	***P. setosa***	**90.3 ± 3.1**	64.2 ± 8.5	22.6 ± 7.3
110040	***D. maculans***	**100 ± 0.0**	59.8 ± 7.7	49.3 ± 15.1
110145	*Penicillium sp.*	39.7 ± 5.8	52.3 ± 9.2	4.15 ± 2.9
110180	*Acremonium sclerotigenum*	0.0 ± 1.4	59.4 ± 10.5	17.3 ± 10.6

[1]Mortality rate at 72 h; [2]Percent setting inhibition (%SI, n = 200 insects); [3]Percent feeding inhibition (% FI, n = 12 insects). Data shows mean ± standard error. In bold, isolates whose extract showed activity.

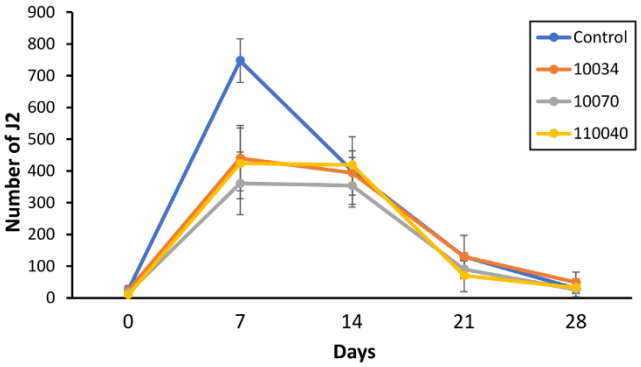
Table 3. Mortality rates and lethal doses (LC50 and LC90) of the active fungal EtOAc extracts[1] against *M. javanica in vitro.*

Extract ID	Species	% Mortality		Lethal dose (mg)		P-value[2]
		48 h	72 h	LC_{50}	LC_{90}	
10033	*Alternaria* sp.	47.2 ± 3.1	100 ± 0.0	0.51	0.87	<0.001***
10034	*A. terricola*	77.6 ± 1.8	90.2 ± 2.0	0.04	1.03	<0.001***
10070	*A. alternata*	3.3 ± 1.1	96.7 ± 0.9	0.05	0.4	<0.001***
110013	*P. setosa*	27.3 ± 2.9	90.3 ± 3.1	0.91	1.53	<0.001***
110040	*D. maculans*	56.8 ± 2.7	100 ± 0.0	0.42	0.8	<0.001***

[1]Extracts were considered active when they showed >90% mortality rate at 72 h.
[2]*P* values from Probit analysis used for the calculation of LC_{50} and LC_{90}.

Active extracts 10034, 10070 and 110040 were selected to test their effect on egg hatching. The three treatments resulted in a reduction in the total number of J2 that hatched from egg masses in comparison to controls, being the effect stronger during the first seven days (Figure 1), with reductions between 11% and 62% at day 0 (after five days of exposition to extract) and between 41% and 52% at day 7. Extract 10070 showed the highest total hatching inhibition rate with 37%, followed by 110040 with 28% and 10034 with 22%.

A



B

Isolate/Extract ID	Total number of hatched-out J2			Total inhibition rate
Control	1335	±	329	-
10034	1036	±	334	22%
10070	847	±	259	37%
110040	954	±	144	28%

Figure 1. Egg hatching inhibition assay. A) Temporal series of the number of second-stage juveniles (J2) hatched out of three egg masses. B) Total number of J2 hatched out at the end of the experiment and total inhibition rate of the different EtOAc extracts from isolates 10034 (*A. terricola*), 10070 (*A. alternata*) and 110040 (*D. maculans*). Egg masses were exposed for five days to the extracts diluted in DMSO:0.6% Tween 20 or DMSO:0.6 % Tween 20 for controls. The number of J2 hatched out of the egg masses was recorded at days 0 (after five days of exposition to extracts), 7, 14, 21 and 28. Data (n = 4) shows means ± standard deviations.

3.2. Insecticidal activity

The EtOAc extracts from the 13 endophytic fungi were screened for their biocontrol properties against the insect pests *M. persicae and S. littoralis.* Four fungal extracts (10006, 10027, 10050 and 10070), representing 31% of the tested extracts, showed high (>70%) SI effect against *M. persicae* at a starting concentration of 100 µg/cm^2 (Table 2). With these active extracts we calculated EC_{50} by conducting the assays at lower concentrations (Table 4). Extract from isolate 10050 (*F. spartum*) exhibited the lowest EC_{50} at 20 µg/cm^2, followed by 10006 (*P. solitum*) with

a EC_{50} of 30 µg/cm^2. The latter was also the extract showing the highest SI rate at 100 µg/cm^2 with 93.6%.

Three fungal extracts (10006, 10013 and 10070) exhibited high FI effect against *S. littoralis* (Table 2). The extract from isolate 10070 (*A. alternata*) was the most effective with 99.4% FI at 100 µg/cm^2 and a EC_{50} of 42 µg/cm^2.

Table 4. Effective concentrations 50 (EC_{50}) of fungal EtOAc extracts with insecticidal activity.

Extract ID	Species	*M. persicae*		*S. littoralis*	
		EC_{50} (µg/cm^2)	*P* value[1]	EC_{50} (µg/cm^2)	*P* value[1]
10006	*P.solitum*	30	<0.001***	76	0.105
10013	*D. macrostoma*	-	-	60	0.001**
10027	*F. citricola*	37	0.001**	-	-
10050	*F. spartum*	20	0.007**	-	-
10070	*A. alternata*	35	<0.001***	42	<0.001***

[1]*P* values from exponential regression models used for the calculation of EC_{50}.

3.3. Metabolomic analysis

As a result of the GC-MS analysis, the presence of 117 compounds (>%1 abundance) was revealed, among which only 42 (36%) could be identified (Supplementary Table S1). The number of compounds in each extract ranged from 9 (isolate 10006, *P. solitum*), to 25 (isolate 110013, *P. setosa*), and the presence of the different compounds was mostly specific of each isolate, with just one compound (No. 100: unidentified) present in the extracts of all nine isolates and three compounds (No. 34: 2,10 bisaboladien-1-one; No. 48: 9-Octadecen-1-ol, (Z)-, and No. 69: Tributyl acetylcitrate) shared by the extracts of five or more isolates. Namely, compounds (No. 34) 2,10-bisaboladien-1-one and (No. 69) Tributyl acetylcitrate were present in all three extracts with activity against *M. persicae* (Supplementary Table S1). Samples could be grouped in three clusters according to their volatile-lipid composition (Figure 2A), with cluster 3 divided further in three subgroups. Clusters GC1 and GC2 grouped the extracts from isolates from Polán population (roots and rosette respectively). Cluster GC3 grouped extracts from isolates from the two populations and different plant organs. The grouping did not correlate with the taxonomy of the isolates or the activity (Figure 2C), indicating that the volatile-lipidic composition of the extracts was not specific for these traits.

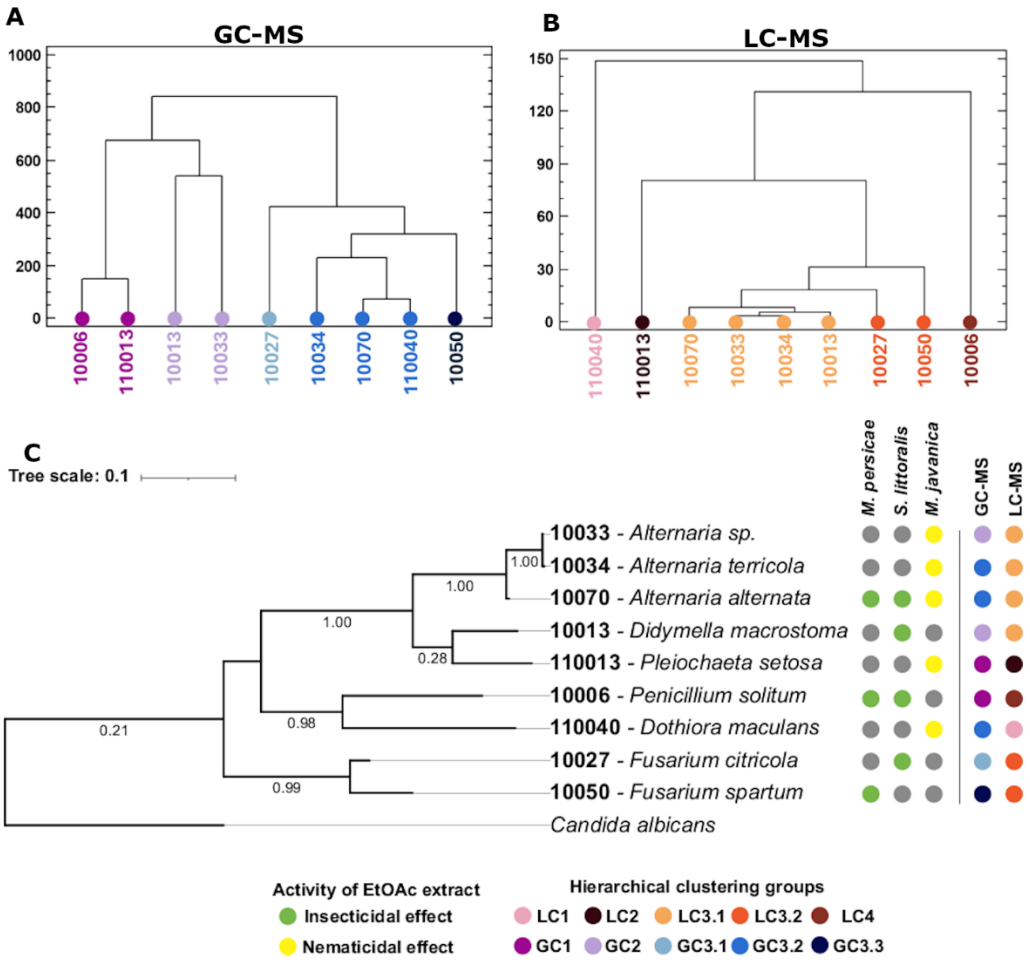


Figure 2. Sample clustering according to metabolomic profiles. Hierarchical clustering of fungal EtOAc extracts based on their (A) GC-MS and (B) LC-MS profile. C) Phylogenetic tree of the fungal isolates based on ITS sequences and classification based on activity of the EtOAc extracts and the clustering of chromatographic profiles.

A non-target LC-MS analysis showed the presence of at least 28 metabolites, grouped by their retention time, with a range of 7 to 12 metabolites per extract (Supplementary Table S2). Three of the metabolites (No. 130, 134 and 136) were present in all the extracts and two metabolites were absent in just one (No. 132 and 139). The clustering of the results (Figure 2B) showed a profile that could be related to the taxonomical proximity of the isolates. A total of four groups could be established, with three including one species: LC1 (110040, *D. maculans*); LC2 (110013, *P. setosa*); and LC4 (10006, *P. solitum*), and one including three genera, which could be divided into two: LC3.1 (10070-10033-10034, *Alternaria* spp. and 10013, *D. macrostoma*) and LC3.2 (10027-10050, *Fusarium* spp.).

4. Discussion

In nature, the preeminent model plant *A. thaliana* hosts a high variety of fungal endophytes (García et al., 2013). Characterizing its natural endophytic mycobiota offers valuable opportunities to deepen our understanding of plant-endophyte interactions. However, characterization of endophytes isolated from *A. thaliana* is relatively limited, particularly in the context of secondary metabolites biosynthesis and their potential biotechnological applications. In this work**,** we obtained EtOAc extracts from 13 *A. thaliana* fungal endophytes and screened

them for their activity against the phytoparasitic nematode *M. javanica* and the plant insect pests *M. persicae* and *S. littoralis*. To our knowledge, this is the first study that explores the potential nematicidal and insecticidal effects of secondary metabolites derived from fungal endophytes of *A. thaliana*.

Our findings show that 9 out of the 13 (69%) EtOAc extracts exhibited nematicidal and/or insecticidal activity, what highlights the enormous potential of fungal endophytes of this model plant to produce bioactive compounds of agricultural interest. The nine isolates with bioactive extracts have different origins regarding population (Polán or Menasalbas) and plant tissue (root, rosette and floral stalk), so their activity is independent from the geographical site or plant organ of provenance. Notably, there where isolates from the roots with activity against leaf pests (i.e. 10006 or 10050), and isolates from the rosette with activity against root nematodes (i.e.10033, 10034 or 110040). Even, the extract from isolate 10070 from the floral stalk was active to both leaf and root attackers. Therefore, the tissue of origin of the isolate did not determine the possible activity of a fungal endophyte.

Nematicidal activity

Isolates 110040 and 110013 from the species *D. maculans* and *P. setosa*, respectively, and the three isolates of *Altern*aria (10033-10034-10070) produced EtOAc extracts with nematicidal activity. *Dothiora* species are found on plants in terrestrial habitats as saprobes and weak pathogens on stressed plants (Crous and Groenewald, 2016, 2017; Hyde et al., 2020). Previous studies reported the production of hormonemates, compounds with cytotoxic activity against tumoral cells, by endophytic *Dothiora* sp. isolated from *Launaea arborescens* (Pérez-Bonilla et al., 2017). *P. setosa* is an important pathogenic necrotrophic fungi of grain legumes (Agudo-Jurado et al., 2023). The only compound described for this species is setosol, which has been shown to inhibit the growth of different fungal phytopathogens, such as the fungus *Magnaporthe oryzae* (Okeke et al., 1994) . However, this is the first report on the nematicidal effects of an organic extract from endophytic species belonging to these two genera.

The fungal genus *Alternaria* is a diverse group of ascomycete fungi which occupy different ecological niches ranging from saprobes to endophytes and pathogens. It is widespread in nature, commonly found in a wide range of hosts and substrates, such as soil, plants, organic matter, wood or textiles (Thomma, 2003; Lawrence et al., 2015). From a human health perspective, *Alternaria* is notable due to its airborne spores, which are among the most prevalent allergens (Kasprzyk et al., 2015). As plant pathogens, *Alternaria* species can cause significant pre- and post-harvest diseases, sometimes leading to mycotoxin accumulation and serious economic losses (Pinto and Patriarca, 2017). Nonetheless, several *Alternaria* species have gained attention for their ability to produce secondary metabolites with a variety of bioactive properties, positioning them as valuable candidates for biotechnological applications in the pharmaceutical and agricultural industries (Lou et al., 2013; Zhao et al., 2023). In this work, the three *Alternaria* isolates showed nematicidal activity. Lou et al. (2016) already showed the potential nematicidal effect of endophytic *Alternaria* sp. isolated from *Salvia miltorrhiza*. The bioactive compound was alternariol methyl ether, which was toxic to the model organism *Caenorhabditis elegans* and the xylophagous nematode *Bursaphelencus xylophilus*. However, although there is promising evidence of the nematicidal effects of secondary metabolites produced by *Alternaria*, this field remains still underexplored.

Insecticidal activity

Isolate 10006 identified as *P. solitum,* produced an EtOAc extract with antifeedant activity against *M. persicae* and *S. littoralis*. *P. solitum* is known for its role in the spoilage of pome fruits during storage (Jurick et al., 2012). In addition, this fungus has been isolated from extremophilic environments, such as the acidic waters of the Berkeley Pit Lake (Stierle et al., 2012) and Antarctica (Gonçalves et al., 2013). There is no direct evidence in the literature supporting the use of *P. solitum* metabolites as biopesticides. However, various secondary metabolites from this fungus have exhibited potential bioactive properties. One example is compactin (also referred to as mevastatin), a precursor for the cholesterol-lowering drug pravastatin (Frisvad and Filtenborg, 1989; Boruta et al., 2018). Additionally, viridicatol, a quinoline alkaloid, exhibited moderate anti-tumor activities against certain cancer cell lines and potent anti-food allergic effects *in vitro* (He et al., 2021). Nonetheless, many studies have explored the metabolites of other *Penicillium* species for biopesticide purposes. Non endophytic species of this genus are known producers of insecticidal compounds such as tryptoquialanines (Costa et al., 2019), indole diketopiperazine alkaloids (Jia et al., 2018), okaramine indole alkaloids (Hayashi et al., 1989), meroterpenoids (Geris et al., 2008), (-)-botryodiplodin (Cabedo et al., 2007), yaequinolones (Uchida et al., 2006) or the terpenoid-pyridine oxalicines, active against *S. frugiperda*, (Li et al., 2005). The closely related species *P. crustosum,* produces neurotoxic penitrems, which have exhibited insecticidal activity (Carmen González et al., 2003). Furthermore, an endophytic *Penicillium* isolated from *Derris elliptica* also was shown to produce the insecticidal compound rotenone against the lepidopteran *Plutella xylostella* and the aphid *Lipaphis erysimi* (Hu et al., 2008). Still, this is the first report documenting the insecticidal potential of secondary metabolites from *P. solitum* in agriculture.

Isolate 10013, *D. macrostoma,* exhibited antifeedant effects against *S. littoralis*. *D. macrostoma* (formerly *Phoma macrostoma*) is a fungal species with diverse ecological roles. It has been recognized as a plant pathogen, notably causing calyx-end rot in pears during cold storage (Wenneker et al., 2023). However, it has recently shown potential as a biocontrol agent against rapeseed clubroot, significantly reducing disease severity and improving crop yields (Li et al., 2024). The genus *Didymella* is well known for producing a variety of secondary metabolites with phytotoxic and cytotoxic activities, which have garnered attention for their potential as bioherbicides. For instance, an endophytic *Didymella* isolated from mangroves synthesizes cytotoxic ascomylactams (macrocyclic alkaloids), didymetone (Yuan et al., 2023) and phomapyrrolidones (Chen et al., 2019). Additionally, cytotoxic naphthalenones and didymelol have been isolated from the endophytic fungus *D. glomerata*, found in *Saussurea laniceps* (Luo et al., 2020). Another example is *D. pinodes*, an aggressive isolate from pea (*Pisum sativum*), which produces pinolidoxin, a phytotoxin affecting several plant species (Cimmino et al., 2012). Despite these findings, this study marks the first report of insect antifeedant effects from an organic extract of endophytic *Didymella*. The compound duroquinone, identified in the GC-MS profile of the EtoAc extract of isolate 10013, may contribute to this effect. Duroquinone has previously been reported to inhibit survival, growth, and pupation in the Black Cutworm (*Agrotis ipsilon*) by reducing ingestion and the efficiency of food conversion (Reese and Beck, 1976). However, further studies are required to confirm its role in the antifeedant activity observed against *S. littoralis*.

Two species of *Fusarium* (isolates 10027-10050) produced active extracts against *M. persicae*. The fungal genus *Fusarium* is a large and significant group of filamentous fungi, primarily found in soil and associated with plants. While certain *Fusarium* species are notorious for causing plant diseases, such as *Fusarium* wilt and root rot in crops like cereals (Yli-Mattila, 2010; Arata et al., 2024; Zuo et al., 2024), legumes (Sampaio et al., 2020), and bananas (Ploetz, 2015; Dita et al.,

2018), as well as for producing harmful mycotoxins (Ji et al., 2019), the majority of strains are saprotrophs or endophytes. In fact, *Fusarium* is one of the most abundant endophytic fungal genera, with certain species providing benefits to the plant (Vu et al., 2006; Pappas et al., 2018; de Lamo and Takken, 2020) . Moreover, *Fusarium* is a rich source of bioactive compounds from various chemical classes, including those with insecticidal properties (Toghueo, 2020; Ahmed et al., 2023). Specifically, *F. sambucinum* from *Nicotiana tabacum* produced the prenylated indole alkaloids sclerotiamides and notoamide with potent insecticidal activity against *Helicoverpa* (Zhang et al., 2019). Moreover, several species of *Fusarium* have been discovered as entomopathogenic in aphids (Santos et al., 2020), which could be related with the antifeedant effect observed in their organic extracts.

Apart from the nematicidal effect, EtOAc extract from isolate 10070, identified as *A. alternata,* also showed antifeedant activity against *M. persicae* and *S. littoralis*. Previous studies have reported that *A. alternata* strains isolated from *Azadirachta indica* gave EtOAc extracts with antifeedant and toxic effects against *S. litura* (Kaur et al., 2016). Also, altenuene, an acetyl cholinesterase inhibitor isolated from an endophytic *A. alternata* strain of *Catharantus roseus*, exhibited insecticidal effect against *S. litura* (Bhagat et al., 2016). The EtOAc extract from the 10070 isolate was the only one showing activity against all three plant pests examined in this study, indicating its broad-spectrum efficacy and underscoring its potential as a versatile source of biopesticide products. Additional research will be necessary to identify the specific molecules responsible for this effect.

Analysis of chromatographic profiles

The extracts, analyzed by GC-MS and LC-MS, exhibited a highly diverse composition, with most metabolites being unknown and unique to each extract. Our results did not show a direct correlation between the secondary metabolite profiles and nematicidal or insecticidal activity. Nonetheless, we could observe that two compounds— (No. 34) 2,10-bisaboladien-1-one and (No. 69) tributyl acetylcitrate—were present in all three extracts that demonstrated activity against *M. persicae* (Supplementary Table S1). Tributyl acetylcitrate is primarily known as a plasticizer for biodegradable polymers (Chaos et al., 2019), and its use as a biopesticide remains unexplored. However, 2,10-bisaboladien-1-one is already known for its antifeedant effects on *M. persicae*, significantly reducing both probing activity and the number of intracellular penetrations (Gutiérrez et al., 1997). This suggests that 2,10-bisaboladien-1-one may be one of the key compounds responsible for the observed bioactivity.

In addition, no clear relationship between the chemical composition of the extracts and the taxonomical proximity of the isolates was observed. The analysis of the less polar fraction (DCM-soluble) of the EtOAc extracts, containing the lipid fraction, did not show a taxonomy-dependent distribution. On the other hand, the secondary metabolites in the EtOAc-soluble fraction (analyzed by LC-MS) revealed chemical profiles that could be related to taxonomy, though only to a limited extent. This lack of a clear pattern could be expected, as secondary metabolite profiles in fungi are complex traits influenced by mono- and polyphyletic factors. Although certain metabolites in fungal groups like the *Xylariaceae* family strongly correlate with phylogenies (Stadler, 2011), the inconsistent distribution of secondary metabolites across the fungal kingdom and the great influence of environmental factors in their biosynthesis makes difficult its correlation with phylogeny (Frisvad et al., 2008). Comparative genomic studies have shown that homologous genes and gene clusters related to secondary metabolism are distributed across a wide phylogenetic range of species (Spatafora and Bushley, 2015). In some cases, this distribution is consistent with inheritance from a common ancestor, as seen with the

ergot alkaloid gene cluster in *Claviceps* and *Metarhizium* (Young et al., 2015). In other cases, the presence of large, complex gene clusters in distantly related taxa is thought to result from horizontal gene transfer (Wisecaver et al., 2014). Within-species genomic comparisons have revealed polymorphisms in secondary metabolite gene clusters, such as gene gain/loss, cluster mobility, and allelic variations, which might explain this evolutionary divergencies (Liu et al., 2015; Young et al., 2015; Lind et al., 2017).

In summary, our study underscores the significant potential of fungal endophytes as sources of bioactive compounds with biocontrol capabilities against agricultural pests. The diverse composition of EtOAc extracts from different fungal isolates does not correlate with the nematicidal and insecticidal activities observed, suggesting similar activities for different metabolites. Future research focusing on isolating and characterizing the specific compounds responsible for these activities will be crucial in harnessing the full potential of these fungal endophytes. Our results also show that a model host as *A. thaliana*, routinary found in many different anthropic ecosystems in temperate regions (García et al., 2013; Thiergart et al., 2019; Mesny et al., 2021) hosts fungal endophytes with the ability to produce high diversity of compounds that are bioactive. The use of a model plant gives opportunities to better study the relationship of the plant with these endophytes and the conditions in which the secondary metabolites of interest are produced in the interaction with the plants.

5. Authors' contributions

S.D.-G., S.S., A.G.-C. and M.A. conceptualized and designed the research. S.S. provided the fungal isolates. C.G.-S refreshed the isolates and conducted the ITS identification. S.D.-G. constructed the phylogenetic tree, obtained the fungal extracts, conducted the bioassays and prepared the first draft of the manuscript. S.D.-G., A.G.-C. and M.A. prepared the tables and figures. S.D.-G., S.S., A.G.-C. and M.A. contributed to the text of the main manuscript.

All authors have read and agreed to the published version of the manuscript.

6. Funding

S.D.-G was supported by a Margarita Salas Grant for junior doctors (RD 289/2021), funded by the Spanish Ministry of Science, Innovation and Universities (MCIN/AEI/10.13039/501100011033) and the European Union – NextGenerationEU and PID2021-123697OB-I00 funded by MCIN/AEI/10.13039/501100011033 and "ERDF A way of making Europe". C.G.-S was supported by grant PEJ-2020-AI/BIO-19580 funded by Comunidad de Madrid and is currently supported by grants PRE2022-103983 and CEX2020-000999-S-20-3 funded by MCIN/AEI/ https://dx.doi.org/10.13039/501100011033 and "ESF Investing in your future". S.S. research is supported by grant PID2021-123697OB-I00 funded by MCIN/AEI/10.13039/501100011033 and "ERDF A way of making Europe".

7. Conflict of interest

The authors declare no conflict of interest.

8. References

Agudo-Jurado, F. J., Reveglia, P., Rubiales, D., Evidente, A., and Barilli, E. (2023). Status of Phytotoxins Isolated from Necrotrophic Fungi Causing Diseases on Grain Legumes. *International Journal of Molecular Sciences 2023, Vol. 24, Page 5116* 24, 5116. doi: 10.3390/IJMS24065116

Ahmed, A. M., Mahmoud, B. K., Millán-Aguiñaga, N., Abdelmohsen, U. R., and Fouad, M. A. (2023). The endophytic Fusarium strains: a treasure trove of natural products. *RSC Advances* 13, 1339–1369. doi: 10.1039/D2RA04126J

Andrés, M. F., Diaz, C. E., Giménez, C., Cabrera, R., and González-Coloma, A. (2017). Endophytic fungi as novel sources of biopesticides: the Macaronesian Laurel forest, a case study. *Phytochemistry Reviews* 16, 1009–1022. doi: 10.1007/s11101-017-9514-4

Arata, A. F., Martínez, M., Castañares, E., Galizio, R. I., Fernández, M. D., and Dinolfo, M. I. (2024). The richness of Fusarium species in maize tassels and their relationship with Fusarium stalk rot. *European Journal of Plant Pathology* 168, 351–362. doi: 10.1007/S10658-023-02760-6/FIGURES/5

Bhagat, J., Kaur, A., Kaur, R., Yadav, A. K., Sharma, V., and Chadha, B. S. (2016). Cholinesterase inhibitor (Altenuene) from an endophytic fungus Alternaria alternata: optimization, purification and characterization. *Journal of Applied Microbiology* 121, 1015–1025. doi: 10.1111/JAM.13192

Bogner, C. W., Kamdem, R. S. T., Sichtermann, G., Matthäus, C., Hölscher, D., Popp, J., et al. (2017). Bioactive secondary metabolites with multiple activities from a fungal endophyte. *Microbial Biotechnology* 10, 175–188. doi: 10.1111/1751-7915.12467

Bokulich, N. A., and Mills, D. A. (2013). Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. *Applied and Environmental Microbiology* 79, 2519–2526. doi: 10.1128/AEM.03870-12/SUPPL_FILE/ZAM999104282SO3.PDF

Boruta, T., Przerywacz, P., Ryngajllo, M., and Bizukojc, M. (2018). Bioprocess-related, morphological and bioinformatic perspectives on the biosynthesis of secondary metabolites produced by Penicillium solitum. *Process Biochemistry* 68, 12–21. doi: 10.1016/J.PROCBIO.2018.02.023

Cabedo, N., López-Gresa, M. P., Primo, J., Ciavatta, M. L., and González-Mas, M. C. (2007). Isolation and structural elucidation of eight new related analogues of the mycotoxin (-)-botryodiplodin from Penicillium coalescens. *Journal of Agricultural and Food Chemistry* 55, 6977–6983. doi: 10.1021/JF071568V/SUPPL_FILE/JF071568VSI20070529_110429.PDF

Capella-Gutiérrez, S., Silla-Martínez, J. M., and Gabaldón, T. (2009). trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* 25, 1972–1973. doi: 10.1093/BIOINFORMATICS/BTP348

Carmen González, M., Lull, C., Moya, P., Ayala, I., Primo, J., and Yúfera Primo, E. (2003). Insecticidal Activity of Penitrems, Including Penitrem G, a New Member of the Family Isolated from Penicillium crustosum. *Journal of Agricultural and Food Chemistry* 51, 2156–2160. doi: 10.1021/JF020983E

Chandler, D., Bailey, A. S., Mark Tatchell, G., Davidson, G., Greaves, J., and Grant, W. P. (2011). The development, regulation and use of biopesticides for integrated pest management. *Philosophical Transactions of the Royal Society B: Biological Sciences* 366, 1987. doi: 10.1098/RSTB.2010.0390

Chaos, A., Sangroniz, A., Gonzalez, A., Iriarte, M., Sarasua, J. R., del Río, J., et al. (2019). Tributyl citrate as an effective plasticizer for biodegradable polymers: effect of plasticizer on free

volume and transport and mechanical properties. *Polymer International* 68, 125–133. doi: 10.1002/PI.5705

Chen, Y., Liu, Z., Huang, Y., Liu, L., He, J., Wang, L., et al. (2019). Ascomylactams A-C, Cytotoxic 12- or 13-Membered-Ring Macrocyclic Alkaloids Isolated from the Mangrove Endophytic Fungus Didymella sp. CYSK-4, and Structure Revisions of Phomapyrrolidones A and C. *Journal of Natural Products* 82, 1752–1758. doi: 10.1021/ACS.JNATPROD.8B00918/SUPPL_FILE/NP8B00918_SI_004.PDF

Cimmino, A., Andolfi, A., Fondevilla, S., Abouzeid, M. A., Rubiales, D., and Evidente, A. (2012). Pinolide, a new nonenolide produced by Didymella pinodes, the causal agent of ascochyta blight on Pisum sativum. *Journal of Agricultural and Food Chemistry* 60, 5273–5278. doi: 10.1021/JF300824D/SUPPL_FILE/JF300824D_SI_001.PDF

Costa, J. H., Bazioli, J. M., de Vilhena Araújo, E., Vendramini, P. H., de Freitas Porto, M. C., Eberlin, M. N., et al. (2019). Monitoring indole alkaloid production by Penicillium digitatum during infection process in citrus by Mass Spectrometry Imaging and molecular networking. *Fungal Biology* 123, 594–600. doi: 10.1016/J.FUNBIO.2019.03.002

Crous, P. W., and Groenewald, J. Z. (2016). They seldom occur alone. *Fungal Biology* 120, 1392–1415. doi: 10.1016/J.FUNBIO.2016.05.009

Crous, P. W., and Groenewald, J. Z. (2017). The Genera of Fungi - G 4: Camarosporium and Dothiora. *IMA Fungus* 8, 131–152. doi: 10.5598/IMAFUNGUS.2017.08.01.10/FIGURES/15

de Lamo, F. J., and Takken, F. L. W. (2020). Biocontrol by Fusarium oxysporum Using Endophyte-Mediated Resistance. *Frontiers in Plant Science* 11, 500488. doi: 10.3389/FPLS.2020.00037/BIBTEX

Díaz, C. E., Andrés, M. F., Bolaños, P., and González-Coloma, A. (2024). Nematicidal and Insecticidal Compounds from the Laurel Forest Endophytic Fungus Phyllosticta sp. *Molecules 2024, Vol. 29, Page 4568* 29, 4568. doi: 10.3390/MOLECULES29194568

Diaz, C. E., Andres, M. F., Lacret, R., Cabrera, R., Gimenez, C., Kaushik, N., et al. (2024). Antifeedant, antifungal and nematicidal compounds from the endophyte Stemphylium solani isolated from wormwood. *Scientific Reports 2024 14:1* 14, 1–10. doi: 10.1038/s41598-024-64467-w

Dita, M., Barquero, M., Heck, D., Mizubuti, E. S. G., and Staver, C. P. (2018). Fusarium wilt of banana: Current knowledge on epidemiology and research needs toward sustainable disease management. *Frontiers in Plant Science* 871, 398832. doi: 10.3389/FPLS.2018.01468/BIBTEX

Doyle, J. J., and Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *PHYTOCHEMICAL BULLETIN*.

Frisvad, J. C., Andersen, B., and Thrane, U. (2008). The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. *Mycological Research* 112, 231–240. doi: 10.1016/J.MYCRES.2007.08.018

Frisvad, J. C., and Filtenborg, O. (1989). Terverticillate Penicillia: Chemotaxonomy and Mycotoxin Production. *Mycologia* 81, 837–861. doi: 10.1080/00275514.1989.12025674

García, E., Alonso, Á., Platas, G., and Sacristán, S. (2013). The endophytic mycobiota of Arabidopsis thaliana. *Fungal Divers* 60, 71–89. doi: 10.1007/s13225-012-0219-0

Geris, R., Rodrigues-Fo, E., du Silva, H. H. G., and da Silva, I. G. (2008). Larvicidal Effects of Fungal Meroterpenoids in the Control of Aedes aegypti L., the Main Vector of Dengue and Yellow Fever. *Chemistry & Biodiversity* 5, 341–345. doi: 10.1002/CBDV.200890032

Gonçalves, V. N., Campos, L. S., Melo, I. S., Pellizari, V. H., Rosa, C. A., and Rosa, L. H. (2013). Penicillium solitum: A mesophilic, psychrotolerant fungus present in marine sediments from Antarctica. *Polar Biology* 36, 1823–1831. doi: 10.1007/S00300-013-1403-8/TABLES/2

Gonzalez-Coloma, A., Gutierrez, C., Miguel del Corral, J. M., Gordaliza, M., De la Puente, M. L., and San Feliciano, A. (2000). Structure- and Species-Dependent Insecticidal Effects of neo-Clerodane Diterpenes. *Journal of Agricultural and Food Chemistry* 48, 3677–3681. doi: 10.1021/JF990843D

Gutiérrez, C., Fereres, A., Reina, M., Cabrera, R., and González-Coloma, A. (1997). Behavioral and sublethal effects of structurally related lower terrenes on Myzus persicae. *Journal of Chemical Ecology* 23, 1641–1650. doi: 10.1023/B:JOEC.0000006428.00568.c5

Hardoim, P. R., van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., et al. (2015). The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. *Microbiology and Molecular Biology Reviews* 79, 293–320. doi: 10.1128/mmbr.00050-14

Hashem, A. H., Attia, M. S., Kandil, E. K., Fawzi, M. M., Abdelrahman, A. S., Khader, M. S., et al. (2023). Bioactive compounds and biomedical applications of endophytic fungi: a recent review. *Microbial Cell Factories* 22, 1–23. doi: 10.1186/s12934-023-02118-x

Hayashi, H., Takiuchi, K., Murao, S., and Arai, M. (1989). Structure and insecticidal activity of new indole alkaloids, okaramines a and b, from penicillium simplicissimum ak-40. *Agricultural and Biological Chemistry* 53, 461–469. doi: 10.1080/00021369.1989.10869287

He, Z. H., Wu, J., Xu, L., Hu, M. Y., Xie, M. M., Hao, Y. J., et al. (2021). Chemical constituents of the deep-sea-derived penicillium solitum. *Marine Drugs* 19, 580. doi: 10.3390/MD19100580/S1

Hu, M. Y., Zhong, G. H., Sun, Z. T., Luo, J. J., Gao, Y., and Weng, Q. F. (2008). Insecticidal metabolites produced by Penicillium spp., an endophytic fungus in Derris elliptica Benth. *Allelopathy Journal* 21, 349–360.

Hyde, K. D., Jeewon, R., Chen, Y. J., Bhunjun, C. S., Calabon, M. S., Jiang, H. B., et al. (2020). *The numbers of fungi: is the descriptive curve flattening?*. Springer Netherlands. doi: 10.1007/s13225-020-00458-2

Ji, F., He, D., Olaniran, A. O., Mokoena, M. P., Xu, J., and Shi, J. (2019). Occurrence, toxicity, production and detection of Fusarium mycotoxin: a review. *Food Production, Processing and Nutrition* 1, 1–14. doi: 10.1186/S43014-019-0007-2/TABLES/4

Jia, B., Ma, Y., Chen, D., Chen, P., and Hu, Y. (2018). Studies on Structure and Biological Activity of Indole Diketopiperazine Alkaloids. *Progress in Chemistry* 30, 1067–1081. doi: 10.7536/PC171231

Jurick, W. M., Vico, I., Gaskins, V. L., Whitaker, B. D., Garrett, W. M., Janisiewicz, W. J., et al. (2012). Penicillium solitum produces a polygalacturonase isozyme in decayed Anjou pear fruit capable of macerating host tissue in vitro. *Mycologia* 104, 604–612. doi: 10.3852/11-119

Kasprzyk, I., Rodinkova, V., Šaulienė, I., Ritenberga, O., Grinn-Gofron, A., Nowak, M., et al. (2015). Air pollution by allergenic spores of the genus Alternaria in the air of central and eastern Europe. *Environmental Science and Pollution Research* 22, 9260–9274. doi: 10.1007/S11356-014-4070-6/TABLES/5

Katoh, K., and Standley, D. M. (2013). MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. *Molecular Biology and Evolution* 30, 772–780. doi: 10.1093/MOLBEV/MST010

Kaur, T., Kaur, J., Kaur, A., and Kaur, S. (2016). Larvicidal and growth inhibitory effects of endophytic Aspergillus niger on a polyphagous pest, Spodoptera litura. *Phytoparasitica* 44, 465–476. doi: 10.1007/S12600-016-0541-2/FIGURES/6

Kaushik, N., Díaz, C. E., Chhipa, H., Fernando Julio, L., Fe Andrés, M., and González-Coloma, A. (2020). Chemical Composition of an Aphid Antifeedant Extract from an Endophytic Fungus, Trichoderma sp. EFI671. *Microorganisms 2020, Vol. 8, Page 420* 8, 420. doi: 10.3390/MICROORGANISMS8030420

Kumar, J., Ramlal, A., Mallick, D., and Mishra, V. (2021). An Overview of Some Biopesticides and Their Importance in Plant Protection for Commercial Acceptance. *Plants 2021, Vol. 10, Page 1185* 10, 1185. doi: 10.3390/PLANTS10061185

Kusari, S., Hertweck, C., and Spiteller, M. (2012). Chemical ecology of endophytic fungi: Origins of secondary metabolites. *Chemistry and Biology* 19, 792–798. doi: 10.1016/j.chembiol.2012.06.004

Lawrence, D. P., Rotondo, F., and Gannibal, P. B. (2015). Biodiversity and taxonomy of the pleomorphic genus Alternaria. *Mycological Progress 2015 15:1* 15, 1–22. doi: 10.1007/S11557-015-1144-X

Letunic, I., and Bork, P. (2024). Interactive Tree of Life (iTOL) v6: recent updates to the phylogenetic tree display and annotation tool. *Nucleic Acids Research* 52, W78–W82. doi: 10.1093/NAR/GKAE268

Li, C., Gloer, J. B., Wicklow, D. T., and Dowd, P. F. (2005). Antiinsectan decaturin and oxalicine analogues from Penicillium thiersii. *Journal of Natural Products* 68, 319–322. doi: 10.1021/NP0496486/SUPPL_FILE/NP0496486SI20041105_042311.CWG

Li, G., Cheng, J., Luo, T., Zhang, J., Wu, M., Yang, L., et al. (2024). Biological Control of Rapeseed Clubroot (Plasmodiophora brassicae) Using the Endophytic Fungus Didymella macrostoma P2. *https://doi.org/10.1094/PDIS-09-23-1921-RE*. doi: 10.1094/PDIS-09-23-1921-RE

Lind, A. L., Wisecaver, J. H., Lameiras, C., Wiemann, P., Palmer, J. M., Keller, N. P., et al. (2017). Drivers of genetic diversity in secondary metabolic gene clusters within a fungal species. *PLOS Biology* 15, e2003583. doi: 10.1371/JOURNAL.PBIO.2003583

Liu, L., Xi, Z., and Davis, C. C. (2015). Coalescent Methods Are Robust to the Simultaneous Effects of Long Branches and Incomplete Lineage Sorting. *Molecular Biology and Evolution* 32, 791–805. doi: 10.1093/MOLBEV/MSU331

Lou, J., Fu, L., Peng, Y., and Zhou, L. (2013). Metabolites from Alternaria Fungi and Their Bioactivities. *Molecules 2013, Vol. 18, Pages 5891-5935* 18, 5891–5935. doi: 10.3390/MOLECULES18055891

Lou, J., Yu, R., Wang, X., Mao, Z., Fu, L., Liu, Y., et al. (2016). Alternariol 9-methyl ether from the endophytic fungus Alternaria sp. Samif01 and its bioactivities. *Brazilian Journal of Microbiology* 47, 96–101. doi: 10.1016/j.bjm.2015.11.004

Lugtenberg, B. J. J., Caradus, J. R., and Johnson, L. J. (2016). Fungal endophytes for sustainable crop production. *FEMS Microbiology Ecology* 92, fiw194. doi: 10.1093/femsec/fiw194

Luo, G., Chen, S., Yu, J., Yuan, J., Zheng, L., Liu, L., et al. (2020). Naphthalenones and Naphthols Isolated from the Saussurea laniceps Endophytic Fungus Didymella glomerata X223. *Chemistry & Biodiversity* 17, e2000315. doi: 10.1002/CBDV.202000315

Macías-Rubalcava, M. L., and Sánchez-Fernández, R. E. (2017). Secondary metabolites of endophytic Xylaria species with potential applications in medicine and agriculture. *World Journal of Microbiology and Biotechnology* 33. doi: 10.1007/s11274-016-2174-5

Mesny, F., Miyauchi, S., Thiergart, T., Pickel, B., Atanasova, L., Karlsson, M., et al. (2021). Genetic determinants of endophytism in the Arabidopsis root mycobiome. *Nature Communications 2021 12:1* 12, 1–15. doi: 10.1038/s41467-021-27479-y

Moo-Koh, F. A., Cristóbal-Alejo, J., Andrés, M. F., Martín, J., Reyes, F., Tun-Suárez, J. M., et al. (2022). In Vitro Assessment of Organic and Residual Fractions of Nematicidal Culture Filtrates from Thirteen Tropical Trichoderma Strains and Metabolic Profiles of Most-Active. *Journal of Fungi 2022, Vol. 8, Page 82* 8, 82. doi: 10.3390/JOF8010082

Mousa, W. K., and Raizada, M. N. (2013). The diversity of anti-microbial secondary metabolites produced by fungal endophytes: an interdisciplinary perspective. *Frontiers in Microbiology* 4, 44840. doi: 10.3389/FMICB.2013.00065/BIBTEX

Okeke, B., Seigle-Murandi, F., Steiman, R., and Kaouadji, M. (1994). Setosol, a Biologically Active Heptaketide-like Metabolite from the Pleiochaeta setosa Phytopathogen. *Bioscience, Biotechnology, and Biochemistry* 58, 734–736. doi: 10.1271/BBB.58.734

Pappas, M. L., Liapoura, M., Papantoniou, D., Avramidou, M., Kavroulakis, N., Weinhold, A., et al. (2018). The beneficial endophytic fungus fusarium solani strain K alters tomato responses against spider mites to the benefit of the plant. *Frontiers in Plant Science* 9, 408405. doi: 10.3389/FPLS.2018.01603/BIBTEX

Pérez-Bonilla, M., González-Menéndez, V., Pérez-Victoria, I., De Pedro, N., Martín, J., Molero-Mesa, J., et al. (2017). Hormonemate Derivatives from Dothiora sp., an Endophytic Fungus. *Journal of Natural Products* 80, 845–853. doi: 10.1021/ACS.JNATPROD.6B00680/SUPPL_FILE/NP6B00680_SI_001.PDF

Pinto, V. E. F., and Patriarca, A. (2017). Alternaria Species and Their Associated Mycotoxins. *Methods in Molecular Biology* 1542, 13–32. doi: 10.1007/978-1-4939-6707-0_2

Ploetz, R. C. (2015). Fusarium wilt of banana. *Phytopathology* 105, 1512–1521. doi: 10.1094/PHYTO-04-15-0101-RVW/ASSET/IMAGES/LARGE/PHYTO-04-15-0101-RVW_T2.JPEG

Poitout, S., and Bues, R. (1970). Rearing of several species of Lepidoptera Noctuidae on a rich artificial medium and a simplified artificial medium. *Annales de Zoologie Ecologie Anim ale* 2, 79–91.

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2 - Approximately maximum-likelihood trees for large alignments. *PLoS ONE* 5, e9490. doi: 10.1371/journal.pone.0009490

Ratnaweera, P. B., and de Silva, E. D. (2017). Endophytic Fungi: A Remarkable Source of Biologically Active Secondary Metabolites. 191–212. doi: 10.1007/978-3-319-66544-3_9

Reese, J. C., and Beck, S. D. (1976). Effects of Allelochemics on the Black Cutworm, Agrotis ipsilon; Effects of p-Benzoquinone, Hydroquinone, and Duroquinone on Larval Growth, Development, and Utilization of Food. *Annals of the Entomological Society of America* 69, 59–67. doi: 10.1093/AESA/69.1.59

Reyes Castillo, N., Díaz, C. E., Andres, M. F., Imperial, J., Valcárcel, F., and González Coloma, A. A. (2024). Optimization of fungicidal and acaricidal metabolite production by endophytic fungus Aspergillus sp. SPH2. *Bioresour Bioprocess* 11, 1–12. doi: 10.1186/S40643-024-00745-9/TABLES/3

Sampaio, A. M., De Sousa Araújo, S., Rubiales, D., and Patto, M. C. V. (2020). Fusarium Wilt Management in Legume Crops. *Agronomy 2020, Vol. 10, Page 1073* 10, 1073. doi: 10.3390/AGRONOMY10081073

Santos, A. C. da S., Diniz, A. G., Tiago, P. V., and Oliveira, N. T. de (2020). Entomopathogenic Fusarium species: a review of their potential for the biological control of insects, implications and prospects. *Fungal Biology Reviews* 34, 41–57. doi: 10.1016/J.FBR.2019.12.002

Schardl, C., Young, C., Pan, J., Florea, S., Takach, J., Panaccione, D., et al. (2013). Currencies of Mutualisms: Sources of Alkaloid Genes in Vertically Transmitted Epichloae. *Toxins* 5, 1064–1088. doi: 10.3390/toxins5061064

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods 2012 9:7* 9, 671–675. doi: 10.1038/nmeth.2089

Schneider-Orelli, O. (1947). Entomologisches Praktikum: Einführung in die land-und. Forstwirtschaftliche Insektenkunde. Aarau Sauerländer, Switzerland.

Spatafora, J. W., and Bushley, K. E. (2015). Phylogenomics and evolution of secondary metabolism in plant-associated fungi. *Current Opinion in Plant Biology* 26, 37–44. doi: 10.1016/J.PBI.2015.05.030

Stadler, M. (2011). Importance of secondary metabolites in the Xylariaceae as parameters for. *Current Research in Environmental & Applied Mycology* 1, 75–133. doi: 10.5943/cream/1/2/1

Stierle, D. B., Stierle, A. A., Girtsman, T., McIntyre, K., and Nichols, J. (2012). Caspase-1 and -3 inhibiting drimane sesquiterpenoids from the extremophilic fungus Penicillium solitum. *Journal of Natural Products* 75, 262–266. doi: 10.1021/NP200528N/SUPPL_FILE/NP200528N_SI_001.PDF

Thiergart, T., Durán, P., Ellis, T., Vannier, N., Garrido-Oter, R., Kemen, E., et al. (2019). Root microbiota assembly and adaptive differentiation among European Arabidopsis populations. *Nat Ecol Evol* 4, 122–131. doi: 10.1038/s41559-019-1063-3

Thomma, B. P. H. J. (2003). Alternaria spp.: from general saprophyte to specific parasite. *Molecular Plant Pathology* 4, 225–236. doi: 10.1046/J.1364-3703.2003.00173.X

Toghueo, R. M. K. (2020). Bioprospecting endophytic fungi from Fusarium genus as sources of bioactive metabolites. *Mycology* 11, 1–21. doi: 10.1080/21501203.2019.1645053

Uchida, R., Imasato, R., Tomoda, H., and Omura, S. (2006). Yaequinolones, New Insecticidal Antibiotics Produced by Penicillium sp. FKI-2140. *The Journal of Antibiotics 2006 59:10* 59, 652–658. doi: 10.1038/ja.2006.87

Vu, T., Hauschild, R., and Sikora, R. A. (2006). Fusarium oxysporum endophytes induced systemic resistance against Radopholus similis on banana. *Nematology* 8, 847–852. doi: 10.1163/156854106779799259

Wenneker, M., Pham, K. T. K., and Kots, K. (2023). First Report of Didymella macrostoma Causing Calyx-End Rot of Pear (Pyrus communis) in the Netherlands. *https://doi.org/10.1094/PDIS-02-23-0372-PDN* 107, 2855. doi: 10.1094/PDIS-02-23-0372-PDN

Wisecaver, J. H., Slot, J. C., and Rokas, A. (2014). The Evolution of Fungal Metabolic Pathways. *PLOS Genetics* 10, e1004816. doi: 10.1371/JOURNAL.PGEN.1004816

Yan, L., Zhu, J., Zhao, X., Shi, J., Jiang, C., and Shao, D. (2019). Beneficial effects of endophytic fungi colonization on plants. *Applied Microbiology and Biotechnology* 103, 3327–3340. doi: 10.1007/s00253-019-09713-2

Yli-Mattila, T. (2010). Ecology and evolution of toxigenic Fusarium species in cereals in northern Europe and Asia. *Journal of Plant Pathology* 92, 7–18.

Young, C. A., Schardl, C. L., Panaccione, D. G., Florea, S., Takach, J. E., Charlton, N. D., et al. (2015). Genetics, Genomics and Evolution of Ergot Alkaloid Diversity. *Toxins 2015, Vol. 7, Pages 1273-1302* 7, 1273–1302. doi: 10.3390/TOXINS7041273

Yuan, Y., Wang, G., She, Z., Chen, Y., and Kang, W. (2023). Metabolites isolated from the mangrove endophytic fungus Didymella sp. CYSK-4 and their cytotoxic activities. *Fitoterapia* 171, 105692. doi: 10.1016/J.FITOTE.2023.105692

Zhang, P., Yuan, X. L., Du, Y. M., Zhang, H. B., Shen, G. M., Zhang, Z. F., et al. (2019). Angularly Prenylated Indole Alkaloids with Antimicrobial and Insecticidal Activities from an Endophytic Fungus Fusarium sambucinum TE-6L. *Journal of Agricultural and Food Chemistry*. doi: 10.1021/ACS.JAFC.9B05827/SUPPL_FILE/JF9B05827_SI_002.CIF

Zhao, S., Li, J., Liu, J., Xiao, S., Yang, S., Mei, J., et al. (2023). Secondary metabolites of Alternaria: A comprehensive review of chemical diversity and pharmacological

properties. *Frontiers in Microbiology* 13, 1085666. doi: 10.3389/FMICB.2022.1085666/BIBTEX

Zuo, S., Wu, X., Guo, L., Jambhulkar, P. P., Bajpai, R., Reddy, H. J., et al. (2024). Assessment of Genetic Diversity and the Population Structure of Species from the Fusarium fujikuroi Species Complex Causing Fusarium Stalk Rot of Maize. *Journal of Fungi 2024, Vol. 10, Page 574* 10, 574. doi: 10.3390/JOF10080574