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

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Abstract

Endophytic fungi produce a diverse range of bioactive secondary metabolites with potential applications in biopesticide development. This study investigates the nematicidal and antifeedant properties of ethyl acetate extracts from endophytic fungi isolated from wild *Arabidopsis thaliana* populations in Spain. The extracts were tested against the plant-parasitic nematode *Meloidogyne javanica*, and two common insect pests, *Myzus persicae* and *Spodoptera littoralis*. Nine of the 13 extracts demonstrated significant nematicidal and/or antifeedant activity, indicating their potential as biopesticides. The active extracts were derived from six genera: *Alternaria* (3 isolates), *Dydimella* (1), *Dothiora* (1), *Pleiochaeta* (1), *Penicillium* (1), and *Fusarium* (2). Five extracts exhibited nematicidal activity above 90%, with three reducing the total number of *M. javanica* second-stage juveniles hatched from egg masses by 22–37%. Four extracts showed strong settling inhibition (>70%) against *M. persicae*, and three exhibited feeding inhibition against *S. littoralis*. Chemical analysis by GC-MS and LC-MS revealed a wide array of unique secondary metabolites in the active extracts, reflecting substantial chemical diversity, regardless of the fungal origin. This study highlights the potential of fungal endophytes from *A. thaliana* as sources of novel biopesticides, paving the way for future research focused on harnessing these fungi for biopesticide development.

Keywords: fungal endophytes, secondary metabolites, biopesticide, insecticide, nematicide, *Myzus persicae*, *Spodoptera littoralis*, *Meloidogyne javanica*, *Arabidopsis thaliana*, organic 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[1]. Biopesticides are PPPs derived from naturally occurring compounds or agents obtained from biological sources like animals, plants, and microorganisms[2]. Among microbial sources, fungi play a significant role in biopesticide development due to their production of biologically active secondary metabolites[3,4]. In particular, fungal endophytes - fungi that naturally colonize

internal plant tissues without causing disease symptoms[5–7], are recognized as valuable reservoirs of bioactive compounds, with terpenoids and polyketides being among the most commonly isolated ones. The potential of these metabolites for medicinal and agricultural applications has also been emphasized, highlighting the need for further research in this field[8]. Protection against pests and herbivores is one of the multiple benefits that these microorganisms can naturally provide to the host plant[9]. These microorganisms enhance plant resistance by producing secondary metabolites that deter or inhibit a wide range of pests and herbivores, including microbial pathogens, insects, and nematodes[10,11]. A well-known example is the endophyte *Neotyphodium* sp. which protects plants against herbivores by the production of alkaloids[12]. Given the pressing need for sustainable pest control solutions, increasing attention has been directed toward the bioprospecting of fungal endophytes as potential sources of novel biopesticides[13.]

Plant-parasitic nematodes pose a major threat to global agriculture, causing hundreds of billion annual losses worldwide[14,15]. Among them, root-knot nematodes are particularly destructive, attacking nearly all vascular plants[14]. *Meloidogyne javanica* is one of the most economically important species, serving as a model for studying plant-nematode interactions[16]. Its second-stage juveniles (J2) are the responsible for infecting the plants by penetrating the roots. Infected plants show reduced plant growth and wilting, severely impacting crop productivity[10]. Due to environmental concerns, traditional nematicides such as methyl bromide have been banned or restricted, necessitating the development of sustainable control alternatives[16,17].

In addition to nematodes, insect pests such as aphids and lepidopterans pose serious threats to agricultural production. Aphids, including *Myzus persicae*, cause significant economic losses through direct feeding and virus transmission, leading to severe yield reductions[18]. *M. persicae* is particularly problematic due to its ability to infest a wide variety of crops and its resistance to multiple insecticide classes, including organophosphates and neonicotinoids[19]. Consequently, biological control methods, including entomopathogens and biological compounds, are being actively explored[19].

Lepidopteran pests, including *Spodoptera* spp., pose an additional challenge to agriculture[20]. *S. littoralis* is a highly invasive species which damages over 40 plant families, including key crops like wheat, maize, rice, cotton, and vegetables[21]. Its larvae strip leaves and bore into fruits, significantly reducing crop yields[20]. The control of *Spodoptera* spp. requires the massive use of insecticides, since these insects have acquired resistance to all chemical families, including organophosphates, carbamates, and pyrethroids, as well as a more recent family, diamides[22].This growing resistance further underscores the need for alternative, sustainable pest management strategies.

In this context, the bioprospection of endophytic fungal cultures isolated from different plant species has yielded a wide variety of promising bioactive compounds against nematodes and insect pests[23]. 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*[24]. 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*[25]. Recent reports on biocidal compounds from fungal endophytes include nematicidal and antifeedant dioxolanones from *Phyllosticta*

sp. (YCC4) isolated from *Persea indica*[26], aphid antifeedant stempholones from *Stemphylium* sp.[27] or acaricidal mellein from *Aspergillus* sp.[28], both isolated from *Bethencourtia palmensis.*

Arabidopsis thaliana, a well-established model in plant research, has greatly advanced our understanding of plant–microbe interactions[29,30]. However, studies on its natural fungal endophytes remain scarce. Among the natural associations of this model plant, one notable example is the fungal endophyte *Colletotrichum tofieldiae. C. tofieldiae* establishes a mutualistic relationship with the plant that promotes plant growth and fertility under phosphate-starved conditions[31]. Beyond Arabidopsis, this endophyte also colonizes tomato and maize, improving their growth and yield[32]. Additionally, *C. tofieldiae* has demonstrated biocontrol potential by reducing the prevalence of mycotoxigenic *Aspergillus* spp. and lowering aflatoxin contamination in maize grains[33]. Other studies have identified additional fungal isolates from *Arabidopsis* that provide benefits under stress conditions, including isolates of *Macrophomina*, *Sordaria*, *Phaeosphaeria*, *Chaetomium*, and *Truncatella*[34]. Despite these promising findings, research on the natural fungal endophytes of *A. thaliana* remains limited, particularly regarding their secondary metabolites and potential as bioactive agents against plant pests[30].

In this study, we explored the biopesticidal potential of fungal endophytes isolated from wild *A. thaliana* plants collected in the central Iberian Peninsula. Culture-filtrate extracts from these endophytes were screened for their nematicidal activity against *M. javanica* and their antifeedant effects on *M. persicae* and *S. littoralis.* The diverse nematicidal and antifeedant effects observed among the 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 by García et al.[35].

The identification of each isolate was conducted by sequencing the internal transcribed spacer (ITS) region with the primer pairs ITS1/ITS4[36]. The fungal isolates were restored in potato-dextrose-agar plates (PDA, Difco™) and incubated at room temperature for seven days. Total fungal genomic DNA was extracted from mycelial fragments scraped from fresh fungal culture plates with CTAB method[37]. The PCR conditions were set as follows: 2 min at 94°C for initial denaturation step (1 cycle); 30 s at 94°C, 30 s at 52°C and 1 min at 72°C for amplification cycles (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)[38] and trimmed with trimmAl v2.0[39]. Phylogenetic tree was calculated with FastTree v2.1.11[40] and plotted with iTOL v6[41].

2.2. Culture conditions and liquid-liquid extraction

The fungal isolates were restored in 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. Spores were scrapped with sterile deionized water from fresh cultures and concentration was determined using a Neubauer Hemocytometer.

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 49 mL of medium each, were inoculated with 1 mL of the fungal spore suspension at the concentration of $5x10^5$ spores/mL, to reach a final concentration of 10^4 spores/mL of medium. In total, 300 mL of inoculated medium was used per isolate. 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 subjected to liquid-liquid extraction using ethyl acetate (EtOAc) three times. The volume of the culture filtrate was first measured and the sample was transferred to a separating funnel. An equal volume of EtOAc was added to the funnel, which was then sealed and vigorously shaken to promote mixing. After shaking, the funnel was allowed to stand until phase separation occurred due to the density difference between the aqueous and organic layers. The two phases were then carefully separated by decanting. The EtOAc fraction was subsequently evaporated at 40ºC using a rotary evaporator. The dried extract was weighed, and the fraction was stored at 4ºC for further use.

2.3. Screening

For the initial screening, the extracts were tested in bioassays against *M. javanica* (see section 2.4.1), *M. persicae* (see section 2.5.2), and *S. littoralis* (see section 2.5.3), as described below. Extracts exhibiting mortality rates greater than 90% and inhibiting settling (for *M. persicae*) or feeding (for *S. littoralis*) by more than 70% were selected for further analysis at lower doses. For the *M. javanica* egg hatching assay, the three extracts with the lowest LC50 values were chosen (see section 2.4.2).

2.4. Nematicidal activity

2.4.1. 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.[42]. Egg masses of *M. javanica* were handpicked from infected tomato roots. Second-stage juveniles (J2) were obtained from hatched eggs by incubating handpicked egg masses in a water suspension at 25ºC for 24 h. The inoculum, was adjusted to a final concentration of 100 J2 nematodes per 95 µL of distilled water. Then, 5 µL of the dissolved extracts or the control solution (DMSO: 0.6% Tween 20) were added to four wells of a 96-well plate containing 95 µL of the nematode suspension, achieving a final extract concentration of 1 µg/µL. Four replicates per treatment were included. The plates were sealed with parafilm to prevent evaporation and were incubated in a growth chamber at 25 ± 1 ºC in the dark. Dead J2s were counted at two experimental times (48 and 72 h) using a binocular microscope and mortality rate (M%) was calculated and corrected with Schneider-Orelli's formula[43].

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

171 Extracts with a nematicidal activity (M% >90%) were selected for subsequent trials at lower
172 doses (0.5 µg/µL, 0.25 µg/µL and 0.125 µg/µL) to calculate effective lethal doses (LC50 and
173 LC90) at 72 h by probit analysis (software Statgraphics 19, Statgraphics Technologies, Inc.).

174 **2.4.2. Egg hatching inhibition assay**

175 Egg hatching assay was performed as described by Andrés et al.[44], with the three most active
176 extracts (LC_{50}< 0.5), which were isolates 10034, 10070 and 110040. Four replicates of three
177 egg masses each (a total of 12 egg masses) were tested for each treatment. The three egg
178 masses of each replicate were placed in a well of a 24-well plate and exposed to a total volume
179 of 400 µL of the extracts diluted in DMSO:0.6% Tween 20 at the concentration of 1 µg/µL or
180 DMSO:0.6 % Tween 20 for controls. Plates were sealed with parafilm to prevent evaporation
181 and maintained in a growth chamber in darkness, at 25 ± 1 ºC and 70% relative humidity. After
182 five days (day 0) the number of juveniles hatched out of the egg masses was recorded. The test
183 solutions were subsequently removed and the wells with egg masses were washed and filled
184 with sterilized distilled water. Egg hatching was monitored for 4 weeks, until hatching was
185 complete in the control treatment, and then the relative hatching percentages (compared to
186 controls) were recorded.

187 **2.5. Antifeedant activity**

188 **2.5.1. Maintenance of *M. persicae* and *S. littoralis* colonies**

189 *M. persicae* and *S. littoralis* were reared on bell pepper (*Capsicum annuum* L.) plants and
190 artificial diet[45], respectively. Host plants together with insect colonies were maintained at 21 ±
191 2ºC, 60-70% relative humidity, and 16 h light: 8 h dark in a growth chamber.

192 **2.5.2. Bioassay of Settling Inhibition of the aphid *M. persicae***

193 Pepper leaf disks of 2 cm^2were cut into two even pieces (1 cm^2 each). The two leaf sections
194 were set on water-agar (1%) coating the bottom of a ventilated plastic box (3x3x1.5 cm). The
195 dry extracts were redissolved in ethanol at an initial concentration of 10 µg/µL. In each box,
196 one leaf section was treated by spreading 10 µL of the fungal extract over its surface, while the
197 other served as a control, receiving 10 µL of ethanol. Once ethanol evaporated, 10 apterous
198 aphids (24-48 h old) were placed in each plastic box. A total of 20 replicates (boxes) per extract
199 were included in this experiment. The percentage of aphids that settled on each leaf section
200 was recorded after 24 h (at the environmental conditions described above), as described by
201 González-Coloma et al.[46]. Settling inhibition index was calculated at an initial concentration of
202 100 µg/cm^2, using the following equation:

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

203 Where:

204 T = percentage of aphids on the treated section.

205 C = percentage of aphids on the control section

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

209 **2.5.3. 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 extract redissolved in ethanol and the other two with 10 µL of the 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[47]. 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). EC_{50} was calculated using a logarithmic regression model with the software Statgraphics 19 (Statgraphics Technologies, Inc.).

2.6. Chromatography

2.6.1. Gas Chromatography – Mass Spectrometry

The extracts were dissolved in dichloromethane (DCM) and 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), equipped with a 30 m × 0.25 mm i.d. capillary column (0.25 µm film thickness) Teknokroma TRB-5 (95%) Dimetil-(5%) diphenylpolisiloxane. Sample injections (1 µl) were carried out by an AOC-20i autosampler. 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.

2.6.2. Liquid Chromatography – Mass Spectrometry

The fungal 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 MiliQ water with 0.1% acetic acid (A) and methanol (HPLC-MS grade) with 0.1% acetic acid (B). 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

251 µg/µl with 5 µl injection through an automatic injector (SIL-20A XR). All extracts (0.5 µg/µl)
252 were dissolved in 100% MeOH for injection.

253 **2.6.3. Hierarchical clustering**

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

257 **3. Results**

258 **3.1. Extraction yields**

259 Different isolates exhibited varying extraction yields of their culture filtrate in ethyl acetate
260 (Supplementary Table S1). Yields (mg of extract per mL of culture filtrate) ranged from 2.6
261 mg/mL for *A. terricola* (isolate 10034) to 10.4 mg/mL for *D. maculans* (isolate 110040), with an
262 average yield of 5.3 mg/mL across all extracts. Notably, yield differences were observed even
263 among closely related isolates. For instance, *Fusarium* isolates 10027 and 10050 differed by 4.6
264 mg/mL. *Penicillium* isolates 10006 and 10062 had similar yields (6.9 and 7.8 mg/mL,
265 respectively), whereas isolate 110145 exhibited a lower yield of 2.9 mg/mL (Supplementary
266 Table S1). These findings suggest that extraction yield is isolate-specific rather than genus-
267 dependent.

268 **3.2. Nematicidal activity**

269 A total of five extracts out of the 13 tested showed activity (>90% M at 72 h) against *M.*
270 *javanica* (Table 2 and Table 3). Extract 10034 (*A. terricola*) exhibited the earliest effect causing
271 a mortality rate of 77% at 48 h, while 10033 (*Alternaria* sp.) and 110040 (*D. maculans*) showed
272 the highest effect at 72 h with a 100% of mortality. The most effective extract in terms of
273 lethal dose at 72 h was 10070 (*A. alternata*) with LC_{50} of 0.05 mg and LC_{90} of 0.4 mg. Extract
274 10034 showed the lowest LC50 at 72 h with 0.04 mg, however LC90 was the second highest.
275
276 The active extracts with the lowest LC50 values (10034, 10070, and 110040) were selected to
277 assess their effect on egg hatching. The three treatments resulted in a reduction in the total
278 number of J2 that hatched from egg masses in comparison to controls, being the effect
279 stronger during the first seven days (Figure 1 and Supplementary Table S2), with reductions
280 between 11% and 62% at day 0 (after five days of exposition to extract) and between 41% and
281 52% at day 7. Extract 10070 showed the highest total hatching inhibition rate with 37%,
282 followed by 110040 with 28% and 10034 with 22%.

283 **3.3. Antifeedant activity**

284 The extracts from the 13 endophytic fungi were screened for their biocontrol properties
285 against the insect pests *M. persicae and S. littoralis.* Four fungal extracts (10006, 10027, 10050
286 and 10070), representing 31% of the tested extracts, showed high (>70%) SI effect against *M.*
287 *persicae* at a starting concentration of 100 $\mu g/cm^2$ (Table 2). With these active extracts we
288 calculated EC_{50} by conducting the assays at lower concentrations (Table 4). Extract from isolate
289 10050 (*F. spartum*) exhibited the lowest EC_{50} at 20 $\mu g/cm^2$, followed by 10006 (*P. solitum*) with
290 a EC_{50} of 30 $\mu g/cm^2$. The latter was also the extract showing the highest SI rate at 100 $\mu g/cm^2$
291 with 93.6%.

292 Three fungal extracts (10006, 10013 and 10070) exhibited high FI effect against *S. littoralis*
293 (Table 2). The extract from isolate 10070 (*A. alternata*) was the most effective with 99.4% FI at
294 100 µg/cm^2 and a EC_{50} of 42 µg/cm^2.

295 **3.4. Metabolomic analysis**

296 As a result of the GC-MS analysis, the presence of 117 compounds (>%1 abundance) was
297 revealed, among which only 42 (36%) could be identified (Supplementary Table S3). The
298 number of compounds in each extract ranged from 9 (isolate 10006, *P. solitum*), to 25 (isolate
299 110013, *P. setosa*), and the presence of the different compounds was mostly specific of each
300 isolate, with just one compound (No. 100: unidentified) present in the extracts of all nine
301 isolates and three compounds (No. 34: 2,10 bisaboladien-1-one (IUPAC: (2E,10E)-bisabola-
302 2,10-dien-1-one); No. 48: 9-Octadecen-1-ol, (Z)- (IUPAC: (Z)-9-octadecen-1-ol), and No. 69:
303 Tributyl acetylcitrate, IUPAC: Tri-n-butyl 2-acetoxypropane-1,2,3-tricarboxylate) shared by the
304 extracts of five or more isolates. Namely, compounds (No. 34) 2,10-bisaboladien-1-one and
305 (No. 69) Tributyl acetylcitrate were present in all three extracts with activity against *M.*
306 *persicae* (Supplementary Table S3). Samples could be grouped in three clusters according to
307 their volatile-lipid composition (Figure 2A), with cluster 3 divided further in three subgroups.
308 Clusters GC1 and GC2 grouped the extracts from isolates from Polán population (roots and
309 rosette respectively). Cluster GC3 grouped extracts from isolates from the two populations and
310 different plant organs. The grouping did not correlate with the taxonomy of the isolates or the
311 activity (Figure 2C), indicating that the volatile-lipidic composition of the extracts was not
312 specific for these traits.

313 A non-target LC-MS analysis showed the presence of at least 28 metabolites, grouped by their
314 retention time, with a range of 7 to 12 metabolites per extract (Supplementary Table S4).
315 Three of the metabolites (No. 130, 134 and 136) were present in all the extracts and two
316 metabolites were absent in just one (No. 132 and 139). The clustering of the results (Figure 2B)
317 showed a profile that could be related to the taxonomical proximity of the isolates. A total of
318 four groups could be established, with three including one species: LC1 (110040, *D. maculans*);
319 LC2 (110013, *P. setosa*); and LC4 (10006, *P. solitum*), and one including three genera, which
320 could be divided into two: LC3.1 (10070-10033-10034, *Alternaria* spp. and 10013, *D.*
321 *macrostoma*) and LC3.2 (10027-10050, *Fusarium* spp.).

322 **4. Discussion**

323 In nature, the preeminent model plant *A. thaliana* hosts a high variety of fungal endophytes[35].
324 Characterizing its natural endophytic mycobiota offers valuable opportunities to deepen our
325 understanding of plant-endophyte interactions. However, characterization of endophytes
326 isolated from *A. thaliana* is relatively limited, particularly in the context of secondary
327 metabolites biosynthesis and their potential biotechnological applications. In this work**,** we
328 obtained extracts from 13 *A. thaliana* fungal endophytes and screened them for their activity
329 against the phytoparasitic nematode *M. javanica* and the plant insect pests *M. persicae* and *S.*
330 *littoralis*. To our knowledge, this is the first study that explores the potential nematicidal and
331 antifeedant effects of secondary metabolites derived from fungal endophytes of *A. thaliana*.

332 Our findings show that 9 out of the 13 (69%) extracts exhibited nematicidal and/or antifeedant
333 activity, what highlights the enormous potential of fungal endophytes of this model plant to
334 produce bioactive compounds of agricultural interest. The nine isolates with bioactive extracts
335 have different origins regarding population (Polán or Menasalbas) and plant tissue (root,
336 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.

4.1. 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 extracts with nematicidal activity. *Dothiora* species are found on plants in terrestrial habitats as saprobes and weak pathogens on stressed plants[48–50]. Previous studies reported the production of hormonemates, compounds with cytotoxic activity against tumoral cells, by endophytic *Dothiora* sp. isolated from *Launaea arborescens*[51]. *P. setosa* is an important pathogenic necrotrophic fungi of grain legumes [52]. 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*[53]. 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[54,55]. From a human health perspective, *Alternaria* is notable due to its airborne spores, which are among the most prevalent allergens[56]. As plant pathogens, *Alternaria* species can cause significant pre- and post-harvest diseases, sometimes leading to mycotoxin accumulation and serious economic losses[57]. 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[58,59]. In this work, the three *Alternaria* isolates showed nematicidal activity. Lou et al.[60] 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 plant parasitic nematode *Bursaphelenchus xylophilus*. However, although there is promising evidence of the nematicidal effects of secondary metabolites produced by *Alternaria*, this field remains still underexplored.

4.2. Antifeedant 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[61]. In addition, this fungus has been isolated from extremophilic environments, such as the acidic waters of the Berkeley Pit Lake[62] and Antarctica[63]. 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[64,65]. Additionally, viridicatol, a quinoline alkaloid, exhibited moderate anti-tumor activities against certain cancer cell lines and potent anti-food allergic effects *in vitro*[66]. 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[67], indole diketopiperazine

alkaloids[68], okaramine indole alkaloids[69], meroterpenoids[70], (-)-botryodiplodin[71], yaequinolones[72] or the terpenoid-pyridine oxalicines, active against *S. frugiperda*[73]. The closely related species *P. crustosum,* produces neurotoxic penitrems, which have exhibited insecticidal activity[74]. Furthermore, an endophytic *Penicillium* isolated from *Derris elliptica* also was shown to produce the antifeedant compound rotenone against the lepidopteran *Plutella xylostella* and the aphid *Lipaphis erysimi*[75]. 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[76]. However, it has recently shown potential as a biocontrol agent against rapeseed clubroot, significantly reducing disease severity and improving crop yields[77]. 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[78] and phomapyrrolidones[79]. Additionally, cytotoxic naphthalenones and didymelol have been isolated from the endophytic fungus *D. glomerata*, found in *Saussurea laniceps*[80]. Another example is *D. pinodes*, an aggressive isolate from pea (*Pisum sativum*), which produces pinolidoxin, a phytotoxin affecting several plant species[81]. 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[82]. 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[83–85], legumes[86], and bananas[87,88], as well as for producing harmful mycotoxins[89], 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[90–92]. Moreover, *Fusarium* is a rich source of bioactive compounds from various chemical classes, including those with insecticidal properties[93,94]. Specifically, *F. sambucinum* from *Nicotiana tabacum* produced the prenylated indole alkaloids sclerotiamides and notoamide with potent insecticidal activity against *Helicoverpa*[95]. Moreover, several species of *Fusarium* have been discovered as entomopathogenic in aphids[96], 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 extracts with antifeedant and toxic effects against *S. litura*[97]. Also, altenuene, an acetyl cholinesterase inhibitor isolated from an endophytic *A. alternata* strain of *Catharantus roseus*, exhibited insecticidal effect against *S. litura*[98]. 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-

428 spectrum efficacy and underscoring its potential as a versatile source of biopesticide products.
429 Additional research will be necessary to identify the specific molecules responsible for this
430 effect.

431 **4.3. Analysis of chromatographic profiles**

432 The extracts, analyzed by GC-MS and LC-MS, exhibited a highly diverse composition, with most
433 metabolites being unknown and unique to each extract. Our results did not show a direct
434 correlation between the secondary metabolite profiles and nematicidal or antifeedant activity.
435 Nonetheless, we could observe that two compounds— (No. 34) 2,10-bisaboladien-1-one and
436 (No. 69) tributyl acetylcitrate—were present in all three extracts that demonstrated activity
437 against *M. persicae* (Supplementary Table S3). While acetyltributylcitrate is commonly used as
438 a plasticizer[99], recent studies have reported it as a naturally occurring component in
439 antimicrobial extracts[100,101]. For example, it has been identified as a bioactive compound in
440 crude extracts of actinomycetes with antibacterial and antifungal properties[101], as well as in
441 the fungicidal compounds from *Michelia champaca* bark extract[101]. However, its use as a
442 biopesticide remains unexplored. The compound 2,10-bisaboladien-1-one is already known for
443 its antifeedant effects on *M. persicae*, significantly reducing both probing activity and the
444 number of intracellular penetrations[102]. This suggests that 2,10-bisaboladien-1-one may be one
445 of the key compounds responsible for the observed bioactivity.

446 In addition, no clear relationship between the chemical composition of the extracts and the
447 taxonomical proximity of the isolates was observed. The analysis of the less polar fraction
448 (DCM-soluble) of the extracts, containing the lipid fraction, did not show a taxonomy-
449 dependent distribution. On the other hand, the secondary metabolites in the EtOAc-soluble
450 fraction (analyzed by LC-MS) revealed chemical profiles that could be related to taxonomy,
451 though only to a limited extent. This lack of a clear pattern could be expected, as secondary
452 metabolite profiles in fungi are complex traits influenced by mono- and polyphyletic factors.
453 Although certain metabolites in fungal groups like the *Xylariaceae* family strongly correlate
454 with phylogenies[103], the inconsistent distribution of secondary metabolites across the fungal
455 kingdom and the great influence of environmental factors in their biosynthesis makes difficult
456 its correlation with phylogeny[104]. Comparative genomic studies have shown that homologous
457 genes and gene clusters related to secondary metabolism are distributed across a wide
458 phylogenetic range of species[105]. In some cases, this distribution is consistent with inheritance
459 from a common ancestor, as seen with the ergot alkaloid gene cluster in *Claviceps* and
460 *Metarhizium*[106]. In other cases, the presence of large, complex gene clusters in distantly
461 related taxa is thought to result from horizontal gene transfer[107]. Within-species genomic
462 comparisons have revealed polymorphisms in secondary metabolite gene clusters, such as
463 gene gain/loss, cluster mobility, and allelic variations, which might explain this evolutionary
464 divergencies[106,108,109].

465 In summary, our study underscores the significant potential of fungal endophytes as sources of
466 bioactive compounds with biocontrol capabilities against agricultural pests. The diverse
467 composition of extracts from different fungal isolates does not correlate with the nematicidal
468 and antifeedant activities observed, suggesting similar activities for different metabolites.
469 Nevertheless, identifying the bioactive compounds is essential for gaining a deeper
470 understanding of the mechanisms underlying the observed effects. Future research will focus
471 on bioguided fractionation of selected extracts for the identification of the active compounds.
472 Our results also show that a model host as *A. thaliana*, routinary found in many different
473 anthropic ecosystems in temperate regions[34,35,110], hosts fungal endophytes with the ability to

474 produce high diversity of compounds that are bioactive. The use of a model plant gives
475 opportunities to better study the relationship of the plant with these endophytes and the
476 conditions in which the secondary metabolites of interest are produced in the interaction with
477 the plants.

478 **5. References**

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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.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

Additional information

Competing interests

The authors declare no competing interests

Supplementary information: "Supplemetary Tables.xlsx"

Statement of Exemption from Permission Requirements for the Collection of *Arabidopsis thaliana* Samples: "Statement of Exemption from Permission Requirements.pdf"

769 **Tables and Figures**

770 **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

771

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

775

Isolate/Extract ID	Species	*M. javanica* (%M)[1]	*M. persicae* (%SI)[2]	*S. littoralis* (%FI)[3]
10006	*Penicillium solitum*	23.2 ± 2.3	93.6 ± 2.2	72.0 ± 7.9
10010	*Cladosporium cladosporioides*	56.6 ± 3.2	59.2 ± 11	51.5 ± 10.0
10013	*Didymella macrostoma*	25.1 ± 5.2	68.8 ± 5.3	81.6 ± 16.4
10027	*Fusarium 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	*Alternaria terricola*	90.2 ± 2.0	52.8 ± 9.6	29.6 ± 16.0
10050	*Fusarium 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	*Alternaria alternata*	96.7 ± 0.9	80.6 ± 8.1	99.4 ± 0.4
110013	*Pleiochaeta setosa*	90.3 ± 3.1	64.2 ± 8.5	22.6 ± 7.3
110040	*Dothiora 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

776 [1]Mortality rate at 72 h. Extracts were considered active when they showed >90% mortality rate at 72 h.
777 [2]Percent settling inhibition (%SI, n = 200 insects). Extracts were considered active when they showed >70% SI.
778 [3]Percent feeding inhibition (% FI, n = 12 insects). Extracts were considered active when they showed >70% FI.
779 Data shows mean ± standard error.
780
781

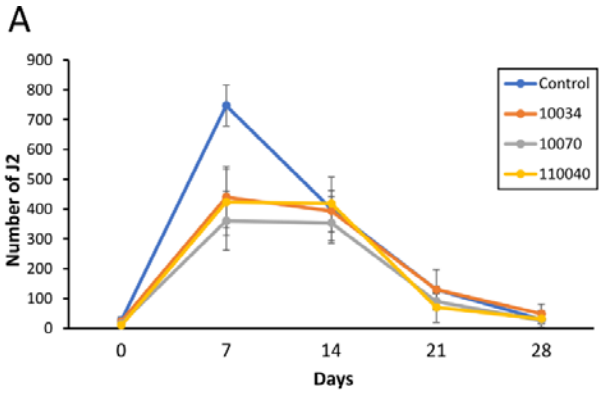
782 **Table 3. Mortality rates and lethal doses (LC50 and LC90) of the active[1] fungal extracts**
783 **against *M. javanica in vitro.***

Extract ID	Species	% Mortality[2]		Lethal doses at 72 h (mg)		P-value[3]
		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	*Alternaria terricola*	77.6 ± 1.8	90.2 ± 2.0	0.04	1.03	<0.001***
10070	*Alternaria alternata*	3.3 ± 1.1	96.7 ± 0.9	0.05	0.4	<0.001***
110013	*Pleiochaeta setosa*	27.3 ± 2.9	90.3 ± 3.1	0.91	1.53	<0.001***
110040	*Dothiora maculans*	56.8 ± 2.7	100 ± 0.0	0.42	0.8	<0.001***

784 [1]Extracts were considered active when they showed >90% mortality rate at 72 h.
785 [2] Data shows mean ± standard error.
786 [3]*P* values from Probit analysis used for the calculation of LC_{50} and LC_{90} at 72 h
787
788 **Table 4. Half maximal effective concentration (EC_{50}) of fungal extracts with antifeedant**
789 **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	*Penicillium solitum*	30	<0.001***	76	0.105
10013	*Didymella macrostoma*	-	-	60	0.001**
10027	*Fusarium citricola*	37	0.001**	-	-
10050	*Fusarium spartum*	20	0.007**	-	-
10070	*Alternaria alternata*	35	<0.001***	42	<0.001***

790 [1]*P* values from exponential regression models used for the calculation of EC_{50}.
791



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 most active extracts, from isolates 10034 (*Alternaria terricola*), 10070 (*Alternaria alternata*) and 110040 (*Dothiora 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 day 0 (after five days of exposition to extracts) and at 7, 14, 21 and 28 days immersed in water. Data (n = 4) shows means ± standard deviations.

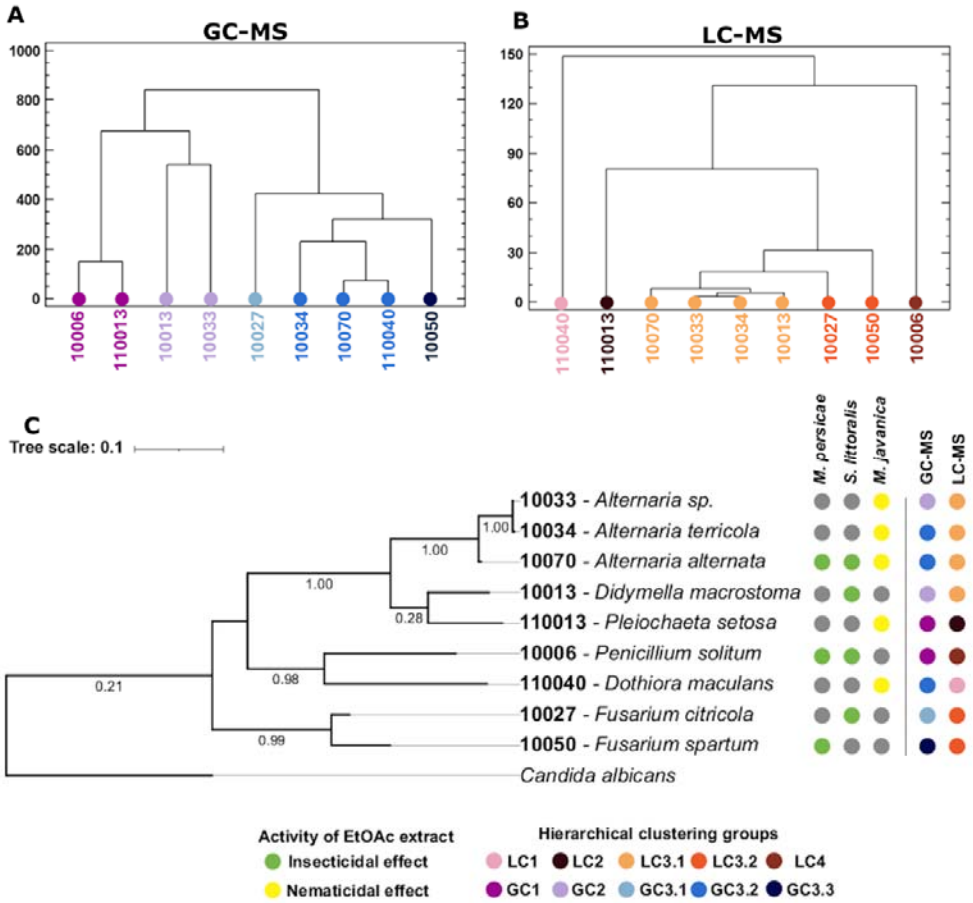


Figure 2. Sample clustering according to metabolomic profiles. Hierarchical clustering of fungal extracts based on their (A) GC-MS (Gas-Chromatography and Mass – Spectrometry) and (B) LC-MS (Liquid Chromatography and Mass – Spectrometry) profile. C) Phylogenetic tree of the fungal isolates based on

804 ITS sequences and classification based on activity of the extracts and the clustering of chromatographic
805 profiles.