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Article

Aphid Antifeedant Compounds from an Endophytic Fungus *Trichoderma* sp. EFI671

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Abstract: In the current study, an ethyl acetate extract from the endophytic fungus *Trichoderma* sp. EFI 671, isolated from the stem parts of the medicinal plant *Laurus* sp. was screened for bioactivity against plant pathogens (*Fusarium graminearum, Rhizoctonia solani, Sclerotinia sclerotiorum* and *Botrytis cinerea*), insect pests (*Spodoptera littoralis, Myzus persicae, Rhopalosiphum padi*) and plant parasites (*Meloidogyne javanica*). The chemical study of this insect antifeedant extract resulted in the isolation of 1-oleoyl-2-linoleoyl-3-palmitoylglycerol (**1**), eburicol (**2**), β-sitostenone (**3**), ergosterol (**4**) and ergosterol peroxide (**5**). The free fatty acids present in compound **1** (oleic, linoleic and palmitic) showed strong dose-dependent aphid antifeedant effects against *M. persicae*. Liquid (PDB, and SDB) and solid (corn, sorghum, pearl millet and rice) growth media were tested in order to optimize the yield and bioactivity of the fungal extracts. Pearl millet and corn gave the highest extract yields. All the extracts from these solid media had strong effects against *M. persicae* with sorghum being the most active. Corn increased the content in linolenic, pearl millet the oleic and stearic and sorghum oleic and linolenic acids compared to rice. Their antifeedant effects correlated with linoleic /oleic acids. The phytotoxic effects of these extracts against *Lolium perenne* and *Lactuca sativa* varied with culture media, with sorghum being the least toxic.

Keywords: Endophyte; *Trichoderma*; fatty acid; *Myzus persicae*; culture media; Biomolecules; Antifeedant

1. Introduction

Endophytes include all microbes that can be isolated from asymptomatic plant tissue. Among them, the study of fungal endophytes has led to the discovery a large quantity of bioactive natural products in recent years [1-7]. Furthermore, fungal endophytes are able to produce compounds found in their host plants such as azadirachtin, podophyllotoxin, hypericin, and taxol [8-13]. Therefore, medicinal plants and their endophytes are good candidates for the isolation of bioactive metabolites.

Botanical and fungal biopesticides, including endophytes, are in high demand given the current restrictive legislations on the use of chemical pesticides. In this context, endophytes isolated from species of the *Lauraceae* family produced extracts with crop protection effects [14]. For example, *Guignardia mangiferae*, *Glomerella acutata* and *Diaporthe* sp. isolated from *Laurus novocanariensis*, *Persea indica* and *Ocotea foetens*, showed potent insect antifeedant effects against *Spodoptera littoralis*, *Leptinotarsa decemlineata* and *Rhopalosiphum padi*. Furthermore, *G. mangiferae* from *P. indica* showed insect antifeedant effects and strong in vitro nematicidal activity against the root-knot nematode

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Meloidogyne javanica, with the dioxalanone guignardianone D being the nematicial agent with antifeedant effects on insect pests [14, 15].

As part of an ongoing search for new bio-pesticides, a series of fungal endophytes have been isolated from selected medicinal plants including *Lauraceae* species. In this work, we reported on the isolation of the endophytic fungal strain EFI671 from *Laurus* sp. and the isolation and characterization of the bioactive compounds present in ethyl acetate extract against *Myzus persicae*. Additionally, liquid (Potato Dextrose Broth, and Sabouraud Dextrose Broth) and solid (corn, sorghum, pearl millet and rice) growth media were tested in order to optimize the yield and bioactivity of the fungal extracts.

2. Materials and Methods

2.1. Plant material

The plant material for endophyte isolation was collected from medicinal plant *Laurus* sp., which was purchased from herbal garden nursery of the University of Agricultural Sciences, Bangalore, India. The samples were placed into sterile polybags and transported, under refrigeration, in a box container until isolation processing within 48 hours of collection.

2.2 Isolation of endophytic fungus Trichoderma sp. EFI 671

Endophytic fungus was isolated from the medicinal plant of Laurus sp. according to Kumar et al., (2013) with surface sterilization method [16]. Surface of stem and leaf was sterilized with 70% ethanol for 2 min followed by 1% sodium hypochlorite for 3 min. Sterilized stem and leaf were dried on sterile blotting sheet and then chopped in sterile petri plate and transferred to Potato Dextrose Agar plates (PDA). These plates were incubated at 24°C for 3–15 days in BOD incubator. Growing fungal colonies were transferred to fresh PDA plates to get pure culture. *Trichoderma* sp. was initially identified by microscopic examination and later identified by molecular methods as described in next section. The culture was maintained on PDA slant by routine sub culturing. The culture has been deposited in the National Centre for Microbial Resources (NCMR), India.

2.3 Molecular characterization of Trichoderma sp. EFI 671

The genomic DNA of pure fungal isolate *Trichoderma* sp. EFI 671 was extracted using DNeasy Plant mini kit (Qiagen GmbH, Hilden Cat. No 69104) by following the manufacture's instruction. The extracted DNA was used for PCR amplification by primer ITS1 and ITS4 according to Kumar et al. (2011) [17]. The Polymerase chain reaction was achieved in 25 µL of reaction mixture, which contained 2.5 µL of 10× PCR Buffer with 15 mM $MgCl_2$ (Applied Biosystem, India), 0.5 µL of dNTP mix (10 mM, Applied Biosystem), 2.5 µL of ITS1, ITS4 primers (10 Pico mole/µL), 1 µL of DNA template, and 0.5 µL of Ampli Teg Gold (5 U/µL). The ITS1 and ITS4 primers were synthesized from Merck nucleotide synthesis services (Bengaluru, India) according to ITS1 5'TCCGTAGGTGAACCTGCGG3', ITS4 5'TCCTCCGCTTATTGATATGC3'sequence. The Polymerase Chain Reaction (PCR) was done on Veriti Thermal Cycler (Applied Biosystem) using following programmes: initial denaturation at 94°C for 2 minutes; 30 cycles of denaturation, annealing, and elongation at 94°C for 1 minute, 57°C for 90 sec, and 72°C for 2 minutes followed by final elongation at 72°C for 4 minutes. The negative control was also run using sterile water. The amplified product was checked on 1.5 per cent agarose gel-by-gel electrophoresis. The amplified product was sequenced by Merck sequencing services, Bengaluru, India. The identification of strain was done by sequence similarity of amplified sequence with NCBI database using Basic Local Alignment Search Tool (nBLAST). The sequence was aligned and trimmed by DNA Baser 4.2 software based on quality read values. The sequence was submitted to NCBI database.

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2.4 Cultivation of endophytic fungi

2.4.1 Small-scale cultivation

The *Trichoderma* sp. EFI 671 was cultivated on 200 g of sterilized rice medium in 1000 ml conical flask for metabolites extraction as per the method of Kumar and Kaushik (2013) [18]. After 15 days of growth at 25ºC under dark conditions, metabolites were extracted using 250 ml of ethyl acetate (EtOAc) solvent according Kumar et al., (2013) [16]. The organic solvent was evaporated to dryness in a rotary evaporator (Heidolph, Germany) yielding 500 mg of ethyl acetate extract (EtOAc). Further, EtOAc (350 mg) extract was partitioned with n-hexane (Hex) and 90% MeOH to separate polar and non-polar compounds. The weight of hexane and methanol extract was 180 mg and 150 mg respectively.

2.4.2 Large-scale cultivation for compound isolation

The fungal strain *Trichoderma* sp. EFI 671 was incubated on 250 g sterilized rice medium in 10 conical flask of 1000 ml volume for 15 days as the same conditions described above for isolation of active compounds. The extraction and partition of fungal extract was done according to Kumar et al. (2013) as described above [16]. A total of 6.0 g of hexane extract was obtained from the partition of 13.46 g of EtOAc extract.

2.5 Optimization of media

The culture conditions were optimized in liquid and solid media. To determine the best liquid media for active component production, Potato Dextrose Broth (PDB) and Sabouraud Broth (SDB), media were used. The fungal strain was inoculated at small scale in 30 ml of medium and incubated for 7 days. Different solid substrates (corn, sorghum, barley, pearl millet and rice) were also used to determine their effect on production of active compounds by the endophytic fungi. In each 1 L flask, 200 g of solid substrates were incubated for 20 days as per the procedure of (Kumar and Kaushik, 2013) [18]. The bioactive compounds were extracted with EtOAc using the same protocol. The fungal growth was measured as biomass estimation and spore count.

2.6 Isolation and Identification of bioactive compounds

Column chromatography (CC) was performed on silica gel 40-70 µm (Merck) and precoated silica gel 60 F254 (Merck) were used for preparative TLC, Compounds were visualized on TLC by heating after spraying with Oleum reagent [H2O: acetic acid: sulfuric acid 16:80:4]. Preparative HPLC was performed on a Varian ProStar with a 20 mm x 250 mm Interstil silica column (10 µm particle size). The bioactive hexane extract was dissolved in Dicholoromethanol(DCM), and the solution neutralized with 0.5N NaOH. The aqueous layer was acidified with 2N HCl at pH 2 and extracted with DCM (3 x 200 mL). Both organic layers were dried with anhydrous Na_2SO_4, filtered and concentrated in vacuum to afford 3.72 g and 110 mg of neutral and acid extracts respectively. The neutral extract was fractionated by Vacuum Liquid Column chromatography (VLC) over silica gel using hexane / ethyl acetate mixtures of increasing polarity (100:0 - 50:50 Hex:EtOAc), to afford 11 fractions. Further preparative HPLC chromatography of fraction 1 (208 mg), eluted with an isocratic mixture of Hex:EtOAc (95:5) at 3ml/min gave compound **1** (28 mg). Fraction 3 (132 mg), was chromatographed by prep HPLC with an isocratic mixture of Hex:EtOAc (97:3) at 3ml/min to give compound **1** (450 mg), compound **2** (5.2 mg) and compound **3** (6.2 mg). Fraction 7 (101 mg), was chromatographed by prep HPLC with Hex:EtOAc (95:5) at 3ml/min to give compound **4** (15.9 mg). Fraction 11 (39.4 mg) was further subjected to prep-TLC and eluted with Hex:EtOAc (95:5) to give compound **5** (5.7 mg).

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The compounds were subjected to mass and NMR spectroscopy for structure elucidation. Optical rotations were measured with a Perkin-Elmer model 343 polarimeter. NMR experiments were recorded on a Bruker Advance-400 and AMX2 500 MHz spectrometers. Chemical shifts were calculated using the solvent as internal standard ($CDCl_3$, at δ_H 7.26 and δ_C 77.0). HRESI MS (positive-ion mode) data were obtained using a Micromass LCT Premier and HREIMS on a Micromass Autospec instrument at 70 eV.

In order to identify the acid esters present in compound **1**, 450 mg of fraction 1 were added to 50 mL of a 20 mg/ml K_2CO_3 solution in MeOH stirred and monitored by TLC. After 18 h, the reaction mixture was extracted with EtOAc and dried over Na_2SO_4 to give an extract (430 mg) that was further chromatographed on a flash column filled with 25 g Si-gel and eluted at 18 mL/min with 1.5-15% Hex-EtOAc to afford fractions 1A (95.3 mg) and 1M (273.4 mg) that were further analyzed by GC-MS.

2.7 Gas chromatography - mass spectrometry (GC-MS) analysis

Analysis were carried out using a Shimadzu GC-2010 gas chromatograph coupled to a Shimadzu GCMS-QP2010 Ultra mass detector (electron ionization, 70 eV) and equipped with a 30 m × 0.25 mm i.d. capillary column (0.25 µm film thickness) Teknokroma TRB-5 (95%) Dimethyl-(5%)-diphenylpolysiloxane. Working conditions were as follows: split ratio (20:1), injector temperature 300ºC, temperature of the transfer line connected to the mass spectrometer 250ºC, initial column temperature 70ºC, then heated to 290ºC at 6ºC min^{-1}. Electron ionization mass spectra and retention data were used to assess the identity of compounds by comparing them with those of standards or found in the Wiley 229 mass spectral database. Further, the retention times of authentic compounds (isolated in this work and purchased from Sigma Aldrich) were also used to confirm the identities of the constituents. The relative amounts of individual components were calculated based on the GC peak area (FID response) without using a correction factor.

2.8 Antifungal bioassay

The antifungal activity of fungal metabolites against different phyto-pathogens like Fusarium graminearum, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Botrytis cinerea* procured from the Indian Type Culture Collection (ITCC), IARI, New Delhi. The activity was determined by food poison assay according Chowdhary and Kaushik (2015) [19]. Dried crude extract and partitioned fungal extract was dissolved in respective solvent to prepare stock solution of 40 mg/ml concentration. From the stock solution 1.00-0.01 mg/ml concentrations were prepared in 10 ml of PDA medium by adding 250µl, 125 µl, 62.5 µl, 25 µl and 2.5 µl of stock. Intoxicated media plates were inoculated with 8 cork borer plugs of plant pathogenic fungi measuring 0.5cm^2 placed in the plate. The solvent control was prepared using respective solvent in 10 ml of PDA media. The plates were incubated at 25±2 ° C for 7 days. The growth inhibition of phyto-pathogen was recorded after 48 h to 7days or before overgrowth of control plates. % GI was calculated as:

$$GI\% = 100 \times \frac{(Diameter\ of\ fungi\ in\ control\ plate\ (A) - Diameter\ of\ fungi\ in\ intoxicated\ plate\ (B))}{Diameter\ of\ fungi\ in\ control\ plate\ (A)}$$

2.9 Insect Bioassay

Insect colonies maintained at ICA-CSIC were used for conducting the bioassays. *S.lilittoralis*, *Myzus persicae* and *Rhopalosiphum padi* colonies were reared on an artificial diet, bell pepper (*Capsicum annuum*) and barley (*Hordeum vulgare*) plants respectively, and maintained at 22 ± 1 ºC and >70% relative humidity, with a photoperiod of 16:8 h (L:D) in a growth chamber [20].

2.9.1 Antifeedant bioassays:

The upper surface of *C. anuum* and *H. vulgare* leaf disks or fragments (1.0 cm^2) were treated with 10 µl of the test substance. The crude extracts and products were tested at an initial dose of 10 or 5 mg/ml (100 or 50 µg/cm2) respectively. Five Petri dishes (9 cm diam.) or twenty ventilated plastic boxes (2x2 cm) with 2 newly molted *S. littoralis* L6 larvae (<24 h) or 10 apterous aphid adults (24-48 h old) each were allowed to feed at room temperature for *S. littoralis* (< 2 h) or in a growth chamber for the aphids (24 h, environmental conditions as above). Each experiment was repeated 2-3 times (SE<10%) and terminated when the consumption of the control disks reached 65-75% for *S. littoralis* or after 24 h for aphids. The leaf disks were digitalized to calculate the area consumed (Image J, http://imagej.nih.gov/ij). Aphid settling was measured by counting the number of aphids on each leaf fragment. Feeding or settling inhibition (%FI or %SI) was calculated as

$$\% FI/SI = [1 - (T/C)\, x\, 100]$$

where T and C are the consumption/settling of treated and control leaf disks, respectively. The antifeedant effects (% FI/SI) were analyzed for significance by the nonparametric Wilcoxon signed-rank test. Extracts and compounds with an FI/SI > 75% were further tested in a dose-response experiment (3-4 serial dilutions) to calculate their relative potency (EC_{50}, the effective dose to give a 50% feeding/settling reduction) from linear regression analysis (% FI/SI on Log-dose) [21].

2.10 Nematode Bioassay

A laboratory (ICA-CSIC) root knot nematode (*M. javanica*) population maintained on tomato (*Lycopersicon esculentum* var. Marmande) plants in pots at 25±1 °C and > 70% relative humidity was used for the experiments. Second-stage juveniles (J2) hatched within 24 h (from egg masses handpicked from infected tomato roots) were used. The experiments were carried out in 96-well microplates (Becton, Dickinson) as described by Andres et al.(2012). The organic extracts and pure compounds were tested at initial concentrations of 1.0 and 0.5 mg/mL (final concentration in the well) and diluted serially if needed. The aqueous extract was diluted serially. The number of dead juveniles was recorded after 72 h. All treatments were replicated four times. The data were determined as percent mortality and corrected according to Scheider-Orelli's (1947) formula [22].

2.11 Phytotoxicity Tests

The experiments were conducted with *Lactuca sativa* and *Lolium perenne* seeds (100 seeds / test) in 12-well microplates as described previously (Martín et al., 2011) [21]. The extracts were tested at a concentration of 0.4 mg/mL (final concentration in the well). Germination was monitored for 6 (*L. sativa*) or 7 days (*L. perenne*) and the root / leaf length measured at the end of the experiment (25 plants were selected randomly for each experiment, digitalized and measured with the application Image J, http//rsb.info.nih.gov./ij/). A non-parametric analysis of variance (ANOVA) was performed on root / leaf length data.

3. Results

3.1 Identification of endophytic fungi

The endophytic fungi *Trichoderma* sp. EFI 671 was isolated from the stem parts of the medicinal plant *Laurus* sp. The axenic culture was identified by rDNA sequencing of internal transcribed region (ITR) as *Trichoderma* sp. EFI 671, based on sequence similarity with NCBI database. The culture showed 99% sequence similarity with *Trichoderma* sp. Accession no. KM458790.1.

3.2 Bioprospecting of endophytic fungi

Bioprospecting of *Trichoderma* sp. EFI671 extracts was carried out against the phyto-pathogenic fungi *F graminearum, R. solani, S. sclerotiorum, B. cinerea*, the nematode *M. javanica* and the insects *S.*

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littoralis, *M. persicae* and *R. padi*. The results of bioactivity of the extracts against phytopathogens and pest insects are given in Tables 1 and 2. The total EtOAc fungal extract and the MeOH partition showed moderate (65 and 50% inhibition, respectively) effects on *S. sclerotiorum* mycelial growth, suggesting the presence of polar antifungal compounds.

Table 1: Screening of bioactivity of *Trichoderma* sp. EFI 671 ethyl acetate (EtOAc), Methanol (MeOH) and Hexane (Hex) extracts against plant pathogens and parasites at 1 mg.ml^{-1} concentration. Data is expressed as % mycelium growth inhibition (fungal pathogens) and % mortality (*M. javanica*).

Extract	*Fusarium graminearum*	*Rhizoctonia solani*	*Sclerotinia sclerotiorum*	*Botrytis cinerea*	*Meloidogyne javanica*
	% Inhibition				% Mortality
EtOAc	03.95 ± 8.46	43.40 ± 05.33	65.4 ± 03.50	09.8 ± 05.26	6.21 ± 1.29
MeOH	10.12 ± 8.95	45.30 ± 05.38	50.5 ± 11.38	22.2 ± 12.90	2.73 ± 0.60
Hex	08.56 ± 6.10	28.05 ± 18.04	05.6 ± 15.50	30.8 ± 06.53	7.50 ± 1.21

Table 2. Insect anti-feedant effects of *Trichoderma* sp. EFI 671 extracts (EtOAc), solvent partitions of the EtOAc extract (MeOH, Hex) and acid partition of the Hex extract (HexN, HexA) against Aphids and pest at 100 µg/cm^2 concentration.

Extract	*Myzus persicae*	*Rhopalosiphum padi*	*Spodoptera littoralis*
	%SIa (100 µg/cm^2)		%FIa (100 µg/cm^2)
EtOAc	82.52 ± 6.19*	72.63 ± 5.99*	17.29 ± 07.80
MeOH	76.89 ± 7.97*	54.25 ± 6.71	11.66 ± 07.30
Hex	87.42 ± 5.32*	39.79 ± 7.80	30.01 ± 15.94
HexN	76.63 ± 7.30*		
HexA	97.10 ± 1.30		

[a]Percent settling (%SI, n = 100 insects) / feeding (%FI) inhibition (n = 20 insects); *Significantly different from the control ($p<0.05$), Wilcoxon paired rank test.

The EtOAc extract showed significant settling inhibition effects on *M. persicae* (82% SI) and *R. padi* (73% SI). Its partition into non-polar (Hex) and polar (MeOH) extracts resulted in 87 and 78 % SI against *M. persicae* and 39 and 54 % SI against *R. padi*, indicating that the hexane extract concentrated most of the anti-feedant compounds against *M. persicae*, while the partition decreased the extract effect against *R. padi* (Table 2). Therefore, a larger scale hexane extract was prepared for bioassay-guided fractionation and compound identification. Both extracts were inactive against root knot nematode *M. javanica*

3.3 Identification of compounds

The acid extraction of the large-scale hexane extract (Hex) gave two aphid antifeedant fractions, neutral (HexN) and acid (HexA), with the acid fraction being more effective (Table 2). The GC-MS analysis of the hexane extract and its partitions showed that the extract contained linoleic as the main component followed by oleic, palmitic and stearic acid. Methyl linoleate and methyl oleate were present as minor components. The HexA extract contained the same acids but not their methyl esters (Table 3).

Table 3. Chemical composition (% abundance) of the hexane extract (Hex), its acid fraction (HexA), the free acids from the hydrolysis of 1 (1A) and their methylated esters (1M).

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Retention time (min)	Compound	Hex	HexA	1A	1M
23.57	Hexadecanoic acid methyl ester (methyl palmitate)	0.78	-	-	19.72
24.19	Hexadecanoic acid (palmitic acid)	15.89	26.70	24.70	-
26.41	Octadecadienoic acid methyl ester (methyl linoleate)	2.89	-	-	36.35
26.50	Octadecenoic acid methyl ester (methyl oleate)	0.67	-	-	39.25
26.90	Octadecanoic acid methyl ester (methyl stearate)	-	-	-	4.68
27.03	Octadecadienoic acid (linoleic acid)	42.60	43.05	20.76	-
27.12	Octadecenoic acid (oleic acid)	32.30	27.47	46.15	-
27.45	Octadecanoic acid (stearic acid)	3.90	2.78	8.39	-

- Could not be detected

The chemical study of the neutral fraction resulted in the isolation of compound **1** as the major component. The spectroscopic data for compound **1** was consistent with the structure of a triglyceride. The composition of fatty acid (1A) and and methylated (1M) fractions obtained from the hydrolysis of compound **1** was confirmed by GCMS and by the comparison of retention times of methyl esters of authentic fatty acids. The GC-MS showed the presence of oleic, linoleic and palmitic acids as the main components, with stearic acid as a minor one (Table 3). On the basis of spectral data analysis, the structure of **1** has been established as 1-oleoyl-2-linoleoyl-3-palmitoylglycerol [23, 24] (Figure 1).

Four known sterol compounds were also isolated from Trichoderma sp. EFI 671 neutral Hex extract. Their structures were determined based on their 1H, 13C RMN and MS spectroscopic data. Their spectroscopic data were in agreement with that reported for eburicol (4,4,14α,24-tetramethyl-5α-cholesta-8, 24(24')-dien-3β-ol (**2**) [26], stigmast-4-ene-3-one (**3**) [25], ergosterol (ergosta-5, 7, 22-triene-3β-ol) (**4**) [26]and ergosterol peroxide (3β,5α,8α,22E)-5,8-epidioxyergosta-6,22-dien-3-ol) (**5**) [27].

The components of the neutral fraction (**1**-**5**) were not active against *M. persicae*, while the mixture of free acids (1A) and their methylated derivatives (1M) showed strong dose-dependent aphid antifeedant effects (Table 4).

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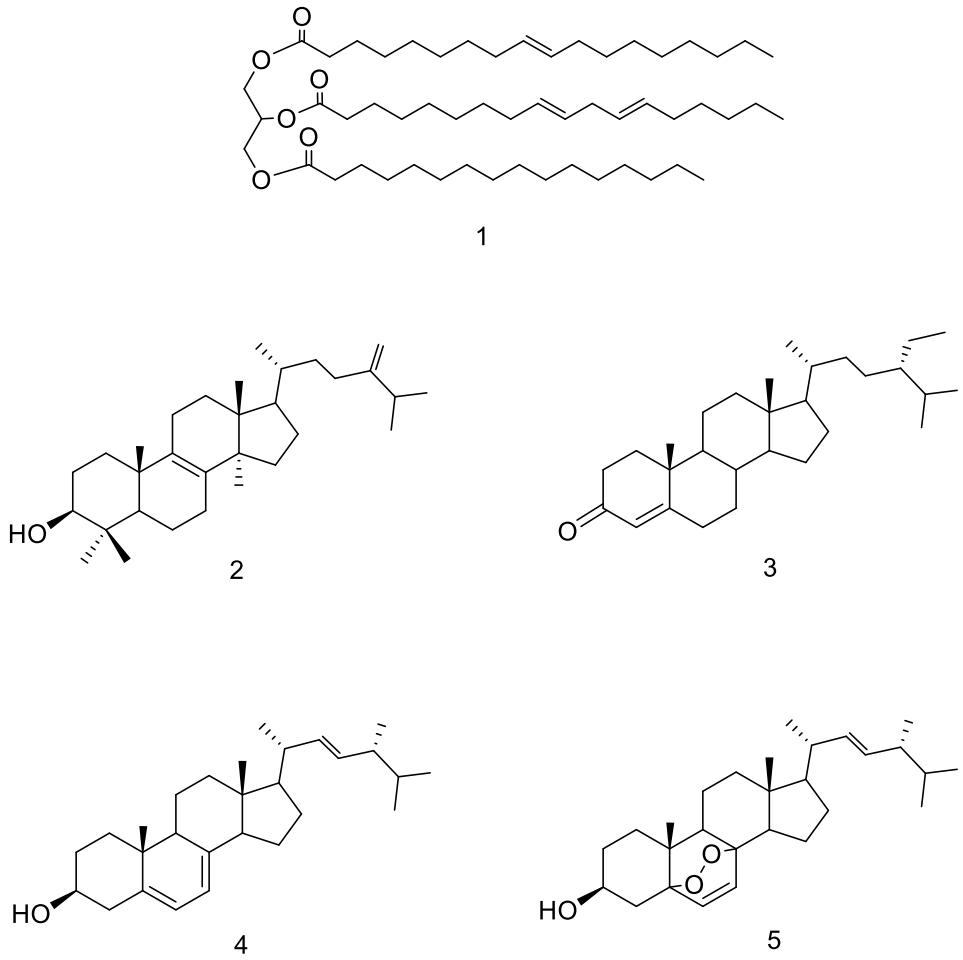

Figure 1: Molecular structures of compounds (**1**) 1-oleoyl-2-linoleoyl-3-palmitoylglycerol, (**2**) (4,4,14*α*,24-tetramethyl-5*α*-cholesta-8, 24(24')-dien-3β-ol (eburicol), (**3**) stigmast-4-ene-3-one (β-sitostenone), (**4**) ergosta-5, 7, 22-triene-3β-ol (ergosterol) and (**5**) (3β,5*α*,8*α*,22E)-5,8-epidioxyergosta-6,22-dien-3-ol (ergosterol peroxide).

Table 4. Antifeedant effects of compounds **1**-**5**, free acids from the hydrolysis of **1** (1A) and their methylated esters (1M) on *Myzus persicae*.

Extract / Compound	*Myzus persicae* %SI[a] (50 μg/cm²)	EC_{50}[b] (μg/cm²)
1	35.86±8.4	
1A	85.56±5.3*	6.87 (4.34-23.8)
1M	72.67±6.9*	1.03 (0.18-5.64)
2	15.35±6.94	
3	18.33±7.09	
4	17.08±6.7	
5	38.76 ± 7.5	

[a]Percent settling (%SI, n = 100 insects) inhibition, [b]Concentration needed to produce 50% setting inhibition (EC_{50}) against *M. persicae* and 95% confidence limits (lower-upper), * Significantly different from the control ($p<0.05$), Wilcoxon paired rank test.

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3.4 Optimization of media

Different liquid and solid growth media were tested on the isolate EFI 671 in order to optimize the bioactivity of the extracts against aphids. The liquid media were the conventional potato dextrose broth (PDB), and Sabouraud Broth (SDB). The solid media included corn, sorghum, pearl millet and rice. Table 5 shows the results of spore count and extract yields. The solid media pearl millet and sorghum gave the highest spore counts while pearl millet and corn gave the highest extract yields (mg/g) (Table 5).

Table 5. Spore count and extract yield from the different culture media (liquid and solid) of EFI 671.

Media	Extract yield (g/ml) / (g/g)	Spore count/ml
PDB	00.02	5.04x 10^6
SDB	00.07	8.96x10^6
Sorghum	09.84	7.92x10^8
Barley	03.56	3.95 x10^8
Corn	13.16	4.27 x10^8
Pearl millet	22.64	9.29 x10^7
Rice	06.48	2.88 x10^8

All the EtOAc extracts from these solid media showed low-moderate settling inhibition effects against *R. padi* and strong effects against *M. persicae*, ranking as follows: sorghum > corn > barley > rice > pearl millet. The conventional liquid media PDB and SDB gave inactive extracts (Table 6).

Table 6. Bioactivity of EFI 671 extracts from different media against *Myzus persicae* and *Rhopalosiphum padi*.

Extract	*Myzus persicae* %SI[a] (100 µg/cm²)	EC_{50}[b] (µg/cm²)	*Rhopalosiphum padi* %SI[a] (100 µg/cm²)
Rice	87.16 ± 3.22*	33.54 (26.12-42.30)	64.49 ± 5.58
Corn	88.6 ± 5.22*	14.63 (08.98-23.82)	30.43 ± 7.23
Sorghum	90.19 ± 2.28*	1.39 (0.01-2.00)	62.62 ± 5.76
Barley	85.38 ± 5.32*	23.10 (14.07-37.58	54.59 ± 7.53
Pearl millet	70.31 ± 5.45*	38.57 (26.46-54.36)	40.62 ± 8.75
PDB	41.08 ± 8.71	~100c	64.17 ± 6.78
SDB	51.08 ± 7.64	~100c	na

[a]Percent setting (%SI, n = 100 insects) inhibition, [b]Concentration needed to produce 50% settling inhibition (EC_{50}) against *M. persicae* and 95% confidence limits (lower-upper). [c] Estimated values. * Significantly different from the control ($p<0.05$), Wilcoxon paired rank test, na= not available as bioassays could not be done due to low amount of extract

The different media resulted in quantitative and qualitative differences in chemical composition (Table 7) thus, explaining the differences in their bioactivity. Overall the major components were linoleic and oleic followed by palmitic. Methylated derivatives of linolenic and oleic acid were only present as minor components in pearl millet and corn. Corn increased the linolenic acid content of the extract, pearl millet the oleic and stearic acids and sorghum increased oleic and linolenic acids compared to rice. A multiple correlation analysis between the content in fatty acids and bioactivity

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showed a strong significant correlation between linoleic /oleic acids and the settling inhibition effect on *M. persicae* of these extracts (Table 7).

Table 7. GC-MS analysis of the different EFI671 solid and liquid media extracts and correlation coefficients (CC, p = significance level) with bioactivity (EC_{50} in µg/cm² for *M. persicae*).

Growth Medium	% Fatty Acid			*M. persicae*
	Linoleic Acid	Oleic acid	Stearic acid	EC_{50} (µg/cm²)
Rice	30.12	28.19	03.01	33.54
Corn	41.67	29.45	03.85	14.63
Sorghum	34.19	36.28	05.50	01.39
Barley	29.49	23.30	03.09	23.10
Pearl millet	26.89	35.16	11.74	38.57
PDB	00.86	00.00	00.00	100.0
SDB	00.96	00.83	00.18	100.0
CC (p)	-0.9693	-0.9317	-0.5373	
	(0.0003)	(0.0023)	(0.2136)	

3.5 Phytotoxicity of bioactive compounds

The phytotoxic effects of the bioactive extracts were tested on *L. perenne* and *L. sativa* germination and growth. None of these extracts affected the germination of *L. sativa* or *L. perenne* (% inhibition <10). All extracts (rice, corn and pearl millet) inhibited the root growth of *L. sativa* (66-83%) except sorghum. Pearl millet significantly increased the growth of root and leaf of *L. perenne* (60%) (Figure 2). Therefore, the phytotoxic effects of the bioactive extracts can be modulated by the culture media.

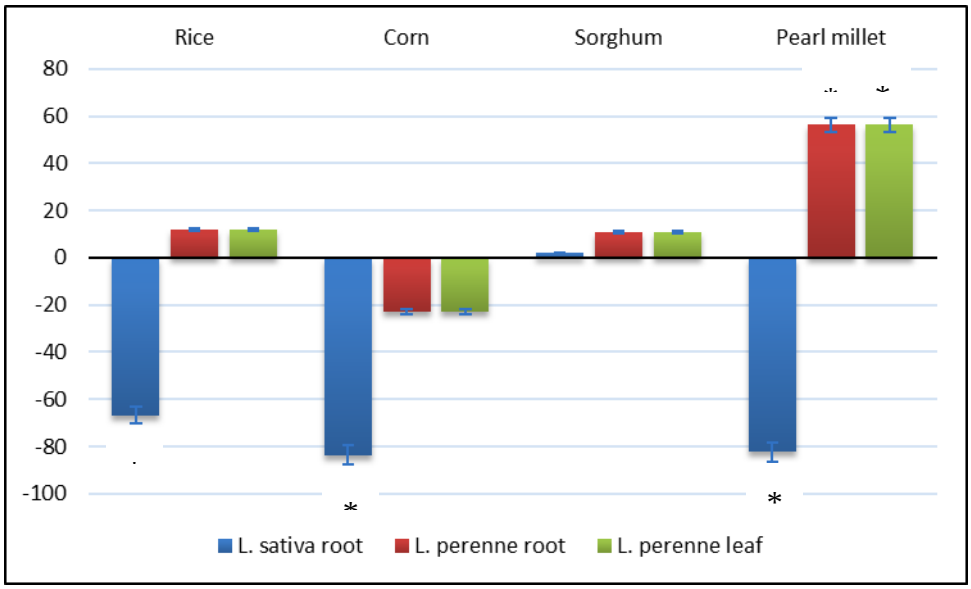

Figure 2: Phytotoxic effects (% inhibition) of the different EFI671 solid media extracts on *Lactuca sativa* and *Lolium perenne*) root and leaf growth (n = 25 plants measured).

4. Discussion

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Species of the genus *Trichoderma* have been widely used as biocontrol agents because of their mycoparasitic capacity and ability to improve plant protection against phytopathogens [28], including *Trichoderma* endophytes [29]. *Trichoderma* sp. is also an insect biocontrol agent [30-32], including endophytic *Trichoderma* acting directly on the insect [33] or indirectly by re-infection of the host plant [34].

The production of effector molecules and secondary metabolites by *Trichoderma* contributes to their beneficial biological activities [35], being this genus a valuable source of a wide variety of secondary metabolites [36]. In this work, five compounds have been isolated from the neutral hexane extract of the *Trichoderma* endophyte EFI671: the triglyceride **1**, previously isolated from the mycelium of *Grifola frondosa* [23] and reported as a synthetic product [24], and four known sterols, eburicol (**2**) [26], β-sitostenone (**3**) [25], ergosterol (**4**) [26] and ergosterol peroxide (**5**) [27].

The most important chemical classes reported in *Trichoderma* are anthraquinones, peptaibols, polyketides, pyrones, terpenes and diketopiperazine-like secondary metabolites [36-38]. Sterol compounds as ergosterol, lanosterol and pyrocalciferol were detected for the first time in the fermentation of a *T. pseudokoningii* strain [37]. Extracts of *Trichoderma* sp. isolated from saline lands contained ergosterol and ergosterol peroxide along with other sterol compounds and two new sorbicillin acid analogues [38]. Ergosterol, stigmasterol and β-sitosterol have been isolated from two strains of *T. harzianum* [40]. Recently, ergosterol, cerevisterol and a tryglyceride derivative have been identified from *Trichoderma* sp. Jing-8 strain isolated from the stem of *Panax notoginseng* [41]. However, this is the first report on the isolation of compounds **2** and **3** from *Thrichoderma* sp.

The bioactive compounds from the *Trichoderma* isolate EFI 679 were the fatty acids (1A) and their methylated derivatives (1M), present in compound **1** and in the extracts, with strong dose-dependent aphid antifeedant effects. Long chain primary alcohols from *Trichoderma citrinoviride*, such as 1-hexadecanol, affected the feeding preference of the aphid *R. padi* [42], but this is the first report on the presence of insect antifeedant fatty acids in a *Trichoderma* endophyte.

Fatty acids are major components of plant lipids, can affect growth and development of insect herbivores and influence host suitability to invasive insects [43]. Additionally, the insecticidal and antifeedant action of fatty acids and esters has been described. Linoleic and oleic acid were insecticidal against fourth instar *Aedes aegyptii* larvae and antifeedant to neonate larvae of *Helicoverpa zea*, *Lymantria dispar*, *Orgyia leucostigma*, and *Malacosoma disstria*, while triglycerides such as 1, 3-dilinoleoyl-2-olein, 1, 3-dioleoyl-2-linolein, and 1, 2, 3-trilinolein were not active [44]. Stearic and palmitic acids have been isolated from *Brassicaceae* bio-oil as antifeedants to Colorado potato beetle [45]. Linolenic and linoleic acids have been reported as antifeedants against *M. persicae* [46]. Furthermore, a mixture of methylated fatty acids was antifeedant to Colorado potato beetle while the individual components (methyl hexadecanoate, ethyl hexadecanoate, methyl octadecanoate, and methyl icosanoate) were inactive, suggesting a synergistic effect. Hexadecanoate methyl (methyl palmitate) and ethyl (ethyl palmitate) esters were antifeedant against the aphids *M. persicae* and *Diuraphis noxia* [47]. Pentacosyl heptacosanoate has been isolated from the natural wax of the plant *Dolichandra cynanchoides* as an insecticidal agent against *S. frugiperda* and *Epilachna paenulata* [48]. Therefore, fatty acids, which are non-toxic substances, are a promising class of molecules for the control of phytophagous insects. The insect antifeedant mode of action of fatty acids is unknown. However, linoleic acid preferentially bound *Bemisia tabaci* chemosensory protein CSP1 in competitive binding assays [49]. CSP proteins (Mp10) are produced by the saliva of the green peach aphid *M. persicae* [50], and therefore could play a role in fatty acid / *M. persicae* interaction. However, further research is needed to support this hypothesis.

Different liquid and solid growth media were tested to optimize the bioactivity against aphids of the fungal extracts. Medium optimization is a useful method to enhance yield of pharmaceutical metabolites in endophytes. Precursor / adsorbent feeding is a strategy to enhance the desired

metabolite yield [51]. For example, tryptamine increased campothecin yield and cellulose (paper disk) increased mycoepoxydiene [52]. The different solid media resulted in quantitative and qualitative differences in chemical composition and had strong settling inhibition effects against *M. persicae* that correlated with linoleic and oleic acids. Therefore, differences in amino acid composition, carbohydrate and cellulose content between the different solid media used could be responsible for the differences in fatty acid yields.

There are no reports on phytotoxic effects of fatty acids. The media-dependent selective phytotoxic effects of the *Trichoderma* extracts could be related to the presence of sterols. For example, ergosterol peroxide (compound **5** produced by EFI 671) has strong allelopathic effects reported against the grass *Echinochloa crus-galli* [53].

5. Conclusions

An endophytic fungi *Trichoderma* sp. EFI 671 was isolated from the stem parts of the medicinal plant *Laurus* sp. The chemical study of its bioactive EtOAc extract resulted in the isolation of 1-oleoyl-2-linoleoyl-3-palmitoylglycerol (**1**), eburicol (**2**), (24R)-stigmast-4-ene-3-one or β-sitostenone (**3**), ergosterol (**4**) and ergosterol peroxide (**5**). The free fatty acids from **1** (oleic, linoleic and palmitic) showed strong dose-dependent aphid antifeedant effects against *M. persicae*. Different liquid (PDB, and SDB) and solid (corn, sorghum, pearl millet and rice) growth media were tested in order to optimize the yield and bioactivity of the fungal extracts. Pearl millet and corn gave the highest extract yields. All the EtOAc extracts from these solid media had strong effects against *M. persicae* with sorghum being the most active. Corn increased the content in linolenic, pearl millet the oleic and stearic and sorghum oleic and linolenic acids compared to rice. Their antifeedant effects correlated with linoleic and oleic acids. The phytotoxic effects of these extracts varied with culture media, with sorghum being the least toxic. Thus, fatty acids can be used as antifeedant for aphid control. However, formulated *Trichoderma* extracts should be tested in vivo followed by up-scaled production and optimization processes.

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