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Secondary metabolites from the endophytic fungus *Talaromyces pinophilus*

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ABSTRACT

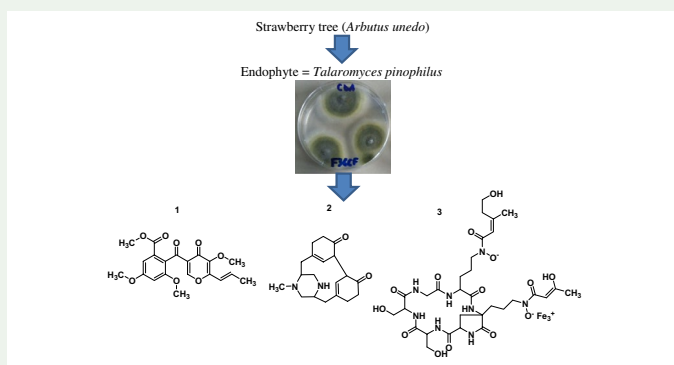
Endophytic fungi have a great influence on plant health and growth, and are an important source of bioactive natural compounds. Organic extracts obtained from the culture filtrate of an endophytic strain of *Talaromyces pinophilus* isolated from strawberry tree (*Arbutus unedo*) were studied. Metabolomic analysis revealed the presence of three bioactive metabolites, the siderophore ferrirubin, the platelet-aggregation inhibitor herquiline B and the antibiotic 3-O-methylfunicone. The latter was the major metabolite produced by this strain and displayed toxic effects against the pea aphid *Acyrtosiphon pisum* (Homoptera Aphidiidae). This toxicity represents an additional indication that the widespread endophytic occurrence of *T. pinophilus* may be related to a possible role in defensive mutualism. Moreover, the toxic activity on aphids could promote further study on 3-O-methylfunicone, or its derivatives, as an alternative to synthetic chemicals in agriculture.

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1. Introduction

Plants live in association with micro-organisms with different levels of interaction. This assumption stimulates insights on plant microbiome, intended as the collective genome of micro-organisms living in contact with plants (Schlaeppli & Bulgarelli 2015), and new concepts in plant evolution have been developed considering a basic role of the associated fungal endophytes (Hardoim et al. 2015). Regarded as an under-explored niche of chemodiversity (Kusari et al. 2014), endophytic fungi have a recognised ability to produce bioactive compounds which may play a role in plant protection against pathogens and pests (Nicoletti & Fiorentino 2015; Gouda et al. 2016).

Colonisation by endophytes may offer significant benefits to their host plants by producing various metabolites that protect against pathogen attack, promote plant (or vegetative) growth, improve crop yields, show herbicide activity and induce resistance. Fungal natural products are currently used in agriculture as active ingredients of different bioformulates (Copping & Duke 2007), and several endophytes are known to have anti-insect properties (Barelli et al. 2016). Although bioinsecticides currently occupy only a small amount of the market, these compounds are very interesting and their use is constantly increasing (Guo et al. 2008).

Endophytic strains of fungi belonging to the genus *Talaromyces* have attracted considerable attention due to their ability to produce a variety of interesting bioactive compounds. Examples of secondary metabolites produced by *Talaromyces* species include tetraene lactones, diphenyl ether derivatives, epiaustdiol, furanosteroid and anthraquinones (Xie et al. 2016; Zhai et al. 2016; Zhi et al. 2016). The endophytic behaviour seems to represent an ecological connotation of several *Talaromyces* species (Li et al. 2010; Bara et al. 2013). Recent studies isolated and characterised novel bioactive compounds produced by *Talaromyces* spp. (Li et al. 2010; Bara et al. 2013) that may have positive implications in crop protection (Kim et al. 2007).

Our work focused on the isolation of bioactive secondary metabolites produced by endophytic fungus *Talaromyces pinophilus* strain F36CF in liquid cultures. The presence of other secondary metabolites released in the culture filtrate has been also investigated by metabolomic analysis. In addition, we report the insecticidal activity on aphids both the organic extracts obtained by strain F36CF and its main secondary metabolite 3-O-methylfunicone.

2. Results and discussion

Strain F36CF of *T. pinophilus* was isolated from a strawberry tree (*Arbutus unedo*) by following a standard protocol (Nicoletti et al. 2013). Fungal strain identification was performed according to morphological and molecular analyses, as previously reported in literature (Yilmaz et al. 2014). The alignment of the ITS sequences of F36CF (KX640963) and the type strain of *T. pinophilus* CBS 631.66 (JN899382) gave an identity of 98.5% (Figure S1A). In addition, a phylogenetic tree based on β -tubulin (*benA*) gene sequence was constructed to confirm the relationships among *Talaromyces* species and *T. pinophilus* strain F36CF (Figure S1B). F36CF clearly clustered with *T. pinophilus* strains, while the closest relative species (*T. liani* and *T. sayulitensis*) formed two distinct groups (*T. dendriticus* was chosen as an out-group).

The ethyl acetate crude extract of F36CF culture filtrate was fractionated by column chromatography and silica-gel TLC to obtain as main compound a metabolite that showed the

NMR signals reported by De Stefano et al. (1999) for (E)-3-methoxy-2-propenyl-5-(2'-carbomethoxy-4'-6'-dimethoxybenzoyl)-4-pyrone named 3-O-methylfunicone (OMF). The identification of OMF was also confirmed by LC-MS/MS qTOF analysis. The metabolite was detected at 10.6 min (Figure S21A) and mass spectra analysis confirmed the presence of the molecular ion $[M - H]^+$ at $m/z = 389.1232$ (Figure S2B), corresponding to the molecular formula $C_{20}H_{20}O_8$ (Calcd 388.1236); other signals were also detected at 799.2203 $[M_2 + Na]^+$; 427.0782 $[M + K]^+$; 411.1049 $[M + Na]^+$; 357.0969 $[M - CO]^+$ (Figure S2B). OMF was not present in the mycelia organic extract that mainly revealed the presence of fatty acids.

The production of bioactive metabolites by F36CF was also investigated by LC-MS approach. The metabolomic analysis of the F36CF culture filtrates showed the presence of three known molecules, herquiline B, ferrirubin and OMF (Figure 1). OMF was found to be the most abundant secondary metabolite biosynthesised by F36CF, yielding 5.5 mg of pure compound per litre of culture filtrate.

The other two compounds identified in the present study, herquiline B and ferrirubin, were detected in *Talaromyces* for the first time and integrate the known chemotaxonomic profile of *T. pinophilus*. These metabolites were confidently identified by comparing the LC-MS qTOF data with known compounds present in an in-house database including over 4000 fungal secondary metabolites. In particular, both herquiline B and ferrirubin yielded a score matching $\geq 95\%$ (Figure S3).

Interestingly herquiline B was previously isolated only from a soil strain of *Penicillium herquei*, and has been characterised as a platelet anti-aggregation factor but showed no antibiotic activity (Enomoto et al. 1996). Conversely, ferrirubin is a ferrichrome-type siderophore reported from many filamentous fungi (Heymann et al. 2000). Besides its primary function related to iron transportation, this class of compounds has also shown a plant growth promoting effect and antibiotic activity, thus suggesting involvement in the plant-fungus endophytic relationship (Bartholdy et al. 2001; Ahmed & Holmström 2014).

The F36C crude extract and OMF were tested on broad bean (*Vicia faba*) leaves against the pea aphid *Acyrtosiphon pisum* (Homoptera Aphidiidae), and demonstrated a mortality (according to Abbott's formula) of 35.7% and 26.2% at 72 h after treatment (Table 1). We observed that the contact activity increased over the time, particularly in the case of the total extract as compared to OMF. This result is typical of insecticides based on natural extracts, whose bioactivity may be related to a single compound or derives from the synergism between different molecules (De Feo et al. 2009).

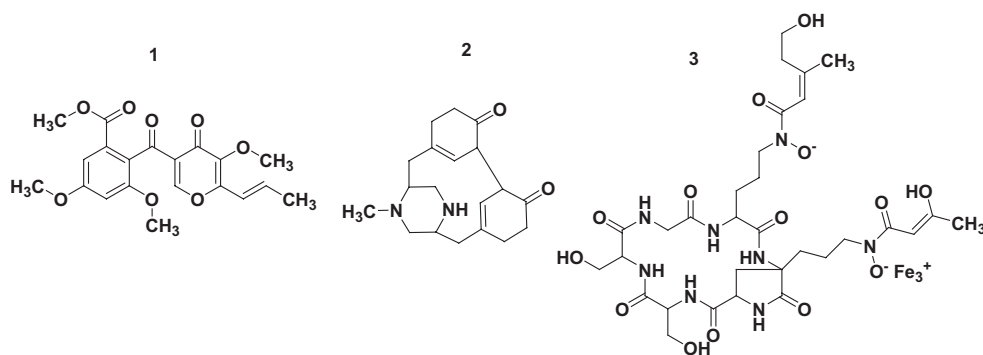


Figure 1. Chemical structures of 3-O-methylfunicone (1), herquiline B (2) and ferrirubin (3).

Table 1. Evaluation of insecticidal activity by *T. pinophilus* extract and OMF as determined by using Abbott's formula to compensate for mortality caused by the control solvent (Rosenheim & Hoy 1989).

Hours after treatment	Abbott value (%) of OMF	Abbott value (%) of <i>T. pinophilus</i> extract
24	14.8	13.0
48	11.3	9.4
72	26.2	35.7

Table 2. Endophytic occurrence of *T. pinophilus* on diverse host plants.

Host plant	Location	Reference
<i>Drosera rotundifolia</i>	Snowdonia National Park (Great Britain)	Quilliam & Jones 2010
<i>Osbeckia chinensis</i>	Meghalaya (India)	Bhagobaty & Joshi 2011
<i>Ceriops tagal</i>	DongZhai Gang, Hainan (China)	Xing & Guo 2011
<i>Laguncularia racemosa</i>	Itamaracá Island, Pernambuco (Brazil)	Costa et al. 2012
<i>Gloriosa superba</i>	Patalkot valley, Madhya Pradesh (India)	Budhiraja et al. 2012
<i>Carex scabrifolia</i>	Suncheon Bay (South Korea)	You et al. 2012
<i>Suaeda japonica</i>		
<i>Artemisia fukudo</i>	Ulleung Island (South Korea)	Kim et al. 2012
<i>Setaria viridis</i>		
<i>Asparagus schoberioides</i>	Dokdo Islands (South Korea)	You et al. 2013
<i>Setaria viridis</i>		
<i>Limonium tetragonum</i>	Muan (South Korea)	Khalmuratova et al. 2015
<i>Suaeda glauca</i>		
<i>Allium fistulosum</i>	Lanzhou (China)	Zhai et al. 2015
<i>Allium schoenoprasum</i>	Nattha Top, Kashmir (India)	Koul et al. 2016
<i>Hypericum mysorensense</i>	Talacauvery, Karnataka (India)	Samaga & Rai 2016
<i>Curcuma amada</i>	Karnataka (India)	Krishnapura & Belur 2016

OMF is a 4-pyrone derivative belonging to a class of fungal compounds mainly isolated from species of *Talaromyces* (Nicoletti et al. 2009), which exhibited notable fungitoxic and antitumour properties (De Stefano et al. 1999; Nicoletti et al. 2014). The observed insecticidal effect expands the range of bioactivities associated with this molecule, stimulating further insights on its biomolecular mechanisms of action. However, our results support the hypothesis that the mutualistic relationship established by some endophytic fungi with their host plant involves the production of microbial compounds able to provide protection against pests and pathogens. In this regard, recent reports from different ecological contexts worldwide have disclosed a widespread endophytic adaptation of *T. pinophilus* (= *Penicillium pinophilum*) (Table 2). This fungus has been involved in different types of interactions with plants and their associated pests and pathogens, and opens novel opportunities for research on biological control agents and related bioactive metabolites (Schulz et al. 2015; Barelli et al. 2016; Keyser et al. 2016).

3. Experimental

3.1. General considerations

All LC–MS analyses were done on an Agilent HP 1260 Infinity Series liquid chromatograph with a DAD system (Agilent Technologies, Torrance, CA, USA) coupled to a Q-TOF mass spectrometer model G6540B (Agilent Technologies) with a Dual ESI source. Separations were performed on a Zorbax Eclipse Plus C18 column, 4.6 × 100 mm, with 3.5 mm particles (Agilent Technologies), operating at a constant temperature of 37°C. The analyses were done at a flow-rate of 0.6 mL/min with a water (H₂O) – acetonitrile (ACN) gradient, starting at ACN

– H₂O (5:95) going to 100% ACN in 12 min, maintaining 100% ACN for 3 min, before returning to the start conditions in 2 min and equilibrating for 3 min. Both solvents were acidified with 0.1% formic acid. The UV spectra were collected by DAD every 0.4 s from 190 to 750 nm with a resolution of 2 nm. The MS system operated in the positive ESI mode and TOF-MS scanning was collected as centroid from *m/z* 100 to 1600 for all samples at a scan rate of 3 spectra/s. To perform the real-time lock mass correction, two reference mass compounds, a lock mass solution including purine (C₅H₄N₄ at *m/z* 121.050873, 10 μmol L⁻¹) and hexakis (1H,1H,3H-tetrafluoropentoxy)-phosphazene (C₁₈H₁₈O₆N₃P₃F₂₄ at *m/z* 922.009798, 2 μmol L⁻¹) were used. The capillary was held at 4000 V, fragmentor at 180 V, cone 1 (skimmer 1) at 45 V. Gas temperature was 350°C during the run at 11 L/min, and the nebuliser was set at 45 psig. The injected sample volume was 5 μL. Solvents were LC-MS grade, and all other chemicals were analytical grade. All were from Sigma-Aldrich (Germany) unless otherwise stated. ESI-TOF tune mix was purchased from Agilent Technologies (Torrance, CA, USA). The chromatograms were analysed by Mass Hunter software (Agilent Technologies) matching their data with known compounds registered in an in-house database including over 4000 fungal secondary metabolites.

¹H and ¹³C NMR spectra were recorded with a 400 MHz Bruker Avance spectrometer, equipped with a 5 mm Bruker Broad Band Inverse probe (BBI), working at the ¹H and ¹³C frequencies of 400.13 and 100.61 MHz, respectively. Peak of chloroform was used as reference to calibrate both ¹H and ¹³C axes.

Column chromatography was performed on silica gel (Merck silica gel 60 GF254), and TLC with glass pre-coated silica gel GF254 plates (Merck Kieselgel 60 GF254, 0.25 mm). The compounds were detected on TLC plates using UV light (254 or 366 nm), and/or by spraying the plates with 5% (v/v) H₂SO₄ solution in EtOH followed by heating at 110°C for 10 min.

3.2. Fungal strain isolation and identification

Strain F36CF was recovered from a secondary branch of a strawberry tree collected in the isle of Favignana, off the coast of western Sicily (37.921545 N, 12.32123 E). The sampled cutting was processed according to a standard protocol for isolation of endophytic fungi (Nicoletti et al. 2013). The fungus was identified according to morphological characters and molecular analyses rDNA-ITS and β-tubulin gene sequence (Yilmaz et al. 2014).

F36CF was inoculated on Potato Dextrose Agar (PDA, Hi-Media, India) plates and cultivated for 10 days at 25°C. Fungal mycelium was collected and genomic DNA was isolated using NucleoSpin® Soil kit (Macherey-Nagel, Düren, Germany), following manufacturer's instructions. DNA quantity was determined using a Qubit 2.0 fluorometer with the dsDNA BR assay (Life Technologies, Grand Island, NY). PCR analysis was carried out in 50 μl total reaction volume, with 0.5 μM primer, 0.2 mM dNTP Mix, 1 × DreamTaq Green Buffer (Thermo scientific), and 1.25 U of DreamTaq DNA Polymerase (Thermo scientific). The internal transcribed spacer region (ITS) was amplified using the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). β-tubulin (*benA*) gene was amplified using the primer pair Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2b (5'-ACCCTCAGTGTAGTGACCC TTGGC-3'). Sequencing reactions were performed by Eurofins Genomics with the same primer sets used for PCR amplification. Sequences alignments were carried out through Clustal Omega multiple sequence alignment program tool of the EMBL-EBI web site (<https://www.ebi.ac.uk/Tools/msa/clustalo>). Molecular phylogenetic analysis by Maximum Likelihood method for *benA* gene was conducted using MEGA7 software (www.megasoftware.net).

Sequences have been deposited in GenBank under the reference numbers KX640963 (ITS) and KX985415 (benA).

3.3. Extraction and isolation

F36CF mycelial plugs from actively growing cultures were used to inoculate 150 mL potato dextrose broth (PDB, HI-MEDIA) in 500 mL Erlenmayer flask (10 conical flasks). The stationary culture was kept incubated in the dark at $26 \pm 2^\circ\text{C}$ for 21 days. The fungal cultures were vacuum filtered through filter paper (Whatman No. 4) to remove the biomass, and the culture filtrate was collected and then concentrated 10-times by lyophilization. The culture filtrate was extracted exhaustively with ethyl acetate and the combined organic extracts were dried (Na_2SO_4) and evaporated under reduced pressure at 37°C .

The crude extract (120 mg) was fractionated by column chromatography isocratically eluted with CH_2Cl_2 -MeOH (98:2, v:v). Seven fractions were collected and pooled on the basis of similar TLC profiles. Fraction 2 was further purified by silica-gel preparative TLC (CH_2Cl_2 -MeOH 98:2 v:v) to yield 8 mg of OMF (**1** in Figure 1).

3.4. Bioassays against aphids

Insecticidal activity was tested against the pea aphid *A. pisum*. Twenty 3rd-instar nymphs, obtained from a synchronised parthenogenetic population reared on broad bean (*Vicia faba*) plants were placed on two circular (35 mm diameter) leaf disks laid upside down on 2% (w/v) water agar in a 90 mm diameter petri dish. One mL of the test solution was sprayed onto the aphids, and the treated insects were incubated at 20 – 22°C and 16:8 h (light/dark) photoperiod. The number of dead aphids was assessed 24, 48 and 72 h after treatment. The classical Abbott's formula (Rosenheim & Hoy 1989) was used to correct bioassay data for control response (solvent-treated aphids) and compute the insecticidal effectiveness of a compound. In bioassays OMF was tested at $50 \mu\text{g mL}^{-1}$, prepared by dissolving the purified compound in $4 \mu\text{L}$ of dichloromethane and adjusting the concentration with distilled water.

4. Conclusion

According to its metabolomic profile and the emerging widespread endophytic behaviour, the strain F36CF of *T. pinophilus*, identified in this work, represents an interesting example of a micro-organism with a multifunctional lifestyle. The production of secondary metabolites is important for the chemotaxonomic characterisation of F36CF isolate. In addition, a novel insecticidal activity of both F36CF organic extracts and its main metabolite OMF on aphids has been demonstrated. Our results may help the development of new biopesticide formulations based on bioactive metabolites and further studies are necessary for future applications in agriculture.

Disclosure statement

No potential conflict of interest was reported by the authors.

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