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Occurrence, isolation and biological activity of phytotoxic metabolites produced *in vitro* by *Sphaeropsis sapinea*, pathogenic fungus of *Pinus radiata*

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Abstract

Sphaeropsis sapinea was repeatedly isolated in Sardinia from symptomatic samples of the upper part of declining pine (*Pinus radiata*) plants. Observed symptoms mainly consisted of foliage chlorosis, drying of needles and cankers on branches. The *S. sapinea* strains were shown to produce phytotoxic metabolites in culture filtrates. Three metabolites were isolated for the first time from this fungus and identified by their spectroscopic and optical properties as *R*-(−)-mellein, (3*R*,4*R*)-4-hydroxymellein and (3*R*,4*S*)-4-hydroxymellein. When assayed for phytotoxic and antifungal activities on host and non-host plants and on some phytopathogenic fungi, the *R*-(−)-mellein showed significant activity, while the other two 3,4-dihydroisocoumarins showed only a synergic activity in both tests.

Introduction

In 1998 and 1999, *Sphaeropsis sapinea* was repeatedly isolated in Sardinia from symptomatic samples taken in the upper part of declining *Pinus radiata* plants. Observed symptoms mainly consisted of foliage chlorosis, drying of needles and cankers on branches. Following these observations a monitoring programme for *Pinus* spp. plantations in Sardinia, was started. Fungi in the genus *Sphaeropsis* Sacc. are well-known for the *in vitro* production of different biologically active metabolites (Sparapano et al., 2004). Several studies were carried out to investigate the phytopathogenic properties of *S. sapinea* f. sp. *cupressi* in many *Cupressus* species. The phytotoxic metabolites characterised belonged to various chemical families (Sparapano et al., 2004). Previous papers confirmed that there are great differences in the morphological characteristics (Swart et al., 1993), pathogenic behaviour (Swart et al., 1993; Linde and Kemp, 1997; Xenopoulos and Tsopelas, 2000), isoenzyme profile (Swart et al., 1993) and RAPD profile markers (Stanosz et al., 1998) between *S. sapinea* and *S. sapinea* f. sp. *cupressi*. Few reports on phytotoxin production by *S. sapinea* are present in the literature. Evidente et al. (1999) described the isolation and chemical characterisation of two 5-substituted dihydrofuranones, sapinofuranones A and B, from liquid cultures of *S. sapinea* isolated from *Cupressus macrocarpa*. This pathogen has been associated with severe diseases on a wide range of forest hosts throughout

the world. We thought it would be interesting to study the metabolites produced by an isolate of *S. sapinea* from *Pinus radiata* previously characterised using morphological, physiological and molecular techniques (unpublished data). For the first time phytotoxins were isolated from liquid cultures of a fungal isolate from *P. radiata*. This paper describes the isolation and identification of the three main toxic metabolites of this fungus. Their antifungal and phytotoxic activities are also reported to improve the understanding of the host–pathogen interaction.

Materials and methods

Chemical analyses and characterization

Optical rotation was measured in chloroform ($CHCl_3$) solution (unless otherwise noted) on a Jasco (Tokyo, Japan) P-1010 digital polarimeter; infrared (IR) spectra were recorded as neat on a Perkin–Elmer (Norwalk, CT, USA) Spectrum One FT-IR spectrometer and ultraviolet (UV) spectra was taken in acetonitrile solution on a Lambda 25 UV–Vis spectrophotometer. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 600, 400 or 300 MHz and at 150, 100 or 75 MHz, respectively, in $CDCl_3$, on Bruker (Karlsruhe, Germany) spectrometers. The same solvent was used as an internal standard. Carbon multiplicities were determined by distortionless enhancement by polarisation transfer (DEPT) spectrum (Berger and Braun, 2004). DEPT, correlated spectroscopy (COSY), hetero single quantum correlated (HSQC) and hetero multiple bond correlated (HMBC) experiments (Berger and Braun, 2004) were performed using Bruker microprograms. Electron ionisation mass (EI-MS) spectra were taken at 70 eV on a Fisons (Beverly, MA, USA) Trio-2000 spectrometer; electrospray ionisation mass (ESI-MS) spectra were recorded on a Perkin–Elmer (Norwalk, CT, USA) API 100 LC-MS with a probe voltage of 5300 V and a declustering potential of 50 V. Analytical and preparative thin layer chromatography (TLC) were performed on Kieselgel 60 F_{254}, 0.25 and 0.50 mm, respectively, silica gel plates (Merck, Darmstadt, Germany) or KC18 F_{254}, 0.20 mm, reverse phase plates (Whatman, Clifton, NY, USA); the spots were visualised by exposure to UV light (254 or 360 nm) and/or dipping the plates in a 10% (w/v) aqueous solution of $KMnO_4$ or by spraying first with 10% H_2SO_4 in methanol and then with 5% phosphomolybdic acid in ethanol, followed by heating at 110 °C for 10 min. For column chromatography Kieselgel 60, 0.063–0.20 mm silica gel (Merck) was used.

Fungal cultures

Pure cultures of *S. sapinea* were isolated from wood of naturally infected adult plants of *P. radiata* from Sardinia (Italy). Single spore isolates were grown on potato–dextrose–agar at 25 °C for 7 days and deposited in the fungal collection of the Dipartimento di Protezione delle Piante, Università degli Studi di Sassari, Italy. Only one of these isolates (isolate PVS 300) was used in this work.

Extraction and purification of fungal metabolites

The cultures of *S. sapinea* (10 l) were obtained as described by Evidente et al. (1996). Combined cultures were filtered, acidified to pH 4.00 with 2 N HCl, then extracted with ethyl acetate (4×2.5 l). The organic phases were combined, dried over Na_2SO_4 and evaporated under reduced pressure at 40 °C. The organic extract was applied to a silica gel column (160 g, 80 cm high, 5 cm id), eluted with a gradient of chloroform-*iso*-propanol (100:1 → 1:2 v/v). Fractions (15 ml each) were monitored by TLC analysis and the resulting homogeneous fractions combined. Fractions were tested for bioactivity against tomato cuttings as described below. Active fractions T_2 and T_4 were purified by preparative silica gel TLC, with chloroform-*iso*-propanol (100:1 and 25:1 v/v, respectively) as the solvent system. The bands at R_f 0.70 and 0.39, respectively, visualised by UV light at 254 nm, were removed from the plates, extracted with ethyl acetate and evaporated. The main component of fraction T_4 was further purified by preparative reverse phase TLC with water-ethanol (1:1 v/v) as the solvent system. The bands visualised by UV light (254 nm) at R_f 0.53 and 0.59 were separately removed and extracted with acetonitrile.

Phytotoxic activity

Phytotoxicity was evaluated using stems of young *P. radiata* seedlings. The apical parts of the stems, approximately 12 cm long and 3 mm diameter at the point of cutting, were used for the experiments. Cuttings (three replicates per treatment) were taken from 2 year-old pine seedlings grown in the greenhouse at 25–27 °C and 60–70% relative humidity (RH).

The same assay was carried out on a herbaceous non-host plant (tomato: *Lycopersicon esculentum* var. Marmande). Seedlings of tomato were grown in growth chambers at 25 °C and 70–80% RH, exposed to a luminous flux of 400 mmol m^{-2} s^{-1} with a 12 h photoperiod. Cuttings (three replicates per treatment) were taken from 21 day-old seedlings. Toxicity was evaluated by placing the cutting (tomato for 48 h and pine for 96 h) in the assay solution (substance dissolved in 0.03 ml of methanol and diluted with 3 ml of distilled water). Tomato and pine cuttings were then transferred to distilled water for 7 and 21 days, respectively. Symptoms on cuttings were observed daily. Culture filtrates, their organic extract and pure metabolites were assayed on *P. radiata* and on tomato cuttings, while chromatographic fractions were assayed only on tomato cuttings. Culture filtrates were tested after 1:10000 v/v dilution with distilled water. Organic extracts were assayed at concentrations of 0.1, 0.25 and 0.5 mg ml^{-1}. Mellein was tested, at 0.002, 0.005, 0.01, 0.05, and 0.1 mg ml^{-1} and at 0.01, 0.05 and 0.1 mg ml^{-1}, on tomato and pine cuttings respectively (different concentrations were used for pine and tomato because of the greater sensitivity of tomato to the treatment). Fraction T_4 was assayed, at 0.01, 0.02 and 0.05 mg ml^{-1} and at 0.02, 0.05, 0.1 and 0.2 mg ml^{-1}, on tomato and pine cuttings, respectively; (3*R*,4*R*)-(−)-4-hydroxymellein and (3*R*,4*S*)-(−)-4-hydroxymellein were tested, at 0.1 mg ml^{-1}, only on tomato cuttings.

Phytotoxicity was also evaluated by means of subcortical injections. Both pure (3*R*,4*R*)-(−) and (3*R*,4*S*)-(−)-4-hydroxymellein, and fraction T_4 (containing both compounds in 6.5:1 ratio) were tested at 0.1 mg ml^{-1}. The assay solutions (0.1 ml), prepared as for the test on cuttings, were injected into the cortical tissue of 2 year-old pine plants, at a distance of 10 cm from the apex.

All assays (both on cuttings and on seedlings) included controls with distilled water and solvent.

Antifungal activity

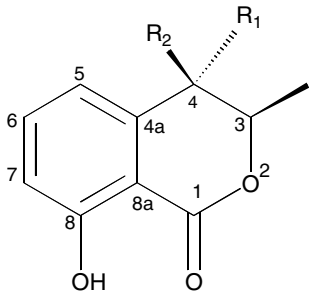
Fraction T_4 (mixture of the two 4-hydroxymelleins) and (3*R*,4*R*)-(−)-4-hydroxymellein were assayed for antifungal activity. The assays were carried out on six phytopathogenic fungi: *Botrytis cinerea, Colletotrichum acutatum, Fusarium oxysporum* f. sp. *radicis lycopersici, Sclerotinia sclerotiorum, Sclerotium rolfsii* and *Verticillium dahliae* as test microorganisms Petri-dishes (9.0 cm) containing PDA (three per fungal species) were inoculated in the centre with a 6 mm agar plug of fungus removed from the margin of a 3–8 day-old colony on PDA. The antifungal activity was tested by direct application of the metabolite in acetone solution (0.1 mg in 0.02 ml) to the inoculum disk surface (Claydon et al., 1987). The cultures were incubated at 25 °C and growth rates recorded for 10 days. Inhibition percentage was evaluated from the equation: $100(y - x)/y$, where y = growth diameter in the untreated control, and x = growth diameter in the treated sample.

Results

Extraction of culture filtrate with ethyl acetate gave a brown oily residue (1.95 g) with high phytotoxic activity. It was fractionated through column chromatography on silica gel, as described above. Homogeneous fractions were combined into 9 groups, T_1–T_9. Fractions T_2, T_4 and T_5 were the most active. Purification of fraction T_2 gave 31 mg (3.1 mg ml^{-1}) of a homogeneous amorphous compound, which was identified as (*R*)-(−)-mellein (**1**, Figure 1) by its spectroscopic and optical properties. Purification of fraction T_4 gave 25 mg of a mixture of two compounds in 6.5:1 ratio. Further purification of fraction T_4 gave 21 and 3 mg (2.1 and 0.3 mg ml^{-1}) of two amorphous homogeneous compounds (T_4A and T_4B) which were identified as (3*R*,4*R*)-(−)- and (3*R*,4*S*)-(−)-4-hydroxymellein (**2** and **3**, Figure 1), respectively, by their spectroscopic and optical properties. Fraction T_5 has not yet been investigated.

*(R)-(−)-Mellein(**1**)*

Compound **1** had: $[\alpha]^{25}D$ −94.0 (*c* 0.4); IR: ν_{max} (cm^{-1}) 3060 (OH), 1666 (C=O), 1616, 1581, 1497, 1461 (Ph); UV: λ_{max} (log ε) 245 (3.87), 312 (3.65).



1 R_1=R_2=H

2 R_1=H, R_2=OH

3 R_1=OH, R_2=H

Figure 1. Chemical structure of *R*-(−)-mellein, (3*R*,4*R*)-(−) and (3*R*,4*S*)-(−)-4hydroxymellein (**1**, **2** and **3**, respectively).

^{1}H-NMR spectrum was very similar to that reported in the literature (Cole and Cox, 1981). ESI-MS (+): *m*/*z*: 217 $[M+K]^+$, 201 $[M+Na]^+$, 179 $[M+H]^+$.

*(3R,4R)-(−)-4-Hydroxymellein (**2**)*

Compound **2** had: $[\alpha]^{25}D$ −36.0 (*c* 0.78, methanol); IR: ν_{max} (cm^{-1}) 3384 (OH), 1669 (C=O), 1617, 1584, 1493 (Ph); UV: λ_{max} (log ε) 244 (3.76), 312 (3.68). ^{1}H-NMR and EI-MS spectra were very similar to those reported in literature (Aldridge et al., 1971; Cole and Cox, 1981). ESI-MS (+): *m*/*z* 236 $[M+K]^+$, 217 $[M+Na]^+$, 195 $[M+H]^+$.

*(3R,4S)-(−)-4-Hydroxymellein (**3**)*

Compound **3** had: $[\alpha]^{25}D$ −24.6 (*c* 0.16); IR: ν_{max} (cm^{-1}) 3400 (OH), 1653 (C=O), 1618, 1583, 1485, 1466 (Ph); UV: λ_{max} (log ε) 245 (3.91), 313 (3.80). ^{1}H-NMR spectrum was very similar to that reported in literature (Devys and Barbier, 1992), while the ^{13}C-NMR spectrum was very similar to that reported for the *cis*-diastereomer (3*R*,4*R*) except for the chemical shift of the Me-11 at δ (ppm) 17.9 (Cole and Cox, 1981). ESI-MS (+): *m*/*z* 236 $[M+K]^+$, 217 $[M+Na]^+$, 195 $[M+H]^+$; ESI-MS (−): *m*/*z* 193 $[M-H]^-$.

Results of phytotoxicity assays are summarised in Tables 1 and 2. On *P. radiata* cuttings, the mellein solution at concentration up to 0.05 mg ml^{-1} induced symptoms on the needles 1 week after treatment. Severity of symptoms increased over the following days: the needles first showed chlorosis, then browning and, finally, necrosis. When a mellein solution from 0.1 to

Table 1. Effects of activity of mellein and fraction T_4 on pine cuttings

	0.2 mg ml^{-1}	0.1 mg ml^{-1}	0.05 mg ml^{-1}	0.02 mg ml^{-1}	0.01 mg ml^{-1}
Mellein	*	Chlorosis after 7 days, necrosis after 15 days	Chlorosis after 7 days, necrosis after 15 days	*	No symptoms
Fraction T_4	Chlorosis, then necrosis after 12 days	Chlorosis, then necrosis after 12 days	No symptoms	No symptoms	*

*: Not tested at this concentration.

Table 2. Effects of activity of mellein, fraction T_4 and hydroxymelleins on tomato cuttings

	0.1 mg ml^{-1}	0.05 mg ml^{-1}	0.02 mg ml^{-1}	0.01 mg ml^{-1}	0.005 mg ml^{-1}	0.002 mg ml^{-1}
Mellein	Wilting after 48 h	Wilting after 72 h	*	Wilting after 4 days	Wilting after 6 days	Wilting after 9 days
Fraction T_4	*	Browning after 48 h, wilting after 72 h	Chlorosis after 6 days, necrosis after 11 days	Chlorosis after 6 days, necrosis after 11 days	*	*
(3*R*,4*R*)-(−)-4-hydroxymellein	No symptoms	*	*	*	*	*
(3*R*,4*S*)-(−)-4-hydroxymellein	No symptoms	*	*	*	*	*

*: Not tested at this concentration.

0.002 mg ml^{-1} was assayed on tomato cuttings, it first induced collapse of the leaves, and then wilting of the cuttings. The positive response in the tomato cuttings test confirms that mellein is a host non-specific phytotoxin.

Fraction T_4, when assayed on *P. radiata* cuttings, caused chlorosis and necrosis on the needles. Symptoms appeared 12 days after treatment with up to 0.1 mg $ml^{-1}$of the fraction. On tomato cuttings, fraction T_4 at 0.05 mg ml^{-1} induced brown discolouration at the leaf margins. Eventually, the affected leaves dried and the entire cutting wilted, the latter occurring from 48 to 72 h after treatment. When injected into cortical tissues of pine seedlings, this fraction produced a brown discolouration on the bark and on the corresponding internal tissues together with a loss of texture (Figure 2).

(3*R*,4*R*)-(−)-4-hydroxymellein produced no symptoms when assayed on tomato cuttings at 0.1 mg ml^{-1}. Likewise, no symptoms were observed when this compound was injected into cortical tissues of pine seedlings. Similar results were obtained when the tests were carried out using (3*R*,4*S*)-(−)-4-hydroxymellein.

In the assay of antifungal activity of fraction T_4 the most sensitive fungal species were *C. acutatum, S. rolfsii* and *B. cinerea*: they showed a moderate inhibition effect on mycelial growth. The least sensitive species were *F. ox.* f. sp. *radicis lycopersici, S. sclerotiorum*, and *V. dahliae* with no growth inhibition. Only pure (3*R*,4*R*)-(−)-4-hydroxymellein was tested on pathogenic fungi: no inhibition of mycelial growth of any of the fungal species assayed was observed. The small amount of pure (3*R*,4*S*)-(−)-4-hydroxymellein obtained from culture filtrates of *S. sapinea* did not allow us to carry out further tests.

Discussion

The (*R*)-(−)-mellein and (4*R*,4*R*)-(−)- and (3*R*,4*S*)-(−)-4-hydroxymellein isolated from *S. sapinea* culture filtrate showed spectral and physical data very similar to those reported for these fungal metabolites in literature (Aldridge et al., 1971; Cole and Cox, 1981; Devis and Barbier, 1992).

Mellein and 4-hydroxymellein are 3,4-dihydroisocoumarins belonging to the family of pentaketides as well as related compounds with different substitution patterns on the phenyl moiety (Garson et al., 1984). Because of their relationship with the ochratoxins group, potent mycotoxins reported for the first time from *Aspergillus ochraceus* but also produced from other fungal genera including *Penicillium*, they are also named ochracins (Cole and Cox, 1981).

Mellein is a widely distributed dihydroisocoumarin derivative in fungi (Turner and Aldridge, 1983). Its production by *Aspergillus melleus* (Garson et al., 1984), *Cercospora taiwanensis* (Camarda et al., 1976), *Septoria nodorum* (Devys et al., 1980), *Hypoxylon* spp. (Anderson and Edwards, 1983), *Botryosphaeria obtuse* (Venkatasubbaiah and Chilton, 1990; Venkatasubbaiah et al., 1991), *Phoma tracheiphila* (Parisi et al., 1993), *Pezicula livida, Plectophomella* spp., *Cryptosporiopsis malicicorticis* and *Cryptosporiopsis* spp. (Krohn et al., 1997), *Microsphaeropsis* spp. (Hoeller et al., 1999) and *Xylaria longiana* (Edwards et al., 1999) has been reported. The biological properties of mellein include:

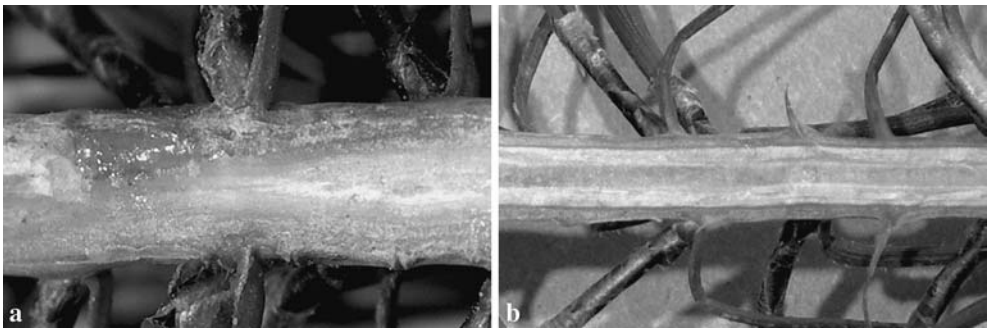


Figure 2. Symptoms of subcortical injection of fraction T_4, [mixture of (3*R*,4*R*)-(−) and (3*R*,4*S*)-(−)-hydroxymellein in 6.5:1 ratio]: (a) longitudinal section of stem tissues showing alterations in texture; (b) control.

phytotoxic activity on tomato cuttings at 0.1 mg ml^{-1} and zootoxic activity to brine shrimp (*Artemia salina*) at 0.2 mg ml^{-1} (Parisi et al., 1993), moderate antifungal activity in agar diffusion assays against *Eurotium repens* at 50 μg per test disk (Hoeller et al., 1999) and weak bioactivity in agar diffusion tests against *E. repens, F. oxysporum* and *Ustilago violacea* at 18 mg ml^{-1} (Krohn et al., 1997).

(3*R*,4*R*)-(−) and (3*R*,4*S*)-(−)-4-hydroxymellein were present in fraction T_4 in a 6.5:1 ratio. (3*R*,4*S*)-(−)-4-hydroxymellein was previously isolated from *A. ochraceus* (Cole et al., 1971), *C. taiwanensis* (Camarda et al., 1976), *Microsphaeropsis* spp. (Hoeller et al., 1999) and *X. longiana* (Edwards et al., 1999). A moderate antifungal activity in agar diffusion assays against *Ustilago violacea* at 50 μg per test disk was reported (Hoeller et al., 1999). Moreover, (3*R*,4*R*)-4-hydroxymellein has been reported in cultures of *Lasiodiplodia theobromae* (Aldrige et al., 1971), *S. nodorum* (Devys et al., 1980), *A. melleus* (Holker and Simpson, 1981), *B. obtuse* (Venkatasubbaiah, et al., 1991; Parisi et al., 1993), *Microsphaeropsis* spp. (Krohn et al., 1997), *X. longiana* (Edwards et al., 1999) and *Apiospora montagnei* (Alfatafta et al., 1994). This hydroxymellein was active in toxicity bioassays on apple and weed leaves (Parisi et al., 1993). It also showed moderate antifungal activity in agar diffusion assays against *E. repens* and *U. violacea* at 50 μg per test disk (Hoeller et al., 1999).

In the present work we have found that, whilst fraction T_4 showed phytotoxic activity, (3*R*,4*R*)-(−) and (3*R*,4*S*)-(−)-4-hydroxymellein caused no symptoms when assayed on tomato cuttings and pine seedlings. Our results showed that the phytotoxic activity of fraction T_4 could be due to a synergistic action between the two metabolites. The assay of antifungal activity suggested that synergism between the two hydroxymelleins may be present also in other mechanisms: in fact the pathogenic fungi tested seemed to be insensitive to any of the substances in fraction T_4. Other studies are required to define the type of mechanism involved in the biological activity and to confirm the synergistic action between the two hydroxymelleins. Synergism between secondary metabolites has been observed previously (Creppy et al., 2004; Carpinella et al., 2005), although little is known about the mechanisms underlying the synergistic or antagonistic interactions. This is the case with the phytotoxic lipodepsipeptides produced by *P. fuscovaginea* in modulation of plant plasma membrane H^+-ATPase (Batoko et al., 1997). Also the two phytotoxins produced by *F. avenaceum* act in a synergistic manner to cause necrotic lesions on detached knapweed (*Centurea maculosa*) leaves (Hershernhorn et al., 1992) as well as the phytotoxins produced by *Bursaphelenchus xylophilus*, when tested in combination for their toxicity on pine seedlings (Hachiro, 1988). In this paper we were able to isolate two metabolites, (3*R*,4*R*) and (3*R*,4*S*)-4-hydroxymellein, from *S. sapinea*, which, in our tests, were not toxic if assayed separately, while they showed biological activity when tested in a mixture resembling that produced by the fungus (6.5:1 ratio).

The occurrence of (*R*)-(−)mellein, (3*R*,4*R*)- and (3*R*,4*S*)-4-hydroxymellein as metabolites of *S. sapinea* isolated from *P. radiata*, should improve our understanding of the host–pathogen interaction.

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