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Lipophilic phytotoxins produced by *Neofusicoccum parvum*, a grapevine canker agent

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Summary. Lipophilic phytotoxins produced by *Neofusicoccum parvum*, a Botryosphaeriaceae species pathogenic to grapevine, were isolated and identified by spectroscopic methods as (3*R*,4*R*)-(-)-4-hydroxymellein, (3*R*,4*S*)-(-)-4-hydroxymellein, isosclerone and tyrosol. When assayed for phytotoxicity on tomato plants, all four metabolites showed phytotoxic activity, with (3*R*,4*R*)-(-)-4-hydroxymellein and isosclerone the most active. Isosclerone is reported for the first time as produced by a Botryosphaeriaceae species.

Key words: esca, Botryosphaeriaceae, melleins, isosclerone, tyrosol.

Introduction

Botryosphaeriaceae are cosmopolitan pathogens, saprophytes and endophytes on a wide range of woody hosts (Arx, 1987; Barr, 1987; Denman *et al*., 2000). In recent years an increasing number of species of Botryosphaeriaceae have been associated with grapevine (*Vitis vinifera* L.) decline worldwide, with several species causing dieback, cankers, and characteristic wedge-shaped necrosis in the arms and trunks as seen in cross-sections of affected plant parts (Van Niekerk *et al*., 2006; Úrbez-Torres *et al*., 2008).

It has recently been reported that species of Botryosphaeriaceae isolated from grapevines produced phytotoxic metabolites (Martos *et al*., 2008; Djoukeng *et al*., 2009), which opens new hypothesis on a possible role of these phytotoxic metabolites in the disease caused by these fungi. A bioassay-guided fractionation of culture filtrates of *Diplodia seriata* De Not. [=*Botryosphaeria obtusa* (Schwein.) Shoem.] led to four dihydroisocoumarins being isolated, named mellein, 4-hydroxymellein, 7-hydroxymellein, and a new 4,7-dihydroxymellein (Djoukeng *et al*., 2009). In another study, five Botryosphaeriaceae species: *Botryosphaeria dothidea* (Moug.:Fr.) Ces. & De Not., *D. seriata*, *Dothiorella viticola* A.J.L. Phillips & Luque, *Neofusicoccum luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, and *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, involved in grapevine decline, were found to produce hydrophilic high-molecular weight phytotoxins whose structures have not yet been determined (Martos *et al*., 2008). Additionally *N. luteum* and *N. parvum* produced lipophilic low-molecular weight phytototoxins, not detected among the remaining species (Martos *et al*., 2008). This prompted the present study of the phytotoxicity of *N. parvum*. In this paper we report on the isolation, the chemical identification and the biological activity of the lipophilic phytotoxins produced by *N. parvum*.

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* This paper is dedicated to the memory of Prof. Carlo Rosini

Materials and methods

Chemical analysis and characterization

Optical rotation was measured in a $CHCl_3$ solution on a JASCO (Tokyo, Japan) P-1010. IR spectra were recorded neat on a Perkin-Elmer (Norwalk, CT, USA) Spectrum One FT-IR Spectrometer and UV spectra were taken in a MeCN solution on a Perkin-Elmer Lambda 25 UV/Vis spectrophotometer. ^{1}HNMR spectra were recorded at 600, 400 MHz, in $CDCl_3$ on a Bruker (Karlsruhe, Germany) spectrometer. ^{13}C NMR spectra were recorded at 150, 100 and 75 MHz, in the same solvent and using the same instruments. The same solvent was also used as an internal standard. Carbon multiplicities were determined by distortionless enhancement by polarization transfer (DEPT) spectra (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. EI spectra were recorded at 70 ev on QP 5050 Shimadzu, and ESIMS spectra on Waters Micromass Q-TOF Micro and Agilent 1100 coupled to a JOEL AccuTOF (JMS-T100LC) (Milford, MS, USA). Analytical and preparative TLC was performed on silica gel (Kieselgel 60 F_{254}, 0.25 and 0.50 mm, respectively, Merck, Darmstadt, Germany) or reverse phase (Whatman, KC18 F_{254}, 0.20 mm, Maidstone, UK) plates; the spots were visualized by exposing to UV light or by spraying previously with 10% H_2SO_4 in methanol and then with 5% phosphomolybdic acid in ethanol, followed by heating at 110°C for 10 min. Column chromatography was performed with Kieselgel 60, 0.063–0.200 mm, silica gel (Merck, Darmstadt, Germany).

Fungus strain and culture filtrate production

Neofusicoccum parvum was obtained from a cankered branch of grapevine (*V. vinifera* cv. Parellada), growing in Catalonia, NE Spain (41°17'18"N, 1°37'59"E, altitude 130 m). A representative culture of this fungus was deposited at the Centraalbureau voor Schimmelcultures (CBS, Utrecht, Netherlands), strain code no. CBS 121486. This strain was grown in stationary conditions in 1 L Roux flasks containing 150 mL of modified Difco™ Czapeck-Dox (Benton, Dickinson and Company, Sparks, MD, USA) medium with 0.5% yeast and 0.5% malt extract (pH 6.8) for 14 days at 25°C. A total of 30 L culture filtrate was obtained, as previously reported (Martos *et al*., 2008), and was lyophilized prior to the phytotoxic activity assay.

Extraction and purification of phytotoxins

The lyophilized residue, corresponding to 30 L of fungal culture filtrate was dissolved in 3 L of ultrapure water, acidified to pH 4.2 with 1 M HCOOH and extracted with EtOAc in three consecutive extractions (3 L for each). The organic extracts were combined, dried (Na_2SO_4) and evaporated under reduced pressure, yielding an oily brown residue (2.32 g). This residue was purified by silica gel column chromatography eluted with $CHCl_3$-*i*-PrOH (9:1, v:v) and then washed with MeOH to give 11 groups of homogeneous fractions. Fractions were tested for their phytotoxicity on tomato plants as described below. The third, fourth and seventh fractions, showing higher phytotoxicity, were further purified. The residues of the third and fourth fraction were purified by preparative TLC on silica gel [eluent EtOAc-*n*-hexane (55:45, v:v)], yielding seven and six fractions respectively. The residues of the third and fourth bands (3.5 and 1.2 mg respectively) were combined and further purified by preparative TLC on reverse phase [eluent EtOH-H_2O (1:1, v:v)], yielding two amorphous solids, **1** (R_f 0.62, 0.8 mg) and **2** (R_f 0.69, 1.3 mg). The residue of the second band (3.5 mg) obtained from the purification of the third fraction of the initial column was further purified by preparative TLC on reverse phase [eluent EtOH-H_2O (6:4, v:v)], yielding a further amorphous solid, **3** (R_f 0.69, 0.5 mg). The residue of the seventh fraction of the initial column (18 mg) was further purified by two successive preparative TLC steps on silica gel [eluent EtOAc-$CHCl_3$-MeOH (2:2:1, v:v:v)], yielding a further amorphous solid, **4** (R_f 0.46, 0.3 mg).

Phytotoxicity assay

The culture filtrate and the organic extract of *N. parvum* were found to be phytotoxic, confirming earlier findings (Martos *et al*., 2008). The organic fractions obtained by column chromatography and the purified compounds **1-4** were assayed for phytotoxicity on tomato cuttings as follows. Samples were taken in solution in 100 μL methanol, the volume adjusted to 6 mL with distilled water and the pH adjusted to 7 either with 1% NaOH (w:v) or 1% HCl (v:v) before the toxicity assays. Czapeck-Dox medium and distilled water receiving the same

treatments were used as controls. Compounds **1** and **2** were tested at various concentrations (0.26×10^{-3} M, 0.13×10^{-3} M and 0.26×10^{-4} M, corresponding to 100, 50 and 10 μg toxin per tomato plant), while compounds **3** and **4** were only tested at one concentration each, 0.31×10^{-3} M and 1.60×10^{-3} M respectively, corresponding to 100 and 220 μg toxin per tomato plant, since the total amounts of these compounds were not enough to test them at a variety of concentrations. The stem of a 2-week-old rootless tomato plant cv. Incas at about the three real leaves stage was immersed in a vial containing 2 mL of each toxin solution for 24 h, and transferred to distilled water for a further 6 h before symptom evaluation. Plants were maintained in a growth chamber at 26°C with a 12 h day during the assay. Lesions on the leaf surface were evaluated 30 h after immersion in the toxic solutions using a 0–3 scale: 0, no symptoms; 1, slight wilting of one leaf; 2, moderate wilting of some leaves; 3, severe wilting of all leaves (with occasional necrotic spotting). Three plants were used for each sample tested. Toxicity on grapevine leaves was not assayed since wilting symptoms on tomato plants are more severe than in grapevine, as reported earlier (Martos *et al*., 2008).

Results

Purification and chemical identification of phytotoxins

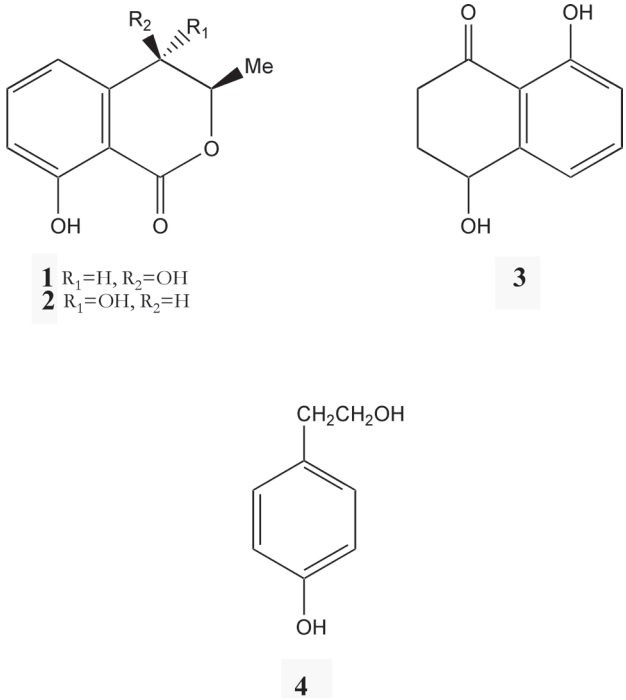
The organic extract that was obtained from the *N. parvum* culture filtrates, and that showed high phytotoxic activity, was fractioned by combined column and preparative TLC chromatography using both silica gel and reverse phase (see Materials and methods for details). Four metabolites showing phytotoxic activity were purified. These pure metabolites were identified by means of spectroscopic and physical data as **1** (3*R*,4*R*)-(-)-4-hydroxy-mellein (0.03 mg L^{-1}); **2** (3*R*,4*S*)-(-)-4-hydroxy-mellein (0.04 mg L^{-1}); **3** isosclerone (0.02 mg L^{-1}); and **4** tyrosol (0.01 mg L^{-1}) (Figure 1). Compounds **1** and **2** had $[\alpha]^{25}_{D}$ and IR, UV, ^{1}H and ^{13}C NMR and ESIMS (+) spectra identical to those reported in the literature (Aldridge *et al*., 1971; Cole and Cox, 1981; Devys *et al*., 1992; Cabras *et al*., 2006). Compounds **3** and **4** had the $[\alpha]^{25}_{D}$ and IR, UV, ^{1}H and ^{13}C NMR and EIMS spectra, and the IR, UV, ^{1}H and ^{13}C NMR and EIMS spectra identical to those earlier reported, (Kimura and Tamura, 1973; Morita and Aoki, 1974; Capasso *et al*., 1992; Evidente *et al*., 2000). The structure of isosclerone was also confirmed by 2D NMR spectra (COSY, HSQC and HMBC).

Phytotoxic assays

When assayed on tomato cuttings all four metabolites (**1-4**) showed phytotoxic activity, with symptoms ranging from slight to drastic leaf wilting (Table 1). (3*R*,4*R*)-(-)-4-hydroxymellein (**1**) and isosclerone (**3**) were the most toxic compounds. For compounds **1** and **2,** as expected, phytotoxicity increased with the concentration.

Discussion

Isolation of the two 4-hydroxymelleins (**1** and **2**), isosclerone (**3**) and tyrosol (**4**) from the culture filtrates of *N. parvum* confirmed preliminary findings that this fungus produced toxic metabolites with a low molecular weight and an acid, lipophilic nature



1 R_1=H, R_2=OH
2 R_1=OH, R_2=H

3

4

Figure 1. Structures of (3*R*,4*R*)-(-) -4-hydroxymellein, (3*R*,4*S*)-(-) -4-hydroxymellein, isosclerone and tyrosol (**1-4**).

Table 1. Phytotoxicity of (3*R*,4*R*)-(-) -4-hydroxymellein, (3*R*,4*S*)-(-) -4-hydroxymellein, isosclerone and tyrosol (**1-4**) on tomato plants[a].

Compound	Concentration (M)	Toxicity rating			Mean toxicity rating ±S.E.
		Rep 1	Rep 2	Rep 3	
1	0.26×10^{-3}	2	2	3	2.3±0.33
1	0.13×10^{-3}	1	1	3	1.7±0.67
1	0.26×10^{-4}	1	1	2	1.3±0.33
2	0.26×10^{-3}	0	1	2	1.0±0.58
2	0.13×10^{-3}	0	1	1	0.7±0.33
2	0.26×10^{-4}	0	1	0	0.3±0.33
3	0.31×10^{-3}	2	3	1	2.0±0.57
4	1.60×10^{-3}	2	2	1	1.7±0.33
Control H_2O		0	1	0	0.3±0.33
Czapek-Dox		0	0	0	0.0±0.00

[a]Lesion symptoms were evaluated using a 0–3 scale: 0, no symptoms; 1, slight wilting in one leaf; 2, moderate wilting on some leaves; 3, severe wilting on leaves (with necrotic spots on leaves occurring occasionally). Three plants (Rep 1 to Rep 3) were used for each sample tested.

(Martos *et al*., 2008). This is the first report on the isolation of 4-hydromellein, isosclerone and tyrosol as phytotoxic metabolites produced by *N. parvum*.

All these toxins have already been reported as being produced by many other phytopathogenic fungi (Aldridge *et al*., 1971; Kimura and Tamura, 1973; Morita and Aoki, 1974; Cole and Cox, 1981; Turner and Aldridge, 1983; Venkatasubbaiah and Chilton, 1990; Devys *et al*., 1992; Evidente *et al*., 2000; Cabras *et al*., 2006). To the best of our knowledge, isosclerone (**3**) is here reported for the first time as produced by a Botryosphaeriaceae species. (3*R*,4*R*)-(-)-4-hydroxymellein (**1**) was known to be produced together with other melleins and tyrosol by a strain of '*B. obtusa*' isolated as the causal agent of frogeye leaf spot and black rot of apple (Venkatasubbaiah and Chilton, 1990). The same 4-hydroxymellein, and its steroisomer (3*R*,4S)-(-)-4-hydroxymellein (**2**) and mellein were also identified as phytotoxins produced by *Diplodia pinea* (Desm.) Kickx, another Botryosphaeriaceae species, which causes decline in *Pinus radiata* D. Don in Sardinia, Italy (Cabras *et al*., 2006). The two hydroxymelleins (**1** and **2**) showed synergic toxic activity when tested on both tomato and pine cuttings (Cabras *et al*., 2006). Mellein and its derivatives belong to the group of dihydroisocoumarins, which are produced by many fungi, including the genera *Aspergillus*, *Cercospora*, *Cryptosporiopsis*, *Hypoxylon*, *Microsphaeropsis*, *Phoma*, *Pezicula*, *Plectophomella*, *Septoria*, and *Xylaria*, with phytotoxic, zootoxic and moderate antifungal activity (Turner and Aldridge, 1983; Cabras *et al*., 2006). Recently, some melleins, namely mellein, 4-hydroxymellein, 7-hydroxymellein and the new 4,7-hydroxymellein, have been reported as phytotoxins produced by *B. obtusa*, isolated as a pathogen of black dead arm (BDA) of grapevine (Djoukeng *et al*., 2009).

Isosclerone (**3**) and scytalone, another naphtalenone pentaketide, are produced by *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & L. Mugnai and by *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams (Evidente *et al*., 2000, Tabacchi *et al*., 2000), two fungi associated to esca disease (Mugnai *et al*., 1999; Surico *et al*., 2006; Surico *et al*., 2008). Esca is a complex disease of grapevine in which a number of fungi have been reported as the causal agents, most frequently

Phaeoacremonium aleophilum, *Phaeomoniella chlamydospora* and *Fomitiporia mediterranea* M. Fischer. *P. aleophilum* and *P. chlamydospora,* are tracheomycotic pathogens commonly associated with all the syndromes in the esca complex (brown wood streaking of the rootstocks, Petri disease, young esca, or 'grapevine leaf stripe disease', and esca proper), while *F. mediterranea* and other species of basidiomycetes cause white rot (Mugnai *et al*., 1999; Surico *et al*., 2006; Surico *et al*., 2008; Surico, 2009). Tiger-striped leaves showing a chlorotic and necrotic pattern resembling those described in esca disease (Mugnai *et al*., 1999) have been attributed by some authors to the late foliar symptoms caused by Botryosphaeriaceae species [(the so called 'black dead arm disease' *sensu* Larignon and Dubos (2001)], although this report is controversial (Lecomte *et al*., 2005, Surico *et al*., 2006, Luque *et al*., 2009). As isosclerone is produced both by the two main esca fungi and by *N. parvum*, it should be clarified whether this toxin is related to the tiger stripe foliar symptoms of esca and the Botryosphaeriaceous fungi infection, which could partially explain the overlapping of foliar symptoms reported (in case the occurrence of foliar symptoms caused by Botryospheriaceous fungi is confirmed). While isosclerone is produced by both the fungi causing esca and by Botryosphaeriaceae species, the melleins have only been reported for this last group (Venkatasubbaiah and Chilton, 1990; Cabras *et al*., 2006; Djoukeng *et al*., 2009). Further investigation is needed to determine whether isosclerone (**3**) is produced by other Botryosphaeriaceae species as well.

Tyrosol (**4**) is a phytotoxic metabolite produced also by plants and by fungi, including *B. obtusa* (Capasso *et al*., 1992; Venkatasubbaiah and Chilton, 1990).

Neofusicoccum parvum also produced a hydrophilic phytotoxic compound of high molecular weight. This compound appeared to be an exopolysaccharide (EPS) consisting essentially of neutral sugars, as shown by GC-MS analysis of the acetylated O-methyl glycosides (Martos *et al.,* 2008). Preliminary NMR showed that the compound was a polysaccharide with a linear repeating unit. Its structure is now being determined.

Studies are also in progress to evaluate the possible synergic effects of the lipophilic and hydrophilic phytotoxic metabolites produced by *N. parvum*.

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