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Phytotoxic polyketides produced by *Phomopsis foeniculi*, a strain isolated from diseased Bulgarian fennel

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Abstract Recently, a new fungal disease caused by *Diaporthe angelicae* (anamorph *Phomopsis foeniculi*) has been found with increasingly frequency on fennel (*Foeniculum vulgare*) in Bulgaria. Using a bioassay-guided isolation and purification procedure, different metabolites were isolated from the fungal culture filtrates. They were identified by spectroscopic methods as nectriapyrone, a pentaketide monoterpenoid, and altersolanols A and J and macrosporin, three octaketides anthracenones. Leaf puncture bioassay was applied on detached tomato leaves to prove the phytotoxic activity of the fractions and of pure compounds. Nectriapyrone and altersolanols A and J showed a modulated phytotoxicity, while macrosporin was not toxic. Altersolanol A was the most active compound.

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Introduction

Fennel (*Foeniculum vulgare* Miller) is a biennial or perennial herbaceous plant belonging to Apiaceae family. The dried fennel fruits commonly called seeds are used in phytotherapy and the pharmaceutical industry because of their antispasmotic, carminative, stomachic, cardiotonic, diuretic, expectorant and stimulant properties (Tisserand 1987; Choi and Hwang 2004). They are also utilized in the alimentary industry. The main constraints upon seed production are fennel diseases. Lately, the development of a new fennel disease has been observed with increasingly frequency in Bulgaria (Rodeva and Gabler 2010). Typical symptoms were umbel browning and stem necrosis. *Diaporthe angelicae* (Berk.) D.F. Farr & Castl. (anamorph *Phomopsis foeniculi* Du Man. et Vegh.) was identified as the causal agent. Diseased umbels could be totally destroyed by umbel browning and produced no fruits. Stem necrosis led to the death of many twigs or whole plants. A rapid spread of the disease symptoms at the distal part of the infection site was observed suggesting the involvement of transposable phytotoxins in the pathogenesis.

P. foeniculi was initially reported on the same host in France (Du Manoir and Vegh 1981), and some

preliminary investigation were performed to ascertain its ability to produce in liquid culture phytotoxic metabolites, which could be involved in pathogenesis (Hunault et al. 1989). Successively, from the culture filtrates of a pathogen strain isolated from diseased fennel near Florence (Italy) foeniculoxin (**1**, Fig. 1), a new phytotoxic geranylhydroquinone (Evidente et al. 1994), and two phytotoxic exopolysaccharides (EPSs), namely a galactan and a branched mannan (Corsaro et al. 1998) were purified.

Considering the economical importance of fennel fruits, the increased disease occurrence in Bulgaria and the fact that the causal agent *P. foeniculi* is a toxigenic fungus, research was undertaken to isolate and characterize the phytotoxins produced by Bulgarian strains when grown in liquid cultures. Moreover, isolates of this pathogen obtained from the same host plant but in different world countries could produce different phytotoxic metabolites.

This paper describes the results of the chemical and biological characterization of the phytotoxic metabolites, produced *in vitro* by a Bulgarian strain of *P. foeniculi*.

Materials and methods

Fungal cultures, nutrient medium and growth conditions

The fungus was isolated from naturally infected umbels and stems of fennel. Five monoconidial strains of *P. foeniculi* (Phf 1-1, Phf 30-2, Phf 76-1, Phf 96-1 and Phf 107-3) representing different morphological groups were used to characterize their capacity to produce phytotoxic metabolites *in vitro*.

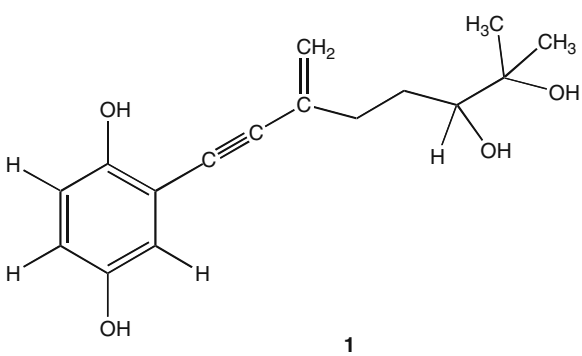

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Fig. 1 Structure of foeniculoxin (**1**)

Three disks of mycelium (5 mm in diameter) were cut from advancing edge of colonies of each isolate and placed in separate 300 ml conical Erlenmeyer flasks containing 100 ml Czapek-Dox broth complemented with 0.02% yeast extract (Oxoid LTD—Basingstoke, Hampshire, England) and 0.02% L-asparagine (Mugnai and Anzidei 1994). The cultures were incubated at 25°C by shaking at 120 rev min^{-1} for 14 days. After incubation, the contents were passed through two layers of cheesecloth to reduce the fungal biomass and further filtered using 45 μm Millipore filters. To determine whether the phytotoxicity of the culture filtrate was host selective or not a seedling immersion assay was applied on uprooted young plants of fennel, cv. Berfena (host) and tomato, cv. Ideal (non-host). The roots were washed and immersed in 2 ml of concentrated and ten-fold diluted culture filtrates of the five isolates. Seedlings were kept at 25°C for 24 h and then transferred into distilled water for a further 48 h. Uninoculated nutrient medium and sterile distilled water served as the controls. The experiment was carried out in triplicate, each consisted of five plants for every plant species. For detailed chemical and biological characterization of *P. foeniculi* secondary metabolites, the strain Phf 1-1 was selected on the basis of the highest phytotoxic activity on host and non-host plants. The strain has been deposited at the National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria, under the number NBIMCC 8645. Mass growth of the fungus for the isolation and identification of the phytotoxic metabolites was obtained by incubation of the latter strain for 14 days using the same growth medium and culture conditions, then filtered, assayed for phytotoxic activity and lyophilized for the successive purification steps.

Chemical analysis and characterization

Optical rotation was measured in methanol, on a Jasco P-1010 polarimeter (Tokyo, Japan); IR spectra were recorded as deposit glass film on a Perkin-Elmer (Norwalk, CT) Spectrum One FT-IR spectrometer, and UV spectra were measured in methanol on a Perkin-Elmer Lambda 25 UV–vis spectrophotometer. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 600 or 300 MHz and at 75 MHz, respectively, in deuterated chloroform-methanol (95:5), unless otherwise noted, on Bruker *DRX-600 AVANCE* spectrometers (Kalsruhe, Germany). The

chloroform was always used as the internal standard. Carbon multiplicities were determined by DEPT spectra (Berger and Braun 2004). DEPT, COSY -45, HSQC, HMBC and NOESY experiments (Berger and Braun 2004) were performed using standard Bruker software. ESI mass spectra were recorded on Waters Q-TOF Micro spectrometer (Milford, MA), and on Agilent Quadrupole 6120 LC/MS (Waghaeusel-Wiesental, Germany). Analytical and preparative thin layer chromatography (TLC) was performed on silica gel (Merck, Kieselgel 60, F_{254}, 0.25 and 0.5 mm respectively,) plates. The spots were visualized by exposure to UV radiation (253 nm), 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. Column chromatography was performed on silica gel column (Merck, Kieselgel 60, 0.063–0.200 mm). Chemical reagents were purchased from Sigma-Fluka, Merck and Serva.

Extraction and purification of phytotoxic metabolites

The lyophilized fungal culture filtrate (12 l, having pH 6.9) was resuspended in distilled water (1.3 l) and extracted with ethyl acetate 3×1 l. The organic extracts were combined, dried (Na_2SO_4) and evaporated under reduced pressure, and yielded a brown oily residue (1.83 g). Showing high phytotoxic activity in the leaf puncture assay, it was purified by silica gel column chromatography eluted with chloroform/*iso*-propanol 87:13, and successively washed with methanol to give 15 groups of homogeneous fractions. They were tested for phytotoxicity on detached tomato leaves as below described and those fractions showing higher phytotoxicity were further purified. The residue (54.4 mg) of fraction 3 was further purified by preparative TLC on silica gel (eluent chloroform/*iso*-propanol 9:1) yielding four groups of homogeneous fractions. The residue (15.0 mg) of the third fraction, obtained as homogeneous white solid, was identified (see below) as nectriapyrone (**2**, R_f 0.86, 1.1 mg/l). The fraction 4 residue (51.5 mg) of the same preparative TLC was further purified as above yielding an anamorphous yellow solid, below identified as macrosporin (**4**, R_f 0.78, 2.3 mg/l). The residues (171, 126.5. and 121.8 mg) of fractions 11–13 obtained from the initial column, were combined and fractioned on silica gel column eluted with chloroform/*iso*-propanol 87:13, yielding seven groups of homogeneous fractions. The fraction 4 residue (50.0 mg) was further purified by two successive steps of preparative TLC on silica gel (eluent chloroform/methanol 9:1) and reversed-phase (eluent ethanol/water 6:4), yielding an orange homogeneous solid, below identified as altersolanol A (**8**, R_f 0.40 and 0.79, respectively 1.0 mg/l). The fraction 3 residue (214 mg) of the last column was fractioned on silica gel column (eluent, chloroform/methanol 92:8) obtaining seven groups of homogeneous fractions. The fraction 3 residue (52.8 mg) of this third column was further purified by two successive steps of preparative TLC on silica gel (eluent chloroform/methanol 92:8 and chloroform/methanol 9:1), yielding another homogeneous solid compound; below identified as altersolanol J (**9**, R_f=0.36, silica gel, eluent chloroform/methanol 9:1, 0.45 mg/l).

Hydrogenation of nectriapyrone

Nectriapyrone (4 mg) dissolved in methanol (300 μl) was added, under stirring, to a H_2 pre-saturated suspension of 10% Pd/C in methanol (300 μl). The reaction was carried out at room temperature and atmosphere pressure. After 1 h the catalyst was removed by funnel filtration, and the clear solution was evaporated under vacuum, yielding an oily residue (3.4 mg). The latter was purified by preparative TLC on silica gel (eluent chloroform/isopropanol 95:5), yielding the tetrahydro derivative of **2** as a homogenous oil (**3**, R_f 0.75, 2.4 mg)

Acetylation of macrosporin

Acetic anhydride (30 μl) was added to macrosporin (**4**, 3 mg) dissolved in pyridine (30 μl). The reaction was carried out overnight at room temperature. The reaction was stopped by addition of methanol and evaporation by a N_2 stream. The residue (18.0 mg) was purified by preparative TLC on silica gel (eluent *n*-hexane/ethyl acetate 1:1), yielding the diacetyl derivative of macrosporin as homogenous oil (**5**, R_f 0.76, 2.1 mg).

Methylation of macrosporin

An ethereal solution of diazomethane was added to macrosporin (**4**, 5 mg), dissolved in methanol

(0.5 ml). The reaction was carried out overnight in the dark at room temperature. The reaction was stopped by evaporation under N_2 stream. The residue (6 mg) was purified by preparative TLC on silica gel (eluent chloroform/*iso*-propanol 98:2), yielding two derivatives as homogeneous yellow solids the monomethyl (**6**, R_f 0.76, 2.0 mg) and the dimethyl (**7**, R_f 0.68, 2.0 mg) ethers of macrosporin.

Leaf puncture assay

A simple leaf puncture bioassay was applied for the rapid determination of phytotoxic activity of the fractions and of pure compounds. Since the feathery leaves of fennel (the host plant) were not suitable to assay performance and the toxicity of the culture filtrate was not host specific, tomato leaves were used for that purpose. The test fractions and pure compounds were first dissolved in a small amount of methanol and then diluted to the desired final concentration (2 mg/ml) with sterile distilled water (3% final methanol concentration). Droplets (20 μl) of assay solutions were applied on punctured leaflets of detached tomato leaves. One repeats consisted of 3 leaves with 3 inoculation points. The inoculated leaves were incubated in a moist chamber and were observed every day for symptoms. The final evaluation was recorded 3 days after application. Phytotoxic activity indexing was on a scale of 0–4, where 0 = no lesion; 1 = lesions 1–2 mm; 2 = lesions 3–5 mm; 3 = lesions 6–7 mm; 4 = lesions 8–10 mm. To test the potential influence of the methanol, a methanol control besides the water one was included in each experiment. Culture filtrate (CF) was included as positive control. All experiments were carried out in triplicate and repeated two times.

Results

A small set of 5 *Phomopsis foeniculi* isolates was chosen for comparison of their phytotoxic profile (Ph1-1, Phf 30-2, Phf 76-1, Phf 96-1 and Phf 107-3). 3 l culture filtrates were prepared of each isolate grown in the same conditions. The chromatographic metabolite profile obtained by the TLC analysis of the organic extracts of their culture filtrates showed that they produced the same metabolites. In the preliminary bioassay on host (fennel) and non-host (tomato) plants the isolate Ph1-1 showed the highest phytotoxicity. On that basis it was chosen for further investigation and the same growth medium and culture conditions were applied to produce increased quantity cultural filtrate (12 l) for the successive purification steps. The phytotoxins produced by *P. foeniculi* were exhaustively extracted from culture filtrates with ethyl acetate. The organic extract, showing a high phytotoxic activity, was purified by a combination of column and thin layer chromatography on silica gel and reverse phase (see Material and Methods section). Four metabolites were isolated and their identification was achieved by comparing their spectroscopic data (ESI MS and COSY, HSQC, HMBC and NOESY NMR spectra) with those previously reported in literature. Notwithstanding the different solvents used to record NMR spectra, a good similarity between our and literature data was observed for the known metabolites.

Among the four metabolites we recognized the nectriapyrone (**2**, Fig. 2).

Nectriapyrone (**2**)

Compound **2** had UV λ_{max} nm (log ε) 227 (4.59) and 321 (4.2), IR ν_{max} 1,682, 1,644, 1,619, 1,554, 1,173, 820, 750 cm^{-1} and ^{1}H- and ^{13}C-NMR spectra were similar to those previously reported (Nair and Carey 1975); ESI-MS (+) *m/z* 217 $[M+Na]^+$.

Its structure was confirmed by preparing the racemate dihydroderivative (**3**, Fig. 2) by catalytic hydrogenation of **2**.

Dihydroderivative of nectriapyrone (**3**)

Compound **3** had: UV λ_{max} nm (log ε) 298 (4.14), 205 (4.74), IR ν_{max} 1,694, 1,644, 1,568, 1,461 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 5.97, (s, H-5), 3.87 (s, OMe), 2.48 (tq, *J*=7.0 and 7.0 Hz, H-7), 1.91 (s, Me-10), 1.72 (dq, *J*=7.0 and 7.0, H-8), 1.22 (d, *J*=7.0, Me-11), 0.88 (t, *J*=7.0, Me-9); ESI-MS (+) *m/z* 219 $[M+Na]^+$, 197 $[M+H]^+$.

This is a new derivative that showed the IR spectrum bands for an extended conjugated carbonyl group (Nakanishi and Solomon 1977), while the UV spectrum showed the absorption maximum of pyrones (Scott 1964). The ^{1}H NMR spectrum, compared to that of nectriapyrone, showed a sec-butyl system instead of a 1-methylpropenyl attached to C-6. In fact, the quartet due to the olefinic H-

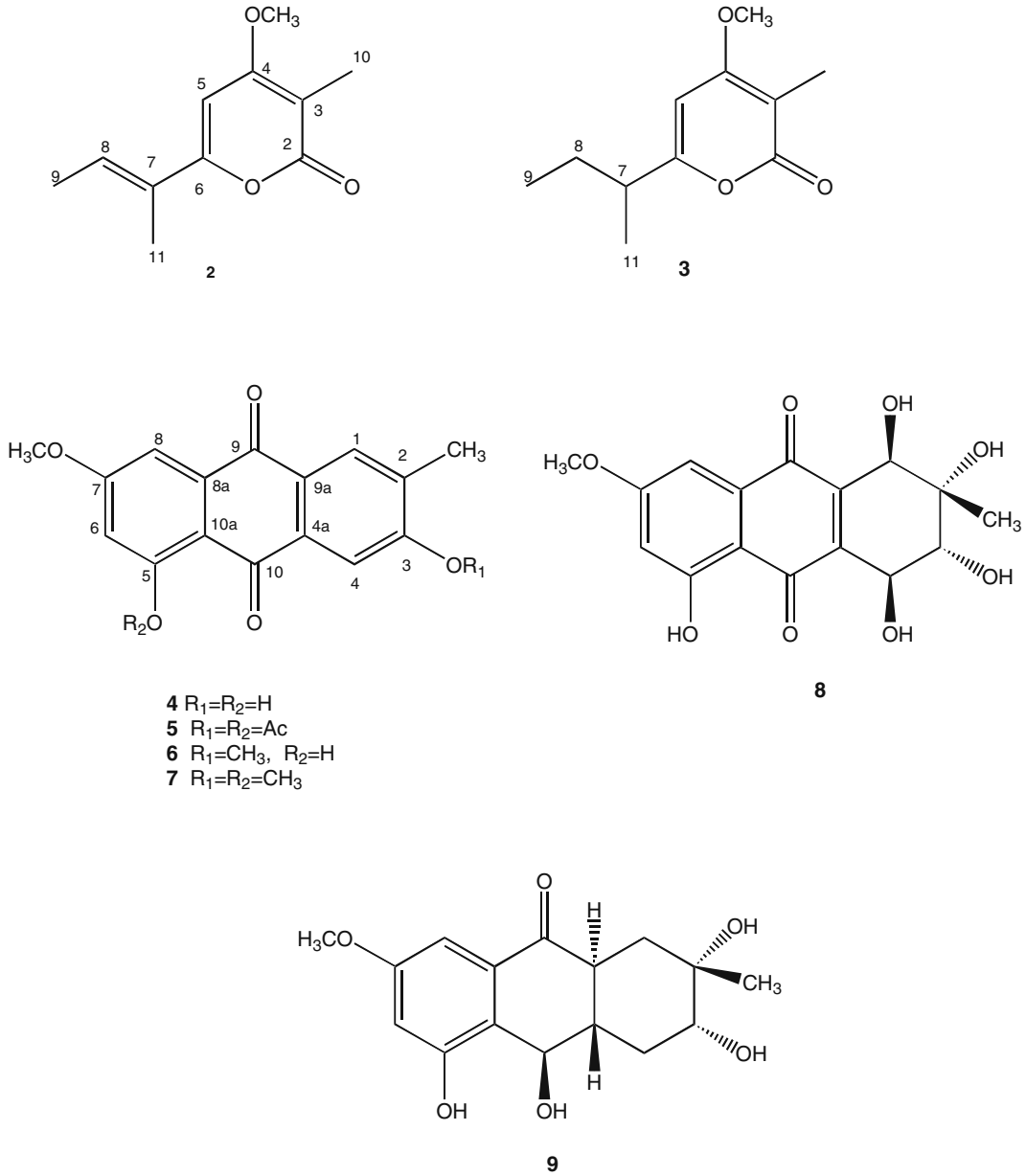

Fig. 2 Structures of nectriapyrone and its dihydroderivative (**2** and **3**), macrosporin and its derivatives (**4**–**7**) altersolanols A and J (**7** and **8**)

8 resonating at δ 6.68 in the ^{1}H NMR spectrum of **2** was absent, while both Me-11 and Me-9 were upfield shifted at δ 1.22 and 0.88, respectively, resonating as a doublet (J=7.0 Hz) and a triplet (J=7.0 Hz). Finally, the additional presence of a triple quartet (J=7.0 and 7.0 Hz, H-7) and a double triplet (J=7.0 and 7.0 Hz) was observed at δ 2.48 and 1.72 and assigned to H-7 and H-8, respectively. This derivative was different from the tetrahydroderivative obtained by hydrogenation of **2** as it has been previously reported (Nair and Carey 1975). In fact, this latter also showed the reduction of the double bond α,β-located with respect to the pyrone oxygen (Nair and Carey 1975). Although, the catalyst was the same, the solvent used was different and this probably justified the different reduced derivatives.

Three other metabolites appeared to be anthracenone octaketides and were identified as macrosporin,

altersolanols A and J (**4, 8, 9**, Fig. 2). Acetylation of **4**, carried out with acetic anhydride and pyridine, yielded the corresponding diacetyl derivative **5** (Fig. 2). By reaction with diazomethane, **4**, differently from the literature (Suemitsu et al. 1959; Stoessl 1969), was converted into the corresponding mono- and di-methyl derivatives (**6** and **7**, Fig. 2). The ESI and ^{1}H NMR data of these three derivatives confirmed their structures and have been reported here for the first time together with the ESI and ^{13}C NMR data of macrosporin.

Macrosporin (**4**)

Compound **4** had: IR ν_{max} 3,289, 1,655, 1,637, 1,572 cm^{-1}; UV and ^{1}H-NMR spectra were similar to those previously reported (Stoessl 1969; Becker et al. 1978), respectively. ^{13}C-NMR [$CDCl_3$-CD_3OD (1:1,*v/v*)]: δ 187.7 (s, C-9), 182.6 (s, C-10), 166.8 (s, C-5), 165.4 (s, C-7), 162.4 (s, C-3), 136.0 (s, C-10a), 134.2 (s, C-8a), 133.0 (s, C-9a), 126.1 (s, C-4a), 111.9 (d, C-4) 111.3 (s, C-2), 108.0 (s, C-1) 106.3 (d, C-6), 56.3 (OMe), 16.6 (Me); ESI-MS (−) *m/z* 283 [M-H]$^{-}$.

Diacetylderivative of macrosporin (**5**)

Compound **5** had: UV λ_{max} nm 334 and 270 (log ε) 3.25 and 4.35; IR ν_{max} 1,772, 1,674, 1,577, 1,435 cm −1: [(Suemitsu et al. 1959), IR 1,672 cm^{-1}; (Stoessl 1969), (EtOH) λ_{max} 331 and 270 nm (log ε 3.71 and 4.67)]; ^{1}H-NMR δ: 8.13 (s, H-4), 7.86 (s, H-1), 7.37 (d, *J*=2.4 Hz, H-8), 6.89 (d, *J*=2.4, Hz, H-6), 3.98 (s, OMe), 2.48 and 2.37 (two s, 2×MeCO) 2.34 (s, Me); ESI-MS (+) *m/z*: 407 [M+K]$^{+}$, 391 [M+Na]$^{+}$.

Monomethylderivative of macrosporin (**6**)

Compound **6** had: UV λ_{max} nm 280 (log ε) 4.18; IR ν_{max} 3,356, 1,641 cm−1: [(Suemitsu et al. 1959) IR 1,667 cm^{-1}; (Stoessl 1969) (EtOH) λ_{max} 410 (inf), 377, 308, 281 and 225 nm (log ε 3.83, 4.12, 4.56, and 4.35)]; ^{1}H-NMR δ: H-1 12.9 (s, OH-C-5), 8.03 (s, H-1), 7.63 (s, H-4), 7.35 (d, *J*=2.2 Hz, H-8), 6.67 (d, *J*= 2.2 Hz, H-6), 4.02 and 3.83 (two s, 2×OMe), 2.35 (s, Me); ESI MS (+) *m/z*: 299 [M+H]$^{+}$.

Dimethylderivative of macrosporin (**7**)

Compound **7** had: IR ν_{max} 1,668, 1,593, 1,444 cm−1; UV λ_{max} nm 282 (log ε) 3.80: ^{1}H-NMR δ: 7.98 (s, H-1), 7.65 (s, H-4), 7.46 (d, *J*=2.1 Hz, H-8), 6.76 (d, *J*= 2.1 Hz, H-6), 4.00, 3.99 and 3.98 (three s, 3 × OMe), 2.33 (s, Me); ESI -MS (+) *m/z*: 313[M+H]$^{+}$.

Altersolanol A (**8**)

Compound **8** had $[\alpha]_D^{25}$, UV IR and ^{1}H- and ^{13}C-NMR spectra similar previously reported (Becker et al. 1978; Stoessl et al. 1983); ESI-MS (+) *m/z* 359 [M+Na]$^{+}$, 337 [M+H]$^{+}$, 301 [M−CH_3]$^{+}$.

Altersolanol J (**9**)

Compound **9** had $[\alpha]_D^{25}$, IR, UV and ^{1}H- and ^{13}C-NMR spectra similar to those previously reported (Holler et al. 2002); ESI-MS (−) *m/z* 307 [M−H]$^{-}$.

The identification of macrosporin, isolated for the first time from *Alternaria porri* Ellis (Suemitsu et al. 1959) was confirmed by preparation of three key derivatives. Acetylation of **4**, carried out with acetic anhydride and pyridine, yielded the corresponding diacetyl derivative **5**. By reaction with diazomethane, **4**, differently from the literature (Stoessl 1969; Thines et al. 1998), was converted into the corresponding mono- (**6**) and di-(**7**) methyl derivatives. The ESI and ^{1}H NMR data of these three derivatives confirmed their structures and have been reported here for the first time together with the ESI and ^{13}C NMR data of macrosporin.

In all bioassays CF showed greatest visible appearance. When nectriapyrone, macrosporin, and altersolanols A and J were tested by puncture assay on tomato leaves in the concentration range 6–64×10^{-3} M they showed a modulated phytotoxicity (Table 1). Altersolanol A (**8**),that appeared to be the most active metabolite, induced a massive, running on the nervature necrosis and a twisting of inoculated leaves (Fig. 3). Altersolanol J (**9**) caused necrosis and wilting of the inoculated leaves (Fig. 4), while nectriapyrone (**2**), which is only slightly active, induced small watery lesions around the inoculated point of the same leaves (Fig. 5).

Table 1 Effect of nectriapyrone, macrosporin and altersolanols A and J (**2**, **4**, **8** and **9**) on tomato leaves in the puncture assay

Compound	Concentration [M]	Toxicity [20 µl/droplet][a]
Nectriapyrone (**2**)	10.3×10^{-3}	1
Macrosporin (**4**)	7×10^{-3}	0
Altersolanol A (**8**)	6×10^{-3}	4
Altersolanol J (**9**)	64×10^{-3}	3
Methanol control[b]	–	0
Water control	–	0

[a] Phytotoxic activity indexing was on a scale of 0–4, where 0 = no lesion; 1 = lesions 1–2 mm; 2 = lesions 3–5 mm; 3 = lesions 6–7 mm; 4 = lesions 8–10. [b] 3% MeOH in distilled water

Discussion

The four lipophilic metabolites isolated from culture filtrates of *P. foeniculi* were four polyketides, namely nectriapyrone, macrosporin, and altersolanols A and J. These differed from foeniculoxin, the main phytotoxin produced by an Italian strain of the same fungus. Furthermore, the Bulgarian strain did not produce the EPSs (the galactan and the branched mannan) synthesized by Italian strain.

Nectriapyrone is a monoterpenoid pentaketide firstly isolated from *Gyrostoma missouriense* Seeler (Nair and Carey 1975). Nectriapyrone was also isolated from different fungal genera, including *Phomopsis oblonga* Desm. (Claydon et al. 1985), *Scytalidum* sp. (Thines et al. 1998), *Phomopsis* sp. (Lee et al. 1999), twelve different *Phomopsis* species —endophytes of *Erythrina crista-galli* L. (Weber et al. 2005), and endophytic fungi isolated from *Viguiera arenaria* Baker and *Tithonia diversifolia* Hemsl. (Guimaraes et al. 2008). Nectriapyrone showed a relevant cytotoxic activity against both human T leukemia and melanoma tumor cell lines (Guimaraes et al. 2008), an inhibitory activity on MAO in a dose-dependent manner (Lee et al. 1999), and stimulated the formation of DOPA melanin in B16-F1 melanoma cells in the absence of melanin-stimulating hormone (Thines et al. 1998). However, this is the first report on isolation of nectriapyrone from *P. foeniculi* and on its as a phytototoxicity.

Macrosporin was isolated for the first time from *Macrosporium porri* Ellis (Suemitsu et al. 1959). This anthracenone was also successively isolated from *Alternaria solani* Sorauer (Stoessl et al. 1983), *Dactylaria lutea* Routier (Becker et al. 1978), *Stemphylium eturmiunum* E. G. Simmons (Andersen and Frisvad 2004), *Alternaria tomatophilia* Ellis (Andersen et al. 2008), *Ampelomyces* sp. (Aly et al. 2008), an undetermined fungicolous hyphomycete resembling *Cladosporium* (Holler et al. 2002) and *Stemphylium globuliferum* (Vestergr) E. G. Simmons (Debbab et al. 2009). It showed a moderate cytotoxic activity towards L5178Y mouse lymphoma cells (Aly et al. 2008) and showed antibacterial activity as inhibition of growth of *Bacillus subtilis* Cohn and *Staphylococcus aureus* S. J. Rosenbach (Holler et al. 2002).

Altersolanol A was also a fungal metabolite, previously isolated from *Alternaria solani* (Stoessl et al. 1979, 1983), *D. lutea* (Becker et al. 1978), *Phomopsis juniperovora* Hart. (Wheeler et al. 1975) and *A. porri* (Suemitsu and Nakamura 1981), and both altersolanols A and J from *Ampelomyces* sp. (Aly et al. 2008), *S. globuliferum* (Debbab et al. 2009) and from the undetermined *Cladosporium* sp. cited above (Holler et al. 2002). Altersolanol A showed an inhibitory effect on lettuce and stone-leek seedlings, an antibiotic activity against *Escherichia coli* Migula, *B. subtilis* and *S. aureus* (Suemitsu et al. 1984) and growth inhibition of *Nicotiana rustica* L. cultured

Fig. 3 Massive running on the nervature necrosis and twisting of tomato leaves inoculated with altersolanol A (**8**)

Fig. 4 Necrosis, wilting and twisting of tomato leaves inoculated with altersolanol J (**9**)

cells (Haraguchi et al. 1996). Altersolanol J showed only a moderate activity towards the same lymphoma cells (Wheeler et al. 1975; Aly et al. 2008).

Fungi are well-known producers of both anthraquinones such as macrosporin and hydrogenated anthranoid congeners such as altersolanol A (Debbab et al. 2009). Biogenetically, monomeric anthranoids have been identified as octaketides produced through condensation of acetate (or malonate) units (Stoessl et al. 1983), and the incorporation of altersolanol A and macrosporin has been demonstrated (Stoessl et al. 1979), indicating that these metabolites are part of a common biogenetic grid (Debbab et al. 2009). The culture filtrate of a pathogen is a cocktail of all toxins and other metabolites produced by the fungus under artificial conditions. Many toxins have been identified

Fig. 5 Small watery lesions around inoculation point induced on tomato leaves by nectriapyrone (**2**)

and characterized. In the present investigation the effect of culture filtrate (CF) on the plant tissue was greater than those of individual fractions or pure compounds demonstrating a synergistic interaction between the components of culture filtrate.

Altersolanols and other naturally occurring anthraquinones such as macrosporin have been produced by fungi belonging to different genera such as *Alternaria*, *Ampelomyces*, *Dactylaria*, *Phomopsis*, *Stemphylium* (Becker et al. 1978; Stoessl, et al. 1979; Suemitsu and Nakamura 1981; Aly et al. 2008; Debbab et al. 2009). They differed in phytotoxicity and other biological activities. Aly et al. (2008) reported that the tetrahydroanthraquinone altersolanol A showed the highest cytotoxic activity, while the anthronol derivative altersolanol J expressed only moderate to weak activity, suggesting that the *para*-quinone moiety could be of great importance for the cytotoxicity. Altersolanol A was the most phytotoxic compound in our investigation and altersolanol J showed moderate phytotoxicity. It is interesting the fact that fungi from different genera showed similar metabolite profile (Becker et al. 1978; Stoessl et al. 1979; Suemitsu and Nakamura 1981; Aly et al. 2008; Debbab et al. 2009), while different isolates of the same fungus could produce different secondary metabolites (Vurro et al. 1998; Evidente et al. 2006a; ibidem 2006b; Cabras et al. 2006). The latter was supported by comparison of phytotoxic metabolites of Bulgarian and Italian isolates of *P. foeniculi* (Evidente et al. 1994; Corsaro et al. 1998).

The *in vivo* production of these metabolites in fennel plants has not been studied as yet, but a rapid spread of the disease symptoms at the distal part of the infection site was observed, suggesting the involvement of phytotoxins in the pathosystem fennel/*Phomopsis*.

In conclusion, we reported here for the first time the finding of nectriapyrone, macrosporin, and altersolanols A and J as metabolites of *P. foeniculi* and the phytotoxic activity for the first and last one. In fact, macrosporin was not toxic and altersolanol A has already been known as an inhibitor of lettuce and stone-leek seedlings (Suemitsu et al. 1984). Furthermore, the isolation of the above metabolites, which differ from foeniculoxin, that is a geranylkhydroquinone, and obviously from the EPSs (Corsaro et al. 1998) isolated from an Italian strain of the same fungus (Evidente et al. 1994), supported the fact that

different isolates of same pathogen, obtained from the same host plant, could produce different phytotoxic metabolites. In the literature there are other examples that support these statements. In fact, different phytotoxins were produced by different isolates of *Alternaria zinniae* Ellis (Vurro et al. 1998) and *Drechslera gigantea* Heald & Wolf (Evidente et al. 2006a) proposed as mycoherbicides for the biocontrol of an exotic annual weed *Xanthium occidentale* Schouw and of a grass weed *Digitaria sanguinalis* L., and *Sphaeropsis sapinea* Dyco & Sutton, a pathogen associated with severe diseases on a wide range of forest hosts throughout the world including cypress and pine (Evidente et al. 1999, 2006b; Cabras et al. 2006).

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