Phytopathol. Mediterr. (2011) 50, 154–158

Phytotoxins produced by *Pestalotiopsis guepinii*, the causal agent of hazelnut twig blight

Muharrem TÜRKKAN[1], Anna ANDOLFI[2], Maria Chiara ZONNO[3], Ismail ERPER[4], Carmen PERRONE[2], Alessio CIMMINO[2], Maurizio VURRO[3] and Antonio EVIDENTE[2]*

[1]Department of Plant Protection, Faculty of Agriculture, University of Ordu, 52200 Ordu, Turkey
[2]Dipartimento di Scienze del Suolo, della Pianta, dell'Ambiente e delle Produzioni Animali, Università di Napoli Federico II, Via Università 100, 80055 Portici, Italy
[3]Istituto di Scienze delle Produzioni Alimentari, CNR, Via Amendola 122/O, 70125 Bari, Italy
[4]Department of Plant Protection, Faculty of Agriculture, University of Ondokuz Mayıs, 55139 Samsun, Turkey
* Associated to the Istituto di Chimica Biomolecolare del CNR, Pozzuoli, Italy

Summary. The main lipophilic phytotoxic metabolite was isolated from the culture filtrates of *Pestalotiopsis guepinii,* the fungus causing twig blight of hazelnut. The metabolite was spectroscopically identified as pestalopyrone, a pentaketide that it was originally identified as a minor toxin produced by *Pestalotiopsis oenotherae*. The toxic activity of pestalopyrone was compared with that of nectriapyrone, a structurally related monoterpenoid recently isolated from *Phomopsis foeniculi*, and that of the new dihydro-derivative of nectriapyrone. The high phytotoxic activity of nectriapyrone and its dihydro-derivative on three non host plants, showed that the double bond of the 1-methylpropenyl group at C-6 of the aromatic ring is inessential for its activity, while the much lower activity of pestalopyrone showed that the methyl group at C-3 of the same ring is an important structural feature. The high molecular weight hydrophilic phytotoxins produced by this fungus are reported for the first time.

Key words: pentaketides, monoterpenoids, pestalopyrone, nectriapyrone, SAR.

Introduction

Turkey is the biggest producer of hazelnut (*Corylus avellana* L.) in the world, with a total cultivation area of more than 600,000 hectares, a total annual yield over 800,000 tonnes, and an average yield of around 1.2 tonnes ha^{-1} in 2008 (FAO, 2008). Several bio-constraints negatively affect hazelnut production, such as pests, diseases and weeds (Anonymous, 1995). Among them *Pestalotiopsis guepinii* (Desm.) Stey causes hazelnut twig blight. *P. guepinii* was first reported on hazelnut in Turkey (Yürüt *et al*. 1994), and then on walnut (*Juglans* spp.) and gum mastic tree (*Pistacia lentiscus* var. *Chia*) (Karaca and Erper, 2001; Göre *et al.*, 2010). It causes symptoms consisting of blight and drying. Small necrotic spots appear on hazelnut leaves and shoots at an early stage; the spots expand if environmental conditions are favorable. Eventually, the leaves wilt and dry, and shoots turn brown, especially those on young branches.

Some plant pathogenic fungi can produce phytotoxins which play an important role in the development of the disease and in the appearance of symptoms, such as chlorosis, necrosis, epinasty, and wilting (Yoder, 1980; Ballio and Graniti, 1991; Upadhyay and Mukerji, 1997; Evidente and Mot-

Corresponding author: A. Evidente
Fax: +39 081 2539186
E-mail: evidente@unina.it

ta, 2001). Different species of *Pestalotiopsis* have been studied for the bioactive secondary metabolites they produce in host-pathogen relationships. *P. longiseta* and *P. theae*, the causal agents of tea gray blight disease, produce three phytotoxins, named oxysporone, epiepoxydon and PT-toxin (Nagata and Ando, 1989; Nagata *et al*., 1992); in the culture filtrate *P. theae* (Kimura *et al*., 1998) also produced pesthetoxin. *P. oenotherae*, the causal agent of leaf spot disease of evening primrose (*Oenothera laciniata* Hill.), produces pestalopyrone, pestalotin, hydroxy-pestalotin and oxysporone (Venkatasubbaiah *et al*. 1991). Recently, it has been reported that *Pestalotiopsis* spp. produce a number of hitherto unknown biologically active compounds such as pestalethols A–D (Li *et al*., 2008a) and pestalachlorides A–C (Li *et al*., 2008b). However, no phytotoxic metabolites have been reported for *P. guepinii*.

In this study, the main phytotoxic metabolite produced *in vitro* by *P. guepinii* was isolated and identified as pestalopyrone by NMR and MS spectroscopy. The phytotoxic activity of pestalopyrone was assayed and compared with that of nectriapyrone, a structurally related metabolite produced by *Phomopsis foeniculi*, and that of its newly described dihydroderivative (Evidente *et al*., 2010b).

Materials and methods

Isolation of *Pestalotiopsis guepinii*

A strain of *P. guepinii* was isolated from blighted hazelnut twigs collected in Trabzon, a province of Turkey, and deposited in the collection of Istituto di Scienze delle Produzioni Alimentari , CNR, Bari, Italy, with accession number ITEM 13203. This single-conidial strain was grown on potato dextrose agar (PDA) at 23°C with a 12 h day for 14 days. The cultures were flooded with sterile distilled water (SDW) and spores were scraped off with a sterile glass spatula. The conidial suspension was filtered through Whatman filter paper to remove fungal mycelial fragments, adjusted to 1×10^6 spores mL^{-1}. and stored in 15% glycerol at -80°C.

Production, extraction and purification of pestalopyrone

To produce the toxic metabolite, the fungus was grown on potato broth medium supplemented with 2% glucose, in 250 mL Erlenmayer flasks each containing 150 mL broth. Each flask was inoculated with 150 µL of a *P. guepinii* spore suspension (10^6 spores mL^{-1}). Flasks were incubated in stationary culture in the dark at 27°C for 7 days. Culture filtrates (3150 mL) were harvested by filtering through four layers of muslin and then centrifuged at 15300 *g* for 15 min., assayed for phytotoxic activity as described below, and lyophilized. The lyophilized culture filtrate was dissolved in distilled water (1/10 of its original volume) and extracted with EtOAc (4 times with 300 mL each). The organic extracts were combined, dehydrated with Na_2SO_4 and evaporated under low pressure, yielding a brown oil (48 mg). This oil was chromatographed by preparative TLC eluted with $CHCl_3$-*i*-PrOH (9.5:0.5, v:v), yielding 11 groups of homogeneous fractions. The residue (2.9 mg) was further purified by preparative TLC eluted with petroleum ether-Me_2CO (8:2, v:v), yielding the main metabolite as an anamorphous solid (1.8 mg, 0.6 mg L^{-1}, R_f 0.34 by TLC, eluent petroleum ether-Me_2CO) identified as pestalopyrone, as reported below (**1**, Figure 1).

Chemical analysis and characterization

Infra-red spectra were recorded as a glassy film on a Perkin-Elmer (Norwalk, CT, USA) Spectrum One FT-IR spectrometer, and UV spectra were taken in a MeOH solution on a Perkin-Elmer Lambda 25 UV/Vis spectrophotometer. ^1H spectra were recorded at 400 MHz, in $CDCl_3$ on a Bruker (Karlsruhe, Germany) spectrometer. MeOH was also used as an internal standard. ESIMS were recorded on Agilent Quadrupole 6120 LC/MS (Waghaeusel-Wiesental, Germany). Analytical and preparative TLC was performed on silica gel plates (Kieselgel 60 F_{254}, 0.25 and 0.50 mm, Merck, Darmstadt, Germany). Spots were visualized under 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. Nectriapyrone (**2**, Figure 1) was isolated from the culture filtrates of a Bulgarian strain of *P. foeniculi*, which was deposited at the National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria, under the accession number NBIMCC 8645. Dihydronectriapyrone (**3**, Figure 1) was prepared by catalytic hydrogenation of **2** (Evidente *et al*., 2010b).

Biological assays

For bioassay-driven purification, the culture filtrates, organic extracts, chromatographic fractions and pure compounds were assayed with a leaf puncture assay on a number of non-host plants: *Cirsium arvense* (L.) Scop., *Sonchus oleraceus* L. and *Chenopodium album* L. The test solutions were dissolved in a small volume of MeOH (final MeOH concentration 4%) and then diluted with distilled water. The three pure compounds (pestalopyrone, nectriapyrone and its dihydro-derivative) were tested at a final concentration of 2 mg mL^{-1}, by applying 20 μL of solution to detached leaves previously punctured with a needle. Leaves were kept in a moistened chamber under continuous fluorescent lighting. Symptoms were estimated visually three days after droplet application.

The pure compounds were also tested for the antifungal activity on *Geotrichum candidum*, for the antibiosis against *Bacillus subtilis* (gram +) and *Escherichia coli* (gram -), and for the zootoxicity against *Artemia salina* (brine shrimp) larvae, according to the protocols described above (Bottalico *et al.*, 1990).

Results

Purification and chemical identification of phytotoxins

The culture filtrate of *P. guepinii* was extracted with EtOAc at pH 5. The organic extract and the resulting aqueous phase were assayed for phytotoxic activity, and both were found to be toxic in the leaf assay, with the aqueous phase being more toxic. The organic extract contained various lipophilic metabolites, the most important of which was purified by two steps of preparative TLC, as reported in the Materials and methods section. It was obtained as an amorphous solid (0.6 mg L^{-1}) and identified as pestalopyrone (**1**, Figure 2) by spectroscopy, essentially ^{1}H NMR and ESIMS. This showed IR, UV, ^{1}H NMR and ESIMS(+) spectra identical to those reported for pestalopyrone, which was first isolated from *P. oenotherae* (Venkatasubbaiah *et al.*, 1991), and later from an unidentified fungus separated from the Indo-pacific sponge *Stylotella* sp., when it was isolated together with nectriapyrone B and named demehtyl nectriapyrone A (Abrell *et al.*, 1994).

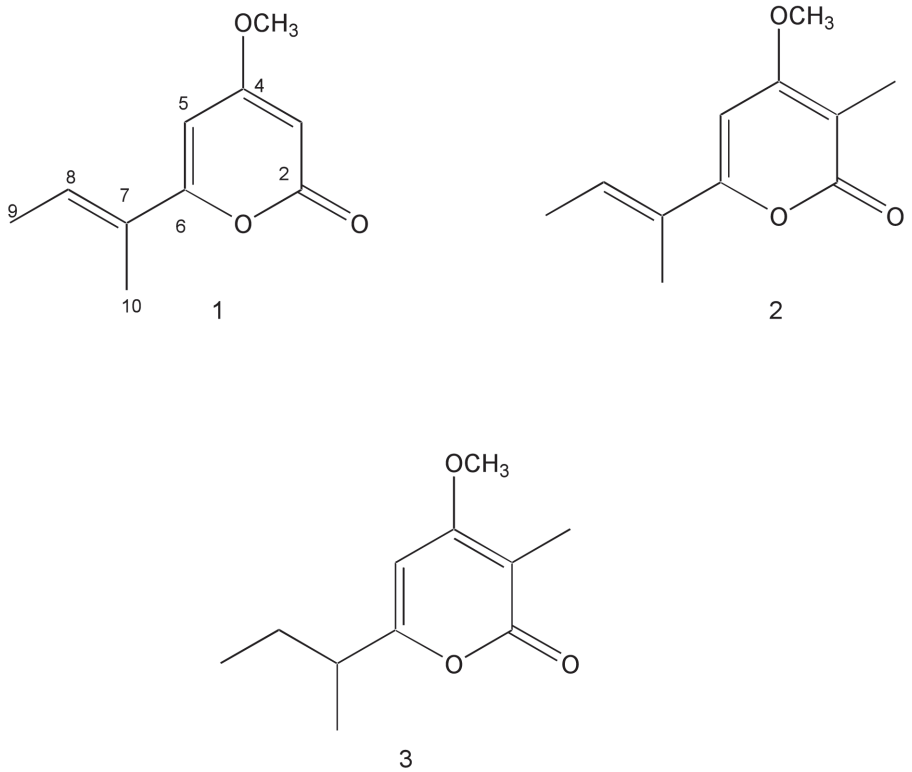

Figure 1. Structures of pestalopyrone, nectriapyrone and its dihydroderivative (**1**, **2** and **3,** respectively).

Biological assays

When assayed on punctured leaves, pestalopyrone was not very toxic on *C. arvense*, more toxic on *S. arvensis*, and it caused extensive necrosis on *C. album* leaves, with a spot having a diameter of several mm. Nectriapyrone and its derivative were both much more toxic than pestalopyrone. The necrotic spots were already large on *C. arvense* and *S. arvensis*, and were much larger on *C. album* leaves. However, none of these compounds had any antimicrobial activity against both bacteria, when tested at up to 50 μg disk^{-1}, nor any zootoxic activity against *A. salina* larvae. Only pestalopyrone had a weak but clear fungistatic activity against *G. candidum* at 50 μg.

Discussion

Both the organic extract and the aqueous phase obtained after the extraction of the culture filtrate of *P. guepinii* were phytotoxic. Besides the toxic metabolite identified in the organic extract, preliminary dialysis experiments carried out on the aqueous phase (data not shown) detected other phytotoxic metabolites having a macromolecular nature, whose identification is in progress. The simultaneous occurrence of hydrophilic and lypophilic phytotoxic metabolites is not surprising, since fungi produce a wide array of secondary metabolites (Evidente and Motta 2001; Evidente *et al*., 2010a; b). The culture medium and the growth conditions also affect metabolite production (Chen and Strange, 1991; Cruz-Cruz *et al*., 2009; Qui *et al*., 2009; Evidente *et al*., 2010a).

The main lypophilic phytotoxic metabolite was identified as pestalopyrone [6-(1'-methylprop-l'-enyl)-4-methoxy-2-pyrone]. This was previously reported as a minor phytotoxic metabolite found in the culture filtrate of *P. oenotherea*, the causal agent of leaf spot of evening primrose, where it occurred together with oxysporone, which is the main phytotoxin biosynthesized by *P. oenotherea* (Venkatasubbaiah *et al*., 1991). Pestalopyrone was also successively isolated together with nectriapytone B from an unidentified fungus separated from the Indo-pacific sponge *Stylotella* sp., and was then named demehtyl nectriapyrone A (Abrell *et al*., 1994). However, neither of these metabolites was reported to have any biological activity, and at least pestalopyrone (=demethyl nectriapyrone A) was inappropriately described as a new α-pyrone.

Pestalopyrone appeared structurally to be closely related to nectriapyrone (**2**, Figure 1), a pentaketide monoterpenoid, from which pestalopyrone differs only in not having the methyl at C-3 on the aromatic ring. Nectriapyrone was first isolated from *Gyrostoma missouriense* (Nair and Carey, 1975) and from *Phomopsis obloganta* (Claydon *et al*., 1985), and more recenly from a Bulgarian strain of *P. foeniculi*, the causal agent of umbel browning and stem necrosis of fennel (*Foeniculum vulgare* Miller).

The phytotoxic activity of pestalopyrone tested on non-host plants was similar to that of nectriapyrone and of its dihydroderivative (**3**, Figure 2). Since both nectriapyrone and its dihydroderivative are significantly more toxic than pestalopyrone, it is possible to hypothesize that the double bond of the 1-methylpropenyl group at C- 6 of the aromatic ring is unessential for phytotoxicity, while the methyl group at C-3 is an important structural feature.

In conclusion: 1) this is the first report dealing with the *in vitro* production of phytotoxins produced by *P. guepinii*, an important pathogen of halzenut in Turkey, the main halzenut producer in the world; 2) it is the first report on pestalopyrone as the main lipohilic phytotoxin produced by *P. guepinii*; 3) the phytotoxic, antimicrobial and zootoxic activity of pestalopyrone was described in detail and its antimicrobial and zootoxic activity was assayed for the first time; the phytotoxic activity was tested on different plants showing its selectivity; 4) the phytotoxic, antimicrobial and zootoxic activity of pestalopyrone was compared with that of nectriapyrone and its dihydroderivative, and with nectriapyrone, a phytotoxin closely related to pestalopyrone first produced by a strain of *P. foeniculi* isolated from infected fennel in Bulgaria; 5) the relation between phytotoxin structure and activity were also discussed; 6) some preliminary experiments on the production of high molecular weight hydrophilic phytotoxins were reported for the first time from *P. guepinii*. These phytotoxins cause significant phytotoxic activity found in the aqueous phase of the culture filtrates after the exhaustive extraction of the lipohilic phytotoxic metabolites including pestalopyrone.

Acknowledgements

The authors thank Prof. Marina della Greca, Dipartimento di Chimica Organica e Biochimica, Università di Napoli Federico II, for recording NMR spectra, and the Council of Higher Education of Turkey for a scholarship. This work was supported in part by a grant from Italian Ministry of University and Research (MIUR). Contribution DISSPAPA N. 239.

Literature cited

Abrell L.M., X-C. Cheng and P. Crew, 1994. New nectriapyrones by salt water cultures of a fungus separated from an Indo-pacific sponge. *Tetrahedron Letters* 35, 9159–9160.

Anonymous, 1995. Agricultural Technical Instructions Struggle. Turkish Ministry of Agriculture and Rural Affairs. General Directorate of Agricultural Research, Ankara, Vol. 1, 291 pp. and Vol. 3, 44 pp. (In Turkish).

Ballio A. and A. Graniti, 1991. Phytotoxins and their involvement in plant disease. *Experientia* 47, 757–826.

Bottalico A., R. Capasso, A. Evidente, G. Randazzo and M. Vurro, 1990. Cytochalasins: structure-activity relationships. *Phytochemistry* 29, 93–96.

Chen Y.M. and R.N. Strange, 1991. Synthesis of the solanapyrone phytotoxins by *Ascochyta rabiei* (Pass.) Labr. in response to metal cations and development of a defined medium for toxin production. *Plant Pathology* 40, 401–407.

Claydon N., J.F. Grove and M. Pople, 1985. Elm bark beetle boring and feeding deterrents from *Phomopsis oblonga*. *Phytochemistry* 24, 937–943.

Cruz-Cruz C.A., K. Garcia-Sosa, F. Escalante-Erosa and L.M. Pena-Rodriguez, 2009. Production of hydrophilic phytotoxins by *Mycosphaerella fijiensis*. *Journal of General Plant Pathology* 75, 191–195.

Evidente A. and A. Motta, 2001. Phytotoxins from fungi, pathogenic for agrarian, forestal and weedy plants. In: *Bioactive Compounds from Natural Sources: Isolation, Characterisation and Biological Properties* (C. Tringali, ed.) Taylor and Francis, London, UK, 473–525.

Evidente A., B. Punzo, A. Andolfi, A. Cimmino, D. Melck and J. Luque, 2010a. Lypophilic phytotoxins produced by *Neofusicoccum parvum*, a grapevine canker agent. *Phytopathologia Mediterranea* 49, 74–79.

Evidente A., R. Rodeva, A. Andolfi, Z. Stoyanova, C. Perrone and A. Motta A., 2010b. Phytotoxic polyketides produced by *Phomopsis foeniculi,* a strain isolated from diseased Bulgarian fennel. *European Journal of Plant Pathology* doi:10.1007/s10658-011-9743-0.

F.A.O., 2008. http://www.fao.org.

Göre M.E., S. Parlak and M.H. Aydın, 2010. *Pestalotiopsis guepinii* newly reported to cause dieback on *Pistacia lentiscus* var. *chia* in Turkey. *Plant Pathology* 21, 8.

Karaca G.H. and I. Erper, 2001. First report of *Pestalotiopsis guepinii* causing twig blight on hazelnut and walnut in Turkey. *Plant Pathology* 50, 415.

Kimura Y., A. Kouge, K. Nakamura, H. Koshino, J. Uzawa, S. Fujıoka and T. Kawano, 1998. Pesthetoxin, a new phytotoxin produced by the gray blight fungus, *Pestolatiopsis theae*. *Bioscience, Biotechnology, and Biochemistry* 62, 1624–1626.

Li E., R. Tian, S. Liu, X. Chen, L. Guo and Y. Che, 2008a. Pestalotheols A-D, bioactive metabolites from the plant endophytic fungus *Pestalotiopsis theae*. *Journal of Natural Products* 71, 664–668.

Li E., L., Jiang, L., Guo, H., Zhang and Y. Che. 2008b. Pestalachlorides A-C, antifungal metabolites from the plant endophytic fungus *Pestalotiopsis adusta*. *Bioorganic & Medicinal Chemistry* 16, 7894–7899

Nagata N., Y. Ando and A. Hirota, 1992. Phytotoxins from tea gray blight fungi, *Pestolatiopsis longiseta* and *Pestolatiopsis theae*. *Bioscience, Biotechnology, and Biochemistry* 56, 810–811.

Nagata N. and Y. Ando, 1989. Oxysporone, a phytotoxin isolated from the tea gray blight fungus *Pestolatiopsis longiseta*. *Agricultural and Biological Chemistry* 53, 2811.

Nair M.S.R. and S.T. Carey, 1975. Metabolites of pyrenomycetes: nectriapyrone, an antibiotic monoterpeneoid. *Tetrahedron Letters* 19, 1655–1658.

Qui J.A., L.A. Castro-Concha, K. Garcia-Sosa, L.M. Pena-Rodriguez and M.L. Miranda-Ham, 2009. Differential effects of phytotoxic metabolites from *Alternaria tagetica* on *Tagetes erecta* cell cultures. *Journal of General Plant Pathology* 75, 331–339.

Upadhyay, R.K. and K.J. Mukerji, 1997. Toxins in Plant Disease Development and Evolving Biotechnology. New Delhi, India, Oxford & IBH Publishing Co. Pvt. Ltd.,. 777 pp.

Venkatasubbaiah P., C.G. van Dyke and W.S. Chilton, 1991. Phytotoxins produced by *Pestalotiopsıs oenotherae,* a pathogen of evening primrose. *Phytochemistry* 30, 1471–1474.

Yoder O.C., 1980. Toxins in pathogenesis. *Annual Review of Phytopathology* 18, 103–129.

Yürüt H.A., Ã. Erkal and M. Gürer, 1994. Hazelnut diseases in Bolu, Zonguldak and Bartin. In: *9th Congress of the Mediterranean Phytopathological Union*, 18–24 September *1994,* Kuşadasi-Aydin, Turkey. *Turkish Phytopathological Society Publications* No.7, 417–-419.

Accepted for publication: February 1, 2011