Phytopathol. Mediterr. (2001) 40, Supplement, S417–S422

Interactions between three fungi associated with esca of grapevine, and their secondary metabolites[(1)]

Lorenzo Sparapano, Giovanni Bruno and Alessandra Campanella

Dipartimento di Biologia e Patologia vegetale, Università, Via G. Amendola 165/A, 70126 Bari, Italy

Summary. The effect of the culture filtrates, crude organic extracts of culture filtrates, metabolites such as scytalone, pullulan and oligosaccharides produced by three fungi associated with esca, *Phaeomoniella chlamydospora* (*Pch*), *Phaeoacremonium aleophilum* (*Pal*) and *Fomitiporia punctata* (*Fop*), on the growth of the fungi themselves, was studied *in vitro*. At 1:1 dilution, the culture filtrates of *Pal* and *Pch* inhibited *Fop* completely, whereas at 1:2 dilution they only increased *Fop* growth latency. *Fop* was not inhibited by crude organic extracts of *Pal* or *Pch*. Growth of *Pal* was slightly stimulated at the lowest concentrations of *Pch* crude extracts, whereas it was inhibited at the highest concentration. Scytalone (at 1 mg ml^{-1}), pullulan (at 0.2 mg ml^{-1}) and oligosaccharides up to 2.5 kDa (2 mg ml^{-1}) did not affect the radial growth of *Fop*.

Key words: esca-associated fungi, secondary metabolites, fungal interactions, antagonism.

Introduction

Several phytotoxic metabolites have been purified from culture filtrates of *Phaeomoniella chlamydospora* (W. Gams *et al.*) Crous & W. Gams, *Phaeoacremonium aleophilum* W. Gams *et al.* and *Fomitiporia punctata* (Fr.) Murrill, three fungi associated with esca and with brown streaking of vinewood (Mugnai *et al.*, 1999; Sparapano *et al.*, 2000, 2000a; Graniti *et al.*, 2001). These metabolites are several forms of α-glucans (pullulans) produced by *Pch* and *Pal,* and two naphthalenone pentaketides (scytalone and isosclerone) produced by *Pal* and *Pch* (Amalfitano *et al.*, 2000; Bruno *et al.,* 2000; Evidente *et al.*, 2000; Sparapano *et al.*, 2000b; Tabacchi *et al.*, 2000). The present paper reports on some *in vitro* experiments to study the effect that the secondary metabolites produced by these fungi have on the fungi themselves.

Materials and methods

Fungal strains and culture conditions

Stock cultures of *P. chlamydospora* strain PVFi56 (University of Florence, Italy) (CBS 229.95), *P. aleophilum* strain PVFi69 (University of Florence, Italy) (CBS 631.94) and *F. punctata* strain DBPV-1 (University of Bari, Italy) isolated from grapevines in Italy were maintained on slants of malt agar (MA) or potato-sucrose agar (PSA) at 4°C.

Fungal strains were grown in stationary cultures in 1-l Roux flasks containing 150 ml Czapek medium (Sparapano *et al.*, 2000b) amended with 0.1% yeast and 0.1% malt extract (CMB) (pH 6.8),

[(1)] Dedicated to Prof. A. Graniti on the occasion of his 75th birthday

Corresponding author: L. Sparapano
Fax: +39 080 5442906
E-mail: sparlor@agr.uniba.it

using 5 ml suspensions of three 10-day-old cultures from each fungus in 50 ml sterile water. The flasks were incubated at 25°C in darkness for 28 days. At harvest, the mycelial mat was removed by filtration on Miracloth (Calbiochem, La Jolla, CA, USA) and on Whatman No. 1 filter paper (Whatman, Maidstone, UK).

Antimicrobial assay

The three fungi were also grown on MA plates at 25°C for two weeks. Plugs (3 mm diam.) were aseptically removed from actively growing colonies and transferred, singly or in groups of two or three fungi, to MA plates (Sparapano *et al.*, 2000a). Moreover, an agar slice was cut from the edge of a 21-day-old colony of *Pal* and transferred to a plate of MA inoculated with *Fop*.

Culture filtrates (CFs), crude organic extracts (COEs) with ethyl acetate (Sparapano *et al.*, 2000b), purified pullulan produced by *Pch*, pullulan hydrolysates up to 2.5 kDa (Bruno *et al.*, 2001), and scytalone (Evidente *et al.*, 2000) were assayed on Petri dishes containing MA for their activity towards the three fungi.

CFs of each strain were added at dilution ratios of 1:1 and 1:2 to the MA medium before the bioassays. Each species was grown singly in Petri dishes containing CFs collected from the liquid cultures of the other single species.

The COEs were first dissolved in sterile distilled water and tested at concentrations ranging from 2 to 0.5 mg ml^{-1}. Pure compounds were assayed at concentrations up to 2 mg ml^{-1}. Each plate was seeded with a 5-mm-diam. mycelial plug of 7-day-old *Fop* or 14-day-old *Pch* or *Pal* cultures. COEs and pure substances were assayed in Petri dishes with MA, inoculated with each fungal strain and containing a cellulose thimble (Whatman 10×15 mm) filled with either 0.5 ml COE or a pure compound solution. An equal number of control plates received 0.5 ml of distilled sterile water in the same way.

The colony diameter of each fungus was measured once every three days for five weeks. Three replicates of five plates each were used for each fungus at each treatment and the inoculated plates were incubated at 25°C in the dark. Mean values ± standard deviation of fungal colony diameter were calculated.

Results

In dual cultures, *Fop* and *Pal* grew antagonistically (Fig. 1A). The antagonism of *Pal* vs. *Fop* was clearly shown on agarised plates where *Fop* colonies were grown together with an agar slice cut from the edge of a *Pal* colony (Fig. 1B and 1C). This experiment suggested that the antagonistic effect of *Pal* against *Fop* was due to the production by *Pal* of substances that freely spread through the medium and reached the *Fop* colony. In triple culture (Fig. 1D), *Fop*, *Pal* and *Pch* also grew antagonistically.

At a 1:1 dilution (CFs:MA), the CFs of *Pal* and of *Pch* completely inhibited *Fop,* whereas at a higher dilution (1:2) they only delayed the onset of *Fop* growth latency (Fig. 2A and 2B). *Fop* started growing 21 days after its inoculation on plates containing diluted (1:2) *Pal*-CFs, and 6 days after its inoc-

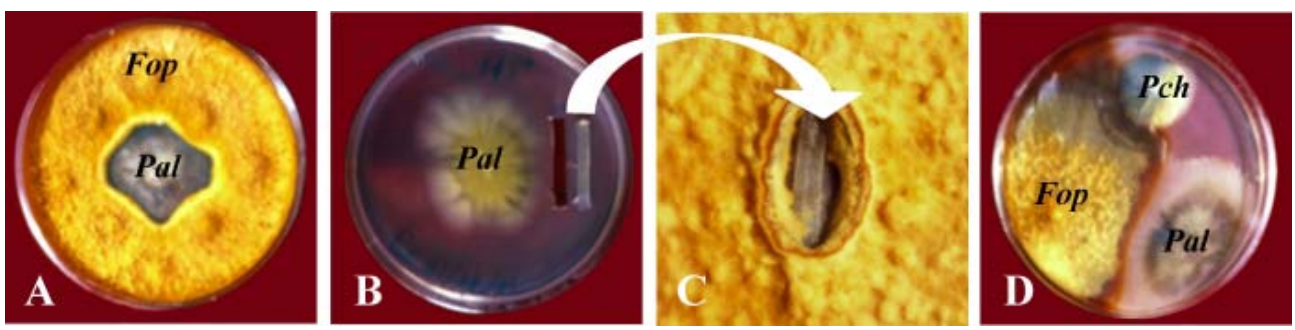

Fig. 1. Antagonism of *Phaeoacremonium aleophilum* (*Pal*) against *Fomitiporia punctata* (*Fop*): dual culture of *Fo*p and *Pal* (A); agar slice cut from the edge of a 21-day-old colony of *Pal* (B) and transferred to a plate inoculated with *Fop* (C); plates of 21-day-old triple cultures of *Fop, Pal* and *Phaeomoniella chlamydospora* (*Pch*) (D).

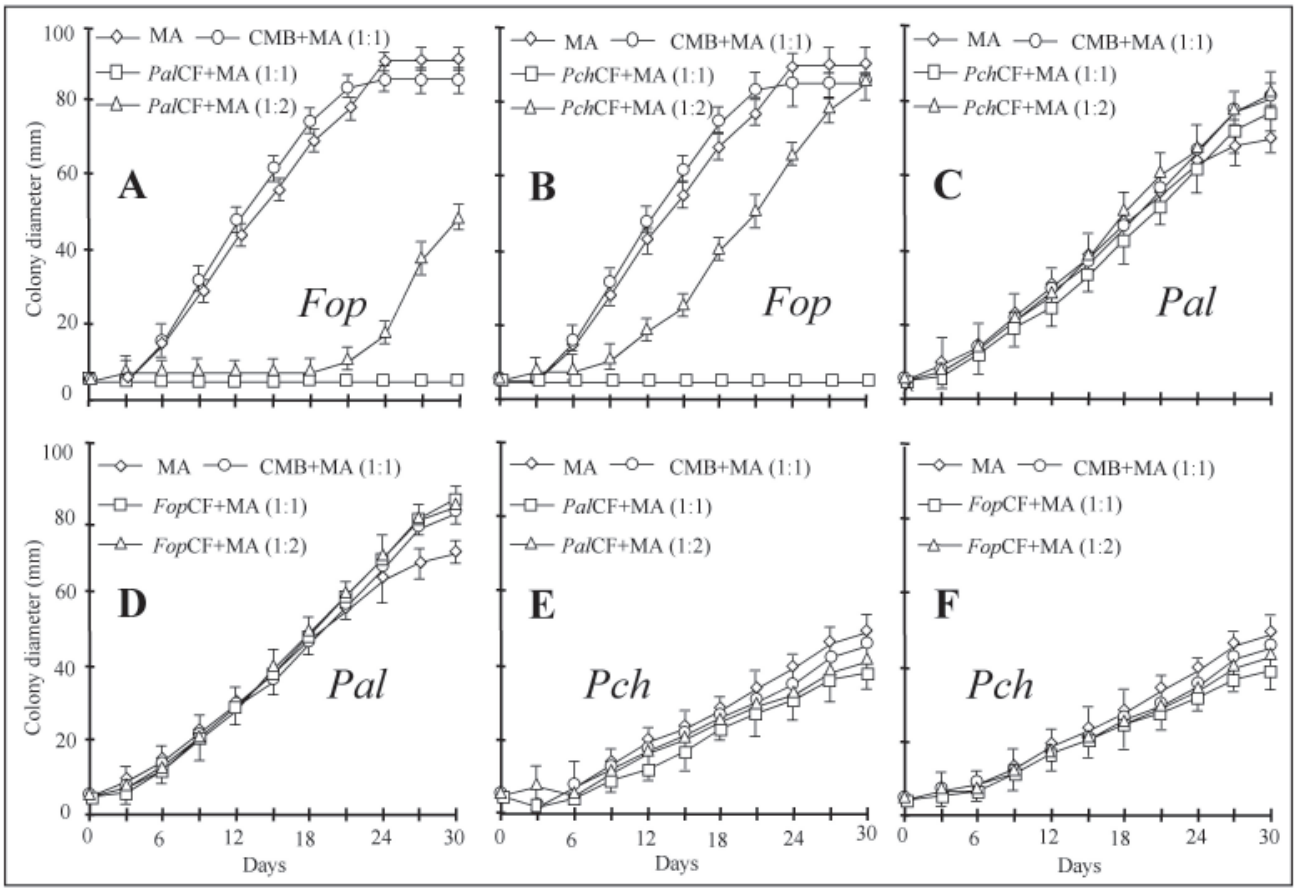

Fig. 2. Effect of culture filtrates (CFs) of each fungus, diluted 1:1 or 1:2 with malt-agar (MA), on growth rates of *Fomitiporia punctata* (*Fop*), *Phaeoacremonium aleophilum* (*Pal*) and *Phaeomoniella chlamydospora* (*Pch*): *Fop* with *Pal*CF (A) or *Pch*CF (B); *Pal* with *Pch*CF (C) or *Fop*CF (D); *Pch* with *Pal*CF (E) or *Fop*CF (F). Controls: malt agar (MA) and amended Czapek medium (CMB) diluted 1:1 with MA. Standard error bars are shown.

ulation on plates containing diluted (1:2) *Pch*-CFs. When *Pal* was grown with CFs of either *Pch* or *Fop* at high or low dilution, its growth was not affected (Fig. 2C and 2D), nor was that of *Pch* grown with the CFs of *Pal* or *Fop* (Fig. 2E and 2F). These experiments demonstrated that *Pal* and *Pch* in liquid stationary culture produced bioactive substances that caused growth inhibition or fungistasis. The CMB medium used for the liquid cultures of all three fungi did not affect fungal growth since growth rates in MA or in MA with CMB were quite similar. The CMB medium did not affect fungal growth in the control plates.

The effect of COEs from the CFs of each fungus on growth of the three fungi is shown in Fig. 3. *Fop* was not inhibited by COEs of *Pal* or *Pch* (Fig. 3A and 3B). *Pal* was slightly stimulated by the lowest concentration of the *Pch*-COEs, but was inhibited by the highest COE concentration (Fig. 3C and 3D). *Pch* was not affected by the COEs of *Pal* or *Fop* (Fig. 3E and 3F). These results suggest that the antagonism of *Pal* and *Pch* was due to hydrophilic substances in the CFs which cannot be extracted by an organic solvent such as ethyl acetate.

Fop was grown in MA medium amended with pullulan from *Pch*-CFs or with oligomers obtained by enzymatic digestion of *Pch*-pullulan. The oligosaccharides exhibited a molecular weight not exceeding 2.5 kDa. Scytalone, the aromatic compound produced by *Pal* and *Pch,* was also tested

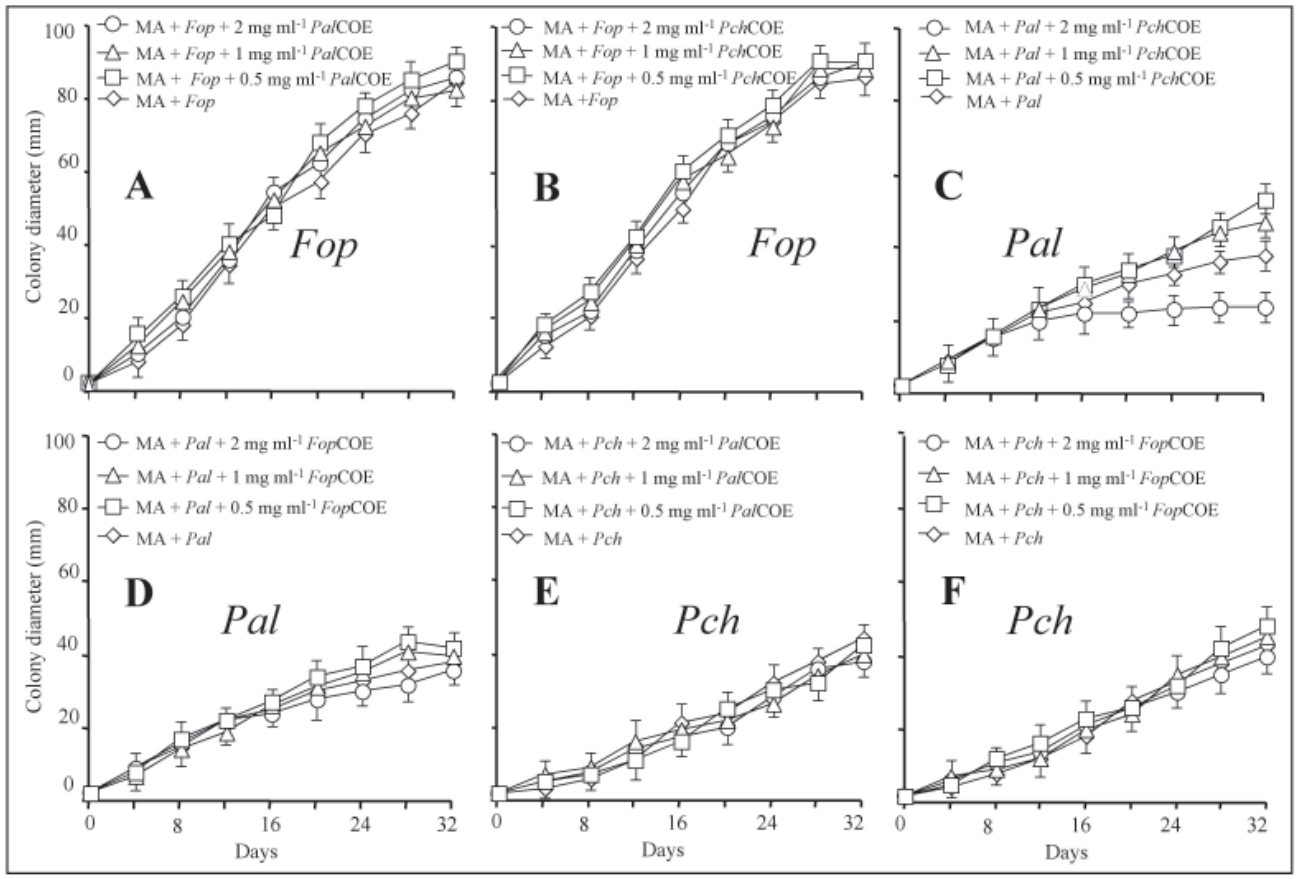

Fig. 3. Effect of crude organic extracts (COEs) of culture filtrates (CFs) of *Fomitiporia punctata* (*Fop*), *Phaeoacremonium aleophilum* (*Pal*) and *Phaeomoniella chlamydospora* (*Pch*) on the growth rate of each fungus: *Fop* with *Pal*COE (A) or *Pch*COE (B); *Pal* with *Pch*COE (C) or *Fop*COE (D); *Pch* with *Pal*COE (E) or with *Fop*COE (F). Control: malt agar (MA). Cultures were grown in MA containing a cellulose thimble filled with 0.5 ml COE solution at concentrations ranging from 2 to 0.5 mg ml^{-1}. Standard error bars are shown.

for bioactivity. Scytalone at 1 mg ml^{-1} and pullulan at 0.2 mg ml^{-1} did not affect radial growth of *Fop* (Fig. 4). Oligomers also did not affect the growth of *Fop*. *Fop* colonies only showed fast colonisation of the substrate, submerged hyphae, surface mycelium, and early hyphal pigmentation.

Discussion

In previous inoculation experiments, an antagonistic effect of *Pal* toward *Fop* had been observed both *in vitro* and *in planta* (Sparapano *et al.*, 2000a). Brown wood-streaking caused by *Fop* in inoculated grapevines was not affected by *Pch,* whereas it was stopped by *Pal*.

This study provides new information on the production of antagonistic compounds in liquid cultures by *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum,* two fungi commonly associated with esca and esca related diseases of grapevine. The findings indicate that *Fop* can be inhibited or reduced by secondary metabolites of *Pal* or *Pch,* even if it is grown on an optimal substrate (MA). In contrast, the same metabolites did not interfere substantially with the growth of each producer fungus (*Pal* or *Pch*).

Most of the plant necrotrophic pathogens produce metabolites toxic to plants, and they may induce the characteristic symptoms of diseases. Our data show that *Pch*, *Pal* and *Fop* produce second-

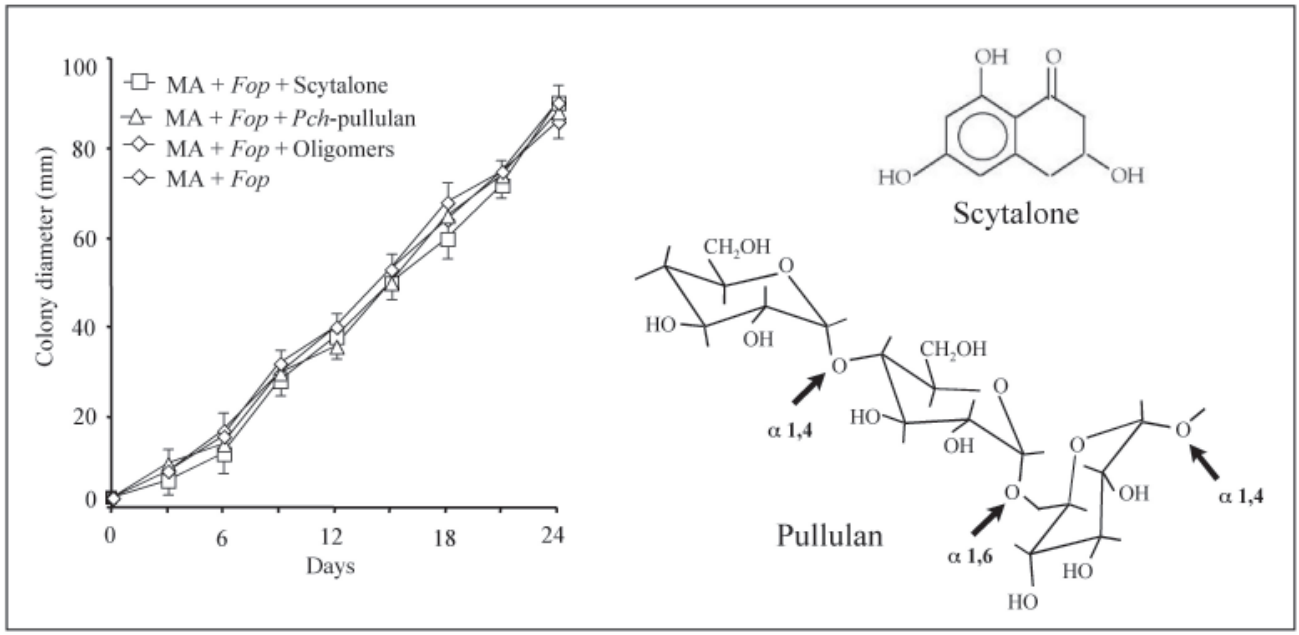

Fig. 4. Effect of scytalone (1 mg ml^{-1}), pullulan produced by *Phaeomoniella chlamydospora* (*Pch*) (0.2 mg ml^{-1}) and oligomers obtained by enzymatic digestion of *Pch*-pullulan (2 mg ml^{-1}) on the growth of *Fomitiporia punctata* (*Fop*). Standard error bars are shown.

ary metabolites which are presumably involved in symptom expression in infected grapevines (Sparapano *et al*., 2000a; 2000b) and they also produce substances which elicit fungitoxic or fungistatic activity against *Fop*.

CFs of *Pal*, and to a lesser extent of *Pch,* caused complete inhibition of *Fop*, but *Pal* or *Pch* were not affected by CFs of *Fop*. COEs obtained from the CFs of each fungus did not show any antimicrobial activity. The finding that *Fop* was able to degrade and utilize fungal metabolites such as pullulans and scytalone confirmed the results mentioned above. Both pullulan and scytalone showed no antifungal activity against *Fop*. The oligosaccharides (≤2.5 kDa) obtained by enzymatic digestion of *Pch*-pullulan stimulated *Fop* growth.

The antimycotic effect of CFs of *Pch* and *Pal* suggests that the substances inhibiting *Fop* were hydrophilic and chemically different from other phytotoxic compounds produced by those fungi. The fungal mycelium in its natural habitat is a heterogenous complex, dynamic entity in which phases of establisment, exploration and explotation of resources and reproduction occur in overlapping sequence. In the antagonism of *Pal* and *Pch* against *Fop* we can assume that mutual exclusion and replacement may occur as a result of a more direct physiological challenge between individuals so that access to one individual's domain was prevented by active defence, or brought about by active mechanisms capturing secondary resources.

In conclusion, the study indicates that antimicrobial compounds, produced in culture by the two tracheiphilous fungi *Pal* and *Pch,* exhibited growth inibition activity against *Fop*. *Fop* growth was affected by secondary metabolites of *Pal* or *Pch*, but *Pal* and *Pch* did not interfere with each other. These metabolites may be involved in the antagonistic activity of *Pal* and *Pch* against *Fop* during their wood colonisation in dual or triple combination, and can help in understanding on how these fungi interact each other and with the host tissue. Studies of interaction patterns may help to provide an insight of the processes involved in how the mycelium of each fungus functions and how the fungi co-ordinate their activities.

The involvement of these metabolites in the pathogenesis of esca and related syndromes on grapevine remains to be investigated.

Acknowledgements

This investigation was supported by a grant from the Italian Ministry of University and Scientific Technological Research (MURST), Rome (PRIN 1999-2001).

Literature cited

Amalfitano C., L. Sparapano, G. Surico and A. Evidente, 2000. Le fitotossine e le fitoalessine coinvolte nel "mal dell'esca" della vite. In: *V Convegno Giornate di Chimica delle Sostanze Naturali*, 21–23 Giugno 2000, Napoli, Italy (abstract).

Bruno G., L. Sparapano, C. Amalfitano, A. Evidente and A. Graniti, 2000. Detection of phytotoxic fungal metabolites in xylem sap of esca-affected grapevines. In: *5th International Congress of European Foundation for Plant Pathology*, 18–22 September 2000, Taormina, Italy (abstract).

Bruno G., F. Delben. A. Forabosco, G. Liut and L. Sparapano, 2001. Analisi di polisaccaridi prodotti dalle specie fungine *Aureobasidium pullulans* e *Cryphonectria parasitica*. In: *XV Convegno Italiano di Scienza e Tecnologia delle Macromolecole*, 24–27 Settembre 2001, Trieste, Italy (abstract).

Evidente A., L. Sparapano, A. Andolfi and G. Bruno, 2000. Two naphthalenone pentaketides isolated from liquid cultures of *Phaeoacremonium aleophilum*, a fungus associated with esca disease syndrome. *Phytopathologia Mediterranea* 39, 162–168.

Graniti A., L. Sparapano and G. Bruno, 2001. Alcuni progressi degli studi sulla patogenesi del "mal dell'esca" e delle "venature brune del legno" della vite. *Informatore Fitopatologico* 51(5), 13–21.

Mugnai L., A. Graniti and G. Surico, 1999. Esca (black measles) and brown wood-streaking: two old and elusive diseases of grapevines. *Plant Disease* 83, 404–418.

Sparapano L., G. Bruno, C. Ciccarone and A. Graniti, 2000. Infection of grapevines with some esca-disease associated fungi. I. *Fomitiporia punctata* as a wood-decay inducer. *Phytopathologia Mediterranea* 39, 46–52.

Sparapano L., G. Bruno, C. Ciccarone and A. Graniti, 2000a. Infection of grapevines with some esca-disease associated fungi. II. Interaction among *Fomitiporia punctata*, *Phaeoacremonium chlamydosporum* and *P. aleophilum*. *Phytopathologia Mediterranea* 39, 53–58.

Sparapano L., G. Bruno and A. Graniti, 2000b. Effects on plants of metabolites produced in culture by *Phaeoacremonium chlamydosporum*, *P. aleophilum* and *Fomitiporia punctata*. *Phytopathologia Mediterranea* 39, 169–177.

Tabacchi R., A. Fkyerat, C. Poliart and G.M. Dubin, 2000. Phytotoxins from fungi of esca of grapevine. *Phytopathologia Mediterranea* 39, 156–161.

Accepted for publication: November 30, 2001