Interactions between three fungi associated with esca of grapevine, and their secondary metabolites

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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 vine-wood (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),...
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 x 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-
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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-CF 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.
for bioactivity. Scytalone at 1 mg ml\(^{-1}\) and pullulan at 0.2 mg ml\(^{-1}\) did not affect radial growth of \textit{Fop} (Fig. 4). Oligomers also did not affect the growth of \textit{Fop}. \textit{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 \textit{Pal} toward \textit{Fop} had been observed both \textit{in vitro} and \textit{in planta} (Sparapano et al., 2000a). Brown wood-streaking caused by \textit{Fop} in inoculated grapevines was not affected by \textit{Pch}, whereas it was stopped by \textit{Pal}.

This study provides new information on the production of antagonistic compounds in liquid cultures by \textit{Phaeomoniella chlamydospora} and \textit{Phaeoacremonium aleophilum}, two fungi commonly associated with esca and esca related diseases of grapevine. The findings indicate that \textit{Fop} can be inhibited or reduced by secondary metabolites of \textit{Pal} or \textit{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 (\textit{Pal} or \textit{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 \textit{Pch}, \textit{Pal} and \textit{Fop} produce second-
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 antmycotic 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 establishment, exploration and exploitation 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 inhibition 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


Accepted for publication: November 30, 2001