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Infection of grapevines by some fungi associated with esca. II. Interaction among *Phaeoacremonium chlamydosporum*, *P. aleophilum* and *Fomitiporia punctata*

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Summary. Cross-inoculation experiments were designed to study the interaction among the three fungi, *Phaeoacremonium chlamydosporum (Pch), P. aleophilum (Pal)* and *Fomitiporia punctata (Fop)* most commonly associated with esca of grapevine. The experiments were carried out in southern Italy on grapevines cv. Italia and Matilde, and the inoculations were performed in January 1999 by infecting trunks (cv. Italia) and branches (cv. Matilde) through wounds. *Fop*, *Pch* and *Pal* were inoculated singly or in groups of two or three in all possible combinations. *Pch*, *Pal* or both were inoculated above or below the inoculation site of *Fop*. From the results obtained in the first eight months of experiments planned for three years, the following conclusions can be drawn. All fungi inoculated singly spread along the woody tissue and caused brown streaking downward and upward along the trunk and branches of inoculated vines. On the basis of internal symptoms, vines cv. Italia were more susceptible than those cv. Matilde; in particular, the brown wood-streaking induced by *P. aleophilum* was much more severe on cv. Italia. Co-infection with the two tracheiphilous species of *Phaeoacremonium* caused wood gummosis and discoloration, whereas the lignicolous basidiomycete *F. punctata* alone caused wood deterioration and decay (white rot). Severity of internal symptoms, assessed as extent and colour of the discoloured wood, varied with the growth and interaction of the inoculated fungi. The wood discoloration caused by *F. punctata* was not hampered by *P. chlamydosporum*, but it was always limited by *P. aleophilum*. A similar interaction was observed *in vitro* with cultures of the three fungi together, which showed a marked antagonistic effect of *P. aleophilum* against *F. punctata*. Although not consistent, foliar symptoms (interveinal and marginal chlorotic areas) developed within six months on 'Matilde' vines co-inoculated with either species of *Phaeoacremonium,* or within three months after a syringe containing a liquid culture of *P. chlamydosporum* was inserted into current-season 'Italia' shoots.

Key words: *Phaeoacremonium chlamydosporum*, *P. aleophilum*, *Fomitiporia punctata*, esca, grapevine, cross-inoculation.

Introduction

Esca of grapevine is a complex disease whose symptoms may develop from the concomitant action of several factors (Mugnai *et al.*, 1999; Graniti *et al.*, 1999, and in this issue). The consistent isolation of fungi from discoloured or decayed wood of esca-diseased grapevines indicates a close relation between particular stages of wood deterioration and individual species of fungi, some of which have been suspected to act as precursors of wood rot (Larignon and Dubos, 1997).

Starting from January 1999, cross-inoculation experiments were performed in southern Italy to study interactions among the three fungi most commonly associated with esca: *Phaeoacremonium*

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chlamydosporum W. Gams *et al.* ([1]), *P. aleophilum* W. Gams *et al.* and *Fomitiporia punctata* (Fr.) Murrill. The results of the first 8 months of experiments, planned to last for 3 years, are reported here.

Materials and methods

Fungal strains

P. chlamydosporum (*Pch*) strain PVFi56 (CBS 229.95), *P. aleophilum* (*Pal*) strain PVFi69 (CBS 631.94) and *F. punctata* (*Fop*) strain 1 (DBPV, University of Bari, Italy), isolated from grapevines showing esca symptoms in Italy, were grown on slants or on plates of malt agar (MA) at 23°C in darkness.

Wound-inoculation of standing grapevines

Grapevines showing neither foliar symptoms of esca nor any sign that they might have wood deterioration (white rot or brown wood-streaking) were selected in 1998 in two vineyards, both located in Apulia, southern Italy: a 6-year-old vineyard cv. Italia near Bari, and a 9-year-old vineyard cv. Matilde near Andria (province of Bari).

Starting from the beginning of January 1999, inoculations with the above fungi were performed by inserting a segment of wooden tooth-picks colonised with one of the fungi in 3-mm diam., 1-cm-deep holes drilled radially through the trunks of 'Italia' or the branches of 'Matilde' vines. *Fop, Pch* and *Pal* were inoculated singly or in groups of two or three in all possible combinations. For dual inoculations, one fungus was inoculated 2 cm above or below the other. For combined inoculations of all three fungi, *Pch* and *Pal* were inoculated about 2 cm above or below the inoculation site of *Fop*. An equal number of control vines received a sterile tooth-pick inserted in the same way**.** The inoculation wounds were protected with moist cotton and Parafilm tape. The experiment was run with 3 replicates per fungus and per combination of fungi. For each type of inoculation, 32 vine plants were used.

In the first year of the experiment (1999), samples from the 3 types of differently inoculated and from non-inoculated control trunks and branches were taken randomly 3, 5, 6 and 8 months after inoculation. Transverse and longitudinal sections of the samples were made, the extent of wood discoloration was recorded, and five small fragments of wood per sample were placed on MA plates for fungal re-isolation.

Wound-inoculation of young grapevines

A deep V-shaped cut was made on the stem of potted, 3-year-old nested grapevines (rootstock 'Paulsen1103' grafted with 'Italia'), about 30 cm above the roots, below the grafting point. A plug from a culture of *Pch*, *Pal* or *Fop* was placed on the wound, which was then protected as above. Each fungus was inoculated on 5 vines, and these were placed in a greenhouse at 22±5°C and 70-90% RH, together with 5 control vines, treated in the same way with a plug of sterile MA. At the end of the experiment (August 1998, 6 months after inoculation) the stem of every vine was sectioned longitudinally and transversely, the extent of the discoloured wood was measured, and re-isolations were performed.

Syringe-infiltration of current-season shoots

At the end of June 1999, syringes with thin (0.5 diam. x 6 mm) insulin-type needles, containing 10 ml of an 8-day-old culture of one of each fungal strain on modified Czapek medium (Sparapano *et al.,* this issue), were inserted into current-season shoots of standing 6-year-old grapevines cv. Italia (5 shoots per strain). Syringes containing either sterile water or modified Czapek medium were used for an equal number of controls. The culture liquid was not injected into the woody tissue, but the syringes were left *in situ* for three months in order to allow a slow continuous absorption. Occurrence of foliar symptoms on the shoots was recorded every two weeks until the end of September, 1999.

Antagonism in culture

The three fungi were 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 two or three fungi together, to plates of a modified Czapek medium (Sparapano

([1]) This species has been redisposed in the new genus *Phaeomoniella* Crous et W. Gams as *P. chlamydospora* (W. Gams *et al.*) Crous & W. Gams. See Crous and Gams in this issue.

et al., this issue). The plugs were placed at the centre of the plates when the fungi were singly grown, or at the corners of a triangle, 2 cm from the centre, when they were grown together. The experiment was run with 10 replicates. All plates were sealed with Parafilm and incubated at 25°C in the dark. Growth of the colonies was measured every three days for three months.

Results

Wound-inoculation of standing grapevines

Wood streaking. All grapevines showed dark-brown wood-streaking extending from the inoculation wounds downward and upward along the trunk or the branches. The columns of discoloured wood in the inoculated vines soon became significantly longer, thicker and darker than those in the control vines.

Severity of internal symptoms, assessed as length and colour of the discoloured wood, varied with the growth and interactions of the inoculated fungi. Cross sections through trunks of cv. Italia or branches of cv. Matilde inoculated with *Pch* or *Pal* or both showed small, dark-brown or black spots that in longitudinal section appeared as streaks or columns of the same colour. An abundant secretion of gums by the parenchyma cells and of exopolysaccharides and melanins by the pathogens, which also filled the lumen of the xylem elements with viscous masses, was observed in the discoloured wood of vines inoculated with *Pch* or *Pal*.

The discoloured woody tissue that developed in the wounded but not inoculated control vines did not show gummosis or formation of slimy masses.

The two grapevine cultivars showed differing susceptibility to the fungi: brown wood-streaking in the vines cv. Italia was more severe than that shown by the more resistant vines cv. Matilde (Fig. 1).

When the fungi were single-inoculated on trunks of grapevines cv. Italia, a substantial (more than 40 mm long) wood discoloration was produced 5 months after inoculation with *Pch* or *Pal*, and 6 months after inoculation of *Fop*. Eight months after inoculation the brown wood-streaking was longer with *Pch* (195 mm) than with *Pal* (152 mm), but was limited with *Fop* (67 mm), while the discoloration shown by the control vines was scanty (27 mm) (Fig. 1 A).

The extent of brown wood-streaking in inoculated branches of cv. Matilde, measured eight months after inoculation, was considerably greater with *Pch* (109 mm) than with *Fop* (57 mm), whereas with *Pal*, at 40 mm, it was not much longer than the controls (Fig. 1A).

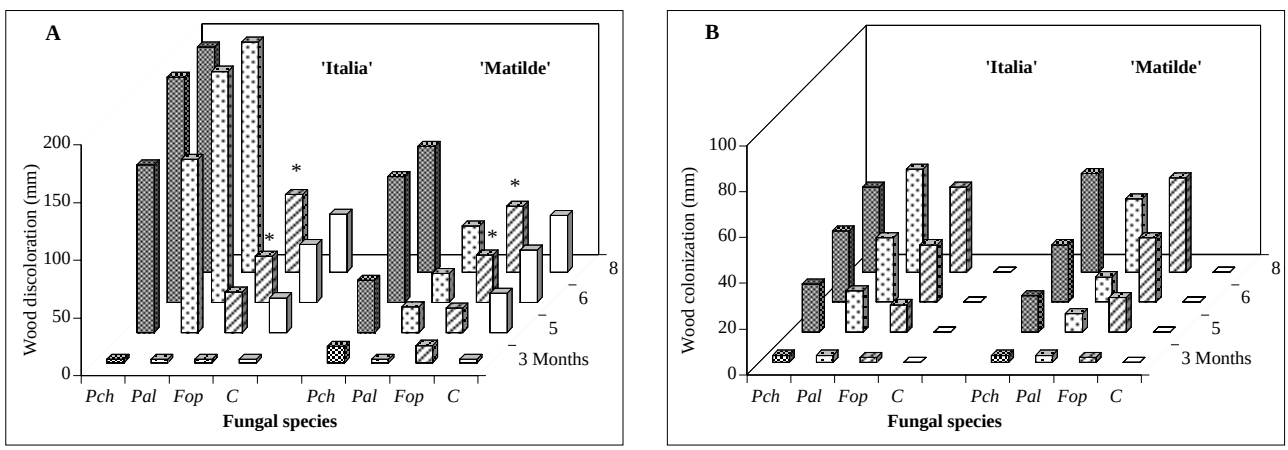

Fig. 1. A. Length of wood discoloration in trunks of 6-year-old grapevines cv. Italia or branches of 9-year-old grapevines cv. Matilde, 3 to 8 months after inoculation with *Phaeoacremonium chlamydosporum* (*Pch* ■), *P. aleophilum* (*Pal* ▩) and *Fomitiporia punctata* (*Fop* ▨); control (*C* □). Data are the means of 5 replicates. An asterisk (*) indicates the appearance of the first signs of spongy wood decay (white rot). B. Corresponding extent of wood colonisation by the above fungi, based on successful re-isolation of the pathogens.

In comparison with the wood discoloration induced when fungi were single-inoculated, dual inoculations *Pch –Pal* or *Pch–Fop* did not change the development of brown wood-streaking substantially. The interaction between *Pal* and *Fop* on the other hand produced a marked reduction in the course and length of the *Fop*-induced wood discoloration. A similar competitive interaction of *Pal* vs. *Fop* was also evident when all three fungi were inoculated on the same grapevine (Fig. 2).

Wood decay. Six months after inoculation, wood-decay (white rot) was actively developing all around the infection dowels in the trunks of cv. Italia and branches of cv. Matilde inoculated with *Fop* (Fig. 1A), whereas in the controls healing processes had more or less to a certain extent begin to somewhat sealed the inoculation wounds.

Foliar symptoms. Although not consistently, foliar symptoms (chlorotic spots and marginal necrotic areas) developed on current-season shoots of 'Matilde' vines branch-inoculated with both species of *Phaeoacremonium*, starting six months after inoculation. On the inoculated branches, the shoots showing symptoms were those closest to the inoculation site. No foliar symptoms were found in vines inoculated with *Fop* alone or in dual inoculations with *Pch* or *Pal*, or in the controls.

Wood colonisation. Fig. 1B shows that all three fungi inoculated, *Pch, Pal* and *Fop,* were able to colonise the woody tissue of the grapevine cultivars 'Italia' and 'Matilde'. Each fungus was re-isolated from the streaks or columns of discoloured wood formed in the inoculated vines, whereas from the brown wood streaks of control vines no species of *Phaeoacremonium* or *Fomitiporia* was isolated. Except at the first stages of infection, fungal colonisation was significantly more limited in its extent than the corresponding wood discoloration.

Wound-inoculation of young grapevines

The results of the greenhouse experiment on potted 3-year-old grapevines cv. Italia indicated that extensive wood discoloration (brown wood-streaking) and sporadic foliar symptoms, i.e. irregularly shaped chlorotic spots followed by necrosis, occurred starting five months after stem-inoculation with *Pch*. Control vines showed only restricted wood discoloration and no foliar symptoms.

Syringe-infiltration of current-season shoots

Chlorotic spots, reddening and areas of necrotic tissue developed on the leaves of current-season shoots of grapevines cv. Italia after three-month absorption of *Pch* culture liquid, gradually supplied

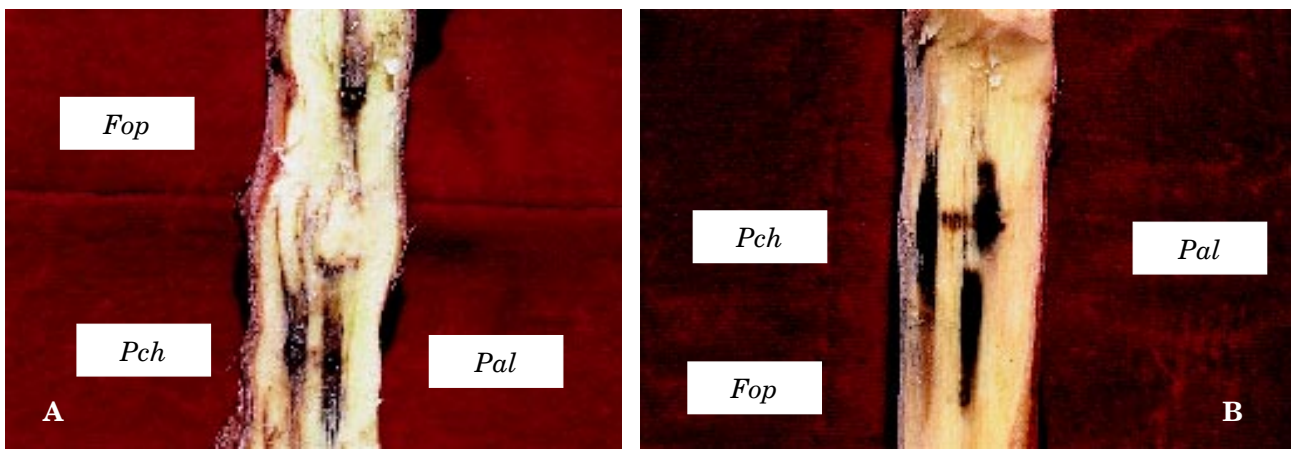

Fig. 2. A. A longitudinal section of a trunk of a 6-year-old grapevine cv. Italia, 8 months after inoculation with *Fomitiporia punctata* (up), *Phaeoacremonium chlamydosporum* (bottom, on the left) and *P. aleophilum* (bottom, on the right). The antagonistic effect of *P. aleophilum* vs. *F. punctata* did not allow the latter to cause a downward discoloration of the woody tissue. B. A longitudinal section of a branch of a 9-year-old grapevine cv. Matilde, 8 months after inoculation with *F. punctata* (bottom), *P. chlamydosporum* (up, on the left) and *P. aleophilum* (up, on the right); The brown wood discoloration caused by *F. punctata* was discontinued upward by the antagonistic effect of *P. aleophilum,* whereas *P. chlamydosporum* caused brown wood streaking in both directions.

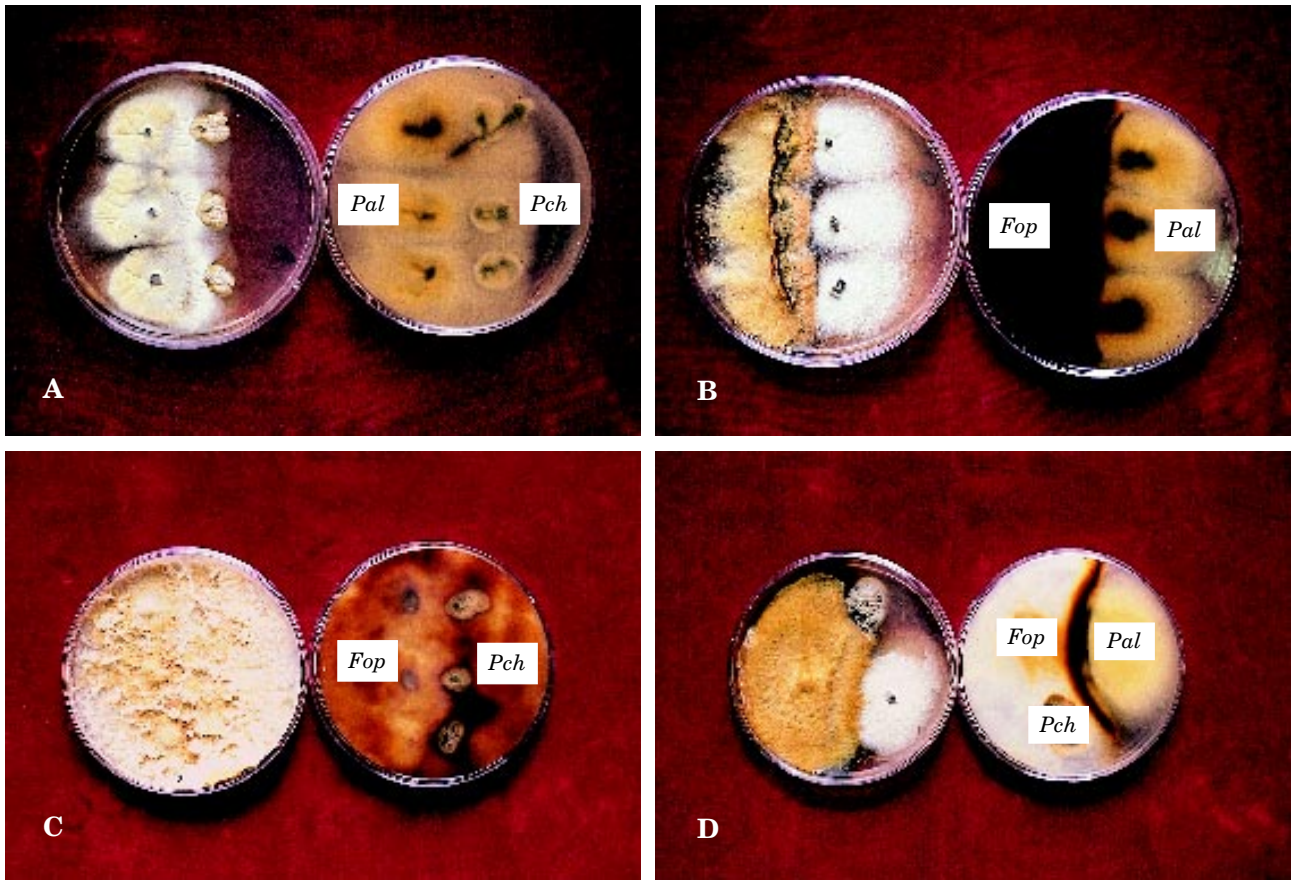

Fig. 3. Plates of 21 to 28-day-old dual cultures (on the left, obverse; on the right, reverse) of: A. *Phaeoacremonium aleophilum* (*Pal*) vs. *P. chlamydosporum* (*Pch*). B. *Fomitiporia punctata* (*Fop*) vs. *Pal*. C. *Fop* vs. *Pch*. When all three fungi were grown together (D), the antagonistic effect of *Pal* vs. *Fop* protected *P. chlamydosporum*, enabling it to grow as an individual colony without being overgrown by *F. punctata*. In this figure the reverse of the plate (on the right) is upside-down.

by a syringe inserted into the shoot. Control shoots which absorbed sterile Czapek medium did not show any symptom.

Competition and antagonism *in vitro*

There was evidence of differential competition among *F. punctata* and the two species of *Phaeoacremonium* when they were grown *in vitro* on MA plates.

When single-grown, *Fop* colonies grew faster than either single-grown *Pch* or *Pal* colonies. In dual cultures, *Pch* and *Pal* grew agonistically (Fig. 3A). Conversely, growth of the *Fop* colonies was abruptly cut short when it came near a *Pal* colony. The margin of the *Fop* colonies then turned brown, became thicker, and aerial hyphae formed a ridge-like barrier between the two (Fig. 3B). Adjacent colonies of *Fop* and *Pch* also started growing agonistically, but eventually the colony of *Pch* was completely overgrown by *Fop* mycelium (Fig. 3C). The antagonistic effect of *Pal* against *Fop* was observed also when *Fop* was grown together with both species of *Phaeoacremonium* in triple culture. In this case, the colony of *Pal* in some way "prevented" that of *Pch* from becoming overgrown by the *Fop* mycelium (Fig. 3D).

Discussion

The results of the inoculation experiments indicated that within 8 months of inoculation on the two cultivars of grapevine, both *P. chlamydospo-*

rum and *P. aleophilum* caused relatively long brown wood-streaking and xylem gummosis, while *F. punctata* caused first wood discoloration, then deterioration and decay of the woody tissue (white rot). By the length and severity of internal symptoms, vines cv. Italia were more susceptible than vines cv. Matilde. In particular, the brown wood-streaking caused by *Pal* was much more severe on cv. Italia than on cv. Matilde.

Grapevine infection by both tracheiphilous species of *Phaeoacremonium*, with their hyphae readily growing within the xylem vessels and the consequent wood gummosis and discoloration, had all the features of a real tracheomycosis, while infection by the lignicolous basidiomycete *F. punctata* caused relatively slow but progressive degradation and rot of the woody tissue.

The interaction (Rayner and Webber, 1984) among the three fungi *in vitro* and *in planta* was shown to be competitive for primary resource capture in the case of *Pch* vs. *Pal* : both fungi competed for the substratum though not directly challenging each other; combative in the case of *Fop* vs. *Pch*: *Fop* tended to overgrow *Pch*; and antagonistic in the case of *Pal* vs. *Fop*: *Pal* inhibited the growth of *Fop*. Lastly, the effect of *Fop* on the woody tissue of both grapevine cultivars was significantly limited by *Pal,* but not by *Pch*.

These findings strongly suggest that all three fungi act as primary wood pathogens and that, to invade and to degrade the woody tissue of grapevine, *F. punctata* does not need prior colonisation by "precursor" species of *Phaeoacremonium* (Larignon and Dubos, 1997; Sparapano *et al.*, this issue).

Foliar symptoms developed, although not consistently, on the current-season shoots six months after inoculation of *Pal* or *Pch* on the branches of grapevines cv. Matilde. Similar symptoms were produced on shoots of cv. Italia after a 3-month-infiltration of culture liquids of *P. chlamydosporum*. This suggests that toxic metabolites are produced by these fungi within the host tissue and may have a role in the expression of esca symptoms (see Sparapano *et al.*, this issue).

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