

Three-year observation of grapevines cross-inoculated with esca-associated fungi

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Summary. Cross-inoculation experiments were carried out in southern Italy (Apulia) on grapevines cv. Italia and Matilde in order to study interactions among the white rot fungus *Fomitiporia punctata* (*Fop*) and two tracheiphilous hyphomycetes, *Phaeoacremonium aleophilum* (*Pal*) and *Phaeomoniella chlamydospora* (*Pch*), commonly associated with esca and brown wood-streaking of grapevine. In January 1999 the three fungi were inoculated through wounds on the trunks and spurs of cv. Italia, and branches and spurs of cv. Matilde. *Pch*, *Pal* and *Fop* were inoculated singly or in groups of two or three in all possible combinations. From the results so far obtained, the following conclusions can be drawn: 1. all three fungi are pathogenic; 2. inoculation of the above fungi on standing grapevines produces symptoms on trunks, shoots, leaves and berries that are reminiscent of those shown by esca-affected vines in the vineyard. In particular, symptoms in the wood (brown streaking, gummosis or “black goo”, and wood decay or white rot), and on the leaves (various forms of chlorosis, reddening and necrosis, distortion of the lamina) and berries (black measles) were reproduced; 3. severity of internal symptoms, assessed as length of brown streaks and intensity of colour change of the infected wood, varied with the growth and interactions between the inoculated fungi; 4. the wood streaking which developed after inoculation with *Pal* or *Pch* on the spurs extended to the supporting shoot or branch, and even to the stem; 5. a non-synergistic, competitive association of the two hyphomycetes, and an inhibition of *Fop* growth by *Pal* was observed *in planta*. A similar marked antagonistic effect of *Pal* against *Fop* was previously shown to occur when examining interactions between the three fungi *in vitro*; 6. the wood discolouration caused by *Fop* was not hampered by *Pch*, but it is always limited by *Pal*; 7. on the basis of internal symptoms, grapevines cv. Italia were more susceptible to the esca fungi than grapevines cv. Matilde.

Key words: *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum*, *Fomitiporia punctata*, esca symptom reproduction, grapevine.

Introduction

Esca of grapevine is a complex disease whose symptoms are thought to arise from the combined effect of several factors (Graniti *et al.*, 1999; Mugnai *et al.*, 1999; Sparapano *et al.*, 2000; Graniti *et al.*, 2001). The consistent isolation of some fungi

from discoloured, and other from decayed wood of esca-diseased grapevines indicates that different fungi are related to particular stages of wood deterioration (Mugnai *et al.*, 1996, 1999; Larignon and Dubos, 1997; Bruno *et al.*, 2000).

Starting January 1999, a cross-inoculation study (Sparapano *et al.*, 2000a) was undertaken in southern Italy to examine interactions between the three fungi most commonly associated with esca: two tracheiphilous hyphomycetes, *Phaeomoniella chlamydospora* (W. Gams *et al.*) Crous & W. Gams and *Phaeoacremonium aleophilum* W. Gams *et al.*,

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and the white rot basidiomycete, *Fomitiporia punctata* (Fr.) Murrill. The results of this study over the last three years are reported here.

Materials and methods

Fungal strains

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 showing esca symptoms in Italy, were grown on slants or plates of malt agar (MA) at 23°C in darkness.

Wound-inoculation of standing grapevines

Grapevines showing neither foliar symptoms of esca nor any wood deterioration (white rot or brown streaking) were selected in 1998 from two vineyards located in Apulia, southern Italy: a 5-year-old vineyard cv. Italia trained to "trellis" in the experimental fields of the Dipartimento di Biologia e Patologia vegetale of the University of Bari, and a 9-year-old vineyard cv. Matilde trained to "tendone" in the experimental farm of the Province of Bari, near Andria. The vineyard cv. Italia was established in 1993 with certified virus-free seedlings, which had also been carefully checked at the nursery for infections of esca-associated fungi. The vineyard cv. Matilde had been surveyed in the foregoing 5 years for external symptoms of esca, and all vines had been sampled for brown wood streaking. Starting from the beginning of January 1999, inoculations with the three esca-fungi were performed on the spurs and trunks (cv. Italia) or on the spurs and branches (cv. Matilde), as described previously (Sparapano *et al.*, 2000a). Each fungus was inoculated singly or in combination with one or both of the other two species (7 combinations for spurs and 10 for branches or trunks, plus controls) in 36 grapevines cv. Italia and 27 grapevines cv. Matilde per fungus or fungal combination. With dual or triple inoculations, one fungus was inoculated either above or below the other(s); except for the dual inoculation *Pch+Pal*, where each fungus was inoculated at right angles on the same level. A total of 1152 inoculations on the spurs and 288 on the trunks of grapevines cv. Italia, and 1728 inoculations on the spurs and 864 on the branches of grapevines cv.

Matilde, including the controls, were carried out.

Samples from spurs, branches and trunks of the variously inoculated vines and from the non-inoculated controls were taken randomly in March, May, July and September of 1999, 2000 and 2001. At each sampling date, 2 entire plants and 6 spurs of vines cv. Italia, 4 branches and 8 spurs of vines cv. Matilde, per fungus or fungal combination, and an equal number of controls were sampled. A total of 576 spur samples and 264 trunk samples of vines cv. Italia, and a total of 768 spur samples and 528 branch samples of vines cv. Matilde were examined during the survey period of three years. Cross and longitudinal sections were made on the samples, the length of wood discolouration was measured, and five small fragments of discoloured wood per sample were placed on MA plates for fungal re-isolation.

Assessments made comprised: 1. extent of wood discolouration above and below the inoculation sites on the spurs, branches or trunks; 2. re-isolation of the inoculated fungi from the discoloured wood tissue; 3. number of successful inoculations, assessed by plating several slices of vinewood showing brown streaking on MA plates; 4. occurrence and type of internal and external symptoms. Mean values \pm standard deviation of data on the length of brown wood-streaking were calculated.

Results

Figure 1 and Tables 1 and 2 show the main results of the inoculation experiments.

Single inoculation: *Phaeoemoniella chlamydospora*

1. on vines cv. Italia

Five months after inoculation, spurs inoculated with *Pch* already showed dark-brown streaks extending downward along the wood tissue. In the following two years the columns of discoloured wood became progressively longer, thicker and darker, all spurs necrotised and the infection passed to the supporting shoots and even to the new growth. In the third year, the wood discolouration spread through the branches and trunks to reach the roots (Fig. 2E). The bark of the shoots holding the inoculated spurs was furrowed by a strip (30 to 40 cm long) of sunken tissue (Fig. 2C). When *Pch* was inoculated in the trunk, the length of the brown wood streak was at first shorter than

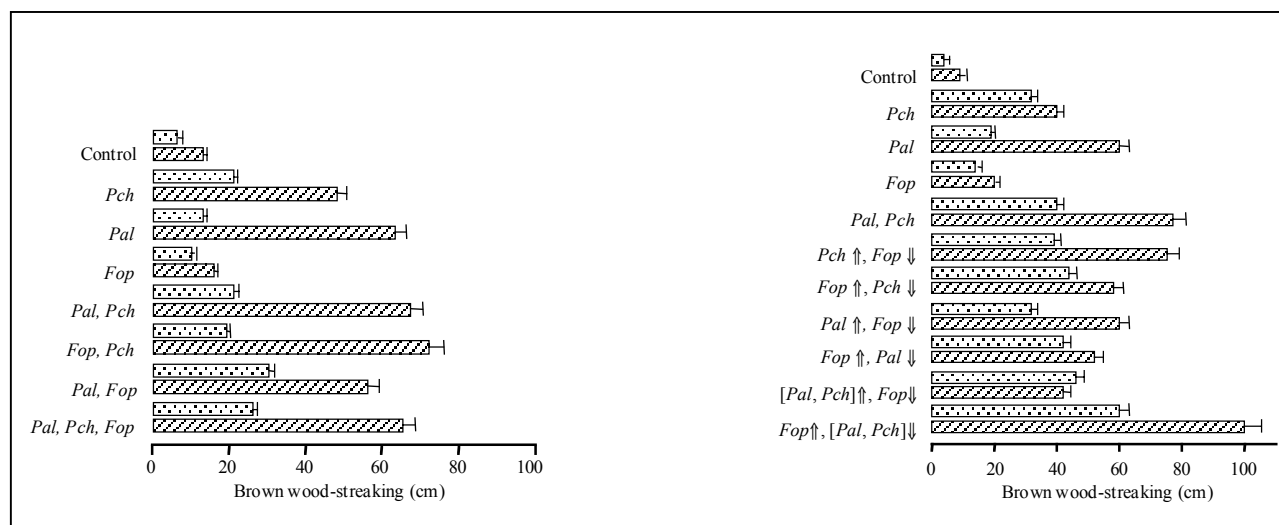


Fig. 1. Mean length of the brown streaks in the spurs (left) or stems and branches (right) of grapevine cv. Italia (▨) and Matilde (▩) inoculated with *Phaeoconiella chlamydospora* (*Pch*), *Phaeoacremonium aleophilum* (*Pal*) and *Fomitiporia punctata* (*Fop*) alone or in combinations, at the end of the survey period (1999–2001). Standard error bars are shown. For dual or triple inoculations, each fungus was inoculated above (↑) or below (↓) the inoculation site of the other(s).

Table 1. Inoculation of grapevines cv. Italia with *Phaeoconiella chlamydospora* (*Pch*), *Phaeoacremonium aleophilum* (*Pal*) and *Fomitiporia punctata* (*Fop*) after two and three years.

Pathogen(s) ^a	No. of vines inoculated on		No. of vines infected		No. of vines showing foliar symptoms of esca ^c					
					Spur-inoculated			Trunk-inoculated		
	spur ^b	trunk	Spur-inoculated	Trunk-inoculated	2 years after inoculation	3 years after inoculation	Total	2 years after inoculation	3 years after inoculation	Total
<i>Pch</i>	36	36	36	36	0	6 (16.6)	6 (16.6)	6 (16.6)	12 (33.3)	18 (50)
<i>Pal</i>	36	36	36	36	0	4 (11.1)	4 (11.1)	14 (38.8)	8 (22.2)	22 (61.1)
<i>Fop</i>	36	36	36	36	0	4 (11.1)	4 (11.1)	9 (25)	8 (22.2)	17 (47.2)
<i>Pch + Pal</i>	36	36	36	36	0	4 (11.1)	4 (11.1)	4 (11.1)	8 (22.2)	12 (33.3)
<i>Pch+Fop</i>	36	n.d.	36	n.d.	2 (5.5)	8 (22.2)	10 (27.7)	n.d.	n.d.	n.d.
<i>Pch</i> ↑+ <i>Fop</i> ↓	n.d.	36	n.d.	36	n.d.	n.d.	n.d.	4 (11.1)	8 (22.2)	12 (33.3)
<i>Fop</i> ↑+ <i>Pch</i> ↓	n.d.	36	n.d.	36	n.d.	n.d.	n.d.	4 (11.1)	2 (5.5)	6 (16.6)
<i>Pal+Fop</i>	36	n.d.	36	n.d.	0	6 (16.6)	6 (16.6)	n.d.	n.d.	n.d.
<i>Pal</i> ↑+ <i>Fop</i> ↓	n.d.	36	n.d.	36	n.d.	n.d.	n.d.	4 (11.1)	4 (11.1)	8 (22.2)
<i>Fop</i> + <i>Pal</i> ↓	n.d.	36	n.d.	36	n.d.	n.d.	n.d.	0	4 (11.1)	4 (11.1)
<i>Pch+Pal+Fop</i>	36	n.d.	36	n.d.	2 (5.5)	16 (44.4)	18 (50)	n.d.	n.d.	n.d.
[<i>Pch + Pal</i>] ↑ <i>Fop</i> ↓	n.d.	36	n.d.	36	n.d.	n.d.	n.d.	4 (11.1)	2 (5.5)	6 (16.6)
<i>Fop</i> ↑ [<i>Pch + Pal</i>] ↓	n.d.	36	n.d.	36	n.d.	n.d.	n.d.	4 (11.1)	2 (5.5)	6 (16.6)
Control	36	36	0	0	0	0	0	0	0	0

^a Each fungus was inoculated singly or in combination with one or both the other two species. With dual or triple inoculations, one fungus was inoculated either above (↑) or below (↓) the other(s); with the dual inoculation *Pch+Pal*, each fungus was inoculated along a line perpendicular to the axis. Spurs were inoculated on the pruning wound.

^b Four spurs per vine were inoculated.

^c Foliar symptoms appeared in the summer of the second and third year. At the end of each annual sampling, the 12 spur-inoculated and the 12 trunk-inoculated vines sampled for assessment of internal symptoms were removed or discarded. Consequently, they were no longer surveyed for external symptoms. Percentages of vines with foliar symptoms are shown in parentheses.

n.d., not determined.

Table 2. Inoculation of grapevines cv. Matilde with *Phaeoconiella chlamydospora* (*Pch*), *Phaeoacremonium aleophilum* (*Pal*) and *Fomitiporia punctata* (*Fop*) after two and three year.

Pathogen(s) ^a	No. of vines		No. of vines showing foliar symptoms of <i>esca</i> ^c		
	Inoculated on spurs and branches ^b	Infected	2 years after inoculation	3 years after inoculation	Total
<i>Pch</i>	27	27	0	3 (11.1)	3 (11.1)
<i>Pal</i>	27	27	0	2 (7.4)	2 (7.4)
<i>Fop</i>	27	27	0	0	0
<i>Pch</i> + <i>Pal</i>	27	27	3 (11.1)	7 (25.9)	10 (37)
<i>Pch</i> ↑ + <i>Fop</i> ↓	27	27	0	0	0
<i>Fop</i> ↑ + <i>Pch</i> ↓	27	27	0	0	0
<i>Pal</i> ↑ + <i>Fop</i> ↓	27	27	0	0	0
<i>Fop</i> ↑ + <i>Pal</i> ↓	27	27	0	0	0
[<i>Pch</i> + <i>Pal</i>] ↑ <i>Fop</i> ↓	27	27	0	0	0
<i>Fop</i> ↑ [<i>Pch</i> + <i>Pal</i>] ↓	27	27	0	0	0
Control	27	0	0	0	0

^a Each fungus was inoculated singly or in combination with one or both the other two species. With dual or triple inoculations, one fungus was inoculated either above (↑) or below (↓) the other(s); with dual inoculation *Pch*+*Pal*, each fungus was inoculated along a line perpendicular to the axis. Spurs were inoculated on the pruning wound.

^b Eight spurs (two per branch) and four branches per vine were inoculated.

^c Foliar symptoms appeared in the summer of the second and third year. At the end of each annual sampling, the 9 vines sampled to assess internal symptoms were removed and discarded. Consequently they were no longer surveyed for external symptoms. Percentages of vines with foliar symptoms are shown in parentheses.

that caused by *Pal*, but subsequently it spread upward and downward from the inoculation site. In cross-section, brown spots were sparsely distributed or grouped together in the xylem rings close to the pith. Three years after infection, the inoculation wounds on the trunk were still open, whereas those on the controls had been sealed by healing processes (Fig. 2F).

Foliar symptoms (yellowing or reddening of large parts of the lamina, marginal leaf necrosis and tiger-stripes) appeared in the summer of the second and third year (Fig. 3A and Table 1). In the third year, a gummy flow exuding from fresh pruning wounds was observed in early spring (Fig. 2A), and the first sign of brown spotting of berries (black measles) occurred in the summer of the same year (Fig. 3I).

2. on vines cv. Matilde

At the end of the first year, the wood of spurs or branches of cv. Matilde showed shorter brown streaks than those recorded for the cv. Italia. Colonisation of woody tissue with *Pch* was slow but continuous. During the third year, the wood

streaks, which had developed independently at the inoculation sites on the spurs and stems, became confluent, and the discolouration spread through the trunk.

An abundant secretion of gums from the wood tissue, and of exopolysaccharides and melanins from the pathogen was recorded; this material filled the lumen of the xylem vessels with viscous masses. Foliar symptoms (light green and chlorotic spots between the veins and along the leaf margins) appeared in the summer of the third year (Fig. 4A and Table 2).

Single inoculation: *Phaeoacremonium aleophilum*

1. on vines cv. Italia

When *Pal* was inoculated on the spurs, substantial wood discolouration occurred within 5 months. In the second year, the entire spur necrotised and the infection spread to the nearest annual shoot. In the third year, the wood streaking was longer than with *Pch*. A depressed or sunken area of bark formed along the spur and the supporting shoot (Fig. 2D).



Fig. 2. Symptoms on 6-year-old grapevines cv. Italia infected with *Phaeoconiella chlamydospora* (*Pch*) or *Phaeoacremonium aleophilum* (*Pal*): A. cross-section of trunk showing gummy flow, 28 months after inoculation with *Pch*, or, B. with *Pal*; C. 3-year-old canes 30 months after inoculation of the spurs with *Pch*, showing sunken bark; D. 3-year-old canes 30 months after inoculation of the spurs with *Pal*, showing browning of the bark; E. cross sections of canes, trunk and roots of vines, 30 months after inoculation of the spurs with *Pch*; F. cross sections of inoculation holes drilled radially through the trunk into which wood dowels colonised by *Pch* and *Pal* were inserted, 32 months after inoculation. Within three years, the inoculation wounds of *Pal*, *Fop* and control, but not of *Pch*, were sealed by the annual rings.

When *Pal* was inoculated in the trunk, the brown wood discolouration that followed was greater than that with *Pch* in the first year, and it further increased substantially during the second and third year. In cross-section, black or brown spots, often oozing a viscous exudate, were sparsely distributed or grouped together around the innermost xylem rings, close to the pith. A gummy flow (Fig. 2B) from pruning wounds on the branches or trunks of standing vines was also observed in the spring of the third year. Foliar symptoms (irregular interveinal spots, rust-coloured necrotic areas, tiger-stripes) appeared in the summers of the second and third year (Fig. 3B and Table 1).

2. on vines cv. Matilde

The cv. Matilde appeared to be more resistant to *Pal* than the cv. Italia. At the end of the third year, the wood streaks in the spurs or branches inoculated with *Pal* were shorter than those inoculated with *Pch*. In a few vines the streaks starting from the inoculated spurs reached the discoloured tissue at the inoculation site of the supporting branch. With time, an elongated depression developed in the bark of the spur and the underlying shoot. In cross section, the xylem tissue showed creamy-yellow and dark-brown discolourations.

The columns of brown wood extended along the

annual rings close to the pith. In cross-section, yellow spots interspersed with black and dark-brown spots were randomly distributed in the innermost xylem rings.

Foliar symptoms (chlorotic areas between the veins and along the leaf margins) appeared in the summer of the third year (Fig. 4B and Table 2). In the same year, spotting of berries (black measles) was recorded in the summer (Fig. 4D).

Single inoculation: *Fomitiporia punctata*

1. on vines cv. Italia

In the inoculated spurs, brown wood streaking measured 5 months after inoculation with *Fop* was considerably shorter than that caused by *Pal* or *Pch*. In the second and third year, *Fop* did not pass to the shoot holding the inoculated spur. A limited degradation of the wood tissue was recorded.

When *Fop* was inoculated into the trunk it promptly colonised the woody tissue. Wood decay started around the infection dowels soon after inoculation; it then progressed, and the dowel itself, adjacent xylem tissue and the pith began to show white rot. The rot spread slowly during the second and third year, and *Fop* colonisation was slower than with *Pal* or *Pch*.

In longitudinal sections of the trunk, the dark-brown columns of degraded wood, surrounded by

layers or masses of rotted spongy tissue, were contiguously distributed in the annual rings.

Foliar symptoms (chlorosis and reddening of the leaf margins; necrosis of large parts of the lamina) appeared in the summer of second and third year (Fig. 3C and Table 1).

2. on vines cv. *Matilde*

Wood streaks in the spurs or branches inoculated with *Fop* were shorter than when these vine-parts were inoculated with *Pch* or *Pal*. Three years after spur-inoculation, the infection was still restricted to the spurs.

All trunk-inoculated vines showed dark-brown wood streaking spreading from the inoculation wound downward and upward as far as the branches. Rotted wood was creamy-yellow or whitish; gradually the hard wood changed to a soft, friable, spongy mass. At the end of the third year, the pith showed white rot extending to a length of about 50 cm.

Dual inoculation: *Phaeoconiella chlamydospora* and *Phaeoacremonium aleophilum*

1. on vines cv. *Italia*

Pch+Pal inoculation caused long wood streaks in both the spurs and the trunks. The columns of discoloured wood resulting from inoculation with each fungus advanced independently along the xylem. In the first year, the infection spread from the inoculated spur to the supporting shoot. In the second and third year, the streaks were longer with both fungi combined than when each fungus was inoculated singly.

A similar pattern was observed when the two fungi were inoculated in the trunks. In the third year, the wood streaks spread upward and merged with the streaks resulting from spur inoculation, and spread downward to the roots. In cross-section, the black or dark-brown spots with their viscous exudate were scattered all over from the bark to the pith.

In the summer of the second and third year, foliar symptoms consisting in yellow or reddish spots scattered over the lamina, roundish or irregular spots between the veins, and tiger-striped leaves, occurred on the stem-inoculated vines (Fig. 3D and Table 1).

2. on vines cv. *Matilde*

Dual inoculation *Pch+Pal* produced wood streaking twice as long as with a single fungus.

After three years, the brown wood-streaking from the inoculated spurs merged with the streaks that had formed in the inoculated branches.

In the branches, the brown streaks caused by *Pch* were mostly in the outer (and more recent) xylem rings, whereas those caused by *Pal* were nearer the pith.

In cross-section, a great number of black or dark-brown and creamy-yellow spots were observed. Hyphae and conidia of both fungi were viewed under the microscope in the xylem vessels.

Foliar symptoms (chlorotic and reddish areas between the veins and along the leaf margins) appeared in the summer of the third year (Fig. 4C and Table 2).

Dual inoculation: *Phaeoconiella chlamydospora* and *Fomitiporia punctata*

1. on vines cv. *Italia*

The wood streaking caused by *Pch* progressed beyond the inoculated spur, whereas that caused by *Fop* remained restricted to the spur. Only *Pch* was re-isolated from both the spur and the supporting shoot.

The outcome of the dual inoculation in the trunk depended on the inoculation sites of the two fungi relative to each other. When *Fop* was inoculated above *Pch*, the wood streaking caused by *Pch* spread farther upward and downward than when *Fop* was inoculated below *Pch*. In the second and third year, the wood streaks reached the branches, shoots and roots.

When inoculated into the trunks, *Fop* grew slowly and caused relatively long columns of dark-brown wood above and below the inoculation site. White rot was evident in the third year. Foliar symptoms (chlorosis and reddening of leaf margins) appeared in the summer of the same year (Fig. 3E and Table 1).

2. on vines cv. *Matilde*

Three years after spur-inoculation, only discoloured streaks produced by *Pch* had spread to the supporting shoot. *Fop* was never re-isolated from the shoots.

Compared with the discolouration induced by *Pch* inoculated singly in the branches, dual inoculation of *Pch+Fop* produced longer streaks. The streaks caused by *Pch* spread upward and downward along the branch, irrespective of whether *Fop*

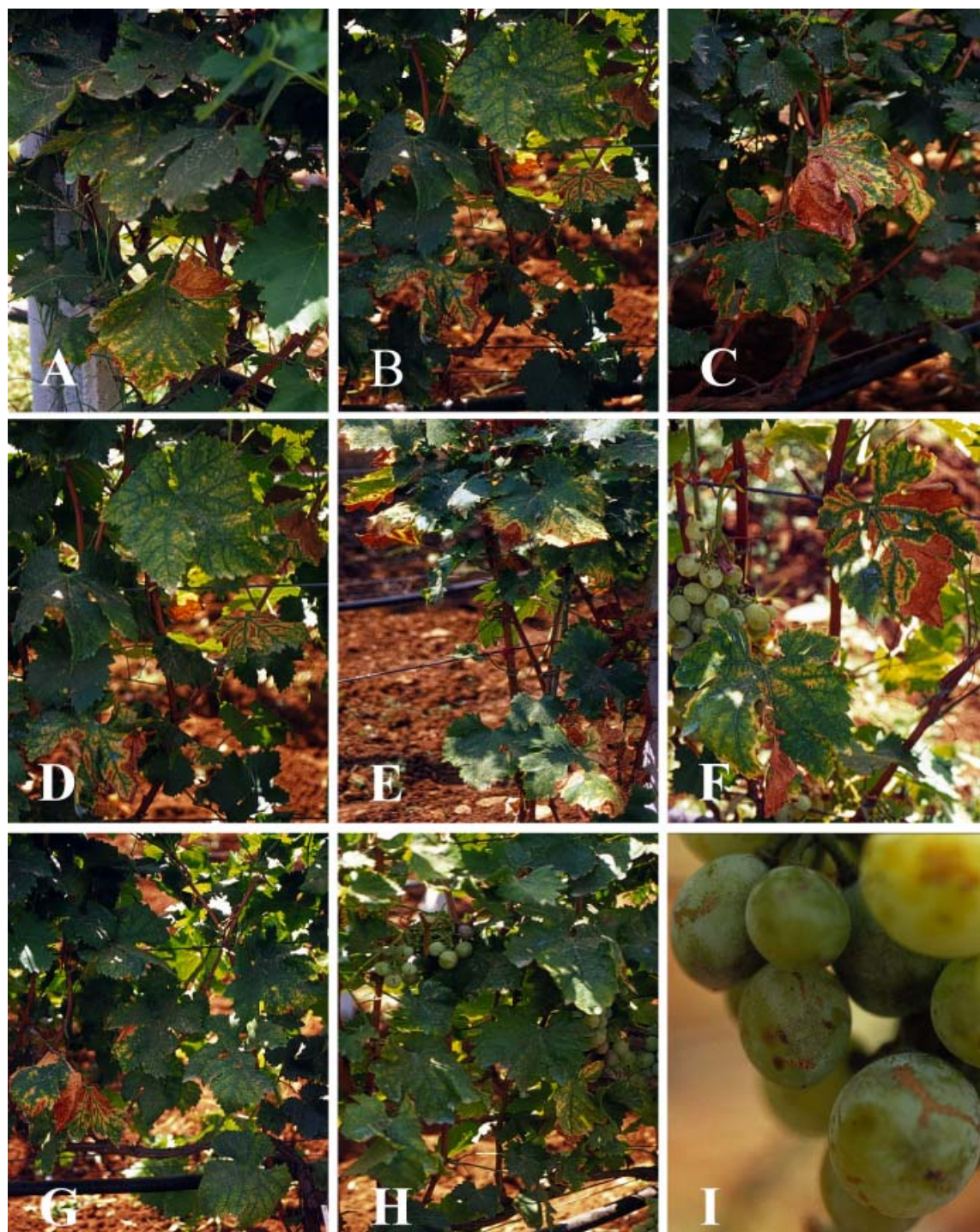


Fig. 3. Symptoms on leaves or berries of grapevines cv. Italia inoculated with: A. *Phaeomoniella chlamydospora* (*Pch*); B. *Phaeoacremonium aleophilum* (*Pal*); C. *Fomitiporia punctata* (*Fop*); D. *Pch*, *Pal*; E. *Pch*↑, *Fop*↓. F. *Fop*↑, *Pal*↓; G. *Pal*↑, *Fop*↓; H. *Fop*↑, [*Pal*, *Pch*]↓; I. *Pch*. With dual or triple inoculations, one fungus was inoculated either above (↑) or below (↓) the other(s); with the dual inoculation *Pch*+*Pal*, each fungus was inoculated at right angles on the same level.

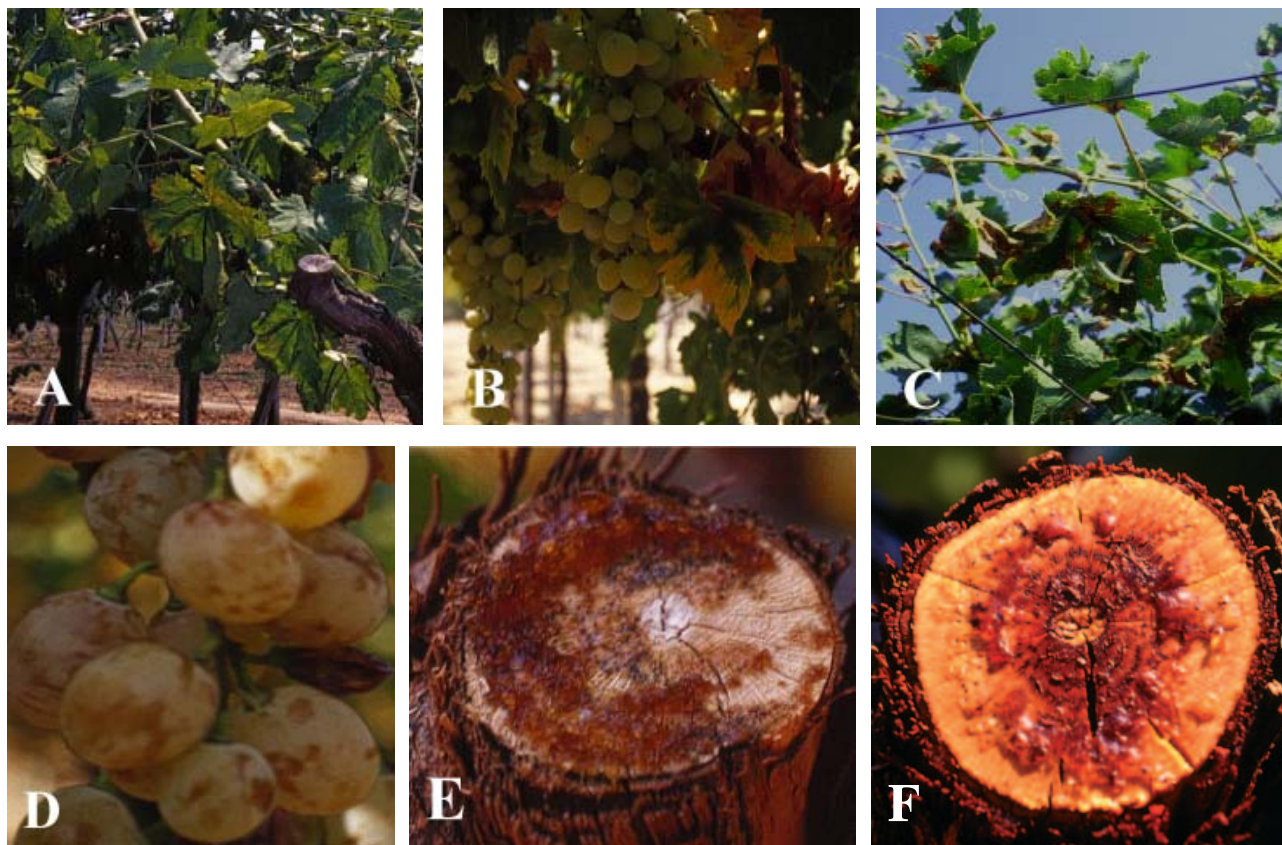


Fig. 4. Symptoms on leaves, berries and branches of grapevines cv. Matilde inoculated with: A. *Phaeomoniella chlamydospora* (*Pch*); B. *Phaeoacremonium aleophilum* (*Pal*); C. *Pal*, *Pch*; D. *Pal*; E. *Pal*↑, *Fomitiporia punctata* (*Fop*)↓; F. [*Pal*, *Pch*]↑, *Fop*↓. With dual or triple inoculations, one fungus was inoculated either above (↑) or below (↓) the other(s); with the dual inoculation *Pch*+*Pal*, each fungus was inoculated at right angles on the same level.

was inoculated above or below *Pch*. Within the three-year observation period, the streaks induced by these fungi became confluent.

White rot occurred in a restricted portion of the woody tissue, around the inoculation wound.

Dual inoculation: *Phaeoacremonium aleophilum* and *Fomitiporia punctata*

1. on vines cv. *Italia*

In the inoculated spurs, only wood streaking caused by *Pal* progressed beyond the spur, whereas that of *Fop* remained restricted to the spur.

From the first year after inoculation, an antagonistic interaction between *Pal* and *Fop* produced a marked reduction in the length of discolouration induced by *Fop*.

When *Fop* was inoculated above *Pal*, the extent of wood discolouration in an upward direction was about 50% less than that in a downward direction. The antagonistic effect of *Pal* against *Fop* here clearly did not allow *Fop* to cause its normal-length streaks. When *Fop* was inoculated below *Pal*, the antagonistic effect of *Pal* against *Fop* was still apparent, and the downward spread of the streaks was about one fifth less than that of the upward streaks. In cross-section, the oldest woody rings were creamy-yellow at the start of infection, and dark brown with viscous exudates later.

Wood decay usually started from the wood dowel used for inoculation, progressed through the xylem and reached the pith, which became whitish and friable.

Foliar symptoms (light-green or chlorotic, rounded or irregular spots between the veins or along the leaf margins, rust-coloured necrotic areas, tiger-stripes) appeared in the summer of the third year (Fig. 3F, 3G and Table 1).

2. on vines cv. *Matilde*

From the first year, the interaction between *Pal* and *Fop* produced a marked reduction in the length of discolouration induced by *Fop*.

Unlike the streaking caused by *Pal*, that caused by *Fop* was restricted to the spurs.

In the branches, irrespective of whether *Fop* was inoculated above or below *Pal*, the discolouration caused by *Pal* spread upward and downward independently from, and alongside the streaks produced by *Fop*.

In cross-section, the xylem vessels of the innermost rings were creamy-yellow at the start of infection, and turned black, filled with a viscous exudate, later.

The white rot usually started from the dowel used for inoculation, and spread to the pith. Rotated tissue became soft and friable.

In the summer of the third year, black goo-like symptoms (Morton, 1997) were observed on sections of the trunk (Fig. 4E).

Triple inoculation: *Phaeoconiella chlamydospora* with *Phaeoacremonium aleophilum* and *Fomitiporia punctata*

1. on vines cv. *Italia*

A competitive interaction between *Pal* and *Fop* was also evident when all three fungi were inoculated on the same grapevine.

In spur-inoculated vines, *Fop* remained restricted to spur tissue, whereas the wood discolouration induced by *Pal* and *Pch* extended to the supporting shoots and branches.

From the first year, trunk inoculation caused discolouration of the wood tissue with xylem gummosis and the formation of slimy masses. Irrespective of whether the *Fop* inoculation site was above or below those of *Pal* and *Pch*, the wood streaks caused by the latter fungi extended upward and downward, by-passing the discoloured wood portions colonised by *Fop*.

Infection eventually spread through the entire vine. In the third year, standing vines with *Fop* inoculated above *Pch* and *Pal* showed brown wood-streaking from the inoculation wounds to the roots.

Foliar symptoms (light green or chlorotic, rounded or irregular spots between the veins or along the leaf margin) appeared in the summer of the second and third year (Fig. 3H and Table 1).

2. on vines cv. *Matilde*

A competitive (*Pal* against *Fop*) or combative (*Pch* against *Fop*) interaction (for terms see Rayner and Webber, 1984) was evident when all three fungi were inoculated in the same vine.

In the spur inoculations, *Fop* remained restricted to spur tissue, whereas the wood discolouration induced by *Pal* and *Pch* spread from the spur to the supporting shoot or branch.

From the first year, the discoloured woody tissue that developed in the inoculated branches showed gummosis and formation of slimy masses.

The interaction between the three fungi was similar to that observed on grapevines cv. *Italia*. Irrespective of the inoculation site of *Fop*, the wood discolouration induced by *Pal* and *Pch* extended upwards and downwards, by-passing the discoloured streaks produced by *Fop*. In the third year, the columns of discoloured wood from the spurs and streaks from the branches became confluent.

In the summer of the third year, black goo-like symptoms were observed on trunk sections (Fig. 4F).

Discussion

Pathogenicity of the inoculated fungi

The pathogenicity of *P. aleophilum* and *P. chlamydospora* and *F. punctata*, was confirmed (Graniti *et al.*, 2001) when these fungi were inoculated singly and in all possible combinations on the spurs, branches or trunks of standing esca-free vines cv. *Italia* and *Matilde*.

All these fungi infected the vines through wounds, colonised and degraded the woody tissue, and caused disease symptoms within three years. However, each fungus produced a distinct symptomatological picture. The severity of internal and external symptoms, for example the length and colour-intensity of the discoloured wood, varied with the growth and interactions of the inoculated fungi.

Most symptoms of esca, both internal (brown wood-streaking, gummosis and deterioration, black goo, wood decay or white rot) (Fig. 4E and 4F), and external, on the shoots (sunken bark), leaves (partial chlorosis or reddening of the lamina, chlorotic

spots, necrotic areas, tiger-stripes, distortion of the leaf lamina), and berries (black measles) were reproduced (Fig. 3 and 4). The inoculated vines exhibited several structural responses such as tylosis, gummy flow (Fig. 2A and 2B), wound repair and healing processes (Fig. 2F).

The inoculated fungi were always re-isolated from the brown streaks that formed in the woody tissue upward and downward of the inoculation sites.

Extent of wood discolouration

The mean length of the brown streaks caused by each fungus in the wood of variously inoculated vines was compared. In spur-inoculated 'Italia' vines, *Pal* alone produced a much longer wood discolouration than either *Pch* or *Fop* singly (Fig. 1). The dual inoculation *Pal+Pch* produced brown streaks longer than those caused by single inoculation of either *Pal* or *Pch*, while the combination *Pch+Fop* gave the longest brown streaks.

With the spur-inoculated vines cv. Matilde, the mean length of wood streaks caused by any type of inoculation was always shorter than that of the grapevines cv. Italia. *Pch* caused longer streaks than *Pal* and *Fop*, while the dual inoculation *Pal+Fop* caused the longest streaks.

The three fungi showed a quite similar behaviour when they were inoculated in the trunk of the vines cv. Italia or in the branches of the vines cv. Matilde. Here the triple inoculation *Pch+Pal+Fop* caused the longest wood streaks (Fig. 1).

Pal inoculated singly on vines cv. Italia was more aggressive than *Pch* and *Fop*, but *Pch* was more aggressive on cv. Matilde.

Fungal interactions

It is probable that when colonizing a vacant resource such as a fresh wound, each invading fungus is competitive in gaining initial access to the resource and is antagonistic in defending itself and trying to exclude other micro-organisms.

In the cross-inoculation trials, *Pch*, *Pal* and *Fop* showed both competition and antagonism during wood colonisation. A non-synergistic, competitive association of *Pal* and *Pch*, and a marked antagonistic effect of *Pal* against *Fop* was observed, thus confirming previous results (Sparapano *et al.*, 2000a).

With the inoculation *Pal+Pch*, each fungus col-

onised the woody tissue independently of the other, producing hyphae and mitospores in a separate column of xylem. With the inoculation *Pal+Fop*, growth of *Pal* was neither helped nor inhibited by the presence of *Fop*, but *Fop* was affected by *Pal*. *Pal* grew and spread through the xylem without constraints, whereas the growth of *Fop* was mainly restricted to the pith tissue.

The interaction between *Pch* and *Fop* was competitive. No deleterious effect of one fungus on the other was found, and often the two fungi grew intermingled.

Microscopic observations of sections of vines cv. Italia and Matilde inoculated with *Pal* or *Pch* singly or in combination showed a high degree of brown or black wood discolouration and sap staining due to the production and accumulation of melanin-like material, which may enhance the competitiveness of the producer fungi against other micro-organisms. *Fop* did not show such a capability.

Susceptibility of tested cultivars

Vines cv. Italia were more susceptible to all three fungi than vines cv. Matilde (Fig. 1 and Tables 1 and 2). Colonisation of the woody tissue by each fungus was faster in vines cv. Italia than in cv. Matilde. Foliar symptoms developed on trunk-inoculated vines cv. Italia as early as the second year with almost all inoculation combinations. The greatest number of vines showing symptomatic leaves were those singly inoculated with *Pal* (Table 1). Conversely, vines cv. Matilde showed foliar symptoms in the second year only with the dual inoculation *Pch+Pal* and in the third year with the single inoculation *Pch* or *Pal* and once again with dual combination *Pch+Pal* (Table 2). Moreover, spur-inoculated vines cv. Italia showed foliar symptoms in the second and third year. Vines with tiger-striped leaves and bunches of grapes with black measles were also more frequent in the vineyard planted with the cv. Italia than in the vineyard with the cv. Matilde.

An irregular occurrence of foliar symptoms in the second and third year was recorded for both cultivars (Tables 1 and 2): the number of vines cv. Italia with foliar symptoms was greater in the second than in the third year (vines inoculated with *Pal*, *Fop* or *Pch+Pal*) or vice-versa (vines singly inoculated with *Pch*).

Symptom production

Infection with both *Pal* and *Pch*, with the hyphae readily growing and sporulating within the xylem vessels, and consequent gummosis, discoloration and melanisation of the woody tissue, had all the features of a real tracheomycosis. When these fungi were inoculated in the branches and trunks, and in the case of the cv. Italia even on the spurs, they spread through the vines and reached the roots within three years. Conversely, infection with *Fop* caused a relatively slow but progressive degradation of woody tissue around the inoculation site, which eventually decayed (white rot). When *Fop* was inoculated on the spurs, it caused local wood decay, which did not however extend to the other parts of the vine plant.

The results of the study indicate that all three fungi produced a number of similar symptoms (e.g., wood streaking, foliar chlorosis), while the hyphomycetes also produced particular symptoms, such as black goo and black measles, and the basidiomycete white rot.

Most symptoms shown by the vines could be caused by toxins, enzymes and other metabolites produced by the pathogens spreading through the vines, together with some products of the host defence reaction (Sparapano *et al.*, 1998, 2000, 2000a, 2000b; Graniti *et al.*, 1999; Bruno *et al.*, 2000; Evidente *et al.*, 2000). Further studies on these bioactive substances are needed for a better understanding of their role in esca.

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