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RESEARCH PAPERS

Bioassays on the activity of resveratrol, pterostilbene and phosphorous acid towards fungi associated with esca of grapevine

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Summary. An examination was carried out under laboratory conditions to determine whether resveratrol, pterostilbene and mixtures of each stilbene with phosphorous acid had an inhibitory activity against some fungi involved in esca: *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora*, *Libertella blepharis* (anamorph of *Eutypa lata*), *Fomitiporia punctata* and *Stereum hirsutum*. An inhibition of mycelial growth by resveratrol was not found but there was a clear correlation between pterostilbene concentration and reduction of radial growth confirming the antifungal activity of this phytoalexin. Although phosphorous acid proved to be generally ineffective, an increase of antifungal activity of stilbenes in the mixtures was observed with all fungi except *F. punctata*. The possible relation between fungal phenol oxidase activity and inhibition by mixtures of stilbenes and phosphorous acid was discussed.

Key words: grapevine, stilbenes, phosphorous acid, esca fungi, phenol oxidase activity.

Introduction

Esca is a very destructive grapevine disease that is widespred in all grape-growing areas. Several micro-organisms are involved in a complex infection process not yet completely elucidated. The wood is colonized by different fungi acting in succession or in combination and producing various types of discoloration and decay (Mugnai *et al*., 1996; Larignon and Dubos, 1997; Graniti *et al*., 2000).

The discoloration normally precedes or occurs on the edge of decayed areas. Non-basidiomycetous fungi are consistently isolated from these discoloured regions, while white rot basidiomycetes are commonly found in zones with visible decay. Discoloration seems a necessary first stage before decay, and the mechanism by which such presumed preconditioning occurs is most commonly suggested to be via the detoxification of phenolic substances produced by the host as an infection response (Rayner and Boddy, 1988).

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The following pathogens are frequently isolated from necrosis typical of esca: *Phaeoacremonium chlamydosporum* (W. Gams, Crous, M.J. Wingf. & L. Mugnai), recently proposed by Crous and W. Gams (2000) as *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams, *Phaeoacremonium aleophilum* (W. Gams, Crous, M.J. Wingf. & L. Mugnai), *Eutypa lata* (Pers.: Fr.) Tul. & C. Tul., *Fomitiporia punctata* (Murril) and *Stereum hirsutum* (Willd.: Fr.) S.F. Gray.

Stilbenes have long been considered important as conferring wood resistance to fungal decay: wood containing stilbenes frequently decomposes more slowly when exposed to various rot fungi (Hart and Shrimpton, 1979). Resveratrol is the major stilbene component in lignified stem tissue of grapevine and this compound probably also acts as a biosynthetic precursor of other *Vitis* stilbenes such as the viniferins and pterostilbene (Langcake and Pryce, 1977). *Trans*-pterostilbene in particular was reported by Langcake *et al*. (1979) to be a methylated resveratrol derivative with an interesting antifungal activity.

Under favourable infection conditions, esca-diseased vines treated with fosetyl Al compounds by wood injection showed positive results with a significant reduction in the severity of foliar symptoms. Although the treatment did not cure the vines, a positive long-term effect was that basic production criteria (weight of harvested bunches and Brix values) were preserved (Mazzullo *et al*., 2000).

The action of fosetyl-Al in stimulating the host defence response as well as in having a direct inhibitory effect on the pathogens through phosphorous acid - to which fosetyl Al is rapidly degraded - was extensively described by Fenn and Coffey (1984).

A proper understanding of the relations between fosetyl-Al, the grapevine defence mechanism and esca pathogens appears to be of basic importance when evaluating of the above mentioned positive preliminary field results, if the potential of fosetyl-Al to control esca is to be correctly interpreted.

The aim of the present study was to determine the effect of resveratrol and pterostilbene on the mycelial growth of the fungi involved in the esca syndrome and to explore the role of these compounds in mixtures with phosphorous acid. Phenol oxidase enzyme tests were also performed.

Materials and methods

Fungi and culture conditions

Cultures of *P. aleophilum, P. chlamydospora, Libertella blepharis* (anamorph of *Eutypa lata*), *F. punctata* and *Stereum hirsutum* isolated from esca-diseased plants were grown and maintained in the dark at 25±1°C on Petri plates containing potato dextrose agar (PDA, Difco, Detroit, MI, USA) at 39 g l^{-1}. An isolate of each fungus was submitted to bioassays.

Bioassays

Bioassays were carried out with resveratrol (3,4',5 - trihydroxy-trans-stilbene, 99%, Sigma, St Louis, MO, USA), pterostilbene (laboratory synthesis) and phosphorous acid (H_3PO_3, 99%, Aldrich, Milwaukee, WI, USA).

Pterostilbene (*trans*-3-5-dimethoxy-4'-hydroxystilbene) was produced in the laboratory by condensing *p*-hydroxybenzaldehyde with dimethoxy-3,5 phenylacetic acid in the presence of piperidine, in accordance with Massarani (1957). The last step of the synthesis involved a Perkin condensation which brought about a *trans* isomere with a coupling constant of 16.5 Hz between the vinyl protons (Pezet and Pont, 1988). The *trans* configuration of the pterostilbene was proved by NMR and mass spectrometry.

Resveratrol and pterostilbene were dissolved in absolute ethanol and the alcoholic solutions were added to a PDA substrate at 40°C. The final concentration of ethanol was 2% (v:v). An identical volume of ethanol was incorporated with the growth medium of the controls (Hart and Hillis, 1974; Bailey *et al*., 1976; Hart and Shrimpton, 1979; Di Marco *et al*., 1999).

Preliminary tests on the stilbenes were carried out with the following amounts of active ingredient in the medium: resveratrol at 10, 100, 500 and 1000 μg ml^{-1}; pterostilbene at 1, 10 and 100 μg ml^{-1}. At a later stage, for the study, phosphorous acid (300 μg ml^{-1}), resveratrol (300 μg ml^{-1}), resveratrol (300 μg ml^{-1}) + phosphorous acid (300 μg ml^{-1}), pterostilbene (10 μg ml^{-1}), pterostilbene (10 μg ml^{-1}) + phosphorous acid (300 μg ml^{-1}) were dissolved in the medium. Resveratrol at concentrations above 100 ppm formed crystals.

The pH of the growth medium was measured by litmus paper (Farmitalia, Carlo Erba, Milano, Italy).

Inoculum plugs cut with a cork borer from the margin of growing cultures were placed at the centre of Petri plates containing treated medium. Control plugs were placed in untreated medium. Plates were incubated at 25±1°C in the dark. For each fungus colony diameters were measured at two points on each plate and averaged.

Treatments were replicated three times, each replicate consisting of one Petri-plate.

For each assessment date, data were analysed statistically using Duncan's multiple range test, P=0.05.

Detection of resveratrol in growth medium

The growth medium of plates treated with resveratrol or resveratrol + phosphorous acid and inoculated with *P. aleophilum* was analysed by Thin Layer Chromatography (TLC) to detect the presence of resveratrol. This analysis was performed because a brownish halo appeared around the edge of *P. aleophilum* colony grown on medium with resveratrol alone (i.e. without phosphorous acid).

Discs of PDA 4 mm in diameter were collected from plates containing medium + resveratrol and medium + resveratrol + phosphorous acid. Discs were cut either adjacent to the outer edge of the colony or away from the front of the mycelium. Samples were extracted in ethanol and analysed by TLC on silica gel plates (Baker-Flex IB2, Phillipsburg, NJ, USA) with cyclohexane/ethyl acetate (9:1) as the usual solvent system. Developed plates were detected by UV lamp (254 nm). Five discs per treatment were set up.

Enzyme assays

For each fungus the tests described below were performed according to Harkin and Obst (1973) and Rayner and Boddy (1988).

Laccase

I. A dilute 0.1% solution of syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine, 99%, Aldrich) in absolute ethanol was added to PDA plates in which discs 5 mm in diameter from the margin of colonies of the pathogens were successively placed.

II. A dilute 0.1% solution of syringaldazine in absolute ethanol was placed dropwise with a capillary pipette at the front of actively growing mycelium of pathogen colonies.

The laccase test reaction was shown by the medium turning deep purple (test I) or by the speed of the colour change (test II) due to syringaldazine oxidation by the laccase.

Peroxidase

The syringaldazine test was similar to laccase test II above. If no colour developed and laccase was absent, a drop of dilute hydrogen peroxide solution was placed on the edge of the colony. Peroxidase activity was shown by the appearance of a purple colour as with the laccase test.

Tyrosinase

A drop of 0.1% *p*-cresol (1.08 g) in 96% ethanol (100 ml) was added to the edge of the colony. Tyrosinase activity was shown by the appearance of an orange-brown coloration in the medium 4-48 hours after application.

Catalase

Catalase activity was carried out by placing a drop of hydrogen peroxide solution on the fungal cultures, and visually assessing the resulting effervescence.

The level of enzyme activity was expressed as "low" or "strong".

Results

Bioassays

Preliminary assays of stilbenes

Effects on mycelial growth by different amounts of resveratrol and pterostilbene active ingredient are summarized in Tables 1 and 2 respectively.

Resveratrol crystals formed when stilbene was used at 500 and 1000 μg ml^{-1}. Crystals were uniformly dispersed in the medium and increased in number with increasing resveratrol concentration.

The morphological and growth characteristics of the colonies did not vary with the amount of resveratrol in the medium, whether as crystals or in diluted form.

Amounts of resveratrol up to 100 μg ml^{-1} significantly reduced mycelial growth of *S. hirsutum* and, to a lesser extent, of *F. punctata*, compared to the control. No effects were observed with *L. blepharis, P. chlamydospora* or *P. aleophilum*. A

Table 1. Mycelial growth[a] of fungi with different amounts of resveratrol in the medium. Mycelial growth (mm) is expressed as colony diameter.

Fungal species	Resveratrol amount (μg ml^{-1})	Days after treatment						
		2	5	8	11	14	17	20
P. chlamydospora	10	7.0 b[b]	11.3 b	14.7 b	19.3 a	25.3 bc	33.3 b	38.7 b
	100	11.0 c	16.0 c	20.3 c	25.7 b	29.0 c	36.0 b	41.0 b
	500	5.0 a	7.3 a	11.0 a	17.0 a	21.7 ab	25.0 a	28.3 a
	1000	5.0 a	7.3 a	10.3 a	15.0 a	17.3 a	21.0 a	25.3 a
	Control	5.0 a	10.7 b	13.3 ab	16.7 a	20.3 a	25.3 a	28.7 a
P. aleophilum	10	5.3 b	9.7 a	13.7 a	17.3 a	23.3 ab	29.7 bc	36.3 c
	100	7.0 c	10.7 a	15.0 a	19.3 a	27.0 b	33.3 c	40.3 c
	500	4.0 a	10.0 a	13.0 a	16.3 a	20.3 ab	25.7 ab	30.0 ab
	1000	4.0 a	8.3 a	10.7 a	14.7 a	17.7 a	21.7 a	26.0 a
	Control	4.0 a	9.0 a	12.0 a	17.0 a	22.3 ab	29.0 bc	35.3 bc
L. blepharis	10	7.7 a	16.7 a	48.0 b	70.3 a	-	-	-
	100	7.7 a	15.3 a	40.0 ab	63.3 a	-	-	-
	500	7.7 a	13.7 a	35.0 a	59.7 a	-	-	-
	1000	8.0 a	13.7 a	32.3 a	56.3 a	-	-	-
	Control	8.3 a	18.0 a	41.0 ab	71.7 a	-	-	-
F. punctata	10	4.0 a	13.0 bc	26.3 b	42.7 b	58.3 b	70.7 c	-
	100	4.0 a	15.0 c	29.7 c	45.3 b	63.0 b	72.7 c	-
	500	4.0 a	10.0 a	21.7 a	34.7 a	53.7 a	67.0 b	-
	1000	4.0 a	11.0 ab	23.0 a	35.0 a	52.0 a	62.7 a	-
	Control	4.0 a	12.7 bc	26.7 b	43.0 b	57.7 b	71.3 c	-
S. hirsutum	10	18.7 c	67.7 c	-	-	-	-	-
	100	16.0 b	62.7 bc	-	-	-	-	-
	500	14.3 ab	55.7 a	-	-	-	-	-
	1000	13.0 a	56.3 ab	-	-	-	-	-
	Control	18.0 c	62.3 bc	-	-	-	-	-

[a] Including the diameter (4 mm) of the inoculum plug.
[b] For each fungus, data in columns followed by the same letter do not differ significantly according to Duncan's MRT ($P = 0.05$).
-, Not recorded because colony had reached the edge of the plate.

statistically significant increase in growth rate was noted at 100 μg ml^{-1} resveratrol for *P. chlamydospora* and, to a lesser extent, for *P. aleophilum* from the 14th day of inspection. In plates containing resveratrol, a brownish halo (pigmentation of the medium) appeared around the outer edge of *P. aleophilum* colony at 10 days from inoculation.

Pterostilbene inhibited mycelial growth of all fungi. On the whole, the amount of inhibition increased with the amount of pterostilbene in the medium. With *L. blepharis* this inhibition was statistically significant at all concentrations. With *S. hirsutum* and *F. punctata* inhibition was significant at 10 and 100 μg ml^{-1}. At the highest stilbene concentration, growth of *P. chlamydospora* was significantly lower than the control, with about 50% reduction. Pterostilbene was reasonably effective in reducing the growth of *P. aleophilum*; this reduction was statistically significant at concentration of 100 μg ml^{-1}.

Resveratrol + phosphorous acid

Growth medium containing resveratrol formed crystals also when phosphorous acid was added.

Addition of phosphorous acid caused a lowering of the pH of the growth medium from 5.5 to 5.

Table 2. Mycelial growth[a] of fungi with different amounts of pterostilbene in the medium. Mycelial growth (mm) is expressed as colony diameter.

Fungal species	Pterostilbene amount (μg ml^{-1})	Days after treatment						
		2	5	8	11	14	17	20
P. chlamydospora	1	4.0 a[b]	11.3 bc	14.0 bc	19.7 b	23.3 bc	25.7 b	28.7 b
	10	4.0 a	8.7 ab	12.0 b	17.0 b	21.7 b	22.0 b	27.0 b
	100	4.0 a	7.7 a	9.0 a	10.0 a	11.3 a	13.7 a	18.7 a
	Control	4.0 a	12.0 c	16.0 c	20.7 b	24.7 c	27.0 b	33.0 b
P. aleophilum	1	4.0 a	8.7 ab	10.7 b	13.3 b	18.3 b	21.7 a	29.0 bc
	10	4.0 a	7.3 a	10.3 ab	12.7 ab	17.7 b	21.7 a	26.3 ab
	100	4.0 a	7.3 a	9.0 a	11.3 a	14.7 a	20.3 a	25.3 a
	Control	4.0 a	9.3 b	11.7 b	15.3 c	18.7 b	24.3 b	31.0 c
L. blepharis	1	9.0 ab	34.7 c	58.7 c	78.3 c	-	-	-
	10	9.0 ab	25.3 b	42.0 b	66.0 b	-	-	-
	100	8.3 a	13.3 a	18.3 a	26.3 a	-	-	-
	Control	10.7 b	39.0 d	67.0 d	89.0 d	-	-	-
F. punctata	1	4.0 a	14.3 a	26.7 b	40.7 b	51.7 b	62.7 b	-
	10	4.0 a	12.7 a	23.3 a	35.0 a	45.3 a	54.7 a	-
	100	4.0 a	13.0 a	23.7 ab	34.3 a	44.0 a	53.7 a	-
	Control	4.0 a	12.7 a	26.0 ab	39.3 b	50.7 b	62.7 b	-
S. hirsutum	1	12.3 b	58.7 bc	-	-	-	-	-
	10	9.3 a	52.0 b	-	-	-	-	-
	100	7.7 a	20.0 a	-	-	-	-	-
	Control	14.0 b	62.0 c	-	-	-	-	-

[a, b,] - See Table 1.

Normally this mixture gave a significant reduction of mycelial growth with all fungi. With *F. punctata,* generally low inhibition only occasionally reached statistical significance (Table 3). The superior inhibition with the mixture compared to the compounds when tested separately, was very evident with *L. blepharis* and *P. aleophilum*, and to a lesser extent with *P. chlamydospora*. The brownish halo on a *P. aleophilum* medium was never observed when the medium was treated with resveratrol + phosphorous acid. Resveratrol crystals were observed at all inspection dates. *P. aleophilum* and *F. punctata* growth was stimulated to various degrees by phosphorous acid.

Pterostilbene + phosphorous acid

The pterostilbene + phosphorous acid mixture gave significant inhibition against all fungi at each inspection dates. Growth of *L. blepharis*, *S. hirsutum* and, to a lesser extent, *P. aleophilum* was significantly lower than with the other treatments, at all inspection dates (Table 4). For *P. chlamydospora* growth inhibition with this mixture was significant from the sixth day of inspection, and *F. punctata* inhibition was also significant but less so. Phosphorous acid inhibited *L. blepharis, P. chlamydospora* and *P. aleophilum* but on the contrary stimulated *F. punctata, S. hirsutum* and *P. aleophilum* growth.

Detection of resveratrol in the growth medium

Resveratrol presence in the medium inoculated with *P. aleophilum* and treated with resveratrol was compared with that treated with resveratrol + phosphorous acid to account for the brownish halo appearance.

Table 3. Effect of resveratrol and phosphorous acid on fungal mycelial growth[a]. Mycelial growth (mm) is expressed as colony diameter.

Fungal species	Treatment	Amount of a.i. (μg ml^{-1})	Days after treatment									
			2	4	6	8	10	12	14	16	18	20
P. chlamydospora	Resveratrol	300	5.0 a[b]	6.5 a	8.7 ab	12.0 a	16.0 a	19.3 ab	22.3 a	24.6 b	26.3 b	31.3 b
	Phosphorous acid	300	5.0 a	7.3 a	11.0 b	13.7 a	15.7 a	18.3 a	20.3 a	24.0 b	25.7 b	27.3 ab
	Resv. + phosph. ac.	300+300	5.0 a	5.7 a	7.0 a	10.0 a	11.7 a	14.0 a	17.0 a	18.0 a	20.7 a	22.3 a
	Control	-	5.3 a	13.7 b	16.7 c	19.7 b	22.7 b	26.0 b	30.3 b	33.0 c	36. 0c	40.7 c
P. aleophilum	Resveratrol	300	4.0 a	7.3 b	9.7 b	12.0 ab	14.3 ab	18.0 ab	21.0 b	22.7 ab	28.7 b	30.0 ab
	Phosphorous acid	300	4.0 a	6.7 b	12.3 c	15.0 b	17.0 b	19.0 ab	19.7 ab	22.7 ab	24.3 ab	26.7 ab
	Resv. + phosph. ac.	300+300	4.0 a	5.0 a	6.7 a	9.0 a	10.3 a	13.3 a	14.7 a	16.7 a	17.7 a	19.3 a
	Control	-	4.0 a	7.0 b	9.3 b	12.0 ab	15.7 ab	21.7 b	27.0 b	29.3 b	31.3 b	33.0 b
L. blepharis	Resveratrol	300	7.3 b	14.0 c	26.0 c	35.0 c	48.3 b	66.6 c	-	-	-	-
	Phosphorous acid	300	4.0 a	5.0 b	6.3 b	9.0 b	11.3 a	19.3 b	-	-	-	-
	Resv. + phosph. ac.	300+300	4.0 a	4.0 a	4.0 a	4.0 a	5.3 a	9.3 a	-	-	-	-
	Control	-	8.3 b	18.7 d	28.0 c	42.0 d	54.7 b	81.7 d	-	-	-	-
F. punctata	Resveratrol	300	5.7 ab	10.0 a	17.3 a	26.0 a	34.0 a	45.7 ab	53.7 a	64.0 a	69.3 b	-
	Phosphorous acid	300	5.3 a	15.0 a	23.3 c	36.3 b	46.7 b	56.7 c	71.7 c	75.0 b	77.7 c	-
	Resv. + phosph. ac.	300+300	5.3 a	12.0 a	17.3 a	26.3 a	33.3 a	43.0 a	52.0 a	60.3 a	66.3 a	-
	Control	-	6.7 b	11.7 a	19.3 b	28.0 a	36.0 a	46.7 b	58.0 b	64.3 a	70.3 b	-
S. hirsutum	Resveratrol	300	18.7 a	47.7 b	78.3 b	-	-	-	-	-	-	-
	Phosphorous acid	300	23.0 b	56.7 c	82.7 b	-	-	-	-	-	-	-
	Resv. + phosph. ac.	300+300	17.3 a	39.3 a	64.0 a	-	-	-	-	-	-	-
	Control	-	22.0 b	56.7 c	83.3 b	-	-	-	-	-	-	-

[a, b,] - See Table 1.

Table 4. Effect of pterostilbene and phosphorous acid on fungal mycelial growth[a]. Mycelial growth (mm) is expressed as colony diameter (mm).

Fungal species	Treatment	Amount of a.i. (µg ml^{-1})	Days after treatment									
			2	4	6	8	10	12	14	16	18	20
P. chlamydospora	Pterostilbene	10	4.0 b[b]	7.3 a	9.3 a	12.3 ab	14.3 ab	18.7 ab	21.3 ab	22.0 ab	25.0 ab	27.3 ab
	Phosphorous acid	300	4.7 b	7.0 a	11.0 ab	13.0 ab	15.0b c	17.7 ab	20.0 ab	23.0 bc	25.7 ab	26.6 ab
	Ptero. + phosph. ac.	10+300	4.0 a	6.0 a	8.3 a	10.3 a	12.3 a	14.0 a	16.0 a	18.0 a	20.7 a	24.0 a
	Control	-	6.0 b	8.7 a	13.7 b	17.0 b	18.3 c	23.7 b	26.0 b	28.3 c	31.0 b	34.3 b
P. aleophilum	Pterostilbene	10	4.0 a	7.0 b	8.3 b	10.7 b	12.3 b	13.3 b	18.0 b	19.3 b	23.7 b	27.0 b
	Phosphorous acid	300	4.0 a	6.7 b	12.3 c	15.0 c	17.0 c	19.0 c	19.7 b	22.7 b	24.3 b	26.7 b
	Ptero. + phosph. ac.	10+300	4.0 a	4.0 a	5.0 a	6.0 a	7.7 a	8.7 a	10.0 a	10.7 a	13.3 a	14.7 a
	Control	-	4.0 a	6.3 b	8.3 b	11.0 b	13.0 bc	14.7 b	18.3 b	22.3 b	26.7 b	33.7 b
L. blepharis	Pterostilbene	10	6.0 b	14.7 c	24.3 c	31.3 c	50.3 c	68.0 c	-	-	-	-
	Phosphorous acid	300	4.0 a	5.0 b	6.3 b	9.0 b	11.3 b	19.3 b	-	-	-	-
	Ptero. + phosph. ac.	10+300	4.0 a	4.0 a	4.0 a	4.7 a	5.3 a	7.7 a	-	-	-	-
	Control	-	8.3 c	24.0 d	44.3 d	56.3 d	71.3 d	88.7 d	-	-	-	-
F. punctata	Pterostilbene	10	7.7 ab	14.0 a	24.3 a	33.0 ab	40.3 b	49.3 b	54.7 a	65.7 a	71.7 a	78.0 a
	Phosphorous acid	300	7.3 ab	15.0 ab	23.3 a	35.3 c	46.7 c	56.7 c	71.7 c	75.0 b	77.7 b	82.7 b
	Ptero. + phosph. ac.	10+300	7.0 a	13.7 a	23.3 a	31.7 a	38.7 a	47.7 a	54.0 a	65.7 a	72.3 a	76.3 a
	Control	-	8.0 b	16.3 b	26.0 b	34.3 b	41.7 b	50.3 b	58.0 b	67.0 a	72.3 a	76.7 a
S. hirsutum	Pterostilbene	10	11.3 a	35.3 b	70.0 b	-	-	-	-	-	-	-
	Phosphorous acid	300	23.0 b	56.7 c	84.0 c	-	-	-	-	-	-	-
	Ptero. + phosph. ac.	10+300	7.3 a	16.7 a	39.3 a	-	-	-	-	-	-	-
	Control	-	22.0 b	51.0 c	85.7 c	-	-	-	-	-	-	-

[a, b,] - See Table 1.

Table 5. Phenol oxidase tests.

Fungal species	Laccase	Peroxidase	Tyrosinase	Catalase
P. chlamydospora	Negative	Negative	Negative	Negative
P. aleophilum	Negative	Negative	Negative	Strong
L. blepharis	Negative	Negative	Negative	Low
F. punctata	Strong	-[a]	Negative	-
S. hirsutum	Negative	Negative	Negative	Low

[a] Not determined.

In the pigmented area of the plates treated with resveratrol no crystals were visible and TLC analysis showed no stilbene was present; conversely, in the non pigmented area, the stilbene was present as crystals and was recovered by TLC analysis. In the medium treated with resveratrol + phosphorous acid there never was a brownish halo, and TLC showed stilbene was present.

Enzyme assays

Both laccase tests showed clearly that *F. punctata* was the only fungus that produced extracellular laccase. Neither peroxidase nor tyrosinase activity were detected with any of the fungi. *P. aleophilum* exhibited strong catalase activity as shown by immediate effervescence when dilute hydrogen peroxide was placed on culture. Catalase activity was weak for *L. blepharis* and *S. hirsutum*; completely absent for *P. chlamydospora* (Table 5).

Discussion

The aim of the study was to investigate the activity of resveratrol, pterostilbene and these same compounds + phosphorous acid towards fungi involved in esca.

A clear inhibition of mycelial growth by resveratrol was not found. Normally, the *in vitro* behaviour of this stilbene depended on the amount of it in the growth medium: at low levels (particularly 100 μg ml^{-1}) it actually had a certain stimulant effect on mycelial growth of *P. chlamydospora* and *P. aleophilum* but at higher levels it inhibited growth, especially with *F. punctata* and *S. hirsutum*. These findings agree with the way in which wood extractives interact with fungal activity either as stimulants or inhibitors of growth (Rayner and Boddy, 1988).

Pterostilbene was described by Langkake *et al.* (1979) as a *V. vinifera* phytoalexin and its antifungal activity was relatively high in comparison with resveratrol. Our data showed a direct correlation between the concentration of pterostilbene in the media and the inhibition of fungal radial growth.

A stilbene + phosphorous acid mixture was generally more inhibitory towards all the fungi except *F. punctata*. According to Mugnai *et. al.* (1997) the insensitivity of this white-rot agent to the stilbenes is probably linked with its strong laccase activity. Besides, the involvement of oxidases in wood attack mechanism is well known: stilbenes in the hardwood of conifers are metabolized only by white-rot fungi producing laccase (Loman, 1970a, b). Phosphorous acid did not impair *F. punctata* growth in the presence of stilbenes.

The greater activity of the mixtures against the other fungi might be due to the lowering of the pH. Probably, resveratrol is taken up more readily by fungal cells when such lowering of the pH occurs (Deverall and Rogers, 1972). Moreover, Hoos and Blaich (1990) established that the strongest activity of resveratrol against *Botrytis* on solid medium was at pH 5.

Despite the lack of laccase, peroxidase and tyrosinase activity, *P. aleophilum* was able to inactivate the resveratrol. Although the way in which resveratrol disappared in the brownish halo is still unclear, the oxidation of this stilbene in the brown area was probably related to the strong catalase activity of this fungus. The superior activity of resveratrol + phosphorous acid appeared to be closely linked to the inability of *P. aleophilum* to metabolize this stilbene in the presence of phosphorous acid.

A clear inhibitory effect of phosphorous acid was not found with any fungus except *L. blepharis*. Phosphorous acid did not act as an antifungal agent as such but enhanced stilbene activity.

Although the relation between the *Vitis* stilbenes (resveratrol and pterostilbene) and resistance to fungal infections is still unclear, the results obtained in this study demonstrated that a mixture stilbenes + phosphorous acid was more active against *L. blepharis*, *P. aleophilum*, *P. chlamydospora* and *S. hirsutum*. Basically, the combina-

tion of phosphorous acid and the stilbenes may contribute to prevent or reduce fungal infection. Further and more extensive *in vivo* investigations are obviously needed to evaluate the potential of fosetyl-Al, to control the esca syndrome, especially considering the spread of young grapevine decline due to *Phaeoacremonium* spp. (Ferreira *et al*., 1994; Morton, 1995, 1997; Bertelli *et al*., 1998; Pascoe, 1998; Scheck *et al*., 1998).

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