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Effects on plants of metabolites produced in culture by *Phaeoacremonium chlamydosporum*, *P. aleophilum* and *Fomitiporia punctata*

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Summary. Several phytotoxic metabolites were extracted and purified from culture filtrates of *Phaeoacremonium chlamydosporum*, *P. aleophilum* and *Fomitiporia punctata*, three fungi associated with esca of grapevine. Those identified and characterised so far were α-glucans of various molecular weight (pullulans) produced by both species of *Phaeoacremonium,* and two naphthalenone pentaketides (scytalone and isosclerone) produced by *P. aleophilum*. Absorbed at very low doses by detached leaves of grapevine or injected into the woody tissue of shoots and branches of standing grapevines, these metabolites produced foliar symptoms similar to those shown by the esca-affected vines. The same results were obtained with preparations of pullulan extracted from the discoloured woody tissue of a grapevine infected with *P. chlamydosporum*, and with samples of commercial pullulan.

Key words: *Phaeoacremonium chlamydosporum*, *P. aleophilum*, *Fomitiporia punctata*, toxins, esca, grapevine.

Introduction

It seems probable that the external symptoms of esca of grapevine (Mugnai *et al.*, 1999) are mainly caused either by substances produced in discoloured woody tissue colonised by *Phaeoacremonium chlamydosporum* W. Gams *et al.* ([1]), *P. aleophilum* W. Gams *et al.* as well as by other fungal species, exibiting brown wood-streaking, or by those produced in the wood parts colonised and decayed by *Fomitiporia punctata* (Fr.) Murrill exibiting white rot. From the diseased wood, extra-cellular metabolites from these fungi, as well as plant degradation or reaction products, then spread to the functioning xylem and are transported to the aerial plant organs in the transpiration stream.

This paper reports recent data on some phytotoxic metabolites produced *in vitro* and *in planta* by *P. chlamydosporum*, *P. aleophilum* and *F. punctata*.

Materials and methods

Fungal strains and culture conditions

Stock cultures of *P. chlamydosporum* (*Pch*) strain PVFi56 (CBS 229.95), *P. aleophilum* (*Pal*) strain PVFi69-257 (CBS 631.94) and *F. punctata* (*Fop*) strain *Fop*1 (DBPV, University of Bari) isolated from grapevines in Italy were maintained on slants of malt agar or potato-sucrose agar at 4°C. All the strains were grown in stationary cultures in 1 l Roux flasks containing 150 ml Czapek medi-

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

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um amended with 0.1% yeast and 0.1% malt extract (pH 5.8). Each flask was seeded with 5 ml of a suspension of three 10-day-old cultures in 50 ml sterile water. The flasks were incubated at 25°C for 28 days in darkness. At harvest, the mycelial mat was removed by filtration on Miracloth (Calbiochem, La Jolla, CA, USA).

Extraction of low-molecular-weight metabolites from fungal cultures

The culture filtrates (3 l per strain) were brought to pH 4 with 2M HCl and extracted 4 times with ethyl acetate (1.5 l each). The combined organic extracts were dried on anhydrous sodium sulphate and evaporated under reduced pressure to give dark-brown oily residues (*Pch*, 100 mg; *Pal*, 150 mg; *Fop*, 120 mg). Chromatographic separation was performed on silica gel plates (Merk F_{254}, 0.50 mm, 20x20 cm; Merck, Darmstaadt, Germany) using a mixture of chloroform and ethyl acetate (3:1) as an eluent. Each band was scraped off and dissolved in ethyl acetate. The solution was first concentrated under reduced pressure and then lyophilised.

Extraction of exopolysaccharides (EPS) from fungal cultures

The culture filtrates from *Pch* and *Pal* were treated with two volumes of absolute ethanol following the procedure described in previous papers (Sparapano and Bruno, 1997, 1998). The resulting precipitates, one for *Pal* and two for *Pch* (upper and lower precipitates) were filtered through Whatman GFC filters (Whatman, Clifton, NJ, USA), dried and weighed.

Extraction of extracellular polysaccharides (EPS) from naturally-infected grapevines

Portions of wood with brown streaks from which *P. chlamydosporum* had been isolated were excised from the trunk of a 30-year-old 'Sangiovese' grapevine showing wood and foliar symptoms of esca. Wood portions of a healthy-looking vine of the same cultivar and age were similarly sampled to serve as a control. The excised woody tissue was ground and extracted following the same procedure as that used for the culture filtrates.

Chemical analysis

HPLC analysis was performed on a Waters liquid chromatograph equipped with a differential refractometer mod. 410. Chromatograms were recorded and analysed on a Waters 740 Data module. The column was a 1,000 Å Nucleogel GFC (300x7.7 mm I.D., Macherey-Nagel, Düren, Germany). Elution was carried out at room temperature under isocratic conditions with pure water that had been filtered through Millipore membrane filter (0.45 μm, Millipore, Bedford, MA, USA), de-aerated and protected from microbial contamination with 0.05% sodium azide, and finally delivered to the column at a flow rate of 1 ml min^{-1}. Before injection, reference compounds, crude EPS and purified samples of pullulan were dissolved in water and filtered through a SPE tube (LC-4,500 Å pores, Supelco, Bellefonte, PA, USA) and then through a Millex FG (0.22 μm, Millipore). The IR spectra were determined with a Perkin Elmer mod. 1720 spectrometer (Perkin-Elmer, Norwalk, CT, USA), using the potassium bromide technique (Barker *et al.,* 1956). Elemental analysis of pure pullulan preparations was also performed.

Molecular weight determination of pullulan

The molecular weight of purified samples of pullulan was estimated by gel permeation chromatography (GPC) with the same column as that used for their separation by HPLC. The column was calibrated using pullulan standards (Macherey-Nagel, Sigma, St Louis, MO, USA and Hayashibara, Okayama, Japan) of known molecular weight, ranging from 5.8 to $8.5x10^2$ kDa, and dextran standards (Sigma) from 9.3 to $2x10^3$ kDa. The reference pullulan (Sigma) was from *Aureobasidium pullulans* (De Bary) G. Arnaud (*Apu*).

Grapevine bioassay

The culture filtrates, the crude organic extracts, the EPS and their pure components, and the other metabolites obtained from each fungal species were assayed on two grapevine cultivars: Italia and Matilde, in a growth chamber on detached leaves, and in the field by injections into the shoots and branches of standing plants.

The grapevine leaves with their petioles were immersed in 3 ml toxic solution until complete absorption, which usually took a few hours and even with the highest molecular weight EPS not more than 24 h, and were then transferred to distilled water. During the assay, the leaves were kept in a

growth chamber at relatively low temperature (23°C), RH (60%) and illumination (150 μE m^{-2} s^{-1}). Toxicity symptoms were recorded 48 h later.

Syringes, each containing 2.5 ml of toxic solution, were used to stick an equal number of shoots and young branches of standing grapevines. Syringes were left *in situ* until complete absorption (7 to 14 days) of toxic solution. Foliar symptoms were recorded every two days for two months.

Aliquots of culture filtrate were assayed after 1:100 dilution with distilled water. Crude extracts from culture filtrates were tested at 0.05, 0.1 and 1 mg ml^{-1}. Residues of preparative TLC bands were tested at 0.1, 0.2 and 0.5 mg ml^{-1}. The crude EPS were tested at 0.01, 0.5 and 1 mg ml^{-1}. Purified pullulan was assayed at 0.05, 0.1 and 0.2 mg ml^{-1}. Controls included distilled water and 1:100 diluted culture media**.**

Callus tissue assay

Sections were removed aseptically from the stems of 40-day-old tobacco cv. White Burley seedlings. The tobacco explants were cultivated on modified Murashige and Skoog's medium (1962) under the conditions stated in a previous work (Sparapano, 1976). After one month, the tobacco calli that had developed at the edge of the pith segments were transferred to fresh medium containing a lower concentration of kinetin (0.01 mg l^{-1}) and 2,4-dichlorophenoxyacetic acid (0.2 mg l^{-1}). The callus produced with two passages of 30 days each was used as inoculum for the experiments.

Sections were also excised aseptically from the pith of grapevine cv. Italia shoots. The explants were grown on modified Linsmaier and Skoog's (1965) medium containing 6-benzylaminopurine (15 mM), pyridoxine-HCl (0.5 mg l^{-1}), sucrose (30 g l^{-1}), thiamine-HCl (0.4 mg l^{-1}), myo-inositol (100 mg l^{-1}), polyvinylpyrrolidone (0.1 g l^{-1}), and agar (10 g l^{-1}). Two months later, calli that had developed at the edge of the pith sections were transferred to the same fresh medium. The callus produced with two passages of 60 days each was used as inoculum for the experiments.

Crude extracts and samples of pullulan were assayed at 0.1, 0.05 and 0.01 mg ml^{-1} by adding them to the culture medium after sterile filtration on a Millipore filter (0.22 μm). After 60 days of subculturing, the callus yield was measured as fresh and dry weight.

Results

Batch cultures

The growth rate of *Pal* in liquid culture was higher than that of *Pch*, whereas *Fop* grew slowly and with mycelial mats.

The two species of *Phaeoacremonium* produced mycelium, conidia and chlamydospore-like structures, which had already formed in abundance even when the culture was 10 days old. The culture liquids of both *Phaeocremonium* species appeared viscous after the first week of incubation. As soon as the colonies of *Pch* became olive-green, the culture medium turned greenish-brown. Later, *Pch* mycelium became covered by a gelatinous material which turned dark grey or black.

When the three fungi were cultivated under the same cultural conditions, the cultures of *Pch* had the lowest pH value, those of *Fop* yielded the highest biomass, and those of *Pal* produced the highest amount of organic extracts (Table 1).

Table 1. Cultural characteristics and crude organic extract yield in liquid cultures of *P. chlamydosporum, P. aleophilum* and *F. punctata*[a].

Fungal species	pH initial	pH final	Mat fresh wt (g l^{-1})	Mat dry wt (g l^{-1})	Organic extract (mg l^{-1})
P. chlamydosporum	6.8	4.4	13.5±1.2	1.23±0.24	62±13
P. aleophilum	6.8	5.5	16.7±1.4	1.54±0.32	177±34
F. punctata	6.8	5.3	19.2±2.1	1.73±0.34	39±12

[a] Values are the means of 5 replicates ± S.D.

EPS production

EPS were produced in large amount *in vitro* by *Pch* (average: *ca*. 6.1 g l^{-1}), to a smaller amount by *Pal* (0.6 g l^{-1}) (Table 2), and not at all by *Fop*.

Recovery of EPS by addition of ethanol to cell-free culture filtrates of *Pal* gave a single precipitate, while two physically distinct polymer fractions (upper and lower precipitates, appearing as separate layers) were formed with *Pch* culture filtrates (Table 2). Similar results (i.e. two distinct precipitates) were obtained with *Apu* by Madi *et al*. (1997).

Regardless of its origin, the main component of the EPS from both species of *Phaeoacremonium* was pullulan, a mixture of α-glucans whose molecular weights, as determined by GPC, are shown in Table 3.

The pullulan produced by *Pal* (0.3 g l^{-1}) consisted of three molecular species with a mol. wt ranging from $2.6x10^3$ to 75 kDa (Tables 2 and 3).

The upper EPS precipitate from cultures of *Pch* (0.28 g l^{-1}) contained three species of pullulan whose mol. wt ranged from $2x10^2$ to $2.5x10^3$ kDa, and the lower EPS precipitate (5.8 gl^{-1}) contained three more species of pullulan with a mol. wt from $1.3x10^2$ to $2.2x10^3$ kDa (Tables 2 and 3).

Purification of the crude extracts from the excised woody tissue of esca-affected 'Sangiovese' grapevines led to the recovery of pullulan, which was never found in extracts from the woody portions of healthy-looking vines. GPC analysis of pullulan found in these extracts yielded five components, two of which had the same mol. wt ($3.5x10^2$ and $8x10^2$ kDa) as the corresponding components of pullulan produced *in vitro* by *Pch* (Table 3).

The GPC data, the IR spectra and the elemental analysis of all pullulan preparations recorded above, compared with pullulan standards (Sigma, Macherey-Nagel and Hayashibara), corroborated that pullulan recovered from culture filtrates and that from infected vines was identical.

Production of low-molecular-weight metabolites

The organic extracts from cultures of *Pch, Pal* and *Fop* contained several low-molecular-weight phytotoxic metabolites whose chemical characterisation is in progress. So far, two naphthalenone pentaketides produced by *Pal* has been identified by chemical and spectroscopic methods: scytalone and isosclerone (Evidente *et al*., this issue) ([2]).

Phytotoxic activity

When detached leaves absorbed crude organic extracts from the culture filtrates of each fungal species at low concentrations in water, symptoms were produced on the foliar lamina. Pure preparations of EPS or pullulan from cultures of both species of *Phaeoacremonium* caused the collapse of marginal and interveinal tissue, which soon desiccated, eventually becoming dry with a paper-like appearance (Fig. 1A).

At very low doses (e.g. 50 μg ml^{-1}), pullulan produced chlorotic interveinal and marginal spots or patches, which slowly became necrotic (Fig. 1B). Similar symptoms were produced when identical concentrations of pullulan extracted from naturally-infected grapevine wood (Fig. 1C), or of commercial pullulan (Fig. 1D) were used.

A range of chlorotic and necrotic spots were induced on detached leaves of grapevine by absorption of low concentrations of organic extracts from culture filtrates of the three fungi (Fig. 2), which contain other metabolites including the above mentioned naphthalenone pentaketides (Evidente *et al*., this issue).

The same type of leaf symptoms was also shown on the leaves of shoots and branches of standing grapevines syringe-injected with the toxic preparations.

Callus tissue assay

When any fungal species was grown together with callus cultures of grapevine cv. Italia or tobacco cv. White Burley, the calli showed reduced growth, gummosis, browning and necrosis (Fig. 3).

The inhibitory effect of all three fungi on callus growth was higher on grapevine than on tobacco (Table 4). *Pal* had the greatest inhibitory effect on tobacco calli weight, causing a 64% reduction (dry wt) compared with the controls. *Pch* was more active against grapevine calli, causing a 85% reduction (dry wt). *Fop* had the least effect, with a 46% reduction of tobacco calli and a 59% reduction of grapevine calli (dry wt).

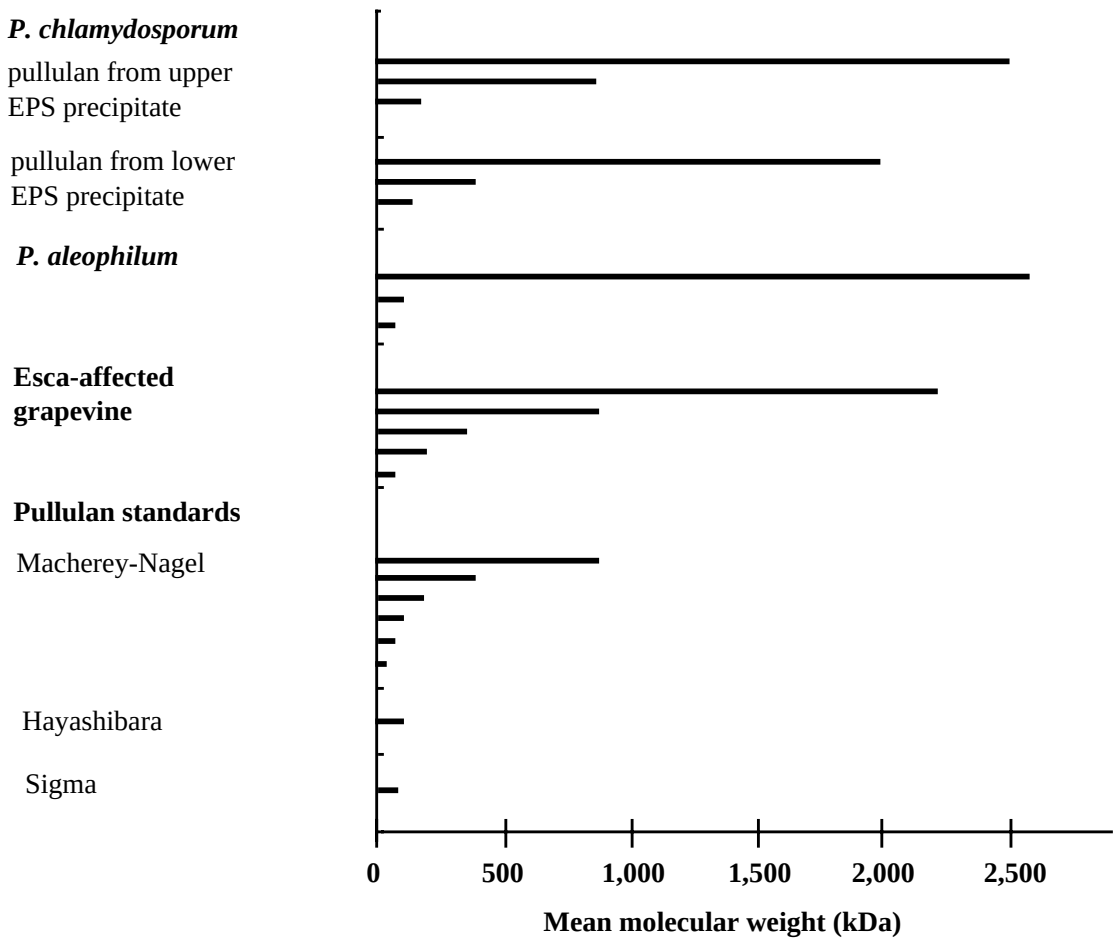
([2]) Two of these metabolites have been independently detected in culture filtrates of *P. chlamydosporum* in Switzerland. See Tabacchi in this issue.

Table 2. Exopolysaccharide (EPS) and pullulan production by *P. chlamydosporum* and *P. aleophilum* in 28-day-old stationary cultures[a].

Fungal species	Biomass (g l^{-1})	EPS (g l^{-1})	Pullulan (g l^{-1})
P. chlamydosporum	12.3±1.13		
upper precipitate		0.28±0.06	0.10±0.02
lower precipitate		5.80±0.22	2.30±0.16
P. aleophilum	15.3±1.15		
precipitate		0.60±0.04	0.30±0.07

[a] Values are the means of 5 replicates ± S.D.

Table 3. Mean molecular weights of pullulan families purified from culture filtrates of *P. chlamydosporum* and *P. aleophilum* and from the woody tissue of an esca-affected cv. Sangiovese grapevine, as compared with commercial pullulan standards.



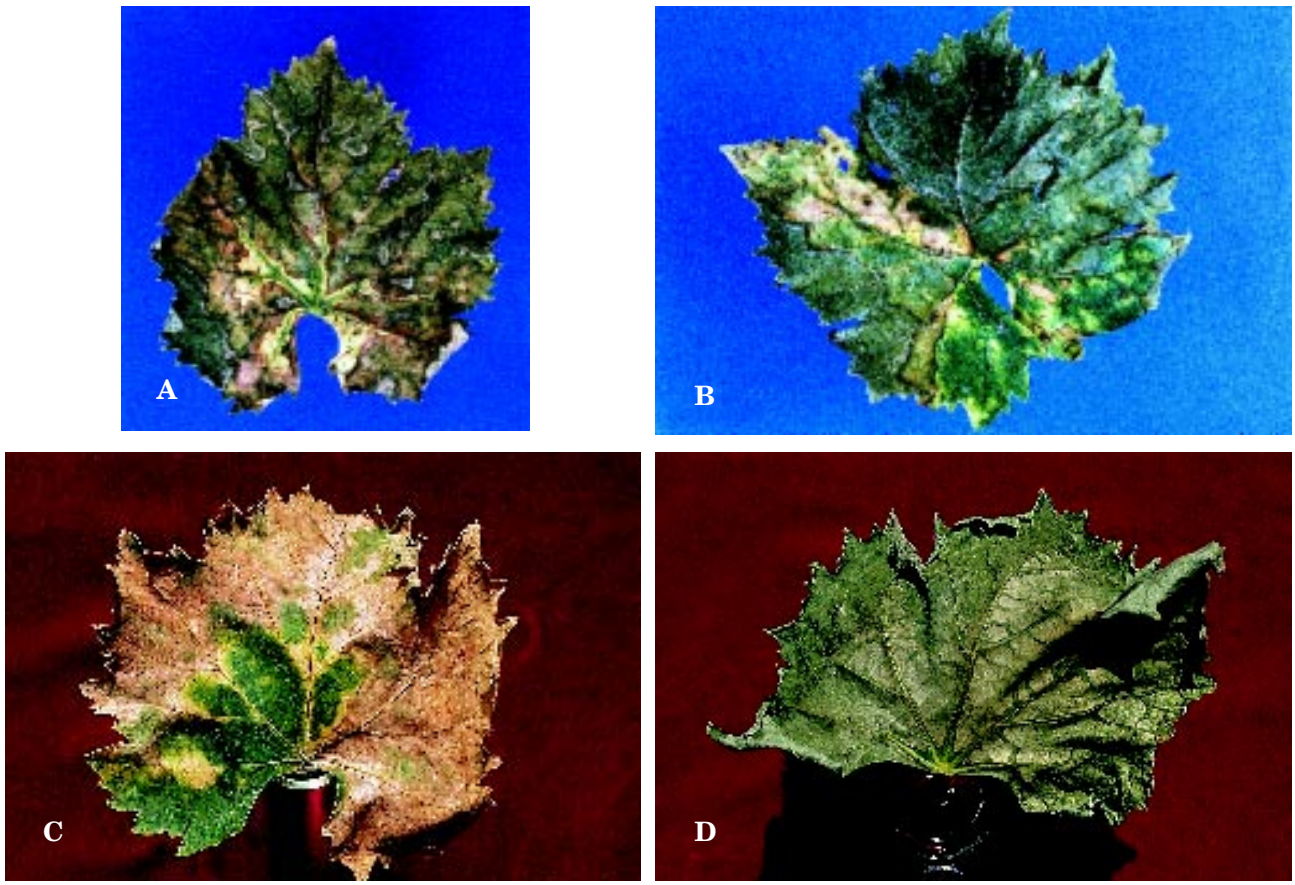


Fig. 1. Effect of the absorption for a few hours of 3 ml each of: A. 0.02 mg ml^{-1} EPS from culture filtrate of 28-day-old *P. chlamydosporum* liquid culture; B. 0.05 mg ml^{-1} pullulan purified from EPS; C. 0.05 mg ml^{-1} pullulan purified from 30-year-old esca-affected 'Sangiovese' grapevine; D. 0.02 mg ml^{-1} Sigma pullulan on detached leaves of grapevine cv. Italia.

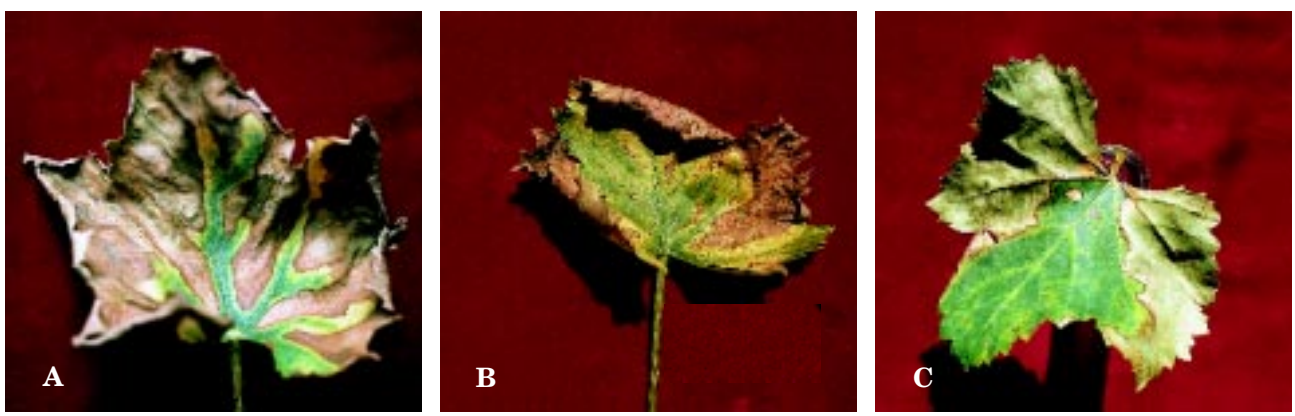


Fig. 2. Effect of the absorption for a few hours of 0.5 mg ml^{-1} crude extract from filtrates of 28-day-old culture of: A. *P. chlamydosporum*; B. *P. aleophilum*; C. *F. punctata* on detached leaves of grapevine cv. Italia.

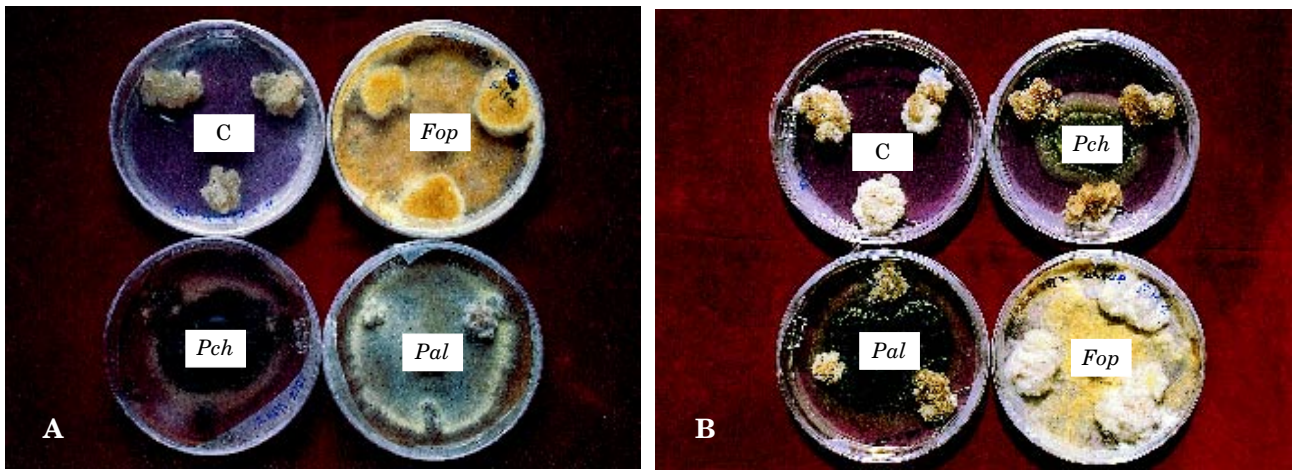


Fig. 3. Callus cultures of grapevine cv. Italia (A) or tobacco cv. White Burley (B) grown in the presence of *P. chalmydosporum* (*Pch*), *P. aleophilum* (*Pal*) and *F. punctata* (*Fop*). C = controls.

Table 4. Yield of 60-day-old callus tissue cultures of tobacco cv. White Burley and of grapevine cv. Italia grown in presence of *P. chlamydosporum*, *P. aleophilum* and *F. punctata*.[a]

Fungal species	Tobacco callus		Grapevine callus	
	fw[b]	dw[c]	fw[b]	dw[c]
P. chlamydosporum	4.97±0.44 (-38%)	0.16±0.06 (-59%)	0.9±0.1 (-88%)	0.06±0.02 (-85%)
P. aleophilum	3.19±0.41 (-60%)	0.14±0.05 (-64%)	2.20±0.36 (-71%)	0.10±0.02 (-74%)
F. punctata	5.92±0.68 (-26%)	0.21±0.07 (-46%)	3.58±0.22 (-52%)	0.16±0.03 (-59%)
None (control)	8.03±0.87	0.39±0.05	7.49±0.82	0.39±0.06

[a] Values are the means of 3 replicates ± S.D. In parentheses, the percentage of weight reduction compared with controls.
[b] Fresh weight (g/plate).
[c] Dry weight (g/plate).

All three fungi invaded callus tissue after overgrowing its external surface entirely. Hyphae were observed within the callus mass, where several cellular aggregates showed melanin-like deposits, particularly when invaded by *Pal*.

EPS were also detected in samples of calli infected by either species of *Phaeocremonium*.

Adding small amounts (0.01-0.1 mg ml^{-1}) of crude organic extracts or of EPS from the culture filtrates of *Pch* to the nutrient medium used for the callus cultures induced a reduction in growth and the browning of the calli. Even in this case however, the inhibitory effect of these substances on callus growth was higher on calli from the host (grapevine) that on those from the non-host (tobacco) (Table 5). At the concentrations tested, the crude extracts were less effective than EPS on calli from either source. The strongest effect of the

Table 5. Yields of 60-day-old callus tissue cultures of tobacco cv. White Burley and of grapevine cv. Italia grown on a medium containing EPS or crude organic extracts from *P. chlamydosporum* cultures[a].

Substances added to the medium	Concentration (mg ml^{-1})	Tobacco callus		Grapevine callus	
		fw[b]	dw[c]	fw[b]	dw[c]
EPS	0.1	5.96±0.85 (-32%)	0.25±0.07 (-22%)	2.75±0.81 (-60%)	0.18±0.05 (-51%)
	0.05	6.76±0.76 (-23%)	0.27±0.05 (-16%)	3.12±0.75 (-54%)	0.16±0.05 (-57%)
	0.01	6.81±1.20 (-22%)	0.29±0.02 (-9%)	4.58±0.66 (-33%)	0.25±0.10 (-33%)
Crude organic extracts	0.1	7.42±0.87 (-15%)	0.34±0.10 (+6%)	4.78±0.78 (-30%)	0.24±0.06 (-35%)
	0.05	8.33±1.10 (-5%)	0.32±0.14 (0)	5.24±0.82 (-23%)	0.28±0.05 (-24%)
	0.01	8.25±0.76 (-5%)	0.32±0.10 (0)	6.22±0.74 (-8%)	0.31±0.12 (-16%)
None (control)		8.72±1.2	0.32±0.08	6.78±0.92	0.37±0.05

[a] Values are the means of 3 replicates ± S.D. In parentheses, percentage of growth reduction compared with controls.
[b] Fresh weight (g/plate).
[c] Dry weight (g/plate).

crude extracts (0.1 mg ml^{-1}) on the grapevine callus was a 35% reduction (dry wt) in growth compared with the controls; the growth of tobacco callus was not significantly affected here (6% increase). The weight of tobacco callus grown on a medium containing 0.1 mg ml^{-1} EPS was reduced by 22% (dry wt), whereas that of grapevine was reduced by 51% (dry wt) compared with the controls.

Discussion

The results of this study provide new information on the production in culture of phytotoxic metabolites by three fungi commonly associated with esca and related diseases of grapevine.

Purification of organic extracts from the culture filtrates of *P. chlamydosporum* and *P. aleophilum* gave 5 and 6 fractions respectively that were toxic in assays with detached leaves of two grapevine cultivars and when the fractions were injected into shoots of standing grapevines of the same cultivars.

Attention was drawn in particular to the EPS fractions, whose major component was pullulan, a mixture of α-glucans with varying molecular weight. Under identical cultural conditions, *Pch* produced about 8 times as much pullulan as *Pal*.

The toxicity to plants of pullulan produced by the above species of *Phaeoacremonium*, as well as by another plant pathogenic fungus, *Cryphonectria parasitica* (Murrill) Barr, has been demonstrated in previous studies (Sparapano and Bruno, 1997, 1998; Sparapano *et al.*, 1998; Corsaro *et al.*, 1998). The findings of the present study indicated that even at a concentration as low as 50 μg ml^{-1}, pullulan extracted from cultures of both species of *Phaeoacremonium* produced severe symptoms in grapevine leaves. At the same low concentration, pullulan extracted from the discoloured wood of a grapevine naturally infected by *Pch*, and that from a non-plant-pathogenic fungus (*Apu*) were both equally phytotoxic.

Solutions of high-molecular-weight plant or microbial polysaccharides are only with difficulty absorbed by the plant cuttings used in bioassays, and usually induce the test plants to wilt. In contrast, absorption of pullulan solutions by the detached leaves of grapevine or its injection into the shoots and branches of standing grape-

vines was relatively easy. In both cases the collapse and drying of large parts of the leaf lamina resulted. The peculiar structure and conformation of pullulan and its rheological and solution properties (Seviour *et al*., 1992; Crescenzi, 1994) may account for the unusually easy transport of these large-molecular compounds in the xylem elements.

On the other hand, pullulan is largely used in the food industry, and to our knowledge there is no report of its toxicity to man or animals. It is possible that the toxic effect of pullulan to the leaf tissue is associated with the formation of thin films in the mesophyll, which are with difficulty permeable by oxygen. If this hypothesis is correct, drying of the leaf tissue would represent a physical rather than a chemical effect of pullulan or some of its byproducts on the plant cells.

The addition of EPS to the nutrient medium caused a reduction in the growth of callus cultures of grapevine and tobacco. Such a reduction was more severe in calli from the natural host of the producer fungi, grapevine, than in those from a non-host like tobacco. The greater sensitivity of grapevine to EPS may indicate the selective toxicity of some particular EPS component, possibly pullulan, to grapevine cells and needs further research.

Purification of the organic extracts from cultures of *Fop* gave 5 phytotoxic low-molecular-weight fractions, whose characterisation is in progress, but no exopolysaccharides.

In conclusion, the findings indicated that several phytotoxic compounds were produced in culture by the three species of fungi most commonly associated with esca, and suggested that at least some of these metabolites may be involved in the pathogenesis and symptom expression of esca and related syndromes on grapevine. The detection of pullulan in the discoloured woody tissue of a grapevine infected with *Pch* and exibiting brown wood-streaking seems to corroborate this hypothesis.

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