A New Flow Cytometry Technique to Identify *Phaeomoniella chlamydospora* Exopolysaccharides and Study Mechanisms of Esca Grapevine Foliar Symptoms

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ABSTRACT

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A flow cytometry technique that unequivocally identifies some of the toxic metabolites produced by *Phaeomoniella chlamydospora*, one of the main fungal pathogens causing esca disease of grapevine (*Vitis vinifera*), was developed. Antibodies raised against exopolysaccharides (EPS)—metabolites produced by *Pa. chlamydospora* that have been reported to be phytotoxic—were used as antigen to immunize rats. The specificity of these antibodies was assayed by flow cytometry against *Pa. chlamydospora* polysaccharides and against EPS with a different structure isolated from other phytopathogenic fungi, including *Phaeoacremonium aleophilum* and the Botryosphaeriaceae species *Neofusicoccum luteum* and *N. parvum*. Using this method, *Pa. chlamydospora* polysaccharides were detected in the symptomatic leaves of esca-affected grapevines, while healthy and asymptomatic leaves from both healthy and diseased vines did not produce a binding reaction. This method potentially could be used to develop a simple kit to study the mechanisms underlying the development of esca foliar symptoms and to indirectly assess the presence of *Pa. chlamydospora* in grapevine material.

Esca is one of the most important diseases of grapevine (*Vitis vinifera* L.) in almost all areas where vines are grown. The disease affects the woody tissues (trunk and branches), which show brown streaking and often also white rot of the wood in the trunk or main branches, and sudden wilting (apoplexy) that eventually leads to the death of the vine (17). The shoots, leaves, and berries are also affected. The leaf symptoms are the most characteristic, consisting of irregular light green or chlorotic areas between the veins or along the leaf margin, which gradually spread from the basal to the distal part of the shoot. As the chlorotic tissue turns yellow-brown or red-brown, the diseased leaves assume a tiger-stripe pattern (17,25). Fruit symptoms include tiny brown spots and sometimes withering of the berries. Of all these symptoms, the most typical and striking is the develop-

ment of tiger stripes on the leaves, but esca can produce a wide spectrum of leaf symptoms and discolorations, some of which happen to be identical with the symptoms of other grapevine disorders (24). Consequently, the visible symptoms of esca are not always diagnostic and in such cases should be confirmed by other means.

Esca is a complex disease in which a number of different fungi have been reported as causal agents, the principal ones being *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingfield & L. Mugnai) Crous & W. Gams, *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingfield & L. Mugnai, and *Fomitiporia mediterranea* M. Fischer (16,17,25). *Pa. chlamydospora* is a tracheomycotic pathogen that is associated with all the syndromes in the esca complex (brown wood streaking of the rootstock, Petri disease, young esca), while *F. mediterranea* and other species of basidiomycetes are agents of white rot (11,16,17,24,25).

One peculiar characteristic of esca is that the expression of leaf symptoms is intermittent and may not appear in every growing season. They can suddenly fail to show up one year or even for a number of years, during which time the vine—although still diseased—is asymptomatic

and to all intents and purposes indistinguishable from a healthy vine (13,14,23). This discontinuity of leaf symptoms makes it almost impossible to determine the true incidence of esca in a vineyard at any given time.

Both the leaf and berry symptoms of esca are suspected to be caused by phytotoxins produced by *Pm. aleophilum* and/or *Pa. chlamydospora*. These phytotoxins have been isolated from culture filtrates of the two fungi and have been identified as scytalone, isosclerone, and pullulans (exopolysaccharides [EPS]) (2,9,22).

The objective of this study was to develop a rapid and specific method for the production of polyclonal antibodies against *Pa. chlamydospora* toxins in order to detect the presence of the toxin produced by *Pa. chlamydospora* in the leaves of esca-affected grapevines. These antibodies could also be used as tools to investigate the manner in which the foliar symptoms of esca are induced. We describe here an immunofluorescence procedure based on flow cytometry and antibodies developed against the *Pa. chlamydospora* exopolysaccharide hapten.

MATERIALS AND METHODS

Fungal strains and culture conditions. The *Pa. chlamydospora* isolate used in this study was obtained from esca-diseased grapevines (*V. vinifera*) in Italy, and is deposited as the *Pa. chlamydospora* type strain (CBS 229.95) at the Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands). In addition, five other isolates of *Pch* (PVFi 7, 31, 135, 551, and 606) and one isolate of *Pm. aleophilum* (PVFi-G1) obtained from different grapevine cultivars and countries, and belonging to the collection of the Dipartimento di Biotechnologie Agrarie-Patologia Vegetale, Università degli Studi di Firenze, were used. Cultures were maintained in slants of malt agar at 4°C and grown to produce metabolites in stationary liquid culture, as described by Evidente et al. for *Pm. aleophilum* (9). Other strains included for comparison were *P. foeniculi* Du Manoir & Vegh (5), *P. exigua* var. *heteromorpha*

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(Schulzer & Sacc.) Noordel. & Boerema (3), *Neofusicoccum luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, and *N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (15). For data on strains and growth conditions of *Phomopsis foeniculi*, *Phoma exigua* var. *heteromorpha*, and *Neofusicoccum luteum* and *N. parvum*, refer to references 5, 3, and 15.

Plant material. Vine leaves with interveinal, marginal discoloration and necrosis, and acropetal symptom gradient (Fig. 1A to C), were collected in July 2007 from a 14-year-old cv. Cabernet Sauvignon vineyard in the province of Florence, Italy, which had been monitored for esca symptoms for 4 years. Three to five leaves per each health class, 15 leaves per plant, were collected. At the same time, healthy control leaves were also collected in the same vineyard from vines that had not shown esca foliar symptoms in any of the survey years (2003 to 2006) or in 2007 (vines that have been asymptomatic for so long are generally presumed to be healthy).

Leaves were harvested at the end of July from two asymptomatic vines (no. 61 and 62) and from eight symptomatic vines (no. 1, 2, 3, 6, 44, 47, 58, and 141) that showed typical esca symptoms (tiger-striped necrosis and chlorosis on the blade). The leaves were selected as follows: (AAA) asymptomatic leaves from asymptomatic shoots on asymptomatic vines; (AAD) asymptomatic leaves from asymptomatic shoots on symptomatic vines; (ACD) asymptomatic leaves close to symptomatic leaves growing on the same shoot; (AFD) asymptomatic leaves distal to symptomatic leaves growing on the same shoot; (D) symptomatic leaves with incipient signs (small yellowish chlorotic spots); and (S) symptomatic leaves with tiger-striped coloration but no necrosis.

Preparation of native EPS from fungal culture filtrates. The pooled culture filtrates of *Pa. chlamydospora* (1.7 liters) was brought to pH 4.0 with 2 M HCl and extracted four times with 700 ml of ethyl acetate each time. The combined organic extracts were dried on anhydrous sodium sulfate and evaporated under reduced pressure, yielding a dark-brown oily residue containing the naphthalenone pentaketides scytalone and isosclerone (9). The aqueous phase resulting from the acid extraction was freeze-dried, dissolved in 100 ml of ultrapure Milli-Q water, cooled to 4°C, mixed by stirring with 5 volumes (500 ml) of cold absolute ethanol, and left overnight at -20°C. The precipitate was separated by centrifugation at 7,000 rpm for 45 min at 4°C. The precipitate was dissolved in 90 ml of ultrapure Milli-Q water and reprecipitated with 450 ml of cold absolute ethanol as described above. The resulting precipitate was dissolved in 200 ml of ultrapure Milli-Q water and dialyzed using Spectra/Por molecular porous membrane

tubing with a 3,500-Da cutoff point (Spectrum Medical Industries Inc., Houston, TX, USA). Tubes were immersed in 10 volumes of distilled water for 48 h at 10°C, with water being exchanged every 6 h. After dialysis, the tube contents were recovered and lyophilized to obtain the native EPS (400.5 mg).

The same procedure was applied to the culture filtrates (100 ml) of the other *Pa. chlamydospora* strains (PVFi 7, 31, 135, 551, and 606) and *Pm. aleophilum* strain PVFi G-1 (6.5 liters), to obtain native EPS (31.4, 31.5, 20.5, 16.5, 19.1, and 375.1 mg, respectively). None of these culture filtrates had undergone the acid procedure to extract the lipophilic toxins. For information on extraction of the EPS from culture filtrates of *P. foeniculi* and *P. exigua* var. *heteromorpha* and from *N.*

luteum and *N. parvum*, refer to references 5, 8, and 15.

Extraction of polysaccharides from grapevine leaves. Fresh leaf samples were weighed (range: 0.56 to 1.19 g), frozen (-20°C), ground, and homogenized with 20 ml of phosphate buffer (1.36 g/liter K₂H₂PO₄ and 1.24 g/liter K₂HPO₄), pH 6.0, in a blender (Waring Products Division, New Hartford, CT, USA), and the homogenate was then lyophilized. The lyophilized residue was dissolved in 5 ml of distilled Milli-Q water and 10 volumes (50 ml) of ethanol with stirring. The suspension was kept in the dark at 4°C for 24 h, after which it was centrifuged at 4°C at 7,000 rpm for 40 min, and the supernatant was collected and evaporated under vacuum. The residue was lyophilized, and residues ranged from 22.6 to 126.6 mg.

Fig. 1. Esca-diseased grapevine leaves with tiger-stripe discoloration: **A**, leaves showing different stages of the symptoms; **B**, a leaf showing the initial interveinal chlorotic spots; **C**, a shoot showing the acropetal symptom gradient.

The UV spectra of these extracts were recorded in water on a Perkin-Elmer Lambda 25 UV/Vis spectrophotometer.

Preparation of antibodies. Native EPS from *Pa. chlamydospora* (CBS.229.95 strain) were used to immunize Fisher rats following the method reported by Del Sorbo et al. (6). Specifically, two approximately 3-month-old New Zealand female rats were injected four times at 1-week intervals with 1 mg of *Pch* EPS dissolved in 1 ml of PBS (0.15 M sodium phosphate, pH 7.2, containing 0.85% NaCl). The first injection consisted of the antigen solution emulsified with an equal volume of

Freund's adjuvant (Sigma Chemical Co., St. Louis, MO, USA) immediately before use. Blood samples (5 ml) were collected from the marginal vein of the ear 10 days before the first injection to obtain preimmune serum as a control and 10 days after the last injection. Sera volumes (2.5 ml) were stored at -20°C .

Standard cytofluorimetric test. To attach EPS to latex particles (10 mm diameter), 10^4 latex particles (Polyscience, Eppelheim, Germany) per tube were incubated overnight at 4°C in shake culture with 1 ml of borate buffer (0.2 M, pH 8.5) containing 50 μg of EPS obtained from

each tested fungus. The remainder of this procedure was carried out at room temperature. The mixture was centrifuged and the pellet was incubated for 30 min with 2% (vol/vol) blocking milk diluted with PBS (100 μl) (Waring Products). The particles were then washed with PBS and incubated for 3 h with anti-EPS serum diluted 1:25, 1:50, and 1:100 in PBS, washed with PBS (two times), and incubated with 100 μl of goat anti-rat immunoglobulin labeled with fluorescein diluted 1:600 (Sigma) for 1 h. Particles were washed once with PBS and analyzed using a flow cytometer (FACScan, Beacon-Dickinson, San Jose, CA, USA) equipped with a 15-mW, air-cooled 488-nm argon ion laser. Green fluorescence (FITC) was collected through a 530/30 nm bandpass filter. Data on 3,000 events were collected for each sample, stored in list mode, and then analyzed using the Consort 32 system (Hewlett-Packard, Sunnyvale, CA, USA). Forward scattering (FSC) and side scattering (SSC) were analyzed on a linear scale, and FITC fluorescence on a logarithmic scale. No gates were set around the particles. The results are presented as the mean channel fluorescence of the sample minus the mean channel control (autofluorescence of secondary antibodies with antigens). Autofluorescence (the average fluorescence intensity of the internal control of machine) varied between 1.5 and 2%. Negative controls (asymptomatic leaves) were treated similarly except that extracts were incubated with PBS only without rat anti-EPS serum. Each sample was analyzed in triplicate, and the experiments were repeated three times.

RESULTS

Production and specificity of *Pa. chlamydospora* EPS antibodies. The native EPS obtained by fractionated ethanol precipitation from the *Pa. chlamydospora* culture filtrates produced specific antibodies when they were used as antigen to immunize rats. As expected, the position on the x-axis of the cytofluorimetric profile for EPS obtained from the standard *Pa. chlamydospora* strain (CBS 229.95) indicated binding activity with the antibody-labeled beads (Fig. 2A). The positions on the x-axis of the profiles for the other *Pa. chlamydospora* strains (7, 31, 135, 551, and 606) (Fig. 2A) indicated similar recognition by the antibody-labeled beads. There was no recognition with the control EPS from *P. foeniculi* and *P. exigua* var. *heteromorpha*, and no recognition was observed when testing the EPS obtained from *Pm. aleophilum* (the other vascular pathogen involved in esca disease) or from *N. luteum* and *N. parvum* (data not shown). The latter are agents of wood necrosis and bark canker of grapevine (26), on which preliminary chemical investigations have been carried out (15).

Fig. 2. A, Cytofluorimetric profiles of *Phaeomoniella chlamydospora* extracts from strains CBS 229.95, 7, 31, 135, and 606. B, Cytofluorimetric profile of leaf extracts of asymptomatic vine no. 61 (AAA) and from the symptomatic vine no. 58. Leaf extracts had been obtained from (AAD) asymptomatic leaves collected from the asymptomatic shoot of the symptomatic vine; from asymptomatic leaves growing on the same shoot near (ACD) or distal (AFD) to a symptomatic leaf; (D) from symptomatic leaves with incipient symptoms (small yellowish chlorotic spots); and (S) from leaves from the same vine with tiger-stripe discoloration but no necrosis. Axis of abscissa is FL1-log height; axis of ordinate is number of particles.

Cytofluorimetric tests on the extracts from symptomatic and asymptomatic grapevine leaves. The weight of the lyophilized ethanol extracts from both the symptomatic and the asymptomatic leaves of esca-diseased vines, and from the asymptomatic leaves of healthy vines, ranged between 22.6 and 126.6 mg. Their UV spectra showed absorbance maxima in the range of 251 to 266 nm, which did not interfere with the cytofluorimetric tests. The results of cytofluorimetric tests on both the hapten (*Pa. chlamydospora* EPS) and the leaf extracts were obtained using the optimal dilution (1:25) of the antibodies. The test, which is a qualitative assay, was carried out using a fixed amount of leaf extract (100 µg). The sensitivity of the method was determined by using different concentrations of purified *Pa. chlamydospora* EPS (5 to 100 µg). With 5 µg of *Pch* EPS, a change of 16.3 ± 0.2 in fluorescence intensity relative to the negative control was observed, while with amounts lower than 5 µg there was no recognition (data not shown); consequently, the detection limit for purified *Pa. chlamydospora* EPS was set at 5 µg. No recognition occurred with amounts of leaf extract lower than 100 µg, whereas when double the amount of leaf extract (200 µg) was tested there was no difference from the result seen with 100 µg (data not shown).

When the leaf ethanol extracts were tested, only the extracts of leaves with esca symptoms were recognized by the antibodies (Table 1, columns D and S). Strong recognition was manifested by fluorescence values ranging from 9.1 to 9.8 for ethanol extracts from the leaves with incipient symptoms (Table 1, column D) and values of 16.1 to 16.5 for extracts from highly symptomatic leaves (Table 1, column S) of vines 1, 2, 3, 6, 44, 47, 58, and 141, which showed different levels of typical esca symptoms. In contrast, extracts from asymptomatic leaves from asymptomatic vines 61 and 62 (Table 1, column AAA) and asymptomatic leaves from

symptomatic vines 1, 2, 3, 6, 44, 47, 58, and 141 (Table 1, columns AAD, ACD, and AFD) all had values <0.7, which indicated no recognition by the antibodies.

The positions on the x-axis of the cytofluorimetric profiles for extracts of asymptomatic leaves taken from the asymptomatic vines 61 (Fig. 2B, sample AAA) and 62 (data not shown) indicated no recognition by the antibody-labeled beads. The positions of profiles showed a similar lack of recognition with the extracts of asymptomatic leaves from asymptomatic shoots of symptomatic vine 58 (Fig. 2B, sample AAD), and the extracts of asymptomatic leaves either near or distal to symptomatic leaves growing on the symptomatic shoot of diseased vine 58 (Fig. 2B, samples ACD and AFD). In contrast, when the extracts of leaves with incipient chlorotic symptoms or leaves with fully developed symptoms from diseased vine 58 were tested (Fig. 2B, samples D and S, respectively), the position of the profiles on the x-axis indicated there was strong recognition by the antibodies.

DISCUSSION

As expected, the antibodies raised against *Pa. chlamydospora* EPS immunogens reached high levels in rats (19) and in our tests showed a very high specificity with the injected antigen compared to the specificity toward EPS obtained from five other pathogenic fungi.

The phytotoxic fungal EPS that were used for comparison with the *Pa. chlamydospora* EPS in our cytofluorimetric tests were isolated from *Pm. aleophilum*, *P. foeniculi*, and *P. exigua* var. *heteromorpha* and from two Botryosphaeriaceae species that are also agents of wood diseases of grapevine (15,26). The polysaccharides from *P. foeniculi* and from *P. exigua* var. *heteromorpha* had previously been found to consist of a mixture of a galactan, a branched mannan (5), and a phosphorylated polysaccharide containing 70% mannose and lower percentages of galactose

and glucose, respectively (8). On the other hand, in the first study to focus on the isolation and chemical characterization of the phytotoxic metabolites produced by five phytopathogenic Botryosphaeriaceae species that are agents of grapevine trunk diseases, we recently showed that all the strains tested produced high molecular weight hydrophilic toxins, which in the case of *N. luteum* and *N. parvum* were EPS. In the latter fungus, preliminary GC-MS analyses indicate that the EPS consist essentially of glucose, galactose, and mannose (15). The phytotoxic polysaccharides produced by *Pa. chlamydospora* and *Pm. aleophilum* contain a mixture of α -glucans of different molecular weights, which have up to now been described as pullulans (2,22).

Results of our antibody studies suggested that the EPS from *Pm. aleophilum* differ in structure from those produced by *Pa. chlamydospora* and deserve to be more thoroughly investigated. Further studies are now underway, and the preliminary results are in agreement with the cytofluorimetric data reported here, where the antibodies raised against the *Pa. chlamydospora* EPS showed the highest binding activity with the immunogen while not recognizing the EPS of *Phomopsis foeniculi*, *Phoma exigua* var. *heteromorpha*, *N. luteum*, *N. parvum*, and *Pm. aleophilum*.

There are several possible explanations for the appearance of esca leaf symptoms in grapevine (17), but the most likely explanation is that such symptoms are caused by an accumulation of extracellular fungal toxin(s) in the vine leaves (7). EPS produced in vitro by *Pa. chlamydospora* and either absorbed at very low doses by detached vine leaves or injected into the woody tissues of vine shoots have been found to reproduce in vivo foliar symptoms that are very similar to esca foliar symptoms (21,22). It has been postulated that these toxins are produced in wood colonized by *Pa. chlamydospora*, from where they migrate into the vessels of the

Table 1. Detection of *Phaeomonella chlamydospora* exopolysaccharides (*Pch*-EPS) in extracts of symptomatic and asymptomatic grape leaves by the cytofluorimetric test

Vine no.	Relative change in sample fluorescence ^a					
	AAA ^b	AAD ^b	ACD ^b	AFD ^b	D ^b	S ^b
1	- ^c	0.5 ± 0.1	0.4 ± 0.2	0.4 ± 0.3	9.5 ± 0.3	16.1 ± 0.4
2	-	0.4 ± 0.2	0.2 ± 0.1	0.5 ± 0.2	9.1 ± 0.4	16.2 ± 0.4
3	-	0.3 ± 0.2	0.4 ± 0.3	0.3 ± 0.1	9.8 ± 0.5	16.3 ± 0.5
6	-	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.3	9.5 ± 0.3	16.2 ± 0.7
44	-	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	9.3 ± 0.4	16.4 ± 0.6
47	-	0.4 ± 0.3	0.5 ± 0.3	0.6 ± 0.4	9.5 ± 0.5	16.2 ± 0.7
58	-	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	9.4 ± 0.2	16.5 ± 0.3
141	-	0.5 ± 0.2	0.3 ± 0.2	0.5 ± 0.2	9.8 ± 0.4	16.5 ± 0.5
61	0.3 ± 0.2	- ^c	- ^c	- ^c	- ^c	- ^c
62	0.4 ± 0.2	-	-	-	-	-

^a Fluorescence values (mean channel fluorescence of the sample less the mean channel fluorescence of the control) are the average of all experiments ± standard deviation. Each experiment used 100 µg of extract with 40,000 antibody-labeled particles.

^b AAA: asymptomatic leaves from an asymptomatic vine; AAD: asymptomatic leaves from the asymptomatic shoot of a symptomatic vine; ACD: asymptomatic leaves growing near a symptomatic leaf on the same shoot; AFD: asymptomatic leaves distal to a symptomatic leaf growing on the same shoot; D: leaves with incipient symptoms (small yellowish chlorotic spots); and S: symptomatic leaves with tiger-stripe discoloration but no necrosis.

^c Samples not evaluated.

vine trunks and branches and are then transported upward by the transpiration stream (1,17,20,22,24). If and when they reach an active concentration in the leaves, symptoms will start to appear. The fact that EPS in symptomatic leaves is of the same type as the EPS produced *in vitro* by *Pa. chlamydospora* supports the toxigenic origin of the foliar symptoms of esca.

EPS extracts from leaves with incipient chlorotic symptoms and with interveinal and marginal discoloration and necrosis were strongly recognized by the anti-EPS antibodies, whereas leaf extracts from asymptomatic leaves collected both from healthy vines (i.e., plants that had not shown esca foliar symptoms in any of the 4 years surveyed) and from diseased vines were not so recognized. Unexpectedly, however, binding activity was also not detected in extracts from asymptomatic leaves growing on symptomatic shoots near symptomatic leaves. Here it should be noted that alteration of the photosynthetic apparatus as a consequence of the disease can be detected in the leaves at least 15 days before initial symptom appearance (4,12,18). It is also known that esca is characterized by an acropetal symptom gradient at shoot level. It was therefore expected that the accumulation of *Pa. chlamydospora* EPS would follow the same pattern, so that they would also be detectable in the asymptomatic leaves growing in close proximity to leaves with symptoms; these leaves would still be asymptomatic but would presumably start showing symptoms in a short time (10). But the failure to detect any binding activity in these leaves does not invalidate the research work. The reason why EPS were not detected in these leaves may be simply that the leaves were examined when the concentration of *Pa. chlamydospora* EPS was still below the minimum detectable threshold by the method of analysis used. It is also possible, however, that the leaves that were asymptomatic when they were collected, and that were near, or distal, to symptomatic leaves, would never become symptomatic at all.

In conclusion, this is the first unmistakable demonstration of the presence of a metabolic product of *Pa. chlamydospora* in the symptomatic leaves of esca diseased vines. This finding supports the hypothesis that the mechanism of foliar symptom formation is related to phytotoxic activity of *Pa. chlamydospora* exopolysaccharides. Data in support of this hypothesis would be a demonstration of a gradient of EPS that parallels the increasing severity of symptoms seen in leaves progressing to the tip of the vine. We also need to improve

the sensitivity of EPS detection so that the antibodies can be utilized to diagnosis esca in the absence of symptoms, and to show the presence of *Pa. chlamydospora* in the wood in cases in which the plant shows atypical foliar symptoms. Work is in progress on these matters.

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