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Interaction between esca-associated fungi, grapevine calli and micropropagated shoot cultures of grapevine[1]

Lorenzo Sparapano, Silvana De Leonardis, Alessandra Campanella and Giovanni Bruno

Dipartimento di Biologia e Patologia vegetale, Università, Via G. Amendola 165/A, 70126 Bari, Italy

Summary. Callus of the grapevine cv. Italia, micropropagated shoot cultures of the cv. Italia, Matilde and Sangiovese and callus of the tobacco cv. White Burley were cultivated on modified LS and MS-media. *Phaeomoniella chlamydospora* (*Pch*), *Phaeoacremonium aleophilum* (*Pal*) and *Fomitiporia punctata* (*Fop*) grew well in the presence of calli and vitroplants of all the grapevine cultivars which stimulated the growth of the fungi, but growth of the calli and vitroplants was reduced. All three fungi inhibited callus growth more strongly in grapevine than in tobacco. *Fop* had the greatest inhibitory effect on grapevine callus. Symptom severity also differed between cultivars. All three fungi invaded callus tissue after entirely overgrowing its surface. Hyphae were observed within the callus mass, where several cellular aggregates showed melanin-like deposits, particularly when invaded by *Pal*. On the foliar lamina of vitroplants symptoms were produced before and after the invasion of the plantlets by each fungus. Symptoms consisted in light green or chlorotic, rounded or irregular spots between the veins or along the leaf margin. The combination *Pal* vs. vitroplant gave the fastest host-pathogen response. The susceptibility or resistance of calli or vitroplants to esca-associated fungi could be a means to select grapevine for resistance to these fungi.

Key words: esca-associated fungi, grapevine, callus tissue, vitroplants.

Introduction

Tissue cultures and micropropagated shoot cultures are a convenient means for use in bioassays to determine susceptibility/resistance or sensitivity/tolerance relationships between plants and pathogens, or between plants and phytotoxins. The objective of this study was to establish callus cultures and micropropagated shoot cultures from grapevine cultivars susceptible (*Vitis vinifera* L., cv. Italia and Sangiovese) and fairly resistant (cv. Matilde) to esca (Sparapano *et al*., 2000a), and from a non-host tobacco cultivar (*Nicotiana tabacum* L. cv. White Burley), and to use these cultures to study the influence of callus cells and vitroplants on the growth of *Phaeomoniella chlamydospora* (W. Gams *et al.*) Crous & W. Gams, *Phaeoacremonium aleophilum* W. Gams *et al*. and *Fomitiporia punctata* (Fr.) Murrill, the three fungal species most commonly found in the woody tissues of esca-infected vines (Larignon and Dubos, 1997; Mugnai *et al*., 1999; Graniti *et al*., 2001).

These same species also produce several phytotoxic compounds (Amalfitano *et al*., 2000; Bruno *et al*., 2000; Evidente *et al*., 2000; Sparapano *et al*., 2000, 2000a, 2000b).

[1] Dedicated to Prof. A. Graniti on the occasion of his 75th birthday

Corresponding author: L. Sparapano
Fax: +39 080 5442906
E-mail: sparlor@agr.uniba.it

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 on plates of malt agar (MA) at 23°C in darkness.

Callus tissue bioassay

Explants of the grapevine cv. Italia were grown on modified Linsmaier and Skoog's medium (LSG1) (Sparapano *et al*., 2000b). Callus produced after two transfers of 60 days each was used as inoculum for the experiments.

The tobacco explants were cultivated on modified Murashige and Skoog's medium (MST1) (Sparapano *et al*., 2000b). Callus produced after two transfers of 30 days each was used as inoculum for the experiments.

Micropropagated shoot cultures

Micropropagated shoot cultures of the grapevine cv. Italia, Matilde and Sangiovese were grown and maintained on a modified Murashige and Skoog medium (MSG2) containing the inorganic and organic compounds of Murashige and Skoog basal medium plus: nicotinic acid 0.5 mg l^{-1}, pyridoxine hydrochloride 0.5 mg l^{-1}, thiamine hydrochloride 0.1 mg l^{-1}, glycine 2 mg l^{-1}, myo-inositol 100 mg l^{-1}, 6-benzylaminopurine 0.4 mg l^{-1}, α-naphthaleneacetic acid 0.01 mg l^{-1}, polyvinylpyrrolidone 100 mg l^{-1}, sucrose 30 g l^{-1}, agar 10 g l^{-1} (all medium components were purchased from Sigma Chemical Co., St. Louis, MO, USA). After the constituents were mixed, pH was adjusted to 5.8 with 1 N NaOH. Vitamins and plant hormones were added aseptically to the autoclaved medium after sterilising by filtration through a Millex-Millipore filter (Millipore, Bedford, MA, USA, 0.22 μm). Buds of each grapevine cultivar were removed from young shoots, left for 10 min under UV light (360 μm), sterilised with a 5% chloramine T solution, washed with sterile distilled water and seeded in Magenta vessels (Sigma). The vessels were transferred to a growth chamber at a constant 25°C with a 16 h day (100 μmol m^{-2} s^{-1})/8 h night.

Fungal inoculation

The *Pch*, *Pal* and *Fop* strains were grown at 25°C in darkness for two weeks in Petri dishes containing MA. Plugs (3 mm diam.) were aseptically removed from the actively growing fungal colonies and were transferred singly or in groups of two or three fungi to plates of LSG1 with calli of cv. Italia or to Magenta vessels with vitroplants of each grapevine cultivar. The plugs were placed in Petri dishes parallel 2, 4 and 6 cm from the callus. The experiment was run with 10 replicates. All dishes were sealed with Parafilm M and incubated at 25°C in the dark. Growth of the colonies was measured every four days for two months. Plugs of each fungus were also seeded in Magenta vessels with one or two plantlets of each cultivar. The Petri dishes were transferred to an incubator and maintained at 25°C in the dark. The vessels were maintained at 25°C in the same growth chambers and in the same enviromental conditions used for vitroplant production. The experiment was run with 3 replicates per fungus and per combination of fungi. Readings of colony growth were taken every four days for one month. Mean values ± standard deviation of fungal colony diameter were calculated.

Results

When *Pch*, *Pal* and *Fop* were grown singly with calli of cv. Italia, their growth rates increased (Fig. 1). The growth rate of *Fop* was higher than those of *Pch* and *Pal*. Tobacco calli did not stimulate growth of these fungi (Fig. 2). Each fungus invaded the callus tissue after completely overgrowing the surface. Hyphae were observed within the callus mass, and several cellular aggregates showed melanin-like deposits, particularly when invaded by *Pal*. *Pch* was more strongly inhibited by *Fop* than by *Pal* (Fig. 1A, B). However, when *Fop, Pal* and *Pch* together were grown with the callus, the growth rate of *Fop* decreased (Fig. 1C, D).

Pal had the greatest inhibitory effect on tobacco calli weight compared with the controls. *Pch* was more active against grapevine calli; *Fop* had less effect on tobacco than on grapevine calli. The invading mycelium of *Fop* produced chlamydospores.

Fop produced the greatest inhibition of grapevine calli, with a 92% reduction compared with the controls (Fig. 3). *Pch* or *Pal,* alone or each com-

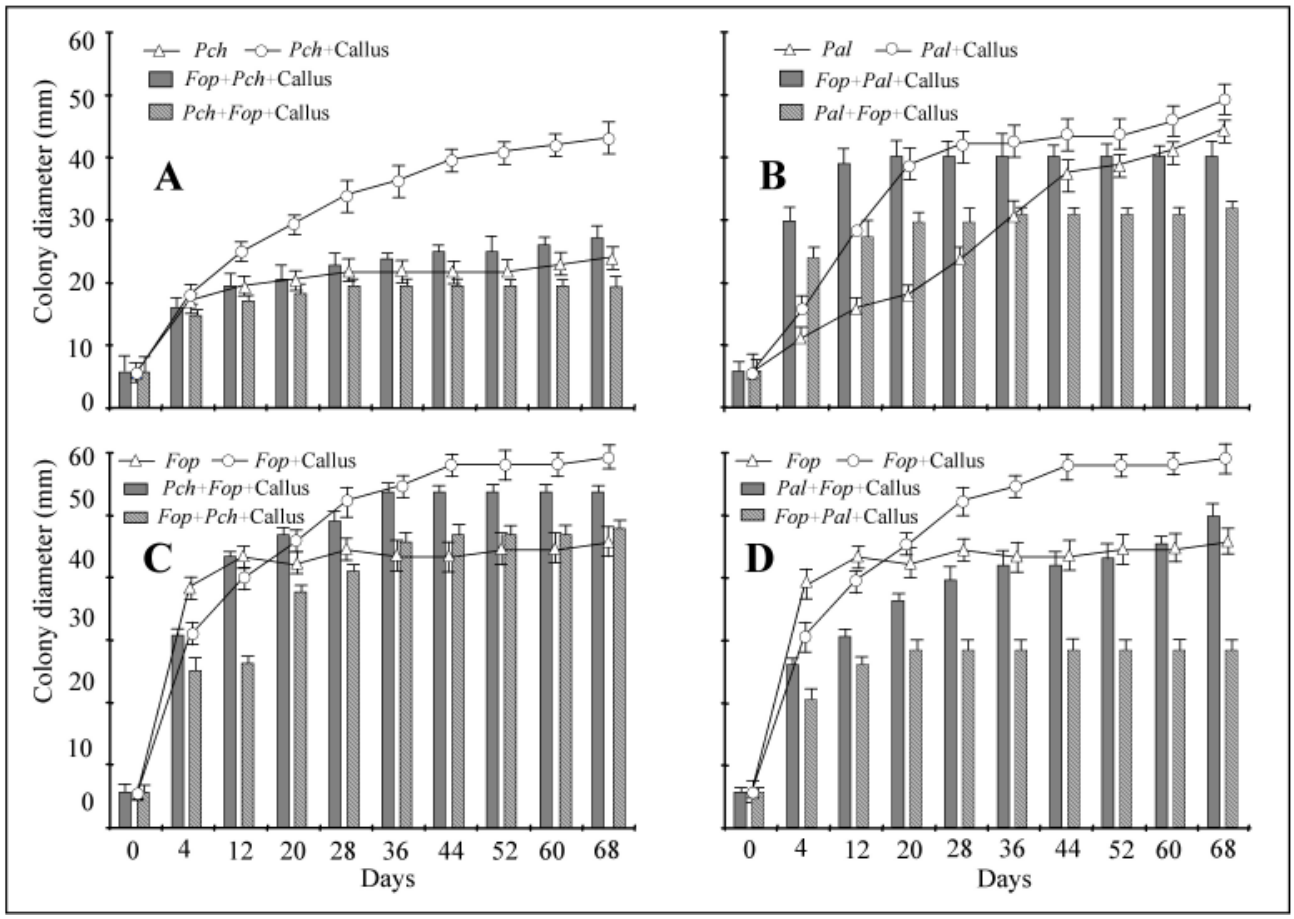

Fig. 1. Comparison of growth rates of: A. *Phaeomoniella chlamydospora* (*Pch*); B. *Phaeoacremonium aleophilum* (*Pal*); C. and D. *Fomitiporia punctata* (*Fop*) grown singly or in dual culture with cv. Italia callus. Standard error bars are shown.

bined with *Fop,* caused similar reductions of callus yield (69–72%). *Pch* and *Pal* were more effective together than singly in reducing callus growth (87 vs. 71%). The inibitory effect of *Fop* combined with *Pal* and *Pch* was the same as that of *Fop* alone (93%), but only in plates where the *Fop* inoculation site was outside those of *Pal* and *Pch*. When the *Fop* inoculation site was located between *Pal* and *Pch* or near the *Pal* site, callus growth inhibition was lower (82%) than it was with *Fop* alone.

Growth of the esca fungi was also stimulated by the vitroplantlets of the cvs. Italia, Matilde and Sangiovese. When *Pch*, *Pal* and *Fop* were grown singly with the vitroplantlets, they had nearly the same growth rate, irrespective of the cultivar, althought the *Fop* growth rates were somewhat higher (Fig. 4).

Differences between cultivars in symptom severity were also observed under experimental conditions, with cv. Italia showing early leaf alterations such as yellowing and reddening (Fig. 5). All three fungi invaded the vitroplantlets after overgrowing the basal surface. Symptoms were produced on the foliar lamina of vitroplants before and after invasion by each fungus. Symptoms consisted in light green or chlorotic, rounded or irregular spots between the veins or along the leaf margins. Plantlets of all three cultivars grown with either *Pal* or *Pch* showed chlorotic tissue turning red-

Fig. 2. Callus culture of grapevine cv. Italia or tobacco cv. White Burley grown in the presence of *Phaeomoniella chlamydospora* (*Pch*), *Phaeoacremonium aleophilum* (*Pal*) and *Fomitiporia punctata* (*Fop*).

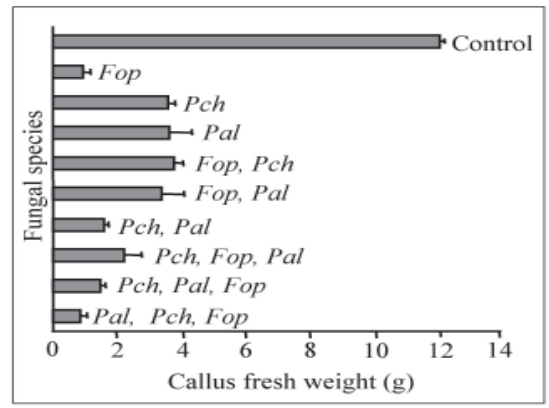

Fig. 3. Effect of *Fomitiporia punctata* (*Fop*), *Phaeomoniella chlamydospora* (*Pch*) and *Phaeoacremonium aleophilum* (*Pal*) or their combinations on the yield of grapevine cv. Italia calli. Standard error bars are shown.

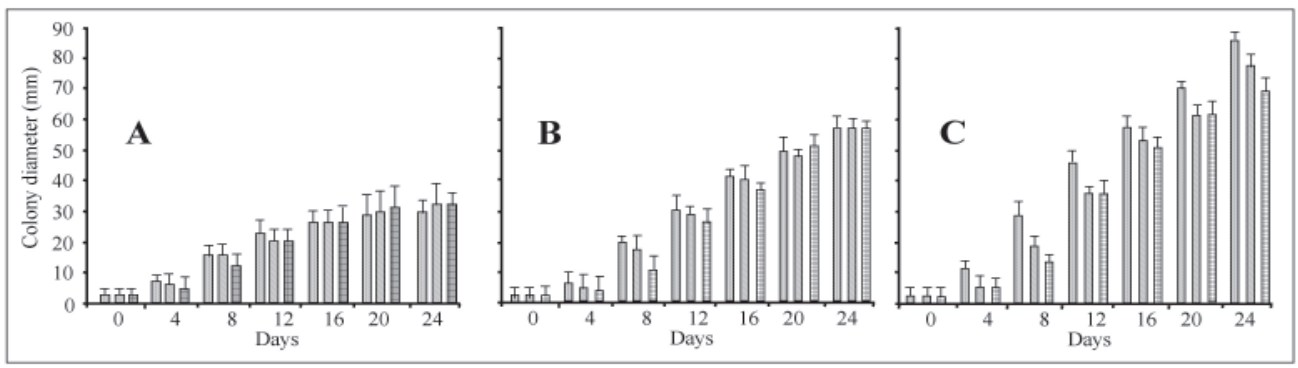

Fig. 4. Growth rates of *Phaeomoniella chlamydospora* (A), *Phaeoacremonium aleophilum* (B) and *Fomitiporia punctata* (C) grown with vitroplants of grapevine cv. Italia (▯), Sangiovese (▯) and Matilde (▯). Standard error bars are shown.

brown along the leaf margins, whereas those grown with *Fop* mostly showed chlorotic areas and reddening along the leaf margins. The combination *Pal* and vitroplant gave the fastest host-pathogen response.

When data of fungal growth with callus cultures and vitroplants of the grapevine cv. Italia were compared, growth of *Fop* colonies was stimulated more by the calli than by the corresponding vitroplants (Fig. 6). *Pch* or *Pal* colonies did not show any substantial increase in growth with calli or plantlets.

Colony pigmentation was usual with *Fop* and *Pal*. With *Pch*, pigmentation started only after the colony reached the callus tissue or the basal part of the plantlets; at the same time there was an increase of exopolysaccharide (EPS) production.

Discussion

Grapevine calli and vitroplantlets responded differently to exposure to *Fop*, *Pal* and *Pch*. Each fungal species also reacted differently, hastening or slowing its growth rate. Incubation of the callus tissues together with living mycelial plugs of *Pch*, *Pal* and *Fop* led to a rapid increase in the

Fig. 5. Plantlets of vine cv. Italia, Matilde and Sangiovese grown together with *Phaeomoniella chlamydospora* (*Pch*), *Phaeoacremonium aleophilum* (*Pal*) and *Fomitiporia punctata* (*Fop*) showing reduced growth, chlorosis and reddening of leaves.

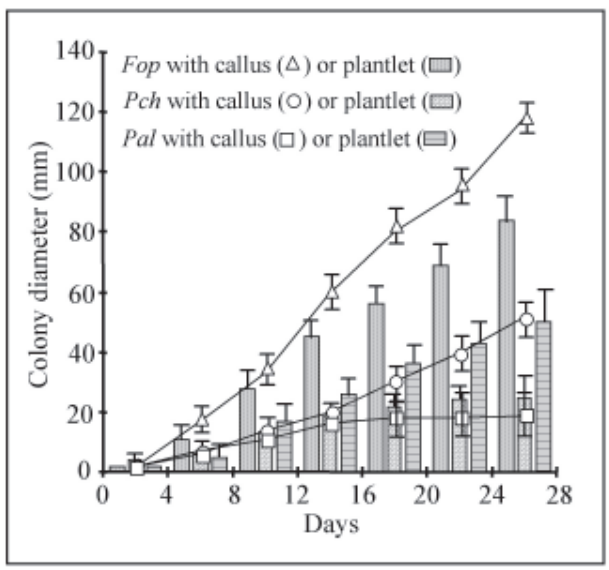

Fig. 6. Effect of calli or vitroplantlets of grapevine cv. Italia on the growth rates of *Fomitiporia punctata* (*Fop*), *Phaeomoniella chlamydospora* (*Pch*) and *Phaeoacremonium aleophilum* (*Pal*). Standard error bars are shown.

colony diameters of the fungi and to a visible inhibition of callus cell growth of the grapevine cv. Italia. Callogenesis decreased progressively during the period when fungal colonies were near calli, and stopped completely when calli became brown to dark. After infection, grapevines synthetise several compounds such as phytoalexins (resveratrol and its derivatives, viniferins), polyphenols and glycolic acid (Feucht *et al.*, 1996; Renault *et al.,* 1996), which accumulate in the cells and cause cell death. The observations are sometimes contradictory and the ability of grapevines to resist to pathogens probably results from various defense mechanisms. This *in vitro* system for co-culturing grape with esca-associated fungi readily allows grape susceptibility and pathogen virulence to be determined and quantified.

Our results indicate that the esca fungi singly or in combination in dual or triple cultures caused leaf yellowness, reddening, necrosis and wilting of vitroplants, depending on the grapevine culti-

var. The grapevines cv. Italia and Sangiovese were most susceptible. Bessis *et al*. (1992) suggested using phytotoxic polysaccharides such as glucans and rhamno-galacto-mannans produced by *Botryotinia fuckeliana* to select *Vitis* for resistance *in vitro*. They may play a role as elicitors of the plant defence response. *Pch* and *Pal* produce α-glucans and the yield of these compounds increased in dual cultures of each with grapevine calli or vitroplants when the colonies were very close. Exopolysaccharides may accumulate in the medium, reach the calli or vitroplants and then induce a host response.

The interactions of the fungi in dual or triple cultures were strictly related to the location of the inoculation site. When *Fop* was located between *Pal* and *Pch* or near *Pal* with the *Pch* colony on the outside, its growth rate decreased and its effect on callus growth was lower. These data confirmed previous observations on how these fungi interact with each other and with the host tissue (Sparapano *et al.*, 2000a, 2000b).

In addition to producing significant and typical esca syndrome symptoms, an *in vitro* culture system (calli or vitroplantlets) should facilitate the evaluation of plant reactions to the fungi. This method of culturing grape with esca fungi allows evaluation of the host/pathogen interactions under controlled environmental conditions. Its primary advantage over previous methods is its capacity for continuous observation without disrupting or interfering with the host-parasite interactions, while excluding secondary interactions with other biotic or abiotic agents. Susceptibility or resistance responses of callus tissues and vitroplants to esca pathogens could be a mean to select grapevines for resistance to esca.

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