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Phenolic compounds have a role in the defence mechanism protecting grapevine against the fungi involved in Petri disease

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Summary. Scanning electron microscopy of stems and roots of vine plants affected by Petri disease demonstrated that the obstruction of xylem vessels and reduction in the flow of xylem sap, two symptoms of this disease, were mainly caused by tyloses forming in the vessels, and, to a lesser extent, by the accumulation of aggregates. Fungal hyphae were also found in the xylem. These hyphae propagated via the xylem and invaded other vessels or adjacent parenchymatic cells through the pit. Analysis of lignin peroxidase, manganese peroxidase and laccase activity (all of which are involved in lignin degradation) in different fungi isolated from Petri-disease-infected grapevines found that *Phaeoacremonium aleophilum* expressed low specific activity for manganese peroxidase and high specific activity for both lignin peroxidase and laccase, while *Phaeomoniella chlamydospora* showed no activity for any of these enzymes. All these enzyme activities were inhibited by the phenolic compounds in grapevine: *p*-coumaric acid, catechin, caffeic acid and tannins. The phenolic compounds also had a direct effect on fungal growth and sporulation. When SO4 vines affected by Petri disease were treated with Brotomax (a product that stimulates synthesis of phenolic compounds) plants showed an increase in growth and a reduction in Petri-disease symptoms. Any new shoots and roots formed after Brotomax treatment did not show any sign of obstruction or tyloses formation.

Key words: tyloses, Brotomax®, lignin peroxidase, manganese peroxidase, laccase.

Introduction

A number of fungi, mainly *Phaeomoniella chlamydospora* but also *Phaeoacremonium aleophilum,* have been isolated from vine plants infected with Petri disease (Crous *et al*., 1996; Mugnai *et al*., 1999; Crous and Gams, 2000). However, the ways in which vines become infected and the identity of the fungi truly responsible for this disease are still under discussion.

However, other fungi such as *Cylindrocarpon* spp., *Botryosphaeria* spp., *Phomopsis* spp., *Eutypa* spp., *Stereum hirsutum* and *Fusarium oxysporum* are reported to be involved in other grapevine trunk diseases (Moller and Kasimaris, 1978; Larignon and Dubos, 1997; Omer *et al*., 1999; Rego *et al*., 2000; Rumbos and Rumbou, 2001).

Since treatment with fungicides, treatment with hot water, and biological control with *Trichoderma* have all had little or no effect on disease spread, increasing the plant's natural defence or resistance may be a means to prevent Petri disease.

In this respect, the phytoalexins, which in vines are phenolic compounds that include tannins, phenolic acids, flavonoids and stilbenes, are certainly involved in vine defence mechanisms, increasing plant resistance. Many phenolic compounds inhibit hyphae development (Nyerges *et al.*, 1975; Schlösser, 1994), or they bind the enzymes released by the fungus during cell invasion, as do, for example, the

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tannins (Laks *et al.*, 1988; Pezet and Pont, 1992; Schlösser, 1994; Dai *et al.*, 1995).

In previous papers, we reported on the use of transmission electron microscopy (TEM) to study tyloses formation in the xylem vessels of vines infected with Petri disease (Del Río *et al.*, 2001a). Tyloses formation was found to be accompanied by softening of the xylem walls, allowing the lumen to be invaded by the protoplasm of adjacent parenchymatic cells. For such softening to take place, hydrolytic enzymes modifying the composition of the cell wall must be excreted by the fungi.

Lignin is a natural composite material found in all vascular plants. It is very abundant in the xylem and gives the plant its strength and ridigity (Brown, 1985; Argyropoulos and Menachem, 1997). Lignin is found throughout the cell wall, but is not distributed uniformly across it: lignin content is lowest in the middle lamella, and greatest in the secondary wall (Fengel and Wegener, 1989; Eriksson *et al.*, 1990). The lignin-degrading system consists of lignin peroxidase, manganese peroxidase and laccase (Kirk and Farrell, 1987; Bourbounnais *et al.*, 1995). Peroxidases need hydrogen peroxide to oxidize lignin, while laccase uses oxygen to do so.

In this study we used scanning electron microscopy (SEM) to determine the causes of xylem vessel obstruction and to study xylem degradation associated with the activity of various enzymes: lignin peroxidase, manganese peroxidase and laccase, which are excreted by several fungi known to be involved in Petri and other trunk diseases. How such enzymatic activity was changed by different levels of phenolic compounds was also examined. In addition, we studied by SEM the antifungal capacity of different phenolic compounds and how they affected sporulation of these fungi. Results were compared with those obtained using Brotomax in SO4 vines affected with Petri disease.

Materials and methods

Plant material and treatment with Brotomax®

Thirty vines showing typical Petri decline external and internal symptoms and thirty control vines (no symptoms) on different rootstocks were used: SO4 (4 years old), Richter 110, 1103 Paulsen and 41-B (1 year old). The plant material was supplied by a Spanish nursery.

In other assays, thirty SO4 vines were treated with 0.3% Brotomax® in March and May (leaf application), and in September (application of 10 ml per plant by drip). An equal number of untreated vines in the same vineyard was used as the control.

Chemicals

Standards of *p*-coumaric acid, catechin, caffeic acid, tannins, Bradford reagent, ABTS [2,2´-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diamonium salt], phenol red (phenolsulfonphthalein), albumin-bovine, sodium succinate and Folin Ciocalteu's phenol reagent were supplied by Sigma (St. Louis, MO, USA). Hydrogen peroxide and dimethyl sulphoxide (DMSO) were purchased from Scharlau (Barcelona, Spain); sulphate manganese and gallic acid from Panreac (Barcelona, Spain); sodium hydroxide from Rectapur (Fontenay, France); potato dextrose agar (PDA) from Microkit (Madrid, Spain) and Brotomax® from Agrométodos (Madrid, Spain).

Fungal cultures and *in vitro* antifungal activity of vine phenolic compounds

Phaeomoniella chlamydospora, Phaeoacremonium inflatipes and *P. aleophilum* were supplied by the Agri-Analysis Company (Davis, CA, USA). *Eutypa lata* and *Stereum hirsutum* were purchased from the Spanish Type Culture Collection (University of Valencia, Valencia, Spain) and *Fusarium oxysporum* was supplied by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Fungi were cultured on PDA at 25ºC. To measure the antifungal activity of the phenolic compounds of the vines, 5-mm-diameter disks of culture medium containing mycelium of these fungi were placed on PDA (control) and on PDA with one of *p*-coumaric acid, catechin, caffeic acid, or tannins added (0.5 to 3 g l^{-1}). Fungus growth was determined at various times by measuring the mycelium diameter (mm). The effect that the phenolic compounds in the media had on hyphal morphology and fungal sporulation was determined by SEM.

Scanning electron microscopy

For SEM examination, transverse and longitudinal sections of stems and roots from vines infected with Petri disease, and samples of mycelium grown in both PDA media (with and without the

phenolic compounds), were fixed with 3% glutaraldehyde for 4 h at room temperature and post-fixed with 1% OsO_4 at 0ºC for 2.5 hours. Samples were then dehydrated by rinsing with acetone at different concentrations before critical point drying with liquid CO_2 (CPD 020) for 2 h. The samples were covered with gold (20 nm) for examination under a JSM-6100 scanning electron microscope (JEOL Ltd., Tokyo, Japan) with an acceleration of 15 Kv.

Enzyme assays

Lignin peroxidase**,** manganese peroxidase and laccase from the fungi were isolated and purified according to Huwiler and Kohler (1985), Glenn and Gold (1985) and Bourbounnais *et al*. (1995) respectively.

Proteins in the supernatant (extracellular proteins) were measured by the Bradford method (1976), using 1 mg ml^{-1} bovine serum albumin as standard. Lignin peroxidase (LiP) activity was measured using the method of Huwiler and Kohler (1985). Oxidation of iodide (I^-) LiP was monitored to determine the absorbance change of triiodide (I_3^-) at 353 nm (ε_{353} = 25,800 $M^{-1}cm^{-1}$). Reaction mixtures for iodide oxidation contained 1.5 mM potassium iodide, 0.05 mM LiP and 250 mM H_2O_2 in 0.1 M sodium tartrate buffer, pH 3.5.

Manganese peroxidase (MnP) activity was measured following the method of Glenn and Gold (1985), which was based on the oxidation of Mn(II) to Mn(III), and used as substrate 2.5 ml of phenol red (0.01%) and $MnSO_4$ (0.1 mM) in sodium succinate buffer (0.1 M). The absorbance was measured at 610 nm (ε_{610}=22,000 $M^{-1}cm^{-1}$).

Laccase activity was measured using the method of Bourbounnais *et al*. (1995), which was based on the oxidation of the substrate ABTS (5 mM). The absorbance was measured at 420 nm (ε_{420}=36,000 M^{-1} cm^{-1}).

In all cases, a unit of enzymatic activity was defined as the quantity of enzyme that produced 1 μmol of oxidized product.

Results and discussion

Microscopic study of the deterioration produced in the different tissues of infected plants

One well-known manifestation of Petri disease is darkening of the xylem of infected plants, seen when the stems or roots are cut (Fig. 1). Xylem darkening is associated with the formation of tyloses (Del Río *et al*., 2001a).

Using SEM, we looked into the causes of xylem obstruction in different vine rootstocks. Inspection of Richter 110 rootstock naturally infected with Petri disease (Fig. 2A) revealed that the most common form of obstruction in diseased plants was with tyloses, both in the rootstock and in the cultivar. Another cause of obstruction was the aggregates that invaded the lumen of the xylem (Fig. 2B), although this was less common and was only observed in the rootstock xylem. Fungal mycelium was seen to adhere to the xylem wall; this was associated with partially blocked xylem vessels (Fig. 3). The mycelium propagated and passed from one element to another through the pit. Similar results were also observed on the other rootstocks tested (data not shown).

This is the first time that fungal mycelium has been observed in the xylem of vine plants naturally infected with Petri disease, and it may explain in part why the xylem became obstructed. The tyloses were formed when the xylem lumen was invaded by the protoplasm of the adjacent parenchyma cells. This invasion was made possible by the softening of the xylem walls, which could not resist the pressure of the parenchymatic cells because of their high hydric potential. For this softening to occur the xylem wall, which is rich in lignin, must become degraded.

Fig. 1. Cross-section through the basal end of a rootstock stem of 1-year-old naturally infected grapevine (Richter 110) showing internal symptoms of Petri disease.

Lignin peroxidase, manganese peroxidase and laccase activity of the fungi involved in Petri and other trunk diseases

The study showed that some of the pathogenic fungi involved in Petri and in other trunk diseases have lignolytic activity.

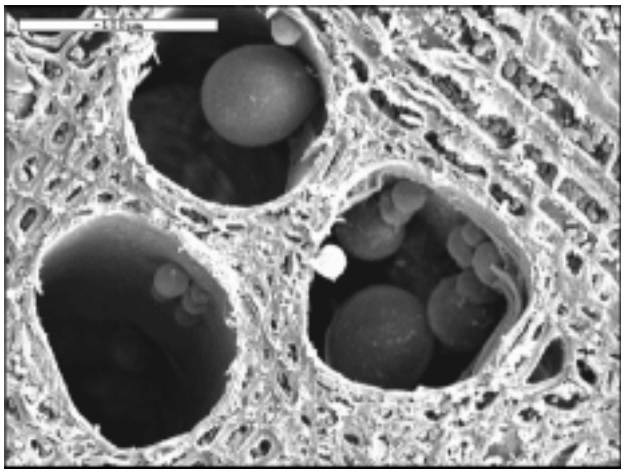
Phaeoacremonium aleophilum showed low specific activity of manganese peroxidase (3.4 UE g^{-1} protein) but high specific activity of laccase and lignin peroxidase (24.4 and 22.5 UE g^{-1} protein respectively). *P. chlamydospora* showed no activity for these enzymes (Table 1). Based on these results, *P. aleophilum* has a greater capacity for degrading the xylem wall, and hence for initiating formation of tyloses inside the xylem vessels in vines with Petri disease.

As regards the other fungi studied, which may be involved in other grapevine trunk diseases, *P. inflatipes* showed high specific activity of manganese peroxidase (33.1 UE g^{-1} protein) and low specific activity of laccase and lignin peroxidase (8.7 and 2.0 UE g^{-1} protein respectively); *F. oxysporum* low specific activity of lignin peroxidase and laccase (7.0 and 1.0 UE g^{-1} protein respectively) and no activity against manganese peroxidase (Table 1); *E. lata* showed low specific activity of lignin peroxidase (3.2 UE g^{-1} protein) and no activity of manganese peroxidase or laccase (Table 1), while *S. hirsutum* showed no activity of any of these enzymes (Table 1).

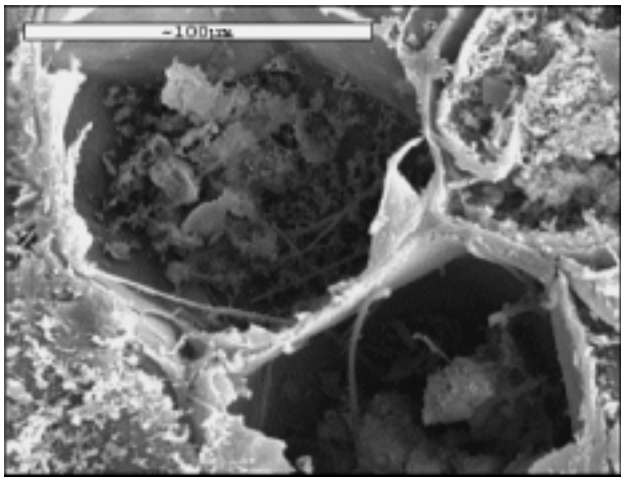
As regards the degrading activity of an oxidative nature exhibited by these enzymes, certain phenols inhibited such activity. For example, the addition of different concentrations (30, 150, 300 and 600 *m*M) of *p*-coumaric acid to the manganese peroxidase reaction medium reduced activity of that enzyme by 50, 87, 97, and 99% respectively) (Fig. 4).

Catechin, caffeic acid, tannin, and other vine phenolic compounds also inhibited manganese peroxidase (data not shown).

These results suggest that the phenolic compounds can protect the plant by inhibiting or inactivating some of the enzymes that degrade the cell wall.



A



B

Fig. 2. Scanning electron micrographs of a cross-section of the same Richter 110 rootstock as in Fig. 1. A. Tyloses formation in the xylem lumen (×450). B. Deposition of aggregates in the xylem lumen (×600).

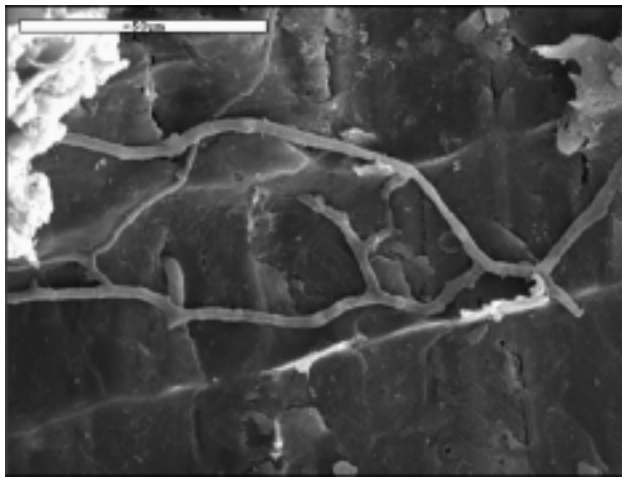


Fig. 3. Scanning electron micrograph of a longitudinal section of naturally infected rootstock Richter 110 (×450), showing fungal mycelium on the inside of a xylem vessel.

Table 1. Lignin peroxidase, manganese peroxidase and laccase specific activity (UE g^{-1} protein) in the fungi involved in Petri disease (a) and in fungi implicated in other trunk grapevine diseases (b). Data correspond to mean ± SE (n=3).

Fungus	Enzyme activity (UE g^{-1} protein)		
	Lignin peroxidase	Manganese peroxidase	Laccase
Phaeomoniella chlamydospora[a]	0.0	0.0	0.0
Phaeoacremonium aleophilum[a]	22.5 ± 2.1	3.4 ± 0.2	24.4 ± 1.9
Phaeoacremonium inflatipes[b]	2.0 ± 0.1	33.1 ± 2.6	8.7 ± 0.8
Fusarium oxysporum[b]	7.0 ± 0.5	0.0	1.0 ± 0.08
Eutypa lata[b]	3.2 ± 0.2	0.0	0.0
Stereum hirsutum[b]	0.0	0.0	0.0

Effect of phenolic compounds on fungal growth

Phenolics are well-known antifungal and antibacterial compounds in plants (Laks, 1988; Sivaprakasan and Vidhyasekaran, 1993; Schlösser, 1994). According to Matern and Kneusel (1988), the first step of the defence mechanism in plants responding to infection is a rapid accumulation of phenols at the infection site, which restricts or slows down pathogen growth.

In vitro studies in which *p*-coumaric acid (3 g l^{-1}) was added to a PDA culture medium inhibited *P. chlamydospora* growth by more than 50% (Fig. 5A). Similar results were obtained with *P. aleophilum, E. lata, S. hirsutum* and *F. oxysporum,* although *P. inflatipes* was less subject to such inhibition. Other fungi, not known to be involved in Petri or trunk diseases of grapevine, were also inhibited by these compounds (data not shown). Similarly, catechin,

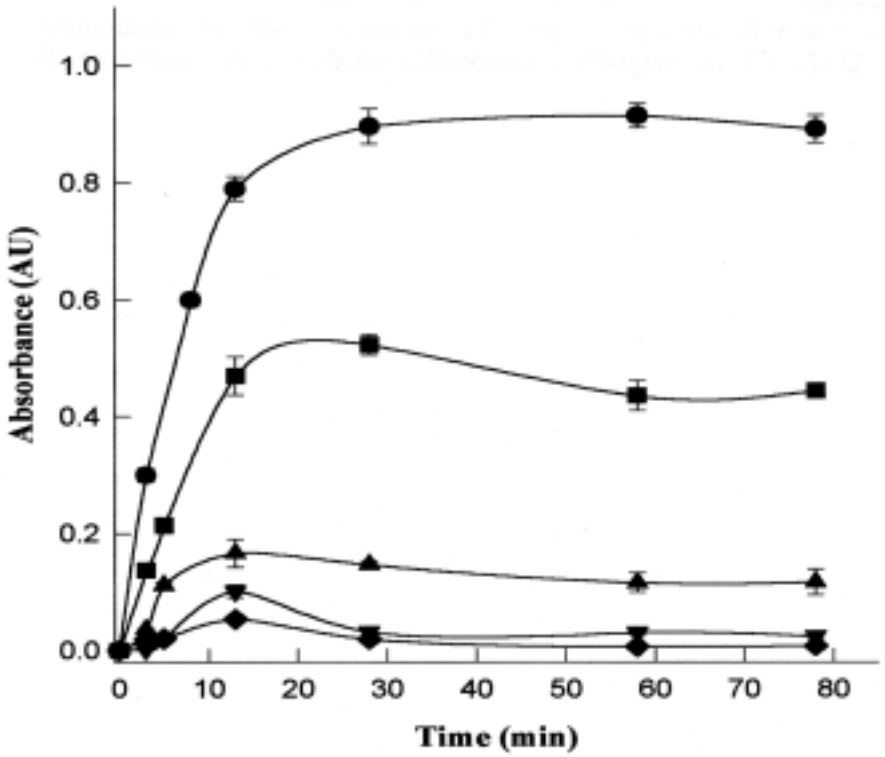


Fig. 4. Effect of different concentrations of *p*-coumaric acid on manganese peroxidase activity: (●) Control, (■) 30μM, (▲) 150 μM, (▼) 300 μM, (◆) 600 μM.

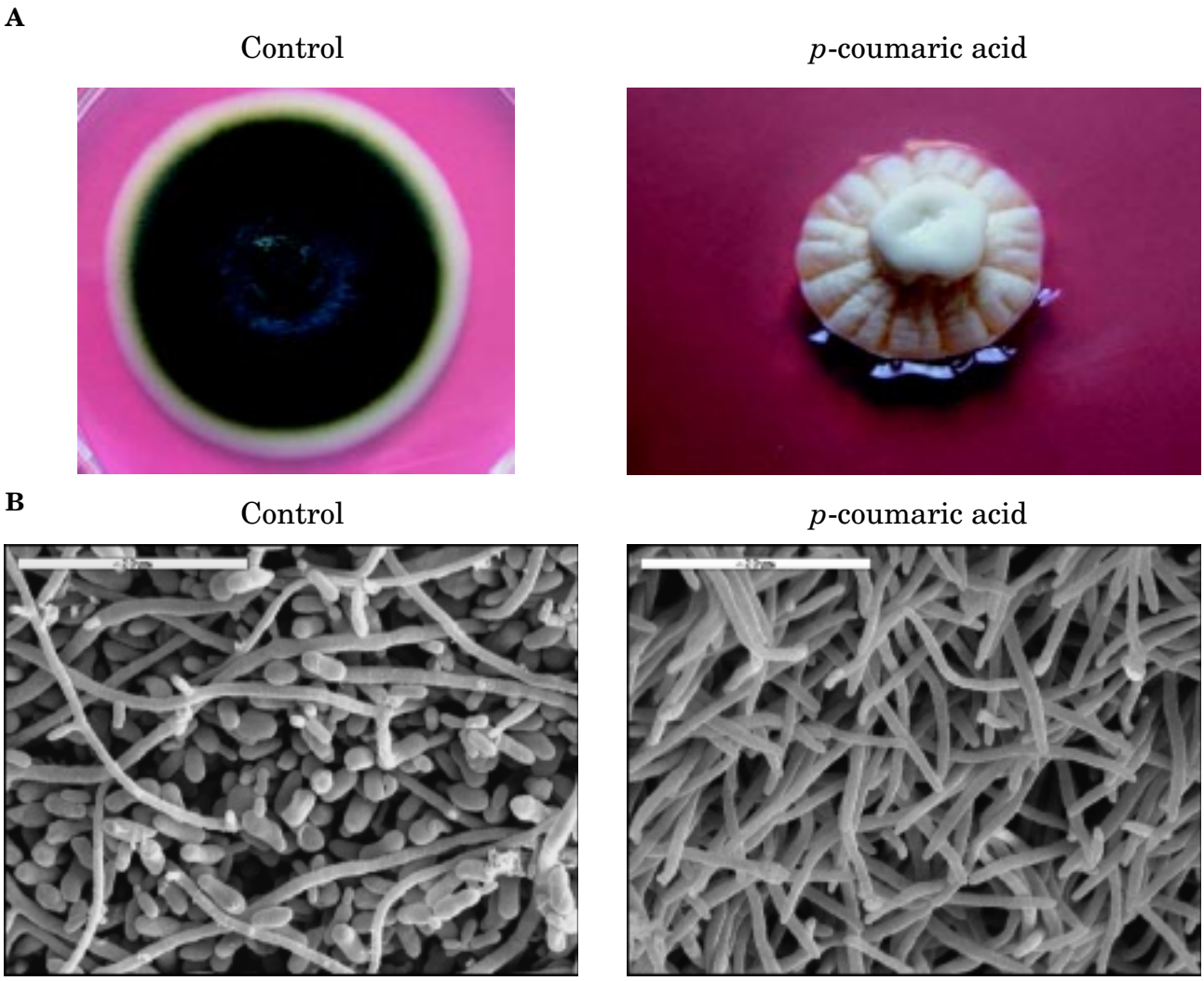


Fig. 5. A. Growth inhibition of *P. chlamydospora* mycelium by *p*-coumaric acid (3 g l^{-1}) added to the culture medium. B. Scanning electron micrograph showing changes in hyphal morphology and sporulation (×2,300).

caffeic acid and tannin (among others) inhibited these fungi (data not shown).

Besides inhibiting the radial growth of the fungus, *p*-coumaric acid also reduced the rate of sporulation (see Fig. 5B). These findings are in close agreement with those described for other fungi (Arcas *et al.*, 2000).

Brotomax enhances resistance to pathogenic fungi

Numerous studies have shown that Brotomax increases phenolic compound concentrations in plants such as citrus, olive and cotton (Fuster *et al.*, 1995; Ortuño *et al.*, 1997; Botía *et al.*, 2001; Del Río *et al.*, 2001a, b) thereby enhancing the disease tolerance or resistance of these plants .

In previous studies, it was found that 0.3% Brotomax applied to vines cv. Flame in commercial plantations increased polyphenolic levels in all plant organs studied, although increases were greater in the leaves than in the stems and the fruits (Del Río *et al.*, 2001a). This phenol-enhancing effect was observed not only in cv. *Flame* but also in other commercial varieties destined for both wine-making and table grapes (data not shown).

Fig. 6. Effect of Brotomax treatment on root development of a SO4 vine with Petri disease. A. Detail of a root cross-section in a control vine. B. Detail of roots and regenerated stems in vines treated with 0.3% Brotomax.

We also found that the addition of leaf extracts to PDA cultures inhibited growth of *P. chlamydospora* (Del Río *et al*., 2001a). Similarly, the radial growth of this fungus was more strongly inhibited in media containing extracts of leaves treated with Brotomax because of the higher polyphenol levels in those leaves. This suggests that phenolic compounds had a role in the plant self defence mechanism and could help explain the field results obtained with Brotomax (Del Río *et al*., 2001a).

Application of 0.3% Brotomax for one year to S04 vines with the typical external and internal symptoms of Petri decline increased growth in all vines compared with the control vines. A study of the effect of Brotomax on root growth in 10% of the treated plants showed that none of these vines had xylem obstruction (see Fig. 6A, B), and SEM detected no fungi inside the xylem.

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