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Tylose formation and changes in phenolic compounds of grape roots infected with *Phaeomoniella chlamydospora* and *Phaeoacremonium* species

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Summary. The role of phenolic compounds in strengthening resistance of grapevine to young grapevine decline was analysed. The formation of tyloses has also been associated with this disease. A microscopic study showed that tyloses originated in parenchyma cells associated with the xylem and from there invaded the xylem lumen. As tyloses formed, there were changes in the cell wall, associated with the accumulation of crystalline structures. The cells surrounding the blocked xylem contained more polyphenolic compounds than the cells of intact xylem. Changes in the level and composition of polyphenolic compounds occurred in relation to the severity of infection. Tannin concentrations also increased with increasing numbers of xylem vessels containing tyloses. Root extracts added to fungal culture media inhibited mycelial growth of *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum* and *Pm. inflatipes*. Inhibition was greater with extracts from roots with tyloses, and was also correlated with polyphenol content. Extracts of the leaves, stems and berries of vines treated with Brotomax, which increases the biosynthesis of phenolic compounds, inhibited mycelial growth of the fungi compared to untreated control plants.

Introduction

Esca and grapevine decline[(1)] are two of the most destructive diseases of woody tissues in grapevine. Several fungi, such as *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum* and *Phaeoacremonium inflatipes* have been implicated in these infections (Crous *et al.*, 1996; Mugnai *et al.*, 1996; Mugnai *et al.*, 1997; Bertelli *et al.*, 1998), where they act as pathogens, and they have been isolated with high frequency from the wood of diseased vines.

Young grapevine decline is characterised by internal brown to black vascular streaking and tylose or gum-formation in the xylem vessels (Petri, 1912). Both tyloses and pectic gum are known to provide effective protection against invasion by fungi and bacteria, and to form part of the vine defence mechanism against vascular wilt (Petri, 1912).

Plants possess preformed substances that inhibit pathogen, germination and growth. These inhibitory substances are of a phenolic nature and

(1) At the general assembly of the 2nd ICGTD meeting held in Lisbon 2001 it was unanimously decided that the disease will henceforth be called Petri disease

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include catechol, protocatechuic acid, chlorogenic acid, different flavonoids, etc. (Kuc, 1995; Del Río *et al.*, 1998). Moreover, it is known that after infection by *Botrytis cinerea* or *Plasmopora viticola*, grapevine synthetizes resveratrol and viniferins, stress metabolites which confer a certain resistance to those fungi (Hart, 1981; Langcake, 1981). It has also been ascertained that an accumulation of resveratrol and ε-viniferin occur in deteriorated wood of esca-diseased vines (Amalfitano *et al.*, 2000).

In this paper, tylose formation in SO4 grapevines with young grapevine decline was examined under the light and electron microscopes. The phenolic compounds in infected roots were determined and their capacity to inhibit mycelial growth of *Pa. chlamydospora*, *Pm. aleophilum* and *Pm. inflatipes* was studied. The use of Brotomax (an activator of disease resistance, marketed in Spain by Agrométodos, S.A. Madrid) to stimulate production of phenolic compounds was shown to be an alternative method for controlling these diseases.

Materials and methods

Plant material and Brotomax treatment

Thirty plants of SO4 grapevines (4 years old) with and without infection with *Pa. chlamydospora* and *Phaeoacremonium* species were used in some tests. Plant material was supplied by a Spanish nursery.

In other tests 60 grapevines (cv. Flame) located at Abarán (Murcia, Spain) were treated with Brotomax. Treatment consisted of a first application of 0.3% Brotomax to the leaves at the beginning of flowering, a second at fruit set and a third at veraison, when 10 cc/plant was applied by drip. An equal number of plants in the same vineyard was used as the untreated control.

Chemicals

The standard phenolic compounds resveratrol, catechin, gallic acid and Folin Ciocalteu's phenol reagent were purchased from Sigma (St. Louis, MO, USA). Cyanidin was supplied by Extrasynthèse, S.A. (Genay, France) and Brotomax by Agrométodos, S.A.

Extraction and measurement of phenolic compounds

Non-infected and infected roots (SO4) and leaves from control vines and from vines treated with Brotomax (cv. Flame) were collected and each of these four sample groups was divided into three approximately equal lots. The sample material was ground and shaken with dimethylsulphoxide (DMSO) (100 mg f wt ml^{-1}) for 12 h for extraction. In some cases, a fraction of the extracts was hydrolysed with HCl 2N to measure the tannins, which were hydrolysed as gallic acid. Both hydrolysed and non-hydrolysed extracts were filtered through a 0.45 μm nylon membrane before analysis by: 1) spectrophotometry, using a UNICAM UV/VIS spectrometer UV2 (Unicam Limited, Cambridge, UK), to estimate total phenols, expressed as gallic acid equivalents (Folin Ciocalteu Method; Singleton and Rossi, 1965), and condensed tannins, expressed as catechin equivalents (Price *et al.*, 1978); 2) HPLC-MS with a Hewlett-Packard liquid chromatograph (model HP 1050) (Hewlett-Packard Co., Palo Alto, CA, USA) fitted with a diode array detector (range scanned: 220–500 nm) to quantify and identify the phenolic compounds. In the HPLC analysis the stationary phase was a Sherisorb ODS-2 column (250 mm×4 mm i.d.) with a particle size of 5 μm at 35ºC. As solvent we used: acetonitrile (A)/water (B), 25 to 95% of A in 50 min. The eluent flow was 1 ml min^{-1}. Absorbance changes were recorded in the UV-V diode array detector at 280 and 353 nm. The amounts of the main phenolic compounds were determined from the area given by the integrator using the response factor of the corresponding standards. The main phenolic compounds in these extracts were collected with a fraction collector (Pharmacia LKB Biotechnology, Uppsla, Sweden) at the exit of the HPLC column for identification by means of a Hewlett Packard mass spectrometer (Model 5989).

Fungal cultures and antifungal activity of grapevine extracts

Isolates of *Pa. chlamydospora*, *Pm. inflatipes* and *Pm. aleophilum* were supplied by the Agri-Analysis Company (Davis, CA, USA) and cultured on potato dextrose agar (PDA) at 25ºC to serve as inoculum. Isolates of infected roots (SO4) cultivated under the conditions described above showed colony growth patterns and morphological features similar to those of the *Pa. chlamydospora*, *Pm. inflatipes* and *Pm. aleophilum* isolate supplied by Agri-Analysis. The antifungal activity of non-hy-

drolysed extracts from infected and non-infected grapevine roots (SO4), from the periderm and vascular cylinder of these roots, and from extracts of control and Brotomax-treated vine leaves (cv. Flame) was determined by *in vitro* tests with the fungi as described in a previous paper (Del Río *et al.*, 1998).

Light and electron microscopy

The microscopic examinations carried out and the conditions and procedures used to process the non-infected and infected roots (SO4) were as reported in previous studies (Ortuño *et al.*, 1990), which also described how semithin and ultrathin sections for light and electronic microscopy were obtained. A Photomicroscope II (Carl Zeiss, Oberkochem, Germany) was used as a light microscope. For ultramicroscopic examination, the root sections were cut (Ultracut Reicher Jung, Vienna, Austria), stained with uranyl acetate and lead citrate, and examined in a Zeiss EM 109 electron microscope with an acceleration of 60 KV. To asses the degree of root infection the number of xylem vessels blocked by tyloses was counted. Roots with 30% blocked xylem vessels were classified as infected, those with 90% blocked vessels as very infected.

Results and discussion

Figure 1A shows a cross-section of infected roots (SO4). The obstruction and discoloration of the xylem can be seen. This obstruction is in the previous year's vessels and is due to tyloses (Fig. 1B) which are formed by expansion of the xylem parenchyma cells, as can be seen in the longitudinal section of a secondary root (Fig. 2A). Here the tyloses are invading the xylem lumen their connection with the xylem parenchyma is clearly visible. A detail of tylose formation formed by expansion of the parenchyma cells can be seen in the micrograph of Figure 2B. The expansion of the parenchyma cells involves changes in the cells and xylem walls. The presence of a big vacuole suggests that growth was due to the inflow of water similar to that in cell expansion. Expanding tyloses have walls similar to the primary walls of meristematic cells. During expansion, polymers can be detected inside the tyloses. These polymers seem to be generated by the cytoplasm. Tyloses undergo many changes in appearance as a result of interactions between them in the xylem. These include changes in the walls, and the formation of aggregated and crystalline structures (Fig. 2C).

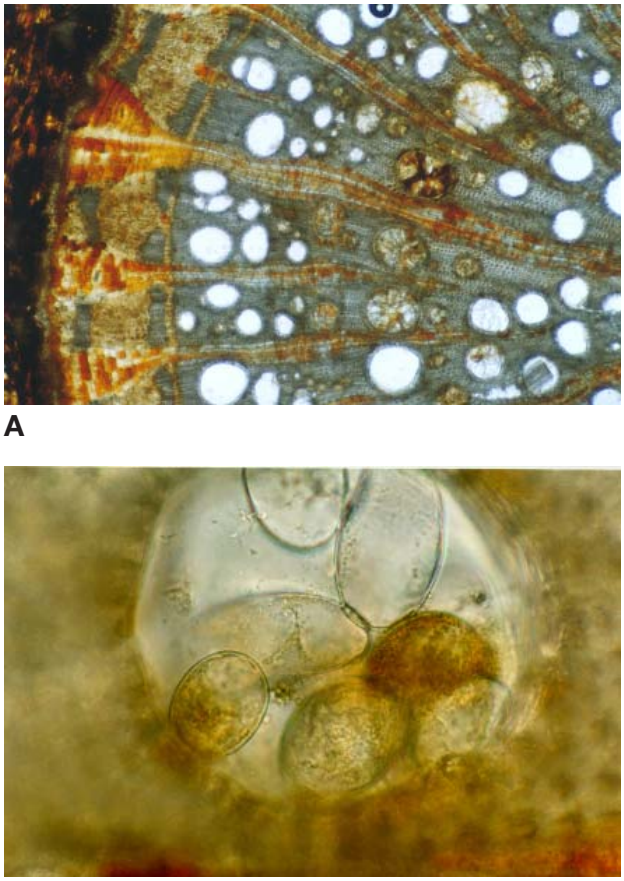
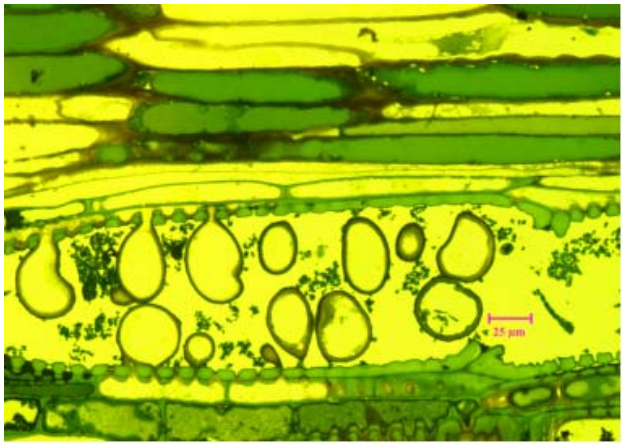


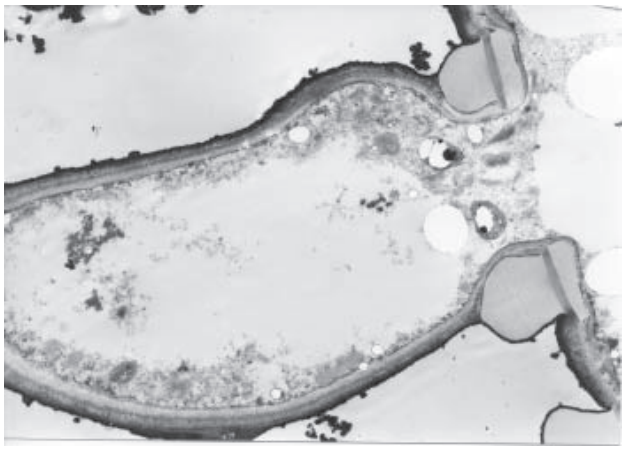
Fig. 1. A. Cross-section of infected SO4 vine roots (×100). B. Xylem with tyloses (×400).

Besides the formation of tyloses, there were also changes in the phenolic compounds. Microscopic examination of cross-sections of infected roots dyed with a phenol-specific dye ($FeCl_3$-HCl), showed stronger stains in areas where vessels were obstructed, and weaker stains in non-infected xylem elements (data not shown).

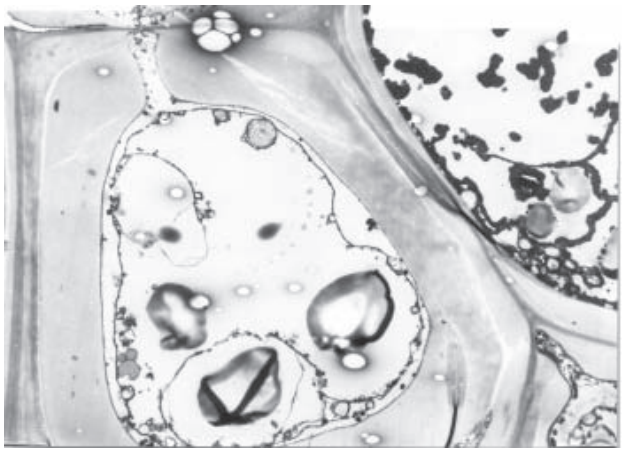
Phenolic compounds were identified mainly as condensed tannins, with cyanidin being the main flavonoid after hydrolysis. Resveratrol was also identified in the root extracts. Table 1 shows the total polyphenol, tannin and resveratrol content



A



B



C

Fig. 2. A. Magnified longitudinal section of infected SO4 vine roots. B. Electron microscopy of a tylose formation in the xylem lumen (× 3,150). C) Electron microscopy of a mature tylose in the xylem lumen (× 3,150).

of roots with varying numbers of blocked/discoloured xylems. There was a correlation between the severity of root infection and total phenol content and, particularly, total tannin content. In very infected roots, 86% of phenols were condensed tannins, 10% were hydrolysable tannins, and the remaining 4% other phenolic compounds. A similar correlation was also observed between degree of infection and resveratrol content: in very infected roots resveratrol levels may be three times as high as in non-infected roots.

When extracts of infected roots were added to the culture medium there was a correlation between the severity of infection and the degree of inhibition. Hence an increase in total polyphenols was strongly related with a greater inhibition of *Pa. chlamydospora* and *Phaeoacremonium* spp. mycelial growth (Table 2).

In the roots, polyphenols were differently distributed between the periderm and the vascular cylinder. However although the periderm contained higher polyphenol levels, the vascular cylinder had higher concentrations of hydrolysable tannins and resveratrol (Table 3). The level of condensed tannins in the periderm and vascular cylinder was similar.

Extracts of the vascular cylinder, which contained lower phenolic concentrations than the periderm, had the same inhibition capacity as extracts of the periderm. The results described suggest that resveratrol and hydrolysable tannins, are involved in vine defence mechanisms.

Higher levels of these compounds in healthy (non-infected) vines would therefore increase the natural defence of vines against the pathogens studied. In this connection, tests with Brotomax have shown that it is possible to increase the levels of these protective molecules in grapevines.

Brotomax applied during the sprouting period to grapevines cv. Flame increased polyphenol levels in different organs (Table 4). Adding phenolic extracts of leaves from vines treated with Brotomax to a culture medium increased the inhibition of mycelial growth of *Pm. inflatipes* and *Pa. chlamydospora* (data not shown).

The results suggest that phenolic compounds in *Vitis* may be involved in protecting vines against pathogen attack, and that increasing these compounds may represent a new approach for the control of trunk diseases.

Table 1. Concentration of total polyphenols (gallic acid g/100 g f wt), total tannins (condensed and hydrolysable, expressed as catechin equivalents g/100 g f wt and gallic acid g/100 g f wt respectively), and resveratrol (g/100 g f wt) in roots with different percentages of blocked/discoloured xylem.

Roots	Active compounds[a]		
	Total polyphenols	Total tannins	Resveratrol
Uninfected	12 ± 3	5 ± 0.8	0.17 ± 0.06
Infected[b]	26 ± 6	16 ± 2	0.35 ± 0.1
Very infected[c]	34 ± 8	31 ± 6	0.44 ± 0.1

[a] Mean ± SE (n = 3).
[b] Thirty % blocked xylem vessels by microscope inspection.
[c] Ninty % blocked xylem vessels by microscope inspection.

Table 2. Mycelial growth inhibition (%) of *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum* and *Pm. inflatipes* grown with infected root extracts. Five ml of root extract (100 mg ml^{-1} dimethylsulphoxide, DMSO) was added to 100 ml of culture medium potato dextrose agar (PDA) of each fungus.

Roots	Fungal species[a]		
	Pa. chlamydospora	*Pm. aleophilum*	*Pm. inflatipes*
Uninfected	12 ± 5	14 ± 6	8 ± 2
Infected[b]	68 ± 7	72 ± 9	35 ± 6
Very infected[c]	89 ± 8	88 ± 8	43 ± 8

[a, b, c] See Table 1

Table 3. Concentration of total polyphenols (gallic acid g/100 g f wt), condensed tannins (catechin equivalents g/100 g f wt), hydrolysable tannins (gallic acid g/100 g f wt), and resveratrol (g/100 g f wt) in different parts of infected vine roots [a] .

Root tissue	Active compounds[b]			
	Total polyphenols	Condensed tannins	Hydrolysable tannins	Resveratrol
Periderm	32 ± 3	15 ± 1	0.26 ± 0.05	0.10 ± 0.06
Vascular cylinder	26 ± 6	16 ± 2	0.88 ± 0.1	0.34 ± 0.1

[a] Thirty% blocked xylem vessels by microscope inspection.
[b] Mean ± SE (n = 3).

Table 4. Total polyphenols (gallic acid g/100 g f wt) in different organs of *Vitis* cv. Flame treated with Brotomax. The analysis was carried out in the first week of August.

Treatment	Plant organ[a]			
	Leaves	Stems	Fruits	Roots
Control	2.24 ± 1.3	2.1 ± 0.8	0.34 ± 0.08	0.28 ± 0.1
Treated	4.6 ± 1.1	2.9 ± 0.5	0.44 ± 0.1	0.45 ± 0.1

[a] Mean ± SE (n = 3).

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