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Phaeomoniella chlamydospora-grapevine interaction: histochemical reactions to fungal infection[(1)]

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Summary. Histochemical reactions of one-year-old potted micropropagated vines (rootstock 1103 Paulsen) to inoculation with *Phaeomoniella chlamydospora* were studied. Microscopic examination of the vine wood showed that the fungus spread through the wood tissue, albeit slowly. Starting from the roots it required nine months to colonise the first 20-25 cm of the grapevine stems. The slow spread of *P. chlamydospora* is thought to be due to the defence response initiated by the vines: production of tyloses, including accumulation of phenols in the vessels and adjacent tissue, and the deposition of unidentified defence-response substances, probably stilbene-like substances from the cell-wall surrounding the infection

Key words: defence reactions, phenols, esca.

Introduction

Plants protect themselves against pathogen attack with an arsenal of defence mechanisms that are either pre-existing (waxy cuticle, reservoirs of antimicrobial compounds, enzyme inhibitors) or become activated after pathogen invasion (oxidative burst, cell wall lignification, callose and suberin deposition, PR protein and phytoalexin biosynthesis). For disease to occur, the pathogen must circumvent the preformed- (passive) defences of the plant and it must also avoid eliciting induced (active) defence responses in the infected tissue or it must inhibit the induction of active defence responses, as by secreting necrotising factors (Alfano and Collmer, 1996; Jackson and Taylor, 1996; Knogge, 1996). There is also an intermediate situation, when the pathogen elicits the plant's defences but still manages to colonise the host, possibly because the pathogen grows or develops faster than the host can activate its defences, because it manages to tolerate the induced defences, or because it is able to disarm them (Isaac, 1992).

The pathogenic fungus *Phaeomoniella chlamydospora* (*Pch*) is considered the causal agent of Petri disease (Crous and Gams, 2000), a vine decline that is associated with some forms of esca (Mugnai *et al*., 1999; Graniti *et al*., 2000). Grapevine wood colonised by this fungus shows higher levels of resveratrol (Amalfitano *et al*., 2000), a phytoalexin belonging to the group of stilbenes, which seems to have a role in the resistance of plants to fungal pathogens (Hart, 1981). However, it has also been shown that resveratrol does not inhibit *Pch* growth *in vitro* (Mazzullo *et al*., 2000).

(1) Dedicated to Prof. A. Graniti on the occasion of his 75th birthday

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The fungus invades the xylem vessels of the vine trunk and advances mostly upwards and also laterally into the adjacent vessels and xylem parenchyma cells. At the same time, tyloses and brown deposits appear in the lumina of the vessels (Pascoe and Cottrall, 2000).

In the present work, we investigated the reaction induced by *Pch* artificially inoculated into the stems of 1-year-old micropropagated grapevine plants as well as the spread of the pathogen in the wood of inoculated vines.

Materials and methods

Plant material and pathogenicity test

Fifty one-year-old, micropropagated vines (rootstock 1103 Paulsen) grown in pots were used in the tests. Conidial inoculum of *Pch* strain 56-94 (CBS229.95) was prepared by placing 6 mm diam. agar plugs from colonies grown on 2% malt agar in 500-ml Erlenmeyer flasks containing 200 ml of 2% malt extract liquid medium. Flasks were placed in an orbital shaker (100 rpm) for 15 days at 25°C. Conidia were collected by centrifugation (5 min, 7000 *g*, 15°C), and adjusted with sterile distilled water (SDW) to 10^6 conidia ml^{-1}.

Plants to be inoculated were uprooted, adhering soil was gently shaken from their roots before they were washed under running tap water. The roots were cut about 10 cm from the collar with a sterile scalpel and soaked in a conidial suspension for l h (Scheck *et al*., 1998). The 35 inoculated and 15 control vines (the latter soaked in SDW) were then transferred to 16-cm-diam. pots containing a 1:1:1 (v:v:v) sand, peat and soil mixture and maintained in a growth chamber at a constant 25°C**,** R.H. 80% with a photoperiod of 14 h (200 μmol m^{-2} s^{-1}).

Fungus isolation and survey of wood alterations in inoculated plants

Nine months after inoculation, 7 *Pch*-inoculated and 3 control vines were collected. Each vine was carefully washed in running water and divided into three parts, namely: roots, base of stem (collar) and stem. The roots were further divided into 2 segments of about 10 cm each: A. roots 5-10 cm from the collar and B. roots 0-5 cm from the collar; and the stems were likewise divided into three segments: A. between the 1th and 4th internode; B. between the 5th and 8th internode; and C. between the 9th and 11th internode.

The roots, base of the stem (5 cm long) and stem segments were surface-sterilised by soaking in 95% ethyl alcohol for 1 min, 4% sodium hypochlorite for 5 min and again 95% ethyl alcohol for 30 sec. For each sample, fragments about 5×5 mm were removed. A total of 336 root fragments, 168 collar fragments and 294 stem fragments were collected from infected vines, and 72 root fragments, 36 collar fragments and 108 stem fragments from control vines. All fragments were transferred to Petri dishes containing malt agar (Difco, Detroit, MI, USA) and incubated at 20–22°C in darkness. The remaining 28 inoculated and 7 control vines were sectioned lengthwise and the stems and collars examined for macroscopic changes attributable to the fungus inoculated.

Histochemistry

Changes produced in the vine collars and stems were examined macroscopically, followed by microscopic examination of fresh plant material sectioned with a freezing microtome. Transverse and longitudinal (tangential and radial) sections were cut in deteriorated areas of infected vine wood and in healthy control vines.

Sections (30 μm thick) were stained with various reagents to locate the fungus in the tissues and to detect structural and biochemical changes in the cells of the host vines. After staining (see below) the sections were examined with a Leitz Dialux 22 microscope (Leitz Wetzlar, Wetzlar, Germany). The filters used included a UV filter set with 365-nm excitation and a 400 nm barrier filter.

The stains used, and the cell components indicated by each stain, are listed below. The colour or reaction produced in the tissue by staining is given in parentheses.

Astra blue (Beccari and Mozzi, 1966): acid polysaccharides, cellulose and pectocellulose walls (blue).

Neutral red (Carol e Peterson, 1979): secondary or modified walls and lignin (red).

DAPI (4', 6-diamino-2-phenylindole) (Williamson and Fennel, 1975): nuclear DNA inside the fungal cells (blue-green fluorescence).

Vanillin-HCl (Sarkar and Howarth, 1976): cat-

echins and condensed tannins. Sections were immersed for 5 minutes in 10% (w:v) vanillin prepared in absolute ethanol and concentrated HCl (1:1, v:v), mounted in the reagent and observed under a light microscope (brilliant red).

Lugol (Jensen, 1962): starch presence (black).

Neu's reagent (Dai *et al*., 1995): flavonoid compounds (yellow fluorescence).

Fungal cell wall was stained by calcofluor White M2R (Maeda and Ishida, 1967), a specific stain for polysaccharides - cellulose and chitin (blue fluorescence). Where the fungal hyphae occurred in cells with lignified cell walls a contrast stain (Schiff reagent) was used to distinguish cellulose fluorescence from lignin fluorescence (Mori and Bellani, 1996).

Methyl blue in lactic acid: chitin in the fungal cell walls (blue).

Fuchsin: lignin (fuchsia pink) (Kraus *et al.*, 1998).

Autofluorescence was monitored under UV light (epifluorescence at 365 nm): modified walls and lignin usually produced a blue fluorescence.

Results

Pathogenicity test

All vines infected with *Pch* showed in longitudinal section brown and black streak that were concentrated in the collar area and in the first internode. Vessels transversally sectioned exuded a characteristic shiny black tar-like gum mainly around the pith. No wood deterioration was ever noted in the stem segments above the second and/or third internode (max. 25 cm). None of the vines displayed any external symptoms.

Pch was isolated from all inoculated vines, and from each of the plant parts examined: roots (14% of the wood fragment analysed), collars (25%) and stems (7.1%) (Table 1). However, *Pch* colonisation differed between vine plant portions, being greatest at root collar level (25%) and at the base of the stem (20.2%), to become less frequent (5.9%) and disappear altogether above the 7th/8th internode. Colonies of saprophytic fungi were sometimes found both in control (5.1%) and in inoculated (3.5%) vines.

Table 4. Results of isolations from seven grapevine plants artificially inoculated with *Phaeomoniella chlamydospora* and from three control plants, nine months after inoculation.

Plant portion			Analysed w.f.[a] No.		w.f. colonised by fungi No./(%)		w.f. colonised by *Pch* No./(%)	
			Control	*Pch* inoculated	Control	*Pch* inoculated	Control	*Pch* inoculated
Stem	C	9–11° internode (25–35 cm from root collar)	36	84	0	0	0	0
	B	5–8° internode (15–25 cm from root collar)	36	84	0	5 (5.9)	0	5 (5.9)
	A	1–4° internode (5–15 cm from root collar)	36	126	2 (5.5)	20 (15.9)	0	16 (20.2)
Root		collar 5 cm above root insertion	36	168	1 (2.8)	48 (28.6)	0	42 (25)
Roots	B	5–2 cm from root collar	36	168	3 (8.3)	23 (13.6)	0	17 (10.1)
	A	10–5 cm from root collar	36	168	5 (13.9)	42 (25)	0	30 (17.8)
Total			216	798	11 (5.1)	138 (17.3)	0	110 (13.9)

[a] w.f., wood fragments.

Histochemistry

The presence of fungal hyphae in the xylem vessels was revealed by staining with calcofluor and DAPI fluorochrome. From the xylem vessels the fungus was able to invade the paratracheal parenchyma, either through the pits of the vessels, or by penetrating the vessel walls, probably after producing degrading enzymes (Fig. 1) (Mugnai *et al*. 1997; Marchi *et al*., 2001) (epifluorescence).

In the nine months following inoculation the fungus advanced up to the 7th/8th internode on average (about 15–25 cm from the collar), moving mainly along the vessels. No conidia or conidiophores were noted in the xylem vessels.

Longitudinal and radial sections viewed under secondary fluorescence showed tyloses in the tracheae, which were almost completely occluded in the areas where there were black streaks (Fig. 2A and 2B). Chemically the cell walls of the tyloses consisted mainly of cellulose (staining with astra blue and calcofluor) associated with substances of a phenolic nature (tannins, gums), which were also found in the lumina of the tyloses (staining with astra blue viewed under white light).

Staining with vanillin-HCl indicated that at the infection sites there was production and accumulation of condensed tannins in the vessels, fibres, parenchyma rays and paratracheal parenchyma cells (Fig. 3). Condensed tannins were also found in healthy vines, but there they were less common and occurred almost exclusively in the late wood.

Inspection of stem cross sections by epifluorescence revealed a white primary fluorescence (autofluorescence) coming from the cell walls of the paratracheal parenchyma, the fibres, and the cells of the rays surrounding the vessels (Fig. 4). This type of fluorescence suggested that defence-response substances perhaps of a flavonoid type, were deposited in the cell walls after infection. The accumulation of defence-response substances around the infection sites was confirmed when these sites tested negative with specific lignin stains (fuchsin) (Fig. 5).

Staining with Neu, which confers a yellow fluorescence on any flavonoids present, was negative.

Lastly, staining with lugol showed much higher levels of starch in the xylem fibres and the parenchyma rays of the wood near the infection sites (not shown).

Some of the biochemical responses found (higher levels of phenolic substances in the vessels and adjacent cells) occurred also in tissue sections from apical portions of the stem not colonised by *Pch,* but the mechanical defence response (production of tyloses) was always associated with the presence of the fungus in the vessels.

Discussion

Histochemical examination of vine plantlets inoculated with *Pch* revealed that the fungus advances in the host, albeit slowly. The host reacted to the fungus by setting up mechanical and biochemical barriers. The main indication that the vine is resisting *Pch* invasion is by its formation of tyloses, with the resulting occlusion of the xylem vessels These extrusions of the parenchyma cells surrounding the vessels were observed only in areas where the fungus was found (from the roots to about 15 cm above the collar) and the cell walls consist not only of polysaccharide material but also of condensed tannins and phenols. It appears that these last two substances also accumulate within the tyloses.

The accumulation of phenols (especially condensed tannins) in the tissue adjacent to the vessels invaded by *Pch* must also be considered a defence response. Condensed tannins normally occur also in healthy vines, but in low concentrations and only in the late wood (Misaghi, 1982). However, it is known that following attack by micro-organisms, plants sometimes have higher levels of condensed tannins because of an increased production of new tannins or the mobilisation of pre-existing tannins towards infection sites, in order for example to inhibit fungal enzymes (Byrde, 1981), to reinforce the structural components of the cell walls (Bell, 1981) or to form a chemical barrier to infections (Dai *et al.,* 1995). When condensed tannins are oxidised by polyphenol oxidases and transformed into chinones, they become very toxic and inhibit the growth of plant pathogens (Vidhyasekaran, 1988).

The inoculated vines revealed another peculiarity: chemical changes in the cell walls of the paratracheal parenchyma and the xylem fibres near the vessels invaded by *Pch*. Unlike normal cell walls, they were impregnated by substances that emitted a white primary fluorescence, and moreover, when treated with acid fuchsin they did not become stained, unlike cells of the same type that

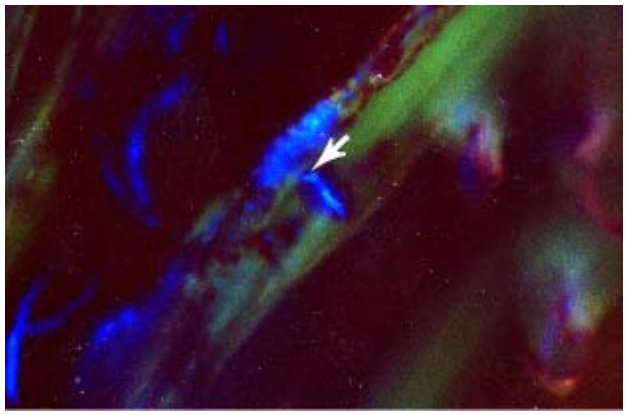

Fig. 1. Radial longitudinal section of the stem of a micropropagated vine inoculated with *Phaeomoniella chlamydospora*. Photograph taken after calcofluor staining. Fungal hyphae in the vessels and perforating the walls. The cells adjacent to the vessels could be the place where materials of various types accumulate, accounting for the yellow fluorescence. Epifluorescence, ×225.

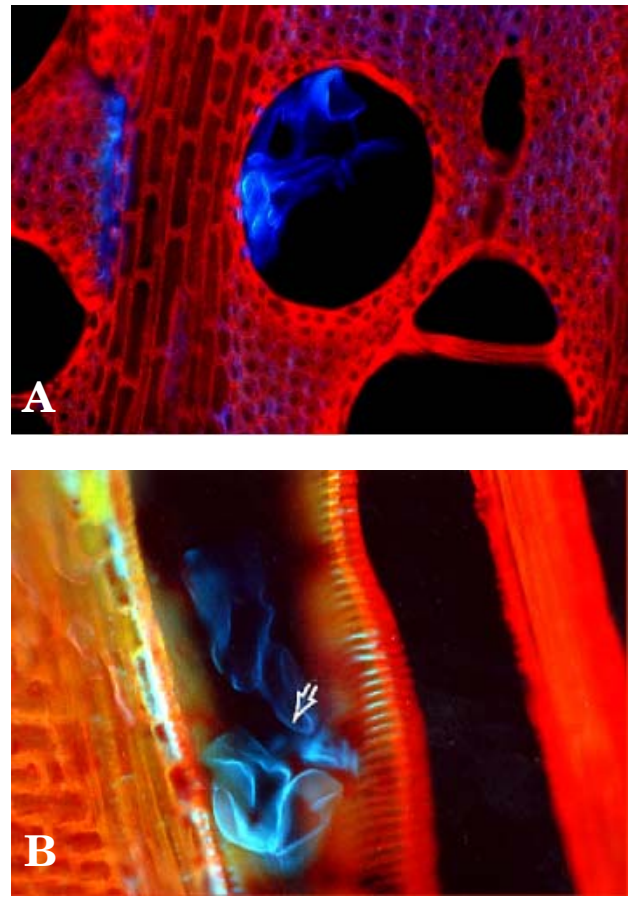

Fig. 2. Base of the stem of a micropropagated vine inoculated with *Phaeomoniella chlamydospora*. Cross (A) and radial longitudinal section (B). Photographs taken after calcofluor and neutral red staining. The lumen of the trachea in both photographs is partly filled with tyloses. The blue fluorescence is caused by cellulose. Epifluorescence, ×225.

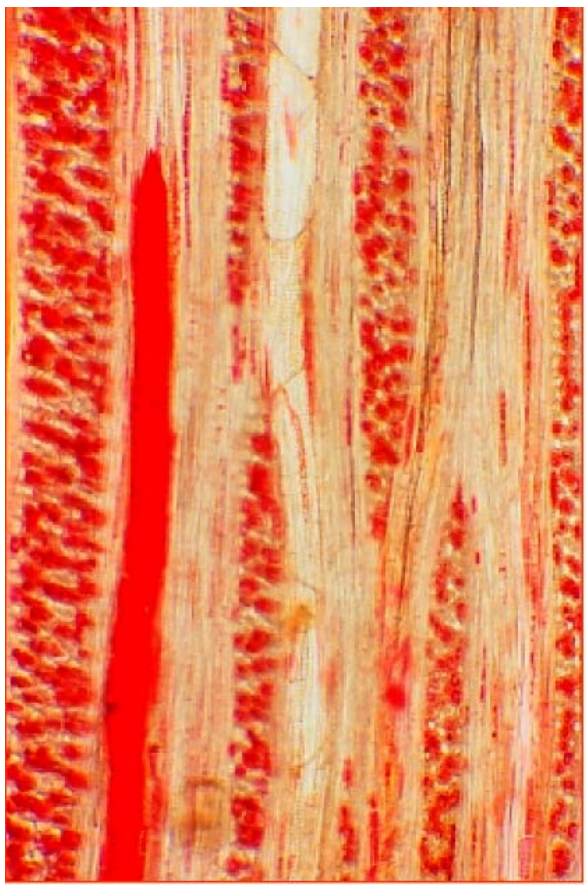

Fig. 3. Radial longitudinal section of the stem of a micropropagated vine inoculated with *Phaeomoniella chlamydospora* stained with vanillin-HCl. The red material consists of condensed tannins in the lumen of the vessels, in the paratracheal parenchyma and in the cells of the parenchymatic rays. Epifluorescence, ×110.

were somewhat farther away from the xylem vessels. It would seem, therefore, that these changes were not attributable to the deposition of lignin in the cell walls, or the formation of papillae, since the examination in fluorescent light indicated that all the cells of the xylem and perixylem tissues already have a lignified secondary wall (blue fluorescence). A more likely explanation of these cell wall changes is that they are a consequence of the deposition of stress metabolites (perhaps flavonoids or stilbenes of the resveratrol type) produced by the vine to block the spread of *Pch*. Dai *et al*. (1995) reported a white fluorescence emanating from the guard cells of the stoma in leaves of *Vitis* spp. colonised by *Plasmopara viticola*. They attributed this fluorescence to the accumulation of phenolic compounds. Perhaps in the vines studied here the compounds deposited in the cell walls were also phenolic.

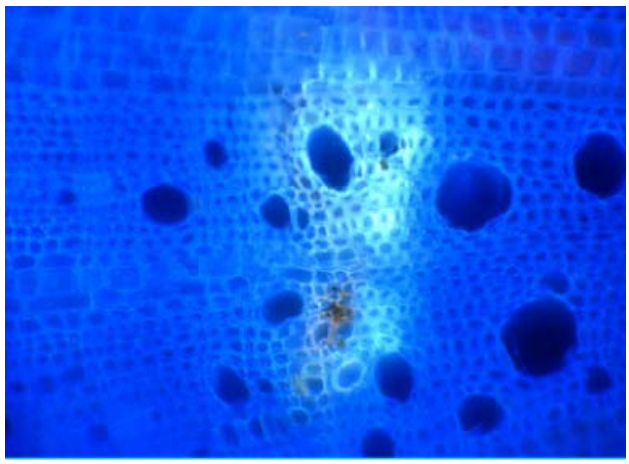

Fig. 4. Cross section of the base of the stem of a micropropagated vine inoculated with *Phaeomoniella chlamydospora*. The walls of the wood cells in an infected area emit a white primary fluorescence that reveals unidentified substances presumably produced in response to infection ×110.

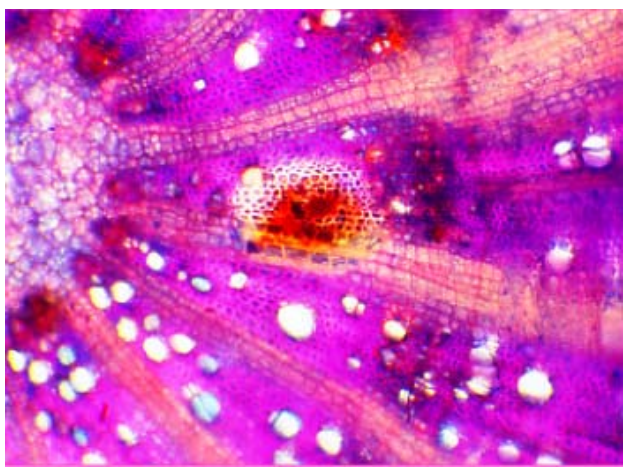

Fig. 5. Cross section of a micropropagated vine inoculated with *Phaeomoniella chlamydospora* and stained with acid fuchsin. The cells around the deteriorated area are not stained. This may indicate the presence newly formed substances that by impregnating the walls prevent the stain from acting. Epifluorescence, ×55.

The defence response of grapevine occurs before the tissues are colonised by the fungal mycelium, since in 30-cm-high plants the inoculated fungus was not found in the stem portions at more than 15 cm above the soil, but chemical changes in the cell walls of the paratracheal parenchyma and the xylem fibres had already occurred.

Tracing the advance of *Pch* in micropropagated vines showed that the fungal mycelium spread both longitudinally and laterally: it was found mainly in the xylem vessels but the hyphae were also clearly seen to spread through the pits of the tracheae and thus to spread radially. These findings are consistent with Pascoe and Cottral (2000) on the histopathology of *Pch* in *in vitro* cultures of *Vitis vinifera* cv. Chardonnay. The fungus initially advances within the cells of the xylem parenchyma, and, once it has caused the formation and extrusion of tyloses in the vessel lumen, it penetrates into the vessels through the intrusion points of the tyloses. Longitudinally, it spreads along the stem with the sap flow.

If account is taken of the fact that the histological examination was carried out nine months after inoculation, and that inoculation was through the roots, the significant finding to emerge from this study is that the spread of *Pch,* even if considered only in a longitudinal direction, was very slow, and this was perhaps because of the defence response of the plant.

Literature cited

Alfano J.R. and A. Collmer, 1996. Bacterial pathogens in plants: life up against the wall. *Plant Cell* 8, 1683–1698.

Amalfitano C., A. Evidente, G. Surico, S. Tegli, E. Bertelli and Mugnai L., 2000. Phenols and stilbene polyphenols in the wood of esca-diseased grapevines. *Phytopathologia Mediterranea* 39, 178–183.

Beccari N. and Mazzi V., 1966. *Manuale di Tecnica Microscopica*. Società Editrice Libraria, Milano, Italy

Bell A.A., 1981. Biochemical mechanisms of disease resistance. *Annual Review of Plant Physiology* 32, 21–81.

Byrde R.J.W., 1963. Natural inhibitors of fungal enzymes and toxins in disease resistance. In: *Perspectives in biochemical plant pathology*. (S. Rich, ed.), *Connecticut Agricultural Experimental Station Bulletin* 663.

Carol A. Peterson, 1979. Selective vital staining of companion cells of potato tuber and parsnip root with neutral red. *Stain technology* 54(3), 135–139.

Crous P.W. and W. Gams, 2000. *Phaeomoniella chlamydospora* gen. et comb. nov., a casual organism of Petri grapevine decline and esca. *Phytopathologia Mediterranea* 39, 112–118.

Dai G.H., C. Andary, L. Mondolot-Cosson and D. Boublas, 1995. Histochemical studies on the interaction between three species of grapevine, *Vitis vinifera, V. rupestris* and *V. rotundifolia* and the downy mildew fungus, *Plasmopara viticola*. *Physiological and Molecular Plant Pathology* 46, 177–188.

Graniti, A., G. Surico and L. Mugnai, 2000. Esca of grapevines: a disease complex or a complex of diseases? *Phytopathologia Mediterranea* 39, 16–20.

Isaac S., 1992. *Fungal-Plant Interaction*. Chapman and Hall, London, New York, USA.

Jackson A.O. and C.B. Taylor, 1996. Plant-microbe interactions: life and death at the interface. *Plant Cell* 8, 1651–1668.

Jensen W.A., 1962. *Botanical Histochemistry*. W.H. Freeman, San Francisco, CA, USA.

Knogge W., 1996. Fungal infection of plants. *Plant Cell* 8, 1711–1722.

Kraus J.E, H.C. de Sousa, M.H. Rezende, N.M. Castro, C. Vecchi and R. Luque, 1998. Astra blue and basic fuchsin double staining of plant materials. *Biothecnic and Biochemistry* 73, 235–243.

Maeda H. and Ishida N., 1967. Specificity of binding of hexopyranosyl polysaccharides with fluorescent brightener. *Journal of Biochemistry* 62, 276–278.

Marchi G., 2001. Susceptibility to esca of various grapevine (*Vitis vinifera*) cultivars grafted on different rootstocks in a vineyard in the province of Siena (Italy). *Phytopathologia Mediterranea* 40, 27–36.

Mazzullo A., S. Di Marco, F. Osti and A. Cesari, 2000. Bioassays on the activity of resveratrol, pterostilbene and phosphorous acid towards fungi associated with esca of grapevine. *Phytopathologia Mediterranea* 39(3), 357–365.

Misaghi I.J., 1982. *Physiology and Biochemistry of Plant-Pathogen Interaction*. Plenum Press, New York, USA.

Mori B. and L.M. Bellani. Differential staining for cellulosic and modified plant cell walls. *Biotechnic and histochemistry* 71(2), 71–72.

Mugnai L., A. Graniti and G. Surico, 1999. Esca (black measles) and brown wood-streaking: two old and elusive diseases of grapevines. *Plant disease* 83(5), 404–418.

Mugnai L., G. Surico and A. Sfalanga, 1997. Produzione di enzimi esocellulari da parte di funghi del legno di viti colpite dal "Mal dell'Esca". *Micologia italiana* 26(1), 11–22.

Pascoe I., Cottral E., 2000. Developments in grapevine trunk diseases research in Australia. *Phytopathologia Mediterranea* 39, 68–75.

Sarker S.K. and R.E. Howarth, 1976. Specificity of the vanillin test for flavanols. *Journal of Agricultural and Food Chemistry* 24, 317–320.

Scheck H.J., S.J. Vasquez, D. Fogle and W.D. Gubler, 1998. Grape growers report losses to black-foot and grapevine decline. *California Agriculture* 52(4), 19–23.

Vidhyasekaran P., 1988. *Physiology of Disease Resistance in Plants*. Vol. II, CRC Press Inc., Boca Raton, FL, USA 128 pp.

Williamson D.H and D.J. Fennel, 1975. The use of fluorescent DNA-binding agent for detecting and separating yeast mitochondrial DNA. *Methods in Cell Biology* 12, 335–351.

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