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RESEARCH PAPERS

Histo-pathology study of the growth of *Trichoderma harzianum*, *Phaeomoniella chlamydospora* and *Eutypa lata* on grapevine pruning wounds

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Summary. Protecting grapevine pruning wounds by inoculating them with *Trichoderma* spp. can prevent infection from trunk disease pathogens. The growth and interactions of both, the biological control agent *Trichoderma* spp. and the vine pathogens, are not well understood. Green fluorescent protein (GFP)-labelled *Trichoderma harzianum* and red fluorescent protein (DsRed)-labelled *T. harzianum*, were dual-inoculated with *Phaeomoniella chlamydospora* (DsRed) or *Eutypa lata* (GFP) on fresh pruning wounds of one-year-old Cabernet Sauvignon and Sauvignon blanc shoots. The inoculated fungi were recovered from varying depths within the shoots at 30-day-intervals for 90 days. *Trichoderma harzianum* suppressed the pathogens and grew deeper in the presence of the pathogens than when it was singly inoculated; possibly an indication of pathogen recognition and competitive response. *Eutypa lata* was completely eliminated from Sauvignon blanc in dual-inoculated canes after 90 days. The mycelium of *P. chlamydospora* (DsRed) grew extensively in the xylem vessels and possibly contributed to vessel occlusion. *Phaeomoniella chlamydospora* and *E. lata* caused blockage of the vessels and thickening of the vessel walls. Grapevine wood produced both tyloses and gums (gels) that blocked xylem vessels as a result of infection. A thickening of the cell walls of xylem fibres occurred only in *E. lata*-inoculated shoots, indicative of a different mode of pathogenesis from *P. chlamydospora*.

Key words: Green fluorescent protein (GFP), Red fluorescent protein (DsRed), histology, biological control.

Introduction

Pruning wounds are important portals by which trunk diseases infect grapevine vascular tissue causing wood necrosis and decay (Chapuis *et al*., 1998; Edwards *et al*., 2007a, b). Grapevine trunk diseases, namely Eutypa dieback, Petri disease, esca, Botryosphaeria canker and Phomopsis dieback induce a slow grapevine decline, reduce yield, shorten the productive life of vines and increase vineyard management costs (Munkvold *et al*., 1994; Mugnai *et al*., 1999; Creaser and Wicks, 2001).

These diseases can possibly be controlled or prevented by avoiding infection or minimising the effect of the pathogens in the wood tissue. Natural epiphytes are known to also colonise grapevine wounds and some inhibit wound infection from grapevine trunk pathogens (Munkvold and Marois, 1993). *Trichoderma* species colonise wood tissue and protect grapevine pruning wounds from infection by *E. lata* (Harvey and Hunt, 2006; John *et al*., 2008) and *Phaeomoniella chlamydospora* (Kotze, 2008). Vascular streaking

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induced by *P. chlamydospora* on nursery vines was significantly reduced in *T. harzianum*-inoculated shoots, where vine growth was also stimulated (Di Marco *et al.*, 2004; Fourie and Halleen 2006; Di Marco and Osti, 2007). *Trichoderma* species are suited for biological control due to their fast growth rates, high reproductive capacity and antagonistic properties that enable them to successfully compete with other microorganisms (Ghisalberti and Sivasithamparam, 1991; Harman *et al.*, 2004). However, little is known about the interaction of *Trichoderma* species with trunk disease pathogens in pruning wounds and vascular tissue of grapevines.

In studies on the growth of fungi in plant tissue histological staining and fluorescent antibody labelling are employed. Due to the lack of histochemical stains that distinguish between the hyphae of different species, previous efforts have used specific fluorescent antibodies (Gendloff *et al.*, 1983). However, fluorescent antibody techniques may have problems in applicability due to the complexity of environmental samples. The feasibility of transforming fungi for the expression of reporter genes allows for the detection and monitoring of pathogens and biological control agents *in vivo* without the exclusion of natural epiphytes and endophytes. Reporter-gene labelling of fungi to express fluorescent proteins that allow visualisation, detection and differentiation between fungi has been used to study pathogenesis and fungal growth in natural habitats (Olivain *et al.*, 2003; Oren *et al.*, 2003).

Green fluorescent protein (GFP) has been expressed in *E. lata* and *P. chlamydospora* (Bradshaw *et al.*, 2005); however, no *in vivo* studies have been done. Landi *et al.* (2009) also transformed an isolate of *P. chlamydospora* with GFP and inoculated five different grapevine cultivars. They detected the green fluorescence microscopically as well as with PCR, and were able to trace the growth of *P. chlamydospora* in grapevine tissue, but they did not see the fungal mycelium. Recently, McLean *et al.* (2009) reported the transformation of *T. harzianum* and *P. chlamydospora* for the expression of the GFP and dsRed-Express (DsRed). The transformed isolates were also subjected to biological fitness studies to ascertain their suitability for subsequent *in vivo* studies (McLean *et al.*, 2009).

Various studies have investigated the growth and spread of vine trunk pathogens in vascular tissues. Mycelium of *E. lata* has been seen in inoculated shoots, where it occurs in the xylem vessels and pith parenchyma (Gendloff *et al.*, 1983; John *et al.*, 2004). Mycelium was not found in young canes or foliage where symptoms were due to translocated toxic metabolites (Rudelle *et al.*, 2005). Edwards *et al.* (2003) isolated *P. chlamydospora* from the entire length of inoculated canes and suggested that the spread of the pathogen inside the host was by transportation of the spores through the vascular system. *Phaeoacremonium* species sporulate inside vascular tissue (Feliciano and Gubler, 2001). Conidia of both *Phaeoacremonium aleophilum* and *P. chlamydospora* occur in pruning wound sap (Bruno *et al.*, 2007). Histological studies on the penetration of *P. chlamydospora* have been carried out only on inoculated non-woody tissue-cultured grapevine plants (Pascoe and Cottral, 2000; Troccoli *et al.*, 2001) where pathogen growth may be different than in mature wood tissue. However, histological studies in mature wood are limited by the impossibility of distinguishing inoculated fungi from other fungi also present in the wood.

Grapevines respond to infection of the vascular tissue by producing tyloses and gels (or gums) that occlude the vessels (Troccoli *et al.*, 2001; Sun *et al.*, 2008) and that provide mechanical and biochemical barriers that compartmentalise the infection and stop or restrict the further spread of the pathogen. Tyloses are cell extrusions formed from the xylem parenchyma cells into the vessel lumen (Esau, 1977), while gels are fluid substances secreted into the vessel from the parenchyma cells (Rioux and Quellette, 1989; Rioux *et al.*, 1995). Ferreira *et al.* (1994) attributed reduced xylem function in Petri diseased vines to xylem blockage as a host response to infection. However, impairment of xylem function could also be a result of the pathogen altering xylem permeability rather than physical blockage alone (Edwards *et al.*, 2007c).

Reporter gene-labelled *T. harzianum*, *P. chlamydospora* (McLean *et al.*, 2009) and *E. lata* (Bradshaw *et al.*, 2005) were inoculated onto one-year-old grapevine canes of two cultivars, Sauvignon blanc and Cabernet Sauvignon. The objective was to monitor the growth of reporter

gene-labelled *T. harzianum,* in mature wood tissue, and to determine its ability to prevent or interfere with colonisation of grapevine pruning wounds by the trunk disease pathogens. The grapevine wood response to *T. harzianum* and the trunk disease pathogens is also reported.

Materials and methods

Isolates used

Two isolates of *T. harzianum*, one expressing GFP (STE-U 6517) and the other DsRed (STE-U 6518), and *P. chlamydospora* expressing DsRed (STE-U 6520) were transformants derived from the transformation of the respective fungi by McLean *et al*. (2009). The isolates are maintained in the University of Stellenbosch, Plant Pathology Department culture collection. An isolate of *E. lata* transformed to express GFP (Bradshaw *et al*., 2005) was kindly provided by R.E. Bradshaw (National Centre for Advanced Bio-Protection Technologies, Institute of Molecular Biosciences, Palmerston North, New Zealand). All the transformed isolates were also co-transformed to express the selectable marker gene hygromycin phosphotransferase (*hph*) and thus could grow on a medium amended with the antibiotic hygromycin B.

The isolates were first plated on potato dextrose agar (PDA; Biolab, Wadesville, South Africa) amended with hygromycin B at 40 mg L^{-1} to verify hygromycin resistance. The fluorescence of the fungi was verified in water-mounted slides of the mycelium with an epifluorescence microscope (Zeiss, Göttingen, Germany). GFP-labelled fungi were examined under UV-light with an Endow GFP BP filter having an excitation filter of 470 nm (blue fluorescent light), an emission filter of 525 nm, and a beam splitter of Q495 Lp (Chroma Technology Corp., Rockingham, North Carolina, USA). DsRed-labelled fungi were detected by a HQ,TRITC filter with excitation filter of 545 nm (green fluorescent light), an emission filter of 620 nm and a beam splitter of Q570 Lp (Chroma Technology).

Inoculum was prepared by plating isolates on PDA in Petri dishes and incubating at 22–25°C. Spore suspensions were prepared using 7–8-day-old cultures of *T. harzianum* expressing GFP and DsRed (hereafter referred to as TG1 and TR1 respectively). The plates were flooded with sterile distilled water and conidia were dislodged with a glass rod. Spore suspensions were adjusted to 10^6 conidia mL^{-1}. The same procedure was used to obtain inoculum for *P. chlamydospora* expressing DsRed (hereafter referred to as PchR1). *Phaeomoniella chlamydospora* conidial concentrations were adjusted to 10^5 conidia mL^{-1}. One hundred µL droplets of conidial suspensions of each treatment were applied to fresh pruning wounds.

For *E. lata* (EL), mycelial plugs made with a 5-mm-diameter cork borer were used for inoculation, as this fungus does not produce conidia in culture. Inoculation was done with 5–7-day-old cultures growing on PDA Petri dishes and plugs were covered with Parafilm.

Single and dual inoculation of grapevine pruning wounds

Inoculation of canes

One-year-old (four nodes in length) grapevine canes of the cultivars Sauvignon blanc and Cabernet Sauvignon were obtained from apparently healthy shoots from a certified nursery. The canes were soaked in water for 4 hours, after which they were surface-sterilised by dipping in a patented quaternary ammonium compound (Sporekill™, International Chemicals (Pty) Ltd, Stellenbosch, South Africa) at 1.5 mL L^{-1} for 5 minutes and dried at room temperature. The shoots were then stored for 2 months at 5°C in air tight plastic bags with moistened perlite. Prior to growth, the canes were pasteurised by hot water treatment (50°C for 30 minutes) and then grown in a hydroponic system at ±25°C until budding. The water in the hydroponic system was changed twice weekly and the hydroponic fertilizer Chemicult® (Chemicult Products (Pty) Ltd, Camp's Bay, South Africa) was added once a week at the recommended rate. After budding, the distal nodes were removed by pruning approximately 10 mm above the third node to create a wound similar to a vineyard pruning wound.

The treatments were as follows, Treatment 1, *T. harzianum* (DsRed) TR1; Treatment 2, *T. harzianum* (GFP) TG1; Treatment 3, *P. chlamydospora* (DsRed) PchR1; Treatment 4, *E. lata* (GFP) EL1; Treatment 5, *T. harzianum* TG1 and *P. chlamydospora* PchR1; Treatment 6, *T. harzi-*

anum TR1 and *E. lata* EL1; Treatment 7, *P. chlamydospora* PchR1 and *T. harzianum* TG1; and Treatment 8, sterile distilled water (untreated control). In the dual-inoculated canes (treatments 5, 6 and 7), *T. harzianum* was applied first, and the pathogen inoculated 24 hours later, except in treatment seven, where *P. chlamydospora* PchR1 was inoculated first, and *T. harzianum* applied to the wound 24 hours later. Each treatment had 21 replicates and canes were randomly assigned to a treatment to give a completely randomised design. Isolations and microscopic examination of the canes were carried out 30, 60 and 90 days after treatment.

Fungal isolation

At each interval, seven canes per treatment were harvested, of which three served for fungal isolations, while four were sectioned (two longitudinal and two transverse) and examined under the microscope. Canes for fungal isolation were surface sterilised and aseptically split in two. Isolations were made on PDA amended with hygromycin B (40 μg mL^{-1}) from both the wood and the pith tissue. Four wood tissue sections, one from either side of the pith of each half of the split cane were aseptically removed and placed equidistantly on one 90-mm Petri dish. Four pith sections, two from either half of the split cane were also aseptically removed and placed on a separate Petri dish. Isolations were made at the following positions from the inoculation point: the dead zone (2–5 mm dead tissue directly below the wound); the interface between dead and live tissue, 5, 10, 15, 20, 30, 40 and 50 mm below the interface. Plates were incubated at 25°C under white light 8 hours and darkness for 16 hours per day, for 2–4 weeks, after which the growing fungi were identified and confirmed by the expression of the respective fluorescence. When a fast-growing fungus grew from one or more piece/s, the other pieces were removed from the Petri dish and plated onto fresh medium to avoid their being over grown. The maximum depth of isolation was noted at 30, 60 and 90 days. The frequency of isolation of the fungi, as a percentage of the total number of tissue pieces isolated from a certain depth in a specific cane was recorded at each isolation date. The frequency of isolation was taken to indicate the extent of fungus wood colonisation.

Microscopic examination of wood sections

Sections for microscopic examination were made with a Leitz (Wetzlar Germany) slide stage freeze microtome. Transverse sections were made 15 μm thick while the longitudinal sections were 20 μm thick. The sections were mounted on Dako fluorescent mounting medium and kept on ice until viewing on the microscope. Three microscopes were used for viewing, an epifluorescence Zeiss Axioscope (Göttingen, Germany) microscope where images were captured using a Nikon digital camera DXM1200 with Automatic Camera Tamer (ACT-1) software, an Olympus live cell imaging epifluorescence microscope with CellR software and a scanning disk Zeiss confocal microscope with ZEN 2007 (Carl Zeiss MicroImaging, Göttingen, Germany) imaging software. The wood sections were examined for the presence of fungal hyphae and any structural changes that could be attributed to the inoculated fungi.

Results

Single and dual inoculation of grapevine pruning wounds

Single inoculations

All shoots died back 3–5 mm from the inoculation point. The inoculated fungi were isolated from the dead wood. The inoculated fungi were recovered from the inoculated shoots at all isolation dates (30, 60 and 90 days), except *T. harzianum* DsRed (TR1), which was not isolated at the first isolation date, after 30 days. On further investigation, it appeared that the *T. harzianum* DsRed isolate had lost its hygromycin resistance and could not grow on PDA amended with hygromycin. Isolations were thus carried out on non-amended PDA at 60 and 90-day isolation dates for all treatments with TR1, and fungal identity was confirmed by fluorescence.

Trichoderma harzianum (TR1) was isolated at a maximum depth of 15 mm and 10 mm from the xylem in cultivar Cabernet Sauvignon after 60 and 90 days respectively. In Sauvignon blanc, it was isolated at 10 mm at these two isolation dates. In the pith, TR1 was isolated only from the dead wound tissue in the two cultivars at 30 and 90 days. *Trichoderma harzianum* GFP (TG1) was isolated at the deepest level (15 mm) from the xylem tissue after 90 days in both cultivars. The

Table 1. The isolation frequency of fungi from the xylem and pith tissue at different depths 90 days after inoculation of Cabernet Sauvignon and Sauvignon blanc canes with either *Trichoderma harzianum* GFP (TG1) or *Phaeomoniella chlamydospora* DsRed (PchR1) and both fungi in dual inoculation.

Isolation depth (mm)	Grapevine pieces from where the fungi were isolated (%)[a]															
	Single inoculated canes								Dual inoculated canes							
	TG1				PchR1				TG1				PchR1			
	Cab. Sauv.[b]		Sauv. blanc[c]		Cab. Sauv		Sauv. blanc		Cab. Sauv.		Sauv. blanc		Cab. Sauv.		Sauv. blanc	
	Xylem	Pith	Xylem	Pith	Xylem	Pith	Xylem	Pith	Xylem	Pith	Xylem	Pith	Xylem	Pith	Xylem	Pith
Dead zone	75.00	33.33	75.00	66.67	41.67	0	66.67	0	100	33.33	91.67	0	0	0	8.33	0
0[d]	65.25	0	70.83	0	79.17	66.67	70.83	0	95.83	0	29.17	16.67	4.17	33.33	50	8.33
5	41.67	0	37.50	25	87.50	66.67	75	0	66.66	0	12.50	0	16.67	33.33	33.33	16.67
10	45.83	0	45.83	8.33	41.67	16.67	66.67	0	62.50	0	33.33	0	25	0	12.50	0
15	8.33	0	20.83	0	45.83	0	50.00	8.33	33.33	0	33.33	16.67	29.17	0	0	0
20	0	0	0	0	0	0	33.33	0	41.67	0	20.83	16.67	4.17	0	0	0
30	0	0	0	0	0	0	8.33	0	4.17	0	0	0	0	0	0	0
40	0	0	0	0	0	0	4.17	0	0	0	0	0	0	0	0	0

[a] Isolations were made up to a depth of 50 mm. Number of isolation pieces: dead tissue , 4 per cane=12 per treatment; xylem tissue, 8 per cane=24 per treatment; pith tissue, 4 per cane=12 per treatment.
[b] Cab. Sauv. –Cabernet Sauvignon.
[c] Sauv. blanc –Sauvignon blanc.
[d] 0, Interface of dead and live tissue.

Table 2. The isolation frequency of fungi from the xylem and pith tissue at different depths 90 days after inoculation of Cabernet Sauvignon and Sauvignon blanc canes with either *Trichoderma harzianum* DsRed (TR1) or *Eutypa lata* GFP (EL) and both fungi in dual inoculation.

Isolation depth (mm)	Grapevine pieces from where the fungi were isolated (%)[a]															
	Single inoculated canes								Dual inoculated canes							
	TR1				EL				TR1				EL			
	Cab. Sauv.[b]		Sauv. blanc[c]		Cab. Sauv.		Sauv. blanc		Cab. Sauv.		Sauv. blanc		Cab. Sauv.		Sauv. blanc	
	Xylem	Pith	Xylem	Pith	Xylem	Pith	Xylem	Pith	Xylem	Pith	Xylem	Pith	Xylem	Pith	Xylem	Pith
Dead zone	100	100	83.33	66.67	83.33	66.67	25	66.67	91.67	33.33	66.67	8.33	0	0	25	0
0[d]	100	0	62.25	0	83.33	83.33	79.17	75	41.67	100	20.83	83.33	45.83	0	29.17	0
5	62.50	0	58.33	0	41.66	58.33	50	75	50	100	25	50	33.33	0	0	0
10	8.33	0	0	0	33.33	83.33	58.33	25	25	100	20.83	100	50	0	0	0
15	0	0	0	0	0	41.67	8.33	0	33.33	100	29.17	66.67	25	0	0	0
20	0	0	0	0	0	0	4.17	8.33	41.67	58.33	25	66.67	25	0	0	0
30	0	0	0	0	0	0	0	0	25.00	25	25	50	0	0	0	0
40	0	0	0	0	0	0	0	0	0 .	0	0	0	0	0	0	0

[a, b, c, d] See Table 1.

bio-control agent was isolated from the pith of the dead die-back area at all isolation dates in both cultivars except Sauvignon blanc where it was isolated from the pith up to 5 mm deep after 90 days. The frequency of isolation of both *Trichoderma* isolates declined with depth and is shown in Tables 1 and 2 for the 90 day isolation date.

Phaeomoniella chlamydospora DsRed was rarely isolated from the pith in Sauvignon blanc. From the xylem, the pathogen was isolated at a similar depth (15 mm) at all isolation dates in Cabernet Sauvignon, while it was isolated up to a maximum depth of 40 mm after 90 days in Sauvignon blanc (Table 1). *Eutypa lata* GFP had a similar growth pattern in both cultivars and occurred in both the xylem and the pith after 30 and 60 days. However, after 90 days it was isolated up to 15 mm deep in Cabernet Sauvignon pith and up to 20 mm deep in Sauvignon blanc xylem and pith (Table 2).

Dual inoculations

The biological control agent and the pathogen were recovered from the inoculated shoots at all isolation dates. The fungi were also isolated from the dead wood of the die-back region. There was no difference in the isolation depths of TG1 and PchR1 between Treatment 5 and Treatment 7, and the results for these two treatments were combined. In both treatments, *T. harzianum* grew more deeply than *P. chlamydospora* in the wood. The maximum depth of isolation of *T. harzianum* at all isolation dates was greater in dual-inoculated canes than when *T. harzianum* was inoculated alone. *Trichoderma harzianum* GFP (TG1) was isolated up to 15 mm deep in the xylem at the first isolation date in both cultivars. After 90 days, TG1 in dual-inoculated shoots was isolated at maximum depths of 30 mm and 20 mm, in Cabernet Sauvignon and Sauvignon blanc respectively (Table 1). The growth of the pathogen was restricted in the presence of *Trichoderma*, but not completely eliminated. In Sauvignon blanc, *P. chlamydospora* was isolated to a maximum depth of 10 mm after 90 days in the presence of *Trichoderma* compared with a depth of 40 mm when it was inoculated alone. Although there was no decrease in the depth of isolation of *P. chlamydospora* after 90 days in Cabernet Sauvignon (Table 1), the frequency of isolation of the pathogen was much lower.

A similar pattern was observed with treatment 7 where TR1 was isolated more deeply than *E. lata*. *Trichoderma harzianum* TR1 was also isolated more deeply than when it was the inoculated alone. *Eutypa lata* GFP was isolated up to 5 mm deep after 30 days in Sauvignon blanc, but it only occurred at the interface of live and dead tissue after 90 days indicating that it had been suppressed by the bio-control agent. In one of the dual-inoculated canes of Cabernet Sauvignon, *E. lata* was isolated from the wood up to 20 mm deep. However, in this cane TR1 was not isolated, and the bio-control agent may well have failed to establish. *Trichoderma harzianum* TR1 also reduced the recovery rate of *E. lata* from the dual-inoculated canes. The isolation rates of TR1 and *E. lata* after 90 days in the two cultivars are shown in Table 2. *Trichoderma harzianum* TR1 grew extensively in the pith when dual inoculated with *E. lata* in both cultivars (Table 2). *Eutypa lata* GFP occurred in the pith when singly inoculated, while TR1 did not occur extensively in the pith when inoculated alone.

Microscopic examination of wood sections

Examination of fungi in wood tissue

Fungal hyphae of both *T. harzianum* isolates (TR1 and TG1) were not seen in the wood tissue. *Phaeomoniella chlamydospora* DsRed was the only fungus seen in the wood sections of inoculated canes. The hyphae of *P. chlamydospora* were seen fluorescing in the xylem vessels in all treatments in which the fungus was inoculated. Extensive growth of *P. chlamydospora* was seen after 90 days in sections from Treatment 3, where it was the only fungus inoculated (Figure 1A). The hyphae occurred in the vessel lumen (Figure 1A) and also grew along the walls of the xylem (Figure 1B). Although hyphae were mainly seen in the xylem vessels, they also occurred in the intercellular spaces (Figure 1C) and grew in vessels blocked by gels (Figures 1D and E). *Phaeomoniella chlamydospora* was also found in sections from canes where it was inoculated together with *T. harzianum*, however here, growth of the fungus was seen in only a few sections as compared to when it was the only fungus inoculated. No conidia or conidiophores of *P. chlamydospora* were found.

Eutypa lata mycelium was not seen fluorescing in the wood tissue. Fungal hyphae were, however, found in the pith and wood of sections of *Eutypa*-inoculated canes, but their identity could not be confirmed as they did not fluoresce when viewed under fluorescent light or confocal laser light.

Structural changes of the wood

Distinct structural differences in the wood tissue sections were seen after the second and third month. Cross-sections made from the point of natural wound healing showed no differences in the wood structure with any treatment including the sterile water treatment. The vessels at the

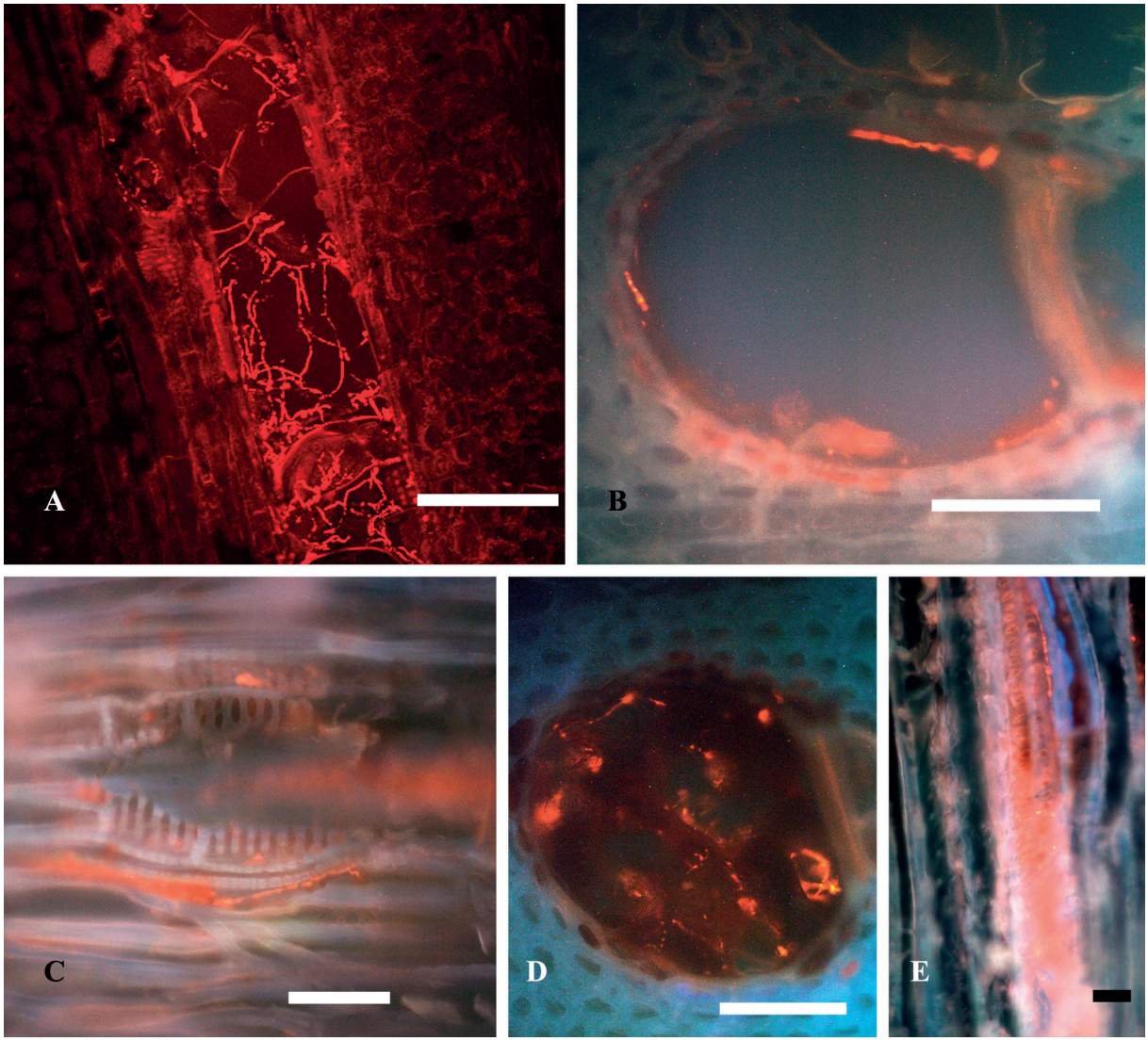

Figure 1. Growth of *Phaeomoniella chlamydospora* expressing the DsRed fluorescent protein in grapevine wood tissue after inoculation of one-year-old canes of Cabernet Sauvignon and Sauvignon blanc. A. Longitudinal section (l.s) of xylem vessel with an extensive mycelium growth in the lumen, 90 days after inoculation. B. Transverse section of xylem vessel with hyphae growing adhering to the vessel walls. C. Hyphae growing in intercellular space (l.s). D and E. Transverse (D) and longitudinal (E) sections of hyphae growing in xylem vessels occluded with gels. Scale bars for A, 100 μm; B–E, 50 μm.

interface of dead and live wood were blocked with tyloses and gums due to the natural wound healing process (Sun *et al*., 2006). Cells on the interface had a very deep red colour after the second and third month compared with cells after the first month.

Sections made from *T. harzianum*-inoculated canes did not differ structurally from the control canes treated with sterile water. A few vessels were blocked close to the scar but there was very little vessel blockage with increasing depth. Wood sections from control canes and *T. harzi-*

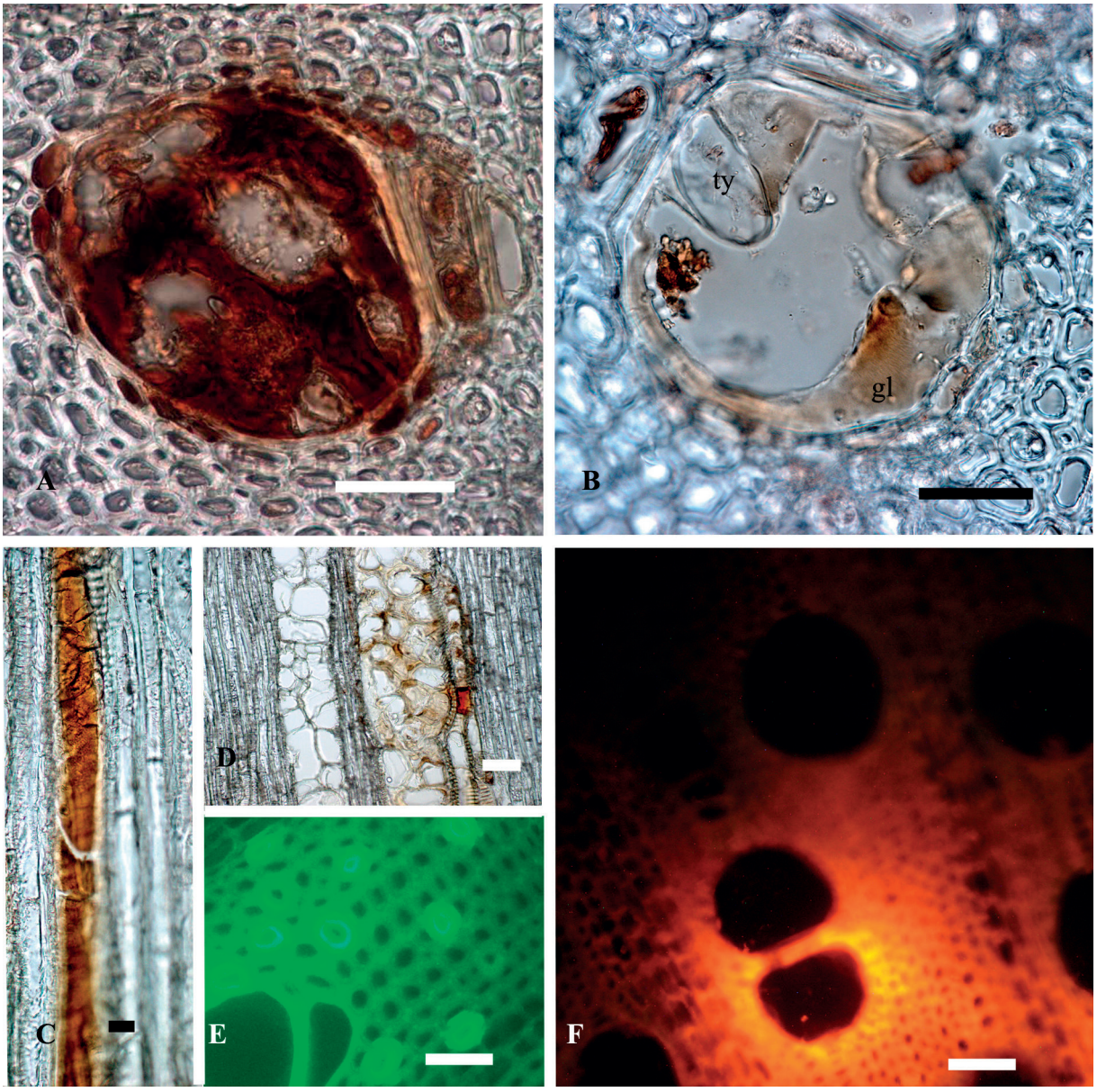

Figure 2. Grapevine wood tissue response to infection of one-year-old canes by *Phaeomoniella chlamydospora* and *Eutypa lata*. A. Transverse section (t.s) of xylem vessels occluded by gum (or gel) in *P. chlamydospora* inoculated cane. B. Tyloses (ty) and gum (gl) co-occurring in the same xylem vessel of a cane inoculated with *P. chlamydospora* (t.s). C and D. Full length vessel elements blocked by gum (C) and tyloses (D) in *P. chlamydospora* infected vessel. F. Increased fluorescence and wall thickening of fibre cells from *E. lata* infected canes. Scale bars, 50 µm.

anum inoculated canes had uniform auto-fluorescence when viewed under blue and green fluorescent light.

The presence of *P. chlamydospora* hyphae in the xylem vessels was associated with tyloses and gels (gum-like substances), which almost totally occluded the vessels. The occluded vessels varied in colour from light-brown to dark-brown to dark-brownish-red (Figure 2A). Cells around the infected vessels had a brown-red discolouration (Figure 2A) which is a host response to infection by the pathogen (Weiner and Liese, 1995). Tyloses and gums occurred together in the same vessel (Figure 2B). When viewed under green or blue fluorescent light, the tyloses had a brighter primary fluorescence (auto-fluorescence) than the surrounding cells. When tyloses occurred, the blockage extended along the whole xylem vessel (Figures 2C and D) and in some cases also continued into the neighbouring vessel. There was no difference in the host reactions observed between the two cultivars at the times assayed.

Eutypa lata also triggered a wood reaction causing vessel occlusion by both tyloses and gels. However, vessel occlusion was less extensive than that observed in sections cut from shoots inoculated with *P. chlamydospora*. Some xylem fibre cells also showed thick cell walls and brighter auto-fluorescence (Figure 2E) than surrounding cells, but without the reddening. This was most likely a result of cell wall thickening and increased lignification. Reddening of the area around some xylem vessels, which produced a bright auto-fluorescence in blue and green light (Figure 2F), was only seen in sections of *Eutypa*-inoculated canes.

Discussion

The transformed trunk disease pathogens and *T. harzianum* were able to colonise grapevine wood by invading through pruning wounds and this allowed their growth to be traced and monitored by selective re-isolation. By plating small wood pieces it was possible to isolate *T. harzianum* and the pathogens from dual inoculated canes at a given isolation depth and also to show the extent of colonisation of each. Harvey and Hunt (2006) took pruned canes, removed the bark, and split the stubs which they placed in 90-mm Petri dishes. They were able to determine the depth of penetration of *Trichoderma* sp., but they found that no other fungi other than *Trichoderma* sp. grew from the wood stubs. Using small pieces for isolation reduced the possibility of competition on the agar medium and enabled the organisms inoculated to be isolated. Furthermore, reporter-gene transformation of the fungi, specifically for hygromycin B resistance, enabled the fungi of interest among those occurring in the tissue to be selectively isolated except for TR1. The loss of hygromycin resistance in TR1 may have been a result of the mitotic instability of the DNA introduced and was therefore lost after sub-culturing (Moar *et al.*, 1998). Another probable reason is that, since *Trichoderma* species form heterokaryons (Moar *et al.*, 1998) some of the multiple genetically different nuclei in the cells carrying the selectable marker were lost in sub-culturing.

Trichoderma harzianum reduced the growth of the inoculated trunk disease pathogens in the canes but did not completely eliminate them. However, it should be noted that the pathogen inoculum levels applied in this study were much higher (10,000 conidia) than would naturally be present on a pruning wound in the vineyard. The biological control agent grew more deeply than the pathogens in dual inoculated canes and restricted pathogen growth compared with single-fungus inoculations. Previous studies have reported a reduction in the number of shoots infected with grapevine trunk disease pathogens after application of *Trichoderma* species to pruning wounds but they did not investigate the pattern of growth in the wood (Di Marco *et al.*, 2004; John *et al.*, 2005; Harvey and Hunt, 2006).

The deeper growth of *T. harzianum* in the dual-inoculated canes could be the result of the *Trichoderma* species recognising the presence of competing fungi. *Trichoderma* species secrete cell wall degrading chitinase and glucanase enzymes that break down chitin and β-glucan (Lorito, 1998; Howell, 2003; Woo and Lorito, 2007), polysaccharides that maintain the integrity of the fungal cell wall. Chitinase and glucanase are thought to be constitutively secreted and *Trichoderma* species sense the presence of a competing fungus by detecting the oligosaccharide products of the hydrolysis of cell-wall polymers (Harman

et al., 2004; Woo and Lorito, 2007; Vinale *et al.*, 2008). *In vitro* and molecular tests have shown that exochitinase and endochitinase genes of *Trichoderma* species are induced by digestion products of the fungal cell wall and colloidal chitin (Woo *et al.*, 2006; Woo and Lorito, 2007). The present study supports the conclusion that *T. harzianum* possesses a recognition and response mechanism to competing fungi.

Trichoderma harzianum was re-isolated more often from the xylem than the pith in single-inoculated canes, but the frequency of re-isolation from both xylem and pith was higher in dual inoculated canes. Recovery of the antagonist from the pith was also higher in canes in which the antagonist was dual-inoculated with *E. lata* than in canes where it was single- inoculated. In single-inoculated canes, *E. lata* was recovered at similar frequencies from the xylem and the pith, but *P. chlamydospora* had a preference for the xylem. The antagonist colonised the pith more frequently in the canes that had the pith colonising fungus *E. lata*, most likely because it sensed the presence of *E. lata* in the pith, compared with the canes inoculated with *P. chlamydospora*, which only rarely occurred in the pith; this further shows competitive sensing of *T. harzianum*.

The wound protecting effect of *Trichoderma* species can be due to its inhibitory interaction with the pathogens, or to its beneficial effects on the plant. The differences between the isolation depth of *T. harzianum* in the two cultivars could be due to differences in the susceptibility of the two cultivars to the fungus, or to natural variation. Di Marco and Osti, (2007) reported a cultivar-dependent response to *Trichoderma*-induced resistance, however, further studies are required to investigate how *Trichoderma* and single grapevine cultivars interact. In dual inoculation where *P. chlamydospora* was applied 24 hours before *T. harzianum*, the biological control agent still out-competed the pathogen and grew to similar depths as when it was applied before the pathogen. This could be due to the fast germination and growth of *T. harzianum* conidia as compared to the pathogen (Harman *et al.*, 2004). However, the fact that *T. harzianum* had similar growth, irrespective of whether it was inoculated before or after the pathogen did not necessarily show that *T. harzianum* had a curative effect on *P. chlamydospora*. *Trichoderma* species can grow in highly competitive environments such as the soil, and this makes them fungi of choice for bio-control purposes (Harman *et al.*, 2004). *Trichoderma* spp. have greater bio-control efficacy when more time is allowed to pass between treatment with *Trichoderma* and pathogen infection (Munkvold and Marois, 1993; John *et al.*, 2005). This is because the bio-control agent needs to establish itself and grow on the wound so that it can impair the pathogen's growth when it is applied later. Colonisation of a wound takes up space and nutrients that would otherwise be available for other wound colonising fungi, including pathogens. John *et al.* (2005) reported that *T. harzianum* was most effective against *E. lata* when it was applied at least 2 days before the pathogen was inoculated.

Phaeomoniella chlamydospora occurred in both the xylem and the pith, but it was less frequent in the pith indicating that it preferred the xylem. The pith of grapevines is made up of undifferentiated parenchyma that may contain starch. However, pith cells die as the wood ages and *P. chlamydospora* may not be able to colonise them at that point. Although the xylem is primarily dead, its function in transporting water and dissolved nutrients from the roots and its close association with paratracheal parenchyma cells (also called vessel-associated cells; Rudelle *et al.*, 2005) may allow sustained colonisation by *P. chlamydospora*. Nutrients in the xylem such as iron enhance the pathogenic capacity of *P. chlamydospora* (Osti and Di Marco, 2010), further explaining why the pathogen prefers the xylem. In both cultivars the pathogen was isolated less frequently as the depth from the inoculation point increased. The difference in the isolation depth of the cultivars could be attributed to a difference in the susceptibility of the cultivars to *P. chlamydospora* (Feliciano *et al.*, 2004). However, both the preference of *P. chlamydospora* for the xylem and the depth related difference in pathogen frequency between the cultivars would require further investigation and cannot be concluded from this study. *Eutypa lata* grew more slowly in inoculated canes than *P. chlamydospora*. The maximum isolation depth of *E. lata* found in this study (15 mm in Cabernet Sauvignon and 20 mm in Sauvignon blanc) was lower than that reported by John *et*

al. (2008) from mature wood, also inoculated with mycelial plugs (isolated from at least 70 mm from inoculation point after 12 weeks). The reason why growth was slower in this study is unclear and could include; the grapevine cultivars tested, their age, or differences in the virulence of the pathogen isolates. In the present study, *E. lata* occurred in both the xylem and the pith, whereas *P. chlamydospora* was isolated more from the xylem. Differences in the growth of *E. lata* and *P. chlamydospora* in one-year-old canes could help explain why Petri disease is more prevalent in nursery grapevines (young wood) and why *E. lata* occurs only in the vineyard (i.e. old wood).

Fluorescence in *P. chlamydospora* (PcR1) results from the expression of the DsRed fluorescent protein, which is an indication of active growth of the fungus in the wood. *Phaeomoniella chlamydospora* hyphae were mainly seen in the secondary and meta-xylem vessels, where they grew more extensively than in the intercellular spaces. Extensive hyphal growth was observed in the lumen and could contribute to obstruction of the xylem flow. Mycelium also adhered to the walls and can thus also block the xylem pits between the vessels, further blocking xylem transport. The loss of xylem function in Petri diseased vines was not only attributed to the physical blockage of vessels, but also to other mechanisms not yet fully understood (Edwards *et al*., 2007c). Although the vessel elements are dead, the plant controls transport in these vessels by means of vessel associated cells. Communication between the vessel elements and the vessel associated cells is through pits on the vessel walls (Fromard *et al*., 1995). Blockage of the pits could result in further damage than would be expected from the obstruction of solute movement alone. The path of invasion from the pruning wound surface is more likely to be through the xylem vessels, along which the hyphae spread down the vessels and laterally to invade the xylem parenchyma and the vessel-associated cells through pits and transfer apparatus. Cell-wall degrading enzymes have been isolated from *P. chlamydospora* (Marchi *et al*., 2001; Santos *et al*., 2006) and these could be employed in penetrating the pit membranes of the xylem vessels during the lateral growth of the fungal hyphae. Troccoli *et al*. (2001) reported that *P. chlamydospora* penetrated the vessel cell walls of one-year-old micropropagated grapevines. Enzymes involved in the breakdown of lignin (lignin and manganese peroxidases or laccases) do not seem to be produced by *P. chlamydospora* (Santos *et al*., 2006; Valtaud *et al*., 2009). Therefore, direct penetration of the vessel walls is less likely in mature wood, since the vessels are thicker and contain more lignin; consequently lateral growth is more likely to be through the vessel piths and transfer apparatus that connect the vessels with the neighbouring cells. However, *P. chlamydospora* can secrete pectic enzymes (Marchi *et al*., 2001; Santos *et al*., 2006) that degrade pectic compounds found in the middle lamella and primary cell walls, thereby enabling it to grow intercellularly.

Since the other transformed fungi were not detected in the wood tissue, the physical interaction between the pathogens and *Trichoderma* species could not be examined. Fungi expressing GFP or the *T. harzianum* DsRed were not found in the wood sections, most likely due to a weak fluorescent signal that was masked by the auto-fluorescence of the wood. Landi *et al*. (2009) also failed to see individual hyphae of a GFP-transformed *P. chlamydospora* isolate. These workers only found an overall stronger fluorescence in tissue which was confirmed with PCR testing for *P. chlamydospora*. The pathogenicity of the *P. chlamydospora* GFP was not affected as it produced the same grapevine wood response as did the *P. chlamydospora* PchR1.

Hyphae of *P. chlamydospora* were associated with occlusion of the xylem lumen and a dark brown-red discolouration of the cells around the xylem vessels. The pathogens in the wood tissue activated a host response as indicated by the formation of tyloses and gels, which then caused the xylem vessels to become occluded. Unlike the fungal mycelium, tyloses and gels can completely occlude the vessels and create physical barriers to xylem transport and fungal spread. Tyloses and gels are produced by parenchyma cells adjacent to the vessels and are formed by grapevines in response to infection or wounding (Stevenson *et al*., 2004; Sun *et al*., 2006, 2008). Tyloses and gels occurred together and sometimes in the same vessel. This finding is consistent with Sun *et al*. (2008) and Valtaud *et al*. (2009) who found that tyloses and gels were both formed as a conse-

quence of similar conditions (or stimuli).

A dark-brown to red discolouration was also seen in occluded vessels and resulted in brighter auto-fluorescence of the discoloured tissue. This is likely to be due to the accumulation of defence compounds in the infected xylem vessels, which creates a biochemical barrier to further growth of the fungus (Weiner and Liese, 1995; Pearce, 1996). Histochemical staining of infected wood revealed that phenolic compounds identified as condensed tannins accumulate in the tyloses, gels and the cells around the infected vessels (Amalfitano *et al.*, 2000; Del Rio *et al.*, 2001; Troccoli *et al.*, 2001). Amalfitano *et al.* (2000) extracted higher levels of the phenolic compounds from the brown-red wood than from healthy wood and identified these compounds as resveratrol and viniferins. These phenolic compounds have an inhibitory effect on *P. chlamydospora* and other grapevine pathogens (Adrian *et al.*, 1997; Santos *et al.*, 2006). Del Rio *et al.* (2004) found that phenolic compounds produced by grapevines inhibited the activity of the cell-wall degrading enzymes secreted by *Phaeoacremonium* species and *E. lata*. *Phaeomoniella chlamydospora* did not secrete the particular enzymes tested by Del Rio *et al.* (2004), but previous studies reported that this fungus is inhibited *in vitro* by leaf extracts containing high levels of phenolic compounds (Del Rio *et al.*, 2001). Although grapevine wood contains preformed phenolic compounds (Amalfitano *et al.*, 2000), the higher levels of these compounds in infected wood indicates that they have a role in protecting grapevines from invading pathogens. The higher levels either result from an increased bio-synthesis of these compounds or from the translocation of the preformed compounds to the sites of infection (Troccoli *et al.*, 2001).

Tyloses and gels were also seen in *E. lata* inoculated shoots but the response level was very low compared to that of *P. chlamydospora*. This may have been due to the fungus growing more slowly, since it occurred at shallower positions (10 mm) than *P. chlamydospora* (30 mm) after 90 days. The response of the grapevine to infection may also have caused the slower growth of *E. lata*, since the activity of *E. lata* enzymes is inhibited by the phenolic compounds of grapevine (Del Rio *et al.*, 2004).

The increase in the thickness of the cell wall and in the auto-fluorescence of the fibre cells of *E. lata* inoculated shoots, was most likely the result of increased lignification (Rudelle *et al.*, 2005). This increased lignification could be a response to lignin degradation by the pathogen. The wood response of *P. chlamydospora*-inoculated canes was different; here the vessels and cells around the infected vessel become more fluorescent. This finding may indicate that there are different mechanisms of plant response to different trunk pathogens, and hence the mode of pathogenesis of *P. chlamydospora* differs from that of *E. lata*. Del Rio *et al.* (2004) isolated lignin peroxidases from *E. lata* and Rudelle *et al.* (2005) reported that fungal hyphae occur in the walls of fibre cells. Lignin-degrading enzymes have not been isolated from *P. chlamydospora*, which therefore may not trigger such a grapevine response. Moreover, Troccoli *et al.* (2001) did not detect an increase in cell wall lignin in vine shoots inoculated with *P. chlamydospora*. It can thus be concluded that the increased fluorescence of fibre cell walls following inoculation with *E. lata* was most likely due to increased cell wall lignification and not to the phenolic compounds.

No visible morphological changes were detected in the wood of *T. harzianum* inoculated shoots. *Trichoderma* species can induce plant resistance (Harman *et al.*, 2004; Woo *et al.*, 2006) and in grapevine this may include an increase in the levels of phenolic compounds, or thickening of the cell-wall by lignification. A possible reason for the absence structural changes is that the increase in phenolic compounds and in lignification was a constitutive increase (expressed in all cells and therefore difficult to detect microscopically) and not a localised response (as in a response to infection).

In vitro studies have shown that the penetration of wood tissue by *Trichoderma* spp. is influenced by competing fungi (Woo *et al.*, 2006; Woo and Lorito, 2007). This was also shown in the present *in vivo* study in which *T. harzianum* grew at a greater depth in dual-inoculated canes than in single inoculated canes. However, it could not be ascertained whether this particular finding was caused by mycoparasitism or whether is merely due to competition for space and nutrients in the wood tissue, as *T. harzianum* could not be visualised in the vine tissue. It was also found that

T. harzianum restricted growth of *E. lata* and *P. chlamydospora* on grapevine pruning wounds. The response of grapevines to pathogens is dependent on the pathogen, for example *P. chlamydospora* caused more extensive vessel blockage by tyloses and gums and higher levels of phenolic compounds, than *E. lata*. *Eutypa lata* increased the lignification of fibre cells, which was not seen with *P. chlamydospora*. Since no obvious host response was observed when *Trichoderma* was inoculated alone, further studies are required to improve the understanding of the interaction between *Trichoderma* spp. and grapevine.

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