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Grapevine cultivar variation to pruning wound protection by *Trichoderma* species against trunk pathogens

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Summary. Using *Trichoderma* species to protect grapevine pruning wounds from trunk disease pathogens is one of the options available for managing grapevine trunk diseases. The growth and persistence of *Trichoderma* species in the pruning wound and the resulting control effect may depend on intrinsic wound factors and hence may vary between cultivars. Cultivar variability to pruning wound protection by *Trichoderma* species was evaluated in eight wine grape (Cabernet Sauvignon, Chardonnay, Chenin blanc, Colombar, Merlot, Pinotage, Sauvignon blanc and Shiraz) and four table grape (Prime, Red Globe, Thompson Seedless and Victoria) cultivars. Two strains of *Trichoderma atroviride* (USPP-T1 and USPP-T2) separately or in combination and Eco 77® a registered pruning wound biocontrol agent based on *T. harzianum*, were applied to fresh pruning wounds of spur-pruned wine grapevines and cane-pruned table grapevines. *Trichoderma* spp. and a variety of trunk pathogens, *Phaeomoniella chlamydospora* and species of *Phaeoacremonium*, *Phomopsis*, Botryosphaeriaceae and Diatrypaceae, were isolated from the pruning wounds eight months after treatment. Significant treatment × cultivar interactions (P<0.01) were found in the incidence of *Trichoderma* spp. in table and wine grapes. *Trichoderma* incidence varied greatly between cultivars and was less between *Trichoderma* treatments within the same cultivar. The highest *Trichoderma* incidence in wine grapes was found in Chenin blanc (71.4–82.5%) and in table grapes in Thompson Seedless (43.5–76.7%). In the remaining wine grape cultivars *Trichoderma* incidence varied between 20–50% while with all treatments in Chardonnay *Trichoderma* incidence was less than 24%. In table grapes *Trichoderma* incidence varied from 20-67% in the other cultivars. *Trichoderma* reduced the pathogen by between 10.3% in Chardonnay to 66.7% in Chenin blanc. *Trichoderma* incidence and pathogen reduction were significantly correlated in most cultivars (r> 0.50; P<0.05) though not in Chardonnay (r=0.37; P=0.11), Pinotage (r=-0.12; P=0.62), Sauvignon blanc (r=0.26; P=0.26) and Victoria (r=0.29; P=0.22). It was concluded that the wound protection effect of *Trichoderma* spp. is also dependent on the *Trichoderma*-grapevine interaction and is not only due to the suppressive effect of *Trichoderma* spp. on the pathogens.

Key words: *Phaeomoniella chlamydospora*, *Phaeoacremonium*, *Phomopsis*, Botryosphaeriaceae, Diatrypaceae.

Introduction

Pruning of grapevines, a cultural practice carried out every winter to maintain the balance between reproductive and vegetative growth, opens a window for infection of grapevines by wood inhabiting pathogens that cause grapevine decline. Grapevine trunk diseases include black dead arm, Eutypa dieback, Petri disease, esca and Phomopsis dieback. Symptoms include stunted growth, cankers, wood necrosis and premature grapevine decline (Munkvold *et al.*, 1994; van Niekerk *et al.*, 2005, 2006, 2010a). In addition to reducing yield and quality of grapes, these diseases increase costs and reduce the life of a vineyard (Munkvold *et al.*, 1994). Grapevine trunk diseases have been reported in most grapevine producing regions of the world and are becoming an increasingly important limiting factor threatening the long-term sustainability of grape and wine production.

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Eutypa dieback caused by *Eutypa lata* (syn. *E. armeniacae*) was considered the main trunk disease pathogen of grapevine (Moller and Kasimatis, 1978). More recently Petri disease, caused by *Phaeomoniella chlamydospora* and some *Phaeoacremonium* species, and esca, which is caused by the Petri disease pathogens plus wood rotting Basidiomycetes species have become more important (Mugnai *et al*., 1999; Fischer, 2006; Mostert *et al*., 2006a). Species of Botryosphaeriaceae and *Phomopsis* have recently also been found to be associated with grapevine cankers, and are considered to be more prevalent in some regions (Taylor *et al*., 2005; Van Niekerk *et al*., 2006; Úrbez-Torres *et al*., 2006, 2010). Recent studies have found that fungi of the family Diatrypaceae which include *Cryptovalsa ampelina* and species of *Eutypa*, *Eutypella*, *Diatrype* and *Diatrypella* are also involved in trunk diseases (Mostert *et al*., 2004; Trouillas and Gubler, 2004). Some of these fungi may originate in the nursery, but here infection rates are low and the higher occurrence in the field is caused by vineyard infections through wounds (Eskalen *et al*., 2007). Wounds are the major sites of infection, therefore, protection of grapevine pruning wounds from infection by trunk disease pathogens is important for vineyard sustainability.

Currently there are no reliable curative control measures, and prevention is the main way to control these diseases. Cultural control practices include pruning late in winter, not pruning in wet weather (Munkvold and Marois, 1995) and double pruning (Weber *et al*., 2007). Since the airborne inoculum of these pathogens has been trapped throughout the pruning period (Úrbez-Torres *et al*., 2010; van Niekerk *et al*. 2010b), the timing of pruning alone will not prevent pruning wound infections. Fungicide wound protection is effective but all fungicides are not equally effective against all trunk pathogens (Sosnowski *et al*., 2008; Rolshausen *et al*. 2010). When fungicides are applied to pruning wounds their effectiveness quickly deteriorates (Munkvold and Marois, 1995); however pruning wounds remain susceptible to infection for up to 16 weeks (Eskalen *et al*., 2007). A means to protect pruning wounds over the long term is therefore required.

Biocontrol agents based on *Trichoderma* species protect grapevine pruning wounds against trunk pathogens (Di Marco *et al*., 2004; John *et al*., 2008; Halleen *et al*., 2010; Kotze *et al*., 2011). Due to a heightened public concern for a safe environment, biocontrol strategies against wood decay fungi are being developed to replace or supplement chemical methods. Pruning wound protection by biocontrol agents can provide sustainable control comparable to fungicides, since the biocontrol agents grow in the wound and offer protection for longer until the wound is healed completely and is no longer susceptible to infection (Munkvold and Marois, 1993). *Trichoderma*-based biocontrol agents are among the few if not the only available/registered grapevine pruning wound protection products in Australia, New Zealand and South Africa (John *et al*., 2005; Sosnowski *et al*., 2008).

The suppressive effect of *Trichoderma* spp. on the pathogens could be due to the species themselves, or be the result of the *Trichoderma*-plant interaction, or be a synergy of these effects. *In vitro* tests have shown that the antagonistic properties of *Trichoderma* to grapevine trunk pathogens include mycoparasitism, antibiosis by volatile and non-volatile compounds and competition for nutrients and space (John *et al*., 2005; Kotze, 2011). *Trichoderma* spp. applied to nursery vines to control Petri disease pathogens reduced infection and also interacted physiologically with the grapevine, improving root development and resistance (Fourie *et al*., 2001; Di Marco *et al*., 2004; Fourie and Halleen, 2004, 2006). Di Marco and Osti (2007) found that *Trichoderma* sp. varied in reducing the streaks caused by *Phaeomoniella chlamydospora* in two grapevine rootstocks. This report indicates that cultivars may vary considerably in their response to *Trichoderma* species used in wound protection.

Two isolates of *T. atroviride* Karsten (USPP-T1 and USPP-T2), which were isolated from grapevines and also been shown to directly inhibit grapevine trunk disease pathogens *in vitro* and to reduce pathogen incidence in pruning wounds *in vivo* (Kotze *et al*., 2011). These isolates persisted in grapevine wood for up to eight months. The study also found cultivar × treatment interactions, indicating that there could be *Trichoderma*-grapevine interactions that affected the effectiveness of the *Trichoderma* isolates. The variation of four *Trichoderma* treatments in response to pruning wound treatment on twelve grapevine cultivars is reported in this study. This is important to under-

stand the mode of action of *Trichoderma* spp. and how these species are affected by different cultivars. It will also assist the development of effective biocontrol agents for protection of grapevine pruning wounds. The objective of this study was to evaluate the ability of *Trichoderma* spp. to colonise and protect pruning wounds from trunk pathogens on eight wine grape and four table grape cultivars.

Materials and methods

Source of isolates and inoculum preparation

Trichoderma atroviride strains USPP-T1 and USPP-T2 were obtained from the Stellenbosch University, Department of Plant Pathology culture collection accessions STE-U 6514 (USPP-T1) and STE-U 6515 (USPP-T2). Spore suspensions for field application were prepared from 7-day-old cultures growing on potato dextrose agar (PDA; Biolab, Wadeville, South Africa) in Petri dishes. The plates were flooded with sterile distilled water and the conidia dislodged with a glass rod, transferred to sterile bottles and the concentration adjusted to 10^6 conidia mL^{-1}. For the combination treatment, equal volumes of the two strains (10^6 conidia mL^{-1}) were thoroughly mixed to obtain a final a concentration of 10^6 conidia mL^{-1}. The registered pruning wound agent Eco-77®, based on *T. harzianum* Rifai (Plant Health Products, South Africa) was applied at the recommended rate of 0.5 g L^{-1} (results in 10^6 conidia mL^{-1}).

Conidia viability of all suspensions was determined by spread-plating one hundred µL of each suspension on PDA dishes and incubated at room temperature for 15 hours. Germination was evaluated on each Petri dish at 40× magnification. The percentage of germination was calculated as the number of conidia with germ tubes longer than the conidial diameter over the total number of conidia within the area of the stereomicroscope ocular, at five randomly chosen places on the Petri dish.

Field trial

The field trial was carried out from July 2008 until March 2009 on eight wine and four table grapevine cultivars. The wine grape cultivars Cabernet Sauvignon (16 years old), Chardonnay (14 years), Colombar (11 years), Merlot (8 years), Pinotage (10 years), Sauvignon blanc (10 years) and Shiraz (13 years) were located at ARC Infruitec-Nietvoorbij (Nietvoorbij Campus, Stellenbosch) and Chenin blanc (7 years) at a wine estate in Stellenbosch. The table grape cultivars Prime (9 years), Thompson Seedless (23 years) and Victoria (11 years) were located in Paarl and the cultivar Red Globe (12 years) in Wellington. The wine grapes were spur-pruned to three buds while the table grapes were cane pruned. All pruning wounds were therefore made in 1-year-old wood. Immediately after pruning, each pruning wound received one of five treatments. The establishment of the trial coincided with the normal pruning time for all the cultivars except Cabernet Sauvignon, which can be pruned later.

The treatments were: *Trichoderma atroviride* isolates USPP-T1, USPP-T2, the combination (USPP-T1+T2), Eco-77® (*T. harzianum*) and a sterile water control. The fresh pruning wounds were inoculated with 2 mL of each treatment with a hand spray. The field trial was laid out in a randomised complete block design with four blocks of ten vines each. There were five pruning wounds per grapevine and each pruning wound received a different treatment. Within blocks treatments were laid out in a 5×5 double latin square design to ensure that the treatments were evenly distributed on the vine arms. Pruning and treatment applications were carried out between 24 and 28 July 2008. Eight months after inoculation the treated pruning wounds were pruned off between the top and second node, and the stubs with the inoculated wound were immediately taken to the laboratory for fungal isolation.

Fungal isolations from pruning wounds

The stubs were surface sterilised by immersion in 70% ethanol for 30 seconds, in 3.5% sodium hypochlorite for one minute and finally in 70% ethanol for 30 seconds. Shoots were then aseptically split longitudinally and isolations were made from two positions, the wound scar interface (top isolation zone) and 10 mm below the first isolation (bottom isolation zone). From each isolation position, four wood tissue sections (0.5×1.0 mm), two from either side of the pith, were plated on one 90 mm Petri dish containing potato dextrose agar (PDA). Isolations were also made from the pith tissue at the top and bottom isolation positions. Two tissue sections, one from

the top and one from the bottom, were placed in one Petri dish each and incubated at 25°C for 4 weeks with sub-culturing where necessary. Fungal cultures were identified by their cultural and morphological characters as *Phaeomoniella chlamydospora* (Crous and Gams, 2000), and species of the Botryosphaeriaceae (van Niekerk *et al*., 2004), *Phaeoacremonium* (Mostert *et al*., 2006b), *Phomopsis* (van Niekerk *et al*., 2005) and *Trichoderma* (Gams and Bisset, 1998). Isolates that could not be identified morphologically were identified by molecular means.

Molecular identification of fungi

Unidentified fungi were hyphal tipped and grown on PDA. Genomic DNA was isolated from the mycelium as described by Lee and Taylor (1990). The primers ITS1 and ITS4 (White *et al.*, 1990) were used for amplification of the internal transcribed spacer region of the nuclear rRNA gene, consisting of the ITS1, the 5.8S rRNA gene and the ITS4 (White *et al*., 1990). PCR conditions were the same as described by Van Niekerk *et al*. (2004). PCR products were purified using a commercial kit (MSB® Spin PCRapace, Invetek, Berlin, Germany) according to the manufacturer's instructions. DNA sequence analysis was performed using the BigDye system (version 3.1 dye terminators, Applied Biosystem, California, USA) on an ABI 3130XL Genetic Analyzer at the Central Analytical Sequencing Facility at Stellenbosch University. Sequences were compared by blast analysis on the NCBI GenBank database to find the closest matches and hence the identity of the fungi.

Data analysis

The incidence of *Trichoderma* and of the pathogens were recorded as percentages of the total number of pruning wounds inoculated with each specific treatment. The frequency of fungal isolation was recorded as a percentage of the pieces of each wound. Pathogen incidence was recorded for each pathogen and for all pathogens. The incidence and severity data were subjected to analysis of variance (ANOVA) and Student's t-test for least significant differences (LSD) at 5% significance ($P<0.05$). As a first step, differences between wine and table grapes (grapevine type) in their response to the treatments between wine and table grapes (grapevine type) were sought by ANOVA. When a significant type × treatment interaction was found ($P<0.05$) the differences between cultivars (of wine and table grapes) were analysed separately for table and wine grapes. Likewise, when no type × treatment interaction was found ($P>0.05$), the analysis for the differences between cultivars was pooled regardless of type.

The percentage of reduction (P_r) of the pathogens with each treatment was calculated as $P_r=100 (P_c - P_t) / P_c$, in which P_c is the mean pathogen infection in the water control and P_t is the mean pathogen incidence in the treatment. Pearson's correlation coefficient and the significance of the correlation were calculated for the relationship between *Trichoderma* incidence and pathogen reduction within each cultivar. Analysis was performed with the SAS version 8.2 statistical software (SAS Institute Inc, Cary, NC, USA).

Results

Conidium viability of the treatment suspensions was confirmed and was at least 95% with all the treatments. *Trichoderma* colonised the wounds (0–82.5%) and was isolated 8 months after pruning. Trunk disease pathogens that were isolated were *Phaeomoniella chlamydospora*, species of *Phaeoacremonium*, of *Phomopsis* and of the families Botryosphaeriaceae and Diatrypaceae. *Phomopsis* species were most common (up to 69.4%), followed by Botryosphaeriaceae species (up to 43.6%), *Phaeomoniella chlamydospora* (up to 27.5%) and the Diatrypaceae species were least common (up to 6.3%). The Diatrypaceae isolates were identified through sequence comparison of which the most were *Cryptovalsa ampelina* (99% similarity with AY920391) and a few isolates were of an unknown species of *Eutypella* (96% similarity with FJ537071).

Incidence of *Trichoderma* species

Analysis of variance revealed a significant ($P<0.01$; ANOVA not shown) treatment × type interaction showing that wine and table grapes differed in their response to the treatments. Within each grapevine type (wine or table) a significant ($P<0.01$) cultivar × treatment interaction was found indicating that the grapevine cultivars of

each type also differed in their response to the *Trichoderma* treatments. The mean percentage incidence of *Trichoderma* spp. in each wine and table grape cultivar is shown in Table 1. The *Trichoderma* incidences varied between cultivars for some treatments and also between treatments within individual cultivars.

In wine grapes, *Trichoderma* incidence was significantly higher (P<0.05) in Chenin blanc with all *Trichoderma* treatments, while in Chardonnay the *Trichoderma* incidence was relatively low (Table 1). Chardonnay was also the only cultivar in which none of the *Trichoderma* treatments differed from the untreated control. The incidence of *Trichoderma* with the *T. harzianum* treatment (Eco-77) was significantly lower than at least one of the *T. atroviride* treatments in the cultivars Colombar and Shiraz, while with Pinotage Eco-77 led to a significantly higher *Trichoderma* incidence than USPP-T2 (Table 1). In Chardonnay, *Trichoderma* was not isolated when treated with USPP-T1+T2, and a higher *Trichoderma* incidence was recorded with the water control (10%).

In table grapes, the cultivar Thompson Seedless treated with USPP-T1+T2 had the highest *Trichoderma* incidence (76.7%) which however was not significantly (P≥0.05) different from the incidence with treatment USPP-T2 (72.6%) of that cultivar, nor from the incidence of *Trichoderma* in Red Globe treated with USPP-T1 (66.7%). *Trichoderma* spp. were also isolated in some cultivars' water control, but at low incidence. In all table grape cultivars, at least one of the *T. atroviride* treatments produced a significantly higher *Trichoderma* incidence than did the *T. harzianum* treatment (Eco-77) except for the cultivar Red Globe where all the *Trichoderma* treatments were similar (Table 1).

Table 1. Mean prevalence of *Trichoderma* spp. (%) isolated from pruning wounds of eight wine and four table grape cultivars eight months after the fresh pruning wounds were treated with a commercial *Trichoderma*-based product (Eco-77), two *Trichoderma atroviride* suspensions (USPP-T1 and USPP-T2) either separately or in combination (USPP-T1+T2), and water as a control.

Cultivar	Treatment (%)[a]				
	Eco-77	USPP-T1	USPP-T1+T2	USPP-T2	Water
Wine grapes[ii]					
Chenin blanc	71.4 a	75.0 a	76.9 a	82.5 a	0.0 j
Cabernet Sauvignon	27.5 e–g	35.0 c–f	35.0 c–f	30.0 e–g	0.0 j
Chardonnay	10.0 h–j	22.8 f–h	0.0 j	23.3 f–h	10.0 h–j
Colombar	17.5 g–i	28.3 e–g	48.9 b–d	23.6 f–h	0.0 j
Merlot	33.8 c–g	50.0 bc	33.3 d–g	29.2 e–g	0.0 j
Pinotage	48.3 b–d	42.5 b–e	53.8 b	27.5 e–g	7.8 h–j
Sauvignon blanc	35.0 c–f	42.5 b–e	35.0 c–f	30.0 e–g	5.0 ij
Shiraz	20.6 f–i	42.5 b–e	23.6 f–h	28.1e–g	0.0 j
Table grapes*					
Prime	21.4 hi	28.9 gh	45.0 d–g	44.7 d–g	5.0 ij
Red Globe	58.6 bcd	66.7 abc	49.7 cde	59.4 bcd	2.5 j
Thompson Seedless	46.6 def	43.5 d–g	76.7 a	72.6 ab	6.1ij
Victoria	20.6 hi	31.1 f–h	36.9 e–h	41.0 e–g	0.0 j

[a] For wine and table grapes separately, means followed by the same letter are not significantly different from each other (P>0.05; [ii] LSD=16.5; *LSD=17.2).

Pathogen reduction

Natural pathogen pressure varied greatly between cultivars, as was shown by the total pathogen incidence in the water control, which varied from 30.5–94.4% between cultivars (Table 2). Because of these variations, pathogen incidence alone could not be used to compare the efficacy of the *Trichoderma* treatments between cultivars. It was therefore normalised by computing the percent reduction in pathogen reduction.

The reduction in pathogen incidence with *Trichoderma* treatments was similar in both grapevine types (table and wine), as revealed by the lack of a treatment × type interaction (P=0.96; ANOVA not shown). Analysis of variance for differences in pathogen reduction between cultivars was thus pooled for all the cultivars regardless of type. No cultivar × treatment interaction was found for total pathogen reduction (P=0.20; ANOVA not shown), but there were significant differences between cultivars and between treatments (P<0.01). USPP-T2 and USPP-T1+T2 had the highest mean pathogen reduction levels (44.6% and 38.1%, respectively). The mean pathogen reduction for USPP-T1 (34.4%) was significantly lower than that for USPP-T2, while that of Eco-77 (27.3%) was significantly lower than that of USPP-T2 and USPP-T1+T2.

Among cultivars pathogen reduction varied from 10.3% in Chardonnay to 66.7% in Chenin blanc (Table 2). There was generally a positive correlation between *Trichoderma* incidence and pathogen reduction. Pruning wounds of Chenin blanc, (which were also the youngest vines used in the trials) were easily colonised by *Trichoderma* spp. and had a very high pathogen reduction. Pathogen reduction was lowest in Chardonnay (10.3%), Colombar (12.6%) and Sauvignon blanc (13.3%; Table 2), which also had a very high pathogen incidence. However, in Colombar there was a significant positive correlation (r=0.54; P=0.01) between *Trichoderma* incidence and pathogen reduction. This correlation was poorer and not significant in Chardonnay (r=0.37; P=0.11) and Sauvignon blanc (r=0.26; P=0.26). Most interestingly, a negative though not significant correlation was found in Pinotage (r=-0.12; P=0.62), which had a lower pathogen pressure than Chardonnay, Colombar and Sauvignon blanc, as estimated by the pathogen incidence in the water control. The correlation was also not significant with Victoria (r=0.29;

Table 2. Mean percentage of pathogen incidence in the water treatment, pathogen reduction achieved by *Trichoderma*-treatment of pruning wounds of 12 grapevine cultivars, eight months after treatment, and the Pearson's correlation coefficient of the relationship between *Trichoderma* incidence and pathogen reduction.

Cultivar	Pathogen incidence in water treatment (%)[a]	Pathogen reduction (%)[a]	Correlation coefficient (r)[b]	Correlation significance
Wine grapes				
Chenin blanc	30.6 g#	66.7 a*	0.88	<0.01
Cabernet Sauvignon	55.0 de	29.1 cd	0.62	<0.01
Chardonnay	94.4 a	10.3 e	0.37	0.11
Colombar	75.0 abc	12.6 e	0.54	0.01
Merlot	35.3 fg	41.2 bc	0.58	<0.01
Pinotage	40.8 fg	17.4 de	-0.12	0.62
Sauvignon blanc	82.5 ab	13.3 e	0.26	0.26
Shiraz	37.5 fg	39.7 bc	0.54	0.01
Table grapes				
Prime	47.2 d-g	21.0 de	0.70	<0.01
Red Globe	52.5 def	44.8 b	0.62	<0.01
Thomson Seedless	61.2 cd	31.7 bcd	0.55	0.01
Victoria	44.4 d-g	18.9 de	0.29	0.22

[a] Means followed by the same letter are not significantly different (P>0.05; #LSD=19.7; *LSD=14.6).
[b] Pearson's correlation coefficient for the relationship between *Trichoderma* incidence (Table 1) and the percentage pathogen reduction for treatments within a cultivar (results not shown).

P=0.22). Generally, *Trichoderma* and pathogen incidence were inversely correlated (results not shown), and the trend was similar to that mentioned above.

Extent of *Trichoderma* species colonisation in the wound

Tests for the normality and homogeneity of the variances revealed that the variances of the extent of *Trichoderma* colonisation data were heterogeneous and hence did not meet the assumptions of ANOVA. The data was transformed (weighted) by the reciprocal of the experimental error of the incidence for each pathogen and cultivar, and thus the final analysis was a weighted ANOVA (John and Quenouille, 1977).

The extent of fungal colonisation in the pruning wound, as assessed by the frequency of isolation, revealed certain patterns in the distribution of *Trichoderma* spp. in the wood. There was no significant type × isolation zone × treatment interaction (P=0.10), but a treatment × type interaction (P<0.01) was found between table and wine grapes and thus the analysis of variance for the differences in the extent of colonisation of *Trichoderma* spp. between cultivars was separated for grapevine types. Significant cultivar × treatment × isolation zone (P<0.01) and cultivar × treatment (P<0.01) interactions were found for extent of colonisation of *Trichoderma* spp. in cultivars of both table and wine grapes. These interactions were attributed to no or very low *Trichoderma* levels obtained from the water treatment, as well as to significant differences between cultivars. This cultivar effect, as well as a growth pattern of the biological control agent in the pruning wound tissue, was more simply demonstrated by significant (P<0.05) two-factor interactions (treatment × isolation zone, and cultivar × isolation zone) that were found in both table and wine grapes. *Trichoderma* occurred in both the xylem and the pith of both cultivar types, but in wine grapes, *Trichoderma* was more common in the xylem than in the pith in all treatments at the same level of isolation (Table 3). This is further shown by the extent of *Trichoderma* colonisation in each cultivar (Table 4): in all wine grape cultivars, except Chardonnay and Colombar, the extent of *Trichoderma* colonisation was higher in the xylem at the wound interface (top isolation zone) than in the pith. Chardonnay and Colombar also had low pathogen reduction by the *Trichoderma* treatments, which could have been a

Table 3. Mean percentage of wound colonisation by *Trichoderma* spp. isolated from wine and table grape pruning wounds eight months after the fresh pruning wounds were treated with a commercial *Trichoderma*-based product (Eco-77), two *Trichoderma atroviride* suspensions (USPP-T1 and USPP-T2) either separately or in combination (USPP-T1+T2) and water as a control. Isolations were made at two isolation positions, a top zone at the wound interface and a bottom zone, 1 cm below the top zone.

Isolation tissue/zone		Treatment [a]				
		Eco-77	USPP-T1	USPP-T1+T2	USPP-T2	Water
Wine grapes*						
Xylem	Top	7.8 bc	11.1a	9.6 ab	8.7 bc	0.4 g
	Bottom	4.5 e	6.9 dc	4.5 e	6.8 cd	0.7 g
Pith	Top	3.7 ef	3.7 ef	5.0 de	4.4 e	1.6 fg
	Bottom	0.9 g	1.2 g	1.7 fg	1.1 g	0.0 g
Table grapes#						
Xylem	Top	13.1 cde	14.3 bcd	16.9 abc	20.3 a	0.7 i
	Bottom	5.8 gh	6.2 hg	13.4 cde	13.0 cde	0.2 i
Pith	Top	9.0 efg	14.7 bcd	18.2 ab	18.3 ab	1.7 i
	Bottom	4.5 ghi	6.2 hg	12.2 def	7.7 gf	0.3 i

[a] For wine and table grapes separately, means followed by the same letter are not significantly different (P>0.05; *LSD=2.2; #LSD=2.0).

Table 4. Mean percentage wound colonisation by *Trichoderma* spp. isolated from pruning wounds of eight wine and four table grape cultivars eight months after the fresh pruning wounds were treated with a commercial *Trichoderma*-based product (Eco-77), two *Trichoderma atroviride* suspensions (USPP-T1 and USPP-T2) either separately or in combination (USPP-T1+T2), and water as a control. Isolations were made at two isolation positions, a top zone at the wound interface and a bottom zone, 1 cm below the top zone.

Cultivar	Percentage of colonisation in isolation zone (%)[a]			
	Xylem		Pith	
	Top	Bottom	Top	Bottom
Wine grapes[*]				
Chenin blanc	29.7 a	15.4 b	12.7 bc	3.0 hi
Cabernet Sauvignon	9.6 cd	3.5 f-i	1.8 hi	0.5 hi
Chardonnay	2.2 i	3.1 ghi	3.1 ghi	0.0 i
Colombar	7.3 de	4.1 e-h	12.1 bc	7.3 de
Merlot	13.7 b	7.4 de	0.8 hi	0.0 i
Pinotage	12.2 bc	9.2 cd	3.1 ghi	0.8 hi
Sauvignon blanc	9.6 cd	6.6 d-g	3.8 e-h	1.3 hi
Shiraz	6.8 def	2.9 hi	2.6 hi	0.5 hi
Table grapes[#]				
Prime	10.8c-g	6.1ghi	7.9f-i	2.7i
Red Globe	15.8cd	7.1f-i	37.4a	4.1i
Thomson Seedless	22.2b	16.0c	13.5c-e	10.7d-g
Victoria	9.7e-h	4.9hi	11.6c-f	7.3f-i

[a]For wine and table grapes separately, mean followed by the same letter are not significantly different (P>0.05; [*]LSD=2.2; [#]LSD =5.2).

result of poor *Trichoderma* colonisation. Interestingly, Pinotage had comparatively high levels of *Trichoderma*, an indication of good colonisation of the pruning wounds of this cultivar, but the reduction in the pathogen was very low. In table grapes, there was no consistent growth pattern for the biocontrol agent except in Thomson Seedless, where *Trichoderma* spp. were more common in the wood than in the pith, while in Red Globe the highest extent of colonisation was in the pith at the wound interface (Table 4).

Extent of pathogen colonisation in the wound

Test for the normality and the homogeneity of the variances revealed that the variances of the severity for the individual pathogens were heterogeneous and hence did not meet the assumptions of ANOVA. The data was transformed (weighted) by the reciprocal of the experimental error of the incidence for each pathogen and cultivar, and thus the final analysis was a weighted ANOVA (John and Quenouille, 1977).

The severity of the trunk pathogens isolated at higher incidences namely *Phaeomoniella chlamydospora*, species of Botryosphaeriaceae and of *Phomopsis* is reported. The effect of the grapevine type on pathogen severity was found only in *Phomopsis* as revealed by a type × isolation zone × treatment (P<0.01; ANOVA not shown) interaction. The cultivar effect was also significant for the three pathogens as shown by a cultivar × isolation zone (P<0.01; ANOVA not shown) interactions found in the analysis of variance. These interactions can be ascribed to the different susceptibility of the cultivars to the pathogens, but unfortunately cannot be normalised for all vineyards since various other factors may have affected the level of natural infection recorded (vine age, microclimate, natural inoculum levels and timing of infection), which could not be controlled and hence are not discussed. However, since all vineyards received similar treatments, the treatment × isolation zone interactions, which would indicate the spatial growth patterns of the pathogens *in plan-*

Table 5. Mean percentage of wound colonisation by *Phaeomoniella chlamydospora* in grapevine pruning wounds of wine and table grapes treated with *Trichoderma* spp. Isolations were made at two isolation positions, a top zone at the wound interface and a bottom zone, 1 cm below the top zone.

Isolation tissue/zone		Percentage of colonisation with treatment (%)[a]				
		Eco-77	USPP-T1	USPP-T2	USPP-T1+T2	Water
Xylem	Top	0.3 b	0.4 b	0.2 b	1.3 a	1.1 a
	Bottom	0.2 b	0.4 b	0.0 b	0.1 b	0.5 b
Pith	Top	0.2 b	0.0 b	0.1 b	0.2 b	0.4 b
	Bottom	0.1 b	0.4 b	0.0 b	0.00 b	0.2 b

[a]Values followed by the same letter are not significantly different (P>0.05; LSD=0.55).

ta, are comparable. The treatment × isolation zone interaction was significant for *P. chlamydospora* (P=0.06; ANOVA not shown), highly significant for *Phomopsis* (P<0.01) and not significant for Botryosphaeriaceae species (P=0.75).

All the pathogens generally occurred more frequent at the wound interface than at the bottom position. *Phaeomoniella chlamydospora* grew more extensively in the xylem than in the pith in most cultivars except Chardonnay, while *P. chlamydospora* occurred more extensively in the bottom pith zone than in the xylem with the water treatment (results not shown). As regards the mean percentage severity of *P. chlamydospora*, this fungus grew significantly more in the xylem than in the pith, with the water treatment and the USPP-T1+T2 treatment (Table 5). *Trichoderma* treatments significantly reduced the severity of *P.*

Table 6. Mean percentage of wound colonisation by *Phomopsis* species in grapevine pruning wounds of table grapes treated with *Trichoderma* spp. Isolations were made at two isolation positions, a top zone at the wound interface and a bottom zone, 1 cm below the top zone.

Isolation tissue/zone		Percentage of colonisation with treatment (%)[a]				
		Eco-77	USPP-T1	USPP-T2	USPP-T1+T2	Water
Wine grapes*						
Xylem	Top	1.1 d-g	1.2 c-g	0.7 fg	0.6 fg	2.4 b-d
	Bottom	0.4 fg	0.2 g	0.2 g	0.4 fg	0.4 fg
Pith	Top	2.1 b-e	2.3 b-d	2.0 b-e	2.5 bc	7.9 a
	Bottom	1.6 b-f	0.9 e-g	1.2 c-g	1.5 b-g	2.7 b
Table grapes#						
Xylem	Top	4.5 b-d	5.5 b	2.5 c-h	3.4 b-g	4.9 bc
	Bottom	0.7 h	0.9 g-h	2.9 c-h	0.5 h	2.3 d-h
Pith	Top	8.2 a	3.6 b-f	4.1 b-e	2.7 c-h	5.7 ab
	Bottom	1.3 f-h	2.5 c-h	1.6 e-h	1.3 f-h	3.3 b–g

[a]For wine and table grapes separately, means followed by the same letter are not significantly different (P>0.05; *LSD =1.4; #LSD=2.5).

chlamydospora in the xylem with all treatments except USPP-T1+T2. No significant differences were seen in the pith.

Due to significant ($P<0.01$) type × isolation zone × treatment interactions for *Phomopsis*, the mean severity differed between the wine and table grape cultivars (Table 6). *Phomopsis* severity was slightly lower in the wine grapes than in table grapes. The growth pattern of *Phomopsis* in the water (control) treatment showed that it was significantly more common in the pith than in the xylem tissue of wine grapes. The same pattern was also seen in the *Trichoderma* treatments. However, *Phomopsis* severity in the pith (top) was significantly reduced with all *Trichoderma* treatments for wine grapes (Table 6). In table grapes, on the other hand, none of the *Trichoderma* treatments significantly or consistently reduced the severity of *Phomopsis* as compared with the water control.

The severity of the Botryosphaeriaceae also revealed growth patterns similar to those of *Phomopsis*. The Botryosphaeriaceae occurred more extensively in the pith than xylem. However, the severity of Botryosphaeriaceae species in the pith of some cultivars was significantly higher with the *Trichoderma* treatments as compared with the water control (results not shown).

Discussion

Trichoderma harzianum in the commercial product (Eco-77) and the two strains of *T. atroviride* (USPP-T1 and USPP-T2) applied as spore suspensions persisted in the pruning wounds for 8 months on all the tested grapevine cultivars. In most cultivars, *Trichoderma* treatment reduced the incidence and severity of grapevine trunk pathogens. A positive correlation between *Trichoderma* spp. and pathogen reduction in most of the cultivars further showed that the presence and persistence of the biocontrol agent in the pruning wounds reduced pathogen infection.

Variations in the incidence of *Trichoderma* spp. among cultivars indicated that the cultivar affected the colonisation and persistence of *Trichoderma* in the pruning wound. Chenin blanc had the highest pathogen reduction, which was attributable to a high *Trichoderma* incidence with all treatments; which in turn is likely to have been due to the young age of this cultivar (7 years). This shows the importance of grapevine age in *Trichoderma* spp. wound colonisation and also the efficacy of the *Trichoderma* biocontrol agents against trunk pathogens. Poor colonisation and establishment in the other cultivars could have been due to intrinsic wound factors, such as the availability of nutrients and the pH of the substratum (Lewisohn *et al*., 1992), which affect the growth of the biocontrol agent. Factors that affect the host resistance and metabolic state of the host also affect the colonisation of the wounds by fungi and may affect the biological efficacy of *Trichoderma* spp. on different cultivars. Chapuis *et al*. (1998) showed that colonisation of pruning wounds by *Eutypa lata* was higher in the dormant season than in the late dormant season, when the vines were becoming metabolically active. There are currently no reports on how metabolic state of grapevines affects susceptibility of wounds to colonisation by *Trichoderma* spp.

The establishment and persistence of *Trichoderma* differed between *Trichoderma* species and strains, as shown by differences in *Trichoderma* prevalence between treatments within cultivars. The efficacy of the biocontrol agents, as shown by the percentage of pathogen reduction varied widely between the *T. harzianum* treatment and the *T. atroviride* strains, and less widely between the *T. atroviride* strains. The *T. atroviride* strains showed a lack of synergy in reducing the pathogen when they were applied together in the combination treatment, although the combined treatment often had a greater pathogen reduction than when each of the strains was applied separately. John *et al*. (2008) demonstrated the advantage of mixing *Trichoderma* biocontrol species or strains in Trichoseal® / Vinevax®, a *Trichoderma* product that is based on seven strains. Despite the considerable differences between *T. harzianum* and the *T. atroviride* treatments, a mixture of these species may protect wounds better and more consistently.

Trichoderma incidence was generally positively correlated with pathogen control (or negatively with pathogen incidence). This shows that the persistence of *Trichoderma* spp. in the pruning wound is essential for protection from infection. Unlike chemical fungicides, biocontrol agents must grow and proliferate to remain active against their target pathogens (Lo *et al*., 1998). *Trichoderma* spp. markedly reduced pathogen incidence when the

pathogen pressure was relatively low. Biocontrol agents are generally expected to perform poorly under high pathogen pressure (Mckenzie *et al*., 1991; Utkheder and Mathur, 2002). In cultivars that had low pathogen reduction but a significant positive correlation between *Trichoderma* incidence and pathogen reduction, such as Colombar and Prime, the implication is that higher deposition and establishment of *Trichoderma* spp. would result in better wound protection. *Trichoderma* deposition and establishment may be improved with the appropriate formulation and application of the biocontrol agent in the field (Schisler *et al*., 2004; Schubert *et al*., 2008).

Cultivar-specific *Trichoderma* interactions in the control of the wound pathogens cannot be ruled out, in view of the correlations that have been found between *Trichoderma* and the percentage pathogen reduction. Poor and non-significant (P>0.05) correlation found in Chardonnay, Pinotage, Sauvignon blanc and Victoria, may indicate that the *Trichoderma* isolates do not reduce pathogen numbers in these cultivars. However, the low reduction in pathogen by *Trichoderma* spp. in Chardonnay and Sauvignon blanc may have been caused by high pathogen pressure, in which biocontrol agents are less effective (Mckenzie *et al*., 1991; Utkheder and Mathur, 2002). In Pinotage, both the incidence of *Trichoderma* spp. and their extent of colonization were high, which indicates that it managed to become established and to persist in the pruning wounds of the cultivar, and yet pathogen reduction was poor. Since the *Trichoderma* spp. treatments reduced pathogen incidence under moderate pathogen pressure, the lack of a significant effect in Pinotage provides evidence that the *Trichoderma*-Pinotage interaction in the pruning wounds affected the degree of wound protection. A comparison of pathogen reduction in Pinotage and Shiraz, showed that the incidence of the pathogen in the water control and that of *Trichoderma* with all *Trichoderma* treatments were much the same, but the incidence of the biocontrol agent and the reduction in the pathogen were significantly correlated only for Shiraz. Similarly, the table grape cultivars Victoria and Prime had comparable incidences of *Trichoderma*, but the incidence of *Trichoderma* and the reduction in the pathogen were correlated only in Prime. This further demonstrates that cultivar-specific *Trichoderma*-grapevine interactions are probable.

In wine grapes, the more extensive growth of *Trichoderma* spp. in the xylem than in the pith could explain the high incidence of Botryosphaeriaceae and *Phomopsis* species, which grew more extensively in the pith than the xylem tissue of both the *Trichoderma* treated wounds and non-treated controls. In an earlier histological study, it was also found that *Trichoderma* grew more extensively in the xylem than in the pith (Mutawila *et al*., 2011). Pith tissue in grapevine canes consists of parenchyma cells that die as the cane gets older and hence cannot resist fungal colonisation. The pith in young canes contains starch granules whose function in food storage is not well understood (Mullins *et al*., 1992), and the starch may still be available as a nutrient in the dead pith of old canes.

Species of Botryosphaeriaceae and *Phomopsis* were isolated at high incidences in all vineyards, showing their increasing prevalence in grapevine growing regions worldwide (Taylor *et al*., 2005; Úrbez-Torres *et al*., 2006, 2010). Botryosphaeriaceae species have a wide host range, however, the virulence of individual species varies on different hosts (Rumbos, 2005). The role of endophytic Botryosphaeriaceae spp. remains unknown. Diatrypaceae species were also isolated from the pruning wounds in the current study but their incidence was very low. The most common species found, *Cryptovalsa ampelina*, has been isolated from grapevines in South Africa, Australia and Spain (Mostert *et al.,* 2004; Luque *et al*., 2005). The virulence of this fungus on grapevine was low. In California, Diatrypaceae species have been isolated from grapevine cankers and from ornamental and native plant species found growing close to vineyards (Trouillas *et al*., 2010). Little is known about the pathogenicity of the lesser known Diatrypaceae species occurring on grapevines, such as *Eutypella*; this topic needs further investigation.

This study shows that the establishment and persistence of *Trichoderma* species and strains in pruning wounds and their protective effect may vary between grapevine cultivars. Factors that could have contributed to this variation include the age of the cultivars, the environmental conditions, and disease pressure in individual vineyards. The main implication of the study is that a *Trichoderma* strain effective in one cultivar

cannot be consequentially expected to be equally effective in other cultivars. These findings, however, need to be verified on different cultivars of the same age, growing in the same location or in a controlled environment (*i.e*. glass house) and with the same pathogen inoculum pressure.

The persistence of *Trichoderma* spp. rather than its mere temporary establishment, on pruning wounds will provide sustained wound protection. *Trichoderma* spp. have several modes of action, one of which, competitive exclusion, is important, and this would require *Trichoderma* to persist in the wood. Knowledge of the factors affecting the establishment and the persistence of the biocontrol agent on and in the pruning wound would make better screening and selection of potential agents possible. A complete understanding of the biology and ecology of the biocontrol agent in the pruning wound is critical for more predictable, consistent and successful wound protection.

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