Phytopathol. Mediterr. (2011) 50 (Supplement), S247–S263

Evaluation of biocontrol agents for grapevine pruning wound protection against trunk pathogen infection

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Summary. Trunk diseases of grapevine are caused by numerous pathogens, including *Eutypa lata*, *Phaeomoniella chlamydospora*, and species of Botryosphaeriaceae (incl. *Botryosphaeria* and aggregate genera), *Phomopsis* and *Phaeoacremonium*. Since infections occur mainly through pruning wounds, that have been shown by previous research to stay susceptible for up to 16 weeks after pruning, long-term pruning wound protection is required for prevention of infection. This study evaluated several biocontrol agents against a range of trunk disease pathogens in dual plate laboratory trials to determine macroscopic and microscopic interactions. The biocontrol agents had a substantial effect on all the pathogens, with a wide range of macroscopic and microscopic interactions observed. The best performing biocontrol agents were tested in two field trials. Fresh pruning wounds were treated with benomyl, *Trichoderma* products (Biotricho®, Vinevax® and ECO 77®) and isolates (USPP-T1 and -T2, identified as *T. atroviride*) and *Bacillus subtilis*. Seven days after treatment the pruning wounds were inoculated by spraying with spore suspensions of *Neofusicoccum australe*, *N. parvum*, *Diplodia seriata*, *Lasiodiplodia theobromae*, *Eutypa lata*, *Phaeomoniella chlamydospora* or *Phomopsis viticola*. Eight months after inoculation, the treatments were evaluated by isolation onto potato dextrose agar. The efficacy of the biocontrol agents was in most cases similar or superior to that observed for benomyl. Isolate USPP-T1, in particular, was very effective, reducing incidence of *Ph. viticola*, *E. lata*, *Pa. chlamydospora*, *N. australe*, *N. parvum*, *D. seriata* and *L. theobromae* by 69, 76, 77, 78, 80, 85 and 92%, respectively. This is the first report of biological protection of grapevine pruning wounds against this group of grapevine trunk disease pathogens.

Key words: *Trichoderma*, *Eutypa*, Esca, Petri disease, *Phomopsis*, Botryosphaeriaceae.

Introduction

Trunk diseases lead to premature decline and dieback of grapevine and are caused by a complex of pathogens (van Niekerk *et al.*, 2011a), including *Eutypa lata* (Moller *et al.*, 1968) and *Phaeomoniella chlamydospora* (Mugnai *et al.*, 1999), species of Botryosphaeriaceae (incl. *Botryosphaeria*, *Neofusicoccum* and *Lasiodiplodia*) (van Niekerk *et al.*, 2004; Crous *et al.*, 2006), *Phomopsis* (van Niekerk *et al.*, 2005) and *Phaeoacremonium* (Mostert *et al.*, 2006). Eutypa dieback, black dead arm, esca, Petri disease and Phomopsis dead arm are grapevine trunk diseases of great economic importance in the wine and grape industries across the world, primarily due to premature decline and dieback of vines (Munkvold *et al.*, 1994; Gubler *et al.,* 2005). Growers also suffer yield loss and poor wine quality as a result of uneven ripening of berries, as caused by *E. lata* (Wicks and Davies, 1999), or delayed ripening and lower sugar content of grapes on vines affected by esca disease (Mugnai *et al.*, 1999). Economic losses in the Stellenbosch region of South Africa due to Eutypa dieback alone has been estimated at ZAR 1570 ha^{-1} in Cabernet Sauvignon vineyards (van Niekerk *et al.,* 2003).

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As pruning wounds have been shown to be the main infection portal for grapevine trunk pathogens (Lehoczky, 1974; Ferreira *et al*., 1989; Adalat *et al*., 2000; van Niekerk *et al*., 2011a, 2011b), it is important to protect pruning wounds against the combined pathogen complex. However, researchers have commonly focussed mainly on protecting pruning wounds against *E. lata* infection by using various fungicides and/or biological control agents (Moller and Kasimatis, 1980; Ferreira *et al*., 1991; Munkvold and Marois, 1993a, b; John *et al*., 2005; Halleen *et al*., 2010). Chemicals shown by *in vitro* and *in vivo* studies to be effective against *E. lata* include benomyl (Carter and Price, 1974; Pearson, 1982; Munkvold and Marois, 1993b; Ramsdell, 1995), carbendazim (Sosnowski *et al*., 2008) and flusilazole (Munkvold and Marois, 1993b), as well as the application of boric acid in a paste form to pruning wounds (Rolshausen and Gubler, 2005). Several species of Botryosphaeriaceae have been found to be most sensitive to benomyl and tebuconazole (Bester *et al*., 2007). Esca, on the other hand, has traditionally been controlled with whole-vine sprays with sodium arsenite (Mugnai *et al*., 1999). Recent studies have recommended drenching of propagation material with a wide-spectrum fungicide, such as captan, iprodione or 8-hydroxyquinaline sulfate, to pro-actively control Petri disease (Mugnai *et al*., 1999; Fourie and Halleen, 2004). In the case of Phomopsis dead arm disease, early-season sprays with copper oxychloride, copper oxychloride/sulphur, copper sulphate/lime, folpet, fosetyl-Al + mancozeb, probineb, sulphur and strobilurin are used (Mostert and Crous, 2000).

Research has shown that pruning wounds stay susceptible to *Ph. viticola* and Botryosphaeriaceae spp. infection for at least 3 weeks (van Niekerk *et al*., 2011b), while infection of pruning wounds by *Diplodia seriata*, *Pa. chlamydospora* and *Pm. aleophilum* could still occur 16 weeks after pruning (Eskalen *et al*., 2007; Serra *et al*., 2008). Moreover, Price and Carter (1975) demonstrated a significant reduction in chemical protection of apricot pruning wounds over time, and Munkvold and Marois (1993a) observed a decrease in efficiency of benomyl when grapevine pruning wounds were inoculated with *E. lata* 2 weeks after treatment. Chemicals should also not be applied during or before rains as it was hypothesized that rain washed away chemical residues from pruning wounds (Munkvold and Marois, 1993a).

The above findings question the ability of fungicides to provide the required long-term protection of pruning wounds. Moreover, the availability of airborne inoculum was demonstrated throughout the grapevine pruning period and the period of wound susceptibility (Larignon and Dubos, 2000; Eskalen and Gubler, 2001; van Niekerk *et al*., 2010). Biological control agents offer the potential to provide long-term protection of pruning wounds. This can be supported by studies that have shown that *Fusarium lateritium* (Carter and Price, 1974) and *Trichoderma* (Hunt *et al*., 2001) were isolated from pruning wounds 15 weeks and 8 months, respectively, after treating the wounds. According to these studies and others such as John *et al*. (2004), *Trichoderma* isolates produce volatile as well as non-volatile products *in vitro* that inhibit *E. lata* leading to *in vitro* and *in vivo* inhibition. *Trichoderma* based treatments have protected pruning wounds against *E. lata* with a 67% reduction of the pathogen in pruning wounds, when the pathogen was inoculated 1 day after *Trichoderma* treatment (John *et al*., 2005).

Several species of bacteria have been shown to have antagonistic activity towards other fungi. An isolate of *Bacillus subtilis*, taken from the pruning wound of a grapevine with symptoms of Eutypa dieback, inhibited mycelial growth of *E. lata* by 88% on culture medium and suppressed the incidence of the pathogen by 100% in pruning wounds (Ferreira *et al*., 1991). A later study by Schmidt *et al*. (2001), showed that transposon mutants of *Erwinia herbicola* completely inhibited growth of *E. lata* on autoclaved grape wood.

Collectively, these studies have demonstrated the potential of antagonistic organisms to protect grapevine pruning wounds. However, most of these studies focussed almost exclusively on *E. lata*, and to a large extent ignored the other pathogens in the grapevine trunk disease complex. The aim of this study was to identify, through *in vitro* and *in vivo* trials, biological control agents that provide protection of pruning wounds against infection by *E. lata* and other important pathogens contributing to the grapevine trunk disease complex.

Table 1. Biocontrol agents and pathogens used in *in vitro* evaluation of potential biocontrol agents against trunk disease pathogens.

Organism	Strain number	Code (Table 2)	Origin
Biocontrol agents			
Trichoderma harzianum	AG 2	AG 2	Vinevax, Agrimm Technologies Ltd, NZ
T. harzianum	AG 11	AG 11	Vinevax, Agrimm Technologies Ltd, NZ
T. harzianum	AGSS 28	AGSS 28	Vinevax, Agrimm Technologies Ltd, NZ
T. harzianum	Biotricho	Biotricho	Agro-Organics (PTY) Ltd., RSA
T. harzianum	ECO 77	ECO 77	Eco 77, Plant Health Products (PTY) Ltd., RSA
T. atroviride	AG 3	AG 3	Vinevax, Agrimm Technologies Ltd, NZ
T. atroviride	AG 5	AG 5	Vinevax, Agrimm Technologies Ltd, NZ
T. atroviride	AG 8	AG 8	Vinevax, Agrimm Technologies Ltd, NZ
Trichoderma spp. 1	STE-U 6514	USPP-T1	Department of Plant Pathology, Stellenbosch University, RSA[a]
Trichoderma spp. 2	STE-U 6515	USPP-T2	Department of Plant Pathology, Stellenbosch University, RSA[a]
Bacillus subtilis	EE 1/10	B.subt.	Nietvoorbij, Stellenbosch, RSA[a]
Pathogens			
Neofusicoccum australe	STE-U 4416		Department of Plant Pathology, Stellenbosch University, RSA[a]
Diplodia seriata	STE-U 4440		Department of Plant Pathology, Stellenbosch University, RSA[a]
Neofusicoccum parvum	STE-U 4589		Department of Plant Pathology, Stellenbosch University, RSA[a]
Lasiodiplodia theobromae	STE-U 4419		Department of Plant Pathology, Stellenbosch University, RSA
Eutypa lata	STE-U 6513		Department of Plant Pathology, Stellenbosch University, RSA[a]
Phaeoacremonium aleophilum	STE-U 5939		Department of Plant Pathology, Stellenbosch University, RSA[a]
Phaeomoniella chlamydospora	STE-U 6505		Department of Plant Pathology, Stellenbosch University, RSA[a]
Phomopsis viticola	STE-U 5602		Department of Plant Pathology, Stellenbosch University, RSA[a]

[a] Isolated from *Vitis vinifera* in the Western Cape Province, South Africa.

Materials and methods

Potential biocontrol agents and selected products

The biocontrol agents used were *Bacillus subtilis* (Ferreira *et al*., 1991), three commercial formulations of *Trichoderma* and two unknown *Trichoderma* isolates, USPP-T1 and USPP-T2 (Table 1). USPP-T1 and USPP-T2 were co-isolated with trunk disease pathogens from cv. Chenin blanc grapevine pruning wounds (van Niekerk, 2008) and demonstrated some antagonism against some

of these pathogens (J.M. van Niekerk, unpublished results). USPP-T1 and USPP-T2 were identified as *T. atroviride* using standard molecular procedures (White *et al*., 1990; O'Donnell *et al*., 1998; Samuels *et al*., 2002) and the sequences were deposited in GenBank (Accession numbers for ITS: FJ232696, FJ232697; EF: FJ232698, FJ232699). The commercial products tested were ECO 77® (Plant Health Products [PTY] Ltd, Nottingham Road, South Africa; 2×10^9 spores g^{-1}), Biotricho® (Agro-Organics [PTY] Ltd, Strand, South Africa; 1.2×10^7 spores g^{-1}) and Vinevax® (Agrimm Technologies Ltd, Christchurch, New Zealand; 5×10^8 spores g^{-1}). The latter product comprised seven strains (six *Trichoderma harzianum* and one *T. atroviride*).

***In vitro* evaluation**

In vitro evaluation of the two potential biocontrol agents (USPP-T1 and USPP-T2), *Bacillus subtilis*, Biotricho®, ECO 77® and six of the seven *Trichoderma* strains in Vinevax® (Ag 2, Ag 3, Ag 5, Ag 8, Ag 11 and AGSS 28) against trunk disease pathogens involved the observation of antagonistic interaction between cultures on dual inoculated plates. For each experimental unit, a mycelial plug of the fungal biocontrol agent or antagonist was cut with a sterile glass tube from a 7-day-old culture grown on potato dextrose agar (PDA; Biolab, Wadeville, South Africa), and placed opposing one another on the outer edges of a 90-mm diameter Petri dish containing PDA. In the case of the bacterial biocontrol agent, *B. subtilis* was streaked out onto the PDA with a sterilised needle eye and the mycelial plug of the pathogen was placed 2 cm from the bacterial streak on each plate. All of the biocontrol agents and pathogens were placed on the dishes simultaneously, except for *Pm. aleophilum* and *Pa. chlamydospora* that were cultured on the dishes for 14 days prior to inoculation with the biological control agents due to their slow mycelium growth rate. The culture plates were incubated in dark conditions at 22–25°C for 7 to 14 days before the interaction between cultures was observed macroscopically and digitally photographed using a high-quality photomicrographic digital camera (Nikon DMX 1200) mounted to a Nikon SMZ 800 stereoscopic zoom microscope.

To investigate the interaction at a microscopic level, the pathogens and biocontrol agents were co-incubated as described above, but on a nutrient deficient medium, synthetic nutrient agar (SNA; Nirenberg, 1976) for 10–14 days at 22–25°C in dark conditions. The two slower growing pathogens *Pm. aleophilum* and *Pa. chlamydospora* were incubated for 21 days prior to the 10–14 days co-incubation with the biocontrol agents. The nutrient deficient medium was used to inhibit sporulation and to reduce the amount of hyphal growth of the fast growing and sporulating *Trichoderma* isolates. Mycelial plugs (±2×2 mm) were removed from the interaction zones at five different positions and placed on a glass slide with sterile deionised water. Hyphal interactions were observed using a Nikon Eclipse E600 microscope at different levels of magnification (×200, ×400, and ×1000 in oil emersion). As sufficient interactions could be observed from the PDA medium, dual culture evaluations were not conducted on SNA medium with *B. subtilis*. Photomicrographs were taken of the microscopic interactions between hyphae using a Nikon DMX 1200 microscope.

***In vivo* evaluation**

Merlot (10 years old) and Chenin blanc (18 years old) vineyards situated between Paarl and Stellenbosch, Western Cape, South Africa (Delvera Estate) were spur-pruned to three buds in August 2006. Immediately after pruning, wounds were treated by spraying with 2 mL of a suspension of either *Trichoderma* isolates USPP-T1 or USPP-T2 (10^6 spores mL^{-1}), *B. subtilis* (10^8 cells mL^{-1}), ECO 77® (0.5 g L^{-1}), Biotricho® (4 g L^{-1} with 0.2% sucrose) or Vinevax® (10 g L^{-1}). As control treatments, wounds were treated by spraying with 2 mL benomyl (Benlate 500 WP, DowAgro Sciences; 10 g L^{-1}) or sterile deionised water (SDW). All *Trichoderma* suspensions were prepared in SDW. Spore suspensions of USPP-T1 and -T2 were prepared from 7-day-old cultures on PDA, which were grown at 25°C in normal light conditions. Plugs of mycelium were placed into 10 mL of SDW and shaken to suspend the spores, and then spore concentration was adjusted using a haemocytometer. The *B. subtilis* suspension was produced according to the protocol described by Ferreira *et al.* (1991). *Bacillus subtilis* was grown at 25°C for 3 days in Czapek-Dox broth using a rotary shaker. The bacterial cells were centrifuged at 3500 *g* for 20 min, and mineral oil (Pharmaoil 20, Mat-Chem, Dur-

ban, South Africa) added to achieve a cell suspension of 10^8 cells mL^{-1}. Just before application, the suspension was supplemented with 1% peptone and 1% sucrose to ensure rapid build up of bacteria in pruning wounds.

Seven days after pruning and application of biocontrol treatments, each pruning wound was inoculated with a 2 mL spore suspension (10^4 spores mL^{-1}) of *E. lata*, *Pa. chlamydospora*, *Ph. viticola* or species of Botryosphaeriaceae (*Neofusicoccum australe*, *N. parvum*, *D. seriata* and *Lasiodiplodia theobromae*). Controls were sprayed with 2 mL SDW. Spore suspensions of Botryosphaeriaceae spp. were prepared using the method by Van Niekerk *et al.* (2005); Botryosphaeriaceae isolates were inoculated onto water agar (WA; 10 g agar, 1 L of sterilised deionised water) containing sterilised pine needles, and plates incubated at 25°C under ultraviolet light for 2 to 3 weeks in a 12 h light-dark regime. Conidia were retrieved from pycnidia formed on pine needles. Inoculum of *Pa. chlamydospora* and *Ph. viticola* was prepared from 14-day-old PDA cultures. *Eutypa lata* ascospore inoculum was acquired from dead grapevine wood containing perithecia of *E. lata,* collected from Remhoogte estate, Stellenbosch, Western Cape, South Africa. Infected grapevine wood was rinsed under running tap water for 15 to 20 min, then the tops of the softened perithecia were removed using a sterile scalpel. The contents of about 15 perithecia were used to prepare the spore suspension (Ferreira *et al*., 1991).

Eight months after pruning and treatment, the pruning wounds were each removed between the top and second node, placed separately into plastic bags and taken to the laboratory for analysis. Pruning wound stubs were surface sterilised by immersion in 70% ethanol for 30 s, 1 min in 3.5% NaOCl and again for 30 s in 70% ethanol, before being split longitudinally under sterile conditions. The incidence of the inoculated pathogens and *Trichoderma* in the xylem tissue beneath the pruning wound scar was determined by isolations onto PDA amended with 0.04 g L^{-1} streptomycin sulphate. Streptomycin sulphate was included to reduce the incidence of bacterial contamination from pruning wounds, therefore *B. subtilis* could not be re-isolated and its incidence determined. Eight pieces of wood tissue (about 0.5×1 mm) were aseptically removed with a scalpel from the interface between apparently healthy and discoloured xylem tissue in each pruning wound and plated onto two Petri dishes containing antibiotic amended PDA. Petri dishes were incubated at 25°C in daylight for 4 weeks. Fungal growth from plated tissue pieces was monitored daily and sub-cultured if necessary. When a fast-growing fungus grew out from one or more tissue piece/s, the other pieces were removed from the Petri dish and plated onto fresh medium to avoid cross-contamination. Fungal cultures were identified using cultural and morphological characters.

For each cultivar, the trial layout was a completely randomised block design with three blocks. Each trial was of 8×8 factorial design (seven pathogen + control treatments × six antagonists + untreated and treated control treatments), with five spur wounds as replicates of each treatment combination randomly arranged within each block. Approximately eight pruning wounds were treated on each vine, with each treated pruning wound regarded as an experimental unit.

The incidences of pathogen and *Trichoderma* spp. in each of the treated pruning wounds (percentages of the eight tissue pieces from each wound that were colonised) were recorded. Data were subjected to analysis of variance and Student's t-test for least significant difference at the 95% confidence level ($P<0.05$) using SAS v8.2 statistical software (SAS Institute Inc).

Results

In vitro evaluation

Macro- and microscopic observations of interactions between antagonists and pathogens are described in Table 2. The macroscopic interactions typically included the formation of inhibition zones (Figure 1A) or overgrowth and sporulation by the antagonist on the mycelium of the pathogen (as described by Antal *et al.*, 2000) (Figure 1B). Other interactions, which were observed to a lesser extent, were defined as "growth inhibition" when growth of the pathogen mycelium was inhibited before coming in contact with the mycelium of the antagonist; in most cases the antagonist overgrew the pathogen culture (Figure 1C). In instances where the interaction was termed "stopped growth", both biocontrol agent and pathogen kept on growing until they came in contact with one an-

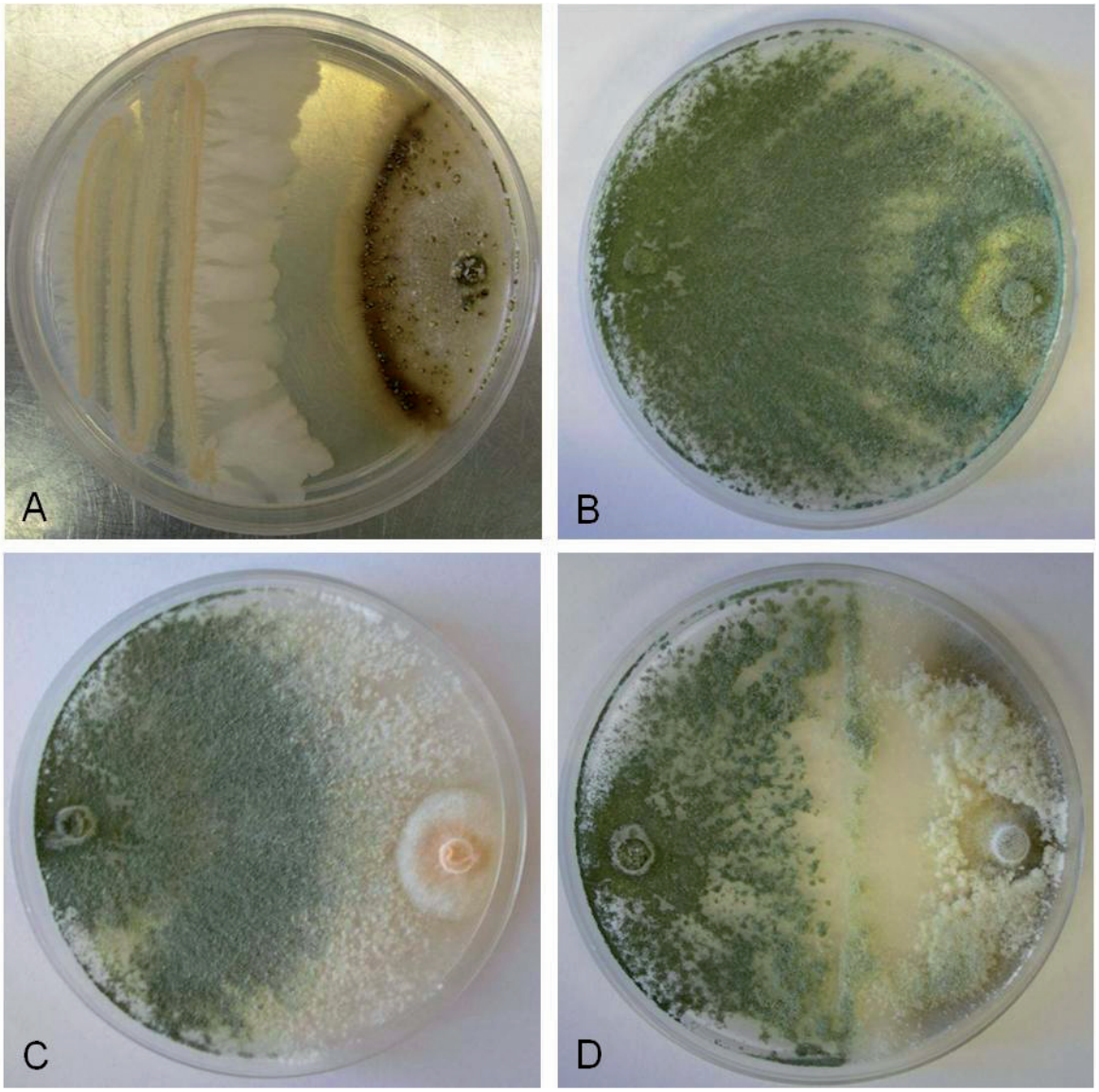

Figure 1. Macroscopic interactions observed on PDA medium. A) Inhibition zone (*B. subtilis* and *Ph. viticola*). B) Overgrowth (Biotricho and *E. lata*). C) Growth inhibition (USPP-T1 and *Pm. aleophilum*). D) Arrested growth (Ag 8 and *L. theobromae*).

other, after which growth of both colonies ceased (Figure 1D).

Microscopic interactions predominantly included "coiling" where hyphae of the antagonist wrapped around those of the pathogens (Figure 2A) and "hyphal disintegration", where holes appeared in the hyphae of the pathogen (as described in Benhamou and Chet, 1997), as well as the hyphae becoming flaccid or shrivelled (as described in John *et al.*, 2004) (Figure 2B). Other observations included "hyphal adhesion" where hyphae of the antagonist attached to the hyphae of the pathogen (Figure 2C). "Hyphal swelling" (Figure 2D) is the loss of turgidity of the pathogen hyphal cells, causing turgescence. This was only observed in interactions between the pathogens and *B. subtilis* and is similar to the observations made by Ferreira *et al.* (1991).

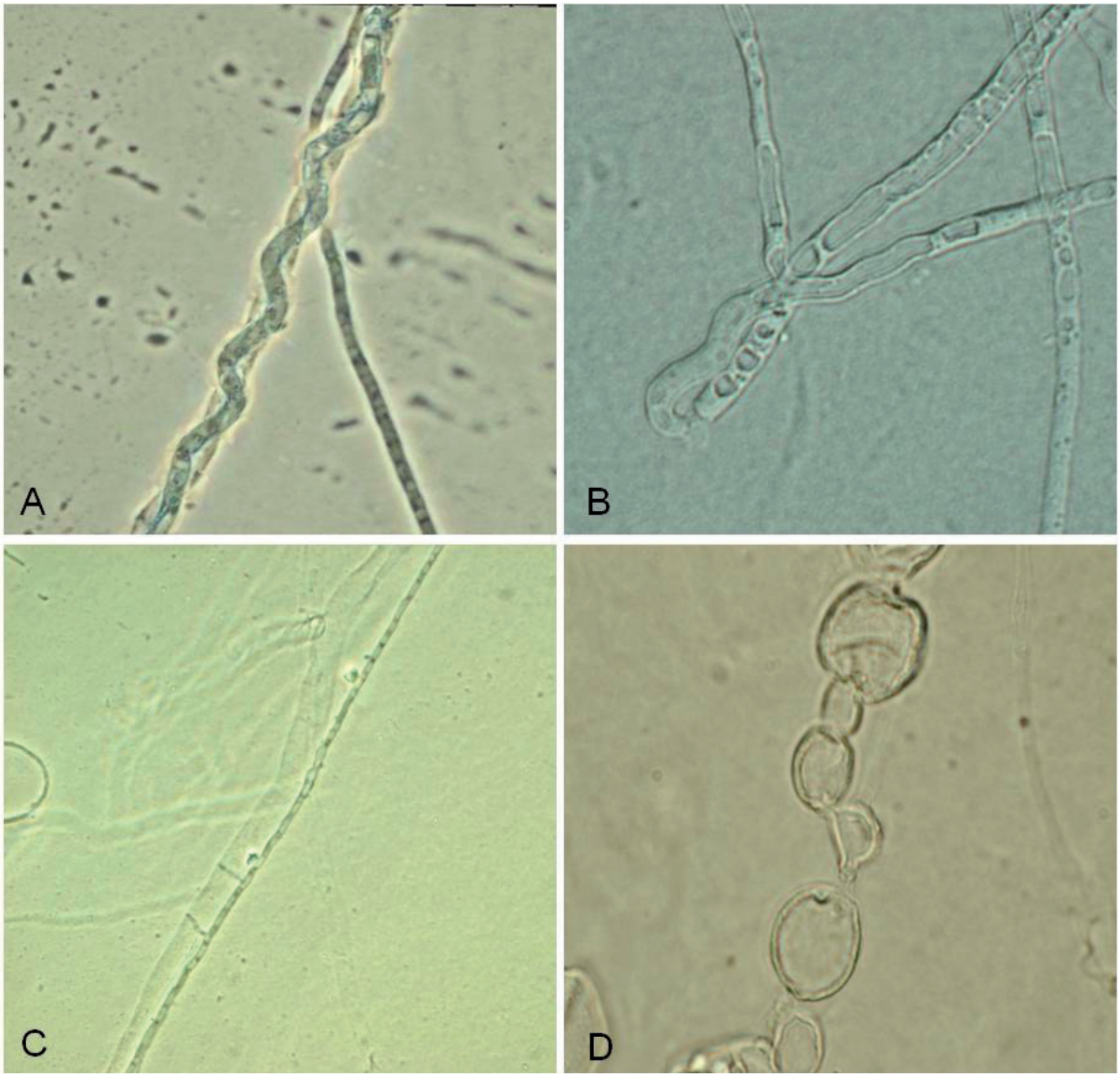

Figure 2. Microscopic interactions as observed at ×400 and ×1000 magnification. A) Coiling of *Trichoderma* hyphae around pathogenic hyphae as observed between USPP-T1 and *L. theobromae* (×1000). B) Hyphal disintegration of *D. seriata* hyphae (×1000). C) Hyphal adhesion observed between USPP-T1 and *N. australe* (×400). D) Hyphal swelling as observed in the interaction between *B. subtilis* and *Ph. viticola* (×1000).

Trichoderma harzianum isolates, Ag 2, Ag 11, AgSS 28, ECO 77 and Biotricho, predominantly grew over each of the pathogens; however, growth of *Pa. chlamydospora* and *Pa. aleophilum*, was first stopped, whereafter they were overgrown. Although isolates Ag 11 and AgSS 28 overgrew most pathogen cultures, growth of *L. theobromae* was stopped, and Ag 11 inhibited the growth of *N. australis* by means of an inhibition zone. Generally microscopical interactions such as "coiling" and "hyphal adhesion" as well as "hyphal disintegration" were associated with overgrowth. The reactions caused by the *T. harzianum* strain in ECO 77 included hyphal cells losing their form and having shrivelled appearance at ×400 magnification (Figure 1B), which could be seen as hyphal disintegration at ×1000 magnification.

The interactions observed for the *T. atroviride*

Table 2. Macro[a]- and microscopic[b] interactions observed between dual cultures of selected grapevine trunk disease pathogens and biocontrol agents.

Biological agent	*N. australis*		*D. seriata*		*N. parvum*		*L. theobromae*		*E. lata*		*Pm. aleophilum*		*Pa. chlam.*		*Ph. viticola*	
	Macroscopic	Microscopic	Macroscopic	Microscopic	Macroscopic	Microscopic	Macroscopic	Microscopic	Macroscopic	Microscopic	Macroscopic	Microscopic	Macroscopic	Microscopic	Macroscopic	Microscopic
Ag 2	OG	PC	OG	N	OG	HD	OG	AH	OG	N	GI,OG	PC	GI,OG	PC	OG	N
Ag 3	OG	HD	OG	N	OG	AH	OG	HD	OG	N	GI,OG	PC	GI,OG	N	OG	N
Ag 5	OG	AH	OG	AH	OG	PC	OG	HD	OG	PC	GI,OG	HD	GI,OG	PC	OG	N
Ag 8	OG	PC	OG	AH	OG	HD	SG	N	OG	N	GI,OG	N	GI,OG	N	OG	N
Ag 11	IZ	N	OG	PC	OG	PC	SG	N	OG	HD	GI,OG	N	GI,OG	PC	OG	N
Agss 28	OG	N	OG	PC	OG	HD	SG	PC	OG	AH	GI,OG	N	GI,OG	N	OG	N
USPP-T1	IZ,OG	AH	IZ	HD	OG	PC	IZ	PC	IZ,OG	PC	GI,OG	PC	GI,OG	PC	OG	N
USPP-T2	IZ,OG	N	SG,IZ	HD	OG	PC	OG	PC	SG,IZ	N	GI,OG	N	GI,OG	N	OG	PC,HD
B. subtilis	IZ	SM	IZ	SM	IZ	SM	IZ	SM	IZ	SM	IZ	SM	IZ	SM	IZ	SM
Biotricho	OG	N	OG	AH	OG	HD	OG	HD	OG	HD	GI,OG	AH	GI,OG	N	OG	N
ECO 77	SG	HD	IZ	N	OG	HD	OG	PC	OG	PC	GI,OG	N	GI,OG	N	OG	N

[a] OG = overgrowth of the pathogen by the biological agent; IZ = inhibition zone formed between the two cultures; SG = growth of the pathogen stopped when they come in contact with the agent; GI = growth of pathogen stopped after incubation with the agent.

[b] AH = adhesion of the biological agents hyphae to that of the pathogens; HD = hyphal disintegration; PC = coiling by the hypha of the agent around hyphae of the pathogen; SM = swelling and malformation of the pathogen's hyphae; N = no interactions have been observed.

Trichoderma harzianum isolates, Ag 2, Ag 11, AgSS 28, ECO 77 and Biotricho, predominantly grew over each of the pathogens; however, growth of *Pa. chlamydospora* and *Pa. aleophilum*, was first stopped, whereafter they were overgrown. Although isolates Ag 11 and AgSS 28 overgrew most pathogen cultures, growth of *L. theobromae* was stopped, and Ag 11 inhibited the growth of *N. australis* by means of an inhibition zone. Generally microscopical interactions such as "coiling" and "hyphal adhesion" as well as "hyphal disintegration" were associated with overgrowth. The reactions caused by the *T. harzianum* strain in ECO 77 included hyphal cells losing their form and having shrivelled appearance at ×400 magnification (Figure 1B), which could be seen as hyphal disintegration at ×1000 magnification.

The interactions observed for the *T. atroviride* isolates, Ag 3, Ag 5 and Ag 8, were similar to those observed for the *T. harzianum* strains, Ag 2 and Biotricho, *i.e.* overgrowth of all the pathogen isolates, except for the Ag 8 and *L. theobromae* combination, which had an inhibitory effect on each other in dual culture. Some antagonists caused a wide range of microscopic interactions on some pathogens, including adhesion of hyphae, coiling, and hyphal disintegration. The two *Trichoderma* isolates, USPP-T1 and USPP-T2, had substantial effects on all trunk disease pathogens, with a wide range of macroscopic and microscopic interactions observed. Macroscopically, inhibition zones were mostly observed, which were later followed by overgrowth of the pathogen by the biological agent, or growth of both organisms ceased. At the microscopic level, coiling was most often observed with both isolates, while hyphal disintegration was observed with both isolates in combination with *D. seriata*.

In dual cultures with *B. subtilis*, all the pathogens were inhibited and showed little mycelium growth and clear inhibition zones (Figure 1A) were observed. At a microscopic level, malformations of the hyphae occurred, typically hyphal swelling (Figure 2A).

In vivo evaluation

For pathogen incidence data, analyses of variance indicated no significant interactions with cultivar (P=0.0940), nor was cultivar significant as a main effect (P=0.3069). Significant interactions

Table 3. Mean incidence of *Eutypa lata* that was isolated from pruning wounds 8 months after the fresh pruning wounds were treated with benomyl, *Bacillus subtilis*, two *Trichoderma* spp. (USPP-T1 and -T2), three commercial *Trichoderma*-based biocontrol products or water as pruning wound protectants, and challenged 7 days later by spray-inoculation with *Eutypa lata*, *Phaeomoniella chlamydospora*, *Phomopsis viticola*, *Diplodia seriata*, *Lasiodiplodia theobromae*, *Neofusicoccum australe* and *N. parvum* spore suspensions, or sprayed with sterile water to evaluate natural infection.

Wound treatment	*Eutypa lata* incidence (%)[a] in treated wounds that were challenged with							
	Natural infection	*E. lata*	*Pa. chlamydospora*	*Ph. viticola*	*D. seriata*	*L. theobromae*	*N. australe*	*N. parvum*
B. subtilis	0.54a	10.71b	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
Benomyl	0.45a	19.58d	0.48a	0.00a	0.00a	0.00a	0.00a	0.00a
Biotricho	0.00a	15.00c	0.00a	0.00a	2.23a	0.00a	0.93a	0.00a
ECO 77	0.00a	10.65b	0.48a	0.00a	0.00a	0.00a	1.56a	1.39a
USPP-T1	0.00a	9.13b	0.00a	0.00a	0.00a	0.00a	0.48a	0.00a
USPP-T2	0.00a	11.00bc	0.00a	0.00a	1.14a	0.00a	0.89a	0.00a
Vinevax	3.45a	8.17b	0.00a	1.56a	0.00a	0.00a	0.93a	0.46a
Water	0.45a	37.50e	0.00a	0.45a	0.00a	0.00a	1.44a	0.00a

[a] Means followed by the same letter within columns and across rows do not differ significantly (P <0.05; LSD=4.147).

were observed between the treatments and pathogens (P=0.0540). The mean pathogen incidences in the Chenin blanc and Merlot pruning wounds treated with six different biocontrol agents, water or benomyl before inoculation with the different pathogens, are given in Tables 3 to 6. Each table shows the mean incidence of the inoculated pathogen as well as the other naturally occurring pathogens. However, for the sake of brevity, only the results for each pathogen's incidence in control wounds (natural infection) and wounds in which it was inoculated will be described.

Eutypa lata was isolated from up to a mean of 3.5% (Table 3) of the pruning wounds that were treated with the biocontrol agents or benomyl and subjected to natural infection, while the fungus was only isolated from 0.5% of the unprotected (water treated) wounds. In pruning wounds inoculated with *E. lata,* incidences were significantly greater (8.2 to 37.5%) than in the non-inoculated pruning wounds. All pruning wound treatments, biological as well as the benomyl treatment, significantly reduced the incidence of *E. lata* in the inoculated pruning wounds compared to the water treated control wounds (mean incidence 37.5%). The most effective treatments for reducing the *E. lata* incidence in inoculated wounds were Vinevax (8.2%), USPP-T1 (9.1%), USPP-T2 (11.0%), ECO 77 (10.7%) and *B. subtilis* (10.7%). Biotricho reduced the incidence of *E. lata* but to a lesser extent (15.0%). *Eutypa lata* was entirely absent or occurred at lower incidence (0.0 to 2.2%) in wounds that were inoculated with the other pathogens. These incidences were not significantly different from the natural *E. lata* infection levels.

The Botryosphaeriaceae isolates were only identified to genus level from the isolations made from the treated pruning stubs. All pruning wound treatments, biological as well as the benomyl treatment, significantly reduced the incidence of the Botryosphaeriaceae isolates in the inoculated pruning wounds compared to the water treated control (10.7%; Table 4). Biotricho and USPP-T1 reduced the natural infection significantly to 0.5% and 0.0%, respectively (P<0.001). Isolations from unprotected pruning wounds that were inoculated with *D. seriata*, *L. theobromae*, *N. australe* and

Table 4. Mean combined incidence of species of Botryosphaeriaceae that were isolated from pruning wounds 8 months after the fresh pruning wounds were treated with benomyl, *Bacillus subtilis*, two *Trichoderma* spp. (USPP-T1 and -T2), three commercial *Trichoderma*-based biocontrol products or water as pruning wound protectants, and challenged 7 days later by spray-inoculation with *Eutypa lata*, *Phaeomoniella chlamydospora*, *Phomopsis viticola*, *Diplodia seriata*, *Lasiodiplodia theobromae*, *Neofusicoccum australe* and *N. parvum* spore suspensions, or sprayed with sterile water to evaluate natural infection.

Wound treatment	Botryosphaeriaceae incidence (%)[a] in treated wounds that were challenged with							
	Natural infection	*D. seriata*	*L. theobromae*	*N. australe*	*N. parvum*	*E. lata*	*Pa. chlamydospora*	*Ph. viticola*
B. subtilis	2.17a-d	13.50g-o	15.50j-p	15.18i-p	16.50l-p	4.46a-g	2.78a-d	8.33a-l
Benomyl	4.46a-g	23.56pq	14.29h-o	12.05e-n	15.52j-p	2.50a-d	0.96ab	0.43a
Biotricho	0.48a	20.09n-p	15.48j-p	10.65c-m	12.50f-n	3.00a-e	3.70a-f	1.92a-c
ECO 77	2.84a-d	14.29h-o	13.46g-o	18.23m-p	7.41a-k	2.31a-d	0.00a	1.63a-c
USPP-T1	0.00a	5.77a-h	2.50a-d	9.62b-m	4.50a-g	6.73a-j	6.25a-i	3.26a-e
USPP-T2	2.40a-d	15.34j-p	8.13a-l	10.27c-m	16.20k-p	4.00a-f	4.63a-g	2.68a-d
Vinevax	3.45a-f	15.76j-p	13.79h-o	11.11d-n	13.89h-o	1.44a-c	3.85a-f	1.56a-c
Water	10.71c-m	37.50rs	32.59qr	44.71s	22.08op	4.33a-f	2.40a-d	5.80a-h

[a] Means followed by the same letter within columns and across rows do not differ significantly (P<0.05; LSD=9.06).

N. parvum yielded 37.5, 32.6, 44.7 and 22.1% incidence of Botryosphaeriaceae species, respectively. Botryosphaeriaceae incidences in all pruning wound protection treatments were significantly less than the untreated inoculated controls, except in the case of *N. parvum* inoculated wounds where *B. subtilis* (16.5%), USPP-T2 (16.2%), benomyl (15.5%) and Vinevax (13.9%) treated wounds yielded statistically similar incidences to unprotected wounds. The most effective treatment against all Botryosphaeriaceae species was USPP-T1 (2.5 to 9.6%), which was also more effective than benomyl, except with *N. australe* inoculated wounds. Markedly lower incidences of Botryosphaeriaceae isolates were obtained from wounds that were inoculated with *E. lata*, *Pa. chlamydospora* and *Ph. viticola* (4.3, 2.4 and 5.8%, respectively) compared to natural infection levels in unprotected wounds (10.7%).

Inoculated unprotected pruning wounds yielded 34.8% incidence of *Ph. viticola* (Table 5). Each of the treated pruning wounds showed significantly lower levels of *Ph. viticola* infection than the inoculated unprotected pruning wounds. Benomyl was the most effective treatment, reducing incidence of *Ph. viticola* to 8.2%. However, benomyl was not statistically more effective than USPP-T2 (17.9%), Biotricho (17.8%), Vinevax (13%) or USPP-T1 (10.9%). Relatively high (6.3 to 12.1%) levels of natural infection by *Ph. viticola* were observed in unprotected pruning wounds that were inoculated with the other pathogens. These levels did not differ statistically, however, from the natural infection levels of *Ph. viticola* (9.8%) in the non inoculated pruning wounds.

Phaeomoniella chlamydospora occurred at relatively low incidence levels in the non-inoculated pruning wounds (5%; Table 6), and none of the treatments reduced the natural infection significantly. The incidence of *Pa. chlamydospora* in the inoculated water controls was statistically greater (34.6%) than in the non-inoculated wounds. All treatments significantly reduced the incidence of infection in the inoculated pruning wounds com-

Table 5. Mean incidence of *Phomopsis viticola* that was isolated from pruning wounds 8 months after the fresh pruning wounds were treated with benomyl, *Bacillus subtilis*, two *Trichoderma* spp. (USPP-T1 and -T2), three commercial *Trichoderma*-based biocontrol products or water as pruning wound protectants, and challenged 7 days later by spray-inoculation with *Eutypa lata*, *Phaeomoniella chlamydospora*, *Phomopsis viticola*, *Diplodia seriata*, *Lasiodiplodia theobromae*, *Neofusicoccum australe* and *N. parvum* spore suspensions, or sprayed with sterile water to evaluate natural infection.

Wound treatment	*Phomopsis viticola* incidence (%)[a] in treated wounds that were challenged with							
	Natural infection	*Ph. viticola*	*E. lata*	*Pa. chlamydospora*	*D. seriata*	*L. theobromae*	*N. australe*	*N. parvum*
B. subtilis	16.85b-f	21.35b	6.25h-n	11.11c-m	4.50j-n	12.00b-l	7.14f-n	16.50b-g
Benomyl	6.70g-n	8.19e-m	3.75k-n	4.33j-n	4.33j-n	6.25h-n	4.46j-n	1.72mn
Biotricho	5.77i-n	17.79b-e	9.00d-n	10.19c-n	19.64bc	14.29b-j	3.24k-n	16.07b-h
ECO 77	13.07b-k	18.48b-d	9.26d-n	15.38b-i	2.68l-n	5.29i-n	4.17j-n	5.09j-n
USPP-T1	6.02h-n	10.87c-l	0.48n	11.46b-m	3.37k-n	4.50j-n	3.85k-n	9.00d-n
USPP-T2	5.77i-n	17.86b-e	7.50f-n	12.96b-k	9.66c-n	7.50f-n	8.93d-n	5.56i-n
Vinevax	10.78c-m	13.02b-j	3.85k-n	1.92l-n	7.07f-n	8.62d-n	7.87e-n	6.48g-n
Water	9.82c-n	34.82a	6.25h-n	10.10c-n	8.00e-n	10.27c-n	6.25h-n	12.08b-l

[a] Means followed by the same letter within columns and across rows do not differ significantly ($P<0.05$; LSD=10.135).

Table 6. Mean incidence of *Phaeomoniella chlamydospora* that was isolated from pruning wounds 8 months after the fresh pruning wounds were treated with benomyl, *Bacillus subtilis*, two *Trichoderma* spp. (USPP-T1 and -T2), three commercial *Trichoderma*-based biocontrol products or water as pruning wound protectants, and challenged 7 days later by spray-inoculation with *Eutypa lata*, *Phaeomoniella chlamydospora*, *Phomopsis viticola*, *Diplodia seriata*, *Lasiodiplodia theobromae*, *Neofusicoccum australe* and *N. parvum* spore suspensions, or sprayed with sterile water to evaluate natural infection.

Wound treatment	*Phaeomoniella chlamydospora* incidence (%)[a] in treated wounds that were challenged with							
	Natural infection	*Pa. chlamydospora*	*E. lata*	*Ph. viticola*	*D. seriata*	*L. theobromae*	*N. australe*	*N. parvum*
B. subtilis	2.17a-c	21.76fg	0.45a	3.13a-c	1.00a	1.00a	0.89a	1.50ab
Benomyl	5.36a-c	12.50de	0.42a	0.00a	1.44ab	0.89a	1.34ab	0.00a
Biotricho	5.29a-c	23.61g	0.00a	1.44ab	0.45a	0.00a	3.24a-c	2.68a-c
ECO 77	1.70ab	14.90e	0.00a	1.63ab	0.45a	0.48a	0.00a	3.24a-c
USPP-T1	1.85ab	7.81cd	2.40a-c	3.80a-c	0.00a	0.00a	3.85a-c	0.00a
USPP-T2	0.48a	15.74e	2.50a-c	1.34ab	1.14a	0.63a	0.00a	2.78a-c
Vinevax	0.00a	7.21b-d	0.00a	4.17a-c	2.72a-c	1.29a	0.93a	0.93a
Water	4.91a-c	34.62h	0.00a	1.34ab	2.00a-c	1.79ab	0.48a	3.75a-c

[a] Means followed by the same letter within columns and across rows do not differ significantly ($P<0.05$; LSD=5.896).

pared to the water treated controls. Low levels (0.0 to 3.8%) of co-infection by *Pa. chlamydospora*, which did not differ statistically from its natural infection levels, were observed in wounds inoculated with the other pathogens.

For *Trichoderma* incidence, analysis of variance showed a significant cultivar × treatment interaction ($P=0.0006$), while the treatment × pathogen interaction was not significant ($P=0.7350$). The mean incidence of *Trichoderma* spp. in Merlot or Chenin blanc was fairly low in wounds that were not treated with *Trichoderma*-based biocontrol products: *B. subtilis* (Table 7; 0.1 and 0.7%, respectively), benomyl (0.1 and 0%, respectively) and water (0.6 and 0.5%, respectively). All tissues treated with the *Trichoderma* products and isolates had greater incidences of *Trichoderma* spp. in the Chenin blanc compared to the Merlot wounds although not significantly, except for Vinevax which had a greater incidence in the Merlot cultivar (16.2% compared with 24.6%). Of the *Trichoderma* based treatments, isolation frequency from the pruning wounds was greatest for USPP-T1 (33.4 and 28.4%, respectively in the two grapevine cultivars), although the differences were not significantly greater than for all the other *Trichoderma* based biocontrol agents.

Discussion

This study demonstrated the potential of biological control agents to provide sustained protection of pruning wounds against a complex of grapevine trunk disease pathogens. Initially, *in vitro* dual culture evaluations were conducted to screen *Trichoderma*-based products, *Bacillus subtilis*, as well as two *Trichoderma* isolates which had previously shown biocontrol potential (Ferreira *et al*., 1991; John *et al*., 2004, 2005; Van Niekerk, 2008) against Botryosphaeriaceae species, *E. lata*, *Pm. aleophilum*, *Pa. chlamydospora* and *Ph. viticola*.

The results of the *in vitro* evaluation tests demonstrated that all of the isolates from the *Trichoderma*-based products, Vinevax®, Biotricho® and ECO 77®, as well as *Trichoderma atroviride* isolates USPP-T1 and -T2, showed varying levels of antagonism towards all or most of the pathogens. Various antagonistic mechanisms were observed. Macroscopically, inhibition zones, most likely in-

Table 7. The mean colonisation incidence of *Trichoderma* isolated from pruning wounds of Merlot and Chenin blanc cultivars 8 months after fresh pruning wounds were treated with benomyl, *Bacillus subtilis*, two *Trichoderma* spp. (USPP-T1 and -T2), three commercial *Trichoderma*-based biocontrol products or water as pruning wound protectants, and challenged 7 days later by spray-inoculation with *Eutypa lata*, *Phaeomoniella chlamydospora*, *Phomopsis viticola*, *Diplodia seriata*, *Lasiodiplodia theobromae*, *Neofusicoccum australe* and *N. parvum* spore suspensions, or sprayed with sterile water to evaluate natural infection.

Wound treatment	*Trichoderma* spp. incidence (%)[a] in treated wounds	
	Chenin blanc	Merlot
B. subtilis	0.13c	0.71c
Benomyl	0.11c	0.00c
Biotricho	10.75bc	6.37c
ECO 77	22.12ab	12.50bc
USPP-T1	33.41a	28.44a
USPP-T2	20.71ab	11.74bc
Vinevax	16.16b	24.64ab
Water	0.56c	0.47c

[a] Means followed by the same letter do not differ significantly ($P<0.05$; LSD=14.449).

dicative of antibiotic production, were most commonly observed and hyphal disintegration was observed microscopically. In cases where *Trichoderma* cultures grew over those of the pathogens, signs of mycoparasitism, as seen from coiling or hyphal adhesion, were observed microscopically. The ability of *Trichoderma* spp. to grow quickly and compete for space contributes to its ability to inhibit the growth of the pathogens in dual cultures (Kucuk and Kivanc, 2004). However, the formation of inhibition zones without contact is most likely due to the ability of *Trichoderma* spp. to produce volatile (John *et al.*, 2004; Kucuk and Kivanc, 2004) and non-volatile (John *et al.*, 2004) substances. The mycoparasitic reactions, such as coiling and adhesion to pathogen hyphae (Almeida *et al.*, 2007), predominantly coincided with the macroscopic physical contact interactions such as overgrowth and ceased growth. With its ability to produce enzymes and antibiotics (Calistru *et al.*, 1997), *Trichoderma* is capable of causing hyphal disintegration (Benhamou and Chet, 1997). During the current study, hyphal malformations and disintegration were observed with several of the paired interactions in which the *Trichoderma* isolates (ECO 77, USPP-T1 and USPP-T2) were not in contact with hyphae of the pathogens. This is, however, in contrast with the study by Benhamou and Chet (1993) who reported that close contact of pathogen and *Trichoderma* isolates coincides with the processes of hyphal disintegration, which could suggest that contact is needed for the secretion of enzymes or antibiotics. Therefore, more research is needed to further identify the different antagonistic mechanisms employed by ECO 77, USPP-T1 and USPP-T2 against these grapevine trunk disease pathogens.

Bacillus subtilis caused hyphal malformations in all of the pathogens, which could be attributed to an antibiotic substance. Ferreira *et al.* (1991) identified two antibiotic products produced by this particular *B. subtilis* isolate, which were capable of inhibiting mycelial growth of *E. lata*. According to Baker and Cook (1982), the antibiotics from *B. subtilis* can penetrate fungal hyphae and cause malformation.

Given the results of the *in vitro* evaluation, Vinevax, Biotricho, ECO 77, USPP-T1 and USPP-T2 (which all contain *Trichoderma* spp.) as well as the *B. subtilis* isolate were selected for further evaluation in field trials. Fresh pruning wounds of Chenin blanc and Merlot grapevine cultivars were treated with the biocontrol agents/products and individually challenged by the grapevine pruning wound invading pathogens 7 days later. This delay is not uncommon since previous investigations studying the optimal establishment of biological

control agents also challenged by inoculating with a pathogen after 7 days (John *et al*., 2008) or even 3 weeks (Schubert *et al*., 2008). However, Halleen *et al*. (2010) who challenged with the pathogen 24 h after treatment with the biocontrol agent concluded that chemical control of pruning wounds was superior to biological control options, although the *Trichoderma* treatments also significantly reduced infection levels of *E. lata*. The study by Halleen *et al*. (2010), therefore, supported the biocontrol efficacy observed in the current study, in which the 7-day incubation period provided the biocontrol agents the opportunity to establish in the pruning wounds and therefore improve their competitiveness. However, further research is needed to investigate the efficacy of bioprotection of pruning wounds when challenged at various time intervals after pruning and treatment.

Benomyl at 10 g L^{-1} (at 20× the recommended dosage for foliar disease management in South Africa; Nel *et al*., 2003), which was used as the chemical control standard in this study, was proven in previous studies to be one of the most effective options for pruning wound protection (Pearson, 1982; Munkvold and Marois, 1993b). Even concentrations as low as 2 g L^{-1} have been shown to protect pruning wounds when applied 1 day after inoculation with *E. lata* (Sosnowski *et al*., 2008). *In vitro* studies by Bester *et al*. (2007) demonstrated that benomyl was effective against Botryosphaeriaceae trunk pathogens. However, the present study showed that when benomyl-treated wounds were challenged by the pathogens 1 week after treatment, its efficacy was poorer than with the 24 h post inoculation conducted by Halleen *et al*. (2010). The reduced efficacy of benomyl observed in the present study might be attributed to breakdown of the fungicide in the wound sites, as was observed by Price and Carter (1975) who found that the extractable amount of methyl benzimidazole-2-yl carbamate in the sapwood had diminished 2 weeks after application to pruning wounds. However, the dosage of benomyl used in the present study (5,000 μg mL^{-1}) was less than the 12,500 μg mL^{-1} recommended by Munkvold and Marois (1993b), and this could have contributed to its lack of efficacy. The present study showed that the efficacy of the biocontrol agents was in most cases similar or superior to that observed for benomyl. USPP-T1 in particular was very effective, frequently being the best treatment against all seven pathogens tested. Carter and Price (1974) proposed treatment of wounds with a combination of a chemical and a biocontrol agent and suggested that benzimidazole-resistant *Fusarium lateritium* be integrated in mixture with benzimidazoles to treat pruning wounds.

Isolations made from untreated pruning wounds that were challenged with the trunk disease pathogens 1 week after pruning, yielded particularly high incidences of the pathogens. Since previous reports have demonstrated that pruning wounds may stay susceptible to trunk pathogen infection for up to 16 weeks after pruning (Eskalen *et al*., 2007; Serra *et al*., 2008), sustained protection of the pruning wounds is clearly required.

An interesting observation was also made with regards to the levels of secondary infections occurring in inoculated pruning wounds, *i.e.* natural infection by other trunk disease pathogens in wounds that were inoculated with a specific pathogen. It is known that *E. lata* causes primary infection (Larignon and Dubos, 1997), as was also reported for *Pa. chlamydospora* and *Phaeoacremonium* spp. (Sparapano *et al*., 2000). Ferreira *et al*. (1989) suggested that there is a succession of fungal colonisation in pruning wounds after infection by primary colonisers. However, although not statistically significant, findings from the current study suggest otherwise, with pruning wounds inoculated with *E. lata,* and to a lesser extent *Pa. chlamydospora,* having little or no secondary infection. This supports the findings of Larignon and Dubos (1997) and Sparapano *et al*. (2000). Further studies need to be conducted to investigate this phenomenon.

Findings in the present study support previous research on protection of pruning wounds by means of *Trichoderma* or other biological control agents (Ferreira *et al*., 1991; Di Marco *et al*., 2004; John *et al*., 2005, 2008). Disregarding the fact that application rates and/or formulations differed, which might have influenced efficacy, *Bacillus subtilis*, the *Trichoderma* products, Vinevax, Biotricho, ECO 77, and isolates USPP-T1 and -T2 generally reduced the incidence of the inoculated grapevine trunk disease pathogens compared to the untreated controls when given 7 days

to colonise the pruning wounds before infection. Moreover, *Trichoderma* was isolated 8 months after treatment from treated pruning wounds at varying levels (6.4 to 33.4%). However, these levels are less than those observed in other studies (*i.e.* 20-76% [Halleen *et al*., 2010]), possibly due to differences in application techniques and incubation times. The time of pruning (August) might also have played a significant role, since this was at the end of the vine dormancy period when sapflow from pruning wounds might flush out some of the *Trichoderma* spores. Di Marco *et al*. (2004) recovered *Trichoderma* from 90% of treated pruning wounds after 7 days, while John *et al*. (2008) re-isolated *Trichoderma* from 43% of vines 20 months after insertion of wooden dowels impregnated with *Trichoderma*. Di Marco *et al*. (2004), nonetheless, found that there was a significant reduction in recovery percentage with longer incubation periods. In the present study, *Trichoderma* spp. were recovered from untreated pruning wounds at low frequencies, indicating low levels of natural *Trichoderma* infection. Alternatively, this could possibly be due to the fungus being present in the pruning wounds endophytically, as *Trichoderma* are known endophytes in the sapwood of woody plants (Samuels *et al*., 2006), or to cross contamination taking place during treatment of the wounds.

Bacillus subtilis was not isolated from pruning wounds in this study due to the PDA being amended with antibiotics. However, pruning wounds treated with *B. subtilis* showed significantly lower incidences of all the pathogens 8 months after inoculation. Ferreira *et al*. (1991) reported that pruning wounds treated with the same strain of *B. subtilis* significantly suppressed infection by *E. lata* 9 months after inoculation. However, Ferreira *et al*. (1991) covered the treated pruning wounds with aluminium foil, whereas the wounds in the present study were left open and exposed to the environment. Schmidt *et al*. (2001) showed that a *B. subtilis* strain reduced mycelium growth of *E. lata* on autoclaved grape wood. Our results therefore significantly contribute to current knowledge of this bacterium as a potential pruning wound protectant against grapevine trunk disease pathogens.

Although not statistically significant, the present study found that a higher incidence of *Trichoderma* was recovered from Chenin blanc than Merlot for all of the *Trichoderma*-based treatments except for Vinevax. Several factors may have contributed to the imbalance. The Merlot vineyard was under regular drip irrigation, while the Chenin blanc vines were grown under dry land conditions, so water stress of the Chenin blanc could have led to the narrowing of the xylem vessels. Lovisolo and Schubert (1998) suggested that not only did the transectional areas of xylem vessels decrease under water stress, but so too did the hydraulic conductivity. Another contributing factor could be nutrient status, as greater conidial germination was observed by Schubert *et al*. (2008) when *Trichoderma* spore suspensions were amended with glucose. The greater incidence of infection in wounds treated with Vinevax in Merlot may be explained by the suggestion that certain *Trichoderma* strains have greater affinities for certain woody hosts than others (Samuels *et al*., 2006). Preliminary studies into the susceptibility of various *Vitis vinifera* cultivars towards *Trichoderma* infection and colonisation clearly showed that there are differences between the various cultivars (Mutawila, 2010).

Considering the ability of *Trichoderma* to colonise pruning wounds and sustain its presence, long term protection of pruning wounds with *Trichoderma*-based products could provide an effective tool in the management of pruning wound pathogens. *Trichoderma atroviride* isolate USPP-T1, and to some extent USPP-T2, were very effective at reducing infection of the pruning wounds when challenged 7 days after treatment with the pathogens evaluated. Since these isolates were originally obtained from grapevine pruning wounds, from the same vineyard used for the present study, they are probably adapted to the grapevine wood environment. Therefore, the potential of these isolates as pruning wound protectants should be studied further, especially with regard to mechanisms of control, cultivar susceptibility and application methods.

Acknowledgements

The authors thank Carine Vermeulen, Linda Nel, Zane Sedeman and Julia Marais for technical assistance and Winetech, Deciduous Fruit Producer's Trust, NRF and THRIP for funding.

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Accepted for publication: April 6, 2011