Protection of Grapevine Pruning Wounds against *Eutypa lata* by Biological and Chemical Methods

F. Halleen[1*], P.H. Fourie[2], P.J. Lombard[3]

(1) ARC Infruitec-Nietvoorbij (The Fruit, Vine and Wine Institute of the Agricultural Research Council), Private Bag X5026, Stellenbosch 7599, South Africa
(2) Citrus Research International, P.O. Box 2201, Stellenbosch 7602, South Africa
(3) Kelp Products (Pty) Ltd, PO Box 325, Simonstown, 7995

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Eutypa dieback, caused by the fungus *Eutypa lata*, is a serious disease of grapevines that infects mainly through pruning wounds. The aim of this study was to evaluate the *in vitro* efficacy of fungicides from various chemical groups against *E. lata*, as well as the *in vivo* efficacy of the most effective fungicides and selected bacterial and fungal antagonists of *E. lata*, in grapevine pruning wound protection trials. *In vitro* studies revealed that flusilazole, tebuconazole, benomyl, fenarimol and myclobutanil were the most effective fungicides to inhibit mycelial growth of *E. lata*. Two field trials were conducted, one subjected to artificial inoculation and the second to natural infection only. In the first, benomyl, flusilazole and commercially available *Trichoderma harzianum*-containing products and an experimental *Bacillus subtilis* strain were applied to fresh pruning wounds. Two Cabernet Sauvignon vineyards were pruned in August 2001 and 2002 and immediately treated and inoculated with a spore suspension of *E. lata* one day later. Isolations were made from the treated pruning wounds after 12 months to assess the effectiveness of the treatments. The fungicides benomyl and flusilazole were the most effective treatments, although the *Trichoderma* treatments T77 and Trichoseal spray caused a significant reduction in *E. lata* infection. In a second trial, pruning wounds of Cabernet Sauvignon, Sauvignon blanc, Red Globe and Bonheur were treated with the Trichoderma products Vinevax (= Trichoseal spray) and Eco77 (= T77) in August 2005 and 2006, subjected to natural infection only and evaluated after seven months. Vinevax and Eco77 not only reduced *E. lata*, but they also reduced the incidence of other grapevine trunk disease pathogens.

INTRODUCTION

Eutypa dieback, caused by the fungus *Eutypa lata*, is a serious disease of grapevine (*Vitis vinifera* L.) in South Africa, as well as in most other grape-producing areas of the world (Carter, 1994). The disease severely reduces the productive lifespan of a vineyard, and this is reflected not only in the loss of yield, but also in the cost of reworking, removing and replanting such a vineyard (Munkvold *et al*., 1994; Wicks & Davies, 1999; Van Niekerk *et al*., 2003). *Eutypa lata* is commonly associated with symptoms like stunted, zigzag shoot growth and dieback of arms or even entire vines. Although symptom expression differs considerably between cultivars, leaves on the affected shoots are small and usually yellow, cupped, tattered, speckled, and often dead around the margins (Carter, 1994). Flower clusters on stunted shoots are normal, but shrivel and die on severely affected shoots. Bunches that appear normal at the beginning of the season may also shrivel and die (Creaser & Wicks, 2000). Yield reduction is primarily due to a diminished number of clusters per vine (Munkvold *et al*., 1994). Reduced wine quality may also occur due to uneven berry maturity on infected vines (Wicks & Davies, 1999).

Infection occurs when ascospores of the fungus enter pruning wounds (Moller & Kasimatis, 1978). Ascospores are released from perithecia one or two hours after the onset of as little as 2 mm of rain (Pearson, 1980; Trese *et al*., 1980). This release will continue as long as it rains, but a period of depletion, a so-called "exhaustion phenomenon", might occur after prolonged release (Petzoldt *et al*., 1983). The ascospores germinate and grow into the wood below the wound, eventually causing progressive dieback symptoms of the plant. Various studies have investigated the susceptibility of pruning wounds to Eutypa infections, and it is generally accepted that susceptibility is dependent on the time of pruning and the age of the pruning wound. Pruning wounds made early in the dormant season are much more susceptible and stay susceptible for much longer periods than pruning wounds made during the mid- and late dormancy periods (Petzoldt *et al*., 1981; Trese *et al*., 1982; Munkvold & Marois, 1995; Chapuis *et al*., 1998; John *et al*., 2005). The decrease in susceptibility is correlated with an increase in suberin and lignin deposition and with degree-day accumulation (Munkvold & Marois, 1995). Populations of non-pathogenic microorganisms on wound surfaces are also more active and increase more rapidly during the mid- and late dormancy periods (Munkvold & Marois, 1995; Chapuis *et al*., 1998). Some of these microorganisms, which can occur naturally on grapevine pruning wounds, may have the ability to reduce *E. lata* infections (Ferreira *et al*., 1991; Munkvold & Marois, 1993b; Schmidt *et al*., 2001). Furthermore, sap flow from pruning wounds, which

*Corresponding author: halleenf@arc.agric.za [Fax: +27 21 8093002]

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often occurs around budbreak, might affect pathogen infections by means of flushing (Munkvold & Marois, 1995; John *et al*., 2005). The exudate also contains carbohydrates, amino acids and organic acids that may promote rapid growth of beneficial microflora in pruning wounds, which in turn increase competition with pathogens such as *E. lata* (Munkvold & Marois, 1995). Due to these factors, it is recommended that pruning be done later in the dormant season.

Various studies have shown that the age of the wood pruned (i.e. one-, two- or three-year-old wood) does not influence susceptibility (Trese *et al*., 1980; Petzoldt *et al*., 1981; Trese *et al*., 1982; Munkvold & Marois, 1995). Furthermore, artificial inoculations showed that wound size and relative position of a wound on the vine did not influence infection (Petzoldt *et al*., 1981). It therefore is clear that all pruning wounds should be protected for an extended period after pruning.

Several field trials have been conducted in the USA to demonstrate the potential of benomyl as a pruning wound protectant against Eutypa infections, either applied as a paint (Moller & Kasimatis, 1980; Pearson, 1982; Gendloff *et al*., 1983; Munkvold & Marois, 1993a), by means of a pneumatic sprayer-pruning shear (Munkvold & Marois, 1993a) or with an air-blast sprayer (Ramsdell, 1995). Benomyl has been registered in the USA as a paint application at 25 g/L for this purpose since 1976. Despite these applications of Benomyl, the incidence of Eutypa dieback was still of great concern in California and questions arose regarding its efficacy (Munkvold & Marois, 1993a). Munkvold and Marois (1993a) identified flusilazole as a possible alternative to benomyl. The literature regarding the efficacy of these two fungicides varies greatly. Munkvold and Marois (1993a) observed that both fungicides were very effective in field experiments, although flusilazole was the only fungicide that could protect pruning wounds against infection 14 days after treatment. In contrast, Creaser and Wicks (2002) found that benomyl (and certain wound sealants) were the only treatments that could protect pruning wounds against infection 14 days after treatment in Australia. Subsequent trials conducted by Sosnowski *et al*. (2004) confirmed these results. However, the registration of benomyl has recently been withdrawn in the USA and it is no longer available in most countries. Sosnowski *et al*. (2004, 2008) evaluated carbendazim, another benzimidazole fungicide, and found it to be as effective as benomyl and that it could protect pruning wounds even if the treated wounds were challenged with Eutypa 14 days after application.

Rainfall has a considerable effect on fungicide effectiveness. Munkvold and Marois (1993a) even recommended that a second application be applied if rainfall occurs shortly after fungicide application. The ideal would be a treatment that could be effective regardless of environmental conditions. Biological control agents that colonise pruning wounds and render prolonged protection despite environmental factors would therefore be of great benefit. Furthermore, environmental protection has also come to the fore in recent years, increasing the demand for biological control agents. Ferreira *et al*. (1991) found a *Bacillus subtilis* isolate that strongly inhibited *E. lata*. However, despite its efficacy in field trials, the isolate was never commercialised due to the lack of market interest at the time. Promising results were also obtained with *Fusarium lateritium* (Munkvold & Marois, 1993b; John *et al*., 2005), as well as *Cladosporium herbarum* (Munkvold & Marois, 1993b). McMahan *et al*. (2001) conducted bioassays with a benomyl-resistant *Fusarium lateritium* strain obtained through UV mutagenesis, and suggested that it could be applied in combination with benomyl at 1 000 µg/mL.

Trichoderma-based products are registered in New Zealand for protection against Eutypa infections (Hunt, 1999). The so-called Trichoprotection® range includes Trichodowels™, Trichojet™, Trichoseal™, Trichoseal-Spray™ and Vinevax™, comprising several *T. harzianum* and *T. atro-viride* strains. Creaser and Wicks (2002) could not prevent *E. lata* infections with Trichoseal-Spray™. However, this study was only conducted during one season and very low levels of Eutypa infection were obtained through inoculations. Subsequent studies conducted over three seasons on three cultivars with Trichoseal-Spray™ and Vinevax™, a replacement product with an identical base formulation, recorded significant reductions in Eutypa infections (John *et al*., 2005). Volatile and non-volatile metabolites produced by *T. harzianum* strains AG1, AG2 and AG3, three of the components of the Trichoprotection® range, reduced the growth of *E. lata in vitro* (John *et al*., 2004). Co-inoculation of *E. lata* and *T. harzianum* strain AG1 in grapevine wood resulted in changes to the integrity of *E. lata* hyphae, including abnormal swellings and collapsed and shrivelled hyphae. Parallel growth and coiling were also observed, which might indicate mycoparasitic activity (John *et al*., 2005). John *et al*. (2008) also demonstrated that *T. harzianum* colonised and persisted in grapevine wood for 20 months and that it had the potential to protect vines from infection by *E. lata*.

Boric acid, formulated as Biopaste (5% boric acid) and Bioshield (5% boric acid formulated in a spore suspension of *C. herbarum*), has been tested in California as a possible replacement for benomyl. Although both products significantly reduced Eutypa infections, bud failure located at the first node below the pruning wound was associated with these treatments (Rolshausen & Gubler, 2005). Formulations of boric acid must therefore be optimised before they can be recommended as safe alternatives.

Despite Eutypa being a disease of major economic importance in the Western Cape Province of South Africa (Halleen *et al*., 2001), most producers do not apply any form of pruning-wound protection. This lack of protection is furthermore compounded by the fact that no fungicide is registered for the control of this disease in local vineyards (Nel *et al*., 2003) and no fungicide has ever been tested for the protection of pruning wounds under vineyard conditions. Moreover, at the onset of this study, several *Trichoderma* products were marketed as grapevine pruning wound protectants, but these products were not registered and nor has their efficacy been determined under local conditions. The aim of this study was therefore to evaluate the *in vitro* efficacy of fungicides from various chemical groups against *E. lata*, as well as the *in vivo* efficacy of the most effective fungicides, *Bacillus subtilis* (Ferreira *et al*., 1991) and selected *Trichoderma* products, in grapevine pruning wound protection trials on grapevines subjected to artificial inoculation, as well as natural infection.

MATERIALS AND METHODS

In vitro evaluation of fungicides

Twelve fungicides were screened *in vitro* for mycelial inhibition of 12 *E. lata* isolates according to the method of Munkvold and

Marois (1993a). The fungicides were benomyl (Benlate 500 WP; Dow Agrosciences Southern Africa PTY, Silverton, South Africa), flusilazole (Olymp 100 EW; Du Pont, Halfway House, South Africa), myclobutanil (Systhane 20 EW; Dow Agrosciences Southern Africa PTY), tebuconazole (Folicur 250 EW; Bayer Cropscience, Isando, South Africa), fenarimol (Rubigan 12% EC; Dow Agrosciences Southern Africa PTY), trifloxystrobin (Flint 50 WG; Bayer Cropscience), kresoxim-methyl (Stroby WG 500 g/kg; BASF South Africa PTY, Halfway House, South Africa), azoxystrobin (Quadris 50 WG; Syngenta, Halfway House, South Africa), spiroxamine (Prosper 500 EC; Bayer Cropscience), fenhexamid (Teldor 500 SC; Bayer Cropscience), mancozeb (Penncozeb WG 750 g/kg; BASF South Africa PTY) and pyrimethanil (Scala SC 400 g/L; Bayer Cropscience).

The *E. lata* isolates were obtained from perithecia found on diseased vines originating from vineyards in the Stellenbosch, Durbanville, Somerset West, Paarl and Wellington areas of the Western Cape Province, South Africa. Isolates were stored on potato-dextrose agar (PDA, Biolab, Midrand, Johannesburg) slants at 4°C in the culture collection at ARC Infruitec-Nietvoorbij (Stellenbosch, South Africa) and transferred to PDA in Petri dishes for propagation when needed. The Petri dishes were subsequently incubated at 23°C for one week, at which time there was sufficient growth to transfer it to fungicide-amended media. The fungicides were suspended in sterile distilled water and added to molten (± 50°C) PDA in sufficient quantities to achieve final concentrations of 0, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 µg/mL. Mycelium plugs (4 mm in diameter), obtained from the margins of actively growing cultures, were transferred to fungicide-amended plates. Three mycelium plugs (three different isolates) were placed equidistant from each other on each plate. There were three replicates of each fungicide concentration, and the experiment was repeated. Concentrations of 50.0 and 100.0 µg/ml were added for the less effective fungicides when the experiment was repeated. The dishes were incubated for five days at 23°C (12 h light per day), after which the diameter of each colony was measured twice perpendicularly.

The experimental design was completely randomised with a 12 × 12 × 8 factorial and three random replications. The factors were 12 isolates, 12 fungicides and eight concentrations. The percentage inhibition was calculated as follows: 100 × [(colony diameter on fungicide-amended plate – 4 mm) – (colony diameter of the control – 4 mm)]/(colony diameter on fungicide-amended plate – 4 mm). The percentage inhibition data of both experiments was pooled and linear regressions were fitted to concentrations for each isolate and fungicide separately, after the extreme tail points had been deleted. The equations fitted were as follows: percentage inhibition = a + bx (a = intercept, b = slope and x = fungicide concentration). The concentration at which mycelial growth was inhibited by 50% (EC_{50} value) was calculated as follows: EC_{50} = b/(50-a). The EC_{50} values and the rate of change (slopes) were subjected to analysis of variance and Student's t-LSD (least significant difference) was calculated at a 5% significance level to compare fungicide means (SAS, 1990). The Shapiro-Wilk test was performed to test normality on residuals (Shapiro & Wilk, 1965). Outliers were discarded until the residuals were normally distributed.

Field experiments

Trial one

Two Cabernet Sauvignon vineyards, located in the Durbanville and Stellenbosch areas respectively, were identified for the field experiment. The incidence of Eutypa dieback is relatively high in both these areas due to environmental conditions favouring disease development (Halleen *et al*., 2001). The vineyards were eight and 10 years old at the time of the first season's experiment.

The fungicides benomyl and flusilazole proved to be the most effective fungicides in the *in vitro* evaluation and were included in the field experiments. The fungicides were applied at 12 500 µg active ingredient/mL according to Munkvold and Marois (1993a). Several potential biological control agents/products were also included. Isolates of *B. subtilis* (Isolate EE, ARC Infruitec-Nietvoorbij, South Africa; Ferreira *et al*., 1991) and *T. harzianum* (Isolate T77; ARC Infruitec-Nietvoorbij, South Africa – subsequently registered as Eco77, Plant Health Products PTY Ltd., Nottingham Road, South Africa), which previously were proven to be antagonistic to *E. lata*, were included. T77 was applied with (+) and without (-) 1% Bio-Stabiliser (Agro-Organics, Strand, South Africa) to investigate the potential benefits of Bio-Stabiliser, a sticker. *Bacillus subtilis* (with 1% peptone and 1% sucrose; Ferreira *et al*., 1991) and T77 were applied at 10^8 and 10^6 spores/mL respectively. Two commercially available products containing *T. harzianum*, namely Trichoseal-Spray (Agrimm Technologies Ltd, New Zealand) and Bio-Tricho (Agro-Organics, Strand, South Africa), were also included. These products were applied according to manufacturer's specifications (Trichoseal-Spray at 10 g/L; Bio-Tricho at 4 g/L).

Both vineyards were pruned in August 2001 and 2002 (two buds/spur). Fresh pruning wounds were spray-treated with chemical and biological control agents immediately after pruning by means of ordinary household hand-held trigger spray canisters (Munkvold & Marois, 1993a). Ten plants were used for each treatment and five pruning wounds were treated on each plant. The control plants were treated with distilled water only (inoculated control). Treated pruning wounds were inoculated with 1 000 *E. lata* ascospores in a 50-µL droplet of sterile distilled water 24 hours after the treatments had been applied to fresh wounds. An uninoculated control treatment was also included to determine the levels of natural infection. The trial design was a randomised block design.

The efficacy of treatments to prevent *E. lata* infection was determined by making isolations from each of the treated pruning wounds 12 months after treatment (Munkvold & Marois, 1993a). Spurs were removed in the vineyard by means of hand pruning shears and immediately taken to the laboratory for surface sterilisation (30 s in 70% ethanol, 5 min in 0.35% sodium hypochlorite and 30 s in 70% ethanol) before isolations were made. Spur sections were split longitudinally to reveal the xylem and pith regions. Twelve pieces of tissue (approximately 1 × 1 mm in size) were aseptically removed with a scalpel from the interface between apparently healthy and discoloured xylem tissue in each pruning wound and placed in Petri dishes containing 2% potato dextrose agar (PDA) and 250 mg/L chloramphenicol. Dishes were incubated in an incubation growth room at ± 23°C. Fungal and bacterial growth from plated tissue pieces was monitored daily for a period of four weeks. The presence of *E. lata* and other pruning wound

invaders (*Phaeomoniella chlamydospora*, Botryosphaeriaceae and *Phomopsis* spp.) was noted. Fungal identification was based on colony characteristics and microscopic morphology.

Trial two

A second trial was conducted with only two biological control agents, Eco77 and Vinevax [Agrimm Technologies Ltd, New Zealand (Vinevax replaced Trichoseal-Spray, but has identical base formulation)]. The treated pruning wounds were not inoculated with *E. lata* as was the case in trial one.

Four vineyards were selected, namely two wine grape cultivars, Cabernet Sauvignon and Sauvignon blanc located in the Simondium area, and two table grape cultivars, Red Globe and Bonheur located in the Wellington area. The vineyards were five, nine, eight and seven years old respectively, and were visually free of Eutypa symptoms. As the incidence of Eutypa dieback in these areas is normally relatively high, climatic conditions were considered to favour disease development.

The vines were pruned in August 2005 and 2006. The Cabernet Sauvignon and Sauvignon blanc vines were pruned to two buds per cane, while the Red Globe and Bonheur vines were pruned to eight buds per cane. The pruning wounds were treated with the biological control agents immediately after pruning with a hand-held trigger spray canister. The trial consisted of three treatments: Vinevax (10 g/L), Eco77 (0.5 g/L) and a distilled water control with 10 replications per treatment. A replication consisted of a row-unit of four to six plants, depending on the vineyard. Although all the pruning wounds of a row-unit were treated, only two spurs per plant (one on each cordon arm) were used for further evaluation. Only the four middle vines per row-unit were used for further evaluations. Thus each treatment was applied on 80 pruning wounds in each of the four trial vineyards. The trial design was a randomised block design.

Evaluation after seven months was done exactly as described in trial one, by plating eight 1 × 1 mm pieces of dissected wood onto PDA in Petri dishes. The incidence of fungi present in each pruning wound was determined as a percentage of the total number of pruning wounds colonised. Complete split-split-plot analyses were performed, with cultivar as the main plot factor, year as subplot factor and treatment as sub-sub-plot factor. Data were subjected to analyses of variance using SAS version 8.1 (SAS, 1990). Student's t-least significant difference values were calculated at the 5% confidence level to facilitate comparison between the treatment means.

RESULTS

In vitro evaluation of fungicides

The growth rates of the 12 isolates were similar and no fungicide x isolate interaction was observed. Flusilazole, tebuconazole, benomyl, fenarimol and myclobutanil were the most effective fungicides, with EC_{50} values of 0.005, 0.01, 0.19, 0.29 and 1.48 µg/mL respectively. The hydroxy-analide fenhexamid, and strobilurin fungicides trifloxystrobin, kresoxim-methyl and azoxystrobin, were the least effective, with EC_{50} values > 98 µg/mL (Table 1).

Benomyl proved to be the most effective fungicide based on the rate of change in EC_{50} for a 1% increase in concentration (178.0%; Table 1), and would therefore be very effective at low concentrations. Compared to benomyl, flusilazole and tebuconazole had significantly lower rates of change (53.97% and 32.10% respectively), although these were significantly higher than the other fungicides (rates of change less than 16.46%).

TABLE 1

Sensitivity of 12 *Eutypa lata* isolates to different fungicides (*in vitro*).

Fungicide	EC_{50} value (µg/mL) [a]	Rate of change[b]
Flusilazole	0.01 [a]	53.97 [b]
Tebuconazole	0.01 [a]	32.10 [c]
Benomyl	0.19 [a]	178.0 [a]
Fenarimol	0.29 [a]	8.72 [de]
Myclobutanil	1.48 [a]	16.46 [d]
Pyrimethanil	4.54 [b]	6.59 [de]
Spiroxamine	5.33 [b]	8.01 [de]
Mancozeb	22.36 [c]	1.34 [e]
Fenhexamid	98.65 [d]	0.36 [e]
Azoxystrobin	100.0 [d]	0.22 [e]
Trifloxystrobin	99.76 [d]	0.20 [e]
Kresoxim-methyl	100.0 [d]	0.17 [e]
LSD ($P = 0.05$)	1.482	12.940

[a] Values within each column followed by the same letter do not differ significantly ($P = 0.05$).

[b] Effective rate of change in EC_{50} value for a 1% increase in concentration.

Field experiments

Trial one

Analysis of variance of percentage incidence data of *E. lata*, *Pa. chlamydospora*, Botryosphaeriaceae, *Phomopsis* and *Trichoderma* spp. isolated from pruning wounds showed no significant season × treatment interaction ($P > 0.05$, ANOVA not shown). Significant effects for treatment were evident for *E. lata* ($P < 0.0001$), *Phomopsis* ($P = 0.0458$), *Trichoderma* ($P < 0.0001$) and *Pa. chlamydospora* ($P = 0.0815$), but not for Botryosphaeriaceae ($P = 0.6026$). These effects will be discussed, and the incidences of these fungi in pruning wounds as they were affected by the various treatments are summarised in Table 2.

Eutypa lata: *E. lata* was isolated from 48.5% of the pruning wounds of the inoculated control treatment (Table 2). The benomyl (5.0%) and flusilazole (5.5%) treatments effected the lowest *E. lata* incidences and, compared to the inoculated control, reduced infection by 89.7% and 88.7% respectively. The incidence of *Eutypa lata* in the *Bacillus* and Bio-Tricho treatments (45.5% and 39.0% respectively) did not differ significantly from the inoculated control treatment. The T77(+), T77(-) and Trichoseal-Spray treatments effected significantly lower incidences (34.0%, 28.5% and 28.5% respectively). Natural infection was very low, with *E. lata* isolated from only 2% of the pruning wounds on untreated, uninoculated control plants.

TABLE 2

Incidence (mean percentage) of the pruning wound invaders *Eutypa lata*, Botryosphaeriaceae spp., *Phaeomoniella chlamydospora*, *Phomopsis* spp. and the biological control agent *Trichoderma* isolated[x] from Cabernet Sauvignon pruning wounds treated with various chemical and biological treatments directly after pruning[y] and inoculated with 1 000 *E. lata* ascospores one day later (Trial 1).

Treatment	Incidence of pruning wound invaders[z]				
	Eutypa lata	***Botryosphaeriaceae* spp.**	***Phaeomoniella chlamydospora***	***Phomopsis* spp.**	***Trichoderma***
Control (inoculated)	48.5 [a]	12.0 [a]	14.5 [ab]	29.0 [a]	0.0 [b]
Bacillus subtilis	45.5 [ab]	10.0 [a]	11.0 [ab]	37.0 [a]	0.0 [b]
Bio-Tricho	39.0 [abc]	10.0 [a]	13.5 [ab]	27.5 [ab]	12.0 [b]
T77 (+ Bio-Stabiliser)	34.0 [bc]	11.5 [a]	20.0 [a]	31.0 [a]	38.0 [a]
T77 (- Bio-Stabiliser)	28.5 [c]	9.0 [a]	13.5 [ab]	30.5 [a]	29.5 [a]
Trichoseal-Spray	28.5 [c]	11.0 [a]	12.5 [ab]	34.0 [a]	45.0 [a]
Flusilazole	5.5 [d]	9.5 [a]	4.5 [b]	17.5 [b]	0.0 [b]
Benomyl	5.0 [d]	13.0 [a]	3.5 [b]	27.0 [ab]	0.0 [b]
Control (uninoculated)	2.0 [d]	18.5 [a]	19.5 [a]	37.5 [a]	0.0 [b]
LSD ($P = 0.05$)	12.07	9.60	11.40	11.17	15.94

[x] Isolations made during July/August 2002 and 2003.
[y] Vineyards hand pruned in August 2001 and 2002.
[z] Values within each column followed by the same letter do not differ significantly ($P = 0.05$).

Botryosphaeriaceae: The mean incidence of Botryosphaeriaceae in the untreated, uninoculated control pruning wounds (i.e. natural infection) was 18.5% (Table 2). Incidences in the treated wounds were slightly lower (9.0% to 13%), but, as mentioned above, none of the treatments caused a significant effect ($P = 0.6026$).

Phaeomoniella chlamydospora: *Pa. chlamydospora* was isolated from a mean of 19.5% of the untreated, uninoculated control pruning wounds. Incidences in the pruning wounds treated with benomyl and flusilazole (3.5% and 4.5% respectively; Table 2) were significantly lower compared to the untreated, uninoculated control.

Phomopsis spp.: *Phomopsis* spp. were commonly isolated from untreated, uninoculated control plants (37.5%; Table 2), and flusilazole was the only treatment that brought about statistically lower incidences (17.5%).

Trichoderma spp.: *Trichoderma* spp. were only isolated from plants treated with *Trichoderma* products (Table 2), and incidences were highest in pruning wounds treated with Trichoseal-Spray, T77(+) and T77(-) (45.0%, 38.0% and 29.5% respectively). The incidence of *Trichoderma* was significantly lower in wounds treated with Bio-Tricho (12.0%).

Trial two

Incidence of Trichoderma in pruning wounds

According to the analysis of variance for the effect of treatment, cultivar and season on the percentage incidence of *Trichoderma*, a three-factor interaction occurred (Cultivar × Year × Treatment, $P = 0.018$, ANOVA not shown). A further analysis was done with the cultivars and seasons separate. From this analysis, clear differences between treatments were observed for all the cultivars and in both seasons ($P \leq 0.0001$, ANOVA not shown). There was a large difference in the incidence of *Trichoderma* in the pruning wounds (Table 3). The incidence of *Trichoderma* was quite high in pruning wounds that were treated with Trichoderma-based products (20–76%), while no *Trichoderma* occurred in the control plants, except in the Cabernet Sauvignon vineyard during the 2005/2006 season, when it occurred in three pruning wounds. During the 2005/2006 season, the incidence of *Trichoderma* was significantly higher in the Vinevax treatments, except in Red Globe, where the incidences were similar. The incidence of *Trichoderma* in the Vinevax and Eco77 treatments was similar during the 2006/2007 season, except for Red Globe, where *Trichoderma* occurred significantly more in the Vinevax treatments (Table 3).

Incidence of pathogens in pruning wounds

The incidence of the most important pruning wound pathogens (*E. lata*, Botryosphaeriaceae *spp.*, *Pa. chlamydospora*, *Phomopsis spp*. and *Phaeoacremonium spp*.) was determined and calculated as a "total pathogen count". Analysis of variance for the effect of treatment, cultivar and season on the percentage incidence of *E. lata*, *Pa. chlamydospora*, *Phaeoacremonium spp.,* Botryosphaeriaceae, *Phomopsis* and total pathogens isolated from pruning wounds showed no significant Cultivar × Year × Treatment interactions ($P > 0.05$, ANOVA not shown), except for *E. lata* ($P = 0.0446$). Significant effects for treatment were evident for *Phaeoacremonium* spp. ($P = 0.0233$, ANOVA not shown) and total pathogens ($P = 0.0488$), but not for *Pa. chlamydospora* ($P = 0.7055$), *Botryosphaeriaceae* ($P = 0.2141$) and *Phomopsis* ($P = 0.2150$). These effects will be discussed and the incidences of these fungi in the pruning wounds as they were affected by the various treatments are summarised in Tables 4 and 5.

TABLE 3

Mean incidence of *Trichoderma* spp. isolated[x] from pruning wounds treated with Vinevax and Eco77 directly after pruning[y] (Trial 2).

Cultivar	Treatment	*Trichoderma* incidence (%)[z]	
		2005-2006	2006-2007
Sauvignon blanc	Control	0.0 [c]	0.0 [b]
	Vinevax	67.5 [a]	56.3 [a]
	Eco77	38.8 [b]	48.8 [a]
	LSD (P = 0.05)	12.32	18.63
Cabernet Sauvignon	Control	5.0 [c]	0.0 [b]
	Vinevax	76.3 [a]	63.8 [a]
	Eco77	47.5 [b]	68.8 [a]
	LSD (P = 0.05)	19.35	10.08
Bonheur	Control	0.0 [c]	0.0 [b]
	Vinevax	70.0 [a]	45.0 [a]
	Eco77	25.0 [b]	37.5 [a]
	LSD (P = 0.05)	14.89	14.89
Red Globe	Control	0.0 [b]	0.0 [c]
	Vinevax	41.3 [a]	32.5 [a]
	Eco77	37.5 [a]	20.0 [b]
	LSD (P = 0.05)	17.23	12.26

[x] Isolations April 2006 and 2007.
[y] Pruning 29 Augustus 2005 and 15 August to 1 September 2006.
[z] For each cultivar, values within each column followed by the same letter do not differ significantly (P = 0.05).

TABLE 4

Mean incidence of the pruning wound invader *Eutypa lata* isolated[x] from pruning wounds treated with Vinevax and Eco77 directly after pruning[y] (Trial 2).

Cultivar	Treatment	*Eutypa lata* incidence (%)[z]	
		2005-2006	2006-2007
Sauvignon blanc	Control	0.0 [a]	2.5 [a]
	Vinevax	0.0 [a]	0.0 [a]
	Eco77	1.3 [a]	0.0 [a]
	LSD (P = 0.05)	2.14	2.86
Cabernet Sauvignon	Control	0.0 [a]	0.0 [a]
	Vinevax	0.0 [a]	1.3 [a]
	Eco77	0.0 [a]	1.3 [a]
	LSD (P = 0.05)	0.0	3.12
Bonheur	Control	0.0 [a]	1.3 [a]
	Vinevax	0.0 [a]	0.0 [a]
	Eco77	0.0 [a]	0.0 [a]
	LSD (P = 0.05)	0.0	2.14
Red Globe	Control	0.0 [a]	11.3 [a]
	Vinevax	0.0 [a]	0.0 [b]
	Eco77	0.0 [a]	1.3 [ab]
	LSD (P = 0.05)	0.0	10.77

[x] Isolations April 2006 and 2007.
[y] Pruning 29 Augustus 2005 and 15 August – 1 September 2006.
[z] For each cultivar, values within each column followed by the same letter do not differ significantly (P = 0.05).

Eutypa lata: The analysis of cultivars and seasons separately did not reveal any differences between treatments for any of the cultivars during both seasons, except for Red Globe during the 2006/2007 season (P = 0.0813, ANOVA not shown), where Vinevax caused a significant reduction (Table 4). Eco77 also reduced Eutypa in Red Globe (1.3%), although it did not differ significantly from the control (11.3%).

Botryosphaeriaceae: The mean incidence in the untreated, control pruning wounds was 9.4% (Table 5), but none of the treatments caused a significant reduction.

Phaeomoniella chlamydospora: The mean incidence in the untreated control pruning wounds was 8.9% (Table 5), but none of the treatments caused a significant reduction.

Phaeoacremonium spp.: Despite the relatively low incidence (0.8%, Table 5) in the untreated control pruning wounds, both treatments caused a significant reduction compared to the untreated control.

Phomopsis spp.: The mean incidence in the untreated control pruning wounds was 8.1% (Table 5), but none of the treatments caused a significant reduction.

Total pathogens: The mean total pathogen incidence in the untreated, control pruning wounds was 24.8% (Table 5). Eco77 was the only treatment that differed from the untreated control and reduced infection by 30%.

DISCUSSION

One of the objectives of this study was to compare the efficacy of benomyl with newer fungicides with different modes of action. However, the *in vitro* results clearly emphasise the efficacy of benomyl. Furthermore, resistance to benomyl was not detected in this study, despite the fact that it had been applied to pruning wounds for several years in at least one of the vineyards from which isolates were obtained. Except for the sterol demethylation inhibitor (DMI) fungicides, none of fungicides from the newer chemical classes showed any promise *in vitro*. It was therefore decided to include flusilazole in the field evaluations. Benomyl is still registered in South Africa for the control of botrytis (*Botrytis cinerea*) and powdery mildew (*Erysiphe necator*), whilst flusilazole is registered for the control of powdery mildew (Nel *et al*., 2003). However, benomyl has recently been withdrawn from the world market and flusilazole is not registered as a pruning wound protectant in South Africa. In recent years, the big question regarding the long-term efficacy of chemical products, especially during extended periods of rainfall, has also come to the fore. Kotze (2008) showed that the inoculation of benomyl-

TABLE 5

Mean incidence of the pruning wound pathogens Botryosphaeriaceae spp., *Phaeomoniella chlamydospora, Phaeoacremonium spp.*, *Phomopsis* spp. and total pathogen isolated[x] from pruning wounds treated with Vinevax and Eco77 directly after pruning[y] (Trial 2).

Treatment	Incidence of pruning wound invaders[z]				
	Botryosphaeriaceae spp.	*Phaeomoniella chlamydospora*	*Phaeoacremonium* spp.	*Phomopsis* spp.	Total pathogen
Control	9.4 [a]	8.9 [a]	0.8 [a]	8.1 [a]	24.8 [a]
Vinevax	6.9 [a]	9.1 [a]	0.0 [b]	6.1 [a]	20.9 [ab]
Eco77	6.3 [a]	7.7 [a]	0.2 [b]	5.0 [a]	17.3 [b]
LSD ($P = 0.05$)	3.70	3.64	0.59	3.56	5.97

[x] Isolations April 2006 and 2007.
[y] Pruning 29 Augustus 2005 and 15 August – 1 September 2006.
[z] Values within each column followed by the same letter do not differ significantly ($P = 0.05$).

treated pruning wounds with Eutypa ascospores seven days after treatment drastically reduced the efficacy of the chemical. As pruning wounds can remain susceptible to infection for long periods (three to four weeks) after pruning (Van Niekerk, 2008), a biological control agent that can colonise pruning wounds and provide long-term protection against infection in spite of climatic conditions would thus be ideal.

The results of the first field trial indicated that the two fungicides benomyl and flusilazole were the most effective treatments against Eutypa. *Bacillus subtilis* was not effective at all. The reasons for this are not known. Ferreira *et al*. (1991) applied *B. subtilis* to wounds on two-year-old canes, inoculated them with an *Eutypa* spore suspension after four hours and then covered the wounds with aluminium foil, whilst the current trials were conducted on the wounds of one-year-old canes and left open. It is possible that the ascospores were affected by antibiotics produced by the *Bacillus*. Ferreira *et al*. (1991) identified at least two antibiotic substances that were responsible for the inhibition of mycelial growth and ascospore germination. In a recent study, Kotze (2008) dual incubated (*in vitro*) *E. lata* with the same isolate and showed that *E. lata* displayed little mycelium growth and a clear inhibition zone between the cultures. Malformation of the hyphae, specifically swelling, was observed at a microscopic level. One-year-old pruning wounds treated with this *Bacillus*, challenged with *E. lata* seven days later and evaluated after eight months showed a significant reduction in *E. lata* incidence (10.7%) compared to the inoculated control (37.5%).

Although the *Trichoderma* treatments were less effective than the fungicides in the first field trial, T77 and Trichoseal-Spray were able to colonise and survive in pruning wounds and cause significant reduction in *E. lata* infections. To what extent this colonisation might prevent or inhibit later infections is uncertain at this stage. The addition of Bio-Stabiliser, a sticker, to T77 did not increase *Trichoderma*'s ability to colonise pruning wounds and did not increase the inhibition of Eutypa infections. Colonisation by Bio-Tricho was very low and could explain in part why it was less effective than the other Trichoderma formulations. Mutawila (2010) found a positive correlation between *Trichoderma* incidence and pathogen reduction. The inoculum dosage could be adjusted in an effort to obtain better colonisation. John *et al*. (2005) found that *Trichoderma harzianum* and *Fusarium lateritium* were much more effective when inoculation with *E. lata* was delayed until 14 days after the wood colonisers were applied, indicating that biocontrol agents might require a period to colonise the wound surface. Similar results were obtained by Munkvold and Marois (1993b) when investigating *F. lateritium* and *C. herbarum*. The artificially high inoculum doses used in the field evaluations would almost always favour chemicals when compared directly to biological agents. Under natural conditions, in which *E. lata* infections are much lower (for example 2% in trial 1), biological control agents might be more effective in protecting wounds against pathogen infections.

Various pathogens (*E. lata*, Botryosphaeriaceae spp., *Phomopsis* spp. and *Pa. chlamydospora*), all of which are able to infect pruning wounds and eventually cause various trunk diseases, naturally infected plants during the course of this study. Although the primary aim of this study was to protect pruning wounds against *E. lata* infections, it is clear that control strategies will have to take these pathogens into consideration as well. The efficacy of the various treatments used in this study against Botryosphaeriaceae, *Phomopsis* and *Pa. chlamydospora* could not be determined in trial 1, because additional pruning wounds were not treated and then inoculated with these fungi, as was done in the case of *E. lata*. However, compared to natural infections that occurred in the pruning wounds of the untreated, uninoculated control plants, benomyl and flusilazole reduced *Pa. chlamydospora* incidence by 82.1% and 76.9% respectively, whilst flusilazole reduced *Phomopsis* incidence by 53.3%.

The second field trial was undertaken to investigate the efficacy of two Trichoderma products subjected to natural infection only. Relying on natural infection is a risk, since infection levels might be extremely low or even absent. Despite these constraints, clear evidence was obtained to show that Trichoderma products were able to significantly reduce pathogens in pruning wounds. However, the results varied between seasons and cultivars. Vinevax was able to reduce Eutypa significantly in Red Globe during the 2006/2007 season, while Eco77 significantly reduced the total pathogen count. However, one might speculate that some of these pathogens, especially Botryosphaeriaceae and *Phomopsis*, might already be present in the one-year-old cane that is being pruned. Isolation studies investigating the presence of pathogens in newly pruned canes during the 2006/2007 season

showed that Botryosphaeriaceae and *Phomopsis*, and to an lesser extent *Pa. chlamydospora*, were in fact already present in some of the pruned canes, while *E. lata* did not occur in any of the pruned shoots (results not shown). Finally, this trial again illustrated the ability of Trichoderma to colonise and survive in grapevine pruning wounds.

CONCLUSION

Pruning wound infections, especially those caused by *E. lata*, are undoubtedly one of the most important factors that limit the productivity of vineyards in the Western Cape Province. Management strategies ,such as strict sanitation practices and the protection of pruning wounds, can be of great benefit to combat this disease.

Producers are urged to apply strict sanitation practices in order to lower inoculum levels. Infected grapevines, or infected parts thereof, should be removed and buried, burnt or composted. The incidence of disease is normally higher in vineyards where perithecia are found (Munkvold *et al*., 1993). In cases where only part of a plant is removed, it is essential that the wound be treated with a fungicide, biological control agent or wound sealant. Compost made from pruning debris is also a good option, because the composted material can be reintroduced to the vineyard without the fear of re-infection by trunk disease pathogens (Lecomte *et al*., 2006).

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