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Evaluation of fungicides as potential grapevine pruning wound protectants against *Botryosphaeria* species

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Abstract. Protection of wounds against infection by trunk disease pathogens is the most efficient and cost-effective means to prevent grapevine trunk diseases. Studies done to determine the effectiveness of chemical pruning wound protectants have mostly focused on the control of *Eutypa lata*. However, other important wound pathogens, such as *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp., *Phomopsis* spp. and species of Botryosphaeriaceae (including *Botryosphaeria* and aggregate genera), pose just as significant a threat to sustainable grape production. Fungicide sensitivity studies have been conducted for *Pa. chlamydospora*, *P. viticola* and *E. lata*. However, no such studies have been conducted for the pathogenic species of Botryosphaeriaceae from grapevines in South Africa. Ten fungicides were, therefore, tested *in vitro* for their efficacy on mycelial inhibition of the four most common or pathogenic species of Botryosphaeriaceae in South Africa, '*B.*' *obtusa*, *Neofusicoccum australe*, *N. parvum* and *Lasiodiplodia theobromae*. Iprodione, pyrimethanil, copper ammonium acetate, kresoxim-methyl and boscalid were ineffective in inhibiting the mycelial growth at the highest concentrations tested (20 μg/mL for copper ammonium acetate, 5 μg/mL for other agents tested). Benomyl, tebuconazole, prochloraz manganese chloride and flusilazole were the most effective fungicides with EC_{50} values for the different species ranging from 0.36–0.55, 0.07–0.17, 0.07–1.15 and 0.04–0.36 μg/mL, respectively. These fungicides, except prochloraz manganese chloride, are registered for use on grapes in South Africa and were also reported to be effective against *Pa. chlamydospora*, *P. viticola* and *E. lata.* Results from bioassays on 1-year-old Chenin Blanc grapevine shoots indicated that benomyl, tebuconazole and prochloraz manganese chloride were most effective in limiting lesion length in pruning wounds that were inoculated with species of Botryosphaeriaceae after fungicide treatment. The bioassay findings were, however, inconclusive due to low and varied re-isolation incidences. Benomyl, tebuconazole, prochloraz manganese chloride and flusilazole can be identified as fungicides to be evaluated as pruning wound protectants in additional bioassays and vineyard trials against species of Botryosphaeriaceae as well as the other grapevine trunk disease pathogens.

Introduction

Several pathogens are capable of causing decline and dieback associated with grapevine trunk diseases. These include *Eutypa lata*, *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp., *Phomopsis* spp. and species of Botryosphaeriaceae (including *Botryosphaeria* and generic aggregates formerly accommodated in the genus by Crous *et al*. (2006) (Ferreira *et al*. 1989; Mugnai *et al*. 1999; Groenewald *et al*. 2001; Mostert *et al*. 2001, 2005*a*; van Niekerk *et al*. 2004; van Niekerk *et al*. 2005*b*; Bester 2006). The occurrence of these trunk disease pathogens appear to be greatly affected by the prevailing climatic conditions (Merrin *et al*. 1995; Munkvold and Marois 1995; Mugnai *et al*. 1999; Larignon and Dubos 2000; Erincik *et al*. 2003; Copes and Hendrix 2004; Edwards and Pascoe 2004; van Niekerk *et al*. 2005*a*; Bester 2006) and management strategies in each area should either be specific with regard to pathogen incidence, or general, but in both cases effective against all trunk disease pathogens.

Wounds, especially pruning wounds, are regarded as primary infection sites for these pathogens (Ferreira *et al*. 1989; Mugnai *et al*. 1999; Larignon and Dubos 2000; Mostert *et al*. 2005*b*; van Niekerk *et al*. 2005*a*). At present, research on pruning wound protection has focused mainly on *E. lata* and is achieved by applying fungicides (Moller and Kasimatis 1980; Munkvold and Marois 1993*a*; Halleen *et al*. 2001; Sosnowski *et al*. 2004, 2005) or biological control agents (Carter and Price 1974; Ferreira *et al*. 1991; Munkvold and Marois 1993*b*; Hunt 2004) as pruning wound protectants. However, protection of pruning wounds against all the trunk disease pathogens that are present in a specific area is essential for effective prevention of these diseases. *In vitro* fungicide sensitivity studies have been performed for *E. lata*, *Pa. chlamydospora* and *P. viticola* only. Groenewald *et al*. (2000) showed that benomyl, fenarimol, prochloraz manganese chloride (mc) and tebuconazole inhibited mycelial growth of *Pa. chlamydospora* at low concentrations. These findings were complimented by Jaspers (2001), who also found pyrimethanil and a mixture of cyprodinil + fludioxonil to be effective against mycelium growth, and folpet and hydroxyquinoline sulfate against spore germination. Mostert *et al*. (2000) found flusilazole to be

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effective against *P. viticola* and studies done by Halleen *et al*. (2001) showed flusilazole, tebuconazole, benomyl and fenarimol to be effective in inhibiting *E. lata.* Fungicide evaluation trials conducted in Australian vineyards showed carbendazim to be the most effective pruning wound treatment in controlling *E. lata* infections (Sosnowski *et al*. 2004). Halleen and Fourie (2005) reported similar findings with benomyl in South African vineyards, showing that not only benomyl, but also flusilazole limited natural infections by *Pa. chlamydospora*, *P. viticola* and species of Botryosphaeriaceae. The specific identity of the species of Botryopshaeriaceae was, unfortunately, not determined.

In a recent survey of table grape vineyards in South Africa, it was shown that '*B.*' *obtusa*, *Neofusicoccum parvum* ('*B.*' *parva*) and *Lasiodiplodia theobromae* ('*B.*' *rhodina*) appeared to be the most common species of Botryosphaeriaceae, but their incidence in climatically different regions varied from prevalent to absent (Bester 2006; Bester *et al*. 2006). van Niekerk *et al*. (2004) demonstrated that *N. australe*, *N. parvum* and, to a lesser extent *L. theobromae* and '*B.*' *obtusa* (this species will formally be known under its *Diplodia* name, which still needs to be resolved, and hence it is referred to in inverted commas), were pathogenic on grapevine. No fungicides have been evaluated in South Africa against these species of Botryosphaeriaceae. In Australia, Savocchia *et al*. (2005) demonstrated the *in vitro* efficacy of tebuconazole, flusilazole, spiroxamine and fluazinam against mycelial growth of '*B.*' *obtusa* and *N. luteum*. EC_{50} values for tebuconazole were 0.01 mg/L for both species and for flusilazole, values were 0.46 and 0.3 mg/L for '*B.*' *obtusa* and *N. luteum*, respectively. EC_{50} values for spiroxamine were 0.06 and 0.38 mg/L for *B. obtusa* and *B. lutea*, respectively, and 0.01 mg/L for fluazinam for both species. The fungicides were, however, not tested against *N. australe*, *N. parvum* and *L. theobromae*.

The aim of this study was, therefore, to determine the *in vitro* efficacy of selected fungicides against the most important species of Botryosphaeriaceae, '*B.*' *obtusa*, *N. australe*, *N. parvum* and *L. theobromae*, occurring on grapevines in South Africa. Bioassays were also conducted with the most effective *in vitro* fungicides in order to determine their potential as pruning wound protectants.

Methods

In vitro *testing of fungicides*

Sixteen Botryosphaeriaceae isolates, which were previously isolated from grapevine (van Niekerk *et al*. 2004) were used in these trials. These isolates included four isolates each of '*B.*' *obtusa* (STE-U 5139, 4444, 4440 and 5037), *L. theobromae* (STE-U 4583, 4423, 4422 and 4419), *N. australe* (STE-U 5040, 4598, 4416 and 4591), and *N. parvum* (STE-U 4589, 4420, 5253 and 5130). The isolates are maintained at the Department of Plant Pathology culture collection at the University of Stellenbosch (STE-U). Based on previous fungicide efficacy studies on grapevine trunk disease pathogens (Groenewald *et al*. 2000; Mostert *et al*. 2000; Halleen *et al*. 2001) and *N. protearum* (Denman *et al*. 2004), eight fungicides, benomyl, fenarimol, iprodione, prochloraz mc, tebuconazole, flusilazole, pyrimethanil and kresoxim-methyl, were selected (Table 1). Boscalid, a new broad-spectrum fungicide in a novel chemical class (www.agro.basf.com, verified 8 November 2006) and copper ammonium acetate was also included. The latter was also included as copper-containing fungicides are recommended as pruning wound protectants against the bacterial blight pathogen, *Xylophilus ampelinus* (Panagopoulos 1988), which is of major economic importance in the South African table grape industry (R. Carstens, pers. comm.). The fungicides were added to molten (50°C) potato dextrose agar (PDA) medium at seven different concentrations: 0 (control), 0.05, 0.1, 0.5, 1, 2.5 or 5 µg fungicide (a.i.)/mL. Copper ammonium acetate was tested at higher concentrations of active ingredient, at 0 (control), 0.5, 1, 2.5, 5, 10 or 20 µg/mL. Mycelial plugs (5 mm in diameter) obtained from margins of actively growing Botryosphaeriaceae cultures were placed on the amended PDA plates. Three plates per concentration were used. These were incubated at 25°C and the radial mycelial growth of the colonies measured after 24 and 48 h. Each colony's diameter was measured twice perpendicularly for each of the three replicates and the control. For each isolate × fungicide × concentration combination, the percentage inhibition was calculated relative to the respective control treatment. The function, logistic dose response [$\%\ \text{Inhibition} = b/1 + (\text{fungicide concentration}/c)^d$], with the intercept (a) equal to 0, was fitted to the data. From these regression lines, EC_{50} and EC_{90} values (the fungicide concentration where colony growth were inhibited by 50 or 90% compared with the control, respectively) were calculated.

Glasshouse bioassays

Subsequent to the *in vitro* study, the four most effective fungicides were selected for use in the bioassays. Copper ammonium acetate was also included. Dormant 1-year-old Chenin Blanc vine cuttings (each containing five internodes) were hot water treated (30 min at 50°C), followed by submersion in cold water containing a suspension of Sporekill (SPOREKILL, ICA International Chemicals Stellenbosch, South Africa; 1.5 mL/L). The cuttings were then placed in a custom-built hydroponic system in 110 × 1500 mm plastic tubes at ~27°C and allowed to bud. At the woolly bud stage, the distal ends of the cuttings were aseptically pruned off 1 cm above the second bud. These pruning wounds were immediately sprayed with 1 mL of the selected fungicides at the registered concentrations (Table 1). Three days later, spore suspensions (10^4 conidia/mL) of three isolates each of the different species of Botryosphaeriaceae, i.e. '*B.*' *obtusa* (STE-U 4440, 4444 and 5139), *L. theobormae* (STE-U 4583, 4419 and 4423), *N. australe* (STE-U 4416, 4591 and 5040) and *N. parvum* (STE-U 4420, 5130 and 4589), were spray-inoculated (1 mL per wound) onto the treated wounds. Spore suspensions were prepared from pycnidia of these isolates that were produced on sterilised pine needles on water agar as described by van Niekerk *et al*. (2004). Inoculated control treatments consisted of cuttings that were treated with sterile deionised water and an unsprayed, inoculated control. The water inside the tubes was replaced with fresh water, amended with a hydroponics fertiliser, Chemicult (1 g/L) at weekly intervals. The treated cuttings were incubated at a temperature range of 20–32°C (average 27°C).

Table 1. Fungicides selected for *in vitro* sensitivity testing

WP, wettable powder; WG, water dispersible granule; EC, emulsifiable concentrate; SC, suspension concentrate; EW, emulsion, oil in water; SL, soluble concentrate. Registered concentrations in South Africa were determined by Nel *et al*. (2003)

Active ingredient	Trade name	Manufacturer	Formulation	Registered concentration in South Africa
Benomyl	Benlate	Dow AgroSciences	500 g/kg WP	50 g/100 L (grapes)
Boscalid	Cantus	BASF	500 g/kg WG	Not registered
Fenarimol	Rubigan	Klub M5	120 g/L EC	20 mL/100 L (grapes)
Iprodione	Rovral Flo	Bayer	255 g/L SC	200 mL/100 L (grapes)
Prochloraz mc	Octave	Bayer	500 g/kg WP	25 g/100 L (apricots)
Tebuconazole	Folicur	Bayer	250 g/L EW	20 mL/100 L (grapes)
Flusilazole	Olymp	DuPont	100 g/L EW	50 mL/100 L (grapes)
Pyrimethanil	Scala	Bayer	400 g/L SC	120 mL/100 L (grapes)
Copper ammonium acetate	Copper Count-N	Hygrotech	316 g/L SL	500 mL/100 L (grapes)
Kresoxim-methyl	Stroby	BASF	500 g/kg WG	15 g/100 L (grapes)

After 3 months, the distal internode was removed from each cutting, split longitudinally and lesion formation in the xylem tissue measured. Data were submitted to statistical analysis of variance and means compared using Student's *t*-test for least significant ($P < 0.05$) differences (Snedecor and Cochran 1967). From each cutting, isolations were also made from the interface between healthy and symptomatic xylem wood. Four small (1×0.5 mm) wood sections were aseptically removed from this zone, placed on PDA medium and incubated at 23°C for 3 weeks. Identification of the fungal cultures was made to genus level, based on morphological characteristics. The incidence of re-isolated species of Botryosphaeriaceae was calculated for each cutting.

Results

In vitro *testing of fungicides*

Iprodione, pyrimethanil, copper ammonium acetate, kresoxim-methyl and boscalid were ineffective in inhibiting mycelial growth at the concentrations tested and the EC_{50} and EC_{90} values for these fungicides could not be calculated. Data for these treatments were, therefore, not included in the analyses. Analyses of variance of the EC_{50} and EC_{90} values for benomyl, tebuconazole, flusilazole, prochloraz mc and fenarimol showed a significant interaction between species of Botryosphaeriaceae and fungicides ($P < 0.0001$; ANOVA table not shown), indicating that the species reacted differently to the fungicides. EC_{50} and EC_{90} values are given in Table 2. Benomyl, tebuconazole and flusilazole were the most effective fungicides against all four species tested. Prochloraz mc and fenarimol exhibited significantly lower EC_{50} values for *N. australe* (1.15 and 1.74 µg/mL, respectively) compared with the other fungicides (0.13 to 0.55 µg/mL). Compared with the other fungicides, fenarimol also affected significantly lower EC_{50} values for '*B.*' *obtusa* (1.50 µg/mL cf. 0.14 to 0.36 µg/mL) and *L. theobromae* (3.01 µg/mL cf. 0.08 to 0.36 µg/mL). Fenarimol EC_{90} values could not be determined for *N. australe*, '*B.*' *obtusa* and *L. theobromae*, and for *N. parvum* it was significantly higher (3.09 µg/mL) than EC_{90} values for the other fungicides (0.53 to 2.05 µg/mL). EC_{90} values of the other fungicides for *N. australe* (0.70 to 1.28 µg/mL), '*B.*' *obtusa* (0.78 to 2.05 µg/mL), *N. parvum* (0.47 to 1.58 µg/mL) and *L. theobromae* (1.40 to 1.97 µg/mL) were fairly similar, except for the benomyl × *N. australe* (4.39 µg/mL), prochloraz mc × *N. parvum* (2.05 µg/mL) and prochloraz mc × *L. theobromae* (3.87 µg/mL) combinations, which had significantly higher EC_{90} values.

Table 2. EC_{50} and EC_{90} mean values calculated for each species of Botryosphaeriaceae and fungicide treatment interaction following *in vitro* mycelium growth studies on fungicide-amended PDA

Means followed by the same letter do not differ significantly at $P = 0.05$

Treatment	EC_{50} (µg/mL)	EC_{90} (µg/mL)
'*Botryosphaeria*' *obtusa*		
Benomyl	0.39bcd	0.78d
Tebuconazole	0.14ab	1.88de
Prochloraz mc	0.36bcd	2.05e
Flusilazole	0.36bcd	1.67de
Fenarimol	1.50f	—
Lasiodiplodia theobromae		
Benomyl	0.36bcd	1.40d
Tebuconazole	0.17ab	1.75de
Prochloraz mc	0.60d	3.87g
Flusilazole	0.08a	1.97e
Fenarimol	3.01h	—
Neofusicoccum australe		
Benomyl	0.55d	4.39h
Tebuconazole	0.13ab	0.98d
Prochloraz mc	1.15e	1.28d
Flusilazole	0.21abc	0.70d
Fenarimol	1.74g	—
Neofusicoccum parvum		
Benomyl	0.47cd	1.58d
Tebuconazole	0.07a	0.53d
Prochloraz mc	0.07a	2.05e
Flusilazole	0.04a	0.47d
Fenarimol	0.45cd	3.09f
l.s.d. ($P = 0.05$)	0.282	0.447

Glasshouse bioassays

Black vascular streaking and necrotic lesions were observed in the longitudinally dissected shoots. Apart from the general wound response, no lesions were observed in the uninoculated

shoots. Analysis of variance of the lesion length data showed no species × treatment interaction ($P = 0.7360$). Significant differences were, however, observed between the mean lesion lengths for species ($P < 0.0001$) and treatments ($P = 0.0209$; ANOVA table not shown). Significantly longer lesions were measured in the shoots that were inoculated with *N. parvum* (6.40 mm), compared with those inoculated with *L. theobromae* (4.98 mm), *N. australe* (4.82 mm) and '*B.*' *obtusa* (4.76 mm), while statistically shorter lesions were measured on the control shoots that were not inoculated (3.59 mm). The longest lesion lengths were measured on shoots that were treated with water before inoculation (6.19 mm). These lesion lengths did not differ significantly ($P < 0.05$) from those measured on shoots that were treated with copper ammonium acetate (5.67 mm) or flusilazole (5.21 mm), but significantly shorter lesions were measured on shoots treated with tebuconazole (4.89 mm), benomyl (4.77 mm), prochloraz mc (4.62 mm) and the uninoculated control (3.59 mm).

The incidence of species of Botryosphaeriaceae that were isolated from lesions in the water-treated controls varied, and ranged from 0 to 39.4%. The data were, therefore, not statistically analysed. No species of Botryosphaeriaceae were isolated from the uninoculated control shoots. The incidence of Botryosphaeriaceae isolated from '*B.*' *obtusa*-inoculated shoots declined from relatively high levels in water-treated shoots (39.4%) to moderate levels in copper ammonium acetate-treated shoots (24.9%), low levels in flusilazole-, prochloraz mc- (12.5% each) and tebuconzole-treated shoots (7.7%), and very low levels in benomyl-treated shoots (1.0%). From the other treatments, re-isolation data did not, however, conform to this expected trend. From shoots that were inoculated with *N. australe*, positive isolations were made only from lesions in flusilazole- (83.3%) and tebuconazole-treated (16.7%) shoots. Few species of Botryosphaeriaceae were isolated from water-treated shoots that were inoculated with *N. parvum* (6.0%), whereas higher levels (15.1 to 24.1%) were isolated from the fungicide-treated shoots. For the *L. theobromae* treatment, the fungus was isolated from the water- (38.5%), tebuconazole- (38.5%) and prochloraz mc-treated shoots (23.1%) only.

Discussion

This study presents the first report on the *in vitro* fungicide efficacy of *L. theobromae*, *N. parvum* and *N. australe*. In this study, we calculated mean EC_{50} values for benomyl (0.44 µg/mL), tebuconazole (0.13 µg/mL), prochloraz mc (0.55 µg/mL) and flusilazole (1.68 µg/mL) that were similar to the respective values of 0.45, 0.28, 0.5 and 1.08 µg/mL reported by Denman *et al*. (2004) for *N. protearum*, the canker pathogen of *Protea magnifica*. Savocchia *et al*. (2005) reported tebuconazole and fluazinam to be the most effective fungicides for reducing *in vitro* mycelial growth of '*B.*' *obtusa* and *N. luteum*. Fluazinam is not available in South Africa. Differences in the EC_{50} values were observed between the different species of Botryosphaeriaceae tested in this study. Benomyl and tebuconazole showed relatively consistent inhibitory action, whereas prochloraz mc, flusilazole and fenarimol varied considerably between the tested species. Tebuconazole was the most effective fungicide *in vitro*. *B. parva* was the species most inhibited by the fungicides tested, with tebuconazole, prochloraz mc and flusilazole showing the best mycelial inhibitory action *in vitro*. *L. theobromae* and '*B.*' *obtusa* were also inhibited at low concentrations, except for two isolates against which fenarimol did not show good inhibition (data not shown). *N. australe* was the species least inhibited by the fungicides. These fungicides, except prochloraz mc, are registered for use on grapes in South Africa and were also reported to be effective against *Pa. chlamydospora*, *P. viticola* and *E. lata* (Groenewald *et al*. 2000; Mostert *et al*. 2000; Halleen *et al*. 2001).

Results from the bioassays indicated that benomyl, tebuconazole and prochloraz mc were the most effective pruning wound protectants against the species of *Botryosphaeriaceae* tested. However, due to the low and varied re-isolation incidences, these findings cannot be considered as conclusive. One reason for the low re-isolation incidences might have been the relatively short incubation period of 3 months during which time the pathogen did not have adequate time to become established inside the grapevine wood. Species of Botryosphaeriaceae are furthermore reported to be opportunistic pathogens of hosts that are predisposed by stress (van Niekerk *et al*. 2005*a*). The absence of stress on the grapevine cuttings might also have attributed to inadequate pruning wound colonisation. It was clear that treatment of pruning wounds with these fungicides did not completely prevent infection by the species of Botryosphaeriaceae, and application of these chemicals at higher dosages should be investigated.

Halleen and Fourie (2005) demonstrated the efficacy of flusilazole and benomyl as pruning wound protectants against *E. lata* in Cabernet Sauvignon vineyards, and also the efficacy of these fungicide treatments against natural infection by Botryosphaeriaceae. Sosnowski *et al*. (2005) also found benomyl, among other pruning wound treatments, to be effective in reducing *E. lata* infection in Australian Cabernet Sauvignon vineyards. From these findings, as well as those reported in literature, benomyl, tebuconazole, flusilazole and prochloraz mc can be identified as fungicides to be evaluated as pruning wound protectants in additional bioassays and vineyard trials against species of Botryosphaeriaceae and the other grapevine trunk disease pathogens.

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