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www.publish.csiro.au/journals/app *Australasian Plant Pathology*, 2004, **33**, 97–102

Evaluation of fungicides for the control of *Botryosphaeria protearum* on *Protea magnifica* in the Western Cape Province of South Africa

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Abstract. A range of fungicides was tested *in vitro* for their effect on mycelial inhibition. Selected products showing potential for disease control were then further tested under field conditions. The most effective fungicides in the *in vitro* tests were tebuconazole, benomyl, prochloraz mc, iprodione and fenarimol. In field trials, a 25–85% reduction in the occurrence of stem cankers caused by *Botryosphaeria protearum* was achieved if fungicides were applied or sanitation pruning was implemented. The best control was obtained with treatments of prochloraz mc alternated with mancozeb. Applications of bitertanol and fenarimol also significantly reduced the occurrence of cankers.

Additional keywords: contact fungicide, EC_{50}, phytotoxicity, spray, systemic fungicide.

Introduction

Proteaceae cut-flowers are an important agricultural crop in South Africa comprising 70% of the entire national cut-flower industry. Most of the produce is exported (80%), particularly to the Netherlands and Germany, but also to other European markets and to the USA (Wessels *et al*. 1997; Middelmann 2000). The value of these flowers lies in their aesthetic beauty and it is essential to produce perfect blooms with unblemished leaves and stems.

Production of one of the most important commercial proteas, the queen protea (*Protea magnifica*) is severely restricted by leaf necrosis and stem cankers caused by *Botryosphaeria* spp. with *B. protearum* being the major stem canker pathogen in the Porterville district of the Western Cape Province (Denman 2002; Denman *et al*. 2003). Disease devalues the appearance of cut-flower stems and leaves, and also causes losses through branch die-back and ultimately death of bushes. Furthermore, since proteas are mostly exported, they are subject to phytosanitary inspections and the presence of infected material could place entire consignments at risk of being rejected. It is, therefore, imperative that disease caused by these pathogens is controlled.

Integrated control using a variety of strategies is generally advocated to reduce the impact of *Botryosphaeria* infection on *Protea* spp. (von Broembsen and van der Merwe 1990; Forsberg 1993). The judicious use of fungicides is an important component of this integrated strategy (von Broembsen and van der Merwe 1990). Lesions caused by *B. protearum* that lead to the development of stem cankers on *P. magnifica* are initiated through leaf infection (Denman 2002). Thus, chemicals applied to foliage could reduce disease by protecting leaves against infection or by inhibiting the pathogen in leaves before cankers develop. Fungicides previously recommended against *Botryosphaeria* on Proteaceae in South Africa included sprays with captab, captafol and mancozeb (Benic and Knox Davies 1983), and monthly applications of benomyl and captab (von Broembsen and van der Merwe 1990). In Australia, McLennan (1993) suggested spraying with either benomyl or iprodione. None of the above mentioned chemicals are registered for use on Proteaceae in South Africa, although benomyl, iprodione and mancozeb are registered for use on other ornamental plants (Nel *et al*. 1999).

Fungicides can be applied as soil drenches. By applying benomyl as a soil drench, Schoeneweiss (1979) obtained

 10.1071/AP03080 0815-3191/04/010097

Table 1. Fungicides tested for mycelial inhibition of *Botryospaeria protearum in vitro*

Fungicide (a.i.)	Trade name	Formulation	Action	Supplier	Registration in South Africa
Benomyl	Benlate	500 g a.i./kg, WP[A]	S[B]	Du Pont	Ornamentals
Clorothalonil	Bravo	500 g a.i./kg, WP	C[C]	Efekto	Ornamentals
Fenarimol	Rubigan	120 g a.i./L, EC[D]	S	Dow Agrosciences	Roses
Fosetyl-Al	Aliette	800 g a.i./kg, WP	S	Rhône Poulenc	Proteaceae
Iprodione	Rovral Flo	255 g a.i./L, SC[E]	C	Rhône Poulenc	Ornamentals
Kresoxim-methyl	Stroby	500 g a.i./kg, WG[F]	S	BASF	Not on proteas or ornamentals
Mancozeb	Sancozeb	800 g a.i./kg, WP	C	Sanachem	Ornamentals
Quintozene	PCNB	750 g a.i./kg, WP	C	Plaaskem	Ornamentals
Prochloraz mc	Octave	500 g a.i./kg, WP	S	AgrEvo	Ornamentals
Tebuconazole	Folicur	250 g a.i./L, EW[G]	S	Bayer	Not on proteas or ornamentals
Thiram	Thiram	750 g a.i./kg, WP	C	Sanachem	Not on proteas or ornamentals

[A]WP = wettable powder. [B]S = systemic fungicide. [C]C = contact fungicide. [D]EC = emulsifiable concentrate. [E]SC = suspension concentrate. [F]WG = water dispersable granules. [G]EW = oil in water emulsion.

effective control of *B. dothidea* on red-osier dogwood (*Cornus sericea*) because the chemical was taken up by roots and transported to the stems and leaves. However, soil drenches as a general practice in production of queen proteas would be uneconomical. Moreover, certain chemicals such as benomyl are considered environmentally unfriendly, especially when applied to the soil (Fry 1982).

Effective chemical control of diseases caused by *B. dothidea* on apples, cranberries, apricots, peaches and pistachio has been reported (Starkey and Hendrix 1980; Parker and Sutton 1993; Li *et al*. 1995; Ma *et al*. 2001). This suggests that opportunities exist for using fungicides to reduce the impact of stem cankers on *P. magnifica*. However, von Broembsen (1989) maintained that Proteaceae are very sensitive to agricultural chemicals. She suggested that the phytotoxic effects of fungicides applied under field conditions need to be assessed before registration can be recommended.

Very little work has been conducted in South Africa on the control of *Botryosphaeria* on Proteaceae. In addition, subsequent to the last studies on chemical control of *Botryosphaeria* on this host (von Broembsen and van der Merwe 1990) new and promising fungicides have become available for this purpose. One of the aims of this study was to test the efficacy of a range of fungicides *in vitro* on mycelial inhibition of *B. protearum*. Secondly, selected products showing potential for disease reduction were further tested under field conditions. Phytotoxic responses to the chemicals were also monitored and evaluated.

Methods

Selection of fungicides for in vitro *tests*

Both contact and systemic fungicides were selected for *in vitro* screening tests (Table 1) because most fungicide application programs that prevent pathogen populations from developing fungicide resistance combine and/or alternate the two types. The registration status of the fungicides with respect to proteas and ornamentals in South Africa is also listed in Table 1.

In vitro *tests on mycelial inhibition*

The fungicides were suspended in sterile water and added to molten 2% potato-dextrose agar (PDA) (Biolab, Midrand, South Africa) at a range of concentrations from 0 to 100 µg a.i./mL. For the controls, PDA without the addition of chemicals was used. All the fungicides were tested at concentrations 0.005, 0.01, 0.05, 0.1, 0.5, 1 and 5 µg a.i./mL. Additionally, chlorothalonil, mancozeb and thiram were tested at 10, 25 and 100 µg a.i./mL (Li *et al*. 1995).

Each fungicide was tested against four isolates of *B. protearum* (STE-U 1799, 1800, 1801, 1802). The isolates originated from stem cankers on *P. magnifica* and are maintained in the Department of Plant Pathology culture collection (STE-U), at the University of Stellenbosch and at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands.

Approximately 20 mL of fungicide-amended medium was poured into Petri dishes (90 mm diameter). Each plate was inoculated with a 5-mm-diameter mycelial disc cut from the actively growing margins of *B. protearum* on PDA. Mycelial growth was recorded by marking the periphery of the fungal colonies along two perpendicular lines on the back of the Petri dishes after 4 days incubation at 22°C in the dark. Three replicate plates per isolate–fungicide–concentration combination were tested in the experiment and the entire experiment was conducted twice.

The mean colony diameters for each isolate and fungicide-concentration were calculated. Percentage inhibition was then calculated by subtracting mean colony diameters from those of the controls. The data were plotted against the chemical concentrations tested. For each isolate × fungicide combination the most appropriate linearising transformation (log, square root or none) was selected and then linear regression models were fitted to the transformed data. The EC_{50} values were computed from the parameter estimates (Finney 1952) and then back transformed to the original scale (Armitage 1971). The differences in fungicides were then investigated using a general linear model and an analysis of variance (ANOVA). Examination of the residuals indicated significant non-normality. The use of logarithmic transformation corrected the problem and, following the ANOVA, the treatment effects were examined using paired *t*-tests.

Table 2. Analysis of variance on the *in vitro* fungicide trial data

Source	DF	SS	MS	*F* value	*P* value
Experiment	1	0.71	0.71	2.25	0.1330
Treatment	8	74.21	9.28	27.89	< 0.0001
Isolates	3	3.75	1.25	3.76	0.0199
Treatment × isolate	26	13.06	0.54	1.64	0.0939
Error	36	11.04	0.32		

Field trials

At a commercial orchard on Osdam Farm, which had a history of Botryosphaeria stem canker, 225 *P. magnifica* plants (five rows each containing 45 plants) were selected. The farm was situated at 32°56.60′S, 19°02.80′E in the Porterville district of the Western Cape Province. At the onset of the experiment, the plants were tagged and any visually unhealthy tissue was removed by pruning. Plants were 3 years old at the beginning of the experiment and had been subjected to four applications of mancozeb and two of prochloraz with the last application being 6 months prior to the experiment.

Four of the fungicides that gave the best inhibition of *B. protearum* mycelial growth *in vitro* (benomyl, fenarimol, iprodione and tebuconazole) were selected for field trials. Bitertanol (Baycor, 300 g a.i./L, EC, Bayer) was also used even though it had not been included in the *in vitro* tests. In addition, two commercially available mixtures of fungicides that had been tested *in vitro* were included in the trial. One mixture comprised systemic fungicides and the other mixture comprised contact fungicides. The mixture of systemic fungicides was Toreador [a combination of carbendazim (133 g a.i./L) and tebuconazole (167 g a.i./L), SC, Bayer]. The mixture of contact fungicides was Dirac Express [a combination of iprodione (78 g a.i./kg) and thiram (532 g a.i./kg), WG, Rhône Poulenc]. The mixtures were applied alternatively to simulate an anti-fungicide-resistance strategy. This treatment is hereafter referred to as t/d. There were 30 plants per fungicide treatment.

The standard general fungicide program used by protea farmers was also tested i.e. alternating mancozeb with prochloraz mc on a fortnightly basis. The rates of fungicide application were followed according to Nel *et al*. (1999) for ornamental plants and are as follows: benomyl (0.5 g a.i./L); bitertanol (0.24 g a.i./L); fenarimol (0.042 g a.i./L); iprodione (0.51 g a.i./L); mancozeb (1.6 g a.i./L); prochloraz mc (0.75 g a.i./L) and tebuconazole (0.375 g a.i./L). Approximately 0.33 L per plant was dispensed on each application date, by spraying plants using a back-pack apparatus. Fungicides were applied every 14 days during bud break (August until November) (eight applications per year during these months) after which they were applied at monthly intervals (eight applications for the rest of the year). Chemical applications began in August 1998 and ended in July 2000.

Cultural control by pruning stem cankers once every month was also tested. The treatment was included because sanitation pruning is an essential component of integrated control of Botryosphaeria stem canker. It was necessary to determine whether by employing sanitation pruning only as a control measure, high quality blooms could be produced. This treatment was referred to as the 'cut only' treatment. Fifteen plants received the 'cut only' treatment.

In the controls, neither pruning nor fungicides were applied. Control plants were sprayed with water on each fungicide application date. Fifteen plants served as controls.

The experiment was laid out as a randomised block design with ten blocks. Treatments were applied to three plants each, within blocks. However, the control, the cut only treatment, and the standard treatment of alternating prochloraz mc with mancozeb, were applied only in five blocks (i.e. in every second block). The experiment was conducted once.

At the onset of the experiment, the 1996 and 1997 flushes (portion of a branch indicating annual growth) were present as main branches on which canker formation could be noted. Within the first 2 months of the experiment, the 1998 flush began to emerge and by the end of the experiment the 2000 flush was present as a very young developing flush.

Plants were inspected every month and notes and sketches were made regarding disease development on every plant. The flush and position of cankers on each plant were recorded. Once cankers had clearly developed, they were cut out (except on the control plants) and brought back to the laboratory where isolations were made as described by Denman (2002).

Plant mortality was determined at the end of the experiment. In each treatment, the total number of cankers yielding isolates of *Botryosphaeria* was recorded. The numbers of cankers were analysed using a Poisson log-linear generalised linear model (McCullagh and Nelder 1989). Treatment effects were tested using an analysis of deviance.

Results

In vitro *trials*

EC_{50} values could not be calculated for fosetyl-Al and kresoxim-methyl because both these fungicides were ineffective in inhibiting the mycelial growth at the concentrations tested. Data for these two treatments were, therefore, not included in the analysis of variance.

The analysis of variance revealed similar results (P = 0.1330) for the two *in vitro* experiments, and the data were thus pooled. There was no significant isolate × treatment interaction (P = 0.0939) (Table 2), so the main effects (fungicide treatments and isolates) could be interpreted. There were significant differences among the treatments ($P < 0.0001$) and among the isolates ($P = 0.0199$) (Table 2).

With respect to the fungicides three groups were identified (Table 3). The most effective fungicides inhibited growth at low EC_{50} values and included tebuconazole, benomyl, prochloraz mc, iprodione and fenarimol. A single fungicide, quintozene, fell into the moderately effective group where 50% inhibition occurred at a relatively high concentration (6.75 µg a.i./mL). Most of the contact fungicides (chlorothalonil, mancozeb and thiram) represented a group where inhibition only occurred at very high concentrations (Table 3). With the exception of iprodione, the fungicides in the first group were all systemic. The two most effective fungicides were tebuconazole and benomyl with EC_{50} values of 0.38 µL a.i./mL and 0.69 µg a.i./mL, respectively (Table 3). Fenarimol had a significantly

Table 3. Grouping of fungicides with similar predicted mean EC_{50} values of *Botryosphaeria protearum in vitro*

Fungicide (a.i.)	Log-transformed EC_{50} value	Back-transformed EC_{50} value (μg/mL)
	Group 1	
Tebuconazole (S)	0.28 a[B]	1.32
Benomyl (S)[A]	0.45 a	1.57
Prochloraz mc (S)	0.50 ab	1.65
Iprodione (C)	0.61 ab	1.84
Fenarimol (S)	1.08 b	2.94
	Group 2	
Quintozene (C)	1.91 c	6.75
	Group 3	
Chlorothalonil (C)	2.77 d	15.96
Thiram (C)	2.78 d	16.12
Mancozeb (C)	2.93 d	18.72

[A]S = systemic fungicide, C = contact fungicide.
[B]Numbers followed by the same letter do not differ significantly from each other ($P > 0.05$).

Table 4. Predicted mean EC_{50} value of isolates of *Botryosphaeria protearum in vitro* fungicide trials

Isolate number[A]	Log-transformed EC_{50} value	Back-transformed EC_{50} value (μg/mL)
STE-U 1799	1.07 a[B]	2.92
STE-U 1800	1.50 b	4.48
STE-U 1801	1.78 b	5.93
STE-U 1802	1.45 ab	4.26

[A]All cultures stored at the Dept. of Plant Pathology, University of Stellenbosch (STE-U).
[B]Numbers followed by the same letter do not differ significantly from each other ($P > 0.05$).

higher EC_{50} value than tebuconazole and benomyl, but was still placed in the most effective group.

In spite of differences in growth rates between some of the isolates (Table 4), the lack of treatment × isolate interaction indicated that all four isolates showed a similar reaction to the different fungicides. Therefore, the fungicide effects apply to the pathogen as a whole, and the differences are probably due to natural variation amongst individuals.

Field trials

Analysis of the counts of Botryosphaeria cankers showed that there were significant effects for the annual growth (flushes) ($P < 0.001$) and the treatments ($P < 0.001$) but no significant interaction (Table 5). Survival data did not affect the model and were thus not used. In general, a higher predicted number of cankers (8.9) caused by *B. protearum* was recorded on the older growth (1996 and 1997 flushes) than on the new growth (1998–2000) (3.1).

Decreases ranging from 25 to 85% in the occurrence of stem cankers were recorded if fungicides or sanitation pruning was applied. A significant reduction in the number of cankers relative to the untreated controls was achieved with applications of bitertanol, fenarimol and prochloraz mc alternated with mancozeb treatments (Fig. 1). Applications of t/d were completely ineffectual and plants receiving any of the other fungicide treatments had fewer cankers than the t/d plants. An analysis of deviance of the effective fungicide treatments (benomyl, bitertanol, fenarimol, iprodione, prochloraz mc and tebuconazole) *v.* the controls, cut-only and t/d treatments, revealed a significant interaction ($P = 0.003$) with significant differences between the flushes for the effective treatments but no significant differences between the flushes for the others (Table 6). None of the fungicides elicited any phytotoxic response under the field conditions of the experiment.

Discussion

Results presented here have confirmed the importance of using fungicides as part of an integrated disease management strategy, because in the absence of fungicide sprays the incidence of Botryosphaeria disease is very high. The most effective fungicides were bitertanol, fenarimol and the alternation of prochloraz mc with mancozeb. However, application of any of the tested fungicides except the t/d combination was better than not applying anything at all. Furthermore, without chemical control, leaf blemishes caused by other fungi completely deface the foliage, rendering the flowers unmarketable.

Table 5. Analysis of deviance on the number of Botryosphaeria cankers on plants receiving different fungicide treatments

Source	DF	Deviance	Mean deviance	Deviance ratio	*P* value
Flush	1	34.76	34.76	34.76	< 0.001
Treatment	8	38.46	4.81	4.81	< 0.001
Flush × treatment	8	14.66	1.83	1.83	0.066
Residual	12	8.22	0.69		
Total	29	96.10	3.31		

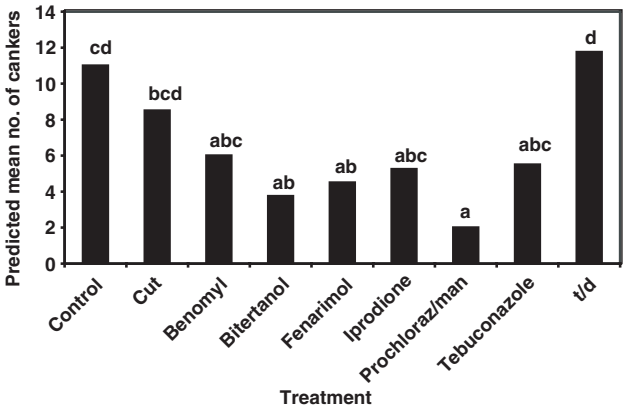

Fig. 1. The predicted mean number of cankers caused by *Botryosphaeria protearum* in various flushes of *Protea magnifica* plants with different treatments. Bars topped by the same letter do not differ significantly from each other ($P > 0.05$).

Previously, von Broembsen (1989) emphasised the importance of applying prophylactic chemical treatments for the control of protea diseases. In our results, the large reduction in the formation of cankers on the young (1998–2000) flushes relative to the controls and to the older (1996–1997) flushes reinforces the importance of applying fungicides preventatively. The young flushes developed during the trial and latent or endophytic infections could, therefore, not have occurred prior to the trial. Thus all infections in these flushes occurred during the trial. Since the number of cankers in the control plants of the older and the younger flushes was the same, the lower number of cankers formed in the new flushes can be attributed to the protective effects of the fungicides. Because *B. protearum* has a latent or endophytic stage in its life cycle (Denman 2002), the higher number of cankers in the older flushes suggests that some infections had taken place prior to the onset of the trial and that the fungicides had been unable to eradicate the pathogen once infection had taken place. Other researchers, working on the control of *B. dothidea* white rot of apples, reported poor curative ability of tebuconazole (Parker and Sutton 1993). Our results are consistent with this finding.

The reduction in the occurrence of cankers achieved only by pruning diseased material out of the bushes was much lower than that obtained with the chemicals. In this treatment, the leaves of the plants were also badly spoiled by leaf spots caused by other fungal pathogens. Nonetheless, by employing sanitation pruning only, a 30% reduction in disease was obtained, and this is highly significant in economic terms. Therefore, by combining sanitation pruning with preventative chemical applications, the highest level of disease control will be achieved and high quality blooms will be produced.

Although none of the chemicals tested in the trial conditions elicited any phytotoxic response, it has been reported that tebuconazole applied under extremely hot conditions (> 30°C) and low humidity levels can burn leaf tips of some proteas (G. Nieuwoud, SAFCOL, personal communication). Farmers are thus advised to apply chemicals under moderate weather conditions and preferably early in the morning or late in the afternoon.

Regular prophylactic use of fungicides, especially if combined with sanitation pruning, will result in an appreciable decreases in disease incidence. Additional studies need to be carried out to optimise the application intervals and to devise a spray program that utilises both contact and systemic fungicides to prevent the development of fungicide resistance.

Acknowledgements

We gratefully acknowledge SAPPEX and the National Research Foundation for their financial support. Thanks are

Table 6. Predicted mean number of cankers caused by *Botryosphaeria protearum* in various flushes of *Protea magnifica* with different treatments applied

Treatment group[A]	Predicted mean number of cankers per flush		Significance level (*P* value)
	Old flushes (1996–1997)	New flushes (2000)	
Effective fungicides	7.55[B]	1.90	<0.001
Controls, cut-only and t/d treatments	13.00	8.50	0.054

[A]Effective fungicide treatments comprise benomyl, bitertanol, fenarimol, iprodione, prochloraz mc and tebuconazole. [B]Predicted mean number of cankers caused by *Botryosphaeria protearum* in various flushes of *Protea magnifica* plants with different treatments.

extended to the Osdam farmers for their assistance with the field trials, and Dr Geoff Morgan of Forest Research, UK, for his help with the statistics.

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Received 19 July 2002, accepted 20 August 2003