In Vitro Screening of Fungicides Against *Phomopsis viticola* and *Diaporthe perjuncta*

L. Mostert, S. Denman and P.W. Crous

Department of Plant Pathology, University of Stellenbosch, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa

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***Phomopsis viticola* is the cause of Phomopsis cane and leaf spot disease of grapevines, while *Diaporthe perjuncta* is associated with bud mortality. The efficacy of nine fungicides (azoxystrobin, flusilazole, folpet, fosetyl-Al + mancozeb, kresoxim-methyl, mancozeb, penconazole, spiroxamine and trifloxystrobin) against isolates of *P. viticola* and *D. perjuncta* was determined *in vitro* using the mycelial growth test. Additionally, azoxystrobin, folpet, kresoxim-methyl, mancozeb, propineb and trifloxystrobin were tested for their ability to inhibit spore germination *in vitro.* Ten isolates of *P. viticola* and three of *D. perjuncta* were used in the mycelium inhibition tests, and five isolates of *P. viticola* in the spore germination tests. The effective concentration at which mycelial growth was inhibited by 50% and at which 50% of the spores (EC_{50} value) were inhibited from germinating was calculated for each isolate/fungicide combination. In the mycelium growth test flusilazole, penconazole and trifloxystrobin gave better inhibition at lower concentrations than folpet and fosetyl-Al + mancozeb. No significant differences in the mean EC_{50} values were detected among azoxystrobin, flusilazole, kresoxim-methyl, penconazole, spiroxamine and trifloxystrobin. There were also no significant differences among the mean EC_{50} values of azoxystrobin, kresoxim-methyl and mancozeb. Flusilazole and penconazole inhibited mycelial growth at the lowest mean EC_{50} values obtained. Kresoxim-methyl and trifloxystrobin inhibited spore germination at lower concentrations than folpet or mancozeb. Folpet required the highest concentration to inhibit 50% germination and was significantly different from mancozeb and propineb. There were also no significant differences among the mean EC_{50} values of mancozeb, propineb and azoxystrobin. The mean EC_{50} values of the strobilurin fungicides were not significantly different from one another. These results indicate that the strobilurin fungicides inhibited mycelial growth and spore germination of *P. viticola.* Trials need to be conducted to verify these findings under field conditions.**

Phomopsis cane and leaf spot of grapevine, caused by the fungus *Phomopsis viticola* (Sacc.) Sacc., can lead to significant yield losses. Serious disease outbreaks with yield losses of up to 50% have been reported in several countries (Pine, 1958; Berrysmith, 1962; Pscheidt & Pearson, 1989).

Several species, including *P. viticola* and *Diaporthe perjuncta* Niessl (anamorph: *Phomopsis* sp.), have been associated with Phomopsis cane and leaf spot (Merrin *et al.,* 1995; Mostert *et al.,* 2000b). These two species have also been isolated from South African grapevines (Mostert *et al.,* 2000a). *P. viticola* was associated with typical cane and leaf spot symptoms, while *D. perjuncta* proved to be non-pathogenic under similar growth conditions (Mostert *et al.,* 2000b).

Phomopsis cane and leaf spot disease occurs sporadically in South Africa. Swart and De Kock (1994) reported that the incidence of *Phomopsis* on table-grape vines had increased over the years. Serious disease incidences have been recorded from vineyards in regions such as Helderberg, Firgrove, Somerset West, Rawsonvile and Slanghoek (Marais, 1981), justifying producers controlling the disease by chemical means.

Chemical control of *Phomopsis* includes the application of protective fungicides on new plant growth early in the growing season and eradicant fungicides could be applied during dormancy in vineyards with a high incidence of *Phomopsis* (Chairman *et al.,* 1982). *Phomopsis* is generally controlled through the application of protectant fungicides at 1–3 cm shoot length, and again at 6–12 cm shoot length (Pine, 1957). When weather conditions favour the disease, an application of contact fungicides every 2 weeks commencing at bud-burst should provide satisfactory protection (Emmet *et al.,* 1992). Up to five sprays might be needed, of which the first two are considered to be the most important. The fungicides used for the protective control of *P. viticola* are aimed at protection of young plant material during critical periods, namely when spores are released after prolonged periods of rain in the spring. A number of fungicides have been registered against this disease in South Africa: copper oxychloride, copper oxychloride/sulphur, copper sulphate/lime, folpet, fosetyl-Al + mancozeb, mancozeb, propineb and sulphur (Nel *et al.,* 1999). In the past fosetyl-Al + mancozeb, folpet and mancozeb were commonly used to control this disease (Swart *et al.,* 1994). Various *in vitro* studies have been carried out to test the performance of different fungicides for the control of *P. viticola* (Dula & Kaptas, 1982; Fareta *et al.,* 1987; Macek & Zgur, 1989; Kuropatwa, 1994). A new fungicide, strobilurin, has since come onto the market. Of the three different strobilurin fungicides tested in this study only trifloxystrobin has been registered against *Phomopsis viticola* (Anonymous, 1998), although not in South Africa.

An integrated approach to grapevine disease control would minimise the use of fungicides. Even though downy mildew and *Phomopsis* could be simultaneously controlled (Swart *et al.,*

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1994), little is known of the effects of powdery mildew fungicides on *Phomopsis*.

The aim of this study was therefore to assess the *in vitro* effectiveness of *P. viticola* to the strobilurin fungicides, and to compare these fungicides with the contact fungicides currently used to control *P. viticola* as well as three powdery mildew fungicides. Isolates representing two species of the *P. viticola*-complex, namely *P. viticola* and *D. perjuncta,* were tested.

MATERIALS AND METHODS

Isolates: isolates were obtained from symptomatic grapevine shoots and leaves from different viticultural regions in the Western Cape province. Three isolates of *D. perjuncta* and ten isolates of *P. viticola* were used in the mycelial growth tests (Table 1). Five isolates of *P. viticola* were selected for the spore germination tests. All isolates are maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch (STE-U).

Fungicides: A range of systemic, contact and quasi-systemic fungicides was tested (Table 2). The contact fungicides included folpet, mancozeb, and propineb. Azoxystrobin, kresoxim-methyl and trifloxystrobin are three different chemical variations of stro-

TABLE 1

Origin of *Phompsis viticola* and *Diaporthe perjuncta* isolates in the Western Cape province, and isolates screened against various fungicides in mycelial growth and spore germination tests.

Accession no.	Species	Location	Test performed[1]
STE-U 2654	*D. perjuncta*	Stellenbosch	M
STE-U 2655	*D. perjuncta*	Stellenbosch	M
STE-U 2632	*D. perjuncta*	Constantia	M
STE-U 3128	*P. viticola*	Stellenbosch	M, S
STE-U 3129	*P. viticola*	Botrivier	M
STE-U 2641	*P. viticola*	Paarl	M, S
STE-U 2648	*P. viticola*	Porterville	M
STE-U 3130	*P. viticola*	Slanghoek	M
STE-U 3131	*P. viticola*	Bonnievale	M, S
STE-U 3132	*P. viticola*	Vredendal	M
STE-U 3133	*P. viticola*	Lutzville	M, S
STE-U 3134	*P. viticola*	Paarl	M, S
STE-U 3135	*P. viticola*	Montague	M

[1]M = mycelial growth; S = spore germination.

TABLE 2

Fungicides tested, corresponding registered diseases, highest recommended spray dosage for *Phomopsis* control and mean EC_{50} values for inhibition of mycelial growth and spore germination.

Active ingredient	Trade name	Recommended against the following local grapevine pathogens[1]	Highest recommended spray dosage/100L H_20 (and equivalent a.i. in µg/mL)[2]	Mean EC_{50} in µg/mL	
				Mycelial growth	Spore germination
Azoxystrobin	Quadris 50% w.p.[3]	*Ucinula necator* *Plasmopara viticola*	35g (175 µg/mL)	0.350	0.123
Flusilazole	Olymp 10% e.c.[4]	*Uncinula necator*	50 mL (50µg/mL)	0.007	
Folpet	Folplan 50% s.c.[5]	*Phomopsis viticola* *Plasmopara viticola* *Elsinoë ampelina* *Botrytis cinerea*	200 mL (1000 µg/mL)	4.489	0.510
Fosetyl-A1/ mancozeb	Mikal M 44/26% w.p.	*Phomopsis viticola* *Plasmopara viticola*	350 g (910 µg/mL for mancozeb)	3.925	
Kresoxim-methyl	Stroby 50% w.p.	*Uncinula necator* *Plasmopara viticola*	15 g (75 µg/mL)	1.665	0.004
Penconazole	Topaz 10% e.c.	*Uncinula necator*	22.5 mL (22.5 µg/mL)	0.023	
Propineb	Antracol 70% w.p.	*Phomopsis viticola* *Plasmopara viticola* *Elsinoë ampelina*	200 g (1400 µg/mL)		0.156
Mancozeb	Dithane 80% w.p.	*Phomopsis viticola* *Plasmopara viticola*	200 g (1600 µg/mL)	2.891	0.250
Spiroxamine	Prosper 50% e.c.	*Uncinula necator*	60 ml (300 µg/mL)	0.321	
Trifloxystrobin	Flint 50% w.p.	*Uncinula necator* *Plasmopara viticola*	15 g (75 µg/mL)	0.051	0.003

[1] According to Net *et al.* (1999).

[2] Recommended dosage for powdery mildew taken for fungicides not registered against *Phomopsis.*

[3] w.p. = wettable powder.

[4] e.c. = emulsifiable concentrate.

[5] s.c. = soluble concentrate.

bilurin and have a quasi-systemic mode of uptake in the plant (Ypema & Gold, 1999). The systemic fungicides tested include penconazole, flusilazole and spiroxamine. Even though fosetyl-A1 has a systemic mode of action, the mixture of fosetyl-A1 + mancozeb was treated as a contact fungicide, since only mancozeb had any effect on *Phomopsis.*

Mycelial growth inhibition: The effects of the following fungicides were tested on mycelial growth: azoxystrobin, flusilazole, folpet, fosetyl-A1 + mancozeb, kresoxim-methyl, mancozeb, penconazole, spiroxamine, and trifloxystrobin. The fungicides were tested at: 0.01, 0.05, 0.1, 0.5, 1.0 and 5.0 µg a.i./mL. Bottles containing prepared malt extract agar (MEA, Biolab, Johannesburg) (40g/L) were kept at 50°C and fungicides were added from stock solutions. MEA was used for the control, and contained no fungicide.

Plates were inoculated within 24 h after they were poured. Three plugs (5 mm diam.) were cut from the margins of actively growing colonies and used to inoculate each plate. Mycelial plugs were inverted and arranged in equal distances from each other. Mycelial growth was recorded after 4 d. The experiment was repeated once.

Inhibition of spore germination: A pilot study was conducted to test whether the fungicides used in the mycelial growth study could be used for spore germination tests. From this preliminary study it was evident that flusilazole, penconazole and spiroxamine did not inhibit spore germination at the concentrations tested. These fungicides were therefore excluded from the spore germination tests. Azoxystrobin, folpet, kresoxim-methyl, mancozeb, propineb and trifloxystrobin were consequently tested *in vitro* for their ability to inhibit spore germination. Kresoxim-methyl and trifloxystrobin were tested at: 0.00005, 0.0001, 0.0005, 0.001, 0.005, 0.01 and 0.05 µg a.i./mL. Azoxystrobin was tested at 0.001, 0.005, 0.01, 0.05, 0.1, 0.2, 0.5 and 1.0 µg a.i./mL. Folpet, mancozeb and propineb were tested at 0.005, 0.01, 0.05, 0.1, 0.2, 0.5, 1.0 and 5.0 µg a.i./mL. Prepared water agar (WA) (12g/L) was kept at 50°C and dilutions were made with fungicide stock solutions. MEA was used for the control and contained no fungicide.

Isolates were cultured on sterilised pieces of grapevine cane on WA. A spore suspension was made by adding cane pieces with sporulating pycnidia to sterile water containing 0.01% Tween 80. The spore suspension was diluted to obtain a concentration of 1 x 10^5 spores/ml. Within 24 h of being poured, plates were inoculated with 800 µL of spore suspension. The spore suspensions were evenly dispersed over the plate with a sterile glass hockey stick. Plates were allowed to dry in the laminar flow cabinet for 20 min and germination determined 24 h after inoculation. In total thirty spores were counted in three fields (40x magnification) per plate. Spores were considered to have germinated if the length of the germ tube was equal to or greater than the length of the spore. The experiment was repeated once.

Statistical analyses:

Mycelial growth inhibition: Isolate growth was determined by calculating the mean of two colony diameters of three replicate colonies. The effective concentration at which mycelial growth was inhibited 50% (EC_{50}) was calculated with inhibition as a proportion to the control. Percentage inhibition was plotted against fungicide concentration for each fungicide/isolate combination. The most suitable regression was fitted to each data set and the EC_{50} values calculated. These values were compared by analyses of variance to determine whether differences between and within isolates of *P. viticola* and *D. perjuncta* were significant. Since the Shapiro-Wilk statistic indicated non-normality, the data were transformed according to the square-root prior to analysis of variance. Student's t-test was carried out to determine whether there was a significant difference between the two species. Since no significant difference could be detected between them, it was decided to use only data of *P. viticola* for further analyses. This decision was further substantiated by the fact that *P. viticola,* rather than *D. perjuncta,* is the cause of Phomopsis cane and leaf spot disease. Differences in inhibitory effects of the various fungicides were determined with an analysis of variance. The mean EC_{50} values were calculated for each fungicide, and pair-wise Student t-tests were conducted on the EC_{50} values.

Inhibition of spore germination: Percentage inhibition was plotted against the log of the fungicide concentration. The EC_{50} values were calculated from the regression functions fitted to each curve. Differences in inhibitory effects of the various fungicides were determined with an analysis of variance. The mean EC_{50} values were calculated for each fungicide, and pair-wise Student t-tests were conducted on the EC_{50} values.

RESULTS

Mycelial growth inhibition: In separate analyses of variance on the $EC5_0$ values no significant differences were found within isolates and replications of *P. viticola* ($P = 0.5615$) or *D. perjuncta* ($P = 0.0704$). The t-test done on the two species indicated that there was also no significant difference between them ($P = 0.2535$). Significant differences were found among the fungicides ($P = 0.0232$). Flusilazole, penconazole and trifloxystrobin gave significantly more inhibition at lower concentrations than folpet and fosetyl-A1 + mancozeb. Azoxystrobin, flusilazole, kresoxim-methyl, penconazole, spiroxamine and trifloxystrobin inhibited mycelial growth equally well (Table 2). There was also no significant difference between azoxystrobin, kresoxim-methyl and mancozeb. Flusilazole and penconazole inhibited mycelial growth at the lowest concentrations. The coefficient of variance for both tests performed indicated that the mycelial growth tests (CV% = 74) were more accurate than the spore germination tests (CV% = 123).

Inhibition of spore germination: No fungicide/isolate interaction occurred and no significant differences were detected among the isolates ($P = 0.7725$). There were, however, significant differences among the fungicides ($P = 0.0001$). Kresoxim-methyl and trifloxystrobin gave significantly more inhibition of germination at lower concentrations than folpet or mancozeb (Table 2). Folpet required the highest concentration among the fungicides tested to inhibited spore germination by 50%. There were no significant differences between the concentrations of mancozeb, propineb and azoxystrobin needed to inhibit spore germination.

DISCUSSION

The fact that the different fungicides were equally effective *in vitro* to isolates of *P. viticola* and *D. perjuncta* indicated that the latter species, which has been associated with bud mortality (Brant *et al.,* 1999), can also be controlled by these fungicides.

Several contact fungicides required concentrations similar to those of the strobilurin fungicides to inhibit mycelial growth and spore germination. Mancozeb was comparable to both kresoxym-methyl and azoxystrobin regarding its ability to inhibit mycelial growth. Furthermore, mancozeb, propineb and azoxystrobin were also similar in their ability to inhibit spore germination.

No significant differences were found between the different strobilurins in their ability to inhibit spore germination, indicating that azoxystrobin, kresoxim-methyl and trifloxystrobin had similar efficacy *in vitro*. The strobilurin data also represent baseline sensitivity of *P. viticola* isolates to these fungicides. Since strobilurins have a single-site mode of action, it would be important to monitor the possible fungicide resistance that may develop due to the continuous use of these fungicides. These fungicides, however, hold various advantages as they are active against a wide range of pathogens, quasi-systemic, easily absorbed by the plant, environmentally safe and have no cross-reactivity with other fungicides currently on the market (Ypema & Gold, 1999).

The sterol biosynthesis-inhibiting fungicides (SBI), penconazole, flusilazole and spiroxamine, inhibited the mycelial growth of *P. viticola* at low concentrations. Previous studies also found that flusilazole inhibited the mycelial growth of *P. viticola* at the lowest concentrations tested (Fareta *et al.,* 1987; Kuropatwa, 1994). Penconazole, flusilazole and spiroxamine are normally recommended for powdery mildew control. Powdery mildew control starts early in the growth season and, if necessary, continues until after harvest with non-systemic fungicides. Results obtained here indicate that these fungicides could also inhibit mycelial growth of *Phomopsis* spp. Furthermore, the strobilurin fungicides were also shown to inhibit spore germination and mycelial growth of *P. viticola.* Field trials are now required to further determine the efficacy of these fungicides *in vivo.*

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