Phytopathol. Mediterr. (2001) 40, Supplement, S453–S458

Effect of fungicides, *in vitro*, on germination and growth of *Phaeomoniella chlamydospora*

Marlene V. Jaspers

Soil, Plant and Ecological Sciences Division, Lincoln University, PO Box 84 Lincoln University, Canterbury, New Zealand

Summary. *In vitro* studies were conducted into the activity of 22 fungicides against *Phaeomoniella chlamydospora,* the cause of Petri disease[(1)]. Mycelial growth of the pathogen was inhibited by the DMI fungicides, cyproconazole, bitertanol, tebuconazole, fenarimol, myclobutanil and prochloraz, which gave EC_{50} values of less than 0.2 mg l^{-1}; by the benzimidazole fungicides, benomyl, carbendazim and thiophanate methyl, which gave EC_{50} values of less than 0.4 mg l^{-1}; by the anilopyrimidines, pyrimethanil and cyprodinil/fludioxonil, which gave EC_{50} values of less than 0.02 mg l^{-1}. The same fungicides had different effects on germination of conidia. All the DMI fungicides tested were relatively ineffective with EC_{50} values greater than 15 mg l^{-1} for germination. Of the benzimidazoles, only benomyl was relatively effective at reducing germination, with an EC_{50} value of 0.09 mg l^{-1}, whereas the anilopyrimidines, alone or combined with phenylpyrrole, were effective with EC_{50} values of less than 0.1 mg l^{-1}. Kresoxim-methyl, which is locally systemic, was effective at inhibiting mycelial growth and germination with EC_{50} values of 0.086 and 0.11 mg l^{-1} respectively. Of the contact fungicides tested, most were effective at reducing germination of *P. chlamydospora* conidia, since their EC_{50} values were much lower than the recommended field rates, but these fungicides were also much less effective at reducing mycelial growth, for which their EC_{50} values were up to 400 times greater. The disinfectant, hydroxyquinolene sulphate was highly effective at reducing germination but less effective against mycelial growth, with EC_{50} values of 0.002 and 8.5 mg l^{-1} respectively. The potential role of these fungicides for disease management in the nursery and vineyard is discussed.

Key words: grapevine decline, fungicides, mycelial growth, germination.

Introduction

Petri disease[(1)] in young grapevines is currently of concern to grapegrowers, throughout the world. Affected young vines are frequently stunted, with chlorotic, sparse and stunted leaves and older vines often decline progressively over a number of years and may even die, although *P. chlamydospora* has not been shown to be the sole cause of their death. Internal examination of trunks and cordons of declining vines, shows longitudinal brown to black streaks and, in cross-section, dark spots which ooze tiny pinheads of shiny, black, tarry substances from cut surfaces (Mugnai *et al.*, 1999). Several species of *Phaeoacremonium* are associated with these symptoms, however *Phaeomoniella chlamydospora* is the pathogen which has been isolated most commonly from affected vines and so is generally considered to be of greatest significance. It was initially identified by Crous *et al.* (1996) as *Phaeoacremonium chlamy-*

[(1)] At the general Assembly of the 2nd ICGTD meeting held in Lisbon 2001 it was unanimously decided that young grapevine decline, 'black goo', Petri vine decline will henceforth be called Petri disease.

Corresponding author: M.V. Jaspers
Fax: +64 3 325 3843
E-mail: Jaspersm@lincoln.ac.nz

dosporum but later renamed *Phaeomoniella chlamydospora* Crous and Gams, after further molecular (Dupont *et al.*, 2000), pathological and morphological studies (Crous *et al.*, 2000).

The development of control strategies for prevention of Petri disease has been restricted because little is known of how the pathogen infects and spreads. The pathogen has been found in apparently healthy grape propagation material (Bertelli *et al.*, 1998) which has led to speculation about possible infection routes for *P. chlamydospora* in young and mature vines. Cuttings may become infected either directly from infected source vines or by contamination of their cut surfaces by conidia of *P. chlamydospora*. Sporulation has been observed on the surface of deep fissures in vine trunks and cordons (Edwards *et al.*, 2001). In addition, Larignon and Dubos (2000) were able to trap airborne conidia within a vineyard and showed that conidia were able to infect through wounds in grapevine wood. There is potential for the prevention of either infection route by fungicides, either as wound protectants or by application to propagation material in vineyards, during grafting or in nurseries. In this study, fungicides were screened for their effects on spore germination and mycelial growth of *P. chlamydospora*, to identify chemicals for further evaluation for control of Petri disease.

Materials and methods

In vitro tests were conducted using three isolates of *P. chlamydospora*, which originated from vines in New Zealand rootstock blocks. The 22 fungicides selected for study were either registered for use on grapes in New Zealand or were known to be effective against fungi related to *P. chlamydospora*. Stock solutions of the commercial fungicide products were made by suspending them directly in 1000 ml in water, while the technical grade chemicals (chlorothalonil, fenarimol, orthocide, thiram and thiophanate methyl) were first dissolved in 10 ml of acetone, before addition of 990 ml of water as recommended by Tomlin (1994).

Mycelial growth assay

For each fungicide, at least six concentrations were made, the most appropriate being initially selected from similar studies reported in the literature and additional concentrations being added as necessary, after evaluation of the first six (Table 1). For all the fungicides except Switch (contains cyprodinil), final concentrations were made in molten (50°C) potato dextrose agar (Difco, Detroit, MI, USA) and 20 ml aliquots were poured immediately into Petri dishes. Within 2–4 h after pouring, the plates were each inoculated with three 5 mm diameter discs cut from the actively growing front of 2 week old colonies of the isolates. For cyprodinil, Czapex-Dox agar (Oxoid, Basingstoke, England) was used because fungicide activity was reported to be reduced within complex media. In addition, the 5 mm inoculum discs were cut from plates on which conidia of *P. chlamydospora* had been spread for germination 48 h earlier, because older mycelia were reported to be resistant to this fungicide (Hilber and Schuepp, 1996). For all fungicides, three replicate plates were inoculated for each fungicide concentration, for each of the three *P. chlamydospora* isolates. Plates were incubated at room temperature (16–24°C), subject to natural light (day length 14–15 h). After 4 weeks, when colony diameters of control plates were approximately 40 mm, the diameters of all colonies were measured and the mean diameter of the three colonies on each plate recorded. Mean colony diameters were used to calculate growth rate inhibition for each fungicide, relative to the control plates.

Conidial germination assay

For each fungicide, a range of concentrations (Table 1) was made by adding stock fungicide solutions prepared as above, to molten (50°C) 1.5% agar (Difco). Ten ml aliquots were poured into three Petri dishes and inoculated within 2–4 h after pouring. For inoculation, conidia were harvested in sterile distilled water containing 0.1% Tween 20, from colonies grown on PDA at 20°C for three weeks. The conidial concentration was adjusted to 1×10^6 conidia per ml before spreading 100 ml aliquots onto plates of fungicide-amended agar. Three replicate plates were inoculated for each of the same three field isolates of *P. chlamydospora* as before, for all fungicide concentrations. Inoculated plates were incubated at 20°C for 48 h in dark (foil-wrapped), humid containers before determining the frequency of germination. From each plate, three 1–2 cm agar squares were cut out, mounted directly on slides and examined with a compound microscope at high magnification. Only those conidia

Table 1. Efficacy of selected fungicides against mycelial growth and conidial germination of *Phaeomoniella chlamydospora*.

Chemical group	Fungicide name	Trade name	Mycelial growth			Germination			Field rate (mg a.i. l^{-1})
			Conc. range (mg a.i. l^{-1}) and [number][b]	EC_{50} (mg a.i.l^{-1})	95% C.I. (+/-)[c]	Conc. range (mg a.i. l^{-1}) and [number][b]	EC_{50} (mg a.i.l^{-1})	95% C.I. (+/-)[c]	
Anilopyrimidines	Cyprodinil/fludioxonil (60:40)	Switch	0.001–100 [8]	0.019	0.021	0.003–10 [8]	0.066	0.061	500
	Pyrimethanil	Scala	0.0001–1.0 [9]	0.011	0.006	0.001–10 [8]	0.074	0.079	800
Benzimidazole	Benomyl	Benlate	0.01–10 [6]	0.079	0.11	0.001–10 [8]	0.090	0.046	250
	Carbendazim	Bavistin	0.001–1.0 [7]	0.078	0.053	0.01–1000 [9]	>1000	n.c.[c]	125–250
	Thiophanate methyl	Tech grade[a]	0.01–1.0 [6]	0.312	0.16	0.1–100 [9]	16.0	2.2	400
Copper	Copper hydroxide	Kocide DF	0.1–1000 [9]	11.5	n.c.[c]	0.1–100 [7]	5.9	0.97	600–1000
Cyclic imides	Folpet	Phaltan 50W	0.01–1000 [7]	21.2	1.3	0.001–10 [10]	0.008	n.c.[c]	1000
	Captan	Tech grade	1.0–1000 [8]	34.1	0.54	1.0–100 [8]	3.1	0.79	960–1200
Dicarboximide	Iprodione	Rovral WG	0.1–100 [6]	10.09	n.c.[c]	0.1–1000 [9]	57.9	2.07	375–500
Dimethylthiocarbamate[d]	Thiram	tech grade	0.1–333 [7]	14.8	0.32	0.1–100 [7]	0.57	0.09	1200–1600
DMI - imidazole	Prochloraz	Sportak EW	0.001–10 [9]	0.019	n.c.[c]	0.01–1000 [10]	15.0	1.29	250
- piperazine	Triforine	Saprol 190EC	0.1–333 [8]	19.3	1.04	0.1–1000 [8]	14.9	0.76	190
- pyrimidine	Fenarimol	tech grade	0.01–10 [7]	0.176	0.14	0.01–1000 [9]	15.7	0.63	24
- triazole	Bitertanol	Baycor 300EC	0.01–10 [6]	0.188	0.27	0.1–1000 [8]	>1000	n.c.[c]	250
	Cyproconazole	Alto 100SL	0.01–10 [6]	0.148	0.17	0.01–100 [7]	114	1.93	15
	Flusilazol	Nustar	0.003–3.3 [7]	0.031	0.044	0.01–1000 [8]	>1000	n.c.[c]	30–40
	Myclobutanil	Systhane 40W	0.001–100 [9]	0.19	n.c.[c]	0.1–1000 [8]	55.6	1.42	48
	Tebuconazole	Folicur 430SC	0.001–3.33 [7]	0.04	n.c.[c]	0.01–1000 [8]	>1000	n.c.[c]	313
Nitrile	Chlorothalonil	Tech grade	0.1–333 [7]	9.93	0.5	0.01–10 [7]	0.07	n.c.[c]	1500–1750
Phosphorous acid	Potassium phosphate salts	Foli-R-Fos 400	1.0–10,000 [7]	2140	4.9	0.1–10,000 [10]	785	2.87	500–2000
Quinolinic	Hydroxyquinoline sulphate	Chinosol W	0.1–100 [7]	8.54	0.5	0.0001–10 [11]	0.0015	n.c.[c]	675–3375
Strobilurin	Kresoxim-methyl	Stroby WG	0.001–10 [7]	0.086	n.c.[c]	0.001–10 [9]	0.11	n.c.[c]	50

[a] Technical grade.
[b] [ ] Number of concentrations tested.
[c] n.c., not calculated. Confidence intervals (CI) could not be calculated by probit analysis for that fungicide.
[d] Also listed as a disulphide.
C.I., inhibitory concentration.

whose germ tubes were at least the length of a conidium were assessed as having germinated. One hundred conidia were assessed for germination in each agar square and mean percent germination, relative to controls, was calculated for each fungicide.

Data analysis

Because data from the three isolates were similar, all data were analysed together, for both the mycelial growth assay and the conidial germination assay. Means were plotted against $\log_{10}$ values of the fungicide concentrations. Probit analysis was used to fit curves and to calculate the EC_{50} values (concentrations of the fungicides which reduced mycelial growth by 50%) and their 95% confidence limits (Table 1).

Results

The systemic fungicides tested were relatively effective at inhibiting *in vitro* mycelial growth of *P. chlamydospora* since most gave low EC_{50} values (Table 1). Of the DMI fungicides, all gave EC_{50} values of less than 0.2 mg l^{-1}, except for triforine, which gave an EC_{50} value of 19.3 mg l^{-1}. The benzimidazole fungicides were effective with both benomyl and carbendazim giving EC_{50} values of less than 0.08 mg l^{-1}, while thiophanate-methyl gave an EC_{50} value of 0.31 mg l^{-1}. For the anilopyrimidines, pyrimethanil and cyprodinil/fludioxonil, EC_{50} values were less than 0.02 mg l^{-1}. In the germination assay, the systemic fungicides gave much higher EC_{50} values, indicating that they were less effective at reducing germination than mycelial growth. Of the DMI fungicides, only prochloraz, triforine and fenarimol gave EC_{50} values less than the recommended rate of dilution for field use on grapes and fruit. Bitertanol, flusilazole and tebuconazole gave very little reduction in germination even at 1000 mg l^{-1}, the highest concentration tested. Of the benzimidazoles, only benomyl was highly effective with an EC_{50} value of 0.09 mg l^{-1}, and carbendazim was ineffective even at 1000 mg l^{-1}. The anilopyrimidines tested gave EC_{50} values of less than 0.1 mg l^{-1}, indicating effective inhibition of conidial germination.

Of the contact fungicides tested, most were highly effective at reducing germination of *P. chlamydospora* conidia, with EC_{50} values that were much lower than the recommended field rates, but these fungicides were much less effective at reducing mycelial growth, for which their EC_{50} values were up to 400 times greater. The phosphorous acid salts tested were ineffective at reducing mycelial growth and germination with EC_{50} values of 2,140 and 785 mg l^{-1} respectively. Since phosphorous acid fungicides are believed to act against a number of pathogens by eliciting induced resistance responses from the host, this result was not unexpected (Fenn and Coffey, 1984). The commonly used disinfectant of grapevine cuttings, hydroxyquinolene sulphate, was highly effective at reducing germination but not mycelial growth, with EC_{50} values of 0.002 and 8.5 mg l^{-1} respectively. The strobilurin compound, kresoxim-methyl was effective at low concentrations with EC_{50} values of 0.09 and 0.11 mg l^{-1} for mycelial growth and germination respectively, however it is not currently registered for grapes in New Zealand.

Discussion

The systemic fungicides tested in this study gave similar results to those described in the literature. A recent mycelial growth study of *P. chlamydospora* sensitivity to 12 fungicides *in vitro* (Groenwald *et al*, 2000*),* found similar EC_{50} values for eight of the fungicides (benomyl, chlorothalonil, fenarimol, iprodione, kresoxim-methyl, prochloraz, tebuconazole and thiram) reported here. Di Marco *et al*. (2000) reported that on PDA amended with 300 mg l^{-1} of the chemical phosphorous acid, mycelial growth of *P. chlamydospora* was reduced by 40%, which indicated a significantly greater effect than that reported here using the fungicide Foli-R-Fos 400, which contains potassium salts of phosphorous acid. The same authors reported that in trials of fungicides for reduction of esca and Petri disease symptoms, the phosphorous acid fungicide Fosetyl Al in foliar application, and both Fosetyl Al and DMI fungicides in trunk injections, gave variable results. It was concluded that these treatments showed promise if used in young vines in the early stages of infection.

In other similar studies with fungi related to *P. chlamydospora*, similar EC_{50} values were reported for mycelial growth. For the anilopyrimidines, the EC_{50} values for mycelial growth of *P. chlamydospora* were within the sensitive range listed for *Bo-*

trytis cinerea (Hilber and Schuepp, 1996). For the benzimidazoles, the EC_{50} values were similar to those described for *Sclerotinia homeocarpa, B. cinerea* (Grindle, 1981; Burpee, 1997) and *Cryphonectria cubensis* (Conradie *et al.,* 1992). The EC_{50} values of the DMI fungicides tested here were also within the ranges reported for sensitive fungi (0.3-0.5 mg l^{-1}) by other authors (Grindle, 1981; Conradie *et al.*, 1992; Parker and Sutton, 1993; Herman and Gisi, 1994; Burpee, 1997; Liggit *et al.*, 1997), except for triforine which gave a higher EC_{50} value (19.3 mg l^{-1}) in this study.

These results indicated that some systemic fungicides may inhibit internal spread of *P. chlamydospora* within vines, thereby reducing symptom development in vines affected by Petri disease. It is possible that regular foliar application of systemic fungicides to young vines, particularly during periods of environmental stress, may enable them to overcome the effects of the pathogen during the establishment stage, when *P. chlamydospora* has been reported to cause greatest losses. Application of systemic fungicides to infected vines in the source blocks from which vine cuttings are taken annually for new plants, may restrict spread of the fungus within vines, thereby preventing infection of all shoots throughout their length. This may allow some healthy cuttings to be harvested from infected stock and so reduce the rate of infection within the planting material entering new vineyards. Greenhouse and field studies should be conducted to determine whether the DMI, benzimidazole and anilopyrimidine fungicides, which are registered and routinely used for control of powdery mildew or Botrytis rot in grapes, are also effective in protecting vines against the effects of Petri disease.

For the contact fungicides tested, the EC_{50} values for mycelial growth of *P. chlamydospora* were greater than those reported for other similar fungi (Conradie *et al.*, 1992; Smith and Black, 1993). However, many of these fungicides were highly effective at reducing germination of conidia, with EC_{50} values much lower than their field rates. The efficacy of these contact compounds in reducing conidial germination indicates a potential for them to be used as wound protectants. Some of the effective systemic compounds, especially the anilopyrimidines and benomyl may also be effective in this role. Benomyl is registered for use as a wound paint in grapevines in the USA, but not in all grape-growing countries world-wide. It is used at high rates (0.5-1.25% active ingredient), applied directly to fresh pruning wounds, and has been shown to reduce infection by *Eutypa lata* by up to 80%. Munkvold and Marois (1993) examined the effects of benomyl and several DMI compounds for activity against *E. lata* both *in vitro* and as wound protectants in grapevines. They found that fenarimol, myclobutanil, flusilazole and benomyl all gave EC_{50} values of less than 0.55 and 1.1 mg l^{-1} for mycelial growth and conidial germination respectively. These compounds were also effective at reducing infection of grapevine wounds except when rain occurred within a few days of application, with only benomyl and flusilazole being sufficiently rainfast to give good protection.

The compound hydroxyquinoline sulphate is used in New Zealand and in the USA as a disinfectant soak for grapevine cuttings, to prevent infection by *B. cinerea* and the crown gall bacteria *Agrobacterium* spp., in cool storage. In this study, hydroxyquinoline sulphate was found to be highly effective at reducing germination of *P. chlamydospora* conidia, with EC_{50} and EC_{95} values of 0.0015 and 0.03 mg l^{-1}, respectively, indicating that it may provide valuable protection against infection during plant propagation processes.

The fungicides identified as being effective against *P. chlamydospora* in this investigation, may have potential to protect vines from the effects of this pathogen. The strategies and methods of fungicide application need to be determined experimentally on vines of all ages. Although the disease cycle for Petri disease has not been fully elucidated, recent studies have reported probable routes of infection and spread and these are sufficient to allow field and greenhouse trials to be attempted. The potential fungicide strategies identified by this study should be investigated in the search for solutions to the problem of Petri disease, for which there is a serious need, worldwide.

Acknowledgements

I am grateful to Chris Frampton, Lincoln University for statistical analysis, and to the agrichemical companies which donated fungicides: Aventis Crop Science; BASF NZ Ltd; Crop Care Holdings Ltd; Cyanamid of NZ Ltd; Dow Agrosciences (NZ)

Ltd; Du Pont NZ Ltd; Fruitfed Supplies; Key Industries Ltd; Novartis Crop Protection; Nufarm Ltd.

Literature cited

Bertelli E., L. Mugnai and G. Surico, 1998. Presence of *Phaeoacremonium chlamydosporum* in apparently healthy rooted grapevine cuttings. *Phytopathologia Mediterranea* 37, 79–82.

Burpee L.L., 1997. Control of dollar spot of creeping bentgrass caused by an isolate of *Sclerotinia homeocarpa* resistant to benzimidazole and the demethylation-inhibitor fungicides. *Plant Disease* 81, 1259–1263.

Conradie, E., W.J. Swart and M.J. Wingfield, 1992. Semiselective medium for isolation of *Cryphonectria cubensis*. *Phytopathology* 82, 1440–1442.

Crous P.W., W. Gams, M.J. Wingfield and P.S. van Wyk, 1996. *Phaeoacremonium* gen. nov. associated with wilt and decline diseases of woody hosts and human infections. *Mycologia* 88, 786–796.

Crous P.W. and W. Gams, 2000. *Phaeomoniella chlamydospora* gen. et comb. nov., a causal organism of Petri grapevine decline and esca. *Phytopathologia Mediterranea* 39, 112–118.

Di Marco S., A.Mazzullo, F. Calzarano and A. Cesari, 2000. The control of esca: status and perspectives. *Phytopathologia Mediterranea* 39, 232–240.

Dupont J., S. Magnin J., Paronnaud and M-F. Roquebert. 2000. The genus *Phaeoacremonium* from a molecular point of view. *Phytopathologia Mediterranea* 39, 119–124.

Edwards J., N. Laukart and I.G. Pascoe, 2001. *In situ* sporulation of *Phaeomoniella chlamydospora* in the vineyard. *Phytopathologia Mediterranea* 40, 61–66.

Fenn M.E. and M.D. Coffey, 1984. Studies on the in vitro and in vivo antifungal activity of fosetyl-Al and phosphorous acid. *Phytopathology* 74(5), 606–611.

Grindle M. 1981. Variations among field isolates of *Botrytis cinerea* in their sensitivity to antifungal compounds. *Pesticide Science* 12, 305–312.

Hermann D. and U. Gisi, 1994. Cross-resistance among DMI fungicides and sensitivity distributions of *Septoria tritici* populations. *Brighton Crop Protection Conference - Pests and Diseases,* 487–492.

Hilber U.W and H. Schuepp, 1996. A reliable method for testing sensitivity to *Botryotinia fuckeliana* to anilopyrimidines *in vitro*. *Pesticide Science* 47, 241–247.

Larignon P. and B. Dubos, 2000. Preliminary studies on the biology of *Phaeoacremonium*. *Phytopathologia Mediterranea* 39, 184–189.

Liggit J., P. Jenkinson and D.W. Parry, 1997. The role of saprophytic microflora in the development of Fusarium ear blight of winter wheat caused by *Fusarium culmorum*. *Crop Protection* 16, 679–685.

Munkvold G.P. and J.J. Marois, 1993. The effect of fungicides on *Eutypa lata* germination, growth and infection of grapevines. *Plant Disease* 77, 50–55.

Mugnai L., A. Graniti and G. Surico, 1999. Esca (black measles) and brown wood-streaking: two old and elusive diseases of grapevines. *Plant Disease* 83, 404–418.

Parke, K.C. and T.B. Sutton, 1993. Effect of temperature and wetness duration on apple fruit infection and eradicant activity of fungicides against *Botryosphaeria dothidea*. *Plant Disease* 77,181–185.

Smith B.J. and L.L. Black, 1993. *In vitro* activity of fungicides against *Colletotrichum fragariae*. *Acta Horticulturae* 348, 509–512.

Tomlin C. (ed.) 1994. *The Pesticide Manual, Incorporating the Agrichemicals Handbook*. British Crop Protection, Surrey, England, 1341 pp.

Accepted for publication: December 17, 2001