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Pollination of *Vitis vinifera* L. cv. Pinot noir as influenced by *Botrytis* fungicides

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Summary

The effects of fungicides on grape (*Vitis vinifera* L. cv. Pinot noir) pollen germination and tube growth were studied *in vitro*, and a related field trial examined effects of time of fungicide application during flowering on seed number and fruitset. Fungicides pyrimethanil, cyprodinil + fludioxonil and chlorothalonil incorporated into *in vitro* germination medium at the recommended mixing rate for field application all prevented pollen germination. When the fungicides were diluted to 10 % of the recommended mixing rate, pollen grain germination was still completely inhibited. Further dilution to 1 %, resulted in some pollen grains germinating but the level was significantly less than the control, and the grains that did germinate had stunted pollen tubes (< 100 µm in length), compared with controls not exposed to fungicide (800 µm in length). In a 0.1 % dilution of the fungicides, germination percent was not significantly different to untreated control, however pollen tube length was still slightly suppressed in media containing pyrimethanil (523 µm) and cyprodinil + fludioxonil (366 µm). In spite of these marked effects on pollen germination and tube growth *in vitro*, plants sprayed with pyrimethanil at either 5 or 80 % cap fall, resulted in no significant reduction in fruitset. Seed set however was affected, with seed number per berry being significantly reduced on inflorescences that were sprayed at 5 % cap fall.

Key words: grape, *Vitis vinifera*, fungicides, pollen germination, pollen tube growth.

Introduction

Fungicide applications in grapevines during full bloom are common practice, even though for other crops it is well known that both active ingredients and other components of commercial formulations can adversely affect growth of pollen tubes (KOPCKE *et al.* 2002; PAVLIK and JANDUROVA 2000; DAG *et al.* 2001; HE *et al.* 1996; ELEZABY and HASSEEB 1995; MARCUCCI and FILITI 1984; GENTILE *et al.* 1978). Several authors have also reported a negative effect of fungicides on the *in vitro* germination of pollen of several species. Fungicides incorporated into the media, or sprayed on the surface of the medium, reduced pollen germination and pol-

len tube growth at concentrations lower than those commercially recommended for successful disease control. HEAZLEWOOD and WILSON (2004 a) showed that pollen germination of Pinot noir occurred only after cap fall, and therefore raised the possibility that fungicide application during flowering may adversely effect fertilisation and in turn reduce bunch size and contribute to the variable yields commonly found with this variety under cool climate conditions (HEAZLEWOOD *et al.* 2004 b)

As reported in NIKOLOV *et al.* (1999) there have been few studies of the influence of fungicides applied during flowering on grapevine growth and development and nature of fruiting; GÄRTEL (1961) established the inhibiting influence of copper containing fungicides on grapevine growth and DYE and HAMMETT (1977) showed no significant changes in Cabernet Sauvignon yield and quality following treatment with benzimidazol during flowering. BOTIVANSKI *et al.* (1998 a and b) showed that synthetic fungicides applied during flowering have a significant effect on generative processes of Bolgar and Cabernet Sauvignon varieties. NIKOLOV *et al.* (1999) found that a selection of fungicides used to control *Botrytis cinerea* and other common diseases had varied effects on pollen germination and pollen tube growth of the grape varieties Bolgar and Cabernet Sauvignon. The fungicides chlorothalonil and cyprodinil reduced Cabernet Sauvignon pollen germination by 10 and 13 % respectively and reduced pollen tube length. Fungicides in the Triazole group did not significantly influence pollen germination. Cuprous fungicides showed a strong inhibiting effect on pollen germination and pollen tube length for cv. Bolgar. As a whole Cabernet Sauvignon pollen was more tolerant to fungicide application.

This present study examined the effects of three commonly used *Botrytis* fungicides in cool climate viticulture on the *in vitro* and *in vivo* germination of pollen of Pinot noir.

Commercial application rates of fungicides and the level of flowering at the time of application were also considered.

Material and Methods

In vitro experiments: Excised anthers of cv. Pinot noir were collected from vines in a commercial vineyard in Southern Tasmania, Australia, desiccated and stored

in Eppendorf tubes at $-80\text{ }^{\circ}\text{C}$ until required. The stored pollen was rehydrated for 30 min at $35\text{ }^{\circ}\text{C}$ at 100 % relative humidity, immediately before testing.

Commercial formulations of three fungicides, at the concentrations listed in Tab. 1, were added to a liquid germination medium containing 10 % (w/v) sucrose, 1mM boric acid and 1mM calcium nitrate. This method was adapted from SHIVANNA *et al.* (1991) with the sucrose concentration determined by experimentation in this study. For each concentration of fungicides the pH was measured and buffered if necessary to the pH of the nutrient solution, pH 3.64.

The hanging drop method employed by ELEZABY and HASSEEB (1995) was used for pollen germination. One drop of the nutrient medium containing the fungicide was placed on a slide cover slip. Three anthers from different flowers were gently stroked across the surface of the drop to deposit pollen. A greased rubber ring approximately 3 mm thick separated a slide and the inverted cover slip, with the drop hanging in the centre of the ring, elevated above the slide (Fig. 1).

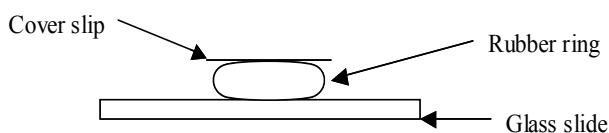


Fig. 1: Hanging drop method for germinating pollen in liquid media.

The slides were left for 10 h in a Contherm Incubator 145 MCP at $20\text{ }^{\circ}\text{C}$ with 15 W fluorescence tubes, producing a photon flux density at specimen level of approximately $40\text{ }\mu\text{mol m}^{-2}\text{s}^{-1}$, before germination and tube length were measured. Pollen was recorded as germinated when the pollen tube length reached a length equal to, or greater than, the diameter of the pollen grain. Germination percentage was determined on a count of 450 grains and the length of 120 pollen tubes was measured for each replicate. Pollen tube morphology was also noted as being normal or abnormal.

The effect of the three fungicides (pyrimethanil, cyprodinil + fludioxonil and chlorothalonil) at 4 concentrations (Tab. 1) on pollen germination was examined in a 3 by 4 factorial design with an external control (no fungicide) in a randomised complete block design. For analysis of variance, dilution rates in which no germination occurred were omitted from the calculation to give an analysed design of only two dilution rates in the factorial. The analysis was carried

out on the general linear models package of SPSS, with Fishers LSD ($P = 0.05$) values calculated after STEEL and TORRIE (1980), for comparison between treatment means.

Field experiment: The effect of spraying pyrimethanil on fruitset during flowering was examined in a commercial Pinot noir vineyard at Berriedale in Tasmania, Australia. Pyrimethanil was applied using hand operated spray equipment at the recommended commercial mixing rate at either 5 % or 80 % cap fall and was compared with untreated control plants. Fifteen vines were chosen at random in a section of the vineyard and assigned to one of the two treatments or untreated control so that each treatment consisted of 5 replicates in a completely randomised design. Spray screens of thick plastic were erected during spraying to protect trial vines from spray drift during fungicide applications. Treated vines were buffered with three additional sprayed vines on either side. Control vines were sprayed with water. For treated vines, a single bunch was chosen at random and tagged for fruitset counts. Flowers on each tagged bunch were counted just before fungicide treatment and 10 d after fungicide treatment, and the number of grapes on the same bunch were counted when the fruit was approximately 2 mm in diameter and again at harvest maturity. Fruitset was calculated as the proportion of flowers setting fruit. Seed counts were performed at harvest and seeds were tested for viability by the water floatation test as outlined by WAKANA *et al.* (2002).

Results

In vitro experimentation: Pollen germination was completely inhibited for each fungicide when added to *in vitro* germination media, at the recommended rate and at 10 % of the recommended rate for disease control in the field (Tab. 2). At lower concentrations, there was a significant interaction between fungicide and dilution rate for both pollen germination and tube length as shown in Tabs 1 and 2.

Dilution to 1 % allowed some pollen germination in each treatment with pyrimethanil allowing significantly ($P < 0.05$) greater percent germination than either, cyprodinil + fludioxonil or chlorothalonil. There was no difference in tube length between the three fungicides at the higher concentration. Tube form also changed, with tubes having swollen ends at higher concentrations (Fig. 2). At the lowest fungicide concentrations percentage pollen germination and mean

Table 1

Fungicide formulation concentrations used for *in vitro* pollen germination study. The concentration refers to the concentration of fungicide, not the concentration of active ingredient

Fungicide		Concentration			
Trade Name	Active Ingredient	Recommended	10 % dilution	1 % dilution	0.1 % dilution
Scala	cyrimethanil	2 ml·l ⁻¹	0.2 ml·l ⁻¹	0.02 ml·l ⁻¹	0.002 ml·l ⁻¹
Switch	cyprodinil + fludioxonil	0.8 g·l ⁻¹	0.08 g·l ⁻¹	0.008 g·l ⁻¹	0.0008 g·l ⁻¹
Elect 720	chlorothalonil	1.6 ml·l ⁻¹	0.16 ml·l ⁻¹	0.016 ml·l ⁻¹	0.0016 ml·l ⁻¹

Table 2

In vitro germination percentage of pollen grains exposed to dilutions of fungicides as shown. Figures with the same superscript were not significantly different based on the LSD ($P = 0.05$) calculated for arcsine square root transformed data. Results with zero germination not included in the statistical analysis

Fungicide	Recommended	Percent germination		
		10 % dilution	1 % dilution	0.1 % dilution
Pyrimethanil	0	0	53 ^b	62 ^b
Cyprodinil + fludioxonil	0	0	25 ^a	48 ^b
Chlorothalonil	0	0	7 ^a	52 ^b
Control	62 ^b			

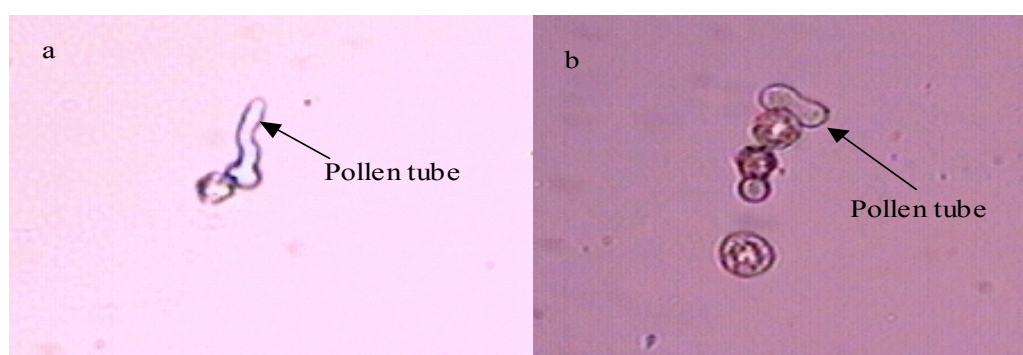


Fig. 2: Pollen tube growth in nutrient solution (a) and malformed pollen tube in 1.0 % fungicide dilution (b).

pollen tube growth were significantly higher compared with the higher concentration for the three fungicides. When diluted there was no significant difference between fungicide effects on germination, and fungicide treatments were not significantly different compared with the control (Tab. 2). Pollen tube length was, however, significantly ($P < 0.05$) reduced by both pyrimethanil and cyprodinil + fludioxonil compared with chlorothalonil and the control (Tab. 3).

Field experiment: The percent fruitset was not affected by the pyrimethanil fungicide application at either stage of flowering, with the percent fruitset in all sample bunches reaching 85 %. The resulting average seed number at harvest however was affected by fungicide application with the average for the control 2.1, and the 5 % cap fall treatment having 1.8 and the 80 % cap fall treatment 2.2 seeds (LSD = 0.29). All seeds in the control and 80 % cap fall treat-

ment were viable, two berries in the 5 % cap fall treatment had non-viable seeds.

Discussion

The results confirm that, for Pinot noir, pollen tube germination and growth are affected by the presence of *Botrytis* fungicides when germination is carried out on a nutrient solution *in vitro*. This is consistent with grapevine cultivars like Cabernet Sauvignon and Bolgar in earlier findings by NIKOLOV *et al.* (1999). In this study strong inhibitory effect *in vitro* did not, however, result in any significant reduction in fruitset in field trials. There was a significant reduction in seed number for the 5 % cap fall treatment but not the 80 % cap fall treatment.

Table 3

In vitro pollen tube length for pollen germinated at the dilutions shown. Figures with the same superscript were not significantly different based on the LSD ($P = 0.05$) calculated for untransformed data. Figures with zero growth were not included in the statistical analysis

Fungicide	Recommended	Pollen tube length (mm)		
		10 % dilution	1 % dilution	0.1 % dilution
Pyrimethanil	0	0	93 ^a	523 ^b
Cyprodinil + fludioxonil	0	0	80 ^a	366 ^b
Chlorothalonil	0	0	60 ^a	750 ^c
Control	800 ^c			

Table 4

In vivo fruitset data for the levels of flowering shown. Figures with the same superscript were not significantly different based on the LSD ($P = 0.05$) calculated for the untransformed data, ns = not significant

	Berries n	Fruitset %	Seeds per grape n	Viable seeds %
5 % cap fall	42.8	85	1.89 ^a	98
80 % cap fall	22.2	85	2.24 ^b	100
untreated	56.4	88	2.11 ^b	100
LSD	ns	ns	0.29	ns

The extended flowering period of Pinot noir and links between cap fall and pollen germination (HEAZLEWOOD and WILSON 2004 a) would result in a relatively small proportion of the total pollen yield in each flower directly exposed to fungicide at the time of application. Pyrimethanil and cyprodinil + fludioxonil are both systemic fungicides, so that although direct exposure might be limited, ongoing exposure to systemic material or surface residue would be expected, especially for the application at 5 % cap fall. Chlorothalonil is a contact fungicide with multi-site activity; it showed stronger recovery rates than the other two fungicides, particularly in pollen tube length where the results were not significantly different from the control. CHURCH *et al.* (1983) suggested that some chemicals can be less toxic to pollen lodged on stigmas than *in vitro*, due to dilution by stigmatic fluid. In either case it would appear that dilution of the fungicide either internally or on the surface of the stigma, may reduce the concentration to which the pollen is exposed below damaging levels.

YI *et al.* (2003) found that fungicide sprays caused detrimental effects on stigma morphology and enhanced exudates production in almond flowers. Percent fruitset was not measured in the YI *et al.* (2003) study, however increased exudates production was raised as possibly causing inhibition of pollen tube growth and germination. It was also suggested that the increased exudates production may be a stress response which could decrease the period of stigma receptivity (YI *et al.* 2003). Cyprodinil promoted a copious increase in exudates secretion and caused the most severe collapse of stigmatic cells of all the fungicides evaluated in the almond study.

The mode of action of the fungicide, systemic or contact, is thought to alter the level of damage caused to pollination. PAVLIK and JANDUROVA (2000) stated, that systemics are transported by translocation in the xylem and phloem, with a very small part of the compound entering into the anthers where the pollen grains are produced. Therefore, pollen grains cultivated *in vitro* should be much more sensitive to potentially toxic compounds than plants grown under field conditions. Contact fungicides would have to make direct contact with the pollen grain or stigmatic surface to cause pollen toxicity. KOPCKE *et al.* (2002) suggest that non-systemic contact fungicides would be unlikely to have an

effect if the pollen tubes have already penetrated into the style.

Previous work by HEAZLEWOOD and WILSON (2004 a) reported that although anthers dehisced in closed flowers, pollen grains already on the stigma did not begin to germinate until the cap was ejected. It is therefore reasonable to assume that recently opened flowers would be the most susceptible to damage caused by fungicide contact, whereas capped flowers and flowers that have been open for some time would not be susceptible to pollen damage.

Pollen tubes growing under fungicide concentrations, which reduced but did not completely inhibit pollen germination *in vitro*, showed markedly shorter tubes, and unusual tube morphology. At 1 % dilution, many pollen tubes exhibited swollen ends (Fig. 2), which is consistent with symptoms observed by HE *et al.* (1996) where the fungicide Benomyl caused tube rupture and swollen ends.

It would be expected in situations where flowering is reduced to a short period, or where cap fall is concentrated into a relatively short period within overall flowering, pollen vulnerability to fungicide may be an issue. Cool moist conditions that favour fungal growth and therefore require greater fungicide use may also delay or even prevent cap fall (MAY 2004). Consequently although the present results indicate little cause for concern, concentration of cap fall in an otherwise long flowering period may result in an unusually high direct exposure of pollen to *Botrytis* fungicides. A further study into the possible damage to fruitset in grapevines by fungicide applications during flowering appears warranted especially under cool damp, high disease pressure conditions.

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Erratum

In the paper

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Greek characters haven't been printed in the abstract section. Units must be read as μm , not mm.

The publishers apologize for this error.