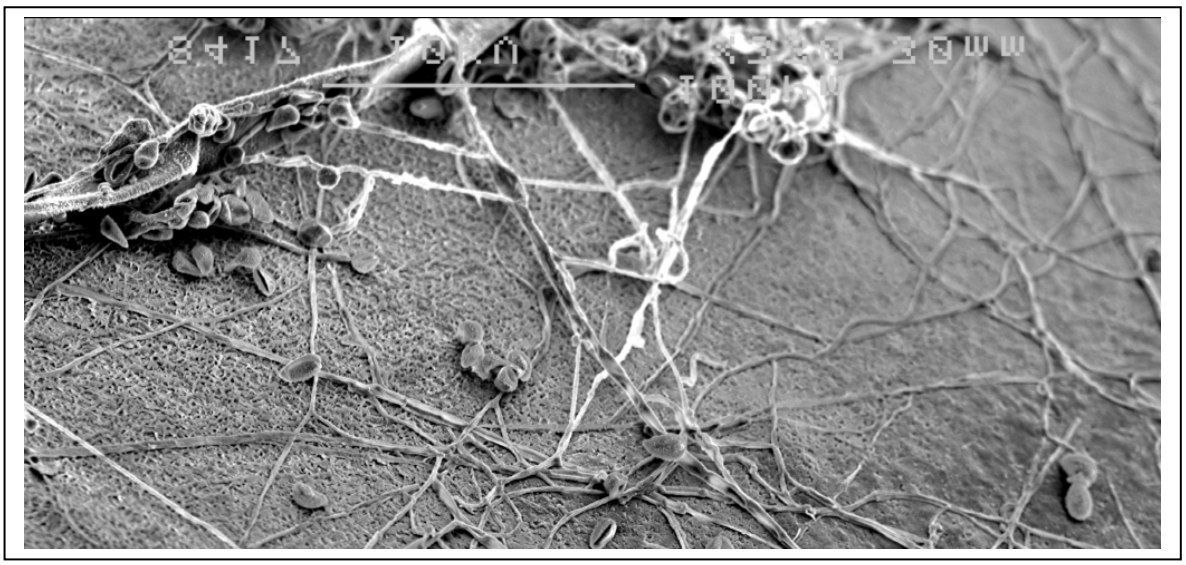
The surface wax of the grape berry: interactions with chemical sprays and subsequent susceptibility to Botrytis infection



Botrytis hyphae growing over surface of grape berry

FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **CSU 02/01**

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Research Organisation: **National Wine & Grape Industry Centre**

Date: **February, 2005**

The surface wax of the grape berry: interactions with chemical sprays and subsequent susceptibility to Botrytis infection

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1. Abstract

Spray adjuvants allow better coverage and penetration of pesticides and are used extensively in viticultural spray programs. Adjuvants, however, may increase a tissue's susceptibility to pathogens that were not a target of the pesticide, or to the target pathogen as the active ingredient wears off over time. Chardonnay, Shiraz and Cabernet Sauvignon berries were treated with four spray adjuvants in the field and subsequent effects on berry waxes, berry microflora and susceptibility to Botrytis infection were monitored. It was found that the adjuvants decreased the efficacy of the fungicide, and Botrytis infection rates were higher than when the fungicide was used alone. This was most likely through (1) degradation of wax platelets allowing easier hyphal penetration and (2) alteration of the natural microflora on the berry's surface.

2. Executive Summary

Botrytis cinerea causes grey mould on grape berries. Despite conventional spray programs, vineyards may be adversely affected by high rates of *B. cinerea* infection. There has been some anecdotal evidence in the Mudgee, NSW region that 'organic' grape growers who did not use fungicides and adjuvants sometimes experienced lower incidences of grey mould than those who applied approved conventional sprays. Furthermore, the waxy, glaucous bloom of berries can be altered in vineyards using conventional spray routines. This glaucous appearance results from light scattering off the surface of the berry, and its loss may be due to disturbance of the orientation, composition or size of the cuticular wax platelets. A range of adjuvants are used in the vineyard to maximise fungicide efficacy. The interactions of the fungicide with the adjuvant, along with the target plant species and organ are not easy to predict. We hypothesise that certain adjuvants may inflict permanent acute damage to the delicate wax layer on the berry and this may facilitate infection by *B. cinerea*. The objective of this study was to examine the effects of spray adjuvants on the berry's (1) epicuticular wax morphology, (2) natural microflora population numbers and (3) susceptibility to infection after inoculation with *B. cinerea*.

Spray adjuvants were applied to berries of Chardonnay (high *B. cinerea* susceptibility), Shiraz (intermediate susceptibility) and Cabernet Sauvignon (low susceptibility). Adjuvants recommended for use on berries were investigated along with others which are routinely used in vineyards in combination with a range of pesticides. They included (1) a wetter-spreader recommended for foliar application of fungicides and insecticides in sensitive crops such as grapevines, (2) a general-purpose wetter-spreader recommended for use with herbicides for inter-row weed control, (3) a general purpose vegetable oil concentrate recommended for use with herbicides for inter-row weed control and for application onto dormant vines at the wooley bud stage, and (4) an activator-penetrant recommended for use with woody weed sprays and contact fungicides and insecticides in situations where a normal wetter spreader is unable to provide the desired coverage. Field treatments consisted of (1) a control (water), (2) fungicide alone, (3 to 6) four adjuvants alone, and (7-10) the four adjuvants combined with the fungicide.

The four adjuvants used in this trial altered epicuticular wax morphology. Under ideal control conditions, waxes of grape berries were arranged in upright platelets. These platelets were intricate with a fine frill-like fringe. Loss in wax platelet sharpness and fine structure was least for the wetter-spreader recommended for sensitive crops, and greatest for the crop oil concentrate and the activator-penetrant. All three grape varieties reacted similarly to the treatments. Waxes did not regenerate over the season after treatment with the adjuvants. Wax disruption may decrease the physical barrier through which the hyphae penetrate, and this may lead to increased infection rates. It may also increase porosity of the cuticle which may lead to greater exudation rates of nutrients and sugars which are used by germinating *B. cinerea* conidia.

Field treated berries were taken to the laboratory and inoculated with *B. cinerea.* Berries were incubated in the laboratory in order to avoid seasonal and canopy variability in temperature and relative humidity. Incidence and severity of infection was higher for Chardonnay than Shiraz or Cabernet Sauvignon berries. The fungicide, Switch® (Syngenta), was effective at controlling *B. cinerea* infection. After 7 days of incubation only 25% of berries treated with Switch® were infected. Berries treated with an adjuvant combined with the fungicide had 45-75% incidences of infection. In the absence of the fungicide, 95-100% of the berries were infected, regardless of which adjuvant was used.

Irrespective of whether a fungicide was used, adjuvant application resulted in lower yeast and fungal populations on Chardonnay berries. Untreated berries had the highest populations, while those berries treated with the fungicide had 17% lower yeast and fungal populations.

Berries treated with the adjuvants had between 38 and 70% lower populations than the untreated berries. The adjuvants did not affect the microflora populations of Shiraz and Cabernet Sauvignon berries, except for the crop oil concentrate which resulted in higher bacterial populations on Cabernet Sauvignon berries. Adjuvants may alter the natural microflora on plant tissues through changes in the immediate physical and chemical environment. Some of the indigenous yeast, fungal and bacterial species are beneficial in that they deter the growth of pathogens on the host, for example, by competition for nutrients. Altering the ecology of the microflora on Chardonnay berries through adjuvants may have contributed to the increased susceptibility to *B. cinerea*.

In summary, the spray adjuvants used in this trial increased the susceptibility of grape berries to *B. cinerea* through epicuticular wax alteration and, in some circumstances, through the reduction of the indigenous microflora on the berry's surface. Further field trials are required to examine the impact of a wider range of adjuvants on berry surface structure, ecology and subsequent susceptibility to pathogens.

Background

Botrytis rot is an ongoing problem in viticulture and conventional spray programs are not effective every year. In some seasons, Mudgee vineyards using conventional spray programs can be worse off than organic vineyards with higher infection rates and greater losses in yield. There are a number of variables which may increase infection rates in conventional vineyards compared to the organic ones, including differences in canopy vigour leading to altered humidity, temperatures and air movement, and different ecology of fungal species due to different weeds and cover crops. The observation by growers that berries in conventional vineyards appeared to have a diminished waxy bloom prior to infection allowed us to zone in on factors that may have altered the waxes on the surface of the berry.

The outer surface of the berry is composed of the cuticular membrane that confines tissues to maintain a firm, compact form and to protect against desiccation while permitting gas exchange. It also serves to protect the plant from injuries and provides a defence mechanism against pathogens such as *Botrytis cinerea.* Epicuticular wax develops after flowering and is made of overlapping platelets that increase in size and number as the fruit ripens. For berry infection to occur from an external origin, a pathogen must either find a weakness on the berry surface where it can bypass the cuticle, or directly penetrate these layers of insoluble material. Factors that may alter the cuticle include heat, UV, and physical damage caused by birds, insects and abrasion with shoots. More likely, the difference in infection rates between the organic and conventional vineyard is agrochemical spray damage. These chemicals often contain surfactants that accelerate rates of permeation through the cuticle by altering the structure of the wax. If sprayed often enough, and in certain combinations, they may cause permanent acute damage to the delicate waxy surface of the berry, facilitating infection by Botrytis.

There have been several studies examining the interaction of Botrytis with the cuticle of the grape berry. The epicuticular wax of a mature berry is semi-crystalline to crystalline in structure and influences the wettability of the berry surface and the adhesive ability of plant pathogens. The thickness and structure of the epicuticular wax layer may contribute to the resistance of grapes to Botrytis. Cabernet Sauvignon berries had more than twice as much cuticle per unit surface area as Grenache, Zinfandel, Carignane and Pinot Noir and is considered relatively resistant to infection by Botrytis compared with these other cultivars (Rosenquist and Morrison, 1989). The thickness of the cuticle of the berry decreases as the berry grows and this may be why young grapes are more resistant to Botrytis than mature berries (Commenil *et a*l., 1997). Anti-Botrytis activity in epicuticular waxes of young berries has also been demonstrated (Commenil *et al*., 1996).

Even though the quantity of epicuticular wax and cuticle proper is predominantly genetically controlled it is influenced by environmental factors. Wax deposition is increased in high light, temperatures and humidity. Physical contact between berries also has an influence on wax development. Marois *et al.* (1986) and Percival *et al* (1993) showed that tight, compressed clusters in some grapevines are associated with the development of Botrytis bunch rot. Thompson Seedless berries showed flattened surfaces where they were in contact with other berries. Scanning electron microscopy showed that on the contact surfaces the epicuticular wax was mostly amorphous with many shallow depressions rather than defined platelets. When contact and intact surfaces of the berry were inoculated with Botrytis conidia the contact surface had a higher proportion of infection. Susceptibility to infection was also increased when the epicuticular wax was removed by dipping the berries in chloroform before inoculation (Marois *et al*., 1986). These results indicate that disturbance of the wax cuticle can lead to increased infection rates.

Project Objectives/Outcomes

Objectives:

1. To examine the effect of various vineyard chemical sprays on berry epicuticular wax structure.
2. To quantify Botrytis infection rates after various types of spray applications at different times during berry development.
3. To examine the berry surface for positive and negative microbial species interactions after spray applications.
4. To develop a more effective management strategy for the control of Botrytis bunchrot.

Outcomes:

1. An understanding of the impact of particular agrochemical spays on berry cuticular development.
2. Information about the potential negative effects of particular agrochemical sprays on Botrytis control.
3. Knowledge about changes in the microbial ecology of grape berries after spraying.
3. Better management strategies to reduce incidence and severity of Botrytis rot.

Outputs and Performance Target

Outputs	Performance Targets
1. Information about spray effects on cuticular waxes.	1. Pilot study completed by June 2003.
2. Information about Botrytis infection rates after various types of spray applications.	2. Pilot study completed by June 2003. Field and laboratory inoculations after spray applications completed by June 2004.
3. Information about the microbial flora on the surface of the berry after particular spray applications.	3. Species identified and assessed for positive or negative effects on Botrytis control by September 2004.
4. Vineyard management strategies to encompass chemical spray effects on berry cuticles.	4. Publication in industry journal and scientific journal by December 2004.

5.0 Methods

Source: Rogiers S.Y., M. Whitelaw-Weckert, M. Radovanovic-Tesic, L.A Greer, R.G. White and C.C. Steel. Effects of spray adjuvants on grape (*Vitis vinifera*) berry microflora, epicuticular wax and susceptibility to infection by *Botrytis cinerea.* Australasian Plant Pathology (in press)

Field treatments

Three grape varieties with different susceptibilities to *B. cinerea* infection were used in this study. Vines of Chardonnay (high susceptibility), Shiraz (intermediate susceptibility) and Cabernet Sauvignon (low susceptibility) (Emmett *et al.* 1992) were located in the same block at the Charles Sturt University Vineyard, Wagga Wagga, NSW. Eight pre-veraison bunches were chosen at random (four from each side of the canopy) within a three-vine plot and treated (December 22, 2003) with one of 10 adjuvant/fungicide combinations, according to recommended label rates. Bunches (still attached to the vine) were dipped in a beaker for three seconds rather than sprayed in order to ensure even and complete coverage of the berries. Each plot was separated by a buffer vine. There were three replicate plots for each variety. Total soluble solid levels at the time of treatment were 3.9 ˚Brix for Chardonnay, 4.2 ˚Brix for Shiraz, and 4.6 ˚Brix for Cabernet Sauvignon.

Field treatments consisted of (1) a control (water), (2) fungicide alone, (3 to 6) four adjuvants alone, and (7-10) the four adjuvants combined with the fungicide. Adjuvants recommended for use on berries were investigated along with others which are routinely used in vineyards in combination with a range of pesticides. The first adjuvant (V) was a wetter-spreader recommended for foliar application of fungicides and insecticides in sensitive crops such as grapevines (Table 1). The second adjuvant (W) was a general-purpose wetter-spreader recommended for use with herbicides for inter-row weed control. The third adjuvant (O) was a general purpose vegetable oil concentrate recommended for use with herbicides for inter-row weed control and for application onto dormant vines at the wooley bud stage. The fourth adjuvant (P) was an activator-penetrant recommended for use with woody weed sprays and contact fungicides and insecticides in situations where a normal wetter spreader is unable to provide the desired coverage. For microscopic observation only, bunches separate to the field trial were treated with chloroform/hexane/ether (1:1:1) and an acidifying adjuvant containing 345 g/L soyal phospholipids and 355 g/L propanoic acid at 1 mL/L as recommended on the product label.

On the day of treatment and at 21, 42 and 56 days after treatment, one bunch was sampled from each plot, with care so as not to disrupt berry surface waxes. Berry waxes were examined by cryo-scanning electron microscopy (SEM), berries were inoculated with *B. cinerea* and assessed for infection rates, and berries were assessed for microflora populations.

Table 1. Fungicide and adjuvants used in the study

Treatment	Code	Active ingredient	Concentration of active ingredient	Rate of application
Fungicide	F	cyprodinil fludioxonil	375 g/kg 250 g/kg	0.8 g/L
Wetter-spreader for sensitive foliage	V	ethoxylated octyl phenol	1 kg/L	0.10 mL/L
General purpose wetter-spreader	W	alcoxylated alcohols	1 kg/L	0.13 mL/L
Vegetable oil concentrate	O	emulsifiable vegetable oil	0.835 kg/L	2.5 mL/L
Activator-penetrant	P	polyether modified polysiloxane	1.02 kg/L	2.0 mL/L

Source: Rogiers S.Y., M. Whitelaw-Weckert, M. Radovanovic-Tesic, L.A Greer, R.G. White and C.C. Steel. Effects of spray adjuvants on grape (*Vitis vinifera*) berry microflora, epicuticular wax and susceptibility to infection by *Botrytis cinerea.* Australasian Plant Pathology (in press)

Cryo-SEM
Berries for SEM observation were stored overnight at 20˚C. A slice of skin (1 cm^2) was cut from the cheek of three berries per bunch, attached to a flat brass stub, quick-frozen at -170˚C on the cold stage of a Jeol 6400 SEM (JEOL Australasia Pty Ltd, Sydney, Australia), gold-coated, then observed.

Botrytis cinerea inoculation
Berries were inoculated with *B. cinerea* in the laboratory as opposed to the field in order to avoid seasonal fluctuations in temperature and RH, and variability in vine microclimate. At each sampling time, four berries selected randomly from each bunch were surface sterilised for 1 min with 0.5% NaOCl. Prior examination by cryo-SEM indicated that solutions of up to 2% NaOCl (results not shown) did not disrupt surface waxes. Berries were rinsed three times with sterile deionized water and placed in a well of an ELISA plate so that the cheek of the berry was facing upwards. A 10 µL drop of inoculum suspension, 10^5 *B. cinerea* spores/mL, was placed on this cheek. Berries were incubated in 100% humidity at 25˚C for 7 days and assessed for presence or absence of hyphae under a dissecting microscope. The number of infected berries were counted, and of those infected, the severity of infection (% surface covered with mycelium) was visually estimated.

Microflora populations count
At each sampling time, two berries per bunch were sonicated (Unisonics Pty Ltd, Sydney, Australia) for 15 sec in 9 mL phosphate buffered saline (pH 7.2) then placed on ice and shaken in a shaking water bath at 60 cycles per min for 1 h. A 50 µL aliquot of the berry washings were then plated on each of three plates of dichloran rose bengal chloramphenicol agar (Oxoid Australia Pty Ltd, Melbourne, Australia) or nutrient agar (Oxoid) amended with 0.03 g/L benomyl. After 10 days incubation at 25˚C, colonies were counted.

Statistics
The Genstat® (fifth edition) software package (IACR, Rothamsted, UK) was used for data analysis. Data were subjected to analysis of variance (ANOVA) for a randomized complete block design with treatments having a factorial structure of 5 adjuvants x 2 levels of fungicide. Linear regressions were carried out with SigmaPlot (version 8.0) software package (SPSS Inc., Gorinchem, The Netherlands).

5. Results

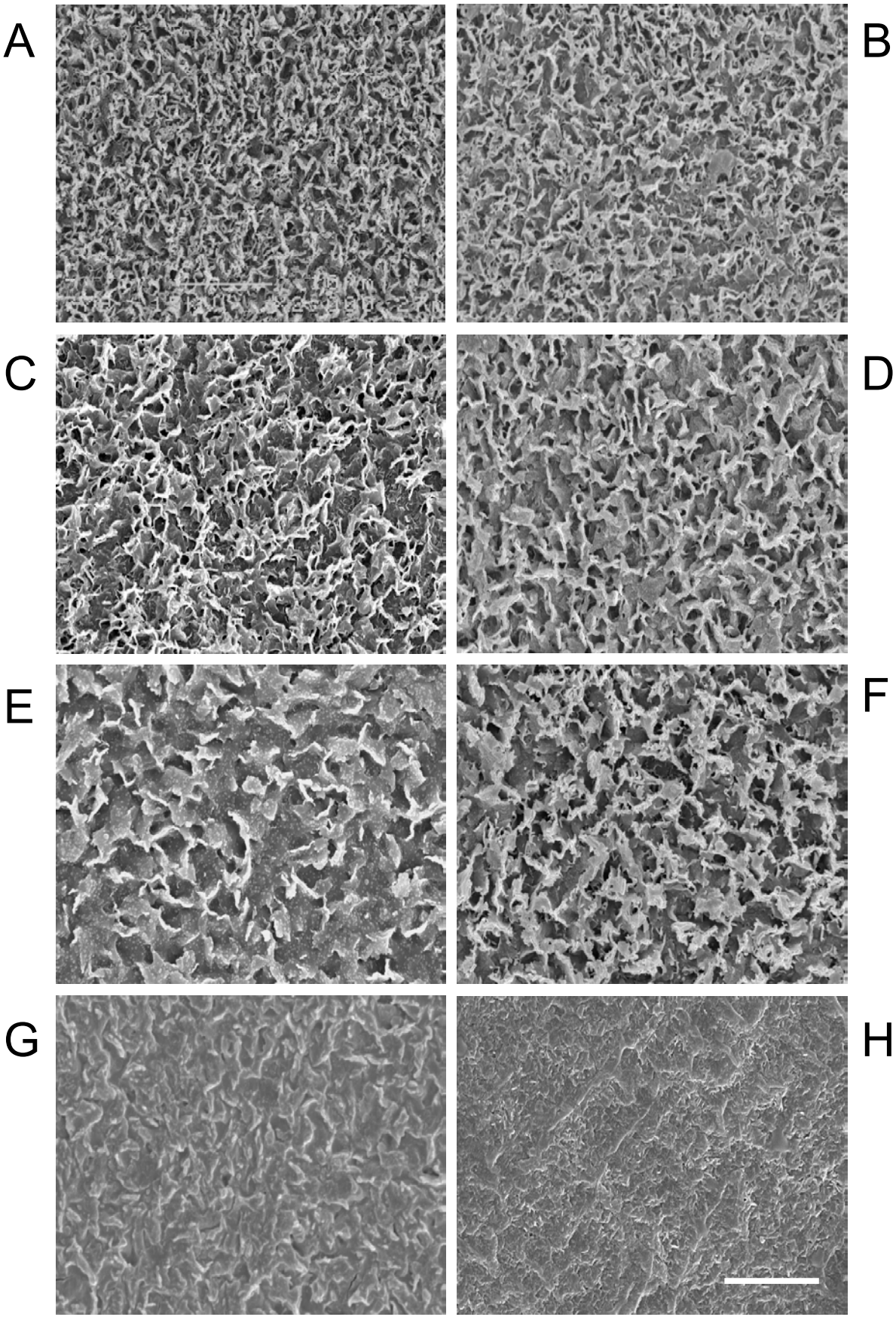
Source: Rogiers S.Y., M. Whitelaw-Weckert, M. Radovanovic-Tesic, L.A Greer, R.G. White and C.C. Steel. Effects of spray adjuvants on grape (*Vitis vinifera*) berry microflora, epicuticular wax and susceptibility to infection by *Botrytis cinerea.* Australasian Plant Pathology (in press)

Epicuticular wax structure
Waxes of grape berries grown in controlled conditions (without exposure to physical abrasion, extreme temperatures, insect damage or chemical sprays) were arranged in intricate upright platelets. A fine, frill-like fringe was often observed to line the ends of these platelets (Fig. 1A). When berries were treated with a fungicide alone, this fringe was not as intricate, with the disappearance of the fine tips (Fig. 1B). The application of adjuvants was more disruptive to the wax platelets (Figures 1C-G), but none was as harsh as a mixture of chloroform/hexane/ether, which is often used to remove waxes from berries (Fig. 1H). Of the four adjuvants tested in the field, the wetter-spreader recommended for grapevine foliage (V) was least disruptive (Fig. 1C). This was followed by the wetter-spreader for weeds (W) (Fig. 1D), the activator-penetrant for woody weeds (P) (Fig. 1F) and the crop oil concentrate (O) (Fig. 1E). The acidifying type of adjuvant (A) (used only for microscopic observation) also caused a loss of intricate platelets (Fig. 1G).

The damage to wax platelet structure did not alter with time after application (up to 9 weeks, results not shown). The waxes did not continue to degenerate further during the 9 weeks, nor did new wax synthesis result in regeneration of original platelet structure. All three grape varieties reacted similarly to the treatments.

SEM observation also indicated that fungal hyphae (unidentified) entered berries through natural openings on the surface of the berry. They entered through stomata of berries shortly after fruit-set when they were still capable of opening and closing (Fig. 2A). As the berry matured, these stomata turned into wax-occluded lenticels. The lenticels were often surrounded by crevices in the cuticle (Fig. 2B). Hyphae entered through these crevices or through the lenticel itself (Fig. 2C). Fungal hyphae also penetrated the berry through the crevices surrounding the stylar remnant (Fig. 2D). No variety differences were observed.

Fig. 1. Scanning electron micrographs (× 2000 magnification) of the effect of fungicide and adjuvants on Shiraz epicuticular wax morphology. Bar = 10 μm. (A) No spray treatment, (B) fungicide (F), (C) wetter-spreader for grape leaves (V), (D) wetter-spreader for weeds (W), (E) oil concentrate for weeds and dormant vines (O), (F) activator-penetrant for woody weeds (P), (G) acidifier, and (H) chloroform/hexane/ether (1:1:1). Source: Rogiers S.Y., M. Whitelaw-Weckert, M. Radovanovic-Tesic, L.A Greer, R.G. White and C.C. Steel. Effects of spray adjuvants on grape (*Vitis vinifera*) berry microflora, epicuticular wax and susceptibility to infection by *Botrytis cinerea.* Australasian Plant Pathology (in press).


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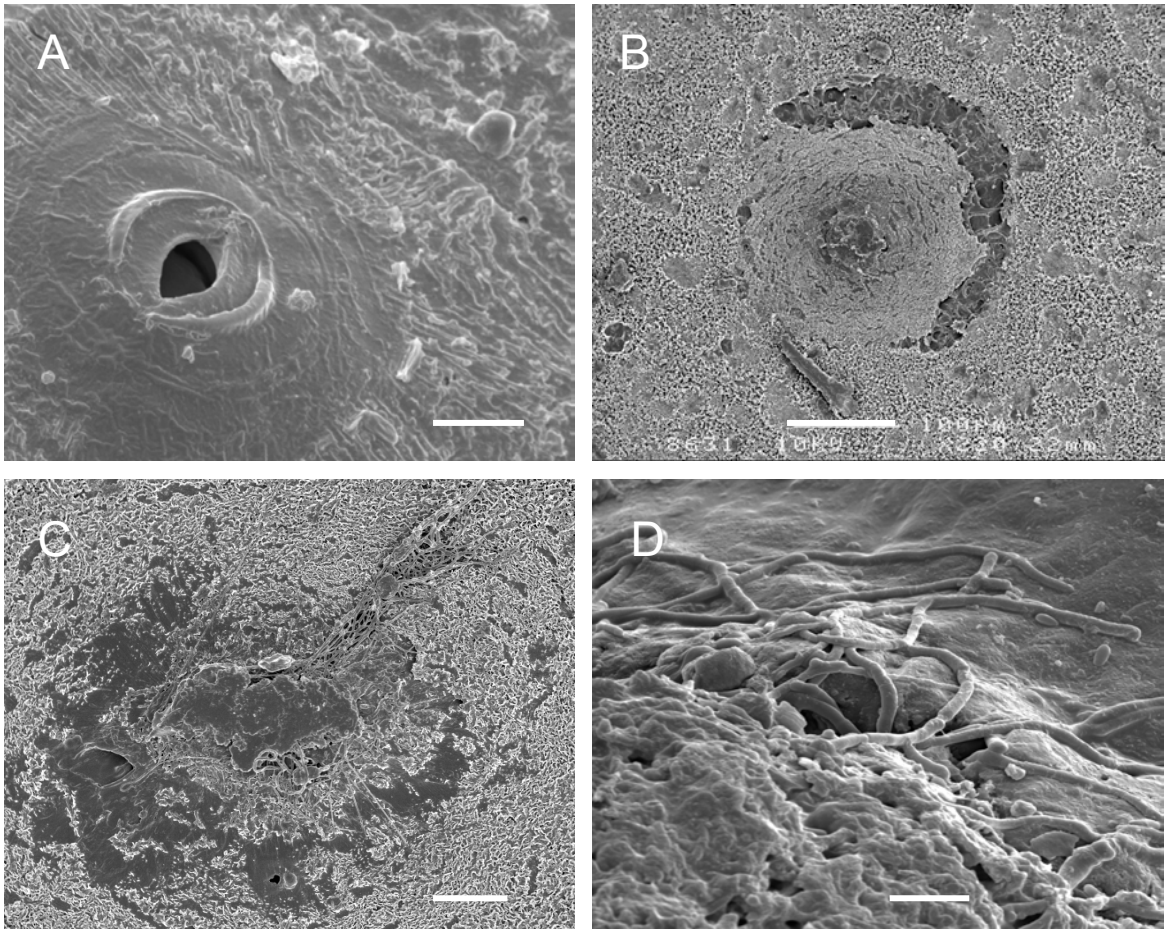


Fig. 2. Natural openings on surface of grape berries: (A) un-occluded stoma on surface of Shiraz berry (Bar = 10 µm), (B) wax-occluded lenticel on surface of Cabernet Sauvignon berry that is partially surrounded by a crevice (Bar = 100 µm), (C) hyphae growing toward Cabernet Sauvignon lenticel and beneath wax plug (Bar = 20 µm), and (D) hyphae growing in crevice surrounding stylar remnant of Shiraz berry, (Bar = 20 µm). **Source:** Rogiers S.Y., M. Whitelaw-Weckert, M. Radovanovic-Tesic, L.A Greer, R.G. White and C.C. Steel. Effects of spray adjuvants on grape (*Vitis vinifera*) berry microflora, epicuticular wax and susceptibility to infection by *Botrytis cinerea.* Australasian Plant Pathology (in press).

Botrytis inoculation

Berry samples taken on the same day of treatment, or three weeks after treatment, and inoculated with *B. cinerea* remained asymptomatic because they were still immature and green. Six weeks after treatment, all the berries had passed veraison, and after inoculation with *B. cinerea*, the incidence of infection was 87±4% for Chardonnay, 68±7% for Shiraz and 62±7% for Cabernet Sauvignon. The severity of infection (% of the berry surface colonised) was 29±5% for Chardonnay, 6±1% for Shiraz and 4±1% for Cabernet Sauvignon.

The adjuvant/fungicide treated berries differed in susceptibility to *B. cinerea* infection. The effect of treatment was significant on percentage of grape berries infected ($P < 0.001$) and percentage surface colonisation ($P< 0.001$). Berries treated with the fungicide alone had the lowest incidence of infection (25%) after 7 days incubation (Fig. 3). Berries treated with an adjuvant and fungicide had higher incidences (44-75%). Regardless of adjuvant treatment, 95-100% of inoculated berries were infected when treated in the absence of the fungicide. Surface colonisation was low (1-3% of berry skin) for berries treated with the fungicide as opposed to berries not treated with the fungicide (20 to 32%). The percent of the berry covered was usually higher if the berry had been treated with an adjuvant (Fig. 3).

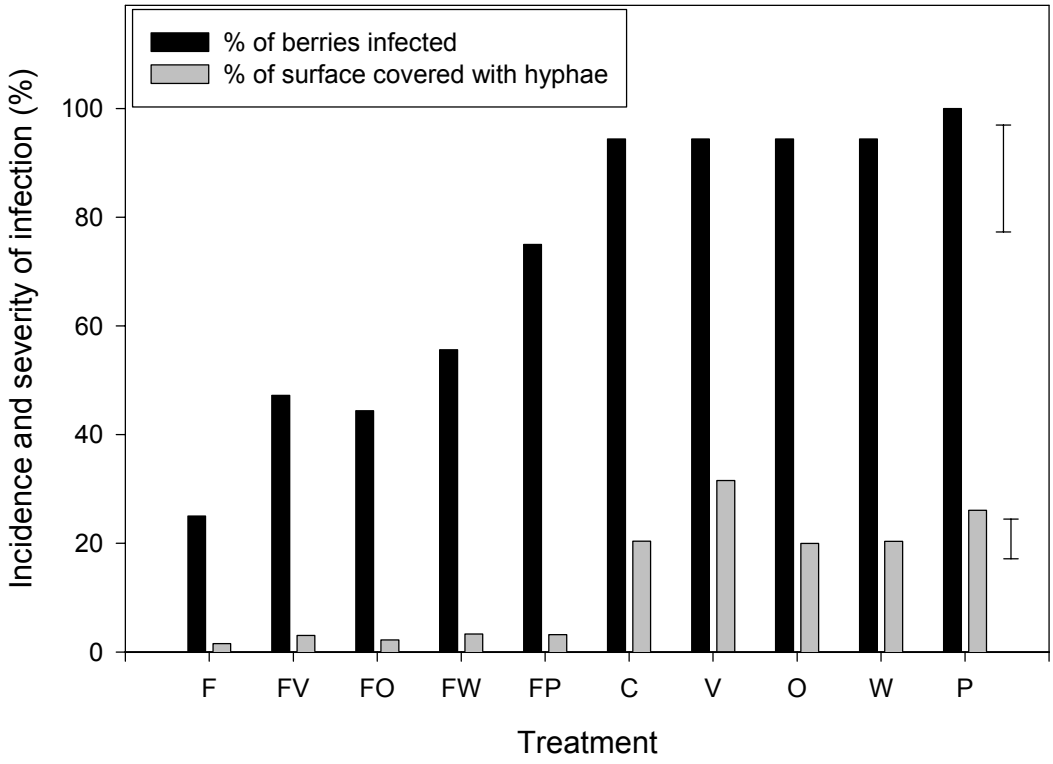


Fig. 3. Percentage of grape berries infected, and of those infected, percentage surface colonisation after inoculation with *B. cinerea* and incubation at 25˚C for 7 days. Berries were treated with adjuvants and/or fungicide in the field prior to veraison and harvested 42 days later. The effect of treatment was significant on percentage of grape berries infected ($P < 0.001$) and percentage surface colonisation ($P< 0.001$). Each data point is the average of three varieties, four berries from each of three bunches ($n = 36$). Bars represent least significant differences of the means (5%) for each adjuvant/fungicide treatment. Treatments are: F = fungicide, FV = fungicide plus wetter-spreader for grape leaves, FO = fungicide plus oil concentrate for weeds and dormant vines, FW = fungicide plus wetter-spreader for weeds, FP = fungicide plus activator-penetrant for woody weeds, C = no spray treatment, V = wetter-spreader for grape leaves, O = oil concentrate for weeds and dormant vines, W = wetter-spreader for weeds, P = activator-penetrant for woody weeds. **Source**: Rogiers S.Y., M. Whitelaw-Weckert, M. Radovanovic-Tesic, L.A Greer, R.G. White and C.C. Steel. Effects of spray adjuvants on grape (*Vitis vinifera*) berry microflora, epicuticular wax and susceptibility to infection by *Botrytis cinerea.* Australasian Plant Pathology (in press).

Microflora

The three grape varieties differed in skin microflora population, and the effect of time on colony numbers was significant for each variety (Fig. 4). The size of the yeast and fungal populations on Chardonnay berries decreased by 46% (from 4700 to 2500 colony forming units (cfu)/ berry) over the 56 days of treatment ($P < 0.001$). The populations varied between 1400 and 300 cfu/ berry for Cabernet Sauvignon berries ($P < 0.001$) and decreased by 86% (from 440 to 60 cfu/ berry) for Shiraz berries ($P < 0.001$). Similarly, as the season progressed, there was a 66% decrease in size of the bacterial populations on Chardonnay berries (from 7680 to 2360 cfu/ berry, $P < 0.001$). Bacterial populations on Cabernet Sauvignon berries increased by 330% (679 to 2230 cfu/ berry) from 0 to 21 days after treatment and then declined ($P < 0.001$). Bacterial populations on Shiraz berries increased by 470% (240 to 1120 cfu/ berry) from 0 to 42 days after treatment and then declined ($P < 0.001$). Linear regressions were not significant ($P > 0.05$) for any variety.

The fungicide/adjuvant treatments altered yeast and fungal population numbers on Chardonnay berry surfaces (Fig. 5). The effect of treatment was significant ($P < 0.001$). Untreated berries had the highest number of populations (6040 cfu/ berry). Those berries treated with the fungicide had 17% lower yeast and fungal populations than untreated berries, while berries treated with any of the adjuvants used in this trial had between 38 and 70% lower populations than the untreated berries. Throughout the season (except at 42 days after treatment), the surface bacterial populations on Chardonnay berries treated with the fungicide (with or without an adjuvant) were (up to 60%) lower than on berries not treated with the fungicide (Fig. 6). The fungicide x time interaction was significant ($P < 0.05$).

The bacterial cfu on Cabernet Sauvignon berry surfaces were higher after treatment with the crop oil concentrate (O). The crop oil concentrate ±fungicide versus no adjuvant ±fungicide treatments were significantly different ($P < 0.01$). Berries treated with the oil concentrate had a population of 2200 bacterial cfu on their surfaces as compared with 1500 cfu on the untreated berries. The bacterial populations of berries treated with the fungicide and crop oil combination were 2640 cfu per berry as compared to 730 cfu/ berry for berries treated with the fungicide alone.

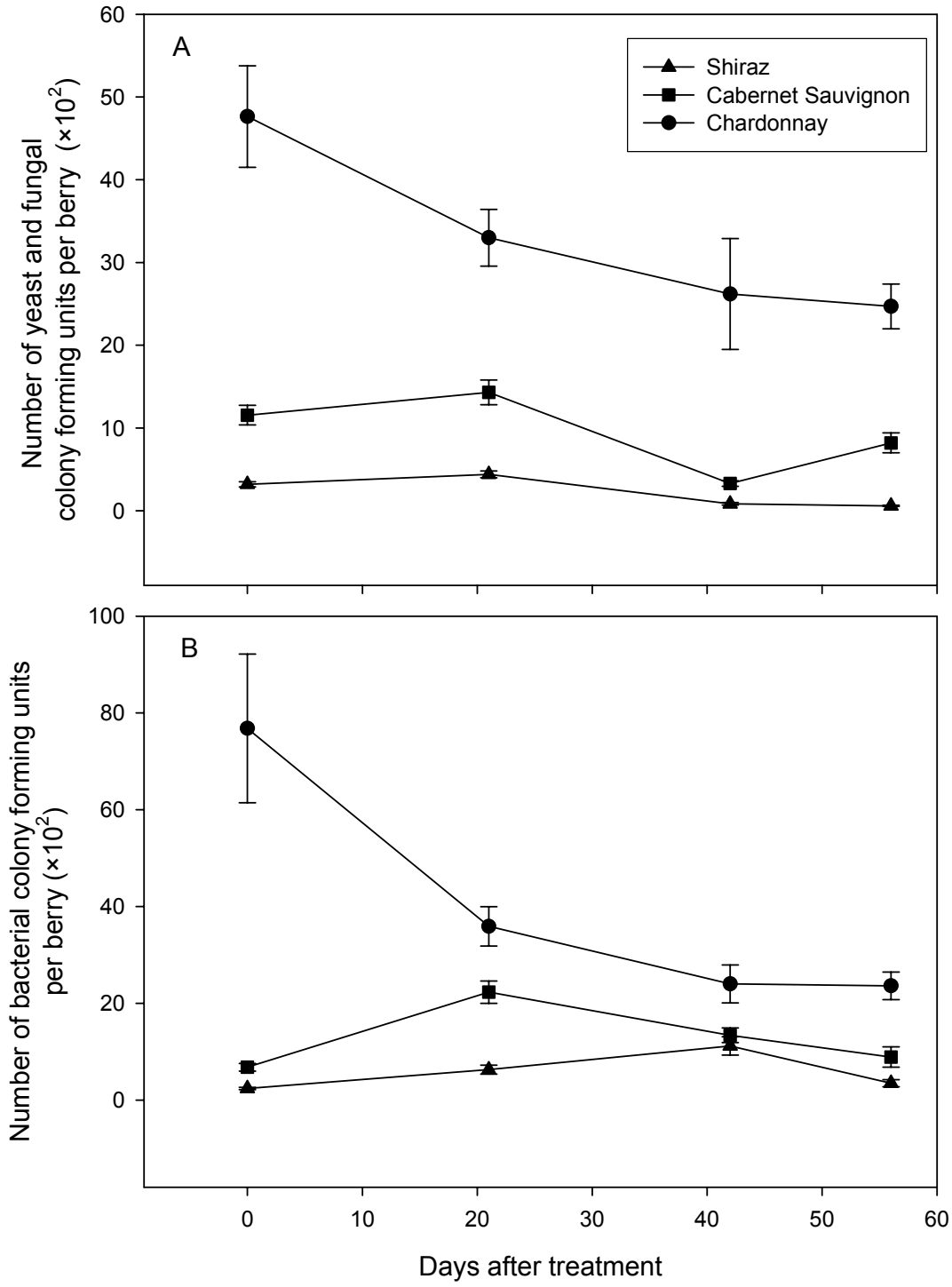


Fig. 4. (A) Number of yeast and fungal colonies ($P < 0.001$) and (B) number of bacterial colonies ($P < 0.001$) on Shiraz, Cabernet Sauvignon and Chardonnay berry surfaces 0, 21, 42 and 56 days after adjuvant/fungicide treatment. Bars represent SE of means ($n = 90$, each point is the mean of 10 treatments). **Source**: Rogiers S.Y., M. Whitelaw-Weckert, M. Radovanovic-Tesic, L.A Greer, R.G. White and C.C. Steel. Effects of spray adjuvants on grape (*Vitis vinifera*) berry microflora, epicuticular wax and susceptibility to infection by *Botrytis cinerea.* Australasian Plant Pathology (in press).

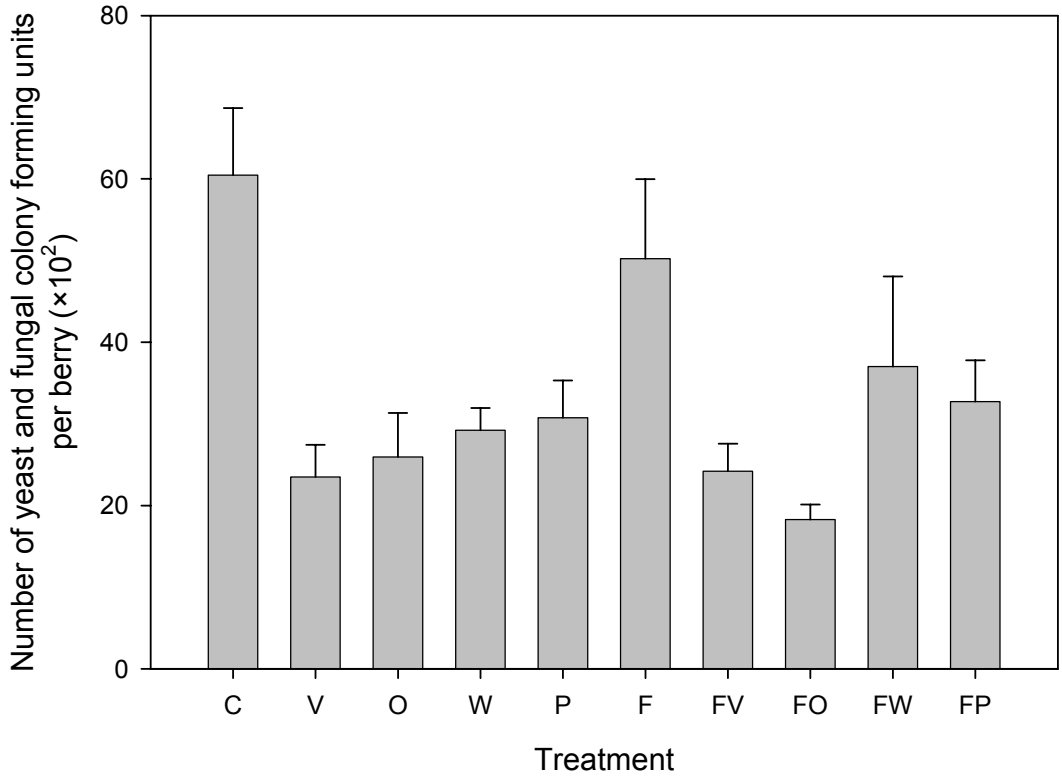


Fig. 5. Number of yeast and fungal colonies on surfaces of grape berries (cv. Chardonnay). Each data point is the mean of 4 sampling times. Bars represent SE of means ($P< 0.001$, $n = 36$). Treatments are: C = no spray treatment, V = wetter-spreader for grape leaves, O = oil concentrate for weeds and dormant vines, W = wetter-spreader for weeds, P = activator-penetrant for woody weeds, F = fungicide, FV = fungicide plus wetter-spreader for grape leaves, FO = fungicide plus oil concentrate for weeds and dormant vines, FW = fungicide plus wetter-spreader for weeds, FP = fungicide plus spreader-penetrant for woody weeds. **Source**: Rogiers S.Y., M. Whitelaw-Weckert, M. Radovanovic-Tesic, L.A Greer, R.G. White and C.C. Steel. Effects of spray adjuvants on grape (*Vitis vinifera*) berry microflora, epicuticular wax and susceptibility to infection by *Botrytis cinerea.* Australasian Plant Pathology (in press).

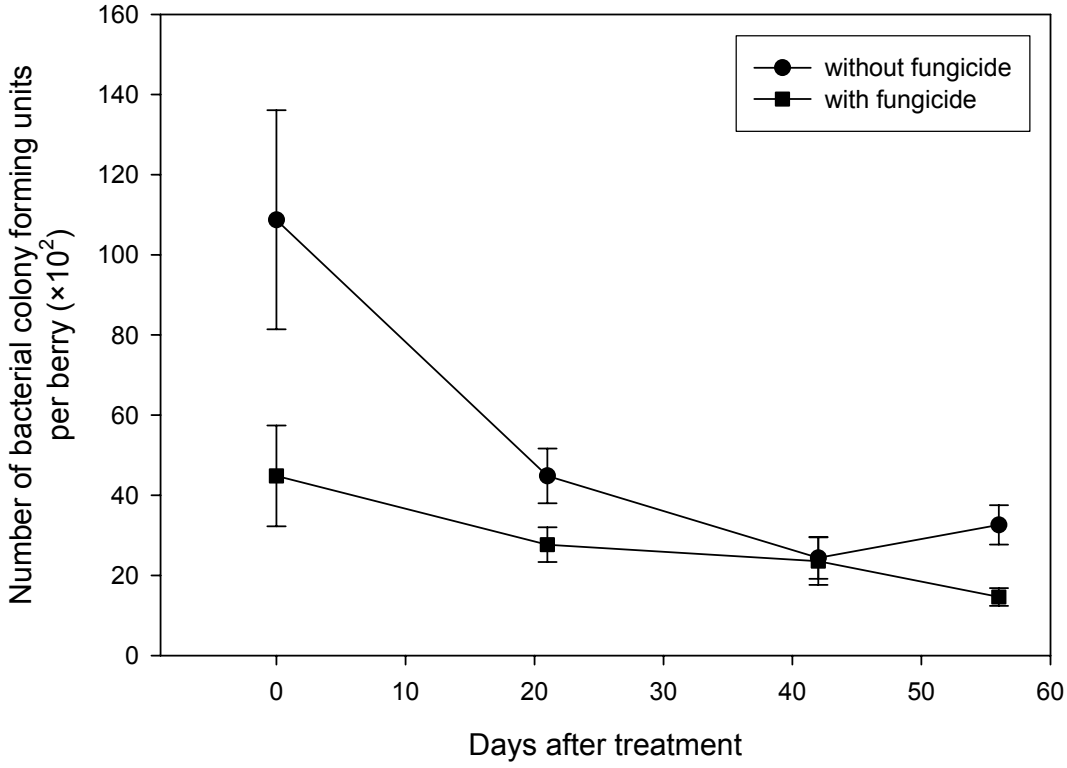


Fig. 6. Bacterial populations on grape berries (cv. Chardonnay) at 0, 21, 42 or 56 days after treatment with or without fungicide. Each data point is the average of five adjuvant treatments. The fungicide by time interaction was significant at the 0.02 level. Bars represent SE of means (n = 45). **Source**: Rogiers S.Y., M. Whitelaw-Weckert, M. Radovanovic-Tesic, L.A Greer, R.G. White and C.C. Steel. Effects of spray adjuvants on grape (*Vitis vinifera*) berry microflora, epicuticular wax and susceptibility to infection by *Botrytis cinerea.* Australasian Plant Pathology (in press).

7. Discussion

Source: Rogiers S.Y., M. Whitelaw-Weckert, M. Radovanovic-Tesic, L.A Greer, R.G. White and C.C. Steel. Effects of spray adjuvants on grape (*Vitis vinifera*) berry microflora, epicuticular wax and susceptibility to infection by *Botrytis cinerea.* Australasian Plant Pathology (in press)

Spray adjuvants assist in the penetration of pesticides through plant surface barriers (Hislop 1993). In the process, adjuvants may disrupt the cuticular layer that covers the plant's surface. Fungal infection is more likely to occur with decreased cuticular thickness, or with an increased number of entry points through the cuticle (Mlikota Gabler *et al*. 2003). Therefore, even though an adjuvant is used to increase the efficacy of a fungicide, it may increase susceptibility to other pathogens that were not a target of that fungicide. Furthermore, as the efficacy of the fungicide diminishes over time, the damaged cuticle may become more vulnerable to the target pest later in the season. Adjuvants may also alter the natural microflora on plant tissues through changes in the immediate physical and chemical environment. Some of the indigenous yeast, fungal and bacterial species are beneficial in that they deter the growth of pathogens on the host (Blakeman and Fokkema 1982), for example, by competition for nutrients (Bashi and Fokkema 1977; Elad *et al.* 1994). If the ecology of these species is altered through the use of adjuvants, the host may become more vulnerable to the pathogen.

There are few publications concerning the interaction of agrochemicals with the grape cuticle. The active ingredient will either remain on the surface of the berry or penetrate through the cuticle. Surfactants accelerate rates of permeation through the cuticle by altering the structure of the wax. The specific interaction between the berry surface, solute (active ingredient) and surfactant are not clear and more detailed studies are required on the ways surfactants modify the cuticle.

One objective of this project was to examine the affect of adjuvant sprays on grape berry waxes. *B. cinerea* may find it easier to penetrate the cuticle and infect the berry once waxes are damaged. The SEM images of this study revealed that the fringe of the wax platelets was disrupted when treated with adjuvants, and the severity of this disruption was dependent on the particular adjuvant used. Furthermore, this fine platelet structure was not regenerated as the season progressed. Other studies (Rosequist and Morrison 1989; Percival *et al.* 1993) have found that wax platelet structure had an influence on the susceptibility to *B. cinerea*. Examination of regions on berries that were in contact with other berries revealed less wax and lack of platelet structure as compared with non-contact areas. Our own observations of Shiraz, Cabernet Sauvignon and Chardonnay berry surfaces have yielded similar results with amorphous waxes predominating.

The results presented here indicate that spray adjuvants can damage berry surface waxes, but did this have any impact on successful *B. cinerea* penetration? There are numerous natural pores in the surface of the berry including the crevices around lenticels (Bessis 1972; Comménil *et al.* 1997) and the stylar remnant. At anthesis, infloresences can become infected through the stigma (McClellan and Hewitt 1973) and cap scar (Keller *et al*. 2003) with the infections remaining latent until the berries start to ripen. Similarly, the hyphae may enter through openings on the mature berry without attempting to traverse the cuticle. Through the use of SEM of mature berries, we observed hyphae of unidentified fungi penetrating through these cracks. Furthermore, grape cultivars highly resistant to *B. cinerea* have few or no pores in the berry surface (Mlikota Gabler *et al*. 2003). The results of the *B. cinerea* inoculation study after treatment with the different adjuvants, however, do indicate that in all three varieties, cuticle disruption does play a role. Berries treated with adjuvant and fungicide had higher incidence of *B. cinerea* infection than berries treated with fungicide alone.

These results indicate that the spray adjuvants included in this study (V,W, O and P) should not be used to assist in the control of *B. cinerea.* Three of the adjuvants used in this study (W, O and P) are not recommended by the manufacturers for use on sensitive grapevine foliage but they are, however, sometimes applied by growers to berries due to insufficient information on the pesticide label, due to economical restraints, or temporary shortages of approved adjuvants. Furthermore, fungicides for the control of *B. cinerea* are often targeted towards berries, and not the foliage, and since berries have denser wax layers than foliage, the grower may believe that a more disruptive adjuvant is required. Spray drift onto berries of adjuvants intended for inter-row weed control can also occur.

Marois *et al*. (1987) also found that, with the exception of one treatment, grape berries were more susceptible to Botrytis bunch rot when treated with adjuvants. Their experiments on water uptake and loss by berries indicated that the epicuticular wax was affected by the sprays. The SEM results provided here confirm those results. Wax disruption may decrease the physical barrier through which the hyphae penetrate, or the wax disruption may increase porosity of the cuticle and this may increase exudation onto the surface of the berry. Nutrients and sugars on the surface of the berry would be a source of energy for germinating conidia (Harper *et al.* 1981) and hence infection rates would increase, as would percentage of surface colonisation by *B. cinerea* as seen in this study.

Surfactants and fungicides may not only have a direct effect on the waxes of the berry but also on the natural microflora on the berry. Often these micro-organisms are beneficial to the health of the berry in that they are parasitic or antagonistic to pathogens (Blakeman and Fokkema 1982). Adjuvants significantly reduced the numbers of yeast and fungi on Chardonnay berries during the season, but not in the other two varieties. Phenols, which are inhibitory to the growth of micro-organisms, are often in lower concentrations in the Chardonnay variety (Padgett and Morrison 1990). Chardonnay bunches are also relatively compact (Bisiach *et al.* 1982), and this perhaps contributes to a more humid berry microenvironment and perhaps an ecology of microflora which is more sensitive to the adjuvants used in this study.

The cyprodinil/fludioxonil fungicide, reduced bacterial numbers on Chardonnay berry surfaces. The fungicide contains two active ingredients, an anilinopyrimidine, and a phenylpyrrole. Anilinopyrimidine fungicides act by inhibiting the secretion of fungal enzymes responsible for plant cell wall degradation (Milling and Richardson 1995) and also possibly methionine biosynthesis (Fritz *et al.* 1997). The mode of action of phenylpyrrole fungicides has not been fully elucidated, although they are likely to interfere with phosphorylation in carbohydrate metabolism (Jespers and de Waard 1994; Pillonel and Meyer, 1997). This being the case, it therefore follows this fungicide may also inhibit the growth of the microflora on the berry surface to some extent and may explain why lower bacterial numbers were recorded from berries treated with the fungicide.

The spray adjuvants affected wax structure on berries of all three varieties and decreased the berry surface microflora in Chardonnay. Most importantly, the adjuvants increased susceptibility of infection by *B. cinerea*. In summary, the particular adjuvants used in this trial counteracted the positive effects of the fungicide by facilitating infection of *B. cinerea*.

8.0 Summary

Source: Rogiers S.Y., M. Whitelaw-Weckert, M. Radovanovic-Tesic, L.A Greer, R.G. White and C.C. Steel. Effects of spray adjuvants on grape (*Vitis vinifera*) berry microflora, epicuticular wax and susceptibility to infection by *Botrytis cinerea.* Australasian Plant Pathology (in press)

Spray adjuvants were tested for their effects on epicuticular wax morphology, grape berry microflora, and susceptibility of berries to Botrytis cinerea on Chardonnay, Shiraz and Cabernet Sauvignon varieties. The four adjuvants used in this trial altered epicuticular wax morphology. Disintegration of the wax platelets was least for the wetter-spreader recommended for sensitive crops, and greatest for the crop oil concentrate and the activator-penetrant. Waxes did not regenerate over the season after treatment with the adjuvants. A cyprodinil/fludioxonil fungicide was effective at controlling *B. cinerea* infection, but when combined with an adjuvant, was less effective in the three grape varieties. Irrespective of whether a fungicide was used, adjuvant application resulted in lower yeast and fungal populations on Chardonnay berries. There were no effects of the adjuvants on the microflora of Shiraz and Cabernet Sauvignon berries, except for the crop oil concentrate which resulted in higher bacterial populations on Cabernet Sauvignon berries. We hypothesise that spray adjuvants increased the susceptibility of grape berries to *B. cinerea* through epicuticular wax alteration and, in some circumstances, through the reduction of the indigenous microflora on the berry's surface.

9.0 Recommendations

Further field trials are required to examine the impact of adjuvants on the berry's susceptibility to Botrytis. The four adjuvants we tested were detrimental to the efficacy of Switch. A wider range of adjuvants in combination with a wider range of fungicides need to be tested to get a better view of the impact of these chemicals in general.

Appendix 1: Communication

Refereed Publications

Rogiers S.Y., M. Whitelaw-Weckert, M. Radovanovic-Tesic, L.A Greer, R.G. White and C.C. Steel. Effects of spray adjuvants on grape (*Vitis vinifera*) berry microflora, epicuticular wax and susceptibility to infection by *Botrytis cinerea.* Australasian Plant Pathology (in press).

Industry Publications

Rogiers SY, Weckert M, Radovanovic-Tesic M, Steel C, Greer L, Steel C. 2004. Spray adjuvants can increase the grape berry's susceptibility to infection by Botrytis. Australian and New Zealand GrapeGrower and Winemaker **489:** 29-30.

Rogiers SY, Greer L, Weckert M, Steel C, Schmidtke L and B Holzpafel. 2003. Agricultural spray adjuvants: effects on grape berry wax, berry microflora and subsequent botrytis infection. Australian and New Zealand GrapeGrower and Winemaker **477:** 19-21.

Rogiers SY, Weckert M. 2004 Effect of adjuvants on susceptibility of grape berries to Botrytis infection. Wagga Wagga Agricultural Institute Annual Report.

Symposium Presentations

SY Rogiers, M Weckert, M Radovanovic-Tesic. 2004. Botrytis, Sprays and Berry Waxes. NWGIC seminar series, June 16, 2004. Wagga Wagga, NSW.

Posters

S. Rogiers, M. Weckert, M. Radovanovic-Tesic, L. Greer, C. Steel, B. Holzapfel and L. Schmidtke. 2004. Effect of adjuvants on susceptibility of grape berries to Botrytis infection. Wine Technical Conference, Melbourne

Rogiers SY, Greer L, Weckert M, Holzapfel B and C Steel. 2003. Spray effects on berry wax, berry microflora and subsequent Botrytis infection. NWGIC annual meeting, June 18-20, Wagga Wagga.

Media

Grapes, gray mold and adjuvants, Agriculture Today, July 29, 2004.

Appendix 2: References

Bessis R (1972) Etude de l'évolution des stomates et des tissus péristomatiques du fruit de la vigne. Comptes Rendus de l'Académie des Sciences de Paris, Série D **274**, 2158-2161.

Bisiach M, Minervini G, Zerbetto F, Vercesi A (1982) Biological and epidemiological aspects of *Botrytis cinerea* and criteria for its control in grapevine cultivation. *Vignevini* **9**, 39-46.

Blakeman JP, Fokkema NJ (1982) Potential for biological control of plant diseases on the phylloplane. *Annual Review of Phytopathology* **20**, 167-192.

Comménil P, Brunet L, Audran JC (1997) The development of the grape berry cuticle in relation to bunch rot disease. *Journal of Experimental Botany* **48**, 1599-1607.

Emmett RW, Harris AR, Taylor RH, McGechan JK (1992) Grape Diseases and Vineyard Protection *In* Viticulture. Volume 2, Practises (Eds BG Coombe, PR Dry) pp. 232-278. (Winetitles: Underdale, South Australia)

Fritz R, Lanen C, Colas V, Leroux P (1997) Inhibition of methionine biosynthesis in *Botrytis cinerea* by the anilinopyrimidine fungicide pyrimethanil. *Pesticide Science* **49**, 40-46.

Hall DM, Matus AI, Lamberton JA, Barber HN (1965). Infra-specific variation in wax on leaf surfaces. *Australian Journal of Biological Science* **18**, 323-332.

Harper AM, Strange RN, Langcake P (1981) Characterisation of the nutrients required by *Botrytis cinerea* to infect broad bean leaves. *Physiological Plant Pathology* **19**, 153-167.

Jespers ABK, de Waard MA (1994) Effect of fenpiclonil on macromolecule biosynthesis in *Fusarium sulphureum*. *Pesticide Biochemistry and Physiolology* **49**, 53-62.

Keller M, Viret O, Cole FM (2003) *Botrytis cinerea* infection in grape flowers: Defense reaction, latency and disease expression. *Phytopathology* **93**, 316-322.

Marois JJ, Bledsoe AM, Bostock RM, Gubler WD (1987) Effects of spray adjuvants on development of *Botrytis cinerea* on *Vitis vinifera* berries. *Phytopathology* **77**, 1148-1152.

McClellan WD, Hewitt WB (1973) Early Botrytis rot of grapes: time of infection and latency of *Botrytis cinerea* Pers. In *Vitis vinifera* L. *Phytopathology* **63**, 1151-1157.

Milling RJ, Richardson CJ (1995) Mode of action of the anilinopyrimidine fungicide pyrimethanil. 2. Effects on enzyme secretion in *Botrytis cinerea*. *Pesticide Science* **45**, 43-48.

Mlikota Gabler F, Smilanick JL, Mansour M, Ramming DW, Makcey BE (2003) Correlations of morphological, anatomical, and chemical features of grape berries with resistance to *Botrytis cinerea*. *Phytopathology* **93**, 1263-1273.

Mullins MG, Bouquet A, Williams LE (1992) Biology of the Grapevine. Cambridge University Press, Cambridge.

Padgett M, Morrison JC (1990) Changes in grape exudates during fruit development on their effect on mycelial growth of *Botrytis cinerea*. *Journal of the American Society for Horticultural Science* **115**, 269-273.

Percival DC, Sullivan JA, Fisher KH (1993) Effect of cluster exposure, berry contact and cultivar on cuticular membrane formation and occurrence of bunch rot (*Botrytis cinerea* PERS.: FR.) with 3 *Vitis vinifera* L. cultivars. *Vitis* **32**, 87-97.

Pillonel C, Meyer T (1997) Effect of phenylpyrroles on glycerol accumulation and protein kinase activity of *Neurospora crassa*. *Pesticide Science* **49,** 229 – 236.

Rosenquist JK, Morrison JC (1989) Some factors affecting cuticle and wax accumulation on grape berries. *American Journal of Enology and Viticulture* **40**, 241-244.

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