REDUCING COPPER USE IN AVOCADO ORCHARDS: RESULTS OF LABORATORY AND POSTHARVEST TESTING OF ALTERNATE FUNGICIDES AND BIOLOGICAL PRODUCTS*

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ABSTRACT

Copper is the only fungicide registered to control avocado rots in orchards. There are concerns about the impact of copper on the environment because it does not readily degrade. In order to reduce copper use on avocado orchards new formulations of copper with more available copper per gram active ingredient, new chemistries, and biological products were tested in a series of laboratory tests and postharvest. Four copper formulations (Kocide® 2000, Kocide® 3000, Cuprofix® Disperss®, Champ™ DP) and dithianon, boscalid/pyraclostrobin and boscalid were tested *in vitro* on poison plates for inhibition of spore germination and mycelial growth of *Colletotrichum acutatum*, *C. gloeosporioides*, *Botryosphaeria parva*, *B. dothidea* and *Phomopsis* sp., the most common pathogens causing postharvest rots of avocados in New Zealand. Those fungicides with lowest EC50 values (effective concentration at which 50% spore germination was inhibited) were dithianon, boscalid/pyraclostrobin and all copper formulations, in order of effectiveness. The fungicide with the lowest EC50 value for mycelial inhibition was boscalid/pyraclostrobin. Three biological products (*Bacillus subtilis* QST713, *Serratia marcescens* HR42, Biostart Target), dithianon, boscalid and boscalid/pyraclostrobin reduced lesion growth of at least one of the five fungi inoculated onto detached fruit. Postharvest application of dithianon statistically significantly reduced rots, prochloraz reduced rots to some extent, but none of the other fungicides tested (boscalid, boscalid/pyraclostrobin, fluazinam, Kocide® 3000) were effective. Because results of laboratory tests are not always directly applicable to field results, fluazinam, boscalid, boscalid/pyraclostrobin, *B. subtilis* QST713 and Biostart® Target should be tested as spray applications in the orchard. Dithianon was an effective fungicide when applied postharvest and should also be field tested.

Keywords: *rots, fungi, Colletotrichum acutatum, C. gloeosporioides, Botryosphaeria parva, B. dothidea, Phomopsis sp., biocontrol, control.*

INTRODUCTION

Replicated spray trials in New Zealand (Everett, 2002) and overseas (Peterson & Inch, 1980; Darvas & Kotze, 1987; Willingham *et al*., 2001) have proven that copper fungicides applied in the orchard provide effective control of postharvest rots of avocados. Alternative chemistries that are as effective as copper in the field include azoxystrobin (Willingham *et al*., 2001; Everett et al., 2005), phosphorous acid and benomyl (Hartill, 1992; Everett *et al*., 1999; Everett, 2002). Benomyl has since been removed from sale. Azoxystrobin requires application in combination with a broad spectrum fungicide in order to prevent the build up of resistant fungal populations. Phosphorous acid is generally not recommended as an orchard spray for the same reason. Postharvest rots of avocados are a serious issue affecting both quality and storage life, and copper is virtually the only chemical that can be used in the orchard for control. However, copper does not degrade readily and can adversely affect earthworm populations (Zwieten et al., 2004) and biomass (Merrington *et al*., 2002) in the soil. Copper levels in New Zealand soils can approach levels that are detrimental for the environment (Guernsey et al., 2004).

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New chemistries are available for testing against the avocado rot fungi, and recently more finely milled copper formulations have become available. These new copper formulations are designed so that less elemental copper needs to be used to achieve good rot control because the copper is more readily available. Overseas and in New Zealand more environmentally sustainable bacterial biological control agents have also shown promise for controlling avocado rots (Everett, 1996; Korsten *et al*., 1997). In New Zealand the bacterium Bacillus subtilis QST713 is commercially available, and there is an experimental HortResearch product, the bacterium Serratia marcescens HR42, available for testing. A further biological product, Biostart® Target, which has an undisclosed composition, is commercially available.

These products and compounds were tested in a series of laboratory tests and as a postharvest application in order to find alternatives to the currently available copper formulations that can be used for rot control.

MATERIALS AND METHODS

Inhibition of spore germination and mycelial growth
A total of seven fungicides were tested. All products in Table 1 were tested except fluazanim, *Bacillus subtilis* QST 713, *Serratia marcescens* HR42 and Biostart® Target.

Single-spore isolates of *C. acutatum*, *C. gloeosporioides*, *B. parva*, *B. dothidea* and *Phomopsis* were grown on Difco® Potato Dextrose Agar under UV and fluorescent lights on a 12:12 hour day/night cycle at about 20°C. After 3-6 weeks, spores were harvested in sterile distilled water and adjusted to a concentration of 10^5 spores/ml with the aid of a haemocytometer. Fungicide stock solutions were made in sterile deionised water to a concentration of 100 and 10 000 µg a.i./ml (parts per million; ppm), by w/v. The appropriate volume of each fungicide stock solution was added to 10 ml of 1.5% w/v water agar in a 5 cm diameter sterile plastic Petri plate to achieve a range of final concentrations of 0, 1, 10, 100 and 1000 ppm. There were three replicate plates per concentration. An aliquot of 100 µl of spores (10^5 spores/ml) was placed on the surface of each Petri plate. After 4 h at 20°C, the total numbers of spores in three microscope fields at a magnification of x 100 were counted and the number of germinating spores was recorded (about 20 spores per microscope field of view). Percentage spore germination per plate was

Table 1. Fungicides for control of avocado fruit rots tested and described according to active ingredient, chemical group and formulation.

Fungicide product[2]	Active ingredient	% a.i.	Chemical group	Formulation[1]
Champ™ DP	copper hydroxide	37.5	copper	WDG
Kocide® 2000DS	copper hydroxide	35	copper	WDG
Kocide® 3000	copper hydroxide	46.1	copper	WP
Cuprofix® Disperss®	copper hydroxosulphate	20	copper	WDG
Delan® 700 WG	dithianon	70	quinones	WDG
BASF 510	boscalid	50	carboxamide	WDG
BASF 516	boscalid/ pyraclostrobin	25.2/ 12.8	carboxamide/ strobilurin	WDG
Shirlan®	fluazinam	50	pyridinamine	SC
Serenade®	*Bacillus subtilis* QST713	10	biological control agent	WP
HR42	*Serratia marcescens* HR42	100	biological control agent	unformulated
Biostart® Target	activators	n.d.	activator	liquid n.d.

[1]WDG = water dispersed granules, WP = wettable powder, SC = suspension concentrate, n.d. = not disclosed.
[2]Champ™ is a trademark of Nufarm Americas Inc., Kocide is a trademark of Dupont, Cuprofix® is a trademark of Cerexagri. Inc., Delan® is a trademark of BASF, Serenade® is a trademark of Agriquest, and Biostart® is a tradename of Biostart New Zealand.

calculated using the mean of three fields of view. At high fungicide concentrations, spores were stained with cotton blue to facilitate visualisation.

For mycelial growth tests, fungal cultures were grown as for the spore germination tests, but mycelial plugs were removed from the edge of actively growing colonies (1-2 weeks of growth) with a sterilised 5 mm diameter cork borer and placed on Difco® potato dextrose agar (PDA) amended with the test fungicides (poison plates). Mycelial diameter was measured every 1-2 days for 10 days and plotted against time. Comparisons were made on the basis of mycelial growth rate, in mm/day, for the linear phase of the fungal growth curve. There were three replicate plates per treatment.

The concentrations at which 50% of spores failed to germinate (EC_{50}) were calculated by the following method. Logit transformations (logit=ln{p/(1-p)}) of spore germination (as a proportion of germination on unamended agar) and mycelial growth (as a proportion of growth on unamended media) averaged over the three replicate plates were plotted against the logarithmic transformation of fungicide concentration to linearise the response. The slope of the linear portion of the transformed data was calculated by linear regression. The EC50 was calculated from each linear regression equation for Y=0, *i.e.* the logit for P = 0.5 or 50%. A constant value of 0.01 was added to non-transformed data to enable 0 and 100% values to be used in the calculations.

Detached fruit tests

Fruit were harvested from a single unsprayed avocado tree from an orchard in Te Puke (HortResearch 412 No. 1 Road) on 22 September 2006. Detached fruit were placed on a plastic paper tray, 9 fruit per container (Figure 1), marked with white correcting fluid, and wounded to a depth of 5 mm with a needle at each marked spot. The test fungicides and biologicals were all those described in Table 1. Fungicides were applied at

Figure 1. Detached avocado fruit marked with correcting fluid, treated with fungicide solutions or biological products, and inoculated with a spore suspension of *Colletotrichum acutatum*, *C. gloeosporioides*, *Botryosphaeria parva*, *B. dothidea* and *Phomopsis* and placed on a paper tray at 20°C

four concentrations of 1, 10, 100 and 1000 ppm, *B. subtilis* QST713 and *S. marcescens* HR42 at a concentration of 10^6 and 10^8 cfu (colony forming units)/ml and Biostart Target at dilutions of 1:10, 1:100, 1:200 and 1:1000 (v/v with sterile deionised water). Fruit were treated by placing 10 µl of each dilution of fungicide or biological on each marked wounded spot and dried by placing in a laminar flow cabinet for 2-3 hours. After drying, fruit were inoculated by placing 10 µl of a suspension of each of the five avocado pathogens at each marked spot on each fruit (Figure 2 a). Spores were applied at a concentration of 10^5 and 10^7 spores/ml. Controls were fruit that were not treated with fungicides or biologicals, but were inoculated. Fruit were placed at 20°C and were assessed when ripe as adjudged by gentle hand squeezing. Fruit were then cut into quarters and peeled, and any lesion diameters were measured and recorded. The trial was repeated by harvesting further fruit from a single

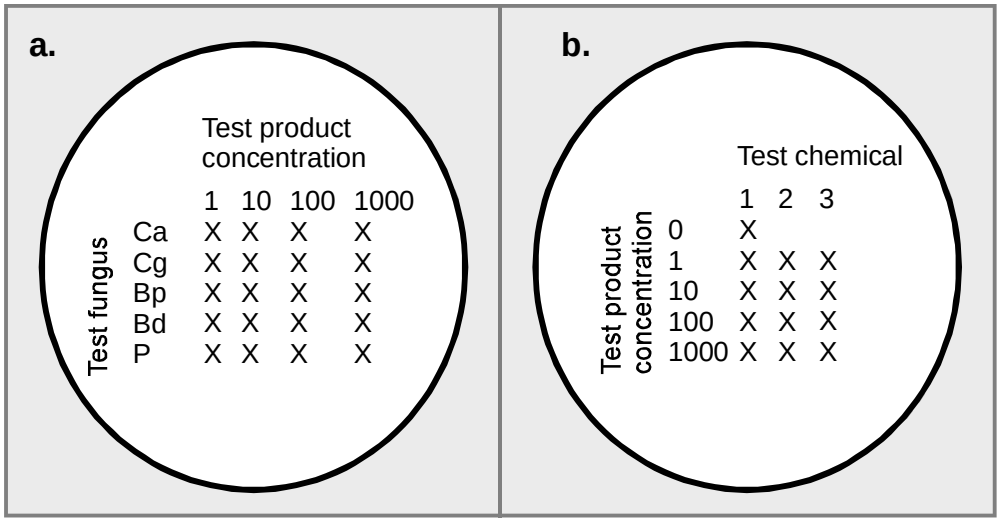

Figure 2. Layout of inoculations for each treated fruit. a) Fungicides or biologicals were placed at the concentrations in the first row on each marked position. Spore suspensions were placed at each marked spot of each fungus (Ca=*Colletotrichum acutatum*, Cg=*C. gloeosporioides*, Bp= *Botryosphaeria parva*, Bd=*B. dothidea*, P=*Phomopsis* sp.) at a concentration of either 10^5 or 10^7 spores/ml on separate fruit b) Fungicides or biologicals were placed as shown, and three test chemicals were placed on each fruit. Fruit were inoculated with 10^7 spores/ml of each fungus. Four fruit were inoculated with each fungus to test all 11 products. There were three replicate fruit for both layouts.

tree on the same orchard on 15th June 2007. A different layout was used (Figure 2 b) and there were two additional dilutions of *B. subtilis* QST713 and *S. marcescens* HR42 (1:1, 1:10, 1:100 and 1:1000 where the original concentrations were 5 x 10^8 cfu/ml and 10^8 cfu/ml, respectively).

Postharvest application of fungicides

Avocado fruit was harvested from an orchard in Te Puke (HortResearch 412 No. 1 Road) on 14th March 2007, treated by immersing in 40L of each of six test chemicals, with agitation, at the rates in Table 2 for 2 min., air-dried on newspaper, then 20 fruit were placed per avocado box, five boxes per treatment, on the same day. The next day these fruit were transported to HortResearch Mt Albert Research Centre and placed in the coolstore at 5.5°C. After 28 days coolstorage fruit was removed and placed at 20°C for ripening. Ripening fruit was tested for firmness by gentle hand squeezing each day after placement at 20° C. When adjudged ripe, fruit were cut into quarters and peeled. Fruit were assessed for internal rots using the methods described in the New Zealand Avocado Industry Council Assessment Manual .

Table 2. Application rates of fungicides tested as a postharvest dip application for control of avocado fruit rots.

Fungicide product	Active ingredient	% a.i.	Rate used/100L
Delan® 700 WG	dithianon	70	18g
Sportak®	prochloraz	45	55ml
BASF 510	boscalid	50	60g
BASF 516	boscalid/ pyraclostrobin	25.2/ 12.8	60g
Kocide® 3000	copper hydroxide	46.1	90g
Shirlan®	fluazinam	50	100ml

Data was analysed using the analysis of variance and means separation tests of Minitab Version 15. Values were arc-sine square root transformed prior to statistical analysis using the GLM procedure of Minitab Version 15. Means were separated by comparison with the untreated control using Dunnett's test.

RESULTS

Inhibition of spore germination and mycelial growth
The fungicide that consistently and most effectively inhibited spore germination for all fungi tested was boscalid/pyraclostrobin at a concentration of 7 ppm and below (Table 3). Dithianon was the next most effective fungicide at a concentration of 18.5 ppm or less. The four formulations of copper inhibited spore germination of all the fungi tested but sometimes at high concentrations (viz. 140.9 ppm for Kocide® 3000 against *C. gloeosporioides*). The copper formulation that was effective at the lowest concentrations against spore germination of *B. parva* was Champ™ DP, the most effective against both species of *Colletotrichum* was copper hydroxosulphate, and the most effective against *B. dothidea* and *Phomopsis* was Kocide® 3000.

Boscalid/pyraclostrobin was the most effective fungicide inhibiting mycelial growth, with efficacy in a concentration range of 0.1-0.8 ppm (Table 4). None of the other fungicides tested was effective at concentrations of this order.

Detached fruit tests
For the first test, all five avocado pathogens caused lesions when inoculated on fruit, but

Table 3. The effective concentration (ppm) at which 50% of spores failed to germinate (EC50) of five avocado postharvest fungal pathogens treated with seven fungicides.

Fungicide	*C. acutatum*	*C. gloeo-sporioides*	*B. parva*	*B. dothidea*	*Phomopsis* sp.
boscalid	<0.1	<0.1	18.2	27.7	74.8
boscalid/ pyraclostrobin	7.0	0.2	2.3	0.2	0.2
Kocide® 2000DS	0.4	8.1	0.6	15.6	1.5
Kocide® 3000	7.3	140.9	0.3	4.8	0.1
Champ™ DP	1.0	57.6	0.1	11.4	2.7
copper hydroxosulphate	0.1	2.3	4.0	98.2	1.5
dithianon	<0.1	<0.1	3.1	18.5	<0.1

Table 4. The effective concentration (ppm) at which 50% of mycelial growth was inhibited (EC50) of five avocado postharvest fungal pathogens treated with seven fungicides.

Fungicide	*C. acutatum*	*C. gloeo-sporioides*	*B. parva*	*B. dothidea*	*Phomopsis sp.*
boscalid	1921.0	>*	837.7	2154.4	>
boscalid/ pyraclostrobin	0.2	0.1	0.2	0.0	0.8
Kocide® 2000DS	2540.7	647.3	304.7	153.2	105.8
Kocide® 3000	867.2	297.1	228.1	118.1	101.8
Champ™ DP	346.7	257.2	1321.7	197.0	193.3
copper hydroxosulphate	910.5	402.1	1645.3	749.9	543.0
dithianon	44.2	201.4	989.6	540.3	17.2

** EC50 value is greater than the highest concentration used*

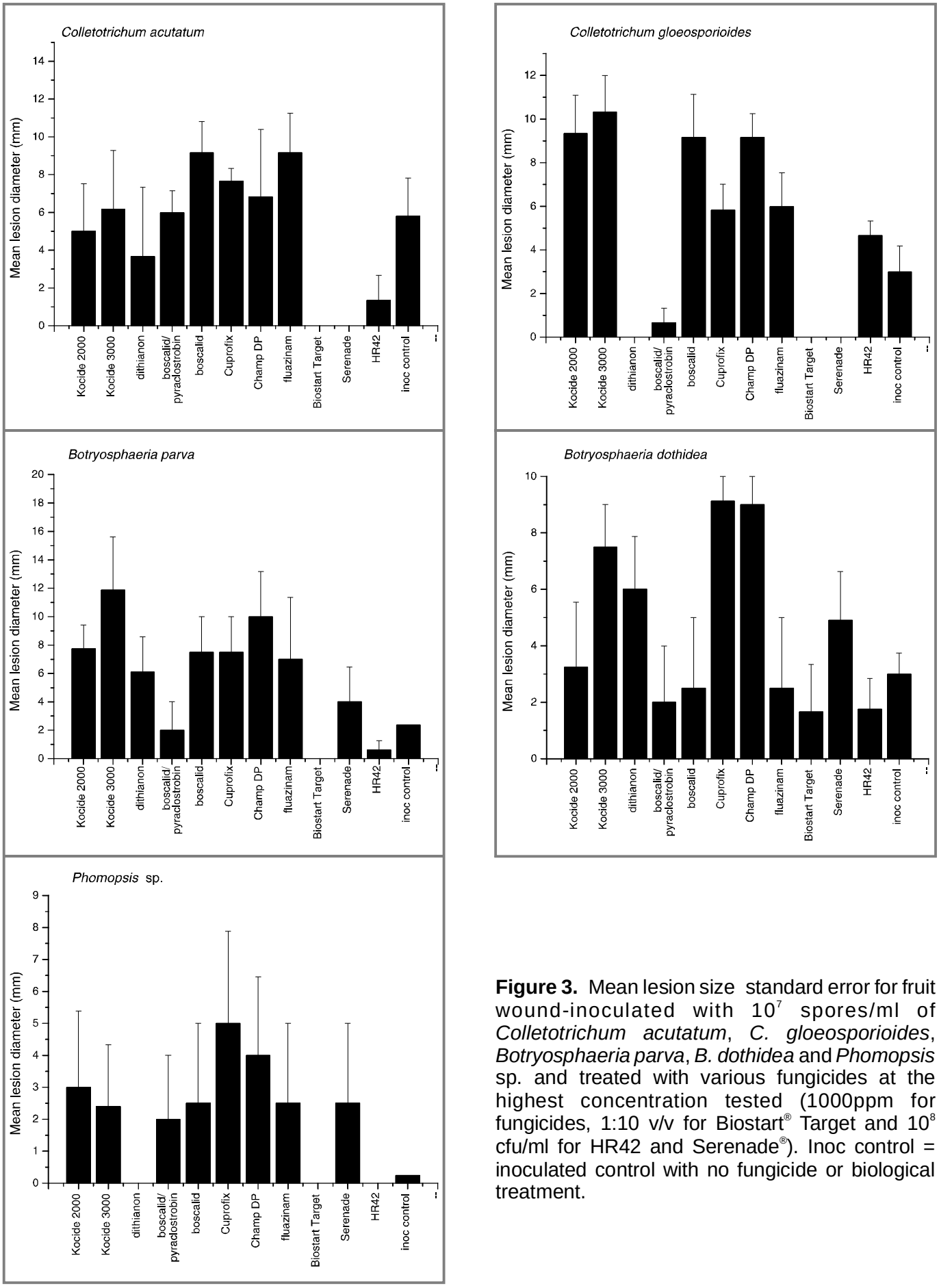

Figure 3. Mean lesion size standard error for fruit wound-inoculated with 10^7 spores/ml of *Colletotrichum acutatum*, *C. gloeosporioides*, *Botryosphaeria parva*, *B. dothidea* and *Phomopsis* sp. and treated with various fungicides at the highest concentration tested (1000ppm for fungicides, 1:10 v/v for Biostart® Target and 10^8 cfu/ml for HR42 and Serenade®). Inoc control = inoculated control with no fungicide or biological treatment.

lesions were inconsistent at the lower spore concentration (10^5 spores/ml) and for this reason these results are not presented. For the second test, only *C. acutatum* and *C. gloeosporioides* caused lesions. The results from the second test for *C. acutatum* and *C. gloeosporioides* are presented, and the results of the first test for the other fungi. Fungicides and biologicals were most effective at the highest concentrations tested (Figure 3).

At this concentration, *B. subtilis* QST713 and *S. marcescens* HR42 most effectively reduced lesion development of *C. acutatum*, dithianon, boscalid/pyraclostrobin, Biostart® Target and *B. subtilis* QST713 lesion development of *C. gloeosporioides*, boscalid/pyraclostrobin, *S. marcescens* HR42, and Biostart® Target *B. parva* and *B. dothidea* and dithianon, Biostart® Target and *S. marcescens* HR42 of *Phomopsis* (Figure 4).

Postharvest tests

Analysis of severity data showed no statistically significant differences between treatments. There was a significant treatment effect for total rots (P=0.039). Further analysis showed that only one

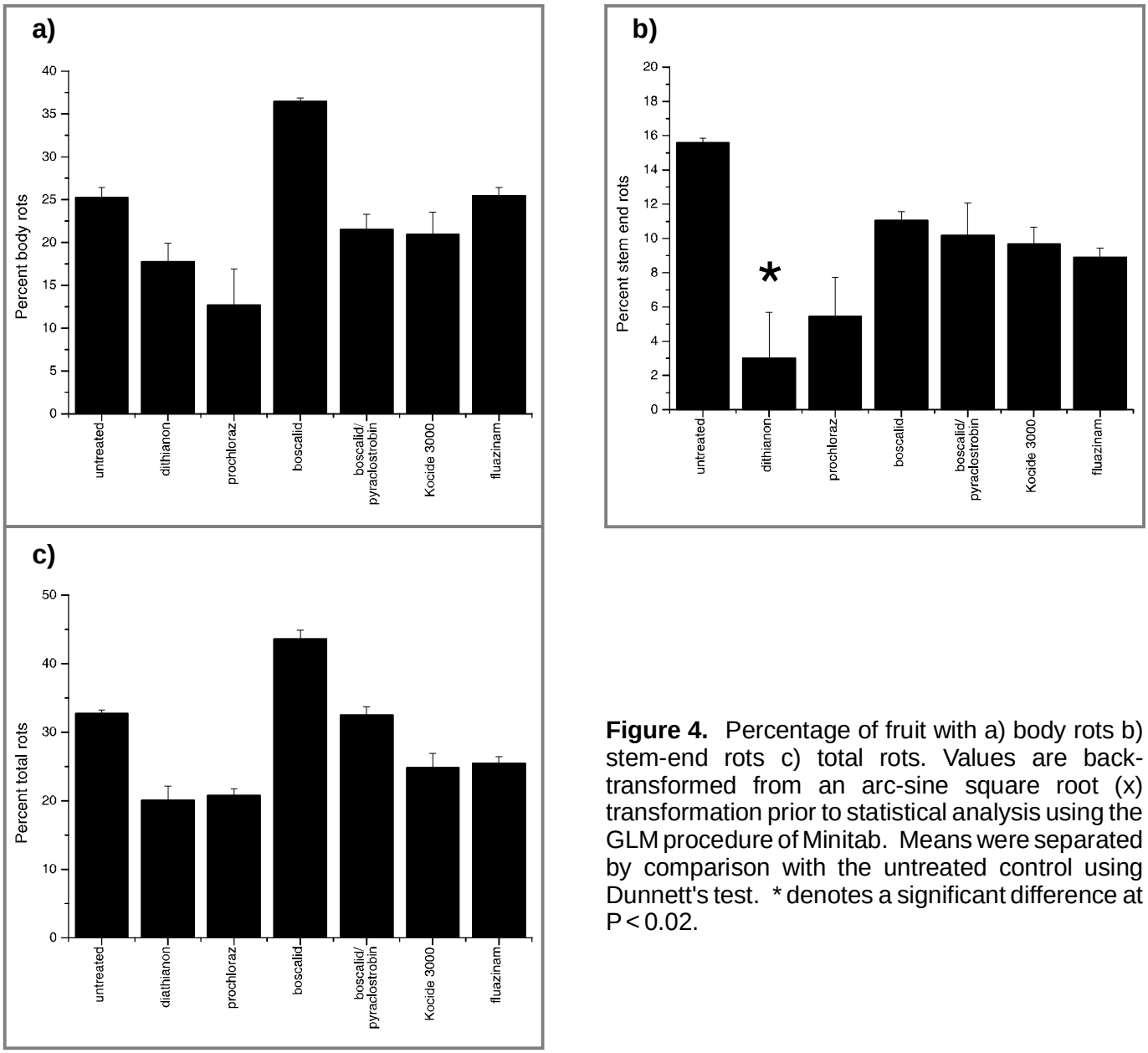

Figure 4. Percentage of fruit with a) body rots b) stem-end rots c) total rots. Values are back-transformed from an arc-sine square root (x) transformation prior to statistical analysis using the GLM procedure of Minitab. Means were separated by comparison with the untreated control using Dunnett's test. * denotes a significant difference at P < 0.02.

treatment effect resulted in significantly fewer rots than untreated control fruit, and that was dithianon on stem-end rots (P = 0.0158; Figure 5). Although prochloraz treated fruit had fewer stem-end rots than untreated controls, the P value was 0.08 which is not significant. For body rots, fruit treated with both dithianon and prochloraz had fewer rots than untreated control fruit, but neither P value was significant (P = 0.4589 and P = 0.1628, respectively; Figure 4).

DISCUSSION

The aims of these experiments were to find fungicides that are more effective against avocado rot fungi than the industry standard, copper, and to find copper formulations that could be used in lower amounts to control avocado rots in the field. Both results could lead to reduced use of copper fungicides in avocado orchards and consequent benefits to the environment (Merrington *et al*., 2002; Zwieten *et al*., 2004).

There were differences between the copper formulations on spore germination and mycelial growth in the series of *in vitro* tests. Although Kocide® 3000 was expected to be a superior product because of its more finely milled particle size, overall it did not perform as effectively as Kocide® 2000DS in spore germination tests. The copper formulations differed in effectiveness against each of the five tested fungi, but overall Kocide® 3000 most effectively inhibited mycelial growth. Different copper formulations inhibited spore germination of different fungal species to varying degrees. For instance Champ™ DP was the most effective fungicide against spore germination of *B. parva*, copper hydroxosulphate against both species of *Colletotrichum*, and Kocide® 3000 against *B. dothidea* and *Phomopsis*. It was therefore difficult to ascertain which copper formulation was most effective at inhibiting spore germination.

The concentration of copper recommended for field use for controlling postharvest rots of avocados are in the order of 1000 ppm. This is clearly well in excess of the concentration of copper required to inhibit spore germination for all five avocado pathogens tested, and for most formulations of copper is sufficient to inhibit mycelial growth. For the other fungicides recommended field rates range from 126 ppm for dithianon to 300 ppm for boscalid. These estimates are based on recommendations for other crops. These field rates are far in excess of EC50 concentrations for spore germination determined in the laboratory. Boscalid is not recommended to be tested alone in poison plate tests using PDA (Spiegel & Stammler, 2006; Stammler & Speakman, 2006), and for this reason field rates of this fungicide may also be in excess of concentrations required to inhibit mycelial growth.

In vitro tests do not always accurately predict the performance of fungicides in the field (Everett *et al*., 2005). For instance, fluazinam was the most effective fungicide using these *in vitro* techniques against all five avocado pathogens, but it did not control postharvest rots when applied in the field (Everett *et al*., 2005). However, poison plate tests and spore germination tests are commonly used as preliminary screening tests for fungicides (Corden & Young, 1962; Anahosur *et al*., 1977; Sharma & Mohanan, 1990; Everett & Neilson, 1996; Everett *et al*., 2005). These tests are useful for eliminating fungicides that are not likely to be effective in the field. Some fungicides can have specific requirements for testing. The method recommended for testing the performance of boscalid and pyraclostrobin is a spore germination method in microtitre plates (Spiegel & Stammler, 2006; Stammler & Speakman, 2006), but it is not known if other fungicides will also perform well in this test. On the basis of these tests, the fungicides boscalid/pyraclostrobin and dithianon have good prospects as alternatives to copper for the control of avocado fruit rots.

In the detached fruit tests dithianon, *B. subtilis* QST713, boscalid/pyraclostrobin, *S. marcescens* HR42 and Biostart® Target performed reasonably well against all fungi. All fungi were able to infect fruit to cause lesions, in contrast to inoculating

leaves (data not shown) where only one fungus (*C. acutatum*) caused lesions. This test was considered superior to the leaf test for this reason, and because high variability in response of individual leaves to inoculations rendered the data unreliable. In previous work, the detached fruit test was used to select S. marcescens HR42 as a potential biocontrol agent. This bacterium significantly reduced postharvest rots when applied as a postharvest application (Everett, 2002). Because dithianon, which performed well in the fruit test, was also effective as a postharvest application, this suggests that the results of the fruit test may predict results in field conditions more reliably than traditional laboratory tests. However, boscalid/pyraclostrobin did not perform well as a postharvest application although it did in the fruit tests. Further field tests, including an orchard spray trial, are required to investigate if boscalid/pyraclostrobin has efficacy outside the laboratory on postharvest rots of avocado.

Because *S. marcescens* HR42 is not a commercially available product it was not tested further, although it also performs well in postharvest dipping experiments (Everett, 2002). *B. subtilis* QST713 has been tested as a postharvest dip and was not effective (Everett 2002). Because results of laboratory tests or postharvest applications are not always directly applicable to field results, fluazinam, boscalid, boscalid/pyraclostrobin, *B. subtilis* QST718 and Biostart® Target should be tested as spray applications in the orchard.

Dithianon performed well in postharvest tests, *in vitro* and on detached fruit. This fungicide is registered for use on apples, grapes and pears, but has never been tested on avocados. It is a broad spectrum fungicide, and if it reduces rots when applied as an orchard spray in the field, it is a suitable candidate for inclusion in an avocado spray programme in combination with copper. Dithianon is not persistent in surface water and does not penetrate into deeper soil layers or into the ground water. It is rapidly decomposed in soil with a high microbial content, and in nutrient rich water. It is not toxic to bees, but needs to be kept out of streams and lakes (Anon., 2005). Because it is readily biodegradable dithianon would have less impact on the environment than copper. The withholding period for apples is 42 days, and for grapes 21 days, and for this reason copper would be used close to harvest in a combined spray programme.

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