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Effect of copper fungicide on *Colletotrichum gloeosporioides* and other microorganisms on avocado leaves and fruit

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Abstract. An investigation into the effect of copper fungicide on non-target microorganisms on avocado (cvv. Hass and Nabal) showed that populations of filamentous fungi, yeasts, and bacteria on leaves were at least 10-fold less after a single application of copper than on unsprayed leaves. This detrimental effect of copper on microorganisms on the phylloplane was confirmed in random samples collected from 5 commercial avocado orchards with regular pesticide spray programs but where isolated unsprayed trees were also available. An intensive 16-month study was carried out on 2 adjacent orchards, 1 that was sprayed with copper fungicide at monthly intervals from October to April each year, and the other that had not been sprayed for at least 6 years. Populations of filamentous fungi, yeasts, and bacteria on leaves and fruit were 10–100-fold lower in the sprayed orchard than in the unsprayed orchard. However, populations in the sprayed orchard recovered during the winter non-spray periods to levels comparable to the unsprayed orchard. In 1993 and 1994, fruit were harvested from both orchards, ripened, and assessed for anthracnose caused by *Colletotrichum gloeosporioides*. In both years, there was significantly less disease in unsprayed fruit (mean disease rating 1.13 and 0.32, respectively) than in sprayed fruit (mean disease rating 1.83 and 2.18, respectively). These results show that copper fungicide is detrimental to phylloplane microorganisms and suggest that those organisms are providing some natural suppression of *C. gloeosporioides* on avocado.

Additional keywords: disease suppression, anthracnose.

Introduction

Anthracnose, caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., results in considerable losses of ripe avocado (*Persea americana* Miller) fruit. The pathogen can infect fruit from fruit-set to harvest. Copper fungicides are widely used to control pre-harvest infections of avocado fruit but despite this, reports of disease-blemished fruit in the market place are relatively frequent (Muirhead *et al*. 1982; Ledger 1993). In contrast, our preliminary observations suggested that fruit from unsprayed orchards had little post-harvest disease. If such a situation was confirmed, it could be due to a disruption by copper and other pesticides of naturally occurring microbial populations that provide some suppression of the pathogen. The older style protectant fungicides that are based on heavy metals or sulfur are most likely to cause such a disturbance (Campbell 1989). Many of these compounds have a broad spectrum activity and are formulated to have maximum persistence on aerial plant surfaces (Dickinson 1981).

There are only limited published data on the effects of copper fungicides on non-target microorganisms on aerial plant surfaces. An increase in apricot dieback caused by *Eutypa armeniacae* Hansf. and Carter occurred when a copper-containing fungicide was introduced into the spray program for the control of shothole disease caused by *Clasterosporium carpophilum* (Lev.) Aderh. (Carter 1971). The author hypothesised that copper most likely depleted the saprophytic microflora that competed with the pathogen at the site of infection. Circumstantial evidence suggested that copper sprays used against coffee berry disease caused an increase in *Colletotrichum coffeanum* Noack. infections (Furtado 1969; Gibbs 1972). It has been suggested that copper alters the competitive relationships between the various microorganisms, including non-pathogenic forms of *Colletotrichum*, that colonise the bark (Furtado 1969; Griffiths and Waller 1971; Mulinge and Griffiths 1974). A similar hypothesis based on natural suppression by saprotrophs has been suggested for the failure to control leaf rust

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(*Hemileia vastatrix* Berk. and Broome) on coffee with copper sprays (Campbell 1989).

The main aim of this study was to determine the effect of copper on *C. gloeosporioides* and on non-target microorganisms on the avocado phylloplane. Since the insecticide endosulfan is also used widely in the Australian avocado industry to control fruitspotting bug, its effect on non-target microorganisms was also examined.

Materials and methods

Media and techniques for enumeration of microorganisms

Bacterial populations were enumerated on 1/3 tryptic soy agar (1/3TSA; Difco) and King's medium B (KMB; King *et al.* 1954), both media containing cycloheximide (75 μg/mL; Sigma). Additionally, bacterial colonies on KMB plates that produced a pigment fluorescent under UV radiation (wavelength about 340 nm) were designated as fluorescent pseudomonads. Numbers of yeasts were estimated on yeast malt-extract agar (YMA; pH 3.8) (Lodder 1970), and filamentous fungi on half-strength potato dextrose agar (PDA; Difco) containing streptomycin (120 μg/mL; Sigma).

Leaves were picked, placed individually in sterile plastic bags, and transported to the laboratory in a portable cooler. The petiole of each leaf was removed, the blade was cut up with sterile scissors, and weighed. Leaves were then processed using a Stomacher Lab-Blender 80 (Seward Medical UAC House, London) for 2 min in 20 mL of phosphate-buffered saline (PBS; pH 7.2) + Tween 80 (0.025% v/v). A 10-fold dilution series was prepared in PBS and 5 replicate 20-μL aliquots per dilution were pipetted as spots on the different media described above. Plates were observed after incubation for 3 days at 25°C. Microorganisms were scored according to a modified version (Stirling 1996) of the method described by Andrews and Kenerley (1978), where a plate-dilution frequency technique (Harris and Sommers 1968) and a most probable number method for 5 tubes (Meynell and Meynell 1970) were used to estimate numbers of propagules in the various categories of microflora.

Effect of copper and endosulfan on non-target microorganisms

To test the effect of a single copper fungicide spray, Kocide (cupric hydroxide 500 g a.i./kg) at 200 g/100 L water was sprayed to run-off onto leaves of 5 branches of a previously unsprayed avocado tree (cv. Nabal). Leaves sprayed with water were used as controls. Twenty-five leaves (5 per branch) were sampled from each treatment prior to spraying and again 4 days after spraying. Numbers of filamentous fungi, yeasts, and bacteria (cfu/g) on individual leaves were determined. Daily rainfall was measured in this and subsequent experiments. This experiment was repeated on avocado cv. Hass.

The effect of a single application of Endosulfan Crop King (endosulfan 350EC) at 200 mL/100 L water on populations of microorganisms was also evaluated on cv. Hass in a similar manner to that described above. Water was used as a control treatment.

In another study, 5 commercial avocado orchards in Queensland with a regular pesticide spray program were selected if there were adjacent trees (usually isolated trees near growers' residences) that were unsprayed. A single random sample of 10 sprayed and unsprayed leaves of the same cultivar was collected and numbers of filamentous fungi, yeasts, and bacteria were determined.

Finally, the effect of a commercial spray program on phylloplane microorganisms was studied on avocado (cv. Hass) trees growing at Maleny, south-eastern Queensland (latitude 26.5°S, and altitude 520 m) in soils of basaltic origin which are described as krasnozems. The climate is cool, mesic subtropical, with a high mean annual rainfall of 2000 mm. Adjacent orchards were selected that were <500 m apart and had a similar aspect and micro-climate. The selected trees were of similar age (approximately 14 years, 5 m in height, with a dense canopy) and were in good health. One orchard had received monthly copper fungicide and endosulfan sprays from October to April for >8 years. The second orchard was well maintained but had not received any copper or insecticide sprays for at least 6 years. During the 16-month study period, Kocide (200 g/100 L water) and Endosulfan Crop King (200 mL/100 L water) were applied to trees in the sprayed orchard using an air-blast sprayer (Silvan), whereas the other orchard was never sprayed.

Leaves were sampled from the sprayed orchard in April 1992, 4 days after the final pesticide application for the 1991–1992 season; in September 1992 (during the non-spray period) and then 4 days after each spraying in November and December 1992, January, February, and March 1993. The final sample of leaves was collected in August 1993, 3 months after the last spray. On the days that leaf samples were taken from the sprayed orchard, similar samples were collected from the adjacent unsprayed orchard. Eight mature leaves were picked from the northern and southern aspects of 6 trees, 4 from the periphery of the canopy (outer), and 4 located about 50 cm from the trunk (inner). The selected leaves were taken from twigs 1.5–2.5 m above the ground. The fifth, sixth, or seventh mature leaf on a twig, counting from the terminal bud end, was removed and numbers of microorganisms were estimated.

The relative abundance of filamentous fungi and yeasts in the 2 orchards was estimated using 'spot frequency analysis' as described by Andrews and Kenerley (1978). Thirty random spots were selected from the undiluted replicates of the PDA and YMA plates and all the filamentous fungi and yeasts from each spot were isolated and identified.

At each sampling date, additional leaves were picked from both orchards and total copper residues were measured by X-ray fluorescent spectroscopy.

During the study, the numbers of microorganisms on the surface of avocado fruit from the sprayed and unsprayed orchards were also estimated periodically. Fruit were picked from the outer canopy of each tree in a band 1.5–2.5 m above the ground and transported to the laboratory in sterile paper bags. Disks (1.5 cm in diameter, 8–10 per fruit) were cut from the peel with a sterile cork borer, processed in PBS using a stomacher-blender, and microorganisms enumerated using the same techniques as for leaves. Five fruit were sampled from 6 trees in each orchard, fruit being collected in December 1992 and in January, March, and July 1993. Daily rainfall and maximum and minimum air temperatures measured using an automatic weather station (Monitor Sensor, Caboolture, Qld) were recorded during the entire 16-month sampling period.

Effect of copper on C. gloeosporioides and other fungi in a commercially sprayed avocado orchard

In the sprayed and unsprayed orchards (above), 5 avocado fruit were sampled from each of the 6 trees in December 1992, and January, March, and July 1993. Pieces of peel were removed at random, surface-sterilised in 70% (v/v) ethanol for 2 min, rinsed with sterile water, and cut into 3 mm squares, and 20 pieces per fruit were placed on PDA+S. After incubating plates at 25±1°C under UV radiation for up to a week, fungal colonies growing from the peel were identified to genus level. Monoconidial selections were made from a random sample of *C. gloeosporioides* isolates from both orchards for further testing.

Copper tolerance of C. gloeosporioides isolates from the unsprayed and sprayed orchard

Eleven isolates from the unsprayed orchard and 13 from the sprayed orchard were tested. Spore suspensions (10^7/mL) of each isolate were prepared in sterile distilled water and 25 μL aliquots were spread on duplicate plates of casitone yeast-extract glycerol agar (Zevenhuizen

et al. 1979) containing 0, 10, 50, 150, 300, and 700 μg/mL of Cu^{2+} (as $CuSO_4.5H_2O$). Plates were incubated at 26°C for up to 2 weeks and observed regularly for spore germination.

Disease assessment of avocado fruit harvested from the sprayed and unsprayed orchards

In 1993 and 1994, mature fruit (total solids >21%), harvested from the 6 selected trees in each orchard, were assessed for symptoms of anthracnose and stem end rot. A representative sample of 20 fruit was picked from the outer canopy of each tree in a band about 1.5–2.5 m from the ground. Fruit were incubated at 24±1°C at about 80% relative humidity (RH) and checked daily to determine whether the eating-ripe stage had been reached. Fruit were then cut into quarters, peeled, and the pulp surface assessed for disease. In 1993, a 5-point disease rating scale was used: 0, no visible lesions; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, >76% of the fruit surface covered with lesions. In 1994, the scale was expanded to an 11-point scheme, increasing in 10% increments, where 0 represented no visible lesions and 10 represented >90% of the fruit surface covered with lesions. Each fruit was also examined carefully for the presence of *C. gloeosporioides* infections that were associated with insect damage. Isolations were made from diseased pulp to confirm the presence of *C. gloeosporioides.*

Pathogenicity of C. gloeosporioides isolates from the sprayed and unsprayed orchards

Each monoconidial isolate (above) was tested for pathogenicity on mature avocado fruit (cv. Hass and/or Fuerte). Isolates were grown on oat meal agar for 8 days and spore suspensions (6×10^5/mL) prepared in sterile distilled water. Surface-sterilised fruit were then inoculated with an isolate by adding 20 μL of a spore suspension onto 3–5 marked spots on each of 5 fruit. The fruit were then incubated in tightly sealed ripening boxes at 25±1°C under 100% RH. After 48 h, fruit were placed in an incubator at 23°C and 80% RH. When ripe, cv. Fuerte was assessed for lesion development on the fruit surface in the marked areas. Fruit of cv. Hass were cut into quarters and observed for lesions that corresponded with the externally marked inoculation sites. *C. gloeosporioides* A111-2 (BRIP 19768) (Coates *et al*. 1993) was used as a positive control and fruit inoculated with water as the negative control.

Statistical analyses

All $\log_{10}$ transformed data for numbers of microorganisms were normally distributed. Microbial populations on sprayed and unsprayed leaves from the 5 commercial orchards were subjected to a 2-sample *t*-test (Snedecor and Cochran 1980) using models in Statistix (Analytical Software, St Paul, MN). Data for the effect of single sprays of copper or endosulfan were subjected to analysis of variance (ANOVA). Since the data on the effect of the commercial pesticide program were obtained from adjacent orchards, no statistical comparison could be made. ANOVA determined whether compass direction, canopy position, or individual trees within each orchard had an effect on microbial populations. The model was a randomised block design containing a 2-factor factorial with sampling as specified by BALF (Biometry Statistical Software, Queensland Department of Primary Industries). Data for disease levels in fruit (mean disease rating) were subjected to non-parametric analysis using the Mann–Whitney rank sum test (Snedecor and Cochran 1980).

Results

Effect of copper and endosulfan on non-target microoganisms

In the experiment on cv. Nabal, a single copper spray applied to a previously non-sprayed tree had no effect on populations of filamentous fungi and yeasts (Table 1), but the bacterial population was significantly reduced on leaves. In the second experiment on cv. Hass, all 3 categories of microorganisms were significantly reduced by a single application of copper (Table 1). Endosulfan did not reduce populations of microorganisms on leaves (data not shown).

Except for yeasts at 2 locations (Site 3 and Site 4), populations of all categories of microorganisms on leaves were lower from sprayed trees than from unsprayed trees in the 5 orchards sampled (Table 2). The effects of spraying were most apparent for bacteria, as populations were consistently 10–100-fold lower on sprayed leaves than on unsprayed leaves.

Analyses of total copper residues on leaves from the 2 orchards where the 16-month study took place showed that the sprayed orchard had about 100 times more copper on leaves than the unsprayed orchard. It also showed that the copper residues in the sprayed orchard persisted even after a 3–4 month break in spraying (Table 3). However, copper concentrations following the non-spray period in 1993 were much lower than for the corresponding period in 1992.

The monthly rainfall and maximum and minimum air temperatures at the study site are shown in Figs 1 and 2, respectively. Periods of low rainfall occurred from June to October in 1992 and from April to June in 1993.

Table 1. Mean number of microorganisms ($\log_{10}$ cfu/g) on avocado leaves after spraying once with copper

	Pre-spray sample			Post-spray sample		
	Control	Copper	l.s.d. ($P = 0.05$)	Control	Copper	l.s.d. ($P = 0.05$)
			Expt 1 (cv. Nabal)			
Bacteria	4.9	4.6	0.46	5.3	4.4	0.51
Yeasts	4.6	4.6	0.18	5.0	4.9	0.29
Filamentous fungi	4.8	4.8	0.43	4.9	4.8	0.22
			Expt 2 (cv. Hass)			
Bacteria	4.9	4.5	0.43	4.5	2.6	0.81
Yeasts	3.9	3.8	0.22	3.7	3.0	0.41
Filamentous fungi	4.1	3.9	0.22	3.6	3.0	0.42

Table 2. Mean number of microorganisms (log_{10} cfu/g) on avocado leaves from adjacent sprayed or unsprayed trees in five orchards

Means were calculated from numbers of microorganisms on ten individually processed leaves. For each site/microorganism comparison, numbers of microorganisms on sprayed and unsprayed leaves followed by the same letter are not significantly different by the two sample *t*-test

	Microorganism	Unsprayed	Sprayed
1. Mt Tamborine, Qld. cv. Hass, sampled 3 months after 7 monthly copper–endosulfan sprays	Filamentous fungi	5.3a	4.2b
	Yeasts	5.6a	4.8b
	Bacteria	6.6a	4.5b
2. Woombye, Qld. cv. Fuerte, sampled 5 days after 1 copper–endosulfan spray	Filamentous fungi	4.8a	3.7b
	Yeasts	5.2a	4.2b
	Bacteria	4.9a	3.1b
3. Mt Tamborine, Qld. cv. Hass, sampled 1 month after 6-monthly copper sprays	Filamentous fungi	3.4a	2.9b
	Yeasts	3.7a	3.7a
	Bacteria	3.8a	1.5b
4. Maleny, Qld. cv. Hass, sampled 1 month after 6-monthly copper–endosulfan sprays	Filamentous fungi	4.4a	3.6b
	Yeasts	3.9a	3.5a
	Bacteria	4.6a	3.3b
5. Maleny, Qld. cv. Hass, sampled 7 days after 1 copper spray	Filamentous fungi	3.6a	3.0b
	Yeasts	3.7a	3.0b
	Bacteria	4.5a	2.6b

Table 3. Total copper residues (mg/kg) from a random sample of leaves collected from the sprayed and unsprayed avocado orchards at Maleny

Sampling times	Sprayed orchard	Unsprayed orchard
April 1992	1697.0	5.7
September 1992	501.2	26.3
November 1992	601.1	6.1
December 1992	789.9	5.7
January 1993	645.2	6.5
March 1993	524.2	6.7
April 1993	461.3	n.d.
August 1993	143.1	11.5

n.d., not determined.

Temperatures were highest in summer, with maximum temperatures peaking at about 27°C in January.

Since data for the 16-month study were collected from adjacent orchards, direct statistical comparison of numbers of microorganisms between orchards was not possible. Nevertheless, the data showed that seasonal population trends were generally similar in the 2 orchards. However, the numbers of filamentous fungi, yeasts, and bacteria were lower in the sprayed orchard (Figs 3 and 4). The extent of the difference between orchards varied with the type of microorganism and sampling date but was greatest with bacteria

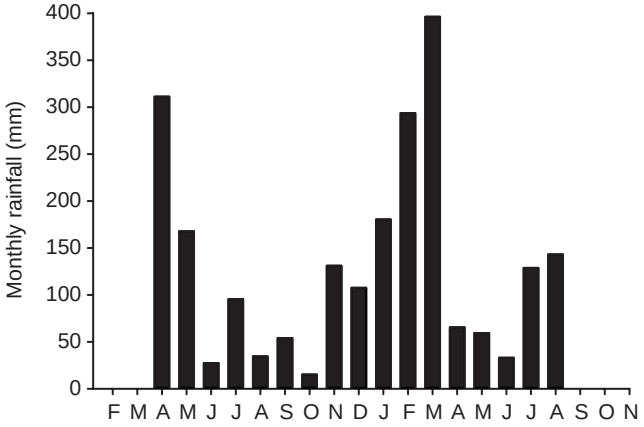

Fig. 1. Total monthly rainfall at adjacent sprayed and unsprayed avocado orchards from April 1992 to August 1993.

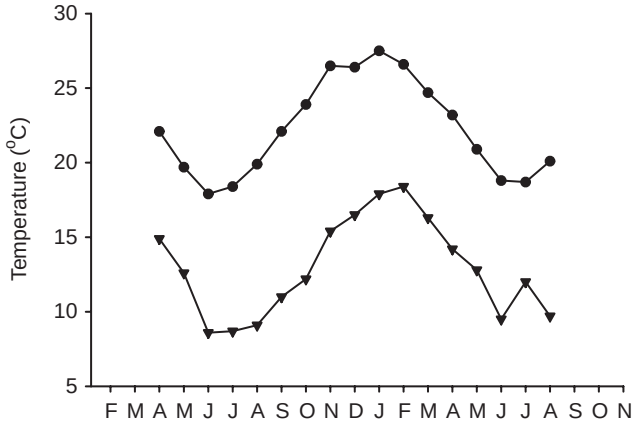

Fig. 2. Mean maximum (●) and minimum (▼) air temperatures at adjacent sprayed and unsprayed orchards from April 1992 to August 1993.

(Fig. 4). A sample taken during the non-spray period in August 1993 showed that populations of all categories of microorganisms had increased by 10–100-fold in the sprayed orchard following the final spray treatment in May 1993. The relative increase in populations of microorganisms in the unsprayed orchard during the same period was much less (Figs 3 and 4). This increase in numbers during the non-spray period for the sprayed orchard was less pronounced in the September 1992 sample for all except the yeasts (Fig. 3).

The effect of the pesticide program on populations of microorganisms on the fruit surface (Fig. 5) was similar to that on leaves. The tendency for population densities to increase in the sprayed orchard during the non-spray period was again observed.

Analyses which took into account the position of leaves within the canopy showed that in the sprayed orchard, location of leaves had a significant effect on the numbers of filamentous fungi (Table 4). Fungal population densities were consistently higher inside the canopy. The inner canopy was

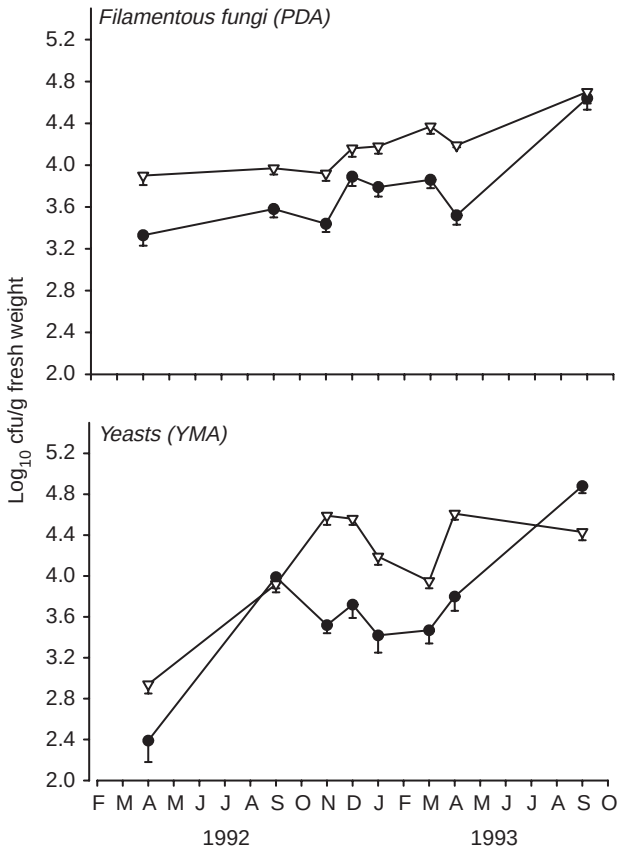

Fig. 3. Populations of filamentous fungi and yeasts from unsprayed (▽) or pesticide sprayed (●) avocado leaves. Points with vertical bars represent means ± s.e. Letters in parentheses represent media used to enumerate microorganisms. Pesticides were applied in March, April, May, November, and December 1992, and in January, February, March, April, and May 1993.

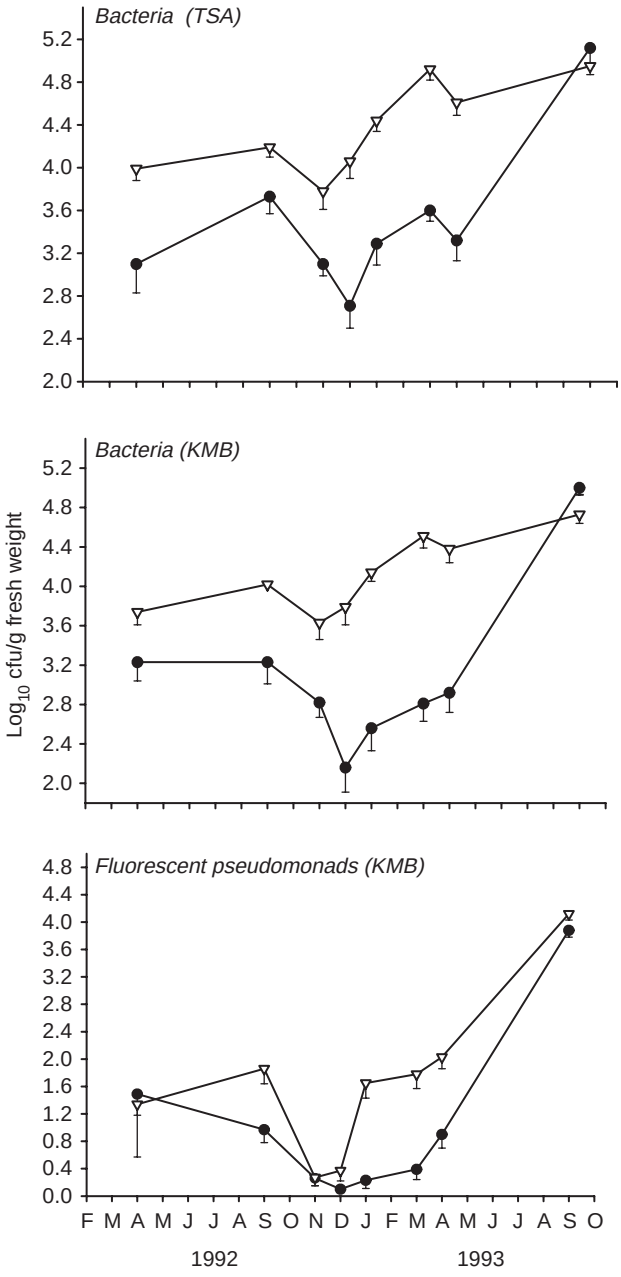

Fig. 4. Populations of bacteria from unsprayed (▽) or pesticide sprayed (●) avocado leaves. Points with vertical bars represent means ± s.e. Letters in parentheses represent media used to enumerate microorganisms. Pesticides were applied in March, April, May, November, and December 1992, and in January, February, March, April, and May 1993.

also more favourable to yeasts and bacteria but differences in population densities between inner and outer canopies were not always statistically significant. Compass direction had a significant effect on the yeast population in the sprayed orchard in January 1993, the southern aspect being more favourable. Both canopy position and compass direction significantly affected numbers of microorganisms in the unsprayed orchard on some occasions (Table 5). There were consistently more microorganisms on the southern aspect when compared with the northern aspect of trees, this effect being most apparent during the summer months from December to April.

A wide range of fungi was isolated by spot frequency analysis, the most predominant being *Cladosporium* spp., *Pestalotiopsis* spp., *Epicoccum* spp., *Leptosphaerulina* sp., *Alternaria* sp., *Nigrospora* sp., *Aureobasidium* spp., *Cryptoccocus* spp., and pink yeasts. Those isolated less frequently were *Fusarium* sp., *Trichoderma* spp., *Phoma* sp., *Curvularia* sp., *Pithomyces* sp., *Penicillium* sp., *Colletotrichum* sp., and *Geotrichum* sp. Several isolates that produced no fruiting structures were also encountered. Although the results were variable for some fungi and numbers were low in both orchards, except for *Cladosporium* spp. and *Aureobasidium* spp., fungal populations in the sprayed orchard were both quantitatively and qualitatively less than in the unsprayed orchard (data not shown). However, the numbers of fungi in the 2 orchards at

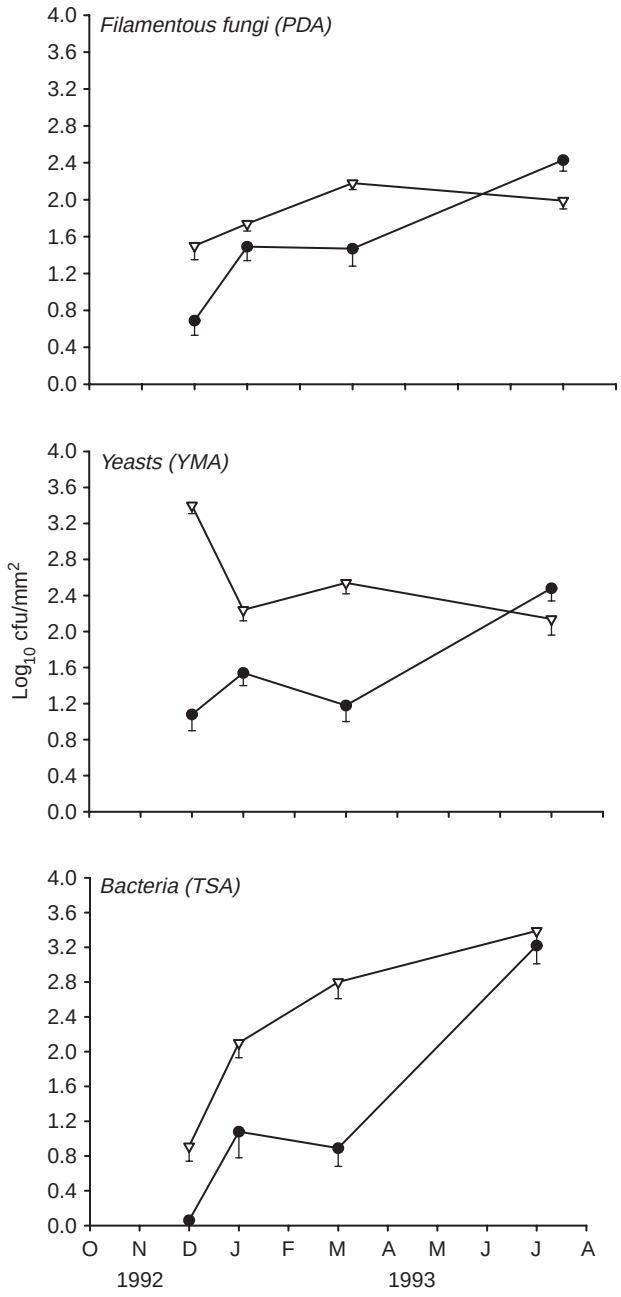

Fig. 5. Populations of microorganisms on unsprayed (▽) or pesticide sprayed (●) avocado fruit. Points with vertical bars represent means ± s.e. Letters in parentheses represent media used to enumerate microorganisms. Pesticides were applied in November and December 1992, and in January, February, March, April, and May 1993.

the end of the non-spray period in September 1992 and August 1993 were similar.

Effect of copper and endosulfan on C. gloeosporioides

There was no significant difference in the frequency of *C. gloeosporioides* isolated from unripe avocado peel from the sprayed and unsprayed orchards (Table 6). Microscopical examination of individual fungi from isolations in 1993 and 1994 showed that, generally, there was a higher percentage of fungi other than *C. gloeosporioides* present on the fruit surface in the unsprayed orchard. In addition, there were more pieces of peel with no fungal growth from sprayed fruit than from unsprayed fruit. In many instances, only *C. gloeosporioides* was isolated from sprayed fruit, whereas a mixture of fungi grew out of peel from most of the unsprayed fruit. All of the *C. gloeosporioides* isolates obtained from green peel from the 2 orchards were susceptible to copper *in vitro* (data not shown). In both 1993 and 1994, significantly more ripe fruit were diseased in the

Table 4. Results of ANOVA indicating a significant effect of tree (Tree), canopy position (Pos), and compass direction (Dir) on microorganisms on the leaf surface in the unsprayed orchard

I > O, numbers significantly higher inside canopy; S > N, numbers significantly higher on southern side

Sampling time	Filamentous fungi	Yeasts	Bacteria (on KMB)	Bacteria (on TSA)
Apr. 1992	n.s.	n.s.	n.s.	n.s.
Sept. 1992	Pos (I > O)	n.s.	n.s.	n.s.
Nov. 1992	n.s.	Tree	n.s.	Pos (I > O) Dir (S > N)
Dec. 1992	Pos (I > O)	Tree	Dir (S > N)	Dir (S > N)
Dir (S > N)	Tree × Dir			
Jan. 1993	Dir (S > N) Tree	n.s.	Dir (S > N) Tree	n.s.
Mar. 1993	n.s.	Pos (I > O)	n.s.	n.s.
Apr. 1993	Pos (I > O)	Pos (I > O)	Tree Dir (S > N) Pos (I > O)	n.s.
Aug. 1993	Tree	n.s.	n.s.	n.s.

n.s., no significant effect of tree, direction, or position.

Table 5. Results of ANOVA indicating a significant effect of tree (Tree), canopy position (Pos), and compass direction (Dir) on microorganisms on the leaf surface in the sprayed orchard

I > O, numbers significantly higher inside canopy; S > N, numbers significantly higher on southern side

Sampling time	Filamentous fungi	Yeasts	Bacteria (on KMB)	Bacteria (on TSA)
Apr. 1992	Pos (I > O)	n.s.	n.s.	n.s.
Sept. 1992	Pos (I > O)	n.s.	n.s.	n.s.
Nov. 1992	Pos (I > O)	n.s.	n.s.	Pos (I > O)
Dec. 1992	Pos (I > O)	Pos (I > O)	Pos (I > O)	n.s.
Jan. 1993	Pos (I > O)	Tree Dir (S > N)	n.s.	n.s.
Mar. 1993	Pos (I > O)	Pos (I > O)	n.s.	n.s.
Apr. 1993	Pos (I > O)	Pos (I > O)	Pos (I > O)	Pos (I > O)
Aug. 1993	n.s.	n.s.	n.s.	n.s.

n.s., no significant effect of tree, position, or direction.

Table 6. Isolations of *Colletotrichum gloeosporioides* from peel of unripe avocado fruit and disease levels caused by *C. gloeosporioides* in ripe fruit from an unsprayed (U) and a sprayed orchard (S)

Disease rating for *C. gloeosporioides* infections in 1993: 0, no lesions; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, >76% of the fruit surface with lesions. In 1994 the scheme was expanded to an 11-point scale: 0, no lesions, followed by 10% increments to 10, >90% of the fruit surface with lesions

Sampling time	Incidence of *C. gloeosporioides* in unripe avocado peel (%)			Mean disease rating (ripe fruit)	
	Orchard U	Orchard S		Orchard U	Orchard S
Dec. 1992	n.i.	4.9	n.a.	—	—
Jan. 1993	7.5	11.5	n.s.	—	—
Mar. 1993	18.4	9.8	n.s.	—	—
July 1993	23.2	23.7	n.s.	1.13	1.83*
July 1994	55.1	68.9	n.s.	0.32	2.18**

n.s., not significant (Mann–Whitney rank sum test); * $P < 0.05$; ** $P < 0.01$.
n.i., no *C. gloeosporioides* isolated; n.a., data not analysed; —, fruit not harvested.

Table 7. Pathogenicity of *Colletotrichum* isolates from peel of unripe avocado fruit obtained from the unsprayed and sprayed orchards

Values in parentheses represent data from a second experiment

Unsprayed orchard		Sprayed orchard	
Isolate accession number	No. of spots with lesions/total no. of spots inoculated	Isolate accession number	No. of spots with lesions/total no. of spots inoculated
C1A	6/8 (6/5)	T1B	13/14
CID	5/7	T1D	12/13 (12/16)
C2B	10/10	T1E	7/10
C2D	11/12 (7/8)	T2E	7/11 (10/12)
C2E	3/4	T3C	6/6 (9/11)
C4B	5/12	T4E	12/12
C6C	2/8 (2/11)	T5B	8/12
C7B	10/10	T5E	11/13
C7E	10/12	T6B	8/12
Control (BRIP 19768)	8/10 (9/12)		

sprayed orchard than in the unsprayed orchard (Table 6). *C. gloeosporioides* was isolated from 80% of the stem-end lesions and all other lesions in fruit from both orchards. *C. gloeosporioides* was rarely (<2%) associated with insect damage on fruit from either orchard. All isolates of *Colletotrichum* from both orchards were pathogenic to avocado fruit (Table 7).

Discussion

Data from single copper sprays on previously unsprayed trees and comparisons of random leaf samples from sprayed and unsprayed trees in several orchards showed unequivocally that copper fungicides were detrimental to bacteria, yeasts, and fungi on the leaf surface. Bacteria were the most affected, with the population being reduced by 10–100-fold. *In vitro* experiments involving plating of leaf washings on copper amended casitone–yeast–extract-glycerol agar (Andersen *et al.* 1991) also showed that bacteria were severely affected by copper sprays (Stirling 1996). The 2 single applications of copper to avocado leaves did not give identical results. In the first experiment, only bacteria were affected, whereas in the second experiment, copper fungicide reduced filamentous fungi, yeasts, and bacteria. However, the first experiment was done during the dry season, and the second experiment during showery weather. This difference may have contributed to the different results as prolonged leaf wetness may enhance the lethal effect of copper by keeping free Cu^{2+} in solution (Menkissoglu and Lindow 1991).

Although only 1 test with endosulfan was carried out, it demonstrated that the insecticide did not reduce populations of microorganisms on the phylloplane. Since minimal rainfall was recorded for the 4-day post-spray period, it is assumed that the pesticide remained on the leaves throughout the experiment. There is only limited published literature on the effects of insecticides on microorganisms on the phylloplane but the available examples suggest that, generally, insecticides are non-toxic (Hislop 1976; Kwee and Teik 1982; Mercier and Reeleder 1987).

The detrimental effect of copper on non-target microorganisms was confirmed during a 16-month study of microbial populations in a commercially sprayed avocado orchard and an adjacent unsprayed orchard. Populations of all categories of microorganisms were consistently lower in the sprayed orchard and since only copper and occasional treatments of endosulfan were applied, it is assumed that the effect was largely due to copper. Although the study was done in 2 separate orchards and it is possible that observed differences could have been due to inherent differences between the 2 orchards, such an effect is unlikely. The 2 orchards were adjacent, they were of similar age, avocado cultivar, canopy structure, leaf form and density, and aspect. Apart from pesticide use they were managed in a similar manner.

When studying the effect of pesticides on microorganisms in the field, it is important to determine whether there are other inherent factors that could affect populations. During this study, it is interesting to note that over 8 sampling times, canopy position frequently had a significant effect on the non-target microorganisms (especially filamentous fungi) in the sprayed orchard. When this occurred, there were always more microorganisms inside the canopy than on the outside. Canopy effect was much less prevalent in the unsprayed orchard. It is tempting to speculate that this effect was partly due to the lack of penetration of the copper sprays into the dense tree canopy. However, one would expect the microclimate inside the canopy to be more conducive to microbial survival because of less exposure to harsh conditions that often prevail on the phylloplane.

In some instances, compass direction affected population numbers in the unsprayed orchard with the cooler southern aspect being consistently more favourable to all categories of microorganisms enumerated in this study. Sunlight and ultraviolet radiation can be detrimental to microorganisms (Bridges 1976; Knudsen 1991) and were probably responsible because significant differences in numbers were generally observed from December to April when levels of radiation are highest.

In August 1993, all categories of microorganisms on the leaf surface in the sprayed orchard reached population levels 10–100-fold greater than they were 3 months previously, when the final spray for the season was applied. The relative increase in the numbers of microorganisms in the unsprayed orchard was much less, suggesting a recovery in microbial populations in response to the declining Cu levels that occurred on leaves during this period. Except for yeasts, there was no corresponding increase during the 1992 leaf sampling. This was most likely due to the detrimental effect of high levels of copper residues that remained on the leaves during the non-spray period. Total copper residue analyses on leaves showed that the levels of copper in April and September 1992 were about 4 times higher than the values for corresponding samples in 1993. This was probably the result of a change by the grower to a new, more efficient low-volume sprayer in November 1992.

Regular spraying could be expected to select microorganisms with tolerance to pesticides. This was observed for the yeasts in the sprayed orchard as they were more tolerant to copper fungicides than those from the unsprayed orchard (Stirling 1996). This may partly explain why of all of the microorganisms, the yeasts were the least affected by copper as the season progressed. Tolerance of yeasts to copper is not a new phenomenon, as some species can develop tolerance to heavy metals after relatively short exposures (Ashida 1965).

Assessment of ripe avocado fruit in 1993 and 1994 showed the presence of significantly more disease in the sprayed orchard compared with the unsprayed orchard. Because the 2 orchards were adjacent and were similar in other respects, it is possible that the differences were due to spraying. The canopies of the avocado trees were of similar densities and differences in leaf wetness should not have been a factor. Difference in tolerance of *Colletotrichum* to copper in the 2 orchards was also not involved as all of the *Colletotrichum* isolates obtained from peel of unripe fruit in both orchards were susceptible to copper *in vitro* (Stirling 1996).

When considering mechanisms which might explain difference in disease levels between the 2 orchards, it is important to note that the frequency of isolation of *Colletotrichum* from peel of unripe fruit was similar in both orchards. The fact that all *Colletotrichum* isolates from the unsprayed orchard were pathogenic to avocado fruit excludes the possibility that differences in disease resulted from the competitive displacement of pathogenic strains of the fungus by saprophytic strains. This has been suggested for suppression of coffee berry disease in Kenya (Furtado 1969; Gibbs 1972; Firman and Waller 1977), where spraying with copper fungicides increased disease. Mulinge and Griffths (1974) suggested that copper disrupted the balance between pathogenic and saprophytic populations of *Colletotrichum*.

A more likely explanation for the increased disease in the sprayed orchard is that copper, being a broad spectrum biocide, had a detrimental effect on the microflora that inhabit the fruit surface. For example, the population densities of fungi associated with the peel of unripe avocado were generally greater in the unsprayed orchard than in the sprayed orchard (Stirling 1996). These fungi included species of *Pestalotiopsis*, *Epiccocum*, *Cladosporium*, *Phoma*, *Fusarium*, *Alternaria*, *Aureobasidium*, and *Leptosphaerulina.* Some of these fungi have been reported to be minor post-harvest pathogens of avocado in South Africa (Darvas and Kotzé 1987) and their presence may give rise to a type of ‘induced resistance’ that prevents the development of anthracnose. Common surface inhabitants such as *Pestalotiopsis* sp. also produce endogenous antifungal sub-

stances (Adikaram and Karunaratne 1997) and they may play a role in inhibition of disease development in the unsprayed orchard.

An alternative hypothesis for there being less disease in the unsprayed orchard is that copper or other elements have direct or indirect effects on tree or fruit physiology, which influence the natural resistance of unripe fruit to anthracnose. Our observations suggested that physiological changes may have been occurring in fruit, as the peel of copper-sprayed ripening fruit was generally more brittle than that of unsprayed fruit. Recent studies (L. Coates, pers. comm.) have also revealed that even within an orchard, the incidence of anthracnose on fruit can vary considerably from tree to tree. Calcium has been implicated as a possible factor, as high calcium levels in fruit can be correlated to low levels of anthracnose. Other minerals such as boron and nitrogen also may indirectly influence disease. Thus, it is possible that nutrient-induced physiological changes in fruit are in some way involved in the differences in disease incidence between sprayed and unsprayed orchards.

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