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Isolation and Selection of Bacteria and Yeasts Antagonistic to Preharvest Infection of Avocado by *Colletotrichum gloeosporioides*

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Abstract

Bacteria and yeasts were isolated from leaves, flowers and fruit of avocado trees that had not been sprayed with pesticides for several years. Of the 1050 microorganisms isolated, 37% inhibited mycelial growth of *Colletotrichum gloeosporioides* on potato dextrose agar. Many of these organisms also significantly reduced spore germination of the fungus on cellophane overlaying weak sugars agar and a greater proportion of yeasts than bacteria were more effective. Some bacteria and yeasts also reduced spore germination of the pathogen on avocado leaf disks. The predominant group of suppressive bacteria was *Bacillus* spp., and the antagonistic yeasts included *Aureobasidium* spp. and a variety of pink and white colony types. Antibiotic resistant isolates of *Bacillus*, carbendazim resistant isolates of two yeasts and an *Aureobasidium* sp. were sprayed on avocado leaves and survived for at least 2 months on the phylloplane. On the basis of performance in these tests, isolates with biocontrol and colonization potential were selected and tested for their capacity to provide disease control on fruit. In repeated tests, several bacteria and yeasts consistently reduced lesion development and lesion size on detached avocado fruit when applied prior to inoculating fruit with the pathogen.

Keywords: Biological control, *Colletotrichum gloeosporioides*, avocado, bacteria, yeasts, anthracnose.

Introduction

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc., the cause of anthracnose in avocado (*Persea americana* Mill.), results in considerable crop losses in all commercial avocado-growing areas of the world. Symptoms of anthracnose are predominantly expressed as a rot in ripening fruit after harvest, but lesions can also develop from infections in unripe fruit for up to 3 months before harvest. This causes premature ripening and subsequent abscission of fruit (Fitzell 1987). Infection of fruit occurs mainly during rainfall by waterborne conidia from dead leaves and twigs entangled within the avocado canopy (Fitzell 1987). The pathogen can infect fruit from fruit-set to harvest (Peterson 1978). Generally these infections remain quiescent in unripe fruit as an appressorium with an infection peg (Coates *et al.* 1993*a*), probably because antifungal dienes suppress fungal development (Prusky and Keen 1993).

In Australia, some disease control is obtained with orchard management practices, such as removal of dead leaves and twigs, canopy ventilation and the use of

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protectant preharvest copper fungicide sprays (Pegg and Coates 1993). Additional postharvest treatment of fruit with fungicides such as prochloraz (Sportak^{R}) (Muirhead *et al.* 1982), and controlled storage and ripening temperatures are also recommended (Pegg and Coates 1993). Insecticides are sprayed preharvest to reduce populations of fruit fly and spotting bug that cause injuries which are associated with increased anthracnose (Fitzell 1987). This heavy reliance on synthetic chemicals for insect and disease control is becoming increasingly unacceptable to growers and consumers, particularly as high levels of postharvest anthracnose continue to occur (Muirhead *et al.* 1982; Ledger 1993). Alternative non-chemical methods of reducing disease severity are therefore required.

To date, there are only a few examples of biological control of anthracnose on tropical fruit crops. In South Africa, phylloplane isolates of *Bacillus subtilis* (Ehrenberg) Cohn, *B. licheniformis* (Weigman) Chester, and *B. cereus* Frankland and Frankland successfully controlled field infections of anthracnose in mango and avocado when applied as preharvest sprays and postharvest dip treatments (Korsten *et al.* 1989; Korsten and Kotzé 1992). *Pseudomonas fluorescens* 558 isolated from mango leaves controlled natural latent infections of *C. gloeosporioides* on mango under packhouse conditions in the Philippines (Koomen and Jeffries 1993). However, preharvest infections were not reduced when bacteria were applied as a spray. *Bacillus cereus* 204, another isolate which showed promise in the initial screening, also did not control natural infections of anthracnose.

Recent observations in unsprayed avocado orchards in Queensland showed that populations of bacteria and yeasts on leaves and fruit were high, and there was little disease (Stirling, unpublished data), suggesting that anthracnose may be under natural suppression by epiphytic microorganisms. The aim of this work was to isolate bacteria and yeasts from these orchards and select those capable of suppressing preharvest infections of avocado by *C. gloeosporioides.* The selection process involved testing organisms for antagonism on agar, leaf disks and fruit. Since the ultimate aim was to find organisms that would colonize aerial plant surfaces and provide disease control after being sprayed on trees, colonization potential was also an important selection criterion.

Materials and Methods

Isolation and Maintenance of Bacteria and Yeasts

Bacteria and yeasts were isolated from 10 avocado orchards. Eight of these had not been sprayed with fungicides for at least 6 years, one had received regular copper fungicides and one was occasionally sprayed with copper. Leaves, flowers and fruit peel were blended separately for 3 min in 20 mL of phosphate buffered saline (PBS; pH 7·2) containing 0·01% (v/v) Tween-80 in a Stomacher Lab-Blender 80 (Seward Medical Company). Samples were then serially diluted in PBS and 0·2 mL aliquots plated on King's medium B (King *et al.* 1954), $\frac{1}{2}$ strength tryptic soy agar ($\frac{1}{2}$TSA; Difco) and glucose-yeast-peptone agar (Stirling *et al.* 1992) to isolate bacteria. Samples were also heat treated (10 min at 85°C) and subsequently plated on $\frac{1}{2}$TSA to isolate spore-forming bacteria. Yeasts were isolated on 5% malt-extract agar and yeast extract-malt extract agar (YMA) (Lodder 1970) acidified with 0·75% (v/v) 1M HCl to pH 3·8. Cycloheximide (75 μg mL^{-1}; Sigma) or streptomycin sulfate (120 μg mL^{-1}; Sigma) were added to media for inhibition of fungi and bacteria respectively. Plates were maintained at 25°C for up to 1 week, and bacteria and yeasts were purified and stored initially in sterile water and also freeze dried for long term storage. For all subsequent experiments, bacteria were grown on $\frac{1}{2}$TSA at 28°C for 36 h, while yeasts were grown on YMA for 3–4 days at 25°C.

In Vitro *Screening for Potential Antagonists*

C. gloeosporioides A111–2 (BRIP 19768) (Coates *et al.* 1993*a*) was grown on oat meal agar (OMA) at 25°C under near UV radiation for all tests. In the first of two agar plate tests that were used to select antagonists, a total of 1050 bacteria and yeasts were tested for inhibitory activity against *C. gloeosporioides* on potato dextrose agar (PDA), while 160 of the same isolates were also tested on weak sugars agar (WSA) (Austin *et al.* 1977). A fungal plug (5 mm diameter, from a 1-week-old culture) was placed at the centre of a 90 mm petri dish, and each organism was spot inoculated 40 mm away on opposite edges of two replicate plates. Inhibition of fungal growth was measured after incubation at 25°C for 9 days (by which time the fungus in the controls had reached the edge of the plate). The degree of inhibition produced by each antagonist was rated as follows: 0, no inhibition zone; +, zone $\leq$ 5 mm; ++, zone 6–12 mm; +++, zone $\geq$ 13 mm. In addition, any bacterium or yeast that did not produce a zone of inhibition but caused the fungus to grow sparsely, or where the antagonist was not overgrown by the fungus (non-zonal inhibition) was selected for further testing.

In the second test, 229 bacteria and 104 yeasts, most of which produced some type of inhibition on PDA, were assessed for their effect on spore germination of *C. gloeosporioides*. Sterile cellophane disks (25 mm in diameter) that had been boiled twice in distilled water were placed on the surface of WSA in 90 mm petri dishes. Once the surface of the cellophane was dry, 25 μL of a bacterial or yeast suspension (approximately 10^6 mL^{-1} in deionized water) was added and gently spread with a sterile glass rod to within 2–3 mm of the edge of the disk. Each petri dish contained three disks which were incubated for 18 h at 25°C. Fungal spores from a 2-week-old culture were filtered through three layers of sterile cheese cloth, adjusted to 5×10^5 mL^{-1} and 20 μL spread on the surface of the disks containing the microorganisms. Disks with fungus only were used as controls. After 8 h the number of germinated spores was counted as described by Austin *et al.* (1977). A spore was considered to be germinated when the germ-tube length was at least equal to the width of a spore and/or an appressorium had formed. Isolates were tested three times, the mean was calculated for each and organisms were ranked for performance.

Identification of Selected Antagonists

Gram-positive bacteria were observed for cell shape, heat resistance (10 min at 85°C in a water-bath), spore formation, position of the spore in the sporangium, motility, catalase, oxidase, acid production from glucose, and reduction of nitrate (Sneath 1986). Three Gram-negative bacteria (isolates 677, 591 and 632) were identified using API 20NE and API 20E (Analytical Profile Index Systems, Bio Merieux SA, France). Five yeast isolates (711, 734, 772, Q6 and Q34) were tested using YT MicroPlate^{TM} (BIOLOG, Hayward, CA).

In Vivo *Selection of Antagonists on Avocado Leaf Disks and Detached Fruit*

The ability of 76 bacteria and 48 yeasts to suppress preharvest infection by *C. gloeosporioides* was tested on detached avocado fruit (cv. Fuerte). Full size or cocktail fruit were washed to remove pesticide residues and then surface sterilized with 70% ethanol (v/v). Because of the presence of latent field infections of *C. gloeosporioides* on most of the fruit, the pathogen was inoculated to marked areas on each fruit. Suspensions (10^8–10^9 mL^{-1}) of each antagonist were prepared in weak sugars broth (WSB; Austin *et al.* 1977) containing 0·3% (w/v) methyl cellulose (used as a sticker) and applied to half the surface of five (full size) or eight (cocktail) replicate fruit with a sterile cotton wool bud. Random samples of fruit peel disks from inoculated areas showed that the numbers of antagonists applied varied between 10^3 and 10^4 mm^{-2} soon after air drying. Once dry, 25 μL of a *C. gloeosporioides* spore suspension (1×10^6 mL^{-1}) was added to the marked areas on the fruit. Control fruit treated with or without WSB and inoculated with fungus were included in all of the experiments. Fruit were randomly placed in ripening boxes and maintained under 100% RH for 48 h at 25±1°C. Fruit were then allowed to ripen at 24±1°C and 80–85% RH, and lesion development and lesion size were noted. Isolations of the pathogen were made from a random selection of lesions in the defined areas. Because of the large number of microorganisms tested, evaluation of all isolates required 11 separate tests and most isolates were tested at least twice. In four of the tests, the response of *C. gloeosporioides* to different batches of avocado fruit was assessed by

measuring spore germination and appressorium formation 8 and 24 h after incubation. Data for disease suppression in each test were calculated as percentage inhibition relative to the control, and all antagonists were ranked according to performance. This gave a measure of consistency of performance for each antagonist and helped in the final selection of the best candidates.

Forty-four antagonists selected on performance in the spore germination test on cellophane and the 11 initial detached fruit tests were tested for their ability to suppress spore germination on leaf disks. Mature avocado leaves (cv. Fuerte) were surface sterilized with 70% ethanol, rinsed with sterile water and wiped dry. Disks (18 mm diameter) cut with a cork borer were placed on sterile, moist filter paper in glass petri dishes. A loopful of each microorganism was added to 1 mL of a *C. gloeosporioides* spore suspension (7×10^5 mL^{-1}) in sterile glass vials. The final concentration of antagonists was approximately 10^8 to 10^9 mL^{-1}, as estimated by comparison with McFarland's barium sulfate standards (Paik 1980) and subsequently confirmed by dilution plating. The suspension was mixed by vortexing for 20 s and 20 μL was then added to five replicate disks and the drops gently spread in a circle about 6 mm in diameter. Fungal spores without yeast or bacteria were used as controls. Plates were enclosed in plastic-wrap and incubated in the dark for 8 h at 25°C. Three disks per treatment with visible moisture were selected and dried in a laminar flow cabinet, and spores were removed from the disk surface by stripping with transparent adhesive tape. After mounting the tape in lactophenol, the proportion of germinated and non-germinated spores was estimated by observing 100 spores at a magnification of 400×. Each antagonist was tested three times and the mean percentage germination was calculated, and means were ranked.

After examining the ranked results from the inhibition test on PDA, the spore germination test on cellophane, the leaf disk assay and the initial detached fruit tests, a single detached fruit test was performed to compare 22 of the most promising antagonists. Organisms tested were *Bacillus* spp. (75, 78, 301, 359, 359ab, 480, 553, 544, 933), *P. fluorescens* (677), *Enterobacter agglomerans* (632), pink yeasts (Q6, Q34, 734), white yeasts (711, 772), the dimorphic fungus *Aureobasidium* sp. (140, 274, 468, 731) and two actinomycetes (202, 312). Cocktail fruit were used and all experimental details were as previously described. Each antagonist was tested on 10 replicate fruit and 40 fruit (4 sets of 10) were used as fungus-only inoculated controls. All fruit for five or six antagonists (50–60 fruit) and 10 controls were placed in each of four boxes.

Colonization Potential of Selected Antagonists

The colonization potential of selected bacteria and yeasts was studied in three experiments. Rifampicin-resistant bacterial mutants *Bacillus* spp. (359R1, 480R2), *E. agglomerans* (632R2) and *P. fluorescens* (677R1), generated as detailed by Stirling *et al.* (1992), were grown in TSB (shake culture) for 36 h at 26°C. *Aureobasidium* sp. 274C1, and yeasts 772C1, 734C2 selected for carbendazim (Bavistin^R) resistance using methods described by Fokkema *et al.* (1987) and wild type Q34 were cultured for 3 days in peptone–yeast–glucose broth (g L^{-1}: glucose 30; yeast extract 2; peptone 10). Each suspension was diluted (100 mL culture in 900 mL autoclaved tap water containing Tween 80 (0·05% v/v)) and sprayed onto avocado leaves until run-off. Once dry, initial numbers of microorganisms on leaves were determined by dilution plating on PDA+carbendazim (40 μg mL^{-1}) or PDA for yeasts and TSA+rifampicin (100 μg mL^{-1}) for bacteria. Further samples were processed 7, 30 and 60 days after spraying. The yeasts were tested on 12-month-old avocado (cv. Hass) seedlings (30–40 leaves per plant) in a shade house with four replicate plants being sprayed with each yeast. At the same time, bacterial isolates were each tested on four replicate twigs (30–40 leaves per twig) on a mature 'Hass' tree in a home garden. In a third experiment, all of the bacterial and yeast isolates were tested on four replicate twigs on mature 'Hass' trees in a commercial orchard. Controls sprayed with diluted growth media were included in each experiment.

Statistical Analysis

Completely-randomized designs were used in all experiments. Data were analysed using Statistix 3·1 (Analytical Software, St Paul, MN). Log_{10} transformed data for numbers of microorganisms g^{-1} leaf in the colonization experiments were normally distributed according the Wilk–Shapiro test and therefore analysed using analysis of variance and means compared

using l.s.d. Since data for percentage lesion incidence in all of the detached fruit tests were non-normally distributed even after angular transformation, Kruskal-Wallis one-way analysis of variance was used, and means compared using the technique described in Conover (1980).

Results

Identification of Antagonists

All the Gram-positive bacteria were rod shaped and heat resistant, produced ellipsoidal, centrally located endospores, and were oxidase negative and catalase positive. They produced acid from glucose, reduced nitrate to nitrite, and were identified as *Bacillus* spp. Extensive phenotypic tests and molecular identification of the most promising isolates to species level have been done (Stirling, unpublished data). Gram-negative isolates 677, 591 and 632 were identified as *Pseudomonas fluorescens*, *Chryseomonas luteola* and *Enterobacter agglomerans* respectively. The YT MicroPlate identification system for yeasts gave unsatisfactory results for all of the isolates tested, and they were therefore grouped according to pigmentation as pink or white colony types.

In Vitro *Screening*

Approximately 37% of the 1050 microorganisms tested showed some form of fungal inhibition on PDA (Table 1). The majority of isolates that were antagonistic exhibited non-zonal inhibition, and about 70% of these isolates were yeasts. The non-zonal inhibition produced by the yeast-like *Aureobasidium* spp. was unique. Each isolate grew in a fanlike spreading fashion from the point of inoculation, and advancement of *C. gloeosporioides* stopped at the edge of the yeast colony. The pathogen did not grow over the yeast colony even when plates were incubated for a further 2–3 weeks at 25°C. Most of the bacteria that strongly inhibited (+++) the fungus on PDA were *Bacillus* spp. Since the 160 antagonists tested on WSA exhibited the same type of antagonism as on PDA, the remaining isolates were not tested on the second medium.

Table 1. *In vitro* inhibition of mycelial growth of *Colletotrichum gloeosporioides* on potato dextrose agar by phylloplane bacteria and yeasts

	Levels of inhibition:[A]					
	0	non-zonal	+	++	+++	Total tested
Number of yeasts	61	140	6	0	0	207
Number of bacteria	598	60	111	37	37	843
Total	659	200	117	37	37	1050

[A] 0, no inhibition zone; +, zone ≤5 mm; ++, zone 6–12 mm; +++, zone ≥13 mm; non-zonal, fungal growth adjacent to antagonist sparse and/or fungus did not overgrow antagonist.

When spore germination was examined on cellophane overlaying WSA, 34 of the 229 bacteria tested reduced spore germination by 80% or more (Table 2). A higher proportion of yeasts (41 out of 104) reduced spore germination to the same extent, with two isolates of *Aureobasidium* completely inhibiting spore germination (Table 2). The reaction of organisms was generally consistent when the test was repeated. Spores on cellophane over WSA in the presence or absence of antagonists mainly produced germ tubes rather than appressoria. A few bacteria greatly enhanced appressorium formation, and these isolates were excluded from further testing.

Table 2. Number of bacteria and yeasts inhibiting germination of *Colletotrichum gloeosporioides* spores on cellophane overlaying weak sugars agar

	Inhibition (%): 0–10	11–79	80–89	90–99	100	Total tested
Number of yeasts	14	49	19	20	2	104
Number of bacteria	48	147	8	11	15	229
Total	62	196	27	31	17	333

There appeared to be a strong relationship between mycelial growth inhibition on PDA and inhibition of spore germination on cellophane overlaying WSA. The 15 bacterial isolates that completely inhibited spore germination exhibited the highest level (+++) of mycelial growth inhibition on agar.

In Vivo *Screening*

Although many bacteria and yeasts suppressed spore germination on cellophane, only nine bacterial isolates reduced spore germination by 80% or more on avocado leaf disks (Table 3). Individual data for eight of these bacteria are included in Table 4.

Table 3. Number of bacteria and yeasts inhibiting germination of *Colletotrichum gloeosporioides* spores on avocado leaf disks

	Inhibition (%): 0–10	11–79	80–89	90–99	100	Total tested
Number of yeasts	0	6	1	0	0	7
Number of bacteria	5	23	4	3	2	37
Total	5	29	5	3	2	44

Table 4. A comparison of the performance of 22 promising antagonists in four selection tests

Isolate	Mycelial inhibition	Mean % of spores germinated on cellophane	Mean % of spores germinated on avocado leaf disks	Fruit test: times successful/ times tested
Bacillus sp. 75	+++	0·0	2·0±0·7[A]	2/2
Bacillus sp. 78	+++	11·2±3·7	60·0±8·5	3/5
Bacillus sp. 301	+++	0·0	0·0	2/4
Bacillus sp. 330	0	20·5±2·7	97·3±0·1	3/3
Bacillus sp. 359	+	20·2±8·9	43·7±4·1	4/4
Bacillus sp. 480	+	65·2±12·4	79·0±2·1	3/5
Bacillus sp. 544	+++	0·0	1·0±1·0	1/3
Bacillus sp. 553	+++	0·0	7·7±3·3	0/2
Bacillus sp. 933	+++	0·0	60·6±3·3	1/2
Actinomycete 202	+++	0·0	3·7±0·9	0/3
Actinomycete 312	+++	14·4±10·1	0·0	0/2
Chryseomonas luteola 591	+++	14·3±5·8	35·2±10·7	2/2
Enterobacter agglomerans 632	+	3·6±1·4	3·5±2·1	2/3
Pseudomonas fluorescens 677	++	16·1±4·4	0·7±0·7	3/4
Pink yeast Q6	0	7·3±1·5	68·1±8·1	2/3
Pink yeast Q34	0	81·3±1·9	73·3±6·7	3/3
Pink yeast 734	0	49·2±14·5	35·3±6·7	3/4
White yeast 711	0	30·0±8·1	69·0±7·0	3/3
White yeast 772	0	27·4±11·8	72·7±11·5	3/4
Aureobasidium sp. 140	NZ[B]	0·0	91·2±5·2	2/4
Aureobasidium sp. 274	NZ	0·0	17·8±3·7	3/3
Aureobasidium sp. 468	NZ	0·0	64·0±9·1	2/3
Control	0	96·0±2·0	86·6±5·1	0/11[C]

[A] ± Standard error. [B] Non-zonal inhibition. [C] Data is from 11 individual tests.

Detached Fruit Test

In the four tests where the behaviour of *C. gloeosporioides* was examined on the fruit surface more than 90% of the spores germinated after 8 h but appressorium formation was variable. The mean percentage conidial germination in experiments 1, 2, 3 and 4 after 8 h were 90, 92·6, 97·3 and 99·0, whereas the percentage of appressoria formed were respectively 15·2 41·1, 75·6 and 94·4. After 20 h, most conidia had produced multiple germ tubes, a mycelial mat and secondary conidia. Results of all 11 detached fruit tests showed that several isolates suppressed lesion development (Table 4). Fruit treated with these isolates had 25–50% fewer lesions than the fungus only controls. However, percentage disease suppression for a given isolate varied considerably between experiments. *Bacillus* spp. 75, 330, 359, pink yeast Q34, white yeast 711 and *Aureobasidium* sp. 274 provided consistently better disease suppression than the control in all of the tests (Table 4), but results were not always significant. The two actinomycetes 202 and 312 failed to suppress fungal infection in any test (Table 4). There was no difference in lesion incidence in the control fruit that were untreated or treated with methyl cellulose+WSB.

In the final detached fruit test, the four sets of controls were not significantly different from each other for lesion development (Table 5), indicating that conditions were similar in all of the ripening boxes. Some isolates failed completely to suppress lesion formation (i.e. mean lesion incidence = 100%), and these isolates were not included in the analysis of variance or in the table. Of the 22 selected bacteria and yeasts, only *Bacillus* spp. 359, 359+359ab and pink yeast Q34 significantly suppressed preharvest infection by the pathogen (Table 5). *Bacillus* sp. 359 was the only isolate to significantly reduce lesion size relative to the controls.

Table 5. Reduction in *Colletotrichum gloeosporioides* lesions on avocado fruit treated with antagonistic bacteria or yeasts

Means followed by the same letter are not significantly different at $P = 0{\cdot}05$

Treatment	Mean lesion incidence (%)	Mean lesion size (mm)
Pink yeast Q34	47·0a	2·9
Bacillus sp. 359	47·5ab	2·4
Bacillus spp. 359+359ab	50·8abc	3·4
Enterobacter agglomerans 632	55·8abcd	3·9
White yeast 711	61·7abcde	5·6
Pseudomonas fluorescens 677	70·0abcde	6·9
Pink yeast 734	72·0bcde	4·7
Bacillus sp. 359ab	75·0bcde	5·7
Aureobasidium sp. 468	80·0cde	8·1
Control	82·5cde	6·5
Bacillus sp. 78	83·3cde	6·9
Bacillus sp. 75	85·0cde	8·0
Control	85·0de	10·6
Control	85·0de	7·4
Bacillus sp. 933	87·5de	8·9
Aureobasidium sp. 274	87·8de	6·8
Control	92·5e	6·3
l.s.d. ($P = 0{\cdot}05$)	ND[A]	3·8

[A] Not done.

Colonisation Potential of Selected Yeasts and Bacteria

All bacterial mutants exhibited stability of resistance to rifampicin and showed similar colony characteristics to their respective wild type isolates. We were unable to obtain satisfactory carbendazim-resistant mutants for pink yeast Q34 and therefore the wildtype was used. No such problems were encountered with yeasts 734, 772 and *Aureobasidium* sp. 274.

In the first two experiments, all except for the bacteria *E. agglomerans* 632R2 and *P. fluorescens* 677R1 maintained relatively high populations for up to 2 months on avocado leaves (Table 6). A similar trend in colonization was evident in a third experiment in an avocado orchard, as 677R1 was not detected 7 days after spraying and numbers of 632R2 were reduced by 1000-fold within a month. Monthly rainfall during the first and second months of the experiments was 175 and 74 mm respectively.

Table 6. Population densities of selected bacteria and yeasts on avocado leaves sampled at various times after they were sprayed on trees

Isolate	Mean $\log_{10}$ cfu±s.e. g^{-1} fresh weight leaf				
	Day 1	Day 7	Day 30	Day 60	l.s.d. ($P = 0{\cdot}05$)[A]
			Experiment 1		
Bacillus sp. 359R1	6·9±0·1	6·3±0·1	5·7±0·1	5·1±0·2	0·5
Bacillus sp. 480R2	7·3±0·1	6·9±0·1	6·7±0·2	4·9±0·2	0·4
Enterobacter agglomerans 632R2	7·1±0·1	3·3±0·1	3·1±0·1	NS[B]	0·3
Pseudomonas fluorescens 677R1	7·4±0·3	1·7±0·2	ND[C]	NS	0·7
			Experiment 2		
Aureobasidium sp. 274C1	6·8±0·1	6·0±0·1	5·4±0·1	4·9±0·2	0·3
White yeast 772C1	6·7±0·1	6·5±0·2	6·3±0·1	6·1±0·1	0·4
Pink yeast 734C2	7·0±0·1	6·7±0·1	6·6±0·2	5·8±0·1	0·4
Pink yeast Q34	7·3±0·1	4·8±0·1	4·5±0·1	NS	0·3

[A] Values are for differences between sampling times for an organism.
[B] Not sampled. [C] None detected.

Discussion

A varied range of yeasts and bacteria antagonistic to *C. gloeosporioides* were selected by screening a large number of isolates using five different tests. The two rapid *in vitro* tests, mycelial inhibition of the pathogen on WSA and PDA, and inhibition of spore germination on cellophane overlaying WSA, although removed from natural conditions, were useful as an initial screen because 1050 isolates were reduced to a manageable number of potentially useful antagonists. The tests also gave some insight into the behaviour of groups of antagonists and possible mechanisms of inhibition. For instance, many of the *Bacillus* spp. and actinomycetes strongly inhibited the fungus on PDA. This type of inhibition is indicative of diffusible antibiotic production (Stirling *et al.* 1992). The *Aureobasidium* spp. exhibited mainly non-zonal inhibition, suggesting that either competition for nutrients or the production of non-diffusible antibiotics may have been involved in the antagonistic reaction. Mycelial inhibition on WSA was included, so that the response of antagonist and pathogen could be studied on a medium with low nutrient status (Whipps 1987) similar to leaf diffusate. Antagonism can differ on media with different nutrient status. However, different responses to those on PDA for the same antagonist were not observed. The strong relationship between mycelial growth inhibition on PDA and reduction of

spore germination on cellophane overlaying WSA for antagonists such as *Bacillus* spp., *Aureobasidium* spp. and the two actinomycetes, suggested that the same antifungal compounds might be involved in both processes.

Although *C. gloeosporioides* does not normally produce disease symptoms on avocado leaves, latent infections have been observed on green leaves of seedlings and mature trees (Peterson 1977). The fungus develops after senescence, producing conidia which are a major inoculum source for subsequent infection of fruit. Therefore, the use of leaf disks as a test system was a logical step, as they simulated natural conditions and were easier to obtain than disks from fruit peel. The mechanism of inhibition of spore germination on leaf disks by some of the antagonists such as *Bacillus* spp. 75, 301, 544, 553, *P. fluorescens* 677, the actinomycetes 202 and 312, and *Aureobasidium* sp. 274 is uncertain, although antibiotics may have been involved. Lenné and Brown (1991) showed that culture filtrates of *Bacillus* sp. and *Pseudomonas* sp. inhibited germination of *C. gloeosporioides* spores on leaves of *Stylosanthes guianensis* and antibacterial compounds were formed by yeasts on artificial leaf surfaces (McCormack 1994), but to date there is little conclusive evidence that antibiotic production occurs on the phylloplane. Transposon mutagenesis of rhizosphere bacteria has shown that antibiotics are produced on the root surface and are responsible for control of take-all of wheat (Thomashow *et al.* 1990). Parallel studies may prove useful in elucidating mechanisms of biological control on the phylloplane.

Our observations on germination and growth of *C. gloeosporioides* on disks of fruit peel showed that appressorium formation varied considerably between batches of fruit. This variation could explain the variability of responses of some antagonists in the detached fruit tests. Testing was carried out over two seasons, and fruit were obtained from a number of districts and growers. Therefore, levels of latent field infection varied considerably, as did evenness of ripening, epicuticular wax content and amount of pesticide residues. Despite these problems of variability, we consider that fruit tests are important when selecting antagonists for preharvest control of anthracnose. A relatively large number of antagonists were tested repeatedly, and the most successful bacterial antagonists were found to be predominantly *Bacillus* spp. This was not unexpected, since there are several examples of biological control of phylloplane pathogens by *Bacillus* spp. (Spurr 1981; Blakeman and Fokkema 1982; Baker *et al.* 1983; Kokalis-Burelle *et al.* 1992), including pre- and postharvest suppression of mango and avocado anthracnose (Korsten *et al.* 1989; Korsten and Kotzé 1992). Many of the yeasts also suppressed anthracnose infection of detached fruit, the most successful being pink yeasts Q34 and 734, white yeasts 711 and 772, and *Aureobasidium* spp. 274 and 468. The mechanisms of suppression by these yeasts are currently being investigated. To date, there are no other reports in the literature of biological control of avocado anthracnose by yeasts, but yeasts have been effective biological control agents of other fruit diseases (Wisniewski and Wilson 1992).

When *Bacillus* spp. 359R1, 480R2, yeasts 734C2, 772C1 and Q34, and *Aureobasidium* sp. 274C1 were sprayed on avocado leaves, they survived for at least 2 months on the phylloplane, a characteristic that is necessary if these microorganisms are to suppress preharvest anthracnose when applied regularly as a spray. *P. fluorescens* 677 and *E. agglomerans* 632 showed some promise in reducing preharvest pathogen infection in detached fruit, but their usefulness

as preharvest spray applications is doubtful in view of their poor survival on the phylloplane. Koomen and Jeffries (1993) reported that *P. fluorescens* 504 isolated from mango leaves reduced postharvest development of anthracnose, but had no effect on preharvest infections when sprayed on mango trees, probably due to its inability to survive harsh conditions on the phylloplane.

In the field, *C. gloeosporioides* spores can germinate and form melanized appressoria within 48 h on fruit, giving rise to latent infections (Coates *et al.* 1993*b*) which may be difficult to control. Latent infections also occur on leaves (Peterson 1977). Therefore for successful disease reduction, prevention of preharvest infections seems a more logical way of proceeding than attacking the problem after harvest. Antagonists capable of suppressing mycelial growth, preventing germination of spores and inhibiting appressorium formation by the pathogen within the avocado canopy, are likely to be useful candidates for biocontrol studies in preharvest situations. Our study has shown that several microorganisms antagonistic to anthracnose are present in the avocado phyllosphere. Some of the promising antagonists that exhibited good colonization potential are currently being evaluated as biological control agents in the field.

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References

Austin, B., Dickinson, C. H., and Goodfellow, M. (1977). Antagonistic interactions of phylloplane bacteria with *Drechslera dictyoides* (Drechsler) Shoemaker. *Canadian Journal of Microbiology* **23**, 710–15.

Baker, C. J., Stavely, J. R., Thomas, C. A., Sasser, M., and MacFall, J. S. (1983). Inhibitory effect of *Bacillus subtilis* on *Uromyces phaseoli* and on development of rust pustules on bean leaves. *Phytopathology* **73**, 1148–52.

Blakeman, J. P., and Fokkema, N. J. (1982). Potential for biological control of plant diseases on the phylloplane. *Annual Review of Phytopathology* **20**, 167–92.

Coates, L. M., Irwin, J. A. G., and Muirhead, I. F. (1993*a*). The use of a benomyl-resistant mutant to demonstrate latency of *Colletotrichum gloeosporioides* in avocado fruit. *Australian Journal of Agricultural Research* **44**, 763–72.

Coates, L. M., Muirhead, I. F., Irwin, J. A. G., and Gowanlock, D. H. (1993*b*). Initial infection processes by *Colletotrichum gloeosporioides* on avocado fruit. *Mycological Research* **97**, 1363–70.

Conover, W. J. (1980). 'Practical Non-parametric Statistics.' p. 493. (John Wiley & Sons: New York.)

Fitzell, R. D. (1987). Epidemiology of anthracnose disease of avocados. *South African Avocado Growers' Association Yearbook* **10**, 113–16.

Fokkema, N. J., Dik, A. J., and Daamen, R. A. (1987). Use of carbendazim and carbendazim-resistant yeasts to create different yeast densities on wheat leaves for field studies on biological control. *Netherlands Journal of Plant Pathology* **93**, 273–83.

King, E. O., Ward, M. K., and Raney, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescin. *Journal of Laboratory and Clinical Medicine* **44**, 301–7.

Kokalis-Burelle, N., Backman, P. A., Rodríguez-Kábana, R., and Ploper, L. D. (1992). Potential for biological control of early leafspot of peanut using *Bacillus cereus* and chitin as foliar amendments. *Biological Control* **2**, 321–8.

Koomen, I., and Jeffries, P. (1993). Effects of antagonistic microorganisms on the post-harvest development of *Colletotrichum gloeosporioides* on mango. *Plant Pathology* **42**, 230–7.

Korsten, L., Bezuidenhout, J. J., and Kotzé, J. M. (1989). Biocontrol of avocado postharvest diseases. *South African Avocado Growers' Association Yearbook* **12**, 10–12.

Korsten, L., and Kotzé J. M. (1992). Postharvest biological control of avocado postharvest diseases. Proceedings of the Second World Avocado Congress. pp. 473–7. April 21–26, 1991. California, USA.

Ledger, S. (1993). Avocado retail survey discovers quality problems. *Talking Avocados* **4**, 15.

Lenné, J. M., and Brown, A. E. (1991). Factors influencing the germination of pathogenic and weakly pathogenic isolates of *Colletotrichum gloeosporioides* on leaf surfaces of *Stylosanthes guianensis*. *Mycological Research* **95**, 227–32.

Lodder, J. (1970). 'The Yeasts.' (North-Holland Publishing Company: Amsterdam.)

McCormack, P. J., Wildman, H. G., and Jeffries, P. J. (1994). Production of antibacterial compounds by phylloplane inhabiting yeasts and yeastlike fungi. *Applied and Environmental Microbiology* **60**, 927–31.

Muirhead, I. F., Fitzell, R. D., Davis, R. D., and Peterson, R. A. (1982). Post-harvest control of anthracnose and stem-end rots of Fuerte avocados with Prochloraz and other fungicides. *Australian Journal of Experimental Agriculture and Animal Husbandry* **22**, 441–6.

Paik, G. (1980). Reagents, stains, and miscellaneous test procedures. In 'The Manual of Clinical Microbiology'. Third Edition. (Ed E. H. Lennette.) pp. 1000–24. (American Society for Microbiology: Washington, DC.)

Pegg, K. G., and Coates, L. M. (1993). Avocado. In 'Diseases of Fruit Crops'. (Ed. D. Persley.) pp. 20–21. (Department of Primary Industries, Queensland.)

Peterson, R. A. (1977). Green leaves as a source of inoculum for anthracnose fungi, with special reference to *Colletotrichum acutatum* on strawberry. M.Agr.Sc. Thesis, The University of Queensland.

Peterson, R. A. (1978). Susceptibility of Fuerte avocado fruit at various stages of growth to infection by anthracnose and stem-end rot fungi. *Australian Journal of Experimental Agriculture and Animal Husbandry* **18**, 158–60.

Prusky, D., and Keen, N. T. (1993). Involvement of preformed antifungal compounds in the resistance of subtropical fruits to fungal decay. *Plant Disease* **77**, 114–19.

Sneath, P. H. A. (1986). Endospore-forming Gram-positive rods and cocci. In 'Bergey's Manual of Systematic Bacteriology'. Volume 1. (Ed. P. H. A Sneath.) pp. 1104–31. (Williams and Wilkins: Baltimore, U.S.A.)

Spurr, H. W. Jr. (1981). Experiments on foliar disease control using bacterial antagonists. In 'Microbial Ecology of the Phylloplane'. (Ed. J. P. Blakeman.) pp. 369–81. (Academic Press: London.)

Stirling, A. M., Hayward, A. C., and Pegg, K. G. (1992). Evaluation of the biological control potential of bacteria isolated from a soil suppressive to *Phytophthora cinnamomi*. *Australasian Plant Pathology* **21**, 133–42.

Thomashow, L. S., Weller, D. M., Bonsall, R. F., and Pierson, L. S. (1990). Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. *Applied and Environmental Microbiology* **56**, 908–12.

Whipps, J. M. (1987). Effect of media on growth and interactions between a range of soil-borne glasshouse pathogens and antagonistic fungi. *New Phytologist* **107**, 127–42.

Wisniewski, M. E., and Wilson, C. L. (1992). Biological control of postharvest diseases of fruits and vegetables: recent advances. *HortScience* **27**, 94–8.

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