AV207
Biological control of anthracnose of avocado

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Queensland Department of Primary Industries

AV207

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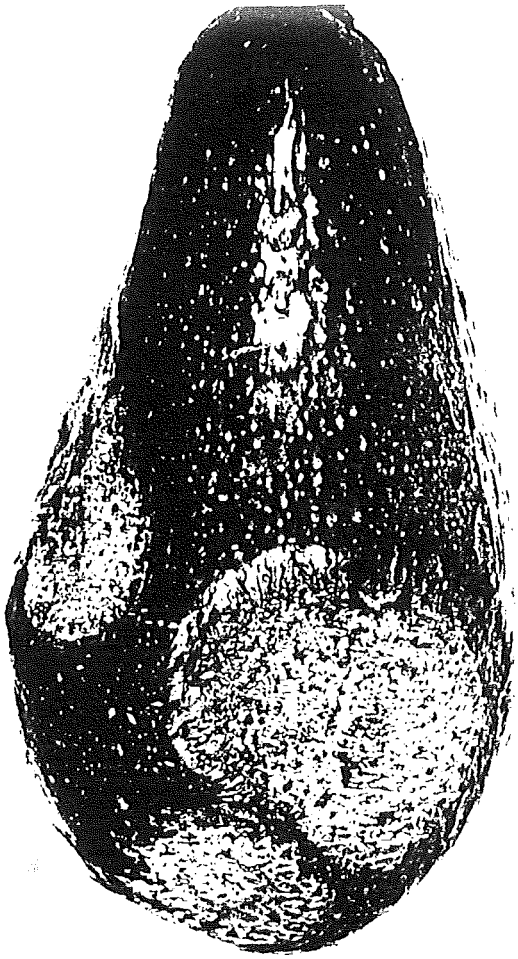
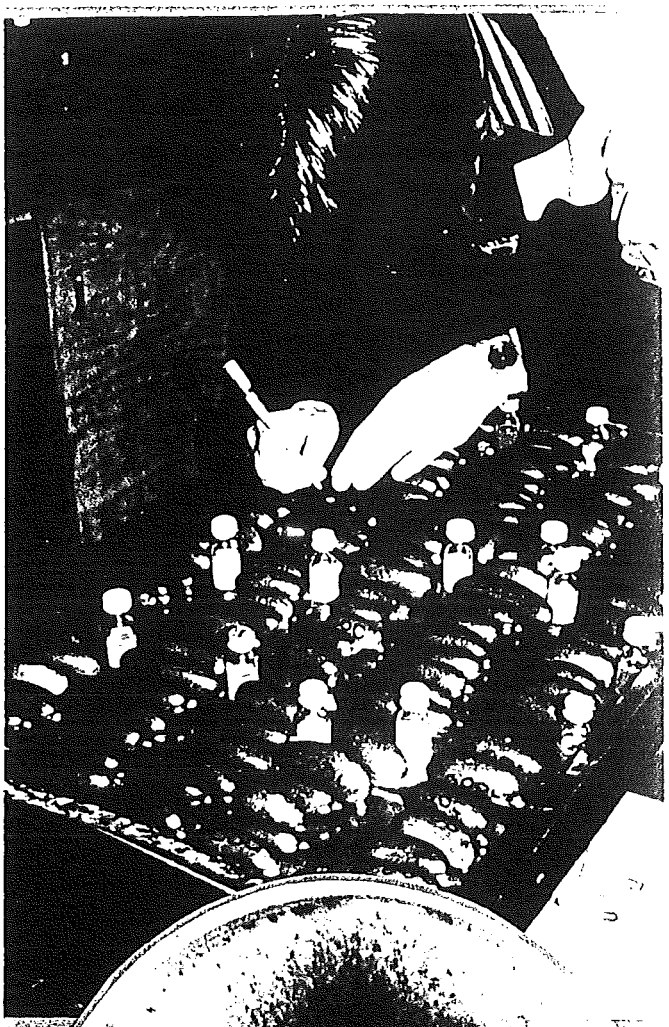
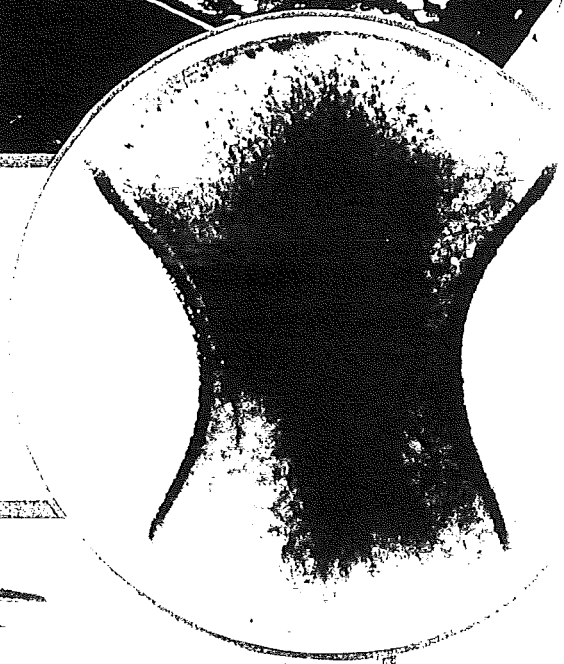




BIOLOGICAL CONTROL OF ANTHRACNOSE IN AVOCADO

FINAL REPORT



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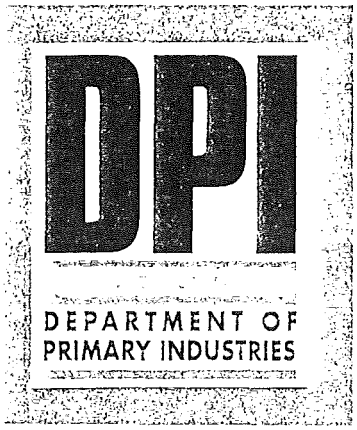

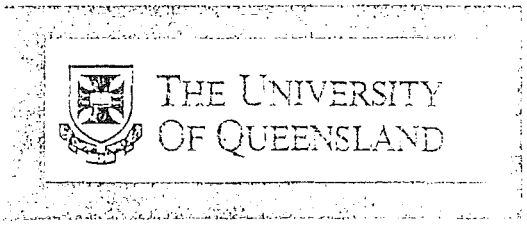

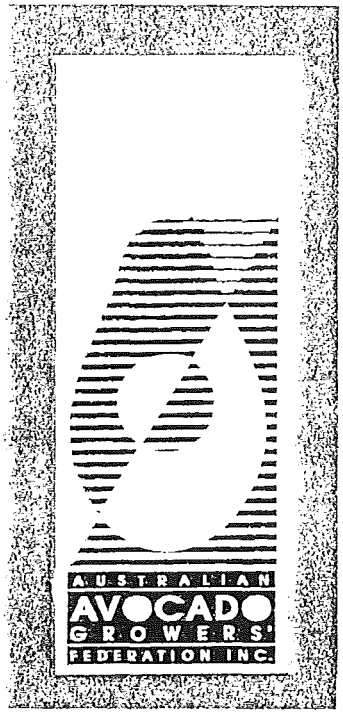

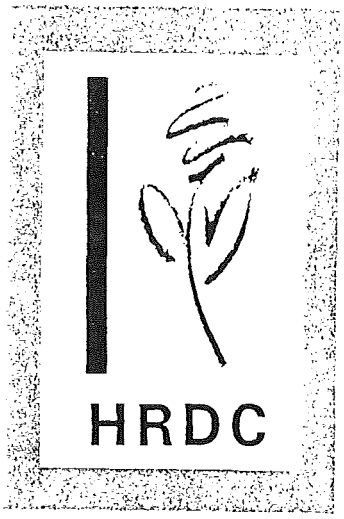

Final Report

Project Number: AV207

Biological control of anthracnose in avocado

July 1992 - June 1995

Project Personnel

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Acknowledgments

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Kerry Everett and Glen Hopkirk (HortResearch, New Zealand)
Kerri Licastro (Crop Care Australia)

1. SUMMARY

Industry Summary

Anthracnose is a major postharvest disease of avocado in Australia and New Zealand. Current control of this disease relies heavily on the use of fungicides, both before and after harvest. Worldwide consumer concern over the use of pesticides in agriculture has prompted the search for non-chemical alternatives to fungicides for disease control in many horticultural crops. Biological control, using naturally-occurring microorganisms isolated from fruit and leaf surfaces, has been shown overseas to have considerable potential for the control of a range of preharvest and postharvest diseases of tropical fruit including avocado. The aim of this project was to evaluate biological control for anthracnose of avocado. Three different approaches were taken to achieving biological control:

1. *Isolation, selection, mass production and application of antagonistic microorganisms.* Potential biocontrol agents (bacteria and yeasts) were isolated from the surface of avocado leaves, fruit and flowers. Isolates were screened for ability to reduce anthracnose using a variety of techniques. On the basis of these evaluations, a small group of isolates antagonistic to anthracnose were selected for further study. In a trial to investigate the field performance of selected biocontrol agents, it was found that one yeast isolate (Q6) gave good control of anthracnose and was equally effective as the standard chemical treatment (copper hydroxide) when applied as a monthly field spray. One bacterial isolate (359) also showed considerable potential. A follow-up field trial was conducted the next season at the same field site although disease levels in untreated fruit were several times lower than in the first trial. None of the treatments, including copper sprays, reduced anthracnose in this trial. More extensive field testing of promising antagonists is currently underway as part of a new project which aims to develop selected biocontrol agents further.

2. *Reducing pesticide use in order to enhance populations of antagonists living on avocado leaf and fruit surfaces.* The effect of copper sprays on microbial populations on the surface of avocado leaves was studied over two seasons on adjacent sprayed and unsprayed orchards. In both seasons it was found that copper reduced numbers of bacteria, yeasts and filamentous fungi, with the effect on bacteria being the greatest. These studies also showed that anthracnose levels were lower in the unsprayed orchard than in the orchard sprayed regularly with copper. The implication here is that in the sprayed orchard, copper is suppressing the naturally-occurring microbes which are antagonistic to anthracnose.

3. *Enhancing populations of antagonists living on avocado leaf and fruit surfaces through the use of nutrient sprays.* The effect of nutrient sprays on microbial populations on avocado leaf surfaces was investigated. Molasses, urea and yeast extract were sprayed onto avocado trees and populations of microbes on leaves were determined. Urea sprays had little effect, but molasses significantly increased populations of bacteria, yeasts and filamentous fungi. The effect of molasses lasted at least one month. A second trial was conducted (on Hass avocado trees which had not been sprayed with copper for several years) to investigate the effect of regular molasses sprays on anthracnose levels as well as leaf microbe populations. No difference was found between anthracnose levels in trees sprayed with molasses and those sprayed with water only. Future studies should focus on the application of molasses to trees previously sprayed with copper.

Technical Summary

The aim of this project was to evaluate biological control for anthracnose of avocado using naturally-occurring phylloplane microorganisms. Three different approaches were taken to achieving this objective:

1. *Isolation, selection, mass production and application of antagonistic microorganisms.* Potential biocontrol agents (bacteria and yeasts) were isolated from the surface of avocado leaves, fruit and flowers. Isolates were screened for inhibition of *Colletotrichum gloeosporioides* and *C. acutatum* on artificial growth media, avocado leaf disks, and detached avocado fruit. On the basis of these evaluations, a small group of isolates antagonistic to *C. gloeosporioides* and *C. acutatum* were selected for further study. In a trial to investigate the field performance of biocontrol agents, two bacteria (isolates 359 and 480) and two yeasts (isolates Q6 and Q34) were applied to Hass avocado trees as monthly sprays. Fruit were harvested at maturity and assessed for anthracnose development when ripe. Isolate Q6 gave good control of anthracnose and was equally effective as the standard chemical treatment (copper hydroxide). Isolate 359 also showed considerable potential. A follow-up field trial was conducted the next season at the same field site although disease levels in untreated fruit were several times lower than in the first trial. None of the treatments, including copper sprays, reduced anthracnose in this trial. Selected isolates were also evaluated for postharvest control of anthracnose (ie applied as a postharvest dip), although none of the isolates tested significantly reduced anthracnose. The mode of action of antagonists which showed potential for preharvest control of anthracnose was studied in some detail. Most isolates of *Bacillus* belonging to the "*subtilis*" group produced antifungal substances in culture, whereas *Bacillus* 359 did not produce any such compounds under the conditions of the experiments conducted in this study.

2. *Reducing pesticide use in order to enhance populations of resident phylloplane microorganisms.* The effect of copper sprays on populations of phylloplane microorganisms was studied over two seasons on adjacent sprayed and unsprayed avocado orchards. In both seasons it was found that copper reduced numbers of bacteria, yeasts and filamentous fungi , with the effect on bacteria being the greatest. These studies also showed that anthracnose levels were lower in the unsprayed orchard than in the orchard sprayed regularly with copper. The implication here is that in the sprayed orchard, copper is suppressing the naturally-occurring microbes which are antagonistic to anthracnose.

3. *Stimulating populations of resident phylloplane microorganisms through the use of nutrient sprays.* The effect of nutrient sprays on populations of phylloplane microorganisms on avocado was investigated. Molasses, urea and yeast extract were sprayed onto avocado trees (which had been sprayed with copper for several years) and populations of microorganisms on leaves were determined. Urea sprays had little effect, but molasses significantly increased populations of bacteria, yeasts and filamentous fungi. The effect of molasses lasted at least one month. A second field trial was conducted to investigate the effect of regular molasses sprays on anthracnose levels as well as leaf microbe populations. No difference was found between anthracnose levels in trees sprayed with molasses and those sprayed with water only. Future studies should focus on the application of molasses to trees previously sprayed with copper.

Publication Schedule

Scientific publications

Coates, L.M., Irwin, J.A.G. and Muirhead, I.F. 1993a. 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. 1993b. Initial infection processes by *Colletotrichum gloeosporioides* on avocado fruit. *Mycological Research*: 97, 1363-70.

Stirling, A.M. 1996. The role of epiphytic microorganisms in the suppression of *Colletotrichum gloeosporioides* on avocado. Ph. D. thesis. University of Queensland.

Stirling, A.M., Coates, L.M., Pegg, K.G. and Hayward, A.C. 1995. Isolation and selection of bacteria and yeasts antagonistic to preharvest infection of avocado by *Colletotrichum gloeosporioides*. *Australian Journal of Agricultural Research*: 46, 985-95.

Extension publications and conference proceedings

Coates, L.M. 1994. Biocontrol control researchers get together in New Zealand. *Talking Avocados*. p26-27 May 1994.

Coates, L.M., Stirling, A.M., Cooke, A.W. and Cannon, K.T. 1995. Biological control of avocado anthracnose. *Proceedings of the Australian Avocado Growers' Federation Conference*, Fremantle, April 30 - May 3, 1995.

Coates, L.M., Stirling, A.M., Cooke, A.W. and Cannon, K.T. 1994. Biological control of avocado anthracnose using phylloplane microorganisms. *Biological Control of Fruit Diseases Workshop*, Brisbane, September 13-15, 1994.

Coates, L.M., Stirling, A.M., Cooke, A.W. and Cannon, K.T. 1993. Progress report on the anthracnose biocontrol project. *Talking Avocados* p26-27 November 1993 (also published in the New Zealand Avocado Grower's Association Newsletter).

Coates, L.M., Stirling, A.M. and Cooke, A.W. 1992. The biology and control of avocado anthracnose. *Proceedings of the Australian Avocado Growers' Federation Conference*, Gold Coast, September 28 - October 2, 1992.

Cooke, A.W. and Coates, L.M. 1994. Anthracnose - Field Control is Essential. *Talking Avocados* p26-27 August 1994.

Stirling, A.M. 1995. Selection of bacteria and yeasts antagonistic to avocado anthracnose. *Australasian Plant Pathology Society Workshop on Biological Control*, Christchurch, August 25-26, 1995.

Stirling, A.M., Coates, L.M., Cooke, A.W., Pegg, K.G. and Hayward, A.C. 1993. Biological suppression of avocado anthracnose. *Australasian Plant Pathology Society Ninth Biennial Conference*, Hobart, July 4-8, 1993. Abstracts of papers, p 34.

Stirling, A.M., Coates, L.M. and Cooke, A.W. 1992. Biological suppression - an alternative approach for controlling avocado anthracnose. *Proceedings of the Australian Avocado Growers' Federation Conference*, Gold Coast, September 28 - October 2, 1992.

Stirling, A.M., Pegg, K.G. and Hayward, A.C. 1995. The effect of preharvest pesticide sprays on microbial suppression of avocado anthracnose. *Australasian Plant Pathology Society Tenth Biennial Conference*. Christchurch, August 28-30. Abstracts of papers.

Stirling, A.M., Pegg, K.G. and Hayward, A.C. 1994. Are copper fungicide sprays detrimental to friendly microorganisms on the avocado phylloplane? *Biological Control of Fruit Diseases Workshop*, Brisbane, September 13-15, 1994.

Manuscripts to be submitted for publication in scientific journals

Coates, L.M., Cooke, A.W. and Cannon, K.T. Suppression of avocado anthracnose using preharvest sprays of antagonistic bacteria and yeasts. To be submitted to *Australasian Plant Pathology*.

Stirling, A.M., Pegg, K.G. and Hayward, A.C. Characteristics of antagonistic *Bacillus* spp. isolated from the avocado phylloplane. To be submitted to *Microbial Ecology*

Stirling, A.M., Pegg, K.G. and Hayward, A.C. The effect of pesticides on *C. gloeosporioides* and non-target microorganisms on the avocado phylloplane. To be submitted to *Canadian Journal of Microbiology.*

Stirling, A.M., Pegg, K.G. and Hayward, A.C. The effect of nutrients on microorganisms on the avocado phylloplane. To be submitted to *Microbial Ecology.*

Seminars (excluding conference and workshop presentations already listed)

"The Biological Control of Avocado Anthracnose" presented by L.M. Coates at the "Focus on Fruit" seminar series at DPI Maroochy Research Station on November 11, 1993

"Progress in research on the biocontrol of avocado anthracnose" presented by L.M. Coates at HortResearch, Te Puke Research Station, New Zealand on February 17, 1994.

"The Development of Biological Control for Avocado Diseases in South Africa" presented by L. Korsten and "Progress Towards Developing Biological Control for Avocado Anthracnose in Australia" presented by L.M. Coates and A.M. Stirling at DPI Maroochy Horticultural Research Station on Sept. 1, 1994.

"Biological Suppression of Anthracnose of Avocado" presented by A.M. Stirling at the University of Queensland, Microbiology Department , 1994.

"Three Possible Approaches to Achieving Biological Control of Anthracnose of Avocado" presented by A.M. Stirling at CSIRO, St. Lucia on July 18, 1995.

2. RECOMMENDATIONS

Extension/adoption by industry

Before industry adoption of biological control can take place, further development and commercialisation must take place. Bacterial and yeast isolates with considerable potential as biocontrol agents for anthracnose have been identified in this project. The next step is to focus on these promising isolates and develop formulations containing these organisms which can be used commercially. In July 1995, we commenced a follow-on project to AV207 (AV504) which aimed to develop these promising organisms further, however funding for this project has been discontinued on the basis of a feasibilty study commissioned by HRDC which concluded that there was little likelihood of commercialisation of these biocontrol agents in the foreseeable future. Given the decision of the industry to discontinue funding as of June 1996, we are completing trials currently in progress and will initiate no new studies. Promising biocontrol agents selected in project AV207 will be retained at QDPI however, and will be made available for future studies should the need arise.

Ecological studies conducted in this project have enhanced our understanding of the importance of microorganisms which naturally occur in avocado orchards. The detrimental effects of copper fungicides on the natural microflora were demonstrated, as were the *potential* benefits of enhancing the microflora through reduced fungicide use and application of nutrients to avocado trees. Although these findings do not suggest that copper sprays should not be used, they do reinforce our thinking that copper sprays need to be applied correctly (ie achieving complete coverage) in order to be effective. This aspect of the project has been publicised widely in industry magazines and at industry conferences.

Directions for future research

In order to achieve biological control of anthracnose, considerable more research and development will be required. If biological control is to be achieved through the application of antagonistic microorganisms, studies will have to be conducted to optimise the mass production, formulation and application of any such organisms. More detailed studies on the mode of action of promising antagonists will also need to be conducted to satisfy requirements for product registration. In terms of copper fungicide studies, there is a need to confirm that suppression of the natural microflora can be correlated with an increase in disease in a wider range of growing regions. The use of nutrients or inoculants to aid in the re-establishment of microflora in orchards which have been sprayed with copper for many years also needs to be further investigated. The integration of nutrient sprays with antagonist sprays may greatly improve the cost efficiency of biocontrol spray programs (through a reduction in the number of antagonist sprays required), and is an area worthy of further investigation.

Financial/commercial benefits

Anthracnose is a serious threat to the avocado industry, causing major losses of fruit during the postharvest handling chain. The outcomes of this project have improved our

understanding of the anthracnose fungus and how it interacts with the natural microflora in avocado orchards. This knowledge will allow us to refine current disease control practices so that the efficacy of such treatments is maximised. This project has also identified antagonistic microorganisms which have the potential to be used in biocontrol programs for anthracnose in the future, providing that work is undertaken to develop a commercial product incorporating these organisms. In this regard, the outcomes of the project have considerable potential financial benefits.

3. TECHNICAL REPORT

Introduction

Anthracnose results in major postharvest losses of avocado fruit in Australia and New Zealand. It also causes considerable preharvest losses of avocado fruit when symptoms of the disease develop prematurely on unripe fruit in the orchard. Although it is very difficult to quantify losses due to anthracnose because of a general lack of published data, a survey conducted by Ledger *et al.* (1993) showed that approximately 23% of Hass and 14% of Fuerte avocados sampled in Sydney retail outlets showed symptoms of the disease. Losses at the consumer level would presumably be higher because of the additional storage time involved.

In Australia, the fungus *Colletotrichum gloeosporioides* is the major cause of anthracnose in avocado, although *C. acutatum* does cause the disease in a minority of cases (Simmonds, 1965). In New Zealand, *C. acutatum* is considered to be of equal importance to *C. gloeosporioides* (Hartill, 1992).

Control of anthracnose in avocado is currently reliant on the use of fungicides, both before and after harvest. In the preharvest situation, trees are sprayed with copper-based protectant fungicides every four weeks from fruit set to harvest. During wet weather, it is recommended that trees are sprayed every fortnight to maintain the protective coating of copper on the fruit surface. It is not uncommon, therefore, for trees to receive in the order of 12 sprays during a wet year. The maintenance of good orchard hygiene and tree ventilation is also an important aspect of field control of anthracnose. In the postharvest situation, prochloraz is applied as a non-recirculated overhead spray on the packing line. Attention to correct storage and ripening temperatures is also a critical component of the overall strategy for anthracnose control.

Worldwide concerns over the use of pesticides in agriculture are rapidly increasing. There is widespread fear that these compounds are contaminating the environment and the food we eat. This is of particular concern in the intensive culture of horticultural crops where fungicides and other pesticides are used in large quantities. These concerns are gradually being translated into regulatory changes regarding the use of pesticides. Many European countries, such as Sweden and The Netherlands, have developed programs with the objective of reducing overall pesticide use within certain time periods, eg. 50% by the year 2000 (Gullino and Kuijpers, 1994). Aside from direct legislative changes, there is a growing consumer demand for chemical-free produce. Clearly there is an urgent need to develop non-chemical disease control measures to replace the fungicides which are currently in use.

A basic understanding of the infection process of the anthracnose pathogen is necessary when considering the relative merits of various disease control strategies. The initial stages of infection by *Colletotrichum* occur in the orchard. Fruit are susceptible to infection from fruit set to harvest (Peterson, 1978) . Spores of the fungus build up in numbers on dead leaves and twigs entangled in the tree canopy, and are spread to the surface of fruit principally by rain (Fitzell, 1987). In the presence of moisture, spores on the fruit surface germinate to produce an infection structure called an appressorium, which becomes firmly attached to the fruit surface. This appressorium then germinates to produce an infection peg which penetrates the

outer wax layer of the fruit and becomes lodged in the fruit cuticle (Coates *et al.,* 1993b). At this point, further development of the infection peg is arrested because of the presence of compounds in the skin which inhibit the development of the fungus (Prusky *et al.,* 1982). During fruit ripening, the concentration of these inhibitory compounds decline, allowing infection to proceed and symptoms to develop.

Infection of avocado fruit by *Colletotrichum* does not always follow this sequence of events, however. In some cases, infection proceeds without a "dormant" phase, leading to the development of disease lesions in unripe fruit which are still hanging on the tree (Fitzell, 1987). The development of these "preharvest" lesions is more prevalent in the cultivar Fuerte than in Hass.

Research conducted in South Africa since 1987 has clearly demonstrated the potential of biological control for tropical fruit diseases. Strains of various *Bacillus* spp., which were originally isolated from fruit and leaf surfaces, were shown to effectively control anthracnose of mango and avocado, stem end rot of mango and avocado, soft brown rot of mango and various postharvest diseases of lychee (Korsten *et al.* 1993). Control was achieved using preharvest *Bacillus* sprays and/or postharvest *Bacillus* dip or spray treatments. In some cases, preharvest spray applications of *Bacillus* also gave control of various preharvest diseases of avocado (such as cercospora spot and sooty blotch) (Korsten *et al.,* 1992; Korsten *et al.,* 1994). Considerable effort has been directed towards the development of a commercial product incorporating these biocontrol agents, and it is expected that a product will be available on the market in South Africa sometime during 1996 (Korsten, personal communication).

Postharvest control of mango anthracnose using a phylloplane isolate of *Pseudomonas fluorescens* was demonstrated by Koomen and Jeffries (1993), although no further studies have been reported to date.

The overall aim of this project was to evaluate biological control for avocado anthracnose. Three different approaches were taken to achieving biological control of this disease:

1. The isolation, selection, mass production and release of antagonistic microorganisms,

2. The enhancement of naturally-occurring beneficial microorganisms by decreasing the use of broad spectrum pesticides,

3. The enhancement of naturally-occurring beneficial microorganisms by using nutrient sprays.

Materials and Methods

Isolation, selection, mass production and application of antagonistic microorganisms

Isolation and maintenance of bacteria and yeasts

Bacteria, yeasts and filamentous fungi 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), ½ strength tryptic soy agar (½ 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 ½ 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 sulphate (120 μg mL^{-1}; Sigma) were added to media for inhibition of fungi and bacteria respectively. Filamentous fungi were isolated on ½ strength potato dextrose agar (PDA) amended with streptomycin sulphate (120 μg mL^{-1}). 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. Filamentous fungi isolated from avocado leaves and fruit were identified to genus level but were not retained for evaluation as biocontrol agents. For all subsequent experiments, bacteria were grown on ½ TSA at 28°C for 36 h, while yeasts were grown on YMA for 3-4 days at 25°C.

In vitro screening of bacteria and yeasts

C. gloeosporioides: *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 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 < 5 mm; ++, zone 6-12 mm; +++, zone > 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, 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 x 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.

C. acutatum: Selected antagonists (i.e. those showing the most promise from the *C. gloeosporioides* plate assays) were screened against a New Zealand isolate of *C. acutatum* (917-11) using the methods described above.

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™ (Biolog, Hayward, CA).

In vivo screening of potential antagonists on avocado leaf disks and detached fruit

Detached fruit tests: The ability of 76 bacteria and 48 yeasts to suppress preharvest infection by *C. gloeosporioides* and *C. acutatum* 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 onto 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 (Plate 1). 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* or *C. acutatum* spore suspension (1 x 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 (Plate 2). 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. 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.

Leaf disk tests: 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 of *C. gloeosporioides* 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 sulphate 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 400x. Each antagonist was tested three times and the mean percentage germination was calculated, and means were ranked.

Comparative detached fruit test: 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.

Statistical analysis: Completely randomized designs were used in all experiments. Data were analysed using Statistix 3.1 (Analytical Software, St Paul, MN). 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).

Evaluation of South African antagonists in a detached fruit test: A joint trial was conducted with Dr Lise Korsten (University of Pretoria, South Africa) to compare the performance of two *Bacillus* sp. antagonists from South Africa (isolates L1 and L2) with a selection of antagonists from our research program (isolates 359, 677, Q34 and Q47). Antagonist suspensions were applied to cocktail avocado fruit cv. Fuerte using methods previously described. Fruit were air-dried and then inoculated with either a South African isolate of *C. gloeosporioides* (2.3) or an Australian isolate (23691). Fruit were incubated at 25^0C until ripe and assessed for lesion development within inoculated areas. Lesion diameter data was log transformed prior to analysis of variance.

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 tryptic soy broth (TSB) for 36 h at 26°C. *Aureobasidium* sp. 274C1, and yeasts 772C1, 734C2 were selected for carbendazim (Bavistin®) 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.

Completely randomized designs were used in all experiments. Data were analysed using Statistix 3.1. Log_{10} transformed data for numbers of microorganisms g^{-1} leaf in the colonization experiments were normally distributed according to the Wilk-Shapiro test and therefore analysed using analysis of variance. Means were compared using the LSD test.

Field evaluation of selected antagonists

Field evaluation of selected antagonists was carried out over two consecutive seasons on a young unsprayed "Hass" avocado orchard located at Mt. Tamborine, South-East Queensland. Because of the large volumes of culture and the large number of trees required for field application, we were limited in the number of antagonists which we could test.

For the first field trial (93/94 season), two bacteria (*Bacillus* 359 and 480) and two yeasts (Q6 and Q34) were selected for field evaluation largely on the basis of performance in the detached fruit tests.

Bacteria and yeasts were grown in yeast-glucose-peptone broth for 36 and 72 hours respectively. Cell concentrations were boosted by adding scrapings of plate inoculum to liquid cultures (Plate 3). To determine viable cell concentrations, one mL samples were taken from each culture, serially diluted in PBS and 0.1 mL aliquots plated on yeast-glucose-peptone agar. Cultures were then diluted in autoclaved tap water (100 mL culture in 900 mL autoclaved tap water containing 0.05% v/v Tween 80). Controls consisting of (i) autoclaved tap water (+ Tween 80) only and (ii) diluted yeast-glucose-peptone broth (+ Tween 80) only, were included in the trial. The currently recommended chemical field treatment for anthracnose, copper hydroxide (Kocide ^{R} 2g L^{-1}), was also included as a treatment in the trial.

A randomised block design (7 treatments x 4 single tree replications) was used in this trial. Treatments were applied to trees at four-weekly intervals commencing in October 1993 (soon

after fruit set) and finishing in August 1994 (commercial harvesting time). The survival of antagonists was monitored for a two month period between the fifth (February 1994) and seventh (April 1994) sprays. Leaf samples were taken from each tree before and after spraying (excluding the "copper" treatment) and processed using methods described previously (see *Isolation and maintenance of bacteria and yeasts*). Serial dilutions of samples were then plated onto 1/3 strength TSA amended with cycloheximide for bacteria and acidified-YMA for yeasts. Following incubation for 2-4 days at 25 ^{0}C, plates were examined and colonies counted. Identification of antagonists on culture plates was made on the basis of gross cultural characteristics only. While it was recognised that this method may lead to the incorrect identification of antagonists in a small number of cases, the method was considered adequate for the purpose of this study, i.e. to confirm that the antagonists were surviving. Previous studies using mutant strains of the antagonists quantified the degree of colonization by the antagonists more precisely. Since the biocontrol potential of the mutant strains was not known, it was considered unwise to use them in the large scale field trials.

Two weeks after the final field spray, all fruit from experimental trees were harvested and transported to the laboratory where they were assessed for insect and mechanical injury, field disease symptoms and fruit size (diameter). Peel samples were taken from 3-5 fruit per tree (excluding trees receiving Q6 and 480 treatments), surface sterilised in 70% ethanol, rinsed in sterile distilled water and plated onto streptomycin-amended PDA. Plates were incubated at 25^{0}C under near UV light for several days. The number of *C. gloeosporioides* colonies which grew on plates was recorded. Remaining fruit (from all treatments) were then stored at 24^{0}C and assessed daily for firmness. When fruit were judged to be ripe, they were cut into quarters, peeled and examined for symptoms of anthracnose and stem end rot (Plate 4). The estimated fruit surface area affected by each of the diseases was recorded.

Where appropriate, data was analysed by analysis of variance and treatment means were compared using the LSD test.

For the second trial, which was conducted during 1994/95 at the same field site, two bacteria (*Bacillus* 359 and an actinomycete Q47) and one yeast isolate (Q6) were tested. Cultures were grown and spray suspensions prepared as descibed for the previous field trial, except Q47 cultures were blended before dilution due to the fungal-like growth of the isolate in liquid culture. As before, controls consisting of water or diluted culture media sprays were included. Two chemical treatments, consisting of copper hydroxide (Kocide^R, 2g L^{-1}) or phosphorous acid (Fos-Ject 200^R, 5mL L^{-1}) were also applied. Treatments were applied as described previously, except five single tree replications were used instead of four.

Effect of antagonist field sprays on the development of sooty blotch in avocado cv. Fuerte

A small scale field trial was conducted to investigate the effect of antagonist sprays on sooty blotch development in Fuerte avocado. Branchlets on a single Fuerte tree were sprayed at monthly intervals with either water, diluted culture media (yeast-glucose-peptone broth diluted 1 in 10 with autoclaved tap water), 359 in diluted culture media, Q6 in diluted culture media, or copper hydroxide (Kocide^R at 2g L^{-1}). In total five sprays were applied between March and June 1995. Fruit were harvested in July, assessed for sooty blotch and ripened at 24^{0}C for assessment of anthracnose and stem end rot.

Postharvest evaluation of selected antagonists

On the basis of results from the screening assays, promising bacteria and yeasts were selected for evaluation in postharvest dipping trials. Bacteria were grown for 36 hours in ½ strength tryptic soy broth, and yeasts for 72 hours in yeast-glucose-peptone broth. Cultures were diluted with autoclaved tap water (containing 0.05% v/v Tween 80) as described before. To determine antagonist concentration, one mL samples were taken from each suspension and serially diluted in PBS before plating onto yeast-glucose-peptone agar.

Freshly harvested Fuerte avocado fruit were obtained from various orchards in South-East Queensland. Fruit were washed to remove traces of copper residue, and then dipped in 4 L suspensions of the antagonists for one minute (3 replicate trays of fruit/treatment). A 30 second prochloraz (Sportak^{R} 0.55mL L^{-1} 45% EC) dip was included as a treatment in each trial. Fruit were air-dried following treatment, ripened at 24^{0}C and then assessed for anthracnose and stem end rot.

Joint trials were also conducted with Dr Lise Korsten (University of Pretoria, South Africa) to evaluate two South African bacterial antagonists (*Bacillus* spp.) for anthracnose and stem end rot control in Hass avocados harvested from South-East Queensland orchards. Freshly harvested avocado fruit (3 replicate trays of fruit /treatment) were dipped in 30 L suspensions of the two antagonists (L1 and L2) for 5 minutes, air-dried, ripened at 24^{0}C and assessed for disease. An antagonist from our research program, isolate Q47, was also included as a treatment in the trial for comparison purposes. The trial was repeated, adding an extra fruit storage treatment of 7^{0}C for 7 days prior to ripening at 24^{0}C.

Mode of action of selected antagonists

Studies were conducted to investigate the mode of action of promising antagonists. The following bacterial antagonists were selected for these studies: *Enterobacter agglomerans* 632, *Pseudomonas fluorescens* 677 and 20 *Bacillus* isolates. Pink yeast 734, white yeast 772 and *Aureobasidium* 274 were also included in the studies (except for the volatiles test). The production of antifungal substances by these antagonists was investigated in a series of experiments.

To test for the production of volatile antifungal substances, bacterial antagonists were spread over the surface of two compartments of a 3-sectioned plastic petri dish containing a range of media types (Stirling, 1996). A mycelial plug of *C. gloeosporioides* was inoculated onto the agar (PDA) surface in the third compartment after the plates had been incubated at 26^{0}C for 24 hours. Plates were then sealed with parafilm to prevent escape of volatiles, incubated at 25^{0}C and examined for fungal inhibition.

To test for the production of diffusable antifungal substances, cell free filtrates of cultures grown in a range of media types (Stirling, 1996) were prepared by centrifugation and filtration. Inhibition of fungal growth of *C. gloeosporioides* was tested by placing 100µL aliquots of each filtrate into wells made in PDA plates and then inoculating the agar with a mycelial plug 10mm away. Colony diameter was measured daily during incubation at 25^{0}C. Germination of *C. gloeosporioides* spores in the cell free filtrates was measured on avocado leaf disks using methods previously described. In addition, cell free filtrates of selected

antagonists mixed with spores of *C. gloeosporioides* were inoculated onto detached cocktail avocado fruit cv. Fuerte. Fruit were incubated and assessed for lesion development when ripe.

Thin layer chromatography (TLC) was used to detect antibiotic production by selected antagonists. TLC was performed on crude extracts of cultures using a modification of the method described by M^{c}Keen *et al.* (1986). The pH of culture supernatants was lowered to 2.5-3.0 using concentrated HCl, any resulting precipitate pelleted by centrifugation and the active fraction extracted from the solid with a minimum quantity (< 25mL) of 80% ethanol. Aluminium backed silica gel plates were loaded with 40 µL aliquots using butanol:acetic acid:water (3:1:1) as the solvent system. Negative and positive controls consisting of ethanol (80%) and cycloheximide (75µg/mL) respectively were run. Solvent traces were removed by air-drying and the entire plate was overlayed with molten PDA containing *C. gloeosporioides* spores and 2,3,5-triphenyl tetrazolium chloride (0.01 % w/v). Plates were examined after incubation for 72 h at 25^{0}C for areas of inhibition of fungal growth which indicated antibiotic production.

Pathogen variability studies

Over 140 isolates of *Colletotrichum* were collected from a number of avocado growing regions in Queensland. Colony characteristics on PDA were described and spore dimensions recorded for each isolate (see Plates 5 and 6 for appearance of spores). Selected isolates were grown in clarified Ca^{2+} V8 juice broth for 3-5 days at 25^{0}C. Fungal mycelium was harvested by vacuum filtration and snap-frozen in liquid nitrogen before being stored at -70^{0}C until needed. DNA was extracted from frozen mycelium using a modification of the method described by Yoon *et* al.,1991 (modifications are described in Hayden *et al.*, 1994). DNA was quantified using UV fluorimetry, and DNA concentrations were adjusted to 25 ng μL^{-1} with TE buffer (10 mM Tris-HCl and 0.1 mM EDTA) in 100 µL samples, before storing at 4^{0}C. RAPD analysis (which is still in progress) was conducted according to the method used by Hayden *et al.,* 1994.

The effect of copper field sprays on populations of resident phylloplane microorganisms

The effect of copper-based fungicides on populations of microorganisms on avocado leaf and fruit surfaces was studied over two seasons on adjacent Hass avocado orchards at Maleny, South-East Queensland. One orchard was sprayed with copper fungicides by the grower at monthly intervals from October to April each year whereas the other had not been sprayed for at least 7 years. Four days after each copper spray was applied to the treated orchard, 48 leaves (8 leaves from each of 6 trees) were sampled from each orchard. Populations of filamentous fungi, yeasts and bacteria on individual leaves were determined by a plate-dilution frequency technique and the most probable number method (Andrews and Kenerley, 1978; Meynell and Meynell, 1970). Samples were also taken in winter when copper sprays were not applied to either orchard. In addition, fruit were harvested soon after spraying in December, January, March and in July (3 months after the last spray) and populations of microorganisms were enumerated as above.

In 1993 and 1994, fruit were harvested from both orchards, ripened and assessed for anthracnose and stem end rot . Each fruit was divided into quarters and rated for disease severity.

Peel samples were taken from unripe fruit in both orchards, surface sterilised in 70% ethanol, rinsed in sterile distilled water and plated onto streptomycin-amended PDA. The incidence of *Colletotrichum* sp. and other fungi recovered from peel samples was recorded. *Colletotrichum* isolates obtained in this way were then screened for pathogenicity on Hass avocado fruit. Fruit were inoculated with a spore suspension of each isolate and incubated at 25^0C until ripe. Lesion development within inoculated areas was recorded.

The effect of nutrient field sprays on populations of resident phylloplane microorganisms

Trials were conducted to investigate the effect of nutrient sprays on populations of microorganisms on avocado leaf surfaces.

Molasses (2.0%), urea (1.0%) and yeast extract (0.5%), either alone or in combination, were sprayed once onto Hass avocado trees. Populations of microorganisms on leaves (total bacteria, yeasts and filamentous fungi) were estimated using a plate-dilution frequency technique and the most probable number method.

To study the effect of molasses on *C. gloeosporioides*, spores of the fungus were inoculated onto avocado leaf disks in the presence of water or molasses (0.02, 0.1, 1.0 and 2.0%). Spore germination, mycelial growth and appressorium formation were measured after incubation at 25^0C for 14 h. Detached avocado fruit cv. Fuerte were also inoculated with *C. gloeosporioides* spores with or without molasses (0.02 and 2.0%) and with or without antagonistic yeasts (isolates 274, 734 and 772). Fruit were incubated at 25^0C and examined for lesion development when ripe.

The effect of monthly molasses (2.0%) sprays on anthracnose development in fruit and on populations of microorganisms on leaves was investigated in a second field trial. Populations of bacteria, yeasts and filamentous fungi on leaves were estimated as described above. Fruit for disease assessment were harvested at commercial maturity, ripened and assessed for anthracnose and stem end rot using a 10 point disease severity scale.

Results

Isolation, selection, mass production and application of antagonistic microorganisms

Isolation of bacteria, yeasts and filamentous fungi

A wide range of filamentous fungi, yeasts and bacteria were isolated from avocado leaf and fruit surfaces. Gram-negative bacteria, such as the fluorescent pseudomonads and yellow-orange pigmented bacteria, were common on avocado leaves. Although *Bacillus* sp. (gram-positive) was consistently isolated from the avocado phylloplane, population densities were much lower than for the gram-negative bacteria. *Aureobasidium* and various types of pink and white yeasts were also common, as were filamentous fungi such as *Alternaria*, *Cladosporium*, *Curvularia*, *Epicoccum*, *Pestalotiopsis*, *Colletotrichum* and *Phomopsis*. There were more fungi and bacteria on mature avocado leaves than on immature leaves, and leaves sampled from the inner canopy of trees had higher numbers of microorganisms than those sampled from the outer canopy

In vitro screening of bacteria and yeasts

Inhibition of C. gloeosporioides: Approximately 37% of the 1050 microorganisms tested against *C. gloeosporioides* 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. Most of the bacteria that strongly inhibited (+++) *C. gloeosporioides* on PDA were *Bacillus* spp.

When spore germination was examined on cellophane overlaying WSA, approximately 15% of bacteria tested reduced spore germination by 80% or more (Table 2). A higher proportion of yeasts (39%) reduced spore germination to the same extent, with two isolates of *Aureobasidium* completely inhibiting spore germination. 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.

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.

Inhibition of C. acutatum: Approximately 58% of isolates tested showed some form of fungal growth inhibition of *C. acutatum* (Table 3). Non-zonal inhibition was the only type of inhibition exhibited by the yeasts, whereas zonal inhibition was predominant in the bacteria. Only 6% of isolates tested against *C. acutatum* reduced spore germination by 80% or more (Table 4).

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

	Levels of inhibition[1]					
	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

[1]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.

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

Table 3. *In vitro* inhibition of mycelial growth of *Colletotrichum acutatum* on potato dextrose agar by phylloplane bacteria and yeasts

	Level of Inhibition (%)					
	0	non-zonal	+	++	+++	Total tested
Number of yeasts	23	19	0	0	0	42
Number of bacteria	22	17	11	14	0	64
Total	45	36	11	14	0	106

[1]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.

Table 4. Number of bacteria and yeasts inhibiting germination of *Colletotrichum acutatum* spores on cellophane overlaying weak sugars agar

	Inhibition (%)					
	0-10	11-79	80-89	90-99	100	Total tested
Number of yeasts	21	19	2	0	0	42
Number of bacteria	12	48	0	3	1	64
Total	33	67	2	3	1	106

Identification of selected 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, 1996). 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 vivo screening of potential antagonists on avocado leaf disks and detached fruit.

Inhibition of spore germination on avocado leaf disks: Although many bacteria and yeasts suppressed spore germination of *C. gloeosporioides* on cellophane, only nine bacterial isolates reduced spore germination by 80% or more on avocado leaf disks (Table 5). Individual data for eight of these bacteria are included in Table 9.

Table 5. Number of bacteria and yeasts inhibiting germination of *Colletotrichum gloeosporioides* spores on avocado leaf disks

	Inhibition (%):					Total
	0-10	11-79	80-89	90-99	100	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

Inhibition of lesion development on detached avocado fruit: Tables 6 and 7 list the antagonists which were most effective in reducing the development of anthracnose lesions in avocado fruit inoculated with *C. gloeosporioides* and *C. acutatum*. Although many isolates inhibited lesion development, very few isolates (eg bacteria Q47, 480, Q30; yeasts Q34, 714, 729, Q6, 700) were effective against both *C. gloeosporioides* and *C. acutatum*. In addition, the degree of inhibition of *C. acutatum* exhibited by isolates in general was greater than for *C. gloeosporioides*. Consistency of response to a given isolate also varied considerably, and was a major factor taken into account when selecting antagonists with potential.

Comparitive detached fruit test: In the final detached fruit test, the four sets of *C. gloeosporioides* controls were not significantly different from each other for lesion development (Table 8), indicating that conditions were similar in all of the ripening boxes. Some isolates failed completely to suppress lesion formation in fruit inoculated with *C. gloeosporioides* (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 *C. gloeosporioides* (Table 8). *Bacillus* sp. 359 was the only isolate to significantly reduce lesion size relative to the controls.

Evaluation of South African antagonists in a detached fruit test

South African antagonists L1 and L2 significantly reduced lesion diameter and lesion incidence in cocktail avocado fruit inoculated with *C. gloeosporioides*, as did the Australian

antagonist 359 (Table 10). All three isolates were equally effective in reducing anthracnose. Australian antagonists Q34, Q47 and 677 were ineffective in this test. There was no significant difference found between inhibition of the Australian isolate of *C. gloeospoioides* and that of a South African isolate, and hence all of the means presented in Table 10 represent the mean of the two isolates.

Table 6. Inhibition of lesion development caused by *C. gloeosporioides* in detached avocado fruit treated with antagonistic bacteria or yeasts

Isolate		Mean Lesion Inhibition (%)[1]	Times Successful/ Times Tested
Actinomycete	Q47	45	1/1
Bacillus spp.	359 + 359 ab	43	1/1
Bacillus sp.	359	41	4/4
Sphingomonas sp.	Q22	29	2/2
Bacillus sp.	359 ab	17	1/1
Enterobacter agglomerans	632	16	1/2
Bacillus sp.	935	16	1/1
Bacillus sp.	717	14	1/2
Sphingomonas sp.	Q19	14	2/2
Bacillus sp.	480	11	3/5
Achromobacter sp.	Q30	10	1/1
Bacterium	Q14	9	2/2
Pink yeast	Q34	24	3/3
White yeast	711	19	3/3
Yeast	878	18	1/1
Yeast	714	16	1/1
Pink yeast	734	15	3/4
Aureobasidium sp.	371	12	1/1
Yeast	729	11	2/2
Yeast	Q1a	11	2/2
Yeast	885	10	1/2
Pink yeast	Q6	10	2/3
White yeast	700	8	2/3
Aureobasidium sp.	731	7	1/3

[1] Relative to controls

Table 7. Inhibition of lesion development caused by *C. acutatum* in detached avocado fruit treated with antagonistic bacteria or yeasts

Isolate		Mean Lesion Inhibition (%)[1]	Times Successful/ Times Tested
Achromobacter sp.	Q30	81	1/1
Bacillus sp.	553	52	2/2
Actinomycete	Q47	45	1/1
Bacterium	509	34	2/2
Bacillus sp.	330	30	2/2
Bacterium	Q24	27	1/1
Bacillus sp.	480	26	5/5
Bacillus sp.	1002	25	1/1
Bacillus sp.	30a	23	2/2
Bacillus sp.	54	23	2/2
Bacillus sp.	75	22	1/1
Bacterium	Q11	22	2/2
Yeast	714	39	1/1
Pink yeast	Q34	36	3/3
Yeast	735	32	1/1
Yeast	Q9	31	2/2
Yeast	Q31	31	1/1
Yeast	263	27	1/1
Yeast	268	27	1/1
White yeast	700	27	3/3
Yeast	740	23	1/1
Yeast	729	21	2/2
Pink yeast	Q6	17	3/3
White yeast.	772	16	3/4

[1] Relative to controls

Table 8. Reduction in *Colletotrichum gloeosporioides* lesions on avocado fruit treated with antagonistic bacteria or yeasts

Treatment	Mean lesion incidence (%)	Mean lesion size (mm)
Pink yeast	47.0 a	2.9
Bacillus sp. 359	47.5 ab	2.4
Bacillus spp. 359 + 359 ab	50.8 abc	3.4
Enterobacter agglomerans 632	55.8 abcd	3.9
White yeast 711	61.7 abcde	5.6
Pseudomonas fluorescens 677	70.0 abcde	6.9
Pink yeast 734	72.0 bcde	4.7
Bacillus sp. 359ab	75.0 bcde	5.7
Aureobasidium sp. 468	80.0 cde	8.1
Control	82.5 cde	6.5
Bacillus sp. 78	83.3 cde	6.9
Bacillus sp. 75	85.0 cde	8.0
Control	85.0 de	10.6
Control	85.0 de	7.4
Bacillus sp. 933	87.5 de	8.9
Aureobasidium sp. 274	87.8 de	6.8
Control	92.5 e	6.3
LSD (P = 0.05)	ND[1]	3.8

[1] Not done

Table 9. 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[1]	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	+	20.6 ± 8.9	43.7 + 4.1	4/4
Bacillus sp. 480	+	65.7 ± 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	++	16.7 ± 4.4	0.7 ± 0.7	3/4
Pink yeast Q6	0	7.8 ± 1.5	68.1 ± 8.1	2/3
Pink yeast Q34	0	81.9 ± 1.9	73.3 ± 6.7	3/3
Pink yeast 734	0	49.10 ± 14.5	35.3 ± 6.7	3/4
White yeast 711	0	30.11 ± 8.1	69.0 ± 7.0	3/3
White yeast 772	0	27.12 ± 11.8	72.7 ± 11.5	3/4
Aureobasidium sp. 140	NZ[2]	0.0	91.2 ± 5.2	2/4
Aureobasidium sp. 274	NZ	0.0	17.8 ± 3.7	3/3
Aureobasidium sp. 468	0	0.0	64.0 ± 9.1	2/3
Control		96.0 ± 2.0	86.6 ± 5.1	0/11[3]

[1] ± Standard error, [2] Non-zonal inhibition, [3] Data is from 11 individual test

Table 10. Comparison of South African bacterial antagonists with selected Australian antagonists for preharvest control of anthracnose in detached Fuerte avocado fruit inoculated with *C. gloeosporioides*.

Antagonist	Bacteria/Yeast (B/Y)	Lesion diameter (mm) log transformed	Lesion incidence (%)	Ripening time (days)
L1	B	0.972 abc[1]	47.5 abc	12.0 a
L2	B	0.777 a	32.3 a	11.2 ab
Q34	Y	1.491 d	68.1 d	11.5 ab
677	B	1.204 bcd	58.0 bcd	11.6 ab
359	B	0.943 ab	41.7 ab	12.0 a
Q47	B	1.325 cd	60.1 cd	11.8 a
control	—	1.471 d	65.6 d	10.8 b

[1] means followed by the same letter **within columns** do not differ significantly at P=0.05 using the LSD test.

Colonization potential of selected antagonists

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 11). 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 11. Colonization of avocado leaves by selected bacteria and yeasts

Antagonist	Antagonist numbers on leaf surface (Mean $\log_{10}$ cfu g^{-1} fresh weight leaf)		
	Day 1	Day 30	Day 60
Bacillus sp. 359R1	6.9	5.7	5.1
Bacillus sp. 480R2	7.3	6.7	4.9
Enterobacter agglomerans 632R2	7.1	3.1	NS[1]
Pseudomonas fluorescens 677R1	7.4	ND[2]	NS
Aureobasidium sp. 274C1	6.8	5.4	4.9
White yeast 772C1	6.7	6.3	6.1
Pink yeast 734C2	7.0	6.6	5.8
Pink yeast Q34	7.3	4.5	NS

[1] NS = Not sampled [2] ND = None detected

Field evaluation of selected antagonists

1993/94 field evaluation: Table 12 shows antagonist concentrations used in monthly field sprays at the Mt. Tamborine field site. Although the concentration of a given antagonist varied from spray to spray, concentrations of the two bacterial isolates were generally higher than that of the two yeast isolates.

Leaf population densities of applied antagonists and resident phylloplane microorganisms are shown in Figs 1-6. All antagonists except isolate 359 declined in numbers during the 30 day period between the fifth and sixth sprays (ie between February and March), although population densities of all isolates could still be considered to be relatively high at the end of this period, with the exception of Q34 (Fig 1). Between the sixth and seventh sprays, isolate 359 declined in numbers in a similar way as the other antagonists (Fig 2).

In terms of natural microflora populations on leaf surfaces, total numbers of bacteria and yeasts were often higher on trees sprayed with diluted media than those sprayed with water only (Figs 3-6). In the case of bacteria, this increase in numbers was not statistically significant. In the case of yeasts, however, the increase in numbers seen in February and March was significant. As expected, total numbers of bacteria and yeasts (which include any

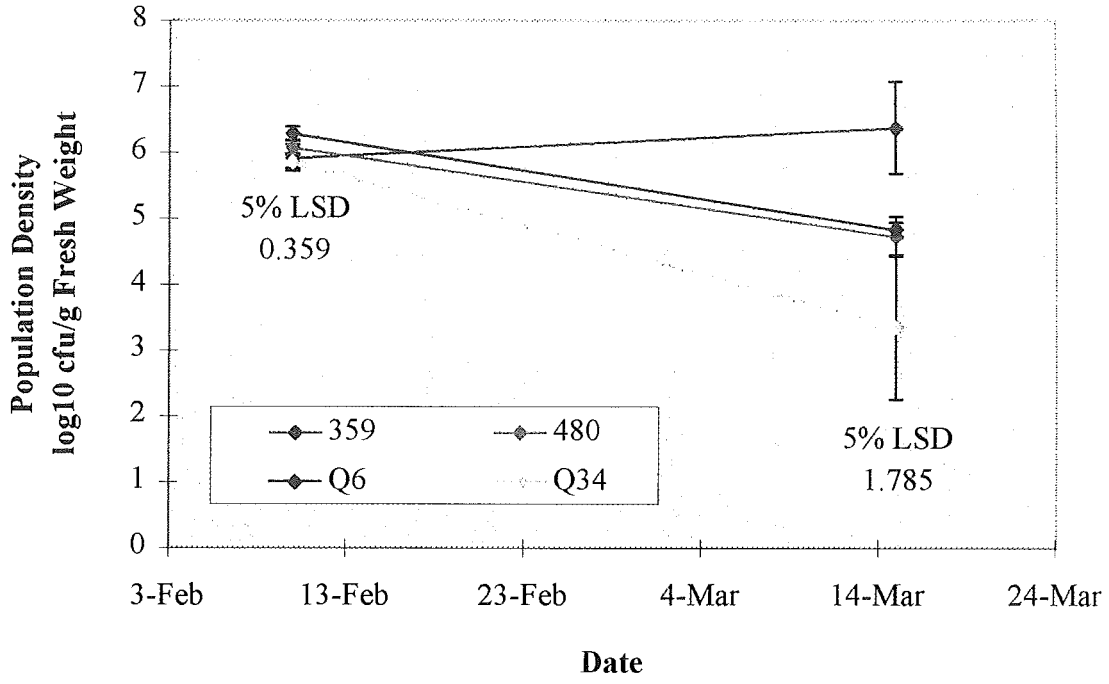

Figure 1. 1993/94 field evaluation - leaf population densities of antagonists applied to Hass avocado trees between the fifth and sixth sprays. Error bars represent the standard error of means.

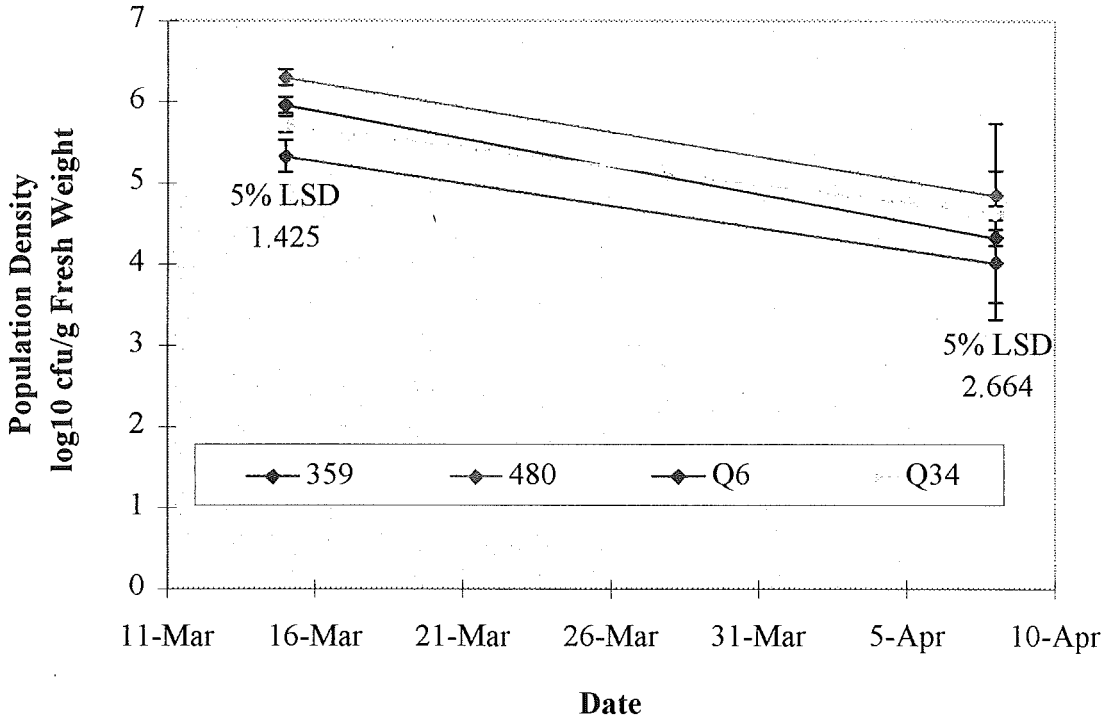

Figure 2. 1993/94 field evaluation - leaf population densities of antagonists applied to Hass avocado trees between the sixth and seventh sprays. Error bars represent the standard error of means.

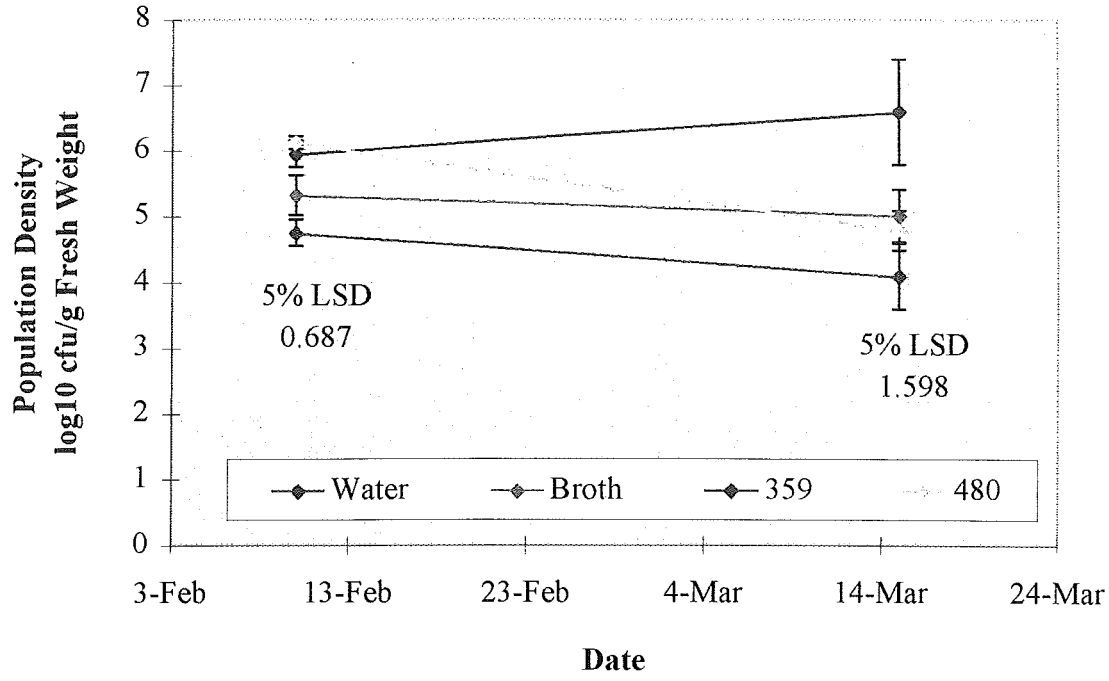

Figure 3. 1993/94 field evaluation - leaf population densities of epiphytic bacteria on Hass avocado trees between the fifth and sixth sprays. Error bars represent the standard error of means.

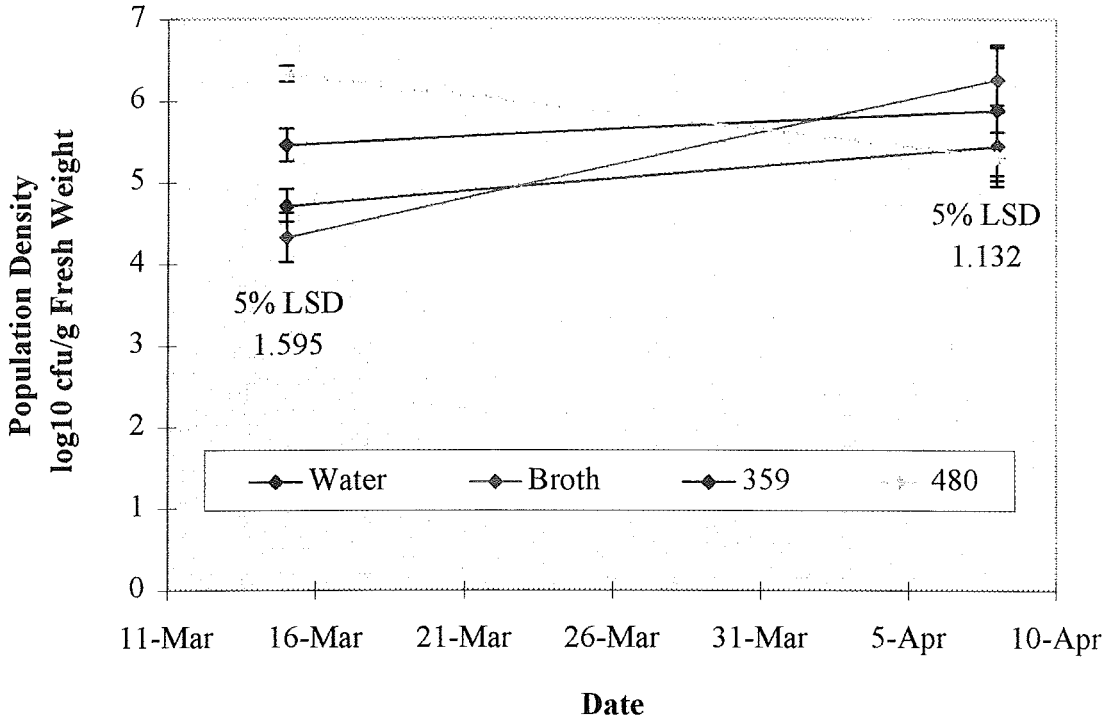

Figure 4. 1993/94 field evaluation - leaf population densities of epiphytic bacteria on Hass avocado trees between the sixth and seventh sprays. Error bars represent the standard error of means.

antagonists sprayed onto leaves) were often highest on the treatments receiving antagonist sprays.

C. gloeosporioides was isolated from a lower frequency of green peel pieces taken from fruit sprayed with either copper, Q34 or 359 than those taken from fruit sprayed with water or diluted media only (Table 13), although these differences were not statistically significant. No samples were taken from fruit sprayed with 480 or Q6.

All treatments, including the 'media only' control, significantly reduced anthracnose severity in comparison to the 'water only' control (Table 14 (a)). The yeast antagonist Q6 was the only treatment to significantly reduce anthracnose in comparison to the 'media only' control, although 359 and copper treatments also had relatively low anthracnose levels. Stem end rot levels were very low in this trial, and there were no significant differences between any of the treatments. *Dothiorella* sp. was the predominant stem end rot pathogen in this trial, causing approximately 58% of stem end lesions. *C. gloeosporioides* and *Phomopsis* sp. were isolated from 28 and 12% of stem end lesions respectively. Percentage marketable fruit generally reflected anthracnose levels, with treatment Q6 having the highest numbers of marketable fruit. There were no significant treatment differences between fruit ripening time, mean fruit diameter, injury and scale levels at harvest. The development of a fine black spotting symptom (pepper spot) was observed on fruit from all treatments. The severity of this spotting symptom was significantly higher in fruit sprayed with water only than in fruit receiving any other treatment.

Stem end rot, fruit ripening time, mean fruit diameter, pepper spot and scale levels varied according to orchard position (Table 14 (b)). Blocks 1 and 2, which were the coolest and most exposed parts of the orchard, had lower stem end rot levels than blocks 3 and 4. Fruit from block 1 also had larger fruit with shorter ripening times than fruit from the other 3 blocks. Scale levels were lowest in the most protected part of the orchard where the trees were largest (block 4).

1994/95 field evaluation: As for the 93/94 field trial, the concentration of isolate 359 in spray suspensions was generally higher than that of isolate Q6 (Table 15). Isolate Q47 (an actinomycete) spray concentrations were consistently lower than both 359 and Q6.

Both 359 and Q6 survived well for the one month period between sprays (Fig 7), although we were unable to recover isolate Q47 from leaf surfaces at any time during the trial, even immediately after spraying trees. For this reason, three small trials were conducted to investigate the problem. In two trials, avocado branchlets sampled from the field site were sprayed with Q47 in the laboratory. Leaf samples were taken soon after spraying and processed as usual. Very low levels of Q47 were recovered from leaves in both trials. In the third trial, leaves were sampled from the field site soon after spraying with Q47 and pressed onto TSA plates. Although the isolate was detected using this leaf impression technique, it did not appear to be present in high numbers on the leaf surface. At this stage a decision was made to continue applying Q47 to trees (because several spray applications had already been made), despite our concerns over the ability of Q47 to attach and survive on avocado leaf surfaces.

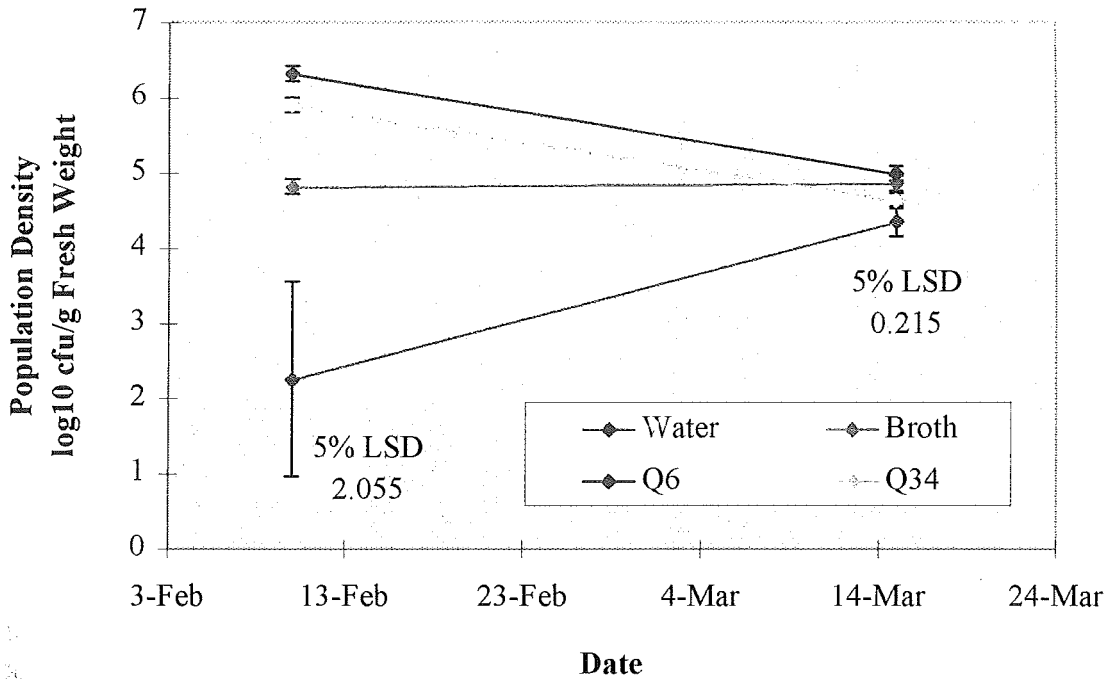

Figure 5. 1993/94 field evaluation - leaf population densities of epiphytic yeasts on Hass avocado trees between the fifth and sixth sprays. Error bars represent the standard error of means.

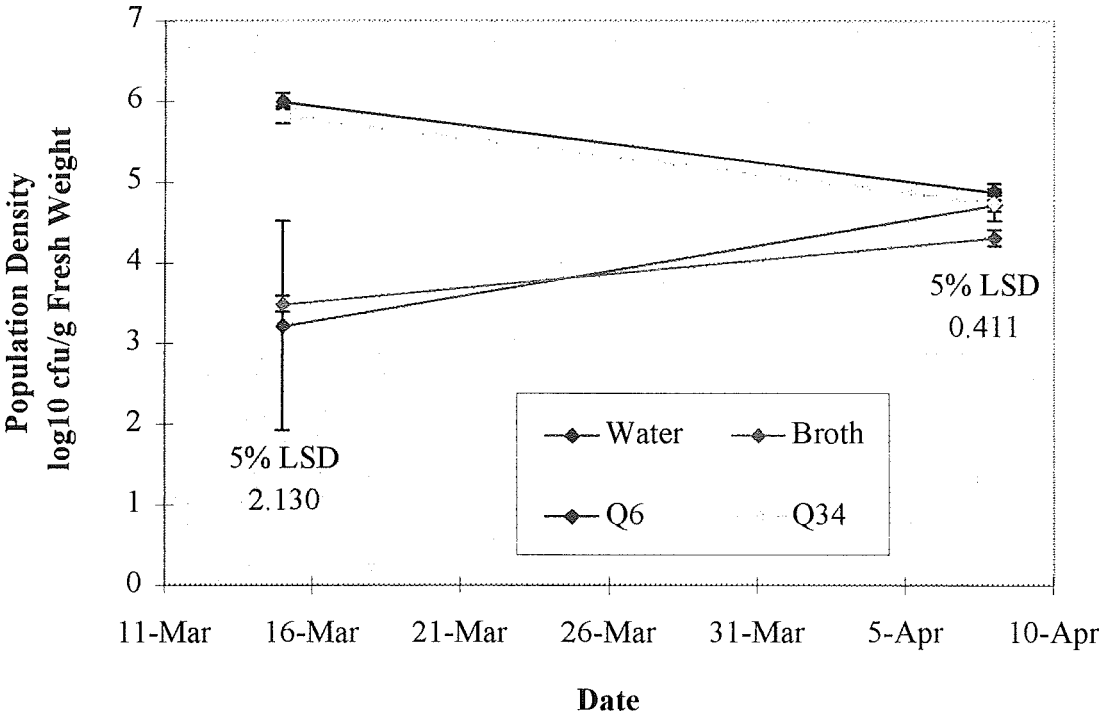

Figure 6. 1993/94 field evaluation - leaf population densities of epiphytic yeasts on Hass avocado trees between the sixth and seventh sprays. Error bars represent the standard error of means.

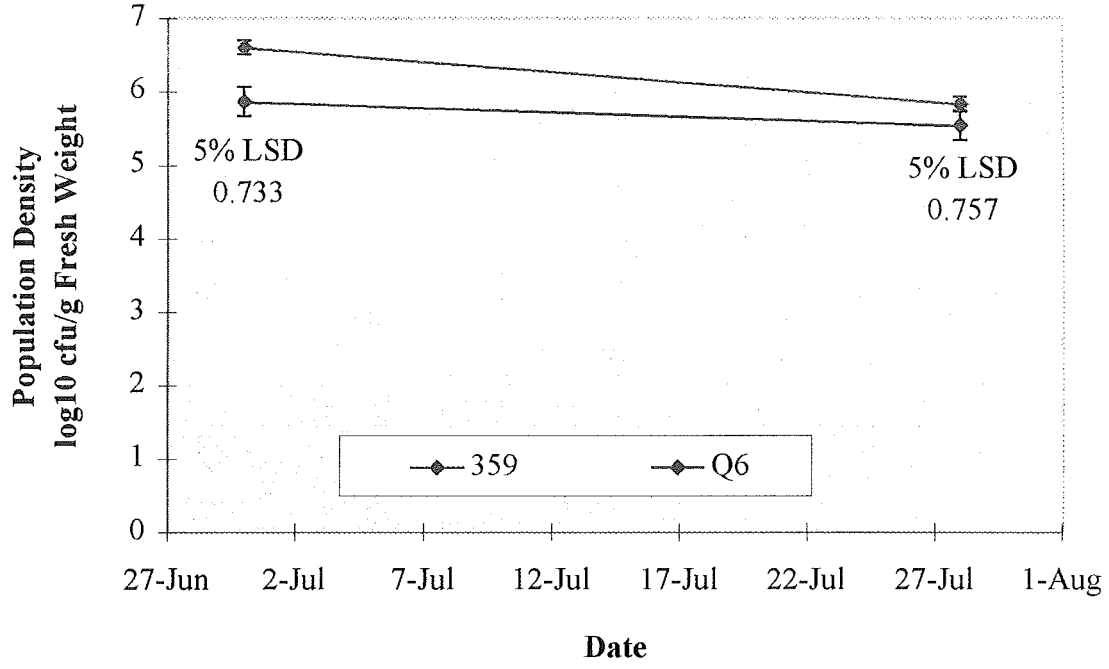

Figure 7. 1994/95 field evaluation - leaf population densities of antagonists applied to Hass avocado trees. Error bars represent the standard error of means.

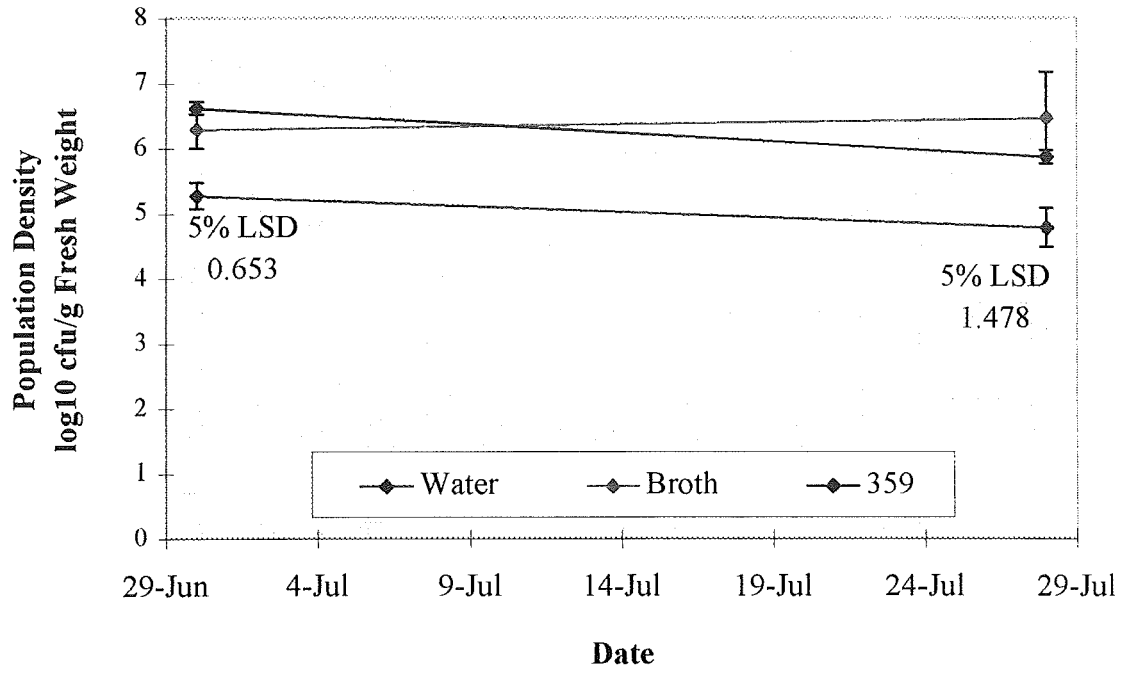

Figure 8. 1994/95 field evaluation - leaf population densities of epiphytic bacteria on Hass avocado trees. Error bars represent the standard error of means.

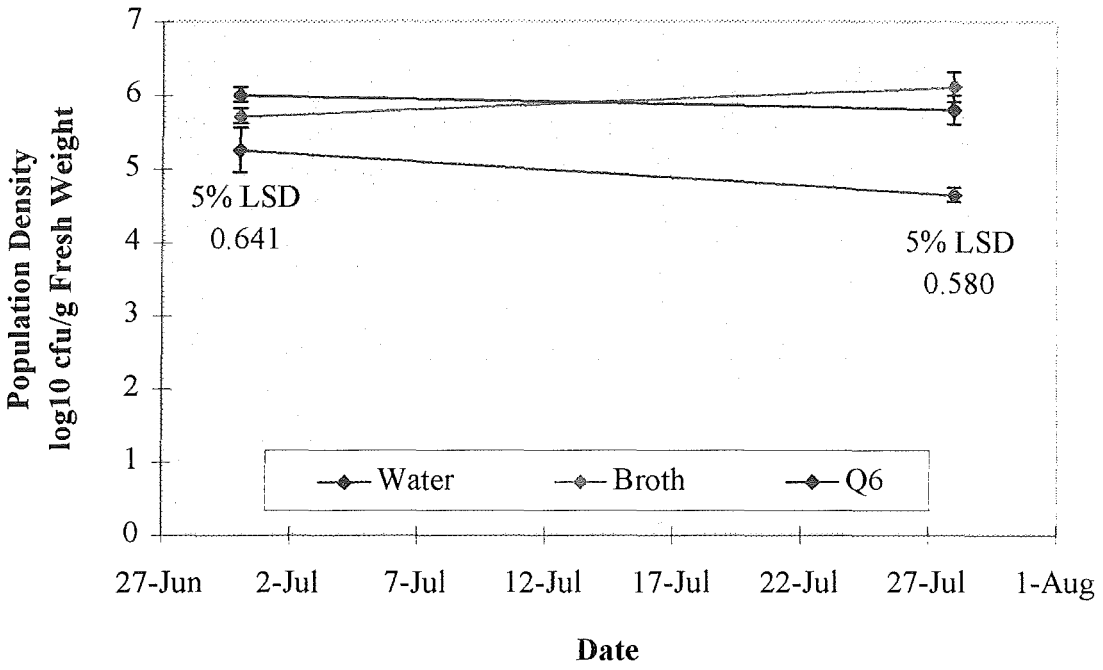

Figure 9. 1994/95 field evaluation - leaf population densities of epiphytic yeasts on Hass avocado trees. Error bars represent the standard error of means.

Leaf population densities of naturally-occurring bacteria and yeasts are shown in Figs 8 and 9 respectively. Total numbers of both bacteria and yeasts were higher on leaves sprayed with diluted culture media than those sprayed with water.

Table 12. Concentration of antagonist suspensions applied to Hass avocado trees at the Mt Tamborine field site - 1993/94 field evaluation.

	ANTAGONIST			
SPRAY DATE	**359**	**480**	**Q6**	**Q34**
20/10/93	N.M.[1]	N.M.	N.M.	N.M.
17/11/93	8.5×10^6	1.2×10^7	2.5×10^5	6×10^5
16/12/93	1.0×10^8	8.8×10^7	3.8×10^7	4×10^7
13/1/94	$>10^7$	3×10^7	1.3×10^7	6.4×10^7
10/2/94	2.4×10^8	6.6×10^8	1.5×10^8	2.1×10^8
11/3/94	N.M.	N.M.	4.5×10^7	2.1×10^7
9/4/94	1.7×10^8	1.2×10^8	2×10^7	6.5×10^7
6/5/94	1.7×10^8	1.3×10^8	6.7×10^7	6.3×10^7
2/6/94	1.4×10^8	4.6×10^7	4.6×10^7	1.6×10^7
1/7/94	7.0×10^7	8.1×10^7	1.8×10^7	3.7×10^7
29/7/94	7.4×10^7	1.3×10^7	1.5×10^7	1.7×10^7
MEAN OF ALL DATES	1.22×10^8	1.31×10^8	4.12×10^7	5.34×10^7

[1] NM=not measured

Table 13. Isolation of *C. gloeosporioides* from green peel of Hass avocados harvested from field trial 1 (1993/94).

Treatment	**Pieces of green peel yielding *C. gloeosporioides* (%)**
Water (control)	33.9 a[1]
Diluted media (control)	26.9 a
Isolate 359	20.9 a
Isolate Q34	13.5 a
Copper hydroxide	11.0 a

[1] Means followed by the same letter **within columns** do not differ significantly at P=0.05 using the LSD test.

Table 14. The effect on monthy preharvest antagonist applications on postharvest disease levels in Hass avocados ripened at 24°C - Field trial 1 (1993/94).

(a) effect of treatment.

Treatment	Anthracnose (% area)	Stem end rot (% area)	Marketable fruit [1] (%)	Ripening time (days)	Mean fruit diameter at harvest (mm)	Pepper spot (% area)	Injury at harvest[2] (% area)	Scale (% area)
Water (control)	36.7 a[3]	0.7 a	18.2 c	13.1 a	67.2 a	5.5 a	2.7 a	0.4 a
Diluted media (control)	22.2 b	3.8 a	36.2 bc	13.1 a	63.3 a	1.2 b	4.1 a	0.1 a
Bacillus 359	13.2 bc	1.6 a	54.4 ab	12.5 a	66.5 a	2.0 b	3.2 a	0.1 a
Bacillus 480	21.8 b	3.4 a	44.8 ab	11.8 a	66.9 a	1.8 b	3.5 a	0.2 a
Pink yeast Q6	8.1 c	2.6 a	61.8 a	11.1 a	67.6 a	0.6 b	4.3 a	0.2 a
Pink yeast Q34	17.0 bc	2.3 a	47.1 ab	11.8 a	66.9 a	1.8 b	3.9 a	0.5 a
Copper hydroxide	14.2 bc	1.5 a	54.7 ab	12.2 a	66.8 a	0.1 b	3.7 a	0.5 a

(b) effect of orchard position.

Block	Anthracnose (% area)	Stem end rot (% area)	Marketable fruit [1] (%)	Ripening time (days)	Mean fruit diameter at harvest (mm)	Pepper spot (% area)	Injury at harvest[2] (% area)	Scale (% area)
1	15.2 a	0.3 c	57.2 a	9.8 b	70.9 a	1.6 b	3.4 a	0.5 a
2	19.1 a	2.1 bc	41.8 a	13.0 a	65.2 b	1.9 ab	3.6 a	0.5 a
3	18.9 a	4.1 a	41.7 a	13.1 a	64.5 b	3.7 a	3.5 a	0.2 ab
4	22.9 a	2.5 ab	40.4 a	13.1 a	65.3 b	0.4 b	4.0 a	0.01 b

[1] Incidence of fruit with 5% or less **total** disease.
[2] Injury includes mechanical (eg. windrub) and insect injury present at harvest.
[3] Means followed by the same letter **within columns** do not differ significantly at P=0.05 using the LSD test.

In this trial, there were no significant differences between any of the treatments in relation to anthracnose severity (Table 16(a)), including the standard copper treatment. Levels of anthracnose were considerably lower in this trial than in the previous field trial. Similarly, none of the treatments significantly reduced stem end rot compared to the "water" control, although trees sprayed with copper or 359 did have very low levels of stem end rot. As for the 93/94 field trial, *Dothiorella* sp. was the predominant cause of stem end rot in this trial, causing approximately 60% of lesions at the stem end of fruit. *C. gloeosporioides* and *Phomopsis* sp. were isolated from 39 and 13% of stem end lesions respectively.

Fruit from copper-treated trees were rated as the most marketable in this trial (Table 16 (a)), although they were not significantly better than controls.

There were also no significant effects of orchard position on any of the parameters assessed in this trial (Table 16(b)), although it should be noted that the selection of trees had to be altered in the early stages of the trial because of storm damage to some trees. Because we were limited in our choice of trees, the new selection was only "loosely" arranged into blocks. This would have had the effect of reducing differences due to orchard position.

Table 15. Concentration of antagonist suspensions applied to Hass avocado trees at the Mt. Tamborine field site - 1994/95 field evaluation

	ANTAGONIST		
SPRAY DATE	**359**	**Q6**	**Q47**
11/11/94	4.6×10^7	1.8×10^7	2.7×10^6
13/12/94	2.8×10^8	2.5×10^7	1.8×10^7
13/1/95	2.0×10^8	2.24×10^7	2.3×10^6
10/2/95	9×10^6	6.8×10^6	1.9×10^6
10/3/95	7.0×10^7	1.2×10^7	1.4×10^6
7/4/95	8.2×10^7	1.25×10^7	2.9×10^6
5/5/95	8.5×10^7	1.2×10^6	5.4×10^6
2/6/95	N.M.	N.M.	N.M.
30/6/95	8.4×10^7	4.5×10^6	2.5×10^6
27/7/95	5.2×10^7	1.17×10^7	3.5×10^6
MEAN OF ALL DATES	1.01×10^8	1.28×10^7	4.61×10^6

[1] ND = not measured.

Table 16. The effect of monthly preharvest antagonist applications on postharvest disease levels in Hass avocados ripened at 24°C - Field trial 2 (1994/95).

(a) effect of treatment.

Treatment	Anthracnose (% area)	Stem end rot (% area)	Marketable fruit[1] (%)	Ripening time (days)	Mean fruit diameter at harvest (mm)	Mean fruit weight at harvest (g)
Water (control)	11.1 a[2]	1.3 bcd	65.7 ab	11.0 a	72.1 a	262.4 a
Diluted media (control)	6.4 a	2.7 ab	63.9 ab	11.4 a	70.8 a	247.3 a
Bacillus 359	5.9 a	0.6 cd	68.3 ab	11.6 a	72.1 a	259.3 a
Pink yeast Q6	5.8 a	2.3 abc	59.6 bc	11.3 a	75.3 a	287.1 a
Actinomycete Q47	5.3 a	3.3 a	69.7 ab	11.0 a	74.7 a	275.6 a
Copper hydroxide	3.5 a	0.1 d	81.6 a	10.7 a	71.8 a	254.0 a
Phosphorous acid	9.5 a	0.9 bcd	45.2 c	10.7 a	72.8 a	271.9 a

Treatment	Dry Matter (%)	Pepper Spot (% area)	Insect injury at harvest (% area)	Windrub injury at harvest (% area)	Scale (% area)
Water (control)	32.8 a	0.8 a	1.4 a	2.5 a	0.3 b
Diluted media (control)	32.0 a	7.8 a	1.1 a	2.5 a	0.5 b
Bacillus 359	31.3 a	2.7 a	1.2 a	4.2 a	0.5 b
Pink yeast Q6	32.9 a	1.1 a	1.3 a	4.2 a	0.2 b
Actinomycete Q47	31.5 a	0.9 a	1.4 a	3.3 a	0.7 b
Copper hydroxide	32.5 a	0.1 a	0.6 a	2.6 a	1.5 a
Phosphorous acid	32.5 a	3.0 a	1.2 a	4.0 a	0.3 b

(b) effect of orchard position.

Block	Anthracnose (% area)	Stem end rot (% area)	Marketable fruit [1] (%)	Ripening time (days)	Mean fruit diameter at harvest (mm)	Mean fruit weight at harvest (g)	Dry Matter (%)	Pepper Spot (% area)	Insect injury at harvest (% area)	Windrub injury at harvest (% area)	Scale (% area)
1	4.5 a[2]	1.1 a	68.2 a	10.8 a	73.0 a	266.2 a	32.5 a	0.2 a	1.0 a	4.0 a	0.4 a
2	10.1 a	1.5 a	60.0 a	10.9 a	72.8 a	267.2 a	32.2 a	3.6 a	0.9 a	2.7 a	0.5 a
3	5.1 a	1.2 a	71.2 a	10.6 a	73.9 a	275.9 a	33.4 a	1.1 a	1.4 a	3.3 a	0.5 a
4	9.0 a	2.4 a	53.6 a	11.5 a	73.7 a	269.3 a	31.1 a	6.2 a	1.0 a	3.7 a	0.9 a
5	5.3 a	1.8 a	71.3 a	11.7 a	70.4 a	248.2 a	27.2 a	0.7 a	1.6 a	2.8 a	0.6 a

[1] Incidence of fruit with 5% or less total disease.

[2] Means followed by the same letter **within columns** do not differ significantly at P=0.05

Total monthly rainfall and number of rain days per month for the duration of the two field trials (September 1993 - September 1995) are shown in Fig 10. Generally speaking, total monthly rainfall during the summer months was higher in 1993/94 than in 1994/95, with the exception of February 1995. Number of rain days per month followed a similar pattern.

Evaluation of antagonist field sprays on the development of sooty blotch in avocados cv. Fuerte

The application of diluted culture media to Fuerte avocado fruit in the orchard did result in an increase in sooty blotch levels (Table 17), although it should be noted that there was no statistical analysis conducted on the results due to the preliminary nature of the trial. There was considerable difference between sooty blotch levels in untreated fruit and fruit sprayed with water, which may indicate that larger sample sizes are required. Despite this, it was clear that copper sprays were completely effective against sooty blotch. Anthracnose levels were also lowest in copper-sprayed fruit. It is important to remember however that sprays in this trial did not commence until March, as our main objective was to assess sooty blotch rather than anthracnose.

Table 17: A preliminary evaluation of antagonist sprays for Fuerte avocado fruit

	Sooty Blotch		Pepper Spot	Anthracnose	Ripening
Treatment	Area (%)	Severity (0-3 scale[1])	Severity (0-3 scale)	Area (%)	Time (days)
Untreated (control)	29.4	1.1	0.5	6.8	8.5
Water (control)	17.3	1.4	0.7	2.1	9.0
Diluted media	44.8	2.0	1.2	5.5	9.4
359 in diluted media	35.9	1.5	0.2	1.0	9.2
Q6 in diluted media	43.5	1.8	0.8	4.2	9.1
Copper	0	0	1.3	0.1	8.9

[1] 0-3 scale where 0=nil and 3=severe

Postharvest evaluation of selected antagonists

Of the ten bacteria and eleven yeasts evaluated, none significantly reduced anthracnose when applied as a postharvest dip to Fuerte avocado fruit (Tables 18-20). Prochloraz reduced anthracnose in all trials, although this reduction was not statistically significant in experiments 2 and 3 (probably due to the large number of treatments tested in the experiment and the overall variability in the data). All treatments, including prochloraz, were ineffective against stem end rot.

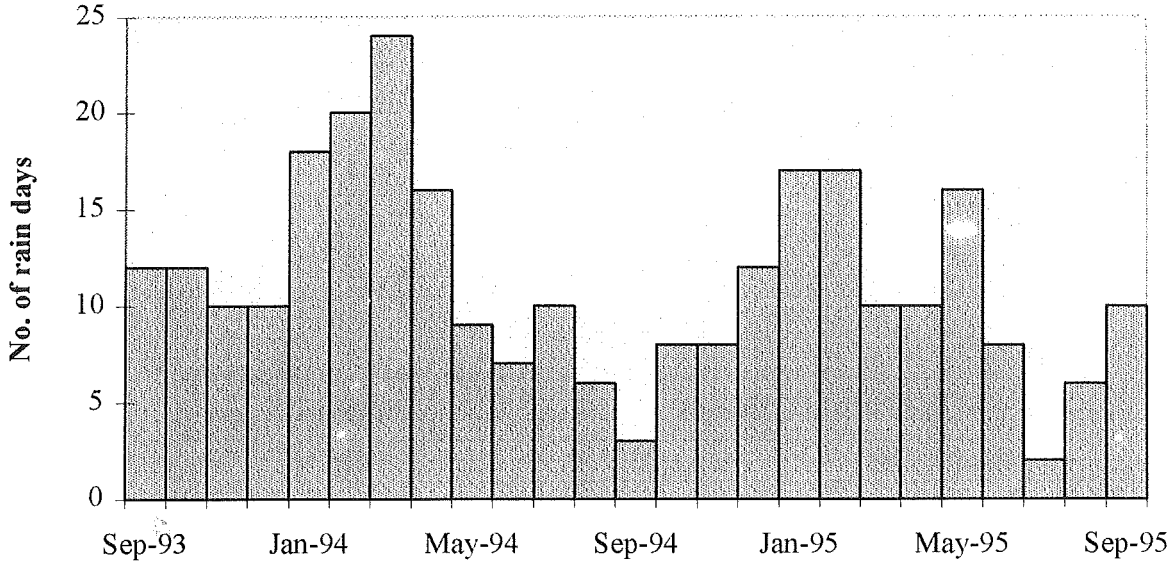

Figure 10. (a) Number of rain days per month at Mt. Tamborine.

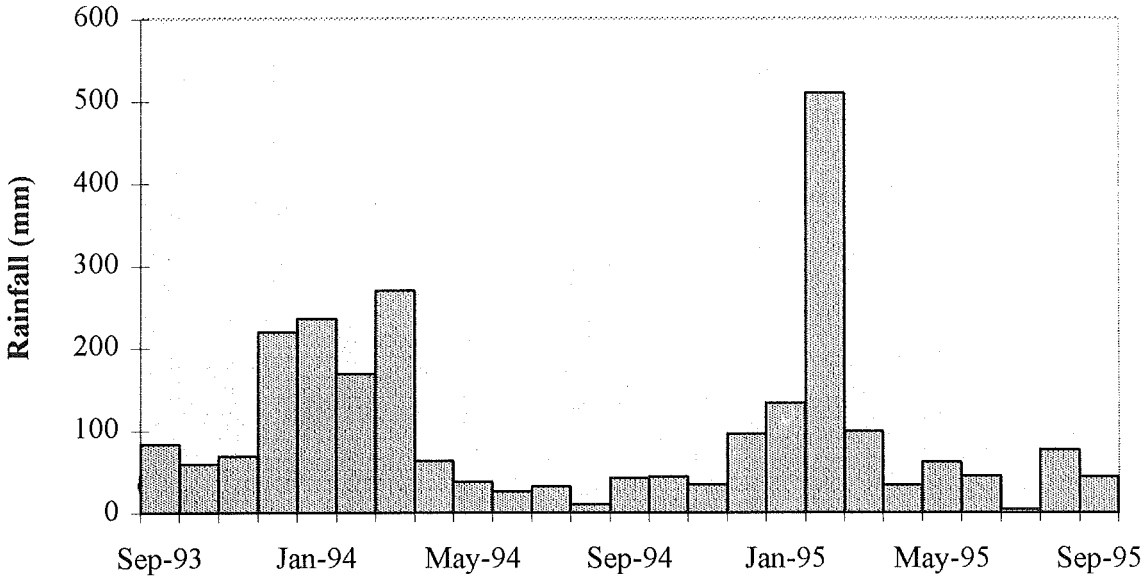

Figure 10. (b) Monthly rainfall at Mt. Tamborine.

Postharvest application of South African antagonists (isolates L1 and L2) to Hass avocados resulted in a significant reduction in anthracnose in experiment 1 (Table 21), as did prochloraz. The Australian antagonist Q47 was ineffective against anthracnose. None of the treatments tested reduced stem end rot.

In experiment 2, only prochloraz and a mixture of the antagonists L1 and L2 reduced anthracnose in fruit stored at 24^0C (Table 22(a)). Once again, none of the treatments reduced stem end rot. Anthracnose levels were very high in fruit stored at 7^0C for 7 days prior to ripening at 24^0C (Table 22(b)), and there were no significant effects of treatments on these fruit.

Table 18. Evaluation of antagonists for postharvest control of anthracnose and stem end rot in Fuerte avocados stored at 24°C: experiment 1 (yeasts).

Antagonist	Antagonist concentration in dip suspension (cfu/mL)	Anthracnose (% area)	Stem end rot (% area)	Ripening time (days)
untreated (control)	—	40.9 abc[1]	12.2 a	15.3 a
water (control)	0	37.2 abc	7.7 a	15.9 a
diluted media (control)	0	32.2 cd	9.0 a	15.6 a
268	3.4×10^7	32.2 cd	7.7 a	15.8 a
468	5.8×10^6	35.1 abcd	8.3 a	15.5 a
700	4.4×10^6	42.6 ab	7.8 a	16.1 a
711	4.0×10^6	32.8 bcd	10.3 a	15.5 a
731	9.6×10^6	32.7 cd	7.5 a	15.2 a
772	5.8×10^6	40.3 abc	9.2 a	15.9 a
878	2.9×10^6	33.4 abcd	9.6 a	15.4 a
885	8.8×10^6	42.7 a	8.0 a	16.0 a
Q6	8.4×10^6	27.3 d	8.1 a	14.3 a
Q34	2.9×10^6	37.3 abc	10.0 a	15.8 a
prochloraz	—	8.7 e	10.8 a	15.7 a

[1] Means followed by the same letter **within columns** do not differ significantly at P=0.05 using the LSD test.

Table 19. Evaluation of antagonists for postharvest control of anthracnose and stem end rot in Fuerte avocados stored at 24°C: experiment 2 (bacteria).

Antagonist	Antagonist concentration in dip suspension (cfu/mL)	Anthracnose (% area)	Stem end rot (% area)	Ripening time(days)
untreated (control)	—	8.5 a[1]	0.5 a	9.6 cde
water (control)	0	7.6 a	1.6 a	9.8 bcde
diluted media (control)	0	7.3 a	1.1 a	10.9 a
21 a	3.6×10^7	6.5 a	0.8 a	9.1 e
358	8.2×10^8	8.9 a	0.8 a	9.2 de
359	3.3×10^8	10.0 a	1.5 a	9.4 cde
480	2.5×10^8	8.2 a	0.7 a	10.2 abcd
553	3.2×10^7	11.8 a	1.2 a	10.2 abcd
717	1.1×10^8	6.8 a	0.8 a	10.2 abcd
933	6.4×10^7	11.1 a	0.7 a	10.4 abc
Q14	3.4×10^7	9.9 a	2.1 a	9.8 bcde
Q28	2.9×10^8	8.2 a	0.7 a	10.8 ab
Q30	5.9×10^8	7.1 a	0.5 a	9.9 abcde
prochloraz	—	3.8 a	0.8 a	9.5 cde

Table 20. Evaluation of antagonists for postharvest control of anthracnose and stem end rot in Fuerte avocados stored at 24°C: experiment 3 (yeasts).

Antagonist	Antagonist concentration in dip suspension (cfu/mL)	Anthracnose (% area)	Stem end rot (% area)	Ripening time (days)
untreated (control)	—	6.7 a[1]	0.4 a	8.6 a
water (control)	0	6.4 a	0.7 a	8.0 a
diluted media (control)	0	4.0 a	0.7 a	8.6 a
268	3.4×10^8	3.3 a	0.2 a	8.5 a
468	2.5×10^7	7.2 a	0.3 a	8.1 a
700	1.6×10^8	3.3 a	0.2 a	8.8 a
711	7.8×10^7	4.2 a	0.6 a	8.2 a
729	8.8×10^6	4.3 a	0.8 a	8.3 a
731	3.5×10^7	6.7 a	0.9 a	8.0 a
772	9.6×10^7	5.7 a	0.2 a	8.2 a
878	2.4×10^7	4.7 a	0.5 a	8.3 a
885	4.2×10^7	3.8 a	0.1 a	8.1 a
prochloraz	—	0.4 a	0.3 a	8.1 a

[1] Means followed by the same letter **within columns** do not differ significantly at P=0.05 using the LSD test.

Table 21. Evaluation of South African bacterial antagonists for postharvest control of anthracnose and stem end rot in Hass avocados stored at 24°C (Experiment 1).

Treatment	Anthracnose (% area)	Stem end rot (% area)	Ripening time (days)
Water (control for L1 & L2)	31.0 a[1]	2.9 a	11.8 bc
Diluted media (control for Q47)	37.4 a	4.2 a	11.8 bc
Bacillus L1 (in water)	17.3 b	1.8 a	11.1 cd
Bacillus L2 (in water)	19.1 b	1.0 a	10.5 d
Actinomycete Q47 (in diluted media)	29.8 a	3.6 a	12.3 b
Prochloraz	14.3 b	1.7 a	14.0 a

[1] Means followed by the same letter **within columns** do not differ significantly at P=0.05 using the LSD test.

Table 22. Evaluation of South African bacterial antagonists for postharvest control of anthracnose and stem end rot in Hass avocados (Experiment 2).

(a) Fruit stored at 24°C.

Treatment	Anthracnose (% area)	Stem end rot (% area)	Ripening time (days)
Water (control)	25.3 a[1]	7.0 a	11.2 a
Bacillus L1 (in water)	14.2 ab	7.0 a	11.5 a
Bacillus L2 (in water)	13.7 ab	3.3 a	11.2 a
Bacillus L1 + L2 (in water)	9.7 b	6.7 a	10.9 a
Prochloraz	4.0 b	4.0 a	11.6 a

(b) Fruit stored at 7°C for 7 days prior to ripening at 24°C.

Treatment	Anthracnose (% area)	Stem end rot (% area)	Ripening time (days)
Water (control)	38.5 a[1]	4.2 a	8.1 b
Bacillus L1 (in water)	51.2 a	3.0 a	8.5 ab
Bacillus L2 (in water)	37.5 a	2.4 a	7.9 b
Prochloraz	42.8 a	4.0 a	9.3 a

[1] Means followed by the same letter **within columns** do not differ significantly at P=0.05 using the LSD test.

Mode of action of selected antagonists

Under the conditions tested in these experiments, none of the antagonists produced any volatile antifungal substances.

In terms of diffusable substances, most *Bacillus* isolates belonging to the "*subtilis* group" produced substances with antifungal activity. Inhibition zones produced on PDA plates between wells containing the cell free filtrates and the fungus remained uninvaded by the fungus, even after incubation for 2 weeks. Since the fungus resumed growth when mycelium adjacent to the zone was transferred onto fresh PDA, it was concluded that the effect was fungistatic rather than fungitoxic. *Bacillus* isolates not belonging to the "*subtilis* group", which included isolate 359, did not produce antifungal substances in any of the media types tested, nor did any of the yeast isolates tested.

Cell free filtrates from *B. subtilis* isolates that were strongly antifungal in the agar-well test also prevented spore germination of *C. gloeosporioides* on avocado leaf disks. In the detached fruit test, however, only filtrates from *B. subtilis* isolates 301 and 504 significantly reduced lesion incidence and size.

TLC studies also confirmed the absence of antibiotic production by *Bacillus* isolate 359 and others not belonging to the "*subtilis*" group.

Pathogen variability studies

Conidial dimensions of *Colletotrichum* spp. isolates collected from a number of different avocado growing regions are shown in Table 23. The general mean of conidial widths for each group of isolates varied from 4.3 to 5.0 μm, and the length from 12.0 to 15.0 μm. Perithecia were produced by some isolates in every group, with the exception of the "A" group which were collected from the Morayfield area. Ascospore dimensions were less variable than conidial dimensions, although isolates from groups T2 and T6 produced relatively wide ascospores. Due to problems experienced with the techniques employed to carry out RAPD analysis of selected isolates, this aspect of the project is still in progress. In order to complete this work, we are currently collaborating with Professor Irwin and Dr Brake from the CRC for Tropical Plant Pathology (The University of Queensland). We anticipate that this work will be finalised by June 30, 1996.

The effect of copper field sprays on populations of resident phylloplane microorganisms

Copper significantly reduced the populations of filamentous fungi, yeasts and bacteria on avocado leaves and fruit , but was most detrimental to bacteria (Figs 11 and 12). Population densities of microorganisms in the sprayed orchard were 10-fold to a 100-fold lower than in the unsprayed orchard. However, populations in the sprayed orchard recovered during the winter non-spray period to levels comparable with the unsprayed orchard.

In both 1993 and 1994 it was found that there was significantly less disease in fruit from the unsprayed orchard compared with the sprayed orchard. The disease ratings for fruit in the unsprayed and sprayed orchards in 1993 were respectively 1.13 and 1.83, whereas for 1994 they were 0.26 and 1 18. The incidence of *Colletotrichum* isolated from the peel of unripe fruit was similar for the sprayed and unsprayed orchard. *Colletotrichum* was the predominant fungus on sprayed fruit, whereas many other fungal species were also present on the peel of unsprayed fruit. All *C.gloeosporioides* isolates obtained from the sprayed and the unsprayed orchards were pathogenic on avocado fruit.

Table 23. Conidial and ascospore dimensions for isolates of *Colletotrichum* collected from various avocado growing regions

Isolate group	Origin of isolates	No. of isolates examined for conidial measurements	Conidial length: range of means (µm)	Conidial width range of means (µm)	Length x width: general mean (µm)	No. of isolates examined for ascospore measurements	Ascospore length: range of means (µm)	Ascospore width: range of means (µm)	Length x width: general mean (µm)
A	Morayfield	11	11.1-14.1	3.9-4.9	12.7x4.3	N/A	-	-	-
B	Maleny	3	10.5-12.8	4.6-5.5	12.0x5.0	2	15.7-16.4	3.9-4.2	16.1x4.1
F	Bli-Bli	5	12.9-14.2	4.3-4.9	13.6x4.6	2	14.4-17.8	4.1-4.2	16.1x4.2
T1	Walkamin	16	12.3-17.1	4.2-5.0	13.8x4.6	5	15.1-16.2	4.1-4.3	15.4x4.2
T2	Childers	10	13.6-15.6	3.9-5.3	15.0x4.4	3	13.9-17.9	5.1-5.6	15.7x5.3
T3	Pomona	18	11.1-14.0	4.0-5.4	13.7x4.6	7	13.1-17.2	3.8-5.2	15.6x4.3
T4	Pomona	8	13.1-14.2	4.3-5.0	13.7x4.8	4	15.0-16.6	4.2-4.6	15.7x4.4
T6	Victoria Pt	22	12.8-15.9	4.0-5.6	14.7x4.8	7	14.8-18.3	5.0-5.7	16.5x5.3
T7	Maleny	17	11.1-14.4	3.5-4.9	13.0x4.5	12	13.8-17.5	4.0-5.7	15.4x4.8
T8	Maleny	2	13.8-15.5	4.3-4.7	14.7x4.5	1	-	-	14.8x4.4
T9	Toowoomba	12	13.4-17.1	4.3-5.3	14.8x4.9	-	-	-	-
T10	Mt. Tamborine	10	13.2-15.4	4.5-5.0	14.1x4.7	-	-	-	-
T11	Victoria Pt.	10	12.8-15.0	4.2-4.8	13.4x4.5	-	-	-	-

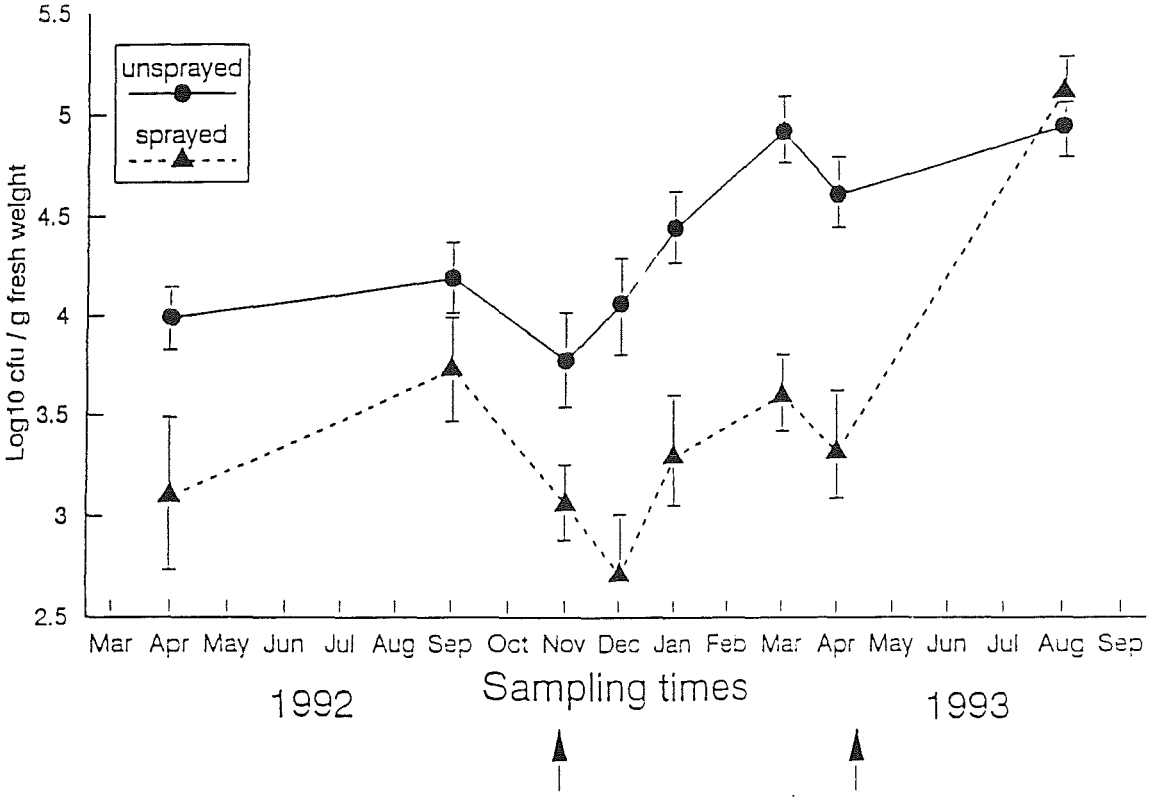

Figure 11. The effect of copper fungicide sprays on the population of bacteria residing on the surface of Hass avocado leaves.

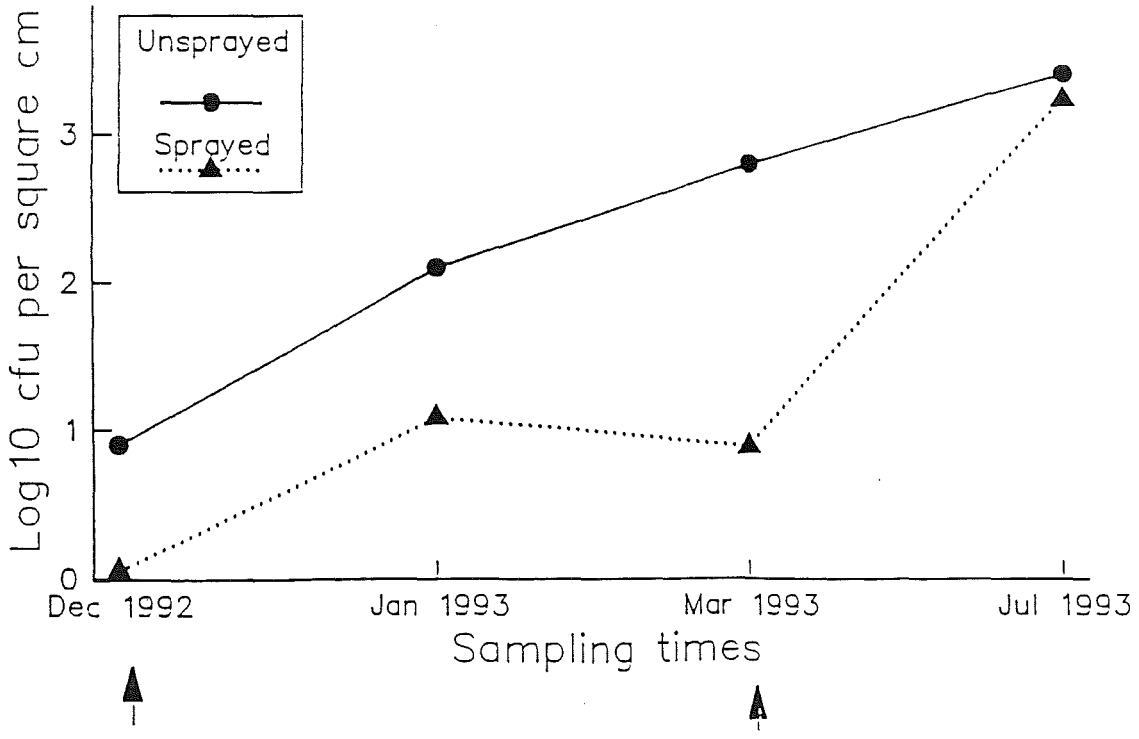

Figure 12. The effect of copper fungicide sprays on the population of bacteria residing on the surface of Hass avocado fruit.

The effect of nutrient field sprays on populations of resident phylloplane microorganisms

Populations of filamentous fungi were significantly higher on urea-treated leaves than on untreated leaves, but populations of bacteria and yeasts were unaffected. All categories of microorganisms increased by 10 to 100-fold in treatments containing molasses. (Fig 13). The effect of molasses lasted for at least one month.

In the second field trial where trees were sprayed monthly with either water or molasses, fruit showed similar levels of anthracnose when harvested and ripened.

Leaf disk studies showed that spores of *C. gloeosporioides* germinated and produced a greater amount of mycelium in molasses than in water, although appressorium formation decreased as the molasses concentration was increased. On detached avocado fruit, molasses increased the virulence of *C. gloeosporioides*, causing lesions to develop on unripe fruit. However, when antagonistic yeasts were applied with molasses, there were fewer lesions.

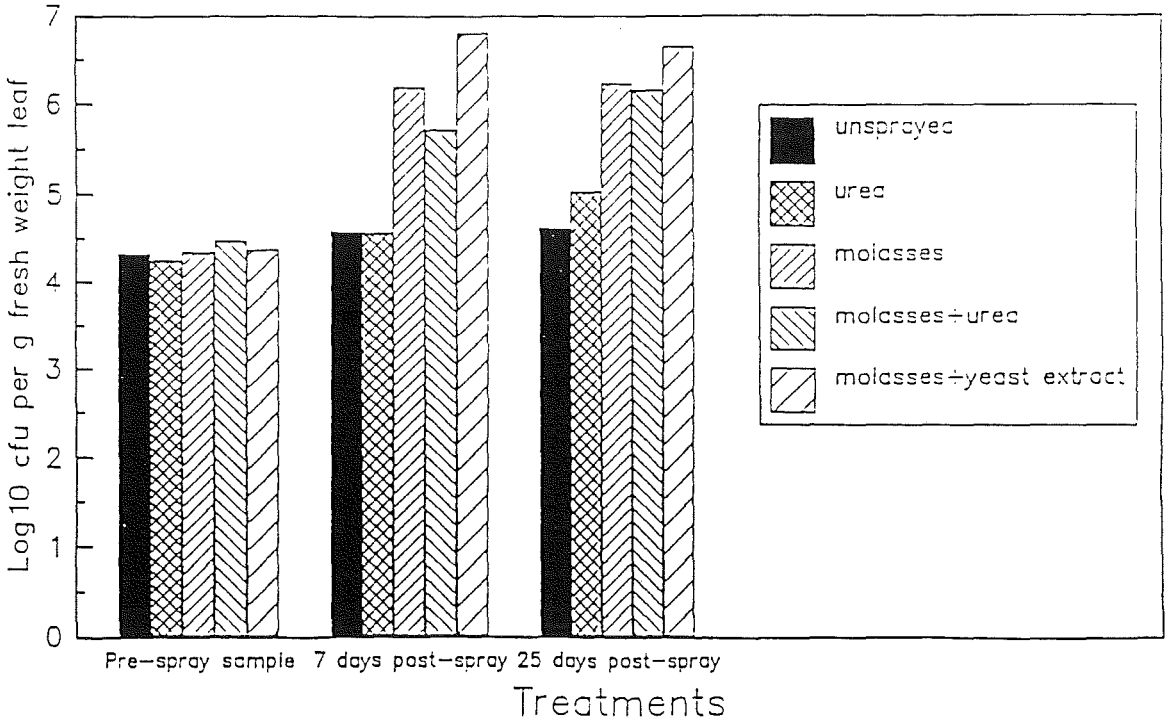

Figure 13. The effect of nutrient sprays on the population of yeasts residing on the surface of Hasss avocado leaves.

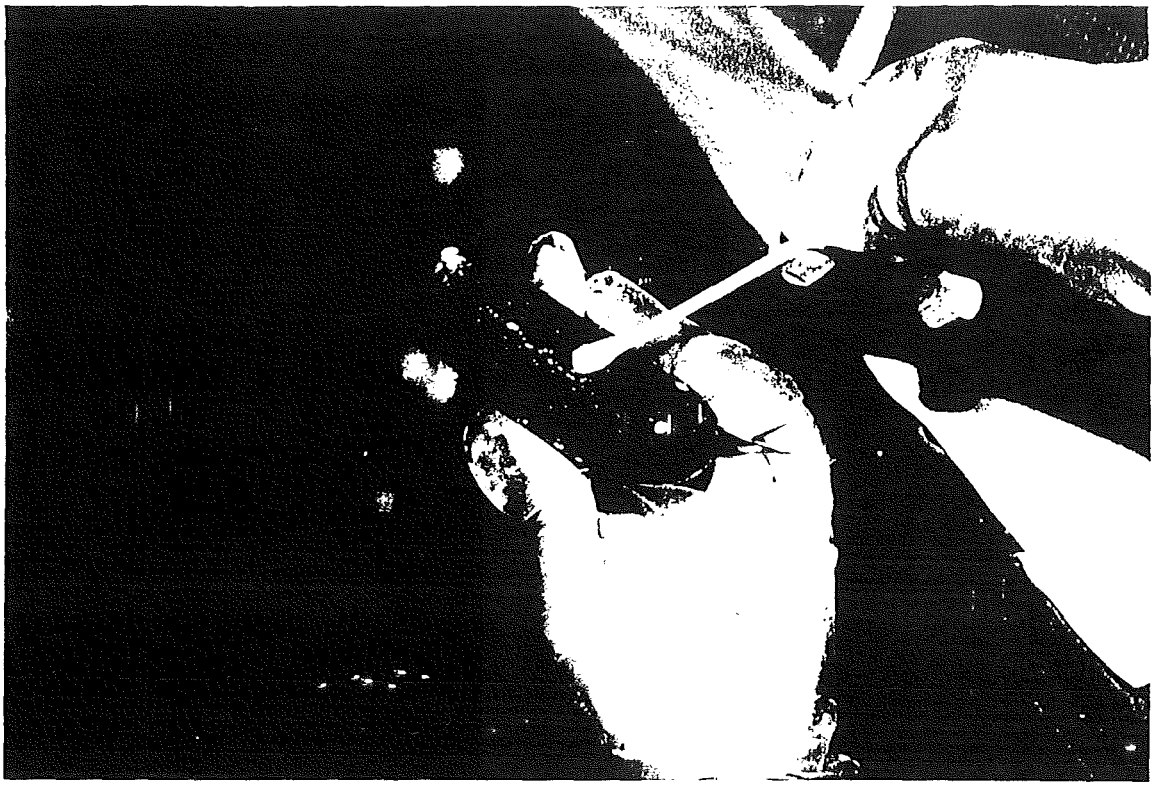

Plate 1: Detached fruit test — application of potential antagonists to cocktail avocado fruit (cv. Fuerte).

Plate 2: Detached fruit test — anthracnose lesion development in ripe fruit.

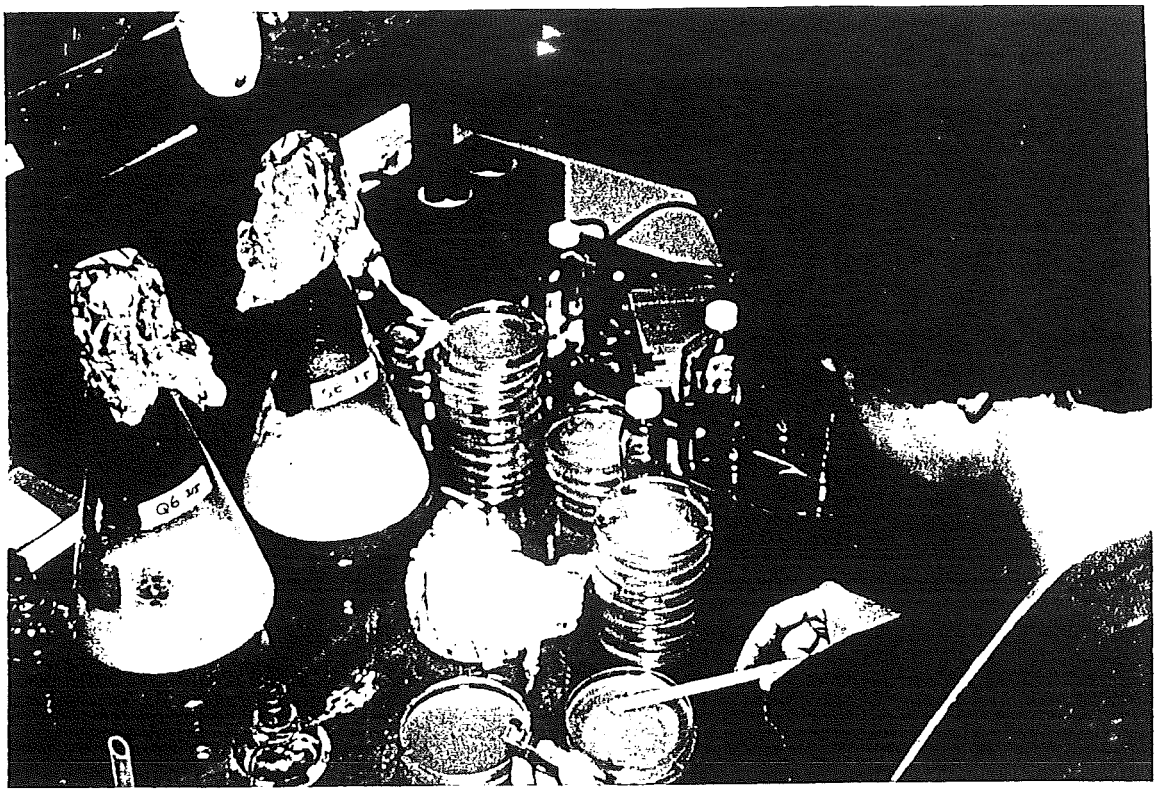

Plate 3: Field evaluation of antagonists — preparation of cultures before the monthly spray application.

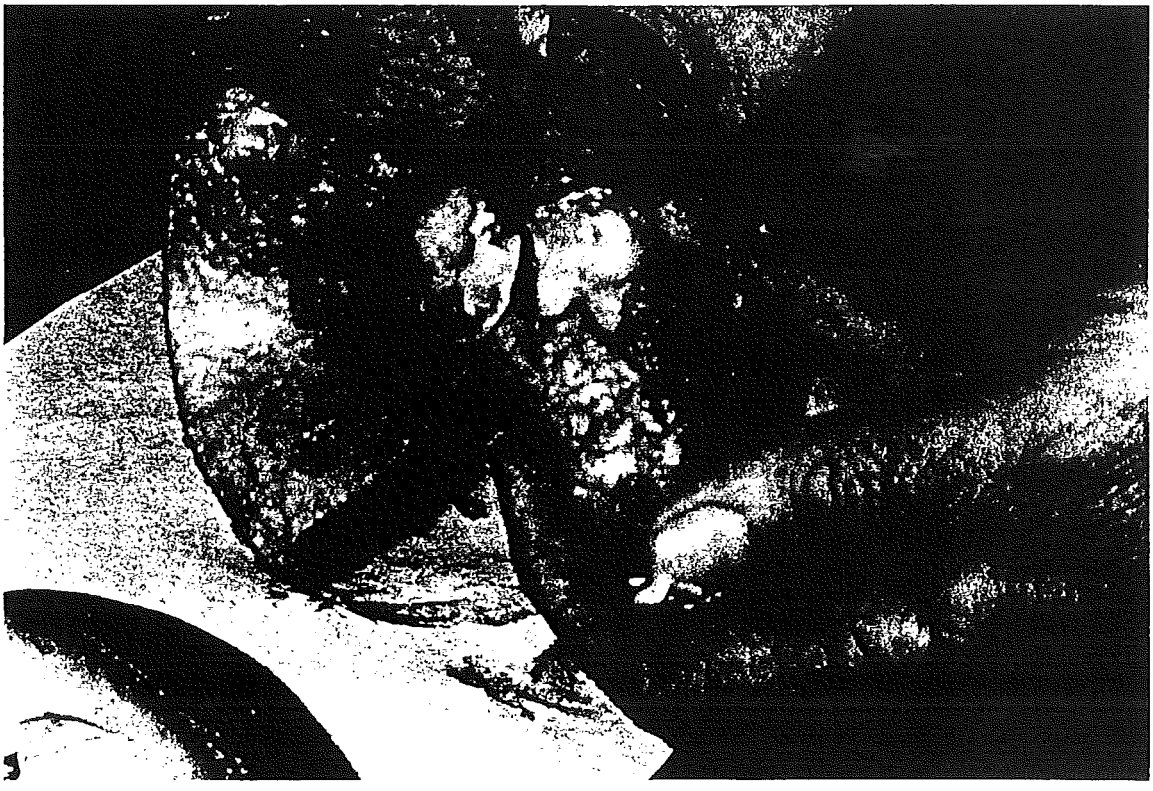

Plate 4: Field evaluation of antagonists — assessment of anthracnose development in ripe avocado fruit (cv. Hass).

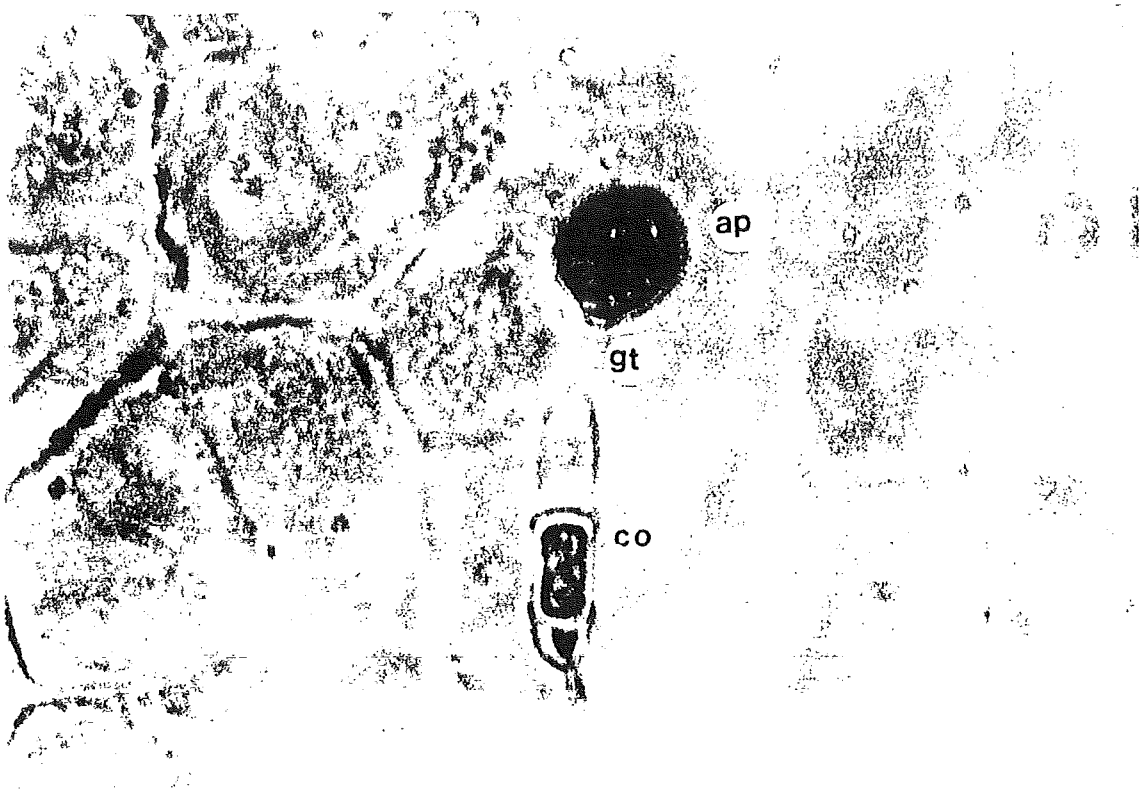

Plate 5: Pathogen variability studies — conidium (spore) germination and appressorium formation of *C. gloeosporioides* on the surface of avocado fruit.
co = conidium, gt = germ tube, ap = appressorium

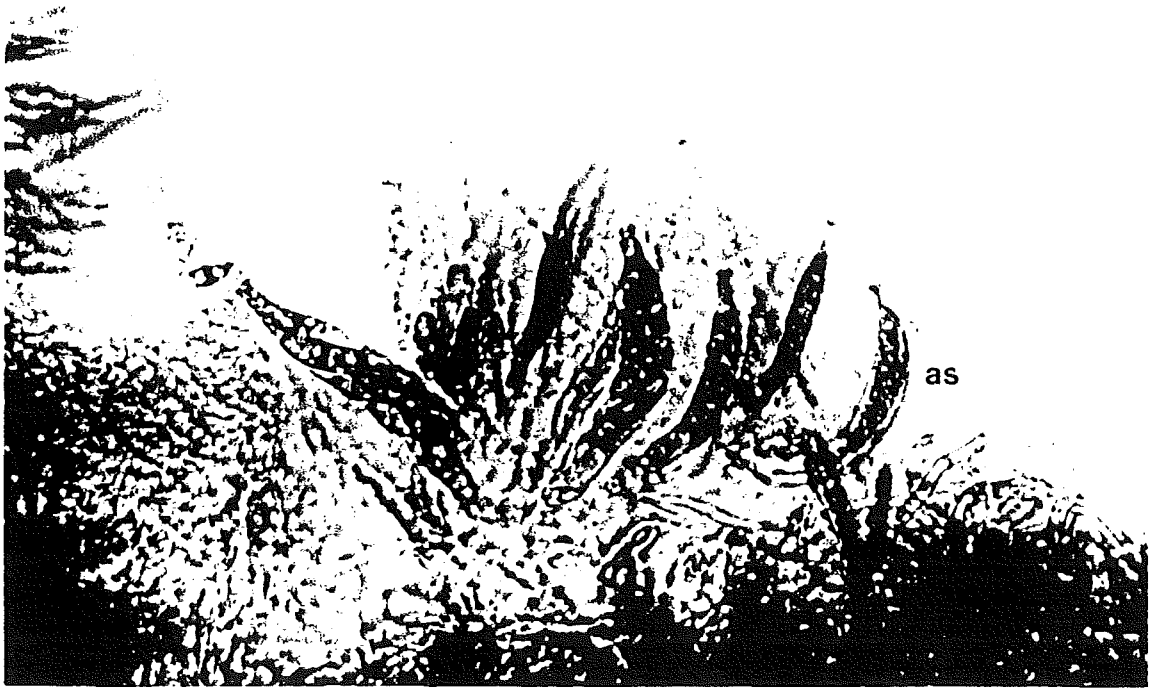

Plate 6: Pathogen variability studies — ascospore production by the sexual state of *C. gloeosporioides* (*Glomerella cingulata*). as = ascus (containing ascospores)

Discussion

In this project, a large number of bacteria and yeasts were screened for their ability to inhibit the anthracnose pathogens, *C. gloeosporioides* and *C. acutatum*. Four different methods were used to screen these isolates. The mycelial inhibition test on PDA and the spore germination test on WSA provided rapid methods for screening a very large number of isolates. From these tests it was possible to reduce a group of 1050 isolates to a manageable number of potential antagonists. It was also possible to derive some information on the possible modes of action of isolates exhibiting antagonistic activity. Isolates showing zonal inhibition of *Colletotrichum* on PDA were clearly producing a diffusable antifungal substance, although it is recognised that such compounds may not necessarily be produced by the isolate under all circumstances, eg. on the surface of avocado fruit and leaves. Given that all of the 160 isolates screened for mycelial inhibition on both PDA (a rich nutrient source) and WSA (a weak nutrient source, similar to the nutrients available on leaf surfaces) showed the same type of antagonism, it is possible that isolates producing a diffusable substance on artificial media may also do so under natural conditions, although there is actually little evidence in the literature that antibiotic production occurs on the phylloplane.

A strong correlation was observed between inhibition of mycelial growth on PDA and inhibition of spore germination on WSA, particularly for *C. gloeosporioides*. This suggests that the same antifungal substances may be involved in the inhibition of both processes. There was not a strong relationship between the response of *C. gloeosporioides* and *C. acutatum* to the antagonists tested, however, particularly in relation to inhibition of spore germination. While the germination of *C. acutatum* spores was reduced by many of the same isolates which reduced germination of *C. gloeosporioides*, the degree of inhibition of *C. acutatum* was generally far less than for *C. gloeosporioides*. This parallels findings from fungicide studies which have shown that *C. acutatum* exhibits an irregular pattern of inhibition and is generally more resistant to the bendazimadoles than *C. gloeosporioides* (Bollen, 1972 ; Muirhead, 1974).

Although plate tests provide a rapid method for the selection of potential antagonists from a large group of isolates, they should not be relied upon as the sole method for screening isolates. The need for using other screening procedures was clearly demonstrated in the results of both the leaf disk and detached fruit tests. Although many bacteria and yeasts suppressed spore germination on cellophane overlaying WSA, only nine bacterial isolates and one yeast isolate reduced germination of *C. gloeosporioides* by 80% or more on leaf disks. Similarly, on detached fruit, isolates which were most effective against *C. gloeosporioides* and *C. acutatum* were not always the best performers in the plate tests. While the detached fruit test is obviously the preferred screening method, it is costly in terms of the number of fruit required and the labour involved in conducting the tests. For this reason, the development of rapid screening tests which correlate more strongly with inhibition of anthracnose development on fruit would be of great value, although probably very difficult to achieve.

The finding that very few isolates were effective against both *C. gloeosporioides* and *C. acutatum* on detached avocado fruit was disappointing, given that a biocontrol agent with broad spectrum activity would be desirable (particularly in New Zealand where both *C. gloeosporioides* and *C. acutatum* are important pathogens of avocado). *Bacillus* 359, which

was consistently effective against *C. gloeosporioides* on detached fruit, did not perform well against *C. acutatum*. It was also found that in general terms, the degree of inhibition of lesions caused by *C. acutatum* was higher than that of lesions caused by *C. gloeosporioides*. This may simply reflect the relatively slow rate of lesion development in fruit inoculated with *C. acutatum* which may give the antagonist greater opportunity for pathogen inhibition.

It was interesting to note that *Bacillus* 359 was equally as effective against *C. gloeosporioides* (on artificially-inoculated detached avocado fruit) as two of the most promising *Bacillus* antagonists from South Africa. It is not known why the yeast isolate Q34 did not control anthracnose in this test, given its previous performance.

Colonization studies showed that *Bacillus* 359 and 480, yeast isolates 734, 772 and Q34 and *Aureobasidium* 274 survived for at least 2 months on avocado leaf surfaces. Without the capacity to survive for such a period, antagonists would be unsuitable for field application. For example, *Pseudomonas fluorescens* 677 and *Enterobacter agglomerans* 632 showed some potential as biocontrol agents in detached fruit tests, yet their usefulness as a preharvest spray treatment for anthracnose is unlikely due to their inability to survive in the field. 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 poor survival capacity on the mango phylloplane.

In the first field trial at Mt. Tamborine, all treatments significantly reduced anthracnose in comparison to the "water" control, although only yeast isolate Q6 reduced anthracnose in comparison to both the "water" and "diluted media" controls. The finding that diluted media sprays resulted in a small but significant reduction in anthracnose may reflect changes in the leaf microflora. Leaf population studies conducted during the trial showed that there was an increase in numbers of resident bacteria and yeasts on leaf surfaces as a result of applying the diluted media to trees. It is possible that this more well developed microflora on the trees sprayed with the nutrients may have better protected the fruit against infection by *C. gloeosporioides*.

None of the treatments tested in the second trial (including copper) significantly reduced anthracnose, although overall disease levels were much lower in this trial than in the previous trial. This may have reflected different summer rainfall patterns or different tree ages (younger trees were selected for the second trial). Given that trees in this orchard had never been sprayed with copper and therefore would have a more well developed microflora than sprayed orchards, there may be more potential for applying biocontrol agents in orchards which have been recently sprayed with copper.

A preliminary field study conducted on Fuerte avocado showed that the spray application of antagonists in diluted media resulted in an increase in sooty blotch levels. This is consistent with reports from South Africa where diluted media was initially used for field trials but soon discontinued because of problems with sooty blotch (Korsten, personal communication). Despite this, we have never experienced any such problems with the cultivar Hass in terms of sooty blotch. Although Hass is the predominant cultivar grown in the Australian avocado industry (and almost the exclusive cultivar grown in New Zealand), Fuerte still remains an important cultivar. To address this problem, therefore, we currently have a field trial in progress (as part of project AV504) at Glasshouse, South-East Queensland where we are

evaluating the application of *Bacillus* 359 in water (rather than in diluted media) to both Fuerte and Hass avocado trees. Initial studies have shown that the antagonist is able to survive adequately without added nutrients, although future studies should focus on the development of a formulation which optimises antagonist survival without accelerating the development of sooty blotch. Reports from South Africa (Korsten *et al.*, 1992; Korsten *et al.*, 1994) indicate some control of sooty blotch with an integrated copper + *Bacillus subtilis* treatment, although no control was achieved with *Bacillus subtilis* alone. There is some evidence, however, that continued application of *Bacillus subtilis* antagonists over a number of seasons has resulted in gradual reductions in sooty blotch levels (Korsten, personal communication).

None of the antagonists evaluated in this project reduced anthracnose when applied as a postharvest dip treatment to Fuerte avocado fruit. This may reflect the initial screening procedures used in this project, which were all based on the application of antagonists prior to the application of the pathogen. Such screening procedures would presumably favour selection of antagonists with potential as preharvest rather than postharvest biocontrol agents. Other factors may have also contributed to the poor result obtained. Recent studies conducted in a related project on the biocontrol of mango anthracnose have shown that application method, antagonist concentration and choice of wetting agent can have a major influence on the efficacy of a given antagonist. For example, dipping mango fruit in a suspension of *Bacillus* 359 prior to inoculation with *C. gloeosporioides* did not result in a reduction in anthracnose, whereas application of the antagonist using a cotton bud was successful in reducing the disease. Once we have optimised application methods and antagonist concentrations, it may be worthwhile to re-evaluate some of our more promising antagonists for postharvest anthracnose control. Certainly *Bacillus* 359 has performed very well in mango leaf disk assays when applied both before *and* after the pathogen (*C. gloeosporioides*), indicating potential for postharvest control.

Postharvest control of anthracnose in Hass avocados stored at 24^0C was achieved by dipping fruit in suspensions of the South African *Bacillus* antagonists L1 and L2. Stem end rot was not controlled by these antagonists, however. In South African packinghouse trials, these antagonists have been successfully incorporated into the "Tag" wax which fruit are coated with after harvest (Korsten *et al.*, 1991).

Mode of action studies conducted on a selected group of antagonists revealed that most *Bacillus* isolates belonging to the "*subtilis*" group produced diffusable antifungal compounds in culture, whereas yeast isolates and *Bacillus* isolates not belonging to the "*subtilis*" group (which included *Bacillus* 359) did not produce antifungal compounds under the conditions of the experiments. This suggests that the principal mode of action of the antibiotic non-producing antagonists may be competition for nutrients and/or space, although further studies need to be conducted to establish this. The finding that *Bacillus* 359 did not produce any detectable antibiotics is encouraging for future development of the antagonist as a biocontrol agent for anthracnose, particularly in relation to product registration. The production of antibiotics by an organism is generally considered an undesirable characteristic in terms of environmental and human safety issues, and may possibly be an obstacle to obtaining registration of a biocontrol agent for commercial use.

There was considerable variability in conidial size of the *Colletotrichum* isolates collected from several avocado growing regions in Queensland. The mean width of conidia has traditionally been used as an important taxonomic character in species delimitation in the genus *Colletotrichum*, and in the case of *C. gloeosporioides,* has been used to delimit sub-specific variation (Simmonds, 1965). The mean width of conidia varied from 4.3 to 5.0 μm in our studies, which is considerably wider than the width of 3.7μm recorded by Simmonds (1965) for isolates belonging to *C. gloeosporioides* var. *minor*, which apparently includes avocado isolates of *C. gloeosporioides.* This problem has been reported in previous studies (Coates, 1991) and is one of the main reasons for attempting to use molecular methods for distinguishing groups within the species *C. gloeosporioides*. Ascospore dimensions recorded in this study were more consistent with the findings of Simmonds (1965) than conidial dimensions.

Copper fungicide studies conducted on adjacent unsprayed and copper-sprayed avocado orchards in South-East Queensland showed that populations of resident phylloplane microorganisms, particularly bacteria, were greatly suppressed by copper sprays. This is consistent with the findings of other studies on the suppression of microflora by fungicides (Andrews and Kenerley, 1978; de Jager *et al.,* 1994). The studies conducted in this project also showed that anthracnose levels were actually lower in the unsprayed orchard than in the sprayed orchard. These findings suggest that *C. gloeosporioides* may have been under natural biological control in the unsprayed avocado orchard. Since copper was detrimental to microorganisms on the leaf and fruit surface, these microorganisms may have been responsible for disease suppression.

These results do not suggest that copper sprays should not be used, but rather imply that copper sprays need to be used correctly if they are to be effective. Complete coverage with copper ensures protection against *Colletotrichum*, even though beneficial microorganisms are suppressed. Incomplete coverage only offers partial (or no) protection, yet also suppresses the beneficials. It is possible that not spraying with copper may be better than applying copper incorrectly, although further studies in a wider range of growing regions are required.

Results from nutrient studies showed that when nutrients such as molasses are sprayed onto avocado trees, the activity of both *C. gloeosporioides* and the natural microflora increases. While one study showed that molasses sprays had no effect on anthracnose levels, the application of diluted culture media to avocado trees at the Mt. Tamborine field site did reduce anthracnose in ripe fruit. This reduction correlated with an increase in the activity of resident microorganisms on the surface of leaves. There may be potential for nutrient sprays to supplement the application of biocontrol agents in the future so that less frequent antagonist applications (which will be costly) can be made.

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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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Appendix 2

Extention Publications

and

Conference Proceedings

AUSTRALIAN AVOCADO GROWERS' FEDERATION INC

Talking Avocados

AHC

The Australian Newsline

Vol 4 Number 4 November 1993

Progress Report On The Anthracnose Biocontrol Project

By Lindy Coates, Tony Cooke and Kathy Cannon, QDPI Division of Plant Protection, Indooroopilly, and Marcelle Stirling, University of Queensland Department of Microbiology, St Lucia

The "Biological Control of Avocado Anthracnose" Project is now in its second year. It is a joint project between Queensland Department of Primary Industries (QDPI) and the University of Queensland and is funded by the AAGF, the New Zealand Avocado Export Council/AGMARDT and HRDC.

The overall aim of the project is to reduce the industries' reliance on fungicides by developing biological control for anthracnose in avocado.

The Problem

Anthracnose causes serious field and market losses of avocado fruit, particularly in susceptible cultivars such as Fuerte. In Australia, the fungus *Colletotrichum gloeosporioides* is the main cause of anthracnose in avocado, whereas in New Zealand both *Colletotrichum gloeosporioides* and *Colletotrichum acutatum* are important causal organisms.

Control of anthracnose is currently reliant on regular field sprays of copper-based fungicides and postharvest treatment with prochloraz (Sportak®). In the not-so-distant future, the use of fungicides may become restricted due to increasing consumer demand for residue-free produce.

The Australian and New Zealand avocado industries have taken the step of preparing for these inevitable changes by supporting the development of alternative disease control strategies through the anthracnose biocontrol project.

Background

Plant surfaces support a wide array of 'epiphytic' micro-organisms. Epiphytic micro-organisms are those which live on the surface of fruit, leaves and other plant organs without causing disease. Many of these epiphytic micro-organisms actually benefit the plant on which they are growing by inhibiting the development of micro-organisms which cause disease. These beneficial micro-organisms are called 'antagonists'.

The aim of the anthracnose biocontrol project is to isolate epiphytic micro-organisms (bacterial and yeasts) from avocado fruit, leaf and flower surfaces and select from these antagonists which inhibit the development of the fungi which cause anthracnose. The best places to look for potential antagonists are orchards which appear to have some degree of natural biological control occurring, for example, unsprayed orchards which have very low levels of anthracnose.

Once we find these organisms, we need to test them extensively for their ability to reduce anthracnose levels in the field and after harvest. It is also very important to determine the mechanism by which these organisms suppress anthracnose development so that we can ensure that they are safe to use.

Progress To Date

Joint QDPI-UQ Studies

To date, over 1 000 bacteria and yeasts have been isolated from avocado orchards in South-East and North Queensland. These isolates were tested for their ability to inhibit fungal growth and spore germination of *Colletotrichum gloeosporioides* on artificial growing media (Figure 1).

It was found that approximately 100 isolates reduced fungal growth of *Colletotrichum gloeosporioides* to varying degrees, and approximately 34 isolates also reduced spore germination by more than 80%.

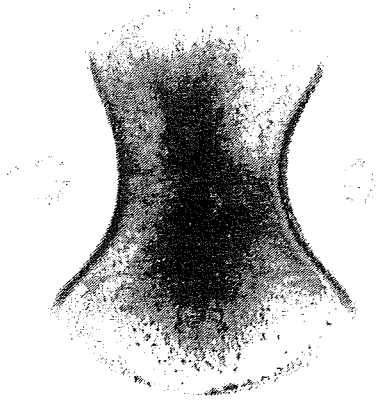


Figure 1. Inhibition of fungal growth of *Colletotrichum gloeosporioides* by a bacterial isolate

Isolates which caused either type of fungal inhibition were then tested for:

a. ability to reduce fungal growth and spore germination of *Colletotrichum acutatum* on artificial growing media. Results obtained so far indicate that approximately 70% isolates tested also reduced fungal growth of *Colletotrichum acutatum* to some degree, although only 8% of isolates reduced spore germination by more than 80%.
b. ability to reduce lesion development in inoculated, detached avocado fruit. Each isolate was applied to the surface of freshly harvested Fuerte avocado fruit (Figure 2). Following this, fruit were inoculated with spores of *Colletotrichum gloeosporioides* or *Colletotrichum acutatum* in circled areas. Fruit were incubated until ripe and then assessed for anthracnose development within circled areas. Approximately 70 bacteria and 50 yeasts have been screened up to 3 times each using this procedure. Isolates which reduced lesion develop-ment (in comparison to controls) are currently being selected for further testing.

UQ studies

The effect of copper sprays on epiphytic micro-organisms is currently being investigated. Intensive sampling over a twelve month period has shown that copper reduces the number of most types of micro-organisms on the surface of avocado leaves, particularly bacteria.

Future Developments

During the next 12 months, we plan to test a selected group of antagonists for both preharvest and postharvest control of anthracnose. This means applying antagonists both in the field (as a spray) or after harvest (as a dip), and assessing fruit for disease development at all stages after treatment. A field sited at Mt Tamborine is currently being selected for the spray trials. Optimum concentrations of antagonists will need to be determined as part of this process. The effect of added nutrients on the colonisation of selected antagonists

on the fruit surface will also be investigated so that we can maximise the effectiveness of applied antagonists. From these tests we will select the best antagonists.

In the final year of the project (1994/95), these isolates will be tested on a larger scale. We don't expect that any antagonists will give complete control of anthracnose, although we do hope to be able to reduce our reliance on current fungicide treatments.

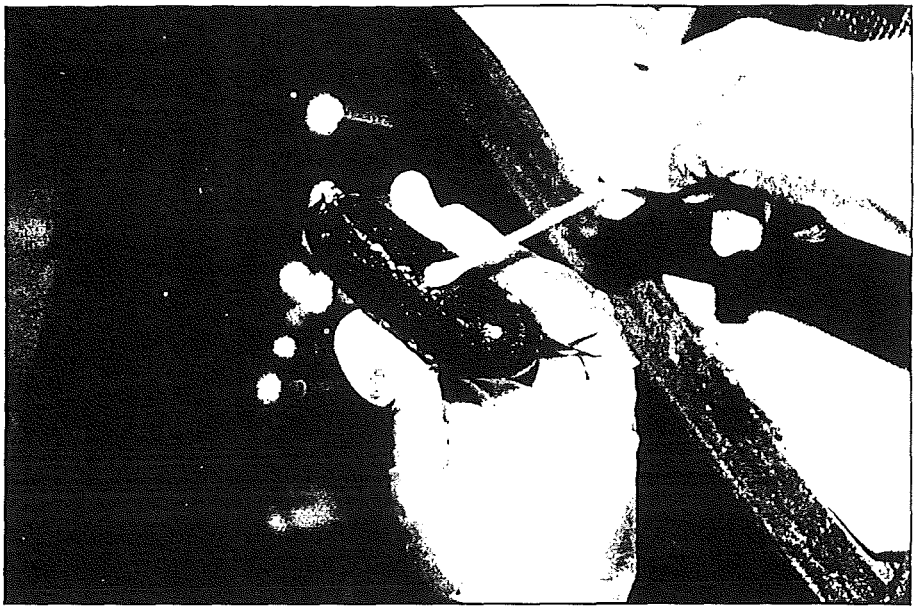


Figure 2: Method of applying antagonists to the surface of 'cocktail' avocado fruit.

A Crop To Shoot To New Heights

From Successful Horticulture, March April 1993

Australian grown bamboo may soon be in demand on the domestic market as well as creating a new $4 million export market to Asia.

Rob Andrews, of Bundaberg is one grower who is successfully pioneering bamboo farming. Mr Andrews has discovered a threefold use for his bamboo plants.

Initially, he planted five sub-species of bamboo in 1989 to provide a 320m wind break on his Bundaberg farm. Since then, he has been trying to discover which type of his bamboo is also edible and provides high quality timber.

"At this stage, not a lot of subspecies do all three," he said.

"It is my idea to have a windbreak that is profitable. If we use bamboo in this way, we don't have to use as many plants and we also have the opportunity to profit from it as shoots and timber products."

Mr Andrews said he had used barna grass in the past as a windbreak for crops, but unlike bamboo, it was nonharvestable.

"There is a necessity to use bamboo at the moment. We have to try to make every inch of ground earn money."

Now, his bamboo plants have reached 8m in height and are expected to grow to 20m.

"The bamboo is just getting to the right size now to make a good wind break. The taller it grows, the thicker it gets," he said.

Mr Andrews, an ex-cabinet maker, said he had been familiar with the uses of bamboo for many years.

"We used bamboo for its decorative purposes. If people wanted to make a room look tropical, we would use bamboo. So I was already familiar with using bamboo for timber purposes when we planted it."

Mr Andrews added that he would have to brush up on his cooking style to make the bamboo more tasty to eat. "Some varieties, although they are edible, I wouldn't classify them as palatable," he said.

"I have tried a few different ways of preparing it, but have decided to see a few Chinese chefs about the best way to cook it."

Mr Andrews said he hoped to sell some of the shoots next year.

"There is a $4 million export market waiting to be attacked," he said.

"It's not as easy as I thought it would be but in time we will get there. We just have to work out which variety will be commercially viable," he said.

The Queensland Department of Primary Industries (DPI) is currently conducting trials to identify suitable bamboo varieties.

DPI horticulturist, Jason Olsen, who is working on the project, said 30 edible varieties of bamboo from Papua New Guinea, Taiwan and Asia were being trialled.

"I'm looking for high yielding, superior quality varieties that will do well in our climate," Mr Olsen said.

Barna Grass Has A Place

By Orf Bartrop

Under some circumstances, barna grass makes an excellent windbreak. If used to enclose very small, reasonable level areas it has the capability to take the sting out of most winds. However, for larger fields, or undulating orchards, barna grass does not measure up as a windbreak because of its lack of height.

A windbreak can only be relied upon to provide protection for a distance of six times its height. In other words, if the windbreak is 20 m in height, it will afford some sort of protection for a distance of 120 m. This figure varies depending upon terrain and what the windbreak is protecting. Orchard trees themselves act as a windbreak and add to the effect of the initial structure.

While barna grass may not be a saleable commodity, in avocado growing it makes an excellent mulch if it can be prevented from shooting and taking root. One proven method is to cut the barna grass like sugarcane and put it through a chaff cutter. The mangled fragments will not take root and grow.

Biocontrol Researchers Get Together In New Zealand

By Lindy Coates, Queensland Department of Primary Industries, Indooroopilly

During a visit to New Zealand in February, I was fortunate enough to attend a workshop on 'Disease Control in Horticulture' which was held at Massey University, Palmerston North. The aim of the workshop was to discuss strategies to reduce pesticide use for postharvest disease control in horticultural crops. The workshop was attended by biocontrol researchers from New Zealand, the United States, South Africa and Australia.

Dr Charles Wilson (US Department of Agriculture, West Virginia), a leading world authority on the biocontrol of postharvest diseases of temperate fruits, described the three approaches that his research team is taking to reduce chemical use after harvest.

Application Of Yeasts

The first approach is the postharvest application of non-antibiotic producing yeasts which control a wide range of postharvest diseases of citrus, pome and stone fruit. Dr Wilson's team is currently collaborating with the biopesticide company Ecogen to commercialise some of the yeast strains which they have found to be effective against postharvest fruit diseases. In some cases they have found that when the yeasts are mixed in with very low concentrations of conventional fungicides (for example, 10% of the normal rate), better disease control is obtained than when the yeasts are applied without any fungicide. They have been able to do this by selecting yeasts which have some resistance to the fungicides which are used. The reason for doing this is to maximise disease control while at the same time minimising fungicide use.

An important part of this work has been to investigate the safety of these organisms to humans and the environment. There is no point in pursuing a biocontrol agent which works wonders against disease but poses a threat to human health. Just like fungicides, extensive toxicology studies must be done before a biocontrol agent can be registered for use on fruit.

Natural Compounds

The second area of research is the application of natural compounds derived from both plants and animals. Dr Wilson's team is currently screening a large number of natural compounds for activity against the fungi which commonly cause postharvest disease in fruit. The main advantage of applying natural compounds is that they biodegrade more rapidly than synthetic compounds. Even so, just because a compound is natural, doesn't mean that it is not toxic. Toxicology testing is a very important part of the evaluation process.

Natural Resistance

The third approach is the enhancement of the fruits' natural resistance to postharvest diseases. Fruit produce a number of compounds which help to reduce infection by disease-causing organisms. The production of these compounds is often related to the stage of fruit ripeness. As the fruit ripens, levels of these compounds may decline, thus decreasing fruit resistance to disease. Treatments which enhance the production of these compounds can therefore help to increase the fruit's resistance to disease.

Dr Wilson's team has been looking at the application of low dose ultraviolet light for the purpose of increasing the fruit's natural resistance to disease. The method has shown good potential for apples, peaches, citrus and sweet potatoes. In some cases the ultraviolet treatment causes delayed fruit ripening.

Dr Lisé Chorister from the University of Pretoria, South Africa, outlined some of her research over the last 7 years on the biocontrol of avocado, mango, lychee and citrus diseases. She has been working with strains of various *Bacillus* species (bacteria which commonly inhabit leaf surfaces) which have been shown to be effective against many of the fungi which cause postharvest disease in these fruit crops. Although treatment with these bacteria is only experimental at this stage in South Africa, large scale field and packing-shed tests have been successfully done. The bacteria can either be applied as a preharvest spray or as a postharvest dip. Dr Korsten has shown that the bacteria can be incorporated in the 'Tag' wax which avocado fruit are coated with after harvest. She is currently working with the private sector to investigate the potential of commercialising the treatment.

While in New Zealand, I also visited the main avocado growing region in the Bay of Plenty. At a growers afternoon which was held at Te Puke Research Station (Horticultural and Food Research Institute of New Zealand), Dr Korsten gave an overview of the South African avocado industry, Dr Bill Hartill (Consultant to HortResearch) gave a New Zealand perspective on postharvest disease control in avocados, and I had the chance to update New Zealand avocado growers on our anthracnose biocontrol project. This a joint project between QDPI and the University of Queensland, and is funded by the AAGF, the New Zealand Avocado Export Council and HRDC. In the first year of the project (1992/93), a large number of bacteria and yeasts were isolated from the surface of avocado leaves and fruit. These isolates were tested against the fungi which cause anthracnose in avocado, *Colletotrichum gloeosporioides* and *Colletotrichum acutatum*. The most effective and consistently performing bacteria and yeasts were selected from this process, and are currently being evaluated in more detail. At a field site at Mt Tamborine, monthly foliar sprays of two bacteria and two yeasts have been applied to Hass avocado trees since last October, and will continue until fruit mature later this year. We have included a treatment of monthly copper hydroxide sprays as a comparison. Disease assessments will be made later this year when fruit are harvested and ripened.

Determining the ability of the selected bacteria and yeasts to colonise and survive on the surface of avocado leaves is an important part of the evaluation process and is being studied in detail by Ms Marcelle Stirling from the University of Queensland. Marcelle has shown that the isolates which we are currently field testing can survive and maintain quite high numbers on avocado leaf surfaces for at least one month.

In addition to field testing, we have now commenced postharvest testing of selected yeasts and bacteria. These isolates are being applied to avocado fruit as a

How Beneficial Bugs Are Produced

Many growers, are familiar with 'beneficial' insects, however, few would realise the complexities involved in producing the critters.

A company "Bugs For Bugs" in the Central Burnett produces two parasitic wasps, *Leptomastix dactylopii* and *Aphytis lingnanensis*, and a variety of ladybird species.

Before goodies can be bred, baddies are required on which they can feed and grow.

Baddie-breeding is a complicated process. Mealybugs and oleander scale are the standard breeding medium. The insects are grown in specially-designed environment controlled breeding chambers, to optimise control over the breeding process. The chambers are maintained at exactly 25°C and humidity of between 50 and 60%.

The 'food' for the beneficial insects are bred and grown on butternut pumpkins. Butternuts have a smooth skin, required to breed the scale, and the shelf-life necessary to survive the lengthy breeding process.

The Leptomastix wasps are bred using mealybugs. The Aphytis wasps are bred on oleander scale. Several ladybird species are bred on the mealybugs or scale.

Established mealybug colonies are maintained in the breeding containers. The crawling insects are transferred to new pumpkins by the 'drip' method. Trays of fresh pumpkins are placed under the 'mother' colonies. Young mealybugs use tiny silk threads to travel from old to new, establishing new colonies of the insects.

Oleander scale, which clings to pumpkins rather like a barnacle to a rock, is actually brushed from established colonies to new pumpkins where they establish themselves.

The scale must be produced at precisely the correct density on the pumpkin. Too light a colony will not provide enough food for the beneficials and too heavy a concentration makes it difficult for the beneficials to establish a foothold.

The pumpkins are then moved into chambers containing established colonies of the parasitic wasps. The wasps' life cycle, including the breeding process, continues unaltered.

For harvesting, wasps are attracted to a fluorescent light, sedated with either ether or carbon-dioxide and then collected and sorted into packages. The ladybirds are collected mostly by hand.

The insectary staff have strict work schedules which allow them to enter each room just once and only in a certain order. This prevents the movement of predators or parasites back into the mealybug breeding rooms on the body or in the clothes of the workers. A few 'beneficial' insects let loose in the breeding room could eat a year of hard work in just weeks.

postharvest dip. Prochloraz treatment (which is the currently recommended fungicide treatment for postharvest disease control in avocado), is being included as a comparison in all evaluations.

Research is also continuing into the effects of copper sprays and applied nutrients on the natural array of microflora which inhabit avocado leaf surfaces. This work is being conducted by Marcelle Stirling (University of Queensland). The aim here is to promote the growth of beneficial micro-organisms which live on leaf surfaces by reducing chemical use and applying nutrients such as molasses. We need to explore as many options as we can for the biocontrol of avocado fruit diseases so that we are prepared for the inevitable limitations placed on chemical use in the future.

For funding this visit, I wish to thank the Australian Centre for International Agricultural Research (ACIAR). For funding our project on the biological control of avocado anthracnose, I would also like to thank the Horticultural Research and Development Corporation (HRDC), AAGF and the NZ Avocado Export Council.

Anthracnose - Field Control Is Essential

Tony Cooke and Lindy Coates QDPI Plant Protection Unit, Indooroopilly

Anthracnose continues to be the major cause of postharvest breakdown in avocados at all levels of the marketing chain, right down to the consumer. This year demonstrated how unseasonal rain in drier growing regions and showery weather can accelerate the development of anthracnose.

Anthracnose is caused by a fungus which infects fruit in the orchard. Under certain conditions, e.g. when the fruit skin is damaged by insects, infection may lead to the formation of dark lesions on fruit which are hanging on the tree. This type of anthracnose, which is often referred to as "pre-harvest anthracnose", is plain to see.

In most cases, however, the anthracnose fungus enters a dormant phase once the initial stages of infection have occurred. This type of anthracnose, which is often referred to as "postharvest anthracnose", cannot be seen in the orchard. It is not until fruit are harvested and ripened that typical anthracnose symptoms develop.

A basic knowledge of the anthracnose life cycle is very important because it helps us to understand how to best control this disease. Therefore, let's have a look at Figure 1 below to see how the infection process works.

The best way to control this disease is to prevent infection from occurring in the first place. The germinating spore has to be stopped. So, how does this spore come to find itself on the fruit? Actually we are talking about large numbers of spores—millions and millions of them are produced on diseased fruit, dead twigs and leaves entangled in the tree canopy.

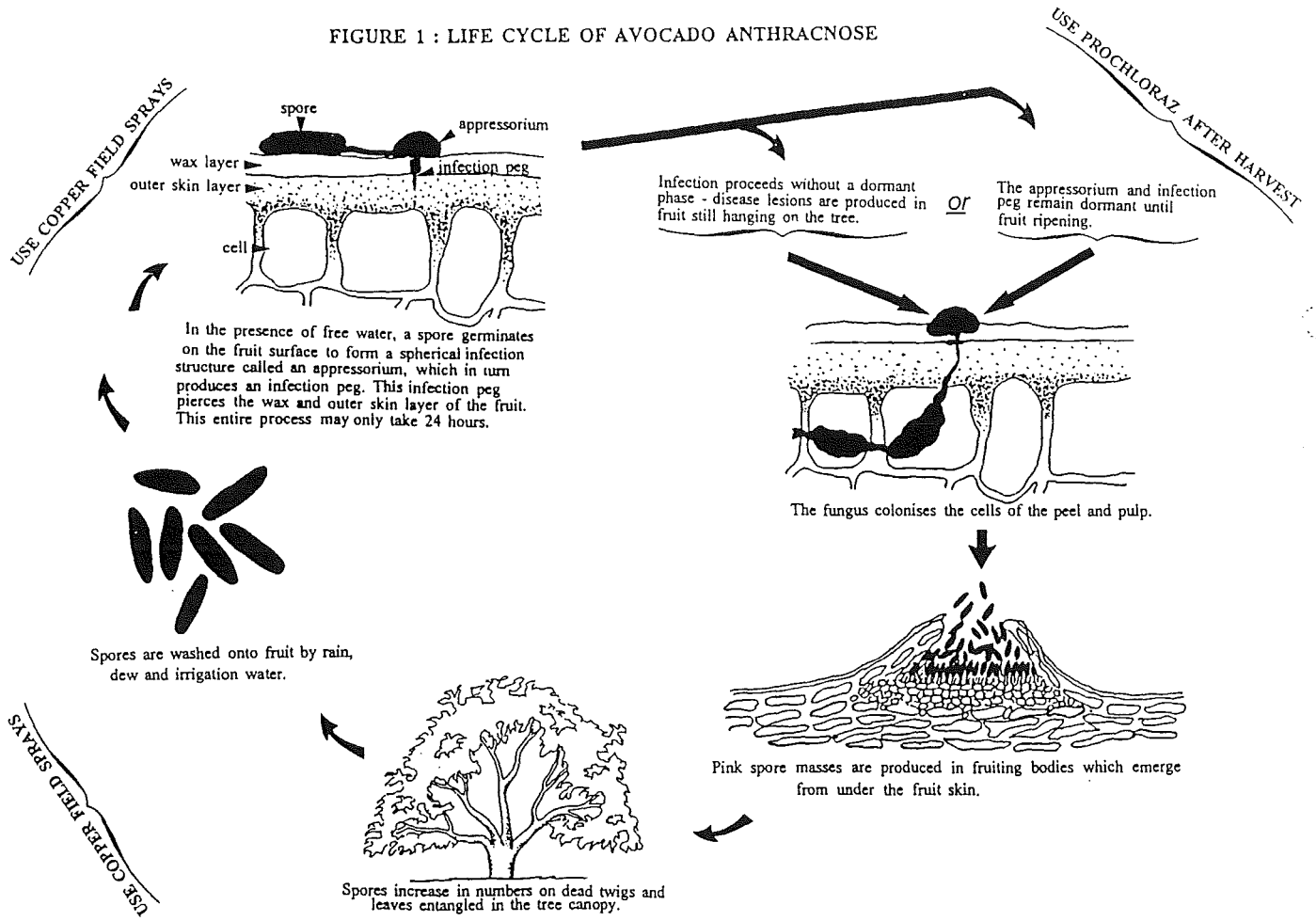
The spores are washed down onto healthy fruit by rain, dew or irrigation water. The more there are, the greater the disease pressure—remember that you can't see any of this happening. You may occasionally see the pink spore masses of anthracnose on leaves and twigs, but generally all of this goes on unnoticed.

At present, the best way to prevent infection is to maintain good orchard hygiene and keep a coating of copper-based fungicide on the fruit and foliage. Copper, being a protective fungicide, works by shielding the fruit against infection and by preventing the build-up of spores in the tree canopy. This is why the use of properly calibrated spray equipment is of such importance.

Complete spray coverage of the fruit and foliage is essential, as is regular application—every 4 weeks in dry weather or every 2 weeks in wet weather. Without this protection, germination of the spore can take place unhindered on the surface of fruit.

Once germinated spores grow to form ar appressorium and infection peg, the disease moves into a totally new phase. From being relatively easy to control, the enemy is now dug in and is so much harder to kill. It is extremely important to prevent the fungus from getting to this stage. **Anthracnose is best controlled by preventing the formation of the infection peg.**

FIGURE 1 : LIFE CYCLE OF AVOCADO ANTHRACNOSE



There is a misconception among some growers that Hass avocados do not need to be copper-sprayed as often as Fuerte avocados. This is not the case. The recent AVOMAN survey of retail outlets in Sydney showed anthracnose to be a major cause of breakdown in Hass avocados displayed for retail sale.

Once infection takes place, the anthracnose fungus plays a waiting game and lies dormant waiting for the right conditions to rot the fruit. The "right" conditions are triggered by the ripening process, which means that this dormant phase may last up to twelve months because infection can take place anytime from fruit set to harvest. Because copper sprays will not kill the fungus in the dormant stage, another battle line has to be drawn and that is done after harvest with an overhead spray of prooraz on the packing line (Sportak®, Mirage®). This does not mean that copper field sprays are not needed—**prochloraz alone will not do the whole job.**

It is important to use copper to reduce infection levels in the first place, and then use prochloraz to attack infections which the copper was not able to stop. If copper is not used to reduce infection levels in the first place, prochloraz won't be able to cope with the high disease pressure, resulting in poor control of anthracnose.

Chemicals alone are not enough. Good orchard hygiene is also essential. It is important to ensure good tree ventilation to allow rapid drying of the foliage after rain. This can be done by pruning lower limbs so that the canopy is at least half a metre above the ground. There appears to be some confusion about this practice, but as a disease control measure it is most beneficial.

It is also important to remove all dead twigs, leaves, branches and diseased fruit from tree canopies, as they encourage the production of spores. Control of fruitspotting bug and fruit fly using recommended practices can also help to reduce preharvest anthracnose which develops around insect injuries on the fruit surface.

In the postharvest situation, apart from applying prochloraz as an overhead spray on the packing line, temperature is a key factor in anthracnose development. Fruit ripened at temperatures of 24°C or higher will develop more anthracnose than fruit ripened at temperatures of 17-20°C. Anthracnose will also have less time to develop if fruit are controlled ripened with ethylene after harvest.

The battle against anthracnose needs to be fought on many fronts by all involved in the production and distribution chain. Because the battle is best fought by preventing infection from occurring in the first place, a major part of the responsibility rests with the avocado farmer. The future of the industry hinges on the production and marketing of high quality fruit, and that means no anthracnose!

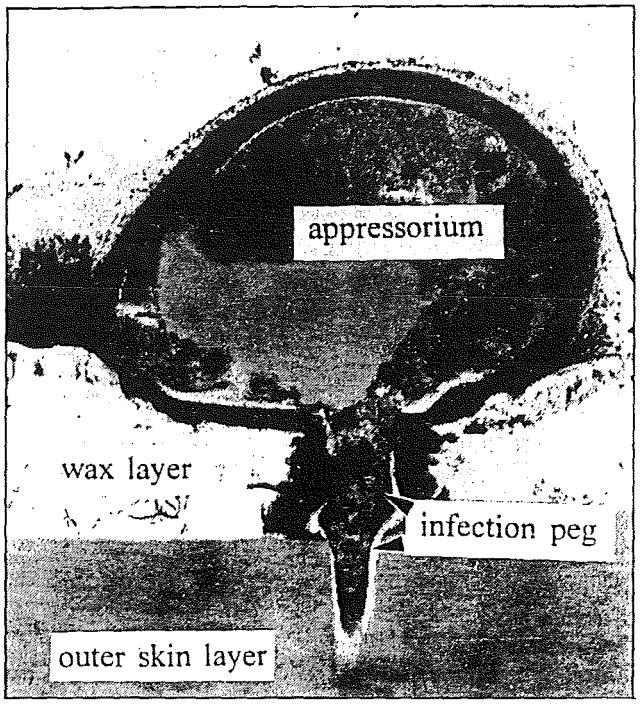


Figure 2 : The infection structure of the anthracnose fungus—an appressorium and infection peg, magnified 10 000 times using the electron microscope. Note that the infection peg becomes embedded in the very thick wax and the outer skin layer. Once infection proceeds to this stage, control is very difficult.

US To Replace Food Safety Standards

From Successful Horticulture March 1994

A food safety package put forward by the Clinton administration may see the Delaney Clause with its zero tolerance standard replaced by a standard based on negligible risk.

The Delaney Clause of the US Food, Drug and Cosmetics Act, written in the late 1950's, has long been criticised as simplistic and outdated.

American scientists say it no longer makes sense, given the improved ability to detect chemical residues at one millionth of the level in the fifties. They say the public would be better served with a tolerance system based on minimal risk.

The current adherence to the Delaney Clause is preventing the EPA from registering new chemicals considered safer than those in use.

Are we winning the post-harvest rot problems?

Derek Smith reports on the technical afternoon held at the Te Puke Research Station on February 17.

The speakers at the research seminar were, from left, Lindy Coates, Bill Hartill and Lise Korsten.

Approximately 35 people attended an excellently organised and presented resume of research findings on post-harvest rots of avocados in South Africa, Australia and New Zealand.

The afternoon was organised and led by industry research manager, Pat Sale. Three guest scientists, Lise Korsten, Department of Microbiology and Plant Pathology, University of Pretoria, South Africa; Lindy Coates, Division of Plant Protection, Queensland Department of Primary Industries and Bill Hartill, consultant to HortResearch, were the keynote speakers.

In opening her first talk of the afternoon, Lise Korsten paid tribute to the HortResearch team she was working with, but made special mention of the research station. Beautiful was the word she used to describe it! In her talk, Lise briefly outlined the main South African avocado growing areas - north and north west - and compared avocado data with that of New Zealand, including weather patterns: rainy season October through to February compared with our October through to September (?) and what it means in terms of fungicide applications. Lise also produced a transparency showing the structure of the industry - where the researchers from the various institutions, the South African Avocado Growers Association (SAAGA), growers and nurserymen fit.

Nurseries produce trees from clonal rootstocks, rooted cuttings and tissue culture techniques.

Circospora spot is a major disease with 60% of diseased fruit being attributed to it.

Lise briefly outlined what she has seen in her short time in New Zealand, mentioning both the bad points and the good. She highlighted areas needing improvement including rootstocks, tree size, production, copper sprays, dipping (wet fruit) and the coolchain. On the plus side she believes the warm air/water trials, sanitation, packhouse accreditation/auditing and quality manual are areas where South Africa could learn from New Zealand.

Lise ended by saying she hoped the collaboration which has come about, working with Kerry Everet, Lindy Coates and others, will not only continue but improve.

New Zealand rot research

Bill Hartill gave an excellent presentation on his research on post-harvest rots, beginning with the three main groups of pathogens which cause the problems; *Collètotrichum* (anthracnose), *Botryosphaeria* and *Phomopsis* (stem end rot).

Bill believes alternatives to dipping have to be found because of the nil tolerance levels for Sportak in some export markets. Trials to date show Benlate and bleach offer some protection, though Sportak is still the most effective for anthracnose control.

Bill also spoke on the phosphonate spray trial which has completed its third year. Summarising, Bill said the current year's trial had not given as promising results as the previous two years' and at this stage no firm recommendation can be given for use of phosphonate in controlling rots.

Bill believes anthracnose is overstated as a problem of New Zealand Hass and more work is needed on neck rots. Anthracnose is more of a problem in thin skinned varieties such as Fuerte, and in Hass is generally only a problem with over-ripe fruit. Results of this work will be published in more detail in the next issue of the *Scientific Research and Technical Supplement.*

Biological control of anthracnose

Lindy Coates gave a very informative presentation on the progress of research on biological control of anthracnose, a joint project involving the Queensland Department of Primary Industries (QDPI) and the University of Queensland with funding from New Zealand's AEC and Agmardt, the Australian Avocado Growers Federation (AAGF) and the Australian Horticultural Research and Development Corporation (HRDC).

The aim of the project is to isolate epiphytic micro-organisms (bacterial and yeasts) from avocado fruit, leaf and flower surfaces and select from these antagonists which inhibit the development of the fungi which cause anthracnose.

Lindy explained that more than a thousand bacteria and yeasts had been isolated of which approximately 10% reduced fungal growth of *Colletotrichum.*

The effect of copper sprays on the micro-organisms is also being investigated. Sampling suggests that copper reduces the number of most types of micro-organisms on the surface of avocado leaves, particularly bacteria.

Lindy said the project has a long way to go, but over the coming months it is hoped to test antagonists in the field. Currently a spray trial is underway, the object being to determine the optimum concentration of antagonists. The effect of added nutrients on the colonisation of antagonists such as urea and molasses on the fruit is also being investigated.

In conclusion, Lindy considered that none of the antagonists will give complete control of anthracnose, but should reduce reliance on the present fungicide treatments.

More on biological control

Lise gave a second talk, also on biological control of avocado disorders. South Africa has only three fungicides registered: copper oxychloride, Benomyl and Sportak, the latter not being approved by the EC.

Lise also highlighted the disadvantages of fungicides; namely environmental, health, pathogen resistance and chemical residues.

She said the European Parliament had voted in favour of banning all post-harvest treatments as soon as possible. The alternatives were: integrated biocontrol, a physical approach, environmental control, bioaugmentation (using antagonists), plant secondary metabolites, stimulated integrated control and host resistance.

Lise then described the current South African research using biocontrol techniques. The most promising antagonist so far has been *Bacillus subtilis* which has significantly reduced both anthracnose and stem end rot. There have been both field and post-harvest trials, the post-harvest trial being *B. sutilis* mixed in with the wax used at time of grading.

While the trials appeared promising, Lise was realistic in her outlook when she said that registration and market acceptability must still be established before their industry can further evaluate this control measure.

A question and answer session on industry matters followed. This was fairly muted, which either goes to show we are a contented grower group or we were completely mentally exhausted by the guest speakers. ❖

Fuerte for Pharaohs?

Egypt eyes avocado culture

By W.H. Brokaw, Director Emeritus, California Avocado Society. Reprinted courtesy of The Avocado Quarterly, the newsletter of the California Avocado Society, Inc.

Interest in avocados is found in many strange places these days, but one rarely associates them with an Arab people in a desert climate. So you can imagine my suprise when invited to consult for three months in Egypt. It seems that the Egyptians (whom I've never seen eat an avocado) want to develop an agricultural industry that will augment the native diet and provide an exportable surplus.

Except for the fact that the native population doesn't recognise avocados, it may not be a bad idea. I wouldn't expect Egypt to compete very effectively with Israel, the Republic of South Africa, or Mexico; but other Arab brothers, if they like avocados, might prefer Egypt as a supplier.

Nilotic Egypt gets hot, never experiences freezing temperatures, is comfortably humid most of the time, and has abundant water. Much of the soil is excellent. Fuerte avocados grow well and bear satisfactorily in a few areas already. Also their Duke trees grow like normal Dukes in California and bear some fruit. Other varieties are not known in the country. Naturally, at this point, Fuerte is their favorite variety.

My job as consultant is to transfer whatever knowledge I might have to improve Egyptian nursery practices, introduce varieties, set up experimental plots and orchards, and impart any other pertinent incidental knowledge that they are in need of.

So far, we've introduced about four new cultivars for them. They've a long way to go in order to make a sound judgement as to what the new industry should look like. I've one more one-month visit to make in order to complete my "contract". It will be principally to introduce some propagation techniques and expand the inventory of varieties.

The very hospitable Egyptian academics and others with whom I associate are very interested in avocados and other (to them) new products. Their chief hurdle, in my opinion, is to transfer knowledge and foster independent judgement among managers and workers in the field. ❖

Third World Avocado Congress

Israel will be hosting the 3rd World Avocado Congress in 1995. Having perused some of the data from the Proceedings of the 2nd World Avocado Congress (held in California in April 1991), I can only support the concept. The information, from every corner of the avocado growing world, is fascinating, if not mind boggling. Murray Carline, an AGA executive member, attended the 91 Congress - and is still wading through the material. ❖

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APPS

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CONFERENCE PROGRAM
AND ABSTRACTS

51. Biological Suppression of Avocado Anthracnose.

A.M. Stirling[a], L.M. Coates[b], A.W. Cooke[b], K.G. Pegg[b] and A.C Hayward[a].

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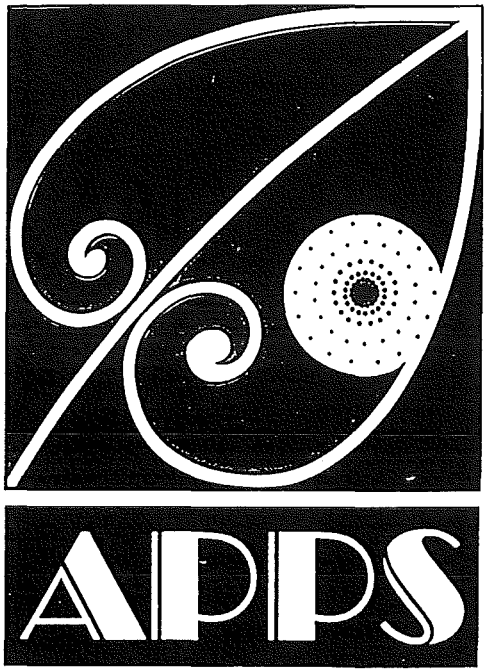
The undesirable side effects of the fungicides used to control *Colletotrichum gloeosporioides* on avocado have prompted investigations into the use of naturally occurring suppressive microorganisms to reduce disease incidence.

Inundative application of mass-reared antagonists: More than 1000 bacteria and yeasts were isolated from leaves, blossoms and fruit and tested *in vitro* for inhibition of mycelial growth and spore germination. Isolates that showed one or both types of inhibition are being tested for suppression of disease on avocado fruit inoculated with *C. gloeosporioides*. Initial results have shown that some yeasts and bacteria prevented lesion development and reduced lesion size.

Enhancement of naturally occurring antagonists: Monitoring of populations of phylloplane microflora on avocado trees frequently sprayed with copper-based fungicides or left unsprayed indicated that numbers of filamentous fungi, yeasts and bacteria were reduced by copper, with the effect on bacteria being the most significant. Experiments will be conducted to determine whether disease control is improved when spray schedules are modified to maintain the phylloplane microflora. Fast growing epiphytic microorganisms which compete with the pathogen could possibly be enhanced by spraying avocado trees with nutrients. The effect of spray treatments such as urea and molasses on phylloplane microorganisms are therefore under investigation.

Key Words: biological control, bacteria, yeasts, fungicides.



10th Biennial Conference
Lincoln University
New Zealand
28-30 August 1995

10th Biennial Australasian Plant Pathology Society Conference

Scientific Programme and Abstracts

Lincoln University
(near Christchurch)
NEW ZEALAND

28 - 30 August 1995
(Workshops 23 - 26 Aug & 31 Aug - 2 Sept)

131. The effect of preharvest pesticide sprays on microbial suppression of avocado anthracnose

Stirling, A.M.[1], *Pegg, K.G.*[2], *and Hayward, A.C.*[1]
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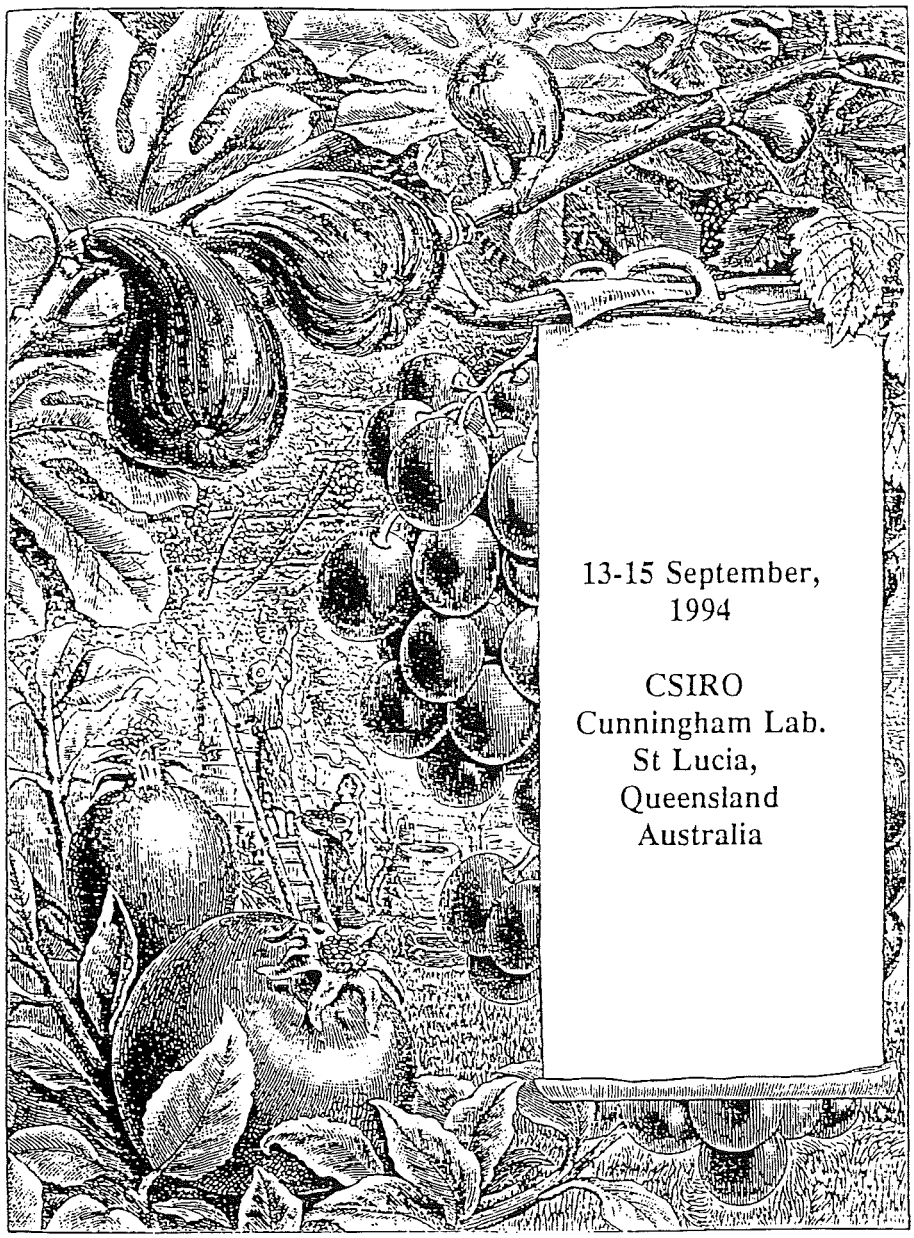
[2] Plant Protection Unit, Department of Primary Industries, Meiers Road, Indooroopilly, QLD 4068, Australia

Anthracnose caused by *Colletotrichum gloeosporioides* is the main disease of avocado fruit in Australia. Disease control strategies involve regular preharvest copper fungicide sprays and the use of insecticides to reduce damage from fruit fly and spotting bug. However, disease levels in sprayed orchards are sometimes high while some unsprayed orchards have relatively little anthracnose. The possibility that pesticides were detrimental to organisms that may have been suppressing the pathogen prompted an investigation on the effect of pesticides on non-target microorganisms on avocado leaves and fruit. Two adjacent orchards (cv Hass) were selected at Maleny, Queensland. One was sprayed with insecticides and copper at monthly intervals from October to April whereas the other had not been sprayed for at least 7 years. Four days after pesticides were applied to the treated orchard, populations of microorganisms on individual leaves (48 per orchard) and fruit (30 per orchard) were determined in both orchards. Samples were also taken during winter when pesticides were not applied. Population densities of filamentous fungi, yeasts and bacteria in the sprayed orchard were 10 to 100- fold lower than in the unsprayed orchard. However, populations in the sprayed orchard recovered during the winter non-spray period to levels comparable with the unsprayed orchard. In 1993 and 1994, fruit were harvested from both orchards, ripened and assessed for anthracnose and stem-end rot. In both years there was significantly less disease in the unsprayed orchard than in the sprayed orchard.

Biological Control of Fruit Diseases

A Three-Day Workshop on Research, Regulation and Registration of Biological Control Agents for Use Against Fruit Pathogens

Program and Abstracts



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Are copper fungicide sprays detrimental to friendly microorganisms on the avocado phylloplane?

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[A] Department of Microbiology, The University of Queensland, Q 4072
[B] Plant Protection Unit, Queensland Department of Primary Industries, Meiers Road Indooroopilly Q 4068

Anthracnose caused by *Colletotrichum gloeosporioides* is the main disease of avocado fruit in Australia and copper fungicides are sprayed regularly to achieve control. However, disease levels in some orchards remain high despite the regular application of preharvest copper sprays. We have also observed that some unsprayed orchards have relatively little anthracnose. The possibility that copper was detrimental to organisms that may have been suppressing the pathogen prompted an investigation on the effect of copper fungicide sprays on non-target microorganisms on avocado leaves and fruit. Two adjacent orchards (cv Hass) were selected at Maleny, Queensland. One was sprayed with copper fungicides by the grower at monthly intervals from October to April each year whereas the other had not been sprayed for at least 7 years. Four days after each copper spray was applied to the treated orchard, 48 leaves (8 leaves from each of 6 trees) were sampled from each orchard. Populations of filamentous fungi, yeasts and bacteria on individual leaves were determined by a plate-dilution frequency technique and the most probable number method. Samples were also taken in winter when copper sprays were not applied to either orchard. In addition, fruit were harvested soon after spraying in December, January, March and in July (three months after the last spray) and populations of microorganisms on the fruit surface were enumerated as above.

Copper significantly reduced the populations of filamentous fungi, yeasts and bacteria on avocado leaves and fruit but was most detrimental to bacteria. Population densities of microorganisms in the sprayed orchard were 10 -fold to a 100- fold lower than in the unsprayed orchard. However, populations in the sprayed orchard recovered during the winter non-spray period to levels comparable with the unsprayed orchard.

In 1993 and 1994, fruit were harvested from both orchards, ripened and assessed for anthracnose and stem end rot. Each fruit was divided into quarters and rated for disease severity using a 5 point rating scale (0 = no rotting to 4 = 75-100% of fruit rotten). In both years there was significantly less disease in the unsprayed orchard compared with the sprayed orchard. The disease ratings for fruit in the unsprayed and sprayed orchards in 1993 were respectively 1.13 and 1.83, whereas for 1994 they were 0.26 and 1.18.

Biological control of avocado anthracnose using phylloplane microorganisms

L.M. Coates[1], A.M. Stirling[2], A.W. Cooke[1] and K.T. Cannon[1]

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2. Department of Microbiology, The University of Queensland, St Lucia Q 4072

Epiphytic bacteria and yeasts isolated from avocado fruit and leaf surfaces were screened for *in vitro* and *in vitro* inhibition of *Colletotrichum gloeosporioides* and *C. acutatum*, the causal agents of avocado anthracnose. Isolates which caused the greatest degree of lesion inhibition in detached avocado fruit were selected for further study. Both pre- and post-harvest applications of promising isolates are currently being evaluated for anthracnose control.

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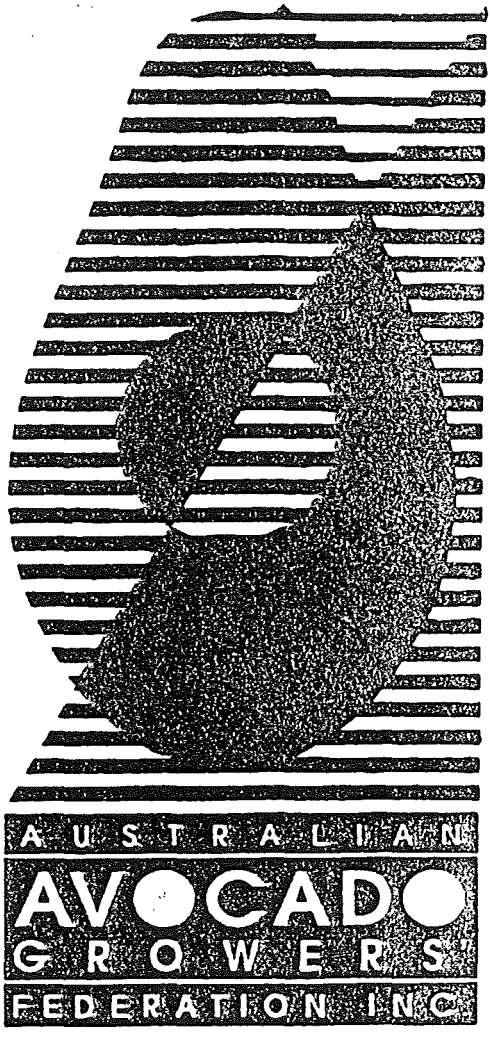

Your Federation - Your Future

BIOLOGICAL SUPPRESSION - AN ALTERNATIVE APPROACH FOR CONTROLLING AVOCADO ANTHRACNOSE

A.M. Stirling[1], L.M. Coates[2] and A.W Cooke[2]
[1] Department of Microbiology, The University of Queensland
[2] Division of Plant Protection, QDPI

Fungicide resistance, lack of adequate replacement fungicides and concerns about the adverse effects of pesticides in the environment have prompted interest in alternative methods for control of fungal plant pathogens.

Epiphytic bacteria, yeasts and filamentous fungi occur on leaves, flowers, twigs and fruit and there is considerable interest in the use of these organisms for biological control of fungi that are pathogens of aerial plant surfaces (Blakeman and Fokkema, 1982; Wisniewski and Wilson, 1992). These epiphytic microorganisms can be used as biological control agents in two basic ways. Firstly, management practices can be modified to enhance the performance of naturally occurring beneficial species. For this to be successful, a knowledge of the ecology of these microorganisms is essential. Secondly, microorganisms can be mass produced and introduced to combat a pathogen at a specific site. So far most of the successes in biological control have been with introduced antagonists. For instance there are several examples illustrating the potential of microorganisms for the biological control of Penicillium sp. in citrus fruit and similar microorganisms have also shown promise in controlling brown rot of stone fruit, grey mould of grapes and pears and blue mould of apples.

BIOLOGICAL CONTROL OF ANTHRACNOSE: CURRENT KNOWLEDGE

There has been little research into the use of biological control agents against Colletotrichum gloeosporioides. This pathogen has a wide host range and is responsible for a number of diseases, including anthracnose of mango and avocado. In mango, it causes damage to leaves, flowers and young fruit and also causes heavy losses due to post-harvest rotting. In avocado, the fungus may be present on twigs and leaves but disease symptoms usually develop only on fruit. In both avocado and mango, infection may occur on immature fruit, but the fungus lies dormant and disease development occurs as the fruit ripens.

Research by Dr. Irene Koomen and co-workers at the University of Kent on anthracnose of mango has shown that a bacterium (Pseudomonas sp.) applied as a post-harvest treatment was able to reduce disease on fruit (Koomen, et al. 1990). In South Africa, Bacillus spp. isolated from avocado leaves reduced anthracnose of avocado when applied as pre-harvest sprays and post-harvest fruit dips (Korsten, et al. 1989).

RESEARCH IN QUEENSLAND

In 1992, a research project was commenced which aimed to evaluate the role of naturally occurring epiphytic microorganisms in the suppression of anthracnose in avocado. The work is a collaborative project between the University of Queensland and the Queensland Department of Primary Industries and encompasses four research areas.

1. Monitoring epiphytic microbial populations in the avocado phyllosphere

This work aims to identify the dominant microorganisms in the avocado phyllosphere and to study their population dynamics, particularly in relation to weather conditions and crop growth cycles. Fungicides can be detrimental to epiphytic microorganisms and may alter the biological equilibrium of the avocado phyllosphere. The impact of copper fungicides used in avocado orchards in interfering with naturally occurring biological control of anthracnose will therefore be determined. Two adjacent orchards, one sprayed and the other unsprayed, will be sampled intensively from September 1992 to June 1993. Leaf samples will be assessed for changes in populations of microorganisms and for the development of copper tolerant strains.

2. Isolation and evaluation of potentially useful biological control agents.

This work has already commenced and over 900 isolates (bacteria and yeasts) have been selected from leaves and mature avocado fruit in south eastern Queensland. Isolations from avocado flowers and immature fruit are ongoing. About 100 of these microorganisms reduced mycelial elongation of C. gloeosporioides to varying degrees in artificial medium. Approximately 34 isolates also reduced spore germination by 80-100% on cellophane overlaying a simulated leaf diffusate medium. The isolates that produced either or both forms of inhibition of the fungus are presently being screened for their ability to reduce C. gloeosponoides infections on detached avocado fruit. Preliminary tests have shown that some isolates were able to retard lesion development and reduce lesion size on fruit. Once a selection of candidate antagonists have been identified, field trials will be carried out to test the antagonistic potential of the organisms in pre- and post-harvest situations.

3. Mechanisms of fungal suppression

Understanding the mechanisms of pathogen suppression by epiphytic antagonists is important in order to exploit the full potential of these antagonistic microorganisms. This knowledge is also important when evaluating their commercial potential. For instance an organism that produces an antibiotic may be unacceptable as a post-harvest treatment but may be useful in preventing the pathogen from establishing as a latent infection prior to harvest. Factors such as the effect of nutrients on the rate and duration of antibiotic production will be studied as it may then be possible to manipulate antibiotic producers to ensure maximum biological control activity. Antagonists with other modes of suppression (eg. nutrient competition, site exclusion, attachment to the pathogen, induced host resistance and direct parasitism) will also be studied because such antagonists are preferred for post-harvest treatment.

4. Colonisation potential and survival of antagonists in the phyllosphere

For pathogen suppression to be successful, it is important to select an antagonist that can survive and maintain itself in the habitat to which it is introduced. The duration of survival will determine the frequency of application of the antagonist during the fruiting season. The colonisation and survival of selected antagonists tagged with detectable markers (eg. antibiotic resistance) will be studied on avocado trees in the glasshouse and the field.

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THE BIOLOGY AND CONTROL OF AVOCADO ANTHRACNOSE

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Despite the widespread use of fungicides throughout the Australian avocado industry, anthracnose continues to be a major impediment to the marketing of quality avocado fruit. Current control recommendations need to be strictly followed in order to minimise both field and postharvest losses due to this disease, particularly in susceptible cultivars such as Fuerte.

DISEASE BIOLOGY

Although anthracnose is predominantly a postharvest disease, symptoms may also develop in fruit prior to harvest. Symptoms which develop before harvest take either of *2* forms:

(1) large spreading lesions resulting from infection through insect or mechanical skin injuries. Fruit with these symptoms are usually shed before reaching maturity.

(2) small disfiguring spots up to 5mm in diameter which sometimes occur around fruit lenticels.

Symptoms of anthracnose which develop after harvest first appear as small brown spots on the surface of fruit. During ripening these spots enlarge rapidly, become sunken in the centre, and change colour to dark-brown or black. Under humid conditions, characteristic salmon-coloured spore masses are produced on the surface of diseased areas.

A detailed understanding of the life cycle of the fungus which causes anthracnose *(Colletotrichum gloeosporioides)* is essential for the development of appropriate disease control strategies.

Spores of the anthracnose fungus are produced in large numbers on dead twigs, leaves and diseased fruit hanging on the tree. During showery weather these spores are spread to developing fruit where under, the right conditions of temperature and moisture availability, they may germinate. Within 24 hours, germinated spores produce a resistant infection structure called an appressorium which becomes firmly attached to the skin surface. The appressorium then germinates to produce an infection peg which pierces the cuticle of the fruit peel. This infection peg stops growing in the cuticle where it remains dormant until fruit ripening. During ripening, the dormant fungus resumes growth and colonises first the peel and then the pulp of fruit. In advanced stages of tissue colonisation, fruiting bodies are produced on the surface of disease lesions. Spores produced in these fruiting bodies are spread by water, and so the life cycle of the fungus is completed.

In the case of damaged fruit, the fungus does not remain dormant until ripening. Instead, the fungus immediately colonises the peel and pulp tissue of unripe fruit, resulting in large spreading lesions which eventually cause premature fruit fall.

CURRENT DISEASE CONTROL RECOMMENDATIONS

Pre-harvest

Because the initial stages of fruit infection by the anthracnose fungus occur in the orchard, field control is an important component of the total disease control strategy. To protect developing fruit from anthracnose infection, spray with copper oxychloride or copper hydroxide at intervals of 28 days from fruit set to harvest. During wet weather, the spray interval should be reduced to 14 days. It is very important to adhere to these guidelines. Missing one spray could result in high levels of anthracnose. It is also important to ensure good coverage of copper on both fruit and foliage.

Because anthracnose infection is favoured by humid conditions, ensuring adequate tree ventilation can also help to reduce infection levels. To improve tree ventilation, prune the lower limbs of trees so that the canopy is at least 50cm above the ground. Dead branches, twigs and leaves entangled in the tree canopy and infected fruit hanging on the tree should be removed at regular intervals as they may harbour large numbers of spores. Controlling insect pests such as fruitspotting bug and Queensland fruit fly will help to reduce fruit damage and therefore reduce the incidence of preharvest anthracnose lesions.

Postharvest

Control of anthracnose after harvest is achieved by a combination of fungicide treatment and attention to correct storage and ripening temperatures.

Prochloraz (Sportak®) is the currently recommended fungicide for postharvest control of anthracnose. Prochloraz is applied to harvested fruit as a nonrecirculated spray on the packing line for a duration of 30 seconds. This treatment is only effective if good field control has already been achieved.

Fruit storage and ripening temperatures have a major influence on the development of anthracnose. Hard, green fruit can be stored at temperatures as low as 7°C without risk of chilling injury. Fruit starting to ripen, however, are more susceptible to chilling injury and therefore need to be held at higher temperatures. To promote uniformity of ripening, fruit should be treated with ethylene for 2 days at a temperature within the range of 16-20°C. Following ethylene treatment, fruit should be ripened at 20°C as temperatures of 24°C and above will accelerate the development of anthracnose.

FUTURE DISEASE CONTROL PROSPECTS

The avocado industry relies heavily on the use of fungicides for anthracnose control. The future availability of these fungicides, however, is threatened by increasing worldwide concerns over the use of pesticides in agriculture. The continuing availability of postharvest chemicals such as prochloraz are particularly at risk because they are applied close to the time of consumption.

We are currently investigating alternatives to fungicides for the control of avocado anthracnose in a collaborative research project between QDPI and the University of Queensland. The project, which is jointly funded by the AAGF, the New Zealand Avocado Export Council and HRDC, aims to develop biological control for anthracnose in avocado.

The broad objectives of the project are to:

1. Isolate and identify naturally occurring non-pathogenic microorganisms from the surface of avocado fruit, leaves and stems.

2. Evaluate these isolates for biocontrol of anthracnose, and

3. Develop promising biocontrol agents for both preharvest and postharvest control of anthracnose.

Although biocontrol shows considerable potential for the control of avocado anthracnose, it is unlikely to give absolute control in highly susceptible cultivars such as Fuerte. Some limited use of chemicals in association with biological control may still be necessary. In addition, the industry may need to consider the gradual phasing out of highly susceptible cultivars.

Australian Avocado Growers' Federation Inc.

Esplanade Hotel, Fremantle 30 April to May 3 ,1995

BIOLOGICAL CONTROL OF AVOCADO ANTHRACNOSE

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Introduction

Anthracnose, caused by the fungi *Colletotrichum gloeosporioides* and *C. acutatum*, is the major cause of postharvest loss in avocado. Control of this disease is currently achieved by a combination of preharvest and postharvest fungicide application, orchard hygiene, and postharvest temperature management. Growing community concern over the use of pesticides in agriculture have prompted the need for research into the development of non-chemical alternatives to pesticides. Aware of this concern, the AAGF in association with the New Zealand Avocado Export Council and HRDC, have funded a joint DPI-UQ project on the biological control of avocado anthracnose since 1992. The aim of this paper therefore is to present the current findings of the project. A final report will be submitted to HRDC by December 1995.

Isolation and selection of antagonists

Potential biological control agents (bacteria and yeasts) were isolated from the surface of avocado leaves, fruit and flowers. Isolates were purified, stored and then screened for inhibition of the anthracnose fungi.

In vitro screening: Each isolate was tested for inhibition of fungal growth of *C. gloeosporioides* on artificial growth media. A fungal plug of *C. gloeosporioides* was placed at the centre of an agar plate, and each antagonist was spotted 40mm away on opposite edges of the plate. Fungal inhibition was measured after incubation at 25°C for 9 days.

Two types of inhibition were observed on artificial growth media. The first type was seen as a distinct zone of no growth between the fungus and the test isolate (zonal inhibition). The second type of inhibition was where the fungus did not overgrow the test isolate despite the absence of a distinct inhibition zone (non-zonal inhibition). A large proportion of the yeasts tested showed non-zonal inhibition of *C. gloeosporioides*, whereas zonal inhibition was more commonly exhibited by bacteria (Table 1). Approximately 37% of all isolates tested showed some form of fungal inhibition.

Most of the isolates which inhibited fungal growth in some way were then tested for inhibition of spore germination of *C. gloeosporioides* on artificial growth media (Table 2). Approximately 23% of all isolates tested reduced germination of *C. gloeosporioides* spores by 80% or more. The most promising isolates were then screened against a New Zealand isolate of *C. acutatum*. Approximately 58% of isolates showed some form of fungal growth inhibition, although only 6% of isolates reduced spore germination of *C. acutatum* by 80% or more (Tables 3 and 4).

Selected isolates were also screened for inhibition of *C. gloeosporioides* on avocado leaf disks. Unlike on artificial growth media where several bacteria and yeasts reduced spore germination, only nine bacterial isolates reduced spore germination on avocado leaf disks by 80% or more.

Screening on detached avocado fruit: A total of 76 bacteria and 48 yeasts were screened against both *C. gloeosporioides* and *C. acutatum* on detached avocado fruit cv. Fuerte. Each antagonist was applied to the surface of either normal-size or cocktail-size avocado fruit, which were then allowed to air dry before inoculation with *C. gloeosporioides* or *C. acutatum*. Fruit were incubated at 25°C and assessed for lesion development when ripe.

Tables 5 and 6 list the twelve most effective bacteria and yeasts against both *C. gloeosporioides* and *C. acutatum*. Only three bacteria and five yeasts were effective against both pathogens.

Preharvest application of selected antagonists

After considering all of the data from the screening assays, a small group of antagonists were selected for further study. Two bacteria (isolates 359 and 480) and two yeasts (isolates and Q34) were chosen for a field trial on a young 'Hass' avocado orchard in south-east Queensland.

Trees were sprayed at four-weekly intervals from October 1993 to August 1994 with diluted liquid cultures of each antagonist. Two controls consisting of 'water only' and 'diluted culture media only' were included in the trial, as was a four-weekly copper hydroxide (Kocide) treatment. In August 1994, all fruit from experimental trees were harvested, ripened at 24°C and assessed for disease.

All treatments, including the 'media only' control, significantly reduced anthracnose severity in comparison to the 'water only' control (see Table 7). The yeast antagonist Q6 significantly reduced anthracnose severity in comparison to both controls ('water only' and 'media only'). Stem end rot was not significantly reduced by any of the treatments.

The finding that anthracnose was reduced to some extent by spraying with diluted media only may be attributable to a stimulation of naturally-occurring beneficial microorganisms on avocado fruit and leaf surfaces. Further work is needed to clarify the role of nutrients in disease suppression.

A follow-up field trial is currently in progress at the same field site. The best performing antagonists from the first field trial (Q6 and 359) are being retested, in addition to a new antagonist (Q47). Chemical treatments consisting of four-weekly sprays of copper hydroxide (Kocide) or phosphorous acid (Fos-Ject) have also been included in the trial.

Table 7. The effect of monthly preharvest spray applications of bacterial and yeast antagonists on postharvest disease levels in avocado fruit cv. Hass

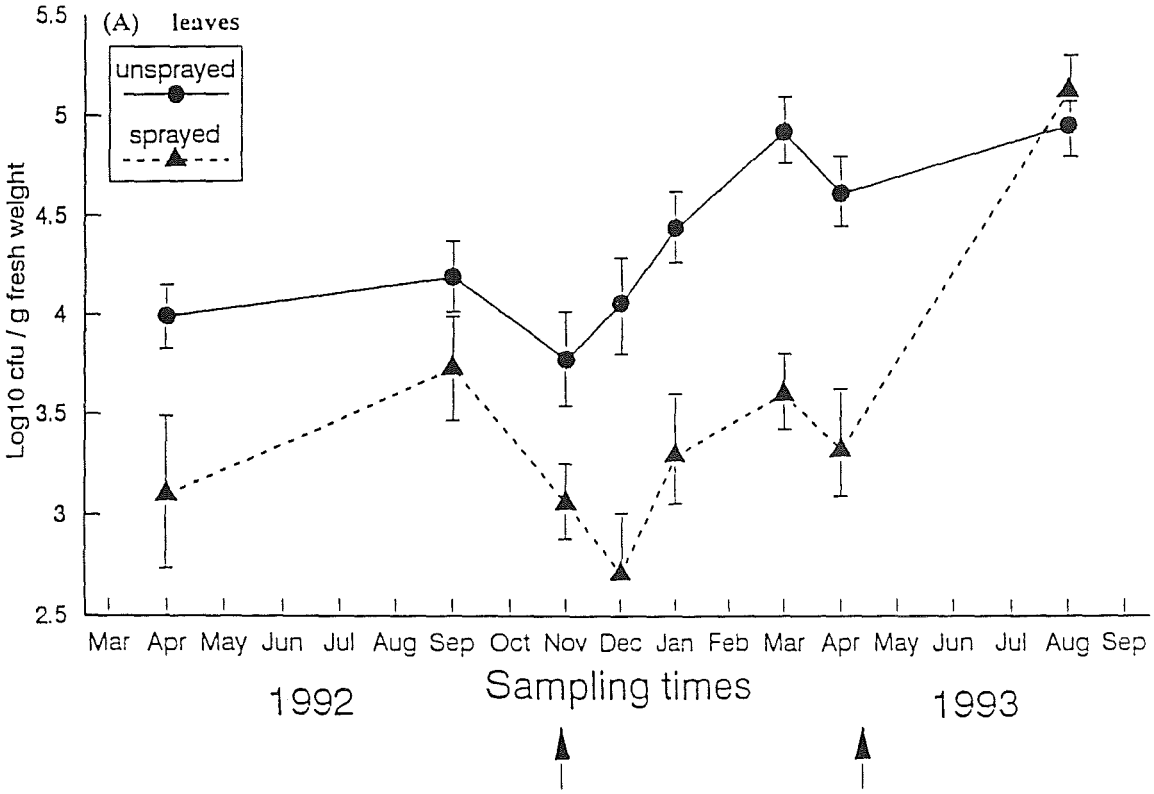
Treatment	Marketable fruit (%)[1]	Anthracnose (% area affected)	Stem End Rot (% area affected)	Ripening Time (days)
Water only	18.2 c[2]	36.7 a	0.7 a	13.1 a
Diluted media only	36.2 bc	22.2 b	3.8 a	13.1 a
Isolate 359	54.4 ab	13.2 bc	1.6 a	12.5 a
Isolate 480	44.8 ab	21.8 b	3.4 a	11.8 a
Isolate Q6	61.8 a	8.1 c	2.6 a	11.1 a
Isolate Q34	47.1 ab	17.0 bc	2.3 a	11.8 a
Copper hydroxide	54.7 ab	14.2 bc	1.5 a	12.2 a

1. % Marketable fruit = fruit with 5% or less disease
2. Means followed by the same letter do not differ significantly at P = 0.05

Table 8. Colonisation of avocado leaves by selected antagonists

Antagonist	Antagonist numbers on leaf surface (Mean $\log_{10}$ cfu g^{-1} fresh weight leaf)		
	Day 1	Day 30	Day 60
Bacillus sp. 359R1	6.9	5.7	5.1
Bacillus sp. 480R2	7.3	6.7	4.9
Enterobacter agglomerans 632R2	7.1	3.1	NS[1]
Pseudomonas fluorescens 677R1	7.4	ND[2]	NS
Aureobasidium sp. 274C1	6.8	5.4	4.9
White yeast 772C1	6.7	6.3	6.1
Pink yeast 734C2	7.0	6.6	5.8
Pink yeast Q34	7.3	4.5	NS

[1] NS = Not sampled [2] ND = None detected



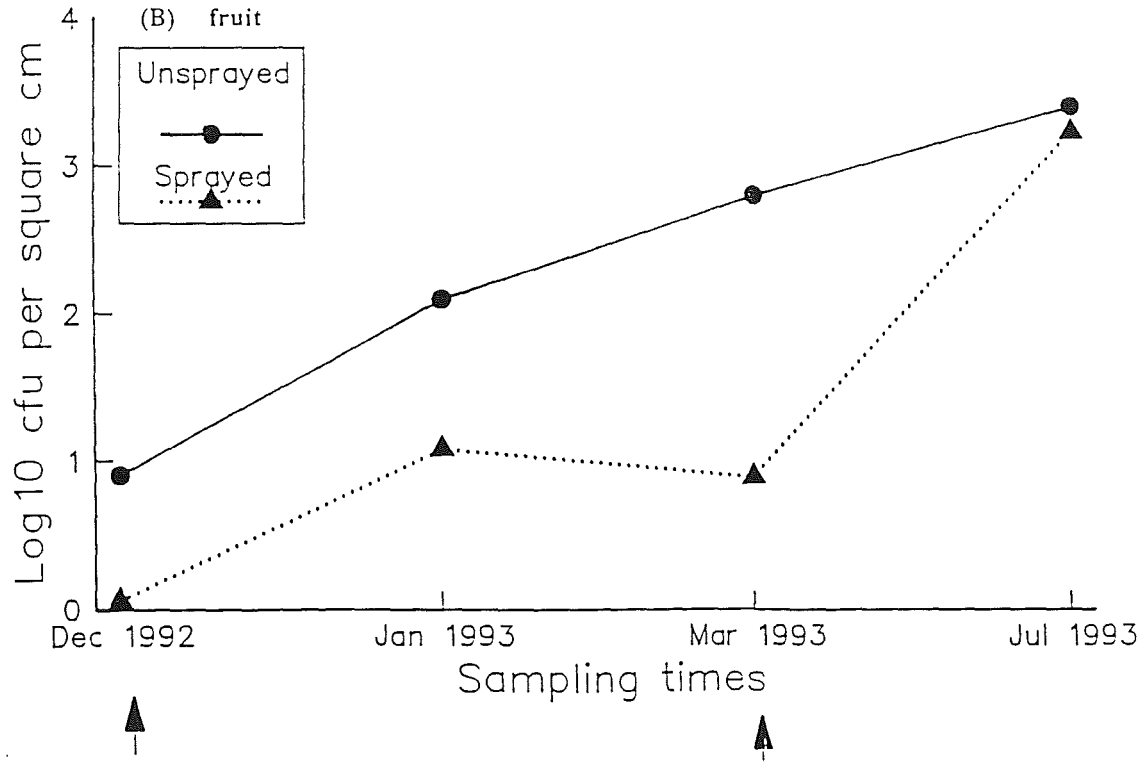


Figure 1: The effect of copper fungicide sprays on the population of bacteria residing on the surface of Hass avocado: (a) leaves (b) fruit

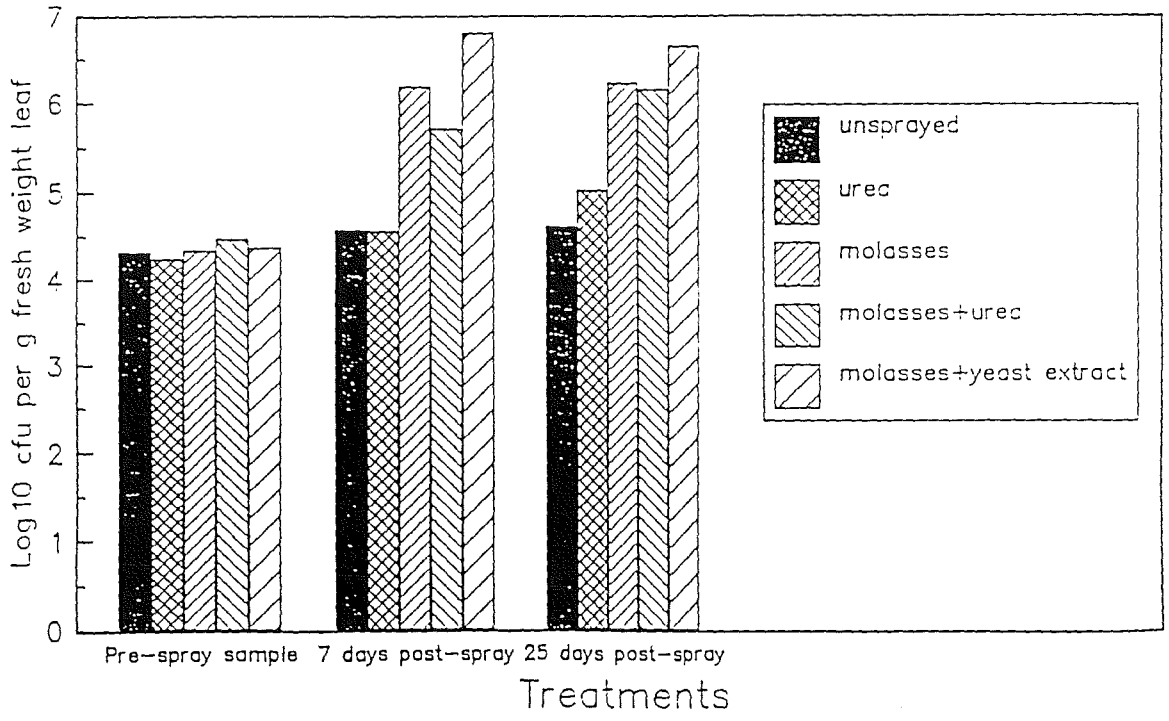


Figure 2: The effect of nutrient sprays on the population of yeasts residing on the surface of Hass avocado leaves

Appendix 3

Other Associated Scientific Publications

Mycol. Res. 97 (11): 1363–1370 (1993) *Printed in Great Britain*

Initial infection processes by *Colletotrichum gloeosporioides* on avocado fruit

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Infection of avocado fruit by *Colletotrichum gloeosporioides* was studied using light and transmission electron microscopy. In unripe fruit *C. gloeosporioides* produced an appressorium and an infection peg which ceased growth in the cuticle. In field-inoculated avocado fruit sampled up to 4 d after inoculation, most infection pegs had penetrated the fruit cuticle to a depth of less than 1·5 μm. In fruit inoculated after harvest, however, the majority of infection pegs had grown down to the subcuticular region of the fruit peel within 48 h of inoculation. There was no further development of these infection pegs until the climacteric rise in respiration when fruit produced amounts of CO_2 in excess of 50 ml CO_2 kg^{-1} h^{-1}. When the fungus resumed growth, infection pegs enlarged either within the walls or within the lumen of epidermal cells. Subsequent intracellular and intercellular development of the fungus resulted in the rapid degradation of cell wall and membrane structural integrity, although cells were not killed in advance of invasion during these early stages of colonization.

Anthracnose, caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., causes serious losses of ripe avocado (*Persea americana* Miller) fruit. Although symptoms of the disease are not usually expressed until fruit ripening, the initial stages of infection occur when fruit are still attached to the tree. Binyamini & Schiffmann-Nadel (1972) reported that *C. gloeosporioides* remains latent on the surface of attached avocado fruit as appressoria. During fruit ripening, they observed the production of thin infection pegs beneath appressoria. These infection pegs penetrated the cuticle and the epidermis of the avocado peel. In contrast to these findings, Prusky, Plumbley & Kobiler (1991) reported that appressoria of *C. gloeosporioides* germinated on unripe avocado fruit to produce infection pegs which penetrated the cuticle to the underlying epidermal cells within 72 h of inoculation, with no further development of the fungus occurring until fruit ripening.

Very few studies have described the ultrastructure of the penetration process of *C. gloeosporioides* in fruit tissue. In a classical ultrastructural study of anthracnose in cv. Robinson tangerines, Brown (1977) found that infection pegs of *C. gloeosporioides* grew through the cuticle of fruit until they approached the wall of an underlying epidermal cell, at which point further development followed one of three patterns. Most commonly, infection pegs (approx. 0·5 μm diam.) enlarged to form hyphae approx. 1–2 μm in diameter, either between or within the wall or the lumen of epidermal cells. These hyphae enlarged further to form hyphae approx. 3–5 μm in diameter, which continued to grow throughout the peel. In some cases, infection pegs enlarged between the cuticle and the epidermal cell wall rather than in the wall or lumen of epidermal cells. Such hyphae grew subcuticularly and intercellularly between and below the epidermal cells. The least common method of penetration observed by Brown (1977) was when the infection peg enlarged directly into a hypha approx. 3–5 μm in diameter either between two epidermal cells or within the lumen of an epidermal cell. Brown (1977) also reported that intracellular hyphae of *C. gloeosporioides* did not kill host cells in advance of penetration or cause severe disruption of invaded cells. It was noted that in the early stages of penetration, hyphae were surrounded by the host plasma membrane and an intervening appositional layer.

Throughout this paper, the term 'primary' hypha has been used to describe the first formed hypha from the infection peg of *C. gloeosporioides*. Brown (1977) used the term 'primordial' or 'large' hypha, depending on the size of the structure, to describe the first formed hypha. The meaning of the term 'primary' hypha in this paper should not be confused with that of the term 'primary' hypha which is used to describe intracellular hyphae of *C. lindemuthianum* which grow from infection vesicles produced inside the lumen of epidermal cells (O'Connell, Bailey & Richmond, 1985).

The purpose of the following study was to re-examine in detail the infection process of *C. gloeosporioides* in avocado fruit using both light and transmission electron microscopy. Special emphasis was placed on determining the form and location of latent infection structures.

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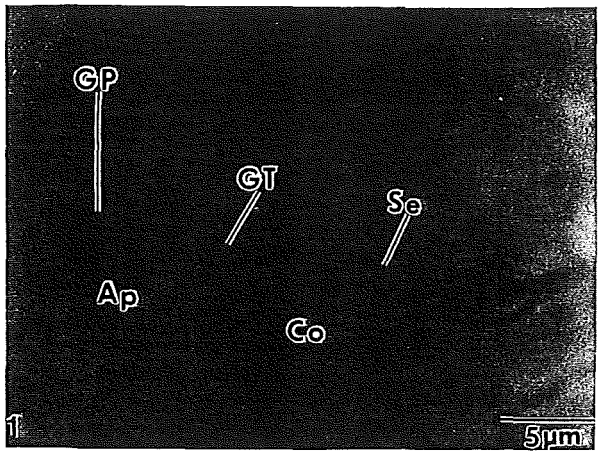
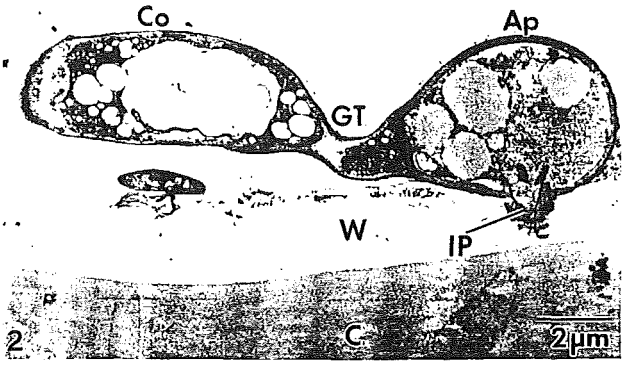
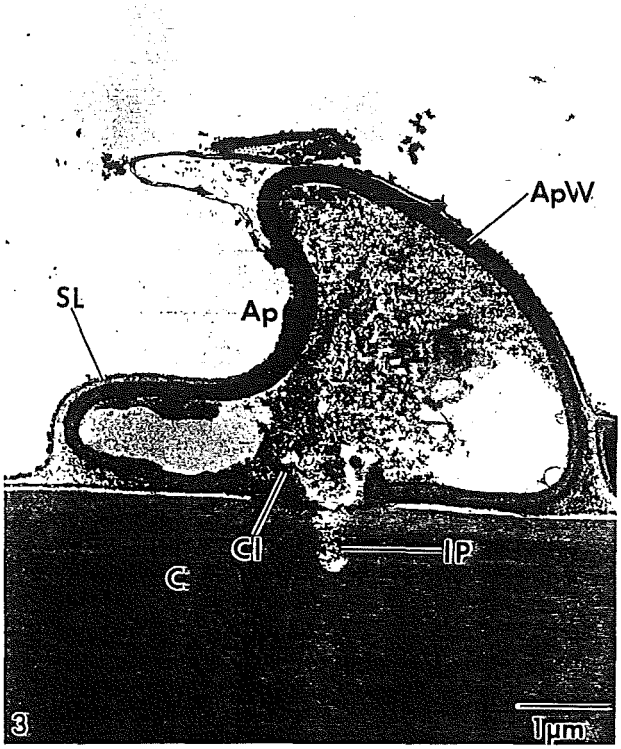
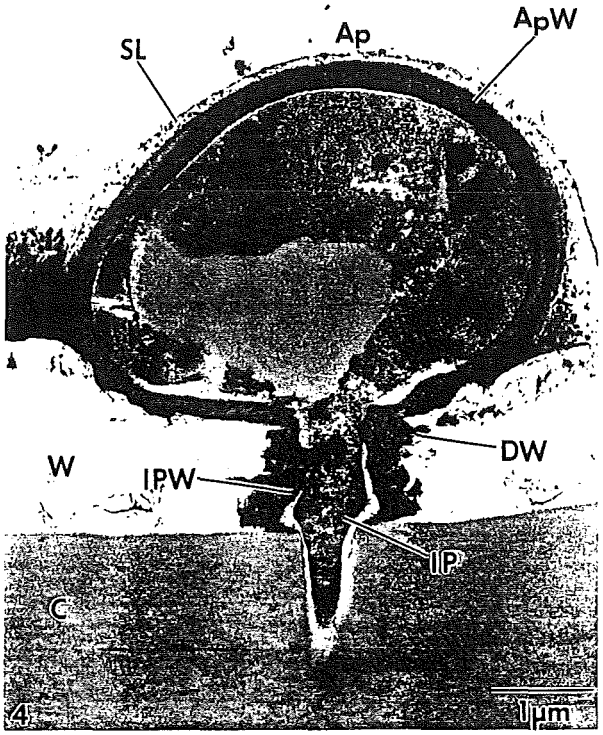
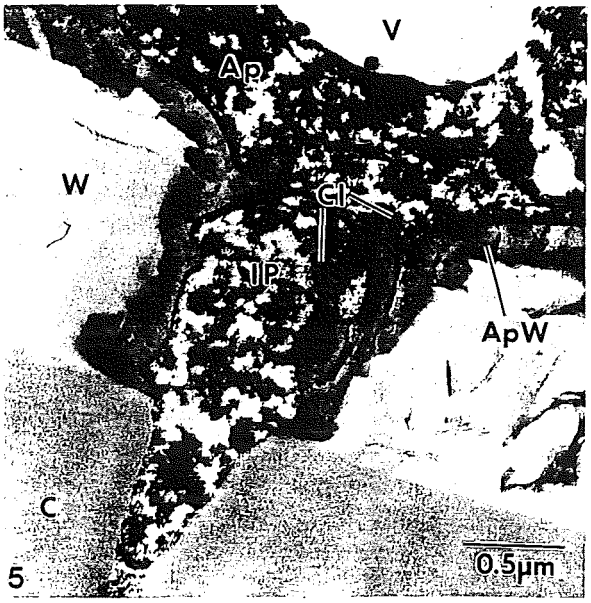










Figs 1–6. For legend see opposite.

MATERIALS AND METHODS

Preparation of inoculum

Conidia for fruit inoculations were obtained from 7-d-old cultures of *C. gloeosporioides* (BRIP 19779 or 19772) growing on oatmeal agar. Suspensions of conidia in sterile distilled water were filtered through muslin to remove most of the mycelial fragments and adjusted to a concentration of 10^6 conidia ml^{-1} unless otherwise stated. Tween-20 wetting agent was added to conidial suspensions at a rate of 0·35 ml l^{-1}.

Fruit inoculation

The cultivar Fuerte was used in all experiments. Prior to inoculation, fruit were wiped with 70% (v/v) ethanol to reduce the epiphytic microflora. Square pieces of sterile blotting paper were saturated in the prepared suspension of conidia and placed onto an area of unwounded fruit peel. Waterproof tape was used to secure the paper squares to the fruit surface. Fruit inoculated in the field in this manner were enclosed in plastic bags for 48 h in order to maintain a high humidity around the inoculation site. Detached fruit inoculated in the laboratory were placed in a humid chamber and held at 25 °C. After 48 h paper squares and waterproof tape were removed from both field-inoculated and laboratory-inoculated fruit.

Tissue preparation for light microscopy (LM)

Tissue for LM was prepared by either of the following methods:

(*a*) Peel slices approximately 200 μm thick, 3 mm long and 1 mm wide were excised from the inoculated areas of fruit. Tissue pieces were fixed in 3% (v/v) glutaraldehyde in 0·1 M phosphate buffer (pH 7), post-fixed in 1% (w/v) osmium tetroxide in 0·1 M phosphate buffer, dehydrated in a graded acetone series and embedded in Spurr's resin. Semi-thin sections approximately 2–3 μm thick were cut with a glass knife on a LKB ultramicrotome and stained at 80° with 1% toluidine blue in 1% (w/v) sodium tetraborate.

(*b*) Peel slices approximately 100 μm thick, 5 mm long and 4 mm wide were excised from the inoculated areas of fruit. Tissue slices were boiled in a 2:1 mixture of 95% (v/v) ethanol and lactophenol cotton blue according to the method of Shipton & Brown (1962), with the exception that tissue pieces were simmered for 5 min instead of 1 min due to the nature of the tissue. Tissue pieces were left in the stain for several days before clearing in a saturated solution of chloral hydrate for 30–60 min.

Tissue preparation for transmission electron microscopy (TEM)

Tissue strips excised from inoculated areas were fixed, dehydrated and embedded in Spurr's resin as for LM [method (a)]. Ultra-thin sections approximately 60–80 nm thick (light gold-silver) were cut with a Delaware diamond knife onto water using a LKB ultramicrotome. Sections were mounted on uncoated 300-mesh hexagonal grids and stained with a saturated solution of uranyl acetate for 20 min and then lead citrate (Reynolds, 1963) for 1 min. Sections were viewed with a Hitachi H7000 transmission electron microscope.

Histopathology of field infections

All field inoculations were conducted on two neighbouring avocado orchards at Maleny (26° 46′ S, 152° 51′ E), south-east Queensland. Fruit were inoculated at monthly intervals commencing in November when fruit were approximately 1·5–2 cm in length (1 month after fruit set). The final inoculation was conducted in April. Tissue samples were excised from the inoculated areas of fruit for LM and TEM at 24, 48, 72 and 96 h after each inoculation. Inoculated fruit that were not sampled at these times were left on the trees until they reached normal harvest maturity in May, at which time they were harvested and stored at 20°. Tissue samples were taken from these fruit for LM and TEM at various stages of ripeness as judged by fruit firmness. Firmness was measured non-destructively using an Instron universal testing machine model 1122 (12 mm diam. cylindrical probe, crosshead speed 20 mm min^{-1}, probe penetration 2 mm) and expressed as the force required (in newtons) to penetrate 2 mm. Fruit were sampled for LM and TEM at each of the following firmness stages (1) $\geqslant$ 53 N (hard fruit), (2) 40–52 N, (3) 27–39 N, (4) 14–26 N and (5) $\leqslant$ 13 N (soft fruit).

Fig. 1. LM of a germinated conidium and appressorium on the surface of avocado fruit sampled at 48 h after inoculation (surface view). **Fig. 2.** TEM of a germinated conidium and appressorium on the surface of avocado fruit sampled at 48 h after inoculation (sectional view). **Fig. 3.** TEM of an appressorium on the surface of avocado fruit sampled at 3 months after fruit set and 48 h after inoculation. **Fig. 4.** TEM of avocado peel sampled at 7 months after fruit set and 48 h after inoculation showing an appressorium and infection peg which has penetrated the thick wax layer and cuticle of the peel. Note degradation of the wax layer around the infection peg. **Fig. 5.** TEM of an appressorium on the surface of avocado fruit sampled at 7 months after fruit set and 48 h after inoculation. Note double collar of wall material. **Fig. 6.** LM of appressoria and infection pegs produced in the cuticle of mature but unripe fruit (firmness rating of 2).

Abbreviations used in Figures 1–17

Ap	appressorium	DW	degraded wax	HR	host reaction	PH	primary hypha
ApW	appressorium wall	EC	epidermal cell	IAH	intracellular hypha	Se	septum
C	cuticle	ECW	epidermal cell wall	IEH	intercellular hypha	SL	slime layer
Ch	chloroplast	GP	germ pore	IP	infection peg	V	vacuole
Cl	collar	GT	germ tube	IPW	infection peg wall	W	wax layer
Co	conidium	H	hypha	M	mitochondria		

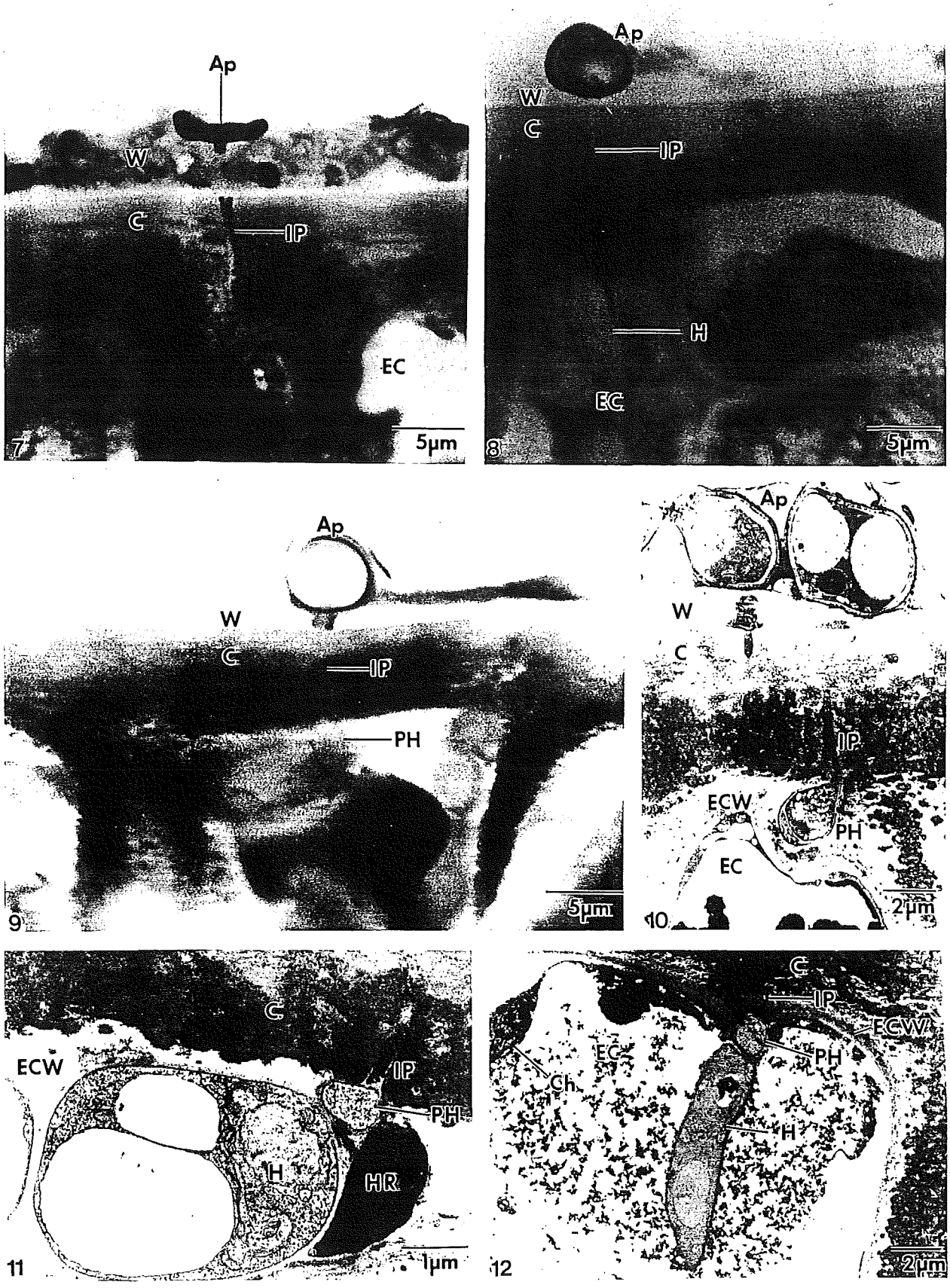


Fig. 7. LM of mature avocado peel sampled at 4 months after inoculation and at a firmness rating of 4 (softening) showing an infection peg which has become detached from the appressorium. **Fig. 8.** LM of mature avocado peel sampled at 4 months after inoculation and at a firmness rating of 5 (soft) showing an appressorium and infection peg which has penetrated an underlying epidermal cell. **Fig. 9.** LM of an appressorium with an infection peg which has enlarged into a primary hypha inside an underlying

Histopathology of postharvest infections

The development of latent infection structures in relation to fruit respiration rates. Twenty-four mature avocado fruit were harvested from an orchard at Maleny and brought to the laboratory where they were inoculated as previously described. Inoculated fruit were incubated at 25° for 48 h under high humidity (⩾ 95% r.h.). At the end of this incubation period, four fruit were removed randomly and tissue slices were excised from within the inoculated areas for LM. The remaining fruit were transferred to individual 1 l respiratory containers for the measurement of carbon dioxide (CO_2) production rates at 20°. Each container was continuously ventilated with ethylene-free, humidified air at a rate of 60 ml min^{-1}. One ml samples were taken from effluent air streams of each container daily using a Hamilton gas-tight syringe. Samples were analysed for CO_2 content using a Shimadzu gas chromatograph model GC-8A equipped with a thermal conductivity detector. Four fruit producing amounts of CO_2 within the range nominated in each of the following categories were removed and tissue slices were excised from within the inoculated areas for LM: (1) ⩽ 38 ml kg^{-1} h^{-1} (preclimacteric minimum), (2) 39–48 ml kg^{-1} h^{-1}, (3) 49–58 ml kg^{-1} h^{-1}, (4) 59–68 ml kg^{-1} h^{-1} and (5) ⩾ 69 ml kg^{-1} h^{-1} (climacteric peak).

Ultrastructure of penetration. Mature avocado fruit were obtained from an orchard at Renmark (34° 10′ S, 140° 45′ E), South Australia and transported to the laboratory within 2 d of harvest. Fruit were inoculated with *C. gloeosporioides* at a concentration of 5×10^6 conidia ml^{-1} using the paper disc method as previously described and then incubated at 25° under high humidity (⩾ 95% r.h.). Tissue samples for LM and TEM were taken from fruit at 48–96 h after inoculation.

RESULTS

Histopathology of field infections

At each inoculation time (November–April), the majority of conidia present on the surface of fruit had germinated to form germ tubes and appressoria within 48 h of inoculation (Figs 1, 2). A septum was visible in all conidia observed. In some cases, a germ tube was produced from each cell in a conidium, although it was rare to find an appressorium produced at the end of each germ tube. In surface view, a central germ pore was visible in all appressoria examined at 48 h after inoculation (Fig. 1). The great majority of appressoria had thick, dark walls by this stage. Only occasionally was a hyaline appressorium observed.

The double-layered wall of the mature appressorium was coated with a mucilaginous substance (Figs 3, 4). The thickness of the epicuticular wax layer of the avocado peel at the time of inoculation was found to have an important bearing on the development of infection structures by *C. gloeosporioides* during the first 48 h after inoculation. In fruit which were inoculated early in development when the epicuticular wax layer was very thin (*ca* 0·1–0·5 μm thick), the great majority of appressoria produced a collar and a short (< 1·5 μm), thin (< 0·5 μm diam.) unmelanised infection peg which came to rest in the cuticle within 48 h of inoculation (Fig. 3). In fruit which were inoculated later in development when the wax layer was thick (*ca* 2 μm), appressoria produced a collar and a thick (*ca* 1·5 μm diameter) melanised infection peg during penetration of the wax layer (Fig. 4). This infection peg was surrounded by a thick layer of wall material which was continuous with the inner wall layer of the appressorium. In some cases, the wall of the thick infection peg formed a second collar (Fig. 5). Surrounding the infection peg wall was a zone of what appeared to be degraded wax material (Figs 4, 5). Once through the wax layer, the infection peg became thin and unmelanized (Figs 4 and 5). As for fruit inoculated early in development, these thin unmelanised infection pegs ceased growth in the cuticle.

In field-inoculated fruit which were sampled after harvest at firmness categories of 1–3 (> 26 N), the majority of appressoria produced infection pegs which were distinctly longer than those observed in fruit which were sampled at 24–96 h after each inoculation. In many cases these infection pegs were resting in the lower region of the cuticle (Fig. 6).

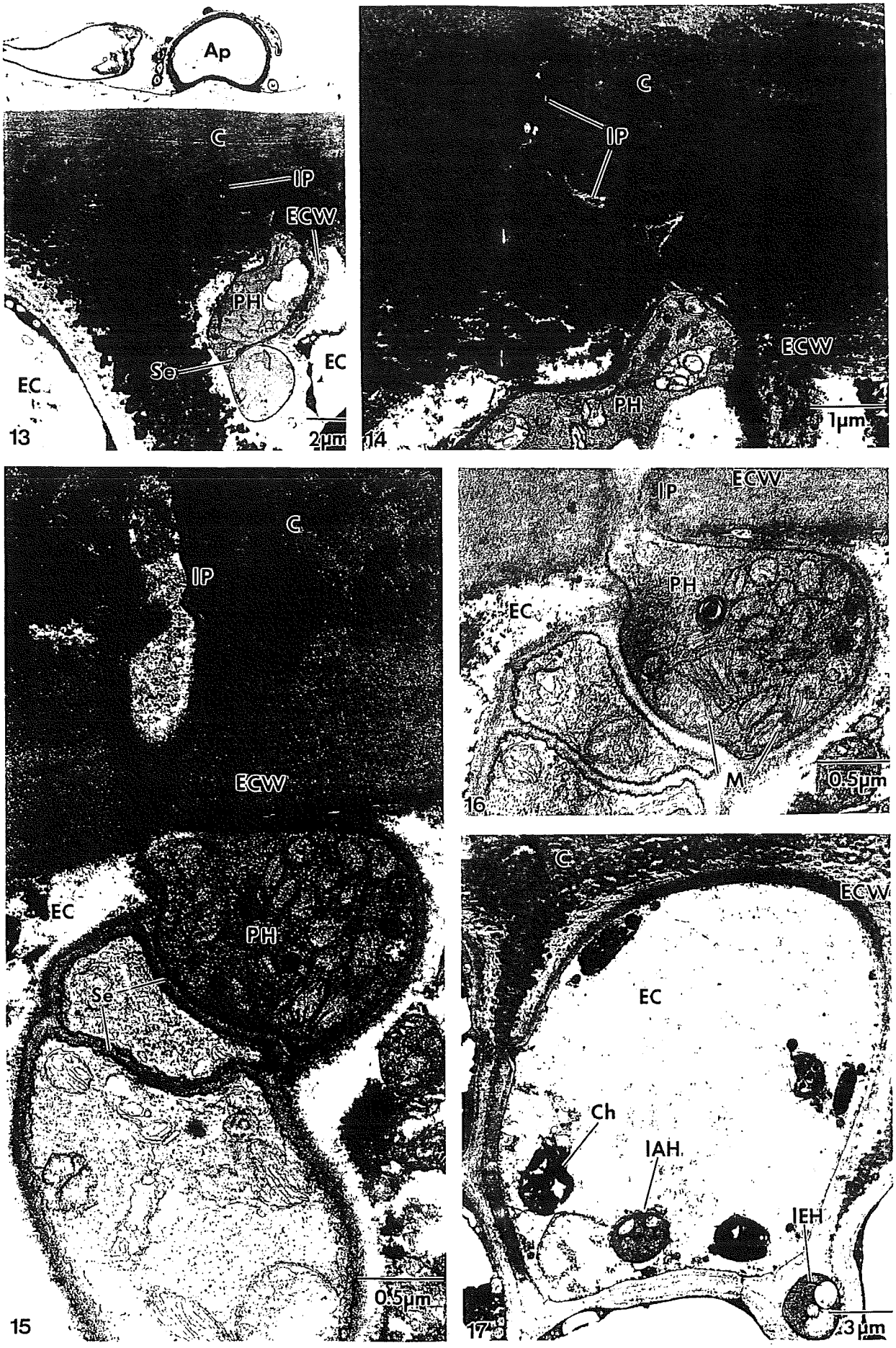
In fruit which were inoculated early in development (November–January) when the wax layer was thin and sampled at various stages during ripening, it was found that many appressoria were resting on the top of a thick epicuticular wax layer (Fig. 7). These appressoria must have been forced upwards during formation of the wax layer. Beneath these appresoria, thin infection pegs appeared to be physically broken in the region of the wax layer.

There was evidence of cell penetration by infection pegs in fruit which were sampled after harvest at firmness categories of 4 or 5 (⩽ 26 N). In some cases it appeared that infection pegs first enlarged within the lumen of underlying epidermal cells (Fig. 8), although in other cases infection pegs appeared to enlarge within the cell wall. Because of the difficulty involved in determining the exact location of infection peg enlargement using LM, numerous attempts were made to study penetration in field-inoculated fruit using TEM. Due to the infrequency of appressoria, the fineness of infection pegs and the thinness of sections, however, infection pegs were extremely difficult to locate. For this reason, a major TEM study of penetration was undertaken using detached fruit inoculated in the laboratory with a higher concentration of conidia than used in this experiment (see Histopathology of postharvest infections).

Histopathology of postharvest infections

The development of latent infection structures in relation to

epidermal cell. **Fig. 10.** TEM of an appressorium with an infection peg which has enlarged into a primary hypha within the epidermal cell wall. **Fig. 11.** TEM of an infection peg and primary hypha which has enlarged within the epidermal cell wall. **Fig. 12.** TEM of an infection peg enlarging into a primary hypha within the wall of an underlying epidermal cell. Further enlargement of the primary hypha took place within the cell lumen.



Figs 13–17. For legend see opposite.

fruit respiration rates. In contrast to the observations made on field-inoculated fruit, most appressoria on the surface of laboratory-inoculated fruit sampled at 48 h after inoculation had produced long infection pegs which came to rest in the cell wall layer of underlying epidermal cells. This was also the case in fruit sampled in the $\leqslant$ 38 and 39–48 ml CO_2 kg^{-1} h^{-1} categories. Cell penetration by infection pegs was evident in two of the four fruit sampled in the 49–58 ml CO_2 kg^{-1} h^{-1} and 59–68 ml CO_2 kg^{-1} h^{-1} categories. Two of the three fruit sampled in the $\geqslant$ 69 ml CO_2 kg^{-1} h^{-1} category also showed evidence of cell penetration. First detectable fruit softening (as judged by applying gentle thumb pressure) usually occurred just before the climacteric peak in CO_2 production (59–68 ml CO_2 kg^{-1} h^{-1}), although occasionally occurred in the 49–58 or $\geqslant$ 69 ml CO_2 kg^{-1} h^{-1} categories.

Ultrastructure of penetration. In semi-thin sections of laboratory-inoculated avocado peel examined using LM, cell penetration by infection pegs was evident as early as 48 h after inoculation (Fig. 9). Infection pegs enlarged to form primary hyphae approx 1–6 μm in diameter. Using LM it was often difficult to determine if these primary hyphae were produced inside cells, within cell walls, or outside cell walls. In a TEM study, it was found that infection pegs often enlarged within the epidermal cell wall to form primary hyphae (Figs 10–14). Where these hyphae formed, there was often considerable distortion of the cell wall (Figs 10, 13). Some infection pegs did not enlarge into primary hyphae until inside the cell lumen (Figs 15, 16).

Colonization of the epidermis and underlying tissues of the peel by hyphae was both intracellular and intercellular. Rapid cell degradation always followed fungal penetration. There was clear evidence of tonoplast degradation in infected cells (Figs 12, 17). Such degradation led to the dispersal of cytoplasmic and vacuolar contents throughout the lumen of cells. Host plasmalemmae and chloroplast membranes were also disrupted in response to fungal invasion (Figs 12, 17).

DISCUSSION

Inoculation studies conducted in the field revealed that appressoria of *C. gloeosporioides* produced infection pegs in the cuticle of unripe avocado fruit. This is in contrast with the previous findings of Binyamini & Schiffmann-Nadel (1972) that appressoria of *C. gloeosporioides* do not germinate to produce infection pegs until fruit ripening.

The question which arises from our findings concerns whether these germinated appressoria represent the latent phase of *C. gloeosporioides* in avocado fruit? Evidence that they do represent the latent phase came from examining the behaviour of the fungus in relation to the thickness of the epicuticular wax layer at the time of inoculation. In fruit inoculated early in development when the wax layer was thin and sampled at maturity when the wax layer was thick, it was found that appressoria were often forced upwards due to the formation of wax beneath them. If ungerminated appressoria represent the latent phase, then one would expect that these upwardly forced appressoria would have to produce thick melanized infection pegs during ripening in order to penetrate the wax layer. No such thick infection pegs were observed in the wax layer of these fruit at any stage during ripening. Instead, thin infection pegs were observed in the very lower region of the wax layer immediately beneath appressoria. It would seem that these thin infection pegs were produced soon after inoculation in immature, unripe fruit and became physically detached from appressoria during formation of the wax layer.

The length of infection pegs observed in the cuticle of unripe avocado fruit was found to vary considerably depending on whether fruit were inoculated after harvest or while still attached to the tree. In the case of fruit inoculated after detachment, infection pegs had reached the wall of underlying epidermal cells by 48 h after inoculation. This is consistent with the findings of Prusky *et al.* (1991). In our studies there was no further development of these infection pegs until fruit started to produce amounts of CO_2 in excess of 50 ml CO_2 kg^{-1} h^{-1} during the climacteric rise. In the peel of field-inoculated fruit sampled up to 4 d after inoculation, however, infection pegs were mostly short ($< 1{\cdot}5$ μm). Such variation in the length of infection pegs could possibly reflect variations in the concentration of antifungal compounds in the peel of fruit before and after harvest. Prusky *et al.* (1991) reported a rapid decline in the concentration of an antifungal diene in the peel of avocado fruit during the first 16 h after harvest. Normal preharvest concentrations of this compound were not restored until 72 h after harvest. This may explain why the inoculation of freshly harvested avocado fruit with *C. gloeosporioides* resulted in the rapid production of long infection pegs. More detailed studies are needed to relate the concentration of antifungal compounds in the peel of avocado fruit to the development of infection pegs.

The only other studies which have claimed that germinated appressoria (as opposed to ungerminated appresssoria or subcuticular hyphae) represent the latent phase of *C. gloeosporioides* in fruit were the studies of Daykin & Milholland (1984*a*, *b*) which examined the infection process of the fungus in grape and blueberry fruit. Although they reported that only a small percentage of appressoria had produced infection pegs (3–11% in blueberry and 36–55% in grape), it was acknowledged that many more infection pegs may have been present but remained undetected due to the thickness of sections and the relatively narrow diameter of infection pegs. It is possible that many histopathological studies may have overlooked the importance of germinated appressoria as latent structures.

The production of thick melanized infection pegs by appressoria of *C. gloeosporioides* has not been reported in any fruit other than avocado. In the study reported here, thick

Figs 13, 14. TEM of an appressorium with an infection peg which has enlarged into a large primary hypha within the epidermal cell wall. **Figs 15, 16.** TEM of an infection peg enlarging into a primary hypha in the lumen of an epidermal cell. **Fig. 17.** TEM of cell degradation following fungal penetration. Note breakdown of the tonoplast and chloroplasts.

infection pegs were seen to be surrounded by a zone of degraded wax material, indicating the production of wax-degrading compounds by the fungus during penetration of the wax layer. Light microscope studies revealed that the walls of these thick infection pegs were heavily melanized. Melanin has been reported to play a role in the penetration of host surfaces by *C. lagenarium* (Kubo *et al.*, 1982) and *C. lindemuthianum* (Wolkow, Sisler & Vigil, 1983). These authors suggested that melanin may provide the rigidity necessary to support the mechanical forces required for penetrating the plant cuticle, although Muirhead & Deverall (1981) found that unmelanized appressoria of *C. musae* were capable of penetrating the cuticle of banana peel. It has also been suggested that melanin may play an osmotic role in the penetration of host surfaces by allowing appressoria to establish and maintain a high internal hydrostatic pressure (Howard & Ferrari, 1989).

Upon resumption of fungal activity during fruit ripening, *C. gloeosporioides* rapidly colonized cells of the avocado peel tissue. Although host cells were not killed in advance of invasion during the early stages of colonization, rapid cell degradation always followed penetration. Brown (1977) reported that intracellular large hyphae of *C. gloeosporioides* did not cause severe disruption of invaded cells in the peel of inoculated tangerine fruit, and noted that such hyphae were surrounded by the invaginated host plasmalemma and an intervening matrix layer during the early stages of penetration. This was not observed in the avocado–*C. gloeosporioides* interaction examined in this study. Such variation in the behaviour of *C. gloeosporioides* in the two different fruit hosts could possibly relate to differences in host physiology. Avocados are climacteric fruit with a relatively short postharvest life, whereas citrus fruit are non-climacteric (Biale, 1960) with a much longer postharvest life. In avocado, *C. gloeosporioides* colonizes fruit tissue at a time when the fruit is just entering its senescence phase. The biochemical and physiological events associated with senescence such as cell wall degradation and increased membrane permeability probably favour necrotrophic development of the fungus. The mode of fungal development observed in the avocado–*C. gloeosporioides* interaction also differs from that of *C. gloeosporioides* in *Stylosanthes* spp. (Vinijsanun, Irwin & Cameron, 1987) and *C. lindemuthianum* in French bean (O'Connell, Bailey & Richmond, 1985), where the fungus reportedly enters an initial period of intracellular biotrophic development prior to necrotrophic growth.

Based on the findings obtained in this investigation, it would be appropriate for the infection process of *C. gloeosporioides* to be re-examined in other fruit such as mango and papaw. Any re-examination of the infection process, however, should include a comparison of field-inoculated and laboratory-inoculated fruit in the light of findings reported for avocado in this study.

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The Use of a Benomyl-Resistant Mutant to Demonstrate Latency of *Colletotrichum gloeosporioides* in Avocado Fruit

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Abstract

A benomyl-resistant mutant of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., generated by irradiating a wild-type isolate of the fungus with ultraviolet light, was used as a marker organism to demonstrate latency under field conditions. This mutant could be easily distinguished from wild-type isolates of *C. gloeosporioides* on the basis of growth rates on benomyl-amended media, and was as virulent in avocado fruit as wild-type isolates. Through the use of this mutant in field inoculations of avocado fruit, it was possible to demonstrate conclusively the existence, for the first time, of latency in the life cycle of *C. gloeosporioides* in this host. It was also shown that the fungus was able to remain latent for periods of at least 6 months.

Keywords: latency, benomyl-resistance, anthracnose, *Colletotrichum gloeosporioides*, avocado, *Persea americana*.

Introduction

Latency is defined as a quiescent or dormant parasitic relationship which, after a time, can change into an active one (Verhoeff 1974). The existence of latent infections of *Colletotrichum* spp. in many subtropical and tropical fruits has long been recognized. Various methods have been used to demonstrate latency of such infections. In early studies, an isolation technique was commonly used to demonstrate latency. In this technique, isolations were made from the surface sterilized peel of apparently disease-free fruit. Wardlaw (1931) isolated *C. musae* from the peel of symptomless banana fruit. Similarly, Baker (1938) isolated *C. gloeosporioides* from the peel of avocado, mango and citrus fruit. At the time, these findings were considered to be good evidence for the existence of latent infection, although Simmonds (1941) pointed out that the technique does not prove the existence of latent infection because it does not show that the fungus resumes parasitic activity when conditions become more favourable.

In recognizing this inadequacy, Simmonds (1941) proceeded to demonstate latency of *C. musae* in banana fruit using an artificial inoculation technique. Banana fruit were inoculated at various stages of development with conidia of *C. musae*. The inoculated areas on each fruit were marked by circles. Fruit were harvested at maturity, ripened and assessed for symptom development within the circled areas. The development of anthracnose lesions within these areas was

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taken as evidence for latent infection. Simmonds (1941) claimed that natural and artificial infections of *C. musae* could be distinguished on the basis of their position and timing of appearance. Using this inoculation technique, Simmonds concluded that infections of *C. musae* could be established shortly after bunch emergence and remain latent for a period of approximately 5 months before developing into typical anthracnose lesions during fruit ripening.

More recently, the inoculation technique was used by Binyamini and Schiffmann-Nadel (1972) to demonstrate latency of *C. gloeosporioides* in avocado fruit. To minimize fruit contact with natural sources of inoculum, inoculated fruit were enclosed in paper bags until they were harvested at maturity.

Dickman and Alvarez (1983) also used an inoculation technique to demonstrate latency of *C. gloeosporioides* in papaw. Entire columns of papaw fruit were spray-inoculated with conidia of *C. gloeosporioides*. Inoculated fruit were harvested when mature, ripened and observed for development of anthracnose symptoms. They found that *C. gloeosporioides* could infect fruit at early stages of maturity and remain latent until fruit reached the climacteric phase.

The main limitation of the inoculation technique is the difficulty in distinguishing between natural and artificial infections. The aim of the work reported in this paper was to develop a new technique for studying latency which would readily distinguish between natural and induced infections using a fungicide-resistant mutant of *C. gloeosporioides*. Benomyl resistance was chosen as a marker for this study because it is relatively easy to obtain, stable, and arises from a single chromosomal gene mutation (Hastie and Georgopoulos 1971). The growth of wild-type strains of *C. gloeosporioides* is strongly inhibited by benomyl (Bollen 1972; Muirhead 1974). Benomyl interferes with fungal mitosis by binding to tubulin, the building block of microtubules (Davidse and de Waard 1984). Resistance to benomyl is apparently conferred by a change in tubulin structure (Davidse and Flach 1978; Sheir-Neiss *et al.* 1978). This paper describes the generation of a benomyl-resistant mutant of *C. gloeosporioides* which could be readily distinguished from wild-type isolates of this fungus and therefore be used subsequently to demonstrate latency in avocado fruit under field conditions.

Materials and Methods

Generation of Benomyl-Resistant Mutants

Conidia of *C. gloeosporioides* (BRIP 19780) were obtained by gently scraping with a wire loop the surface of 7-day-old cultures growing on oatmeal agar (OMA). Suspensions of conidia in sterile distilled water were filtered through muslin to remove most of the mycelial fragments and adjusted to a concentration of $2{\cdot}5\times10^5$ conidia/mL using a haemocytometer. Plastic petri dishes each containing 10 mL of the suspension were exposed to ultraviolet (UV) irradiation at 254 nm wavelength (30 W tubes at a distance of 38 cm) for 5 min. This UV irradiation exposure time was chosen on the basis of a preliminary experiment which showed that a 5 min exposure to UV irradiation resulted in a 90% kill of conidia. Drops (0·2 mL) of the treated suspension were spread over potato dextrose agar (PDA) plates containing either 0, 1 or 5 μg/mL benomyl. Preliminary studies had shown that the growth of wild-type isolates of *C. gloeosporioides* was almost completely inhibited by 1 μg/mL benomyl. Plates were incubated at 25°C under 12 h/day irradiation from near UV light (peak at 365 nm, range 310–410 nm) and 12 h/day darkness. Colonies which grew on benomyl-amended media were regarded as benomyl-resistant. Benomyl-resistant colonies of *C. gloeosporioides* were subcultured onto PDA and OMA and held at 25°C under near UV light (12 h/day).

Growth Characteristics of the Benomyl-Resistant Mutants In Vitro

Plugs of mycelium 5 mm in diameter were taken from the margin of 7-day-old cultures of three benomyl-resistant mutants and six wild-type isolates of *C. gloeosporioides* (ex avocado) growing on PDA. Plugs were transferred to PDA plates containing either 0, 1 or 10 μg/mL benomyl. Plates were incubated at 26±0·5°C under near UV light (12 h/day). Colony diameters were measured 7 days after inoculation.

Pathogenicity of the Benomyl-Resistant Mutants in Avocado Fruit

Avocado fruit cv. Reed were washed to remove traces of copper-based fungicide and then wiped with 70% (v/v) ethanol to reduce the epiphytic microflora. Paper discs saturated with a concentrated suspension of benomyl (2 mg/mL) were placed on the freshly cut surface of fruit pedicels to control stem end rot caused by *Dothiorella aromatica*. Conidium suspensions (4×10^5 conidia/mL) were prepared from 7-day-old cultures of three benomyl-resistant mutants of *C. gloeosporioides* and the parental wild-type isolate (BRIP 19780). Fruit were inoculated by placing 25 μL drops of each conidium suspension onto an area of unwounded peel. Inoculated fruit were incubated under high humidity ($\geq$ 95% RH) at 24±1°C. At 8–12 days after inoculation, fruit were removed and lesion diameters were measured. The firmness of fruit was estimated by applying gentle thumb pressure (firmness scale: 1, hard; 2, first detectable softening; 3, between first detectable softening and eating-ripe; 4, eating-ripe; 5, over-ripe). Isolations were made from the advancing margins of lesions onto PDA. Isolates growing on PDA were subcultured onto benomyl-amended PDA. Plates were incubated at 25°C under near UV light (12 h/day) and colony diameters were measured 6 days after inoculation.

Fruit Inoculations in the Field using a Benomyl-Resistant Mutant of C. gloeosporioides

On the basis of *in vitro* growth characteristics, the benomyl-resistant mutant BRIP 19779 was selected for use in field inoculations. Suspensions of conidia (10^6 conidia/mL) were prepared as previously described. Tween-20 wetting agent was added to conidium suspensions at a rate of 0·35 mL/L.

All fruit inoculations were conducted on the cultivar Fuerte on two neighbouring avocado orchards at Maleny (26° 46′S., 152° 51′E.), south-east Queensland. No attempt was made to control diseases on orchard 1, whereas trees on orchard 2 were regularly sprayed with copper oxychloride for anthracnose control.

Fruit were inoculated at monthly intervals, commencing in November when fruit were approximately 1·5–2 cm in length. The final inoculation was performed in April of the following year when fruit were mature. Fruit were inoculated by saturating a 1 or 2 cm^2 (depending on fruit size) piece of sterile blotting paper in the prepared conidium suspension of *C. gloeosporioides*, and placing this onto an area of unwounded peel. The paper squares were attached to the surface of fruit using waterproof tape, and the location of each square was marked on the surface of fruit. An equal number of fruit was inoculated with water containing wetting agent only (controls). Following inoculation, each fruit along with a few leaves on the same shoot was enclosed in a plastic bag containing a small amount of wet cotton wool to help maintain a high humidity around the inoculation site. A white paper bag was then placed on the outside of the plastic bag to protect fruit from high temperatures. Plastic and paper bags were removed after 48 h.

Preharvest Sampling of Fruit

At 24, 48, 72 and 96 h after each inoculation, three inoculated fruit and three control fruit were removed from orchard 1 and taken to the laboratory within 2 h. Slices of peel approximately 3 mm long×3 mm wide×200 μm thick were cut from the inoculated area of each fruit using a razor blade. Four slices of peel were cut from each fruit. Peel slices were surface sterilized in 1% (v/v) sodium hypochlorite for 1 min, rinsed 3 times in sterile distilled water, and dried on sterile blotting paper. Surface sterilized peel slices were transferred to PDA plates which were incubated at 25°C under near UV light (12 h/day) for 4 days. Mycelial plugs 3 mm in diameter were taken from colonies growing from peel slices and transferred to

PDA containing 0, 1 or 10 μg/mL benomyl. Colony diameters were measured 3 and 6 days after inoculation. On the basis of these growth rates, isolates were classified as being either benomyl-resistant or benomyl-sensitive.

Preharvest Symptom Development

At various intervals after inoculation, observations were made on the development of disease symptoms on fruit still attached to the tree.

Postharvest Sampling of Fruit

All inoculated fruit remaining on trees in May were harvested, brought to the laboratory within 2 h and placed in storage at 20°C. Fruit were sampled at various stages during ripening. Fruit firmness was the parameter used to define different stages of ripeness and was measured using an Instron universal testing machine (12 mm diameter cylindrical probe, crosshead speed 20 mm/min, probe penetration 2 mm). In a preliminary experiment, changes in the firmness of Fuerte avocado fruit during ripening at 20°C were measured. From these results (Fig. 1), five firmness categories were devised as follows: (1) $\geq$ 53 newtons (N) (hard fruit); (2) 40–52 N; (3) 27–39 N; (4) 14–26 N; (5) $\leq$ 13 N (soft fruit). First detectable softening, as judged by using gentle thumb pressure, coincided with firmness readings in the range of 15–20 N.

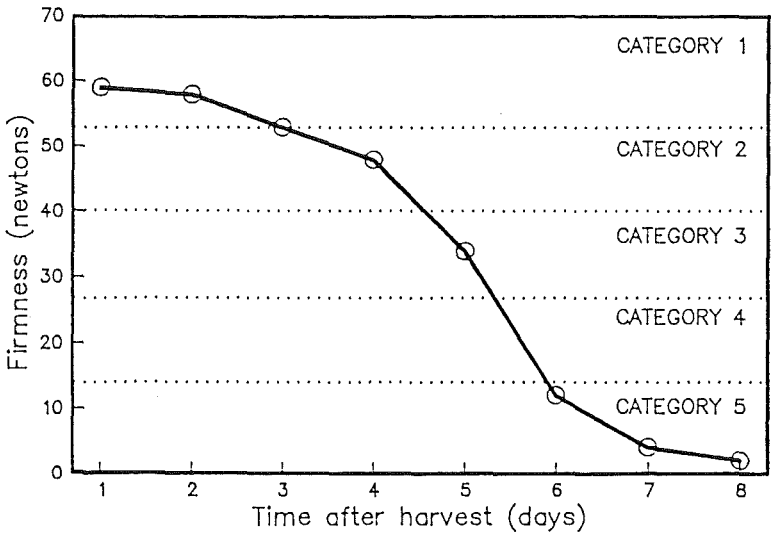

Fig. 1. Postharvest firmness changes in 'Fuerte' avocado fruit ripened at 20°C.

Each day, a subsample of field-inoculated fruit was removed from storage for measurement of firmness. Two firmness readings were taken for each fruit. Fruit were sampled when they attained the desired degree of firmness. For each inoculation time (i.e. November–April) and each inoculum concentration (i.e. 0 or 10^6 conidia/mL), 3–5 fruit were sampled per firmness category, depending on the total number of fruit available. Where fruit were soft and lesions had formed in the inoculated area, isolations were made by plating out small pieces of diseased pulp tissue from lesion margins onto PDA. In all other fruit (unripe fruit and ripe fruit with no lesions), peel slices were taken from the inoculated areas of fruit as previously described and transferred to PDA. Colonies of *C. gloeosporioides* growing from peel or pulp pieces were subcultured onto benomyl-amended media as previously described.

Results

Generation of Benomyl-Resistant Mutants

A total of 10 colonies of *C. gloeosporioides* was found growing on benomyl-amended media after treatment of conidia with UV irradiation for 5 min. Three

of these mutants (BRIP Nos 19777, 19778 and 19779) were chosen for further study on the basis of growth rates on benomyl-amended media and ability to produce abundant conidia.

Growth Characteristics of the Benomyl-Resistant Mutants in vitro

The mycelial growth of three benomyl-resistant mutants and six wild-type isolates of *C. gloeosporioides* on benomyl-amended media is shown in Table 1. On PDA containing no benomyl, the growth rates of mutants BRIP 19777 and 19779 were significantly lower ($P < 0{\cdot}05$) than that of the parental wild-type isolate (BRIP 19780), whereas the growth rate of mutant BRIP 19778 was higher. On PDA containing 1 μg/mL benomyl, the growth rates of all mutants were significantly higher than those of all wild-type isolates of *C. gloeosporioides*. Mutant BRIP 19778 grew the fastest on 1 μg/mL benomyl. On PDA containing 10 μg/mL benomyl, mutants BRIP 19777 and 19779 grew significantly faster than all wild-type isolates of *C. gloeosporioides*, but mutant BRIP 19778 did not.

Table 1. Mycelial growth of benomyl-resistant mutants and wild-type isolates of *C. gloeosporioides* at 26°C on benomyl-amended PDA

Means followed by the same letter within columns do not differ at $P = 0{\cdot}05$ using the l.s.d. test

Isolate	Colony diameter (mm)[A]		
Benomy concentration (μg/mL):	0	1	10
BRIP 19777 (mutant)	65·1c	18·3c	11·5b
BRIP 19778 (mutant)	80·0a	64·3a	0·8d
BRIP 19779 (mutant)	66·3c	46·9b	38·8a
BRIP 19780 (parental wild-type)	73·3b	4·5de	2·0cd
BRIP 19768 (wild-type)	73·3b	2·9de	2·0cd
BRIP 19769 (wild-type)	80·0a	2·9de	2·5cd
BRIP 19770 (wild-type)	71·5b	1·9de	2·5cd
BRIP 19773 (wild-type)	72·3b	5·1d	4·4c
BRIP 19774 (wild-type)	71·5b	1·0e	1·0d

[A] Mean of four replicates at 7 days after inoculation.

Pathogenicity of the Benomyl-Resistant Mutants in Avocado Fruit

There were no significant differences between the mean diameters of lesions in fruit inoculated with any of the mutants or the wild-type isolate. On benomyl-amended media, the growth characteristics of all isolates obtained from lesions were consistent with the growth characteristics of isolates used to inoculate fruit. The pathogenicity of mutants BRIP 19777, 19778 and 19779 in avocado fruit cv. Reed was established in this experiment.

Selection of a Benomyl-Resistant Mutant for Use in Field Inoculations of Fruit

On the basis of growth characteristics on PDA containing 10 μg/mL benomyl, mutant BRIP 19779 was selected for use in field inoculations.

Preharvest Sampling of Field-Inoculated Avocado Fruit

The benomyl-resistant mutant of *C. gloeosporioides* (BRIP 19779) was isolated from the peel of inoculated avocado fruit at each sampling time (24–96 h) after

inoculation (Table 2). Out of a total of 72 fruit sampled from November to April, only three fruit (4·2%) did not yield the mutant. As expected, the mutant was not isolated from the peel of uninoculated control fruit.

Table 2. Recovery of the benomyl-resistant mutant of *C. gloeosporioides* from the peel of field-inoculated avocado fruit cv. Fuerte sampled between 24 and 96 h after inoculation

Month of inoculation	No. fruit yielding mutant/Total no. fruit sampled[A]							
Time after inoculation (h):	24		48		72		96	
	C[B]	I	C	I	C	I	C	I
November	0/3	3/3	0/3	3/3	0/3	3/3	0/3	3/3
December	0/3	3/3	0/3	3/3	0/3	2/3	0/3	3/3
January	0/3	3/3	0/3	3/3	0/3	3/3	0/3	3/3
February	0/3	3/3	0/3	3/3	0/3	3/3	0/3	3/3
March	0/3	3/3	0/3	2/3	0/3	3/3	0/3	3/3
April	0/3	3/3	0/3	3/3	0/3	3/3	0/3	2/3

[A] Sampled from orchard 1 only.
[B] C, uninoculated control fruit; I, inoculated fruit.

Preharvest Symptom Development in Field-Inoculated Avocado Fruit

Fruit inoculated in November showed no symptoms of disease in the marked area prior to harvest. Some of the fruit inoculated at each of the other times, however, did show symptoms of disease before harvest. These symptoms appeared as small, dark, circular or irregularly shaped lesions on the surface of fruit ranging from 1 to 5 mm in diameter. Unlike typical anthracnose lesions, these lesions were restricted to the peel of fruit and did not extend into the flesh. Because the diameter of these lesions rarely exceeded a few millimetres, they

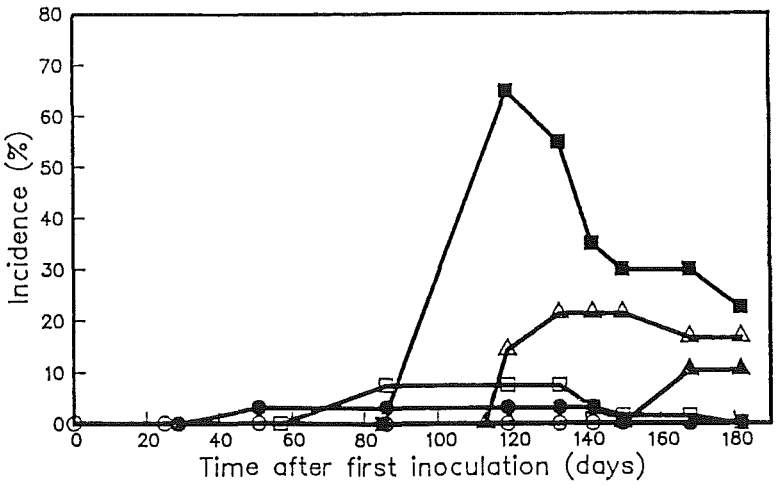

Fig. 2. Incidence of limited anthracnose lesions in avocado fruit cv. Fuerte inoculated with *C. gloeosporioides* at various stages of development (orchard 1 and 2 data combined). ○ November-inoculated fruit; ● December-inoculated fruit; □ January-inoculated fruit; ■ February-inoculated fruit; △ March-inoculated fruit; ▲ April-inoculated fruit.

have been referred to as 'limited lesions'. They were not associated with any macroscopically visible wounds in the fruit peel, although they were often formed around lenticels. In fruit where these limited lesions formed, symptoms usually appeared in the inoculated area within 10 days of inoculation.

The incidence of these limited lesions in inoculated avocado fruit is shown in Fig. 2. Fruit inoculated in February clearly showed the highest incidence of limited lesions. A high incidence of these lesions was also recorded in March-inoculated fruit. In many cases, the incidence of limited lesions decreased during the later part of the season. This is because some of the fruit displaying these symptoms fell from trees at various times. In some cases, it appeared that the limited lesions might be the cause of fruit fall. In other cases, however, the cause of fruit fall was spreading preharvest anthracnose lesions which resulted from natural infections of *C. gloeosporioides* through skin injuries.

Postharvest Sampling of Field-Inoculated Avocado Fruit

Recovery of the benomyl-resistant mutant of *C. gloeosporioides* from the peel of fruit sampled at various stages of ripeness is shown in Table 3. The rate of mutant recovery from peel was very high in fruit from each of the inoculation times. Out of a total of 90 inoculated fruit sampled, only 10 fruit (11%) did not yield the mutant from peel.

Table 3. Recovery of the benomyl-resistant mutant of *C. gloeosporioides* from the peel of field-inoculated avocado fruit cv. Fuerte sampled at various stages of ripeness (orchard 1 and 2 data combined)

Month of inoculation	No. fruit yielding mutant/Total no. fruit sampled									
	FC[A]: 1		2		3		4		5	
	C[B]	I	C	I	C	I	C	I	C	I
November	0/4	1/3	0/4	3/4	0/4	3/3	0/4	2/2	0/3	—[C]
December	0/4	2/4	0/4	4/4	0/4	4/4	0/3	4/4	0/5	2/2
January	0/4	3/4	0/4	4/4	0/4	3/4	0/4	4/5	0/4	—[C]
February	0/4	3/3	0/4	3/3	0/4	2/2	0/5	3/3	0/4	—[C]
March	0/4	4/4	0/4	3/4	0/4	3/3	0/4	2/2	0/4	1/1
April	0/4	4/4	0/4	5/5	0/4	5/5	0/4	0/1	0/5	3/3

[A] FC, firmness category: 1, ≥ 53 N (hard); 2, 40–52 N; 3, 27–39 N; 4, 14–26 N; 5, ≤ 13 N (soft).
[B] C, uninoculated control fruit; I, inoculated fruit.
[C] Pulp isolations made only (see Table 4).

Table 4. Recovery of the benomyl-resistant mutant of *C. gloeosporioides* from diseased pulp of field-inoculated avocado fruit cv. Fuerte (orchard 1 and 2 data combined[A])

Month of inoculation:	No. fruit yielding mutant/Total no. fruit sampled												
	Nov.		Dec.		Jan.		Feb.		Mar.		Apr.		Total
FC[B]:	4	5	4	5	4	5	4	5	4	5	4	5	
	1/2	2/4	—	3/11	—	13/14	—	6/6	—	9/9	2/2	5/5	41/53

[A] Inoculated fruit only.
[B] FC, firmness category: 4, 14–26 N, 5, ≤ 13 N.

Recovery of the benomyl-resistant mutant from the diseased pulp tissue of field-inoculated fruit sampled at firmness categories of 4 and 5 (softening fruit) was also very high (Table 4). Out of a total of 53 inoculated fruit sampled, only 12 fruit (23%) did not yield the mutant from diseased pulp. The lowest rate of mutant recovery from diseased pulp was recorded in December-inoculated fruit.

The benomyl-resistant mutant was not isolated from the peel or pulp of any uninoculated control fruit.

Discussion

The latency of *C. gloeosporioides* in avocado fruit cv. Fuerte was demonstrated clearly in this study. The isolation of the benomyl-resistant mutant of *C. gloeosporioides* from the peel of fruit between 24 and 96 h after inoculation confirmed the presence and the viability of the fungus in or on the peel of fruit. The isolation of the mutant from the peel of field-inoculated fruit after harvest indicated that the fungus was still viable after several months. The isolation of the mutant from the diseased pulp of field-inoculated fruit confirmed that the latent fungus was able to resume growth and cause disease in ripe fruit. It was shown that the fungus could remain latent in or on the peel of avocado fruit for at least 6 months. This is consistent with the findings of Binyamini and Schiffmann-Nadel (1972).

Performance of the benomyl-resistant mutant as a marker organism under field conditions was excellent at all times. Of all the isolations made from the peel or diseased pulp of uninoculated control fruit, no isolate of *C. gloeosporioides* was able to grow on benomyl-amended media to the same extent as the benomyl-resistant mutant. Occasionally, *C. acutatum* was isolated from the peel of uninoculated control fruit, but this too was clearly distinguishable from the mutant on the basis of growth rates and cultural characteristics.

Fruit inoculated in December on both orchards showed a relatively low rate of mutant recovery from diseased pulp. This occurred despite the fact that isolations made from peel after inoculation and after harvest confirmed the existence of the fungus in a viable state in or on the peel of December-inoculated fruit. For some reason, the fungus did not develop further to cause disease lesions. The lesions that were present in the marked area of December-inoculated fruit must have been caused by natural infections of *C. gloeosporioides*. Simmonds (1941) stated that 'areas of artificially inoculated banana peel have yielded isolations of *Gloeosporium musarum* ($\equiv$ *C. musae*) after surface sterilization from areas free from any lesion although the time for the normal appearance of such had definitely passed'. Simmonds (1941) could not offer an explanation as to why some latent infections of *C. musae* did not develop further to cause disease lesions.

Although anthracnose is predominantly a postharvest disease of avocado, Fitzell (1987) reported that lesions can develop in immature, unripe fruit still on the tree. Two types of preharvest anthracnose lesions were described by Fitzell (1987). 'Type 1' lesions were described as large (1–4 cm diameter), spreading lesions which were usually associated with some form of peel injury. 'Type 2' lesions, on the other hand, were small (0·1–0·5 cm diameter), limited lesions which were rarely associated with peel injury, but in 69% of cases were associated with fruit lenticels. The small, limited anthracnose lesions which we observed in

field-inoculated avocado fruit prior to harvest closely fitted Fitzell's description of Type 2 lesions. In the studies conducted by Fitzell (1987), however, these Type 2 lesions were never observed in artificially inoculated avocado fruit. Because such lesions were able to be reproduced in the field study reported here without artificial wounding of the fruit peel, infection must have occurred either through areas of undamaged peel or through lenticels.

The timing of appearance of limited lesions in this field study is worthy of comment. Limited lesions occurred in fruit inoculated between December and April, which correlates well with the periods of highest rainfall during the season. The highest incidence of these lesions, however, occurred in fruit inoculated in February. Although the monthly rainfall during February was high (129 mm), there were many other months when rainfall was much greater (e.g. April, 858 mm). Perhaps other factors such as duration of fruit surface wetness, which was not monitored in this field trial, play an important role in the development of limited lesions. Fitzell (1987) suggested that the appearance of Type 2 lesions may be related to a low concentration of antifungal compounds in the peel of fruit, allowing either direct lenticel infections (no latent phase) or normal latent infections to proceed prematurely. There is very little published information available on preharvest changes in the concentration of antifungal compounds in the peel of avocado fruit. It is quite possible that levels of these compounds vary at different stages during fruit development. Such changes could help to explain the seasonal variation in fruit susceptibility to infection.

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