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Pathogenic diversity of avocado and mango isolates of *Colletotrichum gloeosporioides* causing anthracnose and pepper spot in Australia

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Abstract. *Colletotrichum gloeosporioides* is a major fungal pathogen of avocado and mango fruit in Australia and overseas. It causes anthracnose and stem-end rot in these crops but has also been identified as the causal pathogen of pepper spot of avocado and tear stain of mango. Research was initiated to determine the pathogenic diversity of pepper spot, with emphasis on avocado. Eighty *C. gloeosporioides* isolates obtained from avocado and mango fruit showing anthracnose and pepper spot symptoms were screened for pathogenicity, comparative aggressiveness and cross-infection potential by inoculating onto detached avocado and mango fruit, avocado leaf petioles and branches of young, grafted nursery trees, as well as avocado fruit and pedicels still attached to the tree. On detached, ripening avocado and mango fruit in the laboratory, it was found that pepper spot isolates were as capable as anthracnose isolates of causing anthracnose lesions. However, avocado isolates were significantly ($P < 0.05$) more aggressive than mango isolates on avocado fruit and mango isolates were significantly ($P < 0.05$) more aggressive than avocado isolates on mango fruit. In field inoculations, pepper spots were formed on developing avocado fruit and pedicels on the tree. Likewise, pepper spots developed on petioles and branches of nursery avocado trees, but not on their leaves. When all isolates were grouped according to symptom or host of origin, significant differences in lesion severity were demonstrated between isolates on avocado petioles in the glasshouse, with avocado pepper spot isolates being the most aggressive, followed by avocado anthracnose isolates then mango isolates from both anthracnose and pepper spot, respectively. On unripe avocado fruit in the field, the pattern was generally similar with the mango isolates being the least aggressive. There were more and less pathogenic strains present in the pathogen populations from both mango fruit and avocado fruit but neither were restricted to anthracnose or pepper spot groupings. Generally, a higher percentage of the most aggressive isolates was from avocado pepper spot. When isolates were grouped according to the orchard of origin, there were significant differences in aggressiveness to avocado both in the glasshouse and the field.

Additional keywords: diene, fungus, *Glomerella cingulata*, Hass.

Introduction

Colletotrichum gloeosporioides (Penz.) Penz & Sacc. causes significant postharvest losses of avocado (*Persea americana* Mill.) fruit due to large, spreading anthracnose lesions which develop during ripening. Before harvest, *C. gloeosporioides* also causes lesions on unripe fruit of cultivar 'Fuerte' (Fitzell 1987). In addition, a preharvest fruit spotting symptom on avocado cultivar Hass has become evident in north-eastern Australia (Willingham *et al*. 2000). This disease, which is more prevalent on sun-exposed surfaces of fruit and pedicels, is also caused by *C. gloeosporioides*. It has been called pepper spot and reduces fruit quality due to the presence of small, shiny, black lesions on the skin. The tear stain (also referred to as pepper spot in this paper) symptom on mango (*Mangifera indica* L.) fruit, also a result of infection by *C. gloeosporioides*, is thought to be a similar phenomenon (Dodd *et al*. 1997). Mango fruit is also susceptible to damaging postharvest anthracnose due to *C. gloeosporioides* infection.

Infection occurs when a conidium lands in a splash droplet on the surface of the fruit in the natural environment, and adheres and germinates to produce a germ tube, which develops a terminal appressorium. There are physical and chemical signals in the surface wax to induce this differentiation (Flaishman and Kolattukudy 1994). An infection peg then emerges and penetrates into the outer wax layer and the cuticle of the fruit skin. At this stage it ceases growth and remains quiescent until fruit ripening (Coates *et al*. 1993). It is thought that *C. gloeosporioides* is unable to colonise further due to the presence of preformed antifungal compounds in the fruit known as dienes (Prusky *et al*. 1982). During ripening, levels of antifungal dienes decline in the peel, which correlates with the resumption of fungal growth, leading ultimately to

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anthracnose development. A similar process is thought to occur with antifungal resorcinols in mango fruit (Droby *et al*. 1987; Hassan *et al*. 2007).

Although we now have the benefits of molecular genetics to increase our understanding of pathogen population genetic structure, conventional methodologies still contribute to our understanding of the complexity of *C. gloeosporioides* populations from avocado (Freeman 2000). Freeman *et al*. (1996) used pathogenicity assays to compare *C. gloeosporioides* isolates from avocado and almond to determine genetic diversity and host specificity between and among populations. Four forms of *C. gloeosporioides* from yam were identified by Abang *et al*. (2002) based on morphology, virulence and molecular studies. Chakraborty *et al*. (1999) analysed the relationship between DNA banding patterns and variations in virulence and aggressiveness of *C. gloeosporioides* strains on *Stylosanthes scabra*. A study by Ansari *et al*. (2004) used pathogenicity testing as well as amplified fragment length polymorphism analysis to investigate pathogenic and genetic variability among isolates of *Colletotrichum lindemuthianum* from *Phaseolus vulgaris* from various global geographic locations. They were able to group the isolates of the pathogen based on their pathogenicity on bean cultivars and then further distinguish isolates using molecular genetics data to study patterns of host/pathogen coevolution. Thus, while artificial inoculation studies can demonstrate the potential of isolates to infect other hosts, they are not usually sufficient to definitively establish host specificity. Freeman *et al*. (1998) noted that while almond isolates of *C. gloeosporioides* from Israel have shown ability to infect other fruit hosts in artificial inoculation studies, there is no evidence from isolations of naturally infected fruit that cross-infection has occurred.

Research indicates that isolates from mango comprise a pathogenically and genetically distinct population of *C. gloeosporioides* (Mills *et al*. 1992; Hodson *et al*. 1993; Alahakoon *et al*. 1994*a*; Hayden *et al*. 1994; Waller and Bridge 2000; Than *et al*. 2008). In cross-inoculation studies, Hayden *et al*. (1994) found that isolates of *C. gloeosporioides* displayed a wide host range with the exception of isolates from mango, which were highly aggressive on mango only. They could distinguish a genetically and pathologically distinct mango biotype of *C. gloeosporioides* from eight other isolates of *C. gloeosporioides* obtained from five different fruit species. These findings for mango were confirmed by Alahakoon *et al*. (1994*b*). In those studies mango isolates were not found on other crops and isolates from other crops were found infrequently on mango. In Australian orchards, mango and avocado crops are often grown in close proximity and it was, therefore, from a disease management perspective, considered important to compare these pathogen populations.

This study was initiated due to the recent detection and perceived spread of localised necrotic lesions (pepper spot) on avocado fruit in 'Hass' avocado orchards. The emergence of the pepper spot symptom is different from the anthracnose symptom as the response is occurring either regardless of quiescence or before quiescence can be established. The overall objective of the study was to compare *C. gloeosporioides* isolates from avocado and mango crops grown in Australia and in some cases, grown in relatively close proximity. The objectives were to compare pathogenicity (ability to cause disease on a given host species), aggressiveness (relative capacity to cause disease on a given host genotype) and cross-infection potential of isolates from different collection locations, from different hosts (avocado/mango) and from different symptom types [anthracnose or pepper spot of avocado and anthracnose or tear stain (referred to as pepper spot) of mango]. The focus of this study was on anthracnose and pepper spot disease of avocado fruit and the scale of the experiments was large. Ideally, all of the experimental work could have been replicated on mango fruit and pedicels, however, resources for a full scale mango study were limited. In an additional project, it was possible to carry out the inoculation studies on detached mango fruit, although, at a reduced scale.

Materials and methods

For these pathogenicity studies, isolates were tested for capacity to cause ripe fruit rot (anthracnose) in the laboratory by inoculating onto detached mature 'Hass' avocado fruit (preliminary studies), detached seedless 'Fuerte' avocado fruit (referred to as 'cocktail' avocados), and detached mature 'Brooks' mango fruit. In the glasshouse, isolates were inoculated onto leaves petioles and branches of young grafted nursery avocado trees to assess capacity to cause pepper spot. Similar tests were also conducted in the field where isolates were inoculated onto 'Hass' avocado fruit and pedicels still attached to the tree.

Collection of isolates, inoculum preparation and disease assessments

Five sites in northern New South Wales [Bangalow (lat. 28°40′S, long. 153°31′E), Cudgen (lat. 28°16′S, long. 153°33′E), Duranbah (lat. 28°18′S, long. 153°31′E), Green Pigeon (lat. 28°29′S, long. 153°04′E)] and south-eastern Queensland [Mt Tamborine (lat. 27°58′S, long. 153°12′E)] were selected for the collection of *C. gloeosporioides* isolates from 'Hass' avocado fruit. Fifty isolates were obtained from each site: 25 anthracnose isolates and 25 pepper spot isolates. Similarly, three sites in northern New South Wales (Bangalow, Green Pigeon) and northern Queensland [Ayr (lat. 19°34′S, long. 147°24′E)] were sampled for the collection of isolates from 'Kensington Pride' mango fruit. Fifty mango isolates were obtained from each site: 25 anthracnose isolates and 25 tear stain isolates. To avoid confusion, mango tear stain isolates are referred to as mango pepper spot isolates. The collection (Table 1) contains 250 *C. gloeosporioides* isolates from avocado fruit and 150 *C. gloeosporioides* isolates from mango fruit. Only fast-growing vigorous cultures typical of *C. gloeosporioides* were selected (Sutton 1980). All isolates involved in this work had conidial morphology and dimensions typical of *C. gloeosporioides* (*sensu* Sutton 1980).

To obtain avocado and mango anthracnose isolates, ~20 fruit were selected randomly from each of five trees. Fruit were left to fully ripen and develop disease. Only five diseased fruit were required from each tree. After peeling the fruit, the fungus was isolated from the margin of a discrete lesion on the inner skin surface and grown at room temperature (~25°C)

Table 1. List of *Colletotrichum gloeosporioides* isolates collected for this study and their code, Queensland Primary Industries and Fisheries herbarium accession code, fruit source, symptom, tree number and geographic origin within Australia

Isolate code	Herbarium code	Fruit	Symptom	Tree	Origin
AAB11 to AAB15	BRIP45430 to 45434	Avocado	Anthracnose	1	Bangalow, NSW
APB11 to APB15	BRIP45455 to 45459	Avocado	Pepper spot	1	Bangalow, NSW
AAB21 to AAB25	BRIP45435 to 45439	Avocado	Anthracnose	2	Bangalow, NSW
APB21 to APB25	BRIP45460 to 45464	Avocado	Pepper spot	2	Bangalow, NSW
AAB31 to AAB35	BRIP45440 to 45444	Avocado	Anthracnose	3	Bangalow, NSW
APB31 to APB35	BRIP4546 to 45469	Avocado	Pepper spot	3	Bangalow, NSW
AAB41 to AAB45	BRIP45445 to 45449	Avocado	Anthracnose	4	Bangalow, NSW
APB41 to APB45	BRIP45470 to 45474	Avocado	Pepper spot	4	Bangalow, NSW
AAB51 to AAB55	BRIP45450 to 45454	Avocado	Anthracnose	5	Bangalow, NSW
APB51 to APB55	BRIP45475 to 45479	Avocado	Pepper spot	5	Bangalow, NSW
AAD11 to AAD15	BRIP45530 to 45534	Avocado	Anthracnose	1	Duranbah, NSW
APD11 to APD15	BRIP45555 to 45559	Avocado	Pepper spot	1	Duranbah, NSW
AAD21 to AAD25	BRIP45535 to 45539	Avocado	Anthracnose	2	Duranbah, NSW
APD21 to APD25	BRIP45560 to 45564	Avocado	Pepper spot	2	Duranbah, NSW
AAD31 to AAD35	BRIP45540 to 45544	Avocado	Anthracnose	3	Duranbah, NSW
APD31 to APD35	BRIP45565 to 45569	Avocado	Pepper spot	3	Duranbah, NSW
AAD41 to AAD45	BRIP45545 to 45549	Avocado	Anthracnose	4	Duranbah, NSW
APD41 to APD45	BRIP45570 to 45574	Avocado	Pepper spot	4	Duranbah, NSW
AAD51 to AAD55	BRIP45550 to 45554	Avocado	Anthracnose	5	Duranbah, NSW
APD51 to APD55	BRIP45575 to 45579	Avocado	Pepper spot	5	Duranbah, NSW
AAC11 to AAC15	BRIP45480 to 45484	Avocado	Anthracnose	1	Cudgen, NSW
APC11 to APC15	BRIP45505 to 45509	Avocado	Pepper spot	1	Cudgen, NSW
AAC21 to AAC25	BRIP45485 to 45489	Avocado	Anthracnose	2	Cudgen, NSW
APC21 to APC25	BRIP45510 to 45514	Avocado	Pepper spot	2	Cudgen, NSW
AAC31 to AAC35	BRIP45490 to 45494	Avocado	Anthracnose	3	Cudgen, NSW
APC31 to APC35	BRIP45515 to 45519	Avocado	Pepper spot	3	Cudgen, NSW
AAC41 to AAC45	BRIP45495 to 45499	Avocado	Anthracnose	4	Cudgen, NSW
APC41 to APC45	BRIP45520 to 45524	Avocado	Pepper spot	4	Cudgen, NSW
AAC51 to AAC55	BRIP45500 to 45504	Avocado	Anthracnose	5	Cudgen, NSW
APC51 to APC55	BRIP45525 to 45529	Avocado	Pepper spot	5	Cudgen, NSW
AAG11 to AAG15	BRIP45580 to 45584	Avocado	Anthracnose	1	Green Pigeon, NSW
APG11 to APG15	BRIP45605 to 45609	Avocado	Pepper spot	1	Green Pigeon, NSW
AAG21 to AAG25	BRIP45585 to 45589	Avocado	Anthracnose	2	Green Pigeon, NSW
APG21 to APG25	BRIP45610 to 45614	Avocado	Pepper spot	2	Green Pigeon, NSW
AAG31 to AAG35	BRIP45590 to 45594	Avocado	Anthracnose	3	Green Pigeon, NSW
APG31 to APG35	BRIP45615 to 45619	Avocado	Pepper spot	3	Green Pigeon, NSW
AAG41 to AAG45	BRIP45595 to 45599	Avocado	Anthracnose	4	Green Pigeon, NSW
APG41 to APG45	BRIP45620 to 45624	Avocado	Pepper spot	4	Green Pigeon, NSW
AAG51 to AAG55	BRIP45600 to 45604	Avocado	Anthracnose	5	Green Pigeon, NSW
APG51 to APG55	BRIP45625 to 45629	Avocado	Pepper spot	5	Green Pigeon, NSW
AAT11 to AAT15	BRIP45630 to 45634	Avocado	Anthracnose	1	Mt Tamborine, Qld
APT11 to APT15	BRIP45655 to 45659	Avocado	Pepper spot	1	Mt Tamborine, Qld
AAT21 to AAT25	BRIP45635 to 45639	Avocado	Anthracnose	2	Mt Tamborine, Qld
APT21 to APT25	BRIP45660 to 45664	Avocado	Pepper spot	2	Mt Tamborine, Qld
AAT31 to AAT35	BRIP45640 to 45644	Avocado	Anthracnose	3	Mt Tamborine, Qld
APT31 to APT35	BRIP45665 to 45669	Avocado	Pepper spot	3	Mt Tamborine, Qld
AAT41 to AAT45	BRIP45645 to 45649	Avocado	Anthracnose	4	Mt Tamborine, Qld
APT41 to APT45	BRIP45670 to 45674	Avocado	Pepper spot	4	Mt Tamborine, Qld
AAT51 to AAT55	BRIP45650 to 45654	Avocado	Anthracnose	5	Mt Tamborine, Qld
APT51 to APT55	BRIP45675 to 45679	Avocado	Pepper spot	5	Mt Tamborine, Qld
MAA11to MAA15	BRIP45680 to 45684	Mango	Anthracnose	1	Ayr, Qld
MPA11 to MPA15	BRIP45705 to 45709	Mango	Pepper spot	1	Ayr, Qld
MAA21to MAA25	BRIP45685 to 45689	Mango	Anthracnose	2	Ayr, Qld
MPA21 to MPA25	BRIP45710 to 45714	Mango	Pepper spot	2	Ayr, Qld
MAA31to MAA35	BRIP45690 to 45694	Mango	Anthracnose	3	Ayr, Qld
MPA31 to MPA35	BRIP45715 to 45719	Mango	Pepper spot	3	Ayr, Qld
MAA41to MAA45	BRIP45695 to 45699	Mango	Anthracnose	4	Ayr, Qld
MPA41 to MPA45	BRIP45720 to 45724	Mango	Pepper spot	4	Ayr, Qld
MAA51to MAA55	BRIP45700 to 45704	Mango	Anthracnose	5	Ayr, Qld
MPA51 to MPA55	BRIP45725 to 45729	Mango	Pepper spot	5	Ayr, Qld

Table 1. (*continued*)

Isolate code	Herbarium code	Fruit	Symptom	Tree	Origin
MAB11 to MAB15	BRIP45730 to 45734	Mango	Anthracnose	1	Bangalow, NSW
MPB11 to MPB15	BRIP45755 to 45759	Mango	Pepper spot	1	Bangalow, NSW
MAB21 to MAB25	BRIP45735 to 45739	Mango	Anthracnose	2	Bangalow, NSW
MPB21 to MPB25	BRIP45760 to 45764	Mango	Pepper spot	2	Bangalow, NSW
MAB31 to MAB35	BRIP45740 to 45744	Mango	Anthracnose	3	Bangalow, NSW
MPB31 to MPB35	BRIP45765 to 45769	Mango	Pepper spot	3	Bangalow, NSW
MAB41 to MAB45	BRIP45745 to 45749	Mango	Anthracnose	4	Bangalow, NSW
MPB41 to MPB45	BRIP45770 to 45774	Mango	Pepper spot	4	Bangalow, NSW
MAB51 to MAB55	BRIP45750 to 45754	Mango	Anthracnose	5	Bangalow, NSW
MPB51 to MPB55	BRIP45775 to 45779	Mango	Pepper spot	5	Bangalow, NSW
MAG11to MAG15	BRIP45780 to 45784	Mango	Anthracnose	1	Green Pigeon, NSW
MPG11 to MPG15	BRIP45805 to 45809	Mango	Pepper spot	1	Green Pigeon, NSW
MAG21to MAG25	BRIP45785 to 45789	Mango	Anthracnose	2	Green Pigeon, NSW
MPG21 to MPG25	BRIP45810 to 45814	Mango	Pepper spot	2	Green Pigeon, NSW
MAG31to MAG35	BRIP45790 to 45794	Mango	Anthracnose	3	Green Pigeon, NSW
MPG31 to MPG35	BRIP45815 to 45819	Mango	Pepper spot	3	Green Pigeon, NSW
MAG41to MAG45	BRIP45795 to 45799	Mango	Anthracnose	4	Green Pigeon, NSW
MPG41 to MPG45	BRIP45820 to 45824	Mango	Pepper spot	4	Green Pigeon, NSW
MAG51to MAG55	BRIP45800 to 45804	Mango	Anthracnose	5	Green Pigeon, NSW
MPG51 to MPG55	BRIP45825 to 45829	Mango	Pepper spot	5	Green Pigeon, NSW

on potato dextrose agar (1/2 strength) (Oxoid) amended with streptomycin (0.1%) (SPDA).

To obtain avocado and mango pepper spot isolates, five fruit with visible symptoms were picked from trees corresponding to the trees from which anthracnose isolates were collected. Fruit were surface sterilised with 70% ethanol and air-dried. Small segments (<0.5 mm^2) were cut from the raised lesions on the outer surface of the skin and submerged in SPDA and incubated at room temperature. One isolate was retained from each fruit.

Single-spore cultures were obtained from each isolate. Isolates were stored under sterile water (Boesewinkel 1976) and were also stored as spore suspensions. A 1-mL aliquot of a 10^6 conidia/mL suspension was transferred to a 1.8-mL Nunc Cryotube™ (Nunc™ Thermo Fisher Scientific) containing 0.42 mL of sterile 50% glycerol solution, resulting in a 15% glycerol suspension. Cryotubes were stored in a freezer at −70°C.

The accession details for each isolate represented the host plant, symptom type, geographic source and location within the particular orchard.

Isolates were subcultured from mycelial agar plugs stored in sterile water onto SPDA. The cultures were grown for ~3–5 days at room temperature under near UV light (~350 nm) and then subcultured to oatmeal agar plates for ~7 days, from which spore suspensions were harvested. Spore suspensions were supplemented with 0.01% v/v Tween 80 and kept in sealable plastic containers which could be used directly for fruit dipping. For the field experiment, spore suspensions were prepared and kept on ice during the 90-min trip to the field site.

In detached avocado fruit experiments disease assessments were based on presence or absence of visible anthracnose lesions and the diameter of lesions. In glasshouse and field experiments a 0–5 scale was used for disease assessments based on the number of pepper spot lesions on petioles and small (up to 4-cm-long) fruit, where 0 = no lesions, 1 = 1–5 lesions, 2 = 6–10 lesions, 3 = 11–20 lesions, 4 = 21–50 lesions, 5 = >50 lesions.

Inoculation of selected C. gloeosporioides *isolates from avocado and mango on detached avocado fruit in the laboratory*

Seedless cocktail 'Fuerte' avocado fruit were harvested in May 2003 from an orchard at Duranbah, New South Wales. Fruit were ~5–6 cm in length and ~2 cm wide. The following day, fruit were rinsed in lukewarm water to reduce superficial chemical residues, surface sterilised with 70% alcohol and air-dried. Three sites for inoculation were identified on the surface of each fruit and marked as circles with a pen. Eighty isolates were screened for pathogenicity. Fifty isolates were from avocado fruit and 30 from mango fruit. Half the isolates from each host were from pepper spot lesions and the other half from anthracnose lesions. Isolates from all geographic regions sampled were included in the study.

Fruit were inoculated by pipetting three single droplets (25 µL) of spore suspension (5×10^6 conidia/mL) of an isolate onto the surface of a fruit on the three allocated areas. This was replicated four times, i.e. four fruit per isolate. Control fruit were treated with water. Fruit were then placed in plastic crates (with all fruit from each replicate in a single crate) lined with moist paper and sealed to maintain high humidity. Crates were incubated at 25°C. After 48 h, the fruit were transferred to avocado packing cartons and kept at 23°C (65% relative humidity). Fruit were assessed for disease incidence from eating-ripe stage, (which was 5 days after inoculation) and the diameters of lesions measured. An average was taken of the three measurements per fruit. The data were analysed as a randomised complete block (with

crates as replicates) and lesion incidence was scored as presence or absence of visible lesions at three inoculation sites per fruit.

Inoculation of selected C. gloeosporioides *isolates from avocado and mango on avocado nursery plants in the glasshouse*

These tests were undertaken to provide an assessment of infection of attached leaves and petioles in a controlled environment. The purpose of this experiment was to provide preliminary data before designing the field experiment. Ideally, nursery trees bearing fruit would have been used but this was not possible. The experiment was carried out using 'Hass' avocado leaves and their petioles (in the absence of fruit) on immature (6-month-old) grafted nursery trees ('Velvick' West Indian rootstock) in pots in the glasshouse in April 2003 (autumn).

Five branches were selected at random on each plant and tagged. Isolates were the same as those in the detached fruit experiments. Spore suspensions were prepared for each isolate (5×10^6 conidia/mL) and, using an artist's airbrush, leaves, petioles and branches were sprayed with spore suspensions containing 0.01% v/v Tween 80 ensuring that the entire surface was saturated. Five isolates were inoculated onto each tree (one isolate per branch) and this was replicated five times. Control leaves were sprayed with water containing Tween 80. Leaves were enclosed in a plastic bag containing a water-soaked cotton wool ball and held in place with staples to maintain high humidity. After 48 h the bags were removed. Leaves were assessed for disease after 2 weeks and then at weekly intervals to assess pepper spot development. Samples of lesions which developed within the treated area were excised and returned to the laboratory for isolation and culturing.

Inoculation of selected C. gloeosporioides *isolates from avocado and mango on avocado fruit in the field*

Field experimentation commenced in November 2003 at the property of G. Anderson at Duranbah, on a block of 3-year-old 'Hass' avocado trees grafted to clonal 'Velvick' rootstock. Fruit were on average 2 cm in length at this stage (4–8 weeks after fruit-set). Isolates were the same as those in the detached fruit and the glasshouse experiments. A control was included on each tree, giving a total of 20 control fruit. Twenty trees were used and 20 fruit per tree were selected at random and tagged, ensuring that fruit were free of any obvious blemishes or disease. Each isolate was inoculated onto five fruit randomly chosen throughout the 20 trees.

Spore suspensions were prepared for each isolate (5×10^6 conidia/mL). Fruit were dipped in a spore suspension containing 0.01% v/v Tween 80 ensuring that the entire fruit surface was saturated. Control fruit were dipped in water containing Tween 80. Fruit were enclosed for 48 h in a plastic bag and paper bag as previously described. Fruit were assessed for disease after 2, 4 and 6 weeks. The experimental design was generated by the program CycDesigN (Whitaker *et al.* 2001) and was a non-resolvable block design, where a block was represented by a tree.

Inoculation of selected C. gloeosporioides *isolates from avocado and mango on detached mango fruit in the laboratory*

Mango fruit (cv. 'Brooks') were harvested in March 2007 from the orchard of C. Jacobs near Bundaberg, Queensland. Fruit had received no chemical treatments throughout the season and were sprayed with 70% ethanol and left to air dry. Fruit were mature green and had been desapped. Isolates were the same as those used to inoculate avocado fruit.

Fruit were inoculated by soaking a filter paper disc in the spore suspension of an isolate (5×10^6 conidia/mL) and then the disc was removed from the suspension with forceps and placed on the surface of the fruit on the allocated area (as indicated with a drawn circle). Each fruit had four discs. Isolates were inoculated in series followed by a water control disc. This was replicated four times. Each isolate was inoculated at a different site on each fruit, i.e. at the top, left middle, right middle and bottom of the fruit, to allow for any variation in susceptibility. Fruit were incubated as for avocado. Fruit were assessed for presence or absence of a visible lesion, and the diameter of the lesion was measured at eating ripe stage 12 days after inoculation.

Statistical analysis

The data were analysed using GENSTAT seventh and eighth editions (Lawes Agricultural Trust, Rothamsted Experimental Station, UK). Unless otherwise described, analyses used ANOVA in randomised blocks incorporating Fisher's pairwise comparison tests.

Results

Inoculation of selected C. gloeosporioides *isolates from avocado and mango on detached avocado fruit in the laboratory*

Lesion incidence

In the analysis of lesion incidence, there was a significant isolate × time interaction ($P < 0.001$, l.s.d. = 0.968) indicating that the pattern of response of the isolates over time was different. Five days after inoculation, however, all potential lesions had developed at inoculated sites and this data is presented in Fig. 1. Isolates were grouped according to symptom or host of origin (mango pepper spot, mango anthracnose, avocado pepper spot and avocado anthracnose) and according to geographic origin (Ayr, Bangalow, Cudgen, Duranbah, Green Pigeon, Mt Tamborine) (Fig. 1). There were significant differences between isolate groups ($P < 0.001$, l.s.d. = 0.666).

More lesions developed on detached avocado fruit inoculated with avocado isolates than mango isolates (Fig. 1). All the avocado isolates produced typical anthracnose lesions which ultimately became sunken over time. Approximately half of the mango isolates, however, produced a blackening of the skin surface of 1–2 mm in diameter and, while they

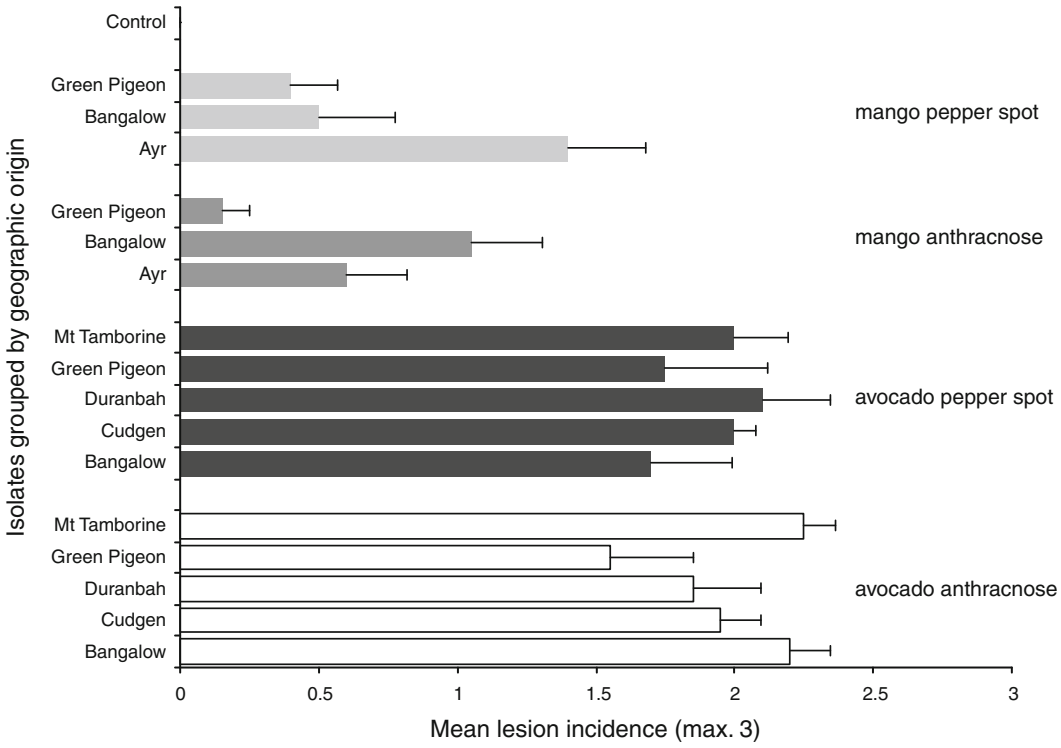

Fig. 1. Mean incidence of anthracnose lesions 5 days after inoculation with avocado isolates of *Colletotrichum gloeosporioides* from pepper spot and anthracnose and mango isolates from pepper spot and anthracnose on detached cocktail 'Fuerte' avocado fruit. Isolates are grouped according to their fruit, symptom and place of origin (±s.e., $n = 60$).

discontinued spreading and did not become sunken, they were counted as lesion incidence in this study. Generally, most sites inoculated with mango isolates remained symptomless until 3 days after inoculation.

Data were further grouped into four categories for isolates based on 'fruit-type' origin and 'symptom-type' origin (i.e. disregarding geographic origin) (Table 2). Comparisons of lesion incidences due to inoculation with *C. gloeosporioides* isolates from avocado pepper spot and anthracnose and mango pepper spot and anthracnose, 5 days after inoculation, show that avocado isolates had a significantly higher mean incidence than mango isolates ($P < 0.001$, l.s.d. = 0.364) but there were no significant differences between isolates from pepper spot and isolates from anthracnose from either fruit.

Table 2. Total mean anthracnose lesion incidence (max. three) and diameter on detached cocktail 'Fuerte' avocado fruit according to fruit and symptom origins of *Colletotrichum gloeosporioides* isolates ($P < 0.001$) 5 days after inoculation

Means with the same letter in each column were not significantly different at $P < 0.05$. $n = 180$ mango; $n = 300$ avocado

Origin of isolate	Mean incidence (max. 3)	Mean diameter (mm)
Avocado anthracnose	1.96a	3.51a
Avocado pepper spot	1.91a	3.70a
Mango anthracnose	0.60b	1.03b
Mango pepper spot	0.77b	1.31b

Lesion diameter

Lesion size, in addition to incidence, was a further indicator of isolate aggressiveness. Again, there was a significant isolate × time interaction ($P < 0.001$) indicating that the pattern of response of the isolates over time was different, but maximum lesion size was attained after 5 days. There were significant differences in aggressiveness manifested at 5 days after inoculation ($P < 0.001$, l.s.d. = 2.449) (Fig. 2). Lesions arising from inoculations with avocado isolates were larger than those from inoculations with mango isolates. It was observed that most of the lesions less than 3 mm were extremely slow to expand and generally did not penetrate deeply into the tissue. This scenario was particularly prevalent with mango isolates.

Data were further grouped into four categories for isolates based on fruit-type origin and symptom-type origin (Table 2). Comparisons of lesion diameters due to inoculation with *C. gloeosporioides* isolates from avocado pepper spot and anthracnose and mango pepper spot and anthracnose, 5 days after inoculation, show that avocado isolates produced significantly larger lesions than mango isolates ($P < 0.001$, l.s.d. = 1.268) but there were no significant differences between isolates from pepper spot and isolates from anthracnose from either fruit. In general, trends in lesion incidence data follow a similar pattern to trends in lesion diameter data; isolates

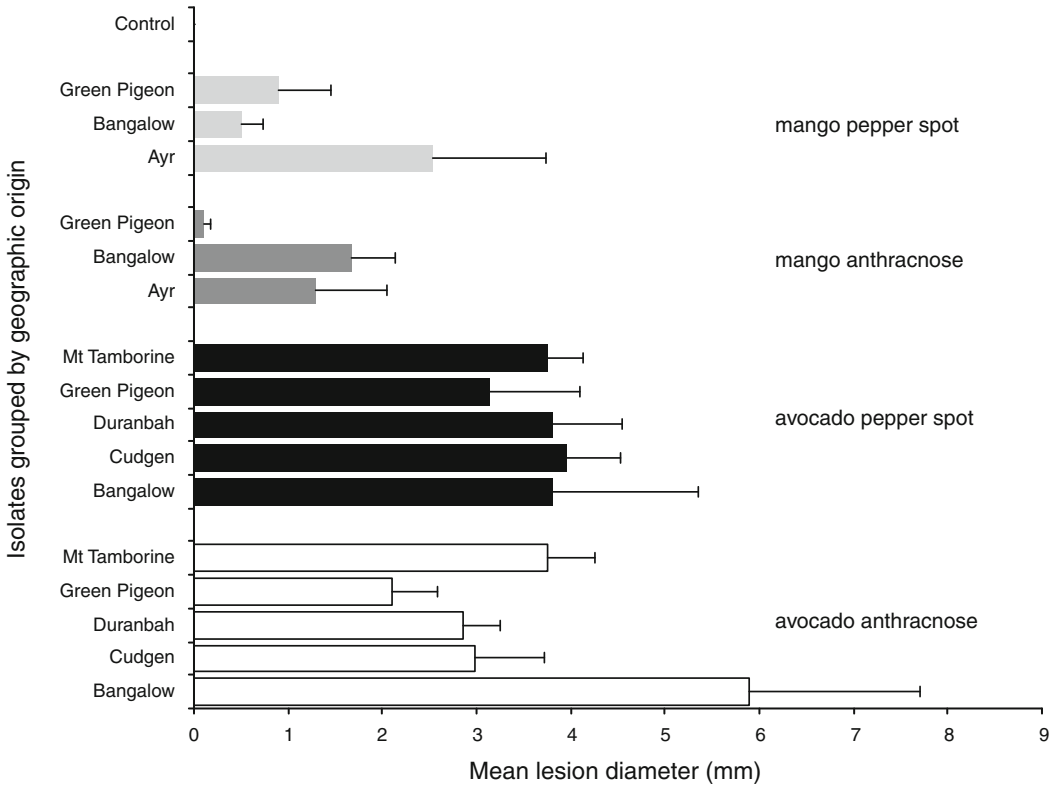

Fig. 2. Mean diameter of anthracnose lesions 5 days after inoculation with avocado isolates of *Colletotrichum gloeosporioides* from pepper spot and anthracnose and mango isolates from pepper spot and anthracnose on detached cocktail 'Fuerte' avocado fruit. Isolates are grouped according to their fruit, symptom and place of origin (±s.e., $n = 60$).

which produced the most lesions also tended to produce the largest lesions.

Inoculation of selected C. gloeosporioides *isolates from avocado and mango on avocado nursery plants in the glasshouse*

Pepper spots were not observed on leaves but they were present on the petioles and branches (Fig. 3) and were identical to those which formed on fruit and pedicels in field tests (Fig. 4). There have been no reports of pepper spots on leaves of avocado. Pepper spot symptoms showed as small, shiny, raised, black lesions measuring less than 0.5 mm in diameter when they become visible to the naked eye. Based on previous studies, symptoms were expected to appear 4–6 weeks after inoculation (S. L. Willingham, pers. comm.); however, this was not the case. After a latent period of 6 months, symptoms appeared, and only on the inoculated petioles and branches (controls remained symptomless). The petioles were then rated for severity of pepper spots, according to the rating scale described in the Materials and methods section. When pepper spot lesions were picked from the petioles and plated on SPDA, *C. gloeosporioides* was isolated from at least 80% of culture sites.

Avocado pepper spot isolates generally produced the most pepper spot disease on petioles (Fig. 5). Avocado pepper spot isolates from Green Pigeon, however, were significantly less aggressive. This contrasts with detached fruit data where these same isolates, although originally isolated from pepper spots, were not significantly more or less aggressive than other avocado isolates when inoculated onto detached fruit and assessed for anthracnose development. Mango isolates produced the lowest ratings. There were no significant differences between mango isolates (anthracnose or pepper spot) from any geographic grouping. Importantly, all control inoculation sites remained symptomless.

To summarise total mean data for isolates from avocado and mango, avocado anthracnose isolates produced significantly less pepper spot lesions than avocado pepper spot isolates ($P < 0.001$, average l.s.d. = 0.549, accounting for uneven replication of avocado and mango isolates) (Table 3). All avocado isolates produced significantly more pepper spot lesions than all mango isolates. Mango anthracnose isolates were not significantly different from mango pepper spot isolates in their capacity to produce pepper spot.

Inoculation of selected C. gloeosporioides *isolates from avocado and mango on avocado fruit in the field*

In young fruit, such as those used in this experiment, pepper spot symptoms tended to appear within 2 weeks. Data collected 6 weeks after inoculation showed that there was a small amount of natural pepper spot infection on water-inoculated

Fig. 3. Pepper spot lesions after inoculation with *Colletotrichum gloeosporioides* on 'Hass' avocado branch (diameter ~7 mm) in the glasshouse.

fruit (mean rating of 0.63) on the trees at the assessment time (January) (Fig. 6). Pepper spot tended to occur only on some branches on a tree and not necessarily on all trees, so it was difficult to predict which fruit would remain free of naturally occurring disease when selecting fruit for inoculation, especially that early after fruit-set as in the case of this experiment. Pepper spot was significantly more severe on fruit inoculated with avocado isolates than with mango isolates ($P < 0.001$, l.s.d. = 0.508).

Mean pepper spot ratings for avocado isolates were significantly higher than for mango isolates ($P < 0.001$, average l.s.d. = 0.266, accounting for uneven replication of avocado and mango isolates), and pepper spot on fruit inoculated with avocado pepper spot isolates was significantly more severe than fruit inoculated with avocado anthracnose isolates (Table 4). These trends were also observed on petioles and branches of nursery trees in the glasshouse (Table 3).

Fig. 4. Pepper spot lesions after dip inoculation with *Colletotrichum gloeosporioides* on 'Hass' pedicels in the field.

Inoculation of selected C. gloeosporioides *isolates from avocado and mango on detached mango fruit in the laboratory*

Lesion incidence

The presence or absence of lesions was recorded on day 12 after inoculation by which time fruit were fully ripe and symptoms fully developed. Isolates were grouped according to symptom or host of origin as well as geographic origin. More lesions developed on detached mango fruit inoculated with mango isolates than avocado isolates (Fig. 7). Significant differences were observed ($P < 0.001$, l.s.d. = 1.064) with all six mango groups recording the highest incidence ratings compared with the avocado groups. The exception was avocado pepper spot isolates from Green Pigeon, which had a significantly higher incidence on mango than most of the other avocado groups. In general, most sites inoculated with mango isolates developed lesions. By contrast, of sites inoculated with avocado isolates, 75% did not develop symptoms.

Data were further grouped into four categories for isolates based on fruit-type origin and symptom-type origin (Table 5). Comparisons of lesion incidences due to inoculation with *C. gloeosporioides* isolates from avocado pepper spot and anthracnose and mango pepper spot and anthracnose, 12 days after inoculation, show that avocado isolates were associated with a lower anthracnose incidence than mango isolates ($P < 0.001$, l.s.d. = 0.597) but there were no significant differences between isolates from pepper spot and isolates from anthracnose from either fruit.

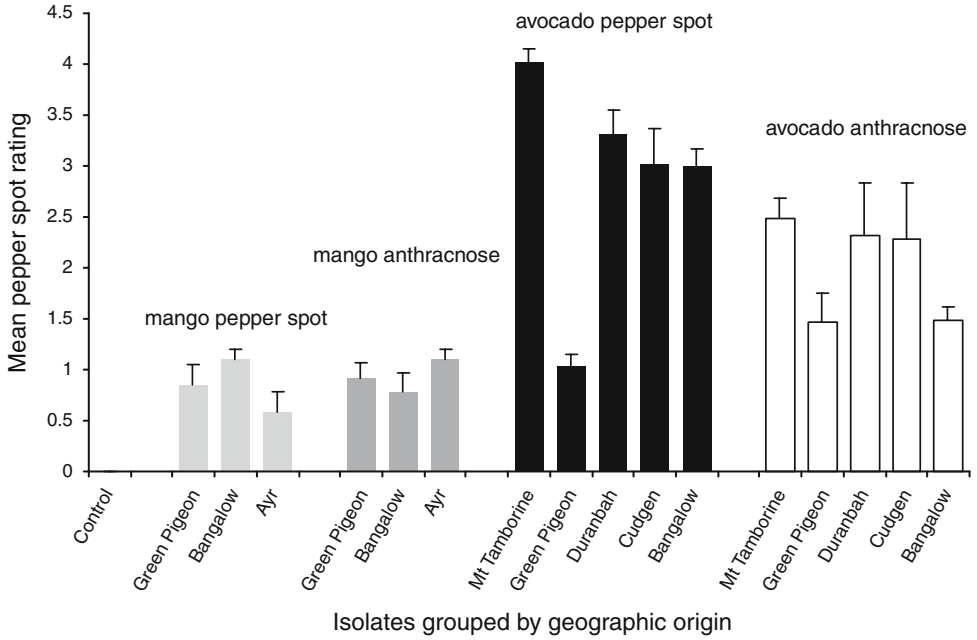

Fig. 5. Mean pepper spot severity (where 0 = no lesions, 1 = 1–5 lesions, 2 = 6–10 lesions, 3 = 11–20 lesions, 4 = 21–50 lesions, 5 = >50 lesions) on petioles of 'Hass' avocado plants in the glasshouse 6 months after inoculation with avocado and mango isolates of *Colletotrichum gloeosporioides* (±s.e., $n = 25$).

Table 3. Total mean pepper spot severity on petioles of 'Hass' avocado plants in the glasshouse 6 months after inoculation with avocado and mango isolates of *Colletotrichum gloeosporioides*

Pepper pot severity: 0 = no lesions, 1 = 1–5 lesions, 2 = 6–10 lesions, 3 = 11–20 lesions, 4 = 21–50 lesions, 5 = >50 lesions. Isolates grouped according to fruit and symptom origin. Means with the same letter were not significantly different at $P < 0.05$. $n = 75$ mango; $n = 125$ avocado

Origin of isolate	Mean pepper spot severity (0–5 rating)
Avocado anthracnose	2.00b
Avocado pepper spot	2.88a
Mango anthracnose	0.94c
Mango pepper spot	0.84c

Lesion diameter

Lesion size was also recorded and there were significant differences between groups ($P < 0.001$, l.s.d. = 4.141). Lesions arising from inoculations with mango isolates were larger than those from inoculations with avocado isolates (Fig. 8). Although the incidence of lesions by avocado pepper spot isolates from Green Pigeon was relatively high, the size of the lesions remained small.

Data were further grouped into four categories for isolates based on fruit-type origin and symptom-type origin (Table 5). Comparisons of lesion diameters due to inoculation with *C. gloeosporioides* isolates from avocado pepper spot and anthracnose and mango pepper spot and anthracnose, 12 days after inoculation, showed that avocado isolates produced significantly smaller lesions than mango isolates ($P < 0.001$, l.s.d. = 2.151). Although there were no significant differences between isolates from avocado pepper spot and isolates from avocado anthracnose, mango anthracnose isolates produced significantly larger lesions than mango pepper spot isolates.

Discussion

Strains of *C. gloeosporioides* isolated from different hosts can vary morphologically and in pathogenicity (Hayden *et al*. 1994; Freeman and Shabi 1996; Afanador-Kafuri *et al*. 2003). The experiments described in this paper were used to characterise populations of *C. gloeosporioides* isolates based on pathogenicity, aggressiveness and cross-infection potential when inoculated onto unwounded avocado fruit, pedicels and petioles and mango fruit under specific conditions. At the high inoculum levels (5×10^6 conidia/mL) used in the experiments, most isolates, irrespective of origin host or symptom type, to varying degrees produced pepper spot symptoms on developing avocado fruit and pedicels on the tree as well as anthracnose symptoms on detached ripening avocado and mango fruit. Likewise, most isolates produced pepper spot symptoms on petioles of nursery avocado trees, but not on their leaves. This research has, however, demonstrated differences in aggressiveness when avocado and mango fruit are inoculated with isolates of *C. gloeosporioides* from avocado and mango. On avocado, the mango isolates were significantly and repeatedly less aggressive than the avocado isolates, and significant differences in pepper spot incidence were demonstrated between avocado anthracnose and avocado pepper spot isolates. On mango, the mango isolates were significantly and repeatedly more aggressive than the avocado isolates, which concurs with previous work (Alahakoon *et al*. 1994*a*; Hayden *et al*. 1994).

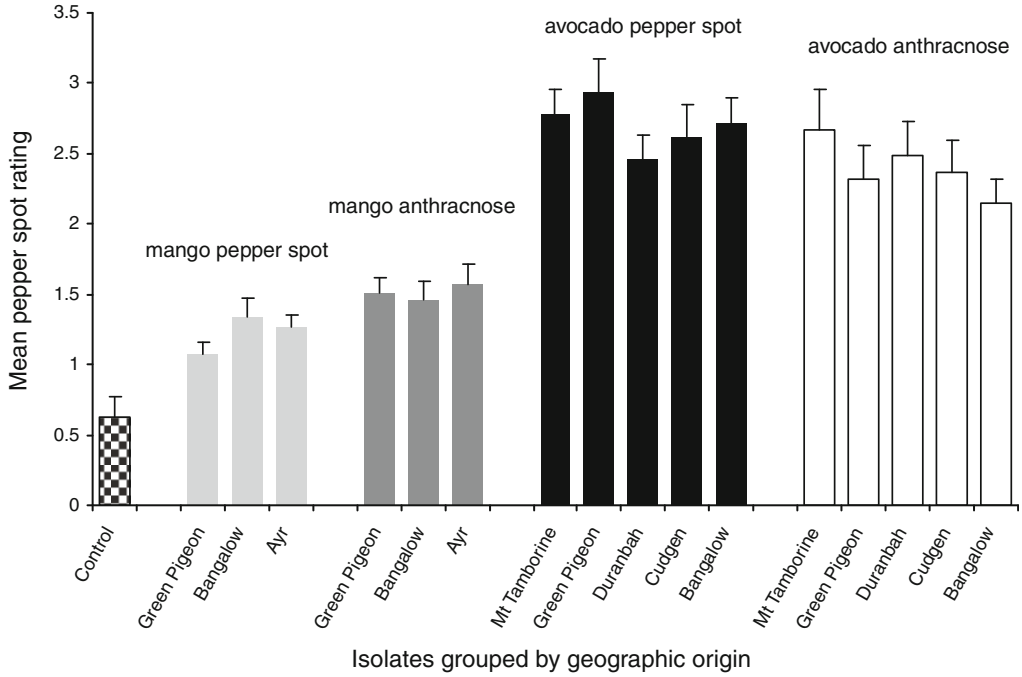

Fig. 6. Mean pepper spot severity (where 0 = no lesions, 1 = 1–5 lesions, 2 = 6–10 lesions, 3 = 11–20 lesions, 4 = 21–50 lesions, 5 = >50 lesions) 6 weeks after inoculation with avocado and mango *Colletotrichum gloeosporioides* isolates on 'Hass' avocado fruit in the field at Duranbah (±s.e., $n = 25$).

Table 4. Total mean pepper spot severity on immature 'Hass' avocado fruit in the field (Duranbah) 6 weeks after inoculation with avocado and mango isolates of *Colletotrichum gloeosporioides*

Pepper spot severity: 0 = no lesions, 1 = 1–5 lesions, 2 = 6–10 lesions, 3 = 11–20 lesions, 4 = 21–50 lesions, 5 = >50 lesions. Isolates are grouped according to fruit and symptom origin. Means with the same letter were not significantly different at $P < 0.05$. $n = 75$ mango; $n = 125$ avocado

Origin of isolate	Mean pepper spot severity (0–5 rating)
Avocado anthracnose	2.40b
Avocado pepper spot	2.70a
Mango anthracnose	1.51c
Mango pepper spot	1.23c

Isolates of *C. gloeosporioides* inoculated on detached avocado and mango fruit in the laboratory were more aggressive, i.e. more isolates from the total population were able to cause disease, than on unripe attached fruit in nature. In the *C. gloeosporioides*-avocado relationship with ripening fruit, this is most likely due to the ripening ethylene levels increasing and antifungal compounds and other defences diminishing (Prusky *et al*. 1982; Bower and Cutting 1988). Therefore, in the detached fruit experiments the fungus was being inoculated onto ripening fruit which were inherently more vulnerable to direct invasion than unripe fruit in the field. However, even with unwounded detached avocado fruit, there were clear differences in aggressiveness between mango isolates and avocado isolates, with disease incidence and severity being lower after inoculation with mango isolates. Inoculation of detached avocado fruit with mango isolates frequently only produced a limited superficial necrosis of the skin. There were no major differences between pepper spot and anthracnose isolates in the incidence and severity of symptoms produced in detached avocado fruit. Generally, the data indicated that pepper spot isolates were as capable as anthracnose isolates of causing anthracnose in ripening fruit. Subsequent experiments in the glasshouse and field also determined that many anthracnose isolates were capable of causing pepper spot to similar levels as pepper spot isolates. Similarly, on detached mango fruit, disease incidence was lower after inoculation with avocado isolates than mango isolates, demonstrating that within this *C. gloeosporioides* collection isolates showed a degree of specialisation towards their host of origin.

In field experiments on avocado fruit, symptoms of pepper spot became visible 1–2 weeks after inoculation. The hypersensitive response (HR) may provide a possible explanation for the development of the pepper spot symptom, whereby the avocado host prevents the establishment of incompatible *C. gloeosporioides* isolates in fruit by hypersensitive cell death (Mittler *et al*. 1996; Heath 1999; Lam *et al*. 2001). The HR involves cell death around the infection site at the point of entry and results in a localised zone of dead cells. The pepper spot lesion appears to restrict further growth of the fungus but does not kill it (as the fungus was readily isolated from the lesions). One possible explanation for the phenomenon is that more aggressive isolates escape or suppress this HR by penetrating more quickly and becoming quiescent before active host responses are manifested. Alternatively the isolates which

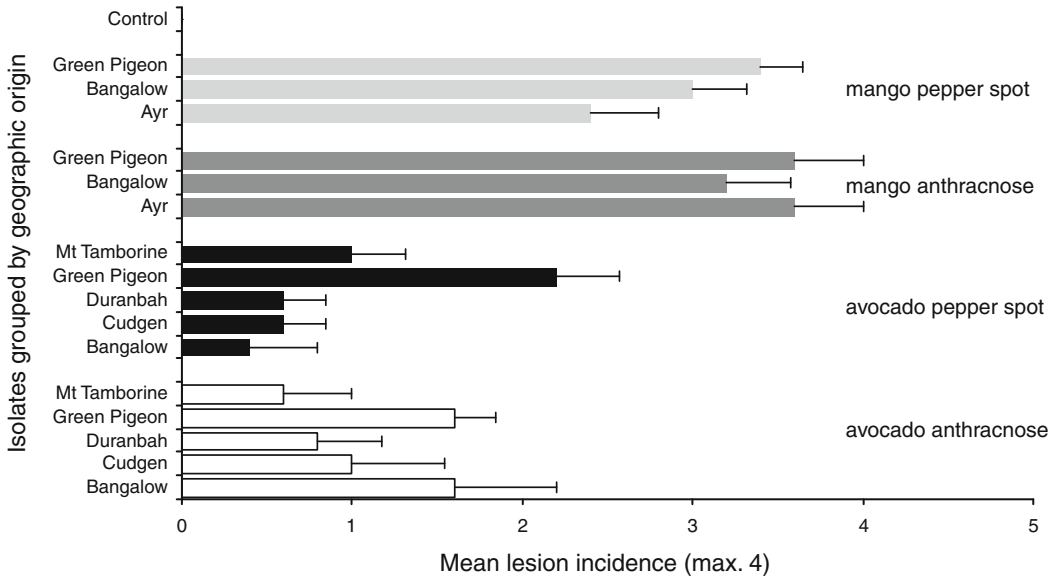

Fig. 7. Mean incidence of anthracnose lesions 12 days after inoculation with avocado isolates of *C. gloeosporioides* from pepper spot and anthracnose and mango isolates from pepper spot and anthracnose on detached 'Brooks' mango fruit. Isolates are grouped according to their fruit, symptom and place of origin (±s.e., $n = 20$).

Table 5. Total mean anthracnose lesion incidence (max. 4) and diameter on detached 'Brooks' mango fruit according to fruit and symptom origins of *Colletotrichum gloeosporioides* isolates ($P < 0.001$) 12 days after inoculation

Means with the same letter in each column were not significantly different at $P < 0.05$. $n = 60$ mango; $n = 100$ avocado

Origin of isolate	Mean incidence (max. 4)	Mean diameter (mm)
Avocado anthracnose	1.12b	1.61c
Avocado pepper spot	0.96b	1.15c
Mango anthracnose	3.47a	13.09a
Mango pepper spot	2.93a	9.96b

become quiescent and ultimately produce anthracnose could be less aggressive and thus do not trigger a HR. In both of these situations, it is implied that constitutive defences (compounds or structures) are not completely effective. One pathogenicity gene has been identified in *C. gloeosporioides* which attacks *Stylosanthes* sp., and studies of mutants of this gene suggested that it had a role in either suppressing or avoiding a host HR during the primary infection process (Manners *et al*. 2000). More research is required to establish if *R* and *Avr* gene products are involved in the initial recognition response between avocado and *C. gloeosporioides*.

In glasshouse experiments, pepper spot symptoms did not appear until 6 months after inoculation of avocado nursery plants. In a previous study, Willingham *et al*. (2000) found that pepper spot symptoms developed after 4–6 weeks on twigs and petioles following inoculation with an avocado pepper spot isolate (3×10^6 conidia/mL). It is possible that the delay in symptom expression in our study was due to an extended period of quiescence resulting from high diene levels in leaves and petioles of the seedlings. As plant tissue became senescent due to the adverse growing conditions, diene levels may have declined, the pathogen resumed growth and there was a hypersensitive host response. Research conducted at The University of Queensland (Carman and Handley 1999) has indicated that diene levels are usually higher in leaf tissue than in fruit of avocado. These higher diene levels may negate the necessity for an immediate HR. It is thought that the plants used in the Willingham *et al*. (2000) study were a Mexican rootstock and were stressed in the glasshouse before inoculation with *C. gloeosporioides* (S. L. Willingham, pers. comm.). Rootstock research by Willingham *et al*. (2001) established that fruit from 'Hass' grafted to West Indian 'Velvick' rootstock are less susceptible to postharvest anthracnose, probably due to a higher concentration of diene in this rootstock/scion combination compared with the 'Hass' on Mexican rootstock combination. In the present experiment the nursery trees were grafted to 'Velvick', which may have delayed further penetration by the fungus through passive antifungal defences. As the plants aged the quiescent fungal structures may have resumed growth, activating a typical HR.

The production of pepper spot lesions (although less severe) by mango isolates on the petioles and branches of 'Hass' avocado nursery trees and 'Hass' fruit and pedicels in the field may again be due to the high inoculum density used in the inoculation experiments. It can be speculated that this is an incompatible host response where isolate growth was inhibited and hypersensitive cell death triggered, whereby active recognition is occurring at some stage of the interaction. The same host response resulting in the production of pepper spot lesions on avocado occurred following inoculation with the avocado isolates but pepper spot production was significantly more severe than with the mango isolates. It is likely that there is no distinction between the pathogenicity of anthracnose *v.* pepper

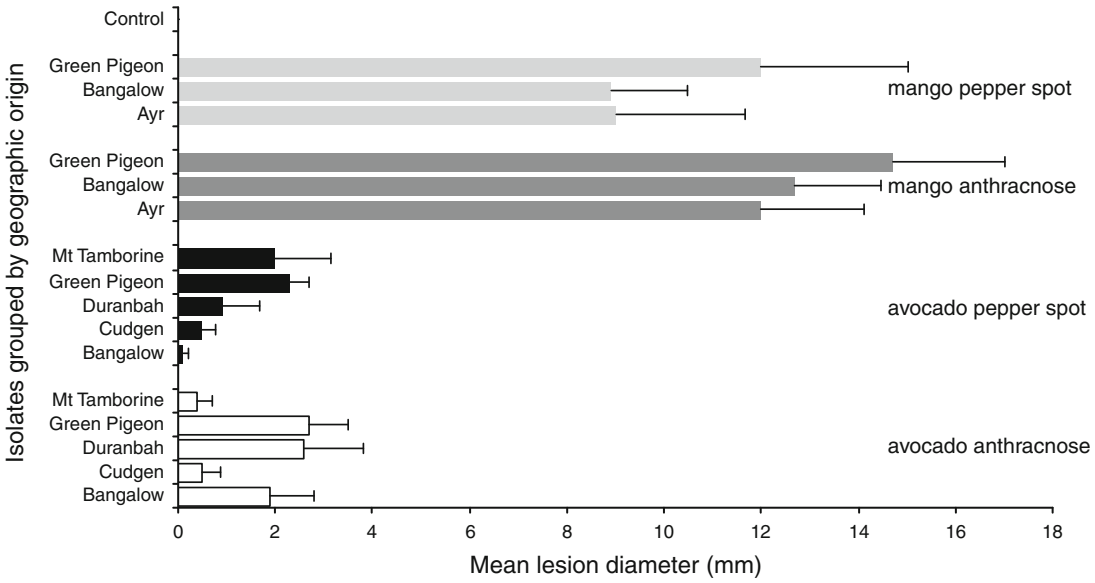

Fig. 8. Mean diameter of anthracnose lesions 12 days after inoculation with avocado isolates of *C. gloeosporioides* from pepper spot and anthracnose and mango isolates from pepper spot and anthracnose on detached 'Brooks' mango fruit. Isolates are grouped according to their fruit, symptom and place of origin (±s.e., $n = 20$).

spot isolates in the induction of pepper spot symptoms. Just as quiescent *C. gloeosporioides* (i.e. infections causing postharvest anthracnose) has likely coevolved to develop a mechanism to use the host's ripening ethylene as a signal to reactivate the infection process at a time when antifungal compound levels have become less effective (Ardi *et al*. 1998), so too it appears that avocado may have further evolved to recognise the invading pathogen causing pepper spot via other defences not yet adequately examined.

Further experimental work involving the inoculation of mango fruit in the field with the same isolates used in these experiments would likely draw some interesting conclusions. Alahakoon *et al*. (1994*a*, 1994*b*); Freeman and Shabi (1996); Freeman *et al*. (1996); and Adaskaveg and Hartin (1997) found that isolates of *C. gloeosporioides* obtained from a specific host were more pathogenic to that crop than others. Our study found that avocado isolates produced some anthracnose symptoms on detached mango fruit but it is not known whether they would produce pepper spot symptoms on young mango fruit still attached to the tree. These studies have shown that the *C. gloeosporioides* from mango comprise a pathogenically distinct subpopulation of *C. gloeosporioides* confirming at the physiological level the findings obtained in a study of the same isolates involving DNA markers (Giblin 2006). Briefly, through DNA amplification fingerprinting of *C. gloeosporioides* isolates, it has been determined that fungal populations from mango are comparatively homogeneous and are genetically distinct from avocado populations (Giblin 2006). This work will be described in more detail in a separate publication. The studies also showed that while the avocado isolates were more aggressive to their host of origin, they were also pathogenic to mango. Additional studies are needed for a better understanding of the physiological mechanisms which condition the responses leading to pepper spot and postharvest anthracnose described in this paper.

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