

POPULATION DYNAMICS OF *COLLETOTRICHUM GLOEOSPORIOIDES* ON DIFFERENT PARTS OF AN AVOCADO (*PERSEA AMERICANA*) TREE

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Abstract: *Colletotrichum gloeosporioides* in different parts of a single avocado tree, and its debris under the tree, was enumerated at monthly intervals, during a period of two years. A positive correlation between quiescent infections-QI on flowers and rainfall (RF), and a negative correlation of QI on twigs and RF were observed. The positive correlation between RF and QI on leaves was significant only when RF was pre-lagged by 3 months, and the level of significance increased progressively when RF was pre-lagged upto 6 months. Disease symptoms were never observed on the vegetative parts of the tree and flowers, but a high number of QI was observed on twigs and leaves. This is probably how *C. gloeosporioides* survives between fruiting seasons. The different relationships between QI on each part of the tree and RF, demonstrate how this pathogen may have adopted itself to survive on the tree. Therefore the results suggest that once the *C. gloeosporioides* propagules find their way to a tree, the pathogen remains there throughout its life time causing anthracnose on fruits. There was a significant drop in the density of QI of *C. gloeosporioides* on larger, mature fruits compared to that of smaller, immature fruits. Throughout this study, the free-living viable fungal propagule counts of debris under the tree varied from 10^1 to 10^3 CFU/g while those of washings of leaves, twigs and flowers varied from 0 to 10^2 CFU/g.

Key words: Anthracnose, avocado, *Colletotrichum gloeosporioides*.

INTRODUCTION

The mechanism of infection of avocado (*Persea americana* Miller) by *Colletotrichum gloeosporioides* (Penz.), the causal agent of anthracnose disease^{1,2}, and the biochemical basis of resistance of unripe avocado fruit to the pathogen^{3,4} have been well documented. This disease is one of the major reasons for the reduced shelf-life of avocado fruits in many parts of the world^{2,5} and in Sri Lanka^{6,7}.

As far back as 1971, it was demonstrated that decay spots could appear on softening fruits even if they have been inoculated with *C. gloeosporioides* as early as 3 months prior to harvest¹. Seven years later it was observed that infections with *C. gloeosporioides* could occur on fruits from fruit set to harvest⁸. More recently it was shown, that the fungus was able to remain quiescent on the avocado fruit for periods of at least 6 months⁹.

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In the literature there are no records of the presence of quiescent infections (QI) of *C. gloeosporioides* on the vegetative parts of the avocado tree. However in mango, it is reported that *C. gloeosporioides* survived actively in diseased leaves, defoliated branch terminals and old inflorescences¹⁰.

The present study was carried out on a single avocado tree. This was only an initial attempt to identify the intrinsic and extrinsic parameters to focus on, in studying the means of survival of *C. gloeosporioides* in between fruiting seasons, and in understanding its infection pattern on the developing avocado fruit.

METHODS & MATERIALS

The avocado tree used for sampling : A *Persea americana* Miller tree of Carribean origin, which is about 15 years old, approximately 20ft tall and healthy, growing in a home garden in Kandy with no special attention given, was studied. The other trees within a 10 m radius were a mango tree and a jak tree. The avocado tree was well exposed to the sun and the wind. Several other avocado trees were present within a 100 m radius, and the tree sampled was the healthiest and robust of them all.

Enumerating inoculum of C. gloeosporioides on avocado leaves, twigs, flowers and debris under the tree: Samples of leaves, pieces of young green twigs, flowers (during seasons) from the tree, and debris under the tree were collected at monthly intervals, and analysed for *C. gloeosporioides* counts immediately. This was done for a period of two years from 21 July 1991 and ending on 26 July 1993, where a total of 25 sets of counts for leaves and twigs, and 15 counts for flowers were taken. Samples of branches about 1m in length from at least five randomly selected locations around the lower region of the trunk were cut by standing under the tree using a knife fixed to a long pole. Samples of debris under the tree were collected into a clean plastic bag at the same time the branches were collected. This consisted of a mixture of dry leaves, twigs and flowers. The samples were taken to the laboratory and the branches were kept fresh by immersing the cut end in water.

Estimation of QI: Approximately 2x2 mm² pieces of green tender peelings of twigs to a depth of approximately 1 mm, leaves or fruit peel of about 1 mm thick were cut and surface sterilized by immediately dipping in 1% NaOCl solution for 2 min¹¹. Individual flowers were split in two before subjecting to surface sterilization. All surface sterilized tissues were plated on Cook's No. 2 agar medium¹² (containing 200 ppm tetracycline). All culture plates made in these experiments were incubated at room temperature (27±3°C) for 48 to 72h and the emergence of *C. gloeosporioides* colonies from the plated tissue was taken as indicative of quiescent infections. Every month, one hundred tissue pieces from each part of the avocado tree (twigs, leaves, flowers when available) were taken

randomly (a total of 300 pieces a month, when flowers were available, or 200 pieces when the tree was not in flower), so that the pieces represented all the cut branches. The plating of all the samples was completed within 3 days of collection.

Estimation of free living viable fungal propagule (FLVFP) counts of C. gloeosporioides: Standard dilution plate technique was used. Randomly obtained pieces (approx. $2 \times 2 \text{ cm}^2$) of leaves, and twigs ($\leq 5 \text{ cm}$ length of approx. 3mm diameter), which made upto 10g fresh weight separately, representing all the branches cut as described above, and debris (10g of a mixture of decaying twigs and leaves, of sizes described above) under the tree were separately placed in 90ml of sterile distilled water in a 250 ml capacity Erlenmeyer flask. The flasks were shaken vigorously for approximately 2 min after placing a previously sterilized rubber lid, and 1 ml from each was transferred to test tubes containing 9 ml sterile distilled water to prepare a series of 10 fold dilutions. One millilitre portions from the dilutions and the original suspension were pipetted out into separate petri dishes to which melted Cook's No. 2 agar medium containing 200 ppm tetracycline, was poured.

Plating was completed on the day the samples were obtained. The number of typical *C. gloeosporioides* colonies that grew on the agar plates (of no dilution to 10^{-2}) after incubating at room temperature for 48-72 h, were counted and the values were expressed as the number of Colony Forming Units (CFU) per gram fresh weight of each part of the avocado tree plated.

At least one representative *C. gloeosporioides* isolate from each part of the tree (twigs, leaves and flowers) and debris in both QI estimation study, and in the FLVFP estimation study, were stored on agar slants at 5° C until pathogenicity was established as described below.

Estimation of QI of C. gloeosporioides on fruits at different maturity stages: Fruits at 6 different maturity stages (distinguished by their size) from very young upto those in harvesting maturity were sampled to determine the quiescent *C. gloeosporioides* infections on them. Soon after plucking from the tree, the average volumes were determined by immersing the entire fruit in water and noting the amount of water displaced by each fruit. The surface area of each fruit was estimated as the two-thirds power of the fruit volume¹¹. Each surface area value thus obtained was rounded off to the nearest integer. Pieces ($2 \times 2 \text{ mm}$) of peel tissues were obtained for surface sterilization followed by plating in such a manner that five pieces per unit surface area of each fruit were considered as the number of pieces to sample from each fruit. Therefore the mean number of pieces obtained from each fruit was: 5 pieces from stage 1, 9 pieces from stage 2, 12 pieces from stage 3, 56 pieces from stage 4, 95 pieces from stage 5, 125 pieces from stage 6. At the first three stages of maturity, 5 fruits each were sampled, whereas at the last three stages of maturity 2 fruits each were sampled. The first three stages represent fruits plucked within six weeks of anthesis and the last three represent fruits at stages 6 weeks prior to harvest.

Representative peel samples were obtained covering all sides of a fruit. These were surface sterilized and plated as described earlier. The plates were incubated at room temperature for 72 h and the number of *C. gloeosporioides* colonies developed were counted, and this value was divided by 5 to obtain the number of quiescent infections per unit surface area (i.e. density) of the fruit. In this way an average density value was obtained for the total number of replicate fruits at a particular maturity stage. The *C. gloeosporioides* isolates obtained were stored on agar slants at 5°C and their pathogenicity on fruits was established as described below.

Determining pathogenicity of isolated C. gloeosporioides cultures : *C. gloeosporioides* isolates obtained were subcultured on freshly prepared Cook's No.2 agar plates and allowed to incubate for 72h at room temperature until the cultures were fully grown. The surface of healthy, mature but unripe avocado fruits were wiped with 70% ethyl alcohol. Suspensions of conidia (approx. 10^5 per ml) were prepared with each isolate in 2% sucrose solutions. Drops (20 μ l) of these suspensions were placed on 5 spots on fruits in one row along the longitudinal axis. A second parallel row of drops was placed on 5 spots which were wounded by piercing the skin to a depth of 2mm using a sterile needle. The inoculated fruits were kept inside moist chambers for 24 h and after that they were allowed to ripen in trays in the laboratory at room temperature until typical anthracnose lesions developed. The pathogen was reisolated into agar plates and the cultural characteristics were compared with the original isolates.

Rainfall (RF) was measured daily with the aid of a rain gauge (Yagami International Trading, Japan), during the two year period of analysis. The monthly RF was determined by totalling the daily values from the day after the previous month's sample was taken, to the day the present sample was taken. The RF values before the study started (i.e. in July 91) were determined by adding up the daily values for 30 day intervals (a total of 6) prior to the first sampling.

Statistical analysis : A five month moving average was used for the time series data of monthly RF and percentages of conidial counts of each part of the tree. The relationship between the independent variable (monthly RF) and each of the dependent variables (i.e. QI of percentage leaf, twig and flower) were determined using different lags, (pre-lags, expressed as $t-n$ where n = number of months) for RF. Regression analysis was performed for each of the dependant variables with RF at different lags and the most influential lags were determined.

RESULTS

Flowering was observed from January to June of 1992 and from December 1992 to July 1993 with a freak flowering period in between in October 1992 (Figure 1). The estimated QIs fluctuated during this period of study and ranged from 3 to 93% on leaves, 13 to 86% on twigs, 1 to 95% on flowers. The total FLVFP counts on the tree were low but consistent throughout the two year period except in July and September 1991, when the counts on the tree as a whole, were zero (Table 1). Of the FLVFP counts taken, the highest were seen in washings of debris under the tree which varied from 0 to 10^3 CFU/g. The FLVFP counts of washings of leaves, twigs and flowers varied from 0 to 10^2 CFU/g. Unlike QIs, there were 6 occasions on leaves, 5 occasions on twigs and 6 occasions on flowers where there were no FLVFP of *C. gloeosporioides* detected. There was no instance where FLVFP counts on the tree and debris were zero in the same month (Table 1). The *C. gloeosporioides* count in debris was zero only on one occasion, which corresponded to a sudden increase of RF in October 1991.

The graphical plots of monthly RF and QI on leaves, twigs, and flowers are given in Figure 1. No significant relationships were observed between the monthly RF values and each of the dependent variables. Therefore, 5 month moving averages were taken to study the trends. When moving averages of each of the dependent variables were compared with that of RF values (using different lags for leaf infections) the relationships listed in Table 2 were observed. There was a significant positive correlation between RF (with no lag) and QI of flowers. The regression coefficient between RF (pre-lagged 3 months, up to 6 months) and QI on leaves was also positive. However, QI of twigs correlated negatively with RF. These statistically significant relationships between RF and each dependent variable are graphically represented in Figure 2. The two flowering seasons including the freak flowering observed in October 1992, are depicted continuously along with the corresponding RF values, disregarding the periods where flowering was absent, as the 5 month moving averages were determined in this manner.

Table 1: Monthly rainfall (RF) and free-living viable fungal propagule (FLVFP) counts [in(CFU/g)x100] of the avocado tree.

Time	RF (mm)	Leaf	Twig	Flower	Debris	Total on tree
1991						
July	4.6	0	0	NFS	22	0
Aug	68.1	0.5	3	NF	3	3.5
Sept	37.8	0	0	NF	11	0
Oct	177.9	0	8	NF	0	8
Nov	181.3	1.2	2.3	NF	3.3	3.5
Dec	0	8	7	NF	27	15
1992						
Jan	23.8	8	0	0	2.6	8
Feb	0	0.7	4	0.5	1.4	5.2
Mar	2	0.1	0	0	42.5	0.1
Apr	191.6	0	1.7	0.2	7.4	1.9
May	187.2	0.5	0.5	0.8	1.5	1.8
June	226.7	0.4	0.4	0.5	14	1.3
July	291	0.5	0.4	NF	16	0.9
Aug	186.6	0.6	0.6	NF	1.2	1.2
Sept	214.7	1	0.7	NF	2.5	1.7
Oct	274.2	0	0.6	0.4	5	1.0
Nov	327.7	1.6	0.9	NF	8	2.5
Dec	81.1	5	0.4	0.5	13	5.9
1993						
Jan	4.5	0.3	0.8	0.2	4.2	1.3
Feb	10.7	6.5	0.5	0	30.5	7.0
Mar	69.3	1.1	0.6	0	12.5	1.7
Apr	208.2	0	1	0	5	1.0
May	141.8	0.1	0.3	9	2.5	9.4
June	813	1.2	0	0	0.6	1.2
July	226.8	1	1.3	4.4	1.9	6.7

§NF = No flowers

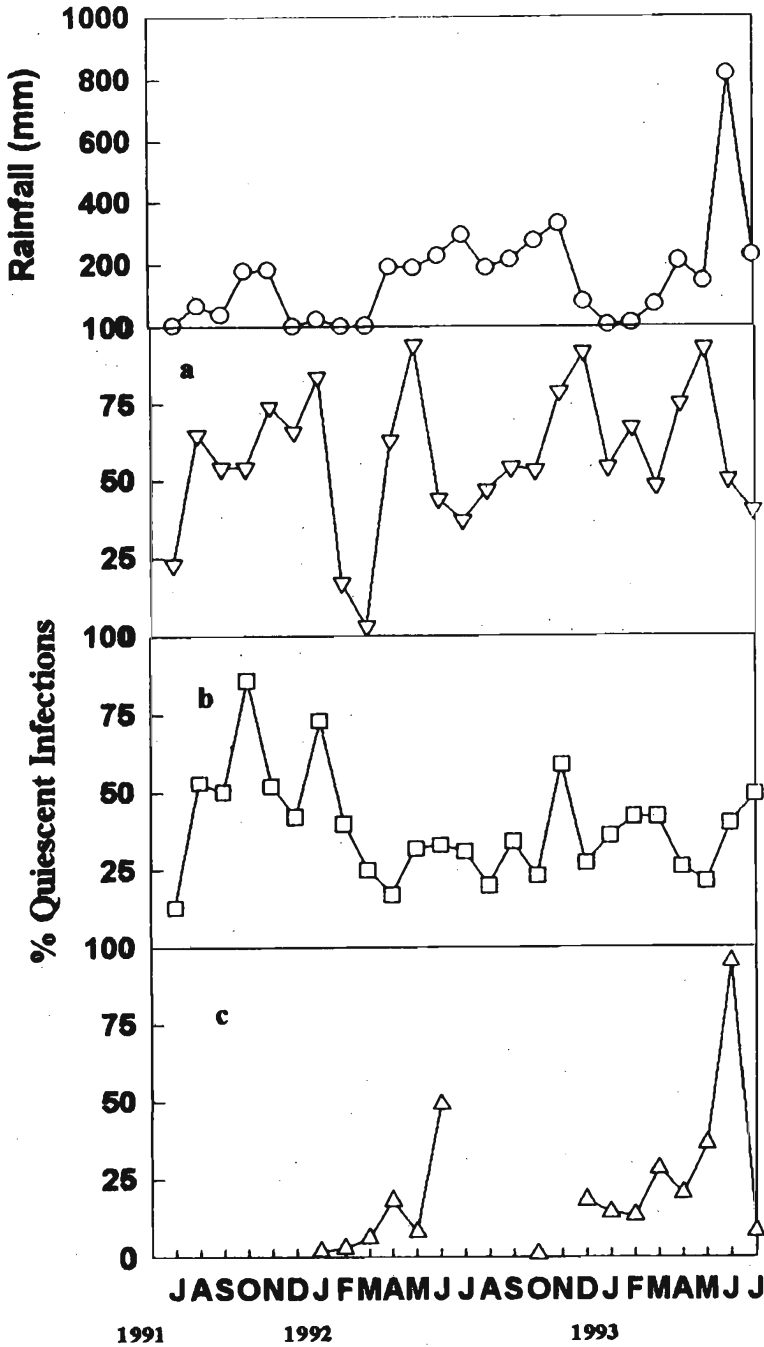


Figure 1 : Monthly rainfall and counts of quiescent infections. The values for (a) leaves (b) twigs and (c) flowers (when in season) of avocado tree are expressed as a percentage during the period between July 1991 to July 1993.

The results of the regression analysis performed between the dependent variables and RF are diagrammatically depicted in Figure 3. The positive linear correlations between RF and QI of flowers, RF (pre-lagged t-3) and QI of leaves and the negative linear correlation between RF and QI of twigs are clearly depicted in these figures.

Table 2: Regression analyses of relationships between rainfall and percentage quiescent infections on flowers, leaves and twigs.

Variables (QI)	Significance (P value for correlation)	R	Standard deviation	Equation
Flower (n=15)				
vs RF	0.003	0.78	0.025	$y=5.31+0.104x$
Leaf (n=25)				
vs RF (t-3)	0.024	0.45	0.023	$y=51+0.058x$
vs RF (t-4)	0.025	0.44	0.024	$y=50.9+0.058x$
vs RF (t-5)	0.002	0.61	0.022	$y=48.6+0.076x$
vs RF (t-6)	0.000	0.73	0.018	$y=46.8+0.09x$
Twig (n=25)				
vs RF (t)	0.003	0.78	0.025	$y=49.7-0.076x$

t= time. Pre-lags are expressed as t-n, where n= number of months.
QI=quiescent infections. RF= rainfall

QI counts of *C. gloeosporioides* on peels of fruits at different stages of maturity showed that the average conidial infections per unit area reduced sharply when the fruits enlarged with age (Figure 4). A Duncan multiple range test ($p<0.05$) revealed that the readings taken from the 3 immature stages of fruits were not significantly different from each other, and the readings of the fruits close to harvesting were not significantly different from each other. However these two sets of three readings each, were significantly different from each other.

Irrespective of the part of the tree from which the isolates was obtained, all *C. gloeosporioides* isolates tested caused typical anthracnose lesions on wound inoculated fruits. None of the inoculations made on unwounded skin of fruits developed into lesions.

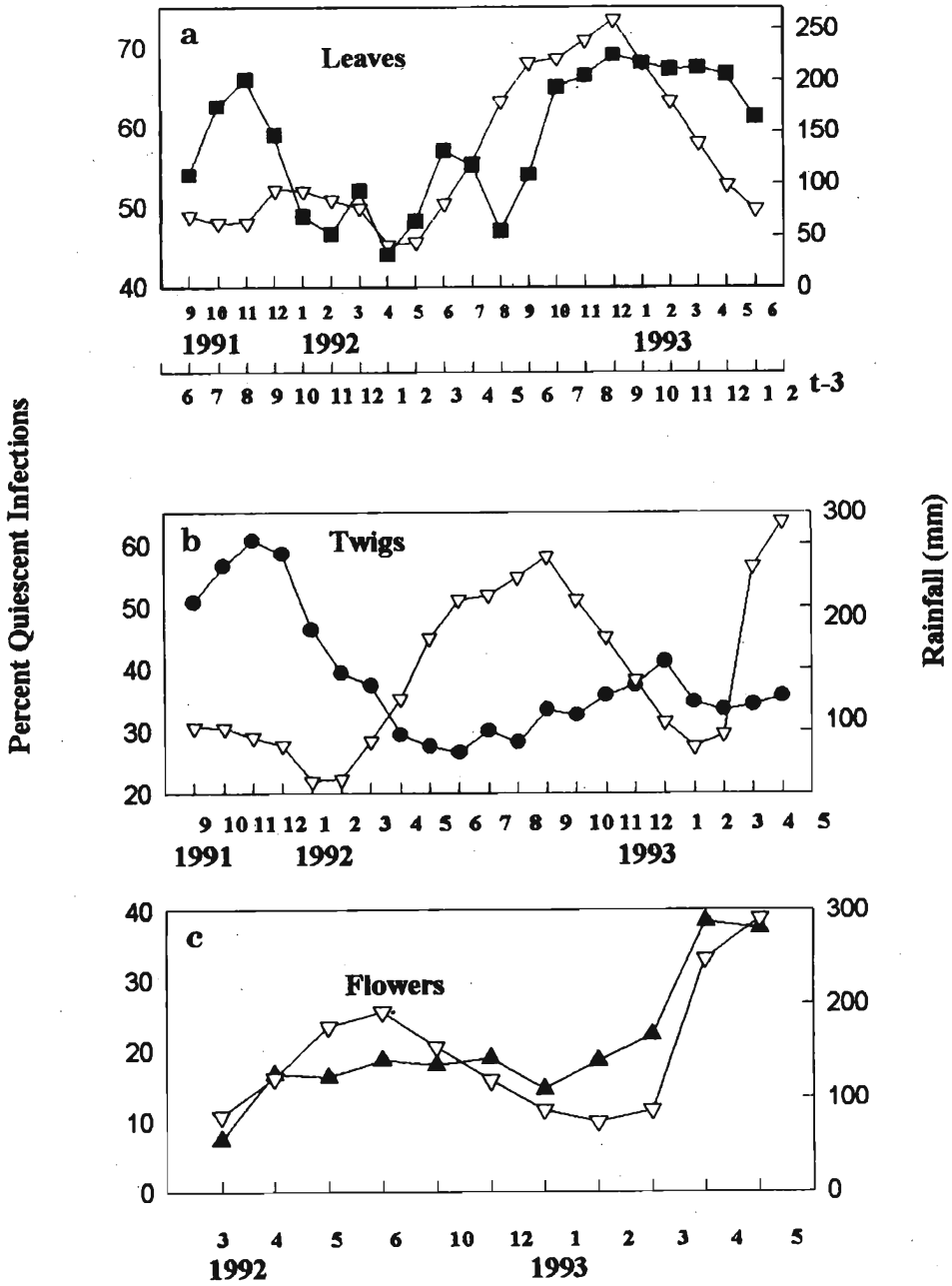


Figure 2: Graphical representations of relationships between rainfall and quiescent infections.

a) RF prelagged by 3 months (t-3) and QI of leaves, b) RF and QI of twigs, c) RF during flowering seasons and QI of flowers. Each 5 month moving average value is plotted centering to the 3rd month

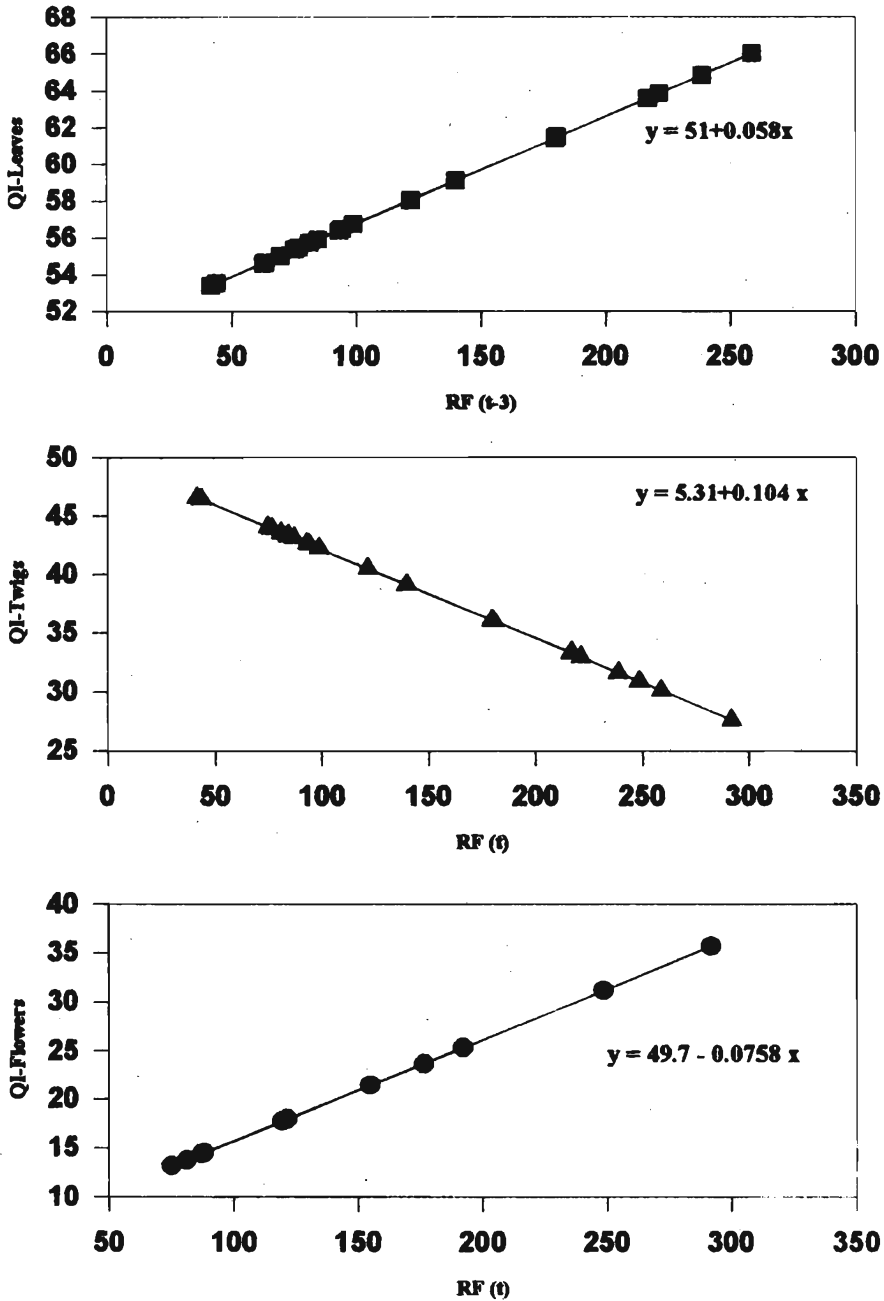


Figure 3 : Linear regression models of rainfall (prelagged 3 months for leaf data) and quiescent infections (QI) of leaves, twigs and flowers expressed as percentages.

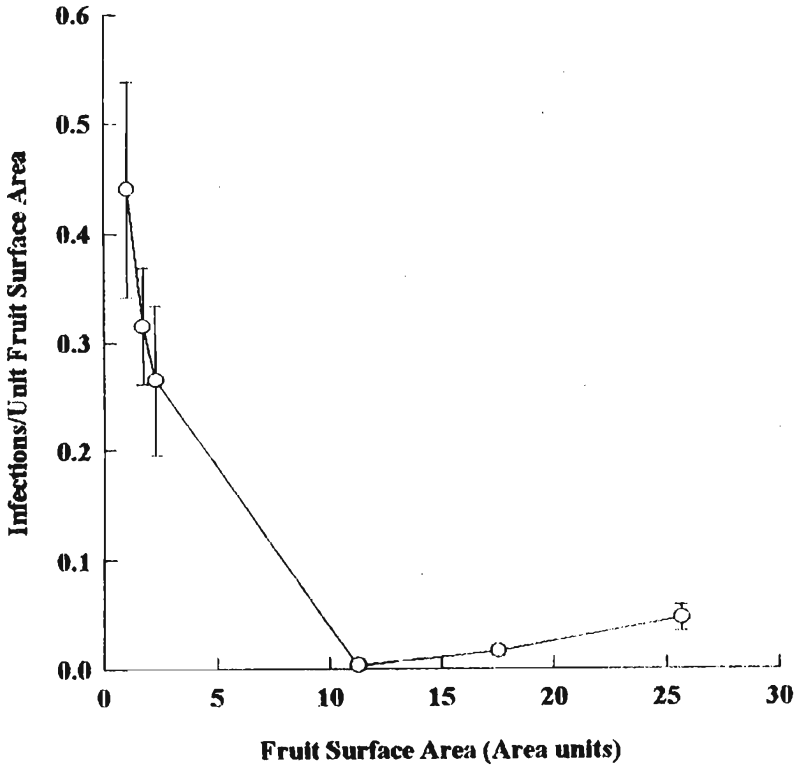


Figure 4 : Density of quiescent infections of *C. gloeosporioides* at different development stages of avocado fruit.

DISCUSSION

The samples for enumerating QI and FLVFP of *C. gloeosporioides*, of leaves, flowers and twigs, were obtained only from the lower branches to eliminate variation caused by sampling from different heights. It is likely that the sunlight falling at different intensities to branches at different heights, among other environmental factors will affect the pathogen counts on branches at different levels of the tree.

The influence of RF on QI in three distinct parts of the tree (i.e. leaves, twigs and flowers) appear to follow three different patterns, making suggestion of control measures (i.e. chemical sprays) complicated. An earlier study on avocado has reported that occurrence of quiescent infections of *C. gloeosporioides* on fruits reached a maximum during the wettest months, but decreased in drier months, and that the data best fitted a non-linear regression model⁵. Although we did not correlate RF with fruit infections, the statistical relationships of QI observed on other parts of the tree were always linear.

The positive correlation of QI on flowers and leaves with RF pre-lagged (t-3), and the negative correlations seen with that of twigs and RF, cannot be explained at this stage. The presence of a thick cuticle on avocado leaves may perhaps be a key to explanation of regression coefficient becoming progressively more significant when the RF is lagged from t-3 to t-6 (Table 2).

Initiation of flowering was observed in January and October respectively in the two years (Figure 1), which are dry months. Flowers have remained on the tree for 6 months in 1992, and 8 months in 1993. The QIs on flowers have increased with the rains. The QI of leaves also increased corresponding to RF of 3 months before (Figure 2). Fruit development occurs spread over a period of approximately 4 months during and after the flowering season (unpublished observations of A. M. Karunaratne). Propagules of *C. gloeosporioides* either as QI (Figure 1) or FLVFP (Table 1) appear to persist on the tree throughout the year with fluctuating numbers. The *C. gloeosporioides* inoculum appears to be nurtured by different parts of the tree, each part hosting the propagules through time in a different pattern, although the vegetative parts and flowers are not debilitated. If the pathogen caused disease in these parts the whole tree would get killed eventually, thus preventing a safe niche for the pathogen to survive in between fruiting seasons. This is different to the survival of *C. gloeosporioides* causing anthracnose of mango, where the conidia were shown to be produced on leaf lesions¹⁰ throughout the year particularly during flowering. In mango, anthracnose is a devastating field disease in addition to being a postharvest disease.

It is possible that the antifungal compounds reported to be present on leaves¹³ prevent them getting diseased by the pathogen. The role of antifungal compounds on different parts of the tree sustaining the propagules of the pathogen needs a detailed investigation. We have tried inoculating avocado leaves and twigs on cut branches with *C. gloeosporioides* in the laboratory, by keeping the cut-end of the branches fresh, by immersing in water. Conidial suspensions were prepared and drops (20 μ l) were placed on the upper surface of intact and wounded leaves and horizontal tender twigs, by the method described above for fruits. They were enclosed inside plastic bags containing wet cotton wool to maintain moisture. None produced lesions although the same conidial suspensions were able to produce lesions on wounded spots of fruits as described above (A. M. Karunaratne, unpublished data).

The FLVFP counts observed on the tree was low. The highest counts observed on the tree were 10² CFU/g. A slightly higher value of 10³ CFU/g was observed in the debris. Due to this narrow variation and low counts in the FLVFP, they were not considered in interpreting data.

The low counts of FLVFP of *C. gloeosporioides* seen in this study may also indicate that the pathogen was unable to cause disease and increase propagule numbers while on the vegetative parts of the tree. Unlike in the present study, in an earlier study on citrus, washings of flower petals have yielded a high number of propagules (i.e. 10^4 to 10^6 per gram fresh weight) of *C. gloeosporioides*¹⁴, and this pathogen is known to cause wither tip in citrus, in addition to anthracnose on fruits.

In avocado it is possible that the debris under the tree, acts as the ultimate source of inoculum. The survival of the conidia in the debris under the tree may also explain the spread of the disease to fruits of younger newly grown trees by rain splash from neighbouring older trees. An earlier study on strawberry anthracnose¹⁵, reported that *C. acutatum* survived in buried strawberry tissue for 9 months, and soil population densities gradually declined over a 11 month period.

As occurrence of anthesis appeared to spread for over a period of four months, we expected that relating the QI on fruits with their age, rather than with RF would give more meaningful data. Since the fruits for analysis were obtained from the same tree, fruit size was taken as a convenient index of maturity in this study although this would not be appropriate in a larger sample where genetical and environmental diversity would have an effect on fruit size. Quiescent infections were higher on fruits soon after anthesis. The levels of antifungal compounds are known to increase gradually and reach a maximum at harvest⁷. However, there is no evidence that antifungal compounds affect the establishment of quiescent infections.

The significant reduction of the density of quiescent infections when the fruit matures (Figure 4), may be explained by any one or a combination of the following hypotheses: a majority of infections are able to establish at an early stage of fruit maturity because of the peel anatomy; the rate of infection cannot keep up with the log phase of expansion of the fruit to maturity.

Several authors have observed that penetration of *C. gloeosporioides* is inhibited by actively growing host cells¹⁶⁻¹⁷. A histopathological study of *C. gloeosporioides* infections to avocado, reported that in fruit inoculated early in development when the epicuticular wax layer was very thin, many appressoria were seen later, resting on top of a thick epicuticular wax layer⁹. In addition, these authors have observed thin infection pegs which appeared to be physically broken in the region of the wax layer directly beneath these appressoria. It is not recorded how many of these detached infection pegs actually were able to cause disease on the ripe fruit. It was observed in the present study and in several other studies, (A. M. Karunaratne, unpublished data), that when intact healthy skin of harvested mature unripe fruits were inoculated with viable *C. gloeosporioides* conidia, lesions did not form in these areas when the fruits ripened. It is possible that the thick wax layer on unripe mature avocados acts as a deterrent to establishment of quiescent infections.

When fruits at a later stage of development were inoculated with *C. gloeosporioides* infection pegs penetrated the wax layer and once through the wax layer the infection peg became thin and unmelanized². While it is apparent that there is a difference in infection patterns when *C. gloeosporioides* inoculations occur at early stages of fruit development and at later stages, whether both types of infections result in lesion formation is not known. In an earlier study¹, fruits covered early after fruit set showed no infections or lesions at ripening, caused by *C. gloeosporioides*. However fruits inoculated with *C. gloeosporioides* at different stages of development (as much as 3 months prior to harvest), developed anthracnose lesions at ripening. These authors do not report how many lesions that formed on the fruit could be identified with the inoculations made by them earlier.

Several other environmental factors and physiological condition of the host will govern conidial counts and infections. An earlier study has shown that disease development on two weeds by *C. gloeosporioides* f. sp. *malvae* was affected by inoculum concentration and plant growth stage at the time of inoculation, post inoculation air temperature and, dew-period duration and temperature¹⁶.

A study which would establish the presence of antifungal compounds on twigs and flowers and the influence of these compounds in different parts of the tree on the pathogen will help in understanding the pathogenicity of *C. gloeosporioides* on avocado. Eventually, control measures could be recommended, based on these findings.

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