Analysis of populations of fungi on avocado leaves and their relationship with avocado fruit rots at harvest

K.R. Everett, J. Rees-George, O.E. Timudo-Torrevilla, I.P.S. Pushparajah, J. Yuan, Y. Wu.
The New Zealand Institute for Plant & Food Research Ltd., PB 92169, Auckland 1142, New Zealand.
email address: Kerry.Everett@plantandfood.co.nz

Avocado leaves were harvested from eight avocado orchards from four growing districts in New Zealand during 2006, 2007, 2008. The number of isolations of fungi from sterilised leaf discs was compared with postharvest fruit rots. There was a strong relationship between time period over which leaf discs were collected and the strength of the relationship between number of isolations and final fruit rots. There was a weak, but significant, relationship between isolations from leaf discs and the mean daily temperature when the samples were collected, DNA was extracted from sampled leaves and analysed using two methods; surface plasmon resonance (SPR) and real-time polymerase chain reaction (RT-PCR). RT-PCR was more sensitive than SPR. The relationship between final fruit rots and crossing threshold (Ct) values was significant if the data were analysed by district.

Keywords: fungi, leaves, fruit, rots, qPCR, SPR

Análisis de las poblaciones micológicas en hojas de aguacate y su relación con podredumbre el aguacate al cosechar

K.R. Everett, J. Rees-George, O.E. Timudo-Torrevilla, I.P.S. Pushparajah, J. Yuan, Y. Wu.
The New Zealand Institute for Plant & Food Research Ltd., PB 92169, Auckland 1142, New Zealand.
Correo electrónico: Kerry.Everett@plantandfood.co.nz

Hojas de aguacate fueron colectadas en ocho huertos localizados en distintos distritos en Nueva Zelanda durante los años 2006, 2007 y 2008. El numero de cepas micológicas aisladas a partir de de pequeños círculos de hoja esterilizados fueron comparados con pudriciones de frutosdurante post - cosecha. Existe una fuerte correlación entre el periodo durante el cual los círculos de hoja fueron colectados y la fuerza de la relación entre aislamientos micológicos y las pudriciones de los frutos. Existe también una débil pero significativa correlación entre las cepas aisladas a partir de los círculos de hoja y la temperatura promedio registrada durante la colecta de las muestras. ADN fue extraído de hojas y analizado utilizando dos métodos Resonancia Plasmon de Superficie (SPR) y Reacción en Cadena de Polimerasa en Tiempo Real (RT-PCR). RT-PCR es más sensible que SPR. La relación entre la podredumbre de la fruta y las cifras del Umbral de Detección (HD) es significativa si los datos son analizados por distrito.

Palabras clave: hongos, hoja, fruta, pudricion, qPCR, SPR

Introduction

The postharvest quality of avocado (*Persea americana*) fruit can be affected by several fungi that cause rots (Snowdon, 1990; Hartill, 1991; Pegg *et al.*, 2002) In New Zealand, the most common of these fungi are *Colletotrichum acutatum*, *C. gloeosporioides*, *Botryosphaeria parva*, *B. dothidea* and *Phomopsis* sp. (Everett, 2003). The two *Colletotrichum* species are known to have a quiescent, or latent, stage during the disease development process, and on avocados can symptomlessly infect fruit during the season to express disease only after harvest (Prusky *et al.*, 1982). Because appressorial formation is stimulated by contact with a hard surface, the possibility of leaves being an indicator of the amount of latent infection on fruit was investigated. It was shown that these fungi can be isolated from leaves in New Zealand avocado orchards (Everett *et al.*, 2003). In a preliminary investigation it was shown that there was a strong correlation between fungi isolated from leaves and final fruit rots. This suggested that leaves could be used to indicate the amount of latent infection on fruit at harvest, and hence the amount of postharvest rot. Since that time several other orchards have been sampled annually and the incidence of final fruit rots compared with leaf isolations and fungi quantified using two DNA techniques, surface plasmon resonance (SPR) and real-time polymerase chain reaction (RT-PCR).

Methods

Orchards sampled

Two orchards were selected from each of the four major avocado growing districts of New Zealand:, the Far North, Whangarei, Western Bay of Plenty and the Eastern Bay of Plenty; a total of eight orchards.

Leaf isolations

During December 2005, January, November and December 2006, and August and September 2007 avocado leaves were collected from eight orchards (Table 1), A total of 16 leaves were collected from each of 10 marked trees in each orchard. Four leaves were collected from each of four quadrats from around the trees, corresponding to north, south, west and east. Leaves were placed in sealed plastic bags. Upon arrival at Mt Albert Research Centre (MARC), two 1-cm diameter leaf discs were cut from every leaf. Leaf discs were surface sterilised by placing in 70% ethanol for 1 min, 1:3 (w/v) 5% sodium hypochlorite for 3 min, sterile deionised water for 30 s, then blotted dry. Leaf discs were placed abaxial side up on Difco® Potato Dextrose Agar. At the same time a further two 1-cm leaf discs were placed in Eppendorf tubes in the -80C freezer for later DNA extractions. After three weeks at room temperature (~20°C) the number of leaves from which colonies of *Colletotrichum acutatum* grew were counted.

Fruit harvest and assessment

In February 2006, January 2007 and January 2008, (Table 2), 10 fruit from each of the 10 labelled trees on every orchard were harvested and placed 10 per box into cardboard avocado boxes containing fibreboard Plix trays. Fruit were transported to MARC in air-conditioned vehicles within 48 h of harvest. Upon arrival at MARC, fruit were placed at 5.5^{o}C for 28 days. Fruit were then placed at 20°C and assessed when ripe as determined by gentle hand squeezing. Fruit were cut into quarters and peeled, and rots assessed as described in the AIC Avocado Assessment Manual (Anonymous, 2001).

DNA extractions

Leaf discs were macerated in liquid nitrogen using a mortar and pestle and DNA extracted using the Qiagen DNeasy kit.

Surface Plasmon Resonance (SPR)

The probe was immobilised onto the CM5 chip for the Biacore Q SPR via a streptavidin coating surface. The DNA extracted from leaf discs was heat denatured then injected into the Biacore Q (25°C) at a flow rate of 5 µl /min.

Real-time polymerase chain reaction (RT PCR)

The 10 µL/well reaction consisted of *1* µL of gDNA, 0.5 µL SYBR Green I Master, *3*µL GIBCO™ water and 5 µM of each forward and reverse primers, and was conducted in the LightCycler® 480 Real-Time PCR System under the following conditions: 95 *°C* for 10min, 45 cycles of 95 *°C* for 5 s, 60 *°C* for 7 s,

72 °C for 7 s, followed by melting-curve analysis with a temperature profile slope from 65 °C to 97 °C with continuous fluorescence measurement.

Table 1. Dates avocado leaf samples were taken.

Orchard*	Year of sampling 2005	2006	2006	2007
FN1	7/12/2005	23/1/2006	21/11/2006	3/9/2007
FN2	7/12/2005	23/1/2006	21/11/2006	3/9/2007
W1	8/12/2005	2/2/2006	13/12/2006	4/9/2007
W2	8/12/2005	31/1/2006	13/12/2006	4/9/2007
WBOP1	12/12/2005	11/1/2006	29/11/2006	31/8/2007
WBOP2	12/12/2005	12/1/2006	30/11/2006	31/8/2007
EBOP1	12/12/2005	11/1/2006	29/11/2006	1/9/2007
EBOP2	12/12/2005	11/1/2006	21/12/2006	31/8/2007

*FN=Far North, W= Whangarei, WBOP = Western Bay of Plenty, EBOP = Eastern Bay of Plenty.

Table 2. Dates fruit samples were taken.

Orchard*	Year of sampling 2006	2007	2008
FN1	9/2/2006	18/01/2007	17/01/2008
FN2	9/2/2006	18/01/2007	17/01/2008
W1	10/2/2006	19/01/2007	18/01/2008
W2	10/2/2006	19/01/2007	18/01/2008
WBOP1	8/2/2006	22/01/2007	23/01/2008
WBOP2	7/2/2006	22/01/2007	24/01/2008
EBOP1	7/2/2006	23/01/2007	25/01/2008
EBOP2	7/2/2006	23/01/2007	24/01/2008

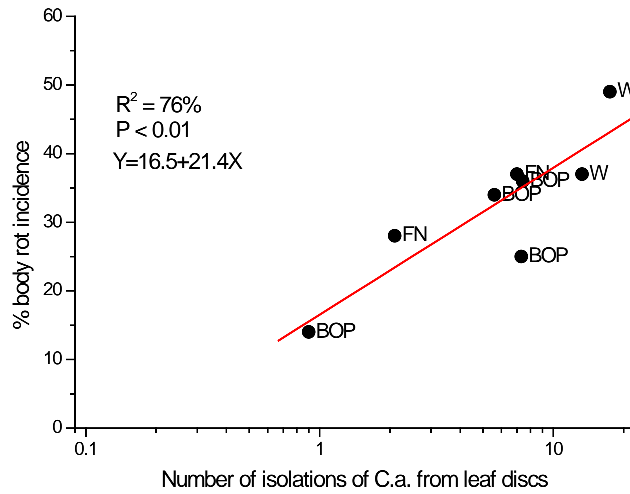
*FN=Far North, W= Whangarei, WBOP = Western Bay of Plenty, EBOP = Eastern Bay of Plenty.

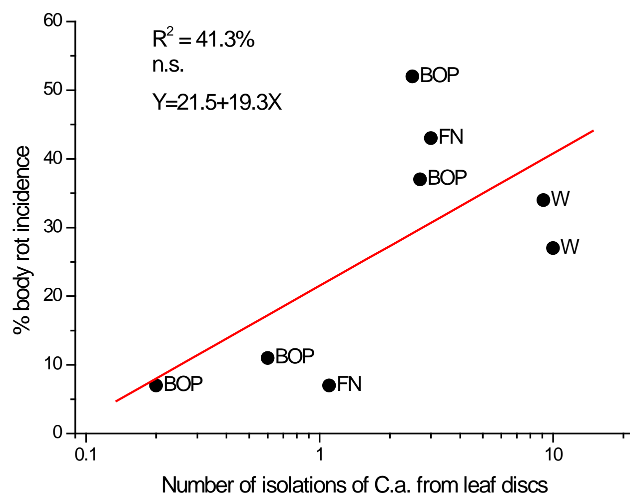
Data analysis

The generalised linear model and linear regression functions of Minitab® and Microsoft® Excel were used for data analysis. Graphs were generated using Microcal® Origin. Data collected during 2005/06 were compared with data collected during 2006/07. The mean daily temperature when each orchard leaf disc sample was collected was compared with the number of isolations of *C. acutatum* from these leaf discs. Temperature at sampling time was summarised using HortPlus™ MetWatch software (www.hortplus2.com) from hourly weather data collected by automatic weather stations (Beresford & Spink, 1992) at sites in Kerikeri, Katikati and Te Puke. In addition, the R^2 values of the linear regressions between numbers of isolations of *C. acutatum* from leaf discs collected in December 2005, November and January 2006 and final total rots were compared with the number of days over which leaf discs were collected for fruit harvested in 2006 and 2007. Data collected in 2002 was also included (Everett *et al.*, 2003).

Results

There was no relationship between stem-end rots and *Colletotrichum acutatum* isolated from leaf discs for all sampling occassions (results not shown), but there was for body rot incidence in December 2005 for fruit harvested in February 2006 (Figure 1), a relationship that was not significant for leaves sampled in November 2006 and fruit harvested in January 2007 and no obvious relationship for leaves sampled in August and September 2007 and fruit harvested in January 2008 (Figure 1).





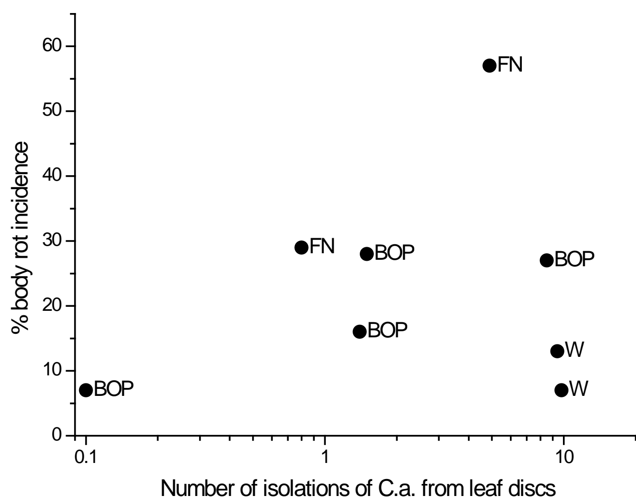


Figure 1. Relationship between *Colletotrichum acutatum* (C.a.) inoculum on leaf discs sampled in (top) December 2005, (middle) November 2006 and (bottom) August/September 2007 and incidence of body rots in fruit harvested in, respectively, February 2006, January 2007 and January 2008. BOP = Bay of Plenty, W = Whangarei, FN = Far North.

.

The 24 hour mean temperature when leaf discs were sampled for every orchard for each of the three occasions during these two years was plotted against the number of isolations of *C. acutatum* from those leaf discs. There was a significant, but weak, linear relationship (R^2 = 19.8%) (Figure 2).

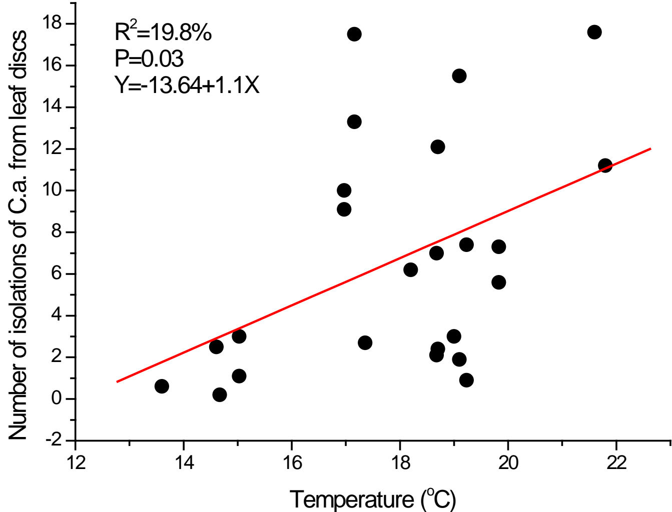


Figure 2. Mean number of isolations of *Colletotrichum acutatum* from leaf discs collected on 24 occasions compared with mean daily temperature on the day when the samples were collected.

When time (in days) over which leaf discs were sampled was plotted against the R^2 value from linear regressions performed on each of the four leaf disc data sets (data from Everett *et al.* (2003), from Figure 1a and 1b, and from leaves collected in January 2006 and fruit harvested in February 2006 where R^2=51% and P=0.01) with final fruit rots, a significant, strong (R^2 = 97.8 %) and inverse relationship resulted (Figure 3).

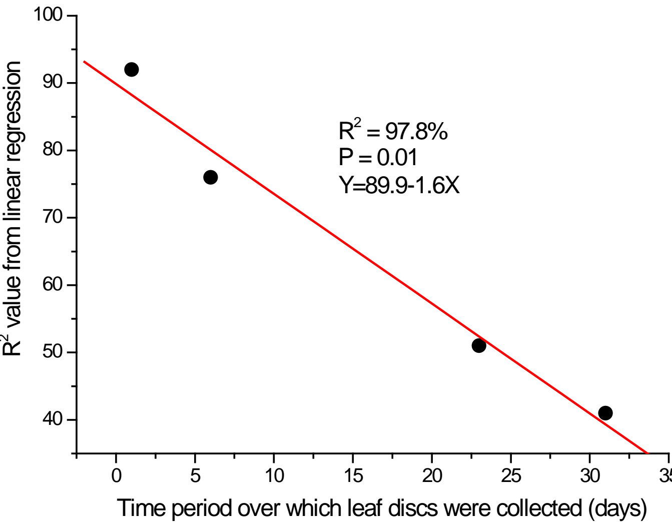


Figure 3. Relationship between R^2 values calculated from linear regression of mean number of isolations of *Colletotrichum acutatum* from leaf discs and final fruit rots and the time period over which leaf discs were collected.

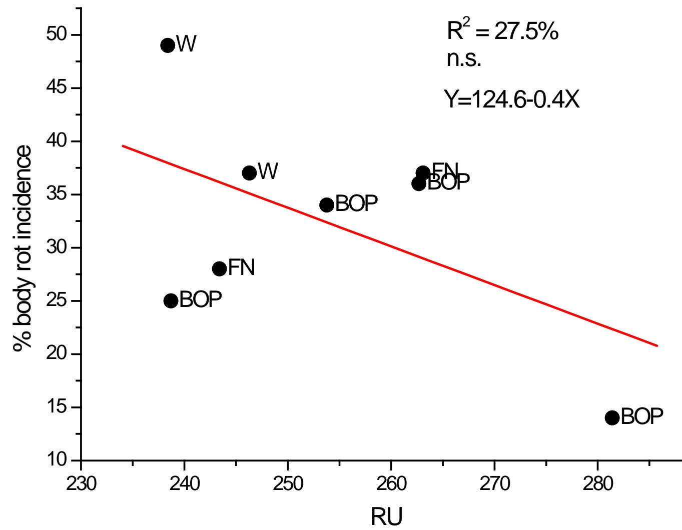


Figure 4. Response unit (RU) from surface plasmon resonance analysis of DNA extracted from leaf discs collected in December 2005 compared with the amount of body rots in fruit harvested in February 2006. BOP = Bay of Plenty, W = Whangarei, FN = Far North.

When DNA was extracted from the leaf discs collected in December 2005, there was a weak relationship between body rot incidence and response unit (RU) values following analysis using surface plasmon resonance, but the relationship was not significant (Figure 4).

When the same DNA was analysed using RT-PCR, there was a significant relationship between body rots and Ct value when the data were separated into two regions, Bay of Plenty and North (Figure 5). Leaf disc material was not stored from the 2006/2007 sampling years, so could not be tested.
DNA extracted from leaves collected in August and September 2007 was also analysed using RT-PCR, and there was a highly significant relationship between Ct values and final body rots for orchards from the Bay of Plenty (Figure 5).

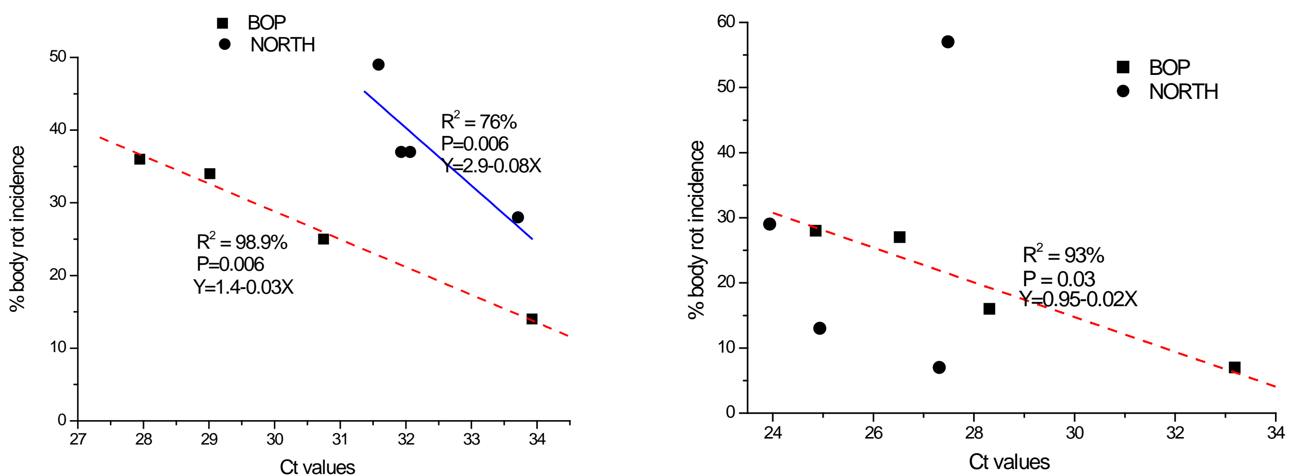


Figure 5. Relationship between Ct values generated using qPCR reactions and body rot incidence for leaves collected in December 2005 and fruit harvested in February 2006 (left) and leaves collected in August and September 2007 and fruit harvested in January 2008 (right). Orchards have been

separately analysed according to location: Bay of Plenty (BOP), or Whangarei and the Far North (North).

Conclusions

Similar to results obtained previously (Everett *et al.*, 2003) there was no significant relationship between stem-end rots in fruit postharvest and inoculum on leaf discs. This result suggests that stem-end rots are not related to latent infections that occur throughout the year, but instead the stem-end wound is most likely infected at harvest.

There was a highly significant relationship between number of isolations of *C. acutatum* on leaves sampled in December 2005 and body rots in fruit harvested in February 2006. However, this relationship was weaker in the other two years of this study. Analysis of the data collected in 2005 and 2006 on 24 separate occasions, suggested that mean temperature on the days on which leaf discs were collected accounted for only 19.8% of the variation in number of *C. acutatum* isolations. However, the number of days between collection of the first and last leaf samples strongly influenced the relationship between number of isolations of *C. acutatum* from leaf discs and final fruit rots. This suggests that inoculum may be constantly deposited on fruit throughout the season, and as long as both fruit and leaves are harvested in as short a time as possible, then the relationship between inoculum on leaves and fruit rots can be detected. From these data it is recommended that all leaf samples should be collected in as short a time frame as possible. However, in the final year of this study, despite leaves being harvested within five days, there was no obvious relationship between isolates of *C. acutatum* and body rots. This may be due to regional differences, and needs to be investigated further.

Surface plasmon resonance successfully quantified DNA from *C. acutatum,* but there were cross-reactions with DNA from *C. gloeosporioides* which could not be corrected due to technical limitations of the machine. This may have impacted on the quantification of *C. acutatum* in DNA extracted from leaf discs and the relationship with final fruit rots.

The amount of DNA extracted from leaf discs quantified by real-time PCR was highly correlated with final fruit rots for the four orchards sampled from the Bay of Plenty, for two of the three study years. This suggests that the quantification of fungal DNA by RT-PCR was more accurate than by leaf isolations, and that DNA extracted from leaves could be used to estimate the amount of body rots in fruit before they are harvested, but this requires further validation.

Acknowledgements

To Roger Barber for selecting orchards, and for assistance with collecting leaves and harvesting fruit; and especially to the growers for provision of leaves and fruit. To Technology New Zealand, Plant and Food Research and Freshco for funding this project.

References

Anonymous, 2001. *Avocado Assessment Manual 2001.* NZ Avocado Industry Council Ltd, PO Box 16004, Bethlehem, Tauranga, New Zealand.

Beresford RM, Spink M, 1992. A national disease forecasting system for apple black spot (*Venturia inaequalis*) in New Zealand. *Acta Horticulturae* **313**, 285-96.

Everett KR, 2003. The effect of low temperatures on *Colletotrichum acutatum* and *Colletotrichum gloeosporioides*, fungi causing body rots of avocado in New Zealand. *Australasian Plant Pathology* **32**, 441-8.

Everett KR, Rees-George J, Parkes SL, Johnston PR, 2003. Predicting avocado fruit rots by quantifying inoculum potential in the orchard before harvest. *5th World Avocado Congress. 19-24th October, 2003*. Granada-Malaga, Spain. pp. 601-606: Viceconsejeria. Servicio de Publicaciones y Divulgacion, 601-6.

Hartill WFT, 1991. Post-harvest diseases of avocado fruits in New Zealand. *New Zealand Journal of Crop and Horticultural Science* **19**, 297-304.

Pegg KG, Coates LM, Korsten L, Harding RM, 2002. Foliar, fruit and soilborne diseases. In: Whiley AW, Schaffer B, Wolstenholme BN, eds. *The avocado; botany, production and uses.* Wallingford, UK: CABI Publishing, 299-338.

Prusky D, Keen NT, Sims JJ, Midland SL, 1982. Possible involvement of an antifungal diene in the latency of Colletotrichum gloeosporioides on unripe avocado fruits. *Phytopathology* **72**, 1578-82.

Snowdon AL, 1990. *A colour atlas of Post-harvest diseases and disorders of fruits and vegetables.* . London, England: Wolfe Scientific Ltd.