Towards commercialisation of avocado rot prediction

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A survey of 32 avocado orchards throughout the three major growing regions of New Zealand was conducted in the 2012/13 season to validate the model for prediction of fruit rots at harvest. The model predicted 80% and 82% of the variation in the data for the Whangarei and Bay of Plenty districts, respectively. In the 2014/15 season leaves were sampled from 100 trees in two avocado orchards. DNA was extracted from leaves and a qPCR analysis of fungal populations was conducted. The variability in the distribution of qPCR crossing thresholds (Ct values) in each orchard was determined using spatial analysis and GPS coordinates. An optimal sampling strategy was designed to maximise the robustness of using Ct values to predict fruit rots at harvest on avocados harvested from individual orchards.

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 infect fruit during the season without showing symptoms only to express disease 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 in 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 real-time polymerase chain reaction (RT-PCR). A highly significant relationship between inoculum on leaves and final fruit rots was shown (Everett *et al.,* 2011) for four orchards in one region for two seasons.

Most avocados in New Zealand are grown in four regions, the Far North (latitude c. 34°.50), Whangarei (latitude c. 35°.50), western Bay of Plenty (WBOP, latitude c. 37°.30) and eastern Bay of Plenty (EBOP, latitude c. 37°.50). One South Auckland (latitude c. 37°.10) orchard was included in WBOP.

A method to predict the rot potential of avocado fruit in the marketplace before harvest offers several benefits for New Zealand marketers and exporters. A high incidence of latent infections reduces the storage life of fruit (Hartill and Everett, 2002). New Zealand fruit with low rot potential could therefore be sent under long-term cool storage to distant markets and still have good quality on arrival. Many of the Asian markets that the avocado industry is targeting for development have relatively poor infrastructure or knowledge to manage avocados effectively. To some extent these problems can be alleviated by sending robust fruit, and this technology would be ideal for selecting appropriate fruit. . Another possible use is to enable growers to use it as a decision support tool to reduce fungicide applications.

Before the technology can be offered as a commercial service, further validation is necessary to examine if the predictive relationship between fungal populations of leaves and fruit rots remains robust if more orchards are included. In addition, the distribution of fungal populations on leaves throughout an orchard needs to be examined in more detail to design a suitable sampling method.

The aims of this study were to 1. Sample leaves from 32 orchards in the three major avocado growing regions in New Zealand and compare fungal populations on leaves with fruit rots and 2. Intensively sample two orchards so that the best sampling strategy can be recommended.

MATERIALS AND METHODS

Sampling and data collection for regional comparison (2012/13 season)

Leaves were collected from 16 orchards in the Bay of Plenty and one orchard in South Auckland in the 7 days beginning on 16th October 2012. Ten trees in each of these orchards were marked with fluorescent tape, and 16 leaves were collected from each of those 10 trees. Leaves from eight orchards in the Far North and eight in Whangarei were collected similarly on 14-16 November (Table 1). After arrival at Mt Albert Research Centre, (latitude 36°.53.456) 4 x 1 cm diameter discs were excised from each leaf and placed in labelled plastic bags in the -80°C freezer for later processing.

The same marked trees were revisited in December, and a total of 100 fruit (10 fruit from each of 10 trees) from each of eight orchards in the Whangarei region were picked 3-5 December 2012, placed in a coolstore in Whangarei, then 100 fruit from each of eight orchards north of Kaitaia were picked 5-7 December. Fruit were packed into boxes on the orchards (10 fruit per box) and both sets of fruit were transported to Auckland (2 hours) on 7 December and placed in a coolstore at 5.5°C. Fruit from the Bay of Plenty and South Auckland orchards were similarly picked 18-20 December and transported (3 hours) to the coolstore at Mt Albert Research Centre. The fruit from one EBOP orchard had already been picked by the owner and was not able to be included in our study. Fruit from Whangarei and the Far North were removed from the coolstore on 4 January 2013 and those from the Bay of Plenty and from South Auckland were removed on 17 January. Both sets of fruit were

evaluated during shelf life at 20°C and assessed for internal disorders following cutting and peeling when ripe, as determined by gentle hand squeezing. Rots were categorised according to a rating system from 0-100% for coverage of fruit surfaces (body rots) or penetration (stem-end rots) according to Dixon (2003). Rots were expressed as mean proportion (percent) of the 10 replicate trays. Spray diaries were obtained from the Avocado Industry Council and compared with these data.

Sampling and data collection for sampling strategy (2014/15 season)
Two large orchards that have been commercially managed for more than 10 years were selected, one in Western Bay of Plenty (WBOP) and the other in Eastern Bay of Plenty (EBOP). One orchard was a mixed kiwifruit, dairy and avocado orchard so that blocks were interspersed with other crops. There was also a mix of tree ages in this orchard. The other orchard was more uniform in both layout and tree age. Both orchardists sprayed fungicides regularly. One hundred trees were sampled in each orchard.

The trees in each block were counted with the aid of aerial maps downloaded from Google Earth, and trees were sampled randomly within each block except that leaf samples were not taken from trees at the edge of blocks. A Global Positioning System (GPS) () location was recorded using a Garmin hand-held GPS device for every tree that was sampled. Leaves were harvested from four points around the tree: north, south, west and east. One leaf was taken from each compass point, placed in a plastic bag for each tree, and transported to the Mt Albert Research Centre within 24 hours of harvest. After arrival at Mt Albert Research Centre, 4 x 1 cm diameter discs were excised from each leaf and placed in labelled plastic bags in the -80°C freezer for later processing.

DNA extractions
Leaf discs were macerated in liquid nitrogen using a mortar and pestle, and DNA extracted using the method of Chang *et al.* (1993). DNA concentration and quality was determined using a NanoDrop® ND-1000 (Biolab) spectrophotometer and by electrophoresis on 1% agarose.

Real-time polymerase chain reaction (RT PCR)
The 10 µL/well reaction consisting of 1 µL of DNA, 0.5 µL SYBR Green I Master, 3µL GIBCOTM 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.

Data analysis
The generalised linear model and linear regression functions of Minitab® and Microsoft® Excel were used for data analysis for the development of the avocado rot prediction model. Graphs were generated using Microcal® Origin.

The border of the area from which trees were sampled was derived from the GPS positions of the trees and the Google maps and, in combination with GPS, positions of trees that were sampled were used to generate spatial distribution maps.

GPS data pertaining to the EBOP and WBOP orchards were collected in latitude and longitude format which were converted into New Zealand northings and eastings. For each orchard, those eastings and northings were zeroed at the south-west corner of the enclosing rectangle by subtracting the corresponding smallest values which resulted in data in metres between zero and approximately 900 metres.

The R (R, Core Team 2014) package spatstat (Baddeley and Turner, 2005) was used to perform tests of Complete Spatial Randomness for each of the point patterns of trees with a Cp above a range of thresholds. Monte Carlo tests (Cressie and Read, 1984) of goodness-of-fit compared the observed quadrat counts with those simulated under the null hypothesis of a uniform Poisson point process. T tests were used to examine variability between blocks.

Using the results of the spatial analysis it was assumed that the historical data are also from orchards with a similar distribution. The standard deviation of Cp threshold results from the EBOP and WBOP orchards were used in two series of simulations of data from the 32 orchards in the 2012–13 season (Everett *et al.,* 2013). These data comprised samples from 10 trees from each of the 32 orchards. DNA from each orchard's 10 trees was pooled. Each DNA sample was replicated in pairs in the qPCR tests (technical replicates). The simulations used the observed mean for each orchard (except for the eight that recorded a threshold of 40 and no variance) with each series assuming the same standard deviation for all orchards. From the simulated data, samples of a range of sizes were taken and their means ranked. Those rankings were compared with the "true" ranking of the 25 orchards.

RESULTS AND DISCUSSION

The Cp values produced following qPCR analysis of avocado leaves predicted the incidence of postharvest rots in fruit from the Whangarei region to a level of accuracy of 80% (Figure 1b). When the number of coppers applied during the season was included in the analysis, the incidence of postharvest rots in fruit from the Bay of Plenty region was predicted to a level of accuracy of 77% (Figure 1a). No significant relationship was found for the fruit harvested from the Far North region, unless it was combined with the Whangarei data (Figure 1c).

Rot prediction based on fungal DNA in leaves appeared to work well for fruit harvested from the Whangarei region. The growing conditions in this region were more homologous than in the Bay of Plenty. In Whangarei, orchards were predominantly from around the base of a large volcano, soils were more homologous, and the geographic spread was less than in the Bay of Plenty.

In the Bay of Plenty fruit were collected from orchards from a larger geographic area, but despite this the relationship when both WBOP and EBOP data were included was very good (R2 = 77%).

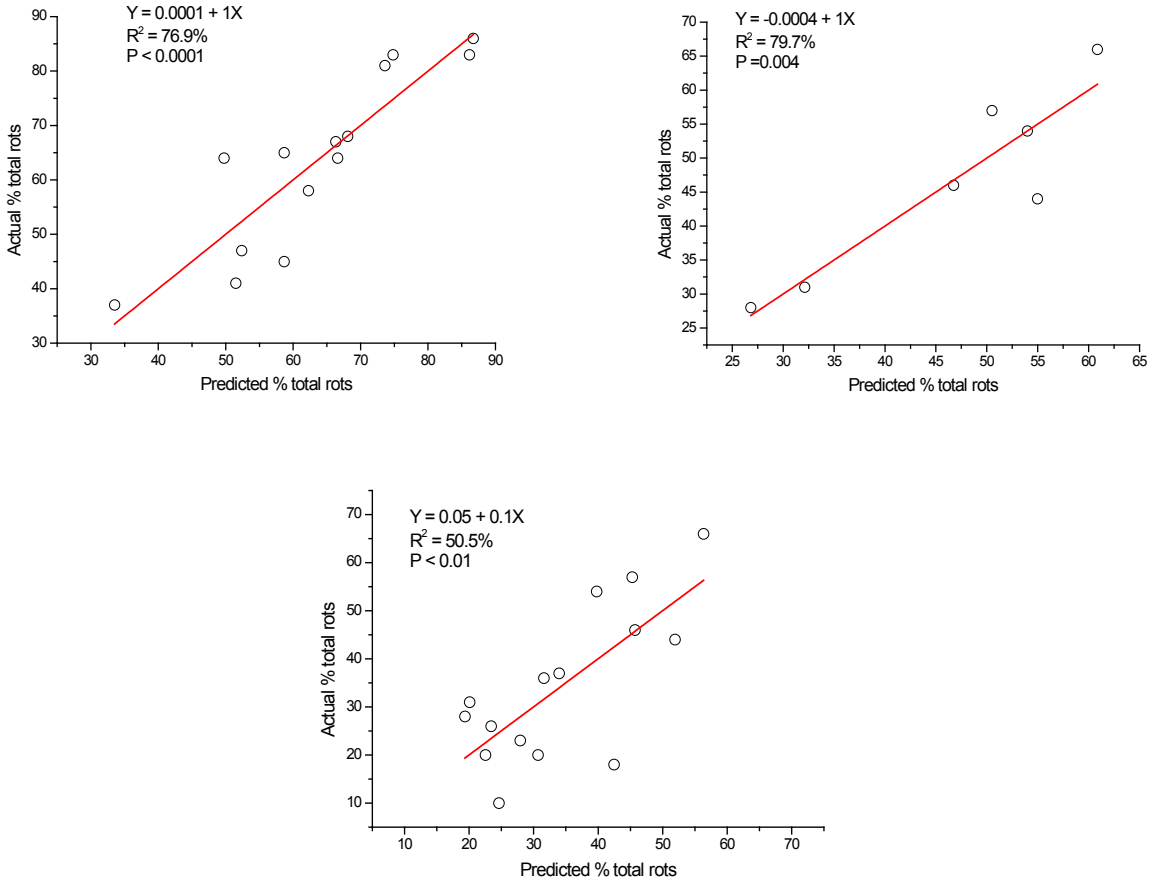

Figure 1. Actual against predicted percent rots using the formula a) Y = 149.6 –2.0Ct – 2.9 copper for the Bay of Plenty Region and b) Y =45.2–0.22Ct for the Whangarei Region and c) Y =141.4 – 2.8Ct – 1.6 copper for the Whangarei and Far North Regions combined. One outlier value was removed from the analysis for each of the Bay of Plenty and Whangarei regions

These results suggest that there was another factor with a strong influence on fruit quality in the Far North that was not accounted for in the analysis. It would be of interest to collect rainfall, temperature, nutrition and maturity data from this region to identify a factor that could be incorporated in the analysis to improve the fit to the rot incidence data. The enhancement of the level of significance following inclusion of the Whangarei data shows that there probably was an effect due to copper applications, but other unknown factor(s) were confounding these results.

This season the model predicted accurately the amount of rots in 15/16 of the orchards from the Bay of Plenty, or 94% of the orchards. That means using the model as it was envisaged will be able to separate lines of fruit by the predicted risk of rots for 94% of the fruit produced in the Bay of Plenty, and 88% (7/8 orchards) of the fruit produced in the Whangarei region. However, it could not be used to predict fruit rots in the Far North region.

Investigation of the distribution of inoculum in two orchards showed that there were some cases of evidence against randomness (Figure 2b, $P < 0.05$) but that was only when all trees or nearly every tree met the Cp threshold criterion (as it was set so high). It shows that the trees were not randomly spaced. Since the distribution of trees meeting lower thresholds was consistent with the hypothesis of randomness, it was considered reasonable to assume that the distribution of inoculum itself was random.

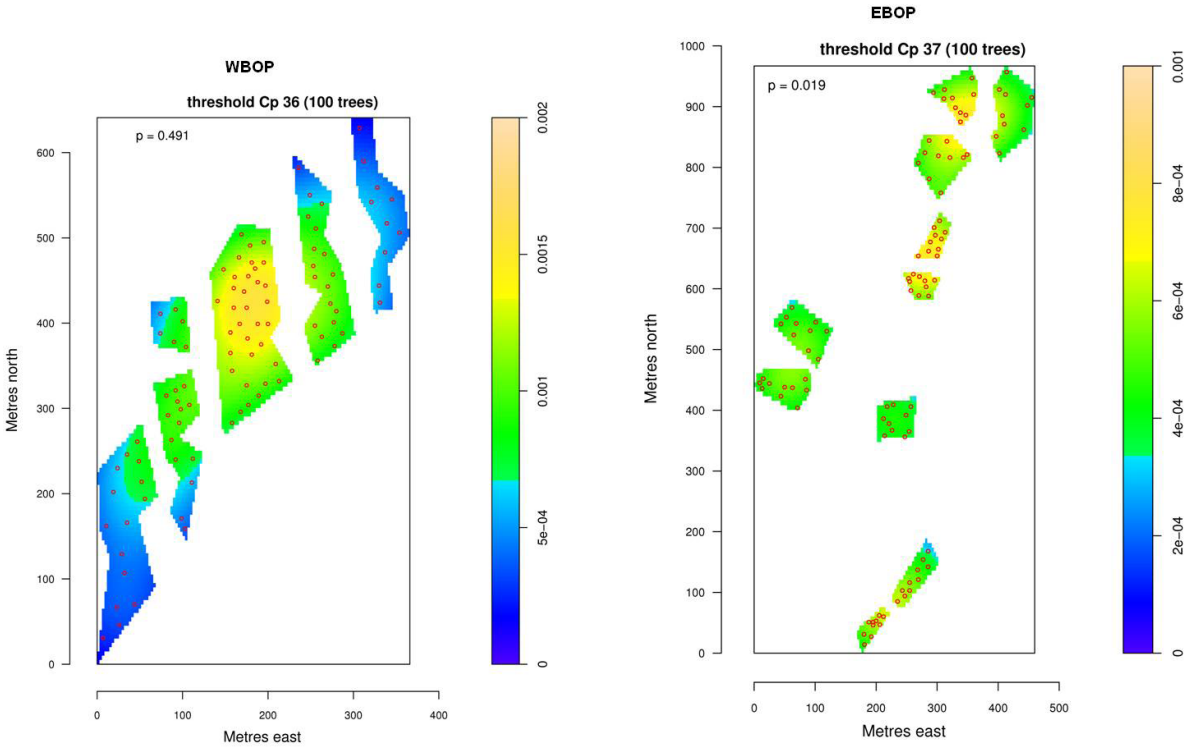

Figure 2. The results of tests of Complete Spatial Randomness for each of the point patterns of trees with a Cp above a threshold for the a) WBOP and b) EBOP orchard. Thresholds are ≤37. Monte Carlo tests of goodness-of-fit compared the observed quadrat counts with those simulated under the null hypothesis of a uniform Poisson point process

The randomness of distribution of inoculum means that samples can be taken using a transect through the orchard. The samples were not taken from trees at the outer edge of blocks in this study, therefore this same strategy needs to be employed when sampling from each orchard once this technique is commercialised.

Since there was no compelling evidence against the hypothesis of Complete Spatial Randomness, it was considered a reasonable assumption that all trees in a block had an identical risk of becoming infected. Under that assumption, Cp values for each block were considered to form a t-distribution which enables the use of basic statistical tests for differences between blocks. Boxplots (Figure 3) of the observed Cp results for the EBOP and WBOP orchards show substantially greater variability between blocks in the former than in the latter. In addition, EBOP blocks tend to be more variable with four of the seven blocks having a higher standard deviation than the highest standard deviation for the Palmer blocks. The standard deviations for each block were used to calculate the sample sizes required to distinguish between blocks for a range of degrees of discrimination. Calculations allowed for a Type 1 error (i.e. probability of declaring a difference that is not true) of 5% and a Type 2 error (i.e. probability of not detecting a true difference) of 20%. Those numbers were plotted in a lattice plot (Figure 4).

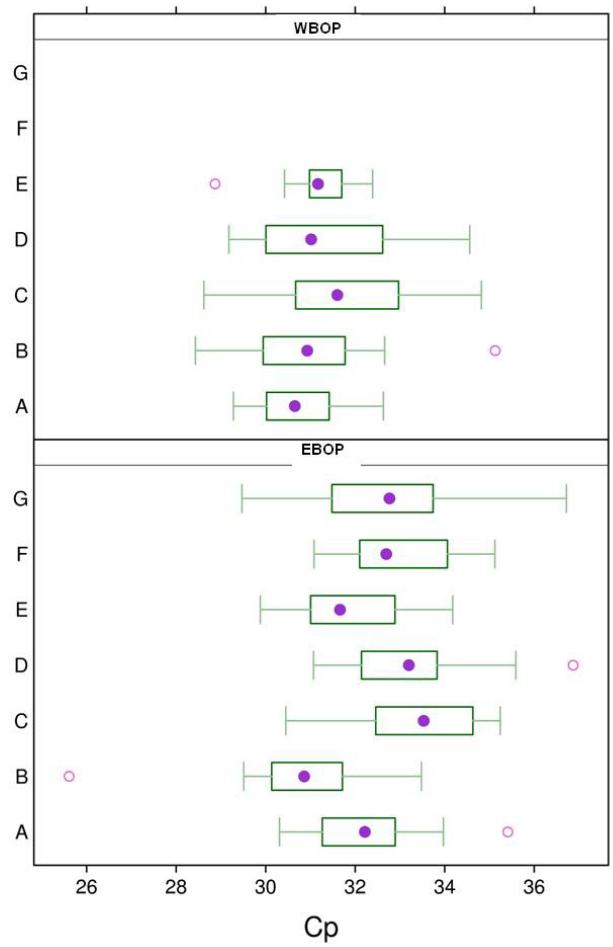

Figure 3. Boxplots of the observed Cp results from four leaf samples per tree for blocks on two orchards (EBOP and WBOP) in the Bay of Plenty. Closed circles are medians, boxes are interquartile range and whiskers are the range of the data except where there are outliers indicated by hollow circles

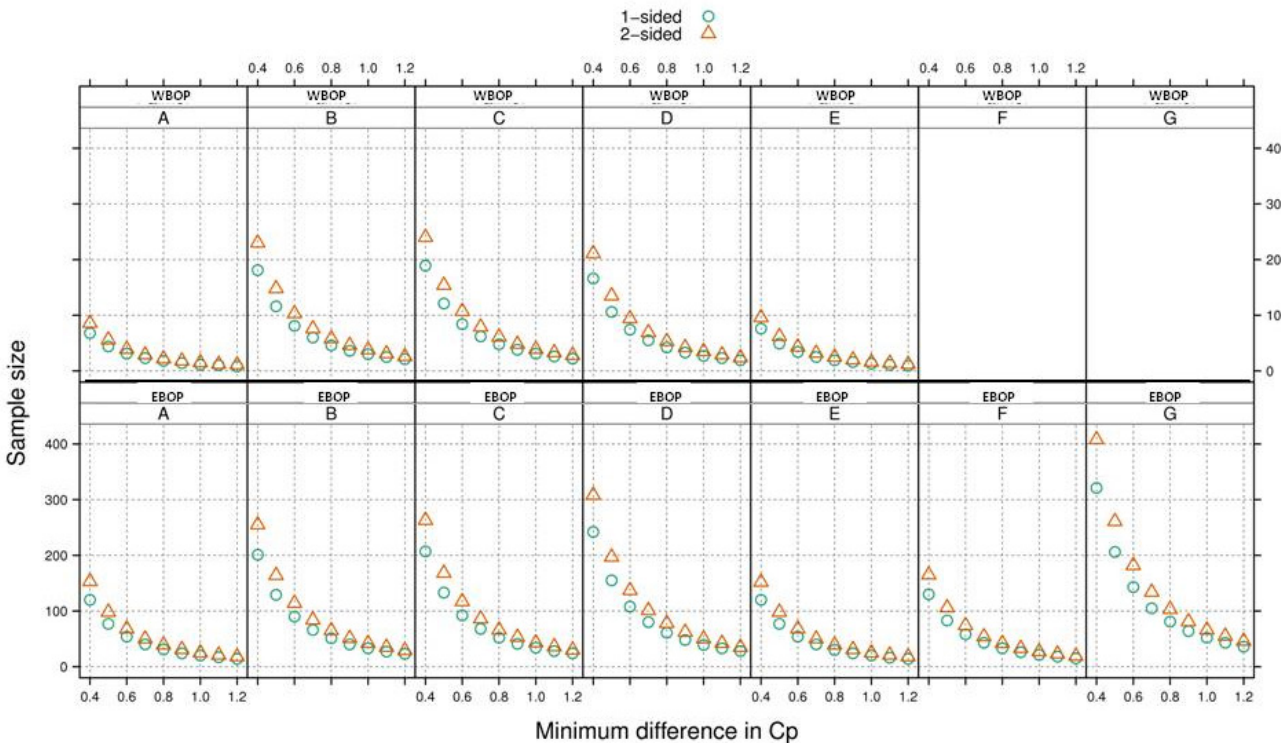

Figure 4. The sample size required for detecting minimum differences in Cp values for a one-sided or a two-sided t test

Assuming that the most variable EBOP block is indicative of the most variable block likely to be encountered, we could draw the conclusion: In a population with a uniform degree of fruit rot potential, a sample of four leaves from each of 50 trees would be sufficient to discriminate between populations with differences as large as 1 Cp. Smaller differences would require larger sample sizes but are unlikely to be practically significant. In most cases, blocks will be less variable than the one used in the calculations, which implies that the allowed for Type 1 and 2 errors will usually be conservative.

If prior information gives confidence that the mean of the Cp values of one block is definitely higher or lower than another, then a 1-sided t test can be used. Because of a lack of consistent prior information, most of the time the 2-sided test should be used.

CONCLUSIONS

The avocado rot prediction model accurately predicted postharvest fruit rots for two avocado-growing regions of New Zealand (Whangarei and Bay of Plenty). Further confidence in the technique can be obtained by sampling leaves and fruit during more seasons.

In an orchard with a uniform degree of fruit rot potential, a sample of four leaves from each of 50 trees would be sufficient to discriminate between orchard populations with differences as large as 1 Cp.

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