AUSTRALIAN Banana GROWERS' COUNCIL INC.

24 August, 2006

Louise Van Meurs
General Manager, Plant Biosecurity
Biosecurity Australia
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CANBERRA ACT 2601

Dear Ms Van Meurs

The Australian Banana Growers' Council recently engaged The Horticulture and Food Research Institute of New Zealand Limited (HortResearch) to undertake two research projects relevant to the bananas IRA.

The first research project involved the molecular detection of the black Sigatoka pathogen in leaf trash associated with Cavendish banana fruit imported into New Zealand from the Philippines. The collection of the tissue samples is outlined in the report entitled *Inspection of Philippine Bananas in New Zealand: December 2005* (March 2006) by Peterson, Piper and Poljak which has previously been provided to Biosecurity Australia. The research project showed that the black Sigatoka pathogen was present in fragments of leaf trash found in cartons of banana fruit imported into New Zealand from the Philippines. I **attach** a report which sets out the results of that research project.

The second research project involved the detection of the black Sigatoka pathogen in the skin of Cavendish banana fruit. The research project showed that the black Sigatoka pathogen can be found in the skin of Cavendish banana fruit grown in an environment where the disease is present. I **attach** a report which sets out the results of that research project. It is apparent from the research project that endophytic fruit infection is a risk scenario for black Sigatoka which needs to be given very serious consideration by the IRA Team.

The Council is contractually obliged to notify you that HortResearch does not give any prediction, warranty or assurance in relation to the accuracy of or fitness for any particular use or application of, any information or scientific or other result contained in the attached reports.

The Council authorises Biosecurity Australia to place both reports on the public file for the bananas IRA.

Would you please ensure that the attached reports are provided to the members of the IRA Team as a matter of priority.

Please do not hesitate to contact me if you have any queries.

Yours sincerely

per: Jann Bonsell

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Executive Summary

Molecular detection of *Mycosphaerella fijiensis* in leaf trash associated with imported Cavendish banana fruit.

S.G. Casonato and R. A. Fullerton
August 2006

HortResearch was contracted by the Australian Banana Growers' Council to investigate whether *Mycosphaerella fijiensis*, cause of black Sigatoka (black leaf streak) disease, could be detected in leaf trash in cartons of bananas imported from a country that has the disease The ability to be carried in this way offers a potential pathway for introduction of the pathogen. This work was undertaken in the HortResearch laboratories at Mt Albert Research Centre, Auckland, New Zealand.

Method
Samples of leaf tissue and banana skin were collected from cartons of a commercial consignment of bananas imported to New Zealand from the Philippines in December 2005. The collection of those samples is outlined in the report entitled *Inspection of Philippine Bananas in New Zealand: December* 2005 (March 2006) by Peterson, Piper and Poljak. The samples were stored at -20C until assayed in July 2006.
DNA extraction. DNA was extracted from the samples using a QIAGEN DNeasy® Plant Mini Kit. The quantity of DNA was determined using a spectrophotometer. DNA extracts were kept at -20°C.
PCR protocol. Samples were initially amplified using primers MF137 and R635 as described by Johanson & Jeger (1993), using an amended protocol from Surridge *et al.* (2003). These primers were found to be not specific for *M. fijiensis* and alternative primers were sought from Dr. Juliane Henderson of the Cooperative Research Centre for Tropical Plant Protection in Queensland. Dr. Henderson provided, on a confidential basis, primer sequences for MFFor and R635-mod, and the PCR conditions for product amplification. These primers and conditions were used for all subsequent tests.
Prior to amplifying the DNA extracted from the tissue samples, the minimum detection limit of *M. fijiensis* for the PCR protocol being used was determined using DNA extracted from a culture of known identity (Mf 748 from the *M. fijiensis* culture collection of Dr Bob Fullerton). This was shown to be 0.243 fg/µL (1fg = 10^{-15}g). Reactions were analysed on 1.0% or 1.5% agarose gels. A PCR product of approximately 1050 bp indicated a positive amplification of *M. fijiensis*. Where a positive result was achieved, the PCR reaction was repeated a minimum of 3 times. In excess of 500 tubes were screened for products.
Sequencing. PCR products were cleaned using a QIAGEN MinElute PCR Purification Kit. Concentration of the product was checked with a spectrophotometer. The PCR product was sequenced using a BigDye terminator reaction mix. Products were sequenced using the universal primers ITS-1 and ITS-4, designed to amplify the internal transcribed spacer region of rDNA. Sequences were blast-searched against sequences lodged in GenBank.

Results
Of the 11 tissue samples subjected to PCR, two samples S56 (stem or petiole) and S36 (particulate trash) (Peterson, Piper and Poljak identification numbers) were sequenced. Both products were 100% homologous with *M. fijiensis* sequences lodged in GenBank. The specific primers also amplified a product from S31 (leaf material). However, this result was not consistent in repeated reactions and the product was not able to be confirmed by sequencing. No pathogen DNA was found in the skin samples obtained from the imported fruit.
Conclusion

- This study has shown that *M. fijiensis* was present in fragments of leaf trash found in cartons of banana fruit imported into New Zealand from the Philippines.

confirmed by both PCR (using species-specific primers provided on a confidential basis by QDPI) and the sequencing of the 16S - - 28S IST region of rDNA.

Results.
Of the five fruit isolates returned to New Zealand, two were positively identified as *M. fijiensis,* with concurring results from spore morphology, PCR and sequencing (99% match E-value 0). Both were obtained from different skin samples from the same fruit. Isolates of *M. fijiensis* from the epidermal strips of infected leaves were also confirmed as *M. fijiensis* by all three methods

Microscopical examination (in Samoa) of a cluster of hyphae on skin of a second fruit (from a different source) revealed a group of dark hyphae strongly resembling the early stages of a stroma of *M. fijiensis*. The structure was insufficiently developed to obtain a positive identification based on morphology, and overgrowth by other fungi prevented its isolation into pure culture.

In all three cases (two confirmed *M. fijiensis*, one suspected), the fungus was associated with minute (~ 1mm dia) red, necrotic flecks on the surface of the skin. Red flecks were relatively common on many of the test fruit. Most did not yield any fungi and others were overgrown before the target fungus would have had time to emerge. It cannot be determined from this study whether there is a constant association between the red fleck symptom and *M. fijiensis*.

Overgrowth of the skin pieces by faster growing fungal species would have masked the presence of *M. fijiensis* in many cases. This problem was probably accentuated by the nutrient rich growth media used for the study. The main species recovered and the average frequency of incidence (% recovery from skin pieces) were *Colletotrichum* spp. (44%) *Cordana musae* (13%), unidentifiable non-sporing species (14%). Other fungi less commonly present were *Penicillium* sp., *Cladosporium* sp., *Curvularia* sp., *Fusarium* sp. *Rhizopus* sp., *Trichoderma* sp., and *Pestalotiopsis* sp.

Conclusions

- *M. fijiensis* can be found within the skin of Cavendish banana grown in an environment where the disease is present.
- The apparently low rate of recovery of *M. fijiensis* in this study may have been due the overgrowth of a large proportion of excised skin pieces by other, faster growing fungal species.
- The degree of overgrowth experienced is likely to be due to the particular growth media used in the study.

Executive Summary

Detection of *Mycosphaerella fijiensis* in the skin of Cavendish banana fruit.

R. A. Fullerton and S. Casonato

3 August 2006

HortResearch was contracted by the Australian Banana Growers' Council to investigate whether *Mycosphaerella fijiensis*, cause of black Sigatoka (black leaf streak) disease, could be carried symptomlessly in the skin of Cavendish type bananas. The ability to survive in this way would offer a potential pathway for introduction of the pathogen to Australia via commercial consignments of fruit from a country that has the disease.

The work was carried out in the plant pathology laboratory of the Samoa Ministry of Agriculture, Forest, Fisheries and Meteorology (MAFFM), Nu'u Crop Research Station, Upolu, Samoa over the period 4-12 July 2006. Cavendish banana is grown extensively in Samoa primarily for cooking. Plantations are relatively small (<1ha), disease pressure from black Sigatoka is extreme, bunches are not covered and no disease control is practiced. It was considered that, if there was fruit infection it would most likely be found under those conditions.

Method.

Source of fruit. Hands of green fruit (the most mature available) were obtained directly from bunches in the field from two sites, Nu'u Research Station (north side of Upolu) and a commercial plot at the village of Siumu (south side of Upolu). In addition, hands of green-mature and ripe fruit were obtained from the Apia market. Fruit was purchased from different stalls, representing different source plantations (stall holders mostly sell their own fruit).

Isolation protocol. Five fruit with minimal damage from either banana scab moth or handling were selected from each hand. Each fruit was cleaned by wiping with an ethanol (95%) soaked paper towel, then flooded with ethanol and allowed to dry in the laboratory. Prior to removal of skin samples, the fruit were again wiped with an ethanol soaked tissue. Squares of skin tissue approximately 5mm square and 0.5-1.0mm thick were excised using a sterile scalpel. Sixteen samples were taken from each fruit, eight from the 'top' (concave aspect) of the fruit, and eight from the 'side' of the fruit. Skin samples were placed onto Petri dishes containing either Potato Dextrose Agar (PDA) or V8 agar, both modified with Streptomycin and Penicillin to inhibit bacterial growth. Plates were incubated under continuous white/near-UV light. After 5 days the plates were examined by stero-microscope. Fungal species growing from the leaf pieces were identified (within the limitations of time available) and recorded. Candidate colonies of *M. fijiensis* were sub-cultured on to fresh plates of antibiotic amended PDA to establish them as pure cultures.

Overall 60 fruit from 13 different sources were examined with a total of 1040 skin pieces being plated out. Six isolations of putative *M. fijiensis* were made of which five were returned to New Zealand for further identification.

In addition, epidermal strips were taken from leaves exhibiting the first (rusty streak) signs of black Sigatoka and plated to PDA. These provided a guide to the expected period to emergence of the target fungus, and also to provide fresh, sporulating cultures for comparative purposes. Isolates from epidermal strips were also returned to NZ for confirmation of identity.

Identification of isolates. The cultures imported from Samoa were held under PC2 containment at the HortResearch Mt Albert Research Centre, Auckland. They were subcultured to PDA and grown under light (white/near UV; 12hr/12hr light/dark) at laboratory temperature (~ 23C) and examined for the presence of spores typical of *M. fijiensis*. The identities of cultures identified as *M. fijiensis* based on spore morphology were