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Department of Agriculture, and Fisheries

Assessment of the end to end sampling and diagnostic process related to Panama disease Tropical Race 4

14 August 2015

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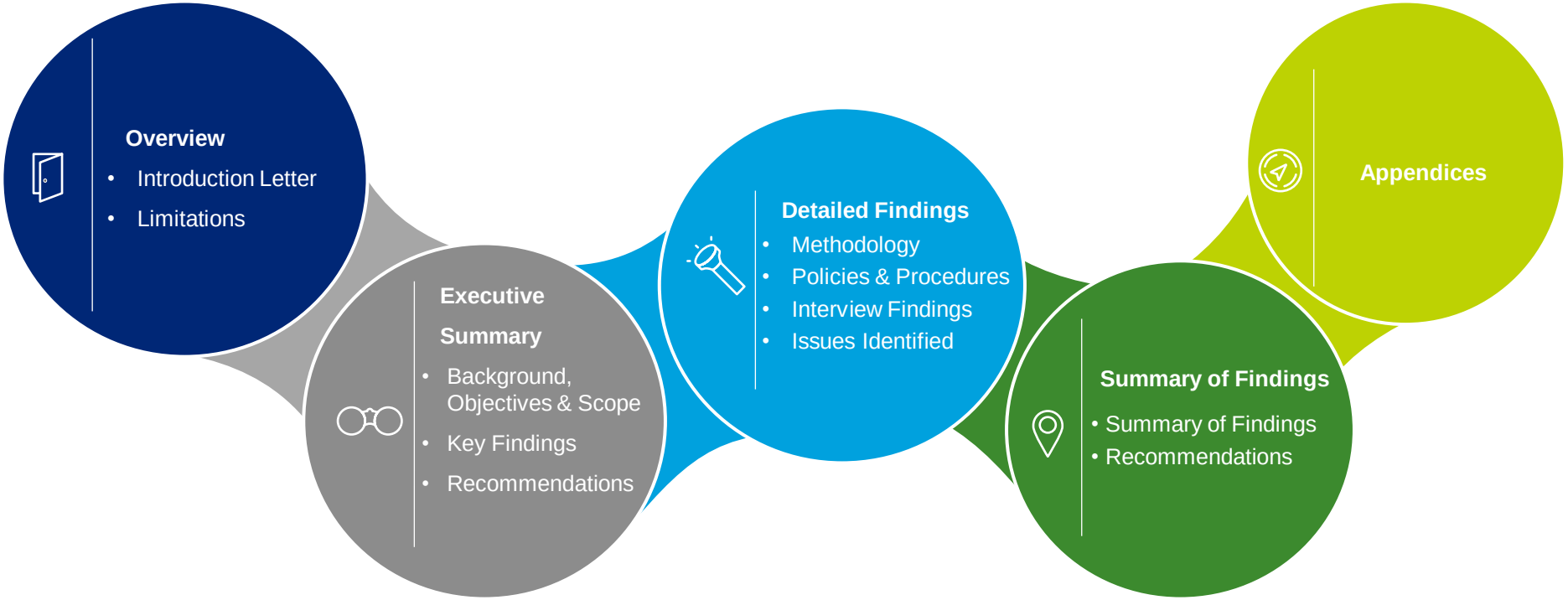
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Deloitte.

Deloitte Touche Tohmatsu
ABN 74 490 121 060

Riverside Centre
Level 25
123 Eagle Street
Brisbane QLD 4000
GPO Box 1463
Brisbane QLD 4001 Australia

Tel: +61 7 3308 7000
Fax: +61 7 3308 7001
www.deloitte.com.au

Ms Jackie Mooney
Account Director, Business Services
Department of Agriculture and Fisheries
80 Ann Street
Brisbane, Qld 4000

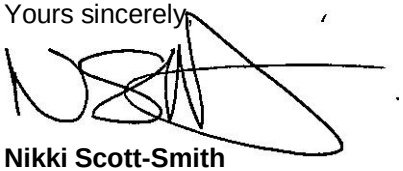
14 August 2015

Dear Jackie,

I refer to our engagement letter dated 15 May 2015, in which you have requested Deloitte Touche Tohmatsu (**Deloitte**) to assist the Department of Agriculture and Fisheries (**DAF**) with an assessment of the end to end process surrounding the sampling and diagnostic process and associated reporting activities used in the response to instances of the Panama disease Tropical Race 4 in North Queensland.

I am pleased to provide you with our report. If you have any questions please do not hesitate to contact me on 3308 7530 or Ian Megom on 3308 7359.

Yours sincerely,



Nikki Scott-Smith
Partner
Deloitte Touche Tohmatsu

Limitations

This report has been prepared exclusively for the Department of Agriculture and Fisheries (**DAF**) as per the terms and conditions set out in our engagement letter, dated 15 May 2015. This report is not intended to and should not be used or relied upon by anyone else and we accept no duty of care to any other person or entity. You should not refer to or use our name or the advice for any other purpose.

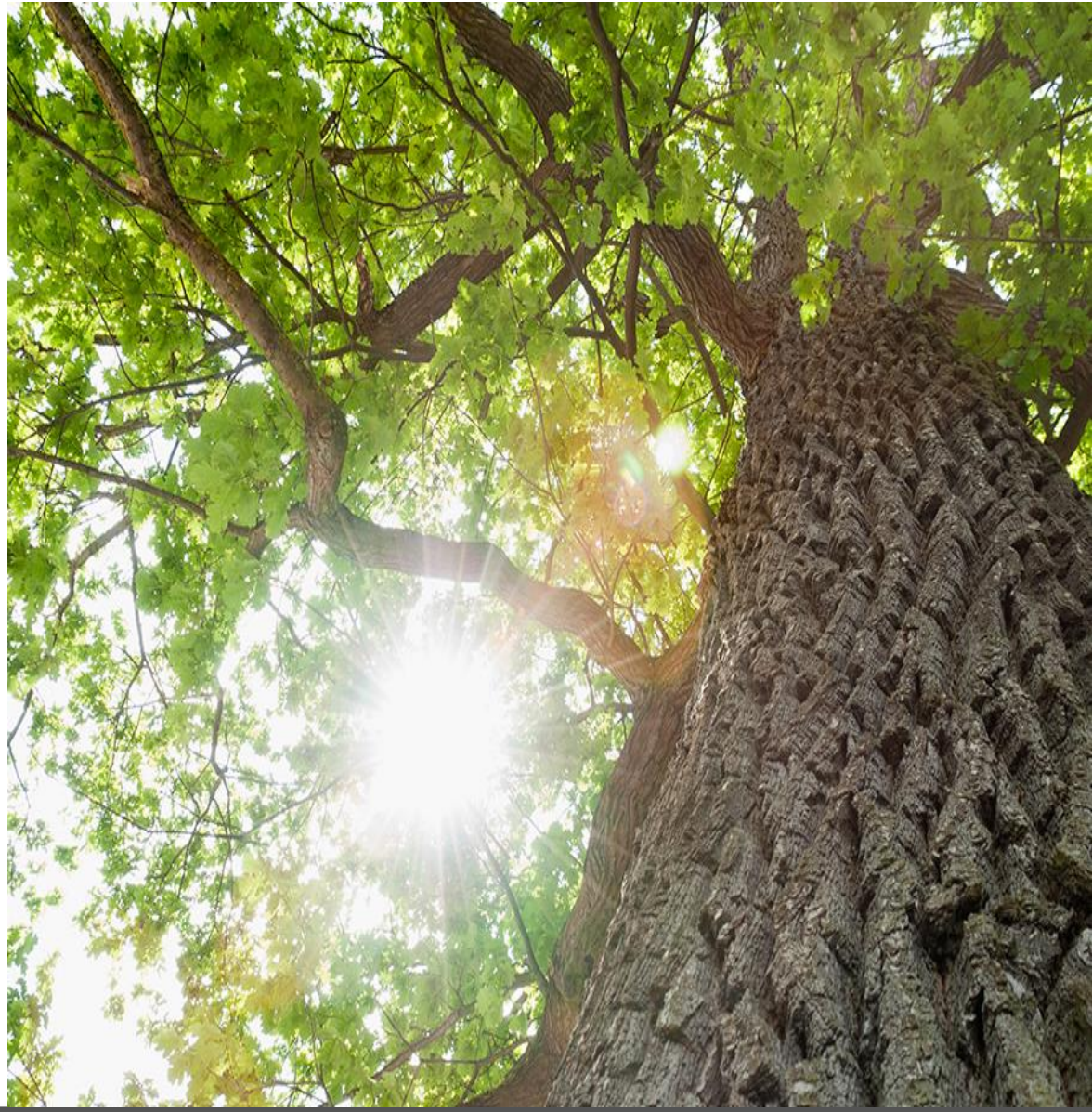
Deloitte Forensic staff are not lawyers, and our report should not be relied upon as legal advice. Our work was conducted in accordance with the Forensic Accounting Standards, APES215, of which we are required to comply. However, our work was not conducted in accordance with any auditing or assurance standards issued by the Audit and Assurance Standards Board, and consequently no opinions or conclusions were made under these standards.

This report is based on the information provided to us by DAF. Other than where specified, Deloitte does not assume responsibility for the validity and accuracy of the information obtained in this regard. For the purposes of preparing this report, reliance has been placed upon the material, representations, documentations, information and instructions obtained. We have not undertaken any audit, testing or verification of the information obtained and we assumed that this information is true, correct and complete and not misleading. If this is not the case or the information changes after we receive it, then our work may be incorrect or inappropriate for you.

Deloitte completed its field work on 15 June 2015 and has not updated its work since that date. The services will be limited by the time available to us, the agreed scope, information available, the accessibility of information sources and clarity or lack of clarity of your objectives. We reserve the right to revise any opinion or conclusion in our work if material information becomes known to us after the date our work is issued.

Executive Summary

- Background, Objectives and Scope
- Key Findings
- Recommendations



Background, Objectives and Scope

Background

The DAF is currently managing a response to Panama disease Tropical Race 4 (**TR4**). TR4 or more specifically *Fusarium oxysporum f. sp. cubense tropical race 4* (***Foc* TR4**) is considered the most destructive disease of banana in modern times. It is a serious threat to the >$500M Cavendish banana industry in Queensland. The DAF has issued Directions under the Plant Protection Act 1989 (s13) , effectively quarantines, on three (3) properties as part of the response

Decisions about actions to eradicate or contain a pest or disease, including issuing Directions, are made on scientific evidence of the presence of a pest or a disease on a property, the likelihood of establishment and spread, and the consequences of that spread

The quarantine of an infected property at Mareeba (**43IP**) was lifted after it was determined the test result, on which the decision to place the property under quarantine was partially based, was a false positive i.e. there was no evidence of the presence of the disease (Foc TR4) in the samples tested from the property

Subsequently, DAF engaged Deloitte to conduct an independent procedural review of the Panama disease diagnostic systems and processes used by DAF and associated facilities.

Objective

The objective of our engagement was to assess the adequacy of the end to end processes surrounding the sampling and diagnostic assessment process and associated reporting and response activities in regards to the recent reported instances of TR4 to address the following specific requirements:

- What standard operating procedures were available to inform decision making in the assessment of samples at the laboratory
- Were the procedures followed
- Were there technical issues that impacted on the accuracy/validity of the results
- At what stage could ambiguity/uncertainty about the accuracy (i.e. the false positives) of the Dita et al[3] methodology become material to decision making
- Was appropriate action taken to address the impact of the ambiguity and uncertainty on decision making
- What issues could have been foreseen
- Did decisions made by DAF to issue Directions based on the information available meet a test of reasonableness
- What recommendations should be considered for improvement.

Scope

The scope of our engagement was to:

- Examine and assess the relevant documentation including Standard Operating Procedures (**SOP**), policies etc.
- Conduct interviews with key personnel involved[1] with the end to end process. Assess the laboratory environment against relevant standards (national and/or international)
- Map the process followed in relation to 43IP and the associated timeframe and reporting activities[2]
- Obtain supporting documentation to verify the escalation of issues/results in relation to 43IP
- Assess the adequacy of the process and associated decision making and response activities
- Identify opportunities for improvement to the existing process and associated reporting and response activities.

[1] Refer to Appendix A for a list of key people interviewed

[2] Refer to Appendix B for a timeline of events

[3] Dita *et al*, 2010, 2011. A molecular diagnostic for tropical race 4 of the banana fusarium wilt pathogen. *Plant Pathology,* 59, 348-357

Key Findings

Detailed findings can be found by clicking on the relevant section in the task bar below. A summary of our key findings is as follows:

Policies and Procedures

- Deloitte identified and reviewed procedures for sample tracking, recording and reporting and laboratory quarantine together with a handwritten diagnostic procedure prepared by the State Coordination Centre (**SCC**) Planning Manager (**SPM**) and a draft *Policy for the Panama TR4 for the distribution of laboratory results (dated 24 March 2015)*. From our review of the documentation and walk through of the process it appears that these policies and procedures were followed.

Methodology and Testing

- There is no single reliable and rapid molecular method of detecting *Foc* TR4. Best practice involves the use of tests such as the Polymerase Chain Reaction (**PCR**) described by Dita *et al*[4] in conjunction with Vegetative Compatibility Group (**VCG)** analyses, internal symptomology and volatile production as was done during this response
- The inconsistency between the negative visual diagnosis, the positive Dita PCR result and failure to grow a culture initially led diagnosticians to believe that sampling was the cause i.e. the amount of infective material in the sample was small. Consequently the diagnosticians requested additional samples. The potential for cross-contamination raised by senior management was discussed with the diagnostic team and discounted due to stringent laboratory practices in the Quarantine Containment Level 3 (QC3) laboratory.
- There was no evidence of prior issues with the Dita PCR protocol and no consideration was given by diagnosticians or management to the possibility of the test being unreliable
- All reasonable steps were taken to verify the original positive result for 43IP sample M591, however, all were unsuccessful
- The molecular-based diagnostic protocols published for Foc TR4 are not robust enough. The Dita PCR protocol did not demonstrate the specificity required to test for Foc TR4 in Australia. There was no evidence that this false positive was caused by human error or poor laboratory practice. The use of the Dita PCR methodology as a diagnostic tool for the detection of TR4 has now been discontinued by DAF.

Decision Points

- The Chief Biosecurity Officer (**CBO**) adopted a risk-based approach consistent with that taken in relation to an infected property at Tully (**1IP**) and declared an emergency response on receipt of a positive Dita PCR result.

[4] PCR analysis as published in: Dita *et al*, 2010, 2011. A molecular diagnostic for tropical race 4 of the banana fusarium wilt pathogen. *Plant Pathology,* 59, 348-357.

Key Findings

Decision Points(Cont'd)

- This was based on a verbally agreed protocol for dealing with containment of *Foc* TR4. All those involved in agreeing the protocol discussed a number of considerations including the implications of not acting in a timely way to minimise the continuation of the risk of spread
- There were also links established between 1IP and 43IP. Deloitte were informed these links added significant weight to the considerations made at the time providing a level of justification to the invoking of the quarantine, as a precautionary response whilst further 'confirmatory' testing took place using VCG.

[4] PCR analysis as published in: Dita *et al*, 2010, 2011. A molecular diagnostic for tropical race 4 of the banana fusarium wilt pathogen. *Plant Pathology,* 59, 348-357.

Recommendations

Based on our review of the end to end process and our discussions with key personnel Deloitte recommend DAF consider the following:

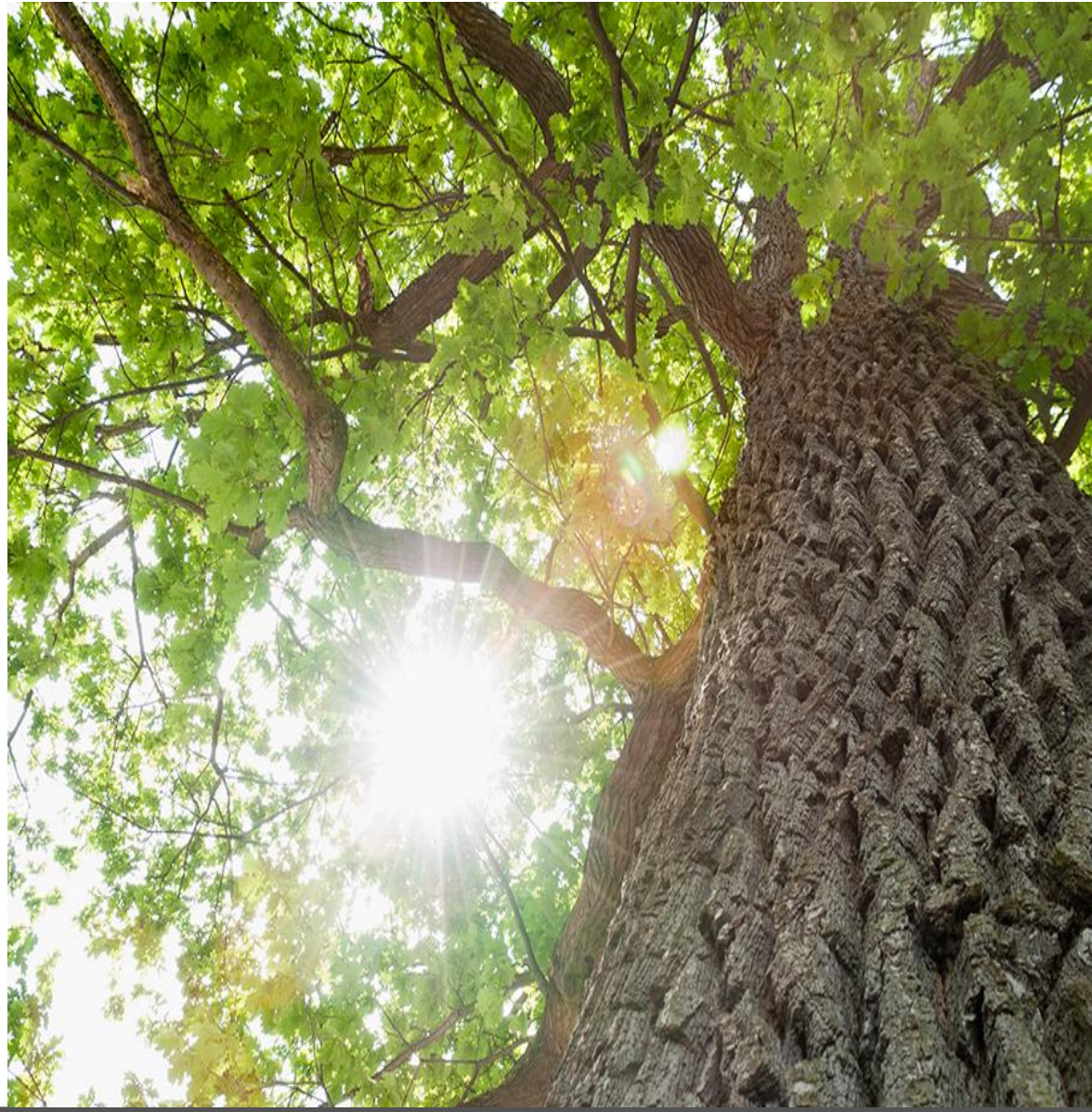
- Develop a quality assurance process to ensure the reliability of diagnosis and reporting by providing for adequate documentation and regular independent review of processes and methodologies, reporting and management
- Document consistent processes and methodologies and establish guidelines for imposing quarantine
- Minute all decisions made involving statutory requirements under the Plant Protection Act which could be reviewable under judicial or administrative processes
- Develop and implement a laboratory management information system. This will facilitate detailed, consistent, systematic and timely reporting of diagnostic results and will aid the decision-making process. The potential for misreporting and loss of information integrity will also be reduced. Uniformity of sample identification, results documentation and recording procedures will also assist in sample tracking and management
- Develop or support the development of a reliable molecular test for TR4 as diagnostic methods developed in other countries need to be tested and validated in the Australian environment
- Perform and record regular servicing and recalibration of equipment.

We recommend the following however, these could be considered under the Terms of Reference for the recently announced Biosecurity Queensland Capability Review:

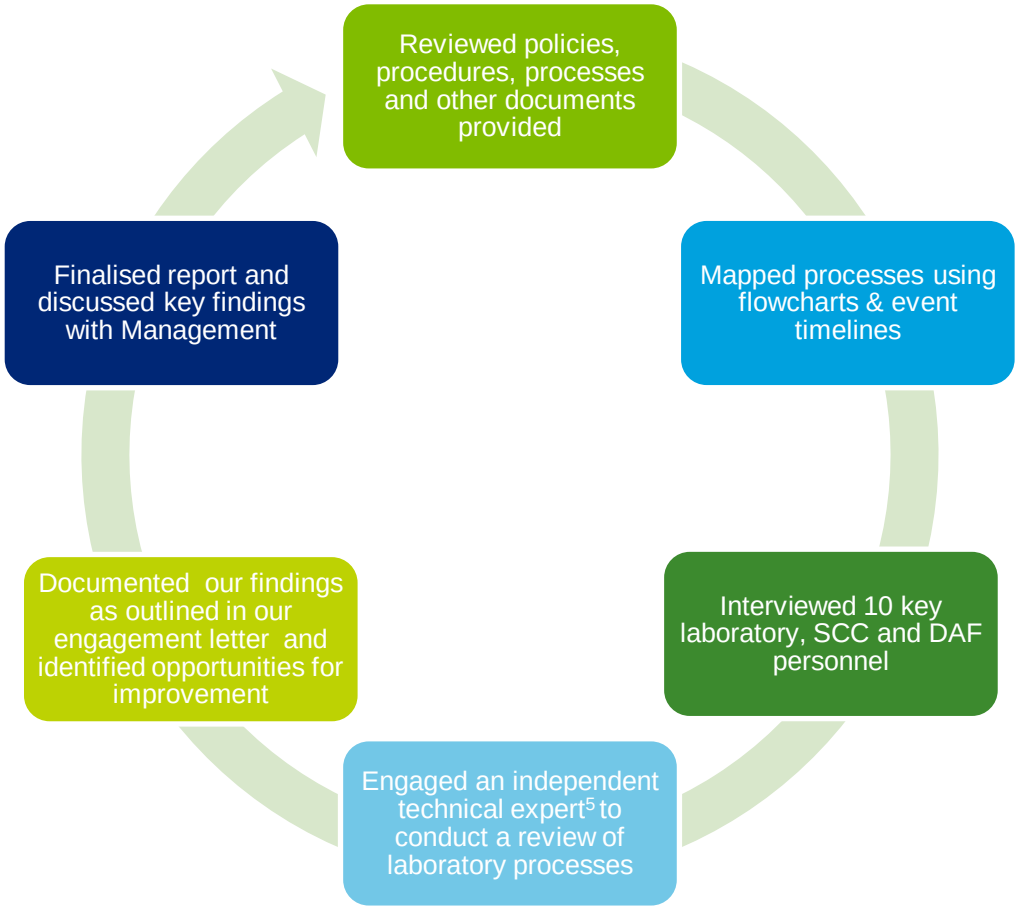
- Assess the core function of the diagnostic laboratory to inform the level of resourcing, specialist support and processes required to meet the objectives of Biosecurity Queensland(BQ)
- Review the laboratory organisational structure whilst considering the unique attributes and complexities of plant diagnostics as there is a requirement for the laboratory to build ‘scale up’ capacity to respond to an emergency event
- Establish appropriate resourcing levels to support laboratory objectives particularly in the event of an emergency response. Laboratory personnel should, where possible, be engaged directly by BQ
- Implement a ‘Preparedness Plan’, to ensure continued efficient and effective laboratory management, that enables scale up of ‘core capacity’ from business as usual to manage diagnostic response in an emergency event (including resourcing)
- Establish a dedicated team to address laboratory management and oversight, plant diagnostics capability (including molecular specialist skills) and quality assurance review capability. Under an emergency response situation, it is preferable to have dedicated facilities and equipment for exclusive use of the diagnosticians.

Detailed Findings

- Methodology
- Policies and Procedures
- Interview Findings
- Issues Identified



Methodology



Weekly status updates were provided to ensure that all key stakeholders were kept informed of the work we performed, our observations and key findings.

[5] Refer to Appendix C for a copy of the independent technical expert report.

Detailed Findings

Policies and Procedures

Deloitte reviewed the policies and procedures provided by DAF and conducted walk throughs of the laboratory processes with the key diagnosticians involved in the response to gain an understanding of the end to end process that was executed and obtain evidence and clarification if required. Deloitte also mapped the processes surrounding application of the following policies/procedures[7]:

1. *Policy for the Panama TR4 Distribution of Laboratory Results (Draft, V1, 24 March 2015)*
2. *Procedure for Receipt, Handling, Tracking of Diagnostic Samples and the Reporting of Diagnostic Results (V4.1)*
3. *Flowchart of Diagnostic Samples*

From our review of the documentation, Deloitte note the following :

- The draft *Policy for the Panama TR4 Distribution of Laboratory Results* which outlines the diagnostic and reporting protocol for *Foc* TR4 was drafted on 24 March 2015 after the response to quarantine 1IP (Tully property) on 04 March 2015
- A diagnostic procedure (a handwritten flowchart that had not been through a review process) prepared by the SPM[8] was provided to diagnosticians around 26 March 2015. This requires suspect samples to be tested by Dita PCR on DNA extracted from plant tissue at the same time as isolations are carried out (i.e. attempts to culture Fusarium from the plant tissue). This is in addition to the draft *Policy for the Panama TR4 Distribution of Laboratory Results* where the sample is firstly cultured on selective media for 14 days and if there is no growth consistent with Fusarium, the culture is recorded as Isolation Negative. If a culture is able to be grown it is then tested by Dita PCR and/or VCG – a test of ability to fuse with a known strain. The handwritten procedure provided by the SPM was used In the case of 43IP. Refer to Appendix D for a map of the two processes
- Our walkthrough with the diagnosticians of the procedures for *Receipting, Handling, Tracking of Diagnostic Samples, Reporting of Diagnostic Results* and the *Containment of Plant Pathogens* indicated the procedures were generally adhered to
- We have not been advised of any additional procedures that relate to the risk based assessment procedures stated above to inform decision making in relation to a response to a suspected *Foc* TR4 positive result
- Deloitte were informed that during a meeting of senior BQ individuals it was agreed that if there was a positive Dita PCR result they would invoke quarantine. It should be noted this meeting was not minuted and we were not advised of additional notes being made by individuals present at that meeting.

[6] Refer to Appendix D for a list of documentation provided

[7] Refer to Appendix E for a process map of the *Policy for the Panama TR4 Distribution of Laboratory Results* and the *Flowchart of Diagnostic Samples*

[8] It should be noted that the informal diagnostic procedure is a hand-drawn flowchart

Detailed Findings

Interview Findings

Deloitte interviewed ten key personnel across BQ, DAF, SCC, Agri-science Queensland (**ASQ**) and Queensland Alliance for Agriculture and Food Innovation (**QAAFI**). The key points from the interviews are outlined under four key themes. Refer to Appendix A for a list of individuals interviewed (by position title).

Policies and Procedures

The key points from the interviews in relation to the policies and procedures are documented below:

- A number of key personnel reported that unlike the animal health world where there are well-known, well-studied diseases with well-established diagnostic methodologies, plant health is a complex area with a large number of plant pests and diseases including, bacteria, fungal infections and viruses, some of which may not be well-understood or studied and thus, not documented or are difficult to document. As a result, standard operating procedures to inform decision making in the assessment of samples at the laboratory are not always available
- The SPM advised there was no impetus to establish a specific response plan in the first few weeks of the emergency response given the pathogen cannot be eradicated and its longevity in the soil. The team discussed developing a response plan after the first Dita PCR result was known at 1IP. Deloitte were advised by the SPM that a response plan for *Fo*c TR4 is currently in development. Deloitte understands from subsequent discussions with DAF that the urgency to develop a strategic response plan for this process did not exist as it was concluded that the pathogen was unlikely to be eradicated from the soil through any practical or available means. On this basis, the mechanisms of the predetermined Exotic Plant Pest Response Deed would not be invoked
- The need to develop and formalise a laboratory response plan was identified by the Acting Director, Animal Biosecurity Science. He stated that a 'preparedness plan' that outlines the mobilisation of additional diagnostic resources in an event should be developed
- In addition, he said that a corrective action plan which outlines how issues, such as questions around the reliability of results, are managed, and rectified should be part of a quality system. This was supported by the SCC Director who stated that a process to capture considerations and the decision making surrounding the response needs to be formalised at the time a decision is made
- The SPM informed us that the decision to quarantine 43IP on a positive Dita PCR result was based on verbal discussions between the CBO, the General Manager (**GM**), the Acting SCC Director and the SPM. This conversation was not minuted and no supporting documentation for this decision could be provided

Detailed Findings

End to End Process

The key points from the interviews in relation to the 'end to end' process are documented below: Refer to Appendix B for a full timeline of the end to end process.

- The diagnostic testing process for *Foc* TR4 involves multiple layers of testing which includes, but is not limited to, visual identification of external and internal symptoms, isolation of *Foc*, various PCR tests performed on tissue and/or culture and a VCG test
- The VCG test is a definitive test that will confirm *Foc* TR4. However, it is a slow biological test that takes approximately three weeks to produce a result as it relies on the growth of a culture. In contrast, the Dita PCR test is quick to perform and can produce results in approximately six hours. Deloitte were informed that the Dita PCR test was therefore relied upon for the early detection of TR4
- In relation to 43IP, the farmer had requested DAF to inspect suspicious plants as they were displaying external symptoms (i.e. yellowing of the leaves). The suspect plants were cut so the vascular tissue could be inspected. The inspection showed no signs of discolouration (i.e. browning of the psyllium). A sample (M591) was collected on 26 March 2015 for testing in the laboratory in accordance with sampling procedural requirements to sample externally symptomatic plants irrespective of a positive or negative visual diagnosis
- After visual diagnosis of sample M591, a Dita PCR test was conducted on the host tissue and returned a positive result for *Foc* TR4 on 28 March 2015 however a culture from the sample could not produce a *Foc*TR4 isolate. According to the QAAFI Research Scientist, the Dita PCR test result was not a strong positive
- The QAAFI Research Scientist re-performed the test on 31 March 2015 and on 1 April 2015 a positive result was delivered. However, she said that due to the inability to grow a culture she was not convinced by the results and requested that the BQ Senior Research Scientist to attempt Foc TR4 isolation from M591 tissue over the Easter weekend. This involved taking another strand of tissue from M591. However, the re-isolation attempt from the tissue, again did not produce a culture
- The QAAFI Research Scientist emailed the BQ Senior Research Scientist and the BQ Principal Plant Pathologist on 8 April 2015 reporting the M591 positive result by Dita PCR and noted the inability to grow a culture from this sample. She stated that sampling may have been an issue due to inconsistencies with the M591 results. She suggested that the plant from which the M591 sample was collected should be resampled for re-testing (refer to Appendix F for email support)
- On receiving this email the BQ Principal Plant Pathologist prepared a Diagnostic Report on M591 that stated the visual diagnosis and isolation were negative but the Dita PCR test (on the host tissue) tested positive for TR4 (refer to Appendix G for diagnostic report)

Detailed Findings

End to End Process cont.

- On 8 April 2015, the BQ Senior Research Scientist sent an email to the Acting SCC Director and the GM, Plant Biosecurity attaching the report noting the positive Dita PCR result along with the negative internal visual symptom and the inability to isolate Fusarium in the culture. The BQ Senior Research Scientist stated that additional sampling would be required from 43IP (refer to Appendix H for email support)
- Many interviewees stated that conversations were held relating to concerns of cross contamination of M591 with samples from 1IP or cross specificity as a result of the previously recorded presence of Panama disease Race 1 (R1) on the same portion of IP43 as the plant from which sample M591 had been taken. The Acting SCC Director discussed the possibility of cross contamination with the SPM. The SPM provided advice regarding the diagnostic testing process including the professionalism and experience of the diagnosticians as well as consultation with them about their laboratory practices and findings. Based on these discussions, concerns surrounding cross contamination were discounted by these individuals
- Based on the report the SCC Director decided to quarantine 43IP in line with the verbally agreed protocol to quarantine a property based on a positive Dita PCR result
- Following discussions between the SCC Director, the Acting CBO, the BQ Principal Plant Pathologist and others *a* decision was made for the quarantine remain in place whilst resampling and VCG analysis was completed
- The QAAFI Research Scientist continued to complete a variety of PCR tests on different gene regions on the same sample (M591)
- 18 additional samples from 43IP were collected between 9 April 2015 and 21 April 2015. These were tested using Dita PCR analysis but again Fusarium could not be isolated and samples tested negative to the Dita PCR testing (refer to Appendix I for a summary of results)
- Samples M734 and M737, which were samples collected by re-sampling the same plant from which M591 (i.e. the original plant) had been collected, eventually produced a culture which enabled VCG testing to be conducted by a Plant Pathologist at ASQ
- The ASQ Plant Pathologist regularly reported his observations to the diagnosticians and the SPM during the VCG testing period via email
- On 18 May 2015, M734 and M737 tested negative to *Foc* TR4 by VCG. The results were emailed to the BQ Principal Plant Pathologist, the BQ Senior Research Scientist and the QAAFI Research Scientist
- On the same day, the BQ Principal Plant Pathologist sent an email to the GM, Plant Biosecurity, summarising the diagnostic testing results for all 18 samples from 43IP. He made a number of observations in relation to their diagnostic investigations and stated "*changes to the diagnostic protocol have been proposed…. These changes recognise that VCG analysis is the most reliable way to identify TR4."* He concluded "there is no reliable evidence to show that TR4 is present on 43IP." (refer to Appendix J for email support).
- Following receipt and consideration of this report the 43IP quarantine was lifted.

Detailed Findings

The Dita et.al Methodology

The key points from the interviews in relation to the Dita methodology are documented below:

- A number of PCR tests have been developed by the international scientific community to detect the presence of Fusarium. Some are specific to *Foc* TR4 and some are specific to *Foc*
- Specifically, a multiplex PCR-based diagnostic tool was developed by Professor Dita *et al* in 2010, 2011 to detect isolates from *Foc* TR4 for fungal or plant samples. The authors concluded that this diagnostic procedure is currently the best option for the rapid and reliable detection and monitoring of *Foc* TR4 to support eradication and quarantine strategies[9]
- As the Dita PCR test is a published and internationally recognised molecular diagnostic, it was selected to form part of the diagnostic process for the current *Foc* TR4 response which involves a number of different tests: visual identification of external symptoms and internal symptoms, PCRs (performed on tissue and/or culture) and a VCG test
- Whilst the Dita PCR test was relied upon for early detection of *Foc* TR4 it was not intended to replace biological testing (i.e. the VCG analysis). Rather, the molecular and biological diagnostics were to be used in conjunction with the entire diagnostic suite in the detection of *Foc* TR4. It was agreed that the Dita PCR test would be conducted first while isolations were being carried out which is consistent with the flowchart process prepared by the SPM[10]
- The SPM advised that the team were familiar with the Dita methodology and had confidence in its application as it is sensitive and specific to detecting *Foc* TR4 of the banana fusarium wilt pathogen. This is a view supported by all those Deloitte interviewed
- The GM, Plant Biosecurity advised that the diagnosticians were using the Dita PCR method appropriately
- The QAAFI Research Scientist conducted the Dita PCR test on M591 twice. However, she explained this was because she was not convinced by the initial positive result when the sample was unable to grow a culture
- The QAAFI Research Scientist told us that at the time she thought sampling may have been an issue due either to insufficient quantity or quality of *Foc* TR4 present in the M591 sample for culture to isolate the pathogen, as they were unable to grow a culture from the M591 sample. Therefore, in her email to the BQ Senior Research Scientist on 8 April 2015, she suggested that M591 should be recollected for re-testing (refer to Appendix F for email support).

[9] Dita *et al*, 2010, 2011. A molecular diagnostic for tropical race 4 of the banana fusarium wilt pathogen. *Plant Pathology*, 59, 348-357
[10] Refer to Appendix E for a process map of the flowchart for diagnostic samples

Detailed Findings

The Dita et.al Methodology cont.

- The inconsistency of the reported results and the potential for cross-contamination during the PCR analysis was discussed by senior management in a meeting held on 8 April 2015 and again on 9 April 2015 when the SCC Director returned from leave. They sought advice from the SPM (an expert in Panama disease) in relation to the level of confidence surrounding the result. The SPM advised that whilst the results were inconsistent, the diagnosticians were confident with the results as the controls were robust. In addition, the possibility of cross-contamination was considered unlikely due to their stringent laboratory practices in the QC3 laboratory. This was confirmed by our walkthroughs of the Quarantine Containment Level 2 (QC2) and QC3 SOPs. It should be noted that no minutes were taken. We were not advised of additional notes being made by individuals present at that meeting
- Deloitte were informed that the inability to grow a culture was also discussed by the Acting SCC Director and the diagnosticians. The Acting CBO informed us that the diagnosticians had no explanation for this. There was consideration given to the potential for cross-reactivity. However, we were told by the SPM that they considered the adequacy of the controls in place to indicate the tests had functioned correctly and therefore excluded potential for cross-reactivity
- Throughout April and early May, the diagnosticians conducted and finalised results for Dita PCR tests on additional samples collected from 43IP. On 18 May 2015 VCG tests were completed and all results were reported to the SCC Director (refer to Appendix I for a summary of results). Based on these results the diagnosticians suggested that the Dita PCR test may be unreliable as a detector of *Foc* TR4 (refer to Appendix J for email support)
- The QAAFI Research Scientist told us she received an email on 23 May 2015 (post quarantine of 43IP) from Professor Miguel Dita, (principal author of Dita *et al.*, 2010,11) highlighting cross-specificity issues in some cases. Deloitte sighted this email during our interview with the QAAFI Research Scientist conducted on 05 June 2015
- In the email dated 18 May 2015, from the BQ Principal Plant Pathologist to the GM, Plant Biosecurity, the diagnosticians proposed the urgent development of a reliable PCR or other molecular test for the detection of *Foc* TR4 in banana tissue, soil and water to ensure effective management and containment of the pathogen
- Deloitte were informed by the BQ Principal Plant Pathologist on 03 June 2015 that the DAF diagnostic protocol no longer includes the Dita PCR test. Deloitte were subsequently advised by DAF that the Dita PCR test may continue to be used, particularly to provide evidence in relation to specificity. However, it will not be relied upon to determine the presence of *Foc* TR4.

Detailed Findings

Key Decision Points

The key points from the interviews in relation to the decision points are documented below:

- Panama disease is a vascular wilt disease in banana plants that has the potential to cause significant damage to Australia's banana industry. The disease can impact all commercial banana varieties, including the dominant Cavendish variety which accounts for 95% of Australian production[11]
- When 1IP tested positive for *Foc* TR4 using the Dita PCR test, the result (as with any positive results for plant pests or diseases) was reported by the GM and the CBO for discussion and to formulate a response. Deloitte were advised that DAF senior management decided that given the nature of the disease and the potential to cause significant damage to the Queensland banana industry, they would take a precautionary approach and quarantine a property if a sample tested positive for *Foc* TR4 by the Dita PCR test until the results were confirmed by further testing (VCG analysis)
- It was confirmed by all those Deloitte interviewed that whilst the VCG test is a definitive test for the detection of *Foc* TR4, it can take approximately three weeks to perform and relies on the growth of a culture. This added weight to the decision to conduct the Dita PCR analysis first (whilst isolations were being carried out to grow a culture) and quarantine a property based on a positive Dita PCR result. The CBO adopted a risk-based approach consistent with that taken in relation to 1IP and declared an emergency response on receipt of a positive Dita PCR result
- Deloitte were told of known links between 1IP and 43IP in relation to the movement of people and earth-moving equipment in 2011 (which is within the lifespan of *Foc* TR4 in the soil). This contextual evidence could have added weight to the decision to quarantine 43IP
- Deloitte were informed by those that were interviewed that the decision to quarantine 43IP was based on a verbally agreed protocol relating to a positive Dita PCR result. This process included discussion of a number of considerations including consideration of scientific evidence of the presence of the pest or disease on a property, the likelihood of establishment and spread, and the consequences of that spread. Consideration was also given to the requisites for imposition of quarantine, impact of imposition of restrictions and the implications of not acting in a timely way to minimise the continuation of the risk of spread.

[11] This information is corroborated by published journal articles/reference papers provided to Deloitte and online research conducted by Deloitte

Issues Identified

Deloitte have identified a number of issues and areas for potential improvement that are summarised below under four key themes:

Resourcing

- Funding for plant health diagnostics has been challenging in recent times and the number of laboratory resources at the time reflects those challenges
- The laboratory was understaffed with four key diagnosticians conducting testing, reporting and managing the laboratory at the same time. However, the need for assistance during the initial stages of the response was not raised early enough. This could potentially result in the diagnosticians making errors caused by fatigue and overload however there was no evidence of errors due to fatigue or overwork in this case
- Deloitte note that additional temporary resources have recently been engaged to assist with the response. This has only addressed the issue of resourcing in the short term.

Laboratory organisational structure

- The *Foc* TR4 response relies on facilities and staff collaboration between three different agencies (BQ, AQS and QAAFI) with different sources of funding. Different diagnostic steps are conducted by the different agencies which has the potential for miscommunication and which relies on the goodwill and cooperation of the relevant staff. Deloitte note there was strong collaboration between the parties using the same facilities and equipment. However, the diagnostic processes in the laboratory are fragmented and potentially inefficient and does not lend itself to effective management and reporting of results. Whilst there is no evidence that these issues impacted the results on this occasion this could be an issue in the future
- The laboratory is a facility shared with PhD students and research projects among others. Whilst there is no reason to believe this impacted the results it could be an issue in the future
- The issues surrounding the laboratory organisational structure also highlight the complexities of the plant health world. As there are a significant number of plant pests and diseases the diagnostic capability is limited and located in disparate parts of the world. Deloitte learned that as a result it would be difficult to establish a single diagnostic laboratory with capabilities to address all the known pests and diseases. This explains why the BQ diagnosticians often have to outsource some of their testing to obtain a diagnosis as they do not have the required capability/expertise on site. Ideally, the focus should be on having access to the right expertise at the right time.

Detailed Findings

Reporting

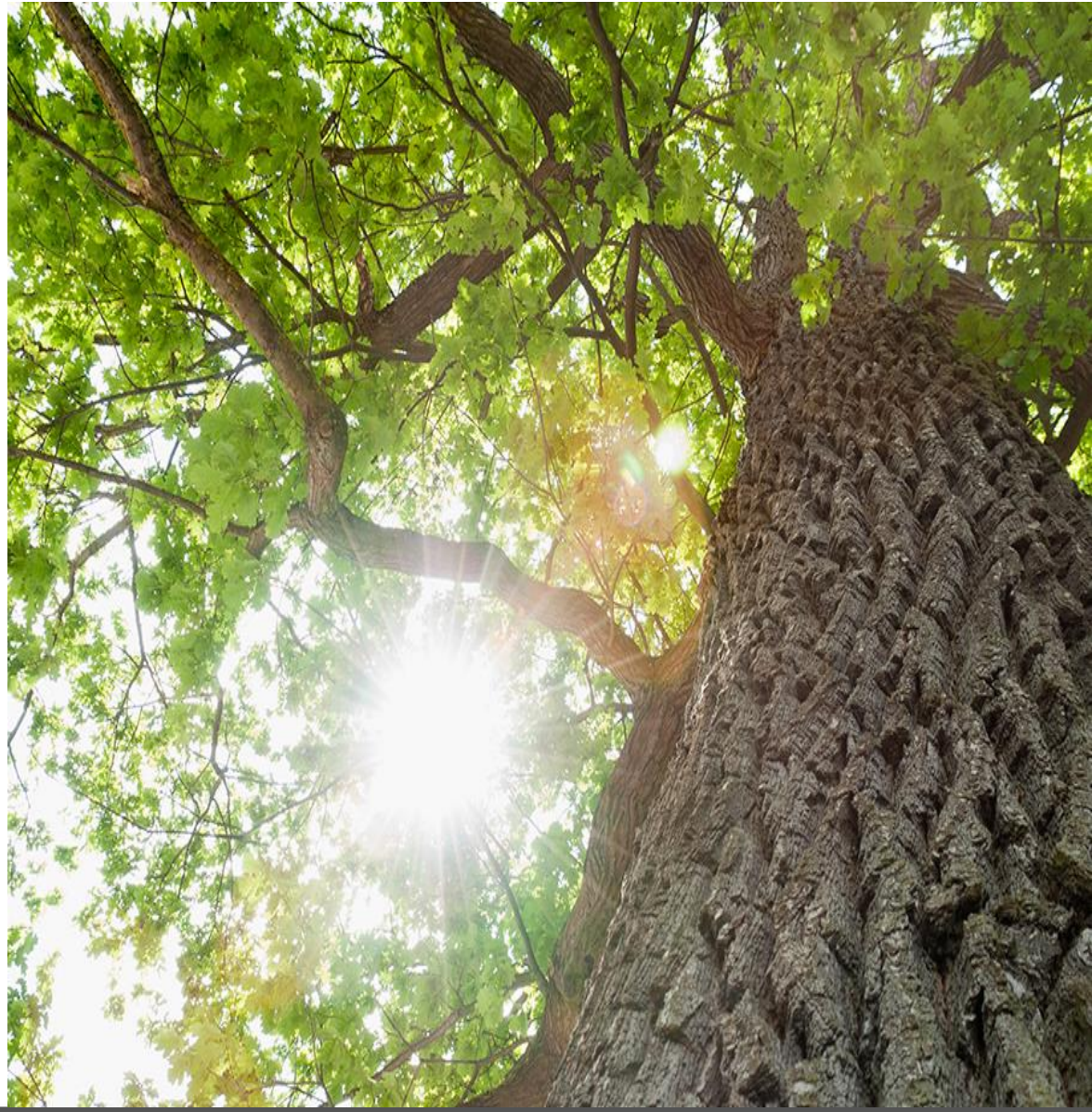
- All interviewees were in agreement that the diagnostic reporting needs improvement. The CBO stated that the reporting of diagnostic results needs to be detailed, consistent, systematic and timely so that senior management can understand the status and results of samples tested. Deloitte understands this is closely linked to the current laboratory structure
- Some people believe the diagnostic reports are inadequate from a decision-making point of view. The involvement of three agencies in the diagnostic reporting process (BQ, ASQ and QAAFI) creates the potential for misreporting and loss of information integrity particularly as comments/nuances were provided via email and then transcribed into the database. Whilst there is no evidence that this issue impacted the results on this occasion it could be an issue in the future
- Sample tracking was made difficult by samples moving between the three agencies as each maintains their own laboratory books and documentation. This was coordinated by the BQ Senior Research Scientist who entered details into the database. Some minor record keeping inconsistencies were noted by the technical expert. For example, there were no records of kit batch numbers that were used or records of freeze-thaw events. These records are important in determining whether the reagents may have had any impact on unexpected test results. (The expert was informed this has since been addressed.)

Quality Assurance Process

- There does not appear to be a formal quality assurance process underpinning the laboratory structure. Deloitte were subsequently advised by DAF that quarantine accreditation processes are maintained and regularly audited for the QC2 and QC3 laboratories
- Routine quality assurance processes such as regular servicing and recalibration of equipment was not performed or recorded.

Summary of Findings

- Summary of Findings
- Recommendations



Summary of Findings

Based on our review of the end to end process and our discussions with key personnel Deloitte note the following:

Policies and Procedures

- Deloitte acknowledge that unlike the animal health world where there are well known, well studied diseases with well established diagnostic methodologies, it is difficult to document a full range of procedures for plant health diagnostics
- Our walkthrough with the diagnosticians of the procedures for *Receipting, Handling, Tracking of Diagnostic Samples, Reporting of Diagnostic Results* and the *Containment of Plant Pathogens* showed the procedures were generally adhered to
- An informal diagnostic procedure was handwritten by the SPM early in the response and this was provided to the diagnosticians to follow. This process adopted was in addition to the process documented in the draft Policy for the Panama TR4 Distribution of Laboratory Results which was created on 24 March 2015 (post 1IP quarantine). The informal procedure was in use throughout the diagnostic process for 43IP
- There were no documented operating procedures available to inform decision making in relation to the response to a suspected *Foc* TR4 incident. At the time of placing 1IP under quarantine, a verbal decision was made during a meeting of senior BQ management to impose quarantine on a positive Dita PCR result. However there are no minutes of this meeting. This approach was applied in the case of both 1IP and 43IP placing them under quarantine on the basis of a positive Dita PCR result.

Methodology & Testing

- The Dita PCR test is internationally recognised and considered a reliable and rapid molecular method for the detection of *Foc* TR4. However, following a review of all laboratory testing related to M591 and 43IP, this test was deemed to be unreliable when used alone. Best practice involves the use of a range of tests such as, gross internal symptomology, volatile production, molecular assays and culture followed by confirmation by VCG analysis as was done during this response
- The molecular-based diagnostic protocols published for *Foc* TR4 are not robust enough. The Dita protocol did not demonstrate the specificity required to test for *Foc* TR4 in Australia
- Deloitte found no evidence of technical issues such as cross-contamination, human error or poor laboratory practice that impacted the accuracy/validity of the results during the course of diagnostic testing
- Deloitte found no evidence to suggest there were questions or concerns raised in relation to the appropriateness and the accuracy of the Dita PCR test at the time of placing 43IP under quarantine. The reliability of the test to detect TR4 was questioned after testing on 43IP samples had been completed.

Summary of Findings

Decision Points

- The CBO adopted a risk-based approach consistent with that taken in relation to 1IP to invoke quarantine restrictions on 43IP
- This decision was based on a verbally agreed protocol for dealing with containment of *Foc* TR4. This process included discussion of a number of considerations including consideration of scientific evidence of the presence of the pest or disease on a property, the likelihood of establishment and spread, and the consequences of that spread. Consideration was also given to the requisites for imposition of quarantine, impact of imposition of restrictions and the implications of not acting in a timely way to minimise the continuation of the risk of spread
- There were also links established between 1-IP and 43IP. Deloitte were told that these added significant weight to the considerations made at the time providing a level of justification to the invoking of the quarantine, as a precautionary response whilst further 'confirmatory' testing took place using VCG
- The protocol was applied consistently to 1IP and 43IP.

Recommendations

Based on our review of the end to end process and our discussions with key personnel Deloitte recommend DAF consider the following:

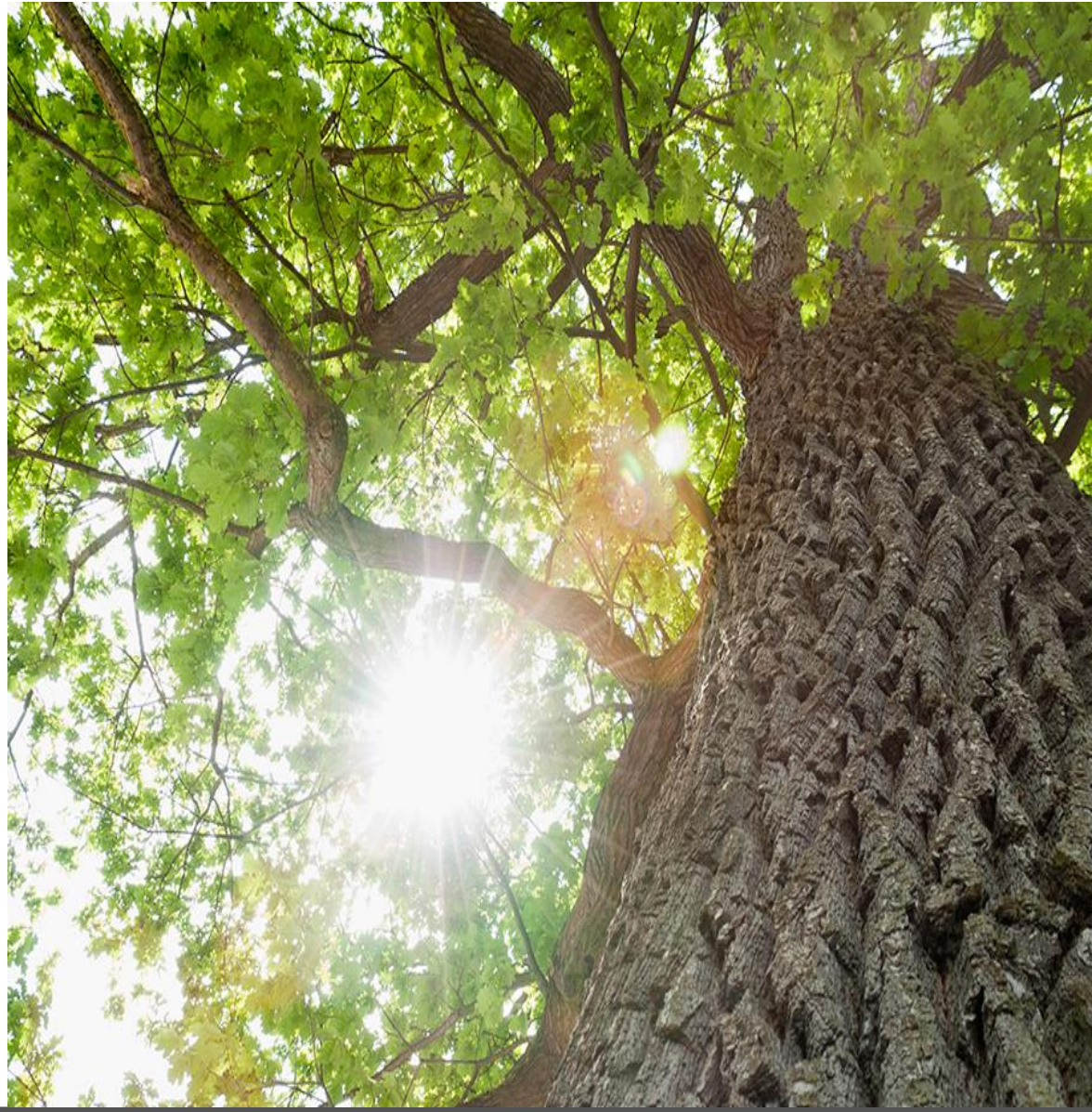
- Develop a quality assurance process to ensure the reliability of diagnosis and reporting by providing for adequate documentation and regular independent review of processes and methodologies, reporting and management
- Document consistent processes and methodologies and establish guidelines for imposing quarantine
- Minute all decisions made involving statutory requirements under the Plant Protection Act which could be reviewable under judicial or administrative processes.
- Develop and implement a laboratory management information system. This will facilitate detailed, consistent, systematic and timely reporting of diagnostic results and will aid the decision-making process. The potential for misreporting and loss of information integrity will also be reduced. Uniformity of sample identification, results documentation and recording procedures will also assist in sample tracking and management
- Develop or support the development of a reliable molecular test for TR4 as diagnostic methods developed in other countries need to be tested and validated in the Australian environment.
- Perform and record regular servicing and recalibration of equipment

We recommend the following however, these could be considered under the Terms of Reference for the recently announced Biosecurity Queensland Capability Review:

- Assess the core function of the diagnostic laboratory to inform the level of resourcing, specialist support and processes required to meet the objectives of Biosecurity Queensland(BQ)
- Review the laboratory organisational structure whilst considering the unique attributes and complexities of plant diagnostics as there is a requirement for the laboratory to build ‘scale up’ capacity to respond to an emergency event
- Establish appropriate resourcing levels to support laboratory objectives particularly in the event of an emergency response. Laboratory personnel should, where possible, be engaged directly by BQ
- Implement a ‘Preparedness Plan’, to ensure continued efficient and effective laboratory management, that enables scale up of ‘core capacity’ from business as usual to manage diagnostic response in an emergency event (including resourcing)
- Establish a dedicated team to address laboratory management and oversight, plant diagnostics capability (including molecular specialist skills) and quality assurance review capability. Under an emergency response situation, it is preferable to have dedicated facilities and equipment for exclusive use of the diagnosticians.

Appendix

- Appendix A
- Appendix B
- Appendix C
- Appendix D
- Appendix E
- Appendix F
- Appendix G
- Appendix H
- Appendix I
- Appendix J



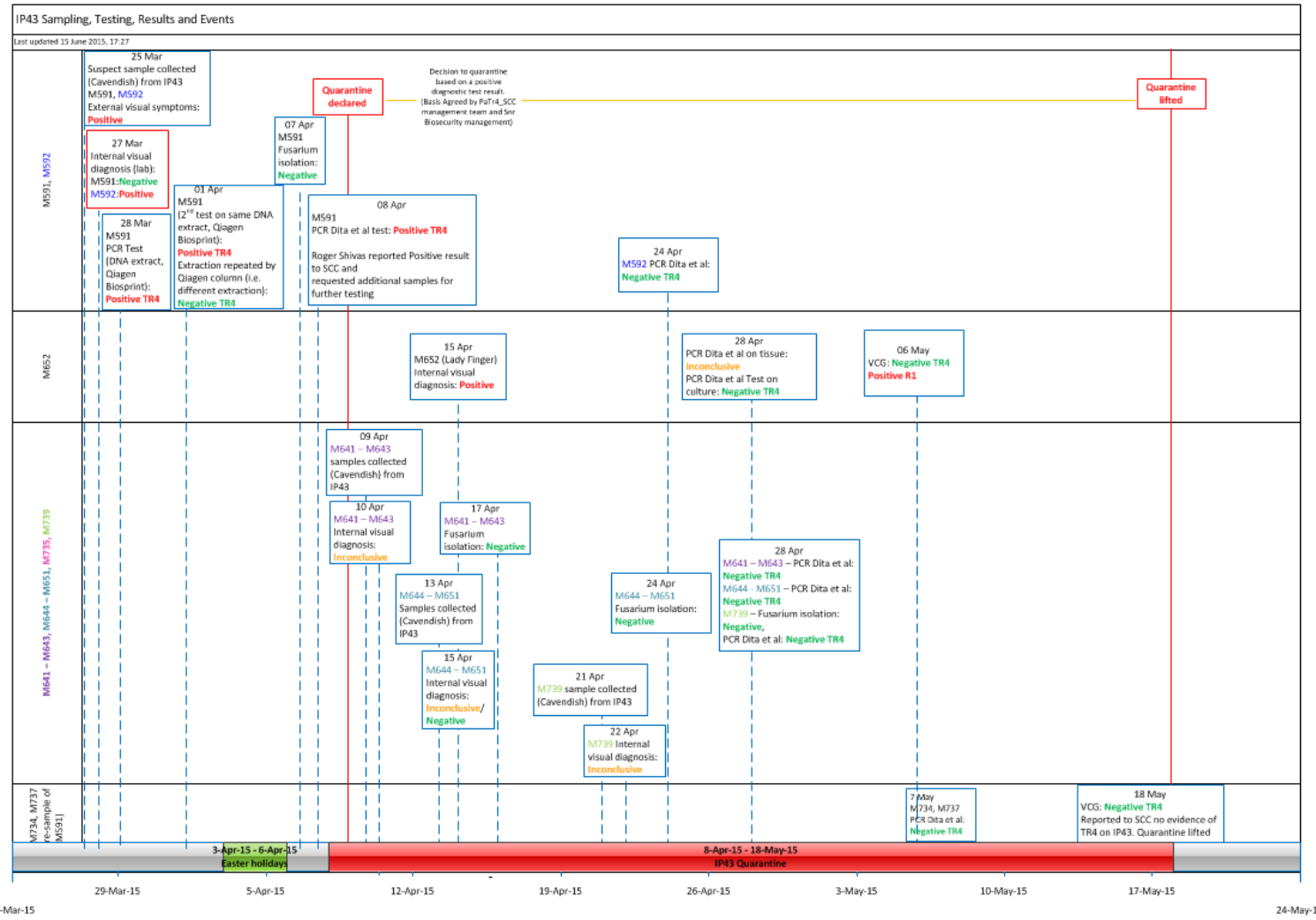
Appendix A

List of Key Personnel Interviewed

Position
General Manager Plant Biosecurity and Product Integrity, Chief Plant Health Officer, Department of Agriculture and Fisheries (DAF)
Senior Research Scientist , Biosecurity Queensland (BQ)
Director State Coordination Centre, Department of Agriculture and Fisheries (DAF)
Research Fellow (Banana Diagnostics) Queensland Alliance for Agriculture and Food Innovation (QAAFI)
Executive Director Regions and Industry Development (acting Chief Biosecurity Officer), Department of Agriculture and Fisheries (DAF)
Plant Pathologist, Agriculture Science Queensland (ASQ)
Planning Manager State Coordination Centre, Biosecurity Queensland (BQ)
Principal Plant Pathologist, Biosecurity Queensland (BQ)
Chief Biosecurity Officer, Department of Agriculture and Fisheries (DAF)
Acting Director, Animal Biosecurity Science

Appendix B

Event Timeline of End to End Process



R1 - Panama disease Race 1 – infects ladyfinger banana

Appendix C

Independent Technical Expert Assessment Report Conducted by Dr Edwards and Dr Dinh, 15 June 2015 (attached overleaf)

Department of Economic Development, Jobs, Transport & Resources

Crop Health Services
Agribio, Centre for AgriBioscience
5 Ring Road
La Trobe University
Bundoora VIC 3083

Expert assessment of the quality aspects of laboratory procedures and of the appropriateness of methodologies used to analyse samples relating to 43IP for Banana Panama Disease Tropical Race 4

Conducted by Drs Jacqueline Edwards and Quang Dinh

Crop Health Services, Victorian Department of Economic Development, Jobs, Transport & Resources, AgriBio, 5 Ring Road, Bundoora, VictoriaSR4

Terms of reference

- Review standards and procedures at Biosecurity Queensland QC2 and QC3 laboratories based at the Ecosciences Precinct Brisbane, and assess these against relevant national and/or international standards.
- Assess the adequacy of the assay methodologies used to diagnose the presence of *Fusarium oxysporum* f. sp. *cubense* in samples collected from the property identified as 43IP and indicate whether any technical issues impacted the accuracy / validity of results.
- Prepare a report detailing the scope of the review, findings and recommendations in relation to both the assessment of procedures and assay methodologies used.

State Government Victoria

Expert assessment of the quality aspects of laboratory procedures and of the appropriateness of methodologies used to analyse samples relating to 43IP for Banana Panama Disease Tropical Race 4

Conducted by Drs Jacqueline Edwards and Quang Dinh

Crop Health Services, Victorian Department of Economic Development, Jobs, Transport & Resources, AgriBio, 5 Ring Road, Bundoora, VictoriaSR4

Laboratory Visited:

Ecosciences Building, 41 Boggo Road, Dutton Park, Brisbane, Queensland, on 15 & 16 June 2015

Staff interviewed:

Dr Roger Shivas and Dr Dean Beasley, Biosecurity Queensland (BQ), Department of Agriculture and Fisheries (DAF), Brisbane

Wayne O'Neill, Agri-Science Queensland (ASQ), DAF, Brisbane

Dr Juliane Henderson, Queensland Alliance for Agriculture and Food Innovation (QAAFI), University of Queensland, Brisbane

Background information on Panama disease

Panama disease of banana , also known as fusarium wilt, is one of the most serious diseases of banana worldwide. Symptoms begin with yellowing and wilting of the older leaves, progressing to the younger leaves until the death of the entire plant. The disease is caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (*Foc*), and is a typical vascular disease causing disruption of water translocation. Therefore, internal symptoms are very important for diagnosis and are characterised by reddish-brown discolouration of the vascular tissues.

Fusarium oxysporum is regarded as a complex of cryptic fungal species with both pathogenic and non-pathogenic strains (Fourie *et al.* 2011). All of them are morphologically similar and cannot be distinguished visually. The more than 150 plant pathogenic forms of *F. oxysporum*, called formae speciales (f.sp.), have been separated on the basis of the hosts in which they cause fusarium wilt, such as tomato, carnation, melon, chickpea, banana and so on. *Fusarium oxysporum* f. sp. *cubense* (*Foc*) is the form that causes disease in banana and its close relatives. However, *Foc* is further subdivided into four races, based on which types of banana and its relatives succumb to infection and disease. All four *Foc* races are present in Australia and the history of distribution of these races in

State Government Victoria

Appendix C

Independent Technical Expert Assessment Report Conducted by Dr Edwards and Dr Dinh, 15 June 2015 (attached overleaf)

Australia is very well documented (Bentley *et al*. 2006). The important economic cultivars of banana in Australia, the Cavendish cultivars, are resistant to Races 1, 2 and 3, and susceptible to Race 4. Race 4 has been even further subdivided into isolates that are found in subtropical climates (subtropical race 4, SR4) and isolates found in tropical climates (tropical race 4, TR4).

- Race 1 infects Lady Finger, Sugar and Ducasse bananas, but not Cavendish bananas.

- Race 2 infects Plantain.

- Race 3 infects *Heliconia*.

- Subtropical Race 4 (ST4) infects all varieties of bananas including Cavendish but only in subtropical regions. This pathogen has been under quarantine control in south east Queensland, northern New South Wales and Western Australia.

- Tropical Race 4 (TR4) infects all varieties of bananas including Cavendish in both subtropical and tropical regions. This pathogen has been under strict quarantine control in Darwin. In Queensland, the infected property in Tully is also under strict quarantine control.

Foc TR4 is regarded as one of the most serious threats to banana production because all cultivars are susceptible, and the major production regions are situated in the tropics. To date in Australia, the economically important banana growing regions of tropical northern Queensland have remained free of *Foc* TR4 until this current detection.

Until five years ago, Queensland had a specialist *Fusarium* laboratory, actively involved in contingency planning and diagnostic development for exotic banana *Fusarium* pathogens. As a consequence, the full VCG tester set was stored on site and able to be resurrected for use. Without this, the testers would have needed to be obtained from overseas, which would have caused considerable delay. World-recognised *Foc* experts, such as Ken Pegg and Suzy Perry (nee Bentley), were available for consultation and advice.

Panama disease diagnostics

Conventional diagnosis of the disease is based on observation of the vascular discolouration and isolation from this tissue onto Nash-Snyder Fusarium-specific medium to confirm *Fusarium oxysporum*.

Given the importance of *Foc* TR4 and the inability to distinguish *F. oxysporum* forms and races by morphology, there have been several methods developed and published over the past few decades in order to differentiate *Foc* TR4 from other strains of *Foc* (summarised in Fourie et al. 2011).

The most reliable is the vegetative compatibility group (VCG) method. This is laborious and time-consuming, with turn-around times usually 6 weeks or longer (six months reported in one instance in Dita *et al.* 2010). It relies on developing mutants of the unknown isolate, then growing these on culture media with known VCG testers to observe the reaction.

More rapid diagnostic methods have been developed based on molecular testing (Bentley *et al*. 2006, Dita *et al.* 2010/2011, Fraser-Smith *et al.* 2014). The Dita *et al*. (2010/11) method published in the internationally recognised journal, *Plant Pathology*, is a molecular (PCR) diagnosis of *Foc* TR4 based on two single nucleotide position (SNP) differences in the specific IGS gene region of the pathogen. Dita *et al*. (2010) tested 82 isolates of *Foc* worldwide and suggested that the PCR test was specific enough to differentiate *Foc* TR4 from the others. Turnaround time was quick, between 6 to 48 hours. This protocol is currently being used in two eradication programs currently underway: Mozambique and Jordan (Perez-Vicente *et al.* 2014). The Fraser-Smith *et al*. (2014) method, also published in *Plant Pathology*, describes a new PCR and sequencing approach using *Foc-SIX8* genes to differentiate *Foc* TR4 from race 1, race 2 and SR4. There is, however, lack of information of this method being applied anywhere in the world.

There are no international or national diagnostic protocols for *Foc* TR4 published and ratified (International Plant Protection Convention, https://www.ippc.int/en/). However, there was a draft National Diagnostic Protocol developed and now available from Plant Health Australia (Bentley *et al.* 2006) and a Workshop Technical Manual funded by FAO (Perez-Vicente *et al.* 2014). Both of these provide detailed instructions on the published methods used to differentiate *Foc* TR4.

History of the current incursion

Queensland *Foc* diagnosis for suspect samples from Tully

In February 2015, Cavendish bananas growing on a property in the Tully region were showing wilt symptoms. An Australian Banana Growers Council inspector took samples and sent them to the Agri-Science Queensland laboratory in Mareeba for diagnosis. Diagnostician Kathy Grice isolated *Fusarium* from the samples and sent them to Dr Juliane Henderson at QAAFI. Dr Henderson's position is funded by the ABGC to provide banana disease diagnostics.

In the absence of a nationally or internationally ratified diagnostic protocol, the Dita *et al.* (2010/2011) diagnostic protocol (hereafter called the Dita method) was chosen to be the best molecular method to use. The authors of the draft National Diagnostic Protocol (Bentley *et al.* 2006) advised that their molecular methodology was outdated and was superseded by the Dita method.

The result from the Dita method was positive for *Foc* TR4 and supported by the results of the other tests prescribed in Bentley *et al*. (2006) *i.e.* extensive internal vascular discolouration, positive VCG testing and the production of volatile compounds. As required by national plant biosecurity agreements, the isolates were sent to a second laboratory for independent confirmatory testing, and Dr Lucy Tran-Nguyen, Department of Primary Industries and Fisheries, at Berrimah Farm, Darwin, confirmed that this was *Foc* TR4 using the molecular test she routinely uses for *Foc* TR4 in the NT. We are not sure which method she uses.

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Delimitation surveillance was carried out. Most of the subsequent suspect samples from this infected property (e.g. M118-22, M549 and M551-4) tested (on 12, 13 and 16 March 2015, on 25 and 26 March 2015, respectively) positive for *Foc* TR4 by all methods. These results are not under question.

Unexpectedly, some samples which tested positive for *Foc* TR4 by the Dita method gave negative results for the VCG tests. For example, samples M509 and M544 tested positive for *Foc* TR4 by the Dita method, but were demonstrated to be *F. oxysporum* but NOT f. sp. *cubense* by VCGs. Sample M574 tested positive for *Foc* TR4 by the Dita method, but confirmed as *Foc* Race 1 by VCGs. These results occurred after the suspect Mareeba sample (M591) had been tested, providing supporting evidence that the method was not sufficiently robust.

Queensland *Foc* diagnosis for suspect samples from 43IP Mareeba (a property 'linked' to the Tully IP)

On the 27th March, a suspect banana sample (M591) from Mareeba was received by Drs Roger Shivas and Dean Beasley at BQ DAF, Brisbane. The plant was wilting, older leaves were necrotic, the younger leaves were chlorotic, and the new leaves were intact. In the QC3 laboratory, however, obvious internal vascular discolouration was not observed. Plant tissues were taken for fungal culturing and DNA extraction. No fungus was recovered from the plant tissues, therefore no VCG test was performed and no culture was sent to a second laboratory for confirmation. DNA extraction from internal plant tissues, however, gave a positive result for *Foc* TR4 when Dr Juliane Henderson applied the Dita method. Sequencing of the cloned PCR products did not provide any useful results. Subsequent retesting of the extracted DNA gave a positive result every time, using the Dita method. The remaining tissues of M591 were repeatedly sampled and tested, but all of these were negative.

Sample M591 was taken from the upper portion of the plant, and subsequent samples were requested to be taken from the lower portions of the plant (*Foc* infects via the roots of the plant and invades upwards through the vascular system).

Subsequent samples (M592) were sent to the lab, resulting in 14 kg of corm being comprehensively examined and sampled. The diagnosticians reported that internal symptoms, when present, were still not characteristic of the disease, but they were able to isolate some *Fusarium* cultures from a portion of soft rot for further testing. These *Fusarium* isolates tested negative for *Foc* TR4 by both the Dita method and by VCG testing, and were concluded not to be *Foc*.

A total of 18 samples were tested from the suspect property. One of these was M625 from a Lady Finger banana plant growing on a different block to M591. Duplicate testing of plant tissue DNA from M625 gave one positive and one negative result for *Foc* TR4 using the Dita method, but the isolated fungus was identified as *Foc* Race 1 by VCGs. All other samples tested negative.

By this stage, the team had lost confidence in the Dita method. Several Tully samples (M509, M544, M574, M575 and M590) also gave positive results by the Dita method that were not supported by

the other tests, or were not able to be repeated in subsequent testing. There was considerable evidence to show that the Dita method was cross-reacting with something other than *Foc* TR4 and therefore unreliable. The team followed legal advice to stop using the protocol.

Laboratory processes and workflow.

On 15th and 16th June 2015, myself and my colleague (Dr Quang Dinh) from Crop Health Services visited the laboratories at the Ecosciences Building Precinct on Boggo Road, Dutton Park, Brisbane where the samples were diagnosed. We met the staff involved in the diagnoses, interviewed them and viewed the laboratories, the processes and workflow for dealing with the samples, and the lab books and database entries.

The response to *Foc* suspect samples relies on facilities and staff collaboration of AgriScience Queensland (ASQ) and Biosecurity Queensland (BQ) from the Department of Agriculture and Fisheries (DAF), and Queensland Alliance for Agriculture and Food Innovation (QAAFI) of the University of Queensland. In brief, suspect *Foc* infected plant samples collected by BQ are processed in the QC3 laboratory of BQ. Plant tissues are subjected to: 1, fungal isolation for pure culture *Foc* and 2, DNA extraction. Fungal cultures were used for VCG tests by ASQ in an adjunct shared PC1 laboratory. DNA extracts were subjected to PCR tests in an adjunct QC2/PC2 laboratory of QAAFI.

All of the staff involved are highly experienced and have had extensive training and experience in these methods. All have up to date Quarantine Approved Premises Training (Commonwealth Department of Agriculture) and the laboratories have AQIS accreditation and are regularly audited.

The workflow for the samples was divided up among them according to their facilities and expertise.

The samples were collected in the field by experienced BQ field staff. Samples were double bagged and tagged and were transferred to BQ facility in Brisbane in sealed carton boxes, along with detailed paper work recording property number, plant number, case number, geographical ID, collector's name and signature.

On arrival, samples were received by Drs Beasley and Shivas and moved into QC3 laboratory. Samples were allocated with specific barcoding numbers on stickers. Stickers were placed on the paper work.

Samples were opened individually, inspected, dissected and tissues removed aseptically for fungal isolations, volatile production tests and for DNA extractions. Between procedures, ethanol and / or chlorine disinfection / sterilisation is used for knives, cutting boards and scalpels.

Plant tissues were plated onto Fusarium selective media (Nash Snyder PDA) in 9 cm diameter Petri plates. Plates were individually sealed with Parafilm, stored in sealed plastic boxes and kept in a closed cupboard under the bench.

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Due diligence and care is taken to ensure there is no opportunity for samples to be mixed. Fungal culturing was performed within the QC3 laboratory. For each fungus that grew out of the isolations, single spore cultures were made following routine laboratory procedures.

Pure fungal cultures were grown for DNA extraction and molecular testing, and stored in glycerine at -80°C in the reference collection. The isolates were also subcultured onto the media used to produce nit mutants for VCG testing, and into rice culture flasks for volatile production.

The nit mutants that were produced were transferred into a shared PC1 laboratory, also adjacent, for testing with the VCG tester for *Foc* TR4. This testing was conducted by Wayne O'Neill, AgriSciences, DAF.

The DNA extraction protocol was commenced in the QC3 laboratory. Fungal / plant tissues were lysed in 2 ml Eppendorf tubes with tungsten balls using a Tissue Lyser. Once the plant tissue or fungal mycelium was in lysis buffer, the samples were taken into the QAAFI QC2/PC2 laboratory for the rest of the steps and subsequent molecular testing by Dr Henderson.

In the QC2 facility, DNA extraction was performed in a separate small equipment room using a BioSprint machine using magnetic-based extraction and / or using normal Qiagen Plant Mini Kit extraction. All the molecular test preparation took place in a separate small dedicated PCR prep laboratory where no DNA is ever taken to prevent any stray DNA contamination via aerosols, etc. The final step of adding DNA templates took place on the benches in the main QC2 laboratory area.

The Dita *et al.* (2010) protocol was run using the short version of *Foc*TR4-R (reverse) primer at 60°C annealing temperature. This temperature allows the plant DNA positive control primers for actin to work in the duplex PCR reactions. All sample templates were duplicated for all PCRs and positive and negative controls used. PCR products were stained and run on gel for positive band visualisation and photos.

All waste was collected in yellow quarantine bags for autoclaving before going to clinical waste incineration.

Quality control

Throughout the process, we looked for any evidence of potential cross-contamination of the positive Mareeba sample through contact with the Tully positive samples. There was no evidence that this occurred.

- The facilities are quarantine approved premises (QAP) including QC3 and QC2/PC2 laboratories.
- All staff have current QAP accreditation.

- There was no possibility of cross contamination between the samples: 1, field collection, sample packaging and transportation, sample processing and laboratory activities followed proper procedures; 2, the Mareeba samples (M591, M592) were handled on different days to the Tully samples.
- DNA extraction kits were of high quality, well-recognised brands and newly purchased
- PCR primers and reagents were kept frozen properly in a dedicated 'PCR setup' room. Preparation of master mixes were done in this room to avoid contamination. No DNA products / by-products were allowed to enter the room.
- PCR machines were used following a booking system. There was no reason to suspect interference in the PCR cycles by other staff.

As soon as the Mareeba positive result occurred, many steps were taken to try and repeat this. DNA was re-extracted from the tissues several times, using different extraction methods and different technical staff. The PCR reactions were set up by different staff. All these gave negative results. The positive result came from plant tissue; there were no associated fungal isolations. Therefore, no isolate was sent to a second lab for confirmatory testing.

Documentation of all procedures was excellent.

Subsequent extensive testing of the Dita *et al.* (2010/11) method and other methods (Fraser-Smith *et al.* 2014, Yang *et al.* 2015) by Dr Henderson on other samples demonstrated that none of them are stringent enough to give consistent results. All have subsequently given false positive results for *Foc* TR4 on isolates known to be *Foc* Race 1, Race 2 and SR4.

Recommendations for system improvement

At the time of the incursion, the response was seriously understaffed. All four staff were required to work very long hours (often 12 hour days and weekends) to keep up with the samples. Drs Shivas and Beasley had to do all the QC3 processing, laboratory management, databasing, documentation and reporting, with no back-filling of their existing duties. We understand this has now been temporarily addressed with six new staff employed to assist until November. However, at the time, there was the potential for error to be caused by staff tiredness and overload.

Although the QC3 laboratory is dedicated for biosecurity work and all users are experienced in working under emergency response conditions, the QAFFI UQ laboratory is a facility shared with other university teams including PhD students, international interns and research project staff. All of the equipment is shared, and most has been purchased from project funds of other teams. Bench space is at a premium, with different teams allocated different benches. Although in this instance there is no reason to believe that this caused the false positive result, it could be an issue in the future. Under incursion scenarios, it is preferable to have dedicated facilities and equipment for use

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during the incursion response to eliminate the chance of tampering. Again, some of this has been recognised and new equipment has been purchased for use by Dr Henderson and her team.

The fact that different steps in the process are undertaken by different agencies means that there is the potential for Biosecurity Queensland to be left out of the loop. It relies on the diligence of the staff to know their reporting responsibilities, their networking skills and goodwill to communicate and co-operate with each other. In this instance this worked smoothly as these staff have a long history of working together. This may not be the case with a new set of staff. Although Biosecurity staff are well versed in the processes and reporting requirements during incursion response, university staff and students are often not aware of these at all.

Sample tracking throughout the complete workflow was made difficult because of samples passing through the different agencies involved. A sheet of sticky barcodes so that every 'child' of the parent sample is given the same barcode sticker, rather than requiring hand labelling, and worksheets that travel with the samples throughout the complete process, are simple improvements that can be made. Each agency and laboratory had their own lab books and note taking, which was all communicated to Dr Beasley via email for him to enter into the official database. Many steps relied on hand-written transfer of labels, etc. We observed some minor inconsistencies in record-keeping that can be improved in future. For example, there were no records of kit batch numbers that were used or records of freeze-thaw events (we were informed this has now been addressed). Methodologies and equipment used for procedures such as DNA extraction were not recorded on the database.

Routine QA processes such as regular servicing and recalibration of pipettes, equipment, etc. are not performed or recorded. This is another area for improvement.

Conclusion

Our conclusion is that the molecular-based diagnostic protocols published for *Foc* TR4 are not robust enough and failed during this current incursion response. The Dita *et al.* (2010/2011) protocol was demonstrated to be not selective enough to test for *Foc* TR4 in Australia. There was no evidence that this false positive was caused by human error or poor laboratory practice.

The use of molecular-based diagnostic tests to discriminate races of pathogens, as opposed to species identification, is still in its infancy. In the case of *Fusarium,* there is recent evidence published in *Nature* that the genes controlling pathogenicity are on mobile chromosomes that can be transferred between different isolates, and that non-pathogenic strains can acquire pathogenicity (Ma *et al.* 2010). This makes development of a molecular test that is specific for a pathogenic race more complex, and helps explain why it has been so difficult to accomplish. The Dita primers were designed before the Ma paper was published and are based on regions of a gene that is not involved with pathogenicity. Over the past two months, Dr Henderson has also tested two recently published protocols (Fraser-Smith *et al.* 2014, Yang *et al.* 2015) that also claim to differentiate *Foc* TR4, one of

them (Fraser-Smith *et al.* 2014) based on the newly-described pathogenicity gene regions *(Foc SIX* genes), but she found that these also cross-reacted with other races when tested during this incursion response.

Additionally, diagnostic methods developed in other countries need to be tested and validated in the Australian environment. Australia has a unique, largely undescribed microflora and it is not uncommon for these to produce false positive results when the diagnostic protocol meets these for the first time. An important controversial case where this occurred is the Fireblight incursion in Victoria in 1997. It was ultimately determined that the molecular primers developed in Europe for *Erwinia amyvora* cross-reacted with unknown native *Erwinia* species, giving a false positive result.

In the immediate timeframe, we conclude that there is no reliable and rapid molecular method of discriminating *Foc* TR4 that can be used alone, and that best practice involves the use of other tests such as VCGs, internal symptomology and volatile production to support the molecular testing, as was done in this case.

References

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Dita MA, Waalwijk C, Buddenhagem IW, Souza MT Jr, Kema GHJ (2010) A molecular diagnostic for tropical race 4 of the banana fusarium wilt pathogen. *Plant Pathology* 59: 348-357.

Dita MA, Waalwijk C, Buddenhagem IW, Souza MT Jr, Kema GHJ (2011) Corrigendum. A molecular diagnostic for tropical race 4 of the banana fusarium wilt pathogen. *Plant Pathology* 60: 384.

Fourie G, Steenkamp ET, Ploetz RC, Gordon TR, Viljoen A (2011) Review: Current status of the taxonomic position of *Fusarium oxysporum formae specialis cubense* within the *Fusarium oxysporum* complex. *Infection, Genetics and Evolution* 11: 533-542.

Fraser-Smith S, Czislowski E, Meldrum RA, Zander M, O'Neill W, Balali GR, Aitken EAB (2014) Sequence variation in the putative effector gene *SIX*8 facilitates molecular differentiation of *Fusarium oxysporum f.sp. cubense*. *Plant Pathology* 63: 1044 – 1052.

Ma LJ, van der Does HC, Borkovich KA *et al.* (2010) Comparative genomics reveal mobile pathogenicity chromosomes in *Fusarium*. *Nature* 464:367-373.

Perez-Vicente L, Dita MA, Martinez-de la Parte E (2014). Technical Manual: Prevention and Diagnostic of Fusarium Wilt (Panama Disease) of Banana caused by *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (TR4). Prepared for the Regional Workshop of Fusarium Wilt (Panama Disease) caused by by *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (TR4): Mitigating the Threat

Appendix C

Independent Technical Expert Assessment Report Conducted by Dr Edwards and Dr Dinh, 15 June 2015 (attached overleaf)

and Preventing its Spread in the Caribbean. Food and Agriculture Organization of the United Nations, May 2014.

Yang LL, Sun LX, Ruan XL, Qiu DY, Chen DH, Cai XQ, Li HP (2015) Development of a single-tube duplex real-time fluorescence method for the rapid quantitative detection of *Fusarium oxysporum* f. sp. *cubense* race 1 (FOC1) and race 4 (FOC4) using TaqMan probes. *Crop Protection* 68:27-35.

Appendix D

List of Documents provided (attached overleaf)

Documents Received from DAF/ASQ/BQ/QAAFI, 25 May 2015

Received from David Waltisbuhl 27/05/15

SOPS

1. WI-PBDSS-01 Sample Tracking and Reporting V4.3
2. Diagnostic process diagram
3. QC2 SOP Version 2.0 (laboratory process)
4. Autoclave SOP
5. Equipment Decontamination SOP – version 2
6. QC3 SOP Version 2.3
7. Autoclave SOP
8. DRAFT PaT4 WI Data capture for diagnostic samples 20150504
9. Database user guides – User Guide-Catalogue Module-Diagnostics Tabs
10. Database user guides – User Guide-Catalogue Module-Version3
11. Database user guides – User Guide-Movements Module

Diagnostic protocols

1. Draft NPDP for Fusarium oxysporum f. sp. Cubense
2. Fusarium Wilt of Banana - Laboratory Diagnostics Manual 2006

References

1. Dita 2010 A molecular diagnostic for tropical race 4 of the banana
2. Dita 2011 Corrigendum
3. Edel 2000 Ribosomal DNA-targeted oligonucleotide probe and PCR specific to Fo
4. Fraser-Smith 2014 Sequence variation in the putative effector gene SIX8 facilitates molecular differentiation of Foc
5. O'Donnell 2009 A two-locus DNA sequence database for typing plant and human pathogens within the Fusarium oxysporum species complex

Laboratory QA

1. Proficiency Testing
    a. ANQAP Enrollment 2014 DAFF QLD
    b. ANQAP 2014 Samples
    c. ANQAP 2014 Results
    d. Lab 75 NPHPTP Fungi report 2014
    e. Lab 75 NPHPTP Insect Report 2014
    f. Lab 75 NPHPTP Nematode report 2014
2. Q2271 QC3
        a. Audits
            i. DA Audit 20140516
            ii. Internal audit 20140814

Appendix D

List of Documents provided (attached overleaf)

b. Certification
   i. OGTR – Dean Beasley – 06 Aug 2013
   ii. OGTR – Julianne Henderson – 28 Jan 2015
   iii. OGTR – Roger Shivas – 18 Aug 2014
   iv. OGTR – Wayne Oneill – 16 Jul 2013

3. Q2272 QC2
   a. Audits
      i. DA Audit 20140516
      ii. Internal audit 20140826
   b. Certification
      i. OGTR – Dean Beasley - 20130821
      ii. OGTR – Julianne Henderson – 20150127
      iii. OGTR – Roger Shivas – 20140124
      iv. OGTR – Wayne O'Neill – 20150227

ASQ VSG Testing

1. VGC Testing Foc
2. VGC Testing results Table
3. Wayne Fusarium Training
4. Wayne Lab Book
5. Wayne emails
   a. Sent 1IP
   b. Sent 43IP
   c. Sent Other
   d. Received 1IP
   e. Received 43IP
   f. Received Other

Reporting Process

1. IP43
   a. IP43 Reporting
1. Distribution policy
   a. Draft Policy TR4 Distribution of Laboratory Results 20150324

Received from Julianne Henderson 29/05/15

Email Correspondence Julianne Henderson

1. Confidential
2. Diagnostic results for IP43
3. Further results for samples with reference numbers M418-M422 inclusive
4. FWM624
5. FW Molecular results for M509 and M544

Appendix D

List of Documents provided (attached overleaf)

6. FW- PCR results at 08.04.15
7. Molecular diagnostic results for M652
8. Molecular results01.05.15
9. Molecular results for M509 and M544
10. Molecular results as at 22.04.15
11. Molecular results as at 29.04.15
12. PCR results as at 08.04.15
13. PCR results as at 16.04.15
14. PCR results for samples with reference numbers M549 – M565 inclusive
15. Results
16. Results for samples with reference numbers M418 – M422

Protocols J Henderson

1. Dita 2010
2. Dita Ammendum 2011
3. DNeasy-Plant-Mini-Kit--April-2012-EN[1]
4. Edel 2000
5. EN-BioSprint-DNA-Plant-Handbook
6. FAO Technical Manual
7. Final PHA Fusarium Wilt Manual Jan 07
8. Fraser Smith 2014
9. O'Donnell 1998

QC2 SOP Documents J Henderson

1. 2C west_Microwave SOP
2. 2CW SOP post staining gels with ethidium bromide
3. APPENDIX 2-1 QAP Register April 2012 APRIL
4. APPENDIX 2-2 AQIS QAP Training Record
5. APPENDIX 3 AQIS imported goods pathway for Foc
6. APPENDIX 3 AQIS imported goods pathway LJS
7. APPENDIX 3 AQIS imported goods pathway Tree Pathology Group - draft 1
8. APPENDIX 3 AQIS pathway for Virology samples 29april2012 (2)
9. APPENDIX 4 ESP QC2 Checklist ver3
10. APPENDIX 5 Part 1 Quarantine Collection and Decontamination SOP
11. APPENDIX 5 Part 2 001 Autoclave 1 Dirty Biological Test
12. APPENDIX 5 Part 3 002 Autoclave 2 Clean Biological Test
13. APPENDIX 5 Part 4 003 Autoclave 4 Media Biological Test
14. APPENDIX 5 Part 5 Quarantine Log BR 15-4-11 to 19-3-12_0001
15. APPENDIX 6 QAP Q2144 Quarantine Waste Material Disposal Register
16. APPENDIX 7 AQIS approved disinfectants
17. Balances_SOP
18. Q2144 SOP Version Control Submitted to AQIS 1 May
19. Transilluminator

Appendix D

List of Documents provided (attached overleaf)

J Henderson Laboratory notes

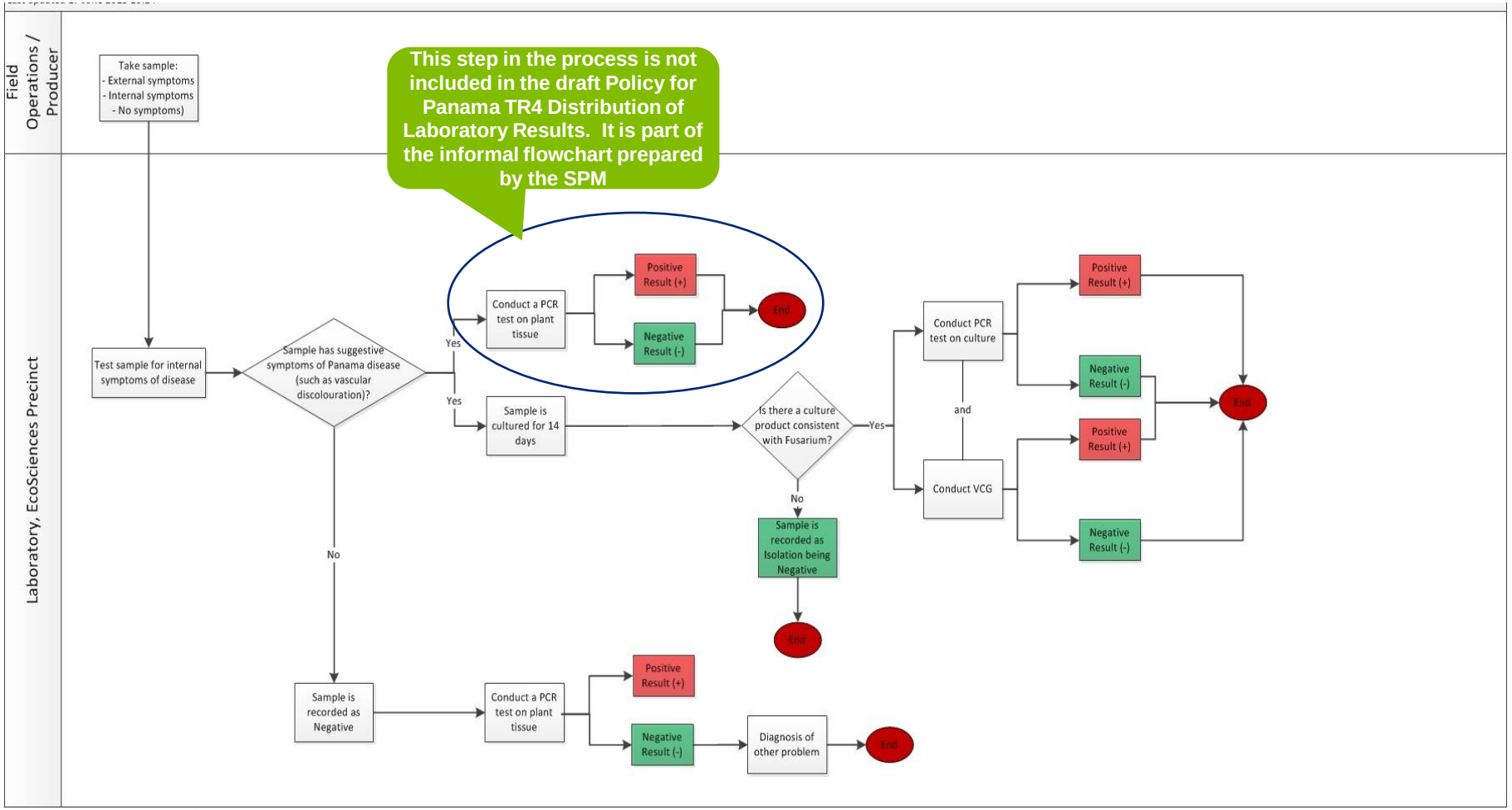
1. Laboratory book 61 2015
2. Laboratory book 62 2015
3. Laboratory book 63 2015
4. Laboratory book 68 2015
5. Laboratory book 69 2015

Qualifications J Henderson

1. KSU Fusarium Workshop 2013 J Henderson
2. OGTR Statement of Completion 2015
3. PCR Proficiency Testing 2007 J Henderson
4. QAPAP Reaccreditation J Henderson 2015

Appendix E

Process Map of Diagnostic Sampling as per the Draft Policy for Panama TR4 Distribution of Laboratory Results



Appendix F

Screenshot of Email from Dr Henderson to De Beasley and Dr Shivas, 08 April 2015

From: HENDERSON Juliane
Sent: Wednesday, 8 April 2015 8:20 AM
To: BEASLEY Dean
Cc: SHIVAS Roger G
Subject: PCR Results as at 08.04.15

Hi Dean,

Please find below, a table summary of the latest finalised PCR results.

Two samples, M566 and M567, have tested positive for TR4 using the Dita TR4/Banana Actin multiplex PCR on fresh pseudostem material. I do not have a record of cultures having been extracted and PCR tested for these samples. Similarly for samples M549, M551, M553, M554 and M555 (reported 26.03.15), I have no record of culture DNA being extracted and tested.

On 26.03.15 I reported the following samples as negative: M550, M552, M560, M561, M564. I have since decided to further test these samples with nested PCR – results to follow.

Finally, sample M591 has tested positive twice by PCR (28.03.15 and 01.04.15) on one DNA extract as prepared by Biosprint extraction. Repeat extraction by Qiagen column (Plant DNeasy Mini Kit) did not produce a TR4 positive, indicating that sampling may be an issue (ie. possibly the amount of infected tissue in whole sample was small). This is supported by the inability to culture from this sample. Ideally, sample M591 should be recollected for re-testing.

Please let me know if you have any questions relating to the results.

Appendix G

Screenshot of Diagnostic Report Authorised by Dr Shivas, 08 April 2015

Plant Biosecurity - Diagnostic Report

Queensland Government

Sample number: TR4-30-1-1

Submitted by: McDougall, G.
Received by: Beasley, D.R. (27 Mar 2015)

Reason for diagnostic tests: Suspect Foc TR4

Job number: M591
Job status: Complete

Host: Musa (Banana) 'Cavendish'
Sample type: Pseudostem
Locality: Block 5, 5455 Kennedy Highway, Mareeba, Queensland
Latitude: -17.06150
Longitude: 145.43915

Tests details

Date	Test type	Organism name	Result	Diagnostician
27 Mar 2015	Visual diagnosis	Fusarium sp.	Negative	Beasley, D.R.
07 Apr 2015	Isolation	Fusarium sp.	Negative	Beasley, D.R.
08 Apr 2015	PCR	Foc TR4	Positive	Henderson, J.

Diagnosis and comments

No evidence of vascular discolouration. Isolation plates negative for Fusarium sp.
Host tissue positive for Foc TR4 by PCR.

Samples have been tested directly on freshly excised vascular tissue using the multiplex PCR method of Dita et al. (2010, 2011). This assay detects Foc Tropical Race 4 and incorporates the banana actin gene as an internal control.

Authorised
Roger Shivas
08 Apr 2015

Note: The purpose of this notice is to inform you of the results of testing done by the Department of Agriculture and Fisheries on sample(s) submitted. This is not a certificate for the purpose of section 19A of the *Plant Protection Act 1989*. The State of Queensland does not warrant, guarantee or make any representations about the accuracy, reliability or currency of any information in this report and will not be held liable for any loss or damage suffered by any person using or relying on any information in this notice. Test results and findings may be provided to authorised staff and used for statistical, surveillance, certification and regulatory purposes. The source of the information will remain confidential unless otherwise required by law or regulatory policies

08 Apr 2015 Page 1 of 1

Appendix H

Screenshot of Email from Dr Beasley to the SCC, 08 April 2015

From: PaT4_SCC_Diagnostics Coordinator
Sent: Wednesday, 8 April 2015 9:06 AM
To: ASHTON Mike; PaT4_SCC_Director
Subject: Suspect sample from Mareeba - TR4-30
Attachments: DiagnosticReport-TR4-30.pdf; PositiveDiagnosticSamplesTR4-30.html

Hi Tim and Mike,

We have a suspect sample collected from Mareeba on the 26 Mar 2015.

This sample has given a positive PCR result, but we did not see any internal visual symptoms and were not able to isolate Fusarium in culture.
Suggest that additional sampling will be required from this location.

Regards
Dean

Dr Dean Beasley
Senior Research Scientist
Biosecurity Queensland
Department of **Agriculture and Fisheries**

Telephone 07 3255 4369 Facsimile 07 3844 4529
Email dean.beasley@daf.qld.gov.au
Website www.daff.qld.gov.au **Call Centre 13 25 23**

Appendix I

Summary of Finalised Sample Results from 43IP, 18 May 2015

17 samples out of 18* displayed inconclusive or atypical internal vascular discolouration

15 samples out of 18* produced a negative result to *Foc* TR4 by Dita *et al PCR* (tissue)

15 samples out of 18* were unable to grow a culture

Final diagnostic test results for Cavendish and Lady Finger bananas on IP43 on 18 May 2015 (results at hand on 08 Apr 2015 shaded in yellow)

Test	Samples from 43IP M591[a]	M652[b]	M734[c,e]	M737[c,e]	Other [c,f]
Field symptoms					
External - Wilt, yellowing	+	+	-	-	+/-
Internal - Vascular discolouration	-	+	-	-	+/-
Laboratory examination					
Internal - Vascular discolouration	-	+	+[d]	+[d]	+[d]
Fungal morphology					
Culture of *Fusarium oxysporum* or *Fusarium oxysporum*-like[e]	-	+	+	+	-
Molecular tests - tissue					
PCR Dita *et al.* (2010, 2011)	+	+/-	-	-	+ (1)/ - (13)
IGS sequencing of PCR product O'Donnell *et al.* (2009)	+				
Molecular tests – fungal culture					
PCR Dita *et al.* (2010, 2011)	n/a	-	-	-	n/a
SIX gene PCR Fraser-Smith *et al.* (2014)	n/a	-	-	-	n/a
Next gen sequencing (MiSeq protocol)	n/a				n/a
IGS sequencing O'Donnell *et al.* (2009)	n/a				n/a
Biological test					
VCG test	n/a	Race 1 VCG 0124	-	-	n/a

[a] banana cultivar Cavendish
[b] banana cultivar Lady Finger
[c] resample of M591
[d] atypical discolouration, often seen in association with bacterial soft rot
[e] diagnostic characters are microconidia formed in false heads on short monophialides
[f] M592, M641 – 651, M735, M739
+/- mixed result, both positive and negative results were obtained
n/a not applicable (as cultures not obtained)

M734 and M737, which were resamples of M591, were the only samples of Cavendish banana to produce a culture. The culture and tissue both tested negative to *Foc* TR4 by the Dita PCR and VCG tests

Summary of Sample Results

The initial positive Dita PCR result from the tissue sample M591 reported on 08 April could not be confirmed by other tests in subsequent samples from the property.

* Note that sample M652 is from the Ladyfinger banana. The sample displayed internal vascular discolouration and was able to grow the Foc culture. The sample produced a negative result to *Foc* TR4 by the Dita *et al PCR* test and a positive result to Race 1 by the VCG test.

Appendix J

Screenshot of Email from Dr Shivas to the SCC, 18 May 2015

From: SHIVAS Roger G
Sent: Monday, 18 May 2015 2:32 PM
To: ASHTON Mike
Cc: LETTS Malcolm; FARRY Tim
Subject: IP43 diagnostics
Attachments: IP43 Diagnostics.docx

Mike

The diagnostic tests for all 18 samples collected from IP43 at Mareeba have concluded and results are shown in the attachment. The initial positive PCR result from a tissue sample (M591) reported on 8 April, has not been confirmed by other tests in subsequent samples from the property, including from the basal corm of the same plant. In the course of diagnostic investigations since then, the following points are relevant

- The diagnostic PCR test (Dita et al. 2010, 2011) used to sample banana tissue samples is an internationally recognised and published protocol.
- UQ and BQ scientists have subsequently obtained evidence that the Dita PCR test is unreliable as it reacts positively with isolates of *Fusarium oxysporum* f. sp. *cubense* that are not Tropical race 4 (TR4). Further the Dita PCR test has sometimes reacted positively with symptomless banana tissue samples.
- The demonstrated unreliability of the Dita PCR test castes doubt over the positive PCR test for M591. Extensive retesting and resampling (attachment) has provided no further evidence that TR4 is present on IP43.
- In particular, the absence of characteristic vascular discoloration in the basal corm of sample M591 is strong evidence that TR4 is not present. The fungus *Fusarium oxysporum* f. sp. *cubense* is a soil-borne pathogen that invades a banana plant via the roots, moving into the vascular system (xylem), through the corm and up into the pseudostem. Disease symptoms are always accompanied by vascular discoloration in the xylem, initially in the roots and moving continuously through the corm to the pseudostem. Symptomless infection of banana by TR4 is not known to occur.
- Changes to the diagnostic protocol have been proposed (Memorandum submitted to you last Friday). These changes recognise that vegetative compatibility group (VCG) analysis is the most reliable way to identify TR4, supported by morphology and other molecular tests.
- The development of a reliable PCR or other molecular test for the detection of TR4 in banana tissue, soil and water is urgently needed to provide effective management and containment of this fungus.

In summary, there is no reliable evidence to show that TR4 is present on IP43.

Cheers, Roger

Dr Roger Shivas
Principal Plant Pathologist
Biosecurity Queensland
Department of Agriculture and Fisheries
GPO Box 267, Brisbane, Qld 4001

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