Hort Innovation

Final Report

Integrated Management of Diseases in Macadamia Industry

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Content

Summary

Husk spot, caused by the fungal pathogen *Pseudocercospora macadamiae* and Phytophthora root rot, caused by the soilborne pathogen *Phytophthora cinnamomi,* were identified as the major constraints contributing to significant crop losses and downgrading of macadamia produce in Australia. The loss in yield reported by macadamia growers when either of these two major diseases is uncontrolled ranges from 30 to 100%, with the majority of growers surveyed reporting losses of 75 to 80%. Therefore, this project was established to deliver an improved, sustainable and proficient disease management strategy for the Australian macadamia industry.

Through extensive research trials under commercial orchard conditions and an active extension program, the project has delivered key outcomes for growers and macadamia industry stakeholders. The adoption of an integrated management system for husk spot disease that extends from risk assessment, cultural practices and applications of crop protection products to the use of resistant varieties has saved the Australian macadamia industry from significant economic loss. The management system of Phytophthora diseases in macadamias, through targeted application of crop protection products and increased focus on soil health has improved productivity in the Australian macadamia industry by an estimated value of over $20 million annually. Working in close collaboration with growers and industry stakeholders including other industry research projects, industry crop consultants, and the peak industry body (Australian Macadamia Society), this project has delivered a range of key outputs and major outcomes including:

- Provided a detailed assessment of the level of susceptibility of commercial macadamia varieties to husk spot and Phytophthora tree decline. This information will assist growers in management decisions and for new plantings. In close cooperation with the macadamia breeding program, the project provided a scientific basis for the selection of disease resistant materials.
- Developed and provided information on effective cultural practices to break the cycle of husk spot disease and reduce the economic impact of this disease.
- Developed practical self-assessment decision support systems for risk assessment for growers to diagnose their level of risk for subsequent disease management decisions for husk spot and Phytophthora diseases.
- Identified a suitable alternative to carbendazim-based fungicides and assisted in the registration of a new crop protection product for husk spot control in macadamia.
- Demonstrated improved management system for Phytophthora root rot and slow tree decline through increased focus on soil health practices. This should reduce reliance on chemical applications.
- Provided efficacy data and assisted in the permit application for trunk spray applications of phosphonates for improved Phytophthora management.
- In collaboration with the industry MacSmart project, developed an information platform to support the adoption of a holistic management system for Phytophthora diseases.
- Provided rapid diagnosis of new and emerging macadamia disease issues that reinforced management decisions of a new significant endemic disease that affect flowering.

Further research and development of cultural management methods and improved decision support systems to manage emerging diseases is required for the macadamia industry to maintain its upward trajectory in developing and implementing integrated disease management systems. Investment in a future disease management project is required to expand and maintain a comprehensive and proactive or preventative disease management approach for all pathogens in macadamia crops from the nursery to postharvest.

Keywords

Branch dieback; flower blight; husk rot; husk spot; Phytophthora.

Introduction

Globally, pathogens are a significant constraint to macadamia productivity. In Australia, husk spot caused by the fungal pathogen, *Pseudocercospora macadamiae*, and Phytophthora diseases caused by the soilborne pathogen, *Phytophthora cinnamomi*, have contributed to significant crop losses and downgrading of macadamia produce. These diseases are endemic in all commercial macadamia plantations in Australia and therefore were the major focus of the MC12007 research project. Previous macadamia industry research projects (Drenth, 2007; Akinsanmi & Drenth, 2012) have produced a strong base that led to significant gains in the efficiency of chemical compounds used to control these diseases, through improved timing of application, spray coverage and volume, rates and the number of applications. However, an improved integrated management system with cultural practices, crop protection compounds and host genetics that is efficient and cost effective to manage these diseases is required.

The main objective of this project was to work with growers and industry stakeholders to develop disease management methods that are efficient and cost effective. With an active extension and adoption program throughout the life of the project and in close collaboration with the growers and industry stakeholders, this project has delivered key outcomes, the detail of which is evidenced in the appendixes, to support disease management decisions to the Australian macadamia industry.

A limited range of fungicides is used to control diseases in the Australian macadamia industry (Akinsanmi et al., 2007). With the continued withdrawal of pesticides from horticultural industries due to health and environmental concerns, it is essential to improve pesticide use or apply products that are better streamlined to reduce off-target impact. The high risks of development of fungicide resistant fungal strains through applications of the same fungicides in commercial production system drive the search for alternative control strategies for the industry. A holistic management program that works towards not only reducing disease incidence but also prevention through research, development and education or extension (RD&E) to macadamia growers is required. Improved soil health practices in preventing Phytophthora occurrence, decision support systems and application of cultural practices to reduce pathogen inoculum in preventing husk spot severity, and early detection of new pathogens of significant importance will reduce the potential economic impact and risk to the industry.

In addition to reducing the economic impact of husk spot and Phytophthora diseases including root rot, tree decline, stem canker and bleeding, this project also examined basic information on the etiology, including the causal agents, timing of infection and conditions for disease development of emerging diseases such as husk rot and flower blights. This information will underpin the development of effective disease management options for the macadamia industry. This project also delivered plant disease diagnostic services and vigilance of pathogens of quarantine or market access concerns for the Australian macadamia industry. A high level of scientific expertise, preparedness and industry support was maintained for the detection and advice on management of any new pathogens of potential significant impact.

Methodology

The methodology used in the project covered the core and specific project activities. The methodology for the core activities was partitioned into three inter-related components that included strategic research, applied research and industry engagement activities.

Strategic research: Basic scientific studies were performed to determine the causal agents, epidemiology and biology of pathogens and host-pathogen interactions of new and emerging macadamia diseases. Consistent technical and scientific input including diagnosis, surveillance, reporting and management of issues on macadamia diseases and crop protection products was provided to the macadamia industry, throughout the life of this project.

Applied research: In order to develop a disease management system, methods used included participatory field research activities with growers and a strong focus on extension of research developments to the Australian macadamia industry stakeholders. Field trials were established in commercial farms where the participants supported the development, monitoring and evaluation of integrated disease management system including fungicide efficacy trials; cultural management systems; soil and tree health management practices to control disease incidences. Extensive field trials were established in farms in Queensland and New South Wales to manage the two major diseases (Husk spot caused by *Pseudocercospora macadamiae* and slow tree decline/Phytophthora disease complex caused by *Phytophthora* species).

Industry engagement: Strong linkages and collaborations with other research projects and the industry adoption, extension and communication projects were established that contributed to the development and evaluation of an integrated disease management system. Engagement with commercial stakeholders was maintained to gain manufacturers' support for registration of new use patterns/label of crop protection compounds. Regular contact with the growers through workshops, industry news bulletin articles, presentations at selected conferences and meetings throughout the year were performed concurrently with the research and development activities.

Methodology for specific activities for each disease management category and the associated key outputs and outcomes:

1. **Husk spot management:** The core activities of husk spot management were developing and communicating to industry a sustainable and integrated disease management strategy.

Specific Activities	Methods	Project Outputs	Project Outcomes
Review of husk spot management.	Comprehensive review of literature.	• One published article in industry journal.	• Gaps in knowledge and management practices identified.
Cultural control - mechanical removal of sticktights.	Replicated field trials using mechanical tree shakers in split plot design with and without fungicide spray applications annually for 3 years.	• One published article in peer reviewed scientific journal. • Oral presentation at the 2014 Australian Macadamia Industry Conference. • Information on conditions for putative biodegradation of macadamia husks	• Demonstrated efficacy of application of mechanical tree shaker to break disease cycle. • Harvesting based on kernel maturation rather than calendar established.
Fungicide efficacy trials.	Replicated field trials at two locations annually for 4 years based on established protocols (Akinsanmi *et al.*, 2007).	• Efficacy data generated. • Technical report on efficacy data for product registration. • Six technical reports on product efficacy to manufacturers.	• Field efficacy of 10 crop protection products tested. • Registration of new crop protection product - DuPont™ Fontelis®.
Monitoring of fungicide resistance.	Sampling of husks for tests using poison plate assay for fungal sensitivities to pyraclostrobin and carbendazim.	• Pathogen sensitivity data to pyraclostrobin and carbendazim. • 200 husk spot isolates were collected to monitor possible pathogenic changes. • 20 isolates stored for future reference and comparison for fungicide resistance.	• Status of field efficacy of commonly sued products established. • Fungicide resistance management system adopted.
Relative susceptibility of commercial cultivars to husk spot.	Laboratory and field evaluation of varietal susceptibility using published methodologies for husk spot (Akinsanmi *et al.*, 2007; Akinsanmi *et al.*, 2008).	• Presentations to growers and industry stakeholders at seven MacGroup and two Best Practice Group meetings in 2013. • One published article in peer reviewed scientific journal. • One published article in macadamia industry News Bulletin.	• Disease severity levels of 25 macadamia commercial cultivars established. • Characterised 25 elite germplasm for their susceptibility to husk spot. • Reduced blanket fungicide sprays due to targeted disease management based on varietal susceptibility.
Management decision support system.	Regression and compartmental models were developed and evaluated.	• Growers' self-risk assessment guide published in macadamia industry News Bulletin. • Husk spot fact sheet produced. • One Honours thesis produced on husk spot forecasting.	• One growers' self-risk assessment guide produced. • Two disease forecasting models developed.

2. **Phytophthora management:** The core activities for Phytophthora management were developing and communicating to industry management strategies for the soilborne pathogen, through targeted application of crop protection products and an increased focus on soil health.

Specific Activities	Methods	Project Outputs	Project Outcomes
***Phytophthora* and soil health management.**	Effect of seven treatments on tree health was examined in replicated field trials under commercial conditions at two locations.	• YouTube video on integrated management of *Phytophthora*. • Presentation workshops to growers and industry stakeholders at seven MacGroups and the Industry consultant meetings in 2016. • Two research articles in scientific outlets. • Two articles in industry publications. • Oral presentations at the International macadamia industry symposium in 2015.	• Demonstrated improved tree health through increased focus on soil health practices. • Increased and increasing adoption of integrated management approaches for Phytophthora diseases • Reduced reliance on chemical applications. • Increased understanding of association with orchard floor management practices.
Phosphonates application.	Comparative field assessments of spray applications of phosphonates with soil health improvement.	• Technical report on efficacy data. • Communication of use pattern to growers at several growers and consultants meetings throughout the life of the project • Focused workshops to industry stakeholders at seven MacGroup and the Industry consultant meetings in 2016. • Isolates of pathogen collected for future analysis of sensitivity to phosphonates.	• Permit for trunk spray applications of phosphonates in macadamia. • Increased understanding of use pattern of phosphonates. • Reduction in chemical use due increased adoption of soil health practices and integrated management approaches.
Varietal susceptibility.	Relative varietal susceptibility to the soilborne *Phytophthora cinnamomi* was determined from field survey and pathogenicity assays for stem canker and Phytophthora root rot in glasshouse trials.	• Information on current common rootstocks was provided to the industry. • Two research articles co-published with macadamia plant breeders in scientific journals. • Over 132 seedlings of clonal or seed propagated materials were examined for their reactions to *Phytophthora* infection.	• Characterised most commercial cultivars for severity to *Phytophthora cinnamomi.* • Improved knowledge of susceptibility levels of commercial cultivars to *Phytophthora.* • Improved knowledge of potential risk to the dominant rootstock to *Phytophthora.* • Potential sources of selection of resistant materials (species and inter-specific hybrids) provided to the breeding program.
Economic impact	Disease severity and yield data from growers were used to estimate economic loss due to *Phytophthora*.	• Report on losses in productivity associated with *Phytophthora.* • Current estimate at $4.50 /kg dry Nut in shell is over $20 million loss in yield per annum due to poor tree decline associated with macadamia.	• Increased awareness of current and potential yield loss due to inadequate control of tree decline associated with *Phytophthora.* • Improved focus on prevention of tree decline associated with *Phytophthora.*

3. Endemic Diseases: The core activities included risk assessment, monitoring and communicating potential strategies for control to industry.

Specific Activities	Methods	Project Outputs	Project Outcomes
Assessment of husk rot.	Disease severity was determined in a survey of affected orchards. A replicated field trial with a susceptible cultivar was established in a commercial orchard in Gympie, QLD to examine disease incidence from 2012-15. Preliminary replicated field trials over 2 years, to determine possible factors including nutritional disorders, such as calcium, and environmental conditions associated with disease incidence.	• First publication of scientific article on husk rot in peer reviewed journal. • Presentation workshops to at MacGroup meetings o 2015 – 7 sessions o 2017 – 26 sessions • Data on associated environmental factors on husk rot incidence. • Data on putative roles or calcium and/or silicon on disease incidence.	• Rapid diagnostics and response to husk diseases. • Improved knowledge of the biotic cause of husk rot. • Improved knowledge of the potential risk and constraints of husk rot to productivity.
Assessment of flower blight.	Diagnostics of causal agents of flower blight complex was performed on diseased samples from different orchards in QLD and NSW. Pathogenicity of the pathogens was proven in controlled field inoculation assays.	• Workshops at MacGroups o 2015 – 7 sessions o 2017 – 26 sessions • Presentations to the crop consultants meeting and industry conference in 2016. • Two research articles on flower blight • First publication of scientific article on dry flower in peer reviewed journal. • Two publications on flower blight complex in industry News Bulletin. • Oral presentations at International macadamia industry symposium in 2015 and International research symposium in 2017. • 100 dry flower pathogens were characterised. • Over 100 flower blight samples received and processed.	• Increased awareness of the threat of flower blight to productivity. • Increased knowledge of the types of flower blight. • Expertise for rapid diagnostics and response to flower issues and diseases.

4. **New and Emerging Pathogens:** Providing diagnostic services to the macadamia industry to identify the cause of diseases of potential high risk to macadamia productivity was the core activity.

Specific Activities	Methods	Project Outputs	Project Outcomes
Assessment and control options for emerging diseases.	Diagnostics and surveillance of new and emerging disease issues.	• One publication in industry New Bulletin on the identity of the cause of branch dieback and tree death. • Diagnostics of poor plant emergence and seedling death for four macadamia nurseries. • Technical report and engagement with crop protection industries on diseases of macadamia. • Technical expertise and response to new pathogen report and implication to macadamia throughout the life of the project. • Over 150 pathogenic isolates identified and stored in the Queensland Plant Pathology Herbarium culture collection. • Farm visits for disease surveillance in average of 20 farms per year. • Average of 50 potential plant disease samples from growers and consultants received per year. • Contributed to the review of Industry biosecurity plan.	• Improved and updated information on new or emerging diseases. • Ready diagnostic assays to identify the causal organism. • Improved awareness and confidence in the industry biosecurity services. • Improved awareness of processes to safeguard industry from severe outbreak of new diseases. • Provided rapid diagnostics of new plant pathology issues

Outputs

List of outputs produced in this project:

Publications in industry magazines and newsletters

1. Akinsanmi, O.A. 2017. Fungi implicated in dry flower disease. *Australian Macadamia Society News Bulletin* **45** (3), 67.
2. Akinsanmi, O.A. 2016. Soil organic amendments improve tree health and suppress *Phytophthora*. *Australian Macadamia Society News Bulletin* **44** (4), 74-75.
3. Akinsanmi, O.A., Searle, C. 2016. Branch dieback: a growing threat. *Australian Macadamia Society News Bulletin* **44** (1), 58-59.
4. Akinsanmi, O.A. 2015. Diseases affecting macadamia flowers: a significant threat to production. *Australian Macadamia Society Ltd. News Bulletin* **43** (2), 68-70.
5. Akinsanmi, O.A. 2014. Research shakes out husk spot disease in macadamia. *Australian Macadamia Society Ltd. News Bulletin* **42** (4), 60 -61.
6. Akinsanmi, O.A. 2014. Pest and disease management feature: Managing husk spot. *Australian Macadamia Society Ltd. News Bulletin* **42** (4), 48 -50.
7. Akinsanmi, O.A., Drenth, A. 2014. Soil health and tree decline *Australian Macadamia Society News Bulletin* **42** (3), 58-60.
8. Metcalf, D., Bright, J., McLean, S., Akinsanmi, O.A. Commens, R. 2014. Biological control of husk spot in macadamia. *Australian Macadamia Society News Bulletin* **42** (3), 56-57.
9. Akinsanmi, O.A., Drenth, A. 2013. Lowdown on Phytophthora. *Australian Macadamia Society Ltd. News Bulletin* **41** (4), 50-53.
10. Akinsanmi, O.A., Drenth, A. 2013. Phytophthora diseases of macadamias. *Australian Nutgrower* **27** (3), 15-17.
11. Akinsanmi, O.A., Drenth, A. 2013. Integrated disease management: a snapshot - husk spot. *Australian Macadamia Society Ltd. News Bulletin* **41** (3), 34-37.
12. Akinsanmi, O.A., Drenth, A. 2013. Australian Macadamia Industry: New Disease Management Project (MC12007). *Australian Macadamia Society Ltd. News Bulletin* **41** (2), 50-51.

Reports to Industry, new protocols and knowledge

1. Review of the current knowledge, research and gaps in knowledge on husk spot in macadamia.
2. Review of the status, gaps in knowledge, and research need on Phytophthora diseases in macadamia.
3. List of relative varietal susceptibility to husk spot published in the industry magazine.
4. Report of phenotypic traits useful as a selection tool for varietal susceptibility to husk spot in the macadamia breeding program.
5. Report on efficacy of a fungicide product recommended as a replacement for carbendazim that fits into existing fungicide-resistance management strategies, and are compatible with industry IPM practices.
6. Report to assist manufacturer to make application for registration of new product for husk spot control in macadamia.

Media reports

1. NBN news report (Macadamia growers set for secure future) – soil health management increased orchard productivity http://www.nbnnews.com.au/2016/07/05/macadamia-growers-set-for-secure-future/ 5th July 2016.
2. Newspaper report (Northern Star) – Fight against disease. Focus on research on soilborne pathogen. http://www.northernstar.com.au/news/fightagainstdisease/1481982/ 20th July 2015.

3. NBN Local News Sunshine Coast TV interview report on management of Phytophthora disease of macadamia. July 2012.

Farm management and research diagnostic tool

1. Diagnostic tool for early detection and assessment of husk spot infection was developed and published for other scientists and a platform for developing on-farm early diagnostic kit.
2. Simple assessment tool based on three principles was developed and provided to growers to support decision for managing soil health that underpin cultural management of Phytophthora in macadamia.

Industry meetings, events and adoption workshops

1. Growers' information workshops on integrated disease management in macadamia for North Queensland, Mackay 5th – 6th October 2017.
2. Growers' adoption workshops on integrated disease management in macadamia at the MacGroup meetings from 18th July to 2nd August 2017 at all major macadamia production regions in Qld and NSW.
3. Annual presentation on integrated disease management at the AMS crop consultant meeting (4th July 2013; 4th June 2014; 11th June 2015; 8th and 9th June 2016; 8th June 2017).
4. Workshop at 'Speed-dating" session at the 2016 AMS industry conference (19th October 2016.
5. Growers' adoption workshops on soil health management for Phytophthora control in macadamia at the MacGroup meetings from 9th to 15th July 2016 at all major macadamia production regions in Qld and NSW.
6. Presentations on the current issues in disease management in macadamia at the Hort innovation IPDM forum for macadamia (13th April 2016)
7. Oral presentation at the 7th International macadamia symposium on macadamia flower blights in South Africa (12th August, 2015).
8. Oral presentation at the 7th International macadamia symposium on prospects of genetic resources for *Phytophthora* resistance in macadamia in South Africa (11th August, 2015).
9. Oral presentation on application of tree shaker technology for husk spot control in macadamias at the 40th Anniversary of AMS and industry conference (16th October 2014).
10. Industry breeding research meeting (February 2013).
11. Macadamia industry benchmarking group
    a. Glasshouse Mountains 17th and Gympie 18th August 2016
12. Macadamia industry best practice group meetings
    a. Glasshouse Mountains 3rd and Gympie 4th December 2014
    b. Glasshouse Mountains 20th and Gympie 21st November 2013;
13. Oral presentation at the 6th International macadamia symposium on *Phytophthora* management in macadamia in Brisbane (19th September, 2012).
14. A comprehensive and effective knowledge transfer from researchers to macadamia growers and crop consultant through face-to-face interactive on-farm visitation.
15. Knowledge transfer from researchers to macadamia growers through a YouTube video to underpin effective change management and adoption of project findings.

Outcomes

List and description of project outcomes on core activities:

1. Husk spot is a major disease of macadamia in Australia, through a review of articles in scientific peer-refereed journals, magazine articles, conference materials and research project reports, knowledge gaps in husk spot disease system that underpin holistic management and future research work were provided.
2. A new fungicide product, DuPont™ Fontelis® was registered for husk spot control in macadamia.
3. Developed and provided to growers information on effective cultural practices to break husk spot disease cycle and reduce reliance on chemical spray applications.
4. Developed practical decision support systems for risk assessment for growers to diagnose their level of risk for subsequent disease management decisions for husk spot and Phytophthora diseases.
5. A comprehensive review of literature and analysis of survey data obtained from the macadamia growers, revealed gaps in knowledge and further research needs for Phytophthora management in the Australian macadamia industry. The review provided key recommendations for future research work.
6. Provided information and assisted in the application for permit for trunk spray applications of phosphonates for improved Phytophthora management.
7. Improved management methods for Phytophthora through increased focus on managing soil health to reduce disease incidence and severity has led to reducing reliance on phosphoric acid in the macadamia industry.
8. Provided a detailed assessment of the level of susceptibility of commercial macadamia varieties to husk spot and Phytophthora tree decline. This information will assist growers in management decisions and for new plantings. The information will be utilised in macadamia breeding program and underpin selection of disease resistant materials.
9. Provided evidence to growers on the improved management system for Phytophthora through increased focus on soil health practices to reduce reliance on application phosphonate products.
10. In collaboration with the industry MacSmart project, developed a YouTube video as a tool or platform to support the adoption of a holistic management system for Phytophthora diseases - (https://www.youtube.com/watch?v=zgAn1T9U7xs).
11. A detailed assessment of the level of susceptibility to husk spot for commercial macadamia varieties to assist growers in management decisions and for new plantings was developed.
12. Provided rapid diagnosis of new and emerging macadamia disease issues that reinforced management decisions of a new significant endemic disease that affects flowering.
13. Assistance with the identification and diagnosis of new and emerging macadamia disease issues was provided to macadamia growers.
14. An active extension and adoption program of disease management strategy was provided to the industry throughout the life of the project.

Evaluation and discussion

1. Industry Plant Pathology Expertise, Engagement and Knowledge Transfer

The ongoing support provided to the industry throughout the life of the project enabled adequate knowledge transfer and quick response to plant pathology issues. Annual trainings provided to the growers and crop consultants through industry workshops and meetings, and personal field visits are a vital resource for continuing development of growers and the industry crop consultants. The systematic diagnostics 'strange' disease-like symptomatic samples and surveillance process safeguard the industry from unexpected outbreaks and incursion of new pathogens. The impact of this activity is significant. The plant pathology expertise and support service provided to the industry have focused complex crop protection processes through one channel. This ensured consistency and up-to-date information on disease management information and protocols, which is cost-effective and non-invasive to growers. The ongoing access to information provided confidence to support disease management decisions shaped the effectiveness and adoption of integrated diseases management strategy in many orchards.

2. Husk Spot Management

a. Cultural control

Cultural control is a vital component of integrated management of husk spot. Three distinct periods in the disease cycle were identified as critical points for application of control measures. These periods coincide with critical stages of macadamia tree phenology and nut development as shown in figure 1 (Miles, 2011). These periods are distinct and easy to identify by crop consultants and growers. Two of the three critical control periods requires cultural intervention. Firstly, cultural control measures to reduce inoculum load (sticktights) after harvest, and second, cultural control measures to prevent loss of harvestable crop by monitoring kernel maturity, as a critical component of decision to schedule harvest rounds rather than by calendar.

The use of tree shaker as a cultural control tool aimed at breaking the disease cycle showed significant ($P<0.001$) reduction in disease severity when sticktights were removed in both cultivars 'A16' and 'A38' trees compared with the untreated control. Results revealed that biennial removal of sticktights provided similar disease control levels compared with the annual removal of sticktights or spray applications of fungicides in the susceptible cultivars. Application of mechanical tree shaker offered additional benefit to growers by increase the amount of nut drop for harvesting compared with natural drop pattern. In other fruit crops and tree nuts, the use of mechanical harvesting system has been reported to reduce harvesting costs by over 50% (Burns *et al.*, 2005). However, further development of mechanical tree shaker is required to improve its efficiency, determine the optimal timing of application and to reduce damage to trunk and possible impact on tree health in macadamia (McConchie, 2005; Akinsanmi & Drenth, 2016).

Sticktights may remain in the tree canopy for several years and its lignin content may influence its biodegradation process. Although major advances in lignin biodegradation research have been realized and each of the principal structural components of lignin is attacked by different microbial organisms, the biochemistry of that attack has not been elucidated (Crawford & Crawford, 1980). Further research is required to advance and develop its application in biodegradation process of sticktights in field conditions.

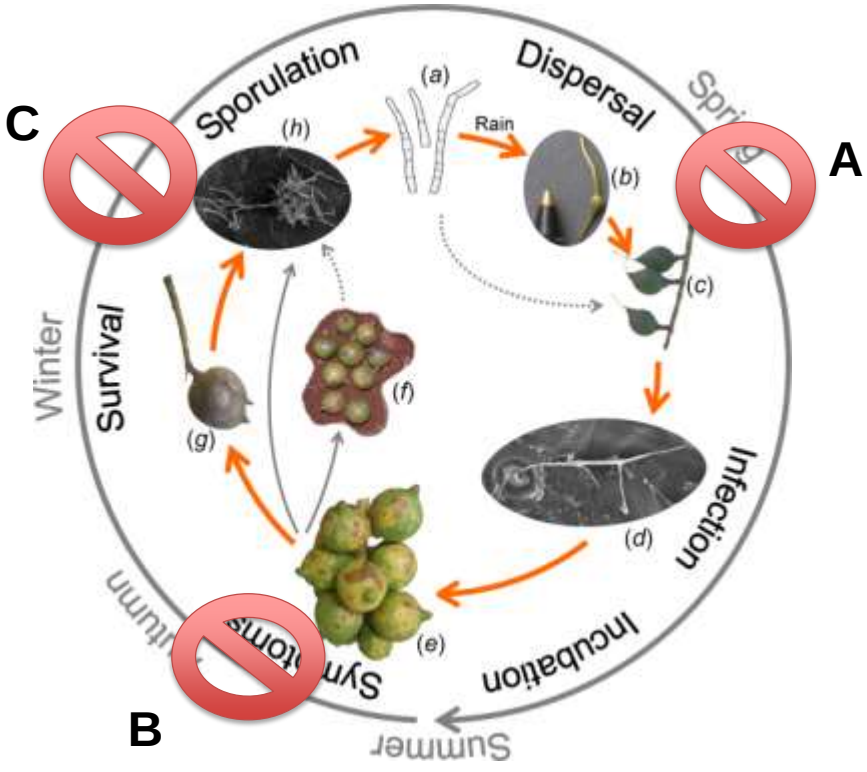

Fig. 1: Husk spot disease cycle adapted from Miles (2011), showing the three periods for targeting disease control. A indicates period of fungicide spray applications; B indicates period for pre-harvest cultural control such as monitoring kernel maturity and pre-harvest clean-up; and C indicates period for postharvest cultural control application such as removal of sticktights.

a. Fungicide control

Carbendazim or difenoconazole in tank mixture with copper and pyraclostrobin products are currently available as a chemical control option for husk spot in macadamia. There is high likelihood of withdrawal of carbendazim-based products in Australia or denial for its use in macadamia produce for export into some countries. The research was developed to identify alternative fungicides to carbendazim and a possible alternative to copper products used in the tank mixture. Multi-location field trials were performed with eight fungicide products, including products in the new chemical group SDHI (succinate dehydrogenase inhibitor), and a multisite action product (mancozeb) as an alternative to copper against husk spot. The results revealed significant differences in the efficacy of the products. The efficacy data generated in this research project were provided to support the registration of a new SDHI-based product that showed near comparable effectiveness with the industry standards (Pyraclostrobin or tank mix of carbendazim and copper) for husk spot control. This product can serve as a replacement fungicide for carbendazim, if it is withdrawn.

Although no fungicide resistance was detected in the current husk spot management program, the inclusion of a new product in different activity group compared to the existing products will contribute to the fungicide-resistance management strategy in the industry. Analysis for resistance of the husk spot fungus to pyraclostrobin and carbendazim showed that both fungicides were active and effective against mycelial growth at low dose rates similar to previous assessments. In future, use of molecular assays should be adopted for early and rapid detection of resistant genotypes of pathogens and understanding of mechanisms of fungicide resistance (Ma & Michailides, 2005).

b. Varietal susceptibility

Varietal susceptibility for 25 commercial cultivars were evaluated. The results showed husk spot symptoms occur

frequently on cultivars 'A16', 'HAES 741', 'A4', 'HAES 344' and 'A38. Knowledge of host resistance and tolerance will contribute to disease control. Understanding of the fundamental processes involved in the mechanisms involved in disease resistance is essential. Therefore, development of molecular information associated with disease resistance in these cultivars and inclusion of phenotypic and physiological factors would aid pre-breeding selection of parents with desirable horticultural traits for disease resistance screening.

c. Disease forecasting and decision support system

A logistic regression and compartmental models were developed to predict husk spot incidence and severity in macadamia (McInerney, 2013). The logistic regression model for predicting risk of disease is based on a qualitative assessment of the effect of the varying characteristics of the host and on number of rainy days from anthesis to fruit physiological maturity period (at approximately 100 days after anthesis). The compartmental model was developed to assess the effects of varying initial conditions on the expected severity of an epidemic of husk spot. Reliability of the two models was evaluated. The logistic regression model predicted relatively higher incidence in the 2014/15 than the 2013/14 season. The results indicate that with every unit increase in the total number of rainy days (>10 days per month), the odds of an outbreak occurring are 1.13 times higher. It was established that there was a positive relationship between the total number of rainy days, and the likelihood of a disease outbreak. The relatively higher temperatures at the early fruit development period (Sept-Oct) in the 2013/14 season contributed to the reduction in disease severity compared to the 2014/15 season. However, the results showed that the odds of a disease outbreak decreased as temperature increased. The compartmental model was more robust when predicting the likelihood of a severe premature nut drop due to husk spot. A critical component of the compartmental model is the initial level of infection and/or source of inoculum available at the beginning of the season. The compartmental model showed that an increase in the initial level of infection resulted in an increase in expected yield loss due to husk spot. This results support the removal of sticktights has an essential disease management strategy to prevent significant yield loss due to husk spot.

3. Phytophthora Management

a. Cultural control of Phytophthora

Large-scale field trials under commercial farming practices in macadamia plantations were used to demonstrate the positive impact of soil organic amendments on tree decline caused by a soilborne pathogen, *Phytophthora cinnamomi* in macadamia. The results provide strong evidence to support reducing the reliance on pesticides to control Phytophthora diseases in macadamia. Significant increase in the number of trees with high disease severity occurred in the untreated control and chemical application at 24 months after the treatments. Yield loss was significantly ($P< 0.05$) reduced in the soil organic amendment plots than the untreated control and chemical application plots between 12 and 24 months after application. Three basic good soil health determinant tools (**BOP**) were identified for growers: **B**alance of soil biology and chemistry, **O**rganic matter (soil content) and **P**lan (Preparation and Maintenance of soil organic amendments).

b. Varietal susceptibility to Phytophthora

Out of 23 commercial cultivars assessed, 'HAES 508', 'HAES 816' and 'H2' were the most susceptible cultivars while 'Daddow', 'A268', 'HAES 788', and 'HAES 748' 'HAES 268' were the least susceptible under commercial orchard conditions. Analysis of species of *Macadamia*, suggests that *M. ternifolia* and *M. jansenii* and their hybrids were more susceptible to root rot with disease severity scores between 73% and 80%. *M. integrifolia* had the greatest stem canker severity and the most extensive lesions above and below the site of inoculation, whereas development of stem lesions were restricted in *M. ternifolia* and *M. jansenii*.

c. Economic impact of Phytophthora diseases

The cost attributable to Phytophthora disease consists of the costs incurred due to disease-induced yield loss at various severity levels. Yield loss was considered as a function of the disease severity and the proportion of yield loss when severity is at its maximum value of 100%. Based on the assumption of dry NIS price at $3.50/kg production estimate of 2.8 NIS t/ha and an average disease severity of 15% in 20,000 ha, the estimated value of loss due to Phytophthora diseases was approximately $19 million.

4. Understanding the Biology and Management of Endemic Diseases

a. Husk rot

Calcium and silicon levels increased in the calcium silicate and potassium silicate+Acadian™ treatments and the proportion of husk rot in these treatments was significantly higher than the untreated control. The levels of other elements such as zinc was highest and potassium was lowest in the potassium silicate-only treatment compared to the other treatments. Potassium solubilizing bacteria improve mineral uptake (Basak & Biswas, 2009), thus, could be the reason why the potassium silicate+Acadian™ treatment has higher potassium levels in the plant tissue than the potassium silicate-only treatment. Timing and the mode of application of potassium silicate may also affects its uptake by plant. The results suggest that the chemical composition of the husk may play a role in husk rot incidence in macadamia. Results from pathogenicity trials showed husk rot symptoms developed only in wounded fruits, suggesting that injury to the macadamia fruit pericarp not only predisposes the pericarp to pathogen infection but is also a prerequisite for infection. Severity of husk rot was strongly correlated to average weekly relative humidity and minimum temperatures, suggesting these environmental factors influence husk rot disease development. This indicates that any biotic agents such as insect pests or abiotic factors such as hail damage, bruising or sunscald, predispose husk to infection under favorable weather conditions. Therefore, effective management of insect pests or situations that cause injury to the husk may reduce husk rot incidence.

b. Flower blight (Botrytis blight)

Macadamia racemes affected by flower blight, Botrytis blight or gray mold were observed in different orchards in Northern New South Wales and Glasshouse Mountains. In some cases, incidence resulted in significant loss of flowers and poor fruit set. Incidence of Botrytis blight often follows wet weather conditions during flowering.

5. Diagnostics and Management Decisions for New and Emerging Pathogens

a. Pestalotiopsis blight (Dry Flower)

Due to lack of adequate knowledge of the specific disease situation, incorrect management decision, extensive poor nut set and significant crop losses were reported. Various pathogens belonging to different genera of fungi (*Cladosporium* and *Botrytis*) and oomycetes (*Phytophthora*) have been reported as causal agents of blossom blight in macadamia. In the 2012-13 production season, a new raceme blight and dieback that resulted in significant crop failure caused by *Pestalotiopsis* was observed in Australia. Characteristic symptoms include necrotic or dieback of the peduncle tip, the entire inflorescence (flowers and peduncle) may be affected as necrotic or dried flowers. Depending on the stage at which infection occurs, dried unopened flowers may remain attached to the peduncle but flowers infected at mid or late bloom stage dried and abscised from the peduncle. Since the first occurrence of the disease, average yield loss due to dry flower ranges between 10-30% in the many orchards in QLD and NSW. Two new species in the genus *Pestalotiopsis* and *Neopestalotiopsis* were identified as causal agents of ‘dry flower’. To our knowledge, this research is the first to report of *Pestalotiopsis* and *Neopestalotiopsis* species, as causal agents of raceme blight and rachis dieback in macadamia. Unlike other flower bights caused by other fungi, dry flower may affect all stages of the raceme development before nut set. Therefore, dry flower poses a serious threat to macadamia production in Australia.

b. Branch dieback

Incidence of macadamia branch dieback caused by species of *Botryosphaeriaceae* increased throughout the life of the

project. In some orchards, infection on the main trunk resulted in complete tree death, Over 300 trees were reported to die due to ‘branch dieback’ disease. Branch dieback poses a significant threat to macadamia production and increased awareness to the threat was highlighted to the industry.

Recommendations

List of general recommendations for current and future improved integrated disease management in macadamia:

1. Macadamia growers should adopt integrated disease management practices. This will reduce reliance on routine and calendar application of crop protection products.
2. Application of cultural practices that contribute to reduction in pathogen inoculum should be encouraged. This would reduce risk of poor disease control when environmental conditions are unfavourable for application of crop protection products.
3. Growers should improve orchard floor and maintain good soil health. This practise would help to produce resilient trees and reduce the impact of soilborne diseases such as Phytophthora root rot.
4. Knowledge of varietal susceptibility to the two major diseases should constitute part of decision for varietal selection in breeding program and new orchard establishment.
5. Fungicide resistance management protocol should be continued.
6. Increased awareness of timing of application of crop protection products is essential to ensure good disease control.
7. Growers should develop and follow good farm biosecurity plan. This will reduce the risk of incursion of new pathogens into the orchards.
8. Disease surveillance is essential for detection of new and resurgence of old pathogens in macadamia orchards. When in doubt, growers should send samples for diagnostics.
9. Industry should continuing the active extension and adoption program. This is ensure early adoption of research findings and development by growers.
10. Development of integrated pest and disease management systems - IPDM is required and very essential to increase orchard productivity.
11. Industry should continue to support access to scientific expertise and diagnostic capability. This is essential to safeguard the industry from plant pathology issues.
12. Industry should continue to fund R&D for integrated disease management program to tackle emerging diseases, plant pathology and crop protection issues.

Scientific refereed publications

Journal article

1. Akinsanmi, O.A., Neal, J., Drenth, A., Topp, B. 2017. Characterization of accessions and species of Macadamia to stem infection by *Phytophthora cinnamomi*. *Plant Pathology* **66**, 186-193.
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Whole book

None to report

Research thesis

1. McInerney C. 2013. *Crop modelling for decision support in agricultural management*. Brisbane: University of Queensland, BSc Honours Thesis.

Chapter in a book or Paper in conference proceedings

1. Akinsanmi OA (2015) Macadamia flower blight caused by a complex of pathogens: a redefinition of disease name. Proceedings of the 7th International Macadamia symposium, Kruger National Park, South Africa August 10 -13, 2015.

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Intellectual property/commercialisation

No commercial IP generated.

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Appendices

List of appendices to the report:

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    i. Cultural practices for husk spot control by mechanical tree shakers.
    ii. Efficacy of fungicide products for husk spot control.
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b. Phytophthora Management
    i. Lowdown on Phytophthora.
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c. Endemic diseases
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    ii. Fungi implicated in dry flower disease.
d. New and Emerging diseases
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Appendix 4 - Posters as brief communication of project activities

a. Growers' self-assessment decision support system for husk spot.
b. Impact of soil health on Phytophthora diseases.
c. Dry flower pathogens.
d. Branch dieback of macadamia in Australia.

Appendix 1

Project Details

Project Code	MC12007
Project Title	Integrated Management of Diseases in Macadamia Industry
Project Type	R&D
Start Date	27 August 2012
End Date	1 December 2017
Service Provider	The University of Queensland
Industry	Australian Macadamia Industry
Key resource persons	Dr Femi Akinsanmi (Project Leader) Ecosciences Precinct, 2C West, GPO Box 267, Brisbane, QLD 4001. Phone: 07 33432453; E-mail: uqoakins@uq.edu.au Prof André Drenth (Team Member)
Date of Report	30 November 2017

Hort Innovation
Strategic levy investment
MACADAMIA FUND

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MACADAMIA FUND

Integrated Management of Diseases in Macadamia Industry

Appendix 2: Details of methodology, results and conclusions of research activity

(a) Husk spot management - Cultural practices using mechanical tree shakers

Dr Olufemi A. Akinsanmi
The University of Queensland

Project number: MC12007

****Part of this research has been published in peer-reviewed journal:**

Akinsanmi, O.A. and Drenth, A. (2016). Sustainable control of husk spot of macadamia by cultural practices. ***Acta Horticulturae*** 1109, 231-236 DOI: 10.17660/ActaHortic.2016.1109.37. https://doi.org/10.17660/ActaHortic.2016.1109.37

Summary

Husk spot, caused by a fungal pathogen *Pseudocercospora macadamiae* causes premature nut drop in macadamia. The fungal inoculum perpetuates between seasons on diseased husk known as 'sticktights' that remain in the tree canopy. Fungicide applications are the most commonly used option for husk spot control. A preliminary study demonstrated significant reduction in husk spot incidence after manually removing sticktights from tree canopy, however, the high costs of manual removal of sticktights from the tree canopy have hindered adoption of the practices for husk spot control. The study examined application of mechanical tree shaker as a suitable replacement of fungicide spray applications for husk spot control. Large-scale trials in commercial orchard with two husk spot susceptible cultivars (A16 and A38) was established in a randomized design. Treatments included trees with and without fungicide spray applications only and in combination with or without mechanical tree shaker. The results showed that mechanical tree shaker is a suitable and effective measure for breaking husk spot disease cycle. Husk spot severity in trees with tree shaker was significantly ($P < 0.001$) reduced compared with the untreated control trees. Similar levels of control was recorded in mechanical tree shaker and trees with fungicide spray applications only. Results revealed that biennial removal of sticktights was effective as the annual removal of sticktights or spray applications of fungicides in susceptible cultivars. These findings demonstrated the use and development of mechanical tree-shaker as an effective cultural practice for husk spot control and as a replacement of fungicide spray control in susceptible cultivars.

Keywords

Abscission; Husk spot; *Pseudocercospora macadamiae*; Nut drop; Tree shaker

Introduction

In Australia, husk spot caused by a fungal pathogen, *Pseudocercospora macadamiae* contribute to significant crop losses and downgrading of macadamia produce. Husk spot symptoms first appear on fruit pericarp (husk) as chlorotic flecks or spots near fruit maturity stages (Miles *et al.*, 2009) and the diseased fruit abscise at any time after symptom expression, depending of the cultivars (Akinsanmi *et al.*, 2007a; Akinsanmi *et al.*, 2012). Infections at early stages of fruit development often lead to premature nut drop. However, in certain cases, husks remain attached to the peduncle. The desiccated husks that failed to abscise and remain in the tree canopy are known as 'sticktights'. Sticktights can remain within the tree canopy for over 2 years (Akinsanmi *et al.*, 2007b), and when the husks are infected before becoming sticktights, *P. macadamiae* survives on the desiccated husks.

The factors that cause sticktights are not well understood, which may involve interaction between environmental and physiological factors. Once the husks split during fruit development and senesces, the normal hormone regulated abscission process is interrupted and the fruit no longer abscise and may remain in the canopy as "sticktights" for more than one growing season (Miles *et al.*, 2010a). Nut drop in macadamia is a regulated process that is governed by plant hormones (Nagao & Sakai, 1985). McConchie et al (2003) suggested that nut drop occurs at the abscission zone from reduced size of cells at a preformed constriction layer in the fruit pedicel. Relative differences in nut drop duration and pattern among macadamia cultivars may be attributed to the rate of reduction in fruit removal force (Trueman *et al.*, 2000). Nut drop process is sometimes interrupted by splitting of the husk and certain cultivars are highly prone to husk splitting early in the season when the fruit are just fully expanded (Akinsanmi & Drenth, 2010; Akinsanmi *et al.*, 2012). Husk spot varietal susceptibility is associated to prevalence and persistence of sticktights in the canopy. Occurrence of sticktights varies among macadamia cultivars. Cultivars such as 'A4', 'A16', 'A38' and 'Purvis' with high amount of sticktights are regarded as susceptible to husk spot, whereas cultivars 'HAES 246', 'HAES 344', and HAES 660' that generally have no sticktights are regarded as tolerant to husk spot (Akinsanmi *et al.*, 2012).

Fungicides are the most commonly used option for husk spot control (Akinsanmi *et al.*, 2007a), but fungicide applications sometimes fail due to prolonged wet weather conditions. Coupled with high pressure to reduce pesticide use, due to public concerns about environmental and health issues, development of alternative control strategies for husk spot is required. Given the fact that *P. macadamiae* inoculum perpetuates between seasons on diseased husks, and a preliminary study has demonstrated that husk spot incidence was reduced after removing sticktights from trees (Miles *et al.*, 2010a), applications of cultural practices that limit formation and retention of sticktights in tree canopy could be an effective control strategy for husk spot in macadamia. This knowledge offers a more targeted and economical approach for the development of effective and sustainable integrated management systems that includes cultural practices for husk spot in macadamia.

Therefore, we tested the hypothesis that husk spot severity, measured as the proportion of nut drop with husk spot symptoms and incidence, measured as the proportion of nuts in the tree canopy with husk spot symptoms are significantly reduce without fungicide applications following mechanical application or tree shaker. Our objective was to develop cultural practices as alternative option to fungicide spray applications for husk spot control. The specific aims were to: (i) examine the effectiveness of mechanical tree shaking to reduce diseased sticktights in the macadamia tree canopy, (ii) determine if application of tree shaker can replace fungicide control practice for husk spot and (iii) determine the frequency of mechanical application of tree shaker, if annually or biennial, to reduce yield loss due to husk spot.

Methodology

Filed site and experimental design

The trees of cultivar 'A16' and 'A38' with history of husk spot and high amount of sticktights in the canopy were selected a commercial macadamia orchard at Bundaberg, Queensland. Trees were about 25 years old, each at about 100 m^3 canopy volume and 6 m high. Four treatments including trees where sticktights were removed and sprayed with fungicide (-ST, +F), trees with sticktights and with fungicide spray applications (+ST, +F), trees where sticktights were removed without any fungicide spray applications (-ST, -F), and trees with sticktights and without fungicide spray applications (+ST, -F) were assigned to plots in a randomized design with four replicates. Each plot contained 15 trees and data were recorded separately from each of the three middle trees from each plot, while the other trees served as buffer trees, to prevent spray drifts between treatments. Field trials were performed from 2012 to 2014.

Application of fungicides and tree shaker

Fungicides were applied following established timing of application and protocols under commercial conditions as described by Akinsanmi and Drenth (2016) using commercial spraying equipment (Akinsanmi *et al.*, 2007a). A spilt-plot design was adopted for -ST treatments, where subplots consisted of annual or biennial removal of sticktights compared with preceding season. Tree shaking was performed using a tractor-mounted tree (TMT) shaker with finger-wheel-like device that rotates on its axis by mechanical transmission when in contact with the tree canopy as the tractor travels forward (Fig. 1). The speed of the tractor was maintained at 3.2 km/h. The TMT shaker concept was developed by Steinhardt Corporation (Macadamias Australia Bundaberg, Queensland).

Data collection

Sticktights prevalence data in the tree canopy (Fig. 1c) were recorded annually as previously described (Miles *et al.*, 2010a; Akinsanmi *et al.*, 2012) and husk spot incidence and severity data were routinely collected following the protocols described by Akinsanmi et al. (2007a). Husk spot severity and incidence were recorded fortnightly from February to June each year (Akinsanmi et al., 2007a). Kernel maturity of the nuts at each harvest was determined as described by Mason and Wills (1983) where 100 oven-dried nuts were cracked using a table-top cracking machine and a standard flotation method was used to determine the percentage of immature kernels (<72% oil content).

Statistical analysis

Data were analysed using statistical software GenStat (release 14; LawesAgricultural Trust, Rothamsted Experimental Station, UK). Disease severity and incidence data were used to produce area under disease progress curve data (Akinsanmi et al., 2008) for analysis of variance and the treatments as main factors. Sticktights prevalence were correlated with husk spot disease data. Significant factors were separated and tested using Fisher's protected least significant difference tests in GenStat.

Fig. 1: (a) Tractor- mounted tree (TMT) shaker with finger-wheel-like device; (b) close up of the finger-wheel-like device in the tree canopy; (c) sticktights and (d) close up of sticktights with husk spot lesion in the tree canopy.

Results and discussion

Efficiency of tree shakers for sticktights removal

Efficiency of tree shakers with two hydro-mechanical systems (Fig. 2 a-b) were compared with TMT shaker (Fig. 1a). Efficiency of sticktights removal using different mechanical tree shakers tested varied between trees (Fig. 2). The hydro-mechanical systems damaged the trunk, by peeling of the bark of the trees, when used after rainfall (Fig. 2 d), whereas, limited limb damage occurred in the TMT shaker. The efficiency of the shakers were similar and ranged between 17% and 100% (Fig. 3). The mean efficiency (%) of the TMT shaker was about 60%. However, the efficiency of the TMT shaker was biased to trees with fewer configuration of big branches. Hydro-mechanical systems are a high throughput tree shakers used in nut and fruit industries to harvest.

Ring barking creates an open tear or bruising of the cambium layer that is responsible for water and nutrient translocation in the tree. Trunk damage is often caused by the use of excessive force and sub-optimal operational practice. Long-term impact of ring-barking on macadamia tree growth has not been determined. It is probable that the failure of phloem materials to reach the root system may have significant consequences on tree heath. The detrimental effect of using the hydro-mechanical systems on the trunk may be reduced with certain treatment of the trees before application of tree shaker. The application of computer control system may help reduce trunk damage (Snell 2008). It is likely, that its application for husk spot control would provide additional benefit for improving harvest efficiency in macadamia.

Fig. 2: Hydro-mechanical tree shakers with (a) forward and (b) backward mounted shaker clamps, (c) 100% removal of nut and sticktights in the tree canopy and (d) damage to the trunk caused by the backward mounted clamps with nuts, sticktights and foliage removed in the process of shaking.

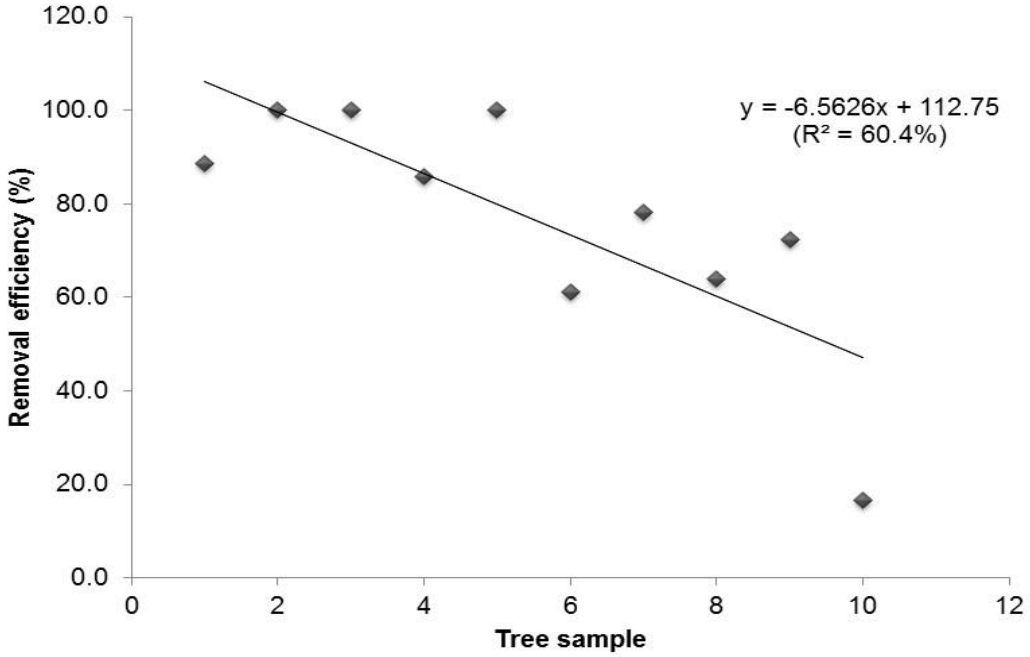

Fig. 3: Mean efficiency of tractor- mounted tree shaker with finger-wheel-like device for removal of sticktights in macadamia tree canopy.

Effect of mechanical tree shaker on husk spot

The results of field trials on mechanical removal of sticktights revealed significant differences among the various treatments (Fig. 4). When sticktights were removed in both 'A16' and 'A38' trees without fungicide spray applications (-ST, -F), husk spot severity was significantly ($P < 0.001$) lower than the untreated control trees (+ST, -F) (Fig. 4). Husk spot severity level in trees sprayed with the standard fungicide spray applications (+ST, +F), was similar with mechanical tree shaking only (-ST, -F) (Fig. 4). The effect of the tree shaker was more pronounced when used in two consecutive years with fungicide spray applications. Results revealed that biennial removal of sticktights is similarly as effective as the annual removal of sticktights or spray applications of fungicides in susceptible cultivars. The use and development of mechanical tree-shaker need to be further fine-tuned so as adapt appropriate tree-shakers to reduce impact on tree health.

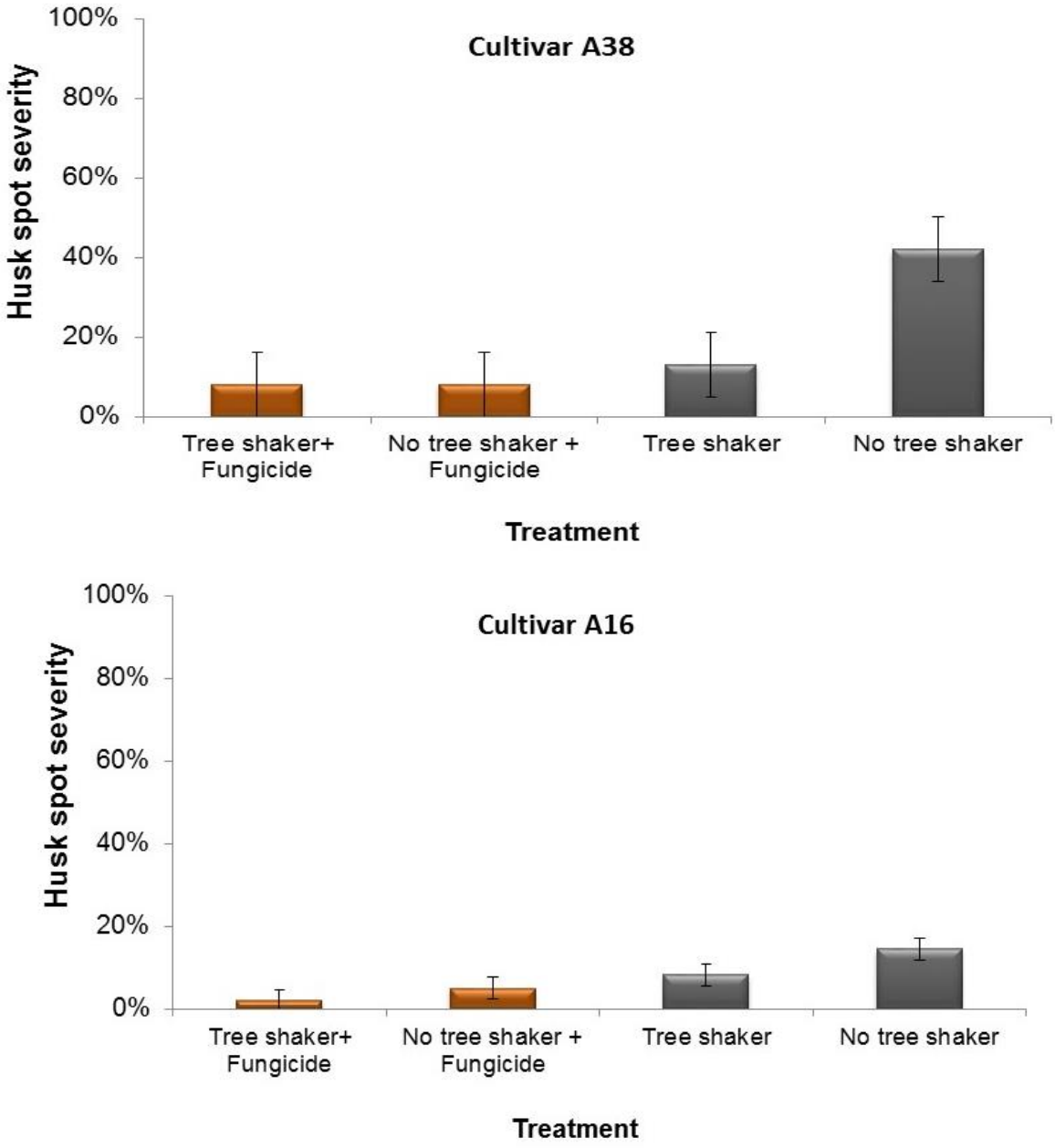

Fig. 4: Mean husk spot severity in macadamia cvs. 'A38' and 'A16' trees with different treatments; trees with or without two spray application of tank mix of carbendazim and copper fungicides where sticktights remained or were removed from the canopy using mechanical tree shaker. Lines on bars indicate standard error of mean.

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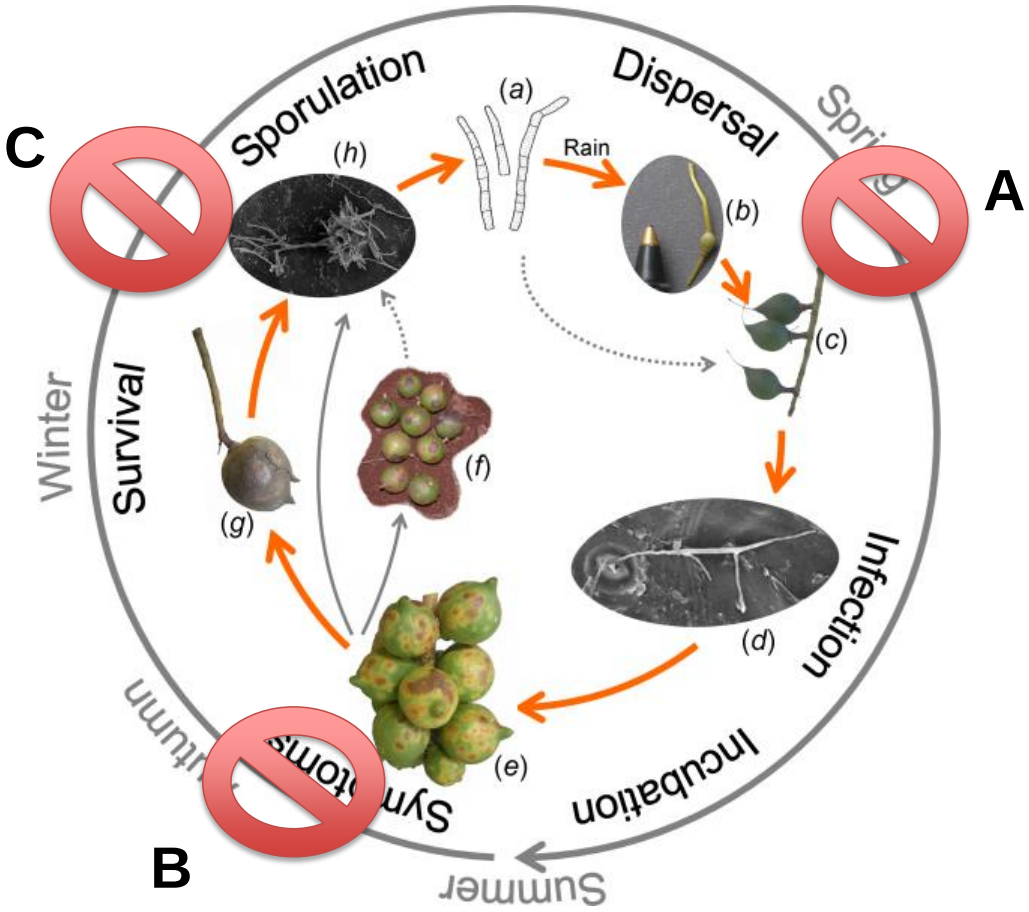

Fig. 5**:** Husk spot disease cycle adapted from Miles (2011), showing the three periods for targeting disease control. A indicates period of fungicide spray applications; B indicates period for pre-harvest cultural control such as monitoring kernel maturity and pre-harvest clean-up; and C indicates period for postharvest cultural control application such as removal of sticktights.

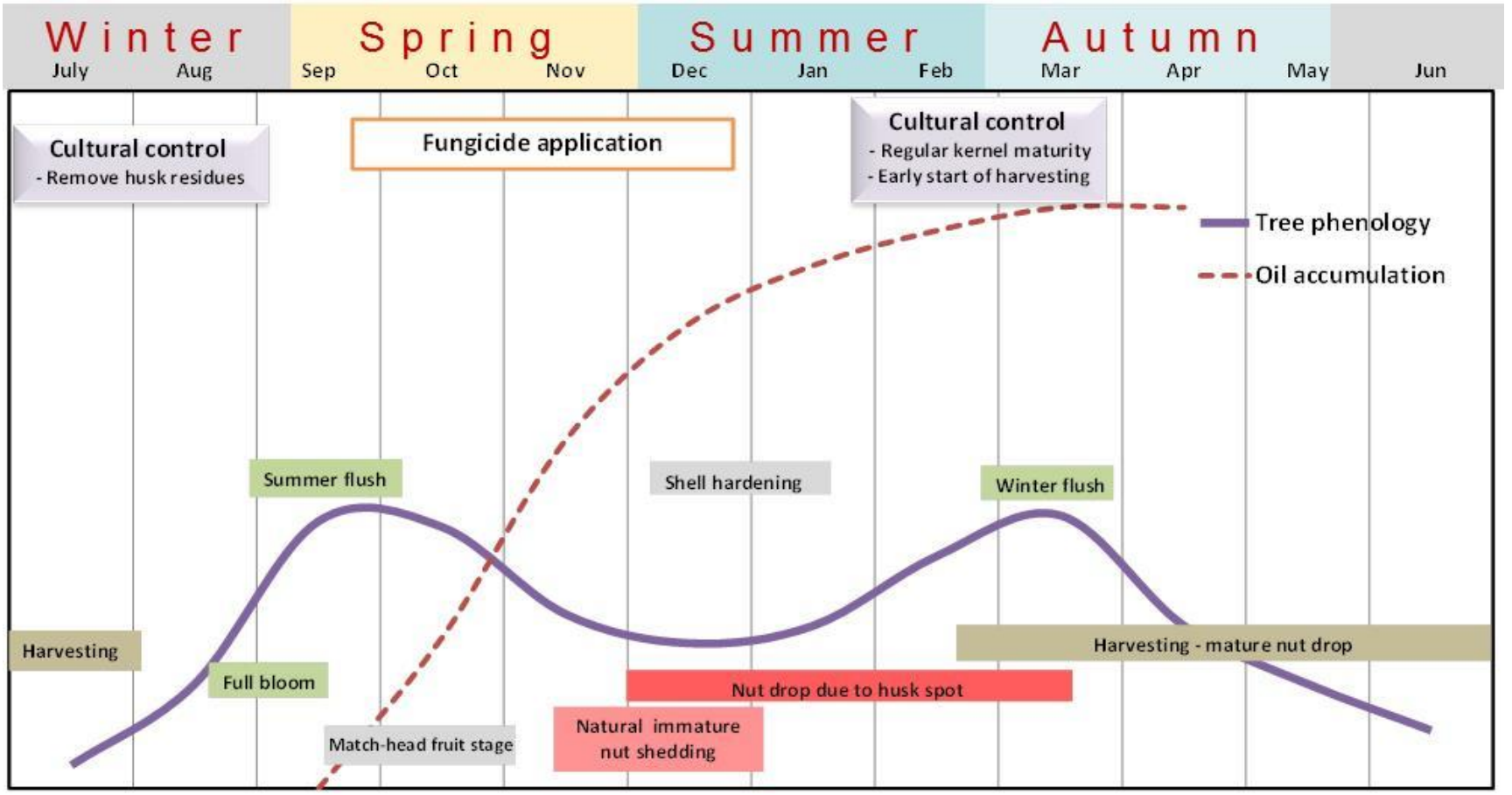

Fig. 6: Layout of macadamia production, tree phenology, fruit development and timing of application of husk spot control in Australia

Acknowledgements

This project has been funded by Hort Innovation, using the macadamia research and development levy and contributions from the Australian Government. Hort Innovation is the grower owned, not-for-profit research and development corporation for Australian horticulture. We acknowledge the assistance provided by many industry consultants. We are particularly Macadamia Farm management for their in kind contributions and for providing access to farms and use of their farm equipment for this study.

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Strategic levy investment

MACADAMIA FUND

Integrated Management of Diseases in Macadamia Industry

Appendix 2: Details of methodology, results and conclusions of research activity

(a) Husk spot management - Cultural practices using mechanical tree shakers

Dr Olufemi A. Akinsanmi
The University of Queensland

Project number: MC12007

****Part of this research has been published in peer-reviewed journal:**

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Summary

Husk spot, caused by a fungal pathogen *Pseudocercospora macadamiae* causes premature nut drop in macadamia. The fungal inoculum perpetuates between seasons on diseased husk known as 'sticktights' that remain in the tree canopy. Fungicide applications are the most commonly used option for husk spot control. A preliminary study demonstrated significant reduction in husk spot incidence after manually removing sticktights from tree canopy, however, the high costs of manual removal of sticktights from the tree canopy have hindered adoption of the practices for husk spot control. The study examined application of mechanical tree shaker as a suitable replacement of fungicide spray applications for husk spot control. Large-scale trials in commercial orchard with two husk spot susceptible cultivars (A16 and A38) was established in a randomized design. Treatments included trees with and without fungicide spray applications only and in combination with or without mechanical tree shaker. The results showed that mechanical tree shaker is a suitable and effective measure for breaking husk spot disease cycle. Husk spot severity in trees with tree shaker was significantly ($P < 0.001$) reduced compared with the untreated control trees. Similar levels of control was recorded in mechanical tree shaker and trees with fungicide spray applications only. Results revealed that biennial removal of sticktights was effective as the annual removal of sticktights or spray applications of fungicides in susceptible cultivars. These findings demonstrated the use and development of mechanical tree-shaker as an effective cultural practice for husk spot control and as a replacement of fungicide spray control in susceptible cultivars.

Keywords

Abscission; Husk spot; *Pseudocercospora macadamiae*; Nut drop; Tree shaker

Introduction

In Australia, husk spot caused by a fungal pathogen, *Pseudocercospora macadamiae* contribute to significant crop losses and downgrading of macadamia produce. Husk spot symptoms first appear on fruit pericarp (husk) as chlorotic flecks or spots near fruit maturity stages (Miles *et al.*, 2009) and the diseased fruit abscise at any time after symptom expression, depending of the cultivars (Akinsanmi *et al.*, 2007a; Akinsanmi *et al.*, 2012). Infections at early stages of fruit development often lead to premature nut drop. However, in certain cases, husks remain attached to the peduncle. The desiccated husks that failed to abscise and remain in the tree canopy are known as 'sticktights'. Sticktights can remain within the tree canopy for over 2 years (Akinsanmi *et al.*, 2007b), and when the husks are infected before becoming sticktights, *P. macadamiae* survives on the desiccated husks.

The factors that cause sticktights are not well understood, which may involve interaction between environmental and physiological factors. Once the husks split during fruit development and senesces, the normal hormone regulated abscission process is interrupted and the fruit no longer abscise and may remain in the canopy as "sticktights" for more than one growing season (Miles *et al.*, 2010a). Nut drop in macadamia is a regulated process that is governed by plant hormones (Nagao & Sakai, 1985). McConchie et al (2003) suggested that nut drop occurs at the abscission zone from reduced size of cells at a preformed constriction layer in the fruit pedicel. Relative differences in nut drop duration and pattern among macadamia cultivars may be attributed to the rate of reduction in fruit removal force (Trueman *et al.*, 2000). Nut drop process is sometimes interrupted by splitting of the husk and certain cultivars are highly prone to husk splitting early in the season when the fruit are just fully expanded (Akinsanmi & Drenth, 2010; Akinsanmi *et al.*, 2012). Husk spot varietal susceptibility is associated to prevalence and persistence of sticktights in the canopy. Occurrence of sticktights varies among macadamia cultivars. Cultivars such as 'A4', 'A16', 'A38' and 'Purvis' with high amount of sticktights are regarded as susceptible to husk spot, whereas cultivars 'HAES 246', 'HAES 344', and HAES 660' that generally have no sticktights are regarded as tolerant to husk spot (Akinsanmi *et al.*, 2012).

Fungicides are the most commonly used option for husk spot control (Akinsanmi *et al.*, 2007a), but fungicide applications sometimes fail due to prolonged wet weather conditions. Coupled with high pressure to reduce pesticide use, due to public concerns about environmental and health issues, development of alternative control strategies for husk spot is required. Given the fact that *P. macadamiae* inoculum perpetuates between seasons on diseased husks, and a preliminary study has demonstrated that husk spot incidence was reduced after removing sticktights from trees (Miles *et al.*, 2010a), applications of cultural practices that limit formation and retention of sticktights in tree canopy could be an effective control strategy for husk spot in macadamia. This knowledge offers a more targeted and economical approach for the development of effective and sustainable integrated management systems that includes cultural practices for husk spot in macadamia.

Therefore, we tested the hypothesis that husk spot severity, measured as the proportion of nut drop with husk spot symptoms and incidence, measured as the proportion of nuts in the tree canopy with husk spot symptoms are significantly reduce without fungicide applications following mechanical application or tree shaker. Our objective was to develop cultural practices as alternative option to fungicide spray applications for husk spot control. The specific aims were to: (i) examine the effectiveness of mechanical tree shaking to reduce diseased sticktights in the macadamia tree canopy, (ii) determine if application of tree shaker can replace fungicide control practice for husk spot and (iii) determine the frequency of mechanical application of tree shaker, if annually or biennial, to reduce yield loss due to husk spot.

Methodology

Filed site and experimental design

The trees of cultivar 'A16' and 'A38' with history of husk spot and high amount of sticktights in the canopy were selected a commercial macadamia orchard at Bundaberg, Queensland. Trees were about 25 years old, each at about 100 m^3 canopy volume and 6 m high. Four treatments including trees where sticktights were removed and sprayed with fungicide (-ST, +F), trees with sticktights and with fungicide spray applications (+ST, +F), trees where sticktights were removed without any fungicide spray applications (-ST, -F), and trees with sticktights and without fungicide spray applications (+ST, -F) were assigned to plots in a randomized design with four replicates. Each plot contained 15 trees and data were recorded separately from each of the three middle trees from each plot, while the other trees served as buffer trees, to prevent spray drifts between treatments. Field trials were performed from 2012 to 2014.

Application of fungicides and tree shaker

Fungicides were applied following established timing of application and protocols under commercial conditions as described by Akinsanmi and Drenth (2016) using commercial spraying equipment (Akinsanmi *et al.*, 2007a). A spilt-plot design was adopted for -ST treatments, where subplots consisted of annual or biennial removal of sticktights compared with preceding season. Tree shaking was performed using a tractor-mounted tree (TMT) shaker with finger-wheel-like device that rotates on its axis by mechanical transmission when in contact with the tree canopy as the tractor travels forward (Fig. 1). The speed of the tractor was maintained at 3.2 km/h. The TMT shaker concept was developed by Steinhardt Corporation (Macadamias Australia Bundaberg, Queensland).

Data collection

Sticktights prevalence data in the tree canopy (Fig. 1c) were recorded annually as previously described (Miles *et al.*, 2010a; Akinsanmi *et al.*, 2012) and husk spot incidence and severity data were routinely collected following the protocols described by Akinsanmi et al. (2007a). Husk spot severity and incidence were recorded fortnightly from February to June each year (Akinsanmi et al., 2007a). Kernel maturity of the nuts at each harvest was determined as described by Mason and Wills (1983) where 100 oven-dried nuts were cracked using a table-top cracking machine and a standard flotation method was used to determine the percentage of immature kernels (<72% oil content).

Statistical analysis

Data were analysed using statistical software GenStat (release 14; LawesAgricultural Trust, Rothamsted Experimental Station, UK). Disease severity and incidence data were used to produce area under disease progress curve data (Akinsanmi et al., 2008) for analysis of variance and the treatments as main factors. Sticktights prevalence were correlated with husk spot disease data. Significant factors were separated and tested using Fisher's protected least significant difference tests in GenStat.

Fig. 1: (a) Tractor- mounted tree (TMT) shaker with finger-wheel-like device; (b) close up of the finger-wheel-like device in the tree canopy; (c) sticktights and (d) close up of sticktights with husk spot lesion in the tree canopy.

Results and discussion

Efficiency of tree shakers for sticktights removal

Efficiency of tree shakers with two hydro-mechanical systems (Fig. 2 a-b) were compared with TMT shaker (Fig. 1a). Efficiency of sticktights removal using different mechanical tree shakers tested varied between trees (Fig. 2). The hydro-mechanical systems damaged the trunk, by peeling of the bark of the trees, when used after rainfall (Fig. 2 d), whereas, limited limb damage occurred in the TMT shaker. The efficiency of the shakers were similar and ranged between 17% and 100% (Fig. 3). The mean efficiency (%) of the TMT shaker was about 60%. However, the efficiency of the TMT shaker was biased to trees with fewer configuration of big branches. Hydro-mechanical systems are a high throughput tree shakers used in nut and fruit industries to harvest.

Ring barking creates an open tear or bruising of the cambium layer that is responsible for water and nutrient translocation in the tree. Trunk damage is often caused by the use of excessive force and sub-optimal operational practice. The detrimental effect of using the hydro-mechanical systems on the trunk may be reduced with certain treatment of the trees before application of tree shaker. The application of computer control system may help reduce trunk damage (Snell 2008). It is likely, that its application for husk spot control would provide additional benefit for improving harvest efficiency in macadamia.

Fig. 2: Hydro-mechanical tree shakers with (a) forward and (b) backward mounted shaker clamps, (c) 100% removal of nut and sticktights in the tree canopy and (d) damage to the trunk caused by the backward mounted clamps with nuts, sticktights and foliage removed in the process of shaking.

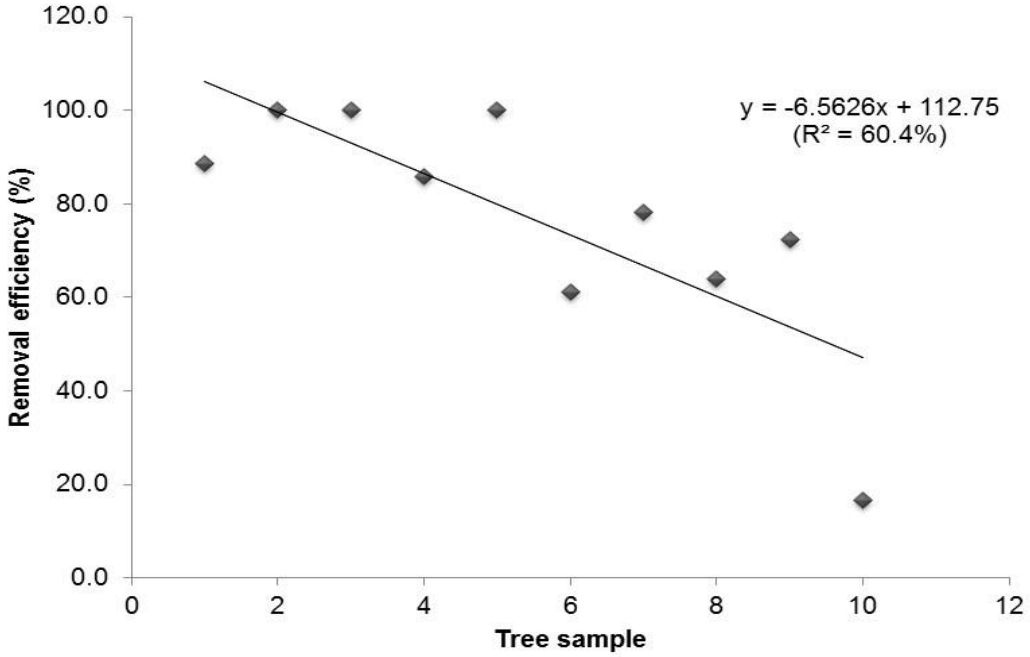
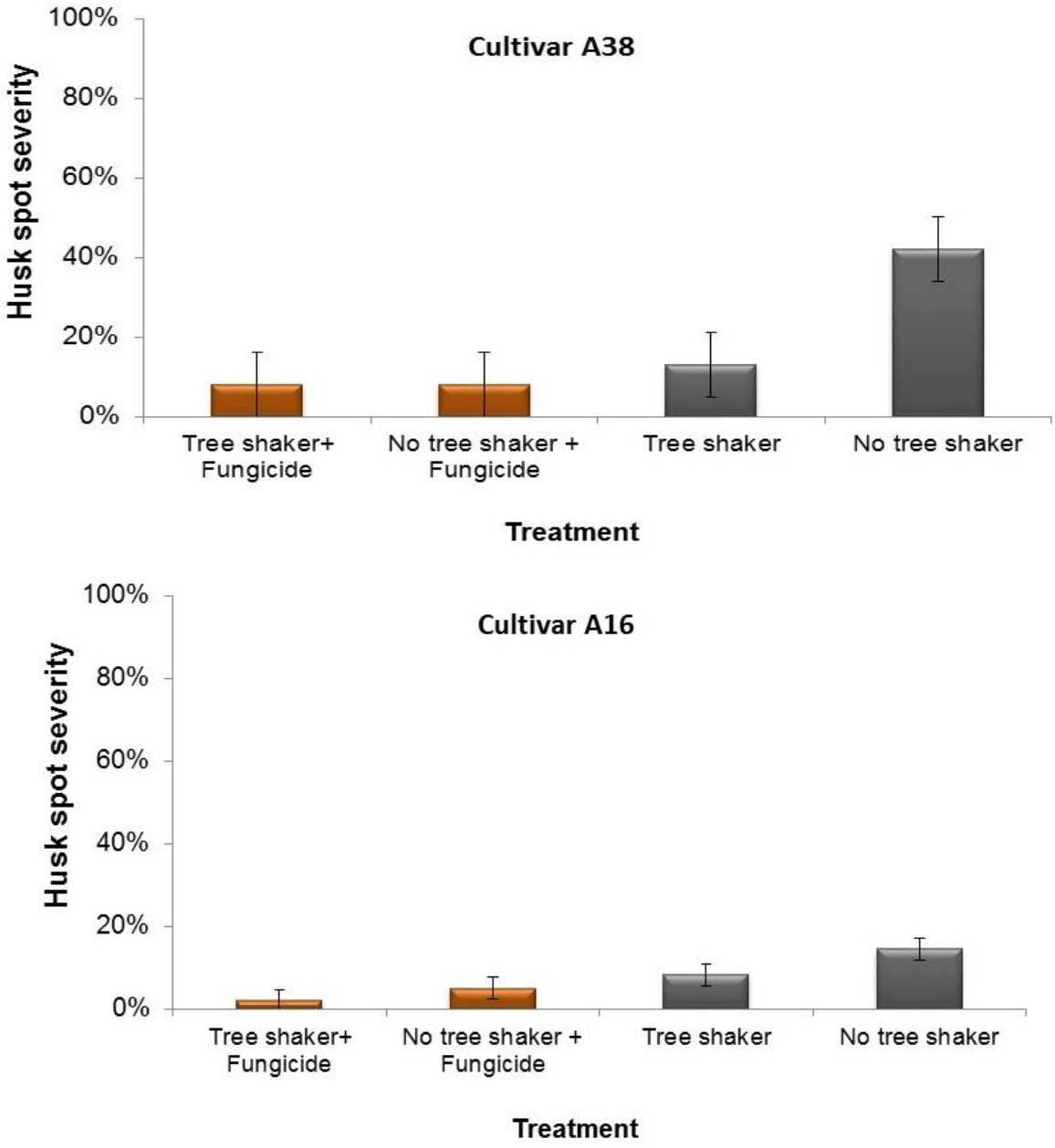


Fig. 3: Mean efficiency of tractor- mounted tree shaker with finger-wheel-like device for removal of sticktights in macadamia tree canopy.

Effect of mechanical tree shaker on husk spot

The results of field trials on mechanical removal of sticktights revealed significant differences among the various treatments (Fig. 4). When sticktights were removed in both 'A16' and 'A38' trees without fungicide spray applications (-ST, -F), husk spot severity was significantly ($P < 0.001$) lower than the untreated control trees (+ST, -F) (Fig. 4). Husk spot severity level in trees sprayed with the standard fungicide spray applications (+ST, +F), was similar with mechanical tree shaking only (-ST, -F) (Fig. 4). The effect of the tree shaker was more pronounced when used in two consecutive years with fungicide spray applications. Results revealed that biennial removal of sticktights is similarly as effective as the annual removal of sticktights or spray applications of fungicides in susceptible cultivars. The use and development of mechanical tree-shaker need to be further fine-tuned so as adapt appropriate tree-shakers to reduce impact on tree health.



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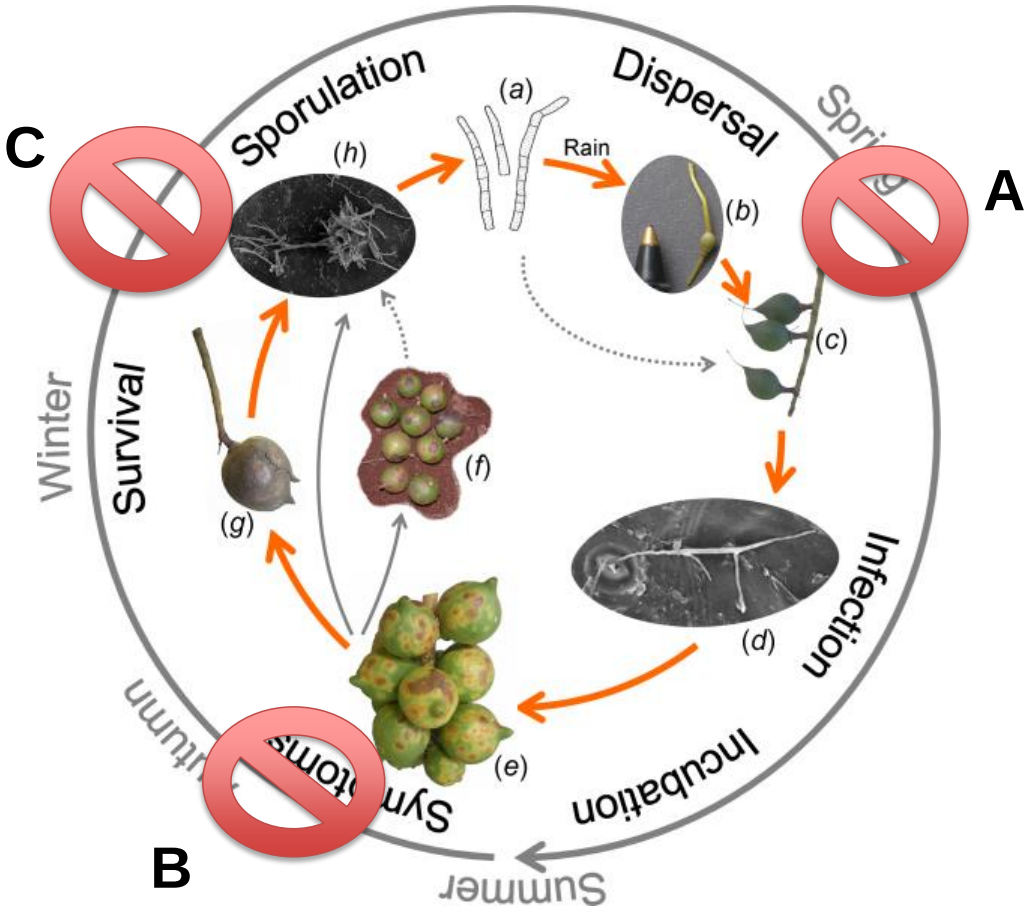

Fig. 5**:** Husk spot disease cycle adapted from Miles (2011), showing the three periods for targeting disease control. A indicates period of fungicide spray applications; B indicates period for pre-harvest cultural control such as monitoring kernel maturity and pre-harvest clean-up; and C indicates period for postharvest cultural control application such as removal of sticktights.

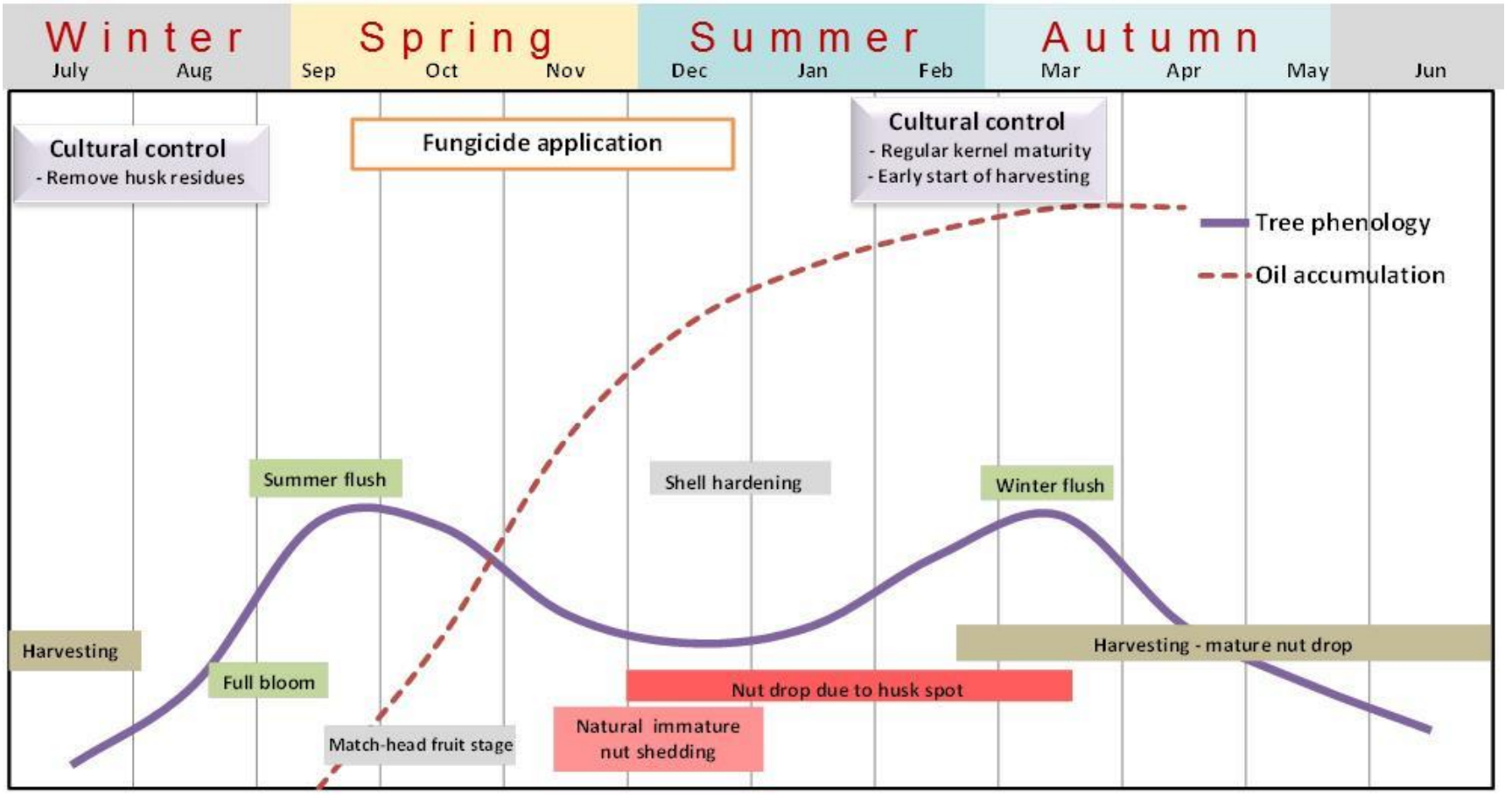

Fig. 6: Layout of macadamia production, tree phenology, fruit development and timing of application of husk spot control in Australia

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Appendix 2: Details of methodology, results and conclusions of research activity

(a) Husk spot management – Rapid detection and diagnostics

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Project number: MC12007

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Characterization and development of qPCR for early detection and quantification of *Pseudocercospora macadamiae* at different stages of infection process

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Abstract Ability to detect *Pseudocercospora macadamiae* infection in macadamia husk at least four months before symptoms become visible will aid the development of disease control measures. This study examined the distinctness of *P. macadamiae* within the phylogenetic lineages of the genus *Pseudocercospora*. In addition, we developed two quantitative PCR (qPCR) assays, as rapid diagnostic tools, for early detection and quantification of *P. macadamiae in planta*. Phylogenetic analysis of concatenated sequences of four gene loci (large subunits, internal transcribed spacer (ITS), translation elongation factor 1-alpha (TEF-1α) and actin of 47 *P. macadamiae* isolates showed that *P. macadamiae* is a distinct species in the genus *Pseudocercospora. P. macadamiae* isolates were partitioned into subunits in the cluster but the grouping of the isolates was regardless of location. Nucleotide diversity (0.02) and the coefficient of genetic differentiation (0.07) were low in the *P. macadamiae* population. Two qPCR primer sets, based on ITS (PMI) and TEF-1α (PME) were designed that consistently amplified *P. macadamiae* in fungal cultures (Ct = 16.93 ± 0.11 and Ct = 21.20 ± 0.11, respectively) and *in planta* (Ct = 32.36 ± 0.28 and Ct = 38.07 ± 1.20, respectively). The PMI primers also detected species in the genus *Pseudocercospora*, while PME was more specific and robust for quantification of *P. macadamiae*. Both primer sets detected *P. macadamiae* in asymptomatic tissue samples and strongly differentiated various stages of disease progression, which revealed approximately 10-fold increase in fungal biomass between each consecutive stage of symptom development.

Keywords Fungal diagnostic · Hyphomycetes · Proteaceae · qPCR · Tree nut

Introduction

Macadamia integrifolia and *M. tetraphylla* are cultivated for their edible nut which is enclosed in very hard shell and husk (pericarp). Macadamias originate from the tropical forest fringes of coastal southeast Queensland (Qld) and northeast New South Wales (NSW) in Australia, where wild trees still exist in the native ecosystem (Storey 1965). Commercial macadamia plantations exist in several countries in Africa, Asia and North and South America. Macadamia trees are affected by about 20 diseases caused by various pathogens such as *Phytophthora cinnamomi* causing stem canker, *Botrytis cinerea* causing flower blight and *Diaporthe* spp. causing Phomopsis

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husk rot (Drenth et al. 2009). Husk spot caused by *Pseudocercospora macadamiae* is a serious disease of macadamia in Australia (Beilharz et al. 2003). Husk spot causes extensive premature fruit abscission, giving rise to nuts with low oil content.

Husk spot symptoms are first visible as tiny chlorotic flecks that slowly progress to necrotic phase (Miles et al. 2009). Each phase of symptom development from chlorotic to the necrotic phase is characterised by the presence of fungal structures ranging from sparse network of hyphae, covering spots at the chlorotic stage, to a denser mycelial mat with visible disruption of the cuticle at the necrotic stage of symptom expression (Miles et al. 2009). The first observations of husk spot symptoms and early herbarium specimens in the Queensland Plant Pathology Herbarium (BRIP) are believed to have been made around the 1950s when the symptoms was erroneously thought to be a sign of kernel maturity (Mayers and Hutton 1987). In the early 1970s, husk spot was associated with a fungal pathogen, and based on morphological characteristics, the fungus was classified as part of the cercosporoid pathogens as described by Deighton (1976). Further morphological characteristics of the fungus resulted in its re-classification as belonging to the genus *Pseudocercospora* (Beilharz et al. 2003). In 1981, severe husk spot epidemic was first reported in commercial orchards in Maleny, Qld, Australia, which resulted in over 40 % yield losses (Newett 1983; Beilharz et al. 2003).

Species of *Pseudocercospora* are well recognised as plant pathogens, endophytes or saprobes on a wide range of plants and are mostly host specific (Leina et al. 1996; Crous et al. 2012). In a recent study of the phylogenetic lineages of *Pseudocercospora*, the belief relating to host specificity was questioned (Crous et al. 2012). It was suggested that more focused studies on *Pseudocercospora* isolates, from a specific host, are required to confirm if a complex of *Pseudocercospora* spp. is involved in a particular disease (Crous et al. 2012). Crous et al. (2012) showed that several *Pseudocercospora* spp. that were identified on the basis of morphological characters or host contain several novel taxa. In macadamia, *P. macadamiae* was described from few diseased macadamia husk samples (Beilharz et al. 2003), however, slight differences in symptom expression on the same cultivars and between growing regions occur on husk spot infected tissues. It is uncertain if a complex of *Pseudocercospora* spp. is involved or responsible for the slight differences in symptoms of macadamia husk spot. Similarly, it is uncertain if *P. macadamiae* isolates constitute a particular group within the phylogenetic lineages of the genus *Pseudocercospora*. Whether *P. macadamiae* population is highly diverse or if multiple species cause husk spot is still not known. A study of genetic diversity of six *P. macadamiae* isolates using RAPD showed a high level of genetic diversity among the isolates, which were grouped according to their geographic location (Wright 1993). In contrast using PCR-RFLP, Miles (2011) demonstrated a low genetic diversity among the *P. macadamiae* population. However, isolates of the same PCR-RFLP profile were more dominant in all macadamia-producing regions and had relatively higher gene diversity and genotypic diversity compared with isolates of other five PCR-RFLP profiles (Miles 2011). Preliminary DNA sequence analysis of the internal transcribed spacer (ITS) gene loci, based on five *P. macadamiae* isolates, also showed genetic similarity with *Pseudocercospora* species from hosts within the *Myrtaceae* family, rather than the species within *Proteaceae* (Miles 2011). Partial sequence of the ITS gene region alone does not differentiate most of the *Pseudocercospora* taxa (Crous et al. 2012). Combined sequence of the ITS gene region with sequences of the translation elongation factor 1-alpha (TEF-1α) and actin (ACT) gene loci is required to separate closely related *Pseudocercospora* species (Crous et al. 2012).

Previous studies have suggested that development or expression of husk spot symptoms is influenced by physiological stage of macadamia fruit (Wright 1993; Akinsanmi et al. 2007; Miles et al. 2010a). Incubation period after infection at the match-head-sized and pea-sized stages of fruit development may last up to three months (Wright 1993; Miles et al. 2010a). Infections at the match-head-sized and pea-sized stages have been reported to cause more severe premature fruit abscission compared with infections at later stages of fruit development (Akinsanmi et al. 2007; Miles et al. 2010a). Consequently, farmers apply prophylactic fungicides sprays at these early stages of fruit development, several months before symptoms become visible. Fungicide spray applications after symptom expression are ineffective in preventing premature abscission of macadamia nut due to husk spot, and hence it is uncommon and unjustifiable to apply fungicide at this late stage (Akinsanmi et al. 2007; Akinsanmi et al. 2008). If environmental conditions are not favourable for infection and disease development, application of

fungicides become an unnecessary production expense. Therefore, management strategy based on prophylactic fungicide spray application strategy for husk spot may lead to reliance on routine pesticide application.

At present, there is a lack of reliable and rapid diagnostic tools to confirm infection and to detect the presence of *P. macadamiae* in macadamia husk. A field diagnostic feature that distinguishes husk spot from symptoms of diseases caused by other pathogens on macadamia husk such as anthracnose caused by *Colletotrichum gloeosporioides* sensu *lato* and husk rot caused by *Diaporthe* spp., is to cut through the lesion on the husk. Unlike other macadamia husk diseases, husk spot lesions are very tough, fibrous, compact and difficult to cut though compared with healthy surrounding tissue. Current laboratory diagnostic practice is to isolate and identify the fungus based on cultural and morphological characteristics and comparison with ITS sequences of other *Pseudocercospora* species. This procedure requires isolation from visibly diseased samples and is time consuming because *P. macadamiae* is a slow-growing fungus that requires up to three weeks of incubation for sufficient fungal culture for DNA extraction.

Real-time quantitative PCR (qPCR) using hybridisation probes (TaqMan) allows for accurate quantification and continuous monitoring of amplification of samples during the PCR of targeted sequence, even at low fungal DNA concentrations in plant tissue. Precise detection and quantification of fungal biomass in host tissue will undoubtedly allow for accurate differentiation of macadamia varieties and other *Pseudocercospora* species in macadamia. Hence, due to its specificity, sensitivity and ability to provide quantitative information on fungal biomass in plant tissue, qPCR is a highly desirable molecular diagnostic assay compared to conventional PCR (Capote et al. 2012). The recent phylogenetic research in the genus *Pseudocercospora* has resolved some ambiguous taxa in the genus (Crous et al. 2012), however, there is no information on how *P. macadamiae* is constituted in the *Pseudocercospora* lineages. Accurate identification of *Pseudocercospora* associated with husk spot in macadamia is a fundamental step in development of tools for detection and control of the disease. The lack of rapid diagnostic tool for *P. macadamiae* in asymptomatic tissue limits early assessment of breeding materials for disease resistance. Therefore, the aims of this study were to define the phylogenetic classification of *P. macadamiae* in the *Pseudocercospora* genus and develop specific and sensitive qPCR assays to detect *P. macadamiae* in macadamia husk tissue. An additional aim was to use the molecular qPCR assays to quantify the *P. macadamiae* biomass in macadamia husk at different stages of disease development. The molecular assays would offer a rapid and more reliable diagnostic tool for assessing disease resistance in macadamia and for early detection of *P. macadamiae* infection before symptom expression.

Materials and methods

Fungal strains for phylogenetic analysis

Details of the fungal isolates used in the study are provided in Table 1. Forty-seven *P. macadamiae* isolates stored in the Queensland Plant Pathology Herbarium (BRIP) culture collection were selected and used in the phylogenetic analysis study (Table 1). Fungal cultures were grown on ¼-strength Potato Dextrose Agar (PDA) plates amended with streptomycin (10 mg/mL) and incubated at room temperature (22 °C ± 2 °C) under 12 h light/12 h dark cycle conditions for 21 days before DNA extraction. Sequences of three gene loci of 43 reference strains of species in the genus *Pseudocercospora* clade 14 as described by Crous et al. (2012) and seven representative species of closely related genera or outgroup species were obtained from GenBank in the National Centre for Biotechnology Information (NCBI). These were included in the phylogenetic analysis study (Table 1).

DNA extraction of fungal isolates

Genomic DNA of each *P. macadamiae* isolate was extracted from approximately 50 mg of mycelium using the Promega Wizard® Genomic DNA Purification Kit (Madison, WI, USA) according to the manufacturer's procedure. Two different types of genomic DNA samples were used to develop the qPCR assays. Firstly, genomic DNA from pure cultures of 13 isolates of *P. macadamiae* (Table 1), and secondly, plant genomic DNA of husk spot diseased macadamia fruits were extracted using DNeasy® Plant Mini Kit (QIAGEN, Netherlands) extraction kit, following the manufacturer's protocol. Prior to DNA extraction, approximately 50 mg fungal mycelial was homogenized in 600 μL of

Table 1 Details of fungal strains and reference strains obtained from NCBI Genbank used in this study

Species	Isolate[b]	Host	Source location[c]	Genbank accession number			
				LSU	ITS	TEF-1α	ACT
Pseudocercospora macadamiae	BRIP 29246	*Macadamia* sp.	NNSW	KU878414	KU666473	KU878459	KU878506
Pseudocercospora macadamiae	BRIP 29248	*Macadamia* sp.	QLD	KU878415	KU666514	KU878460	KU878507
Pseudocercospora macadamiae	BRIP 29253	*Macadamia* sp.	NNSW	KU878416	KU666474	KU878461	KU878508
Pseudocercospora macadamiae	BRIP 29255	*Macadamia* sp.	SEQLD	KU878417	KU666475	KU878462	KU878509
Pseudocercospora macadamiae	BRIP 29256	*Macadamia* sp.	Unknown	KU878418	KU666476	KU878463	KU878510
Pseudocercospora macadamiae	BRIP 45377	*Macadamia* sp.	SEQLD	KU878419	KU666516	KU878464	KU878511
Pseudocercospora macadamiae	BRIP 47614	*Macadamia* sp.	SEQLD	KU878420	KU666477	KU878465	KU878512
Pseudocercospora macadamiae	BRIP 47615	*Macadamia* sp.	SEQLD	KU878421	KU666478	KU878466	KU878513
Pseudocercospora macadamiae	BRIP 47616	*Macadamia* sp.	SEQLD	KU878422	KU666479	KU878467	KU878514
Pseudocercospora macadamiae	BRIP 47617	*Macadamia* sp.	SEQLD	KU878423	KU666480	KU878468	KU878515
Pseudocercospora macadamiae	BRIP 47618	*Macadamia* sp.	SEQLD	KU878424	KU666481	KU878469	KU878516
Pseudocercospora macadamiae	BRIP 47620	*Macadamia* sp.	SEQLD	KU878425	KU666482	KU878470	KU878517
Pseudocercospora macadamiae	BRIP 47622	*Macadamia* sp.	SEQLD	KU878426	KU666483	KU878471	KU878518
Pseudocercospora macadamiae[a]	BRIP 47626[a]	*Macadamia* sp.	SEQLD	KU878427	-	KU878472	KU878519
Pseudocercospora macadamiae	BRIP 47627	*Macadamia* sp.	SEQLD	KU878428	KU666484	KU878473	KU878520
Pseudocercospora macadamiae	BRIP 47628	*Macadamia* sp.	SEQLD	KU878429	KU666485	KU878474	KU878521
Pseudocercospora macadamiae[a]	BRIP 47632[a]	*Macadamia* sp.	SEQLD	KU878430	KU666486	KU878475	KU878522
Pseudocercospora macadamiae	BRIP 47648	*Macadamia* sp.	SEQLD	KU878431	KU666487	KU878476	KU878523
Pseudocercospora macadamiae[a]	BRIP 47782[a]	*Macadamia* sp.	NNSW	KU878432	KU666506	KU878477	KU878524
Pseudocercospora macadamiae	BRIP 47784	*Macadamia* sp.	NNSW	KU878433	KU666488	KU878478	KU878525
Pseudocercospora macadamiae	BRIP 47785	*Macadamia* sp.	NNSW	KU878434	-	KU878479	KU878526
Pseudocercospora macadamiae[a]	BRIP 47786[a]	*Macadamia* sp.	NNSW	KU878435	KU666489	KU878480	KU878527
Pseudocercospora macadamiae	BRIP 47787	*Macadamia* sp.	NNSW	KU878436	KU666490	KU878481	KU878528
Pseudocercospora macadamiae	BRIP 47788	*Macadamia* sp.	NNSW	KU878437	KU666491	KU878482	KU878529
Pseudocercospora macadamiae	BRIP 47789	*Macadamia* sp.	NNSW	KU878438	KU666492	KU878483	KU878530
Pseudocercospora macadamiae[a]	BRIP 47790[a]	*Macadamia* sp.	NNSW	KU878439	KU666493	KU878484	KU878531
Pseudocercospora macadamiae[a]	BRIP 47791[a]	*Macadamia* sp.	NNSW	KU878440	KU666494	KU878485	KU878532
Pseudocercospora macadamiae	BRIP 47792	*Macadamia* sp.	NNSW	KU878441	KU666495	KU878486	KU878533
Pseudocercospora macadamiae	BRIP 47793	*Macadamia* sp.	NNSW	KU878442	KU666496	KU878487	KU878534
Pseudocercospora macadamiae	BRIP 47794	*Macadamia* sp.	NNSW	KU878443	KU666497	KU878488	KU878535

Table 1 (continued)

Species	Isolate[b]	Host	Source location[c]	Genbank accession number			
				LSU	ITS	TEF-1α	ACT
Pseudocercospora macadamiae	BRIP 47795	*Macadamia* sp.	NNSW	KU878444	KU666498	KU878489	KU878536
Pseudocercospora macadamiae	BRIP 47796	*Macadamia* sp.	NNSW	-	KU666499	KU878490	KU878537
Pseudocercospora macadamiae[a]	BRIP 47797[a]	*Macadamia* sp.	NNSW	KU878445	KU666500	KU878491	KU878538
Pseudocercospora macadamiae[a]	BRIP 47799[a]	*Macadamia* sp.	NNSW	KU878446	KU666501	KU878492	KU878539
Pseudocercospora macadamiae	BRIP 47800	*Macadamia* sp.	NNSW	KU878447	KU666502	KU878493	KU878540
Pseudocercospora macadamiae	BRIP 47801	*Macadamia* sp.	NNSW	KU878448	KU666503	KU878494	KU878541
Pseudocercospora macadamiae[a]	BRIP 47803[a]	*Macadamia* sp.	NNSW	KU878449	KU666504	KU878495	KU878542
Pseudocercospora macadamiae[a]	BRIP 47811[a]	*Macadamia* sp.	NNSW	KU878450	KU666515	KU878496	KU878543
Pseudocercospora macadamiae	BRIP 47814	*Macadamia* sp.	NNSW	KU878451	KU666505	KU878497	KU878544
Pseudocercospora macadamiae	BRIP 47825	*Macadamia* sp.	CEQLD	KU878452	KU666507	KU878498	KU878545
Pseudocercospora macadamiae	BRIP 47826	*Macadamia* sp.	CEQLD	KU878453	KU666508	KU878499	KU878546
Pseudocercospora macadamiae	BRIP 47827	*Macadamia* sp.	CEQLD	KU878454	KU666509	KU878500	KU878547
Pseudocercospora macadamiae[a]	BRIP 47829[a]	*Macadamia* sp.	CEQLD	KU878455	KU666510	KU878501	KU878548
Pseudocercospora macadamiae[a]	BRIP 47836[a]	*Macadamia* sp.	CEQLD	KU878456	KU666511	KU878502	KU878549
Pseudocercospora macadamiae	BRIP 47845	*Macadamia* sp.	CEQLD	KU878457	KU666512	KU878503	KU878550
Pseudocercospora macadamiae[a]	BRIP 55526[a]	*Macadamia* sp.	SEQLD	KU878412	KU666513	KU878504	KU878551
Pseudocercospora macadamiae	BRIP 29237	*Macadamia* sp.	NNSW	KU878413	KU666472	KU878458	KU878505
Pseudocercospora proiphydis[a]	BRIP 58545[a]	*Proiphys amboinensis*	QLD	KM055434	KM055430	KM055437	-
Pseudocercospora jagerae[a]	BRIP 58549[a]	*Jagera pseudorhus*	QLD	KM055435	KM055431	KM055438	-
Pseudocercospora airliensis[a]	BRIP 58550[a]	*Polyalthia nitidissima*	QLD	KM055433	KM055429	KM055436	-
Pseudocercospora anacardii[a]	BRIP 59434[a]	*Anacardium occidentale*	QLD	-	-	-	
Pseudocercospora sp.[a]	BRIP 56007[a]	*Dodonaea* sp.	NT	-	-	-	-
Pseudocercospora sp.[a]	BRIP 56011[a]	*Grevillea* sp.	WA	-	-	-	-
Pseudocercospora sp.[a]	BRIP 56558[a]	*Mitrasacme connata*	NT	-	-	-	-
Pseudocercospora sp.[a]	BRIP 59658[a]	*Psidium* sp.	QLD	-	-	-	-
Cercospora zebrina	CBS 118790	Unknown	Unknown	KF442549.1	JX390615.1	KF253107.1	KF253609.1
Cyphellophora eucalypti	CBS 124764	*Eucalyptus sp.*	Australia	GQ3033305	GQ303274	GU384510	JQ325009
Mycosphaerella fori	CMW 9095	Unknown	Unknown	DQ204748.1	AF468869.1	DQ211664.1	DQ147618.1
Mycosphaerella gracilis	CBS 243.94	Unknown	Unknown	DQ204750.1	DQ267582.1	DQ211660.1	DQ147616.1
Pallidocercospora acaciigena	CBS 120740	*Eucalyptus sp.*	Australia	GU253698	GU269649	KF903106.1	GU320357

Table 1 (continued)

Species	Isolate[b]	Host	Source location[c]	Genbank accession number			
				LSU	ITS	TEF-1α	ACT
Passalora eucalypti	CBS 111318	*Eucalyptus saligna*	Brazil	GU253860	GU269845	GU384558	GU320548
Pseudocercospora angolensis	CBS 112933	*Citrus sp.*	Zimbabwe	GU214470	GU269836	GU384548	JQ325010
Pseudocercospora atromarginalis	CBS 114640	*Solanum sp.*	New Zealand	GU253706	GU269658	GU384376	GU320365
Pseudocercospora basiramifera	CBS 111072	*Eucalyptus pellita*	Thailand	GU352709	GU269661	KF903174.1	GU320368
Pseudocercospora basitruncata	CBS 114664	*Eucalyptus grandis*	Colombia	GU253710	GU269662	DQ211675	DQ147622
Pseudocercospora chrysanthemicola	CPC 10633	*Chrysanthemum sp.*	South Korea	GU253722	GU269675	GU384392	GU320381
Pseudocercospora cladosporioides	CBS 117482	*Olea europaea*	Tunisia	JQ324944	GU269678	GU384395	GU320383
Pseudocercospora coprosmae	CBS 114639	*Coprosma robusta*	New Zealand	JQ324946	GU269680	GU384397	GU320386
Pseudocercospora crispans	CPC 14883	*Eucalyptus sp.*	South Africa	GU253825	GU269807	GU384518	GU320510
Pseudocercospora crocea	CPC 11668	*Pilea hamaoi*	South Korea	JQ324947	GU269792	GU384502	GU320493
Pseudocercospora crousii	CBS 119487	*Eucalyptus sp.*	New Zealand	GU253729	GU269688	GU384403	GU320392
Pseudocercospora cymbidiicola	CBS 115132	*Cymbidium sp.*	New Zealand	GU253733	GU269692	GU384408	GU320397
Pseudocercospora dianellae	CBS 117746	*Dianella caerulae*	New Zealand	GU253736	GU269695	GU384411	GU320400
Pseudocercospora eucalyptorum	CPC 12406	*Eucalyptus globulus*	Australia	GU253811	GU269793	GU384503	GU320494
Pseudocercospora fori	CPC 14880	*Eucalyptus sp.*	South Africa	GU253824	GU269806	GU384517	GU320509
Pseudocercospora fraxinites	CPC 10743	*Fontanesia philyraeoides*	South Korea	GU253720	GU269672	GU384389	GU320378
Pseudocercospora fukuokaensis	CPC 14689	*Styrax japonicus*	South Korea	GU253750	GU269713	GU384429	GU320417
Pseudocercospora haiweiensis	CPC 14084	*Eucalyptus sp.*	China	GU253821	GU269803	GU384514	GU320506
Pseudocercospora hakeae	CBS 112226	*Grevillea* sp.	Australia	GU253805	GU269784	GU384495	JQ325017
Pseudocercospora indonesiana	CBS 122473	*Musa sp.*	Sumatra	GU253765	GU269735	GU384448	GU320437
Pseudocercospora ixorae	CBS 118760	*Ixora sp.*	Taiwan	GU253759	GU269726	GU384440	GU320429
Pseudocercospora kiggelariae	CPC 11853	*Kiggelaria africana*	South Africa	GU253762	GU269730	GU384443	GU320432
Pseudocercospora leucadendri	CPC 1869	*Leucadendron sp.*	South Africa	GU214480	GU269842	GU384555	GU320545
Pseudocercospora libertiae	CBS 114643	*Libertia ixioidea*	New Zealand	JQ324959	GU269733	GU384446	GU320435
Pseudocercospora lilacis	CPC 12767	*Ligustrum japonicum*	USA	GU253767	GU269737	GU384449	GU320439
Pseudocercospora longispora	CBS 122470	*Musa sp.*	Malaysia	GU253764	GU269734	GU384447	GU320436
Pseudocercospora luzardii	CPC 2556	*Hancornia speciosa*	Brazil	GU214477	GU269738	GU384450	GU320440
Pseudocercospora lyoniae	MUCC 910	*Lyonia ovalifolia* var. *elliptica*	Japan	GU253768	GU269739	GU384451	GU320441
Pseudocercospora metrosideri	CBS 118795	*Metrosideros collina*	New Zealand	GU253774	GU269746	GU384458	GU320448
Pseudocercospora myrticola	MUCC 632	*Myrtus communis*	Japan	GU253777	GU269749	GU384460	GU320451

Table 1 (continued)

Species	Isolate[b]	Host	Source location[c]	Genbank accession number			
				LSU	ITS	TEF-1α	ACT
Pseudocercospora nandinae	CBS 117745	*Nandina domestica*	New Zealand	GU253778	GU269750	GU384461	GU320452
Pseudocercospora nogalesii	CBS 115022	*Chamaecytisus proliferus*	New Zealand	JQ324960	GU269752	GU384463	GU320454
Pseudocercospora norchiensis	CBS 120738	*Eucalyptus sp.*	Italy	GU253780	GU269753	GU384464	GU320455
Pseudocercospora palleobrunnea	CBS 124771	*Syzygium sp.*	Australia	GQ303319	GQ303288	GU384509	GU320500
Pseudocercospora proteae	CPC 15217	*Protea mundii*	South Africa	GU253826	GU269808	GU384519	GU320511
Pseudocercospora rhabdothamni	CBS 114872	*Rhabdothamnus solandri*	New Zealand	JQ324964	GU269768	GU384480	GU320471
Pseudocercospora robusta	CBS 111175	*Eucalyptus robur*	Malaysia	KF902020	KF901678	KF903213	KF903437
Pseudocercospora rumohrae	CBS 117747	*Marattia salicina*	New Zealand	GU253796	GU269774	GU384486	GU320477
Pseudocercospora securinegae	CPC 10793	*Flueggea suffruticosa*	South Korea	GU253797	GU269776	GU384487	GU320479
Pseudocercospora tereticornis	CBS 124996	*Eucalyptus nitens*	Australia	GQ852647	JQ324982	GU384377	JQ325025
Pseudocercospora theae	CBS 128.30	*Camelia sinensis*	Italy	GU253838	GU269821	GU384534	GU320524
Scolecostigmina mangiferae	CBS 125467	*Mangifera indica*	Australia	GU253877	GU269870	GU384578	GU320566
Teratosphaeria alcornii	CBS 313.76	*Eucalyptus tessellaris*	Australia	GU253876	GU269866	GU384577	GU320565
Zasmidium nabiacense	CPC 12748	*Eucalyptus sp.*	Australia	KF901932.1	KF901678.1	KF903213.1	KF903437.1

[a] Isolates used in qPCR assays

[b] BRIP indicates Queensland Plant Pathology Herbarium, CBS –Centraal Bureau voor Schimmelculturen, Utrecht, The Netherlands MAC = UQ Macadamia Plant Pathology culture collection

[c] Source of isolates from northern New South Wales (NNSW), South east Queensland (SEQLD), central Queensland (CEQLD), Western Australia (WA) and Northern Teritory (NT)

nuclei lysis solution in 2 mL safe-lock tube (Eppendorf AG, Hamburg, Germany), containing an autoclaved stainless steel bead, using TissueLyser (Qiagen Pty Ltd., USA) for 2 min at 30 Hz.

PCR amplification and DNA sequencing

Partial sequences of the large subunit (LSU), ITS, ACT and TEF-1α gene loci were used for the phylogenetic analysis. The ITS gene region including the ITS-1, ITS-2 and the 5.8S nrRNA gene regions was amplified using ITS-5 and ITS-4 primers (White et al. 1990). The partial gene region of TEF-1α was amplified using EF1-728F and EF1-986R primers (Carbone and Kohn 1999), while primers ACT-512F and ACT-783R (Carbone and Kohn 1999) were used to amplify the ACT gene region. The first 900 bp of the 28S and domains D1-D3 of the rDNA operon (Crous et al. 2012) was amplified with the primers LR0R and LR7 (Vilgalys and Hester 1990). Purified genomic DNA of each *P. macadamiae* isolate served as the template for the PCR amplification in a 20 μL reaction mixture containing 10 μL of 10 mM Phusion Master Mix (Thermo Fisher Scientific Inc.) and 1 μL each of forward and reverse primers. Amplification was performed in a SuperCycler Thermal Cycler (Kyratec, Australia) programmed for initial denaturation for 60 s at 98 °C followed by 35 cycles at 98 °C for 10 s, 62 °C for 30 s and 72 °C for 45 s, with a final extension step at 72 °C for 5 min. PCR amplicons were separated in 1 % agarose gel (BIOLINE, Australia) stained with gel red in 0.5 % Tris-borate EDTA buffer solution and viewed under UV light using a Molecular Imager® GelDoc™ (Bio-Rad Laboratories Inc.). The amplicon sizes were determined against a 1 kb HyperLadder (BIOLINE, Australia) and then the targeted PCR amplicons were purified using a Roche™ High Pure PCR Product Purification Kit (Roche Applied Science, Manheim, Germany) according manufacturer's instructions**.** DNA sequencing was carried out at Macrogen Inc. South Korea, using the same primers used for amplification.

Phylogenetic analysis and genetic variation of *P. macadamiae*

MEGA (Molecular Evolutionary Genetics Analysis) v. 6 (Tamura et al. 2013) software was used to manually assemble the forward and reverse DNA sequences into consensus fragments. In order to provide consistency and quality of the sequences, the chromatograms of the sequences were manually checked, aligned and primer sequences were trimmed off at both ends of the sequences. NCBI BLAST megablast search (Altschul et al. 1990) procedure in NCBI was used to determine the identity of each set of nucleotide sequences. All sequences obtained in the study have been submitted to Genbank. In order to study the relatedness of *P. macadamiae* isolates to other members of the genus *Pseudocercospora*, multiple nucleotide sequence alignments and phylogenetic analyses were performed with the 43 reference strains from *Pseudocercospora* clade 14 (Crous et al. 2012) and the seven related genera (Table 1). Prior to phylogeny reconstruction, concatenated sequences of all four gene loci of the 47 *P. macadamiae* isolates were tested for maximum parsimony analysis in MEGA 6. Final maximum parsimony analysis of the reference strains with the *P. macadamiae* isolates based on the combined ITS, TEF-1α and ACT sequence was performed in MEGA 6, using the heuristic search option with 100 random taxon additions and tree bisection and reconnection as the branch swapping algorithm. Tree stability was tested by 1000 bootstrap replicates and tree was rooted to *Passalora eucalypti* (CBS 111318). In order to estimate the genetic variation within the *P. macadamiae* population, DnaSP version 5.10.1 (Librado and Rozas 2009) was used to calculate the coefficient of genetic differentiation (G_{ST}) (Nei 1973), nucleotide diversity (π) was calculated to determine the average proportion of nucleotide differences between all possible pairs of sequences. Estimates of gene flow (*Nm*) (McDermott and McDonald 1993) was determined by calculating the number of segregating sites and Tajima'D statistics was used to test the goodness of fit of the sequences to infinite-sites model, which assumes each mutation event alters only a single nucleotide site (Tajima 1989).

Sampling and DNA extraction of diseased husks at different stages of symptom development

Two sets of samples of macadamia fruit pericarp with visible husk spot symptoms at different stages of symptom expression on cultivar A38 that are very susceptible to husk spot (Miles et al. 2010b; Akinsanmi et al. 2012), were collected from different commercial orchards. Samples of asymptomatic macadamia fruit that served as stage 1 infection, were collected in close proximity to samples with visible husk spot symptoms on the same

tree. Visibly diseased samples were categorised into three stages of symptom expression on the husk as described by Miles et al. (2009). Stage 2 refers to fruits with chlorotic flecking to spot symptoms; stage 3 refers to fruits with advanced chlorotic lesions at a diameter ~ 1 mm with brownish centre; and stage 4 refers to fruits with necrotic tan spot symptoms at a diameter ~ 3 mm (Fig. 1). Sections of symptomatic tissue were obtained from the husk with a 5 mm diameter cork-borer. The cork borer was flame sterilized between samples. Three pieces of each tissue sample were collected into 1.5 ml Eppendorf tubes (Eppendorf AG, Hamburg, Germany) and freeze dried overnight. Approximately 20 mg of the freeze dried husk material was ground in liquid nitrogen using a sterile mortar and pestle from which DNA was extracted using the DNeasy® Plant Mini Kit. In addition, genomic DNA was extracted from four healthy husk tissues that served as uninfected control samples using the same protocols as described above. DNA concentrations were measured using BioDrop DUO (BioDrop, UK). Based on the result of the phylogenetic analysis, a subset (13) of the *P. macadamiae* isolates were selected and used to develop the qPCR assays. Isolates of *Diaporthe* sp. and *C. gloeosporioides* were obtained from macadamia tissue with husk rot symptoms, grown on PDA for 7 days and were used in the qPCR assays for design and cross-specificity testing. At least two independent DNA extractions were performed on the plant samples and fungal cultures for qPCR assays.

Design of primers and probes for qPCR assays

Two sets of primers and probes were designed. Firstly, aligned ITS sequences of the 13 *P. macadamiae* isolates and an annotated BRIP 45377 isolate (EU547234) was used to locate the 18S, ITS-1, 5.8S, ITS-2 and 28S regions on the ITS gene. A TaqMan® assay was designed using RealTimeDesign™ Software (Biosearch Technologies). In order to select the most suitable and unique primer sets and hybridization probe for *P. macadamiae*, the recommended primer sets and probes were analysed using BLAST and were also compared manually against ITS sequences of other *P. macadamiae* isolates. The ITS-based hybridization probe assay (PMI) which consisted of forward primer (PMI-f), reverse primer (PMI-r) and hybridization probe (PMI-p) that were more specific to *P. macadamiae* than other *Pseudocercospora* species or fungi (*Diaporthe* sp. and *C. gloeosporioides*) were selected (Table 2). In order to

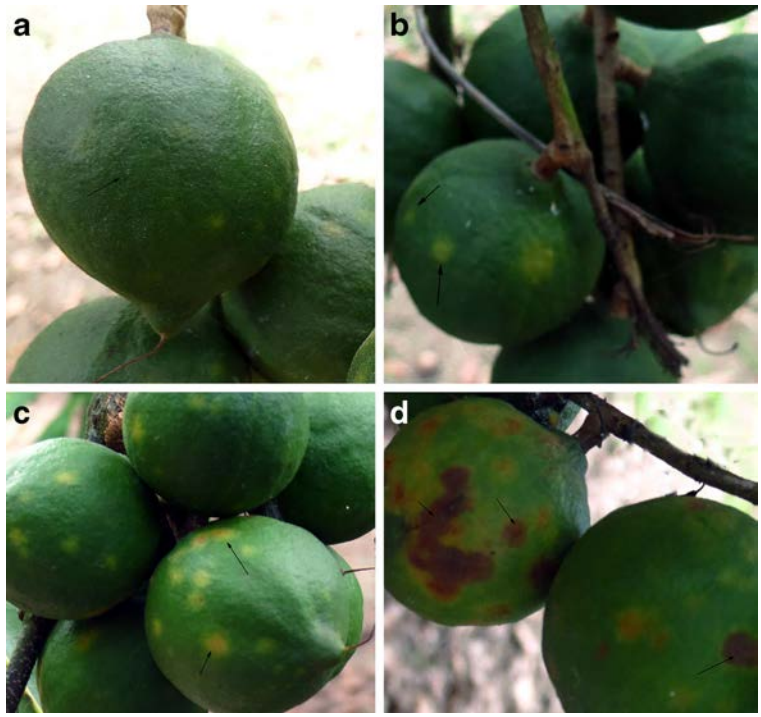

Fig. 1 Macadamia fruits at different stages of husk spot symptoms caused by *Pseudocercospora macadamiae*. Arrows indicate symptoms of each stage on the husks: (**a**) Stage 1 = asymptomatic; stage 2 = chlorotic spot to flecking; stage 3 = advanced chlorotic lesions with brownish centre; and stage 4 = necrotic spots

Table 2 Primers and hybridisation probes developed in this study based on internal transcribed spacer (ITS) and translation elongation factor 1-alpha (TEF-1α) gene loci for the detection and real time quantification of DNA concentration of *Pseudocercospora macadamiae* in macadamia husk

Name	Sequence (5′-3′)	sGene region
PMI-f	TCCACAACGCTTAGAGACGAAT	ITS
PMI-r	GGTCTCCAAACACTGCATC	ITS
PMI-p	(d FAM)CCAGGCTTGAGTGGTGA AATGACGCT(BHQ-1)	ITS
PME-f	CAGCCAATGACTTCACCTCAC	TEF-1α
PME-r	AGGCATCTCGTCAGCAGCTAT	TEF-1α
PME-p	(d FAM)CACTGCACATTCTTCCA CCTCCATGA(BHQ-1)	TEF-1α

achieve accurate quantification of *P. macadamiae*, a further hydrolysis-probe assay was designed based on the single-copy gene, TEF-1α. The same procedures as described above were followed to design the TEF-1α assay (PME) and the best optimal primer set of forward (PME-f) and reverse primers (PME-r), and probe (PME-p) were selected (Table 2). In both assays, dual-labelled BHQ probes were used, which consisted of a 5′ 6-FAM (fluorescein) reporting dye and 3′ black-hole quencher (BHQ-1) dye (Biosearch Technologies, California) (Table 2). Real-time PCR was performed on a Rotor-Gene 6000 (Corbett Research) using QuantiTect® Probe PCR Kit (QIAGEN, Netherlands). Real-time PCR for PMI assay was carried out in a final volume of 20 μL, consisting of 2.0 μL of DNA template, 0.4 μM PMI-f and PMI-r, 0.2 μM PMI-p and 10 μL 2× QuantiTect Probe PCR Master Mix (QIAGEN, Netherlands). Three replicates of no-template controls (NTC) were included in the reaction where DNA template was replaced with nuclease free water in the NTC. Cycling conditions for the assay consisted of initial denaturation at 95 °C for 15 min. Followed by 45 cycles of denaturation at 94 °C for 15 s and combined annealing/extension at 60 °C for 60 s. Fluorescence signal was acquired on green channel and optimisation gain was set for the positive control tube. Assay validation for PME-f, PME-r and PME-p was tested using the same reaction set-up as described for PMI assay.

Specificity and of the qPCR assays for *P. macadamiae*

The TaqMan® assays were optimised and their specificity for *P. macadamiae* was performed against other pathogens which infect macadamia husk (*C. gloeosporioides* and *Diaporthe* sp.) and eight *Pseudocercospora* species from other Australian native plants (Table 1). Sensitivity of the qPCR assays were validated using an eight serial dilution process up to 10^{-7} of DNA from both pure cultures and diseased tissues. Reproducibility of the qPCR assays was verified by replicates in intra-assay and between repeat runs. The efficiency (ef) values of the reaction were obtained from the quantification report generated by Rotor-Gene Q Series Software 2.3.1, calculated based on the eq. $ef = 10^{(-1/m)-1}$, where m represents the gradient of best fit line between cycle threshold (Ct) and DNA concentration. The relationship between Ct values and DNA concentrations from serial dilution was used to determine the sensitivity of the assays. The resulting Ct values and concentrations were used to deduce the reproducibility of the qPCR assays. Statistical analysis based on analysis of variance (ANOVA) procedure in GenStat version 16.1 (VSN International Ltd., UK) was performed to evaluate the intra- and inter-assays and to compare the reproducibility and consistency of Ct values and the estimated DNA concentrations at different orders of magnitude for both PMI and PME assays. Amplifications of the qPCR products were also confirmed in gel electrophoresis in 1 % agarose and visualized under UV light.

Quantification of P. Macadamiae in planta at different stages of disease development

Quantification of *P. macadamiae* biomass in tissue samples at different stages of symptom development was carried out using both the PMI and PME assays. The amount of fungal DNA in the tissue samples was calculated using the standard regression curves derived from the serial dilution runs of genomic DNA of pure culture of *P. macadamiae*. This procedure was repeated three times to obtain the mean of Ct values and concentrations.

Results

Phylogenetic analysis of *P. macadamiae*

BLAST searches of individual *P. macadamiae* isolate revealed differences in identity among the loci, in sequence homology with other members of the

genus *Pseudocercospora*. The ITS sequence analysis revealed 91–100 % nucleotide sequence homology of *P. macadamiae* with other members of the genus *Pseudocercospora.* ACT gene sequence showed 85–100 % nucleotide sequence similarity with other species within the genus *Pseudocercospora*, while LSU sequences showed 83–100 % identity to species of genus *Pseudocercospora*. TEF-1α sequence analysis of *P. macadamiae* isolates showed the least (76–99 %) sequence homology with other *Pseudocercospora* species. Phylogenetic analysis of the concatenated sequences of three gene loci showed that the *P. macadamiae* isolates clustered separately from the other *Pseudocercospora* species (Fig. 2). The phylogenetic analysis showed that the 47 *P. macadamiae* isolates were not grouped on the basis of source of isolation (location) because isolates from Queensland and New South Wales were grouped together (Fig. 2). Analysis of the genetic variation among the *P. macadamiae* isolates for each of the gene region sequenced showed that the lowest nucleotide diversity ($\pi = 0.004$) was observed in the ITS gene region and highest ($\pi = 0.180$) in the LSU gene region (Table 3). Similarly, the lowest coefficient of genetic differentiation ($G_{ST} = 0.060$) was observed in the ITS gene region whereas TEF-1α gene region had the highest G_{ST} (Table 3). Although the number of segregating site was lowest ($S = 12$) in the ACT gene region, the relative percentage of S with the number of nucleotide site ($n = 158$) was the highest (7.6 %), compared to the LSU gene region with the highest number of segregating site ($S = 27$) and n of 1153 (Table 3). The ITS region had the lowest (3.7 %) relative percentage of segregating site, the largest Tajima's D statistic value with the most unequal frequencies between rare alleles and the most common alleles among the populations. Generally, the Tajima's D statistic values were relatively similar among the four gene loci (Table 3). Estimates of gene flow *Nm* among *P. macadamiae* population in the ITS and LSU gene loci were

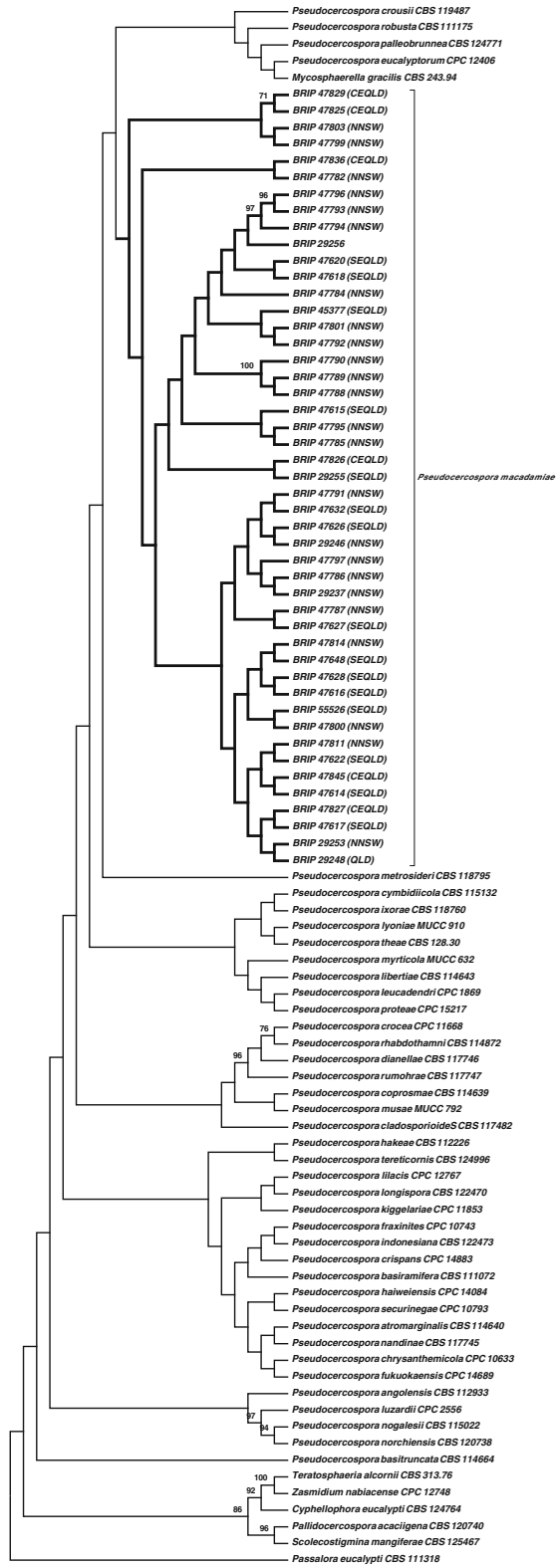

Fig. 2 Maximum parsimony phylogram constructed based on the combined partial sequence of ITS, ACT and TEF-1α gene loci for *Pseudocercospora macadamiae* isolates within the *Pseudocercospora* lineage clade 14. Source of each *P. macadamiae* isolate is shown in bracket in front of the accession name. NNSW indicates northern New South Wales; SEQLD and CEQLD indicate south east and central Queensland, respectively. Bootstrap value >50 is shown based on bootstrap test with 1000 replicates

Table 3 Statistical analysis results showing the number of segregating site (*S*), nucleotide diversity (π), Tajima's D statistics, coefficient of differentiation (G_{ST}) and gene flow (*Nm*) of the nucleotide sequences of the large subunits (LSU), internal transcribed spacer (ITS), translation elongation factor 1-alpha (TEF-1α) and actin (ACT) gene loci of 47 *Pseudocercospora macadamiae* strains

Gene region	No. of nucleotide site	S	π	Tajima's D	G_{ST}	*Nm*
ACT	158	12	0.012 ± 0.003	- 1.66	0.235	0.81
ITS	738	27	0.004 ± 0.002	- 2.43	0.060	3.93
LSU	1153	72	0.180 ± 0.026	- 1.72	0.067	3.49
TEF-1α	348	24	0.014 ± 0.003	- 1.83	0.302	0.58
Combined loci	2372	206	0.021 ± 0.005	- 2.46	0.074	3.13

comparatively lower than those of ACT and TEF-1α gene loci (Table 3). Although all the *P. macadamiae* isolates shared the same phylogenetic node, the isolates were distributed into four subsets (Fig. 2).

Design of specific qPCR primers and probes for *P. macadamiae*

The multiple ITS sequence alignment of *P. macadamiae* isolates revealed a conserved region from the other husk-infection fungi (*Diaporthe* sp. and *C. gloeosporioides*). The sequence specific locations within ITS2 region for PMI-f and ITS1 region for PMI-r was targeted to design the specific primers while the hydrolysis probe (PMI-p) was located in the 5.8S region (Table 2). The selected PMI primers amplified a target region of 295 bp size from all *P. macadamiae* isolates and eight Australian native *Pseudocercospora* species tested. The alignment of representative sequences of TEF-1α gene region for *P. macadamiae* isolates with the *Diaporthe* sp. (MAC1303a) and *C. gloeosporioides* (BRIP 27489) strains showed sequence specific locations for PME-f, PME-r and PME-p for *P. macadamiae* (Table 2). The selected PME assays amplified a target region of 122 bp size from all the *P. macadamiae* isolates. Both PMI and PME qPCR assays showed good amplification efficiencies at the recommended annealing temperature (60 °C) for the enzyme.

Specificity of qPCR assays

Both PMI and PME assays amplified *P. macadamiae* and none of the other macadamia fungi associated with macadamia husk. The identity of the fungal DNA amplified *in planta* in the real time PCR assay was confirmed as *P. macadamiae* by ITS sequencing of the amplicon using ITS4/5 primers. The PMI assay amplified other species of *Pseudocercospora* tested in the study, whereas in the PME assay, no amplification was observed for other *Pseudocercospora* species (Table 4). Amplifications in the PMI assay occurred at earlier cycle threshold than the PME assays (Fig. 3; Table 4). The mean Ct values of PMI assay of DNA of pure culture of *P. macadamiae* isolates was 16.93 ± 0.71, while the mean Ct values of PME assay was 21.20 ± 0.80. Similarly, fungal DNA was detected *in planta* earlier using PMI assay (Ct value =32.36 ± 0.28) than PME assays (Ct value =38.07 ± 1.20). Ct values for other species of *Pseudocercospora* using the PME assay were >42, outside the window of linearity for detection in the qPCR assay (Table 4).

Sensitivity and reproducibility of the qPCR assays

The PMI assay detected *P. macadamiae* in pure culture up to a dilution of 10^{-6} ng/μL DNA concentration, while the PME assay detected *P. macadamiae* at 10^{-5} ng/μL DNA concentration. Similarly, both assays detected *P. macadamiae* fungal DNA in asymptomatic macadamia husks but not in healthy plant tissues. The efficiencies of both qPCR assays were within the recommended range for qPCR (Brisson et al. 2004) and their R^2 and slope (m) values indicated that both assays functioned in the optimal range (Fig. 4). The qPCR assays were highly reproducible between repeat runs. ANOVA confirmed the consistency of the Ct values and at different orders of magnitude, the Ct values were significantly ($P < 0.001$) different but not the estimated DNA concentrations ($P = 0.279$).

Table 4 Cycle threshold (Ct) values of real time PCR assays of DNA concentration at 1 ng/ul of 13 representative *Pseudocercospora macadamiae* strains and other *Pseudocercospora* species evaluated using the internal transcribed spacer (PMI) and translation elongation factor-1a (PME) primers and probes

Species	Accession no.	Ct values (mean ± SD) of PMI	Ct values (mean ± SD) of PME
Pseudocercospora macadamiae	BRIP 47626	17.82 ± 0.08	21.64 ± 0.36
Pseudocercospora macadamiae	BRIP 47632	16.46 ± 0.49	20.11 ± 0.15
Pseudocercospora macadamiae	BRIP 47782	16.06 ± 0.23	22.85 ± 0.04
Pseudocercospora macadamiae	BRIP 47786	17.44 ± 0.07	22.08 ± 0.10
Pseudocercospora macadamiae	BRIP 47790	17.26 ± 0.68	22.19 ± 0.04
Pseudocercospora macadamiae	BRIP 47791	17.14 ± 0.65	22.00 ± 0.17
Pseudocercospora macadamiae	BRIP 47797	17.57 ± 0.12	21.40 ± 0.24
Pseudocercospora macadamiae	BRIP 47799	17.87 ± 0.32	21.95 ± 0.06
Pseudocercospora macadamiae	BRIP 47803	16.37 ± 0.36	23.10 ± 0.20
Pseudocercospora macadamiae	BRIP 47811	17.16 ± 0.06	21.01 ± 0.25
Pseudocercospora macadamiae	BRIP 47829	15.64 ± 0.04	22.54 ± 0.44
Pseudocercospora macadamiae	BRIP 47836	17.88 ± 0.06	21.77 ± 0.04
Pseudocercospora macadamiae	BRIP 55526	15.77 ± 0.11	20.61 ± 0.54
Pseudocercospora sp.	BRIP 56007	18.51 ± 0.34	>42
Pseudocercospora sp.	BRIP 56011	18.65 ± 0.45	>42
Pseudocercospora sp.	BRIP 56558	18.71 ± 0.04	>42
Pseudocercospora proiphydis	BRIP 58545	18.48 ± 0.32	>42
Pseudocercospora jagerae	BRIP 58549	15.15 ± 0.36	>42
Pseudocercospora airliensis	BRIP 58550	13.98 ± 0.84	>42
Pseudocercospora anacardii	BRIP 59434	13.28 ± 0.06	>42
Pseudocercospora sp.	BRIP 59658	17.74 ± 0.73	>42

Correlation between symptom developmental stages and P. Macadamiae biomass

The mean of Ct values and DNA concentrations in the tissue samples at the different stages of symptom expression were quantified using both PMI and PME assays (Fig. 5). Both assays detected *P. macadamiae* in asymptomatic tissue samples. The concentration of *P. macadamiae* biomass in the plant tissue increased with the disease progression (Fig. 5). There were approximately 10-fold increases in DNA concentrations between each stage of symptom development from stage 1 through to stage 3, reaching a plateau phase at stage 4 (Fig. 5). Significant differences ($P < 0.0001$) occurred between the Ct values and estimated fungal DNA concentrations at different symptom stages for both the PMI and PME assays. Multiple comparison of means using Turkey's test revealed that Ct values ($P = 0.0541$) and concentrations ($P = 0.0599$) of *P. macadamiae* at stage 3 and 4 were not significantly different in the PME assay.

Discussion

This study revealed that *P. macadamiae* that cause husk spot of macadamia in Australia is a distinct species within the phylogenetic lineages of genus *Pseudocercospora* as described by Crous et al. (2012). This confirmed that *P. macadamiae* belongs to the defined clade 14 in the phylogenetic lineages of *Pseudocercospora.* The contribution of each gene region to the phylogenetic structure of the *P. macadamiae* isolates showed that the inclusion of TEF-1α sequences strongly influenced the clustering pattern. High-level multi-furcation of the internal branches was observed in the phylogenetic re-construction of each of the other three loci (LSU, ITS and ACT). Hence, concatenated sequence of the multiple gene loci were needed to

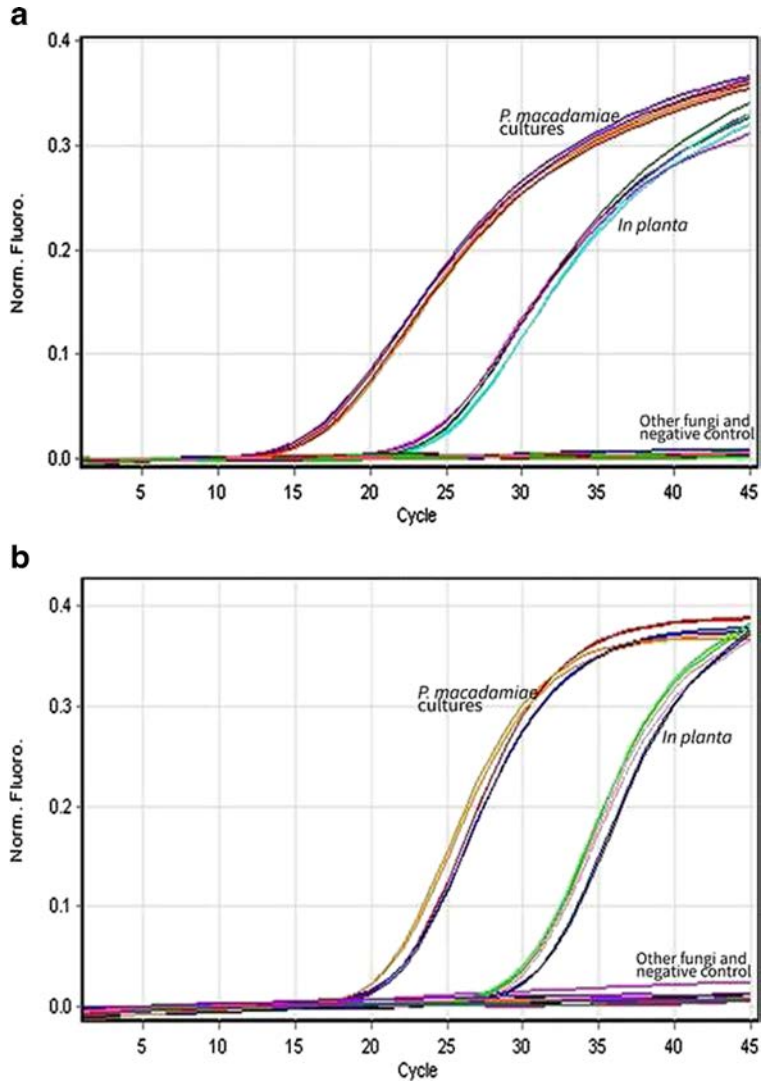

Fig. 3 Real time PCR Fluorescence (norm) curves of 45 cycles using (**a**) internal transcribed spacer and (**b**) translation elongation factor 1-alpha assays for detection and DNA quantification of *Pseudocercospora macadamiae* isolates in pure cultures and *in planta* in macadamia husks with visible husk spot symptoms. Other fungi (*Diaporthe* sp. and *Colletotrichum gloeosporioides* BRIP 27489) and no template control served as negative controls

resolve the *Pseudocercospora* species. Our results support the report by Crous et al. (2012), who reported limited genetic diversity and only 17 % of success rate in ITS sequences for distinguishing species of genus *Pseudocercospora* (Crous et al. 2012). Consequently, the concatenated sequences of multi gene loci including ITS, ACT and TEF-1α gene were used to resolve the species within the genus (Crous et al. 2012). *P. macadamiae* isolates clustered independent of their geographical origin, thus, showed a narrow pattern of isolation by distance consistent with long-term gene flow between the regions. The low G_{ST} values across the four gene loci suggests no population differentiation in the *P. macadamiae* population. Our results did not support high genetic diversity in the *P. macadamiae* populations. The low G_{ST} value of the concatenated sequences is consistent with the findings from previous studies on genetic structure of *P. macadamiae* using PCR-RFLP (Miles 2011) and AFLP (Akinsanmi et al. unpublished), which revealed high genetic similarity (0.79 to 0.95) between the isolates. The estimates of gene flow (*Nm*) among the populations was 3.13 migrants per generation, which indicated substantial migration among the *P. macadamiae* populations, thus, preventing genetic divergence in the population (Hartl and Clark 1997). However, our findings are in contrast to Wright (1993) who used RAPD analysis and obtained high level of diversity among six isolates of *P. macadamiae* and grouped the isolates on the basis of their geographic location.

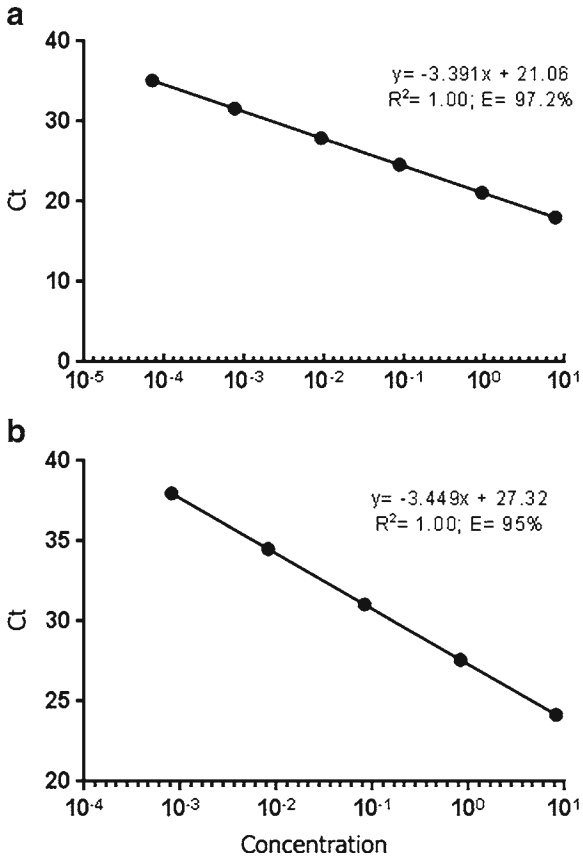

Fig. 4 Standard curves of cycle threshold (Ct) values for real time hybridization probe assays of (**a**) internal transcribed spacer and (**b**) translation elongation factor 1-alpha gene loci for quantification of *Pseudocercospora macadamiae* DNA concentration extracted from pure culture in 10-fold serial dilution. E = efficiency values based on the equation $10^{(-1/m)-1}$, where m represents the gradient of best fit line between cycle threshold (Ct) and DNA concentration

The two qPCR assays developed in this study based on the ITS (PMI) and TEF-1α (PME) are robust for early detection and quantification of *P. macadamiae* in macadamia husk. Both assays showed by 10-fold increase in *P. macadamiae* biomass in macadamia husk between consecutive stages of husk spot symptom expression. Both PMI and PME assays distinguished the target *P. macadamiae* from other fungi (*Diaporthe* sp. and *C. gloeosporioides*) that also infect macadamia husk. Due to its fixed copy number as a highly conserved protein-coding gene, the PME assay was more robust for quantifying *P. macadamiae* biomass than the PMI assay. In addition, the PME assay strongly differentiated *P. macadamiae* from other *Pseudocercospora* species compared with the PMI assay. TEF-1α gene region has gained increasing utility in phylogenetic analysis and has been proven to be useful in distinguishing closely related species in the genus *Fusarium* (Seifert and Levesque 2004; Kristensen et al. 2005) and genus *Armillaria* (Hasegawa et al. 2010; Ross-Davis et al. 2012). This could explain the more specificity of the PME assay compared with the PMI assay in the detection and quantification of *P. macadamiae*. Regardless of the cross-specificity of the PMI assay to other *Pseudocercospora* species, there is lack of evidence of coexistence of multiple *Pseudocercospora* species in macadamia fruit pericarp. By nature, majority of species within the genus *Pseudocercospora* are host-specific, therefore, it is highly unlikely for different species to share a common host plant (Crous et al. 2012). Consequently, due to the relatively high sensitivity of detection of *P. macadamiae* in macadamia husk, positive detection using the PMI assay may be regarded as conclusive detection of *P. macadamiae* in macadamia. The PMI assay has additional utility for the detection of *Pseudocercospora* in other hosts. Therefore, the PMI assay may be extended to act as internal control assay for studies involving other *Pseudocercospora* species.

Both qPCR assays showed high sensitivity in serial dilution runs, while optimal detection level within the window of detection was 10^{-6} ng/μL for the PMI assay and 10^{-5} ng/μL for the PME assay. Consistency of both PMI and PME assays was demonstrated in the intra- and inter-assays. This highlights the need for complementary assays which have different roles, in this case, the PMI assay to provide genus-level detection, and the PME assay to allow for accurate quantification of *P. macadamiae*. This study is the first report for two TaqMan®-based qPCR assays, using ITS and TEF-1α gene loci, developed for the genus *Pseudocercospora*. A semi-quantitative qPCR assay based on SYBR Green of the ITS gene region that showed high specificity to the genus *Pseudocercospora* had also been developed (Zahn et al. 2011).

Quantification of *P. macadamiae* biomass in plant tissue was strongly correlated to the four stages of symptom development. The results showed significant ($P < 0.0001$) differences in the Ct values and concentrations of *P. macadamiae* biomass in the diseased tissue at different stages of symptom expression. The description of the host colonisation process of

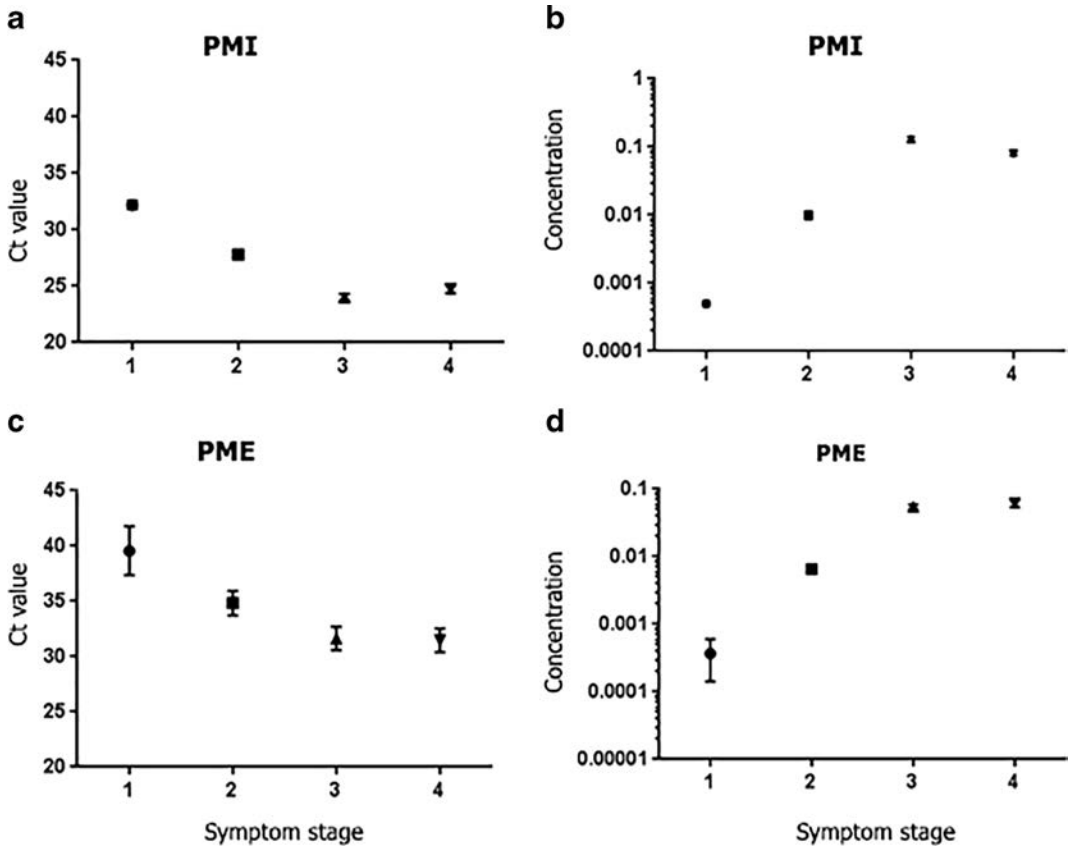

Fig. 5 Mean of cycle threshold (Ct) values and DNA concentrations of *Pseudocercospora macadamiae*, in macadamia husks at different stages of husk spot symptom development, obtained in real time hybridization probe assays using primers of the internal transcribed spacer (PMI) and translation elongation factor 1-alpha (PME) gene loci. Stage 1 = asymptomatic; stage 2 = chlorotic flecking to spots; stage 3 = advanced chlorotic lesions with brownish centre; stage 4 = necrotic lesions

P. macadamiae in macadamia is supported by the Ct values and estimated concentration of fungal DNA in infected plant tissues (Miles et al. 2009). Therefore, our results support the findings by Miles et al. (2009) who associated chlorotic flecks stage in husk spot of macadamia with sparse network of hyphae, while a denser mycelial mat was associated with more advanced necrotic lesions. A rapid increase in *P. macadamiae* biomass of approximately 10-fold was observed between earlier stages of husk spot symptom development. Significant increase in fungal biomass was observed between asymptomatic (stage 1) and chlorotic spot to flecking (stage 2), and from stage 2 to advance chlorotic lesions (Stage 3), but not between stage 3 and the necrotic lesion (stage 4). This suggests that the further fungal growth between at stage 3 and stage 4 may be limited, where the development of necrotic symptom is a reflection of the necrotrophic characteristic of *P. macadamiae.*

Early detection of plant pathogenic fungi before visible symptoms appear is crucial and underpins the development and its application in disease control. Appearance of husk spot symptoms in macadamia normally takes place at the later physiological stages of fruit development. Infection that occurs at early stages of fruit development (at match-head sized and pea-sized stages), results in long incubation period before symptom expression (Akinsanmi et al. 2007; Miles et al. 2010a). In contrast, infection at later stages of fruit development has shorter incubation period (Wright 1993). The mechanism that triggers symptom expression in the husk spot pathosystem is not well understood. It is possible that anatomical or biochemical changes in lignifications, sugars, enzymes activation and phenols in macadamia fruit development (Francis 1928; Strohschen 1986; Miles et al. 2009) may influence husk spot symptom expression. The qPCR assays developed in this study can therefore

serve as rapid detection tools for *P. macadamiae* infection without the necessity for fungal isolation and culturing. Using these assays would offer significant advantages for future research on husk spot infection process, which is currently limited before symptom expression. The plant samples used in this study were obtained from the same cultivar (A38), further studies are needed to examine if there are differences between *Macadamia* species and cultivars. These qPCR primer sets would be useful in future research as internal control assay for *Pseudocercospora* species and serve as tools for monitoring the infection and progression of diseases caused by *Pseudocercospora*. The combination of both PMI and PME assays will provide strong and conclusive inference for detection and quantification of *P. macadamiae* in macadamia.

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MACADAMIA FUND

Integrated Management of Diseases in Macadamia Industry

Appendix 2: Details of methodology, results and conclusions of research activity

(b) Phytophthora management – impact of soil health

Dr Olufemi A. Akinsanmi
The University of Queensland

Project number: MC12007

Soil health management is a precursor to sustainable control of *Phytophthora* in macadamia

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Abstract

Different commercial cultivars of *Macadamia integrifolia* were examined for their relative resistance to *Phytophthora cinnamomi* in different soil conditions at the seedling stage in the glasshouse and grafted trees under field conditions. Results showed differences in susceptibility to *P. cinnamomi* exist among macadamia cultivars at both the seedling and tree growth stages. Significant variations were observed in the above-ground parameters (seedling, height and vigour, stem diameter and leaf area). Disease severity in the below-ground parameters (root weight and root volume) was influenced by the level of *P. cinnamomi* in the soils. Under field conditions, severity of trunk canker and tree dieback was significantly higher in the untreated control trees than trees that were treated once with metalaxyl and potassium phosphonate before planting in the *Phytophthora*-infested soil. At 4 years after planting, tree height was 40-60% lower in the untreated control trees compared to the trees treated at planting. Possible influence of soil nutrition and effect of rootstock × scion interaction on performance of the scions in soils infested with *P. cinnamomi* is discussed.

Keywords: hypoxia, macadamia, *Oomycetes*, *Phytophthora*, *Proteaceae*, soilborne, tree nut

INTRODUCTION

A number of *Phytophthora* species have been reported to cause various diseases in commercial macadamia, *Macadamia integrifolia* and *M. tetraphylla*, and their hybrids. In some cases, a single or multiple *Phytophthora* species can cause similar or different disease symptoms in macadamia (Akinsanmi and Drenth, 2013b; Desegura, 1970; Keith et al., 2010; Ko and Kunimoto, 1994, 1995; Mbaka et al., 2009; Pegg, 1973; Serfontein, 2008; Zentmyer, 1962). *Phytophthora* infects almost all parts of the macadamia tree, including roots, leaves, flowers, stems and the trunk. In the USA, *P. capsici* (*P. tropicalis*) is associated with macadamia quick decline syndrome, stem canker and bleeding from the trunk (Keith et al., 2010). *P. capsici* was found to infect macadamia racemes, tree bark and wood tissue and caused girdling of mature trees leading to their death (Ko and Kunimoto, 1995) while *P. cinnamomi* was associated with stem canker only (Ko and Kunimoto, 1994; Zentmyer, 1962). In Costa Rica, *P. palmivora* was reported to attack macadamia (Desegura, 1970). In Kenya, *P. cinnamomi* caused root rot and trunk canker (Mbaka et al., 2009). In South Africa and Australia, *P. cinnamomi* is associated with stem canker, tree decline and dieback (Pegg, 1973; Serfontein, 2008). Among all the species affecting macadamia, *P. cinnamomi* is the most widespread, affecting macadamia in all producing countries.

In macadamia, it appears that stem canker and decline symptoms caused by *P. cinnamomi* progress slowly; consequently, at the early stage, expression of disease symptoms is often misdiagnosed. In Australia, above-ground symptoms of macadamia tree decline often appear following prolonged water-logging or drought (Akinsanmi and Drenth, 2013a). Soil fertility, poor irrigation, poor drainage, tree age and rootstock-scion interactions are suspected to influence tree decline severity (Akinsanmi and Drenth, 2013a). Previous observations indicate that macadamia roots are resistant to *P. cinnamomi* (Zentmyer, 1960), but subsequent reports suggest that *P. cinnamomi* causes root necrosis and root rot in macadamia (Ko and Kunimoto, 1976; Serfontein, 2008).

At present, there is no information on the relative differences in resistance to *P. cinnamomi* among commercial cultivars. However, a preliminary study suggests putative differences in resistance to *P. cinnamomi* exist among the two *Macadamia* species, *M. integrifolia* and *M. tetraphylla* (Zentmyer and Storey, 1961). The use of rootstocks with genetic resistance or tolerance to *Phytophthora* in combination with good soil management offers an economical approach to managing *Phytophthora* tree decline and trunk canker in macadamia. This study was undertaken to evaluate the relative resistance of different commercial macadamia cultivars to *P. cinnamomi* and to determine whether disease severity may be reduced in the presence of the pathogen by managing soil health or soil conditions against *P. cinnamomi* in macadamia at the seedling production and tree planting stages.

MATERIALS AND METHODS

Effect of *Phytophthora cinnamomi* on macadamia seedling development

In order to test the effect of *P. cinnamomi* on macadamia root and seedling development, three macadamia cultivars 'H2', '246' and 'A4' were planted in soil containing different levels of *Phytophthora* inoculum. Germinated macadamia nuts (with developing hypocotyl from the cracked shell) were planted in pots containing three different soil mixtures: *Phytophthora*-infested soil only, 1:1 ratio mixture of *Phytophthora*-infested and sterilised soil and sterilised soil only. The *Phytophthora*-infested soil was obtained from the base of an avocado tree with severe Phytophthora root rot caused by *P. cinnamomi* (Smith et al., 2011). Each germinated nut was planted at approximately 30 mm depth in 100-mL pots with a perforated bottom and 20 nuts of each cultivar were used per treatment. At planting, each pot was watered with 15 mL sterile distilled water, thereafter the pots were watered weekly and kept in the glasshouse at 25±3°C. Above- and below-ground parameters were recorded. These included percent seedling emergence, seedling height measured from the soil line to the tip of topmost leaf, leaf area measured as cumulative perimeter of all leaves on each seedling and stem diameter measured at the soil line. These above-ground parameters were recorded monthly for 5 months. Seedling vigour was calculated as $(h_f/h_i)^{[1/(tn\text{-}1)\text{-}1]}$, where h_f and h_i are the final and the initial seedling heights, respectively and t_n is the number of days between h_f and h_i. At the end of the trial, the seedlings were removed from the pots, the roots washed under slow running tap water, blot dried and the weight of the whole seedling (with leaves) and the root weight (below soil line level) recorded. Roots were observed for symptoms of root rot and necrosis. Presence or absence of *P. cinnamomi* in the roots was confirmed through isolation from root tissue on V8-juice agar medium while the soils were baited for the presence of *P. cinnamomi* using germinated New Zealand blue lupins seedlings.

Effect of *Phytophthora cinnamomi* on macadamia tree development

Effect of *P. cinnamomi* on grafted trees of cultivars '816' and '842' on 'H2' seedling rootstocks was assessed under field conditions. One set of the trees were planted with soil application of 60 g of metalaxyl-M (Ridomil® Gold 25 G, Syngenta) and bark application with potassium phosphonate applied at 20% v/v phosphite in 2% v/v bark penetrant Pulse® (Nufarm Australia Ltd.). The second set of trees was planted without pesticide application and these served as control. The field trial was established as *P. cinnamomi*-infested site partitioned into three blocks with six trees of each cultivar randomised per block. Disease severity was assessed at 18 months and 4 years after planting using a modified *Phytophthora* diseased tree health rating scale of Darvas et al. (1984) and Gabor et al. (1990). The 0-5 rating scale was used, where 0 = vigorous and healthy; no stem canker symptoms; 1 = full canopy with mild stem canker symptoms; 2 = sparse canopy with severe stem canker symptoms; 3 = sparse and mild dieback canopy and severe stem canker symptoms; 4 = very sparse and severe dieback canopy, severe stem canker symptoms with offshoot from rootstock and 5 = dead tree. The height of each macadamia tree was measured from the soil level to the canopy apex and the tree canopy volume was classified as dense, light or sparse. The effect of the treatment on the trees was determined as percent growth

reduction calculated as [100((h_t - h_n)/h_t)] using the mean height of the treated trees (h_t) and untreated control trees (h_n) of each cultivar. Samples of proteoid and secondary roots were obtained from each tree, observed for root rot and necrosis symptoms and the presence of *Phytophthora* was tested as described above. Soil samples around the root zones were collected and infestation of *P. cinnamomi* confirmed using the lupin baiting method.

Statistical analyses

Seedling vigour data were transformed with $(x+1)^{0.5}$ while the other parameters (seedling height, stem diameter and leaf area) were transformed with $\log_{10}(x+1)$ to stabilise variances and normalise each parameter measured. Data were subjected to generalized linear model (GLM) procedure with normal distribution in GenStat (release 14; Lawes Agricultural Trust, Rothamsted Experimental Station, UK). Significant factors were separated and compared using the least significant difference test at $P<0.05$ significance.

RESULTS AND DISCUSSION

Effect of *Phytophthora cinnamomi* on macadamia seedling development

Seedling emergence was significantly lower in *Phytophthora*-infested soil compared to sterilised soil. Comparison of the cultivars showed that mean percent seedling emergence was highest (68%) in 'H2', followed by 35% in 'A4' and 20% in 246 in the infested soils. Although the three cultivars are *M. integrifolia*, this study on in situ germination of macadamia nuts and the subsequent emergence of seedlings from a *Phytophthora*-infested soil clearly showed that 'H2' is more tolerant than 'A4', while '246' is the least tolerant to *Phytophthora* infection. Therefore, the high seedling emergence in cultivar 'H2', even in *Phytophthora*-infested soil, may have inadvertently contributed to the use of 'H2' as the most common rootstock in the Australian macadamia industry. In the 1970s, *M. tetraphylla* was favoured for seedling rootstock due to perceived superior nursery performance including more even and consistent germination, growth and stronger root systems (Hardener et al., 2009; Trochoulias, 1992). However, due to several rootstock scion incompatibility problems, *M. integrifolia* rootstock from cultivar 'H2' now constitutes the majority rootstocks in macadamia orchards (Hardener et al., 2009; Huett, 2004; Nagao et al., 1992; Trochoulias, 1992).

Generally, seedling vigour was reduced when planted in *Phytophthora*-infested soil, compared to sterilised soil. Nevertheless, cultivars rather than the presence of *Phytophthora* in the soil accounted for the majority (72%) of the variations observed in the above-ground parameters including stem diameter, seedling height, seedling vigour and leaf area, which indicates that these parameters may be a good measure of severity of root rot due to *P. cinnamomi* in macadamia orchards and indicative of the relative susceptibility of the cultivars on the same 'H2' rootstock. Root density was severely reduced in *Phytophthora*-infested soil, followed by 1:1 mixture and sterilised soil treatments. A negative correlation (r=-0.678) was observed between root weight and seedling vigour in infested soils. Root weight and whole seedling weight were significantly correlated (r=0.951) in sterilized soils. The results showed that the presence of *P. cinnamomi* significantly ($P<0.05$) influenced disease severity of the below-ground parameters. Therefore, the negative correlation between root weight and seedling vigour may be due to carbohydrate partitioning from reserves in the seedling (Stephenson, 2004). Lupin baiting of the soil obtained from the field trial confirmed the soil contained *P. cinnamomi* at the start and end of the trial.

Effect of *Phytophthora cinnamomi* on macadamia tree development

Differences in tree health of the treated and untreated macadamia trees were evident from 18 months after planting. Tree health ratings of treated macadamia cultivars were significantly (P=0.05) different from the untreated trees. At 18 month after planting the mean tree health rating in both treated macadamia cultivars was zero, while tree health rating ranged from 0.6-1.3 in the untreated trees. Disease severity in the untreated '816' was higher than '842'. This suggests that '816' may be more susceptible to *P. cinnamomi* than

'842'. Relative differences in susceptibility between the cultivars were more evident at 4 years after planting when the mean tree health rating was very poor (5.0) in the untreated '816' and was 3.0 in the untreated '842'. Tree growth was significantly (P=0.02) reduced with extensive trunk canker and sparse tree canopy in the untreated trees. This resulted in 60% growth reduction in '816' and 40% reduction in '842' of the untreated trees (Figure 1). The results showed that *P. cinnamomi* is able to severely reduce macadamia productivity if not controlled.

Figure 1. Heights of macadamia trees of cultivars 816 (a, c) and 842 (b, d) planted in *Phytophthora cinnamomi* 'killing field' after treated with Metalaxyl-M and potassium phosphonate (c, d) and untreated (a, b) at planting.

CONCLUSIONS

This study provides impetus for the selection of *Phytophthora*-resistant rootstocks in macadamia and application of chemical treatment to reduce severity of infection by *P. cinnamomi* after planting during plantation establishment. It appears that *P. cinnamomi* is able to reduce tree performance. The results also showed the importance of the use of *Phytophthora*-free soil mixtures in the production of macadamia seedlings in the nursery. The results showed relative resistance of macadamia cultivars to *P. cinnamomi*. Differences in root architecture and efficiencies of nutrient utilization among macadamia cultivars (Stephenson and Cull, 1986) may also influence the performance rootstock × scion interactions and impact on disease expression.

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MACADAMIA FUND

Integrated Management of Diseases in Macadamia Industry

Appendix 2: Details of methodology, results and conclusions of research activity

(b) Phytophthora management - varietal susceptibility to Phytophthora root rot

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Variation in susceptibility among macadamia genotypes and species to Phytophthora root decay caused by *Phytophthora cinnamomi*

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ABSTRACT

Phytophthora cinnamomi is a major pathogen of cultivated macadamia (*Macadamia integrifolia*, *Macadamia tetraphylla* and their hybrids) worldwide. The susceptibility of the two non-edible *Macadamia* species (*Macadamia ternifolia* and *Macadamia jansenii*) to *P. cinnamomi* is not well-understood. Commercial macadamia trees are established on grafted seedling (seed propagation) or own-rooted cutting (vegetative propagation) rootstocks of hybrids of the cultivated species. There is little information to support the preferential use of rootstock propagated by either seedling or own-rooted cutting methods in macadamia. In this study we assessed roots of macadamia plants of the four species and their hybrids, derived from the two methods of propagation, for their susceptibility to *P. cinnamomi* infection. The roots of inoculated plant from which *P. cinnamomi* was recovered showed blackening symptoms. The non-cultivated species, *M. ternifolia* and *M. jansenii* and their hybrids were the most susceptible germplasm compared with *M. tetraphylla* and *M. integrifolia*. Of these two species, *M. tetraphylla* was less susceptible than *M. integrifolia*. Significant differences were observed among the accessions of their hybrids. A strong association ($R^2 > 0.75$) was recorded between symptomatic roots and disease severity. Root density reduced with increasing disease severity rating in both own-rooted cuttings ($R^2 = 0.65$) and germinated seedlings ($R^2 = 0.55$). *P. cinnamomi* severity data were not significantly ($P > 0.05$) different between the two methods of plant propagation. The significance of this study to macadamia breeding and selection of disease resistant rootstocks is discussed.

1. Introduction

Four species of macadamia (*Macadamia integrifolia*, *Macadamia tetraphylla*, *Macadamia ternifolia* and *Macadamia jansenii*) originate in Eastern Australia and represent a significant resource for the macadamia industry, as potential sources of resistance to biotic and abiotic stress, and for improving yield and quality. The wild germplasm is vulnerable to extinction due to habitat loss and fragmentation (Pisanu et al., 2009; Neal et al., 2010; Powell et al., 2010). Under the Australian Environment Protection and Biodiversity Conservation Act, 1999, three species (*M. integrifolia*, *M. tetraphylla* and *M. ternifolia*) are listed as vulnerable while *M. jansenii* is listed as endangered (Shapcott and Powell, 2011). *M. ternifolia* and *M. jansenii* do not produce edible nuts and mostly exist in the wild ecosystem (Hardner et al., 2009). *M. integrifolia* and hybrids with *M. tetraphylla* constitute the current commercial macadamia production worldwide (Hardner et al., 2009). Genetic diversity that exists in the wild populations of *Macadamia* has not been explored for resistance to pathogens and pests, yield, quality or tolerance to abiotic stresses.

Macadamia trees in commercial orchards are propagated on grafted rootstocks derived from either vegetatively propagated clonal cuttings or germinated open-pollinated seeds (Hardner et al., 2009). Most rootstocks are selected based on ease of germination, propagation and grafting rather than their resistance to biotic or abiotic stress. In Australia, nearly all the grafted trees in commercial

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macadamia orchards are established on seedlings rootstocks of the cultivar 'H2', a *M. integrifolia* & *M. tetraphylla* hybrid. In South Africa, clonal cuttings of cultivar 'Beaumont' (HAES 695) a *M. tetraphylla* & *M. integrifolia* hybrid are preferred as rootstocks (Trochoulias, 1992; Huett, 2004; Hardner et al., 2009). Phenotypic characteristics of 'H2' are similar to the *M. integrifolia* characteristics, whereas, the 'Beaumont' characteristics are similar to *M. tetraphylla*. There is little information to support the choice of either genotype as rootstock in macadamia. The root systems of seedling populations are likely to have a high degree of variation in terms of root architecture and efficiencies of nutrient utilization (Stephenson and Cull, 1986), which may influence their performance with regard to impact on disease expression. The root systems of vegetatively propagated cutting-derived rootstocks of a particular variety are more likely to be very similar and equally susceptible to invading root pathogens since they are clonally propagated.

Phytophthora cinnamomi is an important soilborne pathogen in macadamia, and is considered a major constraint of macadamia production worldwide (Drenth et al., 2009; Akinsanmi and Drenth, 2013a). Since *P. cinnamomi* was introduced into Australia, it now occurs widely in all states and territories where it causes disease in a several plant species (Cahill et al., 2008; Hee et al., 2013). Information on the susceptibility of the Australian native *Macadamia* species is scanty. The diseases caused by *P. cinnamomi* are of significant economic importance in many horticultural crops including macadamia therefore research, focused on its control, continues to be a priority in many Australian horticultural and forestry industries.

P. cinnamomi can cause a range of symptoms in macadamias. At the early stage of *P. cinnamomi* infection, disease symptom expression is often misdiagnosed, or appears as general tree decline due to poor nutrition. Tree decline associated with *P. cinnamomi* is usually expressed as pale or yellow green leaves instead of dark green. Under conditions of moisture stress, in the advanced stages of infection, the leaves of infected trees rapidly wilt and readily abscise from the tree, giving rise to a sparse canopy appearance. The later stages of symptoms are more evident in macadamia following prolonged water-logging or drought conditions (Akinsanmi and Drenth, 2013b). Most times, fresh leaf flushes and shoot growth are absent or sparse and branches die back from the tip (Pegg, 1973). *P. cinnamomi* may directly infect macadamia stems or branches causing numerous small stem or trunk cankers. Infections of the stem are first characterized as gummosis or bleeding of the trunk and cracking of the bark which, in more advanced stages of the disease may develop in irregular areas of dead bark that extend from the soil line to several feet high. This often results in furrowed deep cankers on the trunk.

Diseases caused by *P. cinnamomi* from root infections are difficult to control (Ali et al., 2000). In macadamia, chemical applications with phosphonates and metalaxyl have been reported to be effective against diseases caused by *P. cinnamomi* (Akinsanmi and Drenth, 2013b). Selection for resistance to *Phytophthora* in macadamia may offer additional advantages and may be an effective means of controlling *Phytophthora*-incited diseases compared with chemical control in macadamia. The resistance system in macadamia genotypes to *P. cinnamomi* is not well understood.

Although early reports suggested that macadamia roots are resistant to *P. cinnamomi* (Zentmyer, 1960), subsequent studies have reported that *P. cinnamomi* is able to infect macadamia roots. These studies suggested that symptoms of root infection may be expressed as root necrosis and soft root rot (Ko and Kunimoto, 1976; Serfontein, 2008; Mbaka et al., 2009). Black to dark necrotic lesions observed on macadamia fine roots are associated with *P. cinnamomi* infection, but there has been no clear or consistent re-isolation of *P. cinnamomi* from roots showing black root symptoms. Although several varieties are affected by *P. cinnamomi* under orchard conditions, there is still confusion as to whether *P. cinnamomi* causes soft root rot or necrosis. Although a preliminary study by Zentmyer and Storey (1961) suggested differences between *M. integrifolia* and *M. tetraphylla* to *P. cinnamomi* infection and variation is observed among varieties of grafted trees in orchard conditions, there is little information on the relative susceptibility of different *Macadamia* species and selections to *P. cinnamomi*.

Selection of *P. cinnamomi* resistant rootstocks depends on the presence and variability in susceptibility to *P. cinnamomi* among different macadamia selections and species. Material with genetic resistance to *P. cinnamomi* may be used directly as rootstocks or as parents in breeding programmes. This will provide a more long-term and economical approach to managing diseases caused by *P. cinnamomi* in macadamia than the current application of agrochemicals as the main control method (Akinsanmi and Drenth, 2013b). In this study, we hypothesize that the non-cultivated species (*M. ternifolia* and *M. jansenii*) are more tolerant to *P. cinnamomi* than the two species (*M. integrifolia* and *M. tetraphylla*) or their hybrids that are cultivated in commercial production systems. We also evaluated the range of susceptibility among the four species of macadamia and their interspecific hybrids to root infections by *P. cinnamomi*. This study provides a more definitive description of symptoms of root infection by *P. cinnamomi* in macadamia. For the purpose of this study, we defined root necrotic lesions as any localized area of root tissue with extended spot, canker or scab, while root rot refers to the softness or decay of the root tissue that compromise its structural integrity.

2. Materials and methods

2.1. Plant materials and inoculation

Plants of four *Macadamia* species (*M. integrifolia*, *M. tetraphylla*, *M. ternifolia* and *M. jansenii*), two accessions of reverse crosses of *M. ternifolia* × *M. jansenii*, three accessions (ITH-1425, ITH-4323, ITH-679) of *M. integrifolia* & *M. tetraphylla* hybrids and one accession (ITH-Beau) of *M. tetraphylla* & *M. integrifolia* hybrid that represent a range of genotypes were selected. A total of 132 plants were established in the glasshouse, propagated as own-rooted cuttings (clonal) or seedlings as described by Topp and Neal (2015) were used in this study. Three cultures of *P. cinnamomi* (UQ7100, UQ7097 and UQ7098) were obtained from the University of Queensland *Phytophthora* culture collection in Brisbane. These three isolates were originally obtained from macadamia trunks expressing symptoms of stem canker and the ability of the three *P. cinnamomi* isolates to cause stem canker in macadamia had been confirmed in a previous study (Akinsanmi and Drenth, 2012). The isolates were grown on V8 juice (Campbell's soups Australia) agar amended with 10 ug/ml Pimaricin, 50 ug/ml Penicillin and 50 ug/ml Polymixin B final concentration (Tsao, 1960; Eckert and Tsao, 1962). The cultures were incubated in the dark at 25 °C for 10 days and used to inoculate sterilized wheat grain culture. The wheat grain culture was prepared by soaking 150 g grain in 40 ml of water in 500 ml conical flasks for 24 h. Thereafter, the flasks were autoclaved twice at 121 °C for 30 min on two consecutive days, before inoculating with three pieces of mycelial agar plugs (10 mm diameter) of each *P. cinnamomi* isolate in separate flasks. Flasks that were inoculated with V8 juice agar plugs without *P. cinnamomi* served as the control. The inoculated flasks were incubated at 25 °C for three weeks, routinely agitated once a week, to prevent clogging of the grains, before grinding the content to produce the inoculum. Approximately 1 g of inoculum per litre of potting mix was placed in the potted macadamia plants. Inoculum for each isolate was placed in a 120 mm deep hole at approximately 10 cm from the stem of each

plant. Thereafter, the whole inoculated pot was immersed in non-chlorinated rain water to about three quarters deep on a 48 h dry and wet cycle for three months. Plants were kept on raised benches in a complete randomized block design with at least three replicates per genotype in a glasshouse at 25 °C ± 3 °C. A repeat run of the trial was performed under the same conditions as the first trial in the same month (August) and glasshouse. Plant materials were of similar age in the repeated trials. Each trial was terminated after three months. At the end of the three months, samples of the potting mix were tested for the presence or absence of *P. cinnamomi*.

2.2. Root disease severity assessments

In order to maintain root structure, the potting mix was gently removed from the roots under running tap water. Roots of each plant were assessed for root rot and necrotic symptoms. Visual disease severity assessment was based on an ordinal rating scale of 0–5, where 0 = no visible disease symptoms and extensive fine root systems; 1 = no obvious root rot and necrosis symptoms but sparse fine root distribution; 2 = slight root rots or necrosis symptoms evidence at the tips of fine roots and sparse fine root distribution; 3 = extensive root rot or necrosis symptoms and sparse fine root distribution; and 4 = very extensive necrosis symptoms and poor root volume with very rare fine root distribution on available roots; and 5 = no root or total plant death occurred. Disease severity was also assessed using an image analysis software tool (ASSESS 2.0, APS, St. Paul Minnesota, USA). Digital photographs of each sample were taken using SLR PowerShot SX40 HS Canon digital camera (Canon Inc. Japan) on a white background. Each digital photograph was downloaded into the image analysis software for assessment of disease severity. Image analytical functions in ASSESS were used in auto mode to examine disease severity (Lamari, 2008). Symptomatic (blackened) roots were considered diseased while non-blackened roots were assessed as healthy. The proportion of diseased symptomatic roots for each sample was recorded. Root biomass (root density) measured as the amount of non-diseased roots of inoculated plants compared with the control was recorded.

2.3. Recovery and detection of P. cinnamomi from inoculated roots

Subsamples of blackened roots were collected from different parts of each sample and stored at 4 °C. The presence or absence of *P. cinnamomi* infection was confirmed using isolates from the roots. In order to isolate *P. cinnamomi* from the roots, 2–5 mm pieces were excised from the margins of symptomatic and asymptomatic sections of blackened roots and arbitrarily from healthy roots. The pieces were surface sterilized in 10% v/v sodium hypochlorite solution for 3 min, then rinsed in three changes of sterile distilled water. The sterilized tissues were blot dried between sterile blotting paper, and plated on the antibiotics-amended V8-juice agar and incubated at 25 °C for three to five days under 12 h light/12 h dark conditions.

The proportion of roots with mycelial growth was determined for five agar plates per sample. Mycelia were sub-cultured onto fresh V8-juice agar plates containing the amended antibiotics and grown to obtain pure cultures for identification. In order to determine if asymptomatic roots were infected, *in planta* detection of *P. cinnamomi* in the inoculated roots was also explored. DNA was extracted directly from root samples. Similar weights of the root subsamples were ground into fine powder in liquid nitrogen using a sterile mortar and pestle. The proportion of positive *P. cinnamomi* detections was recorded and the identities of the isolates were confirmed by Polymerase chain reaction (PCR) amplification and Restriction fragment length polymorphism (RFLP) procedures described below.

2.4. DNA extraction and PCR-RFLP amplification

DNA was extracted directly from the pure cultures or ground root tissue using a Genomic DNA Purification Kit (Promega Corporation, Madison, Wisconsin, USA). Approximately 40 mg mycelium was lysed in a 2 ml safe-lock tube (Eppendorf AG, Germany) containing a 5 mm sterile stainless steel bead and 600 μL nucleic lysis solution using Tissue Lyser (Qiagen, Hilden, Germany) for 1 min at 30 Hz. The total genomic DNA was extracted following the Promega Wizard® kit manufacturer's protocol. DNA concentration was determined with a BioDrop Duo spectrophotometer (BioDrop, England). PCR amplification was performed with *Phytophthora* specific primers with A2 primer (5′-ACTTTCCACGTGAACCGTTT-CAA-3′) used as forward primer and the I2 primer (5′-GATAT-CAGGTCCAATTGAGATGC-3′) used as reverse primer (Drenth et al., 2006). The purified genomic DNA of each of the *P. cinnamomi* isolate served as the DNA template for the PCR amplification in a 20 μL reaction mixture containing 10 μL of 10 mM Phusion Master Mix (Thermo Fisher Scientific Inc.) and 1 μL each of forward and reverse primers. In order to enhance PCR amplification DMSO was added to the PCR mixture. The amplifications were conducted in SuperCycler Thermal Cycler (Kyratec, Australia) programmed for initial denaturation for 60 s at 98 °C followed 35 cycles at 98 °C for 10 s, 62 °C for 30 s and 72 °C for 45 s, with a final extension step at 72 °C for 3 min. PCR amplicons were separated in 1% agarose gel (BIOLINE, Australia) stained with gel red in 0.5% Tris-borate EDTA buffer solution (0.5 × TBE) and viewed under UV light using Molecular Imager® GelDoc™ (Bio-Rad Laboratories Inc.). The amplicon sizes were determined against a 1 kb HyperLadder (BIOLINE, Australia) and then the targeted PCR amplicon size of 500 base pairs was purified using Roche™ High Pure PCR Product Purification Kit (Roche Applied Science, Manheim, Germany) according to the manufacturer's instructions before the restriction digestion process. Restriction digests of the amplified products were used to confirm the identity of the isolates. The PCR products of the A2/I2 primers were subjected to restriction digestion using three restriction enzymes: *Msp1*, *Rsa1*, and *Taq1* (Drenth et al., 2006). The products containing *Msp1* and *Rsa1* were incubated at 37 °C for 60 min while Taq1 was incubated at 65 °C for 60 min. The digested products were separated on gel electrophoresis on 3% agarose in 0.5 × TBE at 100 V for about 45 min. The bands were observed under UV light and the obtained restriction digest fragment patterns compared with standard restriction digest patterns for different standard *Phytophthora* species (Drenth et al., 2006).

2.5. Statistical analysis

In order to stabilize variance the disease severity rating and proportion of symptomatic roots data were $\log_{10}(x+1)$ transformed, whereas data of the root density and proportion of *P. cinnamomi* recovered parameters were square root transformed before analysis. Roots of all the control treatments were healthy without any visible symptoms of *P. cinnamomi* infection. Therefore, only data of the inoculated plants was included in further analyses. Generalized Linear Model (GLM) procedure in GenStat Release 16.1 (VSN international Ltd, Hertfordshire, UK) was performed on the data. Analysis including the two runs showed no significant ($P > 0.05$) differences between the variance of the repeat runs, therefore, data of both runs were combined in the subsequent analyses with germplasm (*Macadamia* species and their interspecific hybrids) and mode of propagation as the main effects. Significant means were compared using the least significant difference

Table 1
F-probability values of Phytophthora root decay severity measures obtained from the accumulated analysis of variance of glasshouse trials with potted plants of the four *Macadamia* species and their interspecific hybrids that were propagated either as own-rooted cuttings or seedlings in soils inoculated with *Phytophthora cinnamomi*.

Source of variation	df	Disease severity rating[a]	Symptomatic roots[b]	*P. cinnamomi* re-isolated[c]	Root density
Trials	1	0.531	0.471	0.272	0.755
Germplasm	7	<0.001	<0.001	0.030	0.031
Propagation	1	0.070	0.139	0.530	0.472
Germplasm × propagation	1	0.919	0.720	0.207	0.704

[a] Ordinal rating scale of 0–5, where 0 = no visible disease symptoms and extensive fine root systems; and 5 = no root or total plant death occurred.
[b] Proportion of symptomatic (blackened) roots of macadamia plants inoculated with *P. cinnamomi*.
[c] Proportion of root samples from which *P. cinnamomi* was re-isolated.

tests. Relationships among the data as suitable predictors of *P. cinnamomi* infection of macadamia roots were examined by regression analysis and Spearman's correlation coefficients in GenStat.

3. Results

3.1. Relative susceptibility of Macadamia species and hybrids to P. cinnamomi

The consistency between the trials is shown in Table 1. Results of the accumulated analysis of variance showed similar ($P > 0.05$) results between the repeat runs, whereas, disease severity levels were significantly ($P < 0.001$) different among the macadamia germplasm of the four *Macadamia* species and their hybrids (Table 1). Varying degrees of root decay were observed in roots infected with *P. cinnamomi* among the macadamia germplasm, from darkening of the primary and secondary feeder roots to complete death or loss of an entire portion of the root system (Fig. 1). Approximately 49% of the inoculated plants had a low (<2) disease severity rating, while 30% had moderate (2–3) and 21% severe (>3) disease severity ratings. There was no significant difference between the propagation type (own-rooted cuttings or seedlings) (Table 1). Comparison of the four *Macadamia* species showed that *M. ternifolia* followed by *M. jansenii* were the most susceptible species, whereas, *M. tetraphylla* had the lowest disease severity rating (Fig. 2a). The proportion of symptomatic roots was significantly lower in the *M. tetraphylla* and *M. integrifolia* species and in both the interspecific hybrids of the non-edible species, *M. ternifolia* and *M. jansenii* (Fig. 2b). Quantitative analysis of root infections showed that *M. ternifolia* had the highest proportion of symptomatic roots (blackened roots) hence, was the most susceptible species to *P. cinnamomi* (Fig. 2b). Among the hybrids, the mean disease severity ratings and symptomatic root were significantly higher in the accessions of hybrids of *M. integrifolia* & *M. tetraphylla* than the reverse crosses of *M. ternifolia* × *M. jansenii* hybrids

Fig. 1. Relative severity of Phytophthora root decay of accessions of different *Macadamia* species and their interspecific hybrids. (a) *M. tetraphylla*, (b) *M. integrifolia* (c, d) *M. integrifolia* & *M. tetraphylla*, (e,f) *M. tetraphylla* & *M. integrifolia*, (g) *M. ternifolia*, (h) *M. jansenii* × *M. ternifolia* and (i) *M. ternifolia* × *M. jansenii*. In each picture, roots on the right were inoculated with *Phytophthora cinnamomi* while roots on the left were non-inoculated controls. Roots derived from own-rooted cuttings (d, f, g).

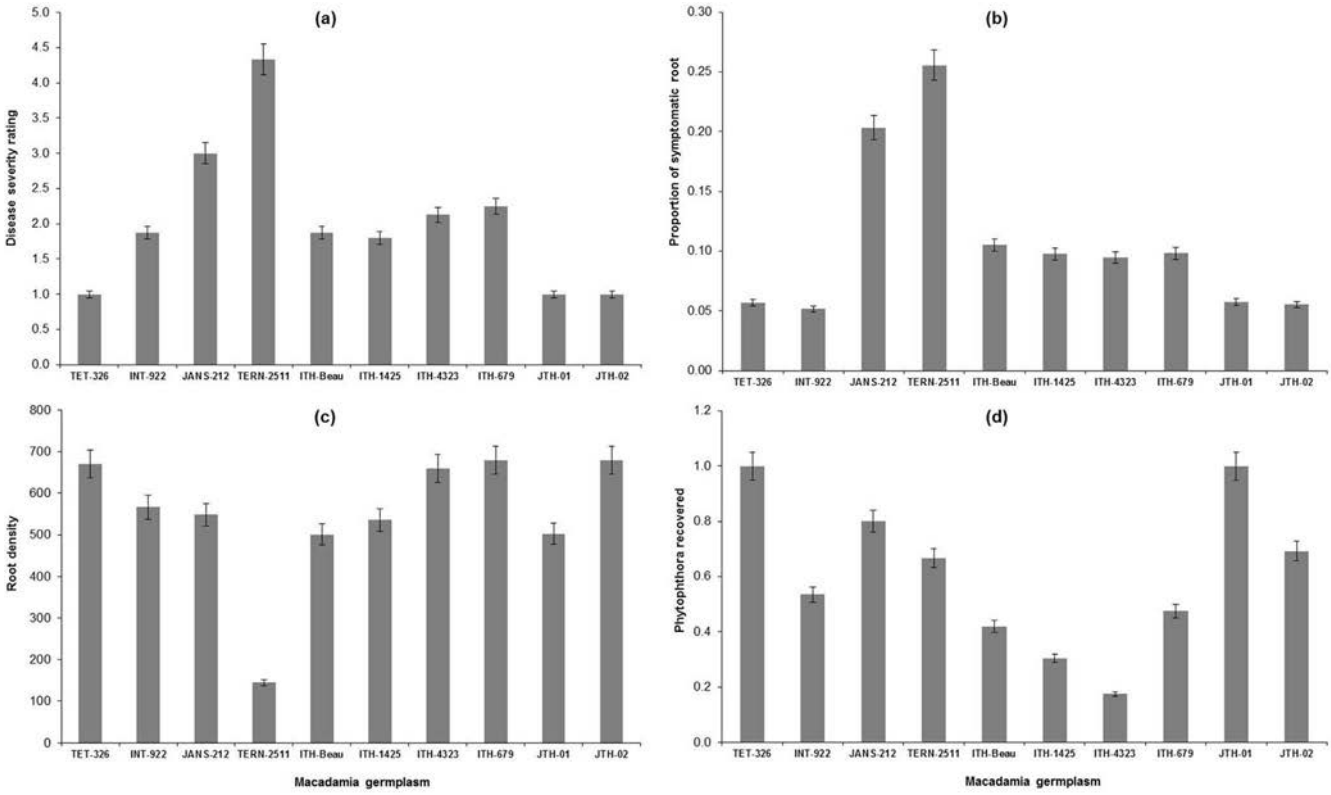

Fig. 2. Means of Phytophthora root decay severity in potted plants of four *Macadamia* species and their interspecific hybrids that were inoculated with *Phytophthora cinnamomi*. (a) Disease severity rating based on scale of 0–5, where 0 = no visible disease symptoms and extensive fine root systems; and 5 = no root or total seedling death occurred. (b) Proportion of symptomatic (blackened) roots of macadamia seedlings inoculated with *P. cinnamomi*. (c) Density of non-symptomatic roots of inoculated plants compared to the untreated control. (d) Proportion of root samples from which *P. cinnamomi* was re-isolated. *M. tetraphylla* (TET-326), *M. integrifolia* (INT-922), *M. jansenii* (JANS-212), *M. ternifolia* (TERN-2511), *M. tetraphylla & M. integrifolia* (ITH-Beau), *M. integrifolia & M. tetraphylla* (ITH-1425, ITH-4323, ITH-679), *M. jansenii* × *M. ternifolia* (JTH-01) and *M. ternifolia* × *M. jansenii* (JTH-02). Lines on each bar represent standard errors.

(Fig. 2). There was no significant variation in disease severity ratings among the accessions of *M. integrifolia & M. tetraphylla* hybrids. Root density was significantly lowest in *M. ternifolia* while the root density of other non-edible species was similar to the cultivated species (Fig. 2c).

3.2. Recovery of *P. cinnamomi* from diseased macadamia roots

Restriction fragment pattern of the *Phytophthora* isolates obtained from inoculated macadamia roots was the same as the pattern expected for *P. cinnamomi* as described by Drenth et al. (2006). *P. cinnamomi* was more frequently (0.1 or 100%) isolated from diseased roots of *M. tetraphylla* species compared with other species (Fig. 2d). Similarly, among the hybrids *M. jansenii* × *M. ternifolia* hybrid had the highest proportion of *P. cinnamomi* recovered from diseased roots compared with the *M. integrifolia & M. tetraphylla* hybrids (Fig. 2d). The frequency of *P. cinnamomi* recovered was lowest in the ITH-4323 accession of *M. integrifolia & M. tetraphylla* hybrids (Fig. 2d).

3.3. Relationships of root disease severity assessment parameters for Phytophthora cinnamomi infection

There was a strong ($r = 0.83$) and significant ($P < 0.001$) positive correlation between disease severity rating and percent symptomatic roots. Root density was also significantly correlated with disease severity rating ($r = 0.79$) and symptomatic roots ($r = -0.54$). Trends of association of the disease assessment parameters in the *P. cinnamomi*-inoculated roots were similar in both cuttings ($R^2 = 0.75$) and seedlings ($R^2 = 0.96$) (Fig. 3a). The slopes of the disease severity rating and density of inoculated roots were also similar for both cuttings and seedlings, where low root density is strongly associated with high disease severity rating (Fig. 3b). The multivariate analysis showed that two principal component axes captured the majority (89.4%) of the variation in the data. The first axis accounted for 66.8% and the second axis accounted for 22.6% of the variation. The first axis was largely influenced by the contribution of root density and disease severity rating that typified the inverse relationship between root decay and root density, hence the eigenvector of root density was negative (−0.550) and disease severity rating was positive (0.554).

4. Discussion

This study revealed the relative susceptibility of the four *Macadamia* species to root infections by *P. cinnamomi* and showed that cultivated species are more tolerant than the non-edible species. *M. tetraphylla* was the most tolerant species while *M. ternifolia* was the most susceptible species to *P. cinnamomi* root infection. The relative tolerance of *M. tetraphylla* compared with *M. integrifolia* may support its use as a preferred rootstock for protection against the soilborne *P. cinnamomi* pathogen in macadamia. The differences observed between *M. tetraphylla* and *M. integrifolia* in this study are generally similar to those observed for stem canker caused by *P. cinnamomi* (Zentmyer, 1960). This is in contrast to field observations in commercial production systems in Kenya where *M.*

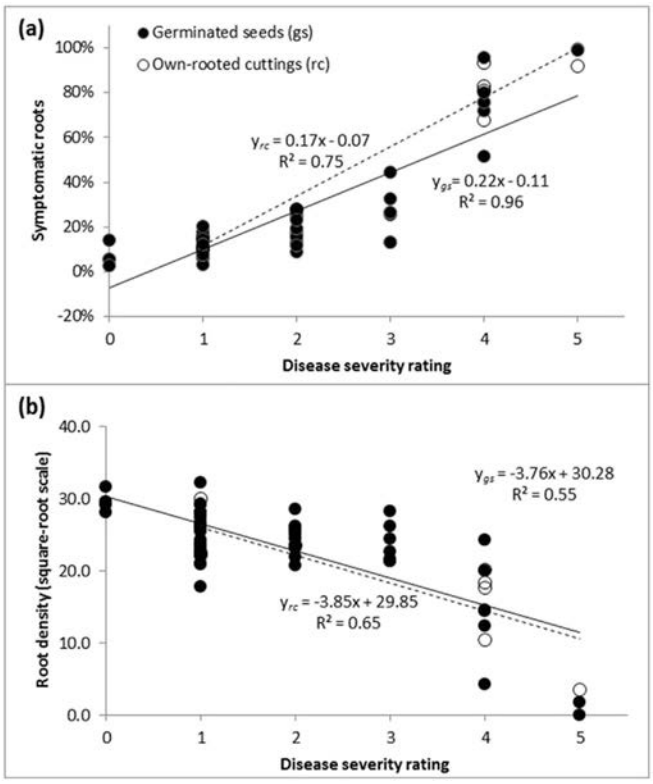

Fig. 3. Relationships based on regression analysis of disease severity rating of macadamia plants inoculated with *Phytophthora cinnamomi* (a) percentage of symptomatic (blackened) roots and (b) root density of inoculated plants propagated as cuttings and seedlings.

tetraphylla trees were observed to be the most severely affected by Phytophthora root rots compared with *M. integrifolia* trees (Mbaka et al., 2009). Mbaka et al. (2009) attributed this to the fact that *M. tetraphylla* trees survived as they were able to tolerate infection for long periods, whereas *M. integrifolia* trees succumbed to the disease and died within a short period.

Among the hybrid, the non-edible hybrids of *M. ternifolia* × *M. jansenii* were the most tolerant while the cultivated hybrids of *M. integrifolia* and *M. tetraphylla* were more susceptible to root infections by *P. cinnamomi*. The relative tolerance of the *M. ternifolia* × *M. jansenii* hybrid compared with those of the cultivated species offers a prospect for further interspecies hybridization as a potential source for high levels of *Phytophthora* tolerance for rootstock in macadamia. The lower severity rating of hybrids of *M. ternifolia* and *M. jansenii* germplasm compared with *M. tetraphylla* and *M. integrifolia* hybrids was surprising. It may be due to relatively low history of association of *P. cinnamomi* with the native uncultivated *Macadamia* species in Australia. One of the key questions concerns the potential contributions *M. ternifolia* and *M. jansenii* germplasm can make to genetic improvement of commercial macadamia varieties with regard to disease resistance, yield, quality, tree size and architecture and resistance to biotic and abiotic stress. It is therefore important to identify and map the sources of resistance and determine the heritability of resistance towards *P. cinnamomi*. In this study, we did not observe significant differences among the four accessions of the *M. integrifolia* and *M. tetraphylla* hybrids, which may be due to the low or relatively small number of accessions used in this study. However, in Australia, significant differences in tree decline symptoms that were associated with *P. cinnamomi* have been reported among trees of *M. integrifolia* and *M. tetraphylla* hybrids on similar seedling rootstocks (Akinsanmi and Drenth, 2013b). In South Africa, cultivars 'Nelmak 2' and 'Beaumont' (*M. tetraphylla* & *M. integrifolia* hybrids) were reported to have higher degree of field resistance to *P. cinnamomi* than another cultivar 'HAES 816' (*M. integrifolia* & *M. tetraphylla* hybrid) (Christie, 2013).

The *P. cinnamomi* isolates used in this study consistently caused severe root infection in the same germplasm between trial runs. This indicates that the bioassay used in this study is suitable for screening macadamia genotypes for their susceptibility to *P. cinnamomi*. These results were consistent with field observations where *M. ternifolia* trees were the most seriously affected species.

We observed blackening of the roots and examination of the diseased roots revealed extensive root decay with necrosis often without loss of structural integrity in symptomatic roots. Isolations from diseased roots consistently produced *P. cinnamomi*, but at times no isolates were obtained from the midst of blackened roots. This indicates that *Phytophthora* is active in the advancing margins and often no longer exists in the dead tissue. Although no obvious above-soil or foliar symptoms were observed in the inoculated plants in the glasshouse studies, a similar situation has been reported under field conditions, where root infections were not apparent in the foliage at an early stage of infection, but became severely expressed following conditions of moisture stress (Akinsanmi and Drenth, 2012). Similar situations of blackened root infections with no above-ground symptoms due to *P. cinnamomi* infections have been reported in junipers (Standish et al., 1982).

In this study we used different methods to examine the severity of *P. cinnamomi* on roots of macadamia plants. The plant disease quantification software ASSESS 2.0 was used to examine the proportion of roots with lesions. The results showed that it provides a more consistent assessment tool for diseased assessment than visual rating. The mechanism is based on the differences of colour between infected roots and healthy roots. In automatic mode, in most cases, the two colour schemes of diseased roots and health roots may overlap, which means some roots in the overlap region were considered as diseased. This problem may be avoided in the artificial mode by setting the colour range. Based on our isolation studies of diseased material, we can now regard blackened roots as a definitive description and expression of *P. cinnamomi* infection of macadamia roots. The degree of detection of *P. cinnamomi* from asymptomatic roots of inoculated soils was low. Future studies are required to study the infection process of *P. cinnamomi* in macadamia roots. Root infection of macadamia trees by *P. cinnamomi* reduced root biomass, and the reduction in root biomass was significantly correlated with disease severity. This suggests that the field observation of tree decline and dieback associated with *P. cinnamomi* (Akinsanmi and Drenth, 2013b) may be attributed to reduced root biomass in infected trees. Since *P. cinnamomi* causes stem canker in macadamia, further research is currently underway to examine if similar trends exist for stem canker as observed for root infections in this study. Further studies should also investigate the relationships between roots and stem resistance to *P. cinnamomi* among the four species and different genotypes of macadamia.

Although there was no significant difference between own-rooted cuttings and seedlings for *P. cinnamomi* susceptibility, the large variation in disease severity in the seedlings may be attributed to the high degree of variation in the root systems of seedling populations. Macadamia is preferentially outcrossing (Sedgley et al., 1990), and therefore the seedling rootstocks are genetically heterozygous for most characteristics. Hence, they would exhibit more phenotypic variability than cuttings which are clonal. This genetic variability in seedling rootstocks may influence their performance against *P. cinnamomi*. In contrast with seed-propagated rootstocks, clonal rootstocks provide uniformity. Clonal rootstocks

which lack a taproot have some setbacks in macadamia, where strong winds can cause severe damage to trees (Trochoulias, 1992; Topp and Neal, 2015). The root architecture and systems of vegetatively propagated cutting-derived rootstocks may also influence their performance to invading root pathogens. Several other factors should be considered to support the choice of mode of propagation in macadamia production systems.

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Integrated Management of Diseases in Macadamia Industry

Appendix 2: Details of methodology, results and conclusions of research activity

(b) Phytophthora management - varietal susceptibility to stem canker

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Characterization of accessions and species of *Macadamia* to stem infection by *Phytophthora cinnamomi*

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Phytophthora cinnamomi is a major pathogen in most macadamia plantations worldwide. Due to stem lesions, stem cankers and leaf defoliation, it results in loss of productivity and tree death. This study examined accessions of the four *Macadamia* species and their hybrids, produced via rooted stem cuttings or germinated seeds, for susceptibility to stem canker and necrotic lesions caused by *P. cinnamomi*. Plants were wound-inoculated with agar containing *P. cinnamomi*. The symptoms produced in inoculated plants were used to characterize host susceptibility variation within and among the population. Lesion length and severity of stem canker were recorded. The four species and hybrids differed significantly in stem canker severity ($P < 0.001$) and lesion length ($P = 0.04$). *Macadamia integrifolia* and *M. tetraphylla* hybrids were the most susceptible. *Macadamia integrifolia* had the greatest stem canker severity and the most extensive lesions above and below the site of inoculation. Restricted lesion sizes were observed in *M. ternifolia* and *M. jansenii*. The effects of basal stem diameter and the method of propagation either from cuttings or from seed were not significant. The genetic variation in the reaction of macadamia accessions to stem infection by *P. cinnamomi* is discussed.

Keywords: host resistance, oomycetes, proteaceae, soilborne pathogen, tree nut, wild germplasm

Introduction

The four species in the genus *Macadamia* are endemic to subtropical eastern Australia and these populations may harbour potential sources of resistance to *Phytophthora cinnamomi*. Macadamia trees in plantations mostly consist of *Macadamia integrifolia* or hybrids of *M. integrifolia* with *M. tetraphylla*. These are routinely managed to reduce the impact of *P. cinnamomi* stem and root infections, through the application of agrochemicals such as metalaxyl fungicides and phosphonic acid (Akinsanmi & Drenth, 2013). In contrast to the cultivated trees, their wild accessions and *M. ternifolia* and *M. jansenii* in the native ecosystem have not been exposed to, or have limited association with *P. cinnamomi*.

Phytophthora cinnamomi infects macadamia stems causing canker or necrotic lesions and gummosis from the trunk (Zentmyer, 1962; Pegg, 1973; Ko & Kunimoto, 1994; Serfontein, 2008; Mbaka *et al.*, 2009). In Australia, thousands of trees have been killed or rendered unproductive as a result of *Phytophthora* infection (Akinsanmi & Drenth, 2013). Diseased trees show a gradual decline syndrome with usually pale or yellow green leaves instead of dark green. Under moisture stress this gives rise to leaf wilting and abscission, resulting in a sparse canopy. Branches may be infected either by *P. cinnamomi* directly or via infection of the main stem or roots, with bleeding symptoms and the potential for subsequent die back.

Trees in plantations are grafted on rootstocks of *M. integrifolia* or hybrids between *M. integrifolia* and *M. tetraphylla*. Rootstocks are used to enable selected scions to be vegetatively propagated through grafting or budding, to shorten time in the nursery, and to reduce the variation that occurs between seedlings (Hardner *et al.*, 2009). Clonally propagated plants are typically produced by grafting cuttings from known cultivars (scions) onto either rooted cuttings or open-pollinated seedling rootstocks. In tree crops such as apple and avocado, different clonal rootstock genotypes are used to alleviate unfavourable soil abiotic and biotic conditions, and achieve desired tree characteristics including increased precocity, and reduced tree size (Gregory *et al.*, 2013). For macadamia there is little information to support rootstock choice, in particular as a source of disease resistance.

In Australia, seedlings derived from open pollinated seeds of *M. integrifolia* cultivar H2 (Hinde) and *M. integrifolia* and *M. tetraphylla* hybrid cultivar D4 (Renown) have been preferentially used as rootstocks (Huett, 2004). The *M. integrifolia* cultivar H2 is favoured because of rapid and consistent seed germination, high grafting success rate and good seedling vigour with broad stem diameter (Huett, 2004; Hardner *et al.*, 2009). In some other macadamia producing countries such as South Africa, stem cuttings of the *M. integrifolia*

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and *M. tetraphylla* hybrid cultivar HAES 695 (Beaumont) are preferred. It is uncertain whether seedling rootstocks produce higher yield and premium kernel per unit canopy area than cutting rootstocks (Trochoulias, 1992; Topp & Neal, 2015). Topp & Neal (2015) observed no significant differences between seedling and cutting rootstock types for yield or tree size traits, and no consistent difference for susceptibility to wind damage. This result differs from that of Trochoulias (1992), where trees propagated through cuttings were found to produce poorer root systems and were more susceptible to wind damage than seedling rootstocks. Trochoulias (1992) tested cultivars HAES 246 and Gower as rootstocks, and also observed increased premium kernel per unit canopy area for Gower seedling over Gower cutting, and no difference between rootstock types for HAES 246. Although findings from studies performed in the 1960s suggest that *M. integrifolia* may be more susceptible to stem canker than *M. tetraphylla* (Zentmyer & Storey, 1961; Pegg, 1973), there is still no information on which rootstock is most appropriate for tolerance or resistance to *P. cinnamomi* in commercial production systems. A preliminary report indicates a high variation in the level and progression of symptoms of diseased trees of the same scion on similar rootstock (Akinsanmi & Drenth, 2016). It is unknown if the genetic variability that is associated with the use of seedling rootstocks contributes to this variation. In certain cases, macadamia trees with obvious stem canker from which *P. cinnamomi* was isolated developed no further canker without treatment. The progression of the initial canker stopped and the trees appeared to recover completely. This situation is not well understood and contributes to the perception among some growers that macadamia is resistant to *P. cinnamomi*. The absence of symptoms on trees that were once severely affected may indicate that trees become more resistant to the disease (Pegg, 1973) or manifest systemic induced resistance. Alternatively, the environmental conditions initially conducive for development of disease may change, potentially limiting expression of disease.

On trunks, branches and large roots, *P. cinnamomi* initiates cankers on wounds, and the pathogen grows vertically and horizontally from the cortex into the outer vascular system (Zentmyer, 1980). In macadamia, the significance of wounding in the formation of stem canker has not been established. Artificial inoculations of soil with *P. cinnamomi* without wounding the roots have resulted in diseased roots and the technique has been successfully used to screen macadamia populations for susceptibility to root decay caused by *P. cinnamomi* (Akinsanmi *et al.*, 2016). Wound inoculations of the stem have been used to examine susceptibility of macadamia cultivars to *P. cinnamomi* (Zentmyer & Storey, 1961; Pegg, 1973; Christie, 2013). The stem inoculation technique has been successfully used to screen germplasm for resistance to *P. cinnamomi* in various host plants (Ogara *et al.*, 1997; Linde *et al.*, 1999a; Lucas *et al.*, 2002; Browne *et al.*, 2005).

This study used a stem inoculation technique as a rapid bioassay for screening macadamia plants for their susceptibility to stem infection by *P. cinnamomi*. Eleven accessions were evaluated, which encompassed all four species and included commercial cultivars and accessions collected from the wild. Three hypotheses were tested: (i) that there is variation in susceptibility among species; (ii) that there is variation in susceptibility among the accessions within species; and (iii) that plants propagated from cuttings are more susceptible to *P. cinnamomi* than seedlings obtained through germinated seeds. The outcomes of this study will underpin the choice of materials for rootstock in macadamia. The study will contribute to the understanding of the genetic variability in macadamia for resistance or tolerance to *P. cinnamomi*.

Materials and methods

Plant materials

Sixty-five macadamia plants were evaluated, representing the four species (*M. integrifolia*, *M. jansenii*, *M. ternifolia* and *M. tetraphylla*), reciprocal crosses of *M. ternifolia* and *M. jansenii*, and four accessions of domesticated hybrids of *M. integrifolia* and *M. tetraphylla* (Table 1). Plants were germinated from seeds or clonally propagated from stem cuttings as described by Topp & Neal (2015) in the glasshouse at Maroochy Research Facility, Nambour, Australia. Clonally propagated cuttings were grown in cutting media mix of 50% Chillagoe Perlite (coarse) and 50% coir fibre peat (Galuku Root Zone Media) for 3–6 months before being potted up into 90 mm pots with Searles premium potting mix (JC & AT Searle Pty Ltd). Seeds were

Table 1 Details of source of *Macadamia* species and hybrids inoculated with *Phytophthora cinnamomi* in the study

Species and hybrids	Source	Propagation type[a]	No. of accessions	No. of plants
Macadamia integrifolia	Domesticated and wild selection	S, C	2	9
M. tetraphylla	Wild selection	S	1	3
M. jansenii	Wild selection	C	1	5
M. ternifolia	Wild selection	C	1	3
M. jansenii × *M. ternifolia*	Breeding progeny	S	1	5
M. ternifolia × *M. jansenii*	Breeding progeny	S	1	7
M. integrifolia & *M. tetraphylla*	Domesticated	S, C	4	33

[a]S, plant produced from seeds; C, plant produced from rooted cuttings.

germinated in 45 × 110 mm trays containing Searles premium potting mix under high humidity conditions at 25–35 °C, before each germinated seed was transplanted into a 90 mm pot. All plants were fertilized with Osmocote Plus Native 8–9 months slow release fertilizer and for the first few weeks watered with Searles Flourish Native Plants Soluble Plant Food (JC & AT Searle Pty Ltd). Plants were maintained in the glasshouse for 8–12 months before inoculation. The seedlings and cuttings were divided into two batches for replicated trials, and a total of 65 plants were inoculated with *P. cinnamomi*. At least three plants per species or hybrid were inoculated (Table 1).

Inoculum preparation and inoculation

In both trials, all plants were inoculated with *P. cinnamomi* isolate UQ7097. Due to the high clonality in the Australian *P. cinnamomi* population (Linde *et al.*, 1999a,b), UQ7097 was used, which has been described as pathogenic on macadamia stems (Akinsanmi *et al.*, 2016). Inoculum was obtained from 10-day-old cultures of the isolate grown on V8 juice (Campbell's Soups Australia) agar, amended with 10 μg mL^{-1} pimaricin, 50 μg mL^{-1} penicillin and 50 μg mL^{-1} polymixin B final concentrations, and incubated at 25 °C in the dark. Plants were inoculated by placing 6-mm-diameter agar disks of V8 juice agar with *P. cinnamomi* on each stem under the bark on the cambial region in fresh wounds created with sterile scalpels. Wounds were wrapped with Parafilm to hold agar in place. Wounds of control plants received sterile V8 juice agar disks. Plants were kept in the greenhouse for 3 months at 16–26 °C and watered daily. Because soil fertility influences disease severity in macadamia (Akinsanmi & Drenth, 2013), no additional fertilizer was applied to the potting mix.

Disease severity assessment

Canker development and the size of any obvious cankers were measured 3 months after inoculation. A disease severity rating scale (0–5) was used to assess canker development, where 0 = no obvious canker and tissue discolouration; 1 = slight discolouration of the tissue below the point of inoculation only; 2 = moderate discolouration of above and below the point of inoculation; 3 = extensive discolouration and girdling around the stem; 4 = girdling around the stem and death of plant above the point of inoculation only; and 5 = complete death of the plant. In addition, the length of the lesion above and below the site of inoculation was measured and the girth of each plant at the soil-line was recorded. The health status of the plants was observed and the plants were categorized into three groups: no canker, canker present but not fatal, or fatal infection.

DNA sampling and analysis

The presence of *P. cinnamomi* in inoculated stems was confirmed by plating tissues from the advancing margin of visible lesions of diseased plants onto V8 juice agar plates amended with antibiotics for identification. The plates were incubated at 25 °C and after 3–5 days, the mycelium growing from the pieces was subcultured into new plates containing the antibiotic-amended V8 juice agar and grown to obtain a pure culture. Genomic DNA was extracted as per Harteveld *et al.* (2013) and was used as template for PCR amplification with *Phytophthora*-specific primers A2/I2 before RFLP analysis with three restriction enzymes (*Msp*I, *Rsa*I and *Taq*I) as previously described by Drenth *et al.* (2006). The identity of each culture was confirmed as *P. cinnamomi* by comparing the PCR-RFLP profile with that of the UQ7097 isolate.

Data analysis

Disease severity ratings and lesion length data were analysed in SPSS STATISTICS v. 22.0 (IBM Corp.). The ordinal rating data were subjected to rank procedure in SPSS, and transformed as $\log_{10}(x + 1)$. Lesion length data were square-root transformed to stabilize variance before further analysis. Trials, species, mode of propagation and treatment were considered as the main effects and analysed by generalized linear model (GLM) procedure in SPSS. Because no significant difference was observed between the trials, data were combined in the subsequent analyses. Accessions were nested within species and their interaction analysed by GLM procedure in SPSS. Relationships among the severity data were examined by regression analysis and Spearman's correlation test. Data of the three categories of plant health status were analysed by chi-square tests with the assumption of independence of stem canker distribution in the macadamia populations. Chi-square tests were performed to determine whether there was a significant difference between the expected frequencies and the observed frequencies in each species and the whole macadamia population. The null hypothesis tested was that the observed classification of stem canker resistance or tolerance is independent of the species. Significant means were compared using the least significance difference test. In order to explore which of the three measured traits were the most influential across species in driving the separation, ordination analysis using principal component analysis (PCA) was performed in PRIMER v. 6.0 software (PRIMER-E Ltd).

Results

Phenotypic response of macadamia to stem inoculation with *P. cinnamomi*

Phytophthora cinnamomi was recovered from the advancing margin of visible lesions of all the plants with symptoms. The PCR-RFLP profile of the isolates was similar to that of the UQ7097 isolate. *Phytophthora cinnamomi* caused lesions of varying lengths and stem canker. None of the control inoculations with V8 juice agar disks developed canker or extended lesions beyond the size of the wound created (Fig. 1). The plant response to *P. cinnamomi* infection varied among the inoculated plants. Lesions developed in the wounds in various forms; some lesions were restricted (Fig. 1b,c), others extended around the stem (Fig. 1d) or above and below the site of inoculation (Fig. 1e). The appearance of stem canker also varied from deep canker to those that developed canker with callus formation around the site of inoculation (Fig. 1f,g). In some cases complete girdling of the stem resulted in the death of the foliage above the site of inoculation (Fig. 1h).

Stem canker and necrotic lesions in macadamia species and hybrids

Stem canker and lesion elongation occurred in all four *Macadamia* species and hybrids. There were significant

Figure 1 Various symptoms expressed on macadamia plants at 3 months after wound-inoculated with *Phytophthora cinnamomi*. (a) Healthy wood of plant inoculated with agar without *P. cinnamomi*; (b) and (c) restricted lesions; (d) lesion girdling stem; (e) extended lesion above and below site of inoculation; (f) deep stem canker with raised callus at site of inoculation; (g) extensive canker with pathogen mycelium; and (h) death of inoculated plants – total death (left plant) and death above site of inoculation (right plant).

($P < 0.05$) differences among the species for lesion length and stem canker severity rating (Fig. 2). In the disease severity parameters, the accessions that contained both *M. integrifolia* and *M. tetraphylla* had the highest mean values and hence were the most susceptible to stem infection by *P. cinnamomi*. Among the four species, *M. integrifolia* had the highest stem canker rating and lesion length (Fig. 2).

There was fatal infection (canker severity rating of 5) in some of the *M. integrifolia* and *M. tetraphylla* accessions, but no fatal infection rating in the accessions of *M. ternifolia* and *M. jansenii* and their crosses. The chi-square test showed that stem canker severity (plants with no canker, canker present but not fatal, or infection fatal), was independent ($\chi^2 = 0.1376$ at d.f. = 10) of species. Significant ($P < 0.05$) differences were observed among accessions in lesion lengths, and significant differences between the expected frequencies and the observed frequencies in the severity categories were observed for accessions in the cultivated species.

Influence of mode of propagation and stem diameter on disease severity

Overall, there were no significant differences at $P < 0.05$ between the two propagation methods and their interaction with species or accessions for stem canker and lesion length. Nonparametric correlations indicated a strong significant association between lesion length and stem canker rating (coefficient = −0.371; $P = 0.009$). Correlations of the disease severity parameters with the basal diameter of the stem were not significant (coefficient = −0.079, $P = 0.68$; and coefficient = −0.109, $P = 0.565$, for lesion length and stem canker rating, respectively; Fig. 3a). Plots of mean lesion lengths per stem canker severity score showed a linear increase, but the trend of the relationship between the disease severity parameters was weak ($R^2 = 0.44$). Hence, low stem canker rating sometimes resulted in high lesion length in infected tissue (Fig. 3b).

The PCA results showed that three axes were needed to explain the variations in the three parameters. The first

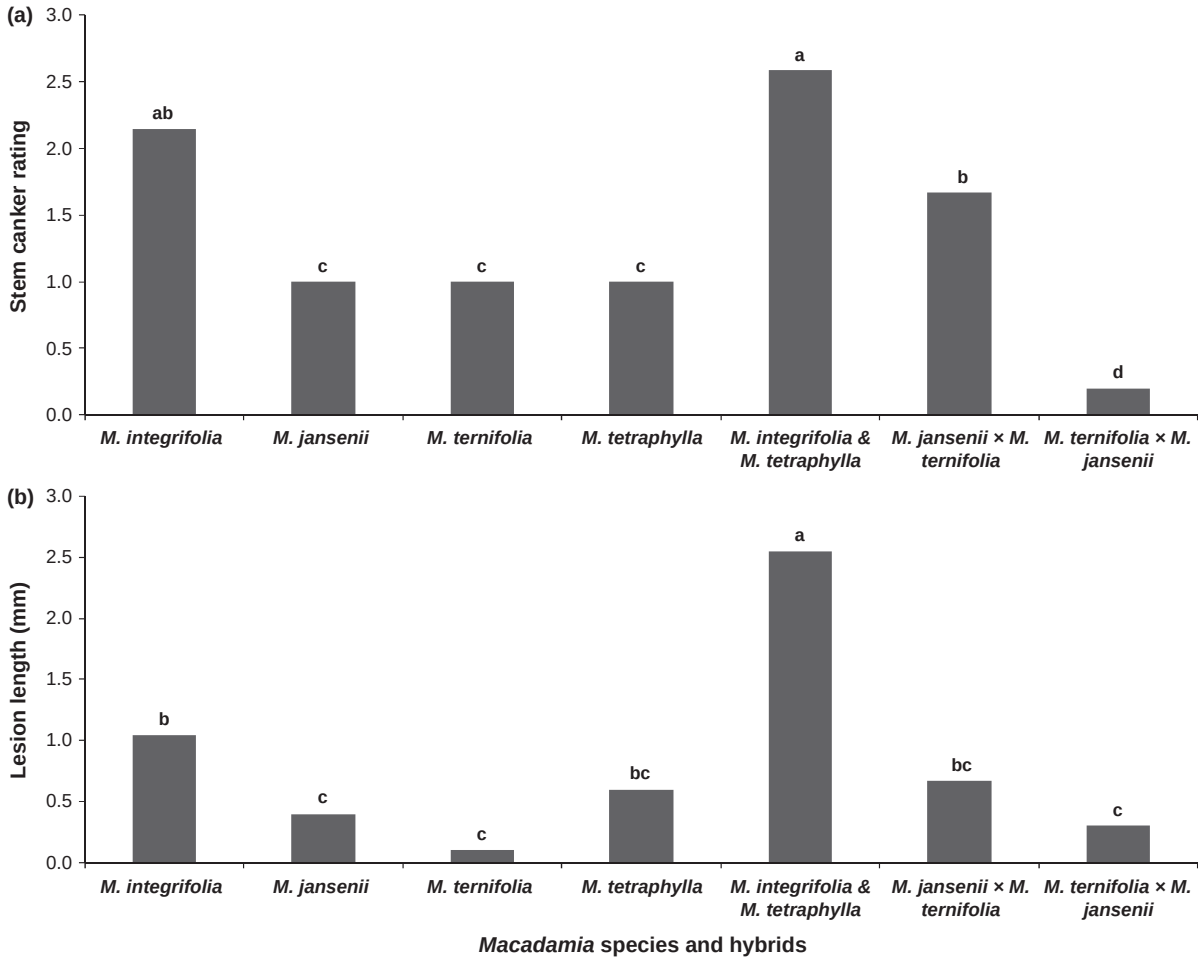

Figure 2 Stem canker ratings (a) and lesion length (b) caused by *Phytophthora cinnamomi* in plants of different *Macadamia* species and hybrids. Rating scale (0–5) where 0 = no obvious canker or tissue discolouration and 5 = complete death of the plant. Bars with the same letters are not significantly different at $\alpha = 0.05$, according to least significant difference test.

axis accounted for the majority of the variation (63.4%) and was influenced by both canker rating (Eigenvector = −0.761) and lesion length (Eigenvector = −0.646), which was largely the contributions of differences among the species. The second axis accounted for 24.1% of the variation and was mostly due to the contribution of stem diameter (Eigenvector = 0.998; Fig. 4).

Discussion

This study demonstrated significant within-species variation in susceptibility to *P. cinnamomi* in accessions of the four different *Macadamia* species. The data clearly indicates the existence of considerable variation in response to *P. cinnamomi* infection via wounding in macadamia stems that is structured mostly at the level of individuals within each species. The results showed that stem canker caused by *P. cinnamomi* is not just a problem in cultivated *M. integrifolia* and *M. tetraphylla* species in plantations, but is a significant threat to macadamia biodiversity. *Phytophthora cinnamomi* was introduced into Australia and has become a pathogen of several native plant species in Australia (Cahill *et al.*, 2008; Hee *et al.*, 2013). There is a very low level of phenotypic and genotypic diversity in the Australian *P. cinnamomi* population (Linde *et al.*, 1999b), therefore, somewhat similar levels of damage may be caused by different isolates on the same plant species.

All four *Macadamia* species usually occur in small populations in the native ecosystem and are at risk of extinction (Pisanu *et al.*, 2009). Unlike *M. ternifolia* and the cultivated species *M. tetraphylla* and *M. integrifolia* where large genetic diversity exists (Steiger *et al.*, 2003; Peace *et al.*, 2008), genetic diversity in *M. jansenii* is low and subpopulations show little genetic differentiation (Shapcott & Powell, 2011). *Macadamia jansenii* is currently known to exist in a single population and has been classified as endangered under the Environment Protection and Biodiversity Conservation Act, 1999. Efforts to safeguard against extinction of the population are currently underway (Shapcott & Powell, 2011). *Phytophthora cinnamomi* infection increases the risk to threatened species

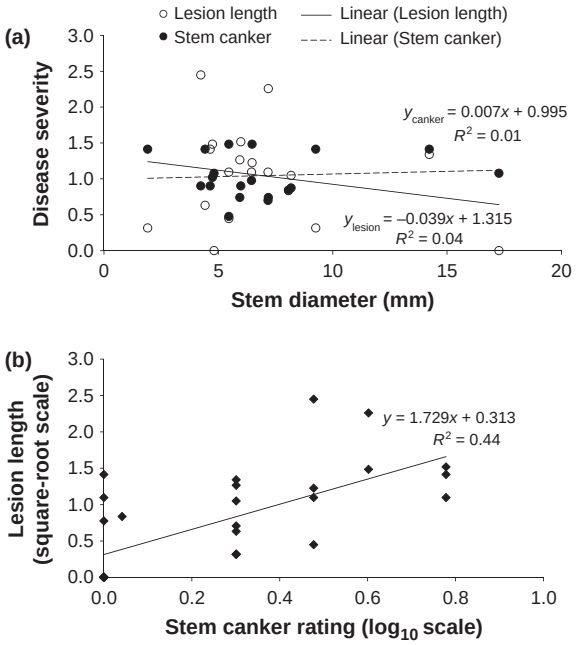

Figure 3 Relationship between (a) disease severity parameters and stem diameter and (b) stem canker rating and length of lesions, on macadamia plants inoculated with *Phytophthora cinnamomi*.

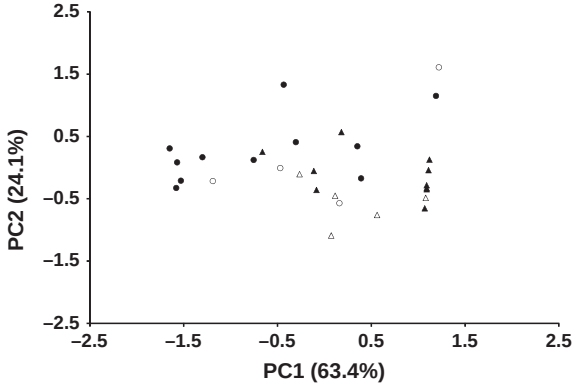

Figure 4 Principal component analysis (PCA) of traits of disease severity (stem canker, lesion length and basal stem diameter) as indicators of variation among plants from stem cuttings (open symbols) and seed germination (closed symbols) of different accessions of cultivated (circles) and wild (triangles) *Macadamia* species inoculated with *Phytophthora cinnamomi*.

including *M. ternifolia* and *M. jansenii* in their native ecosystems. Although all accessions of *M. tetraphylla* and *M. integrifolia* used in commercial productions have been selected from the native environments in Australia, wild macadamia populations represent a significant resource for the macadamia industry as potential sources of resistance to biotic and abiotic stress, and for improving yield and breeding efficiencies. These wild accessions are vulnerable to extinction due to habitat loss and fragmentation (Pisanu *et al.*, 2009; Neal *et al.*, 2010; Powell *et al.*, 2010). In addition to these two major threats, a large number of native plant species are threatened by *P. cinnamomi* in Australia (Kueh *et al.*, 2012). The susceptibility of the wild and cultivated macadamia accessions to *P. cinnamomi* revealed in this study showed that the pathogen poses further serious threat to the remaining native macadamias. Measures to safeguard the species should therefore include strategies to address the risk posed by *P. cinnamomi*.

The significant variation observed in the plant response to *P. cinnamomi* inoculation suggests there are important differences in genetic resistance. While clones of some accessions consistently produced similar lesion sizes and cankers, in other cases the levels of variation of within-accession symptoms were large. Lesion lengths varied significantly among accessions; in addition to the differences between species, greater variance in lesion length occurred within species. There was little variation at the whole population level, where the observed distribution of stem canker severity was consistent with that expected ($\chi^2 = 0.1376$ at d.f. = 10) in the populations. At the within-species level, large significant ($P < 0.01$) variations were only observed in *M. integrifolia* and *M. tetraphylla*. *Macadamia integrifolia* had the highest values for both disease parameters used in this study, showing that it is the most susceptible species to the disease. It should be noted that for three of the species there was only one accession and thus conclusions about the general performance of a species are difficult. The relative resistance in the macadamia accessions observed through the artificial stem inoculations reflects field observations of relative susceptibility of the cultivated species (Zentmyer & Storey, 1961; Pegg, 1973). Lesion lengths were significantly smaller in the nondomesticated species and their hybrids than the cultivated species. Further studies are needed to examine the genetic control of response for lesion length and stem canker severity.

The results indicate that rootstocks propagated as stem cuttings or seedlings respond similarly to stem infection by *P. cinnamomi*. Thus, this study suggests that stem canker has minimal impact for the choice of rootstocks based on mode of propagation either as cuttings or seeds in macadamia. The lack of significant differences between plants propagated as stem cuttings and seedlings for *P. cinnamomi* stem infection may be due to the low number of accessions used in this study. Because macadamia is preferentially outcrossing (Sedgley *et al.*, 1990), a higher degree of variation is expected in the seedling population compared with cuttings that are clonal, which should exhibit relatively more similar phenotypic characteristics. The genetically heterozygous seedling population of each species may influence their performance against *P. cinnamomi*. The results here support the report by Topp & Neal (2015) where there was little evidence for any interaction between rootstock cultivar and propagation method (seedling and cuttings) for susceptibility to *P. cinnamomi*. In the present study the plants were not grafted; however, in some tree crops rootstock genotypes have profound effects on many scion

characteristics (Gregory *et al.*, 2013). A preliminary study in macadamia suggests that scion significantly influences tree performance to *P. cinnamomi* root infection (Akinsanmi & Drenth, 2016). In the study, tree growth was reduced by 33–60% in cultivar 816 and by 9–40% in cultivar 842 grafted on seedling H2 rootstock. This suggests that the significant rootstock × scion interaction might be a key mechanism in allowing macadamia trees to recover from *P. cinnamomi* root infection (Akinsanmi & Drenth, 2016).

The results showed that basal stem diameter had no significant effect on stem canker and lesion length caused by *P. cinnamomi* in macadamia. Low genetic correlation observed between stem diameter and other traits such as yield per tree suggests these traits may be under the control of different genes (Hardner *et al.*, 2002). Although in this study the influence of scion on rootstock performance was not considered, some studies have demonstrated that scion exerts a stronger influence on the variation in several yield and tree performance traits than rootstock selection (Trochoulias, 1992; Topp & Neal, 2015). Therefore, future studies are needed to examine if scion may influence susceptibility of macadamia rootstocks to *Phytophthora*.

This study is the first report of variation in susceptibility among the wild and cultivated species of macadamia to stem infection by *P. cinnamomi*. It suggests that significant variations in susceptibility may exist within each species. Further studies are needed which increase the number of accessions that are evaluated for each species and to examine the genetic control of susceptibility to *P. cinnamomi* in a wider range of material. It is uncertain whether variation in susceptibility of macadamia to *P. cinnamomi* is associated with specific gene loci or controlled by multigenic loci. It would be beneficial to determine the genetic control of resistance or tolerance to *P. cinnamomi* and thus allow recommendations to industry on the value of open-pollinated seedling rootstocks that are derived from resistant or tolerant accessions. Vulnerable and endangered plant species that often occur in fragmented and small populations with restricted geographical distribution are at risk from *P. cinnamomi* (Kueh *et al.*, 2012); an effective management strategy may therefore be required to avoid extinction of the wild *Macadamia* species due to stem canker by the *Phytophthora* pathogen.

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MACADAMIA FUND

Integrated Management of Diseases in Macadamia Industry

Appendix 2: Details of methodology, results and conclusions of research activity

(c) Endemic diseases - Flower blight (Dry flower)

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Project number: MC12007

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Dry Flower Disease of *Macadamia* in Australia Caused by *Neopestalotiopsis macadamiae* sp. nov. and *Pestalotiopsis macadamiae* sp. nov.

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Abstract

Incidence of dry flower disease of macadamia (*Macadamia integrifolia*), expressed as blight of the flowers and necrosis and dieback of the rachis, is increasing in Australia. In the 2012–13 production season, incidence of dry flower disease resulted in 10 to 30% yield loss in the affected orchards. Etiology of the disease has not been established. This study was established to characterize the disease and identify the causal pathogen. A survey of the major macadamia-producing regions in Australia revealed dry flower disease symptoms regardless of cultivar or location at all stages of raceme development. Based on colony and conidial morphology, the majority (41%) of fungal isolates obtained from tissue samples were identified as *Pestalotiopsis* and *Neopestalotiopsis* spp. The phylogeny of the combined partial sequence of the internal transcribed spacer, β-tubulin, and translation elongation factor 1-α gene loci segregated the isolates into two well-supported clades, independent of location or part of the inflorescence affected. Further morphological examination supported the establishment of two new species, which are formally described as *Neopestalotiopsis macadamiae* sp. nov. and *Pestalotiopsis macadamiae* sp. nov. Using spore suspensions of isolates of both species, Koch's postulates were fulfilled on three macadamia cultivars at all stages of raceme development. To our knowledge, this is the first report of species of *Neopestalotiopsis* and *Pestalotiopsis* as causal agents of inflorescence disease in macadamia.

Macadamia (*Macadamia integrifolia* and *M. tetraphylla*, along with their hybrids) is grown in commercial plantations in tropical and subtropical frost-free regions worldwide. Macadamia fruit is a dehiscent pericarp (the husk) that encloses a shell and an edible cream-colored kernel (the embryo). Each kernel is derived from one of the six pairs of flowers at each node of the rachis (Trueman and Turnbull 1994). Macadamia flowers are about 15 mm long, including a 3-mm pedicel (Moncur et al. 1985; Sakai and Mike 1985). Between 100 and 300 flowers are borne on each pendant raceme (inflorescence) and a mature tree may produce over 10,000 racemes (McFadyen et al. 2012; Sedgley 1981; Trueman 2013). In Australia, macadamia raceme elongation starts in mid- to late winter (June to July), with the peak anthesis in late winter or early spring (September) (Heard 1993; Moncur et al. 1985; Trueman and Turnbull 1994; Wallace et al. 1996). Four developmental stages of macadamia raceme (Fitzell 1994) occur during this period. At stage 1, small green florets (or buds) develop on the rachis. At stage 2, the flowers turn light green to white and are partially to fully open and the stamens pull away from the stigmas. At stage 3, the flowers are fully opened for approximately 5 to 10 days and the sepals turn brown. In the final stage, the sepals fall off and the fertilized embryos, still attached to the raceme, begin to swell. Most of the flowers abscise within 2 weeks after fertilization and the remaining fertilized embryos develop rapidly on the rachis, increasing in fruit diameter for up to 15 to 16 weeks after anthesis (McFadyen et al. 2012; Sedgley 1981; Trueman 2013; Trueman and Turnbull 1994; Wallace et al. 1996).

A number of pathogens have been reported to affect macadamia inflorescences, and the diseases they cause are mostly raceme blights (Drenth et al. 2009; Manicom 2003; Zentmyer 1962). There are some distinguishable symptoms of raceme blight caused by different pathogens. Raceme blight or gray mold caused by *Botrytis cinerea* affects mature flowers at stage 3, with signs of the fungus visible on the senescent flower parts as gray mycelia that hold the collapsed flowers together on the rachis. Gray mold has been reported on racemes from most major macadamia-producing countries (Holtzmann 1963; Hunter et al. 1972; Mayers 1993; Nagao and Hirae 1992; Zentmyer 1962). *Phytophthora capsici* has been reported to cause macadamia flower blight in Costa Rica and Hawaii, where the disease is characterized by extensive and irregular dark necrotic lesions on affected plant parts, including the whole raceme, new leaf flush, young shoots, and immature fruit (Aragaki and Uchida 1980; Kunimoto et al. 1976). Severe raceme blight epidemic, caused by *Cladosporium cladosporioides*, has been reported from South Africa (van den Berg et al. 2008). Symptoms of raceme blight caused by *C. cladosporioides* are characterized by small, water-soaked specks on the flower that later become necrotic, with the diseased racemes covered in olive gray patches of mycelia and conidia (van den Berg et al. 2008).

In Australia, a new disease of macadamia racemes, termed dry flower, was first observed in an orchard in the Bundaberg production region in Queensland in 2009. The disease was initially considered a disorder caused by poor tree nutrition, such as phosphorus and boron deficiencies. However, in the 2011–12 production season, the disease resulted in complete crop failure in an orchard and, in the 2012–13 production season, approximately 10 to 30% yield losses occurred in several orchards in Queensland (Akinsanmi and Drenth 2013). Dry flower disease is increasing in incidence and prevalence in macadamia orchards in Queensland and northern New South Wales (Akinsanmi and Drenth 2013). A preliminary report associated the new disease with *Pestalotiopsis* spp. (Akinsanmi and Drenth 2013).

Dry flower disease is characterized by the dry appearance of the raceme. Necrotic blight symptoms appear on infected flowers and sometimes on the rachis from early bloom to full anthesis (Fig. 1 A–D). Immature buds or florets may become blighted within a few days, turning brown to dark brown, and remain attached to the green rachis. At later stages of inflorescence development, the floral parts became blighted, while necrotic flower parts may remain attached to the rachis or are easily dislodged when shaken. In certain cases, dried racemes persist in the tree canopy between seasons, and may serve as a source of inoculum in the following season. In some cases, dieback of the rachis at the distal end with advancing necrosis at the tip was seen on diseased racemes.

Dry flower disease poses a threat to macadamia production and, in order to develop and implement effective control measures, it is

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essential to first establish the cause of the disease. The objectives of this study were to (i) identify the causal agent of the disease and fulfill Koch's postulates and (ii) establish the prevalence of the disease. Precise knowledge about the timing of infection, potential sources of inoculum, and disease-conducive environmental factors are important for the development of integrated disease management practices.

Materials and Methods

Collection of samples and isolates. In order to determine the prevalence and parts of the macadamia inflorescences that were affected, 250 samples, comprising 176 racemes (stage 1), 30 racemes (stage 2), 27 racemes (stage 3), and 17 racemes (stage 4), were collected during a disease survey of commercial macadamia orchards in eastern Australia. Samples were obtained from 10 orchards in Queensland (North, $n = 3$; Central, $n = 4$; and Southeast, $n = 3$), as well as 7 orchards in New South Wales (Northern Rivers, $n = 5$ and Mid-North coast, $n = 2$). Both asymptomatic and symptomatic inflorescences with blight symptoms were collected, of which 137 had obvious and distinct raceme blight symptoms. Pieces of tissue were obtained from different sections (distal end, midsection, and basal) of the rachis and flowers from each sample. The pieces were surface sterilized in 2% sodium hypochlorite solution for 2 min, then rinsed in three changes of sterile distilled water. The tissue was dried using sterile blotting paper before plating on half-strength potato dextrose agar (PDA; Difco Laboratories) plates in four replicates for each sample. The plates were incubated at 25°C under a regime of 12 h of light and 12 h of darkness for 7 days. The percentage of tissue with fungal growth was recorded from the four replicates. Differences in fungal isolates between locations and among plant parts and sections of racemes were compared by one-way analysis of variance. All fungal colonies were subcultured on fresh PDA plates after 7 days of incubation. Preliminary identity of the fungal isolates was based on cultural and morphological characteristics. Single-spore cultures were derived from selected isolates as described by Akinsanmi et al. (2004) and stored at −20°C in sterile 15% glycerol solution. Representative isolates were deposited in the Queensland Plant Pathology Herbarium (BRIP), Ecosciences Precinct, Dutton Park, Australia.

DNA extraction and polymerase chain reaction amplification. Genomic DNA of each isolate was extracted from 10-day-old pure cultures using the Promega Wizard Genomic DNA Purification Kit (Promega Corp.), with minor modifications. For each fungal isolate, approximately 40 mg of the mycelium was transferred into a clean 2.0-ml safe-lock tube (Eppendorf AG) containing sterile stainless-steel beads in 600 µl of Nuclei Lysis solution. The content was homogenized using TissueLyser (Qiagen Pty. Ltd.) for 2 min at 30 Hz, before following the DNA extraction procedure in the Wizard Genomic DNA Purification Kit. DNA concentrations were determined using a BioDrop Duo spectrophotometer (BioDrop) and the working stock concentration was adjusted to 25 ng/µl. Representative fungal isolates were identified to the genus level based on sequencing of the internal transcribed spacer region (ITS) gene region. Polymerase chain reaction (PCR) amplifications were performed with ITS4 as reverse primer and ITS5 as forward primers, which included the ITS-1, ITS-2, and 5.8S ribosomal RNA gene regions (White et al. 1990).

For isolates tentatively identified as *Pestalotiopsis* spp. by conidial morphology, partial sequences were amplified of two secondary gene loci; namely, translation elongation factor 1-α (TEF) with EF1-728F and EF1-986R primers (Carbone and Kohn 1999) and β-tubulin (TUB) with primer pairs BT2A and BT2B (Glass and Donaldson 1995; O'Donnell and Cigelnik 1997). PCR amplification was carried out in a 30-µl reaction mix as described by Harteveld et al. (2013) using a SuperCycler Thermal Cycler (Kyratec). The PCR program consisted of an initial denaturation step at 98°C for 60 s; followed by 35 cycles at 98°C for 10 s, 62°C for 30 s, and 72°C for 45 s; and a final extension step at 72°C for 5 min.

The PCR amplicons were separated in 1% agarose gel (BIOLINE) stained with gel red in 0.5% Tris-borate EDTA buffer solution and viewed under UV light using Molecular Imager GelDoc (Bio-Rad Laboratories Inc.). The amplicon sizes were determined against a 1-kb HyperLadder (BIOLINE) and then the targeted PCR amplicon was purified using Roche High Pure PCR Product Purification Kit (Roche Applied Science) according to the manufacturer's instructions before DNA sequencing with 3730xl DNA analyzer at Macrogen Inc. using the same primers used for amplification.

Phylogenetic analysis. Molecular Evolutionary Genetics Analysis (MEGA6) software (Tamura et al. 2013) was used to manually assemble the forward and reverse sequences into consensus fragments. In order to provide consistency and quality of the sequences, the chromatograms of the sequences were checked and aligned using Clustal W, and primer sequences were trimmed off at both ends of the sequences. For each set of nucleotide sequences, the identity of each isolate was determined by comparison against ex-type cultures available in the National Center for Biotechnology Information GenBank database using the BLAST search procedure. Phylogenetic analyses of the 32 *Pestalotiopsis* and *Neopestalotiopsis* isolates (Table 1) were performed by the maximum-parsimony (MP) method in MEGA6 with the concatenated sequences of all the three gene loci (ITS + TUB + TEF), with the selected strains of different species in the phylogenetic clades of the genus *Pestalotiopsis, Neopestalotiopsis*, and *Pseudopestalotiopsis* as described by Maharachchikumbura et al. (2014). Sequences of the reference strains were obtained from GenBank (Table 2). The MP phylogram was constructed in MEGA 6 using the Subtree-Pruning-Regrafting algorithm (Nei and Kumar 2000) with search level 1, where the initial trees were obtained by the random addition of sequences. All positions containing gaps and missing data were eliminated. Tree stability was tested

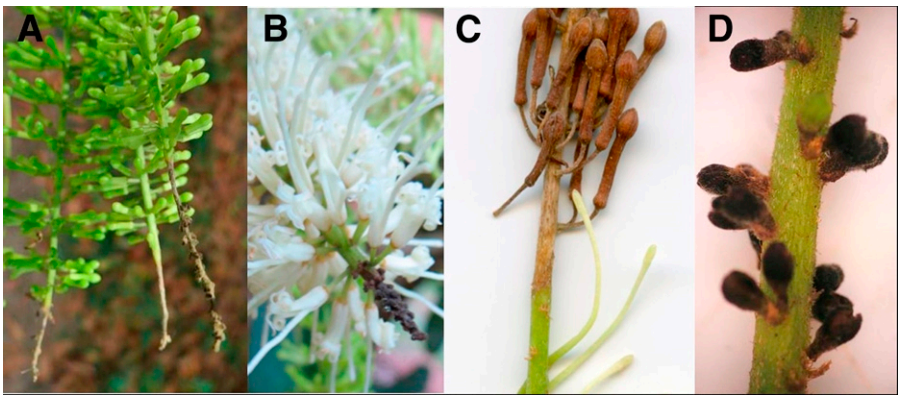

Fig. 1. Symptoms of diseased macadamia racemes associated with species of *Pestalotiopsis* and *Neopestalotiopsis* in Australia. **A,** Macadamia rachis dieback and necrosis; **B,** rachis dieback; **C,** flower blight and rachis necrosis at raceme stage 1 developmental period; and **D,** blight of newly formed flowers.

by 1,000 bootstrap replicates. All generated sequences were submitted to GenBank.

Morphology. Isolates were grown on 2% PDA, autoclaved pine needles on water agar, and oat meal agar at 25°C under 12 h of light and 12 h of darkness for 14 days prior to examination of conidiomata, conidia, and conidiophores under a dissecting microscope and on slide mounts in clear 100% lactic acid. Fungal structures were viewed and measured at ×1,000 magnification by differential interference contrast using a Leica DM2500 microscope. Conidial sizes were expressed as 95% confidence levels derived from 20 observations, with extremes of conidial measurements given in parentheses. Colonies were described after 7 days of incubation on PDA using the color charts of Rayner (1970). Novel species were registered in MycoBank (Crous et al. 2004).

Pathogenicity tests. In order to determine the ability of representative fungal isolates to cause disease, pathogenicity was tested on racemes of 'A203' macadamia under field conditions. Spore suspension of each isolate was obtained from 7-day-old culture on PDA, as described previously (Harteveld et al. 2013). A drop of Tween 80 (Sigma-Aldrich) was added to the final spore suspension (10^5 conidia ml^{-1}). Three racemes at each of the three different stages (1 to 3) of development on four trees were tested against each isolate. Each raceme was lightly sprayed with the spore suspension until run-off using a spray bottle. Racemes sprayed with sterile distilled water served as negative controls. Inoculated racemes were enclosed for 24 h at high humidity in moistened polythene bags covered by white paper bags. After 24 h of incubation, the polythene bags were removed from the paper bags that were left to cover the racemes for 7 to 10 days. Inoculated racemes were removed from the tree and examined for dry flower symptoms. In order to confirm infection and fulfill Koch's postulates, pieces of symptomatic and asymptomatic tissue of the rachis and flowers of each inoculated raceme were surface sterilized and plated on half-strength PDA plates, and the identity of fungal isolates was confirmed as described above. A second set of pathogenicity assays was made with additional isolates of fungi that had successfully infected host material from the first assay. In the second set, three additional isolates, based on the phylogenetic analysis, were included for pathogenicity test on 'HAES 695', A203, and 'HAES 741' macadamia (Table 1).

Results

Incidence of *Pestalotiopsis* and *Neopestalotiopsis* isolates on macadamia racemes. There was no difference in the frequency of *Pestalotiopsis* and *Neopestalotiopsis* spp. isolated from the samples obtained from the various locations. Rachis and flowers at stage 1

Table 1. Details of *Pestalotiopsis* and *Neopestalotiopsis* isolates obtained from macadamia racemes collected from New South Wales (NSW) and Queensland (QLD) states in Australia included in this study

						GenBank accession number[a]		
Species	**BRIP[b]**	**Loc[c]**	**RS[d]**	**PP[e]**	**Symptoms[f]**	**TUB**	**ITS**	**TEF**
Neopestalotiopsis macadamiae	63736a	NSW	3	FL	FB	KX186651	KX186601	KX186623
N. macadamiae	63736b[g]	NSW	3	FL	FB	KX186652	KX186603	KX186625
N. macadamiae	63737b	NSW	4	OV	FB	KX186653	KX186600	KX186626
N. macadamiae	63737c[g,h]	NSW	4	OV	FB	KX186654	KX186604	KX186627
N. macadamiae	63738a	NSW	1	RA	RD	KX186655	KX186602	KX186624
N. macadamiae	63740a[g]	NSW	3	FL	RN, FB	KX186656	KX186617	KX186628
N. macadamiae	63742a	NSW	3	OV	RN, FB	KX186657	KX186599	KX186629
N. macadamiae	63743a	NSW	2	FL	RN, FB	KX186658	KX186598	KX186631
N. macadamiae	63744a	QLD	1	FL	AF-RD	KX186659	KX186615	KX186632
N. macadamiae	63745a	QLD	2	FL	AF-RD	KX186660	KX186614	KX186633
N. macadamiae	63746a	QLD	2	RA	FB, RN, RD	KX186661	KX186605	KX186634
N. macadamiae	63747a	QLD	1	RA	FB, RN, RD	KX186662	KX186613	KX186635
N. macadamiae	63748a	QLD	1	RA	FB, RN, RD	KX186663	KX186612	KX186636
N. macadamiae	63748b	QLD	1	RA	FB, RN, RD	KX186664	KX186611	KX186637
N. macadamiae	63749a	QLD	1	RA	RN, FB	KX186665	KX186596	KX186638
N. macadamiae	63750a	QLD	1	FL	RN	KX186666	KX186610	KX186639
N. macadamiae	63751a	QLD	1	FL	RN	KX186667	KX186597	KX186640
N. macadamiae	63752a	QLD	2	RA	RN	KX186668	KX186595	KX186641
N. macadamiae	63752b	QLD	2	RA	RN	KX186669	KX186609	KX186642
N. macadamiae	63753a	QLD	2	RA	RN	KX186670	KX186608	KX186643
N. macadamiae	63754a	QLD	4	OV	FB	KX186671	KX186606	KX186644
N. macadamiae	63755a	QLD	1	RA	RN	KX186672	KX186594	KX186645
N. macadamiae	63756a	QLD	3	RA	FB, AR	KX186673	KX186593	KX186646
N. macadamiae	63757a	QLD	3	RA	FB, AR	KX186674	KX186592	KX186647
N. macadamiae	63759a[g]	NSW	1	RA	AF, AR	KX186675	KX186591	KX186649
N. macadamiae	63760a[g]	NSW	1	FL	FB, RN, RD	KX186676	KX186590	KX186650
Neopestalotiopsis sp.	63742b	NSW	3	OV	RN, FB	…	KX186616	KX186630
Neopestalotiopsis sp.	63758a	QLD	4	RA	RN	…	KX186607	KX186648
Pestalotiopsis macadamiae	63738b[g,h]	NSW	1	RA	RD	KX186680	KX186588	KX186621
P. macadamiae	63739a[g]	NSW	1	RA	RD	KX186681	KX186589	KX186622
P. macadamiae	63739b[g]	NSW	1	RA	RD	KX186679	KX186587	KX186620
P. macadamiae	63741a[g]	NSW	1	FL	RN, FB	KX186678	KX186586	KX186619
Pestalotiopsis sp.	63737a[g]	NSW	4	OV	FB	KX186677	…	KX186618

[a] Abbreviations: β-tubulin (TUB), internal transcribed spacer (ITS), and translation elongation factor 1-α (TEF).
[b] Queensland Plant Pathology Herbarium (BRIP) accession numbers.
[c] Location.
[d] Developmental stages of macadamia racemes.
[e] Plant part: FL = flower, OV = ovary, and RA = rachis.
[f] Symptoms: FB = flower blight, RN = rachis necrosis, RD = rachis dieback, AF-RD = asymptomatic flower but rachis dieback, AR = asymptomatic rachis, and AF = asymptomatic flowers.
[g] Isolates used in pathogenicity assays.
[h] Ex-type cultures. Taxonomic novelties are in bold print.

raceme development were most frequently infected with *Pestalotiopsis* and *Neopestalotiopsis* spp. compared with other raceme stages (Fig. 2). Regardless of the raceme stage, flowers at the midsection of the racemes were significantly more frequently infected than those at the distal end (tip) or basal section of the raceme (Fig. 2). In contrast, isolations from rachis at the basal section and distal end were generally more infected with *Pestalotiopsis* and *Neopestalotiopsis* spp. than the middle section (Fig. 2).

Pathogenicity assay. In the first series of pathogenicity assays on macadamia A203, both blight and necrotic symptoms were reproduced on racemes inoculated with *Pestalotiopsis* and *Neopestalotiopsis* isolates at stages 1 to 3 of raceme development within 7 days after inoculation. No symptoms were produced on racemes inoculated with isolates of *Alternaria, Epicoccum*, *Fusarium, Penicillium, Aspergillus*, and *Diaporthe* and a water control. Collapsed flowers with extensive fungal growth occurred on racemes inoculated with *Botrytis* and *Cladosporium* isolates at raceme stage 3 from 7 days after inoculation. Koch's postulates were proven for all the plant materials inoculated with *Pestalotiopsis* and *Neopestalotiopsis* isolates. The second series of pathogenicity tests using the additional isolates of *Pestalotiopsis* and *Neopestalotiopsis* on different macadamia cultivars showed that isolates from each genus were pathogenic on macadamia racemes, causing raceme blight and necrosis of the rachis on the three cultivars tested.

Identification and classification of fungi. Fungi were recovered from approximately 82% of the symptomatic and 32% of the asymptomatic tissues sampled. The percentage of isolates of *Pestalotiopsis* and *Neopestalotiopsis* was significantly ($P < 0.0001$) higher than other fungi, which are listed in decreasing order of frequency of isolation as follows: genera *Alternaria, Epicoccum*, *Fusarium, Botrytis*, *Cladosporium*, *Penicillium, Aspergillus*, and *Diaporthe* (Fig. 3). The majority (41%) of the 826 isolates recovered were identified as *Pestalotiopsis* and *Neopestalotiopsis* spp. based on their cultural characteristics on quarter-strength PDA and conidial morphology. Species of *Pestalotiopsis* have four-septate conidia with concolored median cells, whereas *Neopestalotiopsis* have four-septate conidia with versicolored median cells (Maharachchikumbura et al. 2014).

The MP analyses of the combined sequence data (ITS + TUB + TEF) of the Australian isolates of *Pestalotiopsis* and *Neopestalotiopsis* together with reference specimens resulted in phylogenetic trees with the same topology. These trees indicated that Australian isolates of

Table 2. Details of strains representing species in the phylogenetic clades of *Pestalotiopsis*, *Neopestalotiopsis*, and closely related genus used in this study[a]

Species	GenBank accession number[b]	Host family	Location
Neopestalotiopsis asiatica	MFLUCC12-0286	…	China
N. australis	CBS 114159	Proteaceae	Australia
N. ellipsospora	MFLUCC12-0283	…	China
N. eucalypticola	CBS 264.37	Myrtaceae	…
N. foedans	CGMCC3.9123	…	China
N. mesopotamica	CBS 336.86	Pinaceae	Iraq
N. rosae	CBS 101057	Rosaceae	New Zealand
N. surinamensis	CBS 450.74	…	Suriname
Pestalotiopsis arceuthobii	CBS 434.65	Santalaceae	United States
P. arengae	CBS 331.92	Arecaceae	Singapore
P. australasiae	CBS 114126	Proteaceae	New Zealand
P. australis	CBS 114193	Proteaceae	Australia
P. biciliata	CBS 124463	Platanaceae	…
P. chamaeropis	CBS 186.71	Arecaceae	Italy
P. clavata	MFLUCC 12-0268	Buxaceae	China
P. colombiensis	CBS 118553	Myrtaceae	Colombia
P. diploclisiae	CBS 115587	Rubiaceae	Hong Kong
P. diversiseta	MFLUCC12-0287	Ericaceae	China
P. grevilleae	CBS 114127	Proteaceae	Australia
P. hawaiiensis	CBS 114491	Myrtaceae	United States
P. hollandica	CBS 265.33	Sciadopityaceae	Netherlands
P. humus	CBS 336.97	…	Papua New Guinea
P. inflexa	MFLUCC12-0270	…	China
P. intermedia	MFLUCC12-0260	…	China
P. jesteri	CBS 109350	Gentianaceae	Papua New Guinea
P. kenyana	CBS 442.67	Rubiaceae	Kenya
P. linearis	MFLUCC12-0272	Apocynaceae	China
P. malayana	CBS 102220	Euphorbiaceae	Malaysia
P. novae-hollandiae	CBS 130973	Proteaceae	Australia
P. oryzae	CBS 353.69	Poaceae	Denmark
P. papuana	CBS 331.96	…	Papua New Guinea
P. parva	CBS 265.37	Fabaceae	…
P. portugalica	CBS 393.48	…	Portugal
P. rosea	MFLUCC 12-0258	Pinaceae	China
P. scoparia	CBS 176.25	Cupressaceae	…
Pestalotiopsis sp.	OP023	…	…
P. spathulata	CBS 356.86	Proteaceae	Chile
P. telopeae	CBS 114161	Proteaceae	Australia
P. trachicarpicola	OP068	Arecaceae	China
P. unicolor	MFLUCC12-0276	Ericaceae	China
Pseudopestalotiopsis cocos	CBS 272.29	Arecaceae	Indonesia
P. indica	CBS 459.78	Malvaceae	India

[a] Phylogenetic clades of genus *Pestalotiopsis*, as described by Maharachchikumbura et al. (2014).

[b] CBS: culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; CGMCC: China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing; ICMP: International Collection of Microorganisms from Plants, Auckland, New Zealand; IFRDCC: International Fungal Research & Development Centre Culture Collection, China; and MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand.

Pestalotiopsis and *Neopestalotiopsis* from macadamia formed separate, well-supported clades (Fig. 4). Phylogenetic inference and conidial morphology indicated that these isolates represent two novel species. Most of the isolates belonged to the genus *Neopestalotiopsis* and there was no association with the part (flower and rachis) from which they were isolated (Table 1).

Taxonomy. *Pestalotiopsis macadamiae* R. G. Shivas and Akinsanmi, sp. nov. MycoBank MB817931 (Fig. 5). Etymology: Named after the host genus from which it was isolated, *Macadamia*.

Conidiomata pycnidial in culture on PDA, globose, 200–400 μm diameter, solitary or aggregated in clusters, pale yellow brown, exudes dark brown slimy conidial droplets. Conidiophores septate and sparsely branched or reduced to conidiogenous cells, up to 40 μm long, hyaline, smooth. Conidiogenous cells cylindrical to lageniform, 10–20 × 2.5–3 μm, hyaline, smooth. Conidia fusiform to narrowly ellipsoidal, straight or curved, (18–) 18.5–22 (–25) × (5.5–) 6–6.5 (–7) μm, four-septate; apical cell conical, 2.5–5 μm long, hyaline, smooth, thin-walled, with 3 (rarely 2) apical tubular unbranched filiform flexuous appendages (12–) 14–21 (–24) μm; basal cell conic with a truncate base, 3–5 μm, hyaline to subhyaline, smooth, thin- walled, with a simple appendage 3–7 μm long; three median cells doliiform, 12–15 μm, concolored or the lower median cell is slightly paler than the other two cells, olivaceous brown, septa darker than the rest of the cell, smooth, second cell from base 3–6 μm long, third cell 3.5–5 μm long, fourth cell 4–6 μm long.

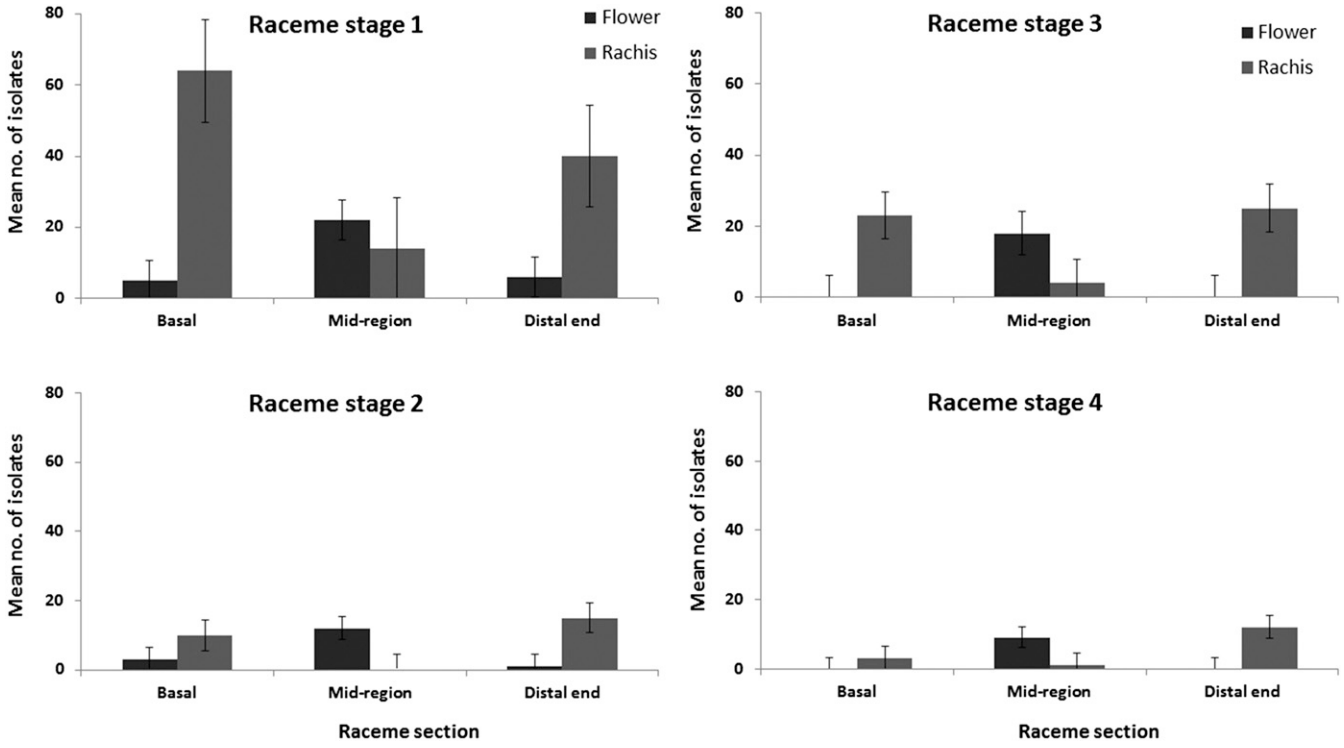

Fig. 2. Mean number of *Pestalotiopsis* and *Neopestalotiopsis* isolates recovered from rachis and flowers at different sections of macadamia racemes at the four stages of raceme development. Bars indicate standard error.

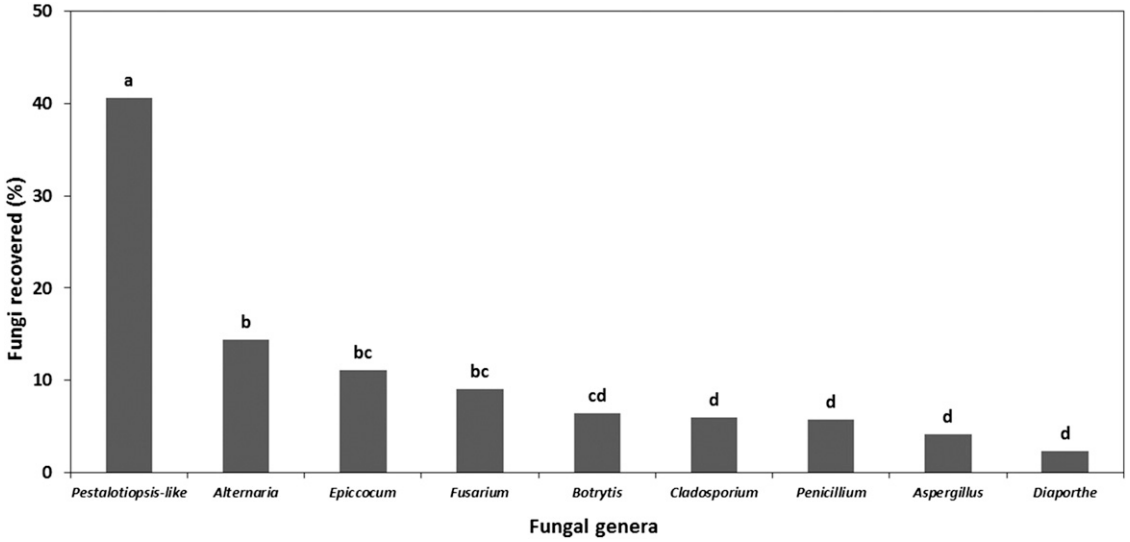

Fig. 3. Percentage of fungi isolated from macadamia racemes collected from different orchards in Australia. Bars with the same letters are not significantly different at $P < 0.05$ level according to Fisher's least significant difference test.

Culture characteristics: Colonies on PDA 6–7 cm after 7 days at 25°C, white, margin undulate, aerial mycelium sparse and irregularly zonate, conidiomata black and gregarious in the central 1.5 cm diameter part, reverse dull white.

Habitat and distribution: Inflorescences of *Macadamia integrifolia* (Proteaceae); Australia.

Type: AUSTRALIA, New South Wales, Lindendale, on rachis of *Macadamia integrifolia* with dieback, 20 Aug. 2014, O.A. Akinsanmi, holotype BRIP 63738b, includes ex-type cultures.

Neopestalotiopsis macadamiae R. G. Shivas and Akinsanmi, sp. nov. MycoBank MB817932 (Fig. 6). Etymology: Named after the host genus from which it was isolated, *Macadamia*.

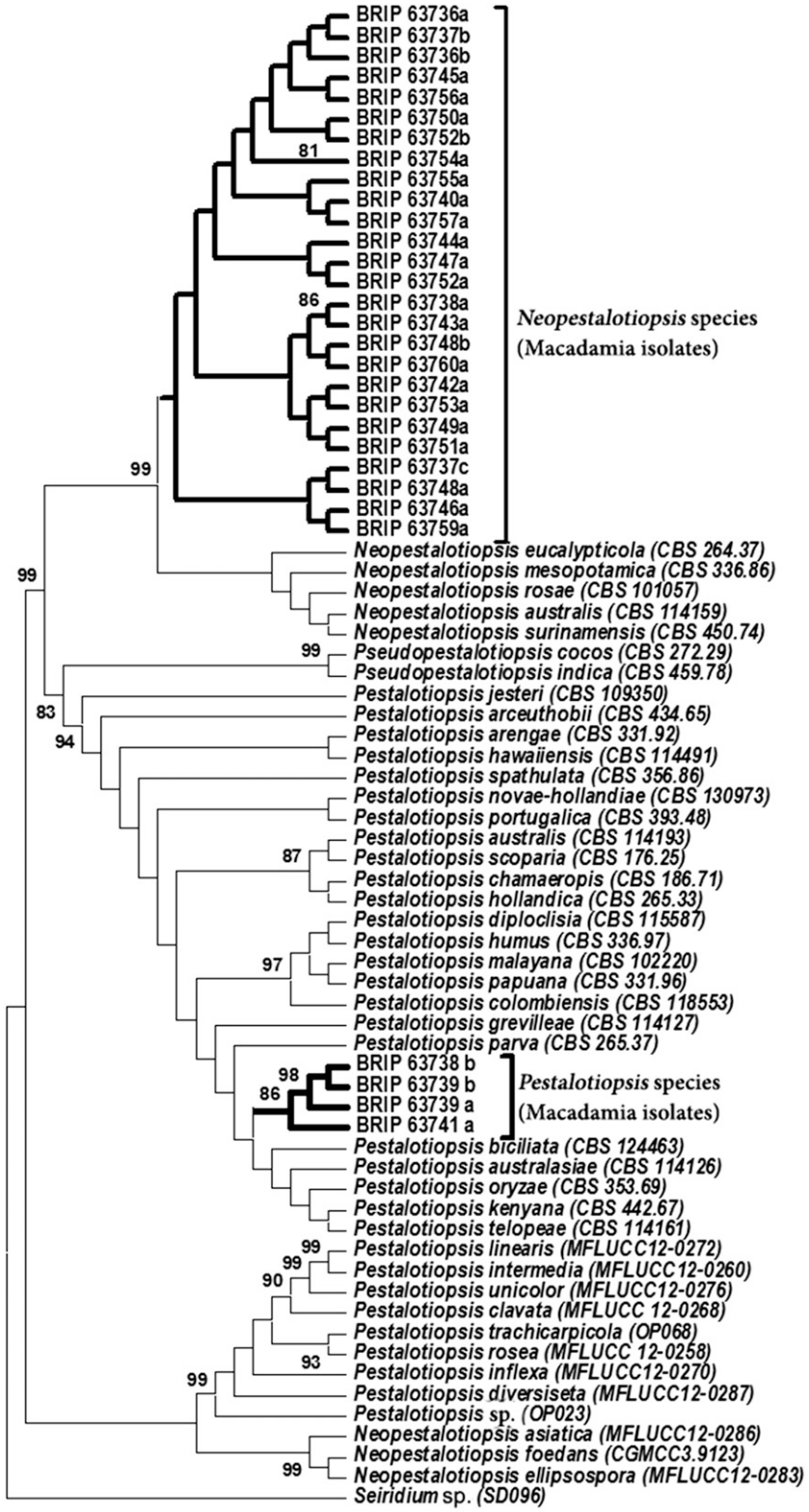

Fig. 4. Maximum-parsimony phylogram of the representative isolates (bold nodes) obtained from macadamia racemes in Australia, with selected strains of different species in the phylogenetic clades of the genera *Pestalotiopsis*, *Neopestalotiopsis*, and *Pseudopestalotiopsis*, as described by Maharachchikumbura et al. (2014). Phylogram is based on more than 50% majority rule inferred from 1,000 bootstrap replicates generated from combined sequences of internal transcribed spacer, β-tubulin, and translation elongation factor 1-α gene loci, and the bootstrap support values over 80% are indicated at the nodes. The tree is rooted to *Seiridium* sp. (SD096).

Conidiomata pycnidial in culture on PDA, globose, 200–500 μm diameter, solitary or aggregated in clusters, pale yellow brown, exudes dark brown slimy conidial droplets. Conidiophores septate or reduced to conidiogenous cells. Conidiogenous cells ampulliform to cylindrical, 5–20 × 2.5–5 μm, hyaline, smooth. Conidia fusiform to narrowly ellipsoidal, straight or curved, (23–) 24–28 (–29) × (6–) 6.5–7.5 (–8) μm, 4-septate; apical cell subcylindrical, 4–6 μm long, hyaline, smooth, thin-walled, with 3 (rarely 2) apical tubular unbranched filiform flexuous appendages (24–) 25–30 (–32) μm; basal cell conic with a truncate base, 3–6 μm, hyaline, smooth, thin- walled, with a simple appendage 3–7 μm long; three median cells doliiform, 14–18 μm, versicolored, olivaceous brown, smooth, septum between median cell and fourth cell from base dark brown and thickened; second cell from base pale brown 3.5–6 μm long, third cell medium to dark brown 4.5–7 μm long, fourth cell medium brown 4–6.5 μm long.

Culture characteristics: Colonies on PDA 7–8 cm after 7 days at 25°C, white to pale buff, margin entire, mycelium adpressed to very sparse, conidiomata not formed, reverse white to pale buff.

Habitat and distribution: Inflorescences of *Macadamia integrifolia* (Proteaceae); Australia.

Type: AUSTRALIA, New South Wales, Lindendale, on rachis with dieback of *Macadamia integrifolia*, 20 Aug. 2014, O.A. Akinsanmi, holotype BRIP 63737c, includes ex-type cultures.

Discussion

The study characterized a new disease of macadamia racemes in Australia termed dry flower caused by two newly described species, *Pestalotiopsis macadamiae* and *Neopestalotiopsis macadamiae*. Both fungal species were recovered from rachises and flowers of macadamia from different locations in eastern Australia. Various sections of the raceme were infected and *P. macadamiae* and *N. macadamiae* were consistently isolated from flowers with blight symptoms, rachises with necrotic symptoms, and dieback at the distal end of diseased macadamia racemes. To our knowledge, this is the first report of *Pestalotiopsis* and *Neopestalotiopsis* spp. as causal agents of raceme blight and rachis dieback in macadamia.

The characteristic symptoms of dry flower disease of macadamia include necrosis or dieback of the rachis (peduncle) tip, and the entire inflorescence (flowers and peduncle) may have dried flowers. All developmental stages of the raceme may be affected, particularly before nut set. Depending on the stage at which infection occurs, dried unopened flowers may remain attached to the rachis, although flowers infected at later stages may dislodge. Sometimes, diseased flowers at an early stage of raceme development cling together by mycelial strands that form on the peduncle. This may be due to secondary fungal colonizers or saprobes on the dead or diseased flowers. A causal relationship between dry flower disease and nut set or yield warrants further study. The key distinguishing characteristics and symptoms of dry flower disease from other raceme blights caused by *B. cinerea* (Holtzmann 1963) and *C. cladosporioides* (van den Berg et al. 2008) are the dry appearance of the diseased raceme, infection at all stages of raceme development, and diseased flowers that easily dislodge from the rachises. Koch's postulates were fulfilled with representative isolates *P. macadamiae* and *N. macadamiae*, including the ex-type cultures.

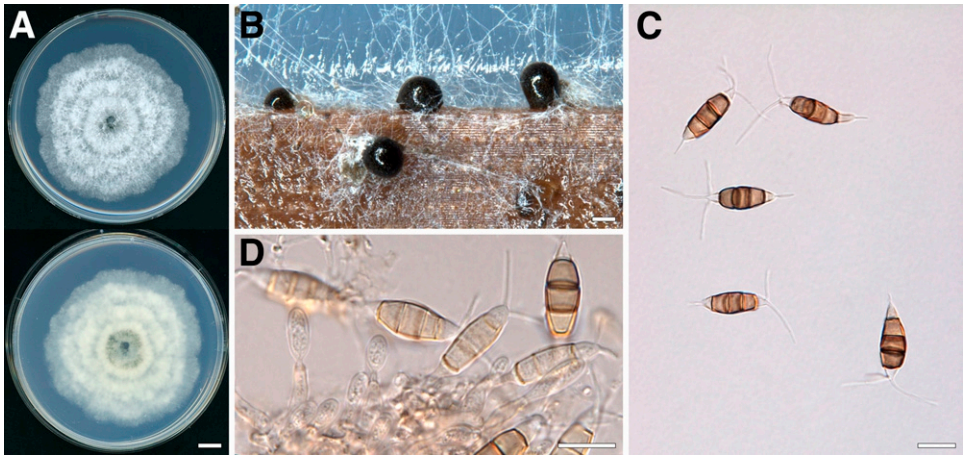

Fig. 5. *Pestalotiopsis macadamiae* (BRIP 63738b). **A,** Colony on potato dextrose agar after 7 days at 25°C (upper and lower surface); **B,** conidiomata on pine needle agar; **C,** conidia; and **D,** conidiogenous cells. Scale bars: A = 1 cm and B to D = 10 μm.

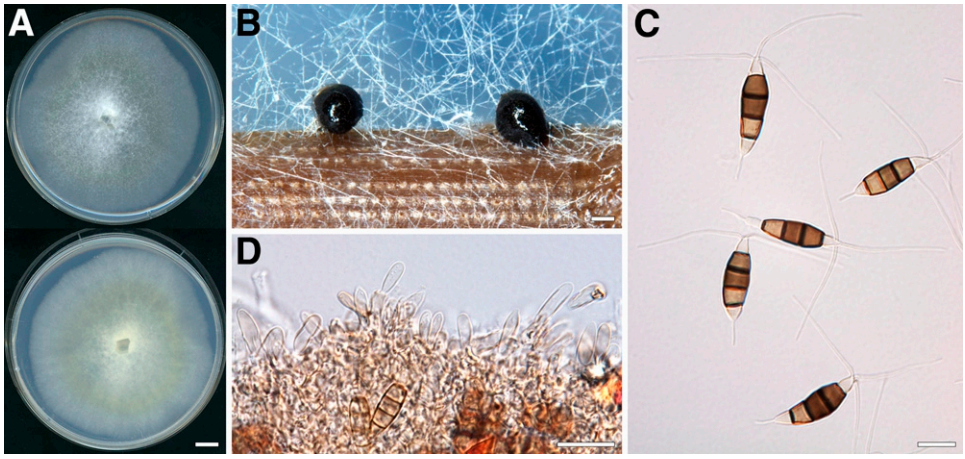

Fig. 6. *Neopestalotiopsis macadamiae* (BRIP 63738b). **A,** Colony on potato dextrose agar after 7 days at 25°C (upper and lower surface); **B,** conidiomata on pine needle agar; **C,** conidia; and **D,** conidiogenous cells. Scale bars: A = 1 cm and B to D = 10 μm.

Pestalotiopsis and *Neopestalotiopsis* are relatively important plant pathogenic genera able to infect wide host ranges (Keith et al. 2006; Maharachchikumbura et al. 2011). They are regarded as weak or opportunistic pathogens (Madar et al. 1991) only able to infect wounded or stressed plants (Keith et al. 2006). They are commonly isolated as endophytes (Tejesvi et al. 2009; Wei et al. 2007) and saprobes (Agarwal and Chauhan 1988; Okane et al. 1998; Osono and Takeda 1999). Many *Neopestalotiopsis* spp. have been isolated as causal agents of leaf spots and tip dieback from several hosts in the Proteaceae family such as macadamia (Maharachchikumbura et al. 2014). Various species of *Pestalotiopsis* and *Neopestalotiopsis* have been reported to cause a range of significant diseases in tree nut and fruit crops, including canker of cypress (Madar et al. 1991) and blueberry (Espinoza et al. 2008), leaf spots of *Caesalpinia echinata* (de Lourdes Mendes and Muchovej 1991) and oil palm (Suwannarach et al. 2013), scab of guava (Keith et al. 2006), shoot dieback of mango (Ismail et al. 2013), blight of *Lindera obtusiloba* (Jeon et al. 2007) and bayberry (Ren et al. 2013), severe chlorosis or necrotic lesions of maize (Tagne and Mathur 2001), and fruit rots of rambutan and grape (Deng et al. 2013; Keith 2008). In macadamia, *P. versicolor* was associated with leaf spots in India (Rawal and Muniyappa 1981). The genus *Pestalotiopsis* consists of over 235 described species, most of which are differentiated primarily based on conidial characteristics and traditionally named according to their host associations (Jeewon et al. 2003, 2004; Maharachchikumbura et al. 2011, 2012). The reason why species of *Pestalotiopsis* that are frequently isolated as endophytes in several horticultural crops are becoming a more common pathogen is still unclear. Anderson et al. (2004) hypothesized that environmental factors may be responsible for causing normally benign fungi to increase in pathogenicity. Species in the genus *Pestalotiopsis* are known to produce numerous secondary metabolites; however, it is uncertain whether this is associated with the dry appearance of diseased racemes. Although we observed minor variations in disease severity among the isolates and species, the focus of this study was to confirm Koch's postulates of the species; further research is needed to establish and compare aggressiveness between the species on macadamia racemes and also determine whether any variations in aggressiveness are linked to secondary metabolites produced by *P. macadamiae* and *N. macadamiae.*

Further cross-pathogenicity studies are needed to determine whether *P. macadamiae* and *N. macadamiae* have the potential to cause significant yield losses in other economically important crops. Further studies are currently underway to determine the environmental factors that are conducive for dry flower disease as well as its control in Australia.

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Integrated Management of Diseases in Macadamia Industry

Appendix 2: Details of methodology, results and conclusions of research activity

(c) Endemic diseases – Husk rot

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RESEARCH ARTICLE

Characterisation of husk rot in macadamia

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Keywords
Diaporthe; epidemiology; fruit disease; *Phomopsis*; Proteaceae; tree nut.

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Abstract

The causal agent of husk rot of macadamia is often attributed to *Colletotrichum gloeosporioides sensu lato*. However, in recent husk rot outbreaks, the characteristic concentric ring of pycnidia of *C. gloeosporioides* that is associated with the disease was often absent. Due to its sporadic occurrence, the importance of husk rot is often underrated and attributed to environmental and physiological factors. In order to determine the significance, prevalence and factors that influence husk rot in macadamia, this study examined the aetiology of husk rot in Australia. The relative incidence and severity of husk rot was evaluated in several macadamia orchards over eight consecutive years. Pathogenicity assays were developed to confirm the identity of the causal agent. A range of fungi from several genera including: *Diaporthe*, *Lasiodiplodia*, *Colletotrichum*, *Pestalotiopsis*, *Aspergillus*, *Alternaria*, *Nigrospora* and *Epicoccum* were isolated from samples of macadamia pericarps with husk rot symptoms from different orchards. Fungi in the genus *Diaporthe* were most frequently isolated, often from symptomatic fruit. Results from pathogenicity trials showed the characteristic soft or spongy black lesions characteristic of husk rot symptoms in wounded fruits that were incubated with the diseased fruit or inoculated with a conidial suspension of *Diaporthe* spp. Our results suggest that injury to the macadamia fruit pericarp not only predisposes the pericarp to pathogen infection but it is a prerequisite for infection. Large variations in husk rot severity were observed over years. Husk rot severity was linked to days after anthesis and was associated with mean weekly relative humidity and minimum temperatures. This study confirmed that *Diaporthe* species cause husk rot in macadamia, hence, a rationale for adopting Phomopsis husk rot as the name of the disease is discussed.

Introduction

Macadamia integrifolia Maiden & Betche, *Macadamia tetraphylla* L.A.S. Johnson and their hybrids are cultivated for their edible cream to white coloured kernel (nut). Macadamia nuts are enclosed in a conical to spherical or slightly flattened hard brown shell that is covered in an approximately 3 mm thick fleshy green fibrous husk (pericarp) (Mast *et al.*, 2008). Macadamia nut contributes an estimated 30 000 metric tons of kernel to the international tree nut trade production (http://www.nutfruit.org) and its production is increasing worldwide in Australia, Brazil, China, Costa Rica, Guatemala, Kenya, Malawi, Zimbabwe, USA and South Africa. In Australia, macadamia is produced in plantations on the Eastern coast of New South Wales and Queensland, with minor plantings in Western Australia.

Husk rot disease is known to be common in most mature orchards in the east coast of Australia, in most cases its importance is considered insignificant and often attributed to environmental and physiological factors. There is scanty information on the infection process, anecdotal reports suggest that infection occurs during periods of 2–5 days of wet weather, with temperatures above 15°C (Zentmyer, 1962; Loebel, 1991; Fitzell, 1994; Quinlan, 2004). The economic significance of husk rot is still unknown (Drenth *et al.*, 2009). Husk rot incidence is increasing in several macadamia orchards in Australia and other countries. Anecdotal reports of significant yield loss due to husk rot have been reported from Queensland and

Figure 1 Symptoms of husk rot caused by *Diaporthe* species on macadamia fruits, (a) diseased fruits in the tree canopy, (b) diseased fruits with visible damage of the pericarp and (c) diseased fruits with pin-hole wounds in pathogenicity assay.

Western Australia. In South Africa, husk rot has emerged as a major threat to macadamia production (Campbell, 2015). Husk rot incidence becomes apparent as premature fruit drop just before or at the fruit physiological maturity stage. In Australia, premature fruit drop attributed to husk rot is observed in macadamia orchards when dry and hot weather conditions prevail at the fruit physiological maturity stage, particularly in the cultivar 'HAES 344'. In South Africa, husk rot diseased fruit may drop prematurely, but in some cases, the diseased husks may remain attached to the pedicel on the tree (Campbell, 2015).

The first report of husk rot in macadamia was in 1957 (Brooks & Olmo, 1957; Zentmyer, 1962). In 1991, husk rot was first reported as a significant threat that reduced yields in Australia where yield losses of up to 20% were attributed to premature fruit drop due to husk rot (Loebel, 1991). Due to a lack of understanding of its aetiology and its sporadic occurrence, until recently, husk rot was considered a relatively minor disease affecting the macadamia fruit pericarp, compared with husk spot caused by *Pseudocercospora macadamiae* Beilharz, Mayers & Pascoe (Mayers, 1998). Husk rot is easily distinguished from husk spot that is characterised by yellowish-brown necrotic lesions, that are very tough, fibrous, compact and difficult to cut through compared with surrounding green healthy pericarp tissue (Miles *et al.*, 2009). In contrast to husk spot, husk rot symptoms are characteristically diffuse soft and spongy black lesions up to 10 mm diameter clearly discrete on the green fruit pericarp. Husk rot lesions may coalesce to form greasy decay of the entire fruit pericarp (Fig. 1a) (Loebel, 1991; Fitzell, 1994). It is not clear how infection of the fruit pericarp actually occurs, but it is assumed that wounds or injuries are important entry points for the pathogen (Fig. 1b).

In Australia, husk rot is sometimes referred to as 'anthracnose husk rot' when concentric rings of pycnidia of *Colletotrichum gloeosporioides sensu lato* occur on the black lesions. Other fungal genera including *Phomopsis* (*Diaporthe*), *Lasiodiplodia* and *Stilbella* have also been associated with husk rot of macadamia (Fitzell, 1994). In conditions of high humidity, fruiting bodies of *Stilbella* and *Colletotrichum* may appear on the infected husk. However, there is little information if any of the fungi that are associated with husk rot are the primary causal agent(s) or are present as saprophytes or secondary invaders.

The cause of husk rot has not been resolved and conditions for infection and disease development have not been determined. In this study, our objective was to test the hypotheses that husk rot is caused by a specific fungal pathogen and damage to the fruit pericarp is a precursor to the infection process. The specific objectives of this study were to: (a) determine the relative prevalence of husk rot and the fungal pathogens associated with the disease, (b) identify the primary causal pathogen(s) of husk rot, (c) determine whether injury to the fruit pericarp and fruit size are conditions necessary for infection and (d) examine the influence of climatic factors on expression of disease symptoms under field conditions.

An understanding of the cause of husk rot and the conditions favourable for infection and development of disease are important for the development of effective control measures for husk rot in macadamia.

Materials and methods

Husk rot prevalence and severity

In order to establish the significance of husk rot in macadamia production areas, 26 macadamia orchards in Southeast Queensland (Glasshouse Mountains and Gympie districts) were surveyed yearly from 2000 to 2008. Husk rot prevalence was recorded as percentage of orchards with husk rot. Husk rot severity was assessed using a rating scale of 0–5; where 0 = no husk rot, 1 = very low (<25% of trees affected), 2 = low (25–40% of trees affected), 3 = medium (41–60% of trees affected), 4 = high (>60–80% of trees affected) and 5 = very high (>80% of trees affected) each year. Macadamia fruits that abscised from the tree canopy with soft or spongy black lesions on the pericarp were assessed as diseased (husk rot). Husk rot severity data were recorded weekly for 10–11 weeks from 100 days after anthesis. The field observations were performed during the fruit physiological maturity phase and the presence or absence of fungal fruiting bodies and damage to the pericarp of diseased fruit was examined. In order to determine if husk rot is always associated with damage (injury) to the pericarp, a more intensive survey was performed in the 2009–10 production season. Three severely (severity ratings >3) affected orchards of cultivar 'HAES 344' were selected and samples of diseased fruits were collected 140 days after anthesis. This period was selected to represent the mid-point of the fruit's physiological maturity phase and husk rot incidence. Fruits with husk rot symptoms on the ground and on the tree within 1–3 m from the ground were examined for damage, and 200 diseased fruits were arbitrarily collected from each orchard. These fruits were individually examined for any injuries to the pericarp at the site of the husk rot lesion. Fruits with no obvious wounds in the lesions were examined for any microscopic injuries under a stereomicroscope at ×40 magnification. The husk rot severity data were $\log_{10}(x+1)$ transformed to stabilise variance and the data were used to calculate the area under disease progress curve (AUDPC) as described by Akinsanmi *et al.* (2007). The AUDPC data were subjected to the generalised linear models (GLMs) procedure in GenStat 16th edition (VSN international Ltd, Hertfordshire, UK) while any significant differences between years and orchards were examined. Significant factors were separated and tested using Ryan-Einot-Gabriel-Welsch multiple range tests.

Prevalence of fungi associated with husk rot

Fungi associated with macadamia fruits were examined on 65 husk rot symptomatic and 40 asymptomatic (healthy) fruits that were randomly selected from the prevalence study. From each symptomatic fruit, approximately 2–5 mm pieces of the pericarp were cut-off from the advancing margins between healthy and diseased sections. The pieces were surface-sterilised in 2.5% sodium hypochlorite (NaOCl) solution for 3 min, rinsed thoroughly in three changes of sterile distilled water and blot dried with sterile blotting paper. From each asymptomatic fruit, similar 2–5 mm tissue of the pericarp was arbitrarily obtained from any portions of the fruit and surface-sterilised. All sterilised tissues were plated on ½-strength potato dextrose agar (PDA, Difco Laboratories Inc., Sparks, MD, USA) plates and incubated in the dark at 25°C for 5–7 days. Percentage of tissues with fungal growth was recorded, and fungal mycelial fragments were subcultured into streptomycin-amended PDA plates and incubated at 25°C in the dark for 7–10 days. Monoconidial isolates were obtained from each pure culture as described by Harteveld *et al.* (2013). Isolates were grouped according to their cultural and morphological characteristics from which representative isolates were selected for molecular analyses.

DNA extraction and PCR amplification

Genomic DNA was extracted from 40 mg mycelium of each monoconidial isolate, using the Promega Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) according to manufacturer's protocol for isolating genomic DNA from plant tissue as described by Harteveld *et al.* (2013). DNA concentration was quantified with a BioDrop Duo spectrophotometer (BioDrop, Cambridge, England), adjusted to 25 ng μL^{-1} and was stored at −20°C. Genomic DNA was used in PCR amplifications of the internal transcribed spacer region (ITS) which included the *ITS-1*, *ITS-2* and the 5.8S nrRNA gene regions, and amplified with ITS-4 as reverse primer and ITS-5 as forward primers (White *et al.*, 1990) for all the fungal isolates. In order to refine the identity of the isolates in the dominant fungal genus isolated from macadamia fruit pericarp, additional gene region of β-tubulin locus (*TUB*) was amplified using the BT2A and BT2B primers (Glass & Donaldson, 1995; O'Donnell & Cigelnik, 1997) for selected isolates. PCR amplification was performed in a 20 µL reaction mixture containing 10 µL of 10 mM Phusion Master Mix (Thermo Fisher Scientific Inc., Australia) and 1 µL each of forward and reverse primers. The amplification was conducted in SuperCycler Thermal Cycler (Kyratec, Fisher biotec,

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Table 1 Details of isolates of *Diaporthe* obtained from husk rot in macadamia used in the phylogenetic analysis

			Genebank Accession No.	
Accession no	Organism	Host Source	*ITS*	*TUB*
BRIP 64227[a]	*Diaporthe* sp.	*Macadamia* sp.		–
BRIP 64228[a]	*Diaporthe* sp.	*Macadamia* sp.	KU985016	KU985029
BRIP 64229[a]	*Diaporthe* sp.	*Macadamia* sp.	–	–
BRIP 64230	*Diaporthe* sp.	*Macadamia* sp.	KU985017	–
BRIP 64231	*Diaporthe* sp.	*Macadamia* sp.	–	–
BRIP 64232	*Diaporthe* sp.	*Macadamia* sp.	KU985018	–
BRIP 64233	*Diaporthe* sp.	*Macadamia* sp.	KU985011	KU985024
BRIP 64234	*Diaporthe* sp.	*Macadamia* sp.	KU985012	KU985025
BRIP64235[a]	*Diaporthe* sp.	*Macadamia* sp.	KU985013	KU985026
BRIP 64236	*Diaporthe* sp.	*Macadamia* sp.	KU985014	KU985027
BRIP 64237	*Diaporthe* sp.	*Macadamia* sp.	KU985015	KU985028
BRIP 64238	*Diaporthe* sp.	*Macadamia* sp.	KU985009	KU985022
BRIP 64239[a]	*Diaporthe* sp.	*Macadamia* sp.	KU985010	KU985023
BRIP 64240	*Diaporthe* sp.	*Macadamia* sp.	–	–
BRIP 64241	*Diaporthe* sp.	*Macadamia* sp.	KU985019	–
BRIP 64242	*Diaporthe* sp.	*Macadamia* sp.	KU985020	–
BRIP 64243[a]	*Diaporthe* sp.	*Macadamia* sp.	KU985008	KU985021
BRIP 64244[a]	*Diaporthe* sp.	*Macadamia* sp.	–	–
BRIP 64245	*Diaporthe* sp.	*Macadamia* sp.	–	–
BRIP 64246	*Diaporthe* sp.	*Macadamia* sp.	–	–
BRIP 54781	*D. fraxini-angustifoliae*	*Fraxinus angustifolia*	JX862528	KF170920
BRIP 54900	*D. litchicola*	*Litchi chinensis*	JX862533	KF170925
CBS 462.69	*D. pseudophoenicicola*	*Phoenix dactylifera*	KC343184	KC344152
CBS 101339	*D. pseudomangiferae*	*Mangifera indica*	KC343181	KC344149
CBS 114979	*D. arengae*	*Arenga engleri*	KC343034	KC344002
BRIP 54847	*D. pascoei*	*Persea americana*	JX862532	KF170924
CGMCC 3.15175	*D. lithocarpus*	*Lithocarpus glabra*	KC153104	KF576311
CBS 115448	*D. hongkongensis*	*Dichroa febrifuga*	KC343119	KC344087
CBS 129519	*D. musigena*	*Musa* sp.	KC343143	KC344111
CBS 161.64	*D. arecae*	*Areca catechu*	KC343758	KC344000
CBS 122676	*D. cynaroidis*	*Protea cynaroides*	KC343058	KC344026
CBS 111886	*D. australafricana*	*Vitis vinifera*	KC343038	KC344006
CBS 129521	*D. acaciigena*	*Acacia retinodes*	KC343005	KC343973
CBS 116311	*D. saccarata*	*Protea repens*	KC343190	KC344158
CBS 116019	*D. phaseolorum*	*Caperonia palustris*	KC343175	KC344143
CBS 121124	*Diaporthella corylina*	*Corylus* sp.	KC343004	KC343972

[a]Isolates used in pathogenicity assays.

Australia). The thermal cycler was programmed for initial denaturation for 60 s at 98°C followed by 35 cycles at 98°C for 10 s, 62°C for 30 s and 72°C for 45 s and a final extension step at 72°C for 5 min. PCR amplicons were separated in 1% agarose gel (Bioline, Australia), stained with gel red in 0.5% Tris-borate ethylenediaminetetraacetic acid (EDTA) buffer solution and viewed under ultraviolet (UV) light using Molecular Imager® GelDoc™ (Bio-Rad Laboratories Inc., Segrate, Milan, Italy). The amplicon sizes were determined against a 1 kb HyperLadder (Bioline) and then the targeted PCR amplicon was purified using Roche™ High Pure PCR Product Purification Kit (Roche Applied Science, Mannheim Germany) according manufacturer's instructions before sequencing using the same primers used for amplifications with 3730xl DNA analyzer at Macrogen Inc. (Seoul, South Korea). Forward and reverse sequences were manually assembled for each isolate in MEGA (Molecular Evolutionary Genetics Analysis) v.6 (Tamura *et al.*, 2013) software from which the consensus fragment was selected.

Phylogenetic analysis and identification of fungal isolates

In order to confirm the identity of the fungi isolates, the BLAST search (Altschul *et al.*, 1990) procedure in NCBI Genbank was performed for the *ITS* sequences. Phylogenetic analyses by maximum parsimony of the concatenated sequences of the *ITS* and β-tubulin gene loci of selected isolates of the dominant genus, aligned

with sequences of reference strains (Table 1), were performed to distinguish the species in MEGA6. The maximum parsimony phylograms were constructed using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei & Kumar, 2000) with search level 1, in which the initial trees were obtained by the random addition of sequences. All positions containing gaps and missing data were eliminated. Tree stability was tested by 1000 bootstrap replicates. All generated sequences have been submitted to NCBI Genbank.

Pathogenicity assays

Macadamia fruits of susceptible cultivar 'HAES 344' were used for the pathogenicity assays. Mature-sized (fully expanded) fruits (Miles *et al.*, 2010) with no visible husk rot symptoms were obtained from the tree canopy of healthy trees (no abscised fruit with husk rot). A total of 160 fruits were used in a hierarchical process to confirm pathogenicity. Each trial consisted of 80 fruits that were subdivided into two lots. The first lot of 40 fruits were surface-sterilised by soaking in 5% NaOCl solution for 5 min, then rinsed in three changes of sterile distilled water, and air dried in the laminar flow, while the second lot of 40 fruits were not surface-sterilised. In order to examine if injury is needed for pathogenicity, 20 fruits in each lot were artificially wounded, with a sterile needle to create10 pin-holes per fruit, whereas, 20 fruits were not wounded. Five fruits in each category were inoculated separately with a conidial suspension (alpha conidia) of *Diaporthe* species or *Colletotrichum* spp. isolated previously. Five fruits were also incubated directly together with husk rot on diseased fruit, and five fruits were inoculated with sterile water as control treatment in separate bags (Table 2). The two fungal genera were used based on the results of fungal isolations from diseased samples described above. Fungal inoculum suspensions were prepared from pure cultures of the selected isolates from fungal isolation above as described by Harteveld *et al.* (2013) and adjusted to a final concentration of 10^6 conidial mL^{-1}. Each fruit was sprayed to run-off with the respective conidial suspension or with sterile distilled water, and kept in different plastic sealed bags, each with wet cotton wool to maintain high relative humidity (>90%). The bags containing the fruits were incubated at 25 ± 2°C and 12 h light/12 h dark conditions for 3–7 days. Each fruit was examined for husk rot symptoms at 7 and 14 days post inoculation. Koch's postulates were conducted for symptomatic fruits.

Disease severity of *Diaporthe* species

Based on the results from successful infection, additional and repeated pathogenicity tests were performed to further examine isolates of *Diaporthe* spp. (Table 1) and to compare their aggressiveness. Fruits of three macadamia cultivars ('Release', 'HAES 705' and 'HAES 344') were inoculated with conidial suspensions of *Diaporthe* spp. representing the two groups (Table 1). Fruits at three different stages of development: small, mid-sized and full-sized that correspond to pea-sized fruit, 50% expanded fruit and fully expanded fruit, respectively, as described by Miles *et al.* (2010) were used in four replicates, each consisted of five fruits and inoculated as described above. Sterile water was used as control treatment. Proportions of inoculated fruits showing characteristic husk rot lesions were used as a measure of aggressiveness. Significance of the treatments was tested using GLM procedure in GenStat.

Table 2 Description and results of pathogenicity assays used to determine the causal agent of husk rot in macadamia

Fruit Condition	Type of Inoculum	Husk Rot
Surface-sterilised		
With injury	Diseased fruit	+
With injury	*Diaporthe* sp.	+
With injury	*Colletotrichum* sp.	–
With injury	Water (control)	–
No injury	Diseased fruit	–
No injury	*Diaporthe* sp.	–
No injury	*Colletotrichum* sp.	–
No injury	Water (control)	–
Non-sterilised		
With injury	Diseased fruit	+
With injury	*Diaporthe* sp.	+
With injury	*Colletotrichum* sp.	–
With injury	Water (control)	–
No injury	Diseased fruit	–
No injury	*Diaporthe* sp.	–
No injury	*Colletotrichum* sp.	–
No injury	Water (control)	–

Influence of climatic conditions on husk rot development under field conditions

Climatic parameters including the mean weekly minimum and maximum temperatures (°C), difference between maximum and minimum temperatures, mean and total rainfall (mm), number of days with rainfall and relative humidity (%) were used to elucidate the effect of each and combined factors on husk rot incidence and severity between 100 and 170 days after anthesis in each production season. The weather data for the field sites were obtained from the Bureau of Meteorology, Australia (station numbers: 040861 and 40093). The effect was analysed using the GLM procedure for the all-subset regression link functions, which was then used to produce several best models among all possible models

as described by Akinsanmi & Drenth (2010). Significant factor was determined at $P < 0.05$.

Results

Field observations

Characteristic husk rot symptoms were observed as soft black diffuse lesions on diseased fruit pericarp in all the macadamia orchards surveyed. Husk rot incidence was irregular between seasons, and appeared to be more commonly observed in certain cultivars such as 'HAES 344', 'A16', 'HAES 216' and 'HAES 816'. Field observations revealed that in most cases, fruit that prematurely abscised with husk rot had visible injury (splitting) of the pericarp, often along the lesion length (Fig. 1b). Fruiting bodies of fungal pathogens were mostly absent on diseased fruit pericarps on the tree canopy, and when present, they occurred in well-advanced (aged) spongy black lesions. At all the orchards surveyed, premature fruit drop was first observed at 100 days after anthesis.

Husk rot prevalence and severity

Large variations in the disease severity ratings occurred between days after anthesis, where cumulative husk rot severity ratings increased with time. Husk rot prevalence varied considerably each year in the orchards, with the highest prevalence in the 2001–02 and the 2005–06 seasons and least in the 2007–08 season (Fig. 2a). Similarly, analysis of the AUDPC showed significant ($P < 0.05$) variations among the years (Fig. 2b), but a weak correlation coefficient ($r = 0.459$) was obtained from analysis of the association of husk rot severity based on AUDPC data and husk rot prevalence (%). In the 2009–10 season, the percentage of fruit that abscised with husk rot symptoms at 140 days after anthesis ranged from 14% to 89%. Results from the intensive survey revealed minor to extensive splitting along the suture lines. Microscopic observations of diseased fruits with no obvious visible wounds showed minute pin-hole sized injuries in the lesions.

Influence of climatic conditions on husk rot development under field conditions

The effect of days after anthesis was highly significant ($P < 0.001$) for husk rot severity with a unimodal distribution between 100 and 170 days after anthesis (Fig. 3). Analyses of the various climatic factors showed that mean weekly relative humidity and minimum temperatures significantly ($P < 0.05$) influenced husk rot severity (Table 3). The correlations of the climatic factors with days after anthesis and husk rot severity showed that significant ($P < 0.05$) correlation between husk rot severity with the mean weekly relative humidity and minimum temperature and days after anthesis (Table 4). In most cases, husk rot occurred ($y > 0$) in approximately 50% of the trees assessed compared with approximately 28% of trees without husk rot ($y = 0$). High level of variability of husk rot severity occurred at each level of the climatic factors, therefore, the trend of the association between husk rot severity and each of the climatic factors was best fitted with a polynomial regression. The terms in the final model ($R^2 = 84\%$) included the mean weekly relative humidity, maximum and minimum temperatures and days after anthesis.

Fungal isolations

Several fungi were obtained from fruit samples with husk rot symptoms. The frequency of fungi isolated from diseased macadamia fruit pericarp varied among samples, but the mean frequency of fungi isolated from fruit with husk rot symptoms with visible damage to the pericarp was 92%, while from symptomatic fruit without obvious or visible damage to the pericarp was 50%. The frequency of isolation of the genus *Diaporthe* from the pericarps with and without visible wounds was significantly higher than any other fungi (Fig. 4). Fungi isolated from diseased fruit pericarps were identified as belonging to the genera *Diaporthe*, *Colletotrichum*, *Alternaria*, *Lasiodiplodia*, *Pestalotiopsis*, *Aspergillus*, *Epicoccum* and *Nigrospora*, in order of frequency of isolation. Results of the BLAST searches of *ITS* sequences of the fungi isolated confirmed that the isolates were similar to *Diaporthe* spp. *C. gloeosporioides sensu lato*, *Pestalotiopsis clavispora* G.F. Atk., *Lasiodiplodia pseudotheobromae* A.J.L. Phillips, A. Alves & Crous and *Nigrospora sphaerica* (Sacc.) E.W. Mason.

Phylogenetic analysis

Phylogenetic analysis of the *Diaporthe* isolates revealed different species were involved in husk rot (Fig. 5). BLAST searches of individual isolate revealed differences in identity of the *ITS* and β-tubulin gene loci. The *ITS* sequence analysis revealed 98–100% nucleotide sequence homology with other members of the genus *Diaporthe* including *Phomopsis eucommii*, *Diaporthe fraxini-angustifoliae* strain BRIP 54781 and *Diaporthe* sp. RP68. β-Tubulin gene sequence showed 97–100% nucleotide sequence similarity with other species within the genus *Diaporthe*, including *Diaporthe lithocarpus* strain LC0785 and *D. fraxini-angustifoliae* strain BRIP 54781. Phylogenetic analyses by maximum parsimony of the concatenated sequences of the *ITS* and β-tubulin gene loci of selected husk rot isolates revealed two distinct clades, named group 1 and group 2 (Fig. 5) within the genus *Diaporthe*.

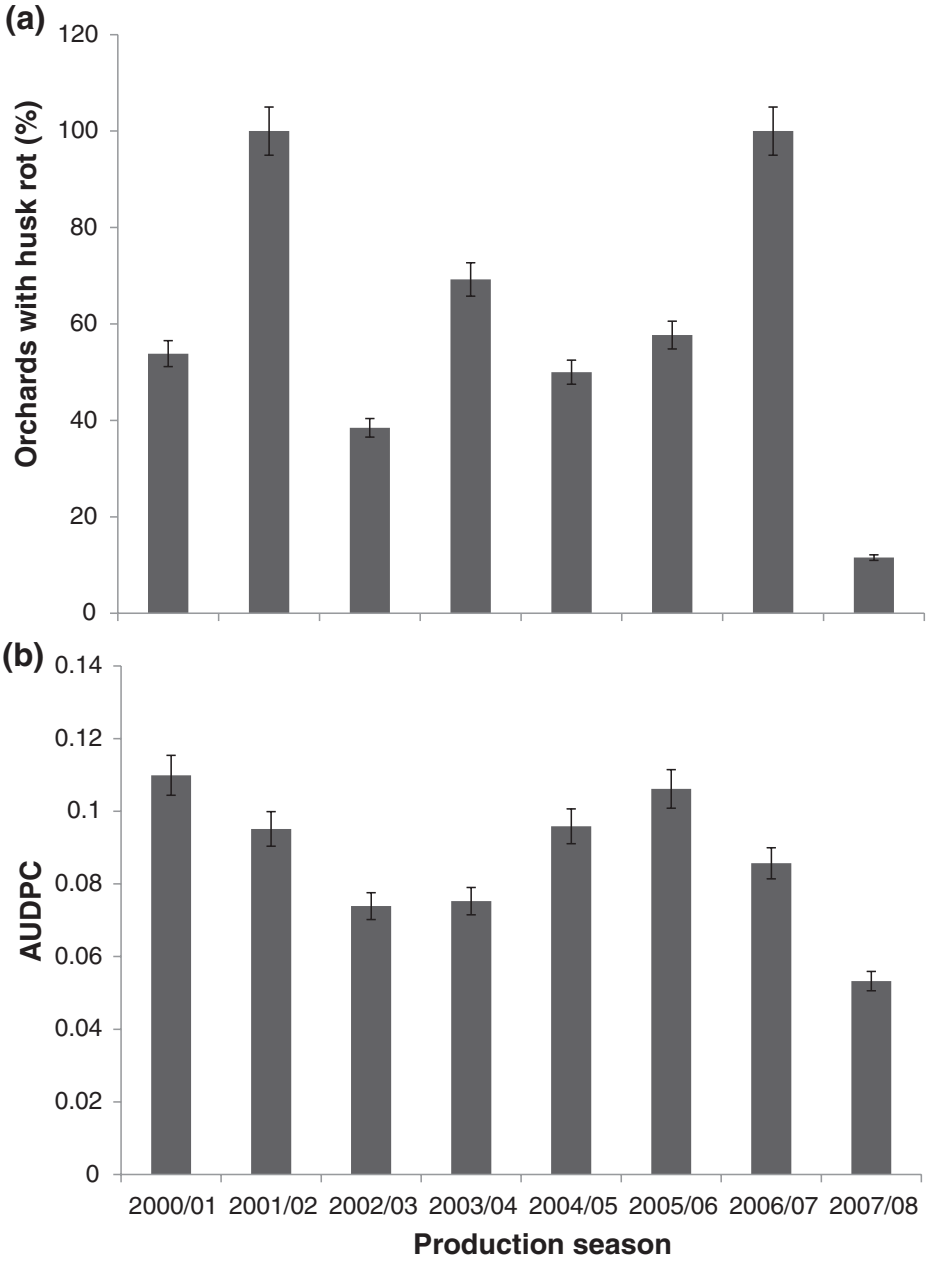

Figure 2 Husk rot (a) prevalence measured as percentage of orchards with husk rot and (b) severity based on area under disease progress curve (AUDPC) from 100 days after anthesis in macadamia orchards. Lines on the bars indicate standard error.

Pathogenicity and virulence on macadamia cultivars

Typical husk rot symptoms of soft black lesions on the macadamia fruit pericarp (Fig. 1c) were consistently reproduced in both surface-sterilised and non-sterilised fruits that were inoculated with a conidial suspension of *Diaporthe* spp. or incubated with diseased fruit (Table 2). None of the fruit treated with water or a conidial suspension of *Colletotrichum* sp. in wounded or unwounded treatments produced the characteristic husk rot symptoms (Table 2). Both spore types of pycnidia with α (elliptical) and β (filiform) pycnidiospores of *Diaporthe* were observed on the diseased area of inoculated macadamia fruit pericarp. Significant ($P < 0.05$) differences in husk rot severity were observed in the inoculated fruits of the three macadamia cultivars and seven isolates used. Species of *Diaporthe* were consistently re-isolated from inoculated fruit pericarp. After 7 days of

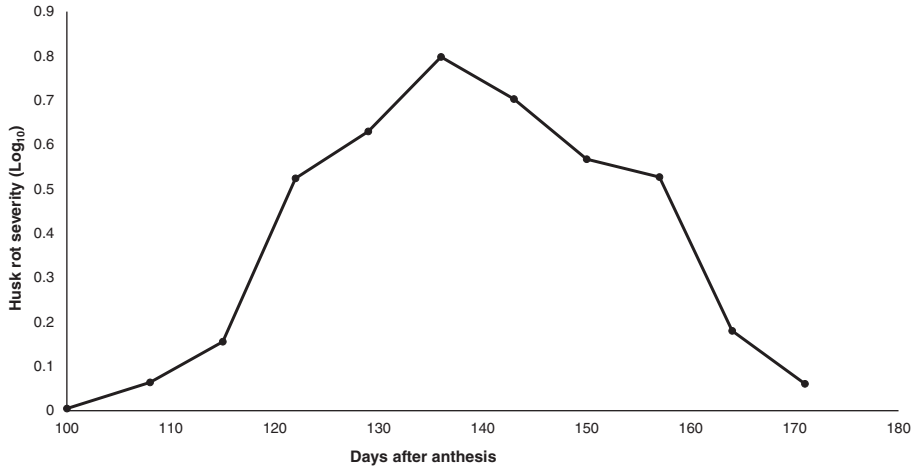

Figure 3 Association of husk rot severity with days after anthesis within macadamia orchards.

Table 3 Values of the accumulated analysis of variance from the regression analysis of climatic factors and days after anthesis of husk rot severity on macadamia

Sources	d.f.	Mean Square	*F pr*.
Temperature difference (°C)[a]	1	0.019	0.853
Mean rainfall (mm)	1	0.015	0.601
Mean maximum temperature (°C)	1	3.427	0.015
Mean minimum temperature (°C)	1	2.613	0.032
Mean relative humidity (%)	1	3.218	0.018

[a]Difference between maximum and minimum temperatures.

incubation of inoculated fruits, the fungi were re-isolated and confirmed as the causative agents. Full-sized fruits were significantly ($P < 0.0001$) more susceptible than other fruit sizes (data not shown). Cultivar 'HAES 344' was the most susceptible to both *Diaporthe* species followed by 'Release' and the husk rot *Diaporthe* isolates in group 1 were more aggressive than the group 2 isolate.

Discussion

This study has provided vital information on the prevalence and aetiology of husk rot of macadamia in Australia. Our disease survey revealed that regardless of cultivar or location, husk rot severity increases from 100 days after anthesis, and premature abscission of diseased fruit continued to be approximately 170 days after anthesis. Husk rot severity showed a unimodal distribution between 100 and 170 days after anthesis (Fig. 3). Husk rot incidence was strongly associated with minor to extensive injury to the fruit pericarp. High husk rot severity was significantly correlated to relative humidity, minimum temperatures and days after anthesis. The susceptible period coincides with when fruit are at full-size stage of development at approximately 100–170 days after anthesis. This study has revealed evidence that different species of *Diaporthe* cause husk rot in macadamia. Phylogenetic analysis suggests that the *Diaporthe* isolates obtained from macadamia husks are different from known species. In addition, significant differences in pathogenicity was recorded for the isolates in both phylogenetically distinct *Diaporthe* species that caused husk rot in macadamia. BLAST searches of each husk rot isolate revealed differences in identity in the *ITS* and β-tubulin gene loci. Sequence homology with different species in the genus *Diaporthe* including *P. eucommii*, *D. fraxini-angustifoliae* strain BRIP 54781, *Diaporthe* sp. RP68 and *D. lithocarpus* strain LC0785 was observed. The concatenated analysis of the two gene loci showed that the husk rot *Diaporthe* isolates belong to different clades. To the best of our knowledge, this is the first report that showed that two novel *Diaporthe* species cause husk rot in macadamia.

Identity of each isolate was confirmed using the combined sequence data of *ITS* and *TUB* gene loci that have been identified as suitable for distinguishing closely related species in the genus (Gomes *et al.*, 2013). The occurrence of either *Diaporthe* species was not associated with host variety or location. However, the results of the phylogenetic analysis showed intraspecific variability among the *Diaporthe* isolates in group 1 (Fig. 5). Recent phylogenetic studies on several *Diaporthe*/*Phomopsis* strains have shown that more than one taxon may be associated with the same or several hosts (Thompson *et al.*, 2011; Chen *et al.*, 2013; Gomes *et al.*, 2013; Lawrence *et al.*, 2015). Hence, fresh collections from studies of microbial ecology of macadamias may reveal more novel taxon in macadamia.

Our results indicate that injury to the macadamia fruit pericarp predisposes the pericarp to pathogen infection

Table 4 Correlation coefficients of climatic factors with days after anthesis and husk rot severity in macadamia

Factors	Temperature Difference (°C)	Total Rainfall (mm)	Min. Temperature (°C)	Mean Relative Humidity (%)	Max. Temperature (°C)	Mean Rainfall (mm)	Husk Rot Severity
Temperature difference (°C)[a]	–						
Total rainfall (mm)	−0.162	–					
Min. temperature (°C)	−0.314**	0.021	–				
Mean relative humidity (%)	−0.301**	0.323**	0.168	–			
Max. temperature (°C)	0.754**	−0.143	0.387**	−0.176	–		
Mean rainfall (mm)	−0.166	0.997	0.022	0.329**	−0.146	–	
Husk rot severity	−0.018	−0.042	0.238**	0.246**	0.147	−0.030	–
Days after anthesis	−0.102	0.011	0.284**	0.403**	0.098	0.018	0.882**

**Significant at $P < 0.05$.

[a]Difference between maximum and minimum temperatures.

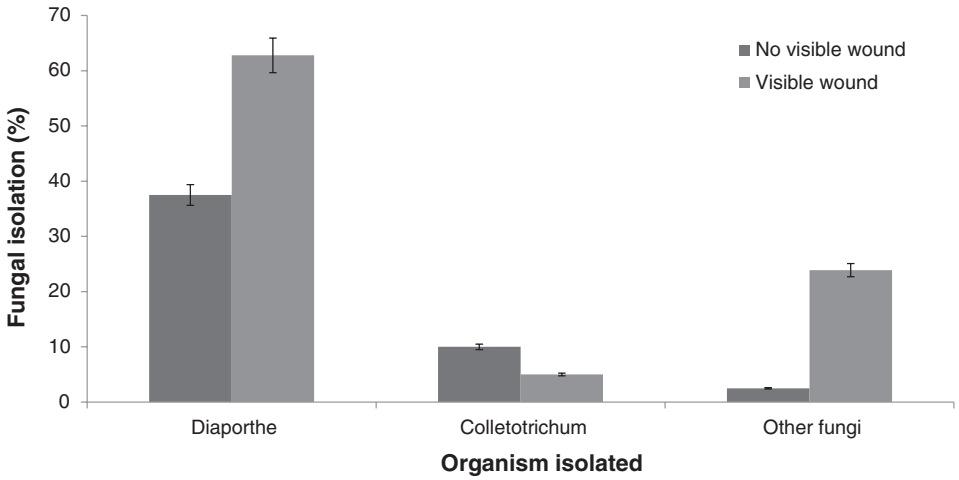

Figure 4 Mean incidence of major fungi obtained from macadamia fruits with husk rot symptoms with visible and no visible wound in the fruit pericarps. Lines on the bars indicate standard error.

such as *Diaporthe*. The infection process of the fungal pathogens should be further examined. Species of *Diaporthe* occur as endophytes and saprobes in plant tissues (Gomes *et al.*, 2013). The potential role of *Diaporthe* as endophytes or saprobes in producing plant tissue degrading enzymes and in infection process is still unclear (Williamson *et al.*, 1991; Udayanga *et al.*, 2011). Husk rot symptoms showed that the *Diaporthe* spp. are necrotrophic, however, most are thought to be hemibiotrophs at least during the latent phase of infection (Udayanga *et al.*, 2011). The source of inoculum was not established in this study, however, spores of *Diaporthe* have been reported to be splashed-dispersed by rain. In peach, inoculum dispersal within the tree canopy has been reported to be via flowing rain and between trees by wind-blown rain (Uddin & Stevenson, 1998). In macadamia, further studies are needed to determine the nature of spread of this fungus. The importance of temperature in husk rot development observed in this study is similar to Phomopsis shoot blight of peach, where temperature was reported to influence growth of *Phomopsis* sp., disease development and dispersal of inoculum (Uddin & Stevenson, 1998).

Several species in the genus *Diaporthe*/*Phomopsis* are important plant pathogens (Washington *et al.*, 1997; Schilder *et al.*, 2005; Polashock & Kramer, 2006; Thompson *et al.*, 2011; Udayanga *et al.*, 2011; Chen *et al.*, 2013), and are responsible for several important diseases such as stem and branch cankers, shoot blight, twig dieback, fruit rot, leaf spot and blight in several tree nut and fruit crops (Arimoto *et al.*, 1986; Udayanga *et al.*, 2011; Lawrence *et al.*, 2015). Based on the fact that the anamorph name is the most commonly applied to many important diseases, herein we suggest Phomopsis husk rot as the name for the disease in macadamia. Hence, the disease can easily be related to similar diseases in other hosts, and distinguished from anthracnose husk rot caused by *Colletotrichum* sp.

Both *Diaporthe* species identified in the samples were pathogenic to the macadamia fruit pericarp, and their

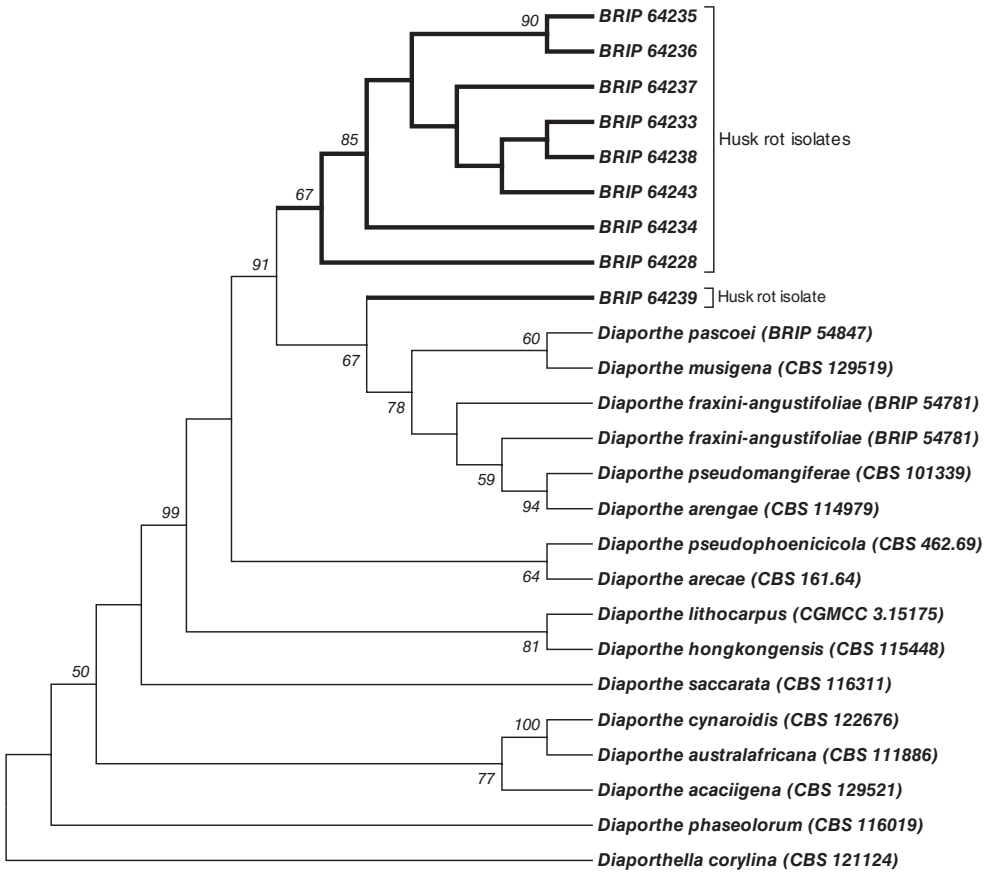

Figure 5 Maximum parsimony phylogram of *Diaporthe* isolates obtained from macadamia fruits with husk rot symptoms in Australia (bold nodes), with selected strains of different species in the phylogenetic clades of the genus *Diaporthe*. Phylogram is based on more than 50% majority rule inferred from 1000 bootstrap replicates generated from combined sequences of *ITS* and *TUB* gene loci and the bootstrap support values over 50% are indicated at the nodes. The tree is rooted to *Diaporthella corylina* (CBS 121124).

presence in symptomatic husks suggests that they cause husk rot in Australia. Pycnidia with α (elliptical) and β (filiform) pycnidiospores were found in inoculated macadamia fruit pericarps. Significant differences were observed between wounded and non-wounded fruit samples. This indicates that damage or injury to the fruit pericarp is an important prerequisite for infection of macadamia fruit pericarp by *Diaporthe* spp. Isolates of *Diaporthe* species produced husk rot symptoms in the inoculated fruits and were re-isolated from diseased fruit pericarps, therefore, Koch's postulates were fulfilled. Under field conditions, certain factors such as insect pests, sun scalding, hail damage and abrasive surface against the fruit may predispose the macadamia pericarp to infection. The significance of these various possible causes of damage to fruit pericarp should be determined. In Australia macadamia are affected by several insect pests such as fruit spotting bug (*Amblypelta* spp.) and nut borer (*Cryptophlebia ombrodelta* Lower) that attack fruit at near maturity stage, the role of these insect pests in inducing the injury, and husk rot incidence is still unclear.

Although disease resistance was beyond the scope of this research, the results from the field survey suggest that differences in susceptibility exist among the commercial cultivars. Future studies are needed to investigate the susceptibility of different cultivars to infection by *Diaporthe* species. Also, aggressiveness and virulence of different *Diaporthe* species should be determined. This study has revealed that husk rot has the potential to become a significant limiting factor for macadamia production and therefore, strategies should be developed to manage the disease.

Acknowledgements

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Strategic levy investment

MACADAMIA FUND

Integrated Management of Diseases in Macadamia Industry

Appendix 3: Communications in the mainstream industry journals and News Bulletin

(a) Husk spot management

Dr Olufemi A. Akinsanmi
The University of Queensland

Project number: MC12007

Integrated disease management: a snapshot

Femi Akinsanmi

A key priority of the macadamia industry has been integrated pest and disease management. Integrated strategies focus on using a range of practices such as crop monitoring, sound orchard management and targeted use of pesticides to control pests and diseases. We asked Femi Akinsanmi about the history of integrated strategies in macadamias and what we might look forward to in the future.

NB. What was the focus of Integrated Disease Management (IDM) when it was first introduced into macadamias?

The term integrated pest management (IPM) is used to describe the use of integrated practices to manage any kind of pests including diseases. IPM is fundamentally a subset of the good agricultural practices needed to produce **profitable and productive crops** in a **sustainable** way.

The IPM concept was developed by entomologists, and plant pathologists have given same level of importance as them to integrated disease management (IDM). This is because there has always been an element of integrated control in plant pathology.

The practice of controlling diseases in macadamias using an integrated approach started in the early days of the industry, for instance several recommendations are documented in the *Australian Macadamia Industry Code of Sound Orchard Practices.*

The focus of IDM is to obtain the maximum effect of disease control and cost-benefit ratios along with environmental benefits. Early detection and understanding of disease development can help farm managers apply appropriate disease management strategies that can reduce the amount of initial inoculum, the amount of time available for disease epidemics to develop and the rate of epidemic development.

NB. Was this the result of an R&D program or was IPM introduced based on experience with other industries?

IDM has been introduced to the macadamia industry as a result of R&D and knowledge that long-term disease control could not be achieved through a 'one-shot' approach relying on one control strategy (usually the application of chemicals). As a result, an integrated approach to disease control using a range of measures that complement one another to stop and manage diseases is recommended and has been accepted by many growers.

NB. What has been the focus of IDM research in macadamias in the last decade?

The focus of IDM research has been to develop integrated management strategies for diseases affecting macadamia, in particular husk spot and phytophthora, through improved and more targeted fungicide spray applications and better understanding the timing of infection, source of inoculum, climatic factors influencing disease development, disease cycle, disease resistance among varieties and chemical control options.

R&D outcomes have provided a strong base for developing and delivering efficient and profitable control methods, including decision tools and disease forecasting to manage plant diseases in macadamia.

NB. What are the elements of current best practice IDM in orchards?

The current best practice IDM in macadamia orchard is to manage diseases of economic significance by combining biological methods, including host plant resistance and microbial agents for disease control, and cultural, physical and chemical tools in a way that minimises economic, health and environmental risks.

NB. What to you predict will be the key opportunity/ies for future R&D into IPM?

These opportunities are being covered in two R&D projects. Major components of IDM are included in project MC12007, *Disease management in the macadamia industry.* These components are:

- host-plant resistance
- cultural practices
- disease forecasting
- chemical control.

Controlling husk spot using microbial agents is another important area with great potential being investigated through project MC12008, *Biological husk spot research.*

Husk spot of macadamia

In this article Dr Femi Akinsanmi, Assoc. Prof. André Drenth and Dr Andrew Miles summarise the current situation with husk spot control.

Symptoms of husk spot, characterised by chlorotic and tan to dark brown spots on macadamia husk.

Husk spot, *Pseudocercospora macadamiae*, is a serious disease of macadamia in Australia, resulting in premature fruit drop and giving rise to nuts with low oil content. It is unique in that it is caused by a fungal pathogen that is believed to have co-evolved with macadamia; all other pathogens causing disease in commercial orchards today also affect a wide range of other crop plant species.

Losses from premature fruit drop due to husk spot are estimated to be up to $10 million annually in Australia, and this does not include its indirect impact on processing, marketing and the environment. Importantly, the impact on individual farms and the industry generally will be far larger if the existing chemical control agents are deregistered, removed from sale, or resistant pathogen strains develop.

Source of husk spot spores in the tree canopy. (A) Husk retained in the tree canopy (sticktights), (B) diseased husks in close proximity to developing fruit, (C) a sporulating husk spot lesion on a sticktight.

Climate is an important factor in the occurrence of husk spot, which is favoured by high relative humidity, moist conditions and warm temperatures (26 °C is ideal). Research has established that husk spot is more prevalent in prolonged wet conditions, and that a combination of rainfall and temperatures above 25°C heightens fruit drop due to husk spot. These climatic conditions delay oil accumulation and kernel maturity, leading to significant yield losses.

Control

The main control methods for husk spot being investigated or which have been shown to be effective are as follows:

- cultural
- chemical
- biological
- integrated strategies.

Cultural control

Sticktights management. Based on the fact that sticktights form a significant source of inoculum, the most effective cultural control method for husk spot would be removing them from tree canopies. Because it is generally a labour-intensive task, this is not commercially realistic, however, mechanical options such as tree shakers and processes that enable rapid degradation of sticktights in the tree canopy are possibilities. A more sustainable approach is to plant cultivars that do not retain sticktights such as HAES cultivars 246, 344, 660 and 800 and avoid those prone to sticktights such as A16, A38, H2 and Purvis.

Harvest based on kernel maturity. The presence of husk spot lesions shifts the fruit drop pattern so that it peaks earlier in the harvest period. For example, about 61% of the total crop load may drop with low kernel quality by March in an orchard with poor husk spot control, compared to 29% in an orchard with good husk spot control. Premium kernel quality occurs between March and July, depending on weather or tree physiological conditions; fruit drop between mid-February and early March can be of average kernel quality and therefore lose its premium grade and be downgraded to 'commercial'. To reduce the amount of harvestable crop lost when the pre-harvest clean-up of the orchard floor coincides with fruit drop of mature kernel, growers should monitor kernel maturity and schedule harvest rounds by maturity rather than by calendar.

Canopy management. Husk spot intensity increases with tree age, possibly because canopies become denser and more sticktights are retained. Open canopy will reduce the development of high humidity microclimates, and reduce the duration of the conditions needed for infection of the husk in the tree canopy. However, husk spot incidence can still be high when sticktights prevail in trees with very open canopy such as A38.

Chemical control

Most of the more than 40 chemical products tested for controlling husk spot, including inorganic, organic, defence promoters and mineral salts, have not been effective. Currently, carbendazim

(SpinFlo®), pyraclostrobin (Cabrio®), difenoconazole (Score®) and various copper formulations are registered for husk spot control in macadamia in Australia. Various copper formulations have been trialled (Tribase Blue® Nordox®, Norshield®, Blueshield® and Kocide®) in the field and all appear to be equally effective. Some products work better when used in combination with other products, e.g. carbendazim and difenoconazole are much more effective when applied in tank mixture with a protectant fungicide such as copper, a broad spectrum fungicide. This is not the case, however, for pyraclostrobin when applied as a spray alone as it is equally effective as mixture of carbendazim and copper.

If a fungicide without any eradicant properties, such as copper, is used alone, any infections early in the season before fungicide spray applications may still cause significant premature fruit drop. This emphasises the importance of using carbendazim, pyraclostrobin or difenoconazole fungicide in the spray application early in the season to control initial infections. Because sole application of pyraclostrobin sprays is as effective as the combination of carbendazim or difenoconazole with copper, including it in the husk spot management program has resulted in a big reduction in the amount of copper used in macadamia orchards.

Spraying program. When to spray to control husk spot is based on epidemiology and infection biology, as follows:

- in a relatively dry season, two spray applications, starting when the fruit is at match-head size stage between (September and October) followed by a second spray application 3 to 4 weeks later, depending on weather conditions.
- when conditions favouring the disease prevail from October through to December, an extra one or two spray applications may be needed 2 to 3 weeks after the second spray application before nut maturity.

The efficacy of spray applications is greatly improved by adding spreader or adjuvant to achieve good spray coverage.

Generally, most growers use only two applications of carbendazim + copper and alternate with a pyraclostrobin-only application. Difenoconazole is occasionally used but it is considered very expensive. In addition, a mixture of difenoconazole with copper is required to achieve similar efficacy compared to pyraclostrobin. A fungicide resistance management program that reduces the risk of selection of fungicide resistance strains has been developed for the Australian macadamia industry.

It is recommended that systemic fungicides be applied alternately with other products with different modes of action or in conjunction with protectant fungicides to prevent selection of fungicide resistant strains.

Biological control

At present, results of laboratory tests of some of the bioagents against *P. macadamiae* are promising, however, the ability of several bioagents including *Bacilus subtilis, Penicillium* spp. (various strains), *Trichoderma harzianum, T. koningii* and *T. viride* and their mixtures, as well as plant derivatives such as Eco oil, Neem in synetrol oil, and pyroligneous acid to directly suppress and control husk spot under field conditions is poor. Further trials to optimise conditions and improve effectiveness of some of the promising products are needed.

Integrated disease management decision tools

An integrated disease management approach that consists of a combination of different control measures and incorporates consideration of specific orchard characteristics and prevailing weather conditions may enable more sustainable husk spot control.

We have developed an initial decision support system for fungicide spray applications that uses a checklist of risk factors that affect infection and husk spot development. These risk factors include:

- history of crop loss due to husk spot
- short-term weather forecast
- previous disease levels
- susceptibility of cultivars
- inoculum load in the canopy
- flowering pattern.

We need a more comprehensive disease forecasting system comprised of an early warning system for predicting optimum number of spray applications needed and an in-season warning system for disease severity for early fruit drop. In the meantime, a combination of any of the cultural control options and targeted fungicide spray applications of high risk cultivars may provide improved husk spot control and safeguard yield and kernel quality.

Adequate knowledge of the orchard layout for targeted monitoring of husk spot incidence and kernel maturity would aid management decision for application of control and harvesting to reduce yield loss in preharvest clean up from early fruit drop of mature nuts. When re-planting or establishing new orchard selection of disease tolerant cultivars should be considered.

The future

Research over the last decade has improved our understanding of husk spot and provided more effective management strategies for the disease. We now have much more information about factors such as the timing of infection, source of inoculum, climatic factors influencing disease development, disease cycle, disease resistance among varieties and chemical control options. This information has generated all the data that enabled the registration of Cabrio and has provided a strong base for developing more efficient and profitable control methods to manage the plant disease.

When re-planting or establishing new orchard selection of disease tolerant cultivars should be considered.

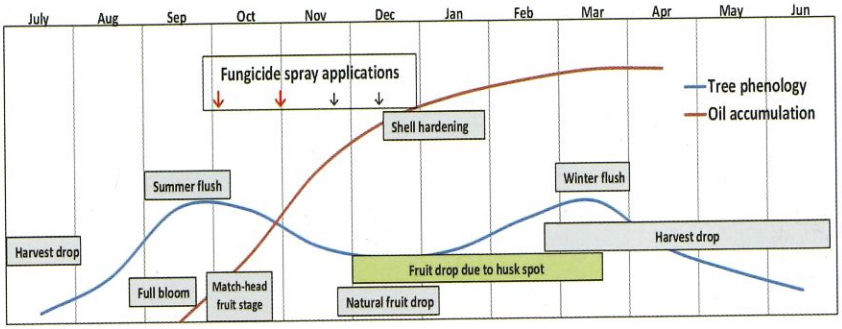

Figure 1. *Seasonal growth pattern of the macadamia tree and reproductive stages with periods of husk spot control indicated.*

We are looking to improve our understanding of the disease cycle and the relative importance of cultivar and weather variables on disease development as these will underpin the development of a disease forecasting system which guides fungicide spray applications. The search for alternative and more effective chemical control options which help the choice of chemical, timing and rate of applications and which take economic thresholds into consideration should continue.

Another area with potential is alternative disease control measures based on plant hormones and other related products that may limit early fruit drop in the presence of husk spot need further investigation.

The most apparent gaps in our knowledge of husk spot of macadamia are in the areas of infection process such as what triggers symptom expression and fruit drop, pathogenic variability and host × pathogen interaction, host resistance, and alternative disease control measures. The long-distance dispersal mechanisms of the pathogen and its distribution, as vital aspects of disease epidemiology, are still largely unknown.

Project MC12007, Disease management in the macadamia industry, is focussing on many of these issues. With the strong foundations and knowledge already generated by research in the last ten years, we are hopeful of expanding the options for controlling husk spot in future.

HAL

Horticulture Australia

Acknowledgments

The authors acknowledge the funding of this project - *Disease management in the macadamia industry* (MC12007) and the two preceding projects (MC03007 and MC07003) using the levy for the Australian macadamia industry with matching funds from the commonwealth government through HAL. We acknowledge all the significant in- kind and cash voluntary contributions to the projects received from private and government agencies. We are grateful for the assistance, access and use of orchards provided by several industry consultants and growers over the past ten years.

Note. This article is a condensed version of an article Husk spot of macadamia: *A review of the current status and future research priorities* by Dr Femi Akinsanmi and Assoc. Prof. André Drenth, Centre for Plant Science, Queensland Alliance for Agriculture and Food Innovation (QAAFI), University of Queensland and Dr Andrew Miles, Department of Agriculture Fisheries and Forestry Queensland, Brisbane.

PEST AND DISEASE MANAGEMENT FEATURE

Managing husk spot

While husk spot has not been a huge issue for the industry in the last couple of years, with the right climatic conditions it could come back with a vengeance and cause significant damage. This article is a reminder of the basics in managing husk spot.

Husk spot management strategy

The husk spot management strategy has been designed to reduce losses and thus increase grower profit margins. Husk spot results in yield loss, mainly due to premature drop in January through to mid-February. It also results in lower quality because of the numbers of immature nuts that are harvested. Fortunately, once the nut is mature, husk spot has minimal effect on yield and quality.

The key to managing husk spot is to ensure the disease is controlled until the nut reaches full size and maturity in the tree. There is no benefit to be had by spraying for husk spot later in the season because infection of nuts close to maturity or at maturity has no direct impact on yield and quality.

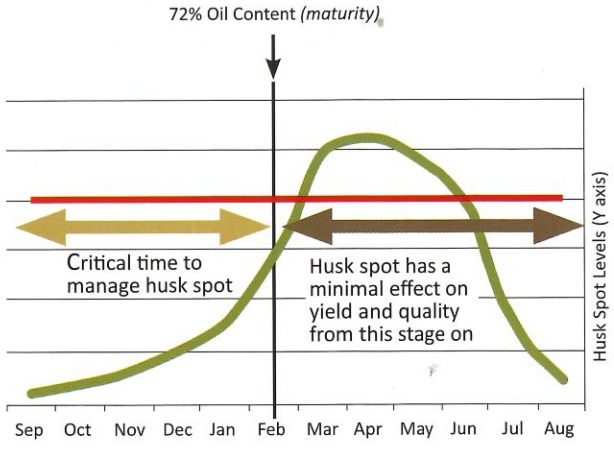

Figure 1. Husk spot lesions when kernels have reached 72% oil content have minimal effect on yield and quality. The goal is to keep husk spot (green line) below the critical level (red line) until after maturity

Yield losses as a result of husk spot are caused mainly by premature nut drop in January and February.

From the figure, it is crucial to stop immature nut drop by protecting the developing nut from match-head size stage to full size stage (approximately in the first three months of nut formation, depending on the variety). It is also important to monitor kernel maturity based on nut from orchard floor using accredited standards and methods. Have the orchard floor ready for harvesting when husk spot symptoms are apparent in early January.

The following factors are important considerations when developing your husk spot management strategy:

- husk spot lesions on green or dried husks, including stick tights, continue to produce viable fungal spores for many years
- husks are only infected when green (starting from match-head size stage to harvest)
- husks (green/living tissue) are infected in wet conditions (dew or rain)
- the fungus causing husk spot does not infect the kernel
- early nut set (before main flowering in August-September) may be infected if unprotected.

Challenges to effective management

The following factors can be issues for your management strategy:

- multiple flowering and nut set, which results in it being hard to adequately protect the crop
- an abundance of sticktights with husk spot lesions in the tree canopy as they increase disease pressure
- long periods of wet weather can result in delayed spray application during key nut development stages
- susceptible varieties that are more prone to infections (usually with high levels of sticktights) or more prone to premature nut drop after infection than others (see table and Figure 2)
- inadequate spray coverage (spray volume; canopy volume and density).

Top 10 most susceptible varieties	
1	Own Venture
2	A 38
3	A 16
4	781
5	H2
6	A 4
7	A268
8	816
9	A29
10	842

Table. The top ten varieties most susceptible to husk spot infection.

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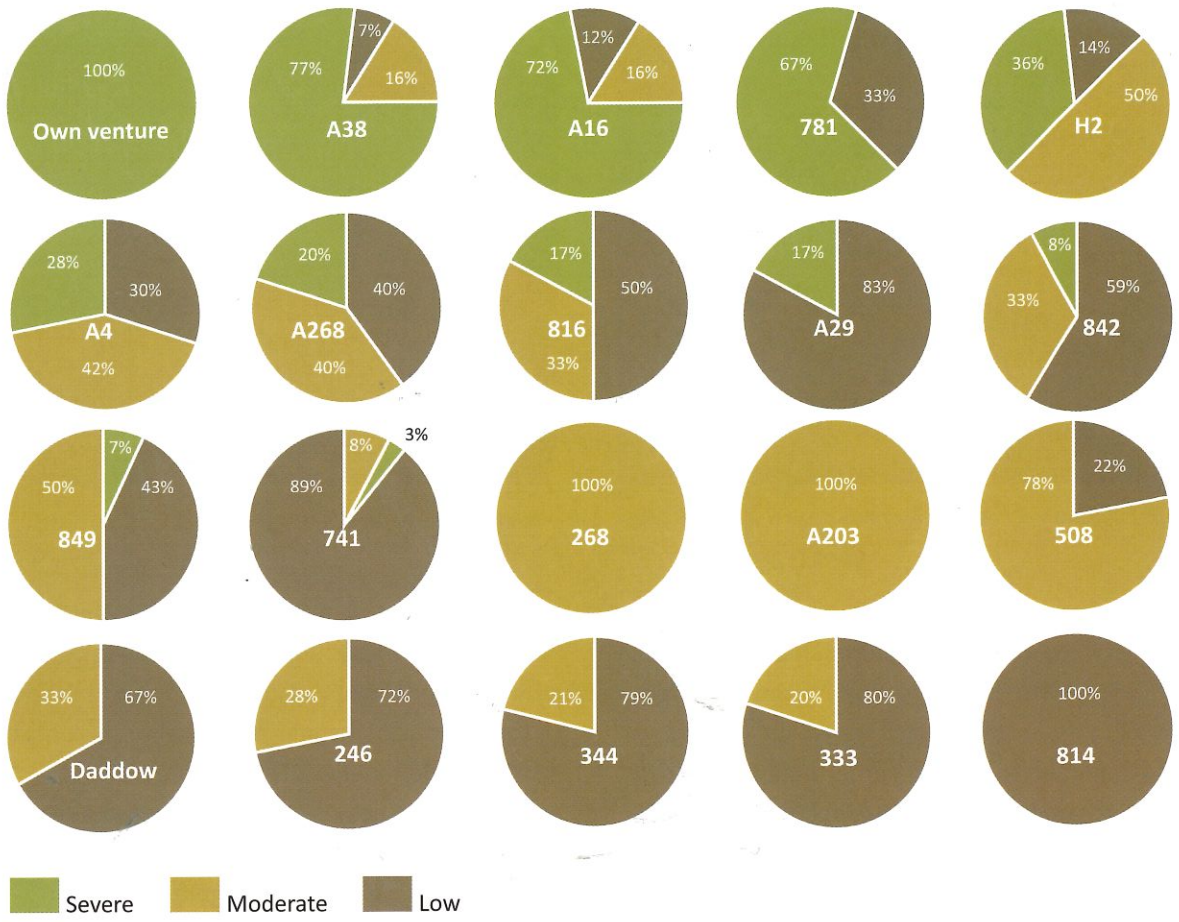


Figure 2. Susceptibility of the most common macadamia varieties to husk spot.

Husk spot – what's the risk in my orchard?

You can assess the risk of husk spot in your orchard for next season by answering the following questions and adding up the score.

Question	Score (0 = NO, 1 = YES)
Does your orchard have a history of husk spot?	
Was the premature nut fall in February due to husk spot greater than 10%?	
Does your orchard have susceptible varieties, i.e. Own Venture, A38, A16, 781, H2, A4, 816, A268, A 29 or 842?	
Do you have stick tight nuts infected with husk spot in your trees?	
Do you have out-of-season flowering and nut set?	
Have you experienced favourable weather conditions for husk spot development, i.e. 20 – 30 degree temperatures during September to December along with continuous wet spells?	
TOTAL	

If you scored 0 or 1 you are at a low risk and require minimal treatment.
If you scored 2 or 3 you are at a moderate level of risk and the standard treatment is suitable.
If you scored between 4 and 6 you are at a high level of risk and additional treatment is needed for the following season.

Information

For more information on husk spot control see article "Research shakes out husk spot disease in macadamia" on page 60. For more information on maintaining quality see the *Australian Macadamia Industry Code of Sound Orchard Practices, the Macadamia Grower's Handbook* and the AMS Website www.australian-macadamias.org

Acknowledgment
This article was taken from AMS Information Sheet No. 7, which was by Dr Femi Akinsanmi and the Crop Protection Industry Reference Group (IRG).

Research shakes out husk spot disease in macadamia

Horticulture Australia

Preliminary results from field trials could pave the way for a new husk spot management regime incorporating cultural practices that will lead to reduction of fungicide spray applications in the industry. The trial is led by Dr Femi Akinsanmi at The University of Queensland as part of research project MC12007 *Disease Management in the Macadamia Industry.*

Results from a season's testing show that mechanically removing sticktights and routine monitoring of kernel maturity alone and sometimes combined with strategic application of fungicides could be as effective as the annual routine chemical application on its own. The advantage is that less chemical needs to be applied, and indications are that, over time, the amount of chemical required for control will decrease.

The problem

Husk spot, caused by the fungus *Pseudocercospora macadamiae,* is a serious disease of macadamia. It affects production and nut quality by shifting abscission (nut drop) forward resulting in an earlier peak in harvest and a higher proportion of immature fruit being harvested with mature kernel. This mix of immature and mature fruit being harvested can result in inefficiencies at sorting and processing and growers being penalised financially.

If not controlled, husk spot will worsen over time. This is because the *P. macadamiae* fungus survives from season to season on split, infected pericarps called sticktights, which do not abscise, rather remain in the canopy. Husk spot severity also increases with tree age, possibly because canopies become denser and more sticktights are retained.

In the past, manual removal of sticktights has been trialled but it is time consuming and, as a result, control in most orchards has been based almost exclusively on a seasonal program of fungicide application.

While applying fungicides is effective, it is not foolproof. It sometimes fails, especially if rain and climatic conditions conducive to the disease, such as humidity, prevail. It also relies on the availability and registration of effective fungicides.

An issue that has provided further impetus to trialling new control methods is community demand to reduce pesticide use in horticulture and agriculture.

Integrated program for husk spot control

With these pressures on growers to use less chemical, an integrated control program has been developed as part of project MC12007 based on:

- mechanical removal of sticktights using a tree shaker
- routinely monitoring kernel maturity as a way of tracking the development of husk spot and fine tuning harvest
- strategic application of fungicide
- host resistance.

The trial is being conducted in a commercial orchard in Bundaberg on A38 and A16 trees, the varieties that have been identified as the most susceptible to husk spot. The reason for choosing these two varieties is that if control measures are effective on them, they are likely to be similarly effective on the less susceptible varieties.

With the advent of modern mechanical tree shakers, the first challenge is to identity an efficient tree shaker. To this end, different mechanical tree shakers are continually being trialled in commercial orchards.

One of the mechanical tree shakers, which was built in Bundaberg, is currently being tested to demonstrate the concept and benefits of application of the technology for husk spot control in macadamia. To determine if using a tree shaker can control husk spot with or without fungicide applications, treatments in trial blocks consist of the following:

- removal of sticktights + fungicide application
- removal of sticktights and no fungicide
- untreated control (trees have sticktights).

There are at least 40 trees in each treatment.

Shakers like these are being used by growers in an effort to establish which is most efficient and effective.

Sticktights are removed using a tractor-mounted tree shaker with a finger-wheel-like device that rotates on its axis by mechanical transmission when in contact with the tree canopy as the tractor moves forward at 3.2 km/hr. This shaker concept was developed by Steinhardt Corporation (Macadamias Australia, Bundaberg).

It is important to note that tree shaking technology has advanced a lot since shakers were used in the industry a number of years ago. These early systems often damaged trees and resulted in soil disturbance. Since then technology and machinery have improved and systems now are being used successfully in a number of tree crops, including pecans, walnuts and apples, to reduce harvesting costs by more than 50%.

This shaker, developed by Steinhardt Corporation, is being used in the research trial.

The bottom line

Data have been collected and analysed for the first year of the trial (2012 -13), and preliminary results are very promising.

They show that the average efficiency of the mechanical tree shaker used in the trial in removing sticktights was about 60% with a range of between 17 and 100% among the trees. Importantly, husk spot was less severe when sticktights were removed in trees of both varieties compared with untreated control trees, i.e. no fungicide application (see figure).

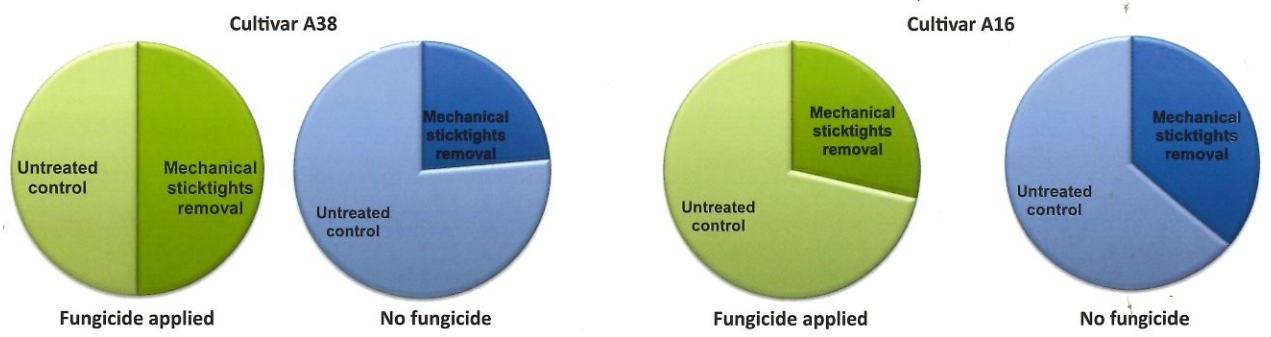
What these results show is that there is potential for incorporating the use of tree shakers into a cultural control component of a husk spot management strategy that could result in less reliance on fungicide spray applications. This strategy would involve the use of mechanical tree-shakers and monitoring of kernel maturity to fine tune the timing of the harvesting.

Tree shakers may be used during autumn and winter to reduce and/or remove sticktights in the tree canopy, potentially as part of harvesting or as post-harvest clean up before the next flowering season to maintain low inoculum density conditions. It is also expected that growers should adopt cultural practice that helps to monitor kernel maturity rather than following a calendar plan for pre-harvest orchard floor clean up and start of harvest.

While it is only early days, indications are that this approach could be incorporated into a holistic management strategy as a way of improving long-term orchard sustainability.

Note. Article adapted by Anne Currey from a presentation by Dr Femi Akinsanmi at the 40th Anniversary Macadamia Industry Conference.

Figure. Proportion of husk spot severity in macadamia tress where sticktights were removed in combination with or without fungicide applications in two varieties at Bundaberg.



Hort Innovation
Strategic levy investment

MACADAMIA FUND

Integrated Management of Diseases in Macadamia Industry

Appendix 3: Communications in the mainstream industry journals and News Bulletin

(b) Phytophthora management

Dr Olufemi A. Akinsanmi
The University of Queensland

Project number: MC12007

Crop protection
The lowdown on phytophthora

Horticulture Australia

Phytophthora is one of the most serious pathogens (microorganisms causing disease) of macadamia. It infects almost all parts of the tree, including roots, leaves, flowers, stems and the trunk. It is responsible for a variety of diseases estimated to cause losses in production in Australia of about $8 million a year and reduce the growth of young trees by between 30 and 60 per cent. The five main diseases that affect macadamia are: phytophthora blight, stem or trunk canker, tree decline and dieback, phytophthora root rot and necrosis, and macadamia quick decline.

There are a number of phytophthora species, and soil is their main habitat where they can survive for a long time in various stages and without a host plant. Soils that stay wet for long periods are associated with diseases caused by phytophthora, as are climatic conditions of high moisture and temperature. Because of the association with wet soils, good irrigation and drainage management is critical.

Disease control

Control measures for phytophthora diseases in macadamia orchards occur at three different stages:

- before orchard establishment
- as a part of routine
- after infection.

Phytophthora stem cankers caused by Phytophthora cinnamomi *as a result of (A) bruising of bark (arrow) from strong winds shaking the tree; (B) prolonged wetting of the trunk (arrow) by irrigation jets close to the tree trunk; (C) ponding from irrigation near the tree trunk which results in (D) poor root development and (E) tree death.*

Before orchard establishment. There are a number of recommended practices before orchards are established.

Site selection and preparation are critical in minimising the risk of phytophthora induced diseases occurring. In orchards where the pathogen is known to exist, the planting hole can be prepared to suppress or eradicate it from the root zone. Chicken manure or fertiliser added into the planting hole can help do this, and it has the added benefit of promoting good root production. Soil with good organic matter content and biological activity reduces disease-promoting conditions and may provide some level of disease-suppression.

Severe stem canker can occur in trees exposed to strong winds that loosen the roots in the soil. This can bruise rootstock at the soil line and result in stem canker development.

Planting stock should be produced with disease-free scion and rootstock in clean soil or potting mix. Using disease-free planting material from a reputable nursery and grafted seedlings is essential.

Good **irrigation and drainage management** is essential. Spray nozzles should be directed away from the base of trees to avoid wetting the bark. Wet bark predisposes the tree to phytophthora infection and to development of stem canker. And it is a good idea to test irrigation water regularly to ensure it is pathogen free.

You can reduce the risk of waterlogging by planting trees on a slight mound. Water will be less likely to pond around the trunk compared to trees planted in areas with that are low lying or flood often, or that have elevated soil moisture. Mounding the soil before planting also promotes good drainage, which is crucial. As well, constructing shallow 'V' drains down the centre of the rows to keep run-off away from tree trunks has been shown to minimise trunk canker in macadamia orchards.

Routine orchard maintenance. Good hygiene in orchards is a fundamental component of effective management, e.g. keeping roadways, inter-rows and equipment clean. It is virtually impossible to eradicate phytophthora from the soil once it is infested.

Generally, mycelium and zoospores survive only for a few weeks, while chlamydospores and oospores may survive for many years, making control challenging. To reduce infections through wounds, cultural practices should be performed with great caution, including mechanical operations that may cause wounds on trees.

Nutrition management is also critical. On one hand, good nutrition enables root growth and proliferation which may be one of the components of resistance to phytophthora. On the other hand, it is believed that high levels of nitrogen exacerbate phytophthora problems. The role of soil nutrition in controlling or suppressing phytophthora diseases is still unclear in macadamia and it is currently under investigation.

Adding **compost, manure or mulching** with macadamia husk has been shown to improve soil fertility in macadamia orchards. Organic amendments are good news generally as they stimulate plant root growth, increase nutrient uptake, decrease evaporation from the soil, increase soil water holding capacity, reduce surface water run-off, facilitate drainage, regulate soil temperature, and provide a high level of nutrients for soil microbes. Depending on their nature, some amendments can either enhance or suppress disease so more investigation is needed to examine their effect on phytophthora control in macadamia.

After infection. Once diseased trees have been found in an orchard, applying corrective treatments is critical. Integrated disease management (IDM) is fundamental to post infection control. Strategies should aim to:

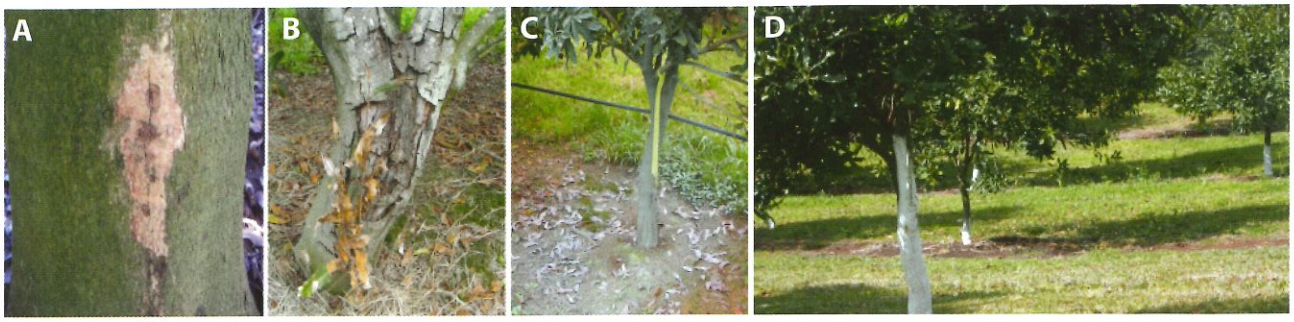
- minimise infection at various points in the disease cycle
- limit the spread of inoculum
- produce high soil biological diversity
- achieve high stability that can provide resilience to stress.

The first step in effective control is the production of **pathogen-free** planting materials. This limits the spread of the pathogen, and ensures that from the outset trees are healthy.

For mature trees, an old and laborious technique is **surgical intervention** to remove diseased tissue, where canker lesions on the trunks are scraped off to expose healthy surfaces before painting the surface with a paste of copper fungicide (in some cases the canker may be too advanced for the treatment to be effective). Copper-based compounds used include a bordeaux mixture, often as a 1:1:1 mixture of copper oxychloride, hydrated lime and acrylic paint and sufficient water, or copper-based protectant fungicides such as copper hydroxide, copper oxide, basic copper sulphate, copper oxychloride and copper ammonium carbonate.

The **chemicals** metalaxyl and phosphonates are commonly used to control phytophthora diseases. Effectiveness of phosphonates in suppressing phytophthora depends on its concentration in the plant tissues which is directly related to the application rate. Unfortunately, we don't know the optimal phosphonates concentration needed to control phytophthora for a long period in macadamias. It has been observed that phytotoxicity on macadamia leaves from foliar application occurs at relatively lower rates than other tree crops.

Many growers apply phosphonates at rates that offer minimal phytotoxicity reactions to control phytophthora trunk canker. Foliar and trunk spray applications of phosphonates or soil drench with metalaxyl-M have been shown to be effective. Timing of application is important; phosphonates accumulate in the prevailing 'sink' at the time of application. For instance, applications at the root flushing period accumulate in the root system and at fruit development period accumulate in the fruit.



Phytophthora stem canker control using surgical intervention and painting with copper fungicide and white emulsion paint. Mild infection scrapped off before painting (A); canker too advanced for surgical intervention (B); trunk with stem canker sprayed with copper oxychloride only (C); and tree trunks painted with mixture of copper fungicide and white emulsion paint (D).

Further studies are needed to confirm the effect of addition of biological control agents on phytophthora stem canker and tree decline in macadamia.

Biological control is another option to control or suppress the development of phytophthora diseases. Adding mulches or composts can be effective. There are several unconfirmed observations that applying biological agents such as *Trichoderma* species as soil drench may control stem canker and tree decline in macadamia. Further studies are needed to confirm the effect of addition of biological control agents on phytophthora stem canker and tree decline in macadamia.

More research needed

Areas identified for further research in macadamia are as follows:

- The pathogenic diversity of species of phytophthora affecting macadamia, i.e. to identify if different species can cause similar symptoms in macadamia and if similar pathogen genotypes cause the same disease in different countries.
- Identifying the genetics and mechanisms of resistance to phytophthora in macadamia by, for example, uncovering good sources of resistance to phytophthora, including from wild germplasm, and their relatives as rootstock. Robust tests for disease resistance screening of cultivars and breeding lines are needed.
- Resolving issues to do with root rot and root necrosis, e.g. whether root rot or necrosis occurs in macadamia roots, whether reduced root density the result of inhibition of root replacement by the pathogen, and what role fertiliser or tree nutrition plays in altering the balance of root loss to root regeneration ratio on tree decline.
- Determining the critical concentrations and dynamics of phosphonic acid in macadamia roots and stems against phytophthora infection.
- Understanding the role of root disease in decline in both young and mature orchards and on disease complexes such as yield declines and replanting diseases.
- Developing effective and sustainable integrated disease management practices that work and which involve cultural practices, soil health and interaction with phytophthora.

Acknowledgment
This article has been condensed from a review, *Phytophthora diseases of macadamias: A worldwide occurrence, management and future research priorities,* by Dr Femi Akinsanmi and Assoc. Prof. André Drenth, Centre for Plant Science, QAAFI, University of Queensland.

Decision Guide for Application of Phytophthora Control in Macadamia

Application strategy	Severity level	Description scale*	Application method
Healthy	None	0	None *(Maintain good management practices)*
Maintenance	Low -Medium	1 - 2	Foliar
Curative	High	3 - 4	Trunk or Foliar
Restoration	Very High	5	Trunk

0 = dark green leaves and healthy tree
1 = mild pale to yellow leaves of the tree canopy;
2 = severe pale to yellow leaves obvious signs of tree stress (shoot regrowth from rootstock)
3 = stem canker, gummosis and sparse canopy with pale to yellow leaves
4 = stem canker, very sparse tree canopy and obvious dieback
5 = extensive branch dieback or dead tree with stem canker present.

Application of chemical control	Foliar spray	Trunk spray
Timing	After leaf flush = root flush *(hardened and non-fleshy new leaf)*	Root flush
Number/year	1 – 4 *(1 - 2 times = low-medium severity; 2 - 4 times for high severity)*	1
Frequency between applications	Depends on dose and timing	1
Dose rate	Maximum of 0.25% Phosphonates *(Use optimum range 250-300 mL of 400 g/L Phosphonates formulation per 100 L)*	20% Phosphonates + 2% bark penetrant (e.g. Pulse) *(500 ml of 400 g/L Phosphonates formulation + 20 mL Pulse per 1 L)*
Spray volume	Dilute	Wet trunk slightly till run-off *(around the trunk to about 1 m above soil level)*

Key points

1. Phytophthora is spread through water, infected plant materials and infested soil
2. Well drained and aerated soil is essential, avoid ponding around tree trunk and do not over irrigate sick trees
3. Tree with good root growth is better able to cope with phytophthora and flowering and fruit set in spring
4. Maintain good tree nutrition (check by having a leaf and soil analysis done)
5. Organic amendments enhance biological activity and improve soil health
6. Apply chemical control for quick recovery of sick trees

Remember: A healthy tree = productive tree

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Queensland Government

Phytophthora diseases of macadamias

Dr Femi Akinsanmi and Assoc. Prof. André Drenth, Centre for Plant Science, Queensland Alliance for Agriculture and Food innovation (QAAFI), University of Queensland, Brisbane.

Introduction

Over 120 *Phytophthora* species infect over 3500 plant species including several tree nuts. *Phytophthora* is a significant impediment to macadamia production worldwide and it infects almost all parts of the tree including root, leaf, flower, stem and trunk (Table 1). In some cases, a single *Phytophthora* species can cause different disease symptoms, and different species can cause the same disease symptoms in macadamia.

Two factors that make *Phytophthora* a significant threat to macadamia worldwide are:

- the slow progression of some disease symptoms which often leads to the disease being misdiagnosed or overlooked by growers at the early stage of infection, and
- the ability of the disease to rapidly become severe or cause tree death when the tree is under stress or under prolonged conditions that are favourable to the disease.

It is estimated that *Phytophthora* causes production losses of over $8 million annually in Australia; in Kenya yield loss is about 60% (Mbaka et al., 2009) and in Hawaii, about 53% of *Phytophthora*-infected trees in field conditions were reported to die within seven months after planting (Keith et al., 2010). Similar losses in production may occur in other tree nuts and therefore, mitigating the impacts of *Phytophthora* diseases requires concerted efforts.

Conditions that favour infection and disease development

Soil is the main habitat for most *Phytophthora* species infecting macadamia. In soil they can survive for a long time in various stages of development and they may also infect other host plants or survive on plant residues until conditions become favourable for infection of macadamia trees.

Phytophthora disease	Symptoms	Associated *Phytophthora* species	Plant part affected	Source
Blight	Extensive dark necrosis of irregular shape on affected plant part.	*P. capsici,* *P. palmivora*	Racemes, young leaf flushes and shoot tissues, immature nut early at fruit set	Hawaii (Aragaki & Uchida, 1980, Hunter et al., 1971, Kunimoto et al,1976) Costa Rica (DeSegura, 1970).
Stem or trunk canker	Furrowed deep cankers on the trunk, irregular areas of dead bark extending from the soil line to several feet high, sap bleeding	*P. cinnamomi*	Trunk, branches	All macadamia-producing nations- (e.g. Australia, South Africa, China, Kenya, Guatemala, USA, etc.) *
Decline and dieback	Pale or yellowish leaves instead of dark green, branches die back and sparse looking	*P. cinnamomi*	Branches/ whole tree	Australia, Guatemala, South Africa
Root rot and necrosis	Dark necrosis of root and rot of rootlets	*P. cinnamomi*	Roots	Hawaii (Ko and Kumimoto, 1976), Kenya (Mbaka et al., 2009), South Africa (Serfontein, 2008), Australia (Pegg, 1973)
Quick decline	Whole canopy changes from dark green to light green, then within 2-3 months turn yellow to light brown, then brown and the tree dies	*P. tropicalis*	Whole tree	Hawaii (Keith et al., 2010)

* Akinsanmi 2008; Hines, 1961; Pegg, 1976;

Table 1. Diseases caused by *Phytphthora* in macadamia.

Phytophthora thrives in wet conditions and in soils with poor drainage and improper irrigation management, and where root systems and tree vigour are impaired. Severity of *Phytophthora* disease is exacerbated in conditions of high moisture, drought and high temperature. Interactions of rootstock and scion make some cultivars at high risk to *Phytophthora* diseases.

Disease control

Integrated disease management (IDM) strategy is the best approach to control *Phytophthora.* Firstly, to protect and prevent infection (Table 2), the IDM strategy is designed to minimise infection at various points in the disease cycle, limit the spread of inoculum, produce a highly diverse soil biology, and achieve high stability to provide resilience to stress. Secondly, to treat trees after infection, the IDM strategy is designed to maintain production and prevent tree death.

Control measures for *Phytophthora* diseases in macadamia orchards occur at difference stages:

1. Before orchard establishment

The first step in effective control is to ensure pathogen-free planting materials. This limits the spread of the pathogen and ensures that from the outset trees are healthy.

- **Orchard site selection and establishment:** Site selection and preparation are critical in minimising the risk of *Phytophthora* infection. At planting, treat the planting hole to suppress or eradicate the pathogen from the root zone and allow the roots to become established before infection.

- **Cultivar selection and resistant rootstock**: Use disease-free planting materials from a reputable accredited nursery and where available, use resistant rootstock. Check that the root distribution in potting bags is adequate and consistent.

- **Irrigation and drainage management:** Irrigate where necessary, spray nozzles should be directed away from the base of trees to avoid wetting the bark and avoid water-logging of the orchard and ponding around the tree trunk (plant on slight mound). Wet bark predisposes the tree to *Phytophthora* infection and to development of stem canker.

	Control measures
• Source of planting materials -	Healthy seeds and buds, resistant plants
• Potting media (soil, potting-mix) -	Pasteurisation or fumigation
• Water -	Sterilisation or filtration
• Grafting tools -	Sterilisation
• Nursery access and operation practices -	Free drainage system, avoid movement of soil water (erosion) or soil materials through the nursery

Table 2. Possible sources of *Phytophthora* in the nursery

2. Routine orchard maintenance and cultural practices

- **Orchard hygiene:** Good hygiene in orchards is a fundamental component of effective pest management. It is virtually impossible to eradicate *Phytophthora* from the soil. Roadways, inter-rows and equipment should be kept clean.

- **Cultural practices:** Reduce infections through wounds. Cultural practices including mechanical operations that may cause wounds on trees, should be performed with great caution. Where *Phytophthora* blight occurs, infected racemes and leaves should be pruned to reduce the source of inoculum.

- **Nutrition management and organic amendments:** Some forms of nitrogen have been shown to favour an increase in disease while other forms suppress disease. Good nutrition enables root growth and proliferation which may be one of the components of tree resilience to *Phytophthora* in macadamia. Generally, adding compost or organic manure to soil and mulching around trees improves soil fertility and soil health, decreases evaporation from the soil, increases soil-water holding capacity, reduces surface water run-off, facilitates drainage, and regulates soil temperature. Increasing the organic content of soil increases the diversity of micro-organisms which has been found to provide a natural protection against *Phytophthora* diseases. Currently, a field trial is being undertaken to evaluate the impact of soil organic amendments on *Phytophthora.*

3. Post infection control measures

Once diseased trees occur in an orchard, applications of corrective treatments are critical.

- **Surgical intervention:** Although this practice is old, laborious, time consuming and now almost obsolete, some macadamia growers still practice this management option. The principle to remove diseased tissue, where canker lesions on the trunk are scrapped off to expose a healthy surfaces before painting the surface with a paste of copper fungicide. In some cases the canker may be too advanced for the treatment to be effective. In order to prevent the copper being washed off by rain, an oil emulsion formulation is added to the fungicide before painting the bark. Any copper-based compounds, such as bordeaux mixture often as a 1:1:1 mixture of copper oxychloride, hydrated lime and acrylic paint and sufficient water, or copper-based protectant fungicides such as copper hydroxide, copper oxide, basic copper sulphate and copper ammonium carbonate are used.

- **Chemical control:** Metalaxyl and phosphonates are commonly used to control *Phytophthora* diseases. Effectiveness of phosphonates in suppressing *Phytophthora* depends on its concentration in the plant tissues which is directly related to the application and varies among plant species. Recent research

outcomes in macadamia showed that rates for foliar application are at relatively lower rates than indicated for other tree crops (Akinsanmi & Drenth, 2013). Many macadamia orchardists apply phosphonates at rates that offer minimal leaf burn. Foliar and trunk spray application rates of phosphonates have been established in macadamia (Akinsanmi & Drenth, 2013) and soil drench with metalaxyl has been shown to be effective in macadamia. Foliar application is mostly applied on an orchard-wide basis while trunk application is specifically targeted to unthrifty looking trees.

- **Biological Control:** Biological control provides an attractive and environmentally friendly option to control or suppress the development of *Phytophthora* diseases. There are several unconfirmed observations that applying biological agents such as *Trichoderma* species as soil drench, or compounds that elicit host resistance, suppress progress and development of stem canker and tree decline in macadamia. Further investigations are needed to confirm the effect of these products on *Phytophthora* stem canker and tree decline in macadamia.

Current status and future outlook

Immediate areas of research are as follows:

- Resolving issues to do with root rot and root necrosis, e.g. whether root rot or necrosis occurs in macadamia roots, whether reduced root density is the result of inhibition of root replacement by the pathogen, and what role fertiliser or tree nutrition plays in altering the balance of root loss to root regeneration ratio on tree decline.
- Determine the critical concentrations, duration and dynamics of phosphonates against *Phytophthora* in young and mature trees.
- Provide a good understanding of the contribution and role *Phytophthora* play to issues of declining productivity in mature orchards.

Macadamia stem with *Phytophthora* canker.

- Developing effective and sustainable integrated disease management practices that involve cultural practices, soil health and interaction with current *Phytophthora* control measures.
- To identify if different species can cause similar symptoms in macadamia and/or other tree nuts, and if similar pathogen genotypes cause the same disease in different countries.
- To identify the genetics and mechanisms of resistance to *Phytophthora* in macadamia by, for example, uncovering good sources of resistance to *Phytophthora,* including from wild germplasm, and their relatives as rootstock.
- Development of robust tests for disease resistance screening of cultivars and breeding lines is needed.

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SOIL ORGANIC AMENDMENTS IMPROVE TREE HEALTH AND SUPPRESS PHYTOPHTHORA

Horticulture Innovation Australia

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Diseases caused by *Phytophthora cinnamomi* are the most common in macadamia orchards worldwide. While cultivars vary in their susceptibility to *P. cinnamomi*, sadly, none of the current ones is immune to the pathogen. In macadamia, Phytophthora diseases are associated with poor soil health management practices including:

- erosion or poor soil water management
- soil compaction and poor soil structure
- poor nutrient retention and lack of organic matter.

These constraints can be managed by appropriate cultural farm practices. Management practices that promote soil health, such as cover crops and green manure and organic amendments, also have generally positive effects on the management of soilborne diseases and production. One of the components of an integrated disease management strategy in macadamia is cultural control. In this article, research and progress made on the importance of soil health, including nutrient availability, sustainability, and soil health management practices, with an emphasis on their implications for phytophthora management, are summarised.

Controlling Phytophthora using cultural practices

In comparison with chemical treatments, organic amendments have the potential to persist in soils for an extended time, promoting longer disease suppression attributes and building soil health. Using organic amendments such as compost, manure and mulch to reduce or suppress plant pathogens and diseases is well-documented (see references 1 to 7). The process benefits biological, chemical and physical properties of the soil. While compost has often been associated with reduced occurrence of soilborne diseases in a wide variety of crops, variation in compost materials, age, maturity and quality mean results can be unpredictable (see references 2, 3 and 5). Maintaining the biological or living system in soil is vital for disease suppression.

Organic matter and Phytophthora control

Soil organic matter, which plays a vital role in many of the soil chemical processes and affects soil structural stability, is crucial to plant productivity. In recent field trials in two macadamia orchards, incorporating soil organic amendments such as mill-mud (sugar mill waste) and garden waste compost greatly improved tree health over three years compared with trees with no control practices. These materials were applied once but their effects lasted for about three years before requiring additional maintenance. The percentage of organic matter in the soils amended with compost was greatly improved compared with soils amended with mill-mud (see photo). Similarly, certain nutrient deficiencies appeared in leaves in the third year after soils were treated with organic amendments, except in soils with higher percentage of organic matter. This demonstrates the need for routine assessments of soil and leaf chemical compositions.

The results suggest that organic amendment helped suppress tree decline caused by Phytophthora and improved yield in macadamia trees. A number of potential mechanisms that influenced this include increasing soil microbial composition, activity and diversity, as well as improved soil nutrient management for macadamia. In some cases, organic amendments may also influence disease suppression by inducing resistance in the trees through increasing or stimulating root-inhabiting bacteria capable of inducing resistance to the soilborne pathogen in the plant (see reference 8).

(l to r) Leaves on trees showing nutrient deficiency at three years after application of organic amendments (compost, sugar factory waste mill-mud) and back spray with phosphorous acid (chemical) compared with untreated soils and their corresponding percent soil organic matter contents.

Soil nutrients and Phytophthora control

Our research has showed that fertile soils tend to suppress *P. cinnamomi* in macadamia. This was confirmed in both glasshouse and field trials. In the glasshouse trial, Phytophthora stem canker developed only in trees that did not receive slow-release fertiliser

compared with trees that received slow-release fertiliser (see photo).

In the field trials, poor tree health resulted in 40 to 60% less growth in young trees planted without any chemical applications at planting in fertile krasnozem soils with high levels of *P. cinnamomi*. A similar situation where disease development was suppressed in fertile soils has been reported in north-eastern Australia in certain avocado farms at Mt Tamborine in Queensland. A reason for soils acting to suppress *P. cinnamomi* has been attributed to horticultural practices that maintained the soil pH at 6 and high levels of organic matter. In the macadamia trials, adding compost-stabilised soil pH at about pH 6 and reduced excessive canopy growth volume compared with soils amended with sugar waste mill-mud (see figure).

Soil systems vary in their complexity

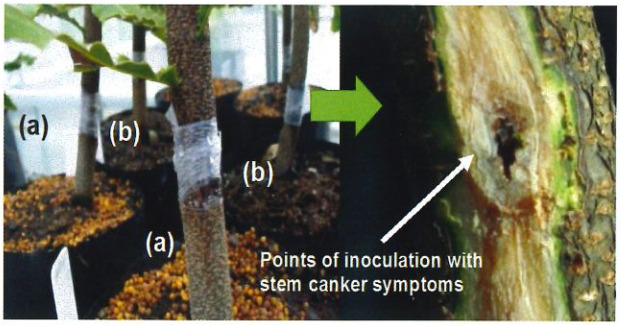
Soil type and physical and chemical situations are important factors that should be considered in any strategy for applying organic amendments as they will affect how well that soil will be in suppressing Phythophthora. Available information clearly indicates that this process involves the activity of a range of diverse microbial populations, rather than one specific group of organisms.

Overall strategies

In addition to establishing good soil health, routine assessments and indicators should be used to identify specific areas and parameters that need to be improved. The guiding principles for building and maintaining good soil health are:

1. Good balance of soil available nutrients, water retention, active microbes
2. Good soil organic matter content
3. Good soil preparation and maintenance of soil management practices

These are general strategies to keep in mind when making management decisions to improve soil health.



Phytophthora stem canker in macadamia trees in planting bags (a) trees that received slow-release fertiliser prevented the formation of stem canker after artificial inoculation compared with (b) trees that received no fertiliser which showed stem canker symptoms three months after inoculation.

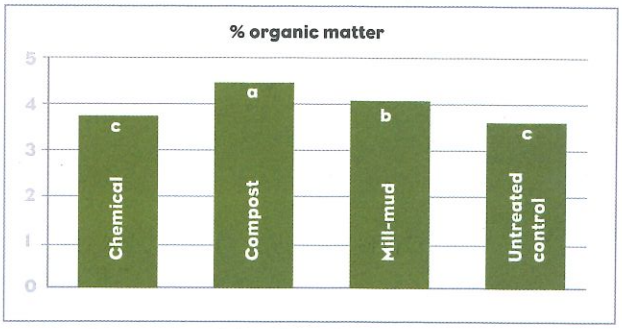


Figure. *Comparison of the effect of compost (composed commercial garden waste), mill-mud (sugar-mill waste), application of phosphorous acid and untreated soils on soil pH and tree canopy size rating (scale 0-10) at three years after application.*

Strategies for managing organic matter could include maintaining living plants and soil cover under the trees. Organic matter serves as the primary food source for soil microorganisms, helps to maintain diverse soil biota, regulates soil pH and nutrient availability, improves soil water retention, infiltration and aeration and improves soil structure.

These characteristics could have contributed to the ability of soil to suppress *P. cinnamomi* and improved tree health in macadamia. Adding organic amendments, however, may not be sufficient to completely control or eliminate disease problems. This is why it is important to adopt an integrated disease management strategy.

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HIA project MC12007

Soil health and tree decline

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A major objective of project MC12007 *Disease management in macadamia industry* is to establish a cost effective, sustainable integrated management strategy for tree decline, dieback and stem canker caused by *Phytophthora cinnamomi*.

The project will test the effect of soil amendments on Phytophthora tree decline and their potential role in an integrated disease management strategy for macadamia. This article outlines preliminary information on the field trials on soil health and Phytophthora management in macadamia.

What is tree decline and dieback?

Decline is a term often used to describe a set of symptoms or syndrome associated with loss of tree vigour. These symptoms include: reduced growth, reduction in size and quantity of foliage, chlorotic foliage, death of twigs and branches and, in some cases, tree death. Dieback can be part of the decline syndrome[1].

Dieback refers to death of branches and can be associated with changes in soil moisture or with pathogens. It can also be the result of attack by insects such as shoot borers that bore into and kill the terminal shoots of trees[1].

What causes decline and dieback?

Several factors may cause decline and dieback symptoms; some factors act alone while others combine to cause tree decline.

In macadamia, there are many possible causes of decline and dieback, including poor nutrition and irrigation (extreme water regimes) and pests and diseases. In Hawaii, decline caused by pathogens is often grouped into **slow decline** resulting from root rot caused by *Kretzschmaria clavus* or *Ganoderma lucidum* and **quick decline** resulting from trunk decay caused by *Nectria rugulosa*, *Xylaria arbuscula*, *Phellinus gilvus*, *Phytophthora tropicalis*, or *Acremonium recifei*[2]. Decline caused by *Phytophthora cinnamomi* is characterised as Phytophthora tree decline and dieback.

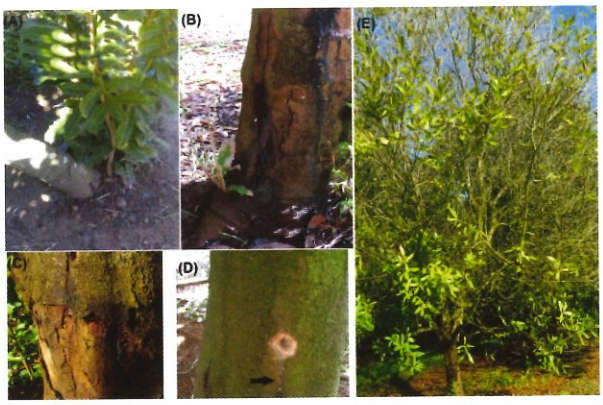
In Australia, symptoms of **Phytophthora tree decline and dieback** in macadamia include a gradual decline (see photo), usually with pale or yellow green leaves instead of dark green. As the disease persists, branches also die back and fruit set is usually poor. In good conditions, macadamia trees in *P. cinnamomi* infested soil are able to grow without any obvious aboveground symptoms [3,4], but under conditions that cause tree stress, leaves of infected trees often wilt and tend to abscise (or fall off) giving the diseased trees a sparse appearance. New leaf flushes and shoot growth usually don't occur or they are sparse. Significant decline of macadamia often follows extreme environmental conditions such as prolonged waterlogging associated with cyclonic weather or drought.

Decline symptoms suggest that the problem could be caused by restricted uptake or impeded transport of water and nutrients by the roots through the vascular system. Affected trees often have very poor root systems. This could develop from poor farming practices and/or be a consequence of nursery practice that results in the development of poor root systems.

Integrated management is the key

Treating decline and dieback by using individual corrective measures, such as increasing fertiliser application, mulching, tree trimming and increasing irrigation on free draining soils, will temporarily alleviate the problem. However, an integrated approach is required for sustainable management to achieve consistent high production.

An important element in disease management is soil health. While both beneficial and pathogenic organisms exist in the soil, an imbalance in the



Symptoms of Phytophthora diseases caused by Phytophthora cinnamomi *in macadamia. (A) water shoot from rootstock a sign of stress on roots, (B) stem canker, (C, D) gummosis from infected stem canker lesions (arrows indicate gummosis) and (E) tree dieback and decline.*

system may lead to pathogenic organisms taking hold of the growing plant. This is often the case with Phytophthora, where infections result in tree decline and/or stem canker in macadamias. Maintaining **soil health** by establishing an appropriate balance of physical, chemical and biological factors is necessary for good production and sustainable farming practices.

Healthy soils will:

- help suppress soilborne pathogens
- induce resistance in the host
- enhance microbial interactions (soil food web) to decompose plant litter, bind soil particles together and release nutrients.

The microbial mechanisms of soil suppression involve the activity of a range of **diverse microbial populations**, rather than one specific population of organisms. Incorporating organic amendments into soil may help soils to suppress soilborne pathogens and may enrich the soil biological processes that are important in natural and induced disease suppression. Previous research has shown the beneficial effects of composted macadamia husk on soil health as a result of increased fibrous root growth and improved soil microbial activity, moisture and pH [5]. In contrast, amendments of gypsum, lime or dolomite were relatively ineffective in reducing decline in macadamias [6].

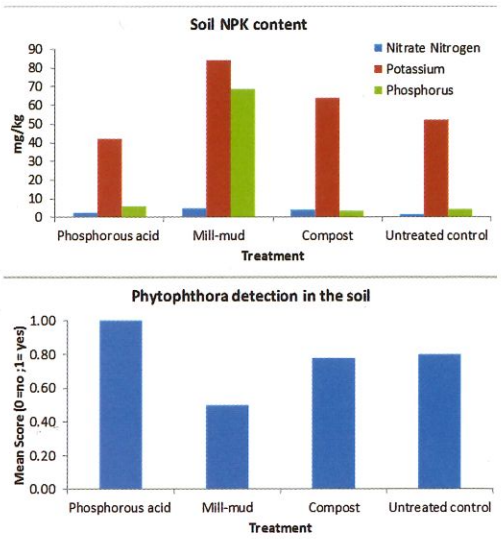


Figure. *Soil chemical composition (P and K test based on Morgan 1 method) and frequency of detection of* Phytophthora cinnamomi *in the soil at 6 months after soil amendment with mill mud and commercial compost on light soil (loam) at Bundaberg.*

Nursery practices that prevent infection and promote development of good root systems are essential for long-term disease management.

Field trials

Large scale field trials were established in commercial macadamia orchards in the Northern Rivers and Bundaberg regions in the 2013-14 production season to examine the influence of soil health on Phytophthora tree decline and stem canker.

These trials tested the effects on tree health of commercial compost, mill mud, and partially composted macadamia husk alone and with urea. These soil amendments were compared to trees treated with potassium phosphonate alone and in combination with the soil amendments and untreated control.

Preliminary data from the Bundaberg trial site (light loam soil) where commercial compost and millmud were used as soil amendments showed that in the first six months after application:

- Overall, the canopy of all the trees sprayed with phosphorous acid and those with soil amendments improved greatly (foliar colour, leaf flush and density) compared to the untreated control trees.
- Presence of Phytophthora from soil that was treated with mill mud was about half compared to the untreated control (see figure).
- Adding commercial compost significantly improved water infiltration from 13 minutes to about 3 minutes in the loam soil.

Phytophthora stem canker control via surgical intervention and painting with copper fungicide and white emulsion paint (A) mild infection scrapped off before painting, (B) too advanced canker for surgical intervention, (C) trunk with stem canker sprayed with copper oxychloride only and (D) tree trunks painted with mixture of copper fungicide and white emulsion paint.

- Amount of phosphorus (P) in mill mud treated soil was nearly 20 times higher than other treatments, whereas N and K levels were comparable with compost amended soil (see figure).
- Soil microbial activity measured as the rate of enzymatic breakdown by microbial enzymes involved in the release of inorganic nutrients from organic matter was significant better in soil amended with compost than soil amended with mill mud and the untreated control.

Implications

This research approach acknowledges the importance of integrated disease management in controlling Phytophthora tree decline and stem canker. It also points to the benefits for long term and sustainable disease control of improving soil health, as indicated by the substantial changes in soil biological and chemical properties and tree health in the treated trees compared to the untreated control.

Since trees with decline symptoms have very poor root systems, selecting root-stocks that are better able to exploit soil reserves or resistant to pathogens also may be important. Nursery practices that prevent infection and promote development of good root systems are essential for long-term disease management.

Future work

Additional data on soil biological, chemical, physical properties, yield and disease from the field trials is currently being collected. Research trials on other components of the integrated management strategy for Phytophthora in macadamia, including development of rapid assay for screening and evaluation of rootstock for Phytophthora resistance, are part of MC12007 project.

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Pest and disease control

Lace bug project to go ahead

The AMS is excited to announce that the University of NSW lace bug research project will now go ahead, thanks to the support of growers and processors who have pledged more than $37,000 to the project.

The AMS extends its sincere thanks to all of these people and businesses for supporting this new 3- to 5- year research project that has the potential to save the industry millions of dollars in lost crop.

The project will research the biology and species/genetic diversity of macadamia lace bugs. In the short term, it will help monitor lace bug levels and guide timing of spraying. For the long term, we are striving for a biological control.

Supporters: Alan and Jan Bingham, Allan Bate, Andrew Reynolds, Andrew Starkey, Ben Neilson, Bill Moore, Bob Evans, Bob Howard, Bob Maier, Brian Pidcock, Chris Warn, Daniel and Belinda Blanco, David Anderson, David Berman, David Bowler, DK & A Jones, Enrique Paredes, Fern Hinchcliffe, G and P Donaldson, Gavin Arthur, Geoff Chivers, Geoff Johns, Gerald Mattinson, Graeme Bilbe, Graeme Fleming, Greg James, Greg Woods, Guido Conte, Henri Bader and Son, Ian and Leoni McRae, Ian Hotson, Ian Perkins , Jacqulene Nash, Jenny McKavanagh, John Avakian, John Boardman, John Bridge, John Shirm, John Stock, Joof Alberts, Lance Emery, Len Green, Leoni Martin Smith, Manuir Singh, Marco Bobbert, Martin Brook, Martin Cresswell, Matthew Knappick, Maxene Hosie, Mike Harty, Miriam G Smith, Pam Woods, Paul Mendels, Dorey family, Peter and Sandra Templeman, Peter Baldwin, Peter Curnow, Peter Fraser, Peter Plunkett Cole, Peter Ranking, Peter Stransky, Peter Zeck, Phil Primrose, Rex Harris, Richard Doggett, Richard Rees, Ross Frederikson, Ross Sillar, Steve Ferndale, Steve Hoole, Sue and Michael Wiley, Tim Ellis and Tony Walker and processing companies Agrimac, Macadamias Direct, MPC and MWT.

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MACADAMIA FUND

Integrated Management of Diseases in Macadamia Industry

Appendix 3: Communications in the mainstream industry journals and News Bulletin

(d) New and emerging diseases

Dr Olufemi A. Akinsanmi
The University of Queensland

Project number: MC12007

Diseases affecting macadamia flowers: a significant threat to production

Dr Femi Akinsanmi, Centre for Plant Science, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Brisbane

Horticulture Innovation Australia

The term 'blossom blight', commonly called Botrytis or Botrytis blight, is incorrectly used to describe all diseases and disorders of macadamia racemes. In addition to *Botrytis cinerea*, other fungi and Phytophthora have been associated with 'blossom blight'. This confusion has often led to incorrect management decisions, which may contribute to increased production costs, significant crop losses and reduced profit margins.

The first reported outbreak of a disease of macadamia raceme, in Hawaii in the early 1960s, was caused by *Botrytis cinerea*. The disease was originally named as raceme blight, but subsequent reports of the disease used blossom blight or Botrytis blight as the preferred names. Major epidemics of Botrytis blight have been reported in Australia and other macadamia producing countries, where total failure of nut set was reported in certain cultivars and seasons. A critical distinguishing feature of Botrytis blight in macadamia is that the fungus affects only the mature and senescing (dying) flower parts, with characteristic grey mycelial strands on the raceme holding the flowers together.

In the late 1960s and early 1970s, raceme blight caused by *Phytophthora* spp. was reported in Hawaii and Costa Rica. Phytophthora was reported to affect the whole raceme, new leaf flush, young shoot and immature nut, causing extensive dark necrosis of irregular shape on affected plant parts. In 2005, 'raceme blight' caused by *Cladosporium cladosporioides* was reported in South Africa. The blight is characterised by initial, small, water-soaked specks on the flower that later become necrotic and the diseased racemes are covered with olive grey patches of mycelial stands.

In 2009, 'raceme blight' caused by *Pestalotiopsis* species, also called 'dry flower' was observed in Bundaberg. In the last three years, the disease has been reported in macadamia orchards in other regions in the south-east Queensland and some orchards in northern New South Wales. In the 2012-2013 production season, it was estimated that the disease contributed to between 10 and 30% loss in macadamia production on affected orchards in Australia.

Distinguishing characteristics, such as timing and stages of flower infection, and disease expression and recognised host variations that are critical for effective disease control are highlighted in the table.

Conditions influencing raceme infection

The incidence of various raceme blights varies between the seasons, depending on flower and immature fruit susceptibility and climatic conditions. Being able to predict and assess the risk of the disease using a decision support system requires a good understanding of disease development and associated factors. The source and amount of inoculum to cause disease at the different growth stages needed to estimate inoculum potential in the orchards during the season should be established for each disease. This information is needed to determine when it is best to implement a control strategy, e.g. the onset of Phytophthora blight was preceded by prolonged periods of rain and diseased and dead racemes attached to the branches in the tree canopy serving as the inoculum source between seasons.

Large numbers of mature flowers and temperatures between 18°C and 22°C are the most important factors contributing to Botrytis development when conditions are wet. Absence of Botrytis blight during disease-conducive periods has been attributed to heavy rain stripping the dead flower parts from the racemes.

Disease name	Causal pathogen	Distinguishing characteristics	Plant part affected	Location
Botrytis blight	*Botrytis cinerea*	Develops only on senescent flower parts, with no evidence of infection of immature buds; infected tissues become brown to dark brown with grey fungal mycelial on senescent parts	Infects mature flowers	Australia; South Africa; Kenya
Phytophthora blight	*Phytophthora capsici; P. palmivora*	Extensive blighting and dark necrosis of irregular shape on affected plant parts	Affects whole raceme, new leaf flush, young shoot and immature nut	Hawaii, Costa Rica
Cladosporium blight	*Cladosporium cladosporioides*	Diseased racemes are covered with olive grey patches of mycelia and abundant spores	Infects flower buds, bud stalks	South Africa
Pestalotiopsis blight	*Pestalotiopsis* species (several)	Symptoms appear from early bloom, immature bud and infect racemes at all developmental stages; diseased flowers dislodge readily from the rachis	Blighting of infected flower and sometimes rachis	Australia

Table 1: Disease name and characteristics of raceme blight caused by different pathogens in macadamia.

conducive periods has been attributed to heavy rain stripping the dead flower parts from the racemes.

Sensitivity and responses of flowering during mild water stress during anthesis, irrespective of the period, the severity or duration of water stress, may vary among cultivars. However, periods of stress at any of the reproductive stages are likely to reduce both yield and quality of macadamia nuts. Some cultivars have broad flowering peaks and are able to adjust to short period of stress without any significant yield losses. In one instance, A4 trees at full flowering were severely affected by Botrytis after five days of continuous rain, but nearby A16 and 344 trees, obviously with different flowering peaks, were not severely affected. It also appears that Botrytis damages flowers in orchards and trees with reduced air flow.

Although mild water stress at floral initiation stage was reported not to harm yield, when stress coincides with flowering peaks, it results in low water potential of racemes, leading to splitting and death of perianth parts and failure of nut set.

Macadamias racemes affected by dry flower blight at stage 1 (left) and stage 5 (right).

Disease management

pplying fungicides is still the primary management tool hen the disease starts to develop. However, because the sporadic nature of the diseases and lack of dequate knowledge of each disease situation, attempts control raceme blight with fungicides during severe utbreaks have mostly not been successful. Applying fferent biological sprays for Botrytis blight has also ot produced significant benefit. Despite these results, ultural control methods will be important to manage e fungi. Growers are encouraged to monitor incidence fungal infection on the raceme from early bloom age to nut set.

uture research should include emphasis on integrating knowledge of how the disease develops and climatic nditions for each disease situation to provide a basis r formulating a practical integrated disease-forecasting stem.

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Acknowledgment

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FUNGI IMPLICATED IN DRY FLOWER DISEASE

Dr Femi Akinsanmi, Centre for Plant Science, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Brisbane

Research has shown that the incidence of a type of flower blight called 'dry flower' is increasing, affecting macadamia orchards in both New South Wales and Queensland, and that several cultivars are susceptible.

Dry flower can affect flowers at all stages. These photos show symptoms at peak flower just before nut set (left) and pre-flowering stage.

Symptoms

Dry flower symptoms, which appear on infected flowers, are characterised by the dry appearance of the raceme and dieback of the raceme stalk (rachis). The flowers can be infected at all stages of development - before nut set, and from pre-flowering to peak flowering stages. Flower buds or florets that are infected early have a blighted brown to dark brown appearance on the green raceme stalk.

Diseased flower parts may remain attached to the rachis but can easily be dislodged when shaken. Diseased racemes may remain in the tree canopy between seasons, and therefore may serve as source of inoculum in the following season. In some cases, advancing dieback at the distal end of the raceme often stops before colonising and destroying the entire raceme.

Causes of dry flower

A recent study described two new species, *Pestalotiopsis macadamiae* and *Neopestalotiopsis macadamiae,* as the causal agents of dry flower. These species were recovered from the raceme stalk and diseased flowers from commercial macadamia orchards.

Various species of *Pestalotiopsis* and *Neopestalotiopsis* are members of relatively important plant pathogenic genera that infect wide host ranges and have been reported to cause a range of significant fungal diseases in tree nut and fruit crops. In India, leaf spots on macadamia were associated with *Pestalotiopsis versicolor.*

Species in the genus *Pestalotiopsis* and *Neopestalotiopsis* are known to produce many secondary metabolites, which may be responsible for the dry appearance of diseased racemes.

Preliminary observations suggest that dry flower is influenced by climatic conditions and disease severity on affected cultivars and varies between the seasons. R&D on the biology and conditions required for pathogen infection and disease spread is currently underway.

Monitoring is important

Because dry flower has the potential to cause significant crop loss, growers are encouraged to monitor its incidence on the raceme from early bloom stage to nut set. R&D to determine a long-term management strategy is underway.

Information
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Hort Innovation Strategic levy investment | MACADAMIA FUND

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Integrated Management of Diseases in Macadamia Industry

Appendix 3: Communications in the mainstream industry journals and News Bulletin

(d) New and emerging diseases

Dr Olufemi A. Akinsanmi
The University of Queensland

Project number: MC12007

Branch dieback: a growing threat

Dr Femi Akinsanmi, Centre for Plant Science, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Brisbane; and Dr Chris Searle, Suncoast Gold Macadamias, Gympie, Queensland

While in the past macadamia branch dieback has usually only been important in the first eight to ten years of tree growth, in recent years the disease has become more common and more severe in mature (>15 years old) trees in Australia. It is now becoming a significant threat to production.

Macadamia branch dieback is caused by *Dothiorella ribis,* which belongs to the fungal family *Botryosphaeriaceae.* Symptoms have been observed on macadamia trees in the major growing regions in Queensland and to a limited extent in the Northern Rivers region. A major epidemic from 2009 to 2010 in the Atherton Tablelands contributed to the demise of a large macadamia orchard at Tolga. Similar scenarios have been reported in macadamia in California and Southern Africa.

Generally, *Botryosphaeriaceae* species are commonly associated with cankers, blight, rot and dieback of woody hosts, including perennial horticultural tree crops such as apples and plum, mango, pistachio, grapevine and blueberries. In the last few decades, fungi in the family *Botryosphaeriaceae* have emerged as serious and devastating pathogens worldwide causing large-scale crop failures and destruction.

What are the symptoms of dieback?

The photographs show the symptoms and extent of infection in some orchards. Usually, a point of gummosis (bleeding) occurs on the affected branch or main trunk and the leaves above this point turn brown, with purplish blotches. The plant parts become 'blighted' and start to die back. In the initial stages the leaves are retained, even though the branch is dead, and take on a light brown colour. Cross-sections through the affected branch showed dark discoloration of the wood. It is uncertain what causes the injury (gummosis point) but this may serve as the point of entry for the pathogen.

In some cases, before obvious dieback symptoms become apparent, the trees might appear to be suffering from a nutritional disorder as the leaf veins turn reddish and there may be no gummosis. In other cases, particularly trees younger than four years, the leaves may take on a dull khaki green colour with the whole tree dying within three to four weeks.

The condition is more common in trees on the ends of rows, in areas where they are growing in poor, shallow soil or where temporary seasonal waterlogging may occur. This observation corresponds to those for diseases associated with *Botryosphaeriaceae,* where

Cross-sections of diseased macadamia branch. Note the dark discoloured wood.

Macadamia trees showing branch dieback symptoms at Mackay. Photo: Graham Wessling

symptoms appear following general stress on the host that increases their ability to cause disease. While this is generally the case, the disease also has been observed in orchards and in 'good' soils where there are no obvious physical or nutritional barriers to growth. Symptoms become more evident from mid-summer to early autumn after prolonged warm humid or unusually hot weather.

Cultivar susceptibility

Field observations from several outbreaks between 2009 and 2014 revealed that a wide range of cultivars are susceptible. The most susceptible, in decreasing order of severity, appear to be A203, A268, HAES 741, HAES 842, Daddow and A16, though this needs to be confirmed by more extensive surveying. It has been observed recently in H2 trees being grown for seed nut.

Trials

Initial pathogenicity of the species isolated from the 2010 epidemics was verified by wound inoculation of seedlings under glasshouse conditions. When the inoculated plants were subjected to water stress (less optimal watering conditions) for three weeks, about 60% of the inoculated test plants withered and became irreversibly desiccated; 30% recovered after an optimal watering regime resumed but declined a few weeks later, reproducing the symptoms observed in the field. Results showed that isolates of *Botryosphaeria* species were more aggressive than isolates of *Lasiodiplodia theobromae,* which was also isolated from the 2010 epidemic.

Botryosphaeriaceae fungi have been isolated from symptomatic plant parts from recent disease outbreaks at Gympie and Bundaberg (2014 and 2015). Preliminary results from samples obtained from Bundaberg in April 2015, based on molecular identification the fungal isolates, showed that *Lasiodiplodia theobromae* was the most common organism recovered from diseased branches. Since the three fungi, *Dothriella*, *Lasiodiplodia* and *Botryosphearia* associated with branch dieback belong to the same family, more research using samples from different regions is needed to confirm if all the three fungi or specific fungus occur under similar or different conditions.

Preliminary control options

For trees showing symptoms of dieback, the following control options are recommended:

- As an interim control measure, cut off the infected branches to a point below where you can see the last discoloured wood in the branch, i.e. cut back into healthy wood. Ensure that you clean the pruning equipment between diseased and healthy trees.
- Paint the exposed cut surface with white paint.

What's next?

These preliminary findings suggest that the emergence of *Botryosphaeriaceae* as a significant pathogen in macadamia may be attributed to the changing climate and adverse conditions occurring more often.

More research is needed to clarify the disease cycle so effective disease management strategies can be developed.

The role of beetles (insects) as a possible causal agent of the injury to the branches and trunk and a potential point of pathogen entry should be explored. Timing of prevalence and identity of the beetle should be determined in association with disease incidence as part of study on the disease cycle.

Acknowledgment
The research into dieback is being conducted as part of HIA funded project MC12007, *Disease management in the macadamia industry.*

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Integrated Management of Diseases in Macadamia Industry

Appendix 4: (a) Posters as brief communication tool of project activities for husk spot

Dr Olufemi A. Akinsanmi
The University of Queensland

Project number: MC12007

Growers' self assessment decision support system for spray application for husk spot in macadamia

Femi Akinsanmi & André Drenth

The University of Queensland, Queensland Alliance for Agriculture and Food Innovation, Centre of Plant Science, Ecosciences Precinct, GPO Box 267, QLD, 4001, Brisbane, Australia. Email: uqoakins@uq.edu.au

Introduction

Pseudocercospora macadamiae is an important pathogen of macadamia in Australia, causing a disease known as husk spot (Fig. 1).

Growers strive to control the disease with a number of fungicide spray applications which are applied prophylactically. Spray applications in non-disease conducive production seasons increase the magnitude of the cost-benefit ratios.

Therefore, the development of an efficient, accurate, and rapid decision support system which facilitates the quantification of disease severity and risk of infection is needed.

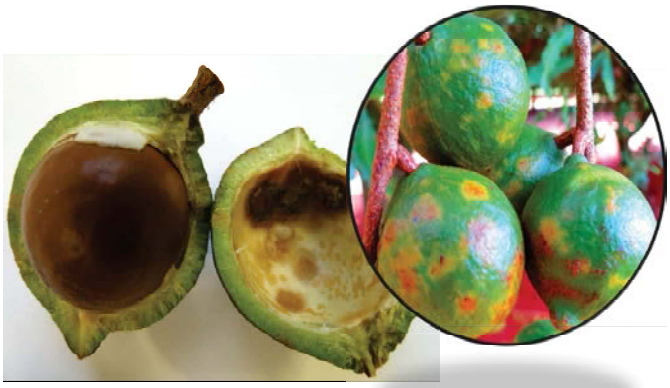

Fig. 1: Husk spot of macadamia. Lesions on fruit pericarp (right), inside of pericarp (middle) and healthy shell (left).

Framework of husk spot development

At present, growers assess their risk of infection and subsequent spray applications, based on history of disease, tree age and cultivar in their orchards.

Framework of a model (Fig. 2) based on the infection process and conditions that influence infection will be used to determine:

- risk of infection,
- disease development &
- risk of crop loss.

This provides the basis for further development of a disease forecasting system for husk spot control.

Factors influencing husk spot development & crop loss

- Weather conditions[1]
- Abundance of inoculum[2]
- Host phenology & susceptibility of cultivar[3]
- Timing of spray applications[4]
- Number and frequency of spray applications

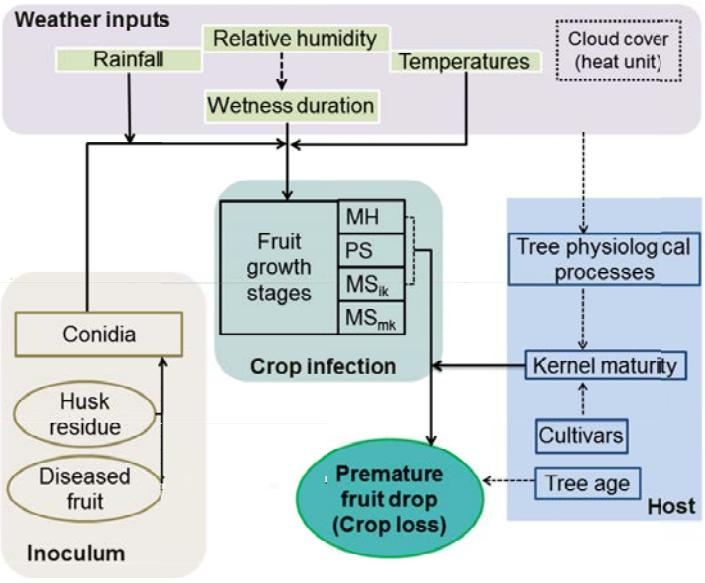

Fig. 2 : Framework of a model to predict husk spot epidemics and crop loss in macadamia. Fruit growth stages: MH - Match-head size; PS - Pea size; MS_{ik} - Mature fruit size immature kernel; MS_{mk} - Mature fruit size mature kernel.

Concluding remarks

This predictive system will provide an additional decision support system to macadamia growers in Australia to control husk spot.

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Integrated Management of Diseases in Macadamia Industry

Appendix 4: (b) Posters as brief communication tool of project activities for *Phytophthora*

Dr Olufemi A. Akinsanmi
The University of Queensland

Project number: MC12007

Positive impact of cultural practices on control of *Phytophthora* diseases and robust biological indicators of soil health in macadamia

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The University of Queensland, Queensland Alliance for Agriculture and Food Innovation, Brisbane.

Introduction

A major challenge for increasing crop productivity is soil health. Soil biology is a key component that influences productivity, soil health and quality. Soilborne pathogens impact soil ecosystem services and modify functions and processes of other soil microbiota. Several cultural practices such as tillage systems, addition of organic composts and the use of cover crops are being promoted as management options for enhancing soil health. These practices directly and indirectly affect soilborne pathogens.

In perennial tree cropping systems, the dynamics and multitrophic interactions that occur in soil microbiota is unabated. Consequently, diseases caused by soilborne pathogens are the most damaging in tropical and subtropical tree crops. Robust measurable indicators of soil health are important and will help assure soil management consultants of the benefits of soil organic amendment and its role in developing suppressive soils against soilborne pathogens and to maintaining good quality soil health.

Case studies of large-scale field trials under commercial farming practices in macadamia plantations are presented. The results illustrate the impact of soil organic amendments on diseases caused by a soilborne pathogen, *Phytophthora cinnamomi* in macadamia (Fig. 1). The results provide strong evidence to support reducing the reliance on pesticides to control diseases in tree crops (Fig. 2). Putative indicators for assessing the effect of soil organic amendment on yield, soil quality and health are presented (Fig. 3).

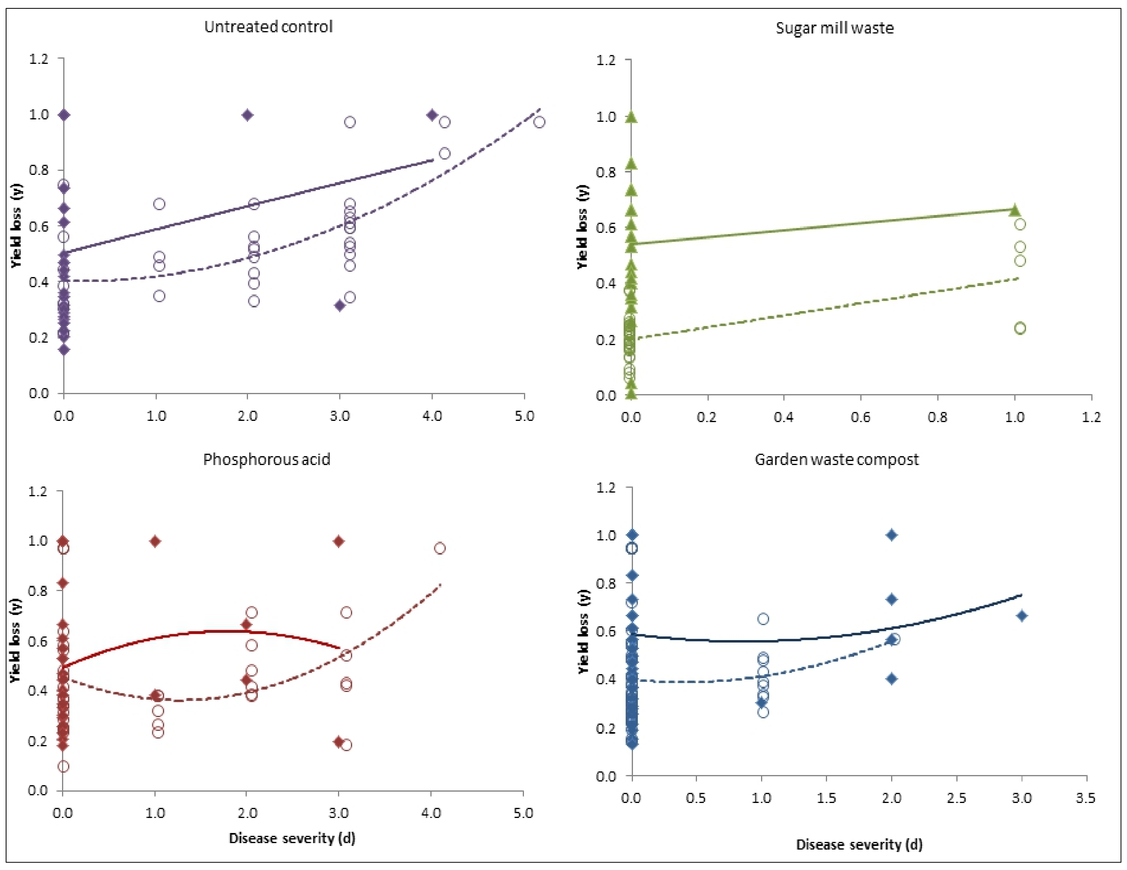

Fig. 1. Relationships between yield loss (y) and disease severity due to *Phytophthora cinnamomi* in macadamia at 12 and 24 months after application of control measures or untreated control in commercial macadamia orchard. Solid and broken lines represent best fit regression models while closed diamond and open circle symbols represent disease severity of each data tree at 12 and 24 months, respectively. $y = (Y_o - Y) / Y_0$, where Y_0 is max yield per season at orchard level and Y is actual yield; d was measured as described by Akinsanmi *et al*.[1]

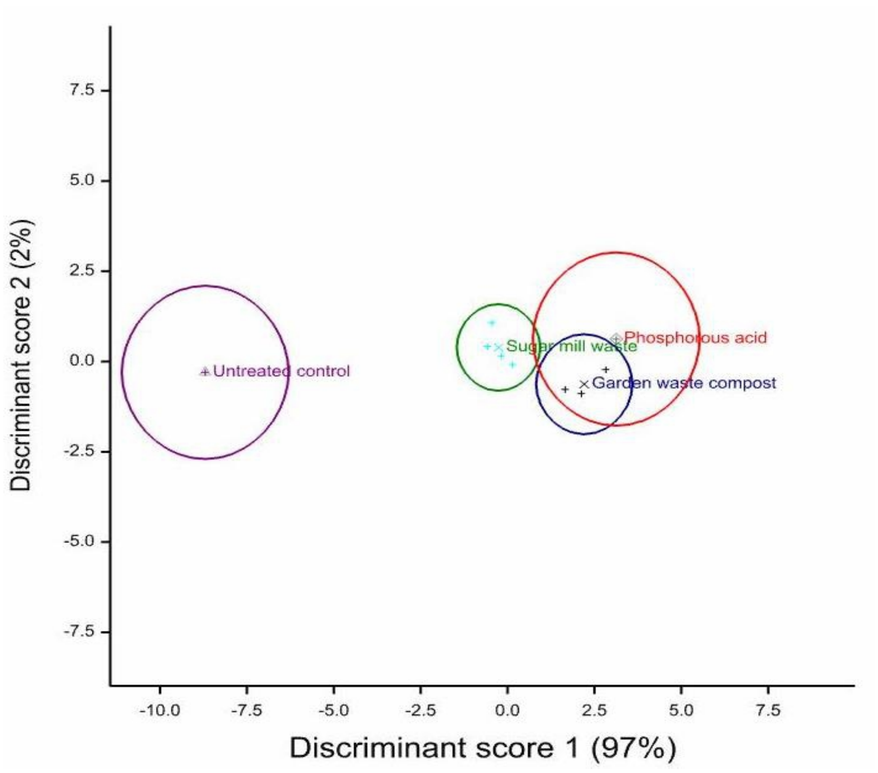

Fig. 2. Multivariate analysis of chemical properties, biological activities and yield in macadamia showing the effects of soil organic amendments in comparison to untreated control and chemical applications on trees affected by *Phytophthora cinnamomi*

Experimental design

- Large-scale RCBD field trials in 4 replicates at commercial macadamia orchards.
- Treatments: soil organic amendments with sugar mill waste (mill-mud) or garden waste compost, trunk spray application of Phosphorous acid and untreated control were applied once.
- Data: annual assessment of disease severity[1]; soil and plant chemical properties, soil biological activities and metagenomics of ITS and 16S rRNA gene loci for soil microbiota diversity and composition; yield; and canopy appearance (density and volume).

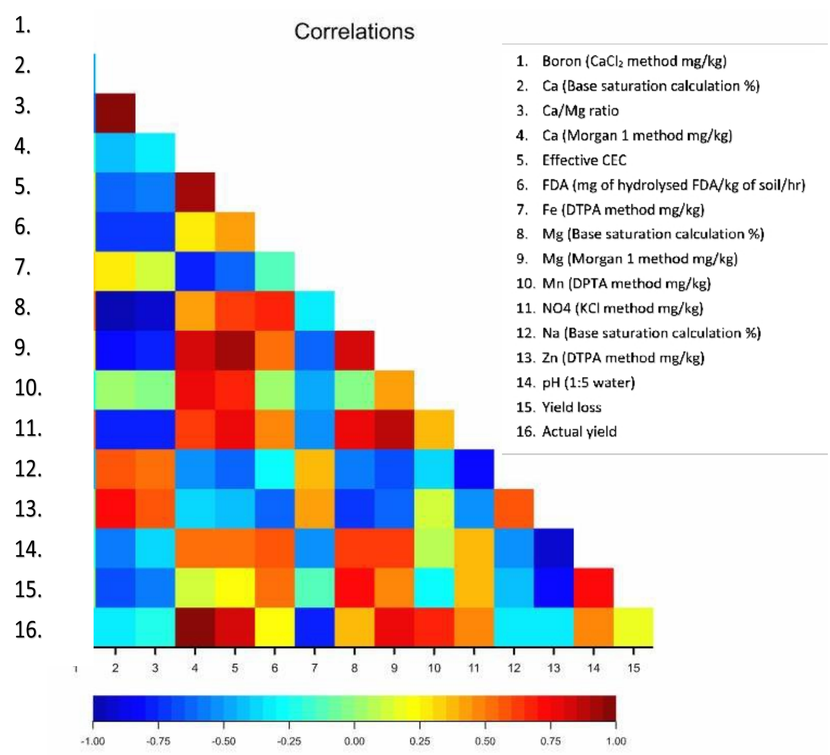

Fig. 3. Correlations of significant (*P*<0.05) chemical and biological parameters that indicate the effect of soil organic amendments on crop yield, soil quality and health.

Remarks

- In most of the trees the disease severity score[1], based on appearance of above-ground foliage, was <1.0 (Fig. 1), but analysis of the root and soil samples confirmed infection and the presence of *P. cinnamomi.* This support previous reports that macadamia infected with *P. cinnamomi* are able to grow without major above-ground symptoms.[1,2]
- Significant increase in the number of trees with high disease severity occurred in the untreated control and chemical application at 24 months after the treatments. Consequently, yield loss was significantly (*P*< 0.05) reduced in the soil organic amendment plots than the untreated control and chemical application plots between 12 and 24 months after application (Fig. 1).
- Application of control measures with either chemical or soil organic amendment improved diseased macadamia trees compared to the untreated control (Fig. 2). However, the soil organic amendment treatments significantly reduced variations among the trees compared to the chemical control or the untreated control trees (Fig. 2).
- Multivariate analysis of the soil chemical and biological properties revealed that a range of nutrients and a measure of biological activity - fluorescein diacetate hydrolysis (FDA) influenced yield and were significantly different among the treatments. Relationships among these parameters and to yield assessments are indicated in Fig. 3.
- The average canopy density significantly increased in the soil organic amendment trees compared to the chemical control and the untreated control trees.
- Preliminary results of the metagenomics analyses revealed depleted soil microbiota diversity and composition in the untreated control over the 24 months period.
- Overall, addition of soil organic materials offers great benefits to the tree and soil health. Combination of some chemical and biological activity properties may serve as more robust indicators of soil quality and health.

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Integrated Management of Diseases in Macadamia Industry

Appendix 4: (d) Posters as brief communication tool of project activities for branch dieback

Dr Olufemi A. Akinsanmi
The University of Queensland

Project number: MC12007

Botryosphaeriaceae associated with macadamia branch dieback in Australia

'Femi Akinsanmi[1], Chris Searle[2] & Andre Drenth[1]

[1]University of Queensland, Brisbane, Australia. [2]Suncoast Gold Macadamias, Gympie, Australia.

Background

Macadamia branch dieback is expressed as conspicuous brown foliage amongst the otherwise healthy foliage (Fig. 1). The vascular tissue under the bark of the diseased branch is often discoloured extending upward from the point of bleeding on the affected branch (Fig. 2).

Previously, branch dieback attributed to *Dothiorella ribis,* was regarded as a minor disease of macadamia, which has been reported to only occur on trees in the first 10 years of tree growth in the orchard. However, in recent years branch dieback has been observed in mature trees (>15 years old) and has resulted in numerous tree deaths. Surveys of macadamia orchards between 2009 and 2014 have revealed wide occurrence of branch dieback in Australia. Preliminary observations of the recent epidemics have showed that the causal agent belongs to the family *Botryosphaeriacae.*

This study was established to characterise the causal agent(s) of branch dieback in macadamia in Australia.

Fig. 1. Macadamia trees with symptoms of branch dieback.

Fig. 2. Cross-sections of wood of diseased plant parts branch and stem of macadamia.

Fig. 3. Disease control includes pruning of diseased branches.

Fungal isolation & identification methods

- Survey of prevalence and severity of branch dieback in macadamia orchards in the major growing regions.
- Fungal isolates were obtained from field samples and grown on potato dextrose agar medium.
- Identification of the isolates was based on phylogenetic analysis of the partial gene sequences of Internal transcribed spacer (ITS) and translation elongation 1-alpha (TEF).

Identity of fungal isolates

- Trees of cultivars A203, A268, HAES741, HAES842, Daddow and A16 were the most affected.
- NCBI BLAST searches & phylogenetic analysis of ITS & TEF sequences revealed similarity of some of the fungal isolates to *Botryosphaeriaceae*
- *Lasiodiplodia theobromae, L. pseudotheobromae*, *L. jatrophicola* and *Botryosphaeria rhodina* were among the species isolated from diseased materials.

Concluding remarks

- It appears that pruning of diseased branches to a point below discoloured wood in the branch may not prevent further disease development (Fig. 3).
- In most cases different species of *Botryosphaeriaceae* were isolated from different samples. However, it is not known if all the species that are associated with the disease are equally aggressive or are present in all macadamia growing regions.
- Although previous trials have proven Koch's postulates with *L. theobromae,* further trials to confirm if the other species of *Botryosphaeriaceae* can cause the disease & the factors that influence disease development are needed.
- Further research is in progress to elucidate the disease cycle that will underpin the development of effective disease management strategies.

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Pathogenicity and identification of *Botryosphaeriacae* spp. causing gummosis and dieback in macadamia

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Introduction

Branch canker and dieback occur sporadically in macadamia trees. The disease is thought to be stress related and only important in young trees, but the severity and distribution of the disease is increasing in mature macadamia orchards in Australia.

Symptomatic trees showed dieback, dried or dead branches with dried leaves still attached (Fig. 1), extending upward from the point of gummosis at the base of the branch and/or main trunk of affected trees (Figs. 2 & 3).

Fig. 1. Macadamia trees with dieback symptoms and fungi isolated from diseased tissues. Healthy tissues (asymptomatic) were free of pathogens.

Pathogenicity test

Wound inoculation → *Lasiodiplodia/Diplodia*

@ 3 months post inoculation

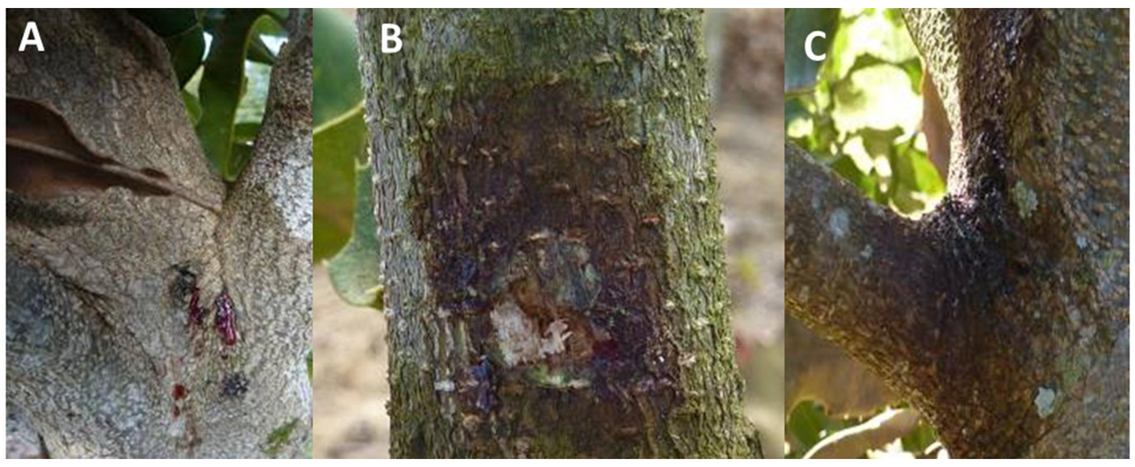

Fig. 2. (A) Point of gummosis on macadamia trees above which branch and leaves turned brown or dead; (B) brown discoloration beneath the point of gummosis on the main trunk and (C) point of gummosis at tree branch region.

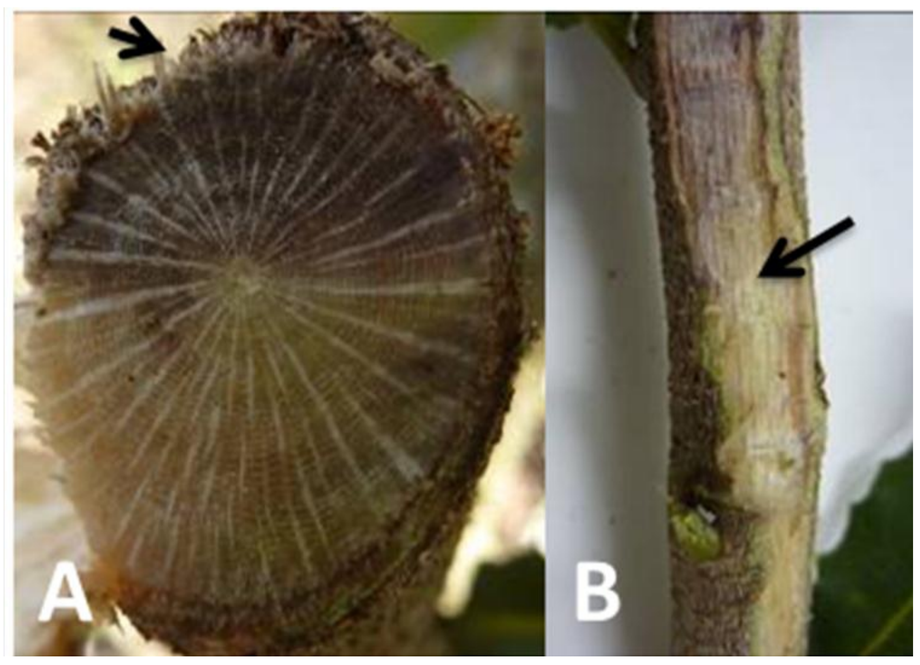

Fig. 3. (A) Cross-section of affected stem above point of gummosis showing discoloration of the vascular bundles; (B) longitudinal section showing discoloration above the point of gummosis. Arrows indicate point of gummosis

Concluding remarks

The incitant of the injury (point of gummosis) is not known, the nature and distribution of diseased trees suggest possible association with beetle attack.

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Hort Innovation
Strategic levy investment

MACADAMIA FUND

Integrated Management of Diseases in Macadamia Industry

Appendix 4: (c) Posters as brief communication tool of project activities for flower blight

Dr Olufemi A. Akinsanmi
The University of Queensland

Project number: MC12007

Multiple *Pestalotiopsis* and *Neopestalotiopsis* species cause flower blight of macadamia in Australia

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Background

Macadamia is produced for its edible cream-coloured nut in several countries in tropical and subtropical frost-free regions worldwide. A mature macadamia tree may produce over 10,000 racemes at peak anthesis. Between 100 and 300 flowers are borne on each long pendant raceme (Fig. 1a & b). There are four developmental stages of macadamia raceme, starting from small green florets (or buds) stage to the fertilized embryos stage.

Flower blights that affect macadamia such as Botrytis blight, Phytophthora blight and Cladosporium blight occur at a specific developmental stage. Recently in Australia, 'dry flower', a new disease affecting flowers and the rachis (Fig 1c & d), was observed at all the four developmental stages of macadamia racemes. Yield losses attributed to dry flower range between 10% and 30%. This study was established to determine the causal agent of dry flower and its prevalence in Australia.

Fig. 1. Macadamia racemes (a) pendant shape racemes in the tree canopy, (b) flower at anthesis, (c) racemes affected by dry flower and (d) rachis tip die-back caused by the same pathogens as dry flower.

Methods

Prevalence study

- A survey of dry flower prevalence in macadamia orchards was performed.
- Sections of the raceme affected was determined from over 200 samples.
- Fungal isolates between locations, among plant parts & sections of raceme were compared.

Identity of isolates

- Identity of isolates was based on morphological & molecular characteristics.
- Phylogenetic analysis of the combined sequences of internal transcribed spacer region (*ITS*), translation elongation factor 1-α (*TEF*) & β-tubulin (TUB) was used to identify 32 closely related *Pestalotiopsis*-like isolates.

Pathogenicity trials

- Representative isolates were tested for their pathogenicity on racemes of three macadamia cultivars (695, A203 & 741) under field conditions.

Results & Discussion

- Dry flower was observed in all the major macadamia production regions in Australia.
- *Pestalotiopsis*-like isolates constituted 41% of the fungi recovered from diseased plant parts.
- Few morphological and cultural differences were observed among the *Pestalotiopsis*-like isolates.

- Maximum parsimony phylogram of concatenated partial sequence (ITS+TEF+TUB) revealed two novel taxa in the *Neopestalotiopsis* and *Pestalotiopsis* genera (Fig. 2).
- The isolates were grouped regardless of location or plant part in the phylogram.

- Koch's postulates were confirmed.
- *Pestalotiopsis* & *Neopestalotiopsis* isolates are the causal agents of dry flower in macadamia.

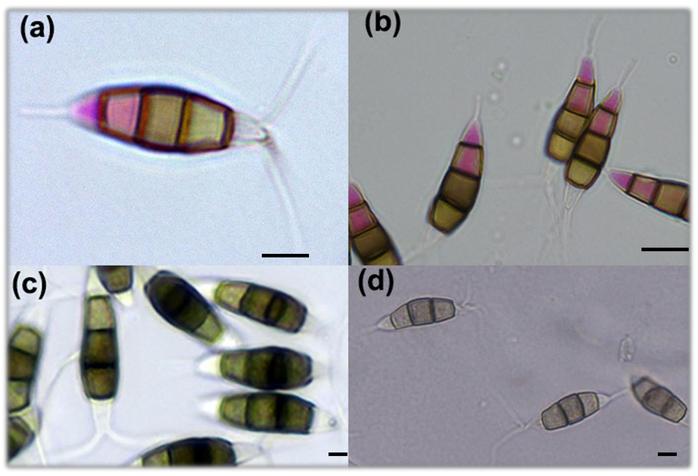

Fig. 2. Spores of *Pestalotiopsis* sp. (a, c) and *Neopestalotiopsis* sp. (b, d) from dry flower disease in macadamia. a & b stained with acid fuchin dye showing the hyaline basal cells. Scale bars = 10 µm.

Concluding remarks

Pestalotiopsis & *Neopestalotiopsis* species are the causal agents of dry flower in macadamia. Several *Pestalotiopsis* species have been reported to produce secondary metabolites. This could be associated with the 'dry' appearance of the diseased racemes in macadamia.

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