Biology and Management of nut rot of chestnut

David Guest
The University of Sydney

Project Number: CH07007

CH07007

This report is published by Horticulture Australia Ltd to pass on information concerning horticultural research and development undertaken for the chestnut industry.

The research contained in this report was funded by Horticulture Australia Ltd with the financial support of the chestnut industry.

All expressions of opinion are not to be regarded as expressing the opinion of Horticulture Australia Ltd or any authority of the Australian Government.

The Company and the Australian Government accept no responsibility for any of the opinions or the accuracy of the information contained in this report and readers should rely upon their own enquiries in making decisions concerning their own interests.

ISBN 0 7341 2854 1

Published and distributed by:
Horticulture Australia Ltd
Level 7
179 Elizabeth Street
Sydney NSW 2000
Telephone: (02) 8295 2300
Fax: (02) 8295 2399

Horticulture Australia

HAL Project Number CH07007 (completed December 2011)
The Biology and Management of Chestnut Rot in South-Eastern Australia

Author: Lucas Alexander Shuttleworth
Contact: lucas.shuttleworth@sydney.edu.au; lshu3875@hotmail.com

Supervisor: Prof. David I Guest
Co-Supervisor: Dr. Edward CY Liew

Research provider: The University of Sydney

HAL Project Number CH07007
The Biology and Management of Chestnut Rot in South-Eastern Australia

Project leader: Prof. David I Guest
Contact details: david.guest@sydney.edu.au
Other key personnel: Dr. Edward CY Liew
Contact details: edward.liew@rbgsyd.nsw.gov.au

Statement of purpose of report: This project investigated the biology and management of Chestnut Rot in South-Eastern Australia, including disease surveys of New South Wales (NSW) and Victoria (VIC) in 2008 and 2009; identification of the organism associated with rotten chestnuts; the infection process and disease cycle; the effectiveness of flotation grading; and recommendations on how growers can manage the problem in their orchards.

Acknowledgements:
This project was funded by Horticulture Australia Ltd. (HAL) using the chestnut industry levy and matched funds from the Australian Government. Thank-you to the University of Sydney, and the Faculty of Agriculture, Food and Natural Resources for providing the University of Sydney Postgraduate Award (co-funded). Thanks to Chestnuts Australia Inc. and the Australian chestnut growers involved with the project particularly David and Margaret Ogilvy, David and Helen McIntyre, Heather and John Kane, Brian and Jane Casey, Sally Robbins and Joe Rinaudo. Thanks to Tanya Edwards, Communications Officer, Chestnuts Australia Inc. for her interest and enthusiasm. Thank-you to the Royal Botanic Garden, Sydney for the use of equipment and facilities. Thanks to Matthew Laurence, Carolyn Blomley, Ameera Yousiph, Suneetha Medis, Rosalie Daniel, Sarah Dunstan, Zoe-Joy Newby, Julie Bates for their help in the laboratory. Thanks to Catherine Wardrop, Royal Botanic Garden, Sydney for completing the scientific illustration and Karen Rinkel, Royal Botanic Garden, Sydney for help with illustrating the phylogenetic trees.

Date of report: December 2011

Table of Contents

Media Summary

Chestnut Rot is a significant problem facing the Australian Chestnut industry. Symptoms manifest as brown lesions on the kernel of the chestnut. The disease is often not visible externally, providing a challenge for growers and consumers alike.

The aims of the project were to survey New South Wales (NSW) and Victoria (VIC) for Chestnut Rot in 2008 and 2009; identify the organism associated with rotten chestnuts; clarify the infection process; determine the effectiveness of flotation grading as a post-harvest method; and provide recommendations to growers on how to manage the disease.

In 2008 Chestnut Rot incidence was up to 72%, and in 2009 up to 35%. The disease was present in all orchards in both years. All the sampled varieties were affected. There was a positive correlation between incidence and December rainfall of the previous year. Surveys of Flemington Markets, NSW showed incidence up to 10% in 2008, and 9% in 2009.

A fungus named *Gnomoniopsis smithogilvyi* sp. nov. was identified living on decaying chestnut burrs and branches, was isolated from diseased chestnut kernels, and was isolated as an endophyte from asymptomatic chestnut flowers, leaves and stems.

Airborne ascospores were captured in the laboratory on agar plates, and in a chestnut orchard, suggesting they are part of the infection process.

The effectiveness of post-harvest flotation grading of chestnuts was tested. Healthy chestnuts have previously been found to sink and rotten ones float. Five water temperature treatments were tested. Rotten chestnuts were found to sink in all temperature treatments, and healthy chestnuts floated in all except 70^{o}C, indicating the method should be used with caution.

Orchard sanitation is key to Chestnut Rot management. Burr removal or the placement of a layer of organic mulch over top of burrs are options. Growing a range of varieties is also recommended to spread out the flowering times and reduce the risk of floral infection.

Surveying South Australia, Western Australia and Tasmania as well as internationally needs to be completed to further determine the distribution of the disease. Burr removal and investigating mulches is an important area of future research. The effectiveness of biological control agents such as *Trichoderma* sprayed on infected burrs should also be investigated.

Technical Summary

Chestnut Rot is a significant problem facing the Australian chestnut industry. Symptoms manifest as brown lesions on the kernel of the chestnut. The disease is often not visible externally, providing a challenge for growers and consumers alike.

The aims of the project were to survey New South Wales (NSW) and Victoria (VIC) for Chestnut Rot in 2008 and 2009; identify the organism associated with rotten chestnuts; clarify the infection process; determine the effectiveness of flotation grading as a post-harvest method; and provide recommendations to growers on how to manage the disease.

Twenty-two orchards were surveyed for Chestnut Rot in 2008 and 21 in 2009, across VIC and NSW. Three-hundred chestnuts per orchard were sampled. Chestnuts were dissected and visually assessed. Incidence up to 72% was found in 2008, and 35% in 2009. All varieties were affected including Decoppi Marone, Purton's Pride and Red Spanish. There was a positive correlation (0.58) between rainfall in December 2007, and 2008 incidence. Surveys of Flemington Markets, NSW showed incidence up to 10% in 2008, and 9% in 2009.

The taxonomy and phylogeny of the Chestnut Rot fungus was determined. Thirteen isolates were analysed from various chestnut tissues including ascospores from decaying burrs (NSW), diseased kernels (VIC and NSW) and endophytes from female and male flowers, leaves and stems (NSW). Morphology (teleomorph and anamorph characters) and phylogenetic analysis of rpb2, ITS, tef1-α, β-tubulin genes were used to identify isolates. All isolates were identified as the novel fungus, *Gnomoniopsis smithogilvyi* sp. nov. Phylogenetic analysis of ITS sequences of the Australian isolates and isolates of *Gnomoniopsis* on *Castanea* sp. from India, Italy and New Zealand showed very high sequence similarity (100% bootstrap, 100% bayesian posterior probability) indicating species of *Gnomoniopsis* are present in these countries with *G. smithogilvyi* likely one of them.

The endophytic phase in the infection process was investigated. Five experiments were conducted in Mullion Creek, NSW to determine how the infection process occurs. These experiments were completed in December 2008, and February, April, August, and December 2009. *G. smithogilvyi* was isolated as an endophyte in all 5 months from various asymptomatic chestnut tissues including female flowers, male flowers, leaves (margins, mid-veins, petioles), current-year and 2 year-old stems, 3rd and 4th year bark, immature and mature burrs, dormant buds, chestnut shells and chestnut kernels. The tissue with the highest isolation frequency was female flowers in December 2008 (82%). In December 2009, isolation frequency in female flowers fell to 10%. This shows the presence of the endophyte is dynamic and changes over time. The drop in isolation frequency from female flowers corresponded to the drop in incidence the following year (incidence in 2009, 10.6%, in 2010, 6%). *G. smithogilvyi* was also isolated as a saprophyte from dead styles, dead male flowers, dead twigs.

Ascospore trapping was conducted in the laboratory to confirm the release of ascospores from perithecia on decaying burrs. Six potato dextrose agar (PDA) plates were set up in a closed chamber experiment with 4 infected burrs. Three colonies grew on the plates in the second week of incubation. One was identified as *G.*

smithogilvyi using morphology and ITS sequencing. This confirms ascospores are released from infected burrs into the atmosphere where they can infect chestnut flowers.

Ascospore trapping was also conducted with a Burkard Volumetric Spore-Trap in an orchard to test if ascospores are part of the infection process. Peak ascospore capture occurred in the hours after sunrise (7-9am) and sunset (8-11pm). This shows ascospore release likely responds to environmental factors such as relative humidity and temperature.

The effectiveness of post-harvest flotation grading was tested. Rotten chestnuts have previously been found to float and healthy ones to sink. Five temperatures were tested 4, 30, 50, 60, 70^{o}C. Rotten chestnuts sank in all temperatures and healthy ones floated in all except for 70^{o}C. The method should therefore be used with caution. Potential losses to mis-identified floating chestnuts were calculated as $800-$1300 per metric tonne.

Orchard sanitation is key to Chestnut Rot management. Targeting infected burrs by removal or placing a thick layer of organic mulch over top to block ascospores are options. Growing a range of varieties is recommended to spread out the flowering times of the chestnut trees and reduce the risk of floral infection.

Surveying South Australia, Western Australia and Tasmania as well as internationally is needed to further determine the distribution of the disease. Determining if there is more then one species of *Gnomoniopsis* present on *Castanea* both in Australia and worldwide is also of interest. The effectiveness of biological control agents such as *Trichoderma* sprayed on infected burrs should also be investigated.

Introduction

The Australian chestnut industry is relatively small, however production is increasing annually. Fresh chestnut production in 2010 was projected in 2007 as 1,880 metric tonnes for the Australian domestic market and 120 metric tonnes for the export market (HAL 2007). The estimated value of the industry in 2010 was $13M ($12.2M per annum domestically and $0.78M per annum in export) using the price of $6.50 per kg of fresh unpeeled chestnuts (HAL 2007). Large commercial groves, mostly of *Castanea sativa* Miller. (European Chestnut) or *Castanea crenata* Siebold and Zucc. (Japanese Chestnut) x *Castanea sativa* hybrids are planted in North-Eastern VIC (Bright, Myrtleford, Buckland Valley, Harrietville, Beechworth, and Stanley). South of the Divide in VIC, chestnuts are grown in the High Country of Gippsland and the Macedon Ranges. Producers are also located in NSW (Central West, Blue Mountains), the Snowy Mountains, the South Coast, the Northern Tablelands, South Australia, Western Australia and Tasmania (McLaren 1999). The main commercial varieties grown presently are Decoppi Marone, Purton's Pride and Red Spanish.

Chestnut Rot is considered as one of the most serious post-harvest problems of chestnuts both in Australia and internationally (Rutter et al. 1990). It was first reported in northern Italy in the 19th Century as 'the mummification of chestnuts' (Spegazzini 1879). Since then it has continued to be reported in Europe in countries

such as Italy (Servazzi 1941; Camici 1948; Cifferi 1951; Gentile et al. 2009a; Gentile et al. 2009b), France (Baudry and Robin 1996; Briesch 2008) and Switzerland (Sieber et al. 2007). It has also been reported in Australia (Anderson 1993; Washington et al. 1993, 1997, 1998, 1999; Ogilvy 1998; Smith and Ogilvy 2008), Chile (Montealegre and Gonzalez 1986), China (Wang et al. 2000; Wu et al. 2001), India (Puttoo et al. 1988), New Zealand (Wadia et al. 2000; Osmonalieva et al. 2001) and the United States of America (Wright 1960; Donis-Gonzalez et al. 2009).

Healthy chestnut kernels have a creamy light yellow appearance, with very firm endosperm and embryo tissue. Symptoms of Chestnut Rot manifest as light, medium and dark brown lesions occurring on the endosperm and embryo of the chestnut (Fig. 1a-d). The disease is often not visible externally, proving a challenge for growers and consumers alike. Disease symptoms mainly occur post-harvest (Rutter et al. 1990; Anderson 1993; Giacalone and Bounous 1993; Washington et al. 1993, 1997, 1998), however observations by Australian growers indicate chestnuts can be affected when they are still attached to the tree.

Previous surveys of Melbourne Markets showed incidence up to 40% (Anderson 1993). This equates to losses of $5.2M in 2010; using projected Australian chestnut production figures (HAL 2007). The Australian agent and retailer threshold for Chestnut Rot is stated as 0-1% (Rinaudo et al. 2009). There became a need for comprehensive surveys determining the scope and distribution of the disease in Australia.

During the 1990s one Chestnut Rot organism was identified in Australia named *Phomopsis castanea* (Sacc.) Höhn. (Anderson 1993; Washington et al. 1993, 1997, 1998, 1999). In 2008, another organism mainly associated with the disease was informally described as *Gnomonia pascoe* sp. nov. (anamorph: *Discula pascoe* sp. nov.) nomen nudum (Smith and Ogilvy 2008). *G. pascoe* sp. nov./*D. pascoe* sp. nov. was reported as causing the majority of Chestnut Rot in Australia, with *P. castanea* reported as causing 4-8% (Smith and Ogilvy 2008). To date there have been no molecular studies completed in Australia on the Chestnut Rot organism, however molecular studies recently completed on fungal endophytes from *Castanea* in Italy (Tamietti et al. 2009), and New Zealand (Sogonov et al. 2008b) identified an organism belonging to the genus *Gnomoniopsis* Berl.

Chestnut Rot organisms have been reported as endophytes of *Castanea* sp. in Australia, New Zealand, and Italy, easily isolated from asymptomatic vegetative and floral tissues including burrs, male flowers, 2-year-old stems, styles, stigmas, dormant buds, current-year stems, 3-year-old stems, peduncles, dead twigs and 3-year-old seedlings (Anderson 1993; Washington et al. 1999; Wadia et al. 2000; Osmonalieva et al. 2001; Gentile et al. 2009a, 2009b). To date no endophyte studies have been conducted on *Castanea* in NSW, only VIC.

Smith and Ogilvy (2008) reported Chestnut Rot organisms existing as saprophytes on decaying burrs on the orchard floor. Until 2008, Chestnut Rot was thought to be caused by a switch from endophytic growth to pathogenic parasitism following harvest and during storage (Anderson 1993; Washington et al. 1993, 1997, 1998, 1999). Ogilvy (1998) found that rainfall during flowering increased incidence, and suggested that this was due to the infection of chestnut flowers from fungal spores

transmitted mainly by rainsplash. He suggested that the critical period of receptivity of flowers to spores was 10 days immediately following the first 7 days of flowering i.e. days 8-17 of flowering. A connection was suggested between poor pollination, misshapen chestnuts and Chestnut Rot. Moisture stress during the harvest period causing delayed abscission of burrs and chestnuts was also suggested as a factor. Smith and Ogilvy (2008) found that the infection of chestnut flowers occurs during December in Australia, and is due to ascospores released from perithecia over-wintered on decaying burrs on the orchard floor (Fig. 2).

Smith and Ogilvy (2008) demonstrated Koch's postulates with *G. pascoe* sp. nov. and Chestnut Rot through direct inoculation of female flowers with ascospore suspensions, and bagging flowers with infected hydrated burrs in Mullion Creek, NSW (Smith and Ogilvy 2008). This study tracked the progression of the infection process from floral infection through to development of Chestnut Rot symptoms post-harvest.

Presently there is still confusion surrounding the infection process. Previous air sampling studies attempting to capture ascospores from the orchard atmosphere to date have returned negative. This was the case in New Zealand between November 1999 and March 2000 when using Burkard high throughput 'jet' spore samplers and the open (agar) plate method (Wadia et al. 2000). These experiments did not capture airborne ascospores of the Chestnut Rot organism. The hypothesis of floral infection by ascospores therefore warrants further investigation. If chestnut flowers are infected through a floral infection, then the presence of ascospores in the orchard air during flowering is essential.

Flotation grading is a method developed in New Zealand, and is used by some growers in Australia to separate rotten chestnuts from healthy ones (Fig. 3) (Klinac et al. 1999; Morris 2006). Chestnuts that float are considered rotten, while those that sink are considered healthy. Rotten chestnuts are usually discarded or sold for a much lower price. It is currently not known how effective this method is at separating rotten chestnuts.

This research project was undertaken to create a better understanding of the scope and distribution of the Chestnut Rot problem in South-Eastern Australia; survey the incidence of Chestnut Rot in South-Eastern Australia at both orchards and markets; observe the effects of rainfall during flowering on the incidence of Chestnut Rot; clarify the taxonomy and phylogeny of the Chestnut Rot organism using both morphological and molecular analyses; examine the Chestnut Rot organism as both an endophyte and saprophyte of *Castanea* in South-Eastern Australia; further clarify the infection process and disease cycle by clarifying if Chestnut Rot is caused by an infection of chestnut flowers by ascospores released from decaying burrs and; investigate if flotation disease grading of fresh unpeeled chestnuts is effective in distinguishing healthy and infected chestnuts.

The results of this research project will help Australian and international chestnut growers to better manage their orchards by equipping them with evidence enabling them to make more informed disease management decisions.

Materials and Methods

Surveys
Twenty-two orchards were surveyed in 2008, 21 in 2009 across Victoria (VIC) and New South Wales (NSW) (Fig. 4). A hierarchical sampling strategy was used (orchards/trees/varieties). Three-hundred chestnuts per orchard were sampled. Chestnuts were dissected, visually assessed for symptoms, and calculated as Chestnut Rot incidence (%). The important commercial varieties were emphasised including Decoppi Marone, Purton's Pride and Red Spanish. Flemington Markets was sampled for Chestnut Rot in 2008 (varieties: Decoppi Marone, Purton's Pride) and in 2009 (varieties: Purton's Pride and Red Spanish).

Taxonomy
Thirteen isolates were analysed from VIC and NSW sourced from various chestnut tissues including diseased kernels, ascospores from perithecia on decaying burrs, and as endophytes from apparently healthy female and male flowers, leaves, and stems. 75 samples per tissue type per month were tested. The $1cm^2$ sections of the various chestnut tissues were cut, triple surface sterilised (Washington et al. 1999), plated on to malt extract agar (MEA) and incubated.

The taxonomy and phylogeny of the organism was clarified by analysing both morphological and molecular characters. Morphological examination of the fungus included perithecia, asci, ascospores, anamorph colony structure, colony growth rate, conidiomata and conidia. PCR amplification and sequencing using various gene loci was completed including RNA polymerase II (rpb2), internal transcribed spacer regions 1 and 2 encompassing the 5.8S rDNA (ITS), translation elongation factor 1-alpha (tef1-α), and beta-tubulin (β-tubulin). These sequences were phylogentically analysed in the context of the *Diaporthales*, *Gnomoniaceae* and *Gnomoniopsis.* ITS sequences from the Australian isolates were also analysed with ITS sequences from *Gnomoniopsis* sp. (sourced from GenBank) on *C. sativa* from Italy (Tamietti et al 2009), *C. sativa* in India (Dar and Rai 2011), and *C. crenata, C. sativa,* and *Castanea* sp. from New Zealand (Sogonov et al. 2008b).

Infection process a. Endophyte isolations
Asymptomatic chestnut tissues were collected over 5 months in 2008 and 2009 (Dec 2008, Feb, Apr, Aug, Dec 2009). The tissue types tested included female and male flowers (living and dead), immature and mature burrs, pedicels, living male flowers, leaves (leaf margins, mid-veins, petioles), dead styles, dormant terminal buds, stems (current-year, 2 year-old, 3 and 4 year-old bark and xylem) and chestnut shells and kernels. The varieties tested included Decoppi Marone, Purton's Pride and Red Spanish. Sections were triple surface sterilised, plated on to MEA and incubated.

b. Laboratory ascospore trapping
Ascospores were captured on PDA plates in a closed chamber experiment with chestnut burrs containing over-wintered perithecia and ascospores of the Chestnut Rot organism. The incubation temperature of the chamber was 23^oC for the duration of the experiment. Colonies were identified using morphology and ITS phylogenetic analysis.

c. Orchard ascospore trapping
A Burkard Volumetric Spore-Trap was set up for one week in December 2010 in an orchard in Mullion Creek, NSW (Fig. 5). It was used to determine daily patterns in ascospore capture from the orchard atmosphere.

Flotation grading
Five water temperature treatments were tested including 4, 30, 50, 60 and 70^{o}C. Tap water was used in all treatments. 100 Purton's Pride chestnuts per temperature treatment were floated for 2 minutes, removed, dissected, the cut surface observed for symptoms and the frequency of rotten chestnuts determined.

Results

Surveys
In 2008, the highest incidence at individual orchards was 72% (Mt Irvine, NSW) (Fig. 6), and in 2009 35% (Mt Wilson NSW) (Fig. 7). Chestnut Rot was present in all of the sampled orchards in both years. Incidence varied widely between and within orchards in both years. The important commercial varieties Decoppi Marone, Purton's Pride and Red Spanish were all affected by the disease. There was a positive correlation between incidence and December rainfall of the previous year, indicating environmental factors are key to the infection process (Table 1, Fig. 8). In 2008 and 2009, surveys of Flemington Markets showed incidence up to 10% in 2008, and 9% in 2009 (Table 2).

Taxonomy
A novel organism was found as mainly associated with Chestnut Rot, described as *Gnomoniopsis smithogilvyi* sp. nov. The 13 isolates including ascospores, isolates associated with rotten kernels in VIC and NSW, and endophytes from NSW were all identified as *G. smithogilvyi.* The rpb2 Maximum Likelihood (ML) phylogenetic tree is included (Fig. 9). The ITS ML phylogenetic analysis showed the Australian isolates, 5 Indian isolates, 17 of the 19 Italian isolates and three of the four New Zealand isolates grouped in the same node of the ITS Maximum Likelihood (ML) phylogenetic tree with 100% maximum parsimony (MP) bootstrap support and 100% Bayesian posterior probability (BP) support (Fig. 10). This indicates these isolates belong to the genus *Gnomoniopsis* and may be *G. smithogilvyi.* Multi-gene phylogenetics needs to be completed with these isolates to determine if they are *G. smithogilvyi* or a number of different species of *Gnomoniopsis.* One of the New Zealand isolates grouped with *Gnomoniopsis paraclavulata* Sogonov. in this analysis indicating that there may be more than one species of *Gnomoniopsis* on *Castanea* in New Zealand.

Infection process a. Endophyte isolations
The Chestnut Rot organism was isolated as an endophyte from various vegetative and floral tissues of *Castanea* in December 2008, and February, April, August, and December 2009 (Table 3). The ranking of highest to lowest isolation frequency in chestnut tissues was female flowers (82%, December 2008), mature burr equators, mature pedicels, living male flowers, dead male flowers, terminal leaf margins (April 2009), dead styles, dormant terminal buds, immature burr equators, pedicels

(February 2009), leaf mid-veins, current-year stems (August 2009, February 2009), and mature shell equators (April 2009). All other tissue types had ≤20% isolation frequency including current-year stems (December 2008, April 2009), 2 year-old stems, petioles, mature kernels, female flowers (December 2009), immature shell equators, living male flowers (December 2009) and 3 and 4 year-old bark. The endophyte was not isolated from 3 and 4 year-old xylem. There was a decreasing trend of isolation with increasing age of chestnut tissues in four of the five months. There was also a 72% reduction in isolation frequency from female flowers between 2008 (82%) and 2009 (10%), indicating a dynamic presence of the organism in chestnut flowers that changes over time. It also suggests a seasonal infection of female chestnut flowers. All tested varieties (Decoppi Marone, Purton's Pride, Red Spanish) had the Chestnut Rot endophyte isolated from their tissues, indicating that they have the potential to be affected by Chestnut Rot.

b. Laboratory ascospore trapping

The observation of Chestnut Rot perithecia on burrs is central to the hypothesis of a floral infection by ascospores. This study observed *G. smithogilvyi* on decaying burrs and branches in Mullion Creek, NSW. This observation of perithecia and ascospores on burrs supports the hypothesis of a floral infection. Ascospore infection of chestnut flowers has previously been found to be the primary stage of infection leading to Chestnut Rot. Three colonies grew from ascospores captured on the PDA plates in the closed chamber laboratory experiment in the second week of incubation. The incubation temperature was stable for the duration of the experiment at 23^{o}C, suggesting fluctuations in temperature are not critical for ascospore release, with moisture and humidity likely to be more important. One Chestnut Rot colony that grew from ascospores was identified using morphological and molecular techniques.

A segment of the ITS region of rDNA was sequenced and analysed. The ascospore isolate grouped in the same node as the Australian Chestnut Rot, ascospores collected from burrs and endophyte isolates in the maximum parsimony (MP) ITS phylogenetic tree with 100% MP bootstrap support, indicating it is a species of *Gnomoniopsis* and likely *G. smithogilvyi*. This experiment indicates that ascospores of *G. smithogilvyi* are released from the decaying burrs into the air where they can potentially infect chestnut flowers, again supporting the floral infection hypothesis. Ascospores were found to be the primary source of inoculum in the infection of chestnut flowers, leaves and stems in December, leading to Chestnut Rot symptoms the following year.

c. Orchard ascospore trapping

Ascospores of the Chestnut Rot fungus were captured by a Burkard Volumetric Spore-Trap in Mullion Creek, NSW. Daily patterns in ascospore capture from the orchard atmosphere were found. The time of peak mean ascospore capture was between 8-11pm and between 7-9am (Fig. 11). The peak times of ascospore capture correspond to sunset and the hours following sunset, and the hours following sunrise. The highest mean hourly frequency of ascospore capture was 33 ascospores per m^3 of air. No rain fell during the one-week sampling period, indicating ascospores are released even in the absence of rain.

Flotation grading
The method was most effective at floating rotten chestnuts in the 70°C treatment (Fig. 12), however 22 out of 80 of the chestnuts that sank in this treatment were rotten. Rotten chestnuts sank in all temperature treatments (Fig.13). The method was observed to work well on chestnuts that are highly dessicated, but less effectively on chestnuts with minor Chestnut Rot symptoms. However, there were many more rotten moist chestnuts than dessicated ones.

Discussion

Surveys
The orchard surveys in 2008 and 2009 showed incidence varied widely between orchards in both years, and within individual orchards between the 2 years. All varieties were affected including Decoppi Marone, Purton's Pride and Red Spanish, indicating they are all susceptible under the right conditions.

There was a positive correlation between 2007 and 2008 December rainfall, and incidence the following year, indicating that rainfall during flowering increases the risk of infection. Orchards in areas with higher rainfall, such as Mt Irvine, NSW, had up to twelve times higher incidence than orchards with low rainfall e.g. Bungendore, NSW. This supports the findings of Ogilvy (1998) and Smith and Ogilvy (2008) that rainfall during flowering increases the incidence of the disease.

The varying levels of incidence between the two years on the same orchard could be due to the timing of the rainfall during flowering. As mentioned, Ogilvy (1998) suggested that there is a critical period of infection by ascospores. This study suggested this was due to an infection of chestnut flowers from fungal spores transmitted mainly by rainsplash. The critical period for floral infection was described as the 10 days immediately following the first 7 days of flowering i.e. days 8-17 of flowering. Different chestnut varieties flower during different periods, for example Red Spanish flowers earlier than Decoppi Marone and Purton's Pride. Therefore varieties have different timing of their critical periods. If rain fell early in the flowering period, Red Spanish would have higher probability of infection than Decoppi Marone and Purton's Pride.

Regions with high rainfall and humidity are reported as higher risk for diseases caused by fungal pathogens. The ascospores of the Chestnut Blight pathogen, *Cryphonectria parasitica* for example are reported as discharged most often after rainfall in Spring (Robin and Heiniger 2001).

An important question with infection risk is whether ascospores from one orchard can infect a neighbouring orchard. If ascospores can travel long distances in air or hitchhike on pollen grains, this would have management implications for growers bordering other chestnut orchards, such as in North-Eastern VIC. Ascospores of many fungal pathogens are wind-borne and may travel long distances. *C. parasitica*, for example, has been documented to travel between 90-120 m from the perithecia source (CABI 2004), and *Sclerotinia sclerotiorum* ascospores have been documented to travel up to 150 m from their source (McCartney 1999).

Incidence at Flemington Market was up to 10% in 2008 and 9% in 2009. This is above the Australian agent and retailer threshold of 0-1% (Rinaudo et al. 2009), indicating Chestnut Rot is still a significant problem for the industry.

Taxonomy

Correctly identifying the Chestnut Rot organism is key to managing Chestnut Rot. There was one species of fungus consistently associated with rotten chestnuts, as an endophyte isolated from asymptomatic floral and vegetative chestnut tissues and as a saprophyte on decaying burrs and branches in South-Eastern Australia, *Gnomoniopsis smithogilvyi* sp. nov. The fungus belongs to the *Gnomoniaceae* family. Sogonov et al. (2008a) reported *Gnomoniaceae* as causing tree diseases such as Oak Anthracnose [*Apiognomonia errabunda* (Roberge ex Desm.) Höhn.], Cherry Leaf Scorch [*A. erythrostoma* (Pers.) Höhn.], Sycamore Canker [*A. veneta* (Sacc.& Speg.) Höhn.] (Sinclair & Lyon 2005), Ash Anthracnose [*Gnomoniella fraxini* Redlin & Stack, now *Plagiostoma fraxini* (Redlin & Stack) Sogonov, anamorph *Discula fraxinea* Redlin & Stack] Sogonov et al. (2008a), and Dogwood Anthracnose caused by *Discula destructiva* Redlin (1991). The genus *Gnomoniopsis* contains economically significant pathogens of species including *Fragaria* sp. (strawberry) and *Rubus* sp. (Blackberry, Raspberry) (Walker et al. 2010; Bolay 1971; Monod 1983; Maas 1998). *Gnomoniopsis clavulata* (Ellis) Sogonov*.,* is reported to cause Oak Anthracnose on *Quercus alba* and *Quercus rubra* (Walker et al. 2010; Sogonov et al. 2007; Cohen 2004).

Infection process a. Endophyte isolations

The organism was isolated from a range of chestnut floral and vegetative tissues in Mullion Creek, NSW. The isolation frequency of the endophyte changed in the same tissue types across all months sampled (except 3 and 4 year-old xylem where it was rarely isolated) indicating that its presence is dynamic and changes over time. The organism was present in all varieties (Decoppi Marone, Purton's Pride, Red Spanish). The highest isolation frequency across all months was in 2008 from female flowers at 82%, changing to 10% in 2009. In April 2009, incidence at this orchard was 10.6%, and in April 2010 it was 6%. This follows the pattern of decreased isolation frequency from female flowers and the decrease in December rainfall between the two years (106.2 mm in 2007, 87.6 mm in 2008). Orchard microclimate has been found to be key to the infection process, particularly rainfall during flowering (Ogilvy 1998). Smith and Ogilvy (2008) found this was due to an ascospore shower during the flowering period, which was increased by rainfall. The decreased December rainfall in 2008 would have reduced ascospore release and the subsequent infection of chestnut flowers. This explains the reduction in incidence in 2009.

Positive correlations have previously been found between increasing precipitation and the frequency of endophytes (Carroll and Carroll 1978). As the organism appears to exist as an endophyte, precipitation would also increase its dispersal and colonisation potential.

There was a pattern of decreased isolation frequency of the endophytic phase of the fungus with an increase in age of chestnut tissue in December 2008, February 2009, April 2009 and August 2009. This may be due to the receptivity of younger tissues to airborne ascospores. Female chestnut flowers and chestnut leaves contain structures

such as stigmas and stomata that are highly receptive. Stigmas are receptive to pollen for fertilisation to occur (Dafni and Motte Maués 1998), and stomata are receptive to air for gas exchange (Knox 2005). Stigmas and stomata are also receptive to ascospores. Germinating fungal spores have been found to invade host plants by active cuticular penetration and through wounds (Juniper 1991; Knox et al. 2005). The older chestnut stem tissue contains bark, which provides a physical barrier to penetration of ascospores unless they enter through wounds or lenticels. This could explain the lower frequency of colonisation by *G. smithogilvyi* in older stem tissues. It also suggests that movement of the endophyte throughout the chestnut tree from one tissue type to another may not be as important as colonisation from airborne propagules invading from the atmosphere.

The teleomorph of *G. smithogilvyi* was also observed growing on decaying burrs and branches on the orchard floor in Mullion Creek NSW. Ascospores of the Chestnut Rot organism, endophytes isolated from floral and vegetative tissues, and the organism associated with rotten chestnuts were found to be all the same taxon, *G. smithogilvyi.*

The disease cycle of the fungus appears to begin with the infection of female flowers, male flowers, leaves, and stems during the flowering period in December. The fungus then exists as an endophyte for the next 4-6 months of chestnut development (December-April/May), and is then associated with rotten chestnuts during maturity and postharvest storage (Fig. 14).

It is paramount that perithecia and ascospores are targeted in any Chestnut Rot management program. Future research areas should also include investigation of the best methods to target infected burrs. The effect of ascospore transmission from infected burrs to chestnut flowers by animals particularly bees, ants, beetles, and earwigs is important, as animals have been reported to transmit pathogens in other tree crops such as Pod Rot and Stem Canker in *Theobroma cocao* L. (Cocoa), caused by *Phytophthora palmivora* (Butler) Butler (Jackson and Newhook 1978; Konam and Guest 2004).

In *C. sativa*, *D. kuriphilus* (Chestnut Gall Wasp) has recently been reported as being associated with *Gnomoniopsis* in Italy (Magro et al. 2010). This insect pest is not present in Australia, but international studies could investigate its potential role in transmission of the Chestnut Rot fungus.

Historically, there has been significant movement of chestnuts and budwood from Europe to Australia. It is therefore possible that the Chestnut Rot organism was imported to Australia from Europe. The organism could also have been introduced from Japan, China, or the USA as *Castanea* from these countries have all been transported to Australia. The organism could also have been transported between orchards in Australia and New Zealand by exchange of chestnuts and budwood between the two countries. There is also a possibility that the organism has an endemic Australian origin. Further work with native plant species needs to be completed to determine if this is the case.

b. & c. Laboratory and orchard ascospore trapping
Ascospores were captured on the PDA plates in the laboratory experiment. This indicates ascospores of the Chestnut Rot organism are released from the decaying burrs into the atmosphere where they can potentially infect chestnut flowers. The colonies isolated from the captured ascospores had cultural morphology identical to that of *G. smithogilvyi* including fast growth rate, colour of mycelia, colour of conidia ooze, and mean dimensions of conidiomata and conidia on MEA, malt yeast agar (MYA) and PDA. The ITS sequence from the ascospore isolate grouped in the same node of the MP ITS phylogenetic tree with the Australian isolates, the Italian isolates (with minor intra-specific variation) and 3 New Zealand isolates. This confirms the morphological evidence that the isolate was *G. smithogilvyi.* It also helps confirm that ascospores are released into the air where they can potentially infect chestnut flowers (Smith and Ogilvy 2008).

The incubation temperature in the laboratory experiment was stable for the duration of the experiment at 23^{o}C, indicating that fluctuations in temperature are not critical for ascospore release.

Ascospores of the Chestnut Rot organism were also captured with the Burkard Volumetric Spore-Trap. The peak capturing times correspond to sunset and the hours following sunset between 8-11pm, and in the hours following sunrise between 7-9am. This pattern is likely due to changes in micro-climate during dusk and dawn such as rising and falling relative humidity. No rain was recorded during the experiment indicating rain is not essential for ascospore release. Moisture (rain, dew, fog) and relative humidity are reported as important factors affecting spore release in numerous other fungal pathogens (Adams et al. 1986; Carroll 1988; Humpherson-Jones 1992; Mondal et al. 2003). Rainfall has been reported to initiate the release of ascospores in *Mycosphaerella citri* Whiteside (Mondal et al 2003). This study found ascospore release was greatest following rain events, declining linearly with horizontal distance from the source. Future experiments in chestnut orchards with the Burkard Volumetric Spore-Trap should test the effects of ascospore release before, during and after rain events to observe any effects on ascospore release. The timing of flowering of chestnut varieties and the timing of ascospore release is likely to be critical for infection to occur.

The findings of both air-sampling experiments supports the hypotheses of Smith and Ogilvy (2008), and Ogilvy (1998) that Chestnut Rot is caused by floral infection by ascospores during the chestnut flowering period. Targeting the ascospores released from infected burrs is critical for controlling the disease.

For more information and detail regarding the experiments conducted in this project

Flotation grading
Flotation grading needs to be used with caution as rotten chestnuts were found to sink in all temperature treatments, and healthy chestnuts found to float in all temperatures except 70^{o}C. Previous studies in New Zealand showed the method to be effective at identifying rotten chestnuts (Klinac et al. 1999). Further research is needed to clarify the effectiveness of the method on other chestnut varieties and on peeled chestnuts.

Technology Transfer

2009

January. Presented findings of 2008 Chestnut Rot survey and taxonomy at chestnuts Australia Inc. (CAI) field day in Hoskinstown, NSW.

September. Presented poster at Australasian Plant Pathology Society (APPS) Conference, Newcastle NSW.

September. Article for HAL Industry Annual Report.
http://www.horticulture.com.au/admin/assets/library/annual_reports/pdfs/PDF_File_97.pdf

October. Presented at The 1st European Congress on Chestnut: *Castanea* 2009.

December. Featured article in *Nuts and Burrs* regarding presentation at *Castanea* 2009 Conference presentation.

2010

June. Chestnut Rot information provided to VIC chestnut grower Jane Casey to post on the Australian Gourmet Chestnuts website.

July. Presented at CAI Open Day. Kiewa Valley, VIC.
September. Article for HAL Industry Annual Report.
http://www.horticulture.com.au/admin/assets/library/annual_reports/pdfs/PDF_File_137.pdf

September. Article for 'The Gardens' magazine. Spring edition. Friends of the Royal Botanic Garden, Sydney.

2011

April. Presented poster at APPS, Darwin.

July. Completed Chestnut Rot progress report for CAI R&D Chair Sally Robbins for R&D Committee meeting.

July. *Nuts and Burrs* article on infection process of Chestnut Rot.

August. HAL Annual Report.
http://cms2live.horticulture.com.au/admin/assets/library/hal_documents/pdfs/PDF_File_23.pdf

September. Completed article for HAL Industry Annual Report. http://cms2live.horticulture.com.au/admin/assets/library/annual_reports/pdfs/PDF_File_178.pdf

October. Final presentation at CAI AGM in Bright, VIC covering key findings of the project, and recommendations on the management of Chestnut Rot. Handout made for meeting covering key recommendations in the management of Chestnut Rot.

December. Article published regarding the recommendations on the management of Chestnut Rot. *Nuts and Burrs* Festive Season Issue.

2012

Publishing of chapters of the thesis as papers in peer reviewed journals including survey work, taxonomy, and infection processes.

Recommendations – Scientific

Direct demonstration of Koch's postulates
The suspected pathogen needs to be isolated from rotten chestnuts; these isolates need to be used to infect female chestnut flowers in summer; Chestnut Rot symptoms need to be observed in the chestnuts that form from these inoculated flowers; and the organism needs to be re-isolated again from the rotten chestnut kernels. This experiment should also be repeated.

Further Chestnut Rot surveys
Other states in Australia would be beneficial, including South Australia, Western Australia and Tasmania as it would further improve knowledge of the extent and distribution of the disease nation wide. International surveys would also help to determine the distribution of the disease worldwide.

Further taxonomy work
Additional taxonomy work with *G. smithogilvyi* should include further determination of its presence in areas where it is known to exist including Europe, Australia and New Zealand. There may be more than one organism responsible for causing the disease including other species of *Gnomoniopsis*. There appears to be more than one species of *Gnomoniopsis* associated with *Castanea* sp. in New Zealand. This could also be the case in other countries. Further taxonomy work needs to be completed in countries where *G. smithogilvyi* has not been reported but where species of *Castanea* are endemic, including North America, Asia, and the Middle-East.

Transport of the Chestnut Rot organism
The transport of infected chestnuts and budwood is an area of research. Chestnut material has been passed from grower to grower locally, interstate and internationally when propagating new chestnut orchards. Exchange of chestnut material has potentially spread the fungus far beyond its natural range and suggests it may have been transported to Australia, India and New Zealand in infected budwood and chestnuts from other continents. Some possibilities for transport of the organism to Australia are from Europe (*C. sativa*), Asia (*C. crenata* from Japan, *C. mollissima* from China) and North America (*C. dentata*), as *Castanea* have been imported into Australia from these areas. The fungus may have also been transported within Australia and between Australia and New Zealand by the exchange of budwood and chestnuts. It is unclear whether *G. smithogilvyi* can exist on closely related species to *Castanea* such as *Quercus* (Oaks). *G. smithogilvyi* could potentially have a local Australian origin in native species and could have infected chestnut trees after importing and planting here. Therefore sampling of native Australian hosts such as *Eucalyptus* sp. would help clarify if Australia may be the source of origin of the fungus.

There are implications with biosecurity and quarantine on the movement of chestnuts and budwood. The recent outbreak of Chestnut Blight in North-East VIC is one example of a breach of quarantine (CAI 2011). This pathogen was presumably brought in from overseas in infected chestnut material. As *G. smithogilvyi* exists asymptomatically in chestnut tissues, this makes detection in quarantine even more challenging.

Environmental effects on ascospore release
Environmental and climatic factors such as light, temperature, relative humidity, and wind patterns and their effects on ascospore release are key areas for research. These factors could be controlled and tested in future chamber experiments with burrs. In future experiments with the Burkard Volumetric Spore-Trap, ascospores should be captured before, during and after a rain event to observe effects on ascospore presence in the atmosphere. The timing of flowering of chestnut varieties and the timing of ascospore release, particularly after rain, is likely to be key to the infection process and warrants investigation.

Effect of ascospore drift and how far ascospores can travel
The effects of ascospore drift from adjacent orchards and how far ascospores can travel is an important area to research, particularly in regions such as North-Eastern VIC where orchards are very close to each other.

Effect of applying water to infected burrs during non-conducive periods
Recent studies have suggested the positive effects of applying water to perithecia in the orchard during non-conducive periods in order to initiate ascospore release when environmental conditions are unfavorable for infection (Mondal et al. 2003). This would potentially reduce the quantity of ascospores during infective periods (December in the case of *Castanea* in Australia). It seems like a very practical control method for growers to use, however it requires further research to determine its effectiveness including variables such as when water should be applied and how frequently.

Rainsplash as a mode of infection
Transmission of the Chestnut Rot organism by rainsplash is an area of interest as it has been suggested as a mode of infection (Ogilvy 1998). However, studies with *P. palmivora* have shown that rainsplash is insignificant above 0.75 m from the ground (Konam and Guest 2004). The importance of rainsplash as a mode of infection in Chestnut Rot needs clarification.

Quantifying the effects of burr removal
Other areas of interest with the infection process include investigating strategies to reduce infection, including burr removal and determination of the percentage of burrs that need to be removed for a significant reduction in disease incidence to occur.

Effects of animals on transmission of the Chestnut Rot organism
The effect of ascospore transmission from infected burrs to chestnut flowers by animals particularly bees, ants, beetles, and earwigs is important, as they have been reported as a key to the infection process in other tree crop diseases such as Pod Rot and Stem Canker in *T. cocao* (Cocoa), caused by *P. palmivora* (Jackson and Newhook 1978). In *C. sativa*, *D. kuriphilus* (Chestnut Gall Wasp) has recently been reported as being associated with *Gnomoniopsis* in Italy (Magro et al. 2010). This insect pest is not present in Australia, but international studies could investigate its potential role in transmission of the Chestnut Rot fungus.

Further flotation grading experiments
Future work with the flotation grading method could include investigating the effectiveness of the method on other varieties; its effectiveness on peeled chestnuts; determining if there are any effects of using heated water on the chestnuts such as a change in flavour; and determination of whether adjusting the salt concentration of the treatment water makes the method more effective. If changing salt concentration does work, what concentration should be used?

Recommendations – Industry

The findings of this study show the key to reducing the incidence of Chestnut Rot is through improved orchard hygiene. Perithecia and ascospores of the Chestnut Rot organism were found growing as a saprophyte on decaying burrs on the orchard floor. Ascospores were determined as the primary source of inoculum in the infection of chestnut flowers, leaves and stems in December. Targeting the perithecia and ascospores on burrs is therefore critical for controlling the disease.

Recommendations for targeting infected burrs include:

Removal of burrs from the orchard floor
Removing and disposing of the primary source of inoculum will reduce the infection of chestnut flowers during the flowering period.

Mulching over top of burrs
Placement of a thick organic compost layer over top of the burrs to provide a physical barrier to ascospores. Organic mulches have also been found to contain antagonistic

microbes that reduce the activity of pathogens such as *Phytophthora cinnamomi* Rands (You and Sivasithamparam 1995). This method has been particularly successful with *Persea americana* Mill. (Avocado) (You and Sivasithamparam 1995). Perithecia and asccospores are microscopic, and can exist on very small fragments of decaying burrs and branches. Therefore the layer of mulch would have to be thick enough and evenly spread enough over top of burrs to have a significant effect on blocking ascospore movement.

Watering burrs during non-conducive periods
Watering dead burrs on the orchard floor during non-infective periods may be an option for growers to reduce ascospore frequency during the flowering period. Research by Mondal et al. (2003) with *M. citri* found that ascospore release can be advanced by irrigating frequently during dry, non-infective conditions stimulating ascospore release when environmental conditions are unfavorable for infection.

Biological control
Biological controls and antagonistic fungi such as *Trichoderma* and *Gliocladium virens* Mill. Giddens and Foster have been found to reduce the activity of chestnut diseases such as *Cryphonectria parasitica* (Chestnut Blight) (Arisan-Atac et al. 1995) and *Phytophthora* Root Rot (Chambers and Scott 1995). *Trichoderma* based products are available in Australia including 'Tri-D25' which is a mix of *Trichoderma koningii* Oudem. and *Trichoderma harzianum* Rifai. (Zadco 2011). There is future scope to test the effectiveness of these control agents on *G. smithogilvyi*.

Variety selection
The results of this study show the selection of one variety over another is not the key to solving Chestnut Rot, even though variety selection has been previously advised (Rinaudo et al. 2009). The important commercial varieties (Decoppi Marone, Purton's Pride, Red Spanish) sampled in the 2008 and 2009 orchard and market surveys were all affected by Chestnut Rot. A more effective method is to plant a diversity of varieties that flower during different periods. This staggers the receptivity period of chestnut flowers and reduces the probability of an epidemic. If only one variety is grown, or varieties that flower at the same time, there is potential for the pathogen to infect all trees if the environmental conditions are conducive, for example, heavy rainfall during the critical period of flowering. This strategy spreads the risk of infection to achieve an overall reduction, rather than eliminating the risk completely.

Fungicides
The use of fungicides on perithecia and ascospores is not recommended for several reasons. The environmental impact of fungicides on the microflora of the soil could potentially make the conditions more favourable to pathogens by reducing the presence and action of antagonistic and beneficial micro-organisms (Jenkins 2005; Schreiner and Bethlenfalvay 2005). Fungicides also place the pathogen under high selective pressure, with surviving offspring possessing fungicide resistance genes quickly being selected and passing the genes on to their offspring (Dekker 1986; Ma and Michailides 2005). The presence of the teleomorph indicates the potential for sexual recombination, a higher genetic diversity and hence a greater probability of resistance genes occurring in Chestnut Rot fungus populations.

The use of these recommendations will hopefully reduce incidence to the target of <10%.

Conclusion

The findings of this project provide the Australian and international chestnut industries with important information on the extent and distribution of the disease in South-Eastern Australia; clarity regarding the taxonomy of the Chestnut Rot organism; and elucidation of the infection process, particularly floral infection and the endophytic phase of the fungus. The effectiveness of flotation grading as a postharvest treatment was also determined.

The management recommendations suggested such as targeting infected burrs, provide growers with practical strategies to reduce the probability of infection occurring in their orchards. This will result in fewer losses to the disease and an increase in the number of healthy chestnuts available to sell to consumers, therefore increasing grower profits. The effect will be a reduction in incidence to below 10% from the current levels of up to 72%.

Chestnut Rot is a diverse area of future research with many questions still to be explored.

For more information and details of the experiments conducted in this project see Shuttleworth 2012.

Figures and Tables

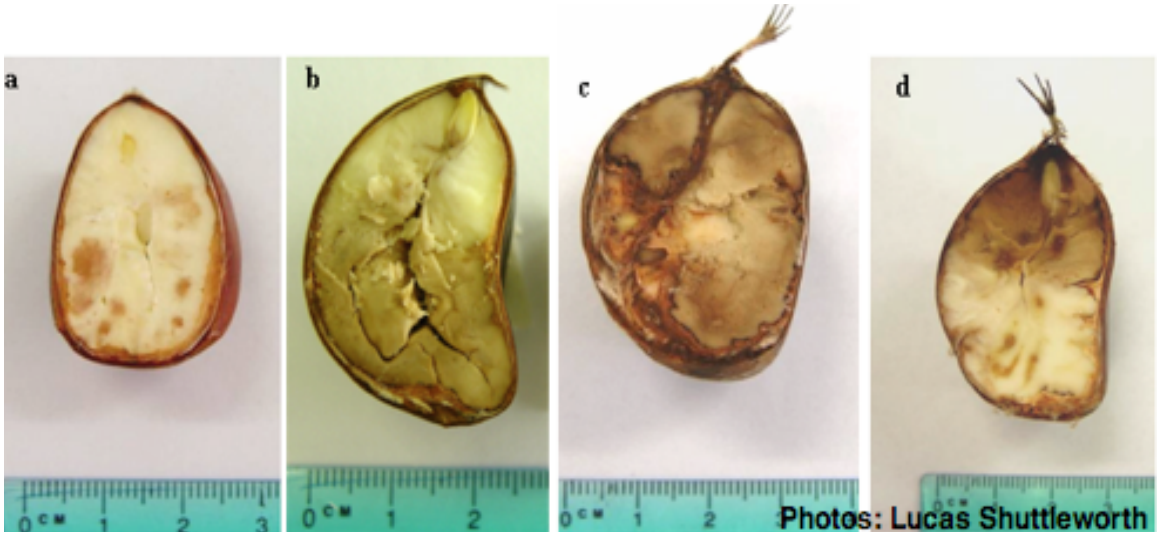

Fig. 1 Chestnut Rot symptoms. a=light brown spotting, b, c=medium brown rot, d=medium and dark brown rot.

Fig. 2 Decaying burrs on the floor of a chestnut orchard in Stanley, VIC. April 2008. Decaying burrs are the primary source of inoculum in the infection process of Chestnut Rot.

Fig. 3 Flotation grading of chestnuts in Fumina, VIC.

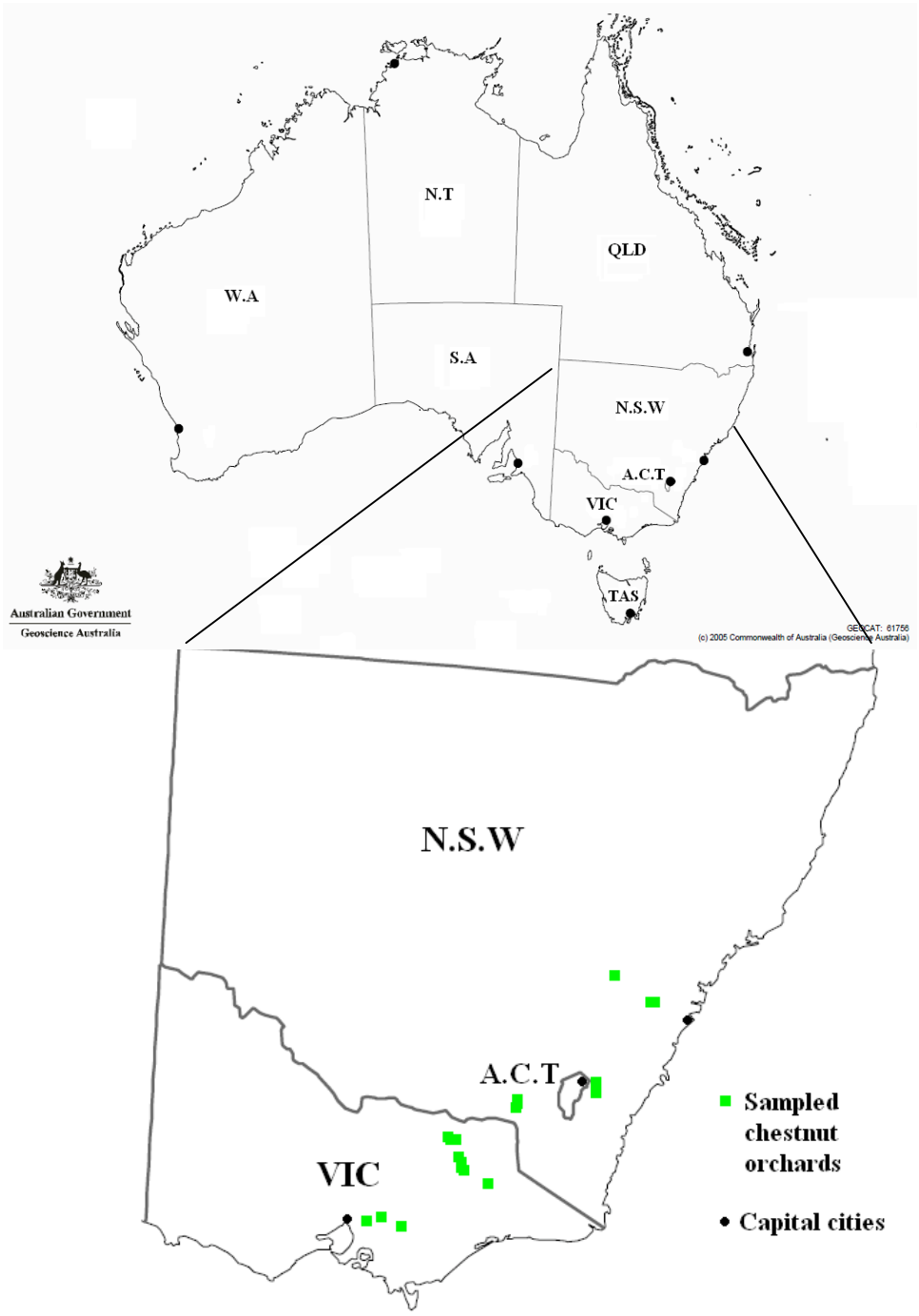

Fig. 4 Location of the 22 chestnut orchards in South-Eastern Australia that were sampled for Chestnut Rot in 2008 and (21 orchards) in 2009 (template map sourced from Geoscience Australia 2006).

Fig. 5 Burkard Volumetric Spore-Trap set up in the chestnut orchard in Mullion Creek, NSW to capture ascospores released from decaying burrs into the orchard atmosphere during the chestnut flowering period. December 2010.

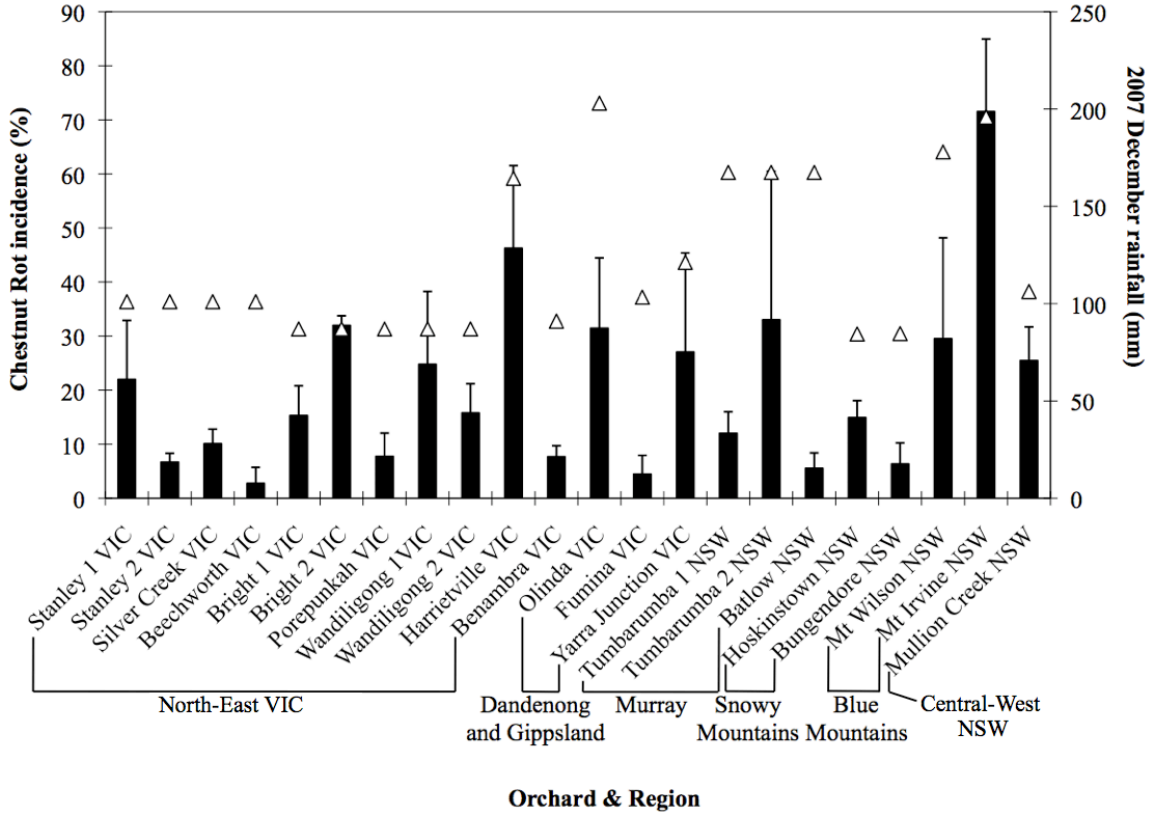

Fig. 6 2008 Mean Chestnut Rot incidence of orchards in VIC and NSW (with regional classifications). 2007 December rainfall is included as triangles on the figure. n= 300 chestnuts per orchard. Error bars indicate the standard error of the mean.

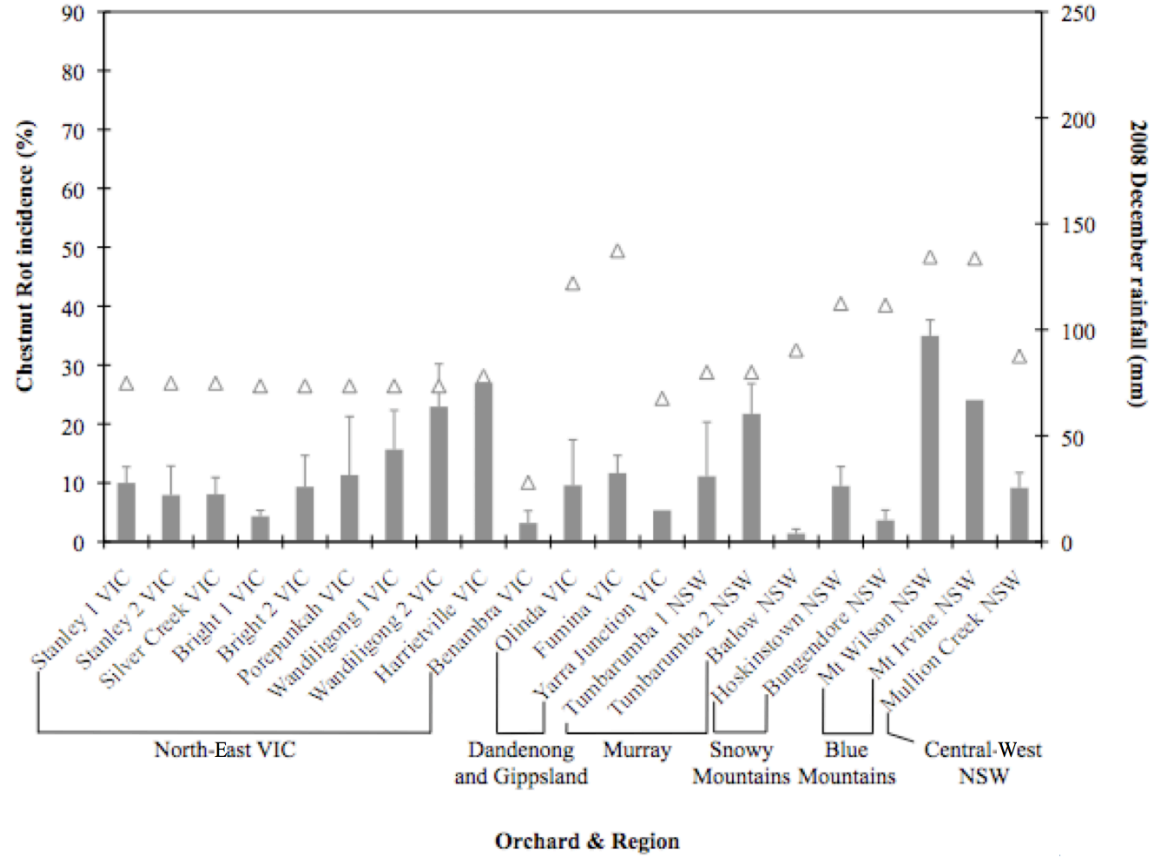

Fig. 7 2009 Mean Chestnut Rot incidence of orchards in VIC and NSW (with regional classifications). 2008 December rainfall is included as triangles on the figure. n=300 chestnuts per orchard. Error bars indicate the standard error of the mean.

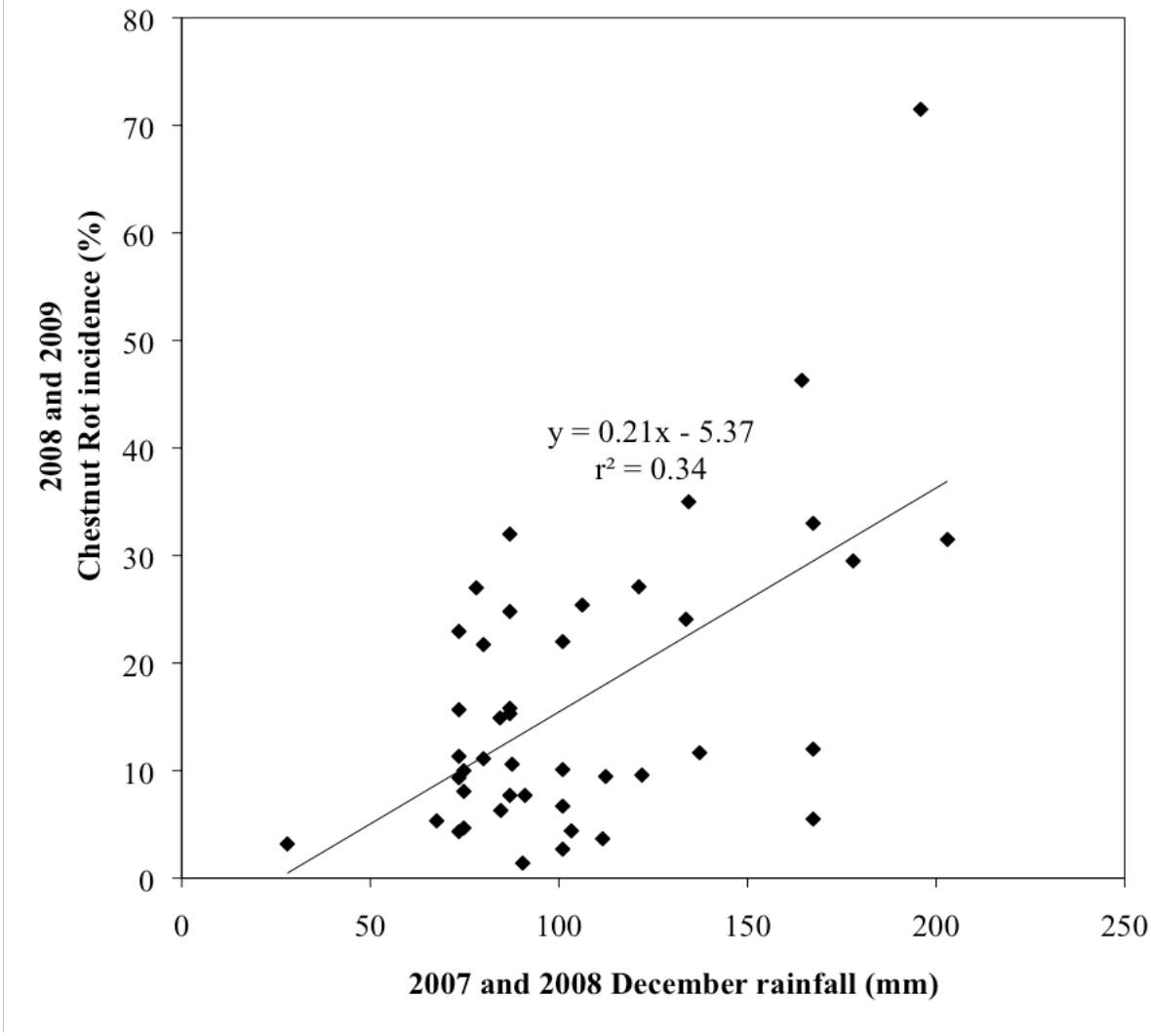

Fig. 8 The pattern of 2008 and 2009 orchard Chestnut Rot incidence and December 2007 and December 2008 rainfall. Correlation Co-efficient (r^2), and the equation of the Regression Analysis line are displayed.

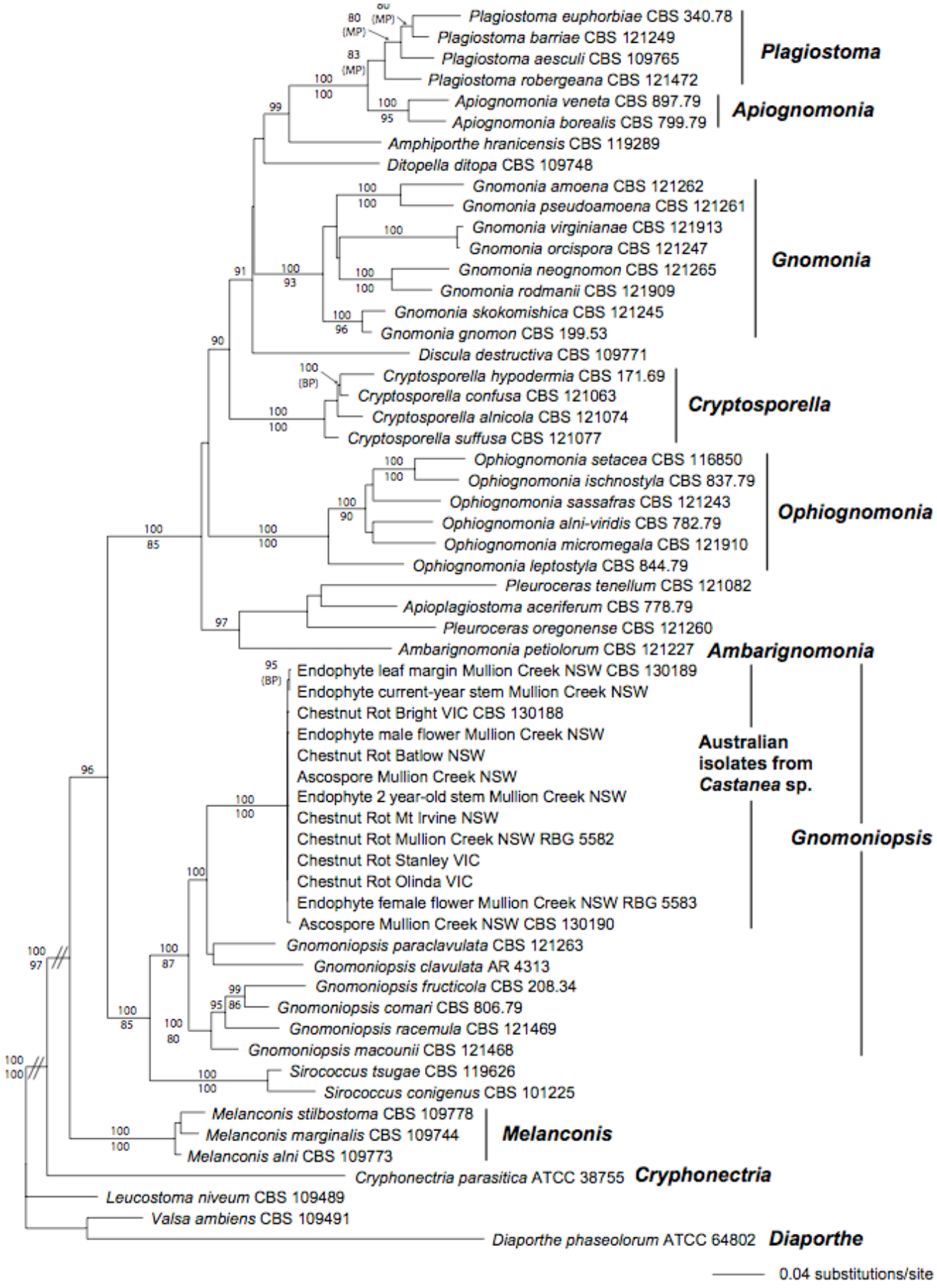

Fig. 9 Maximum likelihood (ML) phylogenetic tree (ML score= -lnL 11769.63) of sequences for the rpb2 analysis of Australian Chestnut Rot, endophyte and ascospore isolates from *Castanea* sp. with reference taxa in the *Gnomoniaceae* including 7 diaporthalean taxa and 39 gnomoniaceous taxa. Bayesian posterior probabilities (BP) ≥90% are displayed above each branch. Maximum Parsimony (MP) bootstrap values ≥70% are displayed below each branch.

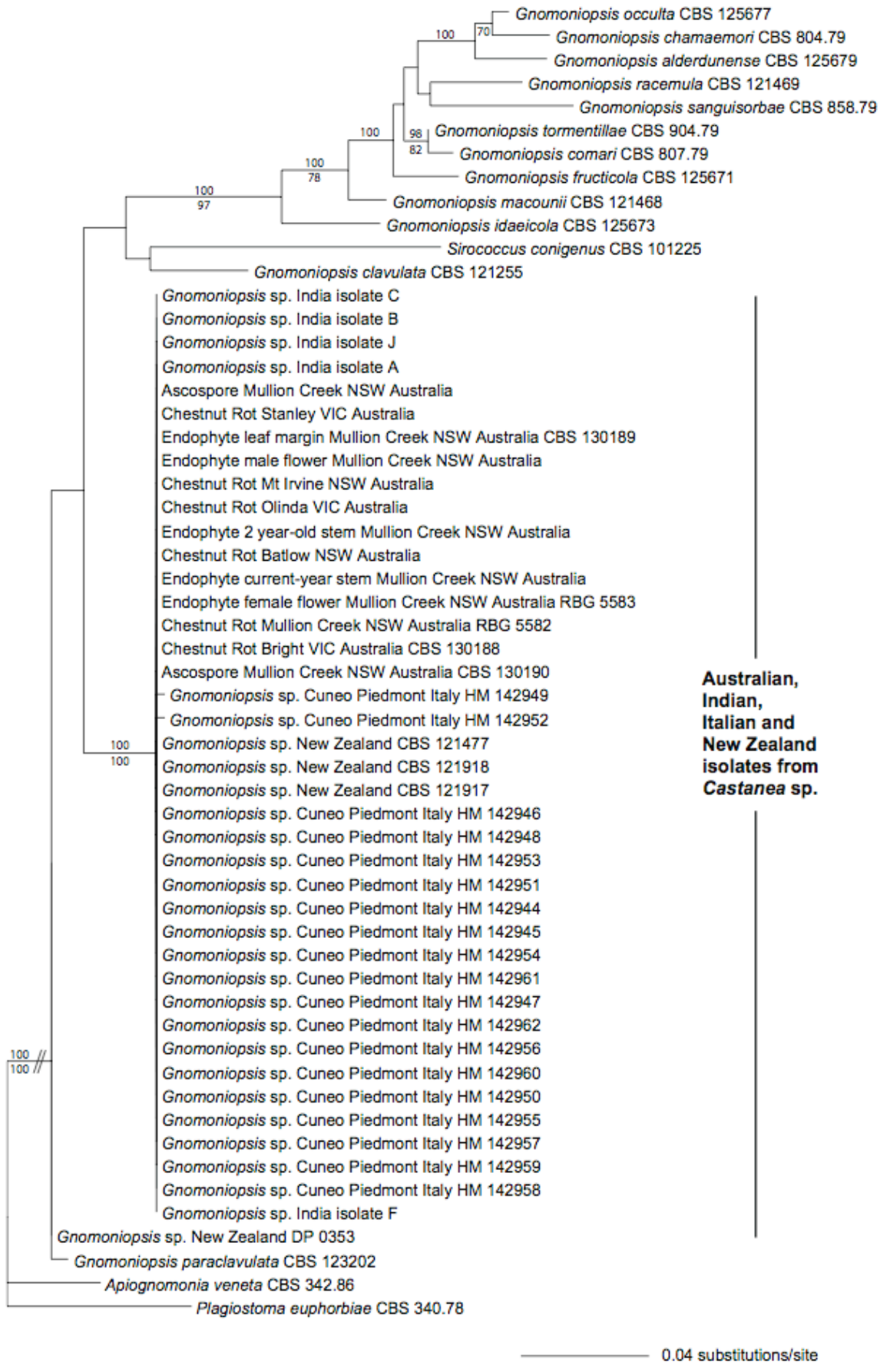

Fig. 10 ML phylogenetic tree (ML score =-ln L score of 1877.21) of the ITS sequences of *Gnomoniopsis smithogilvyi* from Australia and isolates from India, Italy and New Zealand with reference taxa from *Gnomoniopsis* and *Sirococcus.* BP ≥90% are displayed above each branch. MP bootstrap values ≥70% are displayed below each branch.

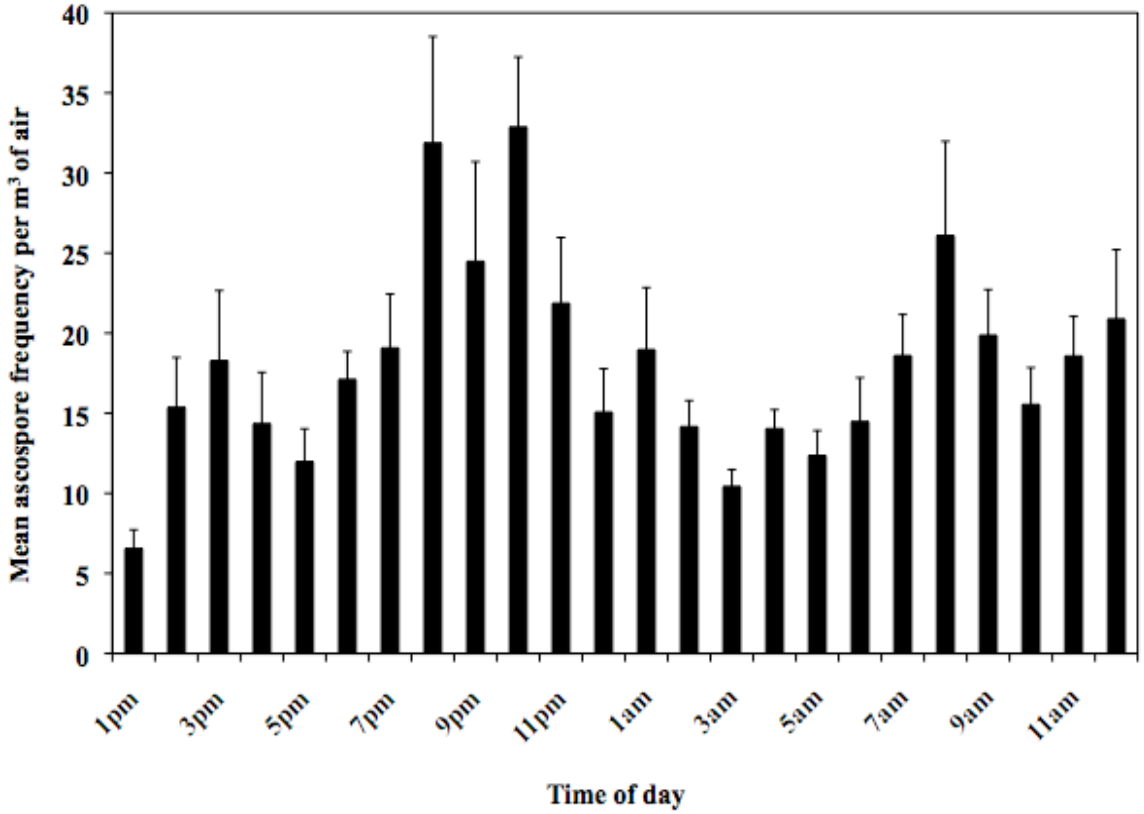

Fig. 11 Mean hourly ascospore frequency over one week from 1pm 11/12/2009 to 1pm 17/12/2009 at Mullion Creek, NSW. Error bars indicate standard error of the mean.

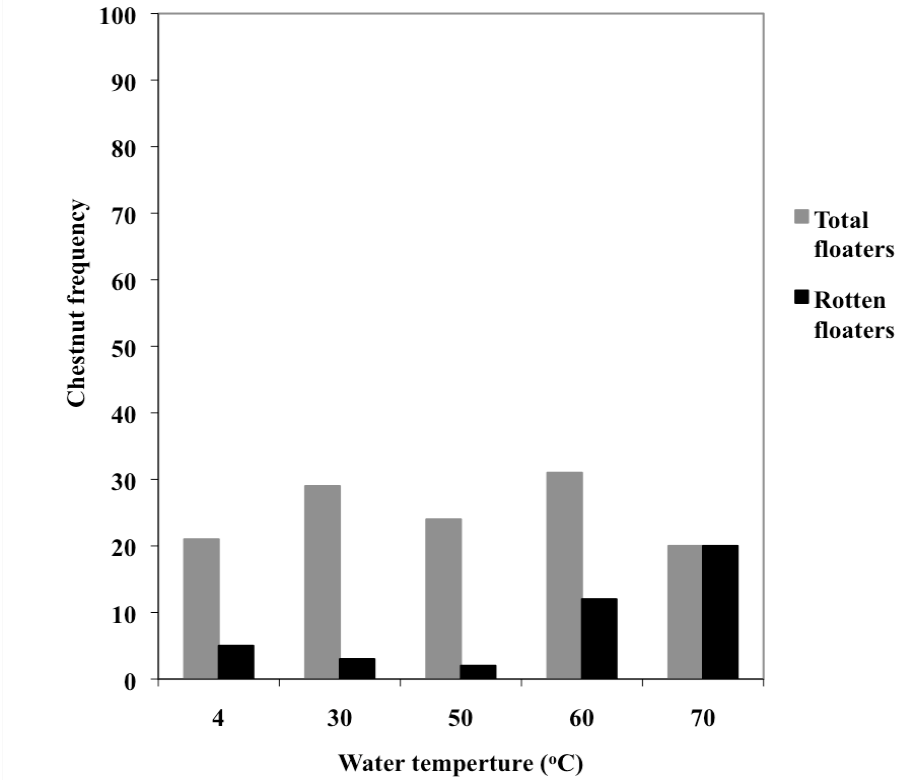

Fig. 12 Floating chestnuts and chestnuts with Chestnut Rot symptoms after flotation grading in water of various temperatures. n=100 Purton's Pride chestnuts per temperature treatment.

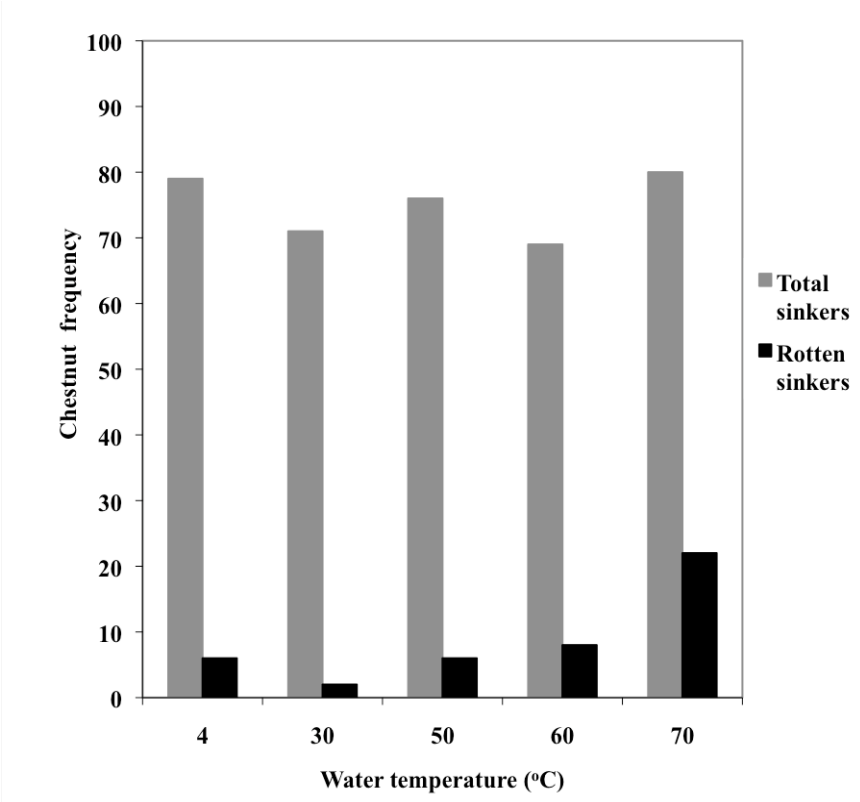

Fig. 13 Sinking chestnuts and chestnuts with Chestnut Rot symptoms after flotation grading in water of various temperatures. n=100 Purton's Pride chestnuts per temperature treatment.

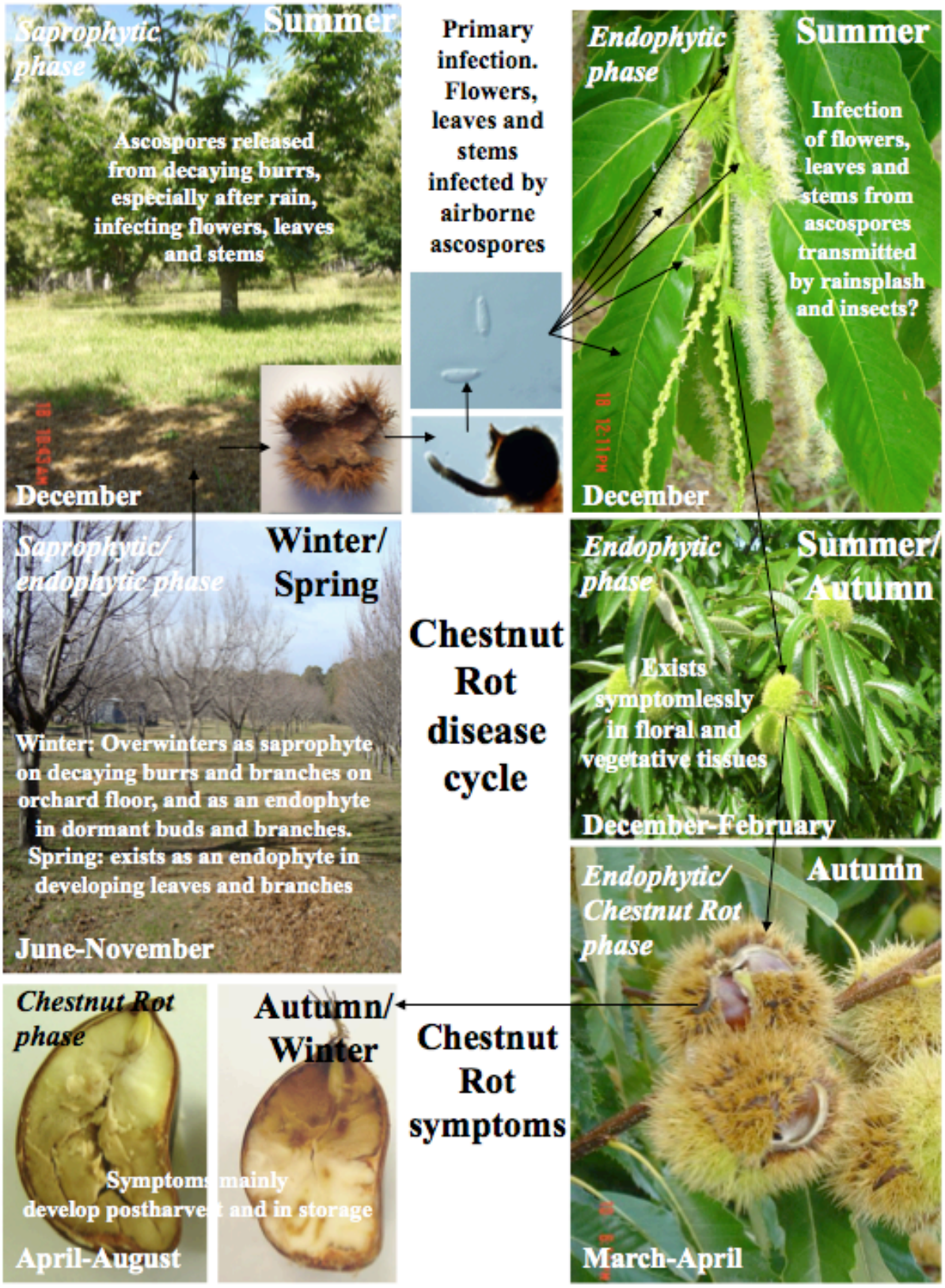

Fig. 14 The disease cycle of Chestnut Rot in *Castanea* sp. in South-Eastern Australia (Washington et al. 1999, Ogilvy 1998, Smith and Ogilvy 2008).

Table 1. Regression Analysis and Pearson Correlation Analysis and ANOVA of 2008 and 2009 Chestnut Rot incidence, and 2007 and 2008 December rainfall.

Regression Statistics	
Pearson Correlation	0.58
r^2	0.34
Standard Error	11.30
Observation	43

	Co-efficients	***Standard Error***	***t Stat***	***P-value***	***Lower 95%***	***Upper 95%***	***Lower 95%***	***Upper 95%***
Intercept	-5.37	5.08	-1.06	0.30	-15.63	4.89	-15.63	4.89
x-variable	0.21	0.05	4.59	0.00	0.12	0.30	0.12	0.30

ANOVA	***df***	***SS***	***MS***	***F***	***Significance F***
Regression	1	2691.7	2691.7	21.0	0.0000
Residual	41	5234.7	127.68		
Total	42	7926.5			

Table 2. Chestnut Rot incidence at Flemington Markets, NSW sampled in July 2008 and July 2009. NS=not sampled. n=100 chestnuts per orchard per variety per year.

Orchard location	Variety	Market Chestnut Rot incidence (%)	
		2008	2009
Bright VIC	Purton's Pride	2	NS
Wandiligong VIC	Purton's Pride	5	NS
Fumina VIC	Purton's Pride	10	NS
Tumbarumba NSW	Purton's Pride	NS	4
Tumbarumba NSW	Red Spanish	NS	9
Batlow NSW	Decoppi Marone	9	NS
Batlow NSW	Purton's Pride	NS	6
Hoskinstown NSW	Decoppi Marone	4	NS

Table 3. Isolation frequency (%) of the Chestnut Rot fungus isolated as an endophyte from various chestnut tissues and varieties (Decoppi Marone, Purton's Pride, Red Spanish) in Mullion Creek NSW. - = tissue type not available due to phenological stage. NS= not sampled. 75 samples per tissue type per month were tested.

Tissue type	December 2008	February 2009	April 2009	August 2009	December 2009
Female flowers	82	-	-	-	10
Male flowers (dead in February and April)	59 (n=30)	28 (n=30)	53 (n=30)	-	3
Dead styles	-	47	40	-	0
Pedicels	28	32	60	-	0
Burr equators	-	36	65	-	0
Shell equators	-	3	21	-	0
Kernels	-	0	16	-	0
Petioles	9	9	17	-	0
Terminal leaf mid-veins	9	21	25	-	0
Terminal leaf margins	33	39	52	-	0
Current-year stems	17	21	16	25	10
2-year-old stems	8	5	1	7	20
3-year-old stems - xylem	0	0	NS	0	0
- bark	3	0	NS	0	0
4-year-old stems - xylem	0	0	NS	0	0
- bark	3	0	NS	1	0
Dormant terminal buds	-	-	-	41	-

Acknowledgements

I would like to acknowledge and thank the University of Sydney, the Faculty of Agriculture, Food and Natural Resources, Horticulture Australia Ltd, Chestnuts Australia Inc. and the Royal Botanic Garden, Sydney for their financial assistance. My supervisors Professor David Guest and Dr. Edward Liew for their guidance and support. Thank-you to all the Australian chestnut growers and members of Chestnuts Australia Inc. who were involved with the project, particularly David and Margaret Ogilvy, David and Helen McIntyre, Heather and John Kane, Brian and Jane Casey. Thanks to Tanya Edwards, Communications Officer, Chestnuts Australia Inc. for her interest and enthusiasm. Thank-you to all the chestnut growers involved in the orchard surveys. Thanks to Matthew Laurence for helping with phylogenetics, Carolyn Blomley for help endophytes, and Ameera Yousiph, Suneetha Medis, Rosalie Daniel, Sarah Dunstan, Zoe-Joy Newby, Julie Bates for their help in the laboratory. Thanks to Catherine Wardrop, Royal Botanic Garden, Sydney for completing the scientific illustration and Karen Rinkel, Royal Botanic Garden, Sydney for help with illustrating the phylogenetic trees.

References

Adams Jr. GC, Gottwald TR, Leach CM (1986) Environmental factors initiating liberation of conidia of Powdery Mildews. Phytopathology 76, 11: 1239-1245.

Anderson CR (1993) A study of *Phomopsis castanea* and post-harvest decay of chestnuts. B. Sc. (Hons.) Thesis. School of Botany, The University of Melbourne. Melbourne, Australia.

Arisan-Atac I, Heidenreich E, Kubicek CP (1995) Random amplified polymorphic DNA fingerprinting identifies subgroups of *Trichoderma viride* and other *Trichoderma* sp. capable of Chestnut Blight biocontrol. FEMS Microbiology Letters. 126: 249–256.

Baudry A, Robin C (1996) Chestnut trees: plants, wood, fruits. A note on their state of health in France. Arboriculture Fruitière 499: 21-28.

Bolay A (1971) Contribution a la connaissance de *Gnomonia comari* Karsten (syn. *G. fructicola* [Arnaud] Fall). Etude taxonomique, phytopathologiqueet recherches sur sa croissance in vitro. Ber Schweizerischen Bot Ges 81: 398–482.

Breisch H (2008) Châtaignes: le point sur les pourritures. Infos-Ctifl 240: 30-33.

CABI (2004) Crop protection compendium. Global Module. Central for Agricultural Bioscience International (CABI). CAB International. Wallingford U.K.

CAI (2011) Chestnut Blight Info. Chestnuts Australia Inc. Accessed 2011. http://www.Chestnutsaustralia.com.au/Chestnut-blight-info.html

Camici L (1948) La "mummificazione delle castagne" da *Phomopsis viterbensis* sp. n. Annali della sperimentazione agraria. Nuova Serie 2: 557-566.

Carroll G (1988) Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. Ecology 69: 2–9.

Carroll GC, Carroll FE (1978) Studies on the incidence of coniferous needle endophytes in the Pacific Northwest. Canadian Journal of Botany 56: 3034-3043.

Chambers SM, Scott ES (1995) In-vitro antagonism of *Phytophthora cinnamomi* and *Phytophthora citricola* by isolates of *Trichoderma* spp. and *Gliocladium virens*. Journal of Phytopathology. 143, 8: 471-477.

Ciferri R (1951) La "mummificazione" delle castagne da *Phomopsis endogena* (Spe.) nobis, n. comb. Notiziario delle Malattie delle piante 8: 36-37.

Cohen SD (2004). Endophytic-host selectivity of *Discula umbrinella* on *Quercus alba* and *Quercus rubra* characterised by infection, pathogenicity and mycelial compatability. European Journal of Plant Patholology 110: 713–721.

Dafni A, Motte Maués M (1998) A rapid and simple procedure to determine stigma receptivity. Sexual Plant Reproduction. 11: 177-180.

Dar MA, Rai, MK (2011) Genetic diversity among isolates of *Cryphonectria parasitica,* cause of Chestnut Blight of *Castanea sativa* in India. PhD Thesis. Department Of Biotechnology, Sant Gadge Baba Amravati University. Amravati, Mahrashtra, India. Unpublished.

Dekker J (1986) Preventing and managing pesticide resistance. Pesticide resistance: Strategies and tactics for management. National Academy Press. Washington DC, USA 347-354.

Donis Gonzalez I.R, Fulbright DW, Ryser ET, Guyer D (2009) Shell mould and kernel decay of fresh chestnuts in Michigan. I European Congress on Chestnut - Castanea 2009. Acta Horticulturae 866: 353-358.

Giacalone G, Bounous G (1993) Tradizione ed innovazione nella trasformazione e nell'utilizzo delle castagne. Monti Boschi 44: 33–41.

Gentile S, Valentino, D, Visentin I, Tamietti, G (2009a) An epidemic of *Gnomonia pascoe* on nuts of *Castanea sativa* in the Cuneo area. I European Congress on Chestnut - Castanea 2009 Acta Horticulturae 866: 363-368.

Gentile S, Valentino D, Visentin I, Tamietti G (2009b) *Discula pascoe* infections of Sweet Chestnut fruits in North-West Italy. The Australian Nutgrower, December 2009. 23-25.

Geoscience Australia (2006) Commonwealth of Australia. Geocat:61756. http://www.ga.gov.au/image_cache/GA5566.pdf

HAL (2007) Chestnut industry strategic plan 2006–2010 Version 8. Horticulture Australia Ltd. Sydney Australia. Accessed 2011. http://cms2live.horticulture.com.au/admin/assets/library/strategic_plans/pdfs/PDF_File_6.pdf

Humpherson-Jones FM (1992) The development of weather-related disease forecasts for vegetable crops in the UK. Problems and prospects. OEPP/EPPO Bulletin 21: 425-429.

Jackson GVH, Newhook, FJ (1978) Sources of *Phytophthora palmivora* inoculum in Solomon Island cocoa plantations. Transactions of the British Mycological Society 71: 239-249.

Jenkins A (2005) Soil fungi. Soil biology basics. Department of Primary Industries, New South Wales. Australia. Accessed 2011. http://www.dpi.nsw.gov.au/__data/assets/pdf_file/0020/41645/Soil_fungi.pdf

Jermini M, Condera M, Sieber TN, Sassella A. Schärer H, Jelmini G, Höhn E (2006) Influence of fruit treatments on perishability during cold storage of Sweet Chestnuts. Journal of the Food and Science of Agriculture 86, 6: 877-885.

Juniper BE (1991) The leaf from the inside and the outside: a microbe's perspective. In: Andrews JH, Hirano SS (Eds), Microbial Ecology of Leaves, Springer-Verlag. New York, USA 21–42.

Klinac DJ, Seelye R, Knowles R, Nguyen H (1999) Chestnut production and chestnut research in New Zealand. Acta Horticulturae 494: 43-48.

Knox B, Ladiges P, Evans B, Saint R (2005) Biology: An Australian Focus. 3rd Edition. McGraw–Hill Australia Pty Ltd. Sydney, Australia. 901.

Konam JK, Guest DI (2004) Role of beetles (Coleoptera: Scolytidae and Nitidulidae) in the spread of *Phytophthora palmivora* Pod Rot of cocoa in Papua New Guinea. Australasian Plant Pathology 33, 1: 55 – 59.

Ma Z, Michailides TJ (2005) Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Protection 24: 853-863.

Maas JL (1998) Compendium of strawberry diseases. 2nd Ed. St Paul. MN. USA APS Press. 1–98.

Magro P, Speranza, S, Stacchiotti M, Martignoni D, Paparatti B (2010) *Gnomoniopsis* associated with necrosis of leaves and chestnut galls induced by *Dryocosmus kuriphilus*. New Disease Reports. British Society for Plant Pathology UK. 21, 15.

McCartney HA, Lacey ME, Li Q, Heran A (1999) Airborne ascospore concentration and the infection of oilseed rape and sunflowers by *Sclerotinia sclerotiorum.* The 10th International Rapeseed Congress. Canberra, Australia.

McLaren D (1999) Industry information. The Chestnut Growers Handbook. Chestnuts Australia Inc. Accessed 2011. http://www.chestnutsaustralia.com.au/growing-chestnuts/industry-information.html

Mignani I, Vercesi A (2003) Effects of postharvest treatments and storage conditions on chestnut quality. Acta Horticulturae. 600: 781-785.

Mondal SN, Gottwald TR and Timmer LW (2003) Environmental factors affecting the release and dispersal of ascospores of *Mycosphaerella citri.* Phytopathology. 93, 8: 1031-1036.

Monod M (1983) Monographie taxonomique des *Gnomoniaceae* (Ascomycétes de l'ordre des *Diaporthales* I). Beihefte Sydowia 9:1–315.

Montealegre JA, Gonzalez SM (1986) Fungi causing rots on *Castanea sativa* fruits. Siemente 56: 166–169.

Morris SC (2006) Recommended Chestnut Storage and Handling Protocols. Sydney Postharvest Laboratory. Sydney, Australia. Accessed 2011. http://www.postharvest.com.au/Chestnut_Postharvest.htm

Ogilvy D (1998) *Phomopsis* when does it strike? The Australian Nutgrower. December 1998-February 1999, 16-18.

Osmonalieva A, McNeil DL, Stewart A, Klinac DJ, Wadia KDR (2001) *Phomopsis castanea* infection of chestnut in New Zealand. The Regional Institute. Australian Society of Agronomy. Accessed 2011. http://www.regional.org.au/au/asa/2001/p/11/osmonalieva.htm

Puttoo BL, Bhat AA, Wattal SK (1988) Dry Rot of edible chestnut in India. Progressive Horticulture 20: 171–172.

Rieger M (2005) Chestnuts. *Castanea* spp. Introduction to fruit crops. CRC Press, USA.

Rinaudo J, Guest P, McIntyre D (2009) Internal rot. Best Practice Protocol. Chestnuts Australia Inc. website. Accessed 2011. http://www.chestnutsaustralia.com.au/growing-chestnuts/internal-rot/best-practice-protocol.html

Robin C, Heiniger U (2001) Chestnut Blight in Europe: Diversity of *Cryphonectria parasitica*, hypovirulence and biocontrol. Forest Snow and Landscape Research 76, 3: 361-367.

Rutter PA, Miller G, Payne JA (1990) Chestnuts (*Castanea*). Acta Horticulturae 290: 761-788.

Schreiner RP, Bethlenfalvay GJ (2005) Mycorrhizae, biocides, and biocontrol 3. Effects of three different fungicides on developmental stages of three AM fungi. Biology and Fertility of Soils 24, 1: 18-26.

Servazzi O (1941) Bol. Lab. Sper. R. Oss. Fitopat. Torino. 17, 1-5 (estr.) (VIII, 3-8). In:

Shuttleworth LA (2012) The biology and management of Chestnut Rot in South-Eastern Australia. PhD Thesis. University of Sydney. Faculty of Agriculture, Food and Natural Resources.

Sieber TN (2007) Endophytic fungi in forest trees: are they mutualists? Fungal Biology Reviews, 21: 75-89.

Sinclair WA, Lyon HH (2005). Diseases of Trees and Shrubs, 2nd Edition. Cornell University, Ithaca, NY. USA

Smith HC, Ogilvy D (2008) Nut rot in chestnuts. The Australian Nutgrower. June 2008. 22,2: 10–15.

Sogonov MV, Castlebury LA, Rossman A, White JF (2007). The type of species of *Apiognomonia, Apiognomonia veneta*, with its *Discula* anamorph is distinct from *Apiognomonia errabunda*. Mycological Research 111: 693–709.

Sogonov MV, Castlebury LA, Rossman AY, Mejia LC, White JF (2008a) Leaf–inhabiting genera of the *Gnomoniaceae*, *Diaporthales.* Studies in Mycology 62, 1: 1–77.

Sogonov MV, Castlebury LA, Rossman AY, Mejia LC, White JF (2008b) Leaf–inhabiting genera of the *Gnomoniaceae*, *Diaporthales.* Studies in Mycology 62, 1: 1–77. GenBank submission numbers: EU254851, EU254854, EU254855.

Spegazzini C (1879) Nova addenda ad Mycologiam Venetam. Michelia 1,5: 453-487.

Tamietti G, Gentile S, Visentin I, Valentino D. (2009) *Discula pascoe* in Italy. GenBank submission (HM142944-HM142962). Accessed 2011. http://blast.ncbi.nlm.nih.gov/Blast.cgi

Wadia KD, Klinac D, McNeil DL, Osmonalieva A, Stewart A, Knowles RD (2000) Occurrence of *Phomopsis castanea* as an endophyte in chestnut trees. New Zealand Plant Protection. 53: 133–137.

Walker D, Castlebury LA, Rossman AY, Sogonov MV, White JF (2010) Systematics of genus *Gnomoniopsis* (*Gnomoniaceae, Diaporthales*) based on a three gene phylogeny, host associations and morphology. Mycologia 102, 6: 1479-1496.

Wang WF, Xiao JH, Li GH (2000) On the occurring dynamics of Chinese Chestnut postharvest disease in Jianxi. Acta Agric Universitatis Jiangxiensis 22, 2: 246-249.

Washington WS, Allen AD, Dooley LB (1993) *Phomopsis castanea* and other organisms associated with chestnut fruit rots in Victoria. Proceedings of the 9th Biennial Conference of the A.P.P.S. Hobart, Australia. Abstract 118.

Washington WS, Allen AD, Dooley LB (1997) Preliminary studies on *Phomopsis castanea* and other organisms associated with healthy and rotted chestnut fruit in storage. Australasian Plant Pathology 26: 37-43.

Washington WS, Hood V, Goubran F, Hepworth G, Stewart-Wade S (1998) Effect of fungicides applied as foliar sprays and trunk injections on nut rot of chestnuts caused by *Phomopsis castanea* in Victoria. Australian Journal of Experimental Agriculture 38: 295–303.

Washington WS, Stewart-Wade S, Hood V (1999) *Phomopsis castanea*. A seed-borne endophyte in chestnut trees. Australian Journal of Botany 47: 77-84.

Wright WR (1960) Storage decays of domestically grown chestnuts. Plant Disease Reports 44: 820-825.

Wu XQ, Lin SY, Xiong CH, Yang XC (2001) Effects of different treatments on the quality and the micro-organisms kinds and quantities of chestnuts in storage. Journal of Nanjing Forestry University 25, 3: 47-51.

You MP, Sivasithamparam K (1995) Changes in microbial populations of an avocado plantation mulch suppressive of *Phytophthora cinnamomi.* Applied Soil Ecology 2, 1: 33-43.

Zadco (2011) Biologicals. Quality Grow Pty Ltd. All Rights reserved. Accessed 2011. http://www.zadco.com.au/