Cooperative Research Centre for Viticulture

Australian Government
Grape and Wine Research and Development Corporation

MANAGING GRAPEVINE TRUNK DISEASES (PETRI DISEASE, ESCA, AND OTHERS) THAT THREATEN THE SUSTAINABILITY OF AUSTRALIAN VITICULTURE



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

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The Cooperative Research Centre for Viticulture is a joint venture between the following core participants, working with a wide range of supporting participants.

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Abstract

Grapevine trunk diseases cause decline and death of vines, thus severely limiting the sustainability of Australian vineyards. Little was known about the contributory factors of these diseases, how to prevent them, or how to minimise their economic impact when this project began. Petri disease, which causes significant losses during the vineyard establishment phase, was shown to be widespread, whereas esca, currently the most serious grapevine disease in Europe, was shown to be rare in Australia. The causal organism, *Phaeomoniella chlamydospora*, is spread from infected mother vines into cuttings, and into newly planted vineyards via infected planting material. Long duration (30 minutes) hot water treatment of dormant cuttings was demonstrated to be an effective method of reducing the risk of producing infected planting material. Best practice protocols for the nursery industry were developed and promoted to encourage production of *P. chlamydospora*-free planting material. Glasshouse studies demonstrated that infected grapevines are more susceptible to water stress. Field trials showed that management practices that reduced stress, such as the use of mulch, reversed the symptoms of decline in infected grapevines. Recently, the number of reports of Petri disease has dropped considerably, suggesting that industry now has the tools both to prevent the spread and to minimise losses attributable to these diseases as a result of this research.

Vale Eve Hilda Cottral (15th September 1976 – 6th October 2003)

This final report is dedicated to the memory of Eve Cottral, our PhD student on the project who sadly passed away before completing her studies.

Executive Summary

Grapevine trunk diseases are caused by xylem-inhabiting fungi that cause the slow decline and loss of productivity in vines at all stages of growth. They either infect propagating material and limit the growth of newly planted vines, or they infect established vines through pruning wounds and cause loss of productivity, just as vines are reaching an elite stage of maturity.

Petri disease, formerly known as black goo, is an emerging problem in international grapevine culture. The description of several possible causal organisms, including *Phaeomoniella chlamydospora*, in 1996 led to the realisation that both the pathogens and the disease are widespread and important. However, there is as yet little understanding of the disease or of its economic effects. The same pathogens are involved in esca, currently the most destructive disease of grapevines in Europe. This project set about to determine which pathogens are the most important, how infection occurs, how it is spread, whether it can be prevented and effective methods of managing infected vineyards.

As a result of this project, it is now known that:

- esca is rare in Australia, whereas Petri disease is widespread.
- the causal pathogen of both diseases is *Phaeomoniella chlamydospora.*
- *Pa. chlamydospora* sporulates in cracks on the grapevine trunks associated with machinery damage
- infection is passed from mother vines into cuttings, resulting in symptomless infected planting material
- *Pa. chlamydospora* spores can be detected in water used during nursery propagation, indicating there is the potential for further infection
- Hot water treatment of dormant cuttings is the most effective method of reducing the incidence of infected planting material
- Best practice nursery protocols were developed and disseminated through Workshops and articles
- *Pa. chlamydospora* infection in grapevines increases stomatal conductance and reduces xylem function, resulting in reduced capacity to respond to water stress
- Vineyards can recover from Petri disease over time with management practices that reduce stress eg mulch, bunch-thinning.

Samples of declining vines were examined during the past 5 years, establishing that *Pa chlamydospora* is the pathogen causing these diseases in Australia. The proportion of grapevines with Petri disease (82%) proved to be much more than those with esca (18%). Other fungi associated with grapevine decline diseases, such as *Phaeoacremonium aleophilum*, species of *Phomopsis, Cylindrocarpon, Botryosphaeria* and heart-rotting basidiomycetes, were not consistently isolated. Thirty-two different grapevine cultivars were represented, and samples were received from most major grape-growing regions of New South Wales, South Australia, Victoria and Western Australia. Sixty-two percent were of ungrafted grapevines. *Pa. chlamydospora* was also found to be present in grapevines with no external symptoms of disease, including mother grapevines used for propagation.

Pa. chlamydospora is a vascular pathogen that colonises the xylem tissues of the grapevine. Examination of infection in tissue-cultured Chardonnay vines clearly showed the fungus travelling both between and inside parenchyma ray cells adjacent to vessels and in the vessels themselves. Hyphae passed between vessels predominantly via pit membranes. Both infected and uninfected plants appeared to have a large amount of cell disruption and wall breakage in this study, and hyphae were observed in close association with wall breaks. If this wall discontinuity is natural, grapevine stems are particularly easy targets for fungal colonisation.

Pathogenicity studies showed that *Pa. chlamydospora* caused poor callus production, graft failure and death in cuttings, while *Pm aleophilum* was less virulent. Rootstock cultivars were more susceptible than *Vitis vinifera* cultivars. Isolates of *Pa. chlamydospora* were genetically similar, suggesting the population is clonal. Considerable variation was found among isolates of *Pm. aleophilum*, and subsequent investigations showed that they represented three *Phaeoacremonium* species: *Pm.*

aleophilum, Pm. parasiticum and a new species *Pm. australiense.* In addition, the sexual state of *Pm. aleophilum* was found on grapevine wood and identified as *Togninia minima*.

In Europe, esca is reportedly caused by the progressive infection of *Pa. chlamydospora, Pm. aleophilum* and *Fomitiporia mediterranea* in mature grapevines, leading to white heart rot and death. During this project, we examined young grapevines with esca symptoms that had no associated heart rot, but were always infected with *Pa. chlamydospora.* The symptoms occurred when the infected vines were subjected to water and heat stress in summer. We also examined mature grapevines with white heart rot, but without esca leaf and fruit symptoms. Several species of unknown basidiomycete fungi were isolated from the white heart rots, the most common of which we described as a new species, *Fomitiporia australiensis. F. australiensis* was shown to also occur in Australian native plants surrounding vineyards, such as native hop-bush and *Eucalyptus* species. There was no evidence of *Fomitiporia mediterranea.* Our conclusion is that *Phaeomoniella chlamydospora* is the causal organism of both esca and Petri disease in Australia, and that *Phaeoacremonium* species and the heart rotting basidiomycetes are incidental and not necessarily indicative of disease.

In the light of these results we concentrated our efforts on *Pa. chlamydospora* and understanding how it is spread. When this project began, it was unknown where *Pa. chlamydospora* produced its spores in the vineyard. We observed abundant sporulation on protected wood surfaces inside deep cracks on several grapevine varieties. Springtails and mites were usually associated with the sporulation, but we could not determine whether they play a role in dissemination of conidia. This was the first report of how and where this fungus produced spores in the field. We demonstrated that both *Pa. chlamydospora* and *Pm aleophilum* are spread in cuttings taken from infected mother vines and result in symptomless infected planting material. A survey of *V. vinifera* mother vines showed that the majority was infected with *Pa. chlamydospora*, and previous research had shown that a majority of rootstock mother vines are also infected. This presents a challenge for those trying to establish clean germplasm collections and provide clean planting material to industry. The evidence for spread occurring through soil was inconclusive.

In order to examine whether further spread occurs during nursery procedures, water, callus media and plant material were sampled from commercial nurseries during three propagating seasons: 2000, 2003, 2005. Various methods to detect *Pa. chlamydospora* in the samples were tested and compared. Nested PCR and quantitative PCR were the most sensitive, although further research is required to optimise their use. All water samples tested positive for *Pa. chlamydospora* with at least one method, indicating that nursery propagation is a potential source of infection.

Several treatments were tested for eradication of infection in cuttings, and hot water treatment was the only consistently effective method. Hot water treatment of infected Cabernet Sauvignon, Chardonnay, Ramsey, Verdelho and Zinfandel cuttings significantly reduced the incidence of infected young vines in five separate experiments from 2000-2005. There is a perception in the nursery industry that hot water treatment causes unacceptable losses. Experiments examining the effects on cellular metabolism and respiration indicated that excessive hydration and incorrect post-treatment handling cause damage to cuttings. We recommend that all grapevine cuttings should be routinely hot water treated to reduce the chances of selling symptomless infected planting material to growers, and that growers use only hot water treated material to minimise Petri disease. Best practice protocols for the nursery industry were developed and delivered through a series of Workshops called 'Making Every Stick Count', and articles in industry magazines.

Petri disease is reported to be a 'stress-related' disease. Examination of field grown Verdelho showed that infection reduced xylem function by 15% for each 1% increase in 'goo'-blocked vessels. In glasshouse experiments using Zinfandel, Cabernet Sauvignon and Chardonnay, stomatal conductance was higher in infected plants, implying that infection causes an increase in respiration and/or interferes with stomatal control. In Zinfandel and Cabernet Sauvignon, leaf water potentials were lower in infected plants subjected to water stress, indicating that infection made it more difficult for the vine to get water to the leaf. This is supported by the relationship shown above for loss of xylem function. This was less apparent in Chardonnay. Clearly, infection alters the grapevine response to water stress and some cultivars are more affected than others.

Four field trials were conducted to develop management strategies for infected vineyards. Treatments included applications of compost, nutrient fertilisers, phosphonate and Brotomax and vine health was monitored over two to five years. To summarise, no single treatment was effective, although compost showed most promise. However, the grapevines recovered from Petri disease over time. The evidence suggests that a combination of stress and infection with *Pa. chlamydospora* is a major factor in development of the disease, particularly stress due to inadequate irrigation and overcropping before the vine has established a decent root system. It is not possible to remove the infection, but it is possible to alleviate the stress, allowing the vines time to recover. It is still unknown, however, whether the recovery is permanent or whether disease expression will re-occur sometime in the future.

Progress towards the development of a rapid non-destructive diagnostic tool for research and industry purposes was cut short by the untimely death of PhD student Eve Cottral. She was investigating the use of a unique hydroxy-benzaldehyde compound as a signature marker of *Pa. chlamydospora* infection. Unfortunately, after she died, we were unable to resolve which isomer of the compound was the appropriate one to use. New developments have shown the potential for using real-time PCR as a method of detecting symptomless infection in plant material. Such a tool is sorely needed and this is an area of research that should be continued.

Many people and organisations contributed to the success of this project.

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- And the Australian viticulture industry.

Background

More than 37,000 hectares of new vineyards were planted in Australia during 1993-1999, with almost 25,000 ha planted over the 3 year period from 1997-1999. This extremely rapid expansion of the wine industry placed unprecedented demand on the availability of planting material and frequently resulted in the use of substandard planting material.

While Petri disease (formerly known as black goo decline) is not a new disease (merely recently recognised), its negative impact on the vigour of cuttings/graftling/rootlings, may, in previous periods of lower demand, have led to rejection of a majority of infected material. However, the demand for planting material has resulted in very poor quality, probably infected, material being planted. The discovery by Pascoe (1999) that a majority of rootstock mother vines are infected with *Phaeomoniella chlamydospora* means that use of a high proportion of infected planting material may be inevitable (no data is available on infection rates of *V. vinifera* mother stocks). Historically, grapevine improvement schemes depended exclusively on indexing for viruses for disease management aspects of the schemes. Neglect of possible fungal infections of source material has resulted in a failure to appreciate the threat posed by fungi such as *Pa. chlamydospora*.

The demand for planting material is such that not using material from infected sources would result in a slowing of the growth of the industry that is so vital to achieving the Strategy 2025 target of "$4.5 billion in annual sales by being the world's most influential and profitable supplier of branded wines, pioneering wine as a universal first choice lifestyle beverage". But using this material may result in a similar slow down in production, at greater cost than simply not planting the material in the first place. At least one nursery has been involved in litigation over supply of infected cuttings, and a number of new vineyards have been almost completely devastated by losses due to the disease.

It is believed that Petri disease has the capacity to very severely impact on the sustainability of the industry's expansion. Involvement of the fungus in parent material for vine propagation means that a very high proportion of new vineyards will be affected. Even if these vineyards do not fail to establish it is likely that vines will begin to decline as they mature. The diagnosis of esca in Australian vineyards in 1998-99 indicates that these vines are also at risk of developing esca as they reach 10 years old and beyond. It is also possible that vines weakened by Petri disease will be more susceptible to infection by *Eutypa lata*.

Anecdotal evidence (supported by diagnostic service data) from some vineyards suggests that as a result of the combined effects of Eutypa dieback, Petri disease, and possibly esca, whole blocks of premium vines have ceased to be economically viable. The recent discovery of esca (for which *Pa. chlamydospora* acts as the pioneering infection) increases the urgency with which research must find a way of preventing infection by *Pa. chlamydospora*. We suggest that an explosion in the incidence and severity of trunk diseases can be expected within 10-15 years.

Preliminary and Previous Research

The name black goo was coined by Morton (1995, 1997) who observed serious declines of newly planted vineyards in America, noted the consistent association with the black goo symptom, and was concerned that there appeared to be no satisfactory diagnosis of the problem available. European work of Larignon and colleagues in France and Mugnai and colleagues in Italy, routinely isolated two undescribed fungi from vines affected by esca. Mugnai was a co-author of the names *Phaeoacremonium chlamydosporum* and *Phaeoacremonium aleophilum* when they were described by Crous et al. (1996). This stimulated worldwide interest in these fungi and their role in grapevine decline. Morton and Chiarappa proposed the formation of the International Council of Grapevine Trunk Diseases and organised its first meeting in California in July 1998 (Morton, 1999). That meeting brought together workers on trunk diseases from around the world and resulted in a synthesis of existing knowledge and experience to provide an enhanced understanding of black goo decline. A comprehensive review of esca disease by Mugnai *et al.* (1999) provides valuable baseline data that is applicable to research on black goo decline. This has led to an acceptance that *Pa. chlamydospora* is

both the causal agent of black goo decline in its own right, and one component of the complex etiology of esca disease. Note that the name black goo decline was not universally accepted amongst grapevine pathologists, and was changed to Petri disease in October 2001.

Only preliminary research on Petri disease has been conducted in Australia or anywhere in the world prior to this current project. The causal agent, *Phaeomoniella chlamydospora,* was first described in late 1996 (although it was renamed and assigned to its own genus by Crous and Gams in 2000) and almost all research dates from that point. Exceptions are Petri (1912) who worked on a disease which he called "legno nero" (black vein) disease and described a fungus which was almost certainly the fungus now known as *P. chlamydospora*, and Chiarappa (1959) who found a fungus associated with black measles which has since been shown to be *Pa. chlamydospora*. The doctoral thesis of Larignon (1991) began a series of European research on esca disease which has led to an acceptance that *P. chlamydospora* is the pioneering fungus in the development of that disease. Esca is believed to be a complex disease caused by initial infection with *Pa. chlamydospora* which preconditions the wood for infection by *Fomitiporia punctata*, which ultimately causes the classic esca symptoms (Mugnai *et al.* 1999). In this context, research on the initiation of esca has provided valuable early data on the development of Petri disease. However, most of that research has concentrated on mature vines and little has been done on infected planting material.

Larignon and colleagues in France (Larignon and Dubos 1997, Larignon 1999) demonstrated that *P. chlamydospora* is capable of infecting vines through pruning wounds and that wounds made early in the dormant season are much more susceptible than wounds made later.

Mugnai and colleagues in Italy (Mugnai *et al.* 1996) have shown that *Pa. chlamydospora* is the most common fungus in esca vines and that it is consistently associated with black streaks. Bertelli *et al.* (1998) were able to detect the fungus in unplanted symptomless grafted cuttings.

Ferreira in South Africa (Ferreira *et al.* 1994) has shown that the fungus (then called *Phialophora parasitica*) is associated with graft failures, and succeeded in causing graft failure by inoculation of graft surfaces during grafting. Ferreira (pers. comm.) and Larignon (pers. comm.) have conducted preliminary experiments which indicate that hot water treatment may eliminate the fungus from infected cuttings.

Pascoe and Mebalds (Pascoe 1999) surveyed a number of rootstock mother vine blocks and demonstrated that the fungus could be isolated from a high proportion of mother vines. Crop Health Services (Knoxfield) has detected *Pa. chlamydospora* from over 50 vineyards since early 1997, and these records cover almost all major grape growing areas in Australia. Most rootstock and scion varieties appear to be affected and there is no indication of any resistant varieties. It appears that infection of the rootstock in grafted vines has the greatest negative impact on the establishment of new vineyards, but the effect of the disease in own-rooted vines is not known. The proportion of infected vines derived from infected propagating material and the role that contamination within the nursery plays is also unknown.

Three scientific papers were published by team members arising from preliminary investigations during the development of this project:

1. Pascoe IG (1999) Grapevine trunk diseases in Australia: diagnostics and taxonomy. In: *Black goo – occurrence and symptoms of grapevine declines – IAS/ICGTD proceedings 1998*, ed. L. Morton, International Ampelography Society, Fort Valley, Virginia, USA.
2. Pascoe IG, Cottral EH (2000) Developments in grapevine trunk diseases research in Australia. *Phytopathologia Mediterranea* 39: 68-75.
3. Denman S, Crous PW, Taylor JE, Kang JC, Pascoe IG, Wingfield M (2000) An overview of the taxonomic history of *Botryosphaeria*, and a re-evaluation of its anamorphs based on morphology and TS rDNA phylogeny. *Studies in Mycology* 45: 129-140.

Project Aims and Performance Targets

Objectives were to develop:

- Sound protocols for the grapevine nursery industry in order to ensure that no new vineyards are planted with infected material.
- Field management strategies that are compatible with practices for growing quality wine-grapes and which can be recommended to owners of infected and uninfected vineyards.
- Enhanced understanding of the interactions between the vine, pathogen and environment and therefore increased knowledge of the epidemiology of these diseases.

Planned project outputs:

Output	Performance Targets
1. A set of nursery management strategies to prevent dispersal of *Phaeomoniella chlamydospora (Pch)* in infected propagation material.	• Complete assessment of the efficacy of hot water treatment on naturally infected cuttings • Develop optimised protocols for problem cultivars • Survey grapevine nurseries to identify potential contamination events during the propagation process and develop nursery hygiene protocols accordingly
2. Vineyard management strategies to minimise losses in Petri disease / esca infected vineyards	• Assess efficacy of products for therapeutic activity on Pch-infected vines • Assess the impact of vineyard management practices on disease expression and grape yield
3. Elucidation of the aetiology and epidemiology of Petri disease / esca in order to devise effective control strategies	• Determine the impact of Pch infection on the performance of different vine cultivars • Determine mode of spread of Pch • Identify other organisms associated with Petri disease and esca
4. Determination of the role that stress plays in disease expression of *Pch*-infected vines	• Study the physiology of disease development in relation to different levels of water stress, including those to be expected under RDI and PRD • Investigate how xylem function is affected by Pch by measuring sap flow restrictions such as cavitation and tyloses

Chapter 1: Distribution of Petri disease and Esca in Australia

Jacqueline Edwards and Ian Pascoe

Summary Over a five year period (1998 – 2002), 124 samples of diseased grapevines sent to the Department of Primary Industries, Knoxfield, in Victoria were diagnosed with the grapevine decline diseases, Petri disease and esca. The proportion of grapevines with Petri disease (82%) proved to be much more than those with esca (18%). In addition, *Phaeomoniella chlamydospora* was isolated from all but two of the samples, whereas *Phaeoacremonium aleophilum* was isolated from only nineteen samples. Other fungi associated with grapevine decline diseases, such as species of *Phomopsis, Cylindrocarpon, Botryosphaeria* and heart-rotting basidiomycetes, were also isolated but not consistently. Thirty-two different grapevine cultivars were represented, and samples were received from most major grape-growing regions of New South Wales, South Australia, Victoria and Western Australia. The majority (62%) were ungrafted grapevines. *Pa. chlamydospora* was also found to be present in grapevines with no external symptoms of disease, including mother grapevines used for propagation.

Introduction

Grapevine trunk diseases are caused by xylem-inhabiting fungi and are insidious causes of slow decline and loss of productivity in vines at all stages of growth. They either infect propagating material and limit the growth of newly planted vines, or they infect established vines through pruning wounds and cause loss of productivity just as vines are reaching an elite stage of maturity.

Petri disease, formerly known as black goo decline, is an emerging problem in international grapevine culture. The description of several possible causal organisms, including *Phaeomoniella chlamydospora*, in 1996 led to the realisation that both the pathogens and the disease are widespread and important. However, there is as yet little understanding of the disease or of its economic effects. The same pathogens are involved in esca, currently the most destructive disease of grapevines in Europe. Although esca is not a major disease in Australia, the recent increase in incidence of Petri disease in young vineyards may indicate that the incidence of esca is likely to increase.

Both *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* have been implicated as components of the aetiology of Petri disease and esca. Petri disease primarily affects young grapevines, causing establishment problems in new vineyards. Symptoms include graft failure, weak growth, shoot dieback, slow decline and gradual death of the grapevine. Often these have been attributed to other causes. The disease has a characteristic symptom of internal black wood streaking, evident when the trunk of an infected grapevine is cut open to reveal black tarry 'goo' in the affected xylem vessels. Esca was until recently believed to affect only mature grapevines fifteen years or older, but over the past decade young grapevines have also been affected (Mugnai *et al.* 1999; Edwards *et al.* 2001). The disease has both a chronic and an acute form. In the chronic form, the leaves develop interveinal chlorosis and necrosis, sometimes called 'tiger stripes', and the fruit becomes shrivelled and discoloured with minute black spots known as 'black measles'. The acute form, called 'apoplexy', is a sudden collapse of the whole grapevine and occurs during hot weather late in the season. Each symptom can occur alone or in concert, and may be present in one season then absent the next.

Although both diseases are known to be present in Australia, their importance and distribution is uncertain. Anecdotal evidence suggested that Petri disease is widespread and causes considerable economic loss, while esca is rarely seen. Certainly, esca is not causing the damage to vineyards in Australia that is occurring in Europe, where the incidence and severity has increased considerably during the past decade (Graniti *et al.* 2000) causing vineyards to be pulled out and replaced within 25 years of planting (L. Mugnai, pers. comm.). The aims of this study were to determine the distribution of these diseases and their causal organisms in Australia, and to identify whether the diseases were restricted to particular cultivars or ages of grapevines. This information is collated here with regard to grapevine samples sent to DPI Knoxfield over a five year period from 1998 to 2002.

Methods

Grapevines with symptoms of decline, dieback, graft failure or esca

From 1998 to 2002, many samples of diseased grapevines were sent from commercial vineyards and nurseries across Australia to DPI Knoxfield for diagnosis. All of the grapevine samples were cut open and examined for signs of discoloured tissue such as black wood streaking or darkened pith. Wood slivers, approx 5 x 1 mm, were taken from discoloured vascular tissue, surface sterilised with 0.5% NaOCl for 1 min, then plated onto potato dextrose agar amended with 50 ppm Achromycin® (a.i. tetracycline hydrochloride; American Cyanamid Company, USA) and incubated at room temperature (18–22°C) for 2–4 weeks. All fungi that grew were identified at least to genus and to species level where possible. Larger pieces of grapevine wood with vascular streaking were cut transversely and longitudinally to half cylinders up to 10 cm long. They were moist-incubated by placing them on moist paper towelling in suitable sized plastic boxes (take-away food containers) with lids, and incubated at room temperature (18–22°C). The paper towelling was moistened periodically as required. Samples were examined under the dissecting microscope after 2–6 weeks and the fungi present were noted.

The following information, where possible, was recorded for each sample that was received: vineyard location, grapevine cultivar, rootstock cultivar if grafted, grapevine age, disease symptoms, presence of *Pa. chlamydospora*, *Pm. aleophilum* and other known grapevine pathogens.

Grapevines were scored as having Petri disease if there were general decline symptoms and internal black wood streaking. Grapevines were scored with esca only if there were typical foliar symptoms of esca, black measles fruit symptoms and/or sudden death.

Grapevines without external symptoms

During 2000–2002, many grapevines that were removed for reasons other than disease were also examined. These included 35 foundation grapevines from Mildura; 17 mother grapevines from source blocks in Western Australia, South Australia and Victoria; nine grapevines from the student training vineyard at Burnley College in Melbourne; seven grapevines from abandoned vineyards in Kyneton and Adelaide, and occasional single grapevines from several commercial vineyards. The trunks were cut open and examined for evidence of black wood streaking and treated in the same manner as already described. Once again, the vineyard location, grapevine cultivar, rootstock cultivar if grafted, grapevine age, presence of *Pa. chlamydospora*, *Pm. aleophilum* and other known grapevine pathogens were recorded.

Results

Grapevines with symptoms of decline, dieback, graft failure or esca

Phaeomoniella chlamydospora and/or *Pm. aleophilum* were isolated from 124 samples of diseased grapevines during 1998–2002 (Table 1.1). The samples were sent in from vineyards and nurseries across most grape-growing regions of Australia, with the exception of Queensland and Tasmania. *Pa. chlamydospora* was isolated from all except two samples. *Pm. aleophilum*, on the other hand, was isolated from only 19 samples. The two diseased samples containing *Pm. aleophilum* but not *Pa. chlamydospora* came from Ti-Tree in the Northern Territory and Myrhee in north-east Victoria.

Species of *Botryosphaeria, Cylindrocarpon, Eutypa, Fomitiporia, Inonotus, Phomopsis* and other *Phellinus*-like fungi were also frequently isolated. *Botryosphaeria* spp. were the most common of these, isolated from 35% of the samples and associated with both Petri disease and esca symptoms. *Cylindrocarpon* spp. were isolated from 10% of the samples, always associated with decline symptoms in young grapevines less than 5 years old (where age was known). *Eutypa* was isolated from 11% of the samples, all 10 years or older (where age was known) and always associated with a wedge of dead wood in the trunk. *Fomitiporia, Inonotus* and *Phellinus*-like fungi, found in 10% samples, were always associated with a white heart rot in grapevines older than 15 years.

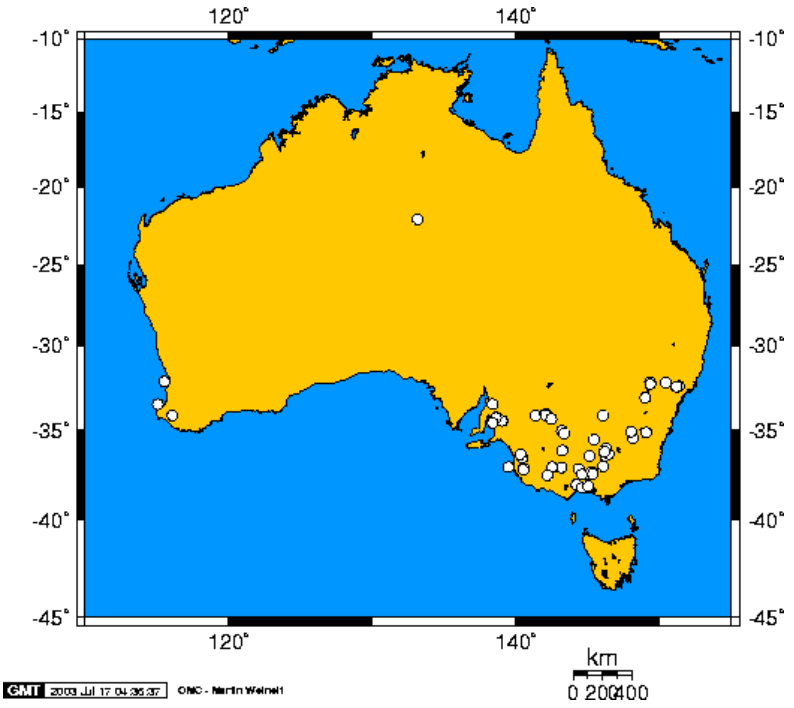


Figure 1.1: Locations of where Petri disease has been diagnosed in Australia

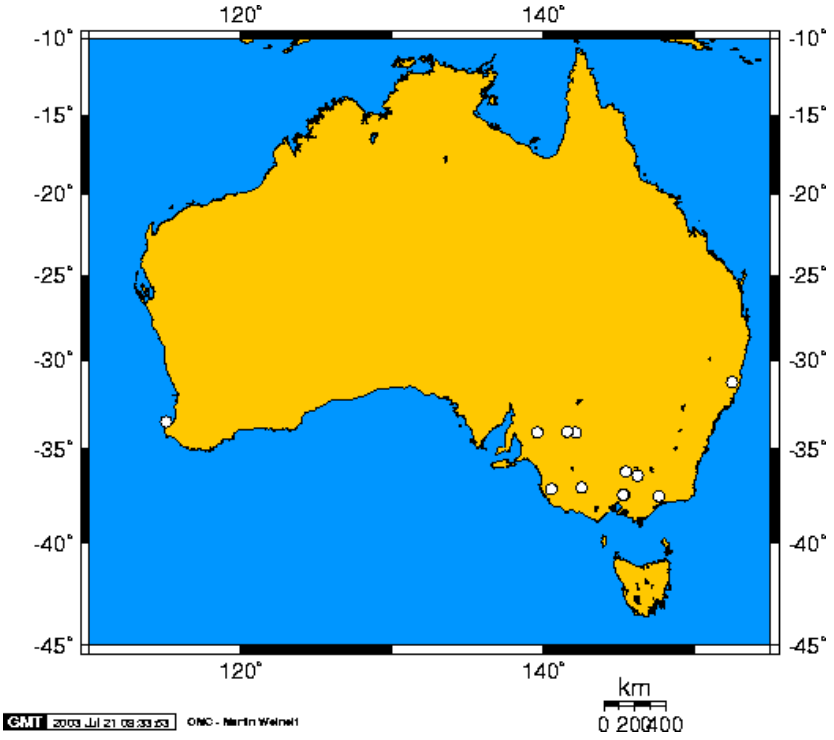


Figure 1.2: Locations of where esca has been diagnosed in Australia

The diseased grapevines ranged in age from less than one year to more than 50 years, with 64% of the samples on their own roots (ie non-grafted). Eighty-two percent of the disease symptoms expressed were characteristic of Petri disease, with only 18% showing symptoms of esca.

In the case of Petri disease (102 samples), 38% were grafted and 62% were own-rooted. The samples came from all major grape-growing regions of Australia (Figure 1.1). Sixty-two percent were less than ten years old, and the other 38% ranged from 10 to 50 years. At least 22 scion and 7 rootstock cultivars were represented (Table 1.2). The most commonly affected white varieties were Chardonnay (17%) and Semillon (13%), red varieties were Shiraz (17%), Cabernet Sauvignon (11%), Merlot (9%) and Pinot Noir (9%), and rootstocks were Ramsey (10%) and Schwarzmann (7%).

Although only 22 samples were diagnosed with esca, their distribution was also widespread and the disease was present in all four major grape-growing states (Figure 1.2). Six out of the 22 samples were grafted. Two were reworked grapevines that had been grafted onto other scion cultivars, and the others were on rootstock varieties AxR 1, Kober 5BB, Schwarzmann and SO4. At least 9 scion cultivars were represented (1.2), the predominant ones being Shiraz (32%) and Cabernet Sauvignon (23%). Seventy-two percent of the samples were 10 years or older, while the other 28% were cases of 'young esca' (Mugnai *et al*. 1999) between 2 and 7 years old.

Table 1.1 Location, cultivar and ages of grapevines with disease symptoms associated with *Phaeomoniella chlamydospora* or *Phaeoacremonium aleophilum.*

Location	Cultivar	Rootstock	Age	Symptoms (Pd/E[A])	Pch[B]	Pal[C]	Other plant pathogenic fungal genera associated
				ACT			
Evatt	Pinot Noir	None	n.k.[D]	Stunted growth (Pd)	+[E]	-	-
				New South Wales			
Barooga	Chardonnay	None	n.k.	Stunted growth (Pd)	+	-	*Botryosphaeria*
	Semillon	None	n.k.	Decline (Pd)	+	-	-
	Semillon	Kober 5BB	n.k.	Decline (Pd)	+	-	*Botryosphaeria*
Buronga	Merlot	Ramsey	n.k.	Dieback (Pd)	+	-	-
Denman	Chardonnay	None	15	Lack of fruit (Pd)	+	-	*Botryosphaeria*
	Semillon	None	2	Poor budburst (Pd)	+	-	*Botryosphaeria, Cylindrocarpon*
	Verdelho	None	2	Poor budburst (Pd)	+	-	*Botryosphaeria, Cylindrocarpon*
Griffith	Pinot Noir	None	n.k.	Decline (Pd)	+	-	*Cylindrocarpon*
	Semillon	Ramsey	4	Decline (Pd)	+	-	-
	Shiraz	None	4	Decline and death (Pd)	+	-	*Botryosphaeria*
	Shiraz	None	10	Decline and death (Pd)	+	-	*Botryosphaeria*
	Shiraz	None	50	Decline (Pd)	+	-	*Botryosphaeria*
	Trebbiano	None	30	Decline and death (Pd)	+	-	*Botryosphaeria*
	Zinfandel	Ramsey	n.k.	Decline and death (Pd)	+	+	*Cylindrocarpon*
Gulgong	Semillon	None	4	Wilting and death (Pd)	+	-	-
Maitland	Chambourcin	None	4	Dieback (Pd)	+	-	-
Mudgee	Chardonnay	None	10	Decline (Pd)	+	-	-
	Chardonnay	None	22	Dieback (Pd)	+	-	-
	Merlot	None	1	Poor growth (Pd)	+	-	-
Orange	Pinot Noir	n.k.	n.k.	Poor vigour (Pd)	+	-	-
Pokolbin	Chardonnay	Shiraz	18	Decline (Pd)	+	+	*Botryosphaeria, Phomopsis*
Tumbarumba	Sauvignon Blanc	None	10	Chlorosis, stunted growth (Pd)	+	-	*Eutypa*
Tumblong	Shiraz	Kober 5BB	2	Dieback (Pd)	+	-	*Botryosphaeria*
Wauchope	Chambourcin	None	10	Necrosis, poor growth (E)	+	-	*Botryosphaeria*
Wentworth	Cabernet Sauvignon	None	3	Esca foliage (E)	+	-	-
	Cabernet Sauvignon	None	6	Esca foliage (E)	+	+	-
	Merlot	Kober 5BB	2	Esca foliage (E)	+	-	*Botryosphaeria*
	Merlot	SO4	2	Esca foliage (E)	+	-	*Botryosphaeria*
	Shiraz	None	4	Esca foliage (E)	+	+	*Botryosphaeria*
				Northern Territory			
Ti-Tree	Menindee Seedless	Schwarzmann	3	Chlorosis, decline (Pd)	-	+	*Botryosphaeria*
				South Australia			
Adelaide Hills	Chardonnay	Unknown	2	Dieback, red wood staining (Pd)	+	-	-
Clare Valley	Semillon	None	2	Poor growth, chlorosis (Pd)	+	-	-
	Merlot	None	2	Poor growth, chlorosis (Pd)	+	-	-
	Semillon	Traminer	10	Decline and dieback (Pd)	+	+	-
	Mataro	None	20	Decline and dieback (Pd)	+	+	-
Coonawarra	n.k.	None	n.k.	Poor growth (Pd)	+	-	*Botryosphaeria*
	Shiraz	None	20+	Esca foliage (E)	+	-	-
	Shiraz	Riesling	5	Decline and death (Pd)	+	-	*Botryosphaeria*
Glen Osmond	Merlot	None	3	Death (Pd)	+	-	-
Mt Pleasant	Cabernet Sauvignon	None	n.k.	Decline (Pd)	+	-	*Botryosphaeria*
Naracoorte	Shiraz	None	30+	Dieback (Pd)	+	-	*Eutypa*

Nuriootpa	Chardonnay	None	n.k.	Dieback (Pd)	+	-	*Eutypa*
	Chenin Blanc	None	n.k.	Decline (Pd)	+	-	*Cylindrocarpon*
	Semillon	None	n.k.	Poor vigour (Pd)	+	-	*Cylindrocarpon*
Padthaway	Cabernet Sauvignon	White Frontignac	20	Decline (Pd)	+	-	*Botryosphaeria, cf.Phellinus*
	Cabernet Sauvignon	None	20	Decline (Pd)	+	+	*Botryosphaeria, Eutypa, cf.Phellinus*
	Traminer	None	20	Decline (Pd)	+	-	*Botryosphaeria, cf.Phellinus*
Penola	Shiraz	Chardonnay	2	Poor budburst, decline (Pd)	+	-	-
Robe	Chardonnay	None	3	Poor growth (Pd)	+	-	-
	Semillon	Ramsey	?	Poor growth (Pd)	+	-	-
Torrens Park	Semillon	Unknown	2	Poor growth, graft failure (Pd)	+	-	-
Waikerie	Shiraz	None	2	Esca foliage (E)	+	+	*Botryosphaeria*
	Shiraz	None	6	Esca foliage (E)	+	+	-
	Grenache	Traminer	32	Early senescence (E)	+	+	*Botryosphaeria, Inonotus*
				Victoria			
Charlton	n.k.	n.k.	2	Poor growth (Pd)	+	-	-
Coldstream	Chardonnay	None	n.k.	Dieback (Pd)	+	-	-
	Cabernet Sauvignon	None	13	Decline and death (Pd)	+	-	-
Dookie	Tarrango	None	20	Esca foliage, death (E)	+	+	*Botryosphaeria, Eutypa, Fomitiporia*
Elmhurst	Marsanne	Schwarzmann	3	Decline and death (Pd)	+	-	*Phomopsis*
	Marsanne	Kober 5BB	3	Decline and death (Pd)	+	-	*Phomopsis*
Geelong	Pinot Noir	Ramsey	10	Shoot dieback, death (Pd)	+	-	*Botryosphaeria*
	Merlot	None	10	Shoot dieback, death (Pd)	+	-	-
Great Western	Chardonnay	None	20	Esca foliage and fruit (E)	+	-	*Botryosphaeria, Fomitiporia*
	Chardonnay	Sauvignon blanc	20	Esca foliage and fruit (E)	+	+	*Botryosphaeria, Fomitiporia*
	Riesling	None	2	Decline (Pd)	+	-	-
	Shiraz	ARX 1	37	Esca foliage (E)	+	+	*Botryosphaeria, Eutypa, Inonotus*
Hanging Rock	Chardonnay	None	15	Decline (Pd)	+	-	-
	Sauvignon Blanc	None	15	Stunted shoots, dieback (Pd)	+	-	*Eutypa*
Healesville	Sauvignon Blanc	None	n.k.	Stunted shoots, dieback (Pd)	+	-	*Botryosphaeria*
Irymple	Cabernet Sauvignon	None	n.k.	Decline and death (Pd)	+	-	-
Lake Cullulleraine	Semillon	Mixed	3	Poor growth and death (Pd)	+	+	*Cylindrocarpon*
Lakes Entrance	Sauvignon Blanc	None	30	Dieback and wood rot (E)	+	-	*Botryosphaeria, cf.Phellinus, Phomopsis*
Launching Place	Cabernet Sauvignon	None	26	Sudden death (E)	+	-	*Inonotus*
Lilydale	Semillon	None	16	Decline (Pd)	+	-	*cf.Phellinus*
Main Ridge	Cabernet Sauvignon	None	1	Poor storage life (Pd)	+	-	-
	Merlot	None	1	Poor storage life (Pd)	+	-	-
	Pinot Noir	None	1	Poor storage life (Pd)	+	-	-
Mansfield	Pinot Noir	None	n.k.	Decline (Pd)	+	-	-
Melbourne	Cabernet Sauvignon	None	3	Poor growth (Pd)	+		*Botryosphaeria*
Merbein	Merlot	None	28	Dieback (Pd)	+	+	*Eutypa*
	Shiraz	SO4	3	Poor growth and death (Pd)	+	-	*Botryosphaeria*
Mildura	Cabernet Sauvignon	None	1	Poor growth (Pd)	+		-
	Cabernet Sauvignon	None	15	Leaf necrosis (E)	+	-	*Botryosphaeria, Eutypa*
	Shiraz	Teleki 5A	2	Poor growth (Pd)	+	-	-
	Shiraz	Paulsen 1103	2	Poor growth (Pd)	+	-	-
	Shiraz	Ruggeri 140	2	Poor growth (Pd)	+	-	-
	Shiraz	Schwarzmann	2	Poor growth (Pd)	+	-	-
	Shiraz	None	15	Leaf necrosis (E)	+	-	*Eutypa*
	Sunmuscat	Paulsen 1103	3	Poor budburst, dieback (Pd)	+	-	-
Mornington	Pinot Noir	None	1	Distorted growth, stunting (Pd)	+	-	-
Moyhu	Chardonnay	Schwarzmann	1	Graft failure (Pd)	+	-	-
	Dolcetto	Schwarzmann	4	Decline and death (Pd)	+	+	-
	Shiraz	Schwarzmann	3	Decline (Pd)	+	+	-
	Pinot Noir	Teleki 5A	1	Graft failure (Pd)	+	-	-
Myrrhee	Chardonnay	Schwarzmann	5	Uneven budburst, death (Pd)	+	-	-
	Pinot Gris	Schwarzmann	3	Shrivelled, spotted fruit (E)	-	+	*Botryosphaeria*
Myrtleford	n.k.	n.k.	n.k.	Decline (Pd)	+	-	-
Nagambie	n.k.	n.k.	n.k.	Stunting (Pd)	+	+	*Cylindrocarpon*
Penshurst	Chardonnay	None	4	Chlorosis (Pd)	+	-	-
Piangil	Shiraz	None	3	Decline and death (Pd)	+	-	*Phytophthora, Cylindrocarpon*
Red Cliffs	Chardonnay	None	n.k.	Dieback (Pd)	+	-	-
Robinvale	Red Globe	Ramsey	<1	Graft failure (Pd)	+	-	*Cylindrocarpon, Phomopsis*
	Sultana	Teleki 5A	<1	Graft failure (Pd)	+	-	*Cylindrocarpon*
	Sunmuscat	Paulsen 1103	2	Death (Pd)	+	-	*Phomopsis*

Rutherglen	n.k.	Unknown	n.k.	Poor growth (Pd)	+	-	-
Seville	Cabernet Sauvignon	None	26	Dieback (Pd)	+	-	*Eutypa*
South Warrandyte	n.k.	None	2	Dieback (Pd)	+	-	-
Steels Creek	Chardonnay	None	19	Poor growth (Pd)	+	-	*Botryosphaeria, Eutypa*
Swan Hill	Shiraz	None	1	Poor growth and death (Pd)	+	-	-
Wandin North	n.k.	None	10	Sudden collapse (E)	+	-	*Eutypa*
Wangaratta	Sauvignon Blanc	None	n.k.	Poor growth (Pd)	+	-	*Eutypa*
Yarra Glen	Verdelho	None	3	Uneven budburst, poor growth (Pd)	+	-	-
Yarra Junction	Pinot Noir	None	n.k.	Rapid decline (Pd)	+	-	*Cylindrocarpon*
				Western Australia			
Baldivis	Cabernet Sauvignon	Ramsey	14	Chlorosis, dieback (Pd)	+	-	*Botryosphaeria*
	Chardonnay	Ramsey	14	Chlorosis, dieback (Pd)	+	-	*Botryosphaeria*
	Merlot	Ramsey	14	Chlorosis, dieback (Pd)	+	-	*Botryosphaeria*
	Semillon	Ramsey	14	Chlorosis, dieback (Pd)	+	-	*Botryosphaeria*
Cowaramup	Cabernet Sauvignon	None	26	Decline and death (Pd)	+	-	*Botrysphaeria, Inonotus*
	Merlot	None	26	Decline and death (Pd)	+	-	*Botrysphaeria, Inonotus*
	Cabernet Sauvignon	None	35	Esca foliage (E)	+	-	*Botryosphaeria*
	Shiraz	None	35	Esca foliage (E)	+	+	*Botryosphaeria, Eutypa*
	Shiraz	None	8	Poor growth (Pd)	+	-	-
Manjimup	n.k.	None	n.k.	Decline (Pd)	+	-	-

[A]Symptoms diagnosed as Petri disease are designated Pd, those as esca designated E
[B]Pch = *Phaeomoniella chlamydospora*
[C]Pal = *Phaeoacremonium aleophilum*
[D]n.k. = information not known.
[E] + = present, - = absent.

Table 1.2. The identity and numbers of grapevine cultivars diagnosed with Petri disease or esca from samples of diseased grapevines sent to DPI Knoxfield during 1998 to 2002.

Grapevine cultivar	**No. diagnosed with Petri disease (out of 102 samples)**	**No. diagnosed with esca (out of 22 samples)**
White		
Chardonnay	17	2
Semillon	13	-
Sauvignon Blanc	4	2
Marsanne	2	-
Sun Muscat	2	-
Traminer	2	1
Verdelho	2	-
Riesling	2	-
Chenin Blanc	1	-
Menindee Seedless	1	-
Sultana	1	-
Trebbiano	1	-
White Frontignac	1	-
Pinot Gris	-	1
Red		
Shiraz	17	7
Cabernet Sauvignon	11	5
Merlot	9	2
Pinot Noir	9	-
Chambourcin	1	1
Dolcetto	1	-
Mataro	1	-
Red Globe	1	-
Zinfandel	1	-
Grenache	-	1
Tarrango	-	1
(Unknown)	(7)	(1)
Rootstock		
Ramsey	10	-
Schwarzmann	7	1

Kober 5BB	3	1
Paulsen 1103	3	-
Teleki 5A	3	-
Ruggeri 140	1	-
SO4	1	1
AxR 1	-	1
(Unknown)	(3)	

Grapevines without external symptoms

Eighty asymptomatic grapevines, representing 24 scion and 3 rootstock cultivars from 11 vineyards, had internal symptoms of black wood streaking from which *Pa. chlamydospora* was isolated (Table 1.3).

Table 1.3. Location, cultivar and ages of asymptomatic grapevines in which black wood streaking and *Phaeomoniella chlamydospora* were present

Location	Cultivar	Rootstock	No. vines sampled	Age	Comments	Other plant pathogenic fungi isolated
			South Australia			
Adelaide	Rondello	None	5	40+	Abandoned vineyard	*Eutypa, Inonotus, Pm. aleophilum*
McLaren Vale	Grenache	None	1	70+	Low yielding but high quality fruit	*Pm. aleophilum*
Rowland Flat	Ruggeri 140	None	2	10+	Rootstock mother vines	-
	Schwarzmann	None	1	10+	Rootstock mother vine	-
			Victoria			
Heathcote	Shiraz	None	2	25	Low yielding but high quality fruit	*Botryosphaeria, Inonotus*
Irymple	Ramsey	None	8	6	Rootstock mother vines	-
	Cabernet Sauvignon	None	4	14	Mother vines	-
Kyneton	Riesling	None	4	32	Never cropped; abandoned vineyard	*Botryosphaeria, Fomitiporia, Phomopsis, Pm. aleophilum*
	Cabernet Sauvignon	None	4	32	"	*Botryosphaeria, Fomitiporia, Phomopsis, Pm. aleophilum*
	Shiraz	None	4	32	"	*Botryosphaeria, Fomitiporia, Phomopsis, Pm. aleophilum*
Melbourne	Cabernet Sauvignon	None	2	40	Burnley College teaching vineyard	*Fomitiporia*
	Chardonnay	None	1	12	"	*Botryosphaeria, Phomopsis, Pm. aleophilum*
	Meunier	None	1	20	"	*Pm. aleophilum*
	Meunier	Trebbiano (40 years)	1	20 (40)	"	*Inonotus*
	Riesling	None	2	40	"	*Fomitiporia*
	Shiraz	None	2	40	"	-
Mildura	Sultana	Ramsey	3	14	Table grapes	*Botryosphaeria, Phomopsis, Pm. aleophilum*
	Barbera	None	1	30+	Foundation vine	-
	Cardinal	None	1	30+	"	-
	Carignan	None	1	30+	"	-
	Chardonnay	None	4	30+	"	*Botrytis*
	Chasselas	None	1	30+	"	-
	Chenin Blanc	None	1	30+	"	-
	Colombard	None	2	30+	"	-
	Malbec	None	1	30+	"	-
	Mataro	None	2	30+	"	*Botrytis*
	Merlot	None	1	30+	"	-
	Muscat Gordo Blanco	None	1	30+	"	-
	Pinot Noir	None	5	30+	"	*Botrytis*
	Riesling	None	2	30+	"	-
	Semillon	None	1	30+	"	*Botrytis, Pm. aleophilum*
	Shiraz	None	2	30+	"	*Botrytis*
	Sultana	None	3	30+	"	*Botryosphaeria, Botrytis, Pm. aleophilum*
	Traminer	None	1	30+	"	-
	Valdiguie	None	1	30+	"	-
	Waltham Cross	None	1	30+	"	*Botryosphaeria*
Taradale	Chardonnay	None	1	10	Healthy vine	-
			Western Australia			
Margaret River	Zinfandel	None	2	24	Mother vines	*Botryosphaeria*

Pm. aleophilum was present in less than a third of these grapevines. Thirty-two out of 35 foundation grapevines examined were infected with *Pa. chlamydospora*. Other suspected grapevine pathogens recovered were species of *Botryosphaeria*, *Eutypa*, *Fomitiporia*, *Inonotus* and *Phomopsis*. Again, *Eutypa* was always associated with necrotic wood and *Fomitiporia* and *Inonotus* with white heart rot. *Botrytis cinerea* was isolated from several Foundation grapevines, always associated with greenish 'water marks' deep inside the trunks. To our knowledge, this is the first time *B. cinerea* has been recovered from inside grapevine trunks.

Distribution of Pa. chlamydospora *and* Pm. aleophilum *across grape-growing regions of Australia*

Pa. chlamydospora was widely distributed throughout south east Australia, limited to the Swan and Margaret River regions of Western Australia and was not present in the sample we examined from the Northern Territory. *Pm. aleophilum* was less common and was not found in samples from the Swan Hill and Gippsland regions of Victoria or the Central Ranges and Southern regions of New South Wales.

Discussion

Over the five-year period from 1998–2002, samples of diseased grapevines were sent to DPI Knoxfield and 124 of these were subsequently diagnosed with either Petri disease or esca. The samples came from most grape-growing regions of New South Wales, South Australia, Victoria and Western Australia. None came from Queensland or Tasmania, although this may be due to samples from those States being sent to other diagnostic services. Both diseases were found present in all four States, but the majority of samples (82%) were diagnosed with Petri disease, rather than esca (18%).

Although several genera of plant pathogenic fungi such as *Eutypa*, *Cylindrocarpon, Botryosphaeria, Phomopsis,* etc. were isolated from the samples, *Pa. chlamydospora* was the only organism consistently associated with the disease symptoms, being isolated from all but two of the samples. In some older grapevines the external symptoms were confounded by dieback and shoot stunting obviously attributable to Eutypa dieback, caused by *Eutypa lata*. However, the internal black wood streaking and presence of *Pa. chlamydospora* indicated that Petri disease could not be discounted. *Pm. aleophilum* was found in only 19 of the samples. *Pm. aleophilum* is reported to be associated with Petri disease and esca (Larignon and Dubos 1997; Scheck *et al.* 1998; Mugnai *et al.* 1999) but these results suggest that it is not a serious pathogen under Australian conditions.

Cylindrocarpon spp. are known to cause black foot disease in grapevines (Larignon 1999; Fourie and Halleen 2001), and to be of particular concern with respect to young vines (Halleen *et al.* 2003). Although the *Cylindrocarpon* spp. reported in the present study were isolated from declining young vines, the purplish-black wood staining characteristic of black foot disease was not observed in any of the samples. The *Botryosphaeria* species were not identified to species level in most cases as they do not sporulate well in culture. Species such as *B. obtusa* and *B. dothidea* are thought to cause black dead arm disease of grapevines in Europe (Larignon *et al.* 2001), but the pathogenicity of other species on grapevine is currently unresolved (P. Crous, pers. comm.). In the present study, *Botryosphaeria* species were isolated from 35% of the samples and did not correlated with any specific symptom or age of grapevine. *Eutypa lata,* however, was always associated with wood necrosis in grapevines older than 10 years. The heart rot fungi were also only isolated from mature vines older than 15 years.

Phaeomoniella chlamydospora was found to be widespread in all grape-growing regions sampled, but was not present in the sample from the Northern Territory. By contrast, *Pm. aleophilum* was limited in its distribution but was present in the sample of diseased table grapes sent from Ti-Tree, Northern Territory. Optimum growth temperatures *in vitro* for each fungus are 25º and 35ºC respectively (Crous *et al.* 1996; Whiting *et al.* 2001), so it is possible that *Pm. aleophilum* is more virulent in hot climates.

Thirty-two cultivars were represented in the samples, including both *Vitis vinifera* scion cultivars and *Vitis* spp. rootstock cultivars. It has been suggested that *Pa. chlamydospora* is spread via infected

rootstock material (Rego *et al.* 2000; Halleen *et al.* 2003), but in this survey over 60% of the samples were own-rooted. However, some possible differences in cultivar susceptibility to Petri disease were evident from these data. As noted above, the most commonly affected white cultivars were Chardonnay, Semillon and Sauvignon Blanc; red cultivars were Shiraz, Cabernet Sauvignon, Merlot and Pinot Noir, and rootstocks were Ramsey and Schwarzmann. According to the Australian Bureau of Statistics, the top six white cultivars in terms of area grown in 2002 were Chardonnay, Sultana, Semillon, Riesling, Sauvignon Blanc and Colombard, and the top six red cultivars were Shiraz, Cabernet Sauvignon, Merlot, Pinot Noir, Ruby Cabernet and Grenache (Winetitles 2003). Over the five-year period from 1998 to 2002, only two samples of Riesling, one of Sultana and none of Colombard, Ruby Cabernet or Grenache were diagnosed with Petri disease at DPI Knoxfield out of the 102 samples diagnosed with this disease.

The demonstrated presence of *Pa. chlamydospora* in symptomless grapevines with internal black wood streaking could be explained by reports (Ferreira *et al.* 1999) that the fungus can behave as an endophyte or latent pathogen until the grapevines are stressed, for example by water deficit, after which Petri disease becomes evident. In the present study, many mother grapevines were found to harbour symptomless infections, with 32 out of the 35 sampled *V. vinifera* Foundation grapevines infected. In 1998, 15 rootstock mother grapevines (hybrids of American *Vitis* spp.) from three sites in Victoria, New South Wales and South Australia were examined, which revealed 13 to be infected with *Pa. chlamydospora* (Pascoe 1999). In Section 3, we demonstrate that cuttings taken from infected mother grapevines can be infected with *Pa. chlamydospora*, and this may explain why the fungus is so widely distributed across Australia.

These results clearly demonstrate that *Pa. chlamydospora* is the organism most closely associated with both diseases and is widely distributed across the major grape-growing regions of Australia. It also confirms that, in contrast with the current situation in Europe, Petri disease is much more prevalent than esca in Australian vineyards.

Chapter 2: Etiology of Esca and Petri Disease in Australia

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Summary *Phaeomoniella chlamydospora* was consistently associated with grapevines symptomatic of either esca or Petri disease. In the case of young esca, symptoms occurred when infected vines were subjected to water and heat stress in summer. The white heart rot observed in many mature grapevines was not consistently associated with esca leaf and fruit symptoms. It was caused by at least six different species of basidiomycete fungi, including a new species we described as *Fomitiporia australiensis*. *F. australiensis* was shown to also occur in Australian native species surrounding vineyards, such as *Dodonea viscosa* and *Eucalyptus* species. Pathogenicity studies showed that *Pa. chlamydospora* caused poor callus production, graft failure and death in cuttings, while *Phaeoacremonium aleophilum* was less virulent. Rootstock cultivars were more susceptible than *Vitis vinifera* cultivars. Isolates of *Pa. chlamydospora* were genetically uniform, strongly suggesting the population is clonal. Considerable variation was found among isolates of *P. aleophilum*, however, and subsequent investigations showed that in fact they represented three *Phaeoacremonium* species: *Pm. aleophilum, Pm. parasiticum* and a new species *Pm. australiense*. In addition, the sexual state of *Pm. aleophilum* was found on grapevine wood and identified as *Togninia minima*. Our conclusion is that *Phaeomoniella chlamydospora* is the causal organism of both esca and Petri disease in Australia, and that *Phaeoacremonium* species and the heart rotting basidiomycetes are incidental and not necessarily indicative of disease.

2.1 Young esca

Esca is a disease generally associated with mature vines more than ten years old. However, sometimes vines less than ten years old express classic external symptoms but no white rot in the trunk and this syndrome has been called young esca (Mugnai *et al.*, 1999). Chicau *et al.* (2000) reported esca-like foliar symptoms associated with black wood-streaking in 3 to 11-year-old *Vinho Verde* grapevines grown in northwest Portugal. In Italy, Serra *et al.* (2000) examined thirty one 5 to 6-year-old grapevines, eight of which had classic esca foliar symptoms and three of which had suffered apoplexy. However, they were unable to decide what pathogen was responsible for the symptoms.

The aetiology of esca has been the focus of research for the past century but remains unresolved as Koch's postulates have never been fully satisfied (Chiarappa, 2000). The external symptoms are erratic and may not show every year, and the factors controlling symptom expression are unknown. Although the internal wood symptoms have been successfully reproduced, the foliar and fruit symptoms have not. Many fungal species have been isolated from esca-affected vines and implicated as causal organisms, leading to debate as to which organism or organisms are necessary for the expression of esca (Mugnai *et al.*, 1999; Graniti *et al.*, 2000).

In Australia, young esca first came to our notice in 1999 when it affected 3 to 7-year-old vines growing in the Riverland-Sunraysia region of Australia. The aim of this study was to determine the cause of young esca affecting three grapevine cultivars in two Australian vineyards.

Methods

Sampling sites

Two vineyards from the Riverland-Sunraysia grape-growing region of Australia, site 1 at Waikerie in South Australia and site 2 at Wentworth in New South Wales, reported esca foliar symptoms on their young vines, which was often associated with shrivelling of the fruit. In this region, the growing season is from September to early March, with periods of very high temperatures (>40°C) over summer (December to February). Vines are grown under irrigation on sandy, alkaline, sodic soils and

are vigorous and high-yielding. At both sites, esca foliar symptoms were observed during three seasons, 1998-99, 1999-00 and 2000-01, first becoming noticeable in late spring (November).

At site 1, the affected vines were own-rooted Shiraz planted in 1994. They had been grown under a restricted-deficit-irrigation (RDI) schedule until February 1998 when severe wilting and fruit shrivelling occurred. RDI was abandoned and irrigation increased, but esca symptoms were again noticed the following season (1998-99).

At site 2, esca symptoms were also noticed during 1998-99, on own-rooted Cabernet Sauvignon planted in 1997. The following season, Merlot grafted onto Kober 5BB, also planted in 1997, showed symptoms.

Spatial analysis

Symptomatic vines at each site were tagged during 1999-00 and 2000-01 in order to follow disease progress. In 2001, the tagged vines were mapped and spatial analysis (ordinary runs and analysis), as described by Surico *et al.* (2000), was performed on the data to determine whether the symptomatic vines were randomly spread throughout the agronomic rows or clustered together.

Preliminary examination of vines

During 1999-00, several vines were removed from each site for examination. At site 1, three symptomatic Shiraz vines were uprooted during autumn, their trunks cut into pieces (both transversely and longitudinally) and isolations made from any areas with wood discolouration, which was mainly black streaking. For this purpose, small slivers (5x50 mm) of internal wood were surface sterilised in 0.5% NaOCl and plated onto potato dextrose agar amended with 50 ppm Achromycin® (a.i. tetracycline hydrochloride; American Cyanamid Company, USA). After incubation at room temperature for 2 to 3 weeks, the plates were examined for fungal growth.

At site 2, a symptomatic Cabernet Sauvignon and a symptomatic Merlot on Kober 5BB vine were removed in autumn (March 2000) for examination as described above, and in the following spring (November 2000) five more symptomatic Merlot on Kober 5BB vines were removed and examined.

Systematic examination of vines

When symptoms were observed at both sites for a third season (2000-01), a more systematic approach to vine examination was taken. In late summer (February and March 2001), from site 1 we chose one asymptomatic vine, one vine which showed symptoms for the first time in 2000-01 and one vine which had been symptomatic during both 1999-00 and 2000-01. From site 2, seven Merlot on Kober 5BB vines were selected: two asymptomatic vines, one that had expressed symptoms only during 1999-00, three showing symptoms for the first time in 2000-01 and one which had been symptomatic during 1999-00 and then died in 2000-01. Four Cabernet Sauvignon vines were also selected: one asymptomatic vine, one which expressed symptoms only during 1999/00, one with symptoms for the first time in 2000-01 and one which had been symptomatic both seasons.

The harvested trunks were cut into 10 cm segments and then surface flame-sterilised. Internal symptoms were described. Small pieces of internal wood were taken from areas with black and/or brown streaking and plated onto potato dextrose agar amended with lactic acid (2 drops lactic acid / 100 ml medium). All remaining trunk pieces were moist incubated and examined at x40 for the presence of esca-associated fungi after 4 to 8 weeks.

Results

Spatial analysis

One dimensional spatial analysis of the symptomatic vines showed that they were randomly distributed at site 1 but clustered at site 2 along the agronomic rows.

Preliminary examination of vines

Internal examination of the trunks showed that all had some black wood-streaking, particularly near the base, which in combination with the foliar symptoms is consistent with young esca. Subsequent isolation and moist incubation confirmed that all of the sampled vines were infected with *Pa. chlamydospora*. Other fungi isolated were species of ubiquitous genera such as *Penicillium, Alternaria, Cladosporium* and *Gliocladium.*

Systematic examination of vines

Site 1: Shiraz

All the own-rooted Shiraz vines had black wood-streaking and *Phaeomoniella chlamydospora* present in the trunk (Table 2.1). Vine 1, symptomatic during both seasons, had black wood-streaking along the entire length of the trunk and *Pa. chlamydospora* present in the bottom 20 cm. Vine 2, symptomatic in 2000-01, had both black wood-streaking and *Pa. chlamydospora* in the lower third of the trunk and again in the top third associated with a deep crack downwards from the cordons. *Pa. chlamydospora* was found sporulating abundantly in this crack, which was very similar to the sites of *in situ Pa. chlamydospora* sporulation found by Edwards *et al*. (2001). Vine 3, asymptomatic, had very little black wood-streaking and some *Pa. chlamydospora* scattered throughout the trunk. The only other esca-associated fungus found was *Phaeoacremonium aleophilum* in a single portion of vine 2.

Table 2.1. Internal symptoms and esca-associated fungi (*Phaeomoniella chlamydospora ,Pch; Phaeoacremonium aleophilum, Pal*) found in the trunks of 7-year-old own-rooted Shiraz vines from site 1, Waikerie, South Australia, Australia.

Trunk portion[a]	Vine 1: esca 1999-00 & 2000-01		Vine 2: esca 2000-01		Vine 3: asymptomatic	
	Symptoms[b]	Fungus	Symptoms	Fungus	Symptoms	Fungus
0	+	*Pch*	+	-	-	-
1	+	*Pch*	+	*Pch*	+	-
2	+	-	+	*Pch*+*Pal*	-	*Pch*
3	+	-	+	*Pch*	-	-
4	+	-	-	-	-	-
5	+	-	-	*Pch*	-	-
6	+	-	-	-	-	-
7	+	-	-	-	-	*Pch*
8	+	-	-	*Pch*	+	*Pch*
9	+	-	-	-	-	-
10			-	-	-	-
11			-	-	-	-
12			+	*Pch*	-	*Pch*
13			-	*Pch*	-	*Pch*
14			+	*Pch*	-	-
15			-	*Pch*	-	-
16			+[c]	*Pch*		
17			+[c]	*Pch*		
18			+[c]	*Pch*		

[a]Trunks were cut up into portions approx. 10 cm length. Portion 0 was below ground, portions 1-18 were sequential beginning at ground level.
[b]Internal wood symptoms: +, wood discolouration, usually black wood streaking; -, no internal symptom observed.
[c]Symptoms in these portions included a large split down the trunk from the top where the cordons differentiated. *Pch* was found sporulating in abundance inside this split.

Site 2: Merlot grafted onto Kober 5BB

All vines had black wood-streaking and *Pa. chlamydospora* in the rootstock just below the graft union (Table 2.2). In the asymptomatic vines (6 and 7) and vine 3 (symptomatic in 2000-01), black wood-streaking and *Pm. chlamydospora* were limited to the rootstock, but in the other vines these were also evident in the scion wood. *Pm. aleophilum* was found in vines 1, 3 and 6.

Site 2: Cabernet Sauvignon

Black wood-streaking and *Pa. chlamydospora* were present in all the symptomatic vines (vines 1-3) but not in the asymptomatic vine (vine 4) (Table 2.3). *Pm. aleophilum* was present in two vines (1 and 4), and an unidentified basidiomycete associated with white heart rot (later identified as a *Fomitiporia* species) was present in the below-ground portion of vine 2.

Once again, the only other fungi isolated from the trunks were species of ubiquitous genera such as *Penicillium, Alternaria, Cladosporium* and *Gliocladium.*

Discussion

In all cases of young esca examined in the present study, the common factor was the presence of *Phaeomoniella chlamydospora* associated with black wood-streaking in the affected trunks. Other esca-associated fungi were rare. *Phaeoacremonium aleophilum* was observed in six of the 19 vines examined (4 symptomatic and 2 asymptomatic), and a *Fomitiporia* species associated with white heart rot was present in only one vine (symptomatic). *Pa. chlamydospora* was also isolated from all except one of the young esca vines examined in Italy (Serra *et al.*, 2000) and Portugal (Chicau *et al.*, 2000). Sparapano was able to reproduce foliar symptoms resembling esca such as interveinal chlorosis and necrosis in 6-year-old vines by injecting liquid cultures of *Pa. chlamydospora* into current-season shoots (Sparapano *et al.*, 2000a). He then demonstrated that similar symptoms could be produced by injecting metabolites extracted from *Pa. chlamydospora* culture filtrates and from discoloured wood infected with *Pa. chlamydospora* (Sparapano *et al.*, 2000b). In 1999, Mugnai posed the question as to whether esca is (a) a disease complex requiring the interaction of two or more organisms to produce the overall syndrome, (b) a complex of two distinct diseases: white heart rot caused by *Fomitiporia punctata* and black wood-streaking caused by *Pa. chlamydospora*, or (c) a disease induced by *Pa. chlamydospora* alone (Mugnai *et al.*, 1999). The results outlined above suggest that esca is a disease induced by *Pa. chlamydospora* alone.

Table 2.2. Internal symptoms and esca-associated fungi (*Phaeomoniella chlamydospora ,Pch; Phaeoacremonium aleophilum, Pal*) found in the trunks of 4-year-old Merlot vines on Kober 5BB rootstock, from site 2, Wentworth, New South Wales, Australia.

Trunk portion[a]	Vine 1: esca 1999-00		Vine 2: esca 1999-00 dead 2000-01		Vine 3: esca 2000-01		Vine 4: esca 2000-01		Vine 5: esca 2000-01		Vine 6: asymptomatic		Vine 7: asymptomatic	
	Symptom[b]	Fungus	Symptom	Fungus	Symptom	Fungus	Symptom	Fungus	Symptom	Fungus	Symptom	Fungus	Symptom	Fungus
0	-	-	+	*Pch*	+	*Pal*	+	*Pch*	+	*Pch*	+	*Pch*	-	-
1	-	-	+	-	(G) +	*Pch*+*Pal*	+	*Pch*	+	*Pch*	(G) +	*Pch*	-	-
2	(G) [c] +	*Pch*	(G) +	*Pch*	-	-	(G) +	*Pch*	(G) +	*Pch*	-	-	+	-
3	+	*Pch*			-	-	+	*Pch*	+	*Pch*	-	-	(G) +	*Pch*
4	+	-			-	-	+	-	-	-	-	-	-	-
5	+	-			-	-	+	-	-	-	-	-	-	-
6	+	*Pal*			-	-	+	*Pch*	-	-	-	*Pal*	-	-
7	+	-			-	-	-	-	-	-	-	-	-	-
8	-	-			-	-	-	-	-	-	-	-	-	-
9	-	-			-	-	-	-	-	-	-	-	-	-
10	+	-			-	-	-	-	-	-	-	-	-	-
11	-	-			-	-	-	-	-	-	-	-	-	-
12					-	-			-	-			-	-
13					-	-								

Table 2.3. Internal symptoms and esca-associated fungi (*Phaeomoniella chlamydospora, Pch*; *Phaeoacremonium aleophilum, Pal*; white heart rot fungus, basidio) found in the trunks of 4-year-old own-rooted Cabernet Sauvignon vines from site 2, Wentworth, New South Wales, Australia.

Trunk portion[a]	Vine 1: esca 1999-2000		Vine 2: esca 1999-2000 & 2000-01		Vine 3: esca 2000-01		Vine 4: asymptomatic	
	Symptoms[b]	Fungus	Symptoms	Fungus	Symptoms	Fungus	Symptoms	Fungus
0	+	*Pch* + *Pal*	+[c]	Basidio	-	-	-	*Pal*
1	+	*Pch*	+	*Pch* + basidio	-	-	-	-
2	+	*Pch*	+	*Pch*	+	-	-	-
3	-	-	+	-	+	-	-	-
4	+	*Pch*	+	-	+	-	-	-
5	-	-	+	-	+	-	-	-
6	-	*Pal*	+	-	+	-	-	-
7	-	-	+	-	+	-	-	-
8	-	*Pal*	+	-	+	*Pch*	-	-
9	-	-	+	-	+	-	-	-
10	-	*Pch*	+	-	+	-	-	-
11	-	-	+	-	+	-	-	-
12					-	-	-	-

[a]Trunks were cut up into portions approx. 10 cm length. Portion 0 was below ground, portions 1-13 were sequential beginning at ground level.
[b]Internal wood symptoms: +, wood discolouration, usually black wood streaking; -, no internal symptom observed.
[c](G), graft union.

2.2 Esca and basidiomycetes associated with white heart rot in grapevines

When this project began, it had been reported that esca was caused by a combination of fungi such as *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum* and *Fomitiporia punctata*, the latter of which was responsible for the white heart rot component of esca. This was widely assumed to be the situation wherever esca 'proper' occurred, esca 'proper' being defined as the combination of both external symptoms and internal heart rot in a mature grapevine (Mugnai *et al.* 1999). However, during the course of this project, we isolated basidiomycetes from white heart rot in grapevines that could not be refered to *F. punctata* or any other known species (Edwards et al., 2001; Fischer, 2001). Corresponding fruit bodies were observed very rarely and usually in such poor condition that confident identifications could not be made. Identification problems also applied to wine growing regions of North America, where vegetative mycelia isolated from white heart rot of grapevine were assigned to the species *Phellinus ignarius* (Chiarappa, 1997), which, however, is a European based species, almost exclusively occurring on species of *Salix* (Fischer, 1995; Fischer, 2000; Fischer & Binder, 2004). In 2002, Fischer described the basidiomycete species associated with esca of grapevines in Europe as a new species, *Fomitiporia mediterranea* (Fischer 2002). Therefore, conclusive data on grapevine-inhabiting basidiomycetes existed for Europe only; information was sparse or essentially non-existing for all other wine growing countries.

Fruiting structures of esca-related basidiomycetes are hard to find or they may occur on non-*Vitis* hosts not well investigated. In general, existence of these fungi can only be demonstrated by the vegetative mycelia that are isolated from the infected wood. Several keys are available for identification of lignicolous fungi in pure culture (Nobles, 1965; Stalpers, 1978), but they are incomplete due to numerous taxonomic novelties and an accurate identification to species level is often not possible.

It was the goal of this study to characterize and, if possible, to identify a number of basidiomycetes of uncertain affinity derived from heart rotted wood of grapevine and other woody species such as *Eucalyptus* and, in one case, a species of *Dodonaea* (native hop-bush) in Australia. When available, fruit bodies were examined using morphological and microscopical characters. For all isolates, molecular sequences were generated for the nuclear encoded ribosomal ITS region. Using a phylogenetic approach, the obtained sequences were compared with selected representatives of the Hymenochaetales belonging to the putatively dosely related genera *Phellinus*, *Inonotus*, *Inocutis*, *Mensularia* and *Fomitiporia*.

Specifically, the following questions were addressed in this study: (i) can the isolates be assigned to basidiomycetes already known to occur on grapevine? (ii) do they represent species known to occur in the southern hemisphere, but not known to be associated with esca-affected grapevine? Or (iii) do they represent taxa so far undescribed?

Methods

Fungal material and culturing

The strains used are listed in Table 2.4. Specimens are deposited at the University of Regensburg Herbarium (REG) in Germany and at the Victorian Plant Research Institute Herbarium (VPRI) in Australia. Mycelial cultures were grown on malt extract medium (ME; agar, 2%; malt extract, 2%; yeast extract, 0.05% in distilled water) under daylight conditions. For determination of temperature requirements all Australian isolates were incubated on ME at 15°C, 21°C, and 30°C. Mycelial growth was measured under 21°C and 30°C conditions by calculating the mean of two perpendicular colony diameters. Two repeats were performed for each isolate.

Comparative microscopy of fruit bodies

Sections of fruit bodies were placed on a slide in a drop of Melzer´s reagent or lactophenol-cotton blue (Meixner, 1975); examinations were at 500x or 1250x under phase contrast optics. A maximum of twenty observations was recorded for measurements of basidiospores.

DNA isolation and PCR amplification

Whole cell DNA was isolated from cultured mycelium as described by Lee and Taylor (1990). Quantity and quality of the DNA were examined on 1% agarose gels. Isolated DNA was diluted 1:100 or 1:1000 in distilled water. The polymerase chain reaction (PCR) was used to amplify a portion of the nuclear encoded ribosomal DNA unit defined by the primer combination prITS5 and prITS4 (for primer sequences, see White et al., 1990). The fragment spans the entire ITS1 region, the 5.8S rRNA gene, and the ITS2 region. The PCR reactions were set up in 50 µl volumes and were overlayed with two drops of mineral oil. Hot start PCR was applied throughout (d'Aquila et al., 1991). Forty cycles were performed on a TRIO-Thermoblock (Biometra, Germany), using the following parameters: 95°C denaturation step (1 min), 50°C annealing step (1 min), 72°C primer extension (1 min). A final incubation step at 72°C (7 min) was added after the final cycle. 5 µl of each PCR reaction were electrophoresed on 1% agarose gels. A 100 bp DNA ladder (MBI Fermentas, Lithuania) was used as standard. The amplified products were purified with the QIAquick PCR Purification Kit (Qiagen, Germany) following the manufacturer's instructions. DNA was suspended in 20 - 50 µl Tris-HCl buffer (10 mM, pH 8.0).

Table 2.4. List of fungal taxa and strains

Species (strain number, date, location)	Substrate	GenBank No.
Phellinus ignarius:		
85-625[1], 25.6.1985, Germany	*Salix caprea*	AF515573
TN5758[1], 25.5.1994, Finland	*Salix*	AF515574
Fomitiporia robusta:		
89-828[1], 28.8.1989, Estonia	*Quercus*	AF515565
96-515[1], 15.5.1996, Germany	*Fraxinus excelsior*	AF515560
Fomitiporia punctata:		
85-74[1], 4.7.1985, Germany (Bavaria)	*Salix caprea.*	AF515563
87-511[1], 11.5.1987, Germany	*Rhamnus cathartica*	AF515564
89-826b[1], 26.8.1989, Estonia	*Sorbus aucuparia*	AF515562
Dai2727[1], 5.10.1997, Finland	*Sorbus aucuparia*	AF515561
Fomitiporia mediterranea:		
CA3[2], VIII-1997, Italy (Tuscany)	*Vitis vinifera*	AF515575
99-105[1], 5.10.1999, Italy (Lazio)	*Corylus avellana*	AF515586
45/23[1], VIII-2001, Germany	*Vitis vinifera*	AF515585
Fomitiporia australiensis:		
VPRI 22409b[1], 10.4.2000, South Australia	*Dodonaea viscosa*	AY624995
VPRI 22451[2], 17.3. 2000, Australia (Victoria)	*Vitis vinifera*	AY624996
VPRI 22485[2], 21.2.2000, Australia (Victoria)	*Vitis vinifera*	AY624987
VPRI 22486[2], 17.3.2000, Australia (Victoria)	*Vitis vinifera*	AY624988
VPRI 22859[1], 19.2.2001, Australia (Victoria)	*Vitis vinifera*	AY624997
Unknown species:		
VPRI 22488[2], 10.4.2000, South Australia	*Vitis vinifera*	AY624989
VPRI 22492[2], 10.4.2000, South Australia	*Vitis vinifera*	AY624990
VPRI 22495[2], 10.4.2000, South Australia	*Vitis vinifera*	AY624991
Inonotus hispidus:		
86-829, 29.8.1989, Germany	*Fraxinus excelsior*	AY624993
Mensularia radiata		
85-107, 7.10.1985, Germany	*Alnus incana*	AY624992
Inocutis rheades		
86-922, 22.9.1986, Germany	*Populus tremula*	AY624994

[1] mycelium isolated from fruit bodies; [2] mycelium isolated from infected wood.

Sequencing

All strains listed in Table 2.4 were included in the sequencing experiments. Instead of mycelium derived from fruit bodies and/or infected wood, single spore isolates were used for strains 45/23 and TN5758, designated 45/23.3 and TN5758.1, respectively. Fragments were sequenced with the

AmpliTaq DNA Polymerase FS Dye Terminator Cycle Sequencing kit (Perkin Elmer, USA), using 2 µl of premix, 1 µl of the primers (8 pmol of prITS1 and prITS4, respectively), and 3.5 µl of the PCR products. The reactions were set up in 11 µl volumes, and were overlayed with one drop of mineral oil. Sequences were generated in two directions and twenty-five amplification cycles were carried out, using the following parameters: 96°C denaturation step (30 s), 59°C annealing step (15 s) for prITS1, 53°C annealing step (15 s) for prITS4, 60°C primer extension (4 min). DNA was precipitated by addition of 2 µl of NaAc (3 M, pH 4.8) and 55 µl of EtOH 100%, and was then washed with 150 µl of EtOH 70%. The DNA pellet was resuspended in 1 : 4 EDTA (50 mM, pH 8.0) : formamide. The electrophoresis was done with an ABI 373A Automatic Sequencer (Perkin Elmer).

Alignment and phylogenetic analyses

After processing the raw data with SeqEd (version 3.0), the sequences were aligned using the ClustalX (version 1.64b) program (Thompson et al., 1997). A final alignment was performed by eye. Alignment gaps were treated as missing data and all positions were included in the final alignment. The sequences obtained have been deposited in GenBank (for numbers, see Table 2.4), sequence alignments have been deposited in TreeBASE as submission no. SN 1893-6146. For neighbor-joining analysis, a distance matrix was generated using DNA DIST, a program from the PHYLIP 3.5c package (Felsenstein, 1995) integrated in ClustalX. The calculation was performed using the Kimura 2 model and a transition:transversion ratio 2 : 1. Bootstrap values for internal nodes were calculated by 1000 replications (Felsenstein, 1985).

Results

Fruit body morphology and mycelial characters of Australian isolates

From eight Australian isolates examined, only two, 22409b (from *Dodonaea viscosa*) and 22859 (from *Vitis vinifera*), were associated with fruit bodies. These fruit bodies were of different shape, being pileate for 22409b, and resupinate for 22859. No clear differentiation was revealed by microscopical means. Basidiospores were slightly smaller in 22409b, with 6 x 5 µm on the average, while they were 7 x 5.5-6 µm in 22859; however, only few spores were found in 22409b.

While the fruit body of 22409b was distinct by its pileate shape, microscopical characters allowed no differentiation between Australian specimens and the European based species, *Fomitiporia punctata* and *F. mediterranea*.

Australian isolates were able to grow at all temperatures between 15°C and 30°C. Appearance of cultured mycelium was not uniform, and two main groups were distinguished. Strains 22488, 22492, and 22495 had fast growing mycelia, with 3.1 - 3.3 and 6.4 - 6.6 cm/wk under 21°C and 30°C conditions, respectively. In such cultures, pigmentation of the medium was weak or lacking; aerial hyphae were well developed, whitish, with yellowish rings or spots, preferably next to the inoculum; colonies became locally velvety and brownish after several weeks.

Strains 22451, 22485, 22486, and 22859 had slow growing mycelia, with 1.8 - 2.4 and 1.7 - 2.6 cm/wk under 21°C and 30°C conditions, respectively. Pigmentation of the medium was modest to strong; aerial hyphae were less developed, resulting in a more woolly appearance of the culture; yellowish to brownish colors were predominant, with whitish colors restricted to the margin of the growing culture.

22409b had even slower growth, with 0.8 and 1.1 cm/wk under 21°C and 30°C conditions, respectively; pigmentation of the medium was very strong (corresponding to the ‘staining type’ as described in Fischer, 1987); however, mycelial type of this culture could vary in subsequent inoculations, and then was similar to the group above.

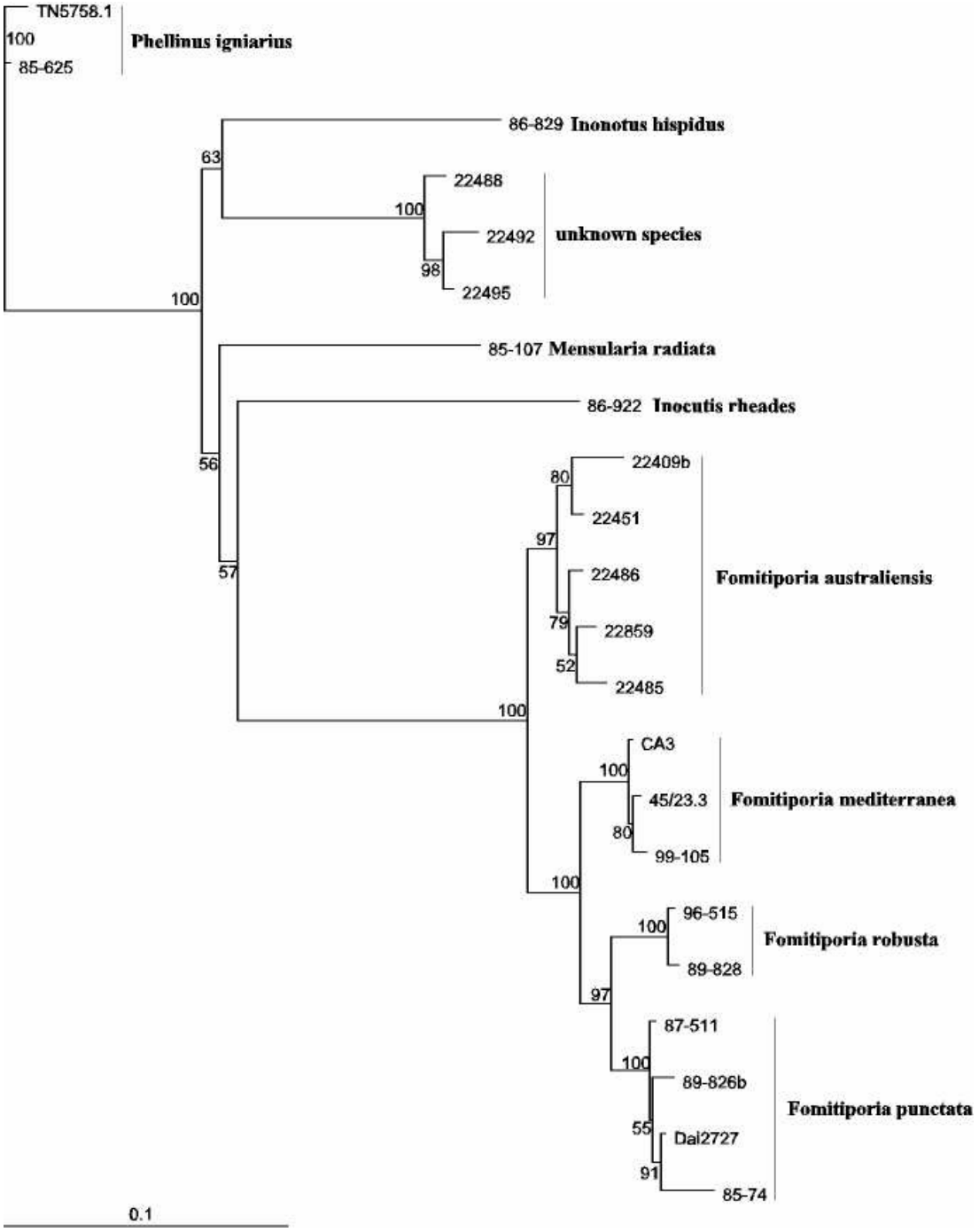


Figure 2.1: Phylogenetic relationships of *Fomitiporia australiensis* and related Hymenochaetales taxa inferred from the nuclear ITS1-5.8S-ITS2 region using the neighbor-joining method. The tree was rooted with isolates belonging to *Phellinus igniarius*. Bootstrap values of 50% or greater are indicated for the corresponding nodes. Branch lengths are proportional to genetic distance. The proposed specific designation is explained in the text.

Molecular sequences and phylogenetic analysis

Molecular sequences of the ribosomal ITS region were generated for all Australian isolates, *Inonotus hispidus*, *Inocutis rheades*, and *Mensularia radiata*. Corresponding sequences of *Phellinus igniarius*,

Fomitiporia robusta, *F. punctata*, and *F. mediterranea* were derived from a former study (Fischer, 2002).

For 86-829 (*Inonotus hispidus*) and 22492 (Australian isolate from *Vitis vinifera*), sequences were incomplete at the 5´ end. As a consequence, this particular section, comprising approximately 35 nucleotides of the ITS1 region, was omitted for all strains in the final alignment.

The Australian isolates fell into two groups: strains 22451, 22485, 22486, and 22859 were 634-638 nucleotides, while strains 22409b, 22488, 22492, and 22495 were 686-692 nucleotides. As a striking phenomenon, sequences of strain 22409b were widely identical with those of the former group, but were separated by three inserts all existing within the ITS1 region.

A phylogenetic analysis using the neighbor-joining method resulted in a subdivision of the Australian isolates into two distinct groups; these were not assignable to any of the other taxa included as references (Fig. 2.1).

The larger group, comprising 5 strains, formed a strongly supported (100%) cluster together with the taxa belonging to the genus *Fomitiporia*, i.e. *F. mediterranea*, *F. robusta*, and *F. punctata*. Both fruit body forming strains, 22409b (pileate) and 22859 (resupinate), are enclosed in this group. As mentioned above, 22409b is characterized by a divergent size of the PCR product, and this is reflected in a peripheral position within the cluster. Together with another strain (22451), 22409b forms a separate subgroup.

The smaller group of Australian strains, comprising three mycelial isolates (22488, 22492, and 22495), came out as related to *Inonotus hispidus*, although only modestly supported by a bootstrap value of 63% (Fig. 2.1).

Taxonomic conclusions

Molecular data show the Australian isolates to be genetically distinct. One of the revealed groups, including strains 22451, 22485, 22486, 22859 (from *Vitis*), and 22409b (from *Dodonaea*) shows a clear affinity to *Fomitiporia*, but is not assignable to any of the included taxa of this genus (see discussion below for further comments). Accepting the molecular data as distinct characters (phylogenetic species recognion; Taylor et al., 2000; Fischer & Binder, 2004), we suggest a specific taxonomic status for these isolates:

Fomitiporia australiensis* M. Fischer et al., *sp. nov.

Basidiomata perrenia, resupinata ad ungulata; superficies pororum brunnea, pori ellipsoideae ad circulares, 2-5 in quoque millimetro; systema hypharum dimiticum, omnia septa fibulis egentia; hyphae skeletales luteobrunneae, 2-5 µm latae, hyphae generativae hyalinae, 2-3 µm latae; sporae ellipsoideae ad subglobosae, hyalinae, crassitunicate, cyanophilicae et amyloideae, 6-8 x 5-6.5 µm.

Holotypus 22859 in Victorian Plant Disease Herbarium (VPRI), collectus a I. Pascoe, J. Edwards, N. Laukart, in Vitis vinifera in Australia, 2001.

Discussion

At the present time, *Fomitiporia* comprises approximately one dozen species worldwide (Gilbertson & Ryvarden, 1987; Ryvarden & Gilbertson, 1994; Fischer, 2002; Fischer & Binder, 2004). Two of these, *F. mediterranea* in Europe and *F. australiensis* in Australia, are associated with white heart rot in esca-affected grapevine. With these data available, *Fomitiporia* represents the economically most important basidiomycetous group related to esca disease. Some other genera of basidiomycetes, such as *Trametes* or *Stereum*, have been mentioned as occurring on diseased *Vitis vinifera*, but they are mostly associated with dead wood and therefore play a subordinate role only (Jahn, 1963; Fischer & Kassemeyer, 2003).

It remains an open question why fruit bodies of *F. australiensis* are rarely found and its presence in an infected host is only detected as vegetative mycelium. Possibly the formation of fruit bodies is correlated with the age and/or overall condition of the host plant, and dead trunks of grapevine most

suitable for bearing fruit bodies have usually been removed from the vineyard. We do not know if a higher number of fruit bodies can be found on non-*Vitis* host plants outside of vineyards in Australia, but in Italy it has been demonstrated that *F. mediterranea* fruit bodies are rarely found within vineyards (Cortesi et al., 2000), yet seem to exist in considerable numbers on hosts outside of vineyards (Fischer, 2002). In vineyards examined in Central Europe, fruit bodies of *F. mediterranea* were only present on 1-3% of esca-affected grapevines older than 15 years, although often more than 50% of the trunks had symptoms of white heart rot (Fischer, unpubl. results). Evidently, grapevines may be affected with white heart rot without showing external symptoms on leaves or berries and they may host the inconspicuous fungus for many years. In view of this, the distribution of fruit bodies is unlikely to be truly indicative of the frequency of basidiomycetes associated with esca.

In this study we have demonstrated the existence of a new basidiomycetous species on grapevine; with the data at hand this species can be demonstrated only by sequence data of the ribosomal ITS region. In addition, another as-yet-undetermined taxon was detected in this study, with distinct molecular sequences and growth behavior of cultured mycelium. In the phylogenetic analysis, this taxon was revealed as somewhat related to *Inonotus hispidus*, an important pathogen on fruit and nut trees in Europe (Ryvarden & Gilbertson, 1994) and North America (Adaskaveg & Ogawa, 1990).

Other basidiomycete fungi isolated from white heart rot in grapevine and native hosts:

Over the course of this project, 46 isolates of basidiomycetes were recovered from heart rot of grapevines and other woody species surrounding vineyards: 42 from grapevine, two from *Eucalyptus* sp., one from *Dodonea viscosa* (native hop-bush), and one from a fence post made from *Eucalyptus* wood. In view of the lack of fruiting bodies, it was decided to determine the affinities of Australian species based on phylogenetic analysis of nuclear large sub unit (28S) rDNA sequence data. The methodology used was that described by Wagner and Fischer (2002). Although DNA was extracted from all isolates, many could not be fully sequenced due to 'slippage', a common problem encountered when sequencing basidiomycete fungal species (Fischer, pers. comm.). Twenty-six isolates were successfully sequenced and compared with other species of Hymenochaetaceae. All except one isolate were identified as species of *Fomitiporia* or *Inonotus s. str.* The single isolate was tentatively identified as *Fuscoporia contigua*. There were two species of *Fomitiporia*: *F. australiensis* (from grapevine, *Dodonea* and the *Eucalyptus* fence post) and an undescribed species (from grapevine and the *Eucalyptus* sp.). Three species of *Inonotus s. str.* were found, all from grapevine. We have not been able to identify any of these three species. One of these taxa was encountered much more frequently than the other two. Given that both *Fomitiporia* species were also isolated from native Australian hosts, it seems likely that the species encountered are native to Australia and are in the bush surrounding vineyards.

2.3 Petri disease

Summary: When the project began, the causal agents of Petri disease were reportedly three *Phaeoacremonium* species: *P. chlamydosporum, P. aleophilum* and *P. inflatipes.* (Scheck *et al.* 1998). Of these, only two had been isolated from diseased Australian grapevines ie. *P. chlamydosporum* (later renamed *Phaeomoniella chlamydospora)* and *P. aleophilum.* From the pathogencitiy tests reported in this section, we show that *Pa. chlamydospora* caused poor callus formation, graft failure and death of young grapevines, whereas *Pm. aleophilum* caused little harm.

2.3.1 The effect of *Pa. chlamydospora* and *Pm. aleophilum* on callus and root production of grapevine scion and rootstock cuttings and on their subsequent survival rate.

Anecdotal evidence suggested that infected grapevine cuttings do not callus properly, resulting in poor planting material, and that some grapevine cultivars are affected less than others. Experiments to determine the pathogenicity of *Pa. chlamydospora* and *Pm. aleophilum* on a range of grapevine scion and rootstock varieties were undertaken with with the help of honours student, John Wallace, from Burnley College, Institute of Land and Food Resources, The University of Melbourne. The aim of these experiments was to determine the relative pathogenicity of *Pa. chlamydospora* and *Pm. aleophilum* and to investigate whether cultivars differed in their response to infection.

Methods

The bases of grapevine cuttings (12 cuttings per treatment/cultivar combination) were inoculated with 100 spores (20 µL of $5x10^3$ spores/mL) of either *Pa. chlamydospora, Pm. aleophilum* or a mixture of both. Control treatment was inoculation with 20 µL water only. The cultivars tested were seven rootstock varieties (Ramsey, 99 Richter, Schwarzmann, Kober 5BB, Paulsen, 101-14 Millardet and SO4) and five scion varieties (Merlot, Cabernet Sauvignon, Pinot Noir, Shiraz PT10 and Shiraz PT23). After inoculation, the cuttings were layered in boxes of moist vermiculite and maintained at 25°C to callus and form roots as per nursery practice. After 10 weeks, callus production, root initiation and internal symptom development were assessed on six cuttings per treatment. Callus formation was assessed in two ways: the proportion of the cut end that had callused and the callus dry weight. Root initiation was assessed as the number of roots per cutting and as root dry weight. Symptom development was assessed as presence or absence of internal brown woood streaking. Treatment effects were determined using ANOVA.

The remaining six cuttings per treatment were potted up and maintained in the glasshouse. Their survival rate was assessed 17 months later.

Results

Callus and root formation

Pa. chlamydospora significantly inhibited callus formation in all cultivars, whether inoculated by itself or jointly with *Pm. aleophilum. Pm. aleophilum* significantly inhibited callus formation in one rootstock cultivar, Schwarzmann, and all scion cultivars, but not to the same extent as *Pa. chlamydospora* (Table 2.5).

Neither fungus affected root initiation (data not shown). Cultivars differed with respect to internal symptom development (data not shown). *Pa. chlamydospora* caused brown wood streaking in the rootstock cultivars, but not the scion varieties. *Pm. aleophilum* did not cause visible internal symptoms in any of the cultivars tested.

Table 2.5. Effect of inoculation with *Phaeomoniella chlamdospora* (Pch) and/or *Phaeoacremonium aleophilum* (Pal) on callus production, measured as % circumference of cut end and as dry weight (mg) of cuttings after 10 weeks.

	Pm. aleophilum		*Pa. chlamydospora*		Pal+Pch		water	
Cultivar	%	mg	%	mg	%	mg	%	mg
Rootstock								
101-14 Millardet	100	56.61	0	0.00	52	8.41	95	70.05
99 Richter	83	34.60	2	0.00	13	7.97	100	73.15
Kober 5BB	100	81.23	7	1.05	14	1.07	97	24.96
Paulsen	100	36.51	1	0.00	64	7.47	100	104.56
Ramsey A11V2	78	0.25	0	0.00	2	0.00	75	1.49
Schwarzmann	2	0.00	0	0.00	0	0.00	47	1.05
SO4	86	5.07	2	0.00	0	0.00	98	14.06
Scion								
Cab Sav G9V3	27	0.00	0	0.00	0	0.00	47	2.11
Merlot D3V14	48	9.31	0	0.15	0	0.00	83	18.49
Pinot Noir D2V5	60	0.00	0	0.00	0	0.00	74	2.30
Shiraz PT10	9	0.71	8	0.40	5	0.00	65	3.22
Shiraz PT23	53	4.70	6	0.00	6	0.00	72	3.39

The remaining six cuttings per treatment were potted up and grown on in the glasshouse for 17 months. By this time, most of the *Pa. chlamydospora*-inoculated rootstocks had died, although the scion varieties were still alive (Table 2.6)

Table 2.6. Survival rate of inoculated cuttings after 17 months.

Variety	**Number of cuttings (out of 6) still alive 17 months after inoculation**			
Scion	**Water**	***Pa. chlamydospora***	***Pm. aleophilum***	**Both fungi**
Cab Sav	5	2	2	2
Merlot	4	2	4	3
Pinot Noir	0	3	1	3
Shiraz PT10	3	4	2	3
Shiraz PT23	2	4	3	3
Rootstock				
Kober	1	1	4	4
Millardet	5	0	4	4
Paulsen	3	0	6	0
Ramsey	3	0	0	0
Richter	5	0	5	3
Schwarzmann	5	2	5	6
SO4	4	0	5	3

2.3.2 The effect of *Pa. chlamydospora* and *Pm. aleophilum* on graft take of omega and wedge graft unions

To test this, we grafted seven rootstock varieties (Ramsey, 99 Richter, Schwarzmann, Kober 5BB, Paulsen, 101-14 Millardet and SO4) to a single scion variety (Shiraz PT10) using both wedge and omega graft types. At the time of grafting, we inoculated the graft unions (12 cuttings per treatment/rootstock/graft type combination) with 100 spores (20 µL of $5x10^3$ spores/mL) of either *Pa. chlamydospora, Pm. aleophilum* or a mixture of both. Control treatment was inoculation with 20 µL water only. After inoculation, the cuttings were layered in boxes of moist vermiculite and maintained at 25°C to callus and form roots as per nursery practice. After 10 weeks, the vines were potted up and allowed to grow on in a shadehouse. Nine months after grafting, the strength of the graft unions was

measured by applying pressure and recording the breaking point of each graft. All grafts that been inoculated with *Phaeomoniella chlamydospora* had failed to take. However, *Phaeoacremonium aleophilum* had no inhibitory effect.

The results of both these experiments demonstrate that *Pa. chlamydospora* is the more virulent of the two fungi.

2.3.3. Investigating secondary metabolite production by *Pa. chlamydospora*

An original intention was to investigate the role of secondary metabolite production in *Pa. chlamydospora* infection and use the results to develop a rapid non-destructive diagnostic tool based to detect the presence of *Pa. chlamydospora* in infected vines. Ideally, this would take the form of a soluble secondary metabolite that could be detected in plant parts such as shoots and leaves remote from the site of infection. PhD student Eve Cottral was working towards identifying a 'signature' biochemical marker for *Pa. chlamydospora* that could be used as a diagnostic tool. She chose 'Compound 10', a secondary metabolite that had been identified by a Swiss group as a novel compound they had found only in cultures of *Pa. chlamydospora*. Eve obtained some synthesised compound 10 and characterised its properties using LC-MS and HPLC. She was then using this equipment to look for the presence of compound 10 in *Pa. chlamydospora* culture filtrates. This work was stalled when Eve died, but was re-examined in 2005. However, when attempting to synthesise further quantities of compound 10, new developments in technology showed that there were several isomers of the compound and it was impossible to know which was the isomer originally identified by the Swiss group. Therefore this avenue of research was halted.

2.4 *Phaeoacremonium* species associated with Petri disease and esca

Summary: In 1999, when this current project began, six species of *Phaeoacremonium* had been described, of which four were known to occur in grapevines (Crous *et al.* 1996). The only two known to occur in Australia were *Pa. chlamydospora* and *Pm. aleophilum.* One of these was later redescribed as *Phaeomoniella chlamydospora* (Crous and Gams 2000). No sexual states of these fungi were known to exist. In the present study, PhD student, Eve Cottral, examined the genetic variation present in populations of the two fungi, *Pa. chlamydospora* and *Pm. aleophilum,* using the molecular technique UP-PCR (universal primed polymerase chain reaction). A total of 31 *Pa. chlamydospora* (22 Australian) and 15 *Phaeoacremonium* (8 Australian) isolates were screened for polymorphisms using UP-PCR. The other isolates were from Italy, New Zealand and the Centraalbureau voor Schimmelcultures (CBS) collection in the Netherlands. *Pm. inflatipes* and *Pm. rubrigenum* (from CBS) were included for comparison with *Pm. aleophilum* isolates. All isolates of *Pa. chlamydospora* (both Australian and overseas) were found to be almost genetically identical indicating that sexual recombination in the field is unlikely and providing evidence for clonal spread via grapevine planting material. However, considerable variation was observed among the isolates of *Pm. aleophilum,* indicating that there could be sexual recombination occurring in this fungus. We later observed the sexual state of the fungus on grapevine wood and determined that the teleomorph (sexual state) was, in fact, *Tognina minima*. In addition, the considerable genetic variation was later explained by Mostert *et al.* (2005) who showed that our isolates, in fact, represented three species: *Pm. aleophilum, Pm. parasiticum* and a newly described species, *Pm. australiense*

2.4.1 Investigation into the genetic variability within Australian isolates of *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* using UP-PCR Analysis.

Phaeomoniella chlamydospora (Pch) and *Phaeoacremonium aleophilum* (Pal) are two fungi commonly isolated from grapevines showing esca symptoms and also from vines infected with Petri disease. Although *Pa. chlamydospora* is easily identified by morphological characters, *Pm. aleophilum* isolates and *Pm. aleophilum*-like isolates have variable morphology. Universal primed polymerase chain reaction (UP-PCR) was used to study the genetic diversity of *Pa. chlamydospora* and *Pm. aleophilum* isolates from different wine-growing regions in Australia and also to compare these isolates with isolates from other countries. In this study 8 UP-PCR primers were used to make comparisons between Australian and overseas isolates of *Pa. chlamydospora* and *Pm. aleophilum.*

Methods

DNA extraction

Genomic DNA was extracted from 31 *Phaeomoniella chlamydospora* and 15 *Phaeoacremonium* spp. isolates. Twenty-two *Pa. chlamydospora* and 8 *Pm. aleophilum* Australian isolates, lodged in the VPRI (Victorian Plant Research Institute) herbarium were used in the study. Three *Pa. chlamydospora* and 5 *Pm. aleophilum* Italian isolates were used. The remaining 5 *Pa. chlamydospora* and 1 *Pm. aleophilum* were from New Zealand and from the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands respectively. Isolates of *Pm. inflatipes, Pm. angustius* and *Pm. rubrigenum* (from CBS) were also included for comparison with *Pm. aleophilum* isolates (Table 2.7).

Single spore isolates of the Australian *Pa. chlamydospora* and *Pm. aleophilum* strains were generated on Potato Dextrose Agar and allowed to grow for 2 weeks. Ten to fifteen 1mm^2 squares were cut from the growing edge of the single spore colony and used to inoculate 100 ml of Potato Dextrose Broth and kept on a shaker at 150 rpm. Six to eight day old mycelia mats were collected on sterile calico cloth on a funnel attached to a buchner flask. Fungal mycelia were rinsed with SDW (sterile distilled water) and vacuum pressure was supplied to the buchner funnel to remove excess water. Eppendorf tubes (1.5mL) were filled 2/3 volume with the fungal mycelia and then centrifuged to drain off excess water, and placed into the freezer. Frozen mycelia were then freeze-dried. DNA was extracted from approximately 0.1g of freeze-dried mycelia using Nucleon Phytopure (Amersham)

according to the manufacturers instructions with the addition of 20mg/ul RNAse (Progen Ltd, Australia). The DNA pellet was vaccuum-dried and resuspended in 50 µL of sterile water upon arrival in NZ prior to use. The DNA concentration was adjusted to 5ng/ul prior to amplification with the UP-PCR primers. To validate the results, DNA was re-extracted from the freeze-dried mycelia using the protocol described by Lee and Taylor (1990), except that 1mg/ml RNAse (Progen Ltd, Australia) was added with the lysis buffer. DNA was quantified by UV visualization on a 1% agarose gel stained with ethidium bromide. The DNA concentration was adjusted to 5ng/ul prior to amplification with the UP-PCR primers.

Table 2.7. Number of isolates used in this study and their geographic origin

Fungus	Origin	Number of isolates used
Phaeomoniella chlamydospora	Australia	23
	Italy	5
	New Zealand	5
Phaeoacremonium aleophilum	Australia	8
	Italy	5
	Yugoslavia	1
Phaeoacremonium inflatipes	USA	1
Phaeoacremonium rubrigenum	USA	1

UP-PCR amplification and reaction conditions

DNA amplification was performed using 8 universally primed PCR primers (Table 2.8). Each 25 µL reaction contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 µM each of dATP, dTTP, dGTP and dCTP, 20 picomoles primer, 2.5 mM $MgCl_2$, 5 ng genomic DNA and 0.25 U Taq DNA polymerase (Roche Diagnostics N.Z. Ltd). The reactions were performed in an Eppendorf MastercyclerR Gradient PCR machine (at Lincoln University, New Zealand) and consisted of 5 min at 94ºC followed by 5 cycles of 50 sec at 94ºC, 2 min at the specified annealing temperature (Table 2.8) and 1 min at 72ºC, followed by 35 cycles of 50 sec at 94ºC, 90 sec at the specified annealing temperature and 1 min at 72ºC, with a final extension of 72ºC for 7 min. Amplification products were separated by electrophoresis on 1% agarose gels in TAE buffer, stained with ethidium bromide and visualized with UV illumination. The molecular weight marker used was 1 Kb DNA ladder (Gibco BRL, USA). All UP-PCR reactions were repeated using a Hybaid PCR Express Thermocycler (Hybaid, UK) at DPI Knoxfield (Australia).

Table 2.8. The oligonucleotide sequence of the UP-PCR primers and their respective annealing temperatures

Name	Primer sequence	Annealing temp (ºC)
AA2M2	5'–CTGCGACCCAGAGCGG-3'	50
AS4	5'-TGTGGGCGCTCGACAC-3'	55
AS15	5'-GGCTAAGCGGTCGTTAC-3'	52
AS15inv	5'-CATTGCTGGCGAATCGG-3'	52
L15	5'-GAGGGTGGCGGTTCT-3'	52
L15/AS19	5'-GAGGGTGGCGGCTAG-3'	52
L45	5'-GTAAAACGACGGCCAGT-3'	51
0.3-1	5'-CGAGAACGACGGTTCT-3'	50
3-2	5'-TAAGGGCGGTGCCAGT-3'	52

DNA sequencing and data analysis

The 5.8S nuclear ribosomal RNA gene and the flanking internal transcribed spacers (ITS1 and ITS2) were amplified using primers ITS4 and ITS5 (White *et al.* 1990). PCR reactions (25µL) comprised of 20 mM Tris-HCl (pH 8.4), 2.5 mM each of dATP, dTTP, dGTP and dCTP, primer, 2.5 mM $MgCl_2$, 5

ng genomic DNA and 0.25 U Taq DNA polymerase (Roche Diagnostics, Australia). The reaction was performed on a Hybaid PCR Express Thermocycler (Hybaid, UK) and consisted of the following program: an initial denaturation for 3 min at 95°C, followed by 30 cycles of 2 min at 95°C, 25 sec at 50°C, and 2 min at 72°C.

Phylogenetic analysis

Amplification products (bands) were scored as either present (1) or absent (0). Faint bands were not scored. A dissimilarity matrix was constructed using data obtained from the UP-PCR analysis on the 31 *Pa. chlamydospora* isolates and 15 *Pm. aleophilum* isolates using Jaccard's coefficient (Jaccard, 1901), using the RAPDistance program (ver. 1.04, Australian National University, Canberra Australia).

The dissimilarity matrix was used to construct a dendrogram using the unweighted pair-group method (UPGMA), with MEGA version 2 (Kumar *et al.* 2001).

Results and Discussion

Genetic variation revealed by all UP-PCR primers was minimal for *Pa chlamydospora*, regardless of geographic origin (Figure 2.2). This strongly suggests that the pathogen is asexual and clonal lineages have been spread around the world via infected planting material (see Section 3).

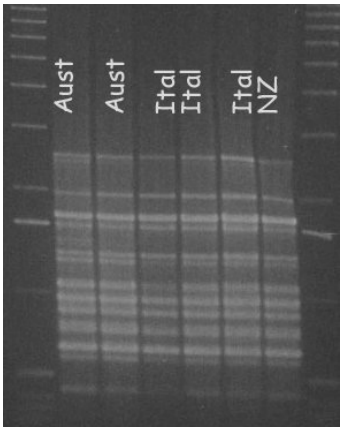


Figure 2.2. Comparison of banding patterns of *Phaeomoniella chlamydospora* isolates generated using UP-PCR primer AA2M2, showing the lack of genetic variation across geographic location.

The Australian *P. aleophilum* isolates formed three distinct groupings (Figures 2.3 and 2.4). The first group was found to have similar DNA banding profiles to the Italian isolates. The second group consisted of a single isolate (VPRI 22940) that had its own unique banding pattern and appeared similar to *Phaeoacremonium inflatipes*. However, ITS sequencing confirmed *Pm. aleophilum* identity (Note – in 2003, it was determined that the culture of *P. inflatipes* had been wrongly identified and was, in fact, *P. aleophilum*). Group 3 contained two isolates (Aust6 and Aust7) which appeared to be more genetically similar to *P. rubrigenum* than *P. aleophilum* (Figure 2.4). However, these two isolates were found to have different banding patterns from the type specimen of *P. rubrigenum* which was included with the study for comparison as an outgroup.

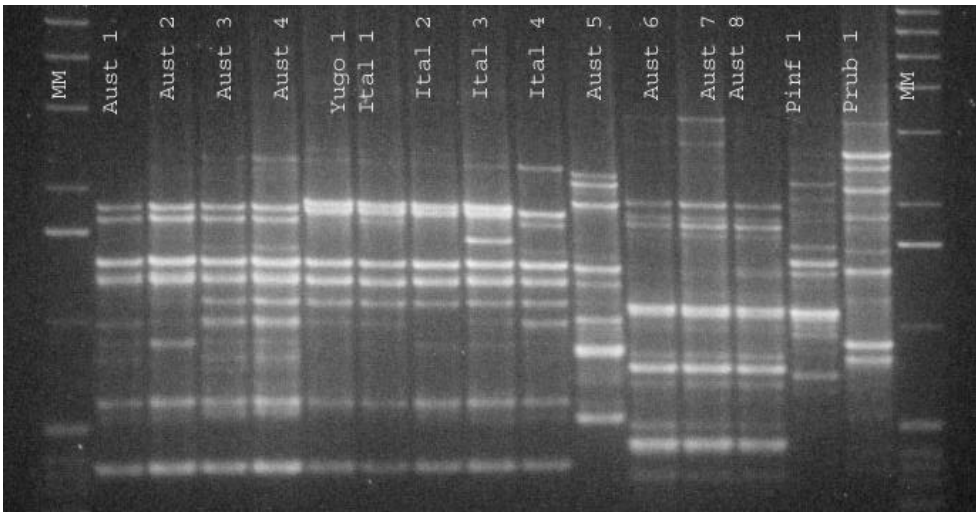


Figure 2.3. Comparison of banding patterns of *Phaeoacremonium* species isolates generated using UP-PCR primer 3-2, showing considerable genetic variation.

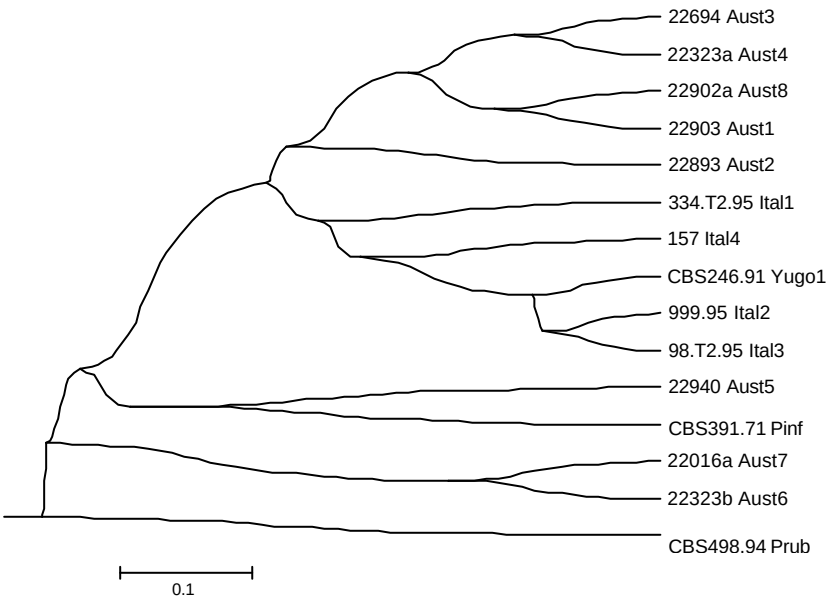


Figure 2.4. Dendrogram showing genetic relationships between 15 isolates of *Phaeoacremonium* spp.using distances produced with Jaccard's coefficient and constructed with the UPGMA alogarithm.

Unfortunately Eve's work was suspended when she fell ill and died in 2003. We sent a selection of *Phaeoacremonium* isolates to Prof Pedro Crous and Lizel Mostert at the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, to be included in a taxonomic review of the genus. Isolates from sources all around the world were compared on the basis of their morphological and cultural characters, and phylogenetic analyses of partial sequences of the actin, β-tubulin and calmodulin genes. When published in 2005, the revised genus of *Phaeoacremonium* included 16 species, of which 11 were known to occur in grapevines (Mostert *et al.* 2005). Mostert *et al.* (2005)

described one of Eve's Group 3 isolates (VPRI 22016a) as the type specimen of a new species, *P. australiense*. In total, the eight isolates from Australian grapevines included in Mostert *et al.*'s study represented three species: *Pm. aleophilum, Pm.parasiticum* and the new species *Pm. australiense*.

2.4.2 The sexual state of *Pm. aleophilum, Togninia minima*

The considerable genetic variation within the *Pm. aleophilum* group observed by Eve Cottral and others indicated that sexual recombination was occurring, but at the time, no record of a sexual stage had been made.

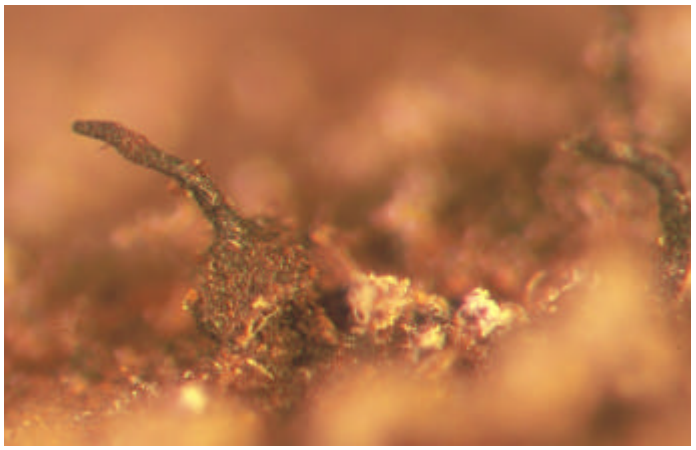


Figure 2.5. Fruiting body of *Togninia minima*, the sexual state of *P. aleophilum.*

During the course of our survey work described above, we found the previously unknown sexual state of *P. aleophilum* (Figure 2.5). Using a combination of morphological and molecular techniques, we were able to assign it to the genus *Togninia*. Unknown to us at the time, the South African group led by Prof. Pedro Crous and the USA group led by Dr. Doug Gubler had also made the same connection. All three groups presented their work at the 3rd International Workshop on Grapevine Trunk Diseases in New Zealand in 2003. Subsequently, we published our finding as a first record within Australia:

> IG Pascoe, J Edwards, JH Cunnington, EH Cottral (2004) 'Detection of the *Togninia* teleomorph of *Phaeoacremonium aleophilum* in Australia' *Phytopathologia Mediterranea* **43**: 51-58

Conclusion

From these studies we conclude that, in the Australian situation at least, the fungus *Phaeomoniella chlamydospora* is the main causal organism of both Petri disease and esca, and others such as *Phaeoacremonium* species and the heart rotting fungi are relatively unimportant. In light of these results, our studies concentrated on understanding the biology, epidemiology and control of the fungus *Phaeomoniella chlamydospora.*

Chapter 3: Epidemiology of Petri disease

Jacqueline Edwards, Natalie Laukart, Ian Pascoe, Soheir Salib

Summary. When this project began, it was unknown where *Pa. chlamydospora* produced its spores in the vineyard. We observed abundant sporulation on protected wood surfaces inside deep cracks on several grapevine varieties. Springtails and mites were usually associated with the sporulation, but it is not known whether they have a role in dissemination of conidia. This was the first report of how and where this fungus produced spores in the field. We also demonstrate that both *Pa. chlamydospora* and *Pm aleophilum* are spread in cuttings taken from infected mother vines and result in symptomless infected planting material. The evidence for spread occurring through soil was inconclusive.

3.1 Sporulation within the vineyard

Although *Pa. chlamydospora* has been recorded in grape-growing regions all over the world, for example in South Africa (Ferreira *et al.*, 1994), France (Larignon and Dubos, 1997), the USA (Scheck *et al.*, 1998), Australia (Pascoe, 1999), Italy (Mugnai *et al.*, 1999), Argentina (Gatica *et al.*, 2000), Austria (Reisenzein *et al.*, 2000), Portugal (Chicau *et al.*, 2000; Rego *et al.*, 2000) and Turkey (Erkan Ari, 2000), the source of inoculum remained unknown. In young vineyards it is believed that the planting material is already infected, either systemically from infected mother vines (Ferreira, 1999; Pascoe and Cottral, 2000) or by contamination during the propagation process (Bertelli *et al.*, 1998; Scheck *et al.*, 1998). In mature vineyards, it is known that infection can occur through wounds (Larignon and Dubos, 2000). However, the fundamental questions remain unanswered: how do mother vines become infected and where is the original source of inoculum in the field? It has been suggested that *Pa. chlamydospora* is soilborne (Ferreira *et al.*, 1994; Bertelli *et al.*, 1998; Mugnai *et al.*, 1999; Sidoti *et al.*, 2000) or that sporulation occurs on dead wood and debris in the vineyard (Mugnai *et al.*, 1999; Ferreira 1999), but neither hypothesis has been supported by conclusive evidence.

Little is known of the reproductive and dispersal behaviour of *Pa. chlamydospora* in the field although *in vitro* ontogeny and morphology of conidia have been well documented (Crous *et al.*, 1996; Crous and Gams, 2000; Pascoe and Cottral, 2000) and Edwards and Pascoe (2001) described the occurrence of pycnidia on grapevine wood in the field. Obviously, further information on reproductive behaviour is critical to a full understanding of the epidemiology of the fungus in vineyards, particularly with respect to the timing, site and mode of spore production and dispersal and the role of these different spore types. The knowledge that *Pa. chlamydospora* sporulates readily on moist incubated, freshly-cut grapevine wood in the laboratory led us to speculate that the fungus may sporulate on fresh wounds in the vineyard. However, sporulation on wound surfaces in the field has never been reported. Larignon and Dubos (2000) were able to demonstrate infection via wounds and trapped *Pa. chlamydospora* spores on vaseline-coated glass slides in vineyards, but they failed to demonstrate the source of these spores.

The aim of this experiment was to determine the timing and environmental conditions for spore production in the vineyard.

M**ethods**

Pa. chlamydospora sporulation on cut surfaces of infected scion and rootstock wood

The trial site consisted of 100 vines : 25 vines in each of four adjacent rows of 10-year-old ‘Pinot Noir’ grapevines grafted onto ‘Ramsey’ rootstocks growing in a vineyard near Geelong, Victoria, Australia. The vines had declined rapidly over the previous three years, the decline being triggered by undetected faulty irrigation drippers that resulted in prolonged waterlogging. Associated symptoms

included reddening of the leaves, poor vigour and fruit set, dieback and death of the vines. Inspection of internal wood symptoms in May 2000 revealed symptoms of black goo decline. Subsequently, the fungus was consistently isolated from symptomatic wood in both rootstock and cordons of all vines tested.

On May 25 (week 0), 88 vines were cut off either at the cordons to expose infected scion wood (40 vines, two cordon cuts each), or below the graft union (48 vines) to expose infected rootstock wood. The vines were assigned to 8 treatments (Treatment 1, one-week old cut surface; treatment 2, two week old cut surface, and so on consecutively).

Table 3.1. Sampling procedure and results of the investigation on sporulation of *Pa. chlamydospora* on freshly-cut infected grapevine wood *in situ*. The treatments' number, from 1 to 8, correspond to different age cut surfaces in weeks. At the time of each survey a wood slice to be examined was cut thus causing a new wound that was then left exposed for the due time for each treatment.

Sampling date	**Treatments**							
	1	2	3	4	5	6	7	8
25 May (week 0) [a]								
1 June (week 1)	-							
8 June (week 2)	-	-						
15 June (week 3)	-		*					
22 June (week 4)	*	*		*				
29June (week 5)	*				*			
6 July (week 6)	*	*	*			*		
13 July (week 7)	*						*	
20 July (week 8)	*	*		*				*
27 July (week 9)	*		*					
3 August (week 10)	+	-			-			
10 August (week 11)	*							
17 August (week 12)	*	*	*	*		*		
24 August (week 13)	*							
31 August (week 14)	*	*					*	
7 September (week 15)	*		*		*			
14 September (week 16)	*	*		*				*

[a] First cut on all the plants (scion or rootstock) of the 8 treatments.

-, No detection of *P.chlamydospora* sporulation.

+, Positive detection of *P.chlamydospora* sporulation.

At regular intervals (from May 25 to September 14) 1 cm thick slices were removed from the relevant scion or rootstock cut surfaces (following the scheme in Table 3.1), every week in Treatment 1, every 2 weeks in Treatment 2, and so on, thus obtaining a series of one- to eight-week-old cut surfaces over the winter months at a time when the vines would normally be pruned. There were ten replicates per each survey date for the scion and six replicates per each survey date for the rootstock.

The slices were microscopically examined (x50) immediately on return to the laboratory on the day of collection and the presence or absence of *Pa. chlamydospora* sporulation on the exposed surface was recorded. They were then moist incubated for up to four months at room temperature (20-22°C) and re-examined several times for the presence of *Pa. chlamydospora* to determine if the wood was infected.

In addition, spore dispersal was monitored by placing ten spore traps consisting of vaseline-coated slides (Larignon, 1999) among the vines each week and collecting them the following week. The vaseline was plated onto potato dextrose agar amended with 50 ppm Achromycin® (a.i. tetracycline hydrochloride; American Cyanamid Company) and examined after two weeks incubation (20-22°C) for the presence of *Pa. chlamydospora*.

Rainfall and temperature data were collected over the period of the trial. We were unable to extend or repeat the trial as the site was unavailable after this period.

Pa. chlamydospora sporulation in cracks and crevices

Deep cracks and crevices were often observed on grapevines where the cordons separated from the trunk and also in rootstocks (Fig. 3.1). These were first noticed on the 10-year-old 'Pinot Noir' scions and 'Ramsey' rootstocks used in the trial described here. The cracks were prised open and the surfaces which had been pressed together microscopically examined (x50). Other vines exhibiting similar symptoms were also examined: these were 10-year-old 'Merlot' on 'Schwarzmann' from Geelong (Victoria), 14-year-old 'Sultana' on 'Ramsey' from Mildura (Victoria) and 6-year-old 'Chardonnay' on 'Ramsey' or 'Teleki' (owner unsure) from Barooga (New South Wales).



Figure 3.1. (a) Cracks in upper trunk at crown of vines in which *Pa. chlamydospora* was found sporulating; (b) Black goo symptoms in infected 'Ramsey' rootstock, with *Pa. chlamydospora* sporulation detected in crack.

Results

P. chlamydospora sporulation on cut surfaces of infected scion and rootstock wood

A total of 410 scion and 252 rootstock cut surfaces from the 88 vines were examined over 4 months, from 1 June to 14 September 2000. Despite having carried out 16 surveys, only once, on 3 August, single sporulating *Pa. chlamydospora* hyphal strands were observed, on 20% of the scion wood slices of Treatment 1, but none of the Treatments 2 and 5 wood slices collected on that date. In addition, sporulating *Pa. chlamydospora* hyphal strands were observed, only at that same date, in the pith region of the rootstock samples (50% of the Treatment 5 samples, but none of the Treatment 1 and 2 wood samples). Sporulation was only observed on the exposed cut surfaces on this sampling date, and there was no evident relationship with rainfall or temperature (Fig. 3.2).

Moist incubation of the pieces at room temperature confirmed that all pieces from all 88 vines were infected with *Pa. chlamydospora.* Examination of the pieces after one and two weeks moist incubation revealed a small amount of growth and sporulation on approximately half of the pieces, but after two to four months moist incubation *Pa. chlamydospora* had overgrown the surface of more than 90% of the pieces.

No *Pa. chlamydospora* cultures grew from the plated-out vaseline of the slide traps.

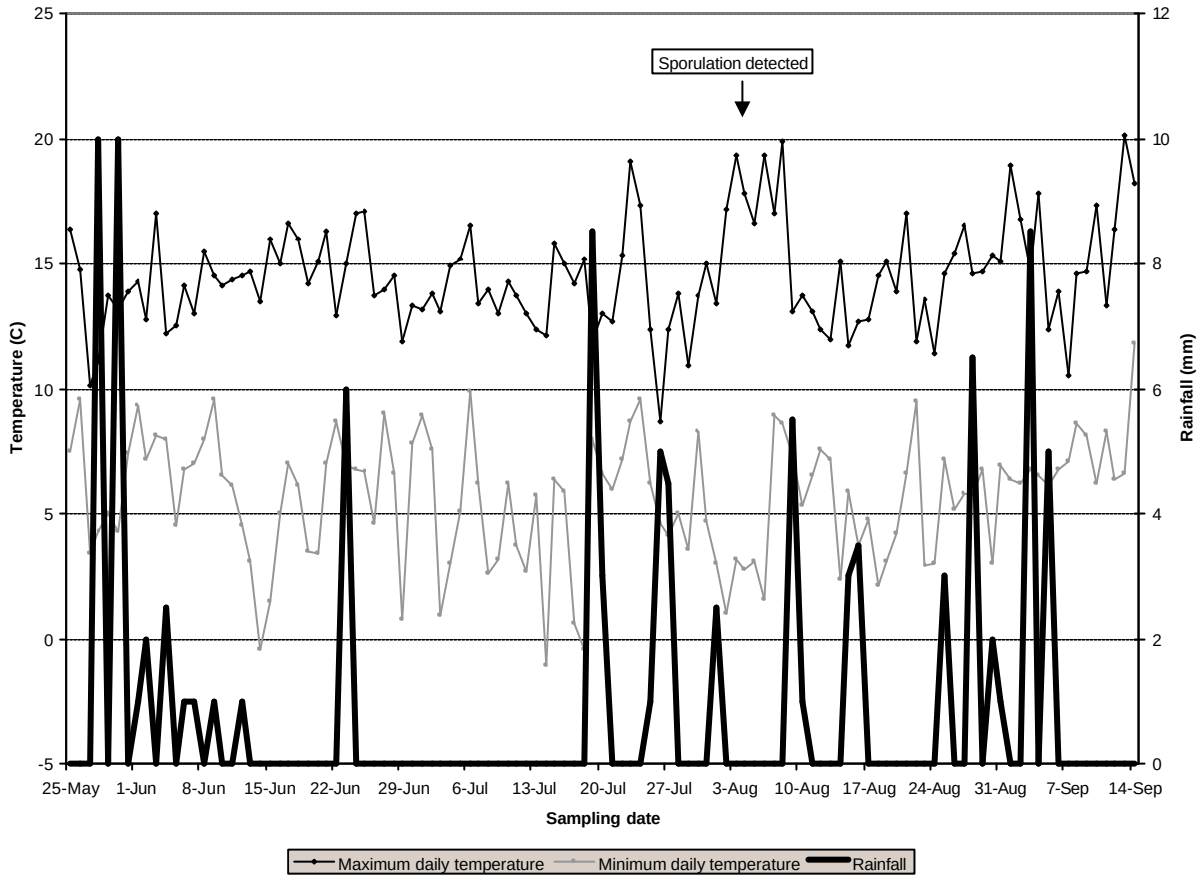


Figure 3.2. Daily temperature (maximum and minimum) and rainfall over the duration of the trial (May to September 2000) and date of detection of *Phaeomoniella chlamydospora* sporulation.

Pa. chlamydospora sporulation in cracks and crevices

In all cases, the two surfaces which had been pressed together were dark and covered with abundantly sporulating *Pa. chlamydospora*, often associated with copious amounts of black sticky 'goo'. A specimen has been lodged in the plant disease herbarium (VPRI) of Agriculture Victoria at Knoxfield as VPRI 22629. At x50 magnification, pycnidia were seen on the wood surface under the hyphomycete state on several of the samples, one of which has been lodged as VPRI 22410. Despite numerous attempts, none of the spores produced by the pycnidial synanamorph, either *in vivo* or *in vitro*, germinated on potato dextrose agar amended with 50 ppm Achromycin®.

Purple collembolans and fast-moving mites were always found among the sporulating hyphae. The collembolans were identified as belonging to *Brachystomella* sp. cf. *platensis* Najt & Massoud from the family Brachystomellidae, members of which are known to feed on various fungi (P. Greenslade, personal communication). The mites were identified as a species of Phytoseiid predatory mite. Both mites and collembolans were observed entering exposed xylem vessels.

Discussion

Contrary to our expectation, *Phaeomoniella chlamydospora* did not sporulate to any significant extent on exposed transversely-cut surfaces during winter. Winter is the most likely time for new infection as this is when pruning is generally conducted. On the one date (3 August) that sporulation was observed on cut surfaces, it was extremely sparse, only one or two hyphal strands. Instead, *Pa. chlamydospora*

was found to sporulate abundantly in deep cracks and crevices on infected grapevines in the field. The cracks and crevices provided a protected humid environment for egress of the fungus, comparable to the moist incubation conditions provided in the laboratory. *Pa. chlamydospora* is a very slow-growing fungus (Chiarappa, 1959) and the amount of sporulation observed within the crevices was consistent with that observed on moist incubated wood after several months. Subsequent moist incubation of the sampled wood slices revealed that they were all infected with *Pa. chlamydospora*. The lack of any *Pa. chlamydospora* spores trapped in the vineyard during the study period implies that the sporulation in the moist chambers was due to internal infection of the wood and not external contamination with spores of the fungus. In view of this, the unexpected low level of sporulation observed on the exposed cut surfaces may be due to the wound surfaces being too dry and exposed to support *Pa. chlamydospora* growth and sporulation, but *Pa. chlamydospora* has been shown to be insensitive to decreasing water potential in laboratory tests (Whiting *et al.* 2001). Another possible explanation is that it was just too cold, as Whiting *et al.* (2001) showed that the temperature range for growth of the fungus in culture was 10-35°C, with an optimum temperature of 25°. This would explain the lack of sporulation observed in this present study, with the amount of *Pa. chlamydospora* growth and sporulation observed within the trunk crevices suggestive of active growth during the preceding summer months (December – March).

Many vines have deep cracks at the junction of cordons and trunk, reported by growers to be the result of damage caused by mechanical pruning and/or harvesting. Vineyard managers in Australia have suggested that the incidence of black goo decline and esca is correlated with increased mechanised viticultural practices. This would be consistent with increased opportunities for egress of the fungus.

Pycnidia were observed among the sporulating hyphae of *Pa. chlamydospora* in several of the cracks examined. This first occurrence of *Pa. chlamydospora* pycnidia in the field was reported by Edwards and Pascoe (2001). Attempts to germinate spores from any of the pycnidium-like structures failed, regardless of whether they were produced *in vivo* or on moist incubated wood, and as such pathogenicity tests could not be carried out.

We believe that the observed association of collembolans and mites with the sporulation in cracks may be relevant to dispersal of *Pa. chlamydospora*. The sheltered nature of the cracks provides limited scope for dispersal methods such as rain or wind, and no spores were detected using the vaseline-coated slide traps. Both the phialidic conidial heads and the pycnidial cirrhi of *Pa. chlamydospora* are presented in such a way as to be readily picked up by small passing arthropods. It is possible that the collembolans and mites could carry fungal spores stuck to their exoskeletons into the cut ends of xylem vessels as they explore fresh wounds. Collembolans and predatory mites have been reported to carry fungal spores on their exoskeletons (Blackwell, 1984; O'Connor, 1984).

The observation that *Pa. chlamydospora* sporulates in cracks and may be arthropod-dispersed may partially explain the apparent increase in importance of Petri disease, coinciding with an increase in vineyard mechanisation. Avoidance of cracking in vines may reduce the inoculum density of *Pa. chlamydospora*, but it is first necessary to compare un-mechanised with mechanised vineyards and verify the suggested correlation between cracks and mechanisation. Further research is also required to determine whether small arthropods are able to transmit this disease.

3.2 Spread by infected planting material.

Infected young grapevines are symptomless (Bertelli et al 1998) and therefore suspected to be the source of disease in new vineyards. In 2000, one-year-old rootlings ready for sale were sampled to determine the percentage that were infected. The cultivars sampled were Cabernet Sauvignon, Merlot and Pinot Noir. They were potted up and grown in the glasshouse to allow any infection to become well established and therefore easier to detect. After 6 months growth, 20 Pinot Noir vines were destructively assessed using moist-incubation to look for the presence of *Pa. chlamydospora* in the trunk wood. 30% were infected. The Cabernet Sauvignon and Merlot vines were assessed after 2 years growth, when 15 and 25 vines respectively were dissected and moist-incubated. Twenty-seven percent of the Cabernet Sauvignon and 40% of the Merlot were infected (Table 3.2).

Table 3.2. Percentage of one-year-old vines sampled from a nursery at point of sale that were subsequently found to be infected with *Phaeomoniella chlamydospora*

Cultivar	Number of vines sampled	Age when examined	% infected
Cabernet Sauvignon	15	3 years	27
Merlot	25	3 years	40
Pinot Noir	20	18 months	30

3.3 Evidence that *Pa. chlamydospora* and *Pm. aleophilum* can be spread from infected mother vines into cuttings.

The source of infection in young grapevine planting material is still unclear. Although the potential exists for infection to occur by contamination events during propagation (Whiteman et al 2004), there has only been indirect evidence that this occurs. Several researchers have demonstrated infection in rootstock mother vines (Pascoe and Cottral 2000, Fourie and Halleen 2004, Ridgway et al 2002, Stewart and Wenner 2004). Other vascular pathogens such as *Ceratocystis*, *Ophiostoma* and *Verticillium* spp are known to spread throughout their hosts via propagules in xylem vessels (Green 1981), and conidia of *Pa. chlamydospora* and *Pm. aleophilum* have been detected in the sap of mature grapevines (Rooney 2002). Larignon and Dubos (2000) and Fourie and Halleen (2002) detected infection in spurs and canes from infected grapevines, and Halleen *et al.* (2003) showed that a percentage of rootstock cuttings taken from infected mother vines were infected.

In addition, Petri disease is generally regarded as a disease of grafted grapevines, with infection carried in the rootstock component. In Australia, however, approximately 80% of all vineyards are planted with own-rooted (ie ungrafted) *Vitis vinifera* cultivars due to the restriction of phylloxera to small clearly-defined Phylloxera Quarantine Zones. We showed that 62% of samples diagnosed with Petri disease at the Victorian Department of Primary Industry's diagnostic arm, Crop Health Services, were own-rooted *V. vinifera* grapevines in section 1. Given the importance of own-rooted grapevines in Australian viticulture, it was decided to investigate infection transfer in both rootstock and scion propagating material.

Methods

Infection in Vitis vinifera mother vines

A block of mother vines was established near Mildura, Victoria, in 1968, consisting of three grapevines each of many *Vitis vinifera* cultivars. Cuttings were harvested annually from these mother vines for a period of 30 years. In July 2001, many of the vines were removed and 35 (comprising 19 cultivars) were made available for examination. The whole trunks, from crown to roots, were cut open and examined for internal symptoms of black wood-streaking. Isolations were made from symptomatic wood. Slivers of wood approx 2 x 5 mm were removed from areas with black wood streaking symptoms, dipped for one sec in 70% ethanol, followed by 60 sec immersion in 5% NaOCl, then plated onto potato dextrose agar amended with achromycin (PDA+A). The isolations were incubated at 21 C and examined after 4 weeks for the presence of *Pa chlamydospora*, *Pm aleophilum* or any other known grapevine pathogens.

Cuttings from infected Ramsey and Zinfandel mother vines

Cuttings were harvested from Ramsey and Zinfandel mother vines, grown in Sunraysia and Margaret River respectively, infected with *Pa. chlamydospora*.

In August 1999, 90 cuttings were harvested from each of 20 dormant Ramsey mother vines known to be infected with *Pa chlamydospora.* The cuttings from each mother vine were divided into two groups of 45 cuttings. The bundles were paired to make matching bundles of 90 cuttings each, ie 10 bundles of 90 cuttings (45 from each of two vines) for the control treatment and matching 10 bundles of 90 cuttings for the hot water treatment. All cuttings were hydrated in chlorinated rainwater (3.0 ppm Cl) for 1 hr. The cuttings allocated to hot water treatment will be reported on in the section on nursery management.

Post treatment, the cuttings were held in cold storage at 1.5ºC for four months until early December 1999. On removal from cold storage, the cuttings were wax-coated to prevent desiccation and planted out in new nursery beds on land that had never previously grown grapevines. There were 20 plots (10 hot water treated, 10 control), with 90 cuttings in each plot, set out in a complete randomised block design.

After 12 and 18 months, ten young grapevines were randomly selected from each plot and cut at ground level. The top growth was discarded, and the stems were removed for assessment. In the laboratory, each stem was flame-sterilised as described above. The stems were cut in half lengthwise and examined for internal wood streaking, then moist incubated for 6-8 weeks prior to examination for growth of *Pa. chlamydospora* and *Pm. aleophilum.*

Zinfandel: In August 1998, 3200 *V. vinifera* cv. Zinfandel cuttings were harvested from a vineyard in Margaret River, WA, for commercial use. When it was realised that the mother vines were infected with *Pa. chlamydospora*, the cuttings were stored at 1.5ºC for 26 months prior to using for experimental purposes. In October 2000, the cuttings were removed from cold storage, divided equally into 4 groups and different treatments were applied. In this section, we will only consider the control treatment, which consisted of hydration in rainwater for 1 hr. The other treatments will be reported on in the section on nursery management.

After treatment, cuttings were placed into polystyrene boxes containing moist vermiculite and maintained at 28 C until callus tissue and roots had developed. In December 2000, they were planted out in new nursery beds that had never previously grown grapevines. The experimental layout was a randomised block design with the four treatments randomised within each of 8 blocks. After 6, 12 and 18 months, 10 young grapevines were randomly sampled from each of the 36 plots, and assessed as described for the Ramsey grapevines.

Mapping infection in canes from infected Ramsey rootstock mother vines

In July 2000, five full-length canes were cut from each of four Ramsey mother vines infected with *Pa chlamydospora* and *Pm aleophilum.* The canes varied in length from 4.5 m to 6.2 m, and were subsequently processed into 4-node cuttings 400-500 mm long. There were 50 cuttings from Vine 1, 44 cuttings from Vine 2, 42 cuttings from Vine 3 and 50 cuttings from Vine 4. The cuttings were tagged to identify their original position in the canes in order to map the location of any infection courts along the full lengths of the canes. The waste pieces from between the cutting lengths were likewise tagged for identification.

The bark was trimmed from the waste pieces, which were then cut into slices of 3 mm thick, surface sterilised (one sec in 70% ethanol, followed by 60 sec immersed in 5% NaOCl), and plated onto PDA+A. The isolations were incubated at 21 C and examined after 4 weeks for the presence of *Pa chlamydospora* or *Pm aleophilum.*

The cuttings were placed in polystyrene boxes containing moist vermiculite and maintained at 28 C in the dark until callus tissue and roots developed. They were then individually potted into 4 cm tubes containing commercial potting mix and grown for two seasons in glasshouse conditions. They were fertilised monthly with slow release fertiliser (Osmocote) and watered as required.

In July 2002, each resulting young vine was destructively assessed. Roots and shoots were removed and dried to determine their dry weights. Bark was removed from the outside of the trunks prior to flame-sterilisation (immersion in 100% ethanol for 1-2 secs, followed by flame to burn off the ethanol). The trunk pieces were cut open longitudinally with secateurs that had been wiped with tissue soaked in 70% ethanol, and examined for internal symptoms. Following this, all trunk pieces were moist incubated on damp paper towelling in plastic take-away food containers, and examined after 6-8 weeks for the presence of *Pa chlamydospora* and *Pm aleophilum.*

Mapping infection in canes from infected Cabernet Sauvignon mother vines

In July 2001, dormant canes were harvested from each of three Cabernet Sauvignon mother vines infected with *Pa chlamydospora* and *Pm aleophilum.* These canes were much shorter than the Ramsey canes described previously, varying in length from 0.7 m to 4.1 m. Approximately half of the canes from each mother vine were left as untreated controls and the rest were hot water treated (see section on nursery management). The canes were processed into 4-node cutting lengths of 300-460 mm, callused as described above and potted into 4 cm tubes containing potting mix. Untreated control = 17 canes (55 cuttings) from Vine 1, 16 canes (50 cuttings) from Vine 2 and 15 canes (59 cuttings) from Vine 3. Waste pieces from between cutting lengths were surface-sterilised and plated onto PDA+A as

described above. All cuttings and waste pieces were tagged to identify their original positions in the canes. The plants were placed in a randomised block design on glasshouse benches and maintained in glasshouse conditions for two seasons, fertilised and watered as required. In July 2003, the resultant young grapevines were assessed in the same manner as described above.

Results

Infection in Vitis vinifera mother vines

Thirty-two out of the 35 mother vines were infected with *Pa. chlamydospora* (Table 3.3)

Table 3.3. Grapevine pathogens isolated from trunks of 32-year-old mother vines.

Mother vine cultivar	Pathogens isolated from trunk
Barbera	*Pa chlamydospora*
Cabernet Sauvignon	None
Cardinal	*Pa chlamydospora*
Carignan	*Pa chlamydospora*
Chardonnay	*Pa chlamydospora* , basidiomycete
Chardonnay	*Pa chlamydospora*
Chardonnay	*Pa chlamydospora, Botrytis cinerea*
Chardonnay	*Pa chlamydospora, Botrytis cinerea*
Chasselas	*Pa chlamydospora*
Chenin Blanc	*Pa chlamydospora*
Colombard	*Pa chlamydospora*
Colombard	*Pa chlamydospora*
Malbec	*Pa chlamydospora*
Mataro	*Pa chlamydospora*, basidiomycete
Mataro	*Pa chlamydospora, Botrytis cinerea*
Merlot	*Pa chlamydospora*
Muscat Gordo Blanco	*Pa chlamydospora*
Pinot Noir	*Pa chlamydospora*
Pinot Noir	*Pa chlamydospora*
Pinot Noir	*Pa chlamydospora*
Pinot Noir	*Pa chlamydospora*
Pinot Noir	*Botrytis cinerea, Phialophora sp., Phaeoacremonium sp.*
Pinot Noir	*Pa chlamydospora, Botrytis cinerea*
Riesling	*Pa chlamydospora*
Riesling	*Pa chlamydospora*
Semillon	*Pa chlamydospora, Pm aleophilum, Botrytis cinerea*
Shiraz	*Pa chlamydospora*
Shiraz	*Pa chlamydospora, Botrytis cinerea*
Shiraz	None
Sultana	*Pa chlamydospora, Pm aleophilum, Botrytis cinerea, B. obtusa*
Sultana	*Pa chlamydospora, Pm aleophilum /Togninia, Botryosphaeria sp.*
Sultana	*Pa chlamydospora, Pm aleophilum, Botrytis cinerea*
Traminer	*Pa chlamydospora*
Valdiguie	*Pa chlamydospora, Pm aleophilum*, basidiomycete
Waltham Cross	*Pa chlamydospora*

Cuttings from infected Ramsey and Zinfandel mother vines

The incidence of infected Ramsey vines propagated from infected mother vines ranged from 12 to 26%, depending on sampling time (Table 3.4). The incidence of infected Zinfandel vines reached 45% when sampled at 12 and 18 months of age (Table 3.4). The increase in incidence with time is a reflection of better establishment of the pathogen in the wood, making it easier to detect, rather than

infections occurring post-planting. The nursery beds had no previous history of grapevines and the nearest vineyards were at least one kilometre away.

Table 3.4. Incidence of infection in Ramsey and Zinfandel plants propagated from infected mother vines.

Cultivar	Incidence of infection (%)	
Age at assessment	*Pa. chlamydospora*	*Pm. aleophilum*
Ramsey		
12 months	26	17
18 months	12	14
Zinfandel		
6 months	13	15
12 months	45	6
18 months	45	6

Mapping infection in canes from infected Ramsey and Cabernet Sauvignon mother vines

Phaeomoniella chlamydospora was found in 9.1% and 5.5% of the Ramsey and Cabernet Sauvignon cuttings respectively, and *Phaeoacremonium aleophilum* in 3.2% and 1.2% of the cuttings (Table 3.5). The incidence varied with the vine that the cuttings were taken from, suggesting that the infection rate of the progeny is linked to the level of infection existing in the mother vine.

Table 3.5. Incidence of infection and symptoms in young grapevines propagated from mother vines infected with the Petri disease pathogens, *Phaeomoniella chlamydospora* (Pch) and *Phaeoacremonium aleophilum* (Pal).

	Number of	Incidence (%)			Incidence in waste cane pieces (%)	
	Progeny vines	Symptoms	Pch	Pal	Pch	Pal
Ramsey						
Vine 1	50	74	4.0	0.0	6.0	46.0
Vine 2	44	50	2.3	2.3	2.3	2.3
Vine 3	42	52	16.7	4.8	0.0	23.8
Vine 4	50	60	14.0	6.0	2.0	0.0
Total	186	59	9.1	3.2		
Cabernet Sauvignon						
Vine 1	55	5.4	7.3	0.0	0.0	2.0
Vine 2	50	8.0	4.0	2.0	6.4	6.4
Vine 3	58	12.1	5.1	1.7	1.8	7.1
Total	163	8.6	5.5	1.2		

The positions of infection along the length of the canes were mapped (Figures 3.3 and 3.4), suggesting that infection is spread by spores in sap, rather than by mycelial growth from the crown of the infected trunk into the canes.

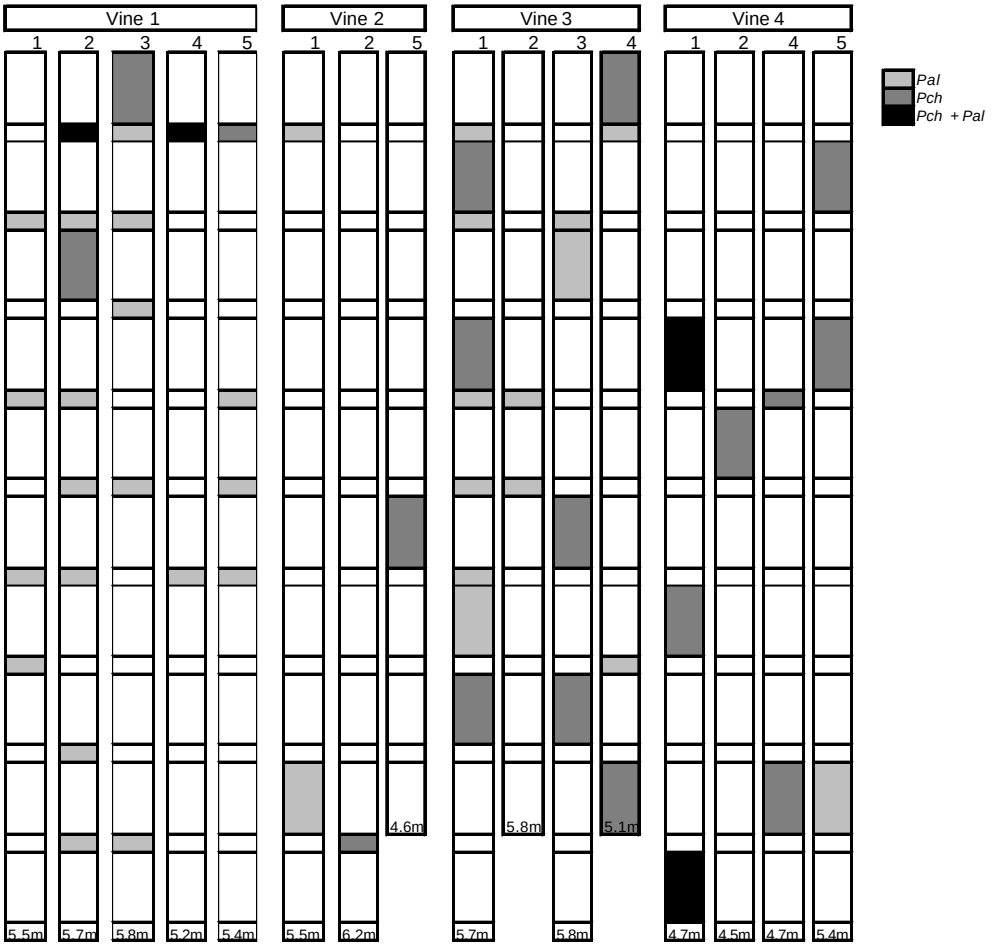


Figure 3.3. Position of *Pa chlamydospora* and *Pm. aleophilum* infections in canes harvested from known infected Ramsey mother vines.

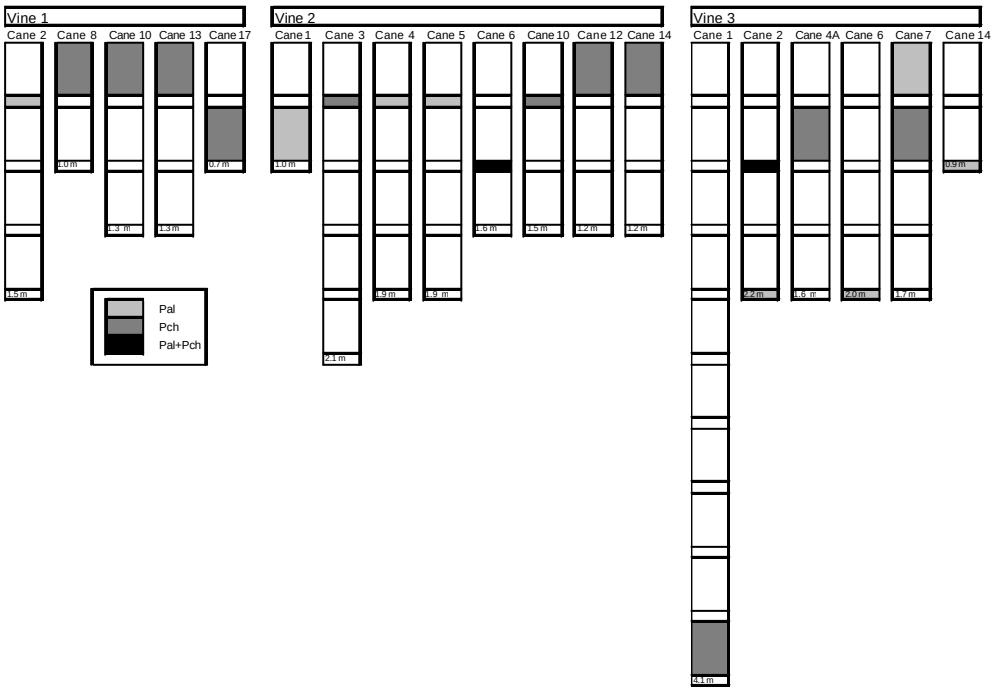


Figure 3.4. Position of *Pa. chlamydospora* and *Pm. aleophilum* infections in canes harvested from known infected Cabernet Sauvignon mother vines

Discussion

Of the 35 *Vitis vinifera* mother vines examined in this study, 32 were confirmed as infected with *Pa. chlamydospora.* Prior to the start of this project, Pascoe (1999) showed that 47 out of 49 rootstock mother vines examined, representing 13 different cultivars growing in three states of Australia, were infected with *Pa. chlamydospora*. Others have also demonstrated infection in rootstock mother vines (Fourie and Halleen 2002, 2004, Ridgway *et al.* 2002). Given this high incidence of infection in mother vines, it was important to establish whether infection could be passed systemically into young grapevines. The incidence of infection in the grapevines propagated from known infected Zinfandel and Ramsey mother vines provides conclusive evidence that infection is passed from mother vine to progeny in the cutting material. In the case of Zinfandel, 45% of the progeny were infected in the samples tested, indicating that approximately half of the plants propagated from those mother vines would have carried symptomless infections.

Several of the grapevines propagated from full-length canes taken from infected Ramsey and Cabernet Sauvignon mother vines were infected when examined after two season's growth. The incidence of young Ramsey and Cabernet Sauvignon vines infected with *Pa. chlamydospora* was 9.1% and 5.5% respectively, and 3.2% and 1.2% for infection by *Pm aleophilum.* There was no potential for contamination of the cuttings during propagation and the plants were grown in commercial potting mix in glasshouse conditions free of contact with other grapevines, proving that infection must have been passed systemically from the mother vines into the canes. Although the incidence of infected vines was relatively low, it was apparent that some mother vines gave rise to more infected progeny than others. For example, the percentage of young Ramsey vines infected with *Pa. chlamydospora* varied from 2.3 - 16.7% depending on the mother vine from which the cuttings had originated. In the case of Cabernet Sauvignon, the variation was 4.0 – 7.3%. This variability probably reflects the level of infection within the mother vine, and a method to determine the level of infection in individual mother vines would be desirable, but is not currently available.

The location of infection courts was mapped along the full length of each cane harvested in order to determine whether infection was restricted to particular sites along the cane. For instance, if only the basal cuttings were infected, then a recommendation could be made to either discard or treat the basal cuttings prior to propagation. However, infection courts of both *Pa. chlamydospora* and *Pm. aleophilum* were scattered randomly along the full length of the canes, suggesting that infection is spread via propagules such as spores or fragments of hyphae carried in the sap flow, rather than by mycelial growth from the crown of the infected trunk into the canes. This method of systemic spread is well documented for vascular pathogens (Green 1981), and conidia of both fungi have been found in sap of infected grapevines by Rooney (2002).

3.4 Spread from vine to vine through soil

It has been suggested that *Pa. chlamydospora* is a soilborne pathogen due to its ability to produce chlamydospores in culture (Ferreira *et al.*, 1994; Bertelli *et al.*, 1998; Mugnai *et al.*, 1999; Sidoti *et al.*, 2000). Gubler *et al.* (2004) also reported that *Pm. aleophilum* is a soilborne pathogen (Gubler *et al.* 2004).

In 2000, soil plus root pieces were collected from the trial site used in Section 3.1, where heavily-infected 10-year-old Merlot vines had just been removed. Half of the 'infested' soil was steam pasteurised as the control treatment and the other half was left untreated. 50 Merlot and 36 Cabernet Sauvignon dormant rootlings were divided into two groups per variety, the roots were trimmed and the canes were pruned to three buds each, as per normal practice at planting. Each vine was planted into 3 L pasteurised commercial potting mix with the addition of 250 mL pasteurised or untreated vineyard soil incorporated through it. After two seasons' growth in the glasshouse, the vines were destructively assessed for infection. The stems were split and moist incubated at room temperature for 2-4 weeks, after which the presence or absence of *Pa. chlamydospora* and *Pm aleophilum* was recorded. Data were analysed using the Mann-Whitney non-parametric test. Although the values were higher for the infested soil treatments (Table 3.6), the differences were not significant ($P<0.05$).

Table 3.6. Incidence of Merlot and Cabernet Sauvignon grapevines infected with *Pa chlamydospora* and *Pm aleophilum* after two years growing in 'infested' soil.

	Incidence of infected young vines (%)			
	Merlot		Cabernet Sauvignon	
	Control	Soil	Control	Soil
Pa. chlamydospora	40	67	27	44
Pm. aleophilum	0	33	7	0

In the United States, researchers from the University of California at Davis demonstrated a close association between grapevine phylloxera presence and soil-borne fungal infection of grapevine roots, including *Phaeoacremonium* species (Omer *et al.* 1995, Granett *et al.* 1998).

In 2004, we had the opportunity to conduct a trial with Dr Kevin Powell (DPI Vic) and Prof. Jeffrey Granett (UC Davis) to investigate whether there was any association between phylloxera infestation and fungal infection in the phylloxera-infested regions of north east Victoria. The results were reported in the Australian and New Zealannd Grapegrower and Winemaker, September 2006 issue, but will not be repeated here as we found very little evidence of grapevine trunk disease pathogens in the roots of the grapevines. There was, however, a clear association between phylloxera infestation and known soilborne pathogens such as *Fusarium* and *Cylindrocarpon* species.

Based on these studies, we have inconclusive evidence to support that Petri disease causal organisms can be spread through soil.

Chapter 4: Nursery management practices for prevention and control of Petri disease

Jacqueline Edwards, Natalie Laukart, Fiona Constable, Soheir Salib, Tonya Wiechel

Summary: Water, callus media and plant material were sampled from commercial nurseries during three propagating seasons: 2000, 2003, 2005. Various methods were tested for their ability to detect *Pa. chlamydospora* in the samples. Nested PCR and quantitative PCR were the most sensitive, although further research is required to optimise their use. When molecular techniques were used, all water samples tested positive for *Pa. chlamydospora*, indicating that nursery practices that involve soaking cuttings are a potential source of infection.

Hot water treatment of infected Cabernet Sauvignon, Chardonnay, Ramsey, Verdelho and Zinfandel cuttings significantly reduced the incidence of infected young vines in five separate experiments from 2000-2005. Hot water treatment of Pinot Noir rootlings also resulted in fewer infected young vines, but the reduction was not significant at P=0.05. No other treatment trialled in these studies was particularly effective. Our recommendation to the nursery industry is that grapevine cuttings should be routinely hot water treated to reduce the chances of selling symptomless infected planting material to growers.

4.1 Methods of detecting *Phaeomoniella chlamydospora* and sources of contamination during grapevine propagation in nurseries

The high level of infection in some affected vineyards suggests that contamination of cuttings may be occurring during nursery procedures, in addition to infection being spread from mother vine into cuttings. In order to test this, samples of water, propagation tools and materials, and plant material were taken from various nurseries over several seasons and tested for the presence of *Phaeomoniella chlamydospora*. The aim of these experiments was (a) to develop a suitable method for detecting *Phaeomoniella chlamydospora* contamination, and (b) to determine where contamination is occurring during nursery propagation procedures.

4.1.1 Year 2000

Methods

Water was sampled from the hydration, hot water and cool down tanks at VAMMVIA, Mildura, during the cutting harvesting season of 2000. Samples were taken at the start of each week when clean water was added to each tank, and at the end of the week after a week's worth of harvested cuttings had passed through the system. The water in both the hydration and cool down tanks was chlorinated to 10 ppm Cl and the chlorine levels were checked daily and topped up if necessary. Sampling took place over 3 weeks. Three 25 mL sub-samples were taken from each water sample, centrifuged at 4500 rpm for 20 min, and the supernatant discarded. The precipitate was resuspended in 5 mL sterile distilled water and plated onto potato dextrose agar amended with achromycin. The plates were incubated for 10 days at room temperature, then fungal colony numbers were counted and colony-forming-units (cfu) per mL tank water were calculated.

Results and Discussion

The hydration tank had very high cfu numbers by the end of the week (Table 4.1), although no *Pa. chlamydospora* colonies were observed. *Pa. chlamydospora* is very slow-growing, unlike the other fungi present such as *Penicillium, Rhizopus, Mucor* and *Cladosporium*, so if present, it was overgrown by the others. Several months were spent unsuccessfully trying to develop a selective medium specifically for *Pa. chlamydospora*.

Table 4.1. Levels of fungal contamination (cfu/mL) of the hydration, hot water and cool down tanks during the cutting harvesting season, July – August 2000.

	Fungal colonies (cfu) per mL of tank water					
	Hydration tank		**Hot water tank**		**Cool down tank**	
	start	**finish**	**start**	**finish**	**start**	**finish**
Week 1	11.3	740.8	0.6	0.0	3.1	70.6
Week 2	0.0	391.7	0.0	0.0	1.0	0.7
Week 3	2.8	1006.9	0.3	0.0	0.0	0.0

4.1.2 Year 2003

At the 3rd International Workshop on Grapevine Trunk Diseases in February 2003, New Zealand researchers reported detecting contamination at various stages of the propagation process using a PCR-based *Pa. chlamydospora* specific probe (Whiteman *et al.* 2003). We decided to explore whether molecular tests such as nested PCR and real time PCR could be used to detect and quantify *Pa. chlamydospora* at various stages along the grapevine propagation process in Australia.

Methods

Samples. During 2003, samples were taken from a commercial nursery at several stages during the propagation process: water from the hydration, hot water and cooldown tanks, vermiculite from boxes of callusing cuttings, wash water from Shiraz and Paulsen cuttings at many stages including the graft union 6 months after grafting (Table 4.2). 10 cuttings per cultivar were sampled (a) at collection from the field, (b) post cold-storage, (c) after hydration, (d) after hot water treatment, (e) after the cooldown tank, (f) post disbudding (Paulsen only), (g) after grafting (50 graftlings). Other samples were vermiculite from callusing boxes (3 reps), 1 L water from each of the hydration, hot water and cooldown tanks, and water used to wash the grafting tool after grafting was completed. The cuttings, graftlings and vermiculite were washed in 1 L distilled water and the wash water collected.

After washing, the plant material was processed as follows. Two cm pieces were cut from each end of the cuttings, surface sterilised and plated out onto PDA+A. Buds from the disbudding process of Paulsen, the disbudded portions of the cuttings, and the graft unions of the graftlings were all surface sterilised and plated out onto PDA. The remainder of the cuttings were split open longitudinally, examined for internal symptoms and then moist incubated as previously described. All of the grapevine pieces were examined for growth of *Pa. chlamydospora* over several weeks.

DNA extraction. DNA was extracted from all water samples (including the washings) using MOBio UltraClean Water DNA Kits (0.22 μm) with 100 mL of water per sample following the manufacturers protocol. The extraction was repeated 3 times. DNA was also extracted from pure cultures of *Phaeomoniella chlamydospora, Phaeoacremonium aleophilum, Botrytis cinerea, Rhizoctonia solani* and *Colletotrichum coocodes* to challenge the *Pa. chlamydospora*-specific primers for their specificity.

Primer design. The internal transcribed spacer regions (ITS1 and ITS2) of *Pa. chlamydospora* were accessed on the GenBank database and sequences of isolates were aligned using ANGIS. Putative specific regions were selected and inserted into Primer 3 software to design specific forward and reverse primers PhaeoFTQ (CCGATCTCCAACCCTTTGT) and PhaeoRTQ (CGATGCCAGAACCAAGAGA). A Taqman fluorescent probe PhaeoTQprobe (ATGTGACGTCTGAACGGTTCCATCA) was also designed and was labelled at the 5' end with the reporter dye FAM (6-carboxy-fluorescein), while the 3' end was modified with the quencher dye TAMRA (6-carboxy-tetramethylrhodamine) (Applied Biosystems).

PCR amplification of the ITS region. PCR amplification of all samples using primers ITS4 (TCC TCC GCT TAT TGA TAT GC) and ITS5 (GGA AGT AAA AGT CGT AAC AAG G) was based on a standard set of conditions [initial denaturation at 95ºC for 3 min, followed by 45 cycles of 95°C for 30, 50ºC for 30 and 72ºC for 30 in a reaction volume of 25 µL. The reaction contained the following components 1 x reaction buffer (), 200 µM dNTPs, 5 mM $MgCl_2$, 0.1 µM of each primer, 2 Units of Taq polymerase and 40 ng of DNA template. The PCR product is 600 bp.

Nested PCR. PCR amplification of all samples using primers Pch1 (CTC CAA CCC TTT GTT TAT C) and Pch2 (TGA AAG TTG ATA TGG ACC C) was based on a standard set of conditions, initial denaturation at 95ºC for 3 min, followed by 45 cycles of 95ºC for 30, 57ºC for 30 and 72ºC for 30 in a reaction volume of 25 µL. The reaction contained the following components 1 x reaction buffer, 200um dNTPs, 5 mM MgCl2, 0.1 µM of each primer, 2 Units of Taq polymerase and 1.5 µL of ITS PCR product as DNA template. The PCR product is 360 bp. PCR products were separated in 1.0% agarose gels and visualised using ethidium bromide staining.

Pa. chlamydospora spiked standards. To prepare spiked water samples with known levels of *Pa. chlamydospora*, spore suspensions of 1, 10, 10^2, 10^3 and 10^4 spores/ 100 mL were prepared using hot water tank water.

Quantitative PCR. Realtime PCR was performed in 0.2 mL tubes in a Rotor Gene 3000 machine (Corbett Research). The 25 µL reaction mix included 5 µL template DNA, 1 x Invitrogen Universal QPCR Master Mix, 0.1 µM of primers PhaeoFTQ and PhaeoRTQ, and 0.2 µM of the Taqman probe (PhaeoTQprobe). The thermal cycle protocol was 50°C for 2 min, 95°C for 10 min and 45 cycles of 95°C for 15 sec and 60°C for 60 secs.

Results and Discussion

The ITS PCR produced a 600 bp product in all samples, indicating that fungal DNA was present. The *Pa. chlamydospora*-specific primers used in the nested PCR assays were specific for *Pa. chlamydospora* and did not amplify any of the other fungal DNA tested. All samples gave positive bands for *Pa. chlamydospora* in at least one replicate of each (Table 4.2), suggesting either that *Pa. chlamydospora* was present in all samples, or that some contamination was happening somewhere during the reaction.

Table 4.2. Quantification of *Pa. chlamydospora* in grapevine nursery water samples (spores/100 mL).

Sample	Nested PCR (Yes/No)	Real-time PCR (sp/100ml)			
		Rep 1	Rep 2	Rep 3	Average
Hydration tank	yes	27	0	0	9.0
HWT tank	yes	0	0	0	0.0
Cooldown tank	yes	73	4	0	25.7
Vermiculite1	yes	42	0.2	18	20.1
Vermiculite2	yes	8	1.8	0	3.3
Vermiculite3	yes	6	1.3	0	2.4
Shiraz collection	yes	9	0	0	3.0
Shiraz hydration	yes	0	0	0	0.0
Shiraz HWT	yes	0	0	0	0.0
Shiraz cooldown	yes	2	0	0	0.7
Shiraz cold storage	yes	2	0	0	0.7
Paulsen collection	yes	3	0	0	1.0
Paulsen hydration	yes	26	0	0	8.7
Paulsen HWT	yes	0	0	0	0.0
Paulsen cooldown	yes	30	0	0	10.0
Paulsen cold storage	yes	20	1.4	59	26.8
Paulsen disbudding	yes	16	0.5	0	5.5
Grafting	yes	4	1.6	0	1.9

The DNA standards of *Pa. chlamydospora* spiked water were amplified consistently in real time PCR with the newly designed primers and probe. The QPCR assay was able to detect *Pa. chlamydospora* at

levels as low as 10 spores per 100 mL of water (Figures 4.1 and 4.2). However, the variation increased as the spore load decreased. Although *Pa. chlamydospora* was detected in all samples using the nested PCR technique, only some samples tested positive using the QPCR technique (Table 4.2). This is the first time that QPCR has been used to detect *Pa. chlamydospora* from samples taken during the propagation process. There was considerable variation between some sample replicates, possibly because the levels were at the limit of detection. Conventional plating techniques are unsuitable for detecting *Pa. chlamydospora* contamination due to the slow growth of the fungus. The nested PCR was successful, but does not quantify the level of *Pa. chlamydospora*. However, it is unknown whether the low levels of contamination detected using QPCR in this study are capable of causing infection.

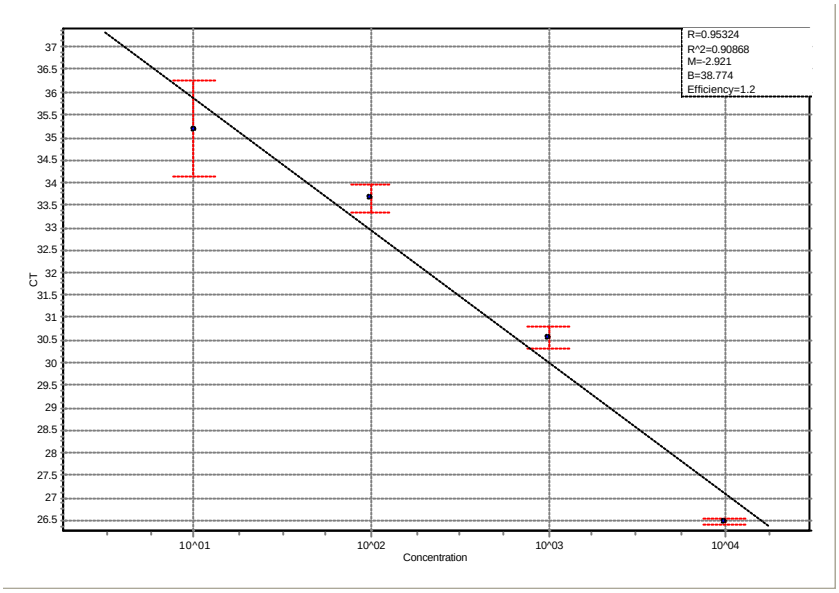


Figure 4.1. Standard curve for the QPCR assay of DNA samples from extracted water spiked with known amounts of *Pa. chlamydospora* (10^4, 10^3, 10^2, 10, 1 sp / mL) replicated 3 times.

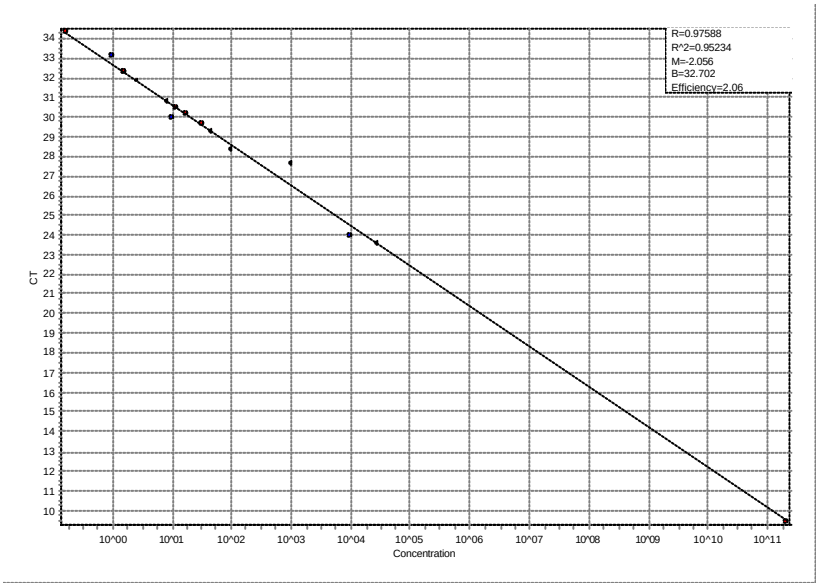


Figure 4.2. Standard curve for the QPCR assay for the quantification of *Pa. chlamydospora* in DNA samples extracted from water collected during the nursery propagation. Blue symbols - standards and red symbols are the unknown test samples.

4.1.3 Year 2005

In 2005, water samples were collected from four commercial nurseries during the propagation season in July. Several molecular techniques and primers (conventional PCR, nested PCR, qualitative PCR using SYBR®Green and TaqMan®) were compared for their usefulness to detect *Pa. chlamydospora* in the water samples.

Methods

Samples. A total of 39 samples (approx. 2 L each) were collected from 13 water sources of four commercial nurseries during the 2005 propagation season (Table 4.3). Three samples were collected from each source. The samples were stored at –20ºC prior to extraction.

Table 4.3. Source of water samples collected for analysis in this study.

ID No.	Nursery	Source of water
1	Nursery 3	cutting soaking water
2	Nursery 1	rain water tank
3	Nursery 2	hydrating water
4	Nursery 4	hot water tank (Time 1)
5	Nursery 3	rain water tank
6	Nursery 3	Chlorine-treated water
7	Nursery 3	hot water tank
8	Nursery 3	bud soaking water
9	Nursery 4	cool down tank (Time 1)
10	Nursery 4	hot water tank (Time 2)
11	Nursery 4	hydration tank (Time 1)
12	Nursery 4	cool down tank (Time 2)
13	Nursery 4	hydration tank (Time 2)

DNA extraction. DNA was extracted from 100mL of each water sample using the UltraClean™ Water DNA Isolation Kit (Mo Bio Laboratories) with a 0.22 micron filter, according to the manufacturer's instructions.

PCR. Primers developed for bacterial phylogenetic studies (Weisberg *et al* 1991) were used in PCR to check the quality of DNA extracts. These primers were used to ensure that nucleic acid was present or that there were no inhibitors in the DNA extracts that retard the activity of the or DNA polymerase during the PCR reactions. PCR primers and the TaqMan® probe sequence are listed in Table 4.4. The location of primers along the 18S ribosomal RNA (rRNA), 5.8S rRNA and internal transcribed spacer regions (ITS1 and ITS2) are shown in Figure 4.3.

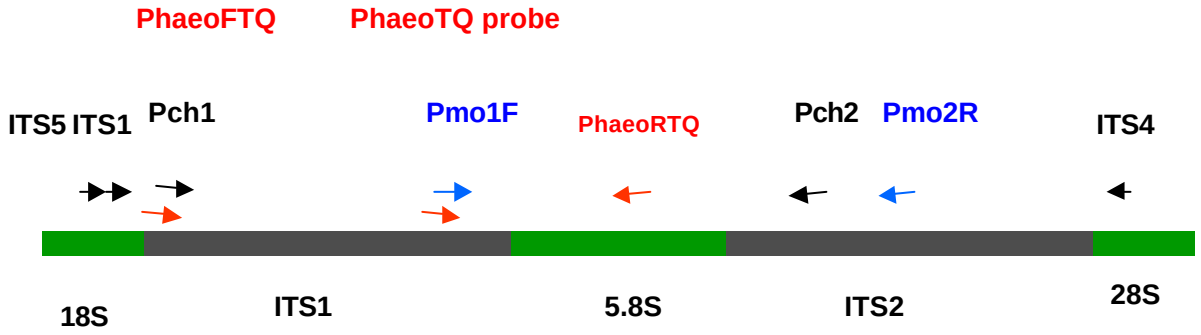


Figure 4.3. The location of each of the PCR primers and the PhaeoTQ TaqMan® probe along the ribosomal DNA region of *Phaeomoniella chlamydospora.*

Table 4.4. Primers used for detection of the bacterial 16S rRNA gene, fungal ribosomal DNA region and *P.chlamydospora* (Pch), the expected product size and the reference for each assay

Assay	Primer name	Primer sequence 5'-3'	Expected product size	
16S DNA house keeping	FD2	AGAGTTTGATCATGGCTCAG	1400-1500 bp	Weisberg *et al* 1991
	RP1	ACGGTTACCTTGTTACGACTT		
Universal fungal ITS primers	ITS-5	GGAAGTAAAAGTCGTAACAAGG	ca. 580 bp.	White *et al.* 1990
	ITS-4	TCCTCCGCTTATTGATATGC		
Pch specific non-quantitative	Pch1	CTCCAACCCTTTGTTTATC	360 bp	Tegli *et al.* 2000
	Pch2	TGAAAGTTGATATGGACCC		
Pch specific non-quantitative and quantitative with SYBR®Green	Pmo1f	GTTACATGTGACGTCTGAACG	320 bp	Overton *et al* 2004
	Pmo2r	CAGTGTATGCTTGATTGCTCG		
Pch specific quantitative TaqMan®	Phaeo FTQ	CCGATCTCCAACCCTTTCT	197bp	See Section 4.1.2 above.
	Phaeo RTQ	CGATGCCAGAACCAAGAGA		
	Phaeo TQ probe	6FAMATGTGACGTCTGAACGGTT CCATCATAMARA		

Quantitative PCR using SYBR® Green. A Platinum® SYBR®Green qPCR SuperMix-UDG kit (Invitrogen) was used for qPCR with the primer pair pMo1F/pMo2R. The total reaction volume was 25 µL and contained 0.4uM of each primer and 5 ul of the DNA template. The PCR conditions were 50ºC for 2 min, 95ºC for 2 min and 35 cycles of 95ºC for 15 s, 55ºC for 30 s and 72ºC for 30s. PCR cycling was followed by a melt curve analysis in which the temperature was increased by 1°C every 5 s from 72°C to 95°C. Each sample was assayed in triplicate in a Corbett Research Rotor-Gene 3000 thermal cycler (Corbett Research). The dF/dT (derivative fluorescence curve with respect to temperature) threshold was set manually and based on the highest dF/dT value of the 'no template' controls at the expected melting temperature (85-86ºC) of the expected amplicon. The presence of non-specific amplicons in each reaction was checked by separating PCR products on a 1% agarose gel and visualising them under ultra violet light following staining in ethidium bromide.

Quantitative PCR using a TaqMan® probe. A Platinum® Quantitative PCR SuperMix-UDG kit (Invitrogen) was used for qPCR with he primer pair PhaeoFTQ/PHaeoRTQ and the TaqMan® fluorescent probe, PhaeoTQprobe (Table 4.4). The total reaction volume was 25 µL and contained 0.3 uM of each primer, 0.1 uM of the probe and 5 ul of the DNA template. The PCR conditions were 50ºC for 2 min, 95ºC for 10 min and 35 cycles of 95ºC for 15 s and 60ºC for 60 s. Each sample was assayed in triplicate in a Corbett Research Rotor-Gene 3000 thermal cycler.

Standards for quantitative PCR. Two sets of standards for qPCR were prepared. The first set of standards was made from a 100 mL spore suspension of 10^4 spores/mL in sterile distilled water was made from a pure culture of *Pa. chlamydospora* and serially diluted to10^3, 10^2, 10 and 10^{-1} spores/mL, each in a total volume of 100mL. The second set of standards was made from a 100 mL spore suspension of 10^6 spores/mL in sterile distilled water was made from a pure culture of *Pa.*

chlamydospora and serially diluted to10^5, 10^4, 10^3 and 10^2 spores/mL, each in a total volume of 100mL. The DNA was extracted from the spore suspensions, as described above and used to produce the standard curves for the SYBR®Green and TaqMan® PCR assays. The DNA standards were stored in aliquots at -20°C. Each standard was assayed in triplicate in one qPCR run to create a standard curve. In addition, one replicate of each standard was incorporated in each qPCR run to ensure reproducibility and to enable comparisons of each sample between runs.

Non-quantitative PCR. Non-quantitative, single PCR assays were done using the primer pairs Pmo1F/Pmo2R or Pch1/ Pch2. A Platinum® *Taq* DNA Polymerase kit (Invitrogen) was used for PCR according to the manufacturer's instructions except that the total reaction volume was 25 µL, with a final primer concentration on 0.4uM and using 5uL of DNA as template. The reaction mix was denatured at 95° C for 2 min, followed by 35 cycles of denaturing at 95° C for 45 s, annealing at 54° C for 45s and 72° C for 45 s and a final extension of 72° C for 10 min. All non-quantitative PCR reactions were conducted in a Palm-Cycler™ (Corbett Research).

Nested PCR was also used for detection of *Pa. chlamydospora* in water samples using the primer pair ITS-5/ITS4 (White et al 1990) and 5 uL of DNA template for the first round and Pch1 and Pch 2 and 1 uL of first round PCR product for the nested PCR. PCR reaction conditions were as described above.

PCR products were separated by 1% agarose gel electrophoresis and visualised under ultra violet light following staining in ethidium bromide.

Sequence analysis. To detemine the specificty of the primers used in each of the PCR assays the primer sequences were analysed using BlastN (Altschul *et al.*, 1997) against the fungal and bacterial databases only.

Effect of HWT on Pa. chlamydospora detection.

A 200 mL spore suspension of 10^6 spores/mL in sterile distilled water was made from a pure culture of *Pa. chlamydospora* and serially diluted to10^5, 10^4, 10^3 and 10^2 spores/mL, each in a total volume of 200mL. Each of these suspensions was separated into 2 aliquots of 100mL and one aliquot of each suspension was placed in a water 50ºC bath. The temperature of a 100mL water control was placed in the bath simultaneously with the suspensions and the temperature of the control was monitored. Once the control reached 50ºC the suspensions were maintained in the water bath for a further 30 min. After treatment the suspensions were removed from the water bath. Three 100ul replicates of each treated and untreated spore suspensions were plated onto PDA and maintained at 21ºC in a growth cabinet. The growth of *Pa. chlamydospora* was monitored and the amount of growth was recorded after 14 days. DNA was extracted from each of the remaining suspensions and each extract was assayed in triplicate using the Sbyr®-green assay as described above.

Results

Standard curve and reproducibility. Standard curves were produced for the SYBR®Green and TaqMan® qPCR assays by measuring three replicates each of the five DNA extracts of 10^4, 10^3, 10^2 10, and 10^{-1}, spores/mL. The TaqMan® probe standard curve exhibited a slope of −3.097 and a correlation coefficient (R^2) of 0.75904. The SYBR®Green standard curve exhibited a slope of −1.25 and a correlation coefficient (R^2) of 0.73449. These standards were not used in the water sample assays due the low R^2, which indicated significant variation in the calculation of spore concentration between the three replicates of each standard and between standards.

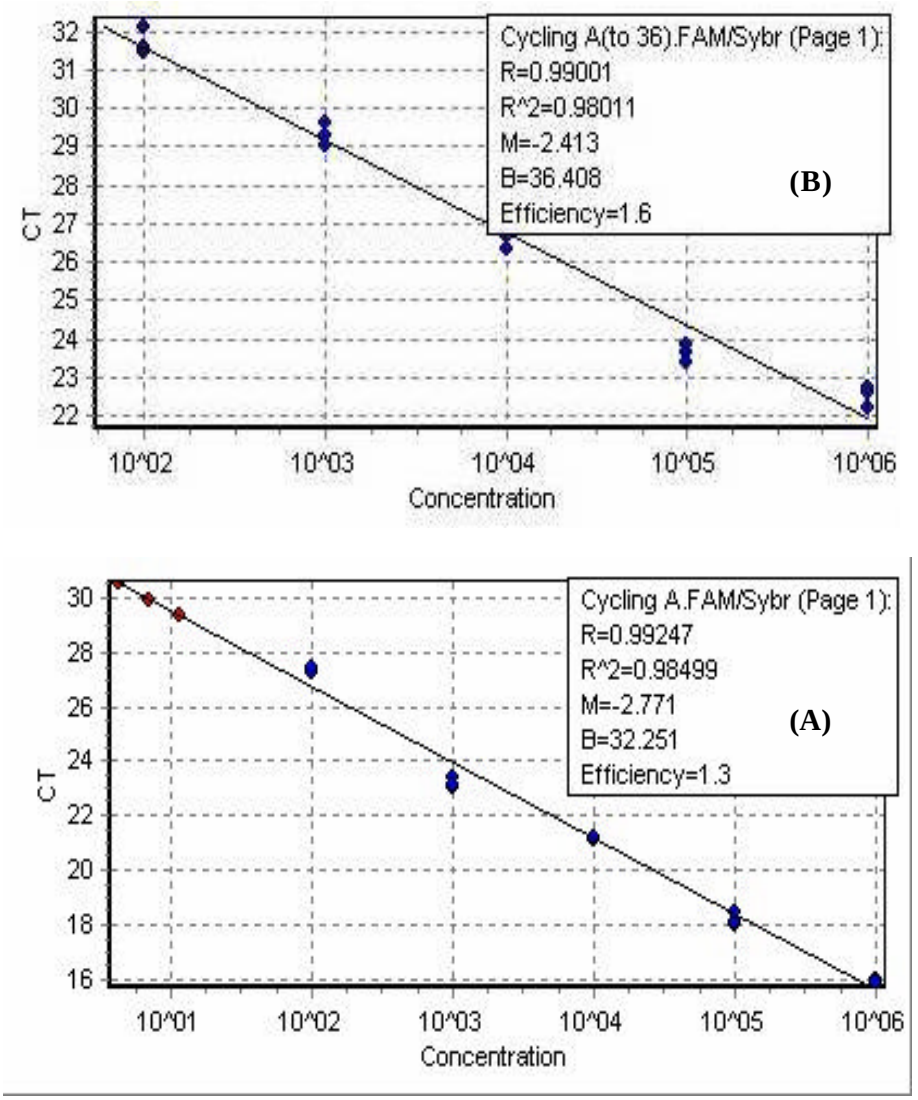


Figure 4.4. Quantification of serial dilution of *P.chlamydospora* spores using the SYBR®Green (**A**) and the TaqMan® (**B**) PCR assays. DNA extracts of 10^6, 10^5, 10^4, 10^3 and 10^2 spores/mL were used. The R^2 value indicates how well the data fit the linear relationship between Ct (the cycle number at which the increase in cDNA is logarithmic) and spore concentration. The M value is the slope, which should be close to −3.32 if the PCR reaction has 100% efficiency. B is the Y axis intercept.

The TaqMan® probe standard curve, produced by measuring three replicates each of the five DNA extracts of 10^6, 10^5, 10^4, 10^3 and 10^2 spores/mL, exhibited a slope of −2.413 and a correlation coefficient (R^2) of 0.98011 (Figure 4.4A). The SYBR®Green standard curve produced three replicates each of the same standards exhibited a slope of −2.771 and a correlation coefficient (R^2) of 0.98499 (Figure 4.4B). Standards incorporated into water sample assays as positive controls gave comparable results (Table 4.5).

The SYBR®Green standard curves showed an increase in fluorescence approx 3.5 cycles earlier than the TaqMan ® standard curves. Similar results were observed for samples, which were considered positive using both assays.

Table 4.5. The slope and correlation co-efficient of standard curves, based on single replicates of each standard which were included as positive controls in each of the SYBR®Green and TaqMan® qPCR assays

	SYBR®Green assay		TaqMan® assay	
Assay	slope	correlation coefficient (R^2)	slope	correlation coefficient (R^2)
1	-2.64	0.9842	-2.542	0.96539
2	-2.74	0.99255	-2.364	0.97029
3	-2.632	0.99667	-2.723	0.97815
4	-2.484	0.97861	-2.327	0.93784

Sequence analysis of Pa. chlamydospora primers. The primers PhaeFTQ, Pmo1 Pch 1 and Pch 2 had similarity with several other fungi other than *Pa. chlamydospora* over all or part of their sequence. Pch 2 had 100% sequence similarity over 14 nucleotides, between nucleotide positions 3 and 14 of the primer sequence, with a putative laccase mRNA of *Botrytis cinerea*. PhaeoRTQ had 100% sequence similarity with many fungi including *Botryosphaeria dothidea* isolated from *Vitis vinifera*, *Quercus* and *Acer* species. *Pa. chlamydospora* was not present in the top 100 blast search sequence results for PhaeoRTQ. Pch1/Pch2. Pmo1F/Pmo2R and Phaeoftq/PhaeoRTQ did not have any fungal species, other than *Pa. chlamydospora*, in common. Pch 1, Pmo 1f, PhaeoFTQ and PhaeoRTQ had some sequence similarity with several bacteria including *Pseudomonas fluorescens*. Pmo2R and the TaqMan® probe, PhaeoTQprobe, did not have similarity with any other published fungi, other than *Pa. chlamydospora*, or with any bacterial sequence.

Non-qPCR and qPCR. The results of the non-quantitative and quantitative PCR are shown in Table 4.7. Thirty three of the 39 samples tested positive with the FD2/RP1 (16S bacterial housekeeping) primers. Five of the 39 samples were positive using the ITS1F/ITS4R1 primers. In single non-quantitative PCR, 16 of the 39 samples were positive using the Pch/1Pch2 primer pair and 22 of the 39 samples were positive using the Pmo1F/1Pmo2R primer pair. In nested non-quantitative PCR, 32 of the 39 samples were positive using the Pch/1Pch2 primer pair and 30 of the 39 samples were positive using the Pmo1F/1Pmo2R primer pair. Based on the calculated concentrations of spores/mL, 35 of the 39 samples were positive using the Pmo1F/1Pmo2R primer pair in qPCR with SYBR®Green and 24 of the 39 samples were positive using the TaqMan® probe in qPCR.

All samples tested positive with at least one PCR assay (Table 4.6). Seven of the 39 samples were positive in all six assays, 12 of the 39 were positive using five assays, seven of 39 were positive with four assays, seven of 39 were positive with three assays, two were positive with two assays and 4 of 39 were positive with one assay. Twelve of the 39 were positive with both qPCR assays. Nested non-qPCR and qPCR produced more positive results than single non-qPCR and 31/39 samples were positive with at least one nested PCR assay and one qPCR assay.

Table 4.6. Comparison of PCR tests for detection of *Pa. chlamydospora* in nursery water samples.

PCR test	No. samples that tested positive	Range of spore concentrations detected
ITS universal	3/39 (7.7%)	
Single PCR – Pch primers	16/39 (41%)	
Single PCR – Pmo primers	22/39 (56.4%)	
Nested PCR – ITS/Pmo primers	30/39 (77%)	
Nested PCR – ITS/Pch primers	32/39 (81.2%)	
SYBR®Green quantitative PCR-Pmo primers	35/39 (89.7%)	8-3452 spores/ml
TaqMan® quantitative primers	24/39 (61.5%)	1-17394 spores/ml

Table 4.7. The results of the single and nested non-quantitative PCR assays and the SYBR®Green and TaqMan® quantitative PCR assays. (*1R=replicate 1 of a sample, 2R=replicate 2 of a sample, 3R=replicate 3 of a sample; 1 = positive for *Pa. chlamydospora)*

Sample*	16S	ITS1F &4	Pch 1& 2	Pmo1F &2R	Pch nested	Pmo nested	SYBR®Green	TaqMan®
1R 1				1	1	1	1	1
1R 2	1			1	1	1	1	1
1R 3	1		1	1	1	1	1	1
1R 4	1			1	1	1	1	1
1R 5	1			1	1	1	1	1
1R 6	1		1	1	1	1	1	1
1R 7	1		1	1	1	1	1	1
1R 8	1			1	1	1	1	1
1R 9	1		1	1	1	1	1	1
1R 10	1			1	1	1	1	1
1R 11	1		1	1			1	1
1R 12	1		1	1	1	1	1	1
1R 13	1			1	1	1	1	
2R 1	1		1	1	1		1	
2R 2	1		1	1	1	1		1
2R 3	1	1	1	1		1	1	1
2R 4		1	1	1	1			1
2R 5	1		1	1	1	1		1
2R 6	1		1	1	1	1	1	1
2R 7	1		1	1	1		1	1
2R 8	1				1	1		1
2R 9	1		1		1	1	1	
2R 10	1				1	1	1	1
2R 11	1		1		1	1	1	1
2R 12				1	1	1	1	1
2R 13	1	1	1	1	1	1	1	1
3R 1	1				1		1	
3R 2	1				1	1	1	
3R 3	1				1	1	1	
3R 4	1						1	
3R 5	1				1	1	1	1
3R 6							1	
3R 7	1				1	1	1	
3R 8	1						1	
3R 9					1	1	1	
3R 10	1	1			1	1	1	
3R 11	1	1			1	1	1	
3R 12							1	
3R 13	1					1	1	

Quantitative PCR. The results of the quantitative PCR assays are shown in Table 4.8. The samples from replicate groups 1 and 2 produced more positive results with all *Pa. chlamydospora* specific assays than the replicate samples of group 3. There was no association between presence of spores or spore level and water type. The qPCR results indicate that samples in the replicate 1 had higher levels of *Pa. chlamydospora* (up to 17395 spores/mL using the TaqMan ® assay) compared to samples in replicate groups 2 and 3 (up to 220 spores /mL using the SYBR®Green assay). The TaqMan® assay indicated higher levels of spores in the replicate group 1 compared to the SYBR®Green assay. In

replicate groups 2 and 3 the SYBR®Green assay indicated a higher levels of spores in most samples compared to the TaqMan ® assay.

The melting temperature (Tm) of PCR products generated in the SYBR®Green PCR assay on the water samples was between 84-86ºC (Table 4.8). The Tm of the water samples was between 85-86ºC when higher levels of spores were detected and between 84-85ºC when low spore levels were detected. The Tm of the *Pa. chlamydospora* isolate used to make the standards was between 85-86ºC (Figure 4.5).

Table 4.8. Average Ct values and average concentration of spores (spores/ml) and melting temperature (Tm) of water samples collected from four nurseries.

	SYBR®Green				TaqMan®	
	PCR results		Tm		PCR results	
Sample	Average Ct	Ave. conc. (sp/ml)	Peak 1	Peak 2	Average Ct	Ave. conc. (sp/ml)
1R1	29.67	74.14	75.5	84.8	31.32	141.04
1R2	25.42	1117.63	75.5	85	27.92	3058.86
1R3	23.95	3451.59	74.2	85.2	26.09	17394.54
1R4	26.81	311.28	76	85.3	28.86	1397.13
1R5	26.07	593.7	75.7	85.3	28.59	1793.09
1R6	28.79	61.06	75.7	85.5	31.69	121.83
1R7	26.46	419.52	75.5	85.7	29.73	631.06
1R8	29.69	27.81	75.5	85.8	31.22	153.33
1R9	28.19	97.5	75.8	85.8	29.65	697.33
1R10	30.86	17.72	76.5	85	33.79	16.21
1R11	29.2	43.75	75.5	84.5	35.385	2.36
1R12	29.02	23.98	75.5	85	33.72	13.47
1R13	29.7	56.81	75.7	85.3		
2R1	28.13	59.69	76.5			
2R2	27.19	128.3	76.7		33.43	24.23
2R3	29.22	21.08	76.2	85	34.58	7.41
2R4	30.5	7.8	76.3		35.29	2.53
2R5	28.41	50.5	76.5		35.73	1.62
2R6	28.97	27.14	76.2	84.3	35.38	2.53
2R7	29.53	15.51	76.2	84	35.115	3.38
2R8	30.17	19.18	76.5		35.42	7.38
2R9	31.09	8.69	76.3	84		
2R10	30.42	14.97	76.2	85	34.01	27.06
2R11	28.71	92.87	76.2	85.2	35.23	8.0
2R12	27.51	220.12	76.5	85.3	32.29	99.60
2R13	28.88	62.05	75.8	85.3	32.13	111.1
3R1	30.73	418.28	76.7			
3R2	33.94	0.58	76	84.3		
3R3	33.38	0.97	75.5	84		
3R4	32.38	13.53	75.8	84		
3R5	30.6	9.85	76.5	85.2	34.07	1.11
3R6	30.83	8.08	76.5	85.2		
3R7	30.2	15.99	76.2	85.2		
3R8	30.86	7.88	76.5	85.5		
3R9	30.93	8.05	76.3	84.8		
3R10	32.07	2.79	75.8	84.5		
3R11	31.64	4.16	75.7	84.5		
3R12	31.54	4.42	75.8	84.2		
3R13	31.27	5.5	75.8	84.2		

Figure 4.5. The melt curve analysis of the *P. chlamydospora* standards (10^6-10^2 spores/mL). The peak at 75-77ºC was associated with primer dimer formation and the Peak at 85-86ºC was associated with the production of the specific *P. chlamydospora* amplicon.

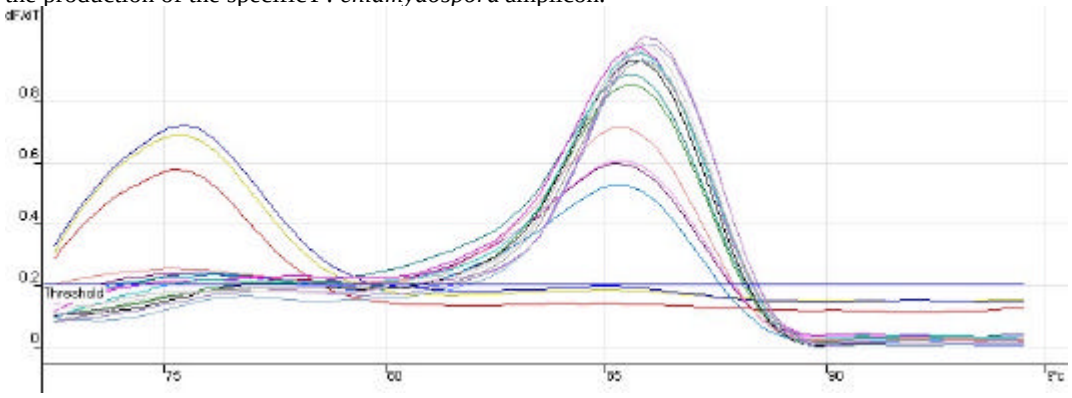


Table 4.9. Detection of *Pa. chlamydospora* in water samples collected from nurseries in July 2005, using qPCR and nested PCR.

ID No.	Nursery	Sample	SYBR®Green			TaqMan®			Nested PCR – Pch primers		
			Rep 1 (sp/ml)	Rep 2 (sp/ml)	Rep 3 (sp/ml)	Rep 1 (sp/ml)	Rep 2 (sp/ml)	Rep 3 (sp/ml)	Rep 1	Rep 2	Rep 3
1	Site 1	Cutting soaking water	74.14	59.69	418.28	141.04	-	-	+	+	+
5		Rain water	593.7	50.5	9.85	1793.09	1.62	1.11	+	+	+
6		Chlorine-treated water	61.06	27.14	8.08	121.83	2.53	-	+	+	-
7		Hot water tank	419.52	15.51	15.99	631.06	3.38	-	+	+	+
8		Bud soaking water	27.81	19.18	7.88	153.33	7.38	-	+	+	-
2	Site 2	Rain water	1117.63	128.3	0.58	3058.86	24.23	-	+	+	+
3	Site 3	Hydrating water	3451.59	21.08	0.97	17394.54	7.41	-	+	-	+
4	Site 4	Hot water 18/7	311.28	7.8	32.38	1397.13	2.53	-	+	+	-
10		Hot water 21/7	17.72	14.97	2.79	16.21	27.06	-	+	+	+
9		Cool down 18/7	97.5	8.69	8.05	697.33	99.60	-	+	+	+
12		Cool down 21/7	23.98	220.12	4.42	13.47	-	-	+	+	-
11		Hydration tank 18/7	43.75	92.87	4.16	2.36	8.0	-	-	+	+
13		Hydration tank 21/7	56.81	62.05	5.5	-	111.1	-	+	+	+

Presence of Pa. chlamydospora in the nursery water samples

Although it was not possible to quantify the amount of *Pa. chlamydospora* present in the nursery samples due to the variability between replicate samples and PCR techniques used, it is clear that all of the samples tested were positive for *Pa. chlamydospora* in more than one of the replicates (Table 4.9). The rainwater (sites 1 and 2) and hydrating water (Site 3) appeared to have the highest relative concentrations of spores.

Effect of HWT on Pa. chlamydospora detection

Hot water treatment reduced the viability of spores by 30-50% in the 10^4-10^6 spores/ml supensions. Some spores from the hot water treated 10^2 and10^3 spores/ml suspensions germinated on PDA media but they did not grow well. The results of the SYBR®Green PCR assay indicated an equivalent reduction in detection of spores from the 10^2, 10^4 and 10^5 hot water treated suspensions compared to the untreated samples, but detected higher concentrations in the 10^3 and 10^6 hot water treated suspensions (Table 4.10) than was evident from observed colony growth of the same samples (data not shown).

Table 4.10. The effect of hot water treatment on detection of *Pa. chlamydospora* spores using SYBR®Green PCR.

Initial sample concentration (spores/ml)	Untreated Ct	Untreated spores/ml	HWT Ct	HWT spores/ml	% reduction in spores/ml
10^2	27.33	59.8	30.01	6.44	89.23%
10^3	23.18	1,872.63	23.45	1,494.24	20.21%
10^4	21.16	10,043.20	22.32	3,826.51	61.90%
10^5	18.19	118,091.80	19.74	32,693.73	72.31%
10^6	15.96	752,984.83	16.07	687,744.04	8.66%

Discussion

"Housekeeping" PCR assays are useful for verify the quality of nucleic acid. This PCR ensures that nucleic acid is present or that there are no inhibitors in the nucleic acid extracts that retard the activity of the DNA polymerase in PCR. The results of this study indicate that the 16S bacterial primers were more reliable than the fungal ITS primers to determine the quality of nucleic acid that was extracted from the water samples. It is possible, in these environments, that bacteria are present in higher numbers than fungi and therefore represent a better target for verifying presence of DNA. Also the 16S bacterial primers are unlikely to amplify template that might cause false positives, due to carry-over contamination, in other PCRs for detection of *Pa. chlamydospora*. The risk of carry over contamination is greater with ITS primers, compared to the 16S primers, as they amplify the same region that all the *Pa. chlamydospora* PCR primers used in this study are based (Figure 4.3).

The results of this study suggest that the nested PCRs are the most sensitive method of detection for *Pa. chlamydospora*, followed by the quantitative assays, and that single PCR is the least sensitive. However, the single, non-quantitative PCR used half the volume of DNA extract that was used in the qPCR reaction. Increasing the volume to the same amount used in the qPCRs may have improved the reliability in detecting low quantities of *Pa. chlamydospora* spores in water samples.

Several samples were positive for *Pa. chlamydospora* using single non-quantitative and qPCR but not by one or both nested PCR assays. Pipetting error may have contributed due to the tiny volume (1µL) of template used in the nested PCR step.

Nested PCR is used to increase yield of the target template or to reduce the effects of inhibition associated with the DNA extraction, by using the cDNA of the first PCR as template for the second

PCR. The primers used in the second round of nested PCR are internal to the primers used in the first PCR assay. The risk of carry-over contamination, resulting in false positive results, is greater with this method than with single PCR, because many copies of any contaminant template DNA made during the first PCR reaction are carried into the second round of PCR (Persing 1991). Consequently the nested PCR technique used in this study for detection of *Pa. chlamydospora* in water samples is not recommended for routine use.

Single PCR can also be affected by carry-over contamination to produce false positive results. This occurs when a PCR assay is carried out routinely from accumulation of amplicons, which are processed in the same area (eg. electrophoresis) and handled with the same equipment that is used to set up PCR reactions (Persing 1991). Quantitative PCR can reduce the risk of carry-over contamination as the accumulation of products is monitored during cycling and very little handling of the post-amplification products is required. However, the positive result obtained with the SYBR®Green assay for samples 3R6 and 3R8 are likely to represent contamination as only one of the three replicates included in the PCR assay of each was positive and they were not *Pa. chlamydospora* positive with any other assay. This may be due to accumulation of PCR products associated with non-quantitative assays that were carried out in the same laboratory as the qPCR assays.

Unlike non–quantitative single and nested PCR, qPCR can indicate how much of a target is present in a sample rather than presence only. The qPCR in this study indicated that HWT at 50ºC for 30 min reduced the number of *Pa. chlamydospora* spores present in water. However, it was also evident that PCR detected non-viable spores when the same samples were plated onto media and monitored for growth. Nested PCR increases the yield of the target DNA and very low levels of viable and non-viable spores will be detected. It is unknown what infection threshold is required for infection of cuttings in nursery water, and it is possible that the nested PCR assay exaggerates the significance of the presence of *Pa. chlamydospora* in water samples. Further studies should be done to determine infection thresholds in water from nurseries on the incidence of *Pa. chlamydospora* and disease in the field. Despite this, we recommend that qPCR be further developed for detection of *Pa. chlamydospora*, based on the reduced risk of carry-over contamination of the amplicons into subsequent PCR assays, the sensitivity of detection of *Pa. chlamydospora* in water samples, and the potential to relate spore level in tanks to the risk of contaminating nursery material and causing disease in the field.

Two sets of standards, 10^4- 10^{-1} spores/mL and 10^6- 10^2 spores/mL, were compared in this study and used to generate standard curves for SYBR®Green and TaqMan® qPCR. The R^2 value of the standard curve indicates how well the data fit the linear relationship between Ct (the cycle number at which the increase in cDNA is logarithmic) and spore concentration. In the SYBR®Green and TaqMan® qPCR assays the R^2 value for the 10^4- 10^{-1} spores/mL standards was lower than the R^2 value for the 10^6- 10^2 spores/mL standards. This indicated that at low spore concentrations, qPCR is unreliable as the results are too variable. Therefore, we used the higher concentration standards as positive controls in the qPCR assays.

The slope of the standard curve is related to qPCR efficiency, and qPCR assays with 100% efficiency have slopes of –3.32, indicating that during each cycle the amount of product doubles (Anon., 2004). Slopes greater than – 3.32 suggest pipetting error or quality problems associated with the DNA (Anon., 2004). In this study, the slopes of the TaqMan® and SYBR®Green qPCR standard curves (10^6- 10^2 spores/mL) assayed in triplicate, were –2.413 and –2.771 respectively. This continued to occur regardless of the user, indicating that the quality of the DNA extracts was reducing PCR efficiency. Alternative DNA extraction methods to improve the quality of DNA extracted from the water samples should be examined.

The reduced PCR efficiency may also have been caused by a deficiency in the annealing ability of the primers and/or the probe to the strain of *Pa. chlamydospora* used in the standards due to genetic variation occurring at the annealing sites of the primers or probe. Our studies to date (Section 2) revealed very limited genetic variation among Australian isolates of *Pa. chlamydospora*, but it should not be discounted.

On average, the SYBR®Green assays increased in fluorescence approx 3.5 cycles earlier than the TaqMan ® assays. This suggests that SYBR®Green is more sensitive than TaqMan®, and may explain the lack of detection of *Pa. chlamydospora* in 12 samples by the TaqMan® assay. However, the lower Ct values for the SYBR®Green assay may have been associated with background fluorescence, due to primer dimer formation throughout the PCR cycling (Chou et al 1992). Alternatively, the lower sensitivity of the TaqMan® assay may have been due to inadequate binding of the primers to Australian isolates of *Pa. chlamydospora.*

Background fluorescence of the SYBR®Green assay and the lower sensitivity of the TaqMan® assay could be associated with annealing of the primers to other templates in the water samples (mispriming, Arnheim and Erlich 1992; Chou et al 1992; Bustin, 2002; Wong and Medrano, 2005). Mispriming and subsequent amplification of a non-specific target, even by single primers (Parks et al 1991), leads to the accumulation of non-specific products and reduces the amount of reaction components available for the amplification of the specific target, resulting in a lower PCR efficiency. A blast analysis indicated that the primers Pch1, Pch2, Pmo1f, PhaeoFTQ and PhaeoRTQ have up to 100% sequence similarity with many organisms and non-specific annealing of these primers to other organisms during amplification may reduce sensitivity of this assay. Further development of the qPCR assays may be required to increase sensitivity and reduce non-specific detection of other nucleic acids. Characterization of Australian *Pa. chlamydospora* isolates will determine if genetic variability is associated with PCR inefficiency.

The melt curve analysis of the SYBR®Green assay can be used to differentiate PCR products that may be of different size or different nucleotide content, which may be associated with genetic variation of the target or mispriming (Ririe et al 1997; Bustin et al 2005). The melt curve analysis of both sets of standards, made from a pure culture of *Pa. chlamydospora*, indicated a Tm between 85-86ºC for *Pa. chlamydospora*, regardless of spore concentration. In some water samples positive results were observed in the SYBR®Green assay associated with low spore concentration but the Tm calculated by the melt curve analysis was between 84-85ºC and when the samples were separated by electrophoresis a product of the correct size was observed. Some of these samples did not test positive for *Pa. chlamydospora* with other PCR primers, including the TaqMan® assay and it is possible that they represented non-specific detection of other nucleic acid. Other samples tested positive with both nested PCR assays and the SYBR®Green assay, but had a Tm of 84-85ºC. These samples may have had very low levels of *Pa. chlamydospora* that could only be detected with nested PCR, and the SYBR®Green detected a variant of *Pa. chlamydospora* that was not detected by the TaqMan® probe. However, the nested PCR results could also indicate carry-over contamination.

Unfortunately, we were unable to optimise the methodology and protocols in the time frame available to us. Although qPCR shows tremendous potential for detecting contamination events during nursery processes, consistency of results remains a challenge. The concentration of spores detected by qPCR always declined from replicate 1 to replicate 3 of the samples collected from the same tank. We were unable to determine the cause of this decline, but it may have been associated with contamination of the DNA extracts of the replicate 1 and replicate 2 groups, or with degradation of the replicate 2 and replicate 3 groups.

To conclude, non-quantitative PCR, both single and nested, are end point analyses and only indicate presence or absence of the target. Quantitative PCR can determine the starting amount of DNA template, when samples are compared to a standard. All assays have a lower limit of detection and are affected by DNA extraction efficiency, inhibition of the DNA polymerase during cycling and primer and probe annealing efficiency. These results indicate that quantitative PCR assays may be the best method of detection for *Pa. chlamydospora* in water samples as they are more sensitive than single non-quantitative PCR, and less likely than nested PCR to produce false positive results due to carry-over contamination.

Recommendations:

- Quantitative PCR using the SYBR®Green or TaqMan® assays should be further developed for detection of *Pa. chlamydospora* in water samples

- The ribosomal DNA region of a large number of pure isolates of *Pa. chlamydospora* needs to be sequenced to determine the extent of variation among Australian *Pa. chlamydospora* isolates. This variation may effect efficiency of the PCR assays to detect *Pa. chlamydospora*.
- The PCR assays should be tested against other organisms, especially fungi, which may also be present in water samples or grapevines, thereby causing mispriming and a reduction in efficiency of the PCR assays to detect *Pa. chlamydospora*. The assays should be tested against individual organisms and against combinations to determine how their presence might alter accurate detection and quantification of *Pa. chlamydospora*.
- Alternative DNA extraction methods should be explored to improve extraction efficiency.

4.2 Control and management of Petri disease - ensuring clean planting material

At present there is no way to screen planting material before it is sold to determine if it is infected and scarce information about treatments to recommend to nurseries to ensure they produce stock free of infection. This leaves suppliers of planting material in a difficult position. Hot water treatment of grapevine propagation material can effectively reduce systemic infections of *Phytophthora cinnamomi* (von Broembsen and Marais 1978), *Agrobacterium tumefaciens* (Ophel *et al* 1990), *Phomopsis viticola* (*Clarke et al* 2004), but there have been conflicting results with regard to effectiveness against Petri disease pathogens (Crous *et al* 2001, Laukart *et al* 2001, Rooney and Gubler 2001, Fourie and Halleen 2004). After harvest, bundles of cuttings are placed for 30 minutes into a hot water bath maintained at 50ºC, then they are removed and immediately plunged into cold chlorinated water to cool down quickly and prevent heat injury to the cuttings. Other treatments that have shown promise are the use of formulations of the biological control agent, *Trichoderma harzianum* (di Marco *et al* 2004, Fourie and Halleen 2004), and applications of phosphorous acid (di Marco *et al* 2000, Laukart *et al* 2001).

Preliminary *in vitro* studies in Italy, the United States and South Africa tested fungicide sensitivity in *Pa. chlamydospora* and identified a number of promising fungicides for further glasshouse evaluation. Phosphonate combined with resveratrol reduced mycelial growth of *Pa. chlamydospora in vitro* (Di Marco *et al.*, 1999) as did prochloraz manganese chloride and tebuconazole (Groenewald *et al.*, 2000). *In vivo*, phosphonate (Di Marco *et al.*, 2000), benomyl and fenarimol (Khan and Gubler, 1999) were successful in reducing symptoms of *Pa. chlamydospora*.

Aim: to determine whether cuttings can be treated during propagation to eradicate symptomless infection.

Methods

Treatment of dormant cuttings harvested from infected mother vines

Cuttings were harvested from Ramsey and Zinfandel mother vines, grown in Sunraysia and Margaret River respectively, infected with *Pa. chlamydospora*.

Ramsey: In August 1999, 90 cuttings were harvested from each of 20 dormant Ramsey mother vines known to be infected with *Pa chlamydospora.* The cuttings from each mother vine were divided into two groups of 45 cuttings. The bundles were paired to make matching bundles of 90 cuttings each, ie 10 bundles of 90 cuttings (45 from each of two vines) for the control treatment and matching 10 bundles of 90 cuttings for the hot water treatment. All cuttings were hydrated in chlorinated rainwater (3.0 ppm Cl) for 1 hr. The cuttings allocated to hot water treatment were treated at a commercial hot water treatment facility by immersion in 50 C hot water for 30 mins, then immediately transferred into a cooldown tank containing chlorinated water (3.0 ppm) at 15 C for 30 mins.

Post treatment, the cuttings were held in cold storage at 1.5ºC for four months until early December 1999. On removal from cold storage, the cuttings were wax-coated to prevent desiccation and planted out in new nursery beds on land that had never previously grown grapevines. There were 20 plots (10 hot water treated, 10 control), with 90 cuttings in each plot, set out in a complete randomised block design.

After 12 and 18 months, ten young grapevines were randomly selected from each plot and cut at ground level. The top growth was discarded, and the stems were removed for assessment. In the laboratory, each stem was flame-sterilised as described above. The stems were cut in half lengthwise and examined for internal wood streaking, then moist incubated for 6-8 weeks prior to examination for growth of *Pa. chlamydospora* and Pal.

Zinfandel: In August 1998, 3200 *V. vinifera* cv. Zinfandel cuttings were harvested from a vineyard in Margaret River, WA, for commercial use. When it was realised that the mother vines were infected with *Pa. chlamydospora*, the cuttings were stored at 1.5ºC for 26 months prior to using for experimental purposes. In October 2000, the cuttings were removed from cold storage, divided equally into 4 groups and the following treatments applied: (a) control *ie.* hydrated in rainwater for 1

hr; (b) hot water treatment *ie.* hydrated in rainwater for 1 hr, then 30 min immersion in 50°C hot water tank, followed by 30 min in 15°C cooldown tank; (c) Trichoflow *ie.* hydrated in rainwater, then dipped in *Trichoderma harzianum* spore suspension as per manufacturer's instructions (Trichoflow-T, Agrimm Technologies Ltd., Christchurch, New Zealand: 100 g per 50 liters); (d) hot water treatment + Trichoflow *ie.* hydrated in rainwater for 1 hr, 30 min immersion in 50°C hot water tank, 30 min in 15°C cooldown tank, then dipped in *Trichoderma harzianum* spore suspension as per manufacturer's instructions.

After treatment, cuttings were placed into polystyrene boxes containing moist vermiculite and maintained at 28 C until callus tissue and roots had developed. In December 2000, they were planted out in new nursery beds that had never previously grown grapevines. The experimental layout was a randomised block design with the four treatments randomised within each of 8 blocks. After 6, 12 and 18 months, 10 young grapevines were randomly sampled from each of the 36 plots, and assessed as described for the Ramsey grapevines.

Cabernet Sauvignon: In July 2001, dormant canes were harvested from each of three Cabernet Sauvignon mother vines infected with *Pa chlamydospora.* Approximately half of the canes from each mother vine were left as untreated controls and the rest were hot water treated (30 min @ 50 C, followed by 30 min @ 15 C). The canes were processed into 4-node cutting lengths of 300-460 mm, callused as described above and potted into 4 cm tubes containing potting mix. (Untreated control = 17 canes (55 cuttings) from Vine 1, 16 canes (50 cuttings) from Vine 2 and 15 canes (59 cuttings) from Vine 3. Hot water treated = 17 canes (46 cuttings) from Vine 1, 14 canes (40 cuttings) from Vine 2 and 15 canes (50 cuttings) from Vine 3.). The plants were placed in a randomised block design on glasshouse benches and maintained in glasshouse conditions for two seasons, fertilised and watered as required. In July 2003, the resultant young grapevines were assessed in the same manner as described above.

Treatment of one year old rootlings

Two hundred dormant 1-year-old Pinot Noir rootlings, suspected of being infected with *Pa. chlamydospora*, were used in this experiment. The rootlings were divided into groups of 20 and each group was subjected to one of 10 treatments (Table 4.11). The treatments were chosen to represent a range of different modes of actions and, where possible, included those identified by other research groups as promising.

Prior to planting, the roots were trimmed to 15 cm length and the canes were cut back to three buds (as per commercial practice). The vines were planted singly in 15 cm diameter pots, placed in a randomized design in the glasshouse, and watered and fertilised as required. The hot water treatment was applied immediately prior to planting. All other treatments were applied as a foliar spray to run-off (40-50 ml/vine) combined with a soil drench to saturation (200-250 ml/pot) at 2 and 19 weeks after planting (growth stages of eight leaves separated and at pre-harvest, respectively).

Vines were destructively assessed at the end of the growing season as leaves began to senesce (22 weeks from planting). Cane and root dry weights, stem fresh weight and diameter were recorded per vine. The stems were surface-sterilised by dipping in 100% ethanol and then flaming, then split longitudinally and examined for the incidence and intensity of brown wood-streaking and dark pith. Intensity was assessed using a scale from 0 to 3, where 0 indicated no discolouration and 3 indicated strong discolouration. Immediately after examination, the stem pieces were moist incubated for 4-6 weeks and re-examined at x 40 magnification for the presence of fungal growth. Differences between treatments with respect to incidence of the above-mentioned symptoms and *Pa. chlamydospora* were determined using the logistic regression. The group average method of cluster analysis (Gordon 1981) was also used to classify the treatments with respect to their effectiveness against the above-mentioned symptoms and *Pa. chlamydospora.*

Visual assessments of the growth stages of the vines indicated that the growth of the hot water treated vines was retarded and that phosphonate was slightly phytotoxic, causing some leaf scorch. However, the fresh and dry weight measurements showed no significant differences between treatments (data not shown), indicating that the affected vines had recovered by the time assessments were made.

Table 4.11. Treatments applied to naturally *Pa chlamydospora*-infected Pinot Noir rootlings

Treatment (a.i.)	Product	Rate	Time of applications (growth stage)
Control	Water		1st: 8 leaves separated 2nd: pre-harvest
Hot water treatment		50°C for 30 min	pre-planting
Benomyl	Benlate DF®	0.4 ml/l	1st: 8 leaves separated 2nd: pre-harvest
Benzothiodiazole	Bion 500WG®	0.08 g/l	1st: 8 leaves separated 2nd: pre-harvest
Fenarimol	Rubigan 120SC®	0.2 ml/l	1st: 8 leaves separated 2nd: pre-harvest
Kresoxim-methyl	Stroby WG®	0.1 g/l	1st: 8 leaves separated 2nd: pre-harvest
Phosphonate	Agri-fos Supol 400®	50 ml/l	1st: 8 leaves separated 2nd: pre-harvest
Prochloraz	Sportak®	0.2 ml/l	1st: 8 leaves separated 2nd: pre-harvest
Tebucanozole	Folicur® 250	2.5 ml/l	1st: 8 leaves separated 2nd: pre-harvest
Triadimenol	Bayfidan 250EC®	0.1 ml/l	1st: 8 leaves separated 2nd: pre-harvest

Hot water treatment of cuttings followed by Agri-Fos applications during first season's growth.

In July 2004, 125 dormant cuttings per cultivar were harvested from infected Verdelho and Chardonnay mother vines. To ensure infection, the cuttings were inoculated by immersion for 30 mins in a tub containing 100 litres of *Pa. chlamydospora* spore suspensions @ 10^2 spores/ml. After air-drying, the cuttings were stored for two weeks at 3 C. Post-storage, the cuttings of each cultivar were randomly divided equally into 2 groups per cultivar. One of each group per cultivar was hot water treated (50 C for 30 mins, followed by 15 C for 30 mins) and the other hydrated only (30 mins at 15 C). All cuttings were callused as previously described, potted into individual 10 cm pots containing commercial potting mix and maintained in glasshouse conditions. In October 2004, 48 of the healthiest young grapevines from each group were selected and the grapevines were further subdivided to give 4 groups of 24 plants per cultivar. Treatments were (a) control: hydration only; (b) hot water treated; (c) Agrifos applied twice during active growth; (d) hot water treated followed by Agrifos applied twice during active growth. The plants were placed in a completely randomised design and grown in glasshouse conditions for one season. AgriFos was applied twice.

Results and discussion

Hot water treatment significantly reduced the incidence of infection in when applied to dormant cuttings (Table 4.12). Incidence was reduced from 26% to 3% in Ramsey, 45% to 4% in Zinfandel, 50% to 12.5% in Verdelho, 62.5% to 8.3% in Chardonnay and 5.5% to 4% in Cabernet Sauvignon. The lattter was not significantly reduced because the original incidence was already low.

The Trichoderma treatment significantly increased incidence of infected young vines (Table 4.12). This was a most unexpected result. The *Trichoderma* organism was certainly viable and was well established throughout the stems of the treated vines even 18 months after treatment. A possible explanation is that the *Trichoderma* caused suppression of some unknown beneficial endophyte that was inhibiting colonisation of *Pa. chlamydospora.*

Hot water treatment of one-year-old rootlings reduced the incidence of infection but not significantly (Table 4.13). This suggests that hot water treatment is most effective before *Pa. chlamydospora* has

had time to establish sizeable infection courts, possibly only present as spores or other propagules lodged in xylem vessels.

Table 4.12. Effects of hot water treatment (HWT), *Trichoderma harzianum* (Trichoflow) and phosphorous acid (Agrifos), in combination or alone, on the incidence of *Phaeomoniella chlamydospora* (Pch) infection in young grapevines.

Cultivar	Incidence of infection (%)					
Age at assessment	Control	HWT	Trichoflow	HWT + Trichoflow	Agrifos	HWT+Agrifos
Cabernet Sauvignon						
24 months	5.5	4	na	na	na	na
Ramsey						
12 months	26a	3b	na	na	na	na
18 months	12a	1b	na	na	na	na
Zinfandel						
6 months	13a	0b	38c	1b	na	na
12 months	45a	4b	70c	3b	na	na
18 months	45a	3b	68c	4b	na	na
Verdelho						
6 months	50.0a	12.5b	na	na	33.3ab	16.7b
Chardonnay						
6 months	62.5a	8.3b	na	na	54.2a	16.7b

Treatment of one year old rootlings

When the effects of the treatments on symptoms and presence of the fungus were analysed independently, all treatments significantly reduced the incidence and intensity of dark pith ($P<0.01$), but none significantly reduced either the brown wood-streaking or the incidence of *Pa. chlamydospora*. No *Pa. chlamydospora* was observed in the phosphonate treatment (Table 4.13).

Table 4.13. Effects of treatments on symptom appearance and presence of *Pa. chlamydospora* (Pch) in 1-year-old Pinot Noir rootlings. Incidence of symptom was measured as the percentage of 20 vines per treatment showing discolouration; intensity of symptom was assessed using a scale of 0-3, where 0 = no discolouration, 1 = faint discolouration, 2 = moderate discolouration and 3 = strong discolouration. The values are expressed as the average over 20 vines per treatment.

Treatment	Wood-streaking		Dark pith		*Pch*
	Incidence (%)	Intensity (0-3 scale)	Incidence (%)	Intensity (0-3 scale)	(%)
Control	95	2.90	95	2.85	30
Hot water treatment	60	2.40	30	1.05	20
Benomyl	65	2.40	45	1.50	10
Benzothiodiazole	50	2.35	40	1.40	25
Fenarimol	70	2.50	30	1.10	10
Kresoxim-methyl	75	2.70	35	1.25	20
Phosphonate	70	2.65	15	0.75	0
Prochloraz	56	2.22	44	1.56	6
Tebuconazole	70	2.55	30	1.00	25
Triadiamenol	75	2.75	30	1.10	15

Cluster analysis operating on a similarity matrix obtained from the proportions in Table 4.13 revealed three distinct groups (Figure 4.6). Group 1 contained the four most effective treatments: phosphonate, prochloraz, benomyl and fenarimol; Group 2 contained kresoxim-methyl, triadimenol, tebuconazole, hot water treatment and benzothiodiazole, and Group 3 contained only the control. All treatments

successfully limited symptom development, particularly pith discolouration, but due to the low level (30%) of *Pa. chlamydospora* present in the untreated control plants, only phosphonate (0% *Pa. chlamydospora*) can be identified from this study as a potentially useful curative treatment for young vines infected with *Pa. chlamydospora*.

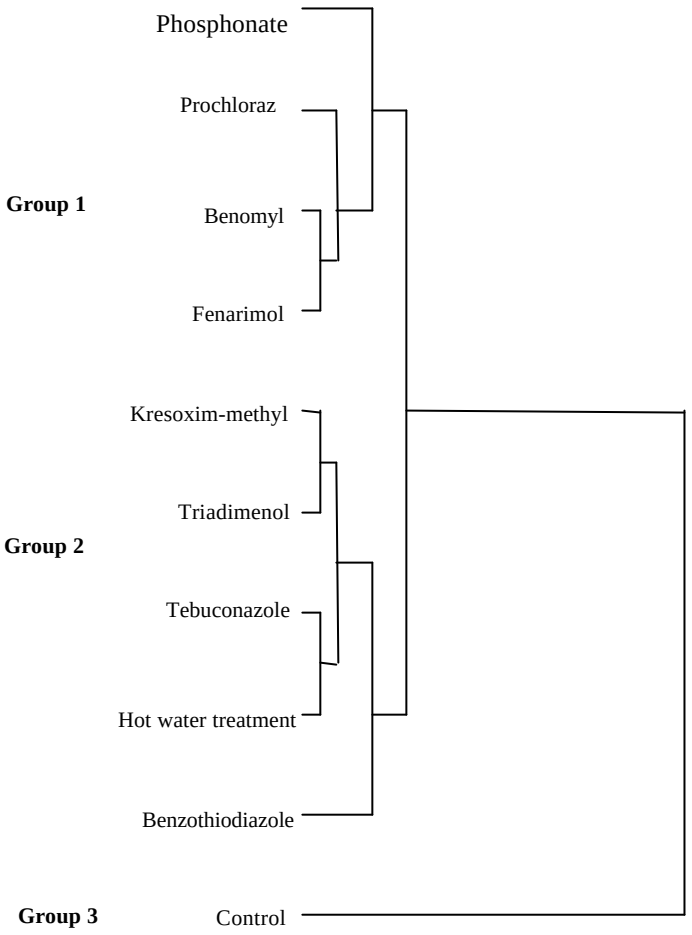


Figure 4.6. Dendrogram of treatments with respect to their relative effectiveness against *Phaeomoniella chlamydospora* infection of 1-year-old Pinot Noir rootlings constructed using cluster analysis determined by the group average method and Euclidean distance.

Hot water treatment of cuttings followed by Agri-Fos applications during first season's growth.

On the basis of the results above, it was decided to test whether hot water treatment of dormant cuttings followed by phosphnate applications during the first seasons growth would be the ideal treatment to recommend to nurseries. However, the results (Table 4.12) did not support this. There was no difference in incidence of infected between hot water treatment alone and hot water treatment combined with phosphonate.

We conclude from these studies that hot water treatment of dormant cuttings is the best option for reducing infection in young vines. No other treatment trialled in these studies was particularly effective. Our recommendation to the nursery industry is that grapevine cuttings should be routinely hot water treated to reduce the chances of selling symptomless infected planting material to growers.

Chapter 5: The effects of Hot Water Treatment on dormant cuttings of *Vitis Vinifera* cvs. Cabernet Sauvignon and Pinot Noir and the development of reliable nursery protocols

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Summary. Three projects were undertaken during the course of this investigation. The most sensitive variety (Pinot Noir) and the least sensitive variety (Cabernet Sauvignon) were used in each experiment in an attempt to understand the causes of the differential sensitivity of the 2 varieties. The treatments used in each of the three experiments were control, HWT, HWT plus hydration and hydration. Hydration was included since there is a perception in the nursery industry that a pre HWT hydration in cold water for several hours improves the quality of cuttings. Previous research indicates that this practice is at best unnecessary and may be harmful.

The first trial used transmission electron microscopy to investigate the effects of HWT protocols on cell ultrastructure. Cuttings were examined 24 hours after treatment and after 4 and 10 weeks cold storage since it is standard industry practice to store cuttings until required in the nursery program and it is thought that there may be an interaction between HWT and cold storage that has a detrimental effect on cuttings. Damaged cells with fractured walls and disrupted amyloplasts were observed in the ray tissue of both varieties and all treatments when the tissue was examined 24 hours after initial treatment. Amyloplasts in untreated intact cells of both Pinot Noir and Cabernet Sauvignon were apparently undamaged, but amyloplasts in intact cells in tissue that had been hydrated, hot water treated or hydrated and hot water treated showed varying degrees of structural change. Damaged amyloplasts were not seen in intact cells after 4 and 10 weeks cold storage. Changes to cell ultrastructure at 4 weeks showed an apparent increase in metabolic activity followed by a return to a less active state by 10 weeks except in HWT and HWT plus hydration Pinot Noir, clearly indicate that cuttings held in cold storage are not quiescent, particularly HWT Pinot Noir that seems to remain active rather than reverting to the quiescent state as did the other treatments and the Cabernet Sauvignon.

The second trial used gas chromatography to investigate the effects of HWT and hydration on respiration in dormant vine tissue by measuring ethanol generation in cuttings of Cabernet Sauvignon and Pinot Noir. Cuttings were examined 24 hours after treatment and after 4 weeks cold storage. Ethanol was detected in significant quantities (>0.1mg/hr/gm) in the atmospheres of both the HWT and HWT + hydration treatments 24 hours after treatment indicating that they had become fermentative. Ethanol production in untreated controls or the hydration only treatment was significantly lower (<0.03mg/hr/gm) indicating that these cuttings remained aerobic. After 4 weeks cold storage ethanol production in all treatments was negligible (<0.005mg/hr/gm) indicating that respiration was aerobic and had returned to normal in the HWT cuttings.

In the third trial, the expression and persistence of heat shock proteins in internode tissue of dormant Cabernet Sauvignon and Pinot Noir cuttings was investigated using acrylamide gel electrophoresis. Internode tissue was chosen in this experiment for consistency with the transmission electron microscopy. Significant problems were encountered with protein solubility and contaminating substances that made quantification of protein difficult and the resulting gels difficult to interpret. However techniques for ridding protein samples of contaminants including phenolics and sugars are currently being investigated and the work is ongoing. However preliminary examination of the gels from fresh Cabernet Sauvignon cuttings collected in 2006 indicates that protein expression in the treated cuttings hydration, HWT + hydration and HWT, did not differ from protein expression in the untreated cuttings 24 hours after treatment. A similar trend was apparent in Pinot Noir, but the gels were not clearly enough resolved to be certain of this interpretation.

Introduction

The quality of planting material is of paramount importance in vineyard establishment. The consequences of using inferior planting material are serious in both the short and long term, but in the rush to plant vineyards in the boom that began in the 1990's many vineyards were established with poor quality vines, frequently infected with endophytic fungal pathogens (Edwards et al. 2004). This created significant problems for growers and resulted in a number of cases of litigation.

Hot water treatment (HWT) of dormant grapevine cuttings and rootlings by immersion in hot water at 50°C for 30 minutes is the only effective and practical means of control for a number of endogenous organisms (including *Pa. chlamydospora*) that affect the short and long term health and viability of vineyards (Goheen *et al.* 1973, Burr *et al.*1989, Bazzi *et al.* 1991, Caudwell *et al.* 1997, Luck *et al.* 2000, Edwards *et al.* 2004). However, the technique has not been universally adopted by nurseries because of concerns regarding the efficacy and safety of HWT.

There have been a number of research projects in the last decade that have investigated the effects of HWT and surrounding handling practices, particularly pre HWT hydration, on different *Vitis vinifera* varieties (Waite 1998; Crocker & Waite 2004; Waite & May 2005). The outcomes of this research have been the impetus for continuous refinements to grapevine propagation practices and HWT protocols that have resulted in a commensurate improvement in the quality and consistency of planting material available to the grape and wine industry and fewer reports of losses associated with HWT. However there continue to be reports of occasional failures of HWT cuttings and rootlings that cannot be explained in the context of our current knowledge and the unpredictable nature of the losses and associated costs have been deterrents to the universal adoption of HWT**.**

It is known that factors such as variety, source area, time of cutting harvest and handling protocols all have an impact on the response of cuttings and rootlings to HWT (Waite 1998; Crocker & Waite 2004; Waite & May 2005), but as yet the mechanisms of these responses are not understood in sufficient detail to enable the cause of all cutting and vine failures attributed to HWT to be determined. Treated material rarely perishes during HWT, or in post HWT cold storage, but is vulnerable to stressful environmental conditions during bud burst and for several weeks immediately following budburst. This period of vulnerability to environmental conditions coincides with a period of stunted and delayed growth in hot water treated material compared to material that has not undergone HWT (Waite 1998). The delayed and stunted growth is particularly marked in sensitive varieties such as Pinot Noir that suffer a significant delay in establishment compared to more tolerant varieties such as Cabernet Sauvignon where the symptoms are less marked. The expression of these symptoms at the time of bud burst when the metabolic rate and energy demands of vine material rapidly increase (Taiz & Zeiger 1998) suggests that the effects of HWT on the tissue are not transient, but persist for some time after treatment and affect the establishment of cuttings in the nursery.

The purpose of this research was to determine the effects of HWT and associated protocols on cutting physiology in order to better understand the causes of the delayed growth and occasional cutting failure so that more reliable protocols could be developed and industry uptake of HWT increased.

5.1 The effects of Hot Water Treatment on the metabolism of dormant cuttings of *Vitis vinifera* cultivars.

Phytosanitary treatment by immersion in hot water at 50°C for 30 min (hot water treatment, HWT) is a chemical free and effective method of controlling a number of endogenous pathogens in dormant grapevine propagating material including crown gall (*Agrobacterium vitis*), phytoplasmas, Petri disease (*Pa. chlamydospora*) and Pierce's disease (*Xylella fastidiosa*) (Goheen *et al.* 1973, Burr *et al.*1989, Bazzi *et al.* 1991, Caudwell *et al.* 1997, Luck *et al.* 2000, Edwards *et al.* 2004). However commercial application of the technique to large batches of dormant cuttings and one-year old vines (rootlings) has met with mixed success. Delayed or restricted growth following HWT is often observed, particularly in Pinot Noir, the most sensitive variety, and there are ongoing reports of occasional losses of large batches of hot water treated cuttings and rootlings. These represent significant financial loss to both nurseries and their customers, and litigation is common. As a result, propagators are reluctant to incorporate HWT into standard nursery protocols in spite of considerable pressure to do so from some segments of the grape and wine industry and the scientific community (Smart 1997).

Previous research (Waite 2002) has shown that many factors including variety, source block management, stage of dormancy, hydration prior to HWT and storage conditions, including exposure to ethylene, all have an impact on the viability of propagating material. However we do not yet fully understand the reasons for some of the responses we observe. Batches of failed or poorly performing HWT cuttings and 1 year-old rooted vines kept in cold storage sometimes have a strong wine like odour suggestive of fermentation and it was thought that oxygen deprivation as a result of sealing cuttings and rootlings in plastic bags may have caused the stored material to ferment. The results of work by Fletcher examining the effects of perforating storage bags with 2 or 3 small holes to improve ventilation showed a reduced incidence of fermentation in stored material and industry practices were subsequently changed to incorporate perforation of storage bags. However irregular reports of fermentation of cuttings and rootlings in storage continued to occur and it was thought that the pre HWT practice of hydrating cuttings and rootlings for several hours, or overnight, by soaking them in water might result in low oxygen levels in the tissue and induction of anaerobiosis, particularly if the soaking water was used for several batches of material, or allowed to stand for long periods, practices that reduce oxygen levels in water. It was also speculated that the very steep increase in respiration rates brought about by HWT (Crocker & Waite 2004) might rapidly deplete the oxygen in the tissue and in the atmosphere of the storage bags, also contributing to the onset of fermentative respiration.

In the experiment reported here we investigated the effects of HWT and hydration on the anaerobic respiration of dormant vine tissue by measuring ethanol generation in cuttings of Cabernet Sauvignon (CS) and Pinot Noir (PN). Nursery industry reports that some varieties of *Vitis vinifera* are more sensitive to HWT than others have been confirmed by research. PN and CS are the most and least sensitive varieties and were chosen for this experiment to represent the broadest range of possible responses.

Methods

Treatments. Dormant 1-year old canes of PN and CS were collected from vineyards at Hanging Rock and Dookie respectively on 13th July 2004. The following day the cuttings were cut into 2 bud cuttings and randomly divided into 12 bundles of 5 cuttings for each variety. The bundles of cuttings were then randomly assigned to 1 of 4 treatments: (a) untreated control, (b) hydration for 8 hours, (c) hydration for 8 hours plus HWT (50°C for 30 min.), and (d) HWT without hydration. The cutting bundles in each treatment were then HWT individually and transported under ambient winter temperatures to the Department of Primary Industries at Knoxfield. Following initial treatment, 1 cutting from each of the 6 bundles and each treatment was randomly selected for immediate testing and the remaining cuttings in their bundles were placed in cold storage in perforated plastic bags at 4-5°C for either 4 or 10 weeks (stages 1,2 & 3 respectively). The temperature of the cool room where the cuttings were stored was higher than optimum (4-5°C rather than 1-2°C), but many industry cool

rooms used to store cuttings are also operated at 4-5°C. After 4 weeks cold storage 1 cuttings from each of the 6 bundles in each treatment was removed for testing. Cuttings were not tested after 10 weeks (see results section).

Gas Chromatography. For each variety (PN & CS) and treatment (a, b, c & d) ethanol production in cuttings was measured 24 hours after treatment and again after 4 weeks cold storage. A syringe was used to sample the atmosphere from 6 individual (replicate) cuttings sealed in test tubes (0.055L) with rubber caps and incubated at room temperature (18-20°C) for 4 hours. Each atmosphere sample was injected into a gas chromatogram (Shimadzu GC 14B) with a Porapak PS column (2m, O.D.3.2mm, mesh 100/120), a gas carrier of N_2 (100kPa) and flame ionization detector (injection and detection temperature 135°C). The column temperature was 115°C and the retention time for ethanol was approximately 3.0 minutes. Ethanol production (A) for each sample was calculated as mg/h/g fresh weight using the following formula.

$$A = \left[\frac{\left(\frac{P}{S} \right) xExV}{T} \right] \div W$$

Where S = ethanol standard peak area, P = sample peak area – blank peak area (blank peak area was 0 as there was no ethanol in the atmosphere), E = standard saturated atmospheric ethanol concentration (mg/L), V = volume of tube (L), T = time of incubation (h) and W = sample weight (g). The ethanol standard was 15.5 mg/L, being the concentration of ethanol in the saturated atmosphere above a solution of 0.1g/L ethanol in water at 20 °C (Harger et al. 1950)

Data Analysis. Data were analysed using the Minitab statistical package (one-way ANOVA) and Microsoft Excel (LSD).

Results and Discussion

Ethanol was detected in the atmospheres of all samples at stage 1, 24 hours after treatment, but only in miniscule amounts at stage 2 (<0.005mg/hr/gm), after 4 weeks cold storage. Because there was negligible generation of ethanol at stage 2 it was decided not to proceed with testing at stage 3 as it was considered unlikely that the cuttings would be generating ethanol because of the low metabolic rate and adequate ventilation in storage. The quantity of ethanol generated at stage 1 by cuttings that had been hot water treated (+/- hydration) was significantly higher (P=0.05) than that generated by cuttings that had no treatment or hydration only (figure 5.1). The increased ethanol production in HWT cuttings might be a result of an oxygen deficit in the tissue of the cuttings that are saturated with water relatively low in dissolved oxygen during hydration and HWT and the elevated respiration rate in HWT material, or simply earlier onset of anaerobic respiration in the sealed tubes caused by elevated rates of respiration induced by the heat shock of HWT. The higher rate of ethanol production in CS cuttings that were not hydrated before HWT lends weight to the former hypothesis. The cold water used in the hydration bath that saturates the tissue before HWT is likely to have higher levels of dissolved oxygen than the water in the HWT bath that is rapidly absorbed during HWT and saturates the tissue of the HWT only cuttings (Gray 1999). It is possible that the lower oxygen water in the HWT bath does not displace the higher oxygen water absorbed during pre HWT hydration thus there is likely to be higher oxygen levels in the tissue of the HWT plus hydration treatment than in the HWT only treatment. However further research investigating ethanol production following HWT in an atmosphere with adequate oxygen for normal aerobic respiration would be required to confirm this.

Pinot Noir cuttings produced more ethanol than CS in the HWT plus hydration treatment ($P<0.05$), and this is likely to be a result of the higher base respiration rate in PN (Crocker & Waite 1994

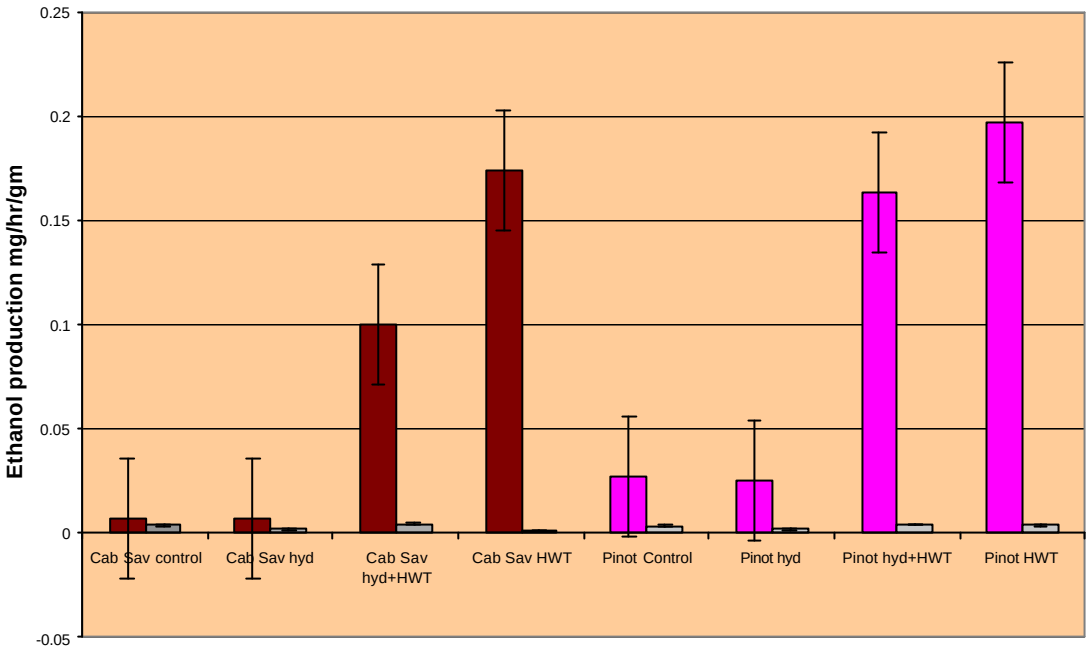


Figure 5.1. Ethanol generation (mg/g/h) in dormant cuttings of CS (burgundy bars) and PN (mauve bars) 24 after treatment (solid bars) and after 4 weeks cold storage (striped bars). (treatments; left to right for each variety: control, hydration, hydration + HWT, HWT). Error bars, LSD at $P = 0.05$.

When the cuttings were examined at stage 2 after 4 weeks cold storage (4-5^{o}C) using the same protocols, ethanol production was negligible (Figure 5.1) in all treatments of both varieties indicating that all cuttings were respiring aerobically. The return to aerobic respiration is most likely to be the result of improved oxygen availability in the tissue as a consequence of drying of the tissue in storage and storage in well-ventilated bags rather than sealed, or partially ventilated bags as is industry practice. Had the cuttings been stored in sealed or poorly ventilated bags, drying of the tissue would not have occurred, the oxygen supply would have been restricted and it is probable that the cuttings would have remained fermentative.

The effects of anoxia and the metabolic by products of fermentation on plants and plant tissues vary between species (Perata & Alpi 1991; Kennedy et al. 1992; Schmull & Thomas 2000; Summers et al. 2000) and include an upsurge in respiration rates (Rychter et al. 1979), inhibition of cell growth (Perata & Alpi 1991) and irreversible damage to tissue leading to death of organs or whole plants (Kennedy et al. 1992). The more pronounced restricted and delayed growth seen in PN and the sensitivity of the variety to post HWT mortality may be a result of greater sensitivity to ethanol and acetaldehyde compared to CS. The likelihood of varietal differences in sensitivity to acetaldehyde, which is also produced by anaerobic respiration, is supported by the work of Wolf (1976) who found that levels of enzymes in berry and leaf tissue including alcohol dehydrogenase (ADH), the enzyme responsible for the conversion of acetaldehyde to the less toxic ethanol (and back again) in grapes and wine, varied between grapevine varieties. Schmull and Thomas (2000) also reported delayed bud burst and reduced root biomass in *Fagus sylvatica* that had been subjected to waterlogging over winter, but attribute the delayed bud burst to the reduced root biomass rather than sensitivity to fermentative metabolites. However, acetaldehyde can bind to proteins and inactivate enzymes and may prevent normal metabolism (Perata & Alpi 1991; Perata et al. 1992) and the delayed bud burst seen in hot water treated cuttings may also be a result of inactivation of essential proteins and enzymes required for cell growth. Furthermore the return to aerobic conditions and the consequent oxidation of ethanol to acetaldehyde by catalase may cause a rapid increase in acetaldehyde

production causing additional damage to the tissue (Monk et al. 1987), and thus explain the stunted and delayed growth observed in cuttings during the callusing phase when they have been removed from the anoxic, or low oxygen, conditions of cold storage.

The significantly higher level ($P<0.05$) of ethanol generation in hot water treated cuttings of PN and CS at stage 1 in the work reported here indicates that HWT results in fermentative respiration in the cutting tissue. The sealed tube technique for detecting ethanol production in cuttings used here means that fermentation caused by the higher rate of respiration in hot water treated cuttings cannot be ruled out as the cause of increased ethanol production. However the higher rate of ethanol generation in the HWT only groups where the tissue is flooded with water very low in oxygen compared with the water flooding the tissue in the HWT plus hydration groups is evidence that fermentation is the result of anoxic conditions in the tissue induced by the HWT rather than the result of the sealed vessel technique used in this experiment.

Since ethanol and its precursor, acetaldehyde, are toxic to plant tissue and can inhibit growth it is probable that the losses and delayed growth observed in hot water treated cuttings are a result of fermentation following HWT combined with a flush of post-anoxic conversion of ethanol to acetaldehyde. The practice in industry of enclosing cuttings in sealed or poorly ventilated bags immediately following HWT (<24hrs) is likely to result in the accumulation of ethanol in the bags and the continuation of fermentation in cold storage in spite of the relatively low respiration rates induced by the cold temperatures. Anecdotal evidence from industry of a strong wine-like odour in cuttings stored in sealed bags and the subsequent poor performance of such cuttings adds weight to this conclusion.

The relative sensitivity of Pinot Noir to HWT compared to Cabernet Sauvignon may be a result of variations in the physiological response to ethanol, or acetaldehyde accumulation in tissue including alcohol dehydrogenase production, or other enzymes. Further investigation into the effects of acetaldehyde on grapevine cutting tissue and the physiological response of both varieties is required to resolve these questions.

Recommendations for industry

The results of the work reported here strongly suggest that it would be prudent for nurseries to change their handling practices to allow at least 24 hours for cuttings to recover from HWT before being placed in storage. This would enable the cuttings to resume aerobic respiration and allow potentially toxic ethanol and acetaldehyde, and/or other metabolites and excess moisture to dissipate before the cuttings are enclosed in the bags where air circulation and oxygen levels are restricted. It would also be prudent for nurseries to increase the perforations in the plastic bags used for storage of cuttings to ensure that oxygen supply is adequate. Cool rooms should also be well ventilated and organized to facilitate air circulation around the stored material. Bagged cuttings should be placed in storage without delay since the relatively high ambient temperatures and the resulting increase in respiration rates are likely to result in the onset of fermentation regardless of treatment. It is also important that bagged cuttings that have been removed from cold storage should be processed without delay to guard against the onset of fermentation as the temperature increases inside the bags.

Anoxia may also be initiated or prolonged during callusing. Normal industry practice is to pack cuttings with moist vermiculite in polystyrene or plastic lined boxes and incubate in a purpose built room at 24-27^{o}C for 10-14 days. The poor ventilation in the boxes coupled with the higher respiration rates in the relatively warm temperature of the callusing room could induce fermentation, or maintain fermentation in already fermentative cuttings, particularly if the cuttings have been hydrated before callusing as is frequently the practice, or the cuttings are packed very tightly in the boxes and air circulation in the callusing room is poor.

In the light of the evidence presented here it is recommended that individual nurseries review their handling and propagation practices to ensure that fermentation caused by HWT is not prolonged and that cuttings have adequate oxygen levels at all stages of storage and propagation. By adopting these measures nurseries should be able to reduce losses and increase the quality of their product.

5.2 The Effects of Hot Water Treatment, Hydration and Cold Storage on Ray Cell Ultrastructure in Dormant Pinot Noir and Cabernet Sauvignon Cuttings

Grapevines store carbohydrate reserves as starch grains (amyloplasts) in the ray tissue of both the xylem and phloem in their roots, trunks, cordons and canes (Mullins, Bouquet & Williams 1992) and it is these reserves that provide the energy required to sustain life during dormancy and for bud burst and shoot growth in spring until the leaves have emerged and are fully functioning (Yang & Hori 1980). However it is only the starch reserves laid down in the ray tissue of the previous season's canes that sustain adventitious root initiation and bud burst in cuttings, since any reserves in the trunk, cordons, or roots are inaccessible by virtue of the cuttings being severed from the mother vine. In grapevines, lignification of canes occurs following secondary thickening of xylem and ray cell walls and deposition of starch in ray cells (Mullins, Bouquet & Williams 1992). Unlignified sections of canes, usually the tips, react negatively to the starch iodine test and are discarded by propagators because there are no stored carbohydrates to support bud burst and the cuttings fail to establish (Hartmann et al.1990; Nicholas, Chapman & Cirami 1992). By contrast, lignified canes react positively to the starch iodine test and generally contain more than adequate carbohydrate reserves for bud burst and root initiation.

It was thought that either HWT, or the practice of soaking propagating material in cold water for variable periods (8-24 hours) prior to HWT might result in rupturing of the amyloplasts or damage to amyloplast membranes that reduces the ability of the cell to mobilize carbohydrate reserves. When starch grains are soaked in water (<50°C) about 30% of the granule weight is absorbed and the grain swells increasing in volume by 5%. If the starch grains are then heated, irreversible changes including gelatinization will occur when the critical temperature is reached. In the case of wheat starch the critical temperature is just above 50°C (Hoseney 1998). However the gelatinization temperature of starch varies depending on the plant species, genotype and growing conditions (Cottrell et al. 1995), but is lowered when starch is plasticized by water absorption (<30% water content) (Billiaderis et al. 1986). The gelatinization temperature of starch in grapevines is unknown, and is possibly affected by water absorption during the pre HWT hydration period and by the growing conditions prior to HWT (Cottrell et al. 1995).

In the experiment reported here, transmission electron microscopy (TEM) was used to investigate the effects of HWT, hydration and cold storage on the structure of amyloplasts and other organelles and membranes in ray tissue in cuttings of Pinot Noir and Cabernet Sauvignon.

Methods

Materials and treatments: Dormant 2 bud hardwood cuttings of Pinot Noir and Cabernet Sauvignon were collected from vineyards at Hanging Rock and Dookie respectively on 13th July 2004 and treated as previously described in Section 5.1.

Embedding. Cuttings were prepared for microscopic examination at Monash Micro Imaging, Monash University, Clayton, Victoria, by cutting small transverse segments of internode approximately 1-2mm thick. Each segment was then dissected radially under Karnovsky's fixative to obtain specimens for embedding approximately 1x1mm. Specimens in Karnovsky's fixative were subjected to gentle vacuum for one hour, then left for a further 24 hours at 4 deg C to complete primary fixation. Following primary fixation the specimens were rinsed with three changes of buffer, and infiltrated with 1% osmium tetroxide for four hours. Following osmication the specimens were rinsed in three changes of distilled water and dehydrated through a graded ethanol series starting at 10% ethanol with 10% increments of one hour each, followed by 3 changes in absolute dry ethanol for 24 hours each. The specimens were then rinsed 100% propylene oxide and infiltrated with firm grade Spurr's (1969) resin. Infiltration began with 25% resin in propylene oxide for 2 days on an inclined rotator, followed by 50% resin in propylene oxide for 2 days and 75% resin in propylene oxide for 2 days. Infiltration at 100% resin was over 7 days with 3 changes at 4°C. Specimens were then placed in moulds with fresh resin and polymerised overnight at 60°C.

For light microscopy specimens were sectioned at 2um using a Reichert Ultracut S, stained with toluidine blue pH 9 and viewed with an Olympus Provis light microscope. For electron microscopy sections were cut at 120nm, collected on 300 mesh copper grids, stained with uranyl acetate and lead citrate (10 minutes each) and viewed at 100kV with a Jeol 200CX TEM.

Results and Discussion

Amyloplasts. Damaged cells with fractured walls and disrupted amyloplasts were observed in the ray tissue of both varieties and all treatments when the tissue was examined 24 hours after initial treatment (fig. 5.2). It is thought that this damage was most likely caused when the specimens were dissected since the cuttings were hard, woody and brittle and difficult to prepare for embedding. However, similar damage was observed in tissue cultured stem tissue (Section 6.1). It may be that the relative thinness and consequent weakness of the cell walls and the numerous pits (areas where the wall is very thin and perforated by plasmodesmata to allow communication between cells) in ray tissue compared to xylem tissue predisposes the ray tissue to damage from shear force when it is dissected. The pattern was different when apparently intact cells were examined (Fig. 5..3). Amyloplasts in untreated intact cells of both Pinot Noir and Cabernet Sauvignon were apparently undamaged, but amyloplasts in intact cells in tissue that had been hydrated, hot water treated or hydrated and hot water treated showed varying degrees of structural change. Some amyloplasts had completely ruptured and appeared as grainy, diffuse entities with no obvious membrane, others were less damaged with partial extrusion of contents into the cytoplasm from a single rupture, but with the membrane visible. Other amyloplasts remained intact, but had a grainy, diffuse electron dense area at the centre with fewer membrane folds. Normal amyloplasts were electron transparent and not grainy at the centre. The most severe damage to amyloplasts was observed in both Pinot Noir and Cabernet Sauvignon tissue that had been hydrated but not hot water treated. It is interesting to note that undamaged amyloplasts were seen in some cells with mildly damaged amyloplasts, and mildly damaged amyloplasts were seen in cells with completely lysed amyloplasts. This is consistent with the observations of Cottrell et al. (1995) who reported that gelatinization was not a uniform or instantaneous event, but happened gradually and was said to have occurred when 50% of starch grains lost birefringence under polarized light. In the experiment reported here, birefringence was not seen in any amyloplasts. The reason for the absence of birefringence in the specimens in this experiment is unknown, but may have been a result of the fixing or embedding techniques or a change in the crystalline nature of starch grains during dormancy.

The grainy appearance of the contents of damaged amyloplasts suggests that gelatinization had occurred and the starch had swollen and lost its crystalline structure (Aurand et al. 1987). The process of gelatinization, a non-equilibrium phenomenon (Biliaderis et al. 1986), appears to begin at the centre of the amyloplast, and when gelatinization has reached a critical point, rupturing of the membrane occurs and the contents spill into the cytoplasm. These results give some credence to the hypothesis that HWT causes an energy crisis in the tissue by disrupting amyloplasts. However as there were no differences observed in the levels of damage to amyloplasts in Pinot Noir and Cabernet Sauvignon these results do not explain the differences that are generally observed in the growth response to HWT protocols.

The picture was further complicated when the tissue was again sampled after 4 weeks cold storage at 4-5^{o}C. Once again cells with torn walls and disrupted amyloplasts were observed, but the trauma of the dissection and embedding processes cannot be ruled out as the cause of the broken cell walls and ruptured amyloplasts.

However, unlike the intact cells at stage 1, amyloplasts in intact cells at stage 2 in all treatments appeared normal and undamaged and although it is possible that these results reflect a sampling error, or artefacts of the embedding processes, other changes in the cytoplasm indicate an increased level of metabolic activity and it is thought that the damage caused to amyloplasts as a result of hydration or HWT may have been repaired during the 4 weeks in cold storage (figs 5.5 – 5.9).

Cytoplasmic activity. When the tissue was first sampled at stage 1, the appearance of the cytoplasm in all treatments was typical of that seen in inactive or dormant tissue by other researchers (Wilson pers. comm. 2005); much reduced and fragmented, with organelles including mitochondria and nuclei not readily apparent (fig. 5.4). However after 4 weeks cold storage (stage 2) there were significant changes to the cytoplasm in all treatments of both varieties. Except in the hydration only treatment of Cabernet Sauvignon (fig. 5.8) and some cells in the hydration plus HWT treatment in Pinot Noir (fig. 5.9), nuclei were distinct and vacuoles had formed (fig. 5.5), or were in the process of forming, apparently by amalgamation of a multitude of smaller vesicles (fig. 5.6), and the amyloplasts were surrounded by halo-like stroma of varying widths that were frequently abutted by membrane stacks resembling smooth endoplasmic reticulum (figs. 5.7 & 5.13). The presence of the visible stroma and the abutting membrane stacks indicate that the starch stored in the amyloplasts is being utilized (Lulai et al. 1986) and would suggest that in spite of being stored at low temperatures, the respiration rate is most likely increasing and the tissue is beginning to emerge from dormancy. It is interesting to note however, that mitochondria were scarce in all treatments of both varieties and the cristae were not distinct. It is also interesting to note that the vesicles in the HWT only Pinot Noir appeared to contain membrane fragments (fig. 5.6). These fragments might be the remains of damaged amyloplast membranes being reprocessed, excess membranes from the amalgamation of vesicles, or artefacts of embedding. By contrast, the amyloplasts were intact in the hydration only treatment of Cabernet Sauvignon and some cells of the hydration plus HWT treatment in Pinot Noir, but the cytoplasm was fragmented and vesicles and vacuoles not apparent. The cell contents of these treatments most resembled those of the hydration plus HWT Cabernet Sauvignon sampled at stage 1, indicating that the tissue may not be as metabolically active as the tissue in other treatments.

After 10 weeks cold storage (stage 3) the picture had changed again. As in stages 1 and 2, cells with torn walls and disrupted amyloplasts were observed in all treatments, but amyloplast were normal in intact cells. In Cabernet Sauvignon in all treatments the vacuoles and vesicles seen at stage 2 were much less distinct and appeared to be disintegrating and the tissue appeared to be returning to a less active state and resembled the tissue sampled at stage 1, except that amyloplasts were intact in all treatments. A similar, but less advanced process was also observed in Pinot Noir at stage 3 (Fig. 5.10) except in the tissue that had received the HWT only treatment. In this treatment (PN + HWT, Stage 3) the vesicles apparent at stage 2 appeared to have amalgamated to form fewer large vesicles or small vacuoles still with membranous inclusions. Amyloplasts were intact with visible stroma and abutting membrane stacks as seen in other treatments (CS: control, HWT, HWT + hydration; PN: control, HWT) at stage 2 indicating that this tissue was more active than tissue in other treatments at stage 3 (fig. 5.11).

It is interesting to observe that, contrary to industry perception, all material in this experiment, including the untreated controls, changed during cold storage, clearly demonstrating that cold storage does not induce a uniform period of artificial quiescence or "deepen dormancy" as is sometime proposed. Dormancy is a variable state and is affected by environmental conditions both before the onset of whole plant dormancy and during the dormant period (depending on the eco or endodormant state of the tissue) (Lavee & May 1997) and although cold storage is superimposed on dormant cuttings it is difficult to determine if the changes observed during cold storage seen in the tissues in this experiment are the result of a response to cold storage, or a result of preconditioning in the tissue. Further investigations including material stored in a sand bed or similar would be required to determine the effects of cold storage.

The effects of HWT on plasmodesmata were also examined in this experiment. Since cuttings needs to utilize the starch stored in the ray tissue to maintain life, closed plasmodesmata would severely restrict the ability of the cutting to utilize the starch reserves (Lalonde et al. 1999). However the plasmodesmata in the ray tissue of all treatments at all 3 stages of sampling appeared to be open and functional with visible connections to the cytoplasm of neighbouring cells (fig. 5.11). It is therefore unlikely that the cause of the retarded growth observed in HWT cuttings is the result of restricted communication between cells. It is interesting to note that Winkler and Williams (1945) thought that translocation of starch via the phloem during winter dormancy was improbable because sieve tube are normally blocked by callus during winter dormancy, but do not mention plasmodesmata as their

function was poorly understood at that time since electron microscopes that enabled the structure of plasmodesmata to be seen for the first time had only been invented in the 1930's (Wall et al. 1973).

The differences observed in cell ultrastructure activity between varieties and treatments in this study support previous research and anecdotal evidence from nurseries that the response of dormant grapevine cuttings to HWT varies between varieties and with differences in protocols. The results also add to evidence indicating that cuttings undergo changes during cold storage. However there is no unambiguous evidence to explain the delayed development observed in HWT cuttings, particularly Pinot Noir, when they are removed from cold storage for callusing. Although damaged amyloplasts were observed in intact cells in the ray tissue at stage 1, this may have been an artefact of the dissecting and embedding process and further investigations would be required to resolve this question. If the amyloplast damage observed at stage 1 was real and not an artefact, the lack of difference in levels of damage between Pinot Noir and Cabernet Sauvignon and the apparent repair or reconstitution of the amyloplasts during the first 4 weeks of cold storage negates the hypothesis that irreparable damage to amyloplasts is the cause of the retarded growth in HWT cuttings. Furthermore there is no evidence to show that intercellular communication in HWT tissue is disrupted by changes to the plasmodesmata that might inhibit the utilization of stored carbohydrates.

While there is no unequivocal evidence arising from this investigation to explain the cause of delayed cutting development in HWT material, delayed development is unlikely to be caused by permanent damage to the amyloplasts and plasmodesmata of ray tissue and factors such as the effects of HWT on meristematic tissue, hormone and enzyme profiles and protein synthesis must now be considered.

The changes to cell ultrastructure at stages 2 and 3 that show an apparent increase in metabolic activity followed by a return to a less active state clearly indicate that cuttings held in cold storage are not quiescent. On the basis of this evidence, it would be useful to investigate the effects of different storage periods on the quality and development of cuttings since it might be preferable to remove cuttings from storage before they re-enter the quiescent state observed at stage 3 (10 weeks cold storage), or modify storage conditions to ensure adequate oxygen supply and efficient dissipation of carbon dioxide during the most active phase at around 4 weeks cold storage (stage 2) when oxygen demand and carbon dioxide emissions are likely to be higher.

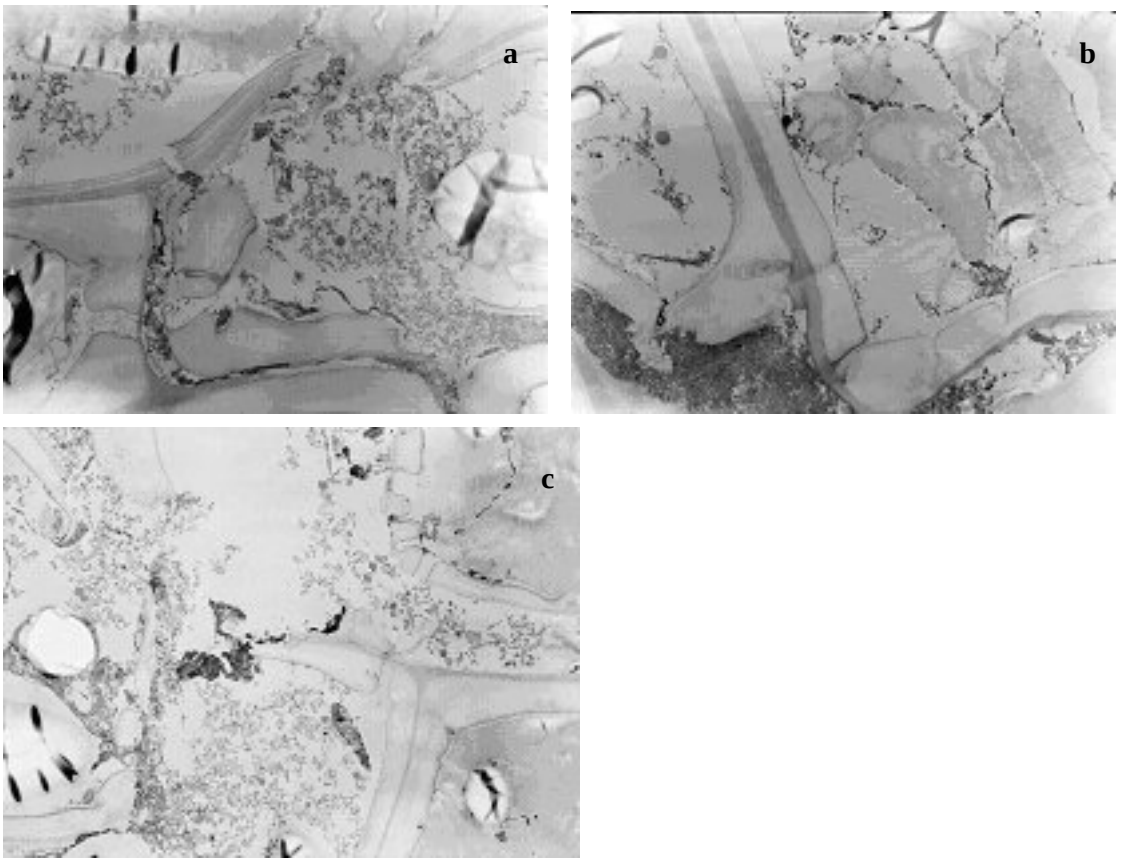


Figure 5.2 (a) Pinot Noir ray tissue (control, stage 1) showing fractured cell walls and whole and disrupted (top centre) amyloplasts x4,100; (b) Pinot Noir ray tissue (HWT + hydration, stage 1) showing fractured cell walls and fully and partially lyse (top right quarter) amyloplasts x4,100; (c) Cabernet Sauvignon ray tissue (HWT, stage 1) showing fractured cell walls and fully lysed (top right quarter) and intact amyloplasts (bottom left) x2,700.

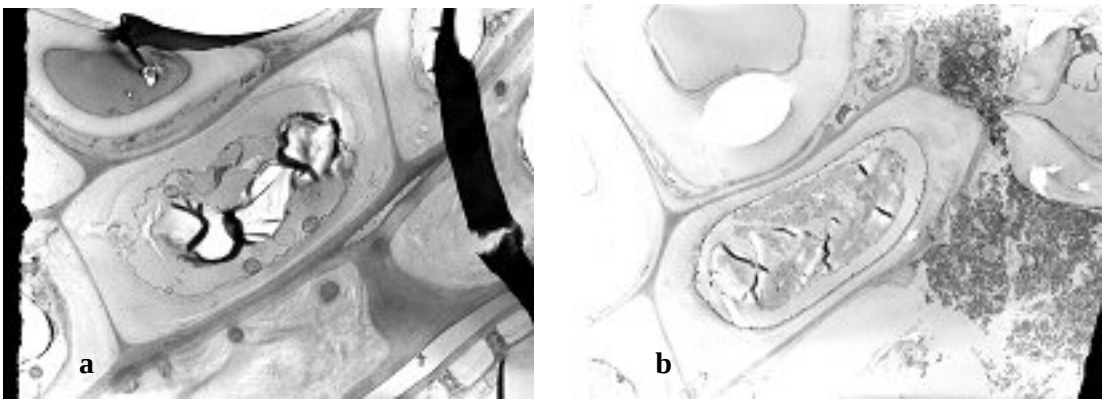


Figure 5.3 (a) Pinot Noir ray tissue (control, stage 1) showing normal amyloplasts in intact central cell x3,000; (b) Pinot Noir ray tissue (hydration stage 1) showing partially lysed amyloplasts in intact central cell x3,000.

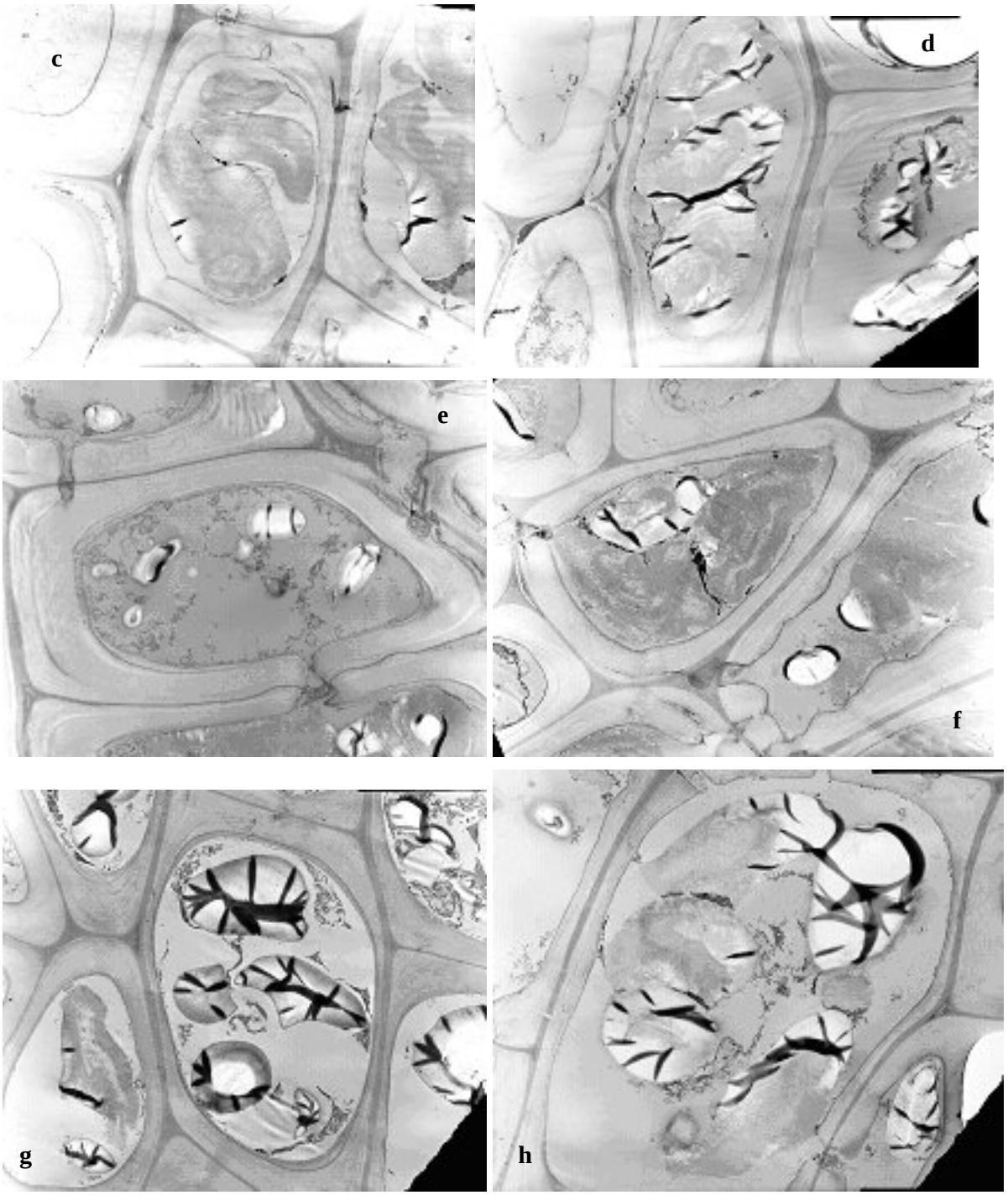


Figure 5.3 (c). Pinot Noir ray tissue (hydration + HWT stage 1) showing lysed amyloplasts in intact central cell x3,000; (d) Pinot Noir ray tissue (HWT stage 1) showing partially lysed amyloplasts in intact central cell x3,000; (e) Cabernet Sauvignon tissue (control, stage 1) showing normal amyloplasts in intact central cell x3,000; (f) Cabernet Sauvignon ray tissue (hydration stage 1) showing partially and fully lysed amyloplasts in intact central cell x3,000; (g) Cabernet Sauvignon ray tissue (hydration + HWT stage 1) showing intact but grainy amyloplasts in intact central cell and lyse amyloplast in cell at bottom left x3,000; (h). Cabernet Sauvignon ray tissue (HWT stage 1) showing partially lysed amyloplasts in intact central cell x3,000.



Figure 5.4. Cabernet Sauvignon ray tissue (control, stage 1) showing much reduced and fragmented cytoplasm typical of dormant tissue x2,700.

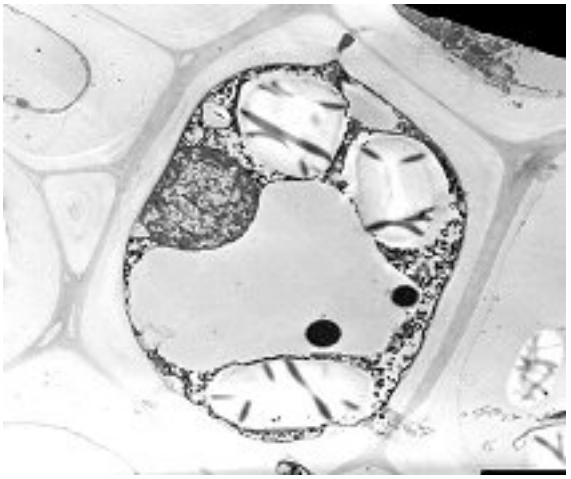


Figure 5.5. Pinot Noir ray tissue (control, stage 2) showing the nucleus, large fully formed vacuole, cohesive cytoplasm and halo-like stroma around amyloplasts x3,000.

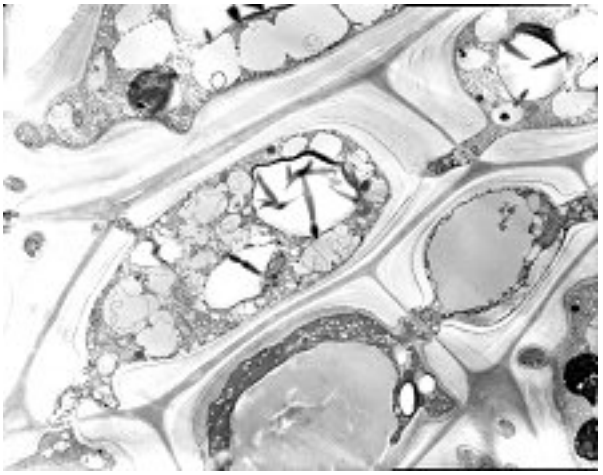


Figure 5.6 Pinot Noir ray cell (centre) (HWT, stage 2) showing possible development of the vacuole from amalgamation of enlarging vesicles containing what appear to be membrane fragments.

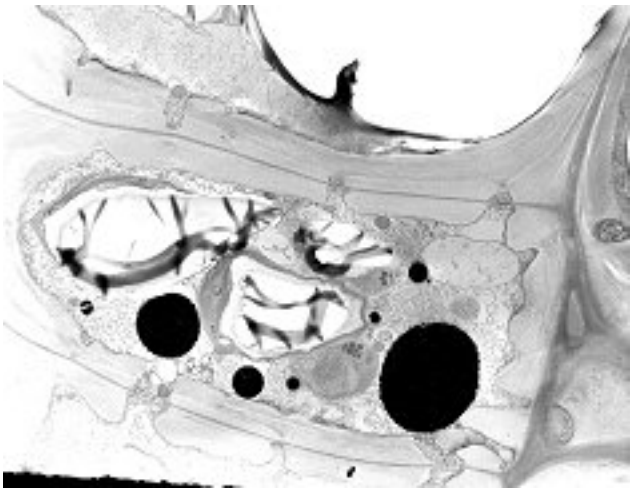


Figure 5.7 Pinot Noir ray cell (hydration, stage 2) showing stroma and membrane stacks abutting amyloplasts x3,000

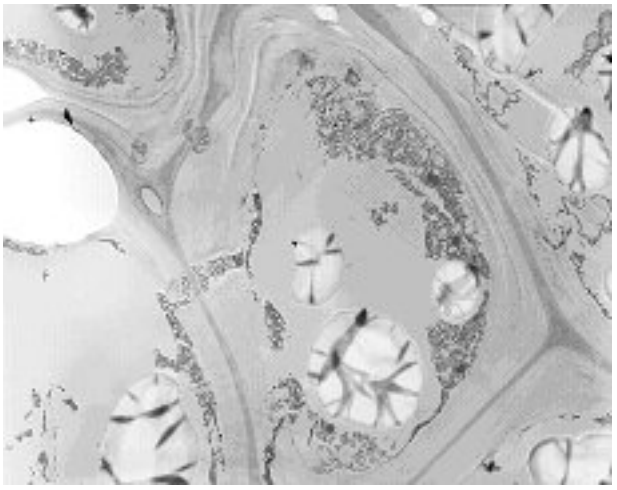


Figure 5.8 Cabernet Sauvignon ray cell (hydration, stage 2) showing intact amyloplasts and fragmented cytoplasm similar to that seen in the untreated control at stage 1 x3,000

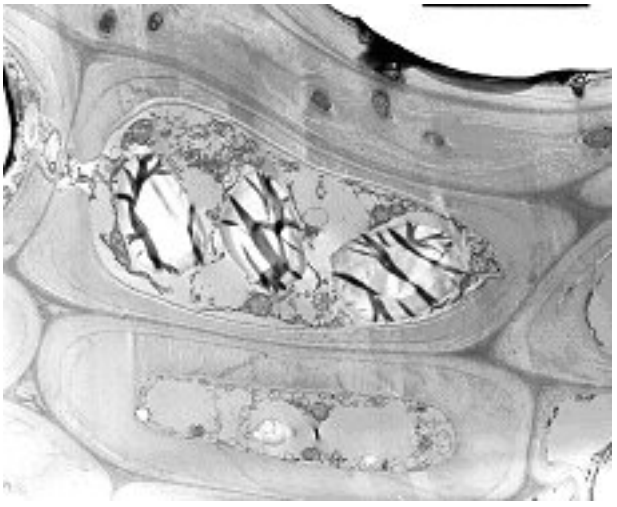


Figure 5.9 Pinot Noir ray cell (HWT + hydration, stage 2) showing showing similat characteristics to untreated controls at stage 1, but also some signs of vesicle formation x3,000

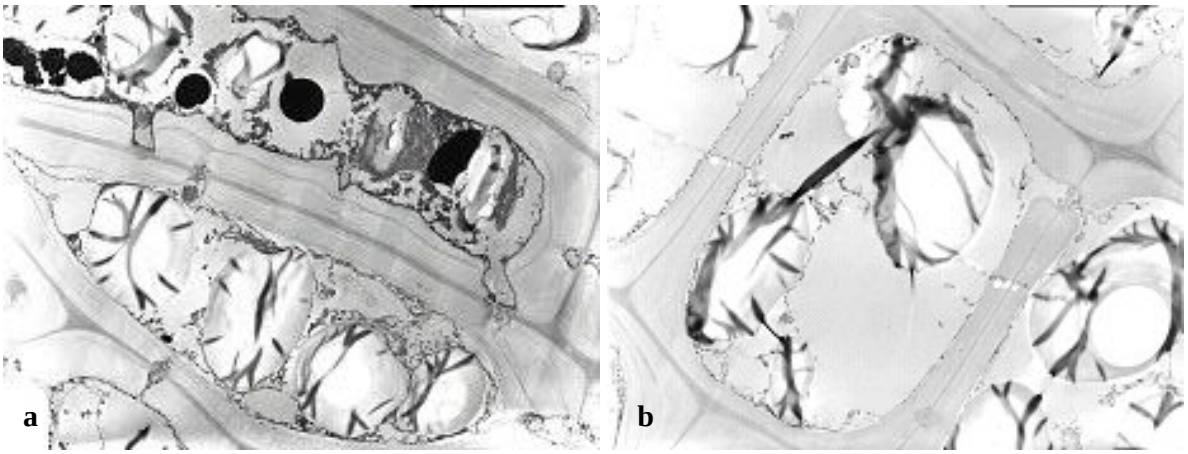


Figure 5.10 (a) Pinot Noir ray cells (control, stage 3) showing fragmented cytoplasm and absence of distinct vacuoles indicating a return to a less active state x3,000; (b) Cabernet Sauvignon ray cells showing fragmented cytoplasm and absence of distinct vacuoles indicating a return to a less active state x3,000.

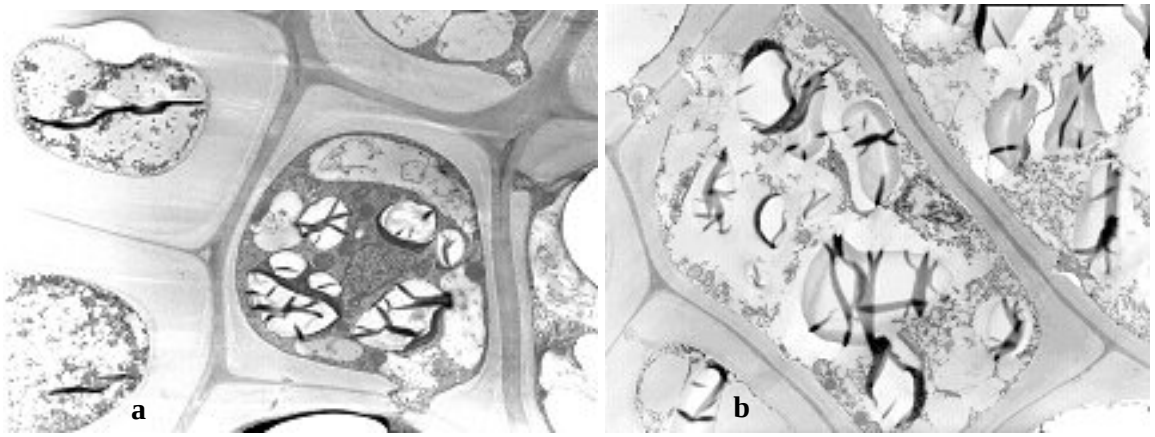


Figure 5.11 (a) Pinot Noir ray cell (centre) (HWT, stage 3) showing intact amyloplasts with narrow stroma, abutting membrane stacks, vacuoles/vesicles with membranous inclusions and distinct nucleus (centre of cell) x3,000; (b) Cabernet Sauvignon ray cell showing intact amyloplasts showing fragmented cytoplasm and marked reduction in distinct vacuoles indicating a return to a less active state compared to Pinot Noir (a) x3,000.

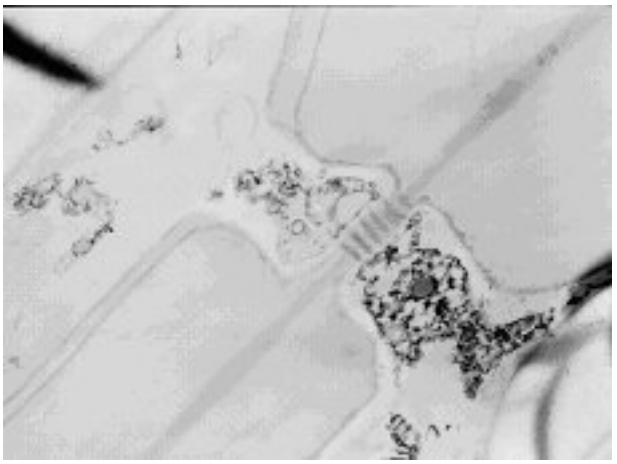


Figure 5.12. Cabernet Sauvignon (HWT+ hydration, stage 1) pit pair showing distinct plasmodesmata with cytoplasmic connections x14,000.

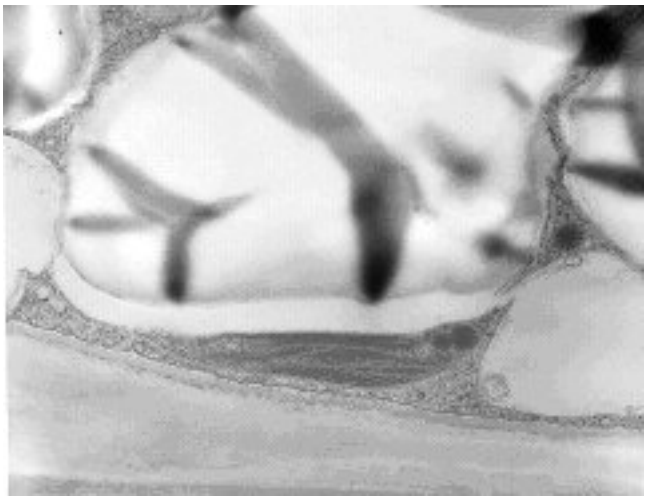


Figure 5.13. Cabernet Sauvignon ray cell (control, stage 2) showing membrane stack (smooth ER?) abutting the stroma surrounding an amyloplast x15,000.

5.3 Heat shock protein expression in dormant cuttings of Cabernet Sauvignon and Pinot Noir

Control of endogenous pathogens in grapevines is problematic. Traditional techniques such as chemical sprays and dips used for the control of surface pathogens do not penetrate dormant grapevine cuttings sufficiently to control organisms inhabiting the phloem and xylem tissue (Ophel *et al.* 1990; Caudwell *et al.* 1997). Long duration hot water treatment (HWT) at 50°C for 30min. is the only effective method for the control of endogenous diseases since the heat is able to completely penetrate the cuttings, killing the pathogens, but not the marginally less sensitive grapevine tissue. However there have been, and continue to be, spasmodic reports of unacceptably high losses when long duration HWT is applied to commercial batches of cuttings and rootlings, particularly Pinot Noir and to lesser extent, Chardonnay. Cabernet Sauvignon is the most resilient variety. It is known that a number of factors affect the response of cuttings to HWT, but it has not always been possible to explain the losses in terms of current knowledge.

Research (Waite 2002) indicates that the viability of propagating material in HWT is affected by a number of interacting factors including variety, time of cutting harvest and the practice of immersing cuttings in water prior to HWT (hydration). In addition it has been observed that Pinot Noir cuttings collected from warm climates are less susceptible to HWT than those from cool climates (Crocker *et al.* 2002). Previous work has also demonstrated that growth and development in HWT cuttings compared to untreated material is delayed in the first half of the growing season. It is very rare for cuttings to be killed outright by HWT, but hot water treated cuttings, particularly those of sensitive varieties, are slower to establish and appear stunted until mid season when they either recover and make up the delayed growth (Waite 1998) or die. At present the cause of this delayed development is unknown, but if the cause can be established it may be possible to prevent losses.

Following exposure to environmental or imposed heat shock, 5-10°C above normal growing temperatures, heat shock proteins (HSP's) are synthesized in plant tissues, including dormant grapevine buds, at the expense of normal protein synthesis (Brodl 1990; Vierling 1991; Brodl & Ho 1992; Morrell et al.1997; Ferguson et al.1998). HSP's are expressed as the temperature increases protecting the tissue from heat-induced damage and, following the return to normal temperatures, effect the repair of heat-induced damage (Brodl 1990; Brodl & Ho 1992; Vierling 1991). Morrell et al. (1997) reported that exposure of Cabernet Sauvignon cuttings to heat shock at 45°C for 30 min or 23°C for 4 hours elicited heat shock proteins (HSP70) in buds that appeared to confer a degree of thermotolerance when the cuttings were subsequently HWT at 54°C, 56°C, 58°C and 60°C. However it is not known if HSP's persist in the tissue for more than 24 hours following treatment, or if their presence is an indicator of a delayed return to normal metabolic activity.

Previous research (Crocker et al. 2004) showed that Pinot Noir cuttings collected from relatively warm climates were more thermotlerant than those collected from cool climates and it is thought that if HSP's are synthesized in the field during hot weather they may persist into dormancy and confer improved thermotolerance when material is HWT. If HSP's persist in dormant grapevine tissue for an extended period after HWT may indicate that normal cellular proteins are not being synthesized while repairs are being effected thus explaining the delayed growth observed in HWT cuttings.

However there may be other explanations for the delayed development and loss of viability in dormant propagating material and HWT may affect protein systems not associated with the expression of HSP's. Work by Boyang *et al.* (1997) and Brodl and Ho (1002) demonstrated that heat shock in barley aleurone layers and carrot root disks resulted in selective degradation of normally stable mRNAs involved in the synthesis of secretory proteins, independent of heat shock protein synthesis, significantly affecting the metabolism of cells and it is possible that there is a similar effect in the tissue of dormant grapevine cuttings.

In this experiment we investigated the expression and persistence of heat shock proteins in internode tissue of dormant Cabernet Sauvignon and Pinot Noir cuttings from warm and cool climates, 24 hours post HWT and after 4 and 10 weeks cold storage. Internode tissue was chosen in this experiment as it was also the tissue chosen for transmission electron microscopy in an earlier experiment to determine

the effects of HWT on cell ultrastructure, particularly amyloplasts that are the source of energy reserves in dormant vines. It was thought that if either the amyloplasts or the cells in which they were contained were damaged a consequent energy crisis might be the cause of the delayed development.

Methods

Plant Material: Dormant canes of Cabernet Sauvignon and Pinot Noir were collected from Victorian and Murray Valley Improvement Association (VAMVVIA) source blocks in the Sunraysia district of north western Victoria (warm climate, MJT 32.8°C, mean no. days >40°C Jan.= 3.8) and from a private vineyard at Kyneton in southern Victoria (cool climate, MJT 27.2°C, mean no. days >40°C Jan.=0) on 11 July 2005. The following day the canes were transported to the laboratory, cut into 2 bud lengths and randomly divided into 20 bundles of 5 cuttings according to variety and source area (4 groups of 20 bundles). Five of each group of 20 bundles was then randomly allocated to 1 of 4 treatments; 1) untreated controls, 2) hydration for 8 hours, 3) hydration for 8 hours followed by HWT and 4) HWT only. The HWT consisted of immersion in hot water at 50°C for 30 min. followed immediately by immersion in cold water at ambient winter temperature for 30min. The cuttings were then transported to the University of Melbourne Laboratories at the Faculty of Land and Food Resources campus at Creswick. On the day following treatment 1 bundle of cuttings was selected from each of the 16 treatment groups for immediate processing and the remainder were placed in plastic bags and stored in a refrigerator at 5°C. At 4 and 10 weeks cold storage 1 bundle of cuttings from each of the 16 treatments was removed and processed using the same method as the bundles that were not stored.

Protein Extraction: Cuttings were debarked and any buds and tendrils were removed and protein was extracted using a method modified from Morrell *et al.* (1997). The cuttings were split longitudinally, the pith was scraped out and the internodes cut into small fragments. Five grams of fragments from each treatment were mixed with 20mL of extraction buffer (120m*M* tris-HCl (pH 6.8), 50 m*M* EDTA-Na_2, 100m*M* KCl, 1 m*M* PMSF, 2% (v/v) 2-ME, 2% (v/v) SDS, 0.7 *M* sucrose, 0.1% (v/v) Triton X-100 and 10% (w/v) PVP) and 1 teaspoon acid washed sand in a Falconer tube and then vortexed. The vortexed tubes were then stored at -80°C to until required for processing. In initial testing of the protein extraction protocols freezing of the fragmented tissue improved lysis of the cells and the yield of protein compared to test specimens that were not frozen before grinding. When required for processing the frozen samples were partially thawed to slurry stage and ground by hand in a mortar for 5 minutes. By grinding the tissue as a semi frozen slurry it was found that grinding was more effective and further improved the yield of protein compared to specimens that were ground without freezing or fully thawed before grinding. Following the initial grinding of the tissue 30mL of water saturated phenol was added to each sample and grinding continued for a further 5 minutes. Twenty milliliters of extraction buffer without PVP was then added to the mortar and the samples vortexed and centrifuged at 4,000x *g* at 4°C for 10 minutes. The phenol phase was aspirated and re-extracted 3 times by vortexing with an equal volume of extraction buffer without PVP and centrifuging at 4,000x *g* at 4°C for 20 minutes. The phenol phase was then collected and mixed with five volumes of methanol containing 1% (v/v) 2-ME and 100 m*M* ammonium acetate and stored at -20°C overnight. The following morning the mixture was centrifuged at 4,000x *g* at 4°C for 20 minutes. The resulting pellet/precipitate was washed twice in the methanol precipitation solution, air dried in the fume hood and stored at -80°C until required for analysis.

Quantification of Proteins and Electrophoresis: Proteins were dissolved in water and quantified for electrophoresis using the Bradford assay (595nm wavelength and Bradford reagent) using bovine serum albumen (BSA) as the standard. However contaminants in the protein, most likely phenolics and soluble carbohydrates, affected the reagent preventing proper colour change and accurate quantification of protein. An alternative method of protein quantification was sought and proteins were then quantified by using 280nm wavelength and the fixed scale analysis. However difficulties were also encountered with variations in the solubility of the protein as a result of storing the pellets at –80°C following extraction, and with the continued interference from the previously described contaminants. A degree of solubility was regained in some samples when they were mixed with water

and stored for at 5°C for 6 weeks. However accurate quantification could not be achieved and the resulting gels (silver stained, 10% SDS-polyacrylamide) showed either too much or too little protein had been loaded. In addition, interference from contaminants resulted in indistinct protein bands making analysis difficult.

Re-extraction of Proteins: As a result of the difficulties experienced in 2005 it was decided to repeat a scaled down version of the experiment in 2006 using fresh cuttings of Cabernet Sauvignon and Pinot Noir from 1 source only, the Northern Melbourne Institute of TAFE (NMIT) vineyard at Eden Park near Whittlesea (MJT 26°C, mean no. days >40°C=0.4) and avoiding freezing during the extraction phase and post extraction. Dormant canes of Cabernet Sauvignon and Pinot Noir were collected from the Eden Park vineyard on 26th June 2006 and transported to the laboratory at NMIT, Epping Campus. On the following day (27/6/2006) the canes were cut into 2 bud segments and randomly divided into 20 bundles of 5 cuttings according to variety and source area (2 groups of 20 bundles). Five of each group of 20 bundles was then randomly allocated to 1 of 4 treatments; 1) untreated controls, 2) hydration for 8 hours, 3) hydration for 8 hours followed by HWT and 4) HWT only. The HWT consisted of immersion in hot water at 50°C for 30 min. followed immediately by immersion in cold water at ambient winter temperature for 30min. On the following day the cuttings were transported to the University of Melbourne Laboratories at the Faculty of Land and Food Resources campus at Creswick where 1 bundle of cuttings was randomly selected from each of the 8 treatment groups for immediate processing and the remainder were placed in plastic bags and stored in a refrigerator at 5°C.

Protein extraction followed the method described above except that the fragmented specimens were not frozen following the initial grinding, the protein pellets were not completely dried after extraction and were stored at 5°C not –80°C in order to avoid problems with solubility and were quantified for electrophoresis using the fixed scale spectrophotometry described above. Electrophoresis and silver staining followed the method described.

Results and Discussion

The changes to the extraction, storage and quantification of proteins described above resulted in improved resolution in electrophoresis. However further work needs to be done to improve accuracy of quantification of proteins and to determine the amount of protein needed to load onto the gels for best resolution. Further investigations into techniques for ridding protein samples of contaminants including phenolics and sugars is in progress at the time of writing. However preliminary examination of the gels from Cabernet Sauvignon cuttings collected in 2006 indicates that protein expression in the treated cuttings hydration, HWT + hydration and HWT, did not differ from protein expression in the untreated cuttings. A similar result was apparent in Pinot Noir, but the gels were not clearly enough resolved to be certain of this interpretation.

Clear single bands in all treatments of both varieties occurred at the estimate molecular masses of 140kDa, 120kDa, 100kDa, 90kDa, 85kDa, 80kDa, 60kDa, 55kDa, 50kDa. There were also 3 distinct bands clustered around the 30kDa band. Bands were poorly resolved below 30kDa either as a result of contaminants or failure of proteins to separate at the end of the gels. The 50kDa band was the most prominent in all treatments followed by the cluster around 30kDa.

The uniformity of protein expression in all treatments of both varieties when examined 24 hours after HWT appear to indicate that if HSP's are expressed during HWT they do not persist in the tissue beyond 24 hours post treatment and consequently the delayed development in HWT cuttings cannot be attributed to the on going presence of HSP's in the tissue. However because of the difficulties experienced with protein solubility and gel resolution, the reliability of these results must be questioned and it would be unwise to draw any conclusions until the difficulties with contaminants and quantification of proteins are resolved.

Conclusion and Recommendations

As a result of this project it is now known that the tissue of cuttings in cold storage changes over the storage period and that there appears to be a period of increased metabolic activity in the first few weeks followed by a return to relative quiescence over the next few weeks except in HWT Pinot Noir which remains active. This may partly explain the poorer performance of Pinot Noir after HWT since a higher metabolic rate may result in a more rapid depletion of carbohydrate reserves if cuttings are stored for extended periods. A higher metabolic rate in Pinot Noir may also result in a return to fermentative respiration if storage bags are not ventilated. The less active state of Cabernet Sauvignon is likely to reduce carbohydrate depletion and delay the onset of fermentation in the low oxygen environment of the storage bags thus reducing the susceptibility of Cabernet Sauvignon to starvation or the toxic effects of ethanol and acetaldehyde.

The discovery that HWT cuttings become fermentative following HWT adds further weight to the proposition that post HWT handling practices be reviewed. Enclosing cuttings in plastic bags within 24 hours of treatment prevents the dissipation of ethanol and acetaldehyde and may prolong fermentation if oxygen levels in storage bags are low. Although it has not been tested, differential sensitivity to the toxic effects of ethanol and acetaldehyde might explain the difference in sensitivity between cabernet Sauvignon and Pinot Noir. The completion of HSP analysis may also show differences in protein expression between the 2 varieties and give further insight into the reasons for the difference in post HWT development.

The results of this project add to the evidence that adequate oxygen supplies are particularly important in cutting propagation. Nurseries are likely to improve the outcomes of HWT by ensuring that HWT material is allowed at least 24 hours to recover from HWT and for ethanol to dissipate before it is packaged for storage. It is also important to ensure that storage bags are well ventilated with several small, evenly spaced holes to prevent oxygen deprivation during storage. It would also be prudent to keep storage time to a minimum to prevent a return to fermentative respiration in the inherently low oxygen atmospheres of storage bags and starvation from carbohydrate consumption.

The results of this research have improved the understanding of the effects of HWT and associated protocols on dormant propagating material and have been the impetus for changes to propagation protocols that have been widely promulgated through industry publications, personal contact and the ‘Making Every Stick Count’ workshop series.

The Australian wine industry faces significant competition in a global market that demands high quality products that represent exceptional value for money. Without the foundation of healthy, high quality vineyards that perform to expectations it will be difficult for Australia to maintain its place in the increasingly competitive global marketplace. It is therefore imperative that the quality of planting material be second to none. Currently the quality of planting material available to growers is of variable quality. While some nurseries produce vines of outstanding quality, the majority produce material of a lesser standard. If the recommendations outlined above are adopted there will be a significant improvement in the quality of planting material available to the grape and wine industries. Nurseries will also be able to reduce wastage and improve profitability in the current climate of low demand when the viability of many nurseries is threatened by the downturn in new plantings.

Chapter 6: The impact of *Phaeomoniella chlamydospora* infection on grapevine response to water stress

Jacqueline Edwards, Eve Cottral, Gunta Jaudzems, Ian Pascoe, Soheir Salib, Fiona Thompson

Summary. *Phaeomoniella chlamydospora* is a vascular pathogen that colonises the xylem tissues of the grapevine. Examination of the host response to infection in tissue-cultured Chardonnay vines using light, fluorescent and transmission electron microscopy clearly showed hyphae travelling both intercellularly and intracellularly in parenchyma ray cells adjacent to tracheids and in tracheids themselves. Hyphae passed between cells predominantly via pits in secondary wall thickenings where they are able to cross the pit membrane and were also located within secondary wall thickenings. Both infected and uninfected plants appeared to have a large amount of cell disruption and wall breakage in this study, and hyphae were observed in close association with wall breaks. If this wall discontinuity is natural, grapevine stems are particularly easy targets for fungal colonisation.

In field grown Verdelho, infection clearly interfered with xylem function, such that xylem function was reduced by 15% for each 1% increase in black goo. In glasshouse experiments using Zinfandel, Cabernet Sauvignon and Chardonnay, infection impacted on stomatal control and leaf water potentials. Stomatal conductance was higher for infected plants of all cultivars, suggesting that infected grapevines have higher respiration rates than uninfected grapevines. In Zinfandel and Cabernet Sauvignon, leaf water potentials were lower in infected plants subjected to water stress, indicating that infection made it more difficult for the vine to get water to the leaf. This is supported by the relationship shown above for loss of xylem function. Chardonnay responded differently, however, and infection did not appear to increase the water stress experienced by the vines. Chardonnay is a cultivar reputedly very sensitive to water stress, so perhaps the main effect of water stress was too large to detect an additional effect of infection. Alternatively, perhaps Chardonnnay is more tolerant of infection. We demonstrated clearly that infection alters the grapevine response to water stress and that some cultivars are more affected than others.

6.1 Impact of *Pa. chlamydospora* infection on grapevine tissue investigated using light and electron microscopy.

Although *Pa chlamydospora* is consistently isolated from vines infected with Petri disease, little is known about the infection path of the fungus. In this study, tissue-cultured plantlets of Chardonnay were artificially inoculated with *Pa. chlamydospora*. Infected tissue pieces were embedded in resin for examination by light and electron microscopy.

Methods

Inoculum

A single spore isolate of *Pa chlamydospora* (VPRI 22079) was grown on PDA under 30W cool white fluorescent lighting (12 hour photoperiod/day) at 22ºC for 4 weeks. The plate was flooded with 10 mL of sterile distilled water, agitated and a conidial suspension of 10^6 conidia/ mL was prepared.

Plant material and inoculation procedure

Ten three month old tissue-cultured grapevine plants (cultivar Chardonnay), maintained on Murashige and Skoog medium, were used for the study. The plantlets were grown at 22ºC, under Growlux 36W fluorescent lighting (emitting 2000 lux). Eight plantlets were inoculated with 10 µL conidial suspension into a wound created by removing one of the branches. Two plants were inoculated with 10 µL of sterile distilled water as controls.

Sample preparation for light microscopy

Three weeks after inoculation, 2-3 mm was taken from the inoculated region of the stems. Sample pieces were fixed in 5%glutaraldehyde + 1% caffeine in 0.03M Pipes buffer pH 6.8-7.0 for at least 2 hours at room temperature with 15 mins vacuum treatment (30 inches mercury), dehydrated in a graded ethanol series starting at 10% and increasing in 10% steps, infiltrated with hard grade LR White resin over a period of 1 week and polymerised for 8-24 hours under UV light in an oxygen-free atmosphere. Sections were cut at 2um using a Reichert Ultracut E ultramicrotome, stained with toluidine blue pH 4.5 and viewed with an Olympus Provis light microscope.

Sample preparation for electron microscopy

Three weeks after inoculation, 2-3 mm was taken from the inoculated region of the stems. Sample pieces were fixed in 5% glutaraldehyde + 1% caffeine in 0.03M Pipes buffer pH 6.8-7.0 for at least 2 hours at room temperature with 15 mins of vacuum treatment (30 inches mercury), followed by secondary fixation in 1% osmium tetroxide in buffer for 2 hours and dehydration in a graded ethanol series starting from 10% and increasing in 10% steps. For scanning electron microscopy, the pieces were then critical point dried with a Balzers CDP 030, sputter coated with gold with a Balzers SCD 005 and viewed with an Hitachi S570 SEM at 10-15kV. For transmission electron microscopy, sample pieces were fixed and dehydrated as for SEM, infiltrated with hard grade Spurr's resin (Spurr 1969) over a period of 2 weeks, polymerised overnight at 60 deg C, and sectioned at 90nm using a Reichert Ultracut E ultramicrotome. Sections were stained with saturated methanolic (50%) uranyl acetate for 10 mins followed by saturated aqueous lead citrate for 10 mins and viewed with a Jeol 200CX TEM at 100kV.

Results and Discussion

Light microscopy: uninfected (control) stem

The cortex of grapevine stems consists of parenchyma ray cells and conducting vessels (tracheids). The ray cells are typically thinner walled and contain abundant starch grains while the tracheids have conspicuous thick walls and are generally empty. Darker staining phenolic substances (lipids and tannins) can be detected both within cells and between cells. The thicker walled tracheids have an abundance of pit pairs between cells (Plate 1).

Light microscopy: infected stem

Ray cells of infected stems appear to contain fewer and smaller starch grains than control stems (Plate 2). Phenolics (tannin droplets) are more abundant within ray cells, and in some cases completely occlude the cells (Plate 3). The true nature of these phenolics is difficult to know, but it is most likely a mix of the black goo produced by *Pa. chlamydospora* and the cells own phenolics. Plant phenolics can be synthesized during the normal development of plant tissues, or they can be synthesized in response to physical injury, infection or other stress (Beckman 2000). Using various methods of light microscopy and specific staining, hyphae were found in tracheids, in ray cells adjacent to tracheids, and apparently between secondary wall thickenings (Plates 4 and 5).

Electron microscopy: uninfected (control) stem

Stems were found to have abundant intercellular spaces and a high amount of broken cell wall. These breaks may have been caused mechanically by needle insertion during inoculation (controls had needle insertion but without inoculation) or during sample trimming with a razor blade. There is an accumulation of electron dense matter, most likely a mix of lysed cytoplasm and cellular tannins, around many of the breaks (Plate 6). Intercellular spaces were also often filled with electron dense

matter (Plates 7, 8). Again, this is most likely lysed cytoplasmic material that had leaked out from cells via breaks in cell walls and via cell wall pits.

Many of the secondary wall pits of tracheids are specialized for transfer of solutes, and have a highly invaginated torus (thickened portion of the pit membrane) (Plate 9). When cut in the opposite plane invaginations in the torus may appear as loose wall material containing spheres of cytoplasm. These spheres are actually fingerlike projections of the cell cytoplasm networking between the torus invaginations. Similarly, pits in the secondary wall appear as cytoplasm-filled spheres. Plasmodesmata can often be seen traversing the pits (Plates 10-12).

Electron microscopy: infected stem

Hyphae were found predominantly in ray cells near tracheids, and were often observed in an apparently secretory phase with large amounts of an amorphous, electron dense substance surrounding the hyphae. This substance is presumably the black goo (Plates 13-17). Many of the tracheids adjacent or near these cells were full of the same substance. However the substance varied in electron density, sometimes appearing totally black (Plates 18-19). Hyphae were also often observed in a non-secretory phase, intracellularly in both tracheids and ray cells. In this case they were surrounded by an electron opaque (clear) layer, possibly a mucilaginous coat (Plates 20, 21). A loose, amorphous substance (possibly black goo) was sometimes seen around the hyphae, but the hyphae did not appear to be actively secreting it (Plates 21-23). Hyphae were also often observed intercellulary, apparently "pushing" their way between cells and causing the cells to become misshapen. Again the hyphae appear to have a mucilaginous coat (Plates 24, 25).

Significantly, hyphae were occasionally observed within secondary walls of tracheids, thus it appears that hyphae are actually able to break down the secondary wall. Various signs of wall breakdown were observed (Plates 26 - 28). In one case a hyphal strand was observed within a pit where the primary wall was broken with a clear pathway between the intercellular space and the tracheid lumen (Plate 29). Scanning electron microscopy also provided some evidence that hyphae are able to pass through pits (Plate 30).

In addition to the hyphal phases described above, vesiculated inclusions were often observed within ray cells possibly representing a further hyphal phase (Plate 31). Occasionally thick-walled spore-like structures were found, indicating that *Pa. chlamydospora* may also move through grapevine stems as spores (Plate 32).

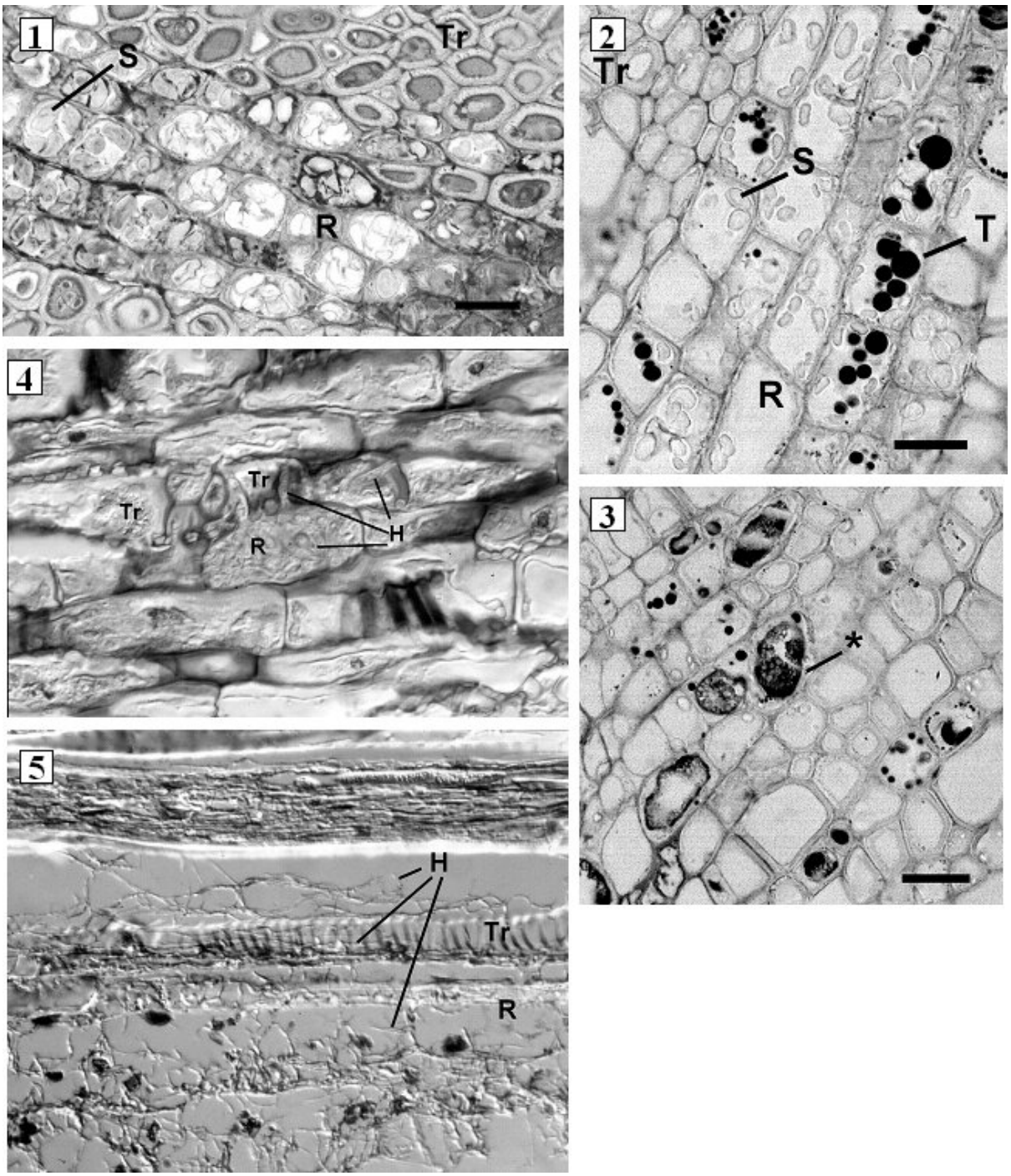


Plate 1. Transverse section through uninfected grapevine stem showing ray cells, tracheids and starch (x100) Tr – tracheids, R- ray cells, S – starch *bar=20um.*
Plate 2. Transverse section through infected grapevine stem showing ray cells with fewer starch grains and more phenolics (x100) Tr – tracheids, R – ray cells, S – starch, T – tannins *bar=20um.*
Plate 3. Transverse section through infected grapevine stem showing ray cells completely occluded by some sort of phenolics (*), probably a mix of the cells' own tannins and the black goo produced by *Phaeomoniella* (x100) *bar=20um.*
Plate 4. Infected stem showing hyphae apparently passing between secondary wall thickenings of a tracheid (X100), Tr – tracheid, R – ray cell, H – hyphae.
Plate 5. Abundant hyphae in tracheids and adjacent cells (x100), H – hyphae, Tr – tracheid, R – ray cell.

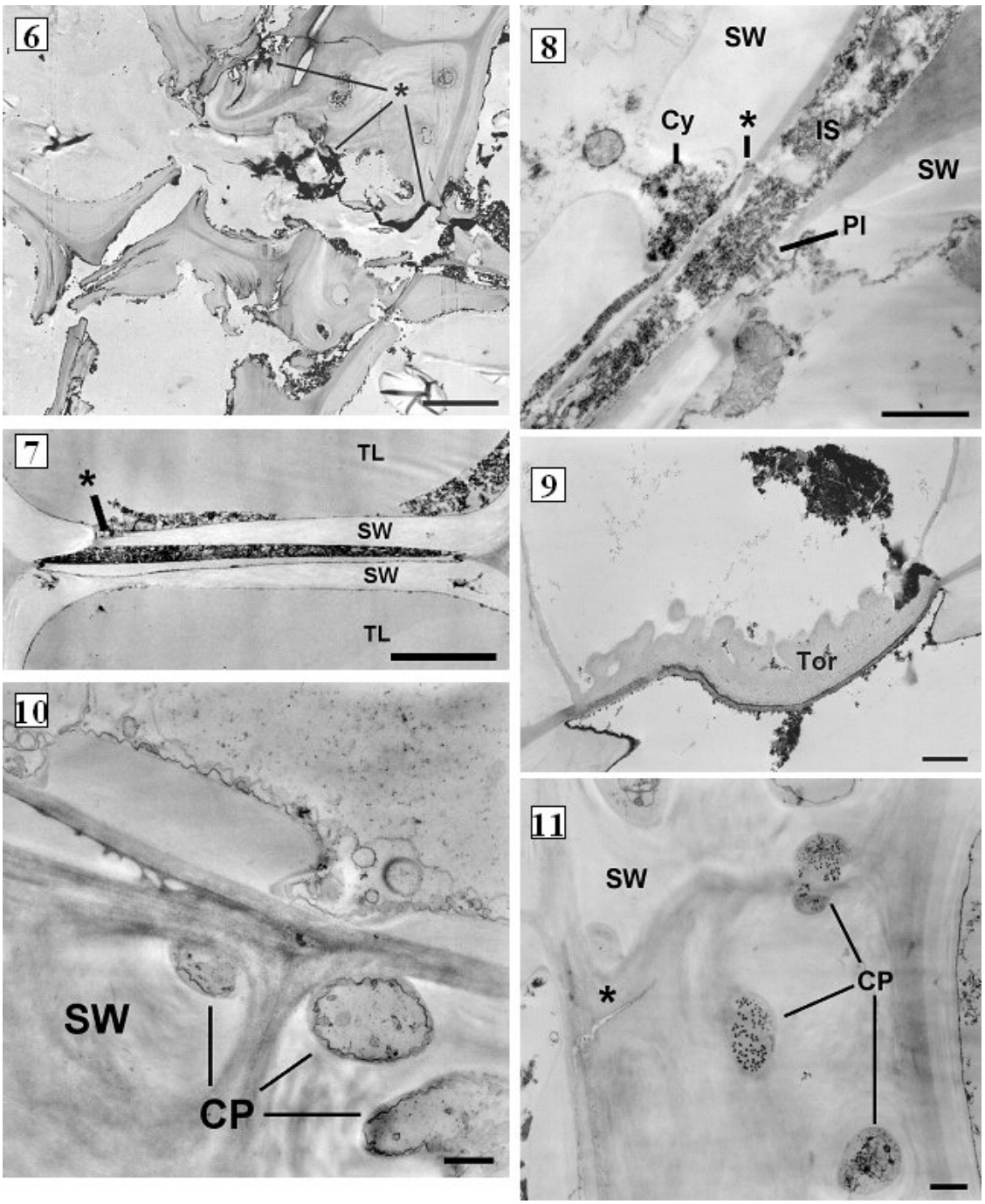


Plate 6 Broken tracheid walls in control stem, with accumulation of electron dense substance around breaks(*) *bar=5um.*

Plate 7 Cytoplasmic material apparently moving from a tracheid into a space formed by a split through the secondary cell wall (*) TL – tracheid lumen, SW – secondary wall *bar=5um*

Plate 8 Leaking of cytoplasmic material into intercellular space via cell wall pits. A break can be seen in the primary wall of the left hand cell (*), and plasmodesmata can be seen in the pit of the right hand cell. Cy – cytoplasm, SW – secondary wall, Pl – plasmodesmata, IS – intercellular space *bar=1um* .

Plate 9 Section through cell wall pit showing highly invaginated torus. Tor – torus *bar=1um.*

Plate 10 Cytoplasmic projections (secondary wall pits) cut transversely. CP – cytoplasmic projections, SW – secondary wall *bar=1um*

Plate 11 Section through secondary wall. Note microscopic crack through wall (*). SW – secondary wall, CP – cytoplasmic projections, *bar=1um.*

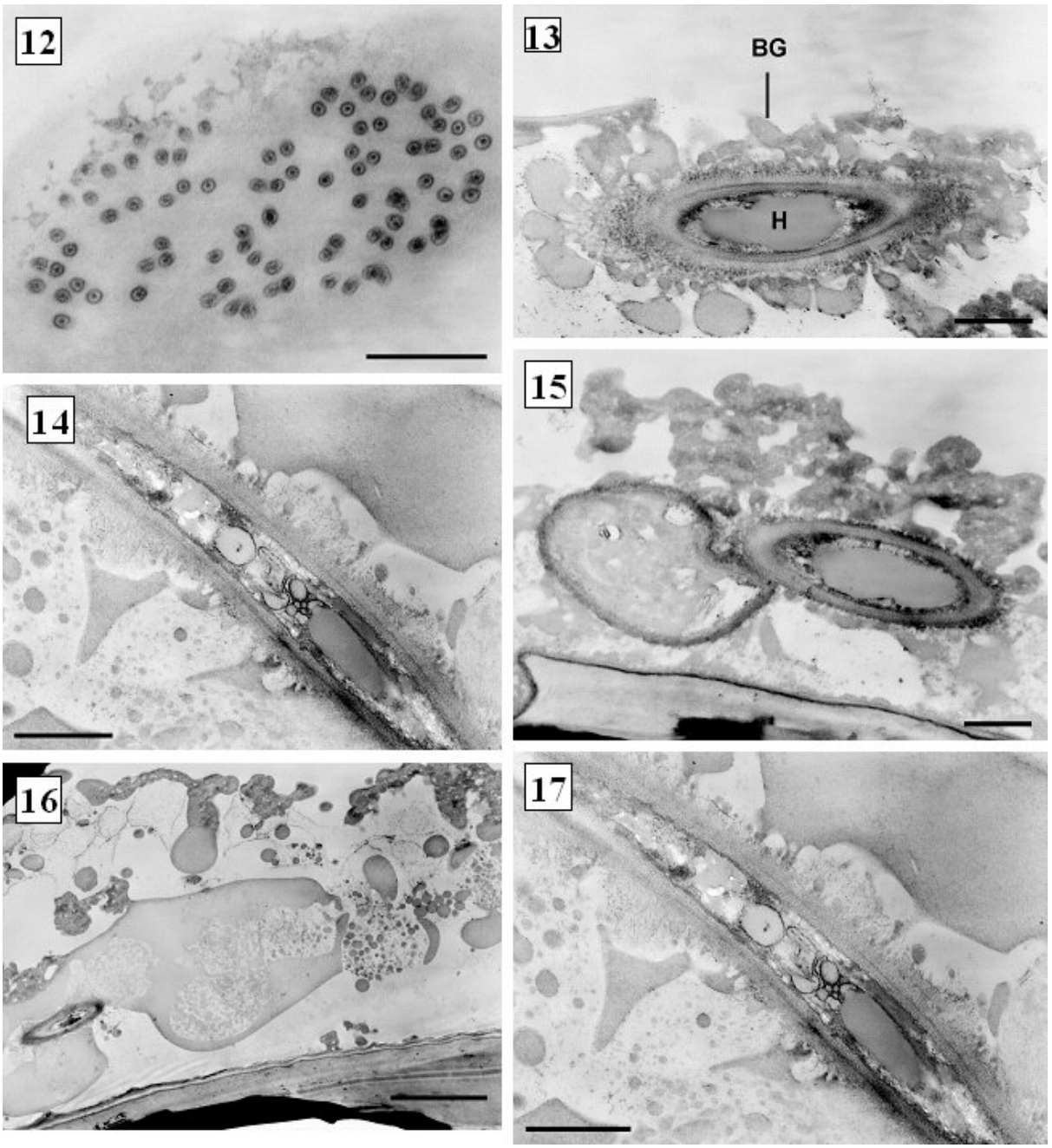


Plate 12 Plasmodesmata through pit membrane/torus *bar=0.5um*
Plate 13 Cross section through hypha found in ray cell, apparently in a secretory phase. BG – apparent Black Goo, H – hypha *bar=1um*
Plate 14 Hypha in a tracheid cell, apparently in a secretory phase. H - hypha *bar=1um*
Plate 15 Hypha in an apparently secretory phase *bar=1um*
Plate 16 Large amounts of Black Goo in a tracheid cell *bar=5um*
Plate 17 Hypha, apparently secreting *(actually same pic as 14) bar=1um*

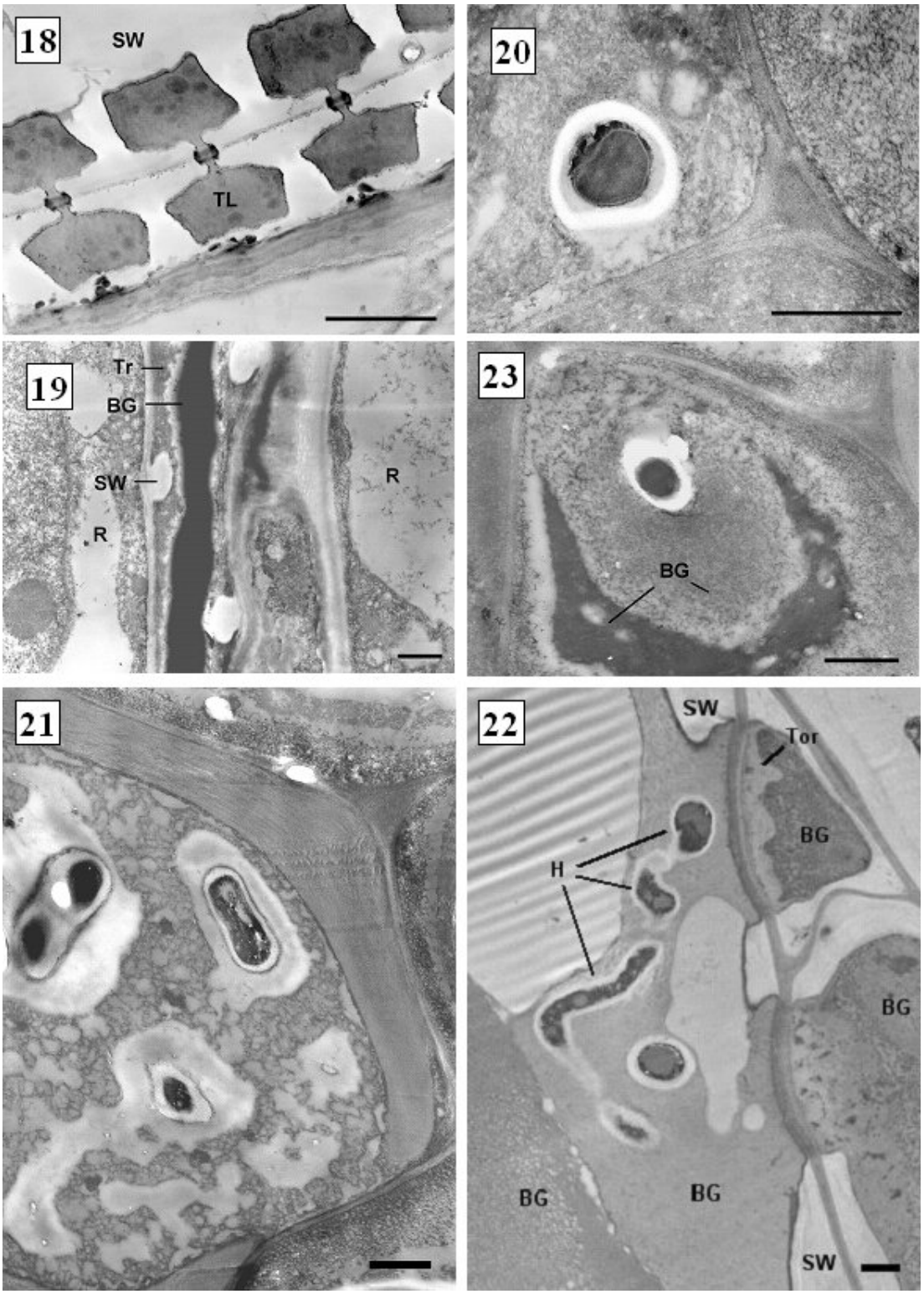


Plate 18 Tracheid lumens completely occluded with amorphous, electron dense substance. SW – secondary wall, TL – tracheid lumen *bar=5um*

Plate 19 Highly electron dense substance in tracheid. Tr – tracheid, BG – black goo, SW – secondary wall, R – ray cell *bar=1um.*

Plate 20 Intracellular hypha in ray cell *bar=1um*

Plate 21 Intracellular hyphae in tracheid, possibly surrounded by black goo *bar=1um*

Plate 22 Intracellular hyphae in tracheid, surrounded by black goo but apparently not actively secreting it. SW – secondary wall, Tor – torus, BG – black goo, H – hyphae *bar=1um*

Plate 23 Intracellular hypha in ray cell. BG – black goo *bar=1um*

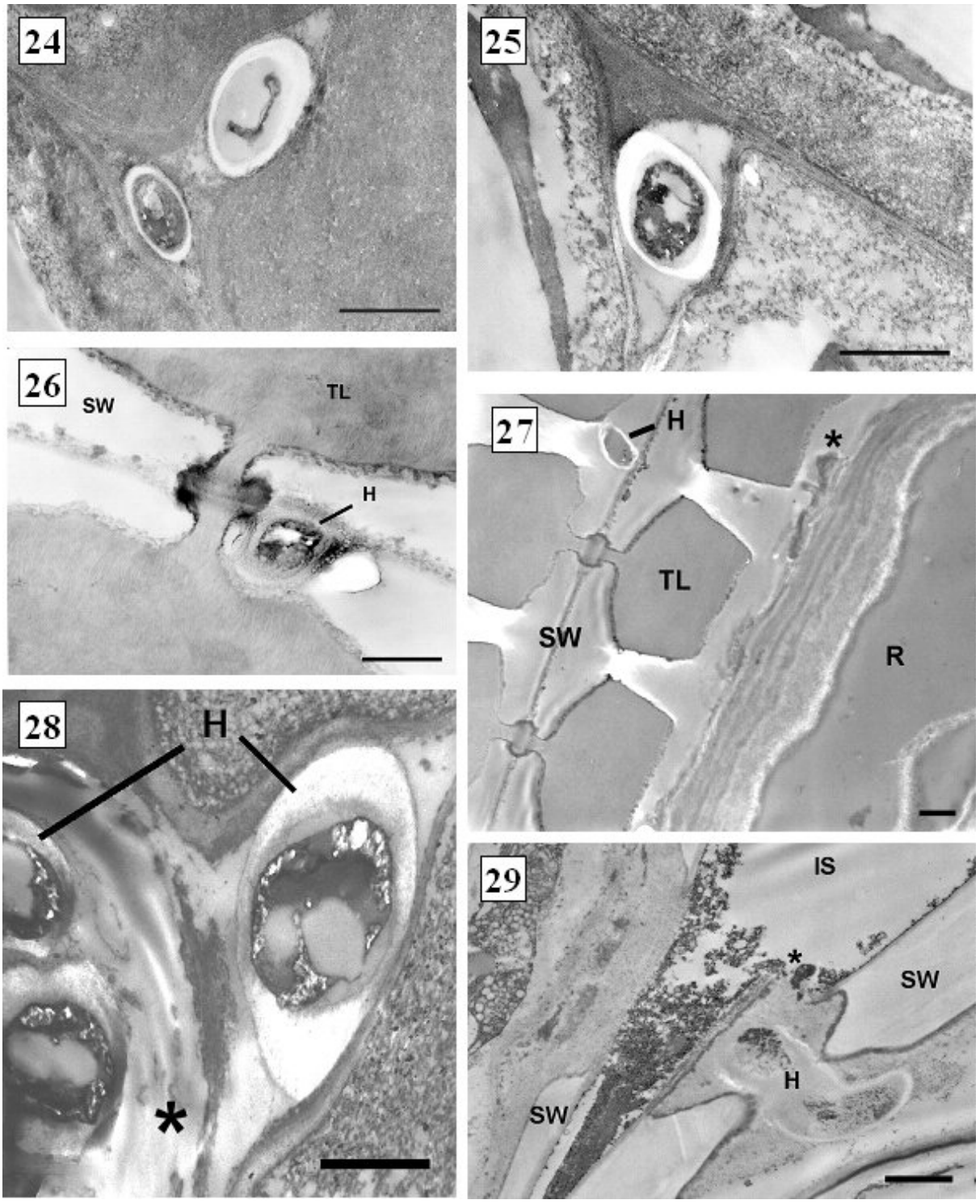


Plate 24 Intercellular hyphae, between ray cells *bar=1um*
Plate 25 Intercellular hypha, between ray cells *bar=1um*
Plate 26 Hyphae-like structure adjacent to pit between two tracheary elements. SW – secondary wall, TL – tracheid lumen, H – hypha *bar=1um*
Plate 27 Hypha in tracheid secondary cell wall. There are also signs of other alterations to the cell wall (*). H – hypha, TL – tracheid lumen, SW – secondary wall, R – ray cell *bar=1um*
Plate 28 Possible signs of wall breakdown (*). H – hypha *bar=1um*
Plate 29 Hyphal strand in secondary wall pit, with broken primary wall leading to an intercellular space (*). SW – secondary wall, IS – intercellular space, H – hypha *bar=1um*

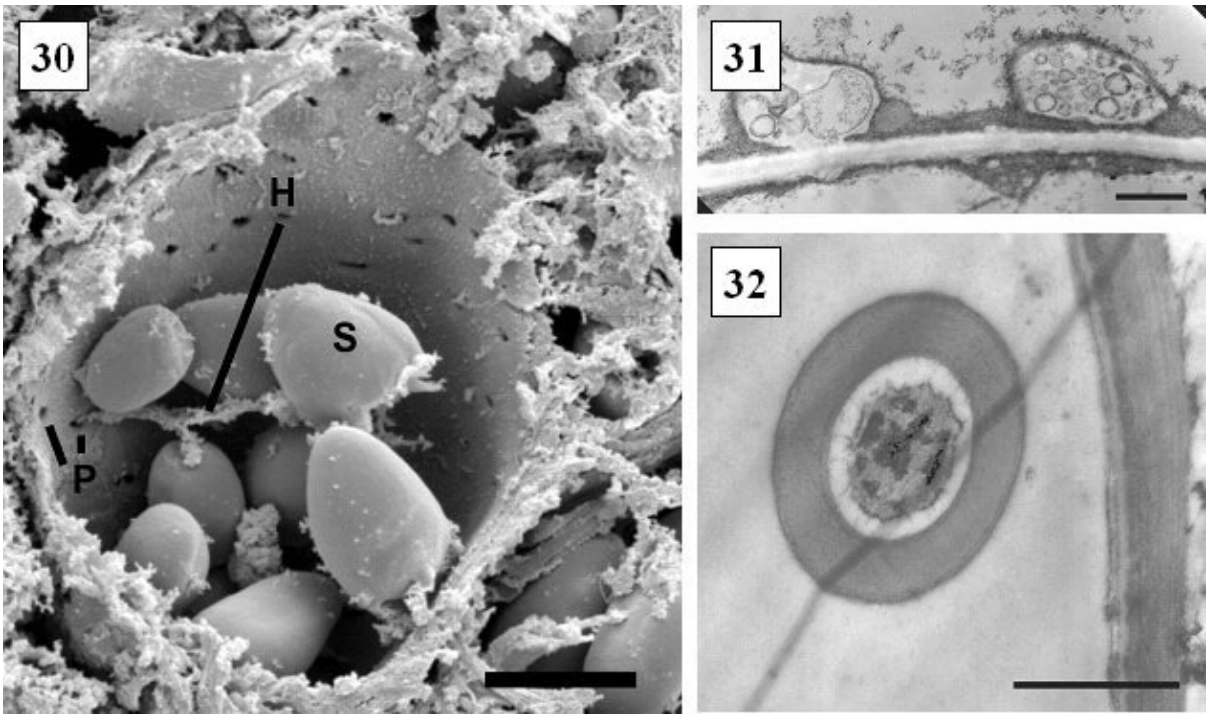


Figure 30 Hyphal filament apparently passing through pit. H- hypha, P – pits, S – starch grain *bar=10um*
Figure 31 Vesiculated inclusions in parenchyma ray cells *bar=1um*
Figure 32 Spore like structure in xylem parenchyma cell *bar=1um*

6.2 Impact of *Phaeomoniella chlamydospora* infection on grapevine xylem function

Phaeomoniella chlamydospora is a xylem-inhabiting fungus and the classic diagnostic symptom for Petri disease is 'black goo', a tarry substance found in the xylem vessels of infected grapevines. It was commonly assumed that disease symptoms such as shoot dieback were due to blockage of xylem vessels by black goo. However, we questioned this because grapevines have a very efficient vascular system and should be able to cope with the blockage of a few vessels. The aim of this trial was to determine whether loss of xylem function was occurring, and if so, could it be solely attributed to blockage of vessels with black goo.

Method

The trial site was a vineyard, cv Verdelho, in the Yarra Valley, Victoria, planted in 1998, which had been diagnosed with Petri disease when the vines were 3 years old. In April 2002, a few days prior to harvest, 10 vines were cut at the base of the trunk, placed in buckets of dilute methylene blue solution (5g in 25L water), and left in the field for 24 hours. On collection, the trunks were sectioned into 10 cm pieces, and % functional (ie blue), non-functional (ie white) and 'goo-blocked' vessels were calculated per grapevine using image analysis software. The experiment was repeated again in April 2004 with five grapevines from the same block.

Results and Discussion

Within a few hours, the blue dye had been translocated right throughout the grapevines and was easily visible at the shoot tips and tendrils. The grapevines were in full canopy and ready for harvest, and the weather was warm (>35°C in 2002 and 27°C in 2004). When the grapevine trunks were recovered after 24 hours, it was immediately apparent that the more goo in the trunk, the less blue dye had been translocated (Fig 6.1).

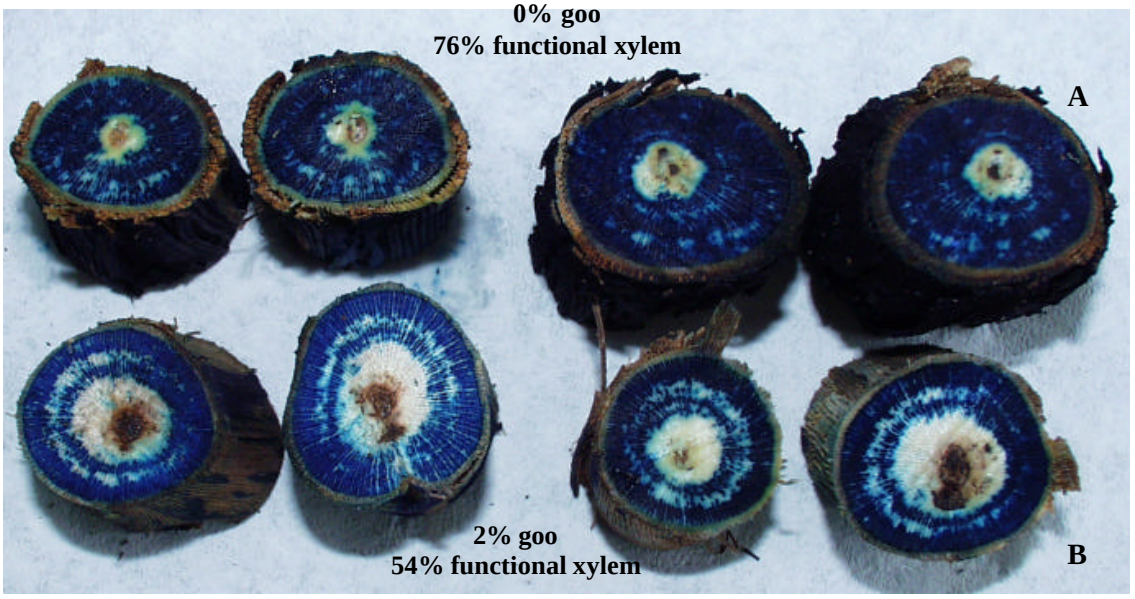


Figure 6.1. Comparison of the functional xylem (blue coloured vessels) of two five-year-old Verdleho grapevines. A: trunk of a grapevine with no black goo – 76% functional xylem; B. trunk of a grapevine with 2% vessels blocked with black goo – 54% functional xylem.

A strong negative correlation was evident between the percentage of functional vessels and those blocked with black goo per grapevine. A similar relationship was seen for data from both 2002 and 2004, so the two data sets were combined (Figure 6.2).

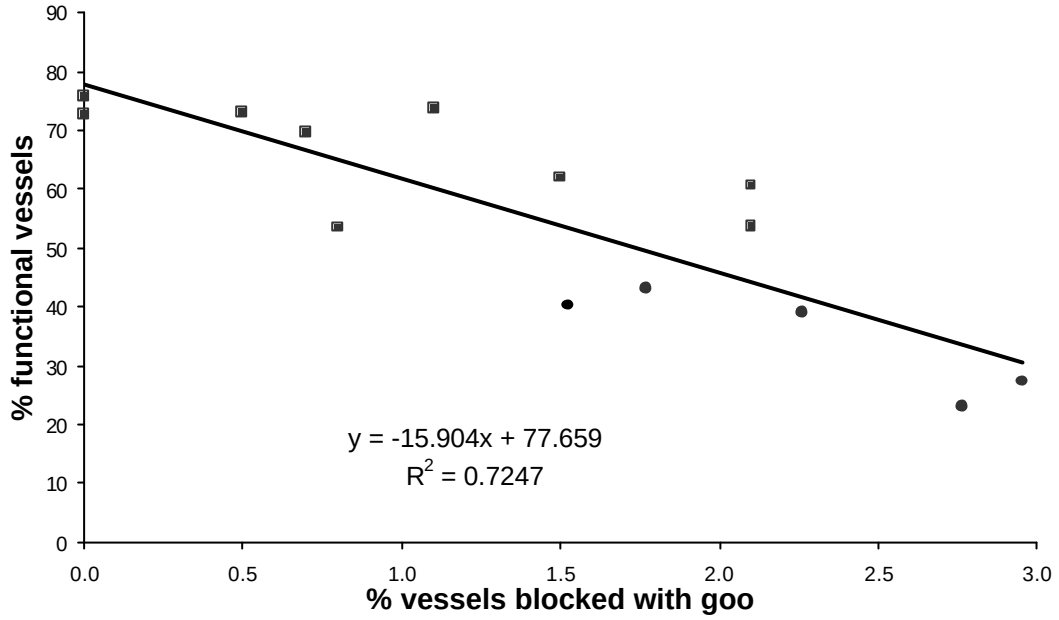


Figure 6.2. Relationship between % functional xylem and % xylem blocked with black goo of Verdelho grapevines infected with *Pa. chlamydospora*. ● = 2004 data, ■ = 2002 data.

It was apparent that *Pa. chlamydospora* infection interfered with the vascular functioning of the grapevine (as expected for a vascular pathogen), but the interference is due to more than just xylem blockage. For example, when only 1% xylem was blocked with goo, there was a corresponding loss in functional xylem from 78% to 60%, indicating that some process other than physical blockage was occurring. It is commonly assumed that vascular pathogens cause water stress in their hosts due to vascular occlusion from tyloses, production of gums (such as black goo) or structures of the pathogen. However, studies on Dutch Elm disease clearly demonstrated that the pathogen, *Ceratocystis ulmi*, was somehow causing air-seeding and inducing embolisms in vessels quite removed from the site of infection (Newbanks *et al.* 1983, Tyree and Sperry 1989). Combining the evidence presented here with the electron microscopy evidence presented above that *Pa. chlamydospora* passes through pit membranes, we suggest that *Pa. chlamydospora* disrupts the xylem function in grapevines also by inducing embolisms and cavitation.

6.3 Impact of *Phaeomoniella chlamydospora* infection on the grapevine's response to water stress

Petri disease is commonly considered a 'stress-related' disease (Ferreira *et al*. 1999, Fourie and Halleen 2004), but the reason for this has not been investigated. *Pa. chlamydospora* colonises the woody xylem tissue of grapevines and, apart from vascular discolouration, infection is generally symptomless until disease expression is triggered by an environmental stress, such as water stress. The research presented here aimed to investigate the impact of infection on the grapevine's capacity to respond to water stress.

Glasshouse trials were conducted over three years. In the first year, naturally-infected and uninfected (ie. hot water treated) potted Zinfandel were subjected to water stress for 3 weeks and their responses (leaf water potential, stomatal conductance and leaf temperature) were compared. The experiments were modified in the second year to increase the level of stress experienced by the grapevines. Again, Zinfandel was the cultivar used. In year three, artificially inoculated Cabernet Sauvignon and Chardonnnay were used in the experiments.

6.3.1 Year 1: three-year-old Zinfandel, 12 Feb –14 Mar 2004

Methods

Treatments. A glasshouse experiment was set up using three year old potted Zinfandel grapevines which had been propagated from infected mother vines and were known to be naturally infected with Pa. chlamydospora. The uninfected grapevines used in this experiment had been propagated from hot water treated cuttings. The vines were grown in standard potting mix in 30 diam pots. The plants were regularly pruned to keep the canopy size to approximately 1 x 1 m. At the end of the trial period, all the vines were destructively assessed to confirm their infection status.

There were four treatments in a 2x2 factorial experiment design, with six replicates per treatment: (1) no water stress, no infection; (2) no water stress, *Pa. chlamydospora* infection; (3) 50% water deficit, no infection; (4) 50% water deficit, *Pa. chlamydospora* infection.

The vines were watered daily with a measured amount of water. Each day, the water use of the vines with no water stress and no infection was calculated to be the required water for the unstressed treatments. The stressed treatments received 50% of this amount. The treatments were applied for three weeks.

Measurements. Leaf water potential, stomatal conductance and leaf temperature measurements per vine were made on three days per week (see table 6.1). Leaf water potential ($?_L$, pressure chamber) was measured destructively on one leaf per pot at approximately 3pm. Using a porometer, stomatal conductance (g_L) was measured on three leaves taken from three different positions and averaged over the three leaves. The leaf temperature (infra-red laser beam) of five leaves was taken at approximately 1pm and averaged over the five leaves. In addition, diurnal leaf water potential measurements were made on Wednesday of each of the three weeks (6 am, 9 am, 12 noon, 3 pm, 6 pm).

Table 6.1. Indicators of water stress in experimental grapevines

Measurement	When?	Per vine
Daily water use	Daily - am	1
Leaf temperature	1-2 pm, x3/week Mon/Wed/Fri	5 leaves, mature sunlit, midshoot
Daily stomatal conductance g_L	1-2 pm, x3/week Mon/Wed/Fri	3 leaves, mature sunlit, midshoot
Daily leaf water potential $?_L$	3-4 pm, x3/week Mon/Wed/Fri	1 leaf, mature sunlit, midshoot
Diurnal $?_L$	6 am, 9 am, 12 noon, 3 pm, 6 pm; x1/week - Wednesdays	1 leaf, mature sunlit, midshoot

At the end of the experiment, the dry weight of the vines was measured as two variables, the above ground dry weight and the below ground dry weight. In addition at the end of the experiment, the

infection 'status' of the vines was checked and revised where necessary. This meant that there were no longer exactly six replicates of each treatment combination.

Results

Stomatal Conductance. A log transformation was required to normalise the data prior to analysis. The main effects of stress and date were significant ($p<0.001$) but infection was not.

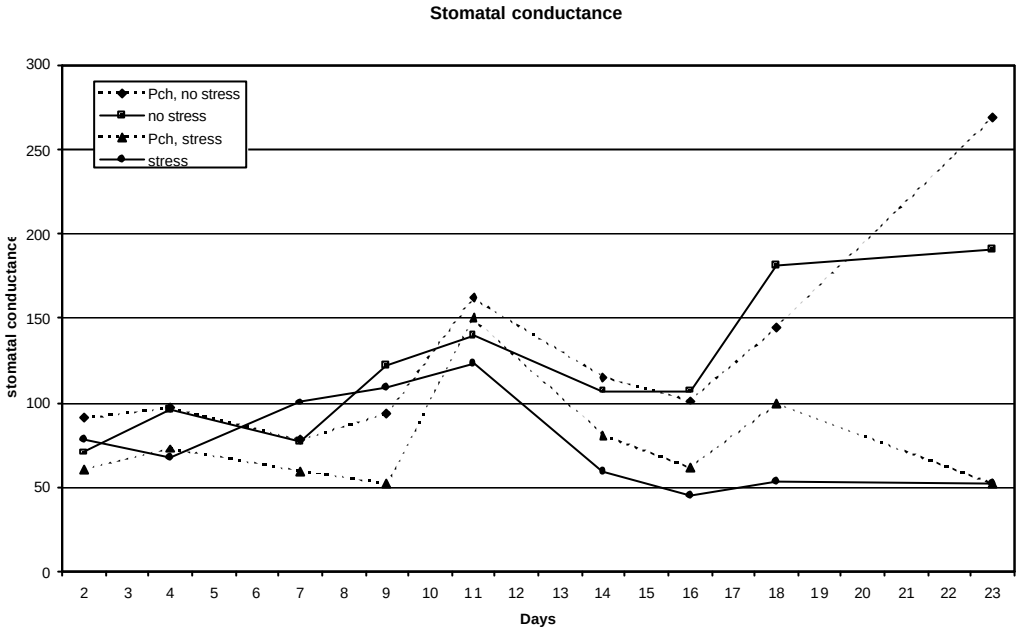


Figure 6.3. Effects of water stress on stomatal conductance of infected and uninfected Zinfandel, 2004.

Leaf Water Potential. There were significant overall main effects of date, infection and stress. In addition, there was a significant interaction between stress and infection ($p=0.049$) and between date and stress.

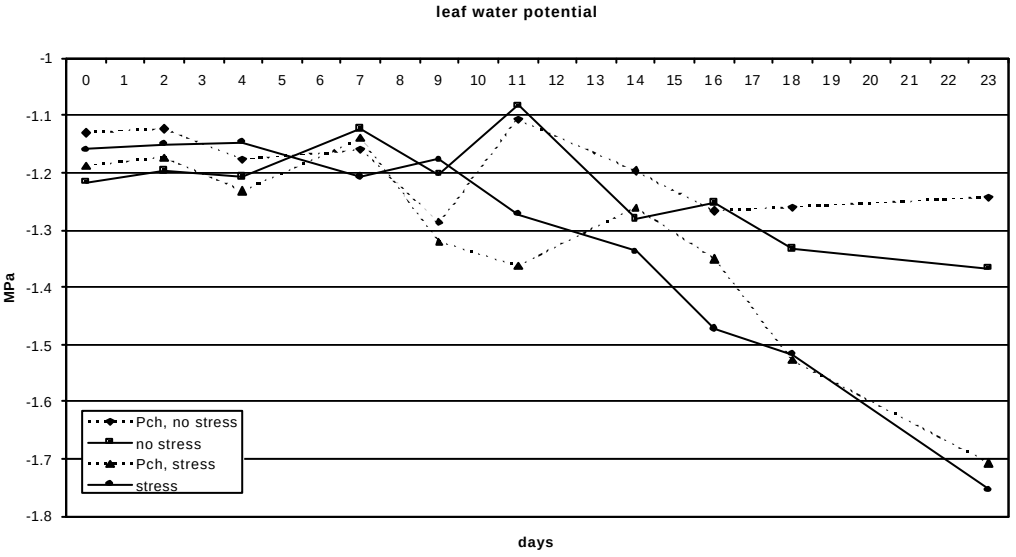
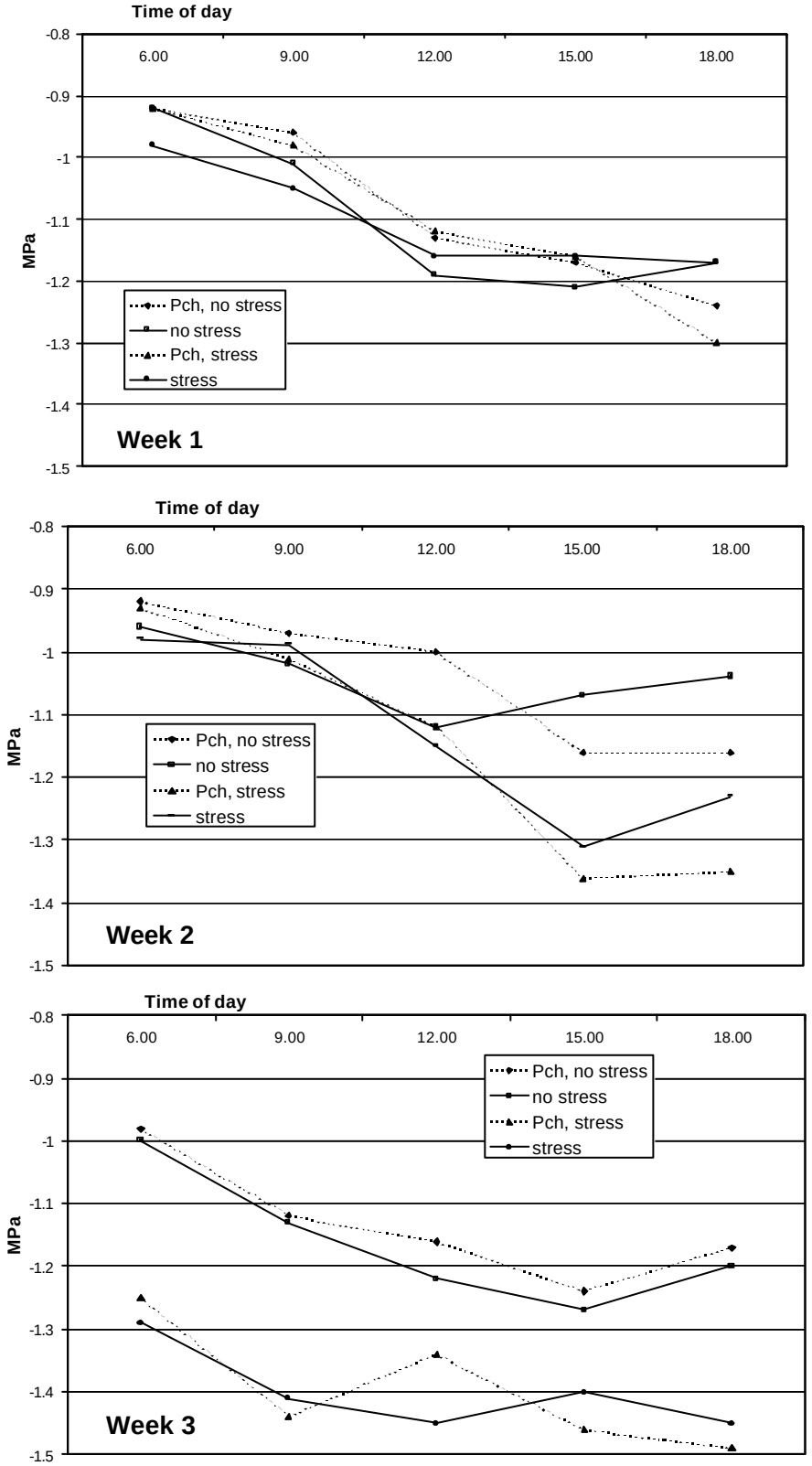


Figure 6.4. Effects of water stress on leaf water potential of infected and uninfected Zinfandel, 2004.

Figure 6.5. Effects of water stress on diurnal measurements of leaf water potential of infected and uninfected Zinfandel grapevines (2004, weeks 1, 2, 3):



By referring to the graphs of each time separately, there is an interesting story (Fig 6.5). For the first three times of day, there is clearly not very much difference in leaf water potential between the four treatments on Week 1 perhaps indicating that plants 'recover' overnight. The 3:00 pm data shows that on Week 1, there appears to be a difference between the four treatments. By Week 2, although the plants are still recovering overnight, the 12 noon measurements clearly show up the effects of stress as the plants are challenged by the heat of the day. By Week 3, it is clear that the stressed plants are unable to recover overnight with a clear difference between the stressed and unstressed pairs of treatments even at 6:00am. The 3:00pm and 6:00pm data show a slight infection effect as infected plants are less able to cope with the stress challenge. There was no significant interaction between stress and infection, however.

Leaf Temperature. The only significant main effect was date ($p<0.001$).

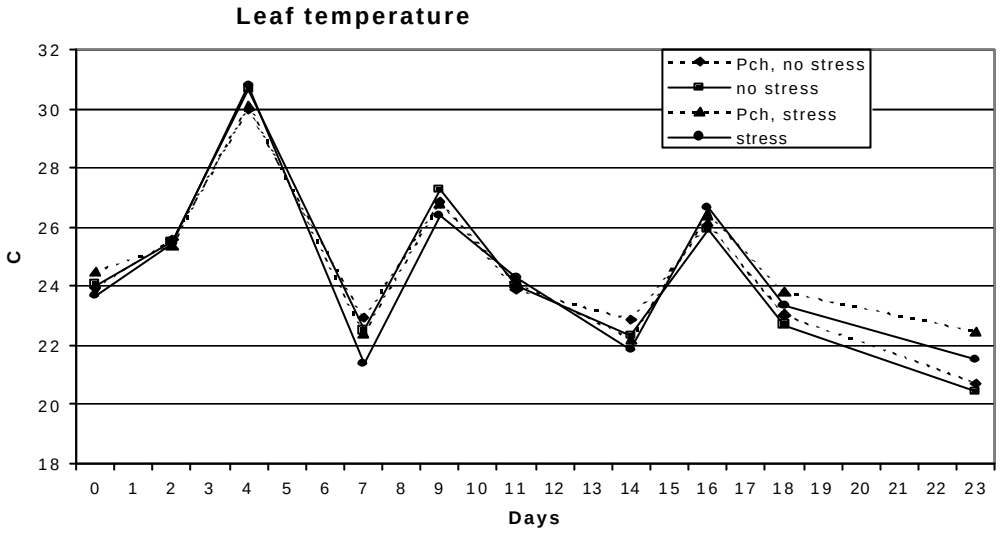


Figure 6.6. Effect of water stress on leaf temperature of infected and uninfected Zinfandel, 2004.

Biomass. There was no significant difference between treatments for root dry weight, but the mean shoot dry weight for the no stress/no infection treatment combination was significantly higher than for the other three treatment combinations.

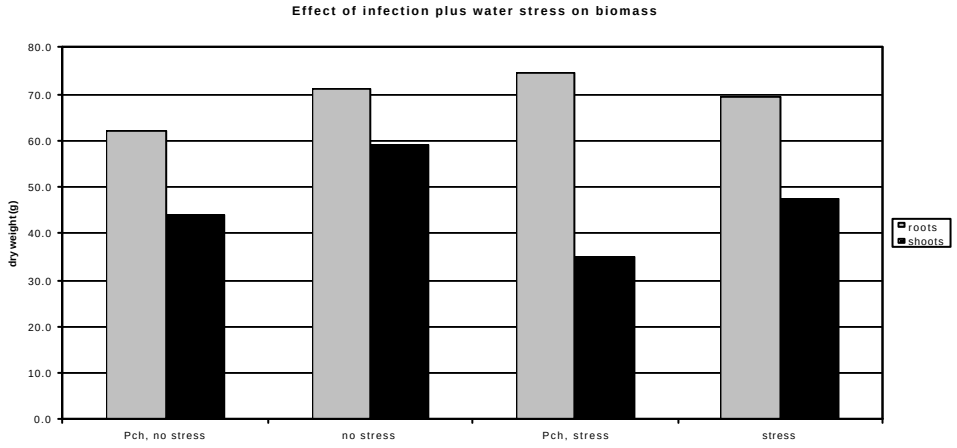


Figure 6.7. Effect of water stress on biomass of infected and uninfected Zinfandel, 2004.

6.3.2 Year 2: four-year-old Zinfandel, 28 Feb – 11 April 2005.

The experiment was repeated again in the following year, with increasing water stress on advice from the Program steering committee.

Methods

Treatments. Once again, there were four treatments in a 2x2 factorial experiment design, with six replicates per treatment: (1) no infection, no water stress; (2) *Pa. chlamydospora* infection, no water stress; (3) no infection, water stress; (4) *Pa. chlamydospora* infection, water stress.

The vines were watered daily with a measured amount of water. Each day, the water use of the vines with no water stress and no infection was calculated to be the required water for the unstressed treatments. 50% of this amount was given to the stressed vines. In the second week, the stressed vines received only 25% of the average water-use. In the third week, the stressed vines were given no water but after three days, it was clear that the vines were shutting down so all vines received 100% of the average water usage. Upon recovery (approximately four days), the stress regime was re-introduced as before, beginning with 50% of average water use for the first week, 25% for the second and third weeks, returning to 100% before the final data measurement. The treatments were applied from 2 March – 9 April.

Watering schedule:
28 February - all vines 100% water
2 March – stress treatments began @ 50% water
9 March – stress increased to 25% water; diurnal measurements taken
11 and 15 March – diurnal measurements taken
16 March – water withheld from stress treatments
19 March – all fully watered and allowed to recover
22 March – stress treatments @ 50% water
29 March - stress treatments @ 25% water
9 April – full water resumed for all vines
11 April – final measurements.

Measurements: Daily water use, leaf water potential ($?_L$, pressure chamber), stomatal conductance (g_L, porometer) and leaf temperature (infra-red laser beam) measurements were made per vine (see table 6.2). In addition, diurnal stomatal conductance and leaf water potential measurements were made on days 7, 9 and 13 of the experiment.

Table 6.2: Indicators of water stress in experimental grapevines

Measurement	When?	Per vine
Leaf temperature	1-2 pm, x3/week Mon/Wed/Fri	5 leaves, mature sunlit, midshoot
Daily stomatal conductance g_L	3-4 pm, x3/week Mon/Wed/Fri	3 leaves, mature sunlit, midshoot
Daily leaf water potential $?_L$	3-4 pm, x3/week Mon/Wed/Fri	1 leaf, mature sunlit, midshoot
Diurnal g_L	6 am, 3 pm, 6 pm; days 7, 9, 11	1 leaf, mature sunlit, midshoot
Diurnal $?_L$	6 am, 3 pm, 6 pm; days 7, 9, 11	1 leaf, mature sunlit, midshoot

The data were analysed using a standard split-plot analysis. The REML procedure was used because not all treatment combinations were present in equal numbers due to the infection status of some vines being revised. A log transformation was required for the conductance data prior to analysis.

Results.

Stomatal Conductance (Zinfandel 2005). For the variable stomatal conductance, a log transformation of the data was necessary. The overall main effects of date, stress and infection were significant. There was also a significant interaction between stress and infection indicating that the infected plants

responded differently to the stress, and between date and stress. In the unstressed grapevines, the stomatal conductance for the infected plants was consistently higher than for the uninfected plants.

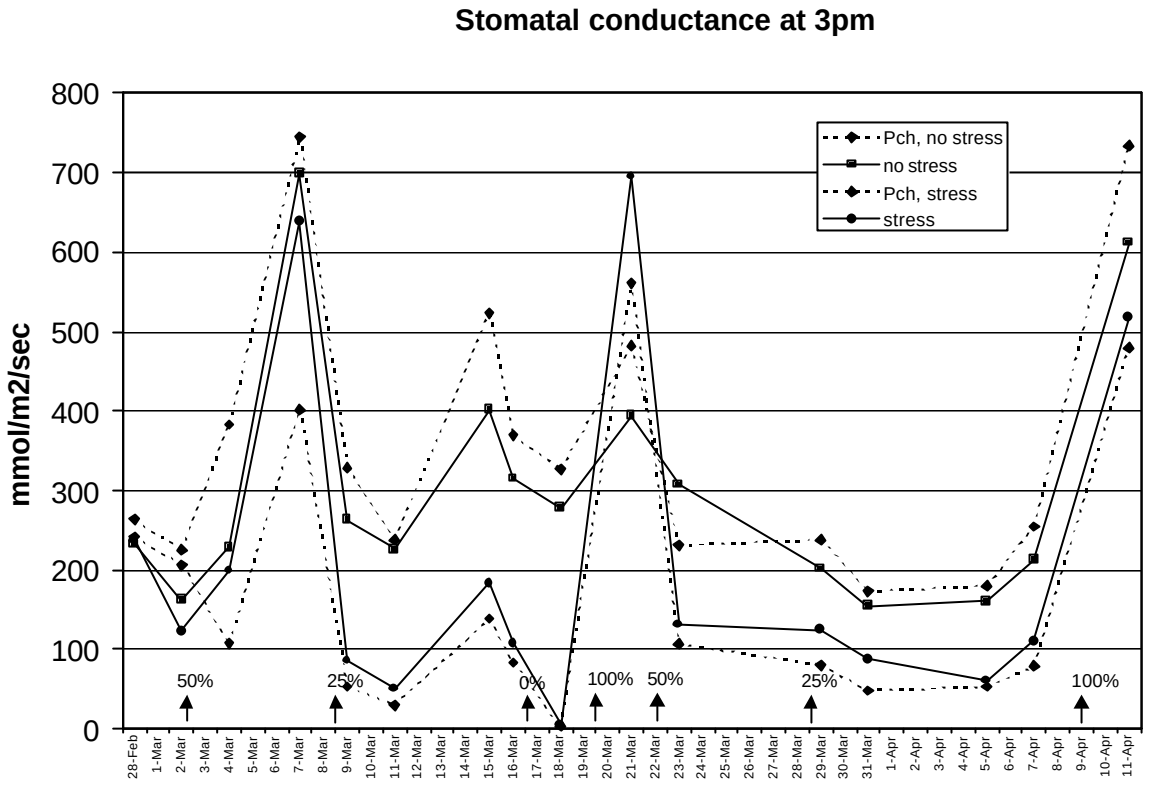


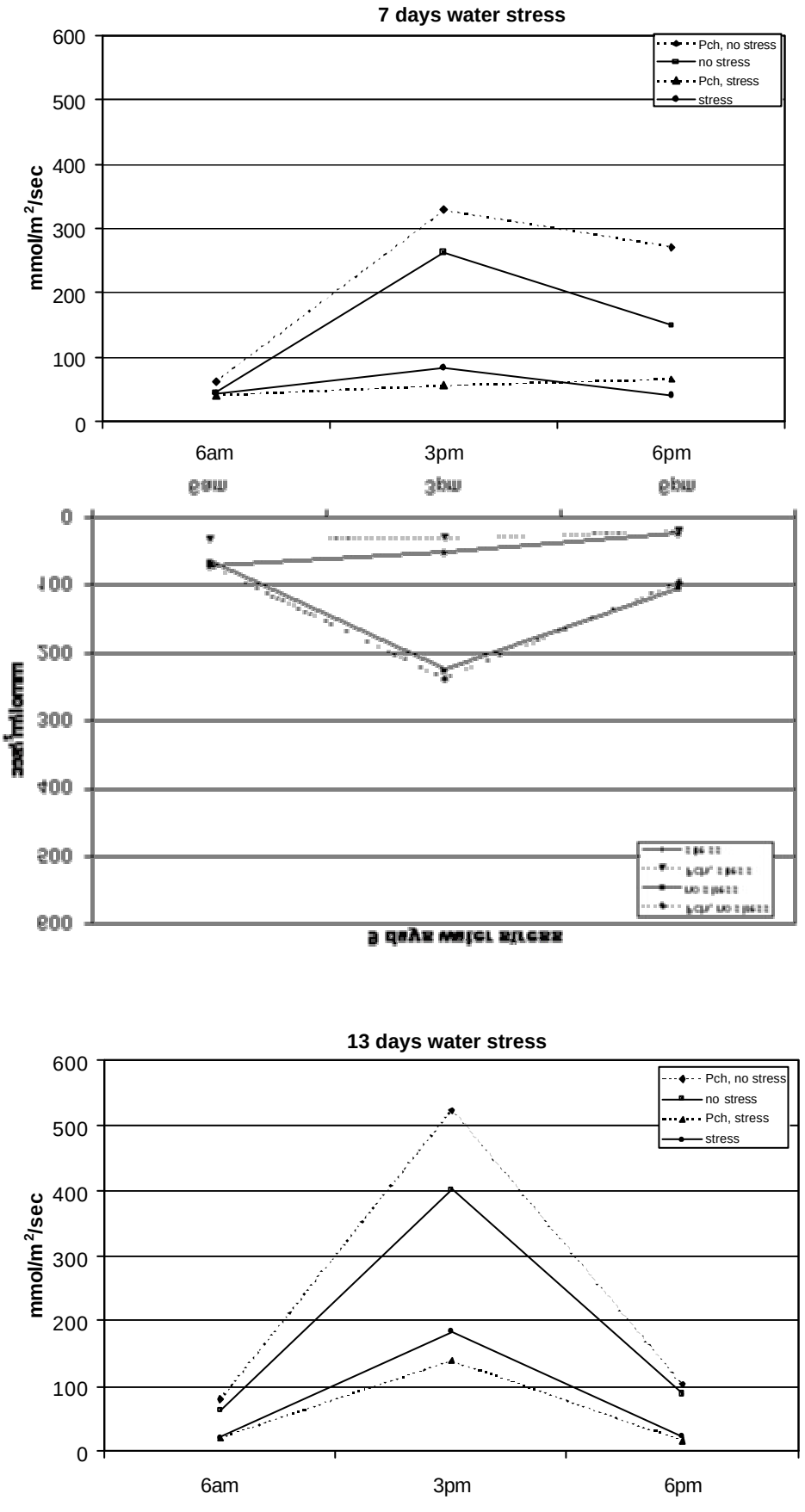
Figure 6.8. Effects of water stress on stomatal conductance of infected and uninfected Zinfandel, 2005.

Diurnal measurements on Days 7, 9 and 13. The analysis showed large main effects of stress and date. The interaction of both stress and infection with date showed that these effects changed significantly over the three days at 6:00am. There was no interaction between infection and stress at 6:00 am.

At 3:00pm, the hottest part of the day, there were significant overall main effects of date and stress, evidence of an overall infection effect ($p=0.054$). The interaction of stress and date was significant and there was also very weak evidence of an overall interaction between stress and infection ($p=0.088$). This was not due to a change in the difference in the effects of infection and stress over time but due to the fact that there was almost no difference between the unstressed infected and unstressed uninfected vines at 3 pm on 11 March. There was a difference on both 9 and 15 March.

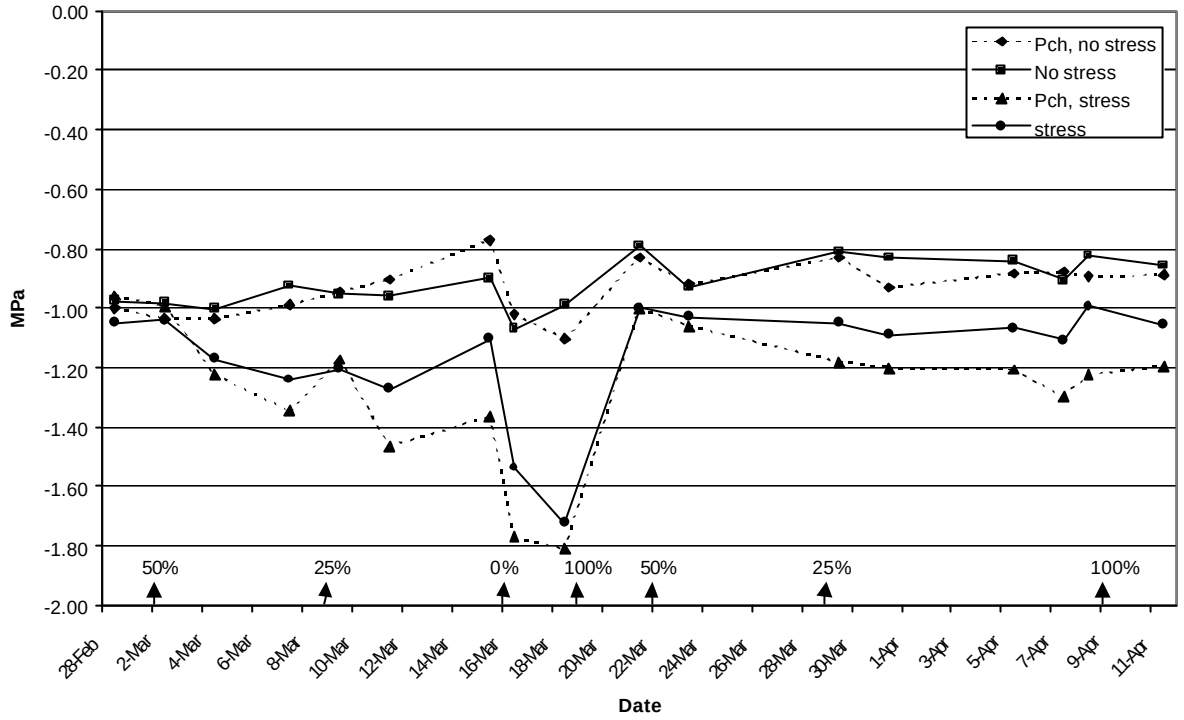
At 6:00 pm, only date and stress effects were significant.

Figure 6.9. Zinfandel, 2005. Stomatal conductance measured at 6 am, 3 pm and 6 pm on days 7, 9 and 13 after stress treatments imposed.



Leaf Water Potential. The main effects of infection, stress, and date were all significant. The interaction between stress and infection was significant and so were the interactions between date and both stress and infection.

Figure 6.10. **Impact of Pch infection on response of potted Zinfandel grapevines to water stress, as measured by leaf water potential at 3 pm**



Diurnal measurements on days 7, 9 and 13.

At 6:00 am, only stress and date are significant.

At 3:00 pm, infection, stress and date are significant main effects whilst the interaction of date with both stress and infection are significant. This can clearly be seen on the figures as there is almost no infection effect on 9 March but a definite effect on the other two dates.

At 6:00 pm, only the overall effect of stress and the interaction of date with stress are significant.

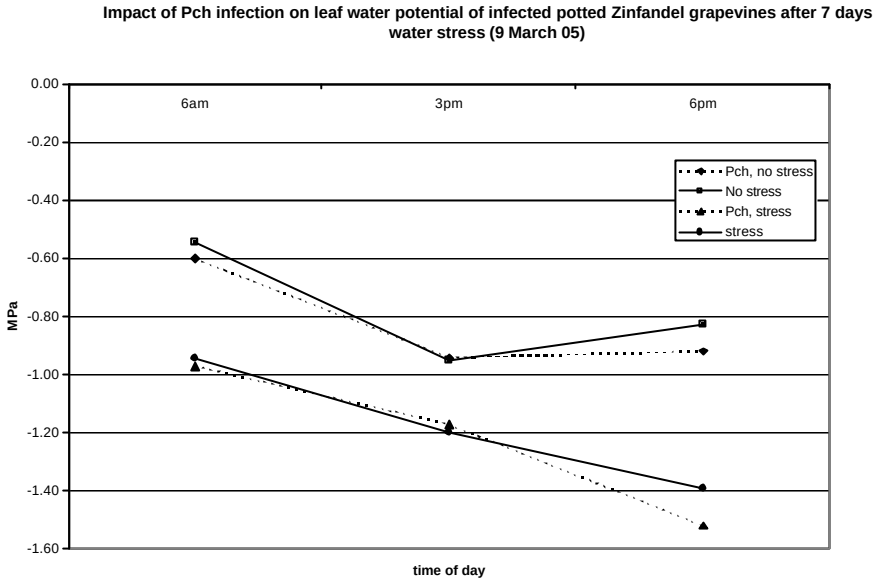
In addition to considering the measurements at particular times over several days, the rate of change ie the slope between 9:00 and 3:00pm was examined. REML was used for estimation as before.

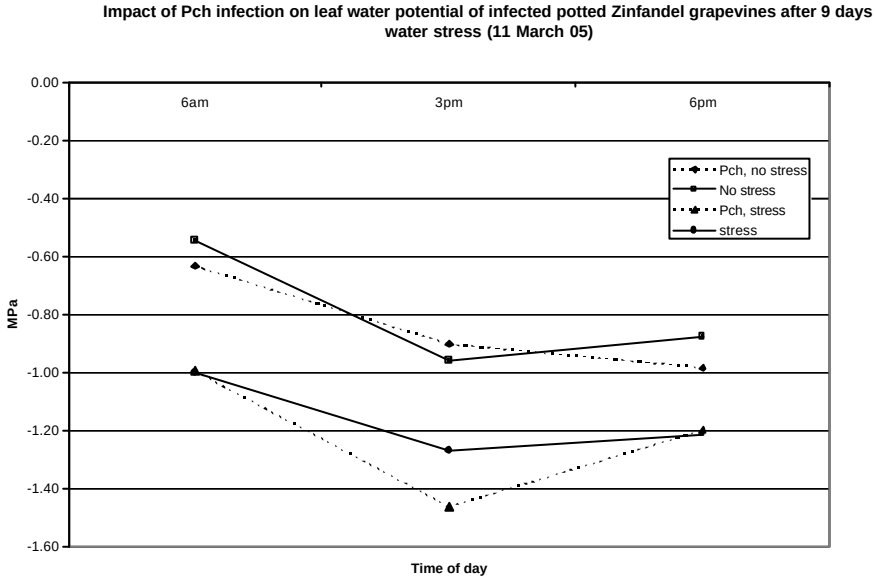
The main effect of date is significant, ie on average the slopes get steeper. The main effect of stress is significant: ie on average over all days the slope of the unstressed vines is steeper. Since the interaction of stress and date is significant, this means that as the plants become more stressed, the difference in the slopes between stressed and unstressed vines becomes greater. As for the significant interaction between date and infection, the slope of the uninfected vines remains constant but the slope of the infected vines becomes steeper. There is an interaction between stress and infection that is evident on the figures when some of the lines cross over treatment lines.

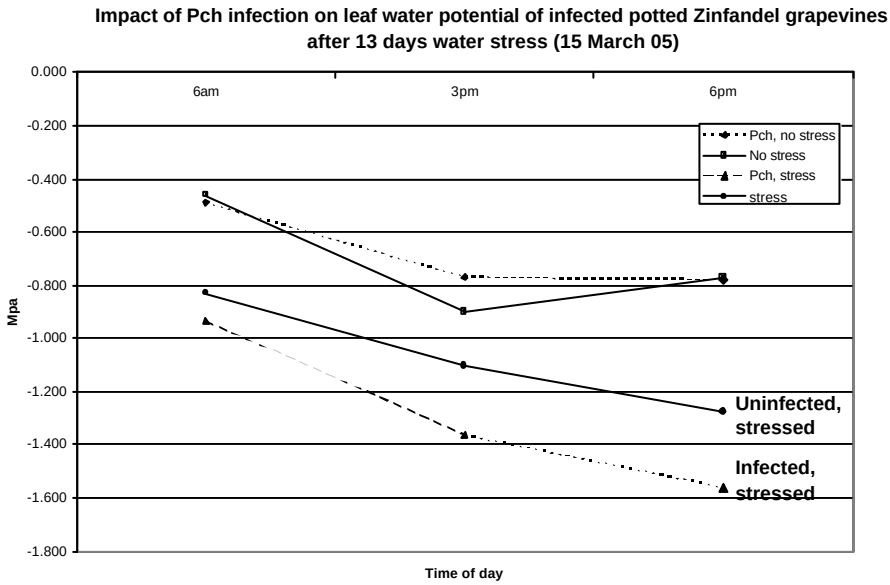
Leaf temperature and dry weights

The effects of date and stress were highly significant, but not infection (data not shown). There were no significant treatment differences for either shoot or root dry weights (data not shown).

Figure 6.11. Zinfandel, 2005. Leaf water potential measured at 6 am, 3 pm and 6 pm on days 7, 9 and 13 after stress treatments imposed.







6.3.3 Year 3: four-year-old potted Cabernet Sauvignon (14 November – 23 December 2005) and Chardonnay (20 February – 31 March 2006)

The experiment was repeated for a third season using artificially inoculated grapevines. The aim of the experiment was to see if there is a difference in the way infected vines and non-infected vines behave under different levels of stress.

Methods

Sets of four year old Cabernet Sauvignon and Chardonnay were inoculated near the base of the trunk, while dormant (mid July 2005), with either 200 µL sterile distilled water or 200 µL spore suspension made up to deliver 50 spores *Pa. chlamydospora*. They were then grown in the glasshouse and, at the time of the experiments, the most uniform plants were chosen to provide 18 infected and 18 uninfected plants of each cultivar.

The variables measured were leaf water potential and stomatal conductance. There were two sets of measurements. The first set consisted of a measurement once a day @ 3pm on three days a week for six weeks and the other set consisted of three measurements per day @ 6 am, 3 pm and 6 pm, for three days in one week.

The factorial set of 6 treatments consisted of three levels of stress, 'no stress', '50% of water requirement' and '25% of water requirement' combined with one of two levels of infection, either 'infected' or 'not infected'. There were 6 reps of the six treatments and the pots were laid out on 6 benches with each bench a replicate.

Water was completely withheld from the Cabernet Sauvignon stress treatments for 4 days from day 23-26, and from the Chardonnay stress treatments for 3 days from day 27-29, after which the treatments wer reapplied. This was to determine whether infection interfered with the grapevines' capacity to recover from a short severe stress. This severe stress was restricted to 3 days for Chardonnay as this cultivar did not tolerate the stress as well as the Cabernet Sauvignon.

The data were analysed using the AREPMEASURE procedure of Genstat, which uses an analysis of variance to analyse the data but allows for the correlation of measurements over time.

Results

Cultivar: Cabernet Sauvignon, stress imposed 14 November – 23 December 2005.

Single daily measurement.

The results were the same for both leaf water potential and stomatal conductance. The overall main effects of stress and infection were significant; there was no overall interaction of stress with infection; the overall time effect was significant; there was a significant interaction of stress with time; there was no significant interaction of infection with time. This indicates that the average difference between infected and uninfected vines is significant, but that the size of this difference does not change significantly over time. However, the difference between the three levels of stress is significant and this difference changed over time.

Figure 6.12. Effect of water stress on stomatal conductance of infected and uninfected Cabernet Sauvignon, Nov 2005.

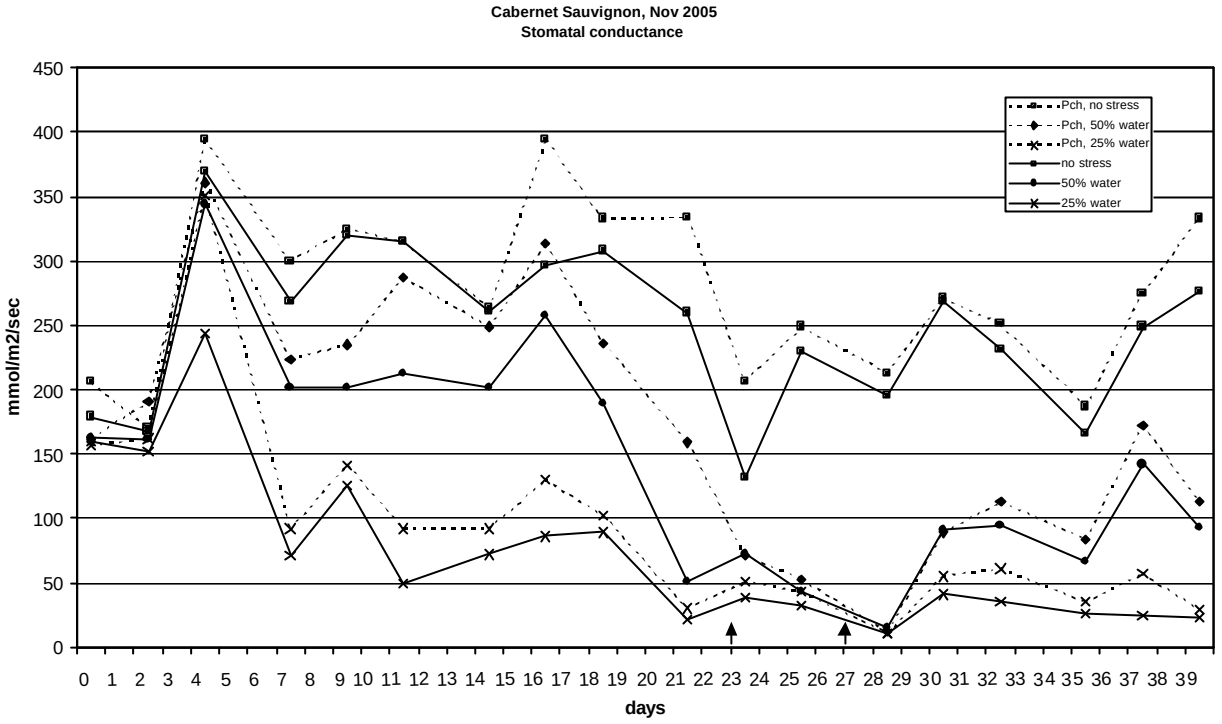
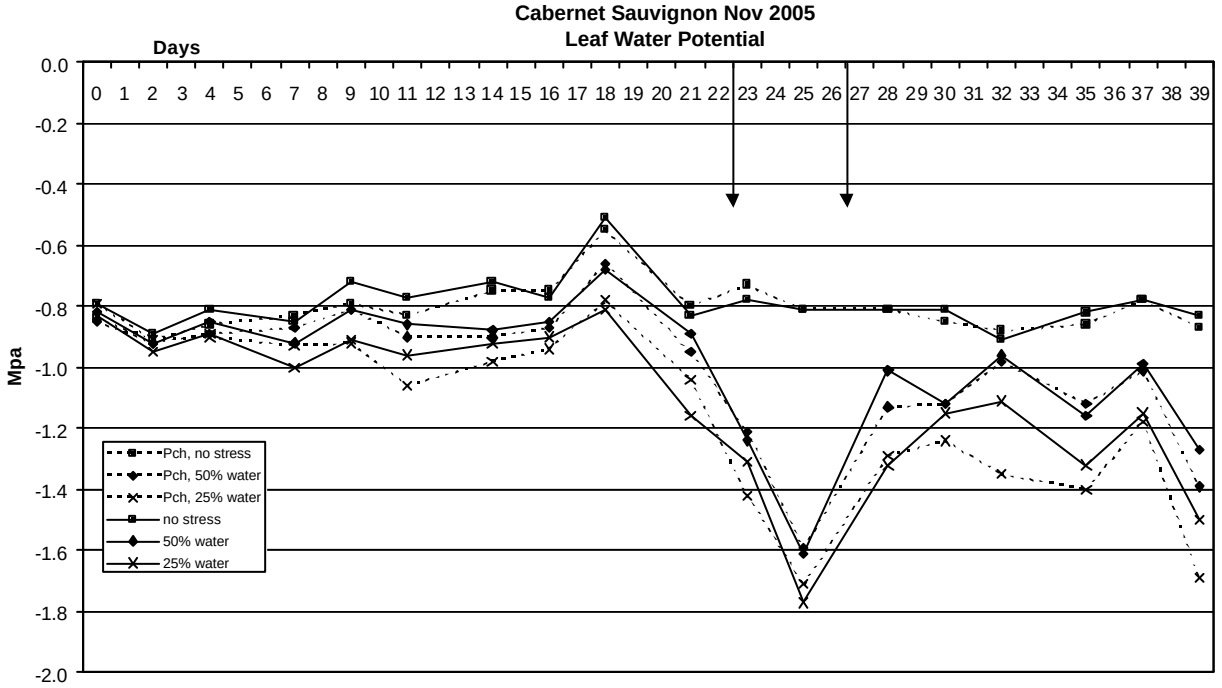


Figure 6.13. Effect of water stress on leaf water potential of infected and uninfected Cabernet Sauvignon, Nov 2005.



The arrows indicate the period when water was completely withheld from the stress treatments.

Diurnal measurements on days 9, 11 and 14

Stomatal Conductance (Fig. 6.14)

At 6 am, there is a significant overall stress main effect but no main effect of infection. The effect of date of measurement is significant and the main effect of stress is different across days. There is weak evidence of the effect of infection varying across days (p=0.076) but both of these are because of the much lower values on Day 14. There is no evidence of stress by infection interaction across the 3 days.

At 3 pm data, the overall effects of both stress and infection are significant with weak evidence of an interaction between these two factors (p=0.091). However, there is no evidence of this interaction effect changing across dates. As for 6 am, the stress main effect does change across days.

At 6 pm, the overall main effects of infection and stress are significant and so is their interaction. But these effects do not change significantly over the three dates of measurement.

Leaf Water Potential (Fig. 6.15)

At 6 am, there is a significant overall stress main effect with only weak evidence of an overall infection effect at this time (p=0.078). The main effect of stress changes across the three days but there is no evidence of the infection affect changing across days. The interaction of stress with Infection is not significant.

At 3 pm, the overall main effects of both stress and infection are significant but neither effect changes across the three dates of measurement and the interaction is not significant.

At 6pm, there is an overall significant stress effect and this effect changes significantly across the three days but infection and the interactions are not significant.

Figure 6.14. Effect of 9, 11 and 14 days water stress on stomatal conductance of infected and uninfected Cabernet Sauvignon, Nov 2005.

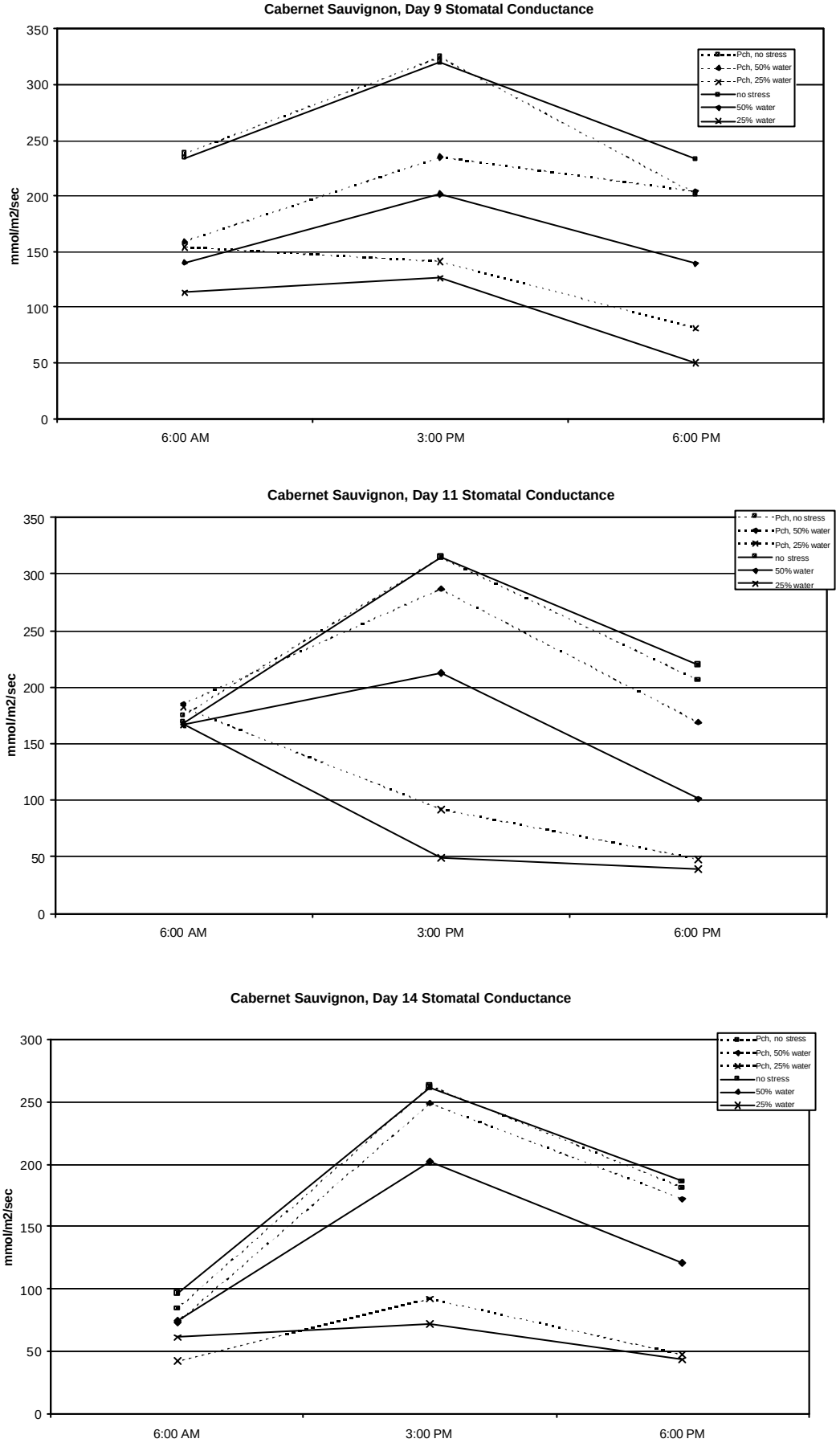
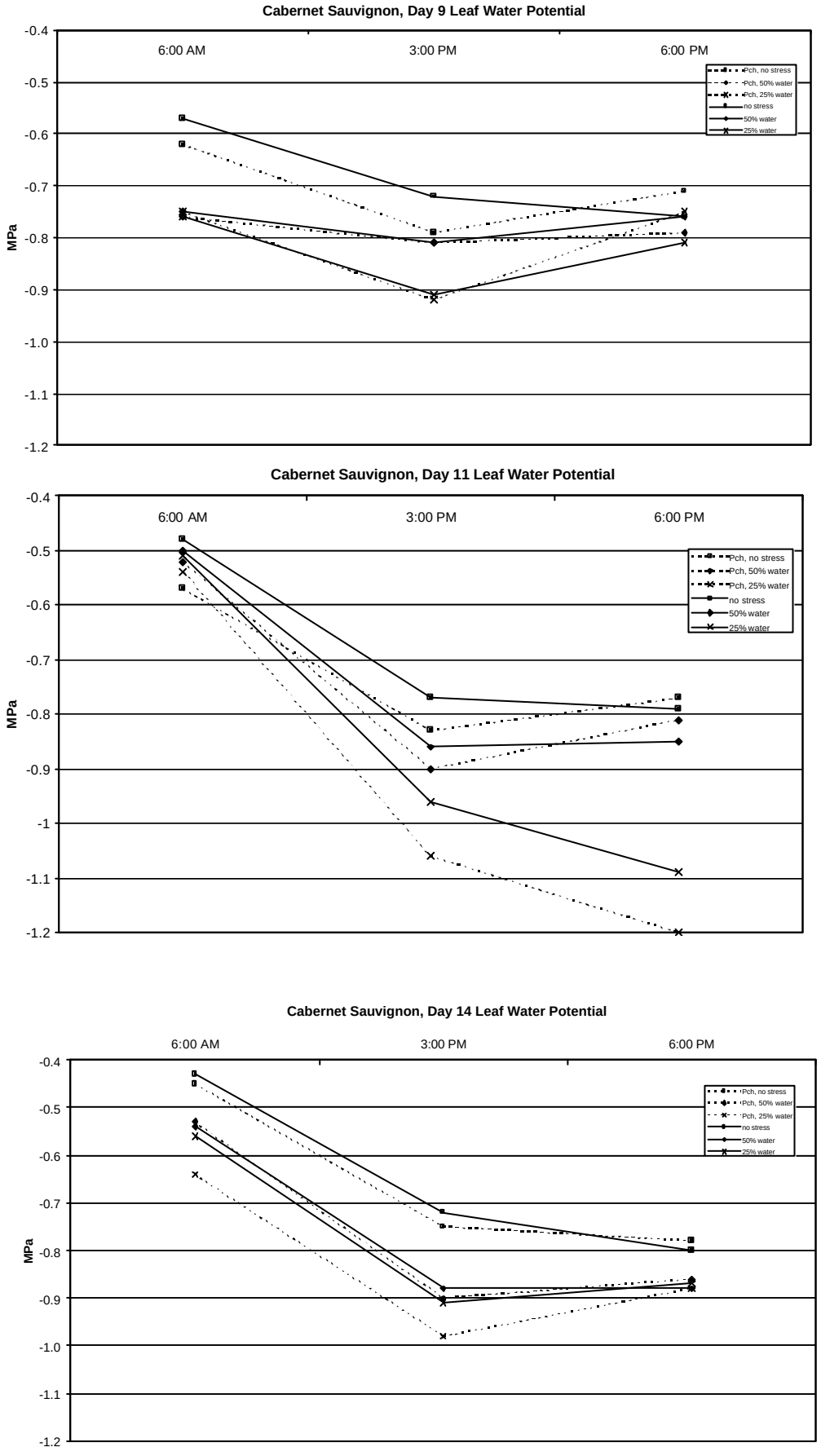


Figure 6.15. Effect of 9, 11 and 14 days water stress on leaf water potential of infected and uninfected Cabernet Sauvignon, Nov 2005.



Cultivar: Chardonnay, stress imposed 20 February – 31 March 2006.

Single daily measurement.

The results were similar for both leaf water potential and stomatal conductance The overall main effects of stress and infection were significant; the interaction of stress with infection was significant. The overall time effect was significant; there was a significant interaction of stress with time; there was a significant interaction of infection with time. The three-way interaction of time.infection.stress was also very slightly significant (p=0.071 for stomatal conductance and 0.083 for leaf water potential) but this is probably due to the catastrophic effect of giving no water to the four stress treatments on days 27-29.

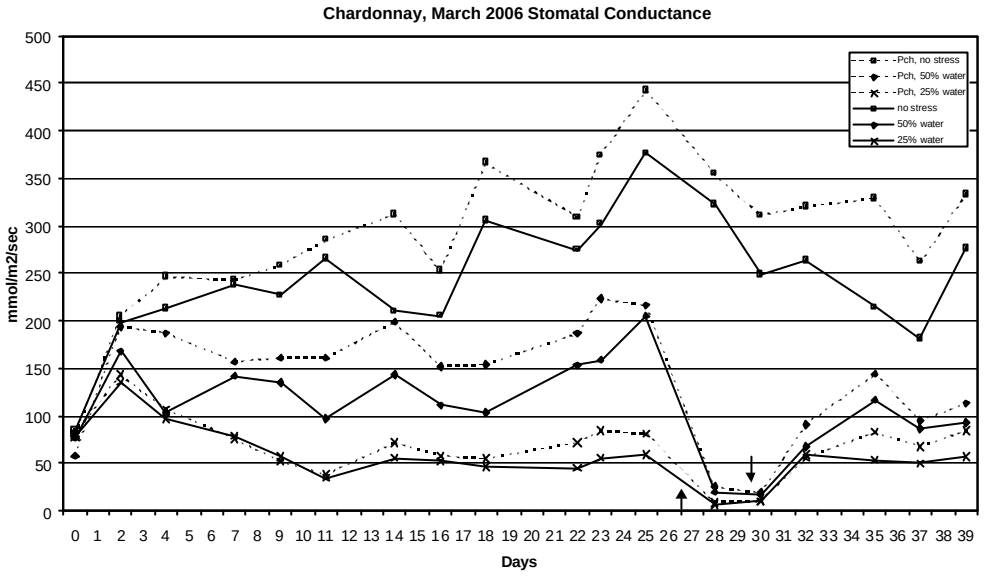


Figure 6.16. Effect of water stress on stomatal conductance of infected and uninfected Chardonnay, March 2006.

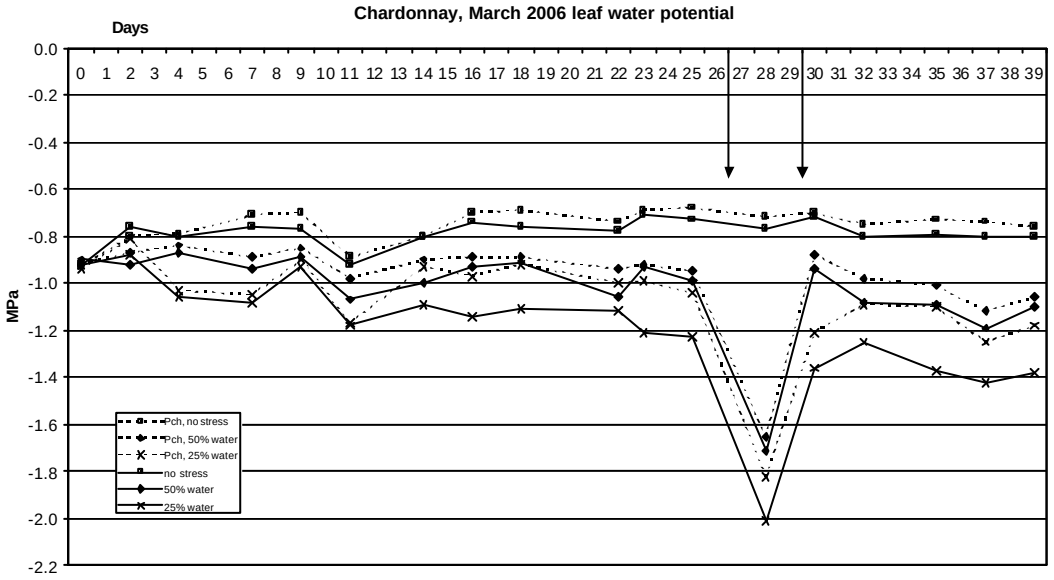


Figure 6.17. Effect of water stress on leaf water potential of infected and uninfected Chardonnay, March 2006.

The possibility of a difference between treatments in the 'recovery' period was considered. On days 27, 28 and 29, the pots receiving the stress treatments received no water at all while the remaining unstressed treatments received the normal allocation. As expected the measurements taken on day 28 showed extreme values for both leaf water potential and stomatal conductance. On day 30, the normal watering recommenced. At 3 pm, the usual measurements were taken and although leaf water potential showed an immediate recovery, stomatal conductance did not show recovery until day 32.

There was a significant difference in the recovery of infected versus uninfected vines, but unexpectedly, the infected vines recovered more rapidly than the uninfected vines.

Diurnal measurements on days 9, 11 and 14

Stomatal Conductance (Figure 6.18)

The three times of day were analysed as separate variables using analysis of variance.

At 6am, there were overall main effects of stress and infection but no interaction between these. The overall effect of date was significant but is more likely due to the changes in daily environment (eg maximum and minimum temperatures) than due to a longer period of stress. The size of the main effects was different on the three days; this difference was significant for infection and stress but not for the interaction.

At 3pm, it was not surprising that the differences were greatest as this is the hottest time of day. The infection and stress interaction was not only significant overall but was significantly different between days. However, once again the length of time the plant has been stressed may not be the explanation; daily maximum temperature is probably the explanation.

The data for 6 pm required a log transformation for the analysis. Overall, the main effects of infection and stress were significant. There is weak evidence of an overall interaction between stress and infection ($p=0.087$). The sizes of the effects of infection and of stress were significantly different between days but there is no evidence that the size of the stress by infection interaction changed over days.

Leaf Water Potential (Figure 6.19)

Once again, the three times of day were analysed as separate variables using analysis of variance.

At 6 am, there were significant main effects of stress and infection but no interaction between stress and infection. There was a significant main effect of date but once again, this is probably due to the day's temperatures rather than the elapse of time. The main effect of infection changes significantly across the three days of measurement but neither the change in the effect of stress nor the change in its interaction with infection over the three days is significant.

At 3 pm and 6 pm, there were significant overall main effects of stress and infection but the interaction of these two factors was not significant. Date was significant but there were no changes in the size of the effects across the three days of measurement.

As mentioned above, the Chardonnay grapevines behaved differently to the Cabernet Sauvignon and Zinfandel grapevines. The leaf water potentials of the stressed uninfected vines were lower than those of the stressed infected vines, implying that the uninfected vines were less tolerant of the stress than the infected vines.

Figure 6.18. Effect of 9, 11 and 14 days water stress on stomatal conductance of infected and uninfected Chardonnay, March 2006

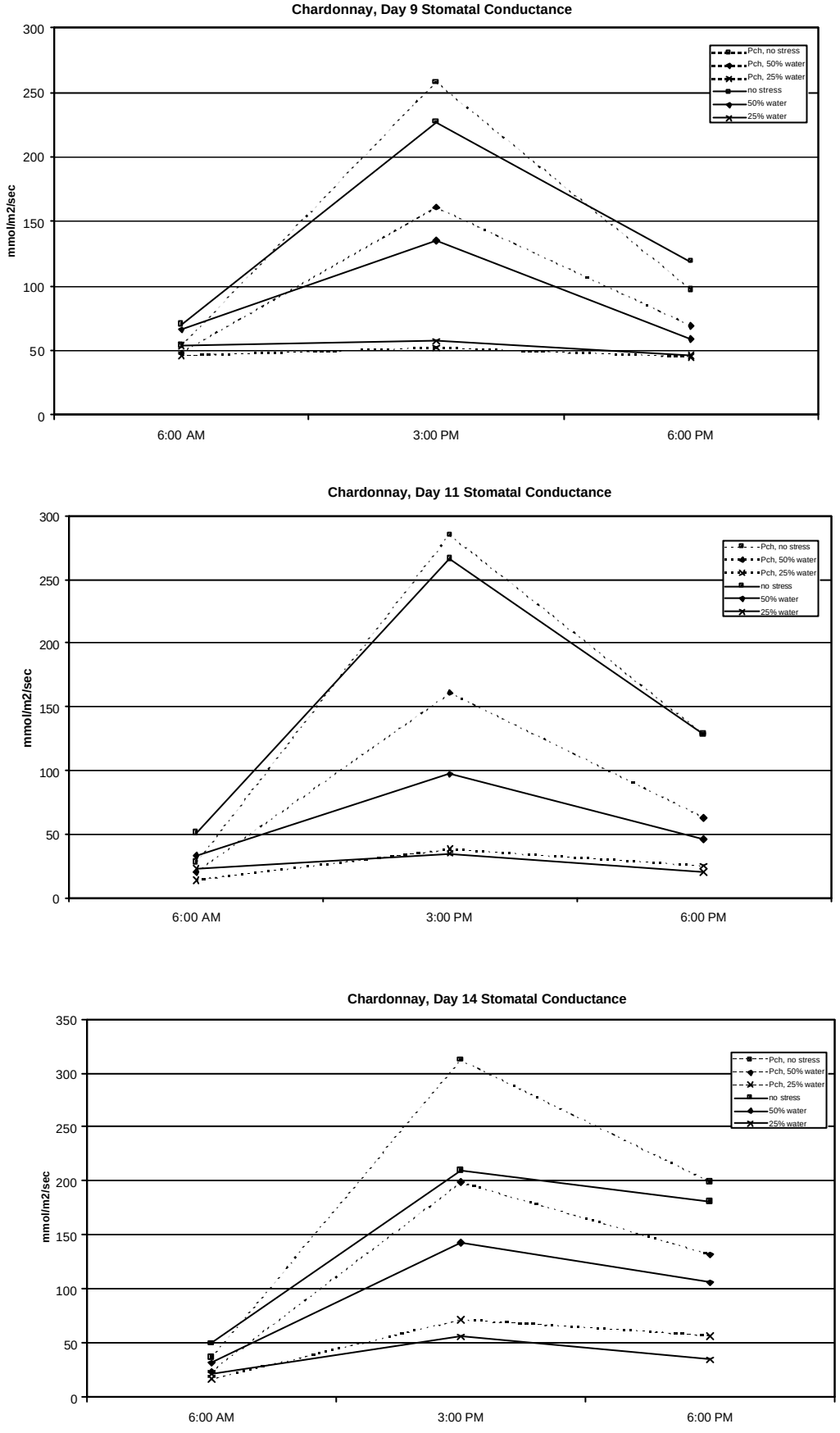
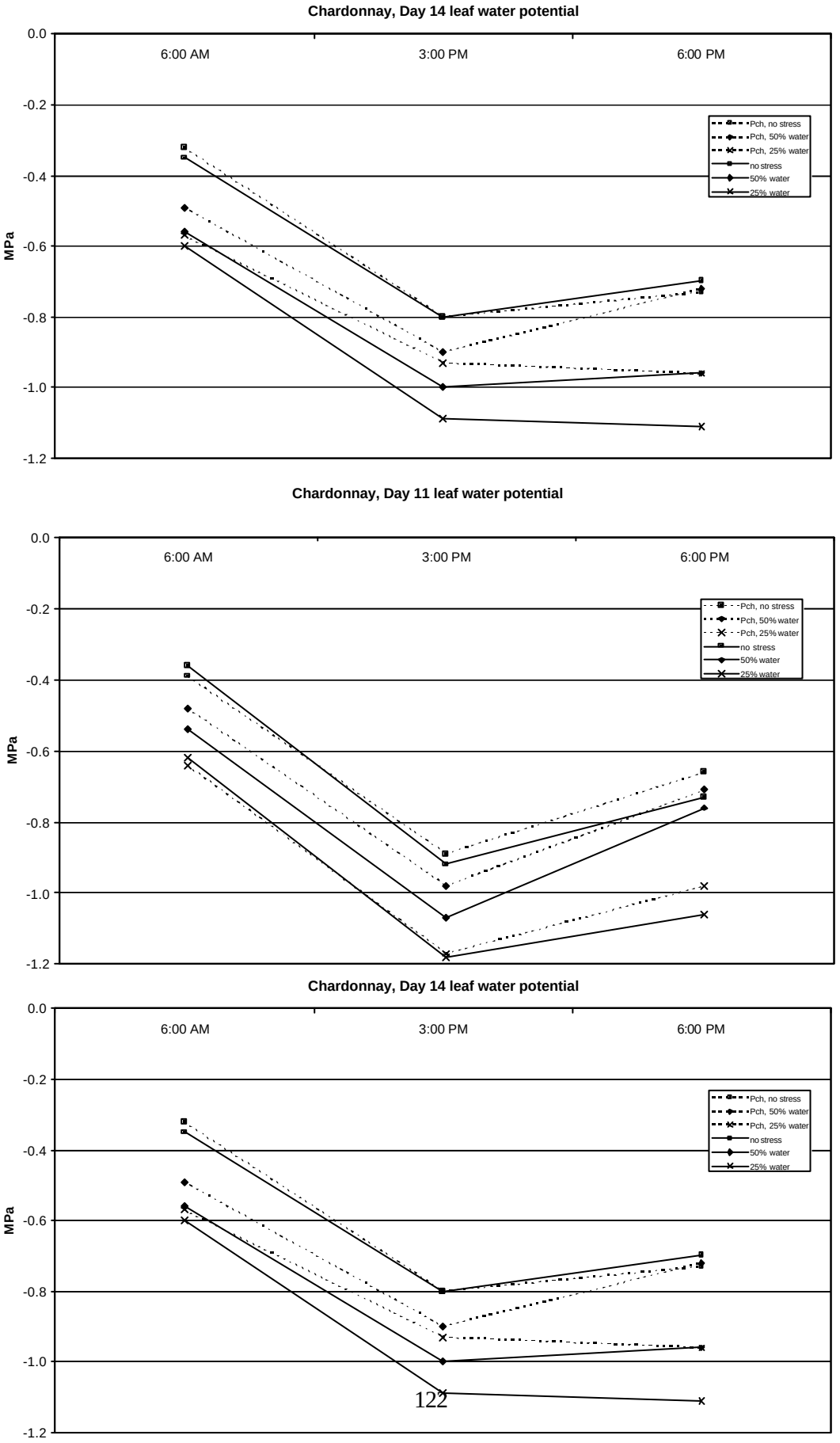


Figure 6.19. Effect of 9, 11 and 14 days water stress on leaf water potential of infected and uninfected Chardonnay, March 2006



Discussion

Three cultivars were trialled: Zinfandel, Cabernet Sauvignon and Chardonnay. Plants were subjected to a single steady stress (years 1 and 3), to a stress that steadily increased over time (year 2) and to the imposition of a short severe stress (years 2 and 3). Under all of these circumstances, grapevines infected with *Phaeomoniella chlamydospora* responded differently to comparable uninfected grapevines.

Stomatal conductance and leaf water potential measurements were the most useful for differentiating infected and uninfected plants. Leaf temperature differentiated stressed and unstressed plants, but was not discriminating enough to detect more subtle differences.

Stomatal conductance was usually higher in infected plants than uninfected plants, including the unstressed treaments, indicating that infection interfered with stomatal regulation, causing the stomates to be more open than normal. The cultivars appeared to differ slightly in reaction, with the most noticeable effect on Chardonnay, followed by Zinfandel and least noticeable in Cabernet Sauvignon.

Infection status did not appear to affect leaf water potentials in unstressed grapevines. Under water stress, however, infected Zinfandel and Cabernet Sauvignon had consistently lower leaf water potentials, but infected Chardonnay had higher leaf water potentials. As a general rule, the lower the leaf water potential, the more stressed the plant. This suggests that Zinfandel and Cabernet Sauvignon were more adversely affected by the combination of infection and stress than Chardonnay.

The diurnal measurements showed that the stress x infection interaction was more pronounced in the afternoon, when plant water demand was highest. In addition, the 6 am measurements showed that over time the infected plants were less able to recover overnight than the uninfected plants. This was particularly noticeable in the Zinfandel 2004 Week 3 measurments. The level of stress was also important. Cabernet Sauvignon experiment, 2005, showed that the biggest impact of infection was in the intermediate (ie 50%) water stress treatment. Under higher stress (ie 25%), all plants were severely stressed whether infected or not.

A short severe stress (ie no water) was imposed to determine if infection affected the plants' ability to recover. In 2005, infected Zinfandel and Cabernet Sauvignon (25% water) were less able to recover than uninfected plants subjected to the same treatments. However, the Cabernet Sauvignon plants at 50% water stress responded similarly, regardless of infection status.

Chapter 7: Control and management of Petri disease in the vineyard

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Summary: This section reports on the results of four field trials investigating amelioration treatments for Petri disease of grapevines. Different treatments trialed included applications of compost, nutrient fertilisers, phosphonate and Brotomax. Vine health was monitored for two to five years. To summarise, successful development of management strategies to deal with infected vineyards has been difficult because no single treatment has proven effective. Despite this, grapevines were seen to recover from Petri disease over time. The evidence suggests that a combination of stress and infection wiith *Phaeomoniella chlamydospora* is a major factor in development of the disease, particularly stress due to inadequate irrigation and overcropping before the vine has established a decent root system. It is still unknown, however, whether the recovery is permanent or whether disease expression will re-occur sometime in the future.

The objective of these field trials was to identify treatments that can be recommended for vineyards affected by Petri disease.

7.1 Multiple inputs: grafted Semillon, Sunraysia

During 1994 to 1995, 600 ha of land in the Sunraysia district of Victoria, previously cropped with wheat, was planted with Semillon grafted onto a variety of rootstocks. The grapevines that were planted in 1994 established quickly and grew well. Those planted in 1995, however, exhibited very poor growth and struggled to become established. During 1997, a total of 21 grapevines were taken out from across the poorly-performing area and sent to the Victorian Department of Primary Industries commercial diagnostic arm, Crop Health Services, for diagnosis. All had internal black vascular staining in the rootstock portion of the trunks, and *Phaeomoniella chlamydospora* was isolated from all samples, confirming Petri disease.

At the time, very little was known about the pathogen or the disease in Australia. Similar problems were being reported from South Africa (Ferreira et al 1994, Crous et al 1996), and anecdotal evidence (Strauss Ferreira, pers comm.) suggested that the grapevines might recover if they were treated with extra water, nutrients and phosphonate.

During 1998 and 1999, the affected vines received 50% more water than the rest of the vineyard, plus 10 tonne/ha composted grape marc applied as a mulch, one tonne Pinnacle (NH_4NO_3) over the whole area per week, and urea, KNO_3, $MgSO_4$, epsom salts and $ZnSO_4$ as determined by petiole sap analysis. They also received four Agri-Fos® (phosphonate) applications (two through the drip irrigation system prior to flowering and two as foliar sprays around veraison) at 5L/ha in 1000 L/ha water.

After two seasons of high inputs, the vines had recovered fully, were extremely vigorous and yielded 47 tonne/ha fruit. However, such high levels of input are unsustainable in the long term and incompatible with growing quality grapes. In 2001, half of the grapevines were returned to normal practice, while the other half remained on the high input regime. 'Normal practice' included 5 tonne/ha composted grape marc, a single Agri-Fos® application via drip irrigation prior to flowering, no nitrogen, and other nutrients applied as required, determined by petiole sap analysis. After one season, the average yield per 'normal practice' grapevine was 12 kg compared with 23 kg for those that continued to receive the 'high' inputs.

After the 2001 harvest, forty vines from each treatment were removed and examined for the presence of *Pa. chlamydospora*. For each vine, the trunk diameter was measured 10 cm above and below the graft union (Table 7.1). The full length of the trunk was cut into 10 cm cylinders, surface sterilised, dissected longitudinally and moist incubated for 12 weeks prior to examination using x40 dissecting

microscope. The presence of *Pa. chlamydospora* and other trunk disease organisms such as *Pm aleophilum* or *Botryosphaeria* species was recorded (Table 7.1).

Table 7.1. Yield, growth and presence of *Phaeomoniella chlamydospora* and other wood-inhabiting fungi in grafted Semillon that had recovered from Petri disease and been returned to normal vineyard practice, compared with that retained on high inputs of water and nutrients.

	Yield (kg)	Number of vines (out of 40) from which these fungi were recovered			Trunk diameter (mm)	
		Pa chlamydospora	*Pm aleophilum*	*Botryosphaeria* spp.	Above graft	Below graft
High input	23	39	15	11	33.9	30.4
Normal input	12	2	4	3	32.3	27.8

All grapevines, regardless of treatment, showed no external symptoms of disease. Growth (as measured by trunk diameter) and yield were lower in the vines returned to normal practice, as expected. However, the incidence of internal symptoms (data not shown) and *Pa. chlamydospora* was also much lower in the vines returned to normal practice. In 39 out of 40 'high input' vines, the pathogen was readily recovered from the full length of the trunks, both below and above the graft union (data not shown). By comparison, the vines that had been returned to 'normal' practice appeared to be relatively pathogen-free, and when pathogens were present, they were confined to the rootstock region below the graft union (data not shown).

In 2002, the whole vineyard was returned to 'normal' vineyard management. Although we have made no further measurements, the vineyard is reported to have performed well for a further three seasons, suggesting that infected vineyards can be remediated.

7.2 Single inputs: grafted Marsanne, central Victoria

In 1996, a new vineyard of Marsanne grafted onto Schwarzmann and Kober 5BB was planted in central Victoria on land that was previously used for dairy cattle. The grapevines struggled to establish, and showed young vine decline symptoms such as graft failure, poor growth, shoot dieback and some esca-like foliar symptoms within two years of planting. During 1998 and 1999, six samples were sent to Crop Health Services, and diagnosed with *Pa chlamydospora* and Petri disease.

In March 2000, twenty vines were chosen from two rows of Marsanne on Schwarzmann (every third vine from each row). The canopy fresh weight, number of bunches and fruit weight per vine were measured, then the vines were cut down and the trunks removed for internal examination. In the laboratory, the trunk diameters were measured at the first internode above and below the graft unions. The vines were then cut at 10 cm above and below the graft union and the presence of black goo symptoms noted. The graft unions were split open longitudinally, small slivers of wood were taken from areas where black streaks were apparent, surface sterilised and plated onto potato dextrose agar amended with antibiotic. The remainder of the graft union pieces were moist incubated. All 20 trunks had black goo symptoms in the rootstock and *Pa. chlamydospora* was present in all 20 graft unions. It had been intended to see if the presence of symptoms or infection affected canopy weight, bunch number or vine yield, but this became irrelevant as all were infected.

In 2000/01, the vineyard was used to test whether any single input used on the Semillon was effective against Petri disease. Two trials (one per rootstock/scion combination) were established across the site to compare the effect of single inputs on recovery or otherwise of the grapevines. The treatments were:

A. control (normal vineyard practice),
B. water stress (half the amount of water for normal practice),
C. green waste compost applied as a 60 cm wide strip of 10 cm deep mulch directly under the vines on 21 Nov 2000. This was a 'once-only' application, sufficient for 3 years (Kevin Wilkinson, pers. comm.)

D. phosphonate (2 x Agri-Fos® applications applied through the drip irrigation lines @ 5L/ha, at flowering and at veraison)
E. P:K fertigation, applied in the same fashion as D. The fertiliser was specially made up to contain the same P and K as Agri-Fos® as a check incase any AgriFos® effect was a nutrient response.

The trial design was a randomised block design with 10 blocks, each with one replicate (15 vines per replicate) of the five treatments.

In April 2001, yield (kg/vine) was measured on 3 vines per rep at harvest, and significant differences between treatments ($P<0.05$) determined using analysis of variance (ANOVA). In addition, each vine was examined for any esca-like foliar symptoms, and the incidence of vines showing symptoms was determined for each treatment – rootstock combination.

The compost treatment gave the highest yields in both rootstock combinations (Figs 7.1 and 7.2), but was only significantly higher than the control for Marsanne/Kober 5BB. The compost treatments also resulted in a higher incidence of esca-like foliar symptoms, particularly in Marsanne/Schwarzmann (Fig 7.3).

The treatments were continued for a second season (2001/02). However, due to a severe downy mildew infection and subsequent fruit thinning, no yield assessments were made. No esca-like foliar symptoms were observed throughout the entire season.

In the third season (2002/03), the region suffered from severe drought and no irrigation or treatments were possible due to lack of water. All grapevines were severely water stressed and fruit was thinned in February 2003, confounding any treatment effect on yield. The vineyard manager, however, reported an average site yield of 3 tonnes/ha of high B grade fruit. Once again, no esca-like foliar symptoms were observed.

In spring of the fourth season (November 2003), we assessed whether any of the treatments applied during the two seasons prior to the drought had impacted on the capacity of the infected grapevines to recover from a season without water. Growth was assessed by measurements of average shoot lengths (5 shoots/vine on 3 vines/rep) and trunk diameters below and above the graft unions (3 vines/rep) in the spring. Treatment differences ($P<0.05$) were determined using ANOVA.

For Marsanne on Kober 5BB, average shoot lengths of vines receiving the compost treatment were significantly shorter than the other treatments, yet for Marsanne on Schwarzmann, the control vines had the shortest shoots and the vines that had received restricted water had the longest shoots (Table 7.2). With respect to trunk diameter, both above and below the graft union, the compost treatment resulted in significantly thicker trunks on Marsanne/Kober 5BB vines, and this trend was also apparent for the Marsanne/Schwarzmann combination, but was not significant at $P<0.05$ (Table 7.2).

Season 4 was another drought year, and no water was applied to the vines for a second year. The vines were bunch-thinned according to canopy size, so no treatment effects on yield could be quantified. Average site yield was 4.5 tonnes/ha of high B grade fruit. No esca-like symptoms were observed.

In October 2004, we investigated whether the vines were still infected with *Pa. chlamydospora*. This involved destructive assessment and the vine from the centre of each replicate was removed and the trunk taken to the laboratory for further examination. A total of 100 trunks was harvested. Trunk diameters were measured 10 cm below and above the graft union, and differences between treatments ($P<0.05$) determined using ANOVA. The graft pieces were examined for black goo symptoms and then moist incubated (as described above) to test for the presence of the fungus.

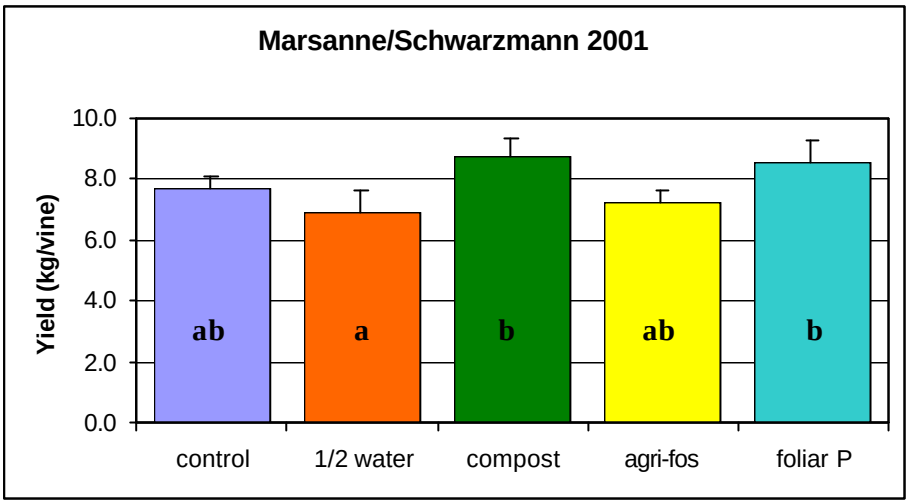


Figure 7.1. Effect of a single season of treatments on yield of Marsanne grafted onto Schwarzmann, previously diagnosed with Petri disease. Columns labelled with the same letter are not significantly different at $P<0.05$.

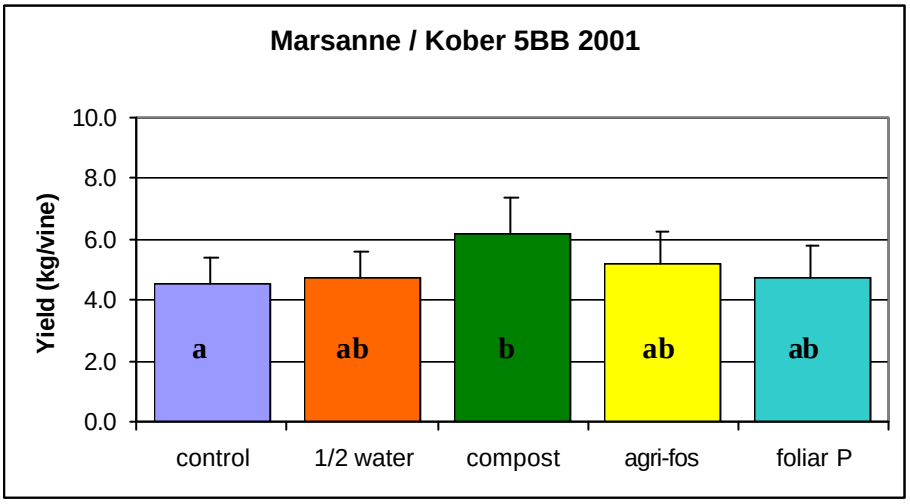


Figure 7.2. Effect of a single season of treatments on yield of Marsanne grafted onto Kober 5BB, previously diagnosed with Petri disease. Columns labelled with the same letter are not significantly different at $P<0.05$.

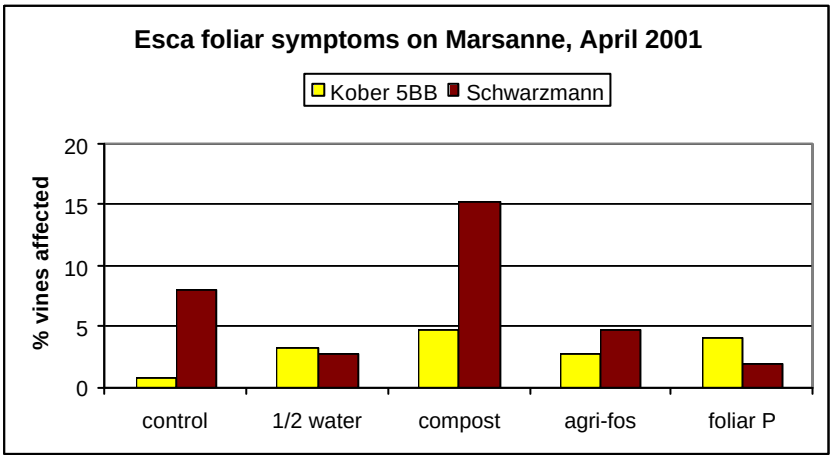


Figure 7.3. Effect of a single season of treatments on the incidence of esca-like foliage (April 2001) on Marsanne/Schwarzmann and Marsanne/Kober 5BB, previously diagnosed with Petri disease.

In both rootstock combinations, the vines that received the water stress treatment had the thinnest trunks and those that had the compost treatment had the thickest trunks in both rootstock combinations, although the difference was not always significant at $P<0.05$ (Table 7.2). The compost treated vines also had the lowest incidence of pathogen recovery, at 20% for both rootstock combinations compared with 60% and 80% respectively for Kober 5BB and Schwarzmann control vines (Table 7.2).

7.3 Therapeutic products, Brotomax® and Agri-Fos®, own-rooted Chardonnay and Verdelho

Two therapeutic products, phosphonate (Di Marco et al. 2000) and Brotomax® (Del Rio et al. 2001), were reported to show promise for treatment of several grapevine trunk diseases at the International Workshops on Grapevine Trunk Diseases held in 1999 and 2001. Following these reports, we established field trials to investigate this. Two vineyards, own-rooted Verdelho (planted 1999) in the Yarra Valley and own-rooted Chardonnay (planted 1987) in central Victoria, had sent samples of poorly-performing grapevines into DPI Knoxfield, which were subsequently diagnosed as infected with *Pa. chlamydospora.* The owners of the vineyards agreed to let us trial the two products, Agri-Fos® and Brotomax®.

Both trials were set up as a replicated block design with 3 blocks, each block containing 3 replicates of the 3 treatments, giving a total of 9 reps per treatment (3 panels per rep). Treatments were:

- control - normal vineyard practice excluding any phosphonate applications,
- 4 x Brotomax® at 1% volume,
- 3 x Agri-Fos® at 4ml/l.

The treatments were applied using a knapsack sprayer, ensuring all foliage, cordons and trunks were thoroughly wetted as Brotomax® is reportly absorbed through the wood as well as foliage. Brotomax® was applied at:

- when shoots were 20-30 cm long,
- three weeks later,
- at veraison and
- three to four weeks post-harvest.

Agri-Fos® was applied only twice during the season at Times 1 and 3, due to current restrictions on the use of phosphonate, followed by the post-harvest application at Time 4. Treatments were applied for three seasons, 2002/03, 2003/04 and 20004/05, with final assessments in spring 2005.

Annual assessments were made of growth and yield (Tables 7.3 and 7.4). Growth measurements included:

- trunk diameter, (5 vines per rep), measured 10 cm above ground level at Time 1,
- average shoot growth rate (5 shoots per vine, 3 vines per rep); determined by measuring shoot lengths at Time 1 and Time 2, and dividing the difference by the number of days between measurements (mm/day),
- and shoot length (5 shoots per vine, 3 vines per rep); determined by measuring shoot lengths at Time 1.

Yield was assessed as kilograms of fruit per vine, measured on two vines per replicate. In season 1 (2002/03), trunk diameter and yield were measured. In seasons 2 and 3 (2003/04, 2004/05), trunk diameter, shoot growth rate and yield were assessed. Unfortunately, yield data was missing for Verdelho in season 3 as the vineyard was harvested earlier than expected. In spring 2005, final measurements were made of trunk diameter and shoot lengths, but no treatments were applied. All the data was analysed using ANOVA, but there were no significant effects produced by either of the products on any of the variables measured (Tables 7.3 and 7.4).

Discussion

Petri disease is caused by the xylem-inhabiting fungus, *Phaeomoniella chlamydospora*. It is mainly a disease of newly-planted vines, causing lack of vigour in new vineyards, but can also affect older vineyards that have been subjected to stress. Like all plant diseases, however, Petri disease is the result of a complex interaction between four elements: the pathogen, the host, the environment and time (Pascoe 2002). Manipulation of any of these can affect the expression of disease symptoms and influence the economic impact of the disease. The common approach taken for disease management is to focus on management of the pathogen, usually by application of a fungicide. This approach is not very practical for trunk diseases as the pathogen is well protected inside mature woody tissues and difficult to target. A different approach is to manage not only the pathogen, but also the host, the environment and time, in order to favour the host. This involves manipulating the environment to provide conditions favourable to the host and manipulating the host to strengthen the host's intrinsic defence mechanisms.

The experience with the infected Semillon clearly showed that if multiple inputs were provided, the grapevines regained full health and productivity despite still being infected with the pathogen. We were perplexed by the higher incidence of pathogen recovery from the grapevines that continued to receive high inputs. A possible explanation is that under normal practice, the balance of water and nutrition provides enough energy for both plant growth and deployment of natural defence mechanisms, allowing the grapevine to restrict growth of the fungus. Under sustained high inputs, however, excess water and nutrients allows the fungus to also grow well, but without causing disease. To extrapolate further, if the grapevine is not receiving enough for its own needs, ie is under stress, then it cannot maintain effective defence mechanisms, fungal growth is not restricted, and the resulting impact on the stressed vine is disease.

The trials with Marsanne, Verdelho and Chardonnay attempted to separate out the inputs and determine if any single input was the key. Phosphonate has been reported to boost the natural defence mechanisms of the host (di Marco et al 2000), as has compost (Hoitink et al 2002) and Brotomax (del Rio et al 2001). In the Marsanne trial, only the compost treatment gave consistently higher growth and also reduced the incidence of the pathogen, supporting the theory proposed by Hoitink et al (2002) that it enhances the defence mechanisms of the host. Neither phosphonate or Brotomax demonstrated any significant effect in the Verdelho and Chardonnay trials.

The Chardonnay vineyard was 15 years old when diagnosed as infected with *Phaeomoniella chlamydospora*, and over the timeframe of the trial it became apparent that the vines were also severely affected by Eutypa dieback which prevented any recovery. However, the other vineyards were all diagnosed with Petri disease within 2 years of planting, and showed signs of recovery over the three to five years of observations, independently of treatment, indicating that time is also an important factor in management of Petri disease. This is supported by Californian research, where growers are now recommended not to crop for the first two years or to apply any water restrictions for up to five years (Gubler et al, 2006).

Table 7.2. The effect of water stress, compost, Agri-Fos and P:K fertigation on the recovery of Marsanne grafted onto Kober 5BB and Schwarzmann from Petri disease.

	April 2001		November 2003			October 2004			
	Yield (kg)	Esca (%)	Shoot length (mm)	Trunk diam. above graft (mm)	Trunk diam. below graft (mm)	Trunk diam. above graft (mm)	Trunk diam. below graft (mm)	Symptoms (%)	*Pa. chlamydospora* (%)
Kober 5BB									
Control	4.48a	0.7	384.7a	32.4a	30.8a	36.5a	26.3ab	70	60
½ water	4.73ab	3.3	363.2ab	33.0a	30.7a	32.7b	24.3a	50	30
Compost	6.20b	4.7	353.1b	35.4b	34.2b	36.1ab	28.4b	10	20
Agri-Fos	5.21ab	2.7	380.2ab	32.6a	29.4a	34.8ab	24.6a	70	30
Foliar P	4.75ab	4.0	369.8ab	32.9a	30.4a	33.7ab	24.1a	70	60
(l.s.d.)	(1.70)		(31.1)	(1.7)	(2.5)	(3.6)	(3.1)		
Schwarzmann									
Control	7.72ab	8.0	425.0a	35.3a	32.6ab	36.5a	27.6ab	80	80
½ water	6.85a	2.7	453.3b	34.1b	31.8ab	35.9a	28.2ab	50	20
Compost	8.79b	15.3	448.1ab	35.0a	32.8b	38.4a	29.4b	70	20
Agri-Fos	7.25ab	4.7	436.9ab	33.2b	30.8a	36.3a	26.1ab	60	30
Foliar P	8.56b	2.0	438.1ab	33.9b	31.1ab	35.0a	25.8a	70	50
(l.s.d.)	(1.57)		(25.6)	(1.5)	(2.0)	(3.7)	(3.3)		

Table 7.3. Effect of Agri-Fos® and Brotomax® on spring growth of Verdelho and Chardonnay grapevines infected with *Phaeomoniella chlamydospora.*

	2002	2003		2004		2005	
	Trunk diam (mm)	**Trunk diam. (mm)**	**Shoot growth rate (mm/day)**	**Trunk diam (mm)**	**Shoot growth rate (mm/day)**	**Trunk diam (mm)**	**Shoot length (mm)**
Verdelho							
Control	21.65	27.87	10.45	33.19	15.58	37.79	135.9
Agri-Fos	20.98	27.22	10.67	32.31	14.71	38.69	140.3
Brotomax	21.05	26.01	9.66	31.33	15.92	36.25	135.8
(lsd)	(1.70)	(2.15)	(1.03)	(2.57)	(1.04)	(2.48)	(11.8)
Chardonnay							
Control	35.41	35.90	9.45	35.30	12.84	37.71	171.1
Agri-Fos	34.12	34.51	8.35	34.54	11.94	36.45	176.7
Brotomax	34.36	34.38	8.41	34.58	11.80	36.89	162.3
(lsd)	(1.73)	(1.68)	(1.26)	(1.59)	(2.19)	(1.69)	(17.0)

Lsd: least signicant difference at $P<0.05$

Table 7.4. Effect of Agri-Fos® and Brotomax® on yields of Verdelho and Chardonnay grapevines infected with *Phaeomoniella chlamydospora*.

	2003			2004			2005		
	Kg/vine	**Bunch no.**	**Bunch wt (g)**	**Kg/vine**	**Bunch no.**	**Bunch wt (g)**	**Kg/vine**	**Bunch no.**	**Bunch wt (g)**
Verdelho									
Control	2.62	49.5	52.7	4.64a	69.1	65.0	na	na	na
Agri-Fos	2.94	53.1	57.5	4.38ab	67.9	65.7	na	na	na
Brotomax	2.58	53.6	48.7	3.20b	61.3	51.4	na	na	na
(lsd)	(0.68)	(10.0)	(10.9)	(1.30)	(12.0)	(15.2)			
Chardonnay									
Control	0.37	10.3	31.7	1.98	27.2	71.4	1.28	23.8	51.6
Agri-Fos	0.40	10.7	33.7	1.68	26.0	58.9	1.12	22.2	49.2
Brotomax	0.24	7.8	26.7	1.82	25.7	58.4	1.14	22.9	49.3
(lsd)	(0.25)	(5.7)	(11.8)	(0.74)	(8.6)	(16.3)	(0.41)	(6.5)	(8.9)

Lsd: least signicant difference at $P<0.05$
na: yield was not assessed on Verdelho in 2005.

Outcome/Conclusions

Petri disease and esca are grapevine trunk diseases that threaten the establishment and sustainability of Australian vineyards. Petri disease, formerly known as black goo, is an emerging problem in international grapevine culture. The description of several possible causal organisms, including *Phaeomoniella chlamydospora,* in 1996 led to the realisation that both the pathogens and the disease are widespread and important. At the start of this project, there was little understanding of the disease or of its economic effects. The same pathogens are involved in esca, currently the most destructive disease of grapevines in Europe. This project set about to determine which pathogens are the most important, how infection occurs, how it is spread, whether it can be prevented and effective methods of managing infected vineyards.

Both diseases are found in the major wine-grape growing regions of Australia, but Petri disease is currently much more common than esca. The fungus, *Pheomoniella chlamydospora,* is the primary cause of both diseases. It inhabits the xylem tissues of mature wood in grapevines and can be spread as spores in sap from infected mother vines into the cuttings harvested from them. Surveys of mother vines and rootstock source blocks indicate that the majority of mother vines are infected with *Pa. chlamydospora.*

Hot water treatment was shown to significantly reduce infection rates of dormant cuttings, but was less effective when used on 1-year-old rootlings. Best practice protocols for hot water treatment of cuttings were developed and disseminated to the nursery industry through a series of workshops and articles.

In the vineyard, the fungus produces its spores in protected cracks in the trunks of infected vines, and is known to be capable of infecting through wounds. By taking care to minimise the amount of mechanical damage caused to vines during production, the grower will also minimise the opportunities for *Pa. chlamydospora* to spread from vine to vine in the field.

Management practices that minimise stress to vines will allow diseased vines to recover from symptoms but not eradicate the fungal infection. The long-term prognosis for infected vines is uncertain at this stage.

During this project, we established that:

- esca is rare in Australia, whereas Petri disease is widespread.
- the pathogen associated with both diseases is *Pa chlamydospora.*
- *Pa. chlamydospora* sporulates in protected cracks on the grapevine trunk
- infection is passed from mother vines into cuttings, resulting in symptomless infected planting material
- *Pa. chlamydospora* spores can be detected in water used during nursery propagation, indicating there is potential for further infection in the nursery
- hot water treatment of dormant cuttings was the most effective method of reducing the incidence of infected planting material
- best practice nursery protocols were developed and disseminated to the nursery industry
- *Pa. chlamydospora* infection in grapevines increased stomatal conductance and reduced xylem function, resulting in reduced capacity to respond to water stress
- vineyards can recover from Petri disease over time with management practices that reduce stress eg mulch, bunch-thinning.

Recommendations

Considerable progress towards understanding the contributory factors of Petri disease and esca, how to prevent them, and how to minimise their economic impact was made during this project. Petri disease, which causes significant losses during the vineyard establishment phase, was shown to be widespread, whereas esca, currently the most serious grapevine disease in Europe, was shown to be rare in Australia. The causal organism, *Phaeomoniella chlamydospora,* is spread from infected mother vines into cuttings, and into newly planted vineyards via infected planting material. Long duration (30 minutes) hot water treatment of dormant cuttings was demonstrated to be an effective method of reducing the risk of producing infected planting material. Best practice protocols for the nursery industry were developed and promoted to encourage production of *P. chlamydospora*-free planting material. Glasshouse studies demonstrated that infected grapevines are more susceptible to water stress. Field trials showed that management practices that reduced stress, such as the use of mulch, reversed the symptoms of decline in infected grapevines.

Several recommendations can be made to industry to help prevent and manage these diseases:

- The nursery industry should consider routine hot water treatment of dormant cuttings prior to propagation to minimise the risk of distributing symptomless infected planting material
- Viticulturists should consider using only hot-water-treated planting material when establishing new vineyards
- Young grapevines in newly-planted vineyards should be allowed time to become well-established (ie 3-4 years) before imposing stresses such as water deficit irrigation or heavy crop loads
- Vineyards affected by Petri disease can be recovered over time with adequate stress management, such as the use of mulch, fertilisers and bunch-thinning.

Recommendations for future research include:

- There is still an urgent need for a non-destructive, rapid, robust diagnostic tool for detecting *Pa. chlamydospora* infection in grapevines for both diagnostic and research purposes. At present, it is only possible to confirm infection by destructive means (ie cutting down the vine and examining inside the trunk), which is costly and impractical for growers and does not allow researchers to adequately assess the progress of treatments. Towards the end of this project, we started to explore whether the molecular technique, real-time PCR, could be used to detect infection in buds and shoots of infected glasshouse-grown grapevines. Unfortunately, there was inadequate time to progress this further.
- Further investigation into the potential for contamination during nursery processes is required. Although we demonstrated the presence of *Pa. chlamydospora* in nursery samples, we did not determine whether that posed a real threat in terms of causing infection that results in infected young vines. Once again, real-time PCR showed the most promise as a tool for this purpose, but further research is required to refine the protocols into a robust, consistent method that can be confidently used, and to determine infection thresholds necessary to cause disease.
- It is unknown how infected grapevines will respond to deficit irrigation schedules, and whether these practices will have detrimental effects on long-term vineyard health. We recommend further research to understand and quantify the impact of reduced irrigation water inputs on the risk of disease development and to understand the mechanisms behind pest/disease host plant interactions under irrigation water deficit regimes.

- Two of the most important driving forces in Australian viticulture today are water shortages and rises in temperature due to climate change. Infections by grapevine trunk disease pathogens are perennial (ie a grapevine is infected for life) and symptomless, with disease expression triggered in response to environmental stress. This current project, along with its sister project, CRCV 2.2.4 Diagnosis and management of Eutypa dieback, has shown that these diseases are 'sleepers' and symptomless infection is widespread. It is important to determine how infected grapevines will respond to the challenges of increased water and heat stress, and how best to manage them for long-term vineyard health and productivity.

Appendix 1: Communications

Refereed scientific papers

Denman S, Crous PW, Taylor JE, Kang JC, Pascoe IG, Wingfield M (2000) An overview of the taxonomic history of *Botryosphaeria*, and a re-evaluation of its anamorphs based on morphology and TS rDNA phylogeny. *Studies in Mycology* 45: 129-140.

Edwards J, Laukart N, Pascoe IG (2001). *In situ* sporulation of *Phaeomoniella chlamydospora* in the vineyard. *Phytopathologia Mediterranea* **40:** 61-66.

Edwards J, Marchi G, Pascoe IG (2001). Young esca in Australia. *Phytopathologia Mediterranea* **40:** 303-310.

Edwards J, Pascoe IG (2001). Pycnidial state of *Phaeomoniella chlamydospora* found on Pinot Noir grapevines in the field. *Australasian Plant Pathology* **30:** 67.

Edwards J, Pascoe IG (2004). Occurrence of *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* associated with Petri disease and esca in Australian grapevines. *Australasian Plant Pathology* **33**: 273-279.

Fischer M, Edwards J, Cunnington JH, Pascoe IG (2005) Basidiomycetous pathogens on grapevine: a new species from Australia - *Fomitiporia australiensis. Mycotaxon* **91**: 85-96

Laukart N, J Edwards, IG Pascoe, NK Nguyen (2001). Curative treatments trialed on young grapevines infected with *Phaeomoniella chlamydospora*. *Phytopathologia Mediterranea* **40:** 459-463.

Pascoe IG, Cottral EH (2000) Developments in grapevine trunk diseases research in Australia. *Phytopathologia Mediterranea* **39**: 68-75.

Pascoe IG, Cunnington J, Edwards J, Cottral E (2004) Detection of the *Togninia* teleomorph of *Phaeoacremonium aleophilum* in Australia. *Phytopathologia Mediterranea* **43**: 51-58.

Waite H, May P. (2005) The effects of hot water treatment, hydration and order of nursery operations on cuttings of *Vitis vinifera* cultivars. *Phytopathologia Mediterranea* **44:** 144-152.

International conference papers

Constable F, Edwards J, Salib S, Wiechel T (2006) Comparison of the molecular tests - single PCR, nested PCR and quantitative PCR (SYBR®Green and TaqMan) – for detection of *Phaeomoniella chlamydospora* spores during grapevine propagation. 5th International Workshop on Grapevine Trunk Diseases, 11-15 September 2006, Davis, California, USA.

Cottral EH, Gaudzems G, Pascoe IG, Edwards J, Taylor PA (2003) Host-pathogen interaction of *Phaeomoniella chlamydospora,* causal organism of Petri disease, in grapevine tissue. 8th International Congress of Plant Pathology, Christchurch, New Zealand, 2-8 February.

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Edwards J (2003). Vine Dieback Diseases Symposium, 3-4 July, I.N.R.A. Centre de Bordeaux, France.

Edwards J, Salib S, Thompson F, Pascoe IG (2006) Effect of *Phaeomoniella chlamydospora* infection on the grapevine's physiological response to water stress. 5th International Workshop on Grapevine Trunk Diseases, 11-15 September 2006, Davis, California, USA.

Edwards J, Cunnington J, Salib S, Pascoe IG (2006) Basidiomycetes associated with heart rot of grapevines in Australia. 5th International Workshop on Grapevine Trunk Diseases, 11-15 September 2006, Davis, California, USA.

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Edwards J, Pascoe IG (2005) Experiences with amelioration treatments trialed on Petri disease in Australian vineyards. 4th International Workshop on Grapevine Trunk Diseases "Esca and Grapevine Declines", 20-23 January, University of Stellenbosch, South Africa. *Phytopathologia Mediterranea* **44:** 112 (Abstr.)

Edwards J, Pascoe IG (2005) The occurrence of Petri disease and esca and their causal organisms in Australia. 4th International Workshop on Grapevine Trunk Diseases "Esca and Grapevine Declines", 20-23 January, University of Stellenbosch, South Africa. *Phytopathologia Mediterranea* **44:** 109 (Abstr.)

Edwards J, Pascoe IG, Laukart N, Cunnington J, Fischer M (2001). Basidiomycetes isolated from esca-like heart rots of grapevines in Australia. 2nd International Workshop on Grapevine Trunk Diseases: Esca and Grapevine Declines, 14-15 September, Lisbon, Portugal.

Edwards J, Pascoe IG, Salib S, Laukart N (2003) Hot water treatment of grapevine cuttings reduces the incidence of *Phaeomoniella chlamydospora* in young vines. 8th International Congress of Plant Pathology, Christchurch, New Zealand, 2-8 February.

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Pascoe IG, Cottral EH (1999) Developments in grapevine trunk disease research in Australia. 1st International Workshop on Grapevine Trunk Diseases, 1-5 October, Siena, Italy.

Pascoe IG, Cottral EH (2000) Grapevine trunk diseases and sustainability of grape production. 5th International Symposium on Cool Climate Viticulture and Oenology, 16-18 January, Melbourne.

Pascoe IG, Cunnington J, Edwards J, Cottral EH (2003) Detection of the *Togninia* teleomorph of *Phaeoacremonium aleophilum* in Australia. 3rd International Workshop on Grapevine Trunk Diseases, 1-2 February, Lincoln, New Zealand.

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Edwards J (2002) What causes the grapevine trunk disease esca? NRE 2002 Horticultural Conference, 21-22 August, Knoxfield.

Edwards J (2003) Preventing Petri disease in grapevines. DPI Horticulture Conference, 26-27 August, Tatura.

Edwards J, Cottral EH, Pascoe IG, Joyce D (2002) *Phaeomoniella chlamydospora* – the common thread in the grapevine trunk diseases, Petri disease and esca. NRE 2002 Horticultural Conference, 21-22 August, Knoxfield.

Edwards J, Cottral EH, Pascoe IG, Joyce D (2002) *Phaeomoniella chlamydospora* – the common thread in the grapevine trunk diseases, Petri disease and esca. CRC for Viticulture 2002 Symposium, 17-18 June, Mildura.

Edwards J, Laukart N, Cottral EH, Pascoe IG (2001). Grapevine trunk diseases – Petri grapevine decline and esca. 11th Australian Wine Industry Technical Conference, 7-11 October, Adelaide.

Edwards J, Laukart N, Pascoe IG, Cottral E (2000). Grapevine trunk diseases. NRE Horticultural Conference, 6-7 September, Knoxfield.

Edwards J, Marchi G, Pascoe IG (2001). Young esca in Australia. 13th Australasian Plant Pathology Society Conference, 24-27 September, Cairns.

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Edwards J, Pascoe IG, Laukart N, Cunnington J, Fischer M (2001). Basidiomycetes isolated from esca-like heart rots of grapevines in Australia. 5th Australasian Mycological Society Conference, 28 September, Cairns.

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Fischer M, Edwards J, Cunnington JH, Pascoe IG (2005) *Fomitiporia australiensis*, a new species of *Fomitiporia* from Australian grapevines found associated with heart rot of grapevines. 15th Australasian Plant Pathology Biennial Conference, 26-29 September, Geelong.

Laukart N, Edwards J, Pascoe IG, Nguyen NK (2001). Can young grapevines infected with *Phaeomoniella chlamydospora* be treated to prevent Petri grapevine decline? A preliminary investigation. 13th Australasian Plant Pathology Society Conference, 24-27 September, Cairns.

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Industry magazines

Edwards J, Powell K, Granett J (2006). Tritrophic interactions between grapevines, phylloxera and pathogenic fungi – establishing the root cause of grapevine decline. *The Australian and New Zealand Grapegrower and Winemaker*: September.

Waite, H. (2006). 'Who is responsible for the quality of planting material in Australian Vineyards? Looking to the future', *The Australian and New Zealand Grapegrower and Winemaker*, April 2006, pp. 40-42.

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Australian and New Zealand Grapegrower and Winemaker 34th Annual Technical Issue: 32-37, August.

Key strategies emerge in the management of Petri disease and esca. *Australian Viticulture* Sept/Oct 2005, pp. 34-36.

Waite, H. 2005, 'Hot water treatment: cooling the confusion', *The Australian and New Zealand Grapegrower and Winemaker*, April 2005, pp. 34-36.

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What's happening in the world of grapevine trunk diseases? *The Australian and New Zealand Grapegrower and Winemaker* 498: 18-21, July 2005.

Edwards J, Pascoe IG (2004). Update on progress towards managing Petri disease and esca *The Australian and New Zealand Grapegrower and Winemaker* 488: 56-60, September.

Waite H (2004) 'Making Every Stick Count'. Coordinated series of articles arising from the Workshops, published in *National Grapegrower*, September – December Issues.

Waite H., Cole M., Jaudzems G, Faragher J. (2004) 'Recent advances in grapevine propagation research', *The Australian and New Zealand Grapegrower and Winemaker*, No 485a, pp. 39-41.

Edwards J, Pascoe IG (2003). The distribution and prevalence of Petri disease and esca in Australian grapevines. *The Australian and New Zealand Grapegrower and Winemaker* 476: 43-46, September.

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'Focus on trunk diseases at recent international workshop' in *CRC for Viticulture Newsletter* Vol 9 No 3 May-June 2003

'Hot water treatment of grapevine cuttings may prevent Petri disease (black goo decline)' *National Grapegrower* Pest and Disease Issue, November 2002.

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Edwards J, Pascoe IG (2002). Hot water treatment of cuttings shows promise as protection against the development of Petri disease. *The Australian and New Zealand Grapegrower and Winemaker* 464: 53-54

Edwards J, Pascoe IG (2002). Progress towards understanding and managing black goo decline (Petri disease) and esca *The Australian and New Zealand Grapegrower and Winemaker 30th Annual Technical Issue*: 81-86

'International grapevine trunk diseases effort' *CRC for Viticulture Newsletter* Vol 7 No 6 November-December 2001

'Trunk diseases are examined from all angles in a new research effort - Research in Progress' *Australian Viticulture* Vol 5 No. 4 July-August 2001.

'CRCV discovery sheds new light on trunk diseases' in *CRC for Viticulture Newsletter* Vol 7 No 4 July-August 2001

'Esca enquiry assisted by CRC' *Australian Viticulture*, Vol. 5, No. 3, May-June 2001, p.16.

'Visiting scientist sheds more light on esca' *The Australian Grapegrower & Winemaker*, No. 449, June 2001, pp. 25-26.

"Slow decline with trunk diseases", "Learning about black goo decline", "National trunk disease project" and "Esca and esca-like syndromes" in *National Grapegrowers*, September 2000.

"Esca or not" in *The Australian Grapegrower & Winemaker*, No. 439, August 2000, p.14.

Pascoe IG (1999) Grapevine Trunk diseases – black goo decline, esca, Eutypa dieback and others. *Australian Grapegrower and Winemaker* 429: 24-26.

Presentations, workshops and industry training/education:

Grapevine Trunk Diseases Workshop. 6th International Cool Climate Symposium for Viticulture and Oenology, Christchurch, New Zealand. 5-10 February 2006

Hot water treatment of grapevine cuttings. Evening discussion group, 6th International Cool Climate Symposium for Viticulture and Oenology, Christchurch, New Zealand. 5-10 February 2006. Attendees from New Zealand, Canada, Australia and Hungary.

'Making Every Stick Count' series of 2 day workshops for the vine nursery industry, Merbein 2004 and 2005, Griffith 2004. Total attendance >40 from Australia, New Zealand and the USA.

'Petri disease and esca research update' CRC for Viticulture Program 2 Industry Review Meeting, CSIRO Plant Industry, Merbein, 8 June 2005.

Development of nursery protocols for the production of healthy planting material. CRCV symposium, June 2005.

Research update to Australian Vine Improvement Association AGM, 2 December 2004, DPI Knoxfield.

Waite H (2004), Presentation on current HWT research to the Australian Vine Improvement Association AGM.

Waite H (2004 & 2005), Dookie research seminar series, presentation of HWT research results and outcomes.

Presentation to Victorian Winegrape Industry R&D Planning Forum, DPI Knoxfield, Vic. 12 October 2004.

National Grapevine Trunk Disease Meeting (CRCV 2.2.1 & S2.2.4), DPI Attwood. 30 July 2004

Pest and Disease Workshops, 12th Annual Wine Industry technical Conference, Melbourne. 24-29 July 2004.

CRCV project review meeting with Rob Walker and Jim Hardie, DPI Attwood. 25 June 2004

Presentation to Australian Vine Improvement Association AGM, DPI Knoxfield. 28 May 2004

GWRDC project review meeting, Knoxfield, 24 Feb 2004

Wicks, Gubler, Edwards (9 February 2004) – presentations to growers, Seppeltsfield Winery, SA

Collaborative meeting with CRCVS2.2.4 + Doug Gubler, and industry representatives, 4 Feb 2004, Plant Research Centre, Waite Campus, SA

Managing grapevine trunk diseases: Petri disease and esca. CRCV Program 2 Year 5 Review, Adelaide, 4-5 September 2003.

'Management of grapevine trunk diseases' Cool Climate Viticulture Forum, DPI Knoxfield, Vic., 21-22 August 2003.

'Trunk diseases and wound treatments' Grapecheque meeting, Lancefield, 11 July 2003.

'Trunk disease management – understanding the big picture' Mudgee, NSW, 30 May 2003.

'Trunk disease management – understanding the big picture' 2003 Hunter Valley Vineyard Seminar, Denman, NSW, 28 May 2003.

'Grapevine trunk diseases and selection of planting material' Orchard and Vineyard IPM Field Day, Dookie College, the University of Melbourne, Dookie, 29 October 2002.

'Trunk diseases (Eutypa dieback and Petri disease)' IPM Viticulture: Research to Practice Training Workshop, Swinburne TAFE, Lilydale, 10-11 October 2002.

'Trunk diseases (Eutypa dieback and Petri disease)' IPM Viticulture: Research to Practice Training Workshop, Rosebud TAFE, 4-5 September 2002.

Greater Victoria Wine Grape R&D Planning Forum, Bendigo, 9 July 2002.

'Petri disease and esca research update' CRC for Viticulture Program 2 Industry Review Meeting, CSIRO Plant Industry, Merbein, 19-20 June 2002.

'Trunk diseases and wound treatments' Grapecheque meeting, Geelong, 24 May 2002.

'Managing grapevine trunk diseases (Petri disease, esca, Eutypa dieback and others) that threaten the sustainability of Australian viticulture' Presentation to the GWRDC Board, 6 May 2002.

Workshop 76 Grapevine trunk diseases. Convenor: R. Sward. 11th Australian Wine Industry Technical Conference, 7-11 October 2001, Adelaide.

Guest lecturer: (2000 & 2001) 24th Oct 2001, 11th Oct 2000; Diploma in Vineyard Management and Winemaking, Northern Melbourne Institute of TAFE Epping Campus, Victoria.

'Grapevine trunk diseases – news from Italy and Portugal' Institute for Horticultural Development Learning Week Seminar, 14 November 2001

'The National Grapevine Trunk Disease Project' Deakin University School of Biological Sciences Seminar Series, 21 September 2001

Review of post entry quarantine protocols for the importation of grapevine budwood into Australia, AQIS Workshop, Knoxfield, 27 June 2001.

'Program 2.2.1 Grapevine Trunk Diseases' CRC for Viticulture Program 2 Two Year Review, 7-8 May 2001, Mildura.

'Epidemiology and control of black goo decline, esca and other grapevine trunk diseases' CRC for Viticulture Program 2 Industry Review Meeting, University of Adelaide, 5-6 October 2000.

'Black goo decline and other grapevine trunk diseases' Elders Workshop, Sunraysia Horticulture Centre, Irymple. 20-21 September 2000.

'Significance of the occurrence of trunk diseases in vineyards' National Vine Improvement Research Forum, Merbein. 30 August 2000.

'Trunk disease fungi and vine decline: A workshop on trunk diseases' Romeo Bragato conference, Port Nelson, August 2000.

'Biology and interactions of the trunk disease fungi involved in esca and black goo decline' Vine Health for Long Term Profitability NSW Wine Association Seminar, Grape and Wine Industry Training Centre, Wagga Wagga. 21 July 2000.

Presentation to visiting French vine improvement delegation, Knoxfield, 3 March 2000 – Pascoe

CRCV Board program overview meeting, Adelaide, 1 March 2000 - Pascoe

Presentation to Grapecheque group from NE Victoria at Knoxfield, 24 Feb 2000 – Pascoe

Program 2 meeting at Merbein, 18 Feb 2000 – Pascoe, Walker and McCarthy

Presentation to southern Grapecheque group, Lilydale, 28 Jan 2000 – Pascoe, Waite.

Presentation to north east Grapecheque group, Dookie, 27 Jan 2000 – Pascoe, Waite

Trunk disease workshop of the 5th International Symposium on Cool Climate Viticulture and Oenology, held at Bianchet Winery, Lilydale, on 19 January 2000

Workshop on '*Recognising grapevine trunk diseases'*, Blenheim NZ on 8 Nov 1999, - Pascoe. The talk and workshop in NZ succeeded in securing funding for a PhD student at Lincoln University, and an agreement to collaborate on research directions.

Presentation to the King Valley Grapegrowers 18 Oct 1999 – Pascoe

Visit to Knoxfield by Marion Chisholm, Cape Mentelle, WA to discuss black goo and esca, Sept 1999.

Presentation to SW Vic Grapecheque group, Portland, 6 Sept 1999 – Pascoe

Book chapters, Reports, other

Course notes for 'Unit: Plant Biology for Viticulture' in Diploma in Vineyard Management and Winemaking, Northern Melbourne Institute of TAFE. July 2000.

Edwards J (2003) 'Grapevine trunk diseases' in Victorian Farmers Federation Industry 2003 Buyers Guide Annual Directory.

Edwards J (2005) Updated version of Petri disease notes + Reviews of other disease notes for Viticare, CRCV (Ed. Donna Aitken).

Edwards J (2005) Updated notes on *Phaeomoniella chlamydospora* and Petri disease for Version 3 of BugMatch™ CD Rom, 2005, produced by CRC for Viticulture and C-Quentec.

Edwards J. (2002) Petri disease *In* IPM Viticulture: Research to Practice Training Manual. (Eds. D. Braybrook, A. Shanks and D. Aitken). 5th Edition. Cooperative Research Centre for Viticulture.

Pascoe IG, Edwards J, Cottral E (2001) Notes on *Phaeomoniella chlamydospora* and black goo decline for Version 2 of BugMatch™ CD Rom, June 2001, produced by CRC for Viticulture and C-Quentec.

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Application by Industry

Ian Pascoe attended and made a presentation to a Southcorp Vine Health group meeting, July 1999, to help Southcorp develop company-wide protocols for managing trunk diseases.

Two Southcorp Fact Sheets were produced in March 2000 for distribution to their vineyards, etc. They acknowledge the expert contributions of Ian Pascoe and Trevor Wicks to the content of these fact sheets.

King Valley growers have developed their own control program for Petri disease based in part on information provided by Ian Pascoe.

Personal contact: Inquiries have been received from nurseries and grape growers in Australia, the USA, Canada, New Zealand, The Ukraine and Romania.

Appendix 2: Intellectual property

Nil.

Appendix 3: References

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