Winegrowing Futures

Final report

Theme 2

Vine health and environment (protecting yield)











The National Wine and Grape Industry Centre is a research centre within Charles Sturt University in alliance with the Department of Primary Industries NSW and the NSW Wine Industry Association

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THEME 2 Vine health and environment (protecting yield)

Abstract

This project investigated three aspects of the biology and management of fungi that limit yield. Firstly we investigated fungi involved with the rotting of grape berries, otherwise known as bunch rots. The work concentrated on those organisms associated with bunch rots in grape growing regions of NSW that are prone to summer rainfall and in particular bitter rot and ripe rot of grapes. The project has led to the development of molecular based techniques for bunch rot identification and improvements in disease management.

Secondly, the identity, prevalence and distribution of Botryosphaeriaceae species in vineyards throughout winegrowing regions of New South Wales (NSW) and South Australia (SA) were also investigated. The presence, pathogenicity and genetic diversity of Botryosphaeriaceae from tissues other than wood were established. Botryosphaeriaceae were isolated from dormant buds, flowers, pea-sized berries and mature berries. Morphological and molecular identification revealed nine Botryosphaeriaceae species on grapevines in eastern Australia as well as *Eutypa lata* and other Diatrypaceae species. Eutypa dieback was more widespread in NSW than first thought. *In vitro* and field trials resulted in several fungicides that may be applied to pruning wounds to reduce Bot canker in vineyards.

Thirdly, the causes and control of young vine decline (YVD): a serious disease complex where newly planted grafted grapevines die soon after planting or have retarded growth and low yields until eventually dying in following seasons. YVD in the Riverina is caused by co-infection by two different wound/root-invading fungi. First *Botryosphaeria* is present in cuttings from infected rootstock mother-vines. These cuttings then become further invaded by *Cylindrocarpon* and probably again by *Botryosphaeria* in the nursery. *Cylindrocarpon* disrupts root function and *Botryosphaeria* invades the xylem. Although management practices, such as composts and the addition of biochar, can reduce stress and alleviate symptoms, the only viable solution is to prevent the sale of diseased young vines through improved nursery practices.

Summary

Bunch rots

This project extended earlier ARC and GWRDC funded research on bunch rot of grapes. The project initially investigated the types of fungi involved in the rotting of grapes, particularly from vineyards in Eastern Australia but with an emphasis on those fruit rots that occur in viticultural regions described as sub-tropical. Aspects of how environmental factors, such as rainfall, heat stress, etc. influence bunch rot occurrence were integral to the project. The work concentrated on two diseases: bitter rot, caused by *Greeneria uvicola* and ripe rot, caused by *Colletotrichum* spp. These two diseases were selected for a more detailed study because they were found to be the predominant bunch rots occurring in sub-tropical vineyards.

The project identified that bunch rot disease is often due to a complex of different organisms with more than one fungal pathogen on the one bunch and even on single berries. Factors that determine which disease is expressed, and more specifically competition on the berry surface between Botrytis and non-Botrytis bunch rots was investigated in relation to berry temperature.

This project has led to a greater understanding of the different organisms involved with bunch rots. Workshops on bunch rot identification have been presented at wine industry meetings and an extension poster on bunch rot identification has been distributed to grape growers. It is hoped that this will help grape growers to identify bunch rot diseases more successfully.

The project has led to the development of a rapid molecular based method (real-time PCR) for ripe rot and bitter rot identification. This tool will assist researchers in future projects, particularly in the unravelling of the epidemiology of non-Botrytis bunch rots, and may be used as a diagnostic tool in the future.

Further characterisation of bitter rot isolates from Australia and the USA is on-going in collaboration with researchers at North Carolina State University. Findings suggest that *G. uvicola* isolates from NSW and QLD tend not to vary as much as those isolates from North Carolina. Other findings from this work indicate that isolates originating from *Muscadinia*

rotundifolia are not as pathogenic on *Vitis vinifera* as those isolates that have originated from *V. vinifera*, irrespective of the country of origin. The collaboration with North Carolina State University has enhanced the findings of this project.

Prior to the commencement of this project, management options for bitter rot and ripe rot of grapes were limited. Findings from the research have demonstrated that grape vine flowers are susceptible to *G. uvicola* and *C. acutatum* infection and that flower infections have the potential to lead to subsequent bitter rot and ripe rot. This has important implications for the management of these two diseases.

Screening of potential fungicides for the chemical management of bitter rot and ripe rot revealed that the strobilurin chemical class were the most effective compounds examined. Standard fungicides used for grey mould or Botrytis bunch rot management were not as effective. Within the strobilurin group, Cabrio (active ingredient pyraclostrobin) was more inhibitory to *C. acutatum* and *G. uvicola* growth than either Amistar (active ingredient azoxystrobin) or Flint (active ingredient trifluoxystrobin).

In a series of trials involving detached flower, pot-grown and field-grown grapevines, Cabrio inhibited the infection of grapevine flowers and subsequent bitter rot and ripe rot by *G. uvicola* and *C. acutatum* respectively. This information will assist grapegrowers in the management of bitter rot and ripe rot of grapes.

Trunk disease

Botryosphaeriaceae species, the causal organisms of Botryosphaeria ('Bot canker') are recognised as important trunk disease pathogens of grapevines both in Australia and overseas. Limited information was available on the presence of Botryosphaeriaceae in Australian vineyards prior to the commencement of this study. This project aimed to identify the prevalence and distribution of Botryosphaeriaceae throughout the major winegrowing regions of New South Wales and South Australia to provide a foundation for improved disease management and prevention and to extend the first surveys conducted in the Hunter Valley by Dr Savocchia and colleagues.

Field surveys conducted in 91 vineyards across NSW and SA resulted in the morphological and molecular identification of eight species belonging to the family Botryospaheriaceae. These included *Diplodia seriata*, *Diplodia mutila*, *Lasiodiplodia theobromae*, *Neofusicoccum parvum*, *Neofusicoccum australe*, *Botryosphaeria dothidea*, *Dothiorella viticola* and *Dothiorella iberica*. The prevalence of individual species varied according to geography and climate. Species of *Diplodia* and *Dothiorella*, characterised by thick-walled, pigmented conidia were the most prevalent and were distributed widely throughout both NSW and SA. Species with hyaline conidia, such as *Neofusicoccum* and *Fusicoccum*, were isolated less frequently and displayed more limited geographic ranges, whilst only a single isolate of *Lasiodiplodia* was recovered, this being from the northern most region of NSW. During the same surveys the fungus *Eutypa lata*, causal agent of Eutypa dieback and other fungi belonging to the same family, Diatrypaceae (*Cryptovalsa ampelina* and species of *Eutypella* and *Diatrypella*) were isolated. Eutypa dieback was found to be more widespread in NSW than first thought, with confirmation that the disease is present both in the Central Ranges and southern NSW districts, regions recognised for their cooler climates and higher annual rainfall, both of which favour the growth of *E. lata*.

The isolation of Diatrypaceae prompted further investigation and collaboration was formed with Dr Mark Sosnowski (SARDI) and Drs Florent Trouillas and Doug Gubler (University of California, Davis) to investigate this further. Funds provided by the NWGIC, SARDI, and a GWRDC travel grant (GWT 09/05) allowed Dr Trouillas to spend three months at SARDI and the NWGIC further surveying and identifying Diatrypaceae of grapevines in NSW and SA. The results of this study can be found in the final report submitted to the GWRDC and in Trouillas *et al*. (2011). In brief, Diatrypaceae may also be contributing to the grapevine trunk disease syndrome and that these should be considered when adopting management strategies.

During the Winegrowing Futures non-*Botrytis* Bunch Rot project Botryosphaeriaceae were isolated from bunches from the Hunter Valley, therefore this prompted an investigation into the role of Botryosphaeriaceae as bunch rot pathogens. An intensive survey conducted by PhD student, Nicola Wunderlich in the Hunter Valley on dormant buds, flowers, pea-sized berries and mature berries resulted in the same eight species as described above being isolated and an additional, *Neofusicoccum luteum*. *Dothiorella viticola*, *Diplodia mutila* and *Neofusicoccum australe* were reported for the first time from grapevines in the Hunter Valley. All species were able to infect and cause symptoms on one year old canes and mature berries. Inoculation of dormant

buds did not affect bud burst or further development of shoots and fruit, however, a small number of Botryosphaeriaceae were re-isolated from bunches at later growth stages. Botryosphaeriaceae appear to be important pathogens of both the vegetative tissues and wood of grapevines. Grapevine wood infected with Botryosphaeriaceae could act as a source of inoculum for reproductive and vegetative tissue. Likewise, the vegetative and reproductive tissues could also act as sources of inoculum for infection of the woody tissues. Control strategies for trunk diseases caused by Botryosphaeriaceae are currently limited to remedial surgery and wound protection. Hence, in regions conducive to Botryosphaeria bunch rots all sources of inoculum should be taken into consideration when developing management strategies for Botryosphaeria bunch rot and Botryosphaeria canker diseases.

An analysis of the genetic diversity of Botryosphaeriaceae isolated from different tissues and vineyards in the Hunter Valley revealed the population to be genetically similar. In addition, isolates from within vineyards were genetically similar to those from outside the vineyard. A genetically uniform population will result in reduced probability that the Botryosphaeriaceae will develop resistance to fungicides.

Although the effect of infected flower and unripe berries on further bunch development and spread throughout the vines still has to be ascertained, there is enough evidence to suggest Botryosphaeriaceae needs to be controlled throughout the entire growing season. We therefore recommend considering Botryosphaeriaceae in Australian vineyards as both trunk disease pathogens and bunch rot pathogens and adjust control methods accordingly. The implications differ from many other bunch rot pathogens because under the right conditions infected wood provides a constant inoculum source and these infections cannot currently be controlled by fungicides generally used to control other bunch rot pathogens.

At the commencement of this project there were no effective fungicides available for the management of this disease in Australian vineyards. Fungicides registered for use on grapevines in Australia were tested in-vitro and then in the field in SA and the Hunter Valley for the protection of pruning wounds against Botryosphaeriaceae. This project had synergies with the Eutypa dieback project conducted at SARDI in that both aimed to find solutions for the protection of pruning wounds. The field trials established in South Australia were conducted in collaboration with researchers at SARDI whereby products were tested simultaneously as pruning wound protectants against Eutypa dieback and Bot canker. In both trials, Bavistin, Folicur, Garrison, Shirlan and Switch were effective in reducing Botryosphaeriaceae. Vinevax was effective in the Hunter Valley and ATCS tree wound dressing, BacSeal Super and Nustar applied at label rates were also effective in the trials conducted in SA. Folicur, BacSeal Super and Garrison are also the most effective protectants of pruning wounds for Eutypa dieback. It must be noted that although these products reduced the Botryosphaeriaceae none provided complete protection. This is most likely due to the endemic and widespread nature of the Botryosphaeriaceae in vineyards.

Spore trapping experiments were established in the Hunter Valley over two seasons to determine the environmental factors associated with the release of Botryosphaeriaceae spores in the vineyard. The data from these experiments is yet to be analysed as the principal investigator, Dr Savocchia, was on maternity leave at the time the trial was completed. The glass slide spore trapping method was adequate for detecting the spores of Botryosphaeriaceae and other wood-inhabiting fungi from the wood surface, however further studies would need to consider trapping spores that may be air dispersed. Other trapping methods such as the use of Burkard spore traps would need to be investigated. Preliminary analysis of the data suggests that spore release is correlated with precipitation. Once the data is fully analysed we hope to make preliminary recommendations on the optimal timing for winter pruning of vines in the Hunter Valley to minimise infection by Botryosphaeriaceae. Future studies should also be conducted in other regions as spore release is most likely influenced by climate.

To increase the awareness of grapevine trunk diseases in Australia, a number of presentations and hands-on workshops were held throughout the life of the project. The workshops focused on the identification and management of Eutypa dieback and Botryosphaeria canker. In addition, the results of this study were presented at national and international conferences, in industry and peer-reviewed publications. The contributors are currently preparing manuscripts for peer-reviewed journals. Future efforts should also be aimed at compiling an extension package for trunk diseases in general.

Young vine decline

Finally, we have shown that the cause of the YVD syndrome in the Riverina for grafted vines is the co-infection by two different wound or root-invading fungi, namely *Botryosphaeria* spp. and *Cylindrocarpon* spp., that infect grapevines at different stages of the propagation process. The general epidemiology of YVD is proposed: first, dormant cuttings, some of which are *Botryosphearia*-infected, are taken from source rootstock plants; next, infected cuttings from those plants contaminate uninfected cuttings during hydration soaking, callusing and/or storage; next, *Botryosphearia*-infected cuttings are invaded by *Cylindrocarpon* and possibly also by *Botrysphaeria* spp. when they are rooted in soil inhabited by those fungi. Consequently the rootstock stem below the graft and the graft union become internally infected with both *Cylindrocarpon* and *Botryosphaeria* while usually the scion, taken from less infection-prone source plants (due to higher above-ground, training and pruning practices) and subsequently subjected to less wounding and hydration soaking, is initially uninfected. As a result, *Cylindrocarpon* disrupts root function, and consequently retards early plant development while *Botyrosphaeria* spp. gradually invades the xylem vessels of the stem, both basipetally and acropetally, and contributes to the plant decline and eventual death. Although both *Botryosphaeria* and *Cylindrocarpon* alone can cause the decline and death of young grapevines, co-infection leads to more severe disease symptoms It is well known that symptoms from *Botryosphaeria* infections are especially severe in cases where the host plant has been subjected to water stress, so the reduced root function caused by *Cylindrocarpon*, which induces severe water stress even under non-water-logged vineyard conditions, probably acts as a stressor for the *Botryosphaeria* infection.

Background

The Vine Health theme addresses diseases and management of the grapevine. The research foci of the team include bunch rots, young vine decline and diseases of the wood.

Bunch rotting of grapes is caused by a wide range of organisms. Frequently several pathogenic organisms can be isolated from the one bunch and even a single berry on occasions. Determining which particular organism is responsible for primary rot is difficult, since visually many of these organisms appear the same when examined by the naked eye, (e.g. Bitter rot/*Greeneria uvicola*, Ripe rot/*Colletotrichum* spp. *Phomopsis viticola* and Bot Canker/*Botryosphaeria* spp.). Similarly a range of different organisms can be isolated from the woody tissues of the vine and identification based on disease symptoms can be difficult. These same organisms also attack vegetative tissues and are readily isolated from canes. While significant advances have been achieved in recent years in our understanding of non-Botrytis bunch rots (Melksham *et al.* 2002; Whitelaw-Weckert *et al.* 2007), disease management options remain far from successful. This problem arises from the fact that although these bunch rot organisms have been identified, a detailed knowledge of their pathology is lacking. For instance studies on the epidemiology of these organisms are scant, and while we know that certain factors pre-dispose berries to infection no models have yet to be developed that have practical benefits to growers. Bunch rot of grapes is frequently caused by a complex of organisms, unravelling the interactions requires an in-depth investigation into the pathogenesis of these diseases. A number of these organisms can cause a serious loss in wine quality, e.g. ripe rot infection levels as low as 1% lead to noticeable off-taints, and colour reduction (Meunier *et al.* 2005). Aside from a loss of yield there is also a loss of quality. This project involved both field trials and laboratory based studies to elucidate the biology and management of bunch rots of grapes. Various commercial vineyards in the Hunter Valley (10 sites), Hastings Valley (3 sites) and Griffith (1 site) were used as part of this study (*Figure 2.1*)

At the commencement of the Winegrowing Futures Program trunk diseases such as Eutypa dieback, Petri disease and Esca had already been identified in Australian vineyards and were known to affect productivity (Creaser and Wicks 2001; Edwards and Pascoe 2004; Lardner *et al.* 2005). Fungi recently isolated from diseased grapevine wood in the NWGIC laboratory include both *Phaeoacremonium* and *Phaeomoniella* species (Petri disease). *Cylindrocarpon destructans* which causes black-foot disease, a serious problem of grapevine in many countries, have also been isolated from vine roots and wood. In California, these fungi have all been implicated in the disease complex 'young vine decline'

More recently, Species of Botryosphaeriaceae fungi were also implicated in grapevine decline (Bot Canker) and research had begun to identify which strains were present in Australian vineyards and which of these are responsible for the decline and

dieback symptoms observed (Savocchia *et al*. 2007; Qiu *et al*. 2011). Symptoms of Bot Canker are similar to those caused by Eutypa dieback and include the distinct wedge-shaped lesion however no foliar symptoms have been recorded in Australia. Stunted shoots and loss of spur positions have also been identified. Bot canker appeared to be predominately a problem of white varieties such as Chardonnay and Semillon whereas Eutypa dieback was known to occur mainly in red varieties. It is difficult to estimate losses caused by trunk diseases in Australia however a recent study has suggested that these diseases result in a mean national economic impact of $6 million per annum (Scholefield and Morison 2010). Bot canker alone can result in 10 - 50% crop loss.

This project involved an integrated management system to restore the productivity of vines infected with *Botryosphaeria* canker. A survey of vineyards provided information on the extent of the disease and which varieties are mostly at risk. Field trials incorporating various control methods were conducted and abiotic environmental factors on disease progression were examined.

The majority of Botryosphaeriaceae found on grapevines are usually considered as secondary pathogens (Phillips 1998). However, it was not known whether this was the case for species found on grapevines in Australia, and whether certain stresses lead to these secondary pathogens becoming primary pathogens. The characterisation and identification of Botryosphaeriaceae species and the management of Bot canker was a major focus of this project

This final report also includes data gathered from a PhD study where the association and pathogenicity of Botryosphaeriaceae fungi was examined on different parts of the grapevine. The genetic diversity of the species isolated was also studied. This project was funded by a CSU PhD scholarship with additional funds provided to the student under the Winegrowing Futures program. This project involved both field trials and laboratory based studies to elucidate the biology, genetic diversity and management of Bot canker of grapevines.

1. Research on vine diseases described in this program was already in progress at NWGIC. Research conducted as part of GWRDC theme CSU 03/01 has revealed that Bitter rot and Ripe rot are the two predominant non-Botrytis bunch rots found along the eastern coastal regions of Australia. Management of bitter rot and ripe rot is currently not successful, although progress in this theme has revealed that a number of fungicide groups may offer some form of control. The timing of fungicide applications is currently under investigation, as is a detailed analysis of factors that predispose berries to infection. Both organisms have been recovered from vegetative parts of the vine, so management strategies to reduce the inoculum load in the vineyard are likely to be important. Further work on the biology of bitter rot and ripe rot fungi has indicated that they tend to infect grape berries at temperatures generally higher than that for the more widely occurring bunch rot pathogen *Botrytis cinerea* (grey mould) and that infection is encouraged under warm humid conditions.
2. Research to date has revealed that there are two species of *Botryosphaeria* associated with the decline symptoms observed in sub-tropical grape growing regions of NSW. Preliminary research on identification, pathogenicity and chemical control of these species is currently being undertaken by a PhD student (ARC Linkage) and GWRDC honours student (theme GWR H04/03) at the NWGIC.
3. Research performed at the NWGIC has established the involvement of the Petri disease fungi *Phaeoacremonium* and *Phaeomoniella* species in young vine decline in NSW. *Cylindrocarpon destructans* (which causes black-foot disease, a serious problem of grapevine in many countries) may also be implicated. We have also isolated *Phaeoacremonium aleophilum* from vineyard soil in the MIA and the sexual stage of *Phaeoacremonium* (Togninia) was recently identified from a moist incubated MIA grapevine wood sample.

Aims

The aims of Theme 2 were to:

1. Elucidate the organisms responsible for bunch rot, young vine decline and trunk diseases of vines
2. Improved detection and identification of vine diseases including where necessary disease surveys
3. Determine biotic and abiotic factors that influence pathogenesis in fungal diseases of vines
4. Development of models to predict disease outbreaks
5. Determine the most effective control methods for vine diseases

	Experiment	Aim/s Addressed
2.1	Fungal Isolate Collection	1
2.2	*In vitro* assessment of fungicidal efficacy	1
2.3	Fungicide field-trials	1
2.4	Flower infection studies	1
2.5	Berry infection studies	1
2.6	Comparison of the biology of the ripe rot fungi, *C. acutatum* and *C. gloeosporioides*	1
2.7	Development of a real time PCR method for the rapid detection of bitter rot and ripe rot	2
2.8	Studies on the overwintering of ripe rot	2
2.9	Comparison of bitter rot isolates from Australia and USA	1
2.10	Distribution of Botryosphaeriaceae species associated with grapevine decline in New South Wales and South Australia	2
2.11	Evidence that *Eutypa lata* and other *diatrypaceous* species occur in New South Wales vineyards	2
2.12	Association of Botryosphaeriaceae grapevine trunk disease fungi with the reproductive structures of *Vitis vinifera*	1 and 3
2.13	Biological factors affecting virulence of Botryosphaeriaceae on grapevines	1 and 3
2.14	AFLP analysis of Botryosphaeriaceae	1
2.15	Evaluation of fungicides for the management of Bot canker of grapevines	5
2.16	Spore trapping of wood-inhabiting fungi	1 and 2
2.17	Spatial variability of soil salinity and grapevine dieback in Chardonnay and Shiraz	6
2.18	Co-infection by *Botryosphaeria* and *Cylindrocarpon* spp. at different stages during propagation	1 and 5
2.19	Root infection of *Vitis Vinifera* by *Cylindrocarpon liriodendri*	1 and 5
2.20	Chemical and biological control of young vine decline	5

6. Develop vineyard soil tests/surveys to predict replant problems

Experiments

Bunch rots

Bunch rotting of grapes is caused by a wide range of organisms, frequently several pathogenic organisms can be isolated from the one bunch and even a single berry on occasions. Determining which particular organism is responsible for primary rot is difficult, since visually many of these organisms appear the same when examined by the naked eye, (e.g. Bitter rot/*Greeneria uvicola*, *Phomopsis viticola* and *Botryosphaeria*). These same organisms also attack vegetative tissues and are readily isolated from canes. While significant advances have been achieved in recent years in our understanding of non-Botrytis bunch rots (Melksham *et al*. 2002; Whitelaw-Weckert *et al*. 2007), disease management options remain far from successful. This problem arises from the fact that although these bunch rot organisms have been identified, a detailed knowledge of their pathology is lacking. For instance studies on the epidemiology of these organisms are scant, and while we know that certain factors pre-dispose berries to infection no models have yet to be developed that have practical benefits to growers. Bunch rot of grapes is frequently caused by a complex of organisms, unravelling the interactions requires an in-depth investigation into the pathogenesis of these diseases. A number of these organisms can cause a serious loss in wine quality, e.g. ripe rot infection levels as low as 1% lead to noticeable off-taints, and colour reduction (Meunier *et al*. 2005). Aside from a loss of yield there is also a loss of quality.

This project involved both field trials and laboratory based studies to elucidate the biology and management of bunch rots of grapes. Various commercial vineyards in the Hunter Valley (10 sites),

Hastings Valley (3 sites) and Griffith (1 site) were used as part of this study (*Figure 2.1*)

Experiment 2.1
Fungal isolate collection

Materials and methods

Fungal isolates were collected from a range of vineyards in NSW and QLD from bunch rot affected grapes and fungi identified according to Sutton and Gibson (1977), Dyko and Mordue (1979) and Barnett and Hunter (1998). Commonly isolated fungi from berries in these regions are shown in *Figure 2.2*. Representative isolates were deposited with the Plant Disease Herbarium of the Agricultural Scientific Collection Unit at NSW Department of Primary Industries in Orange, NSW.

Results and discussion

The organisms associated with bunch rots were examined in vineyards in the Hunter Valley between 2005 and 2008. The 2005–06 and the 2006–07 growing seasons were relatively dry, and the rainfall in the months from November to February were approximately half that of the 18 year long term average. Furthermore there were more days than average where the daily temperature equalled or exceeded 30°C. In both of these seasons, grey mould was largely absent from Hunter vineyards, although bitter rot caused by *Greeneria uvicola* was frequently isolated. On the other hand the 2007–08 season was a relatively wet one with the district experiencing double the average rainfall during the summer months and less days above 30°C than average (*Figure 2.1c*). Of note in this season was the occurrence of grey mould on both pre-véraison and post-véraison berries. The other major bunch rot pathogens occasionally encountered in vineyards were *Colletotrichum acutatum*, responsible for ripe rot (Steel *et al*. 2007), *Phomopsis viticola* (Savocchia *et al*. 2007) and Botryosphaeriaceae (Wunderlich *et al*. 2011). Photos of some of the bunch rots isolated are presented in *Figure 2.3*.

Experiment 2.2
In vitro assessment of fungicidal efficacy

Materials and methods

Five single-spore isolates of each organism were screened for sensitivity to a number of fungicides belonging to the anilide (boscalid), anilinopyrimidine (pyrimethanil), chlorophenyl (chlorothalonil), dicarboximide (iprodione), dinitroaniline (fluazinam), phthalimide (captan) and strobilurin (azoxystrobin, pyraclostrobin, trifluxystrobin) chemical groupings by determining radial growth rates on fungicide-amended Potato Dextrose Agar (PDA) (Oxoid) using a range of concentrations as previously described (Steel and Nair 1993) using three replications and four measurements per agar plate. Active ingredients were obtained from Riedel de Haën (Sigma-Aldrich, Sydney, Australia) and results expressed with respect to the percentage inhibition of radial growth at 1 µM.

Results and discussion

Captan, chlorothalonil, iprodione and pyrimethanil were all found to be weak inhibitors of *C. acutatum* and *G. uvicola* radial growth on agar plates (*Table 2.1*). Although fluazinam inhibited the growth of *C. acutatum* and *G. uvicola* this fungicide is only registered in Australia as a dormant spray for grapevines. Consequently fluazinam was not

Table 2.1 *In vitro* sensitivity of *Colletotrichum acutatum* and *Greeneria uvicola* isolates to fungicides. Results are expressed as percentage inhibition of radial growth at 1 µM. Isolate numbers refer to DAR isolates lodged with the Plant Disease Herbarium of the Agricultural Scientific Collection Unit at NSW Department of Primary Industries in Orange, NSW, Australia.

	***C. acutatum* isolates**					***G. uvicola* isolates**				
Fungicide	75574	77282	77283	77284	77285	77258	77261	77263	77271	77275
Azoxystrobin	21.3	23.4	23.4	29.9	39.5	48.1	32.1	32.3	31.8	41.2
Boscalid	11.2	7.6	24.4	5.5	1.3	0.0	0.0	0.0	0.0	0.0
Captan	24.2	1.5	26.6	0.0	0.0	10.5	11.1	1.3	1.0	0.7
Chlorothalonil	12.0	3.4	7.8	23.4	3.6	9.9	4.7	19.2	15.0	11.8
Fluazinam	87.1	82.6	88.7	85.6	92.3	100.0	100.0	100.0	99.3	98.8
Iprodione	0.0	0.0	12.0	18.7	4.2	10.7	21.9	18.1	16.7	13.6
Pyraclostrobin	82.7	67.0	80.2	96.7	83.0	69.0	100.0	66.5	74.4	50.3
Pyrimenthanil	9.2	17.9	14.4	36.2	3.3	0.0	7.5	0.0	0.0	0.0
Trifluroxystrobin	23.9	36.2	30.0	45.8	43.4	34.5	25.3	26.3	33.6	32.2



Figure 2.1 Examples of experimental vineyards where research was conducted. (a) Hastings Valley, (b) Hunter Valley, (c) Hunter Valley, wet conditions.



Figure 2.2 Non-Botrytis fungi commonly associated with bunch rots.

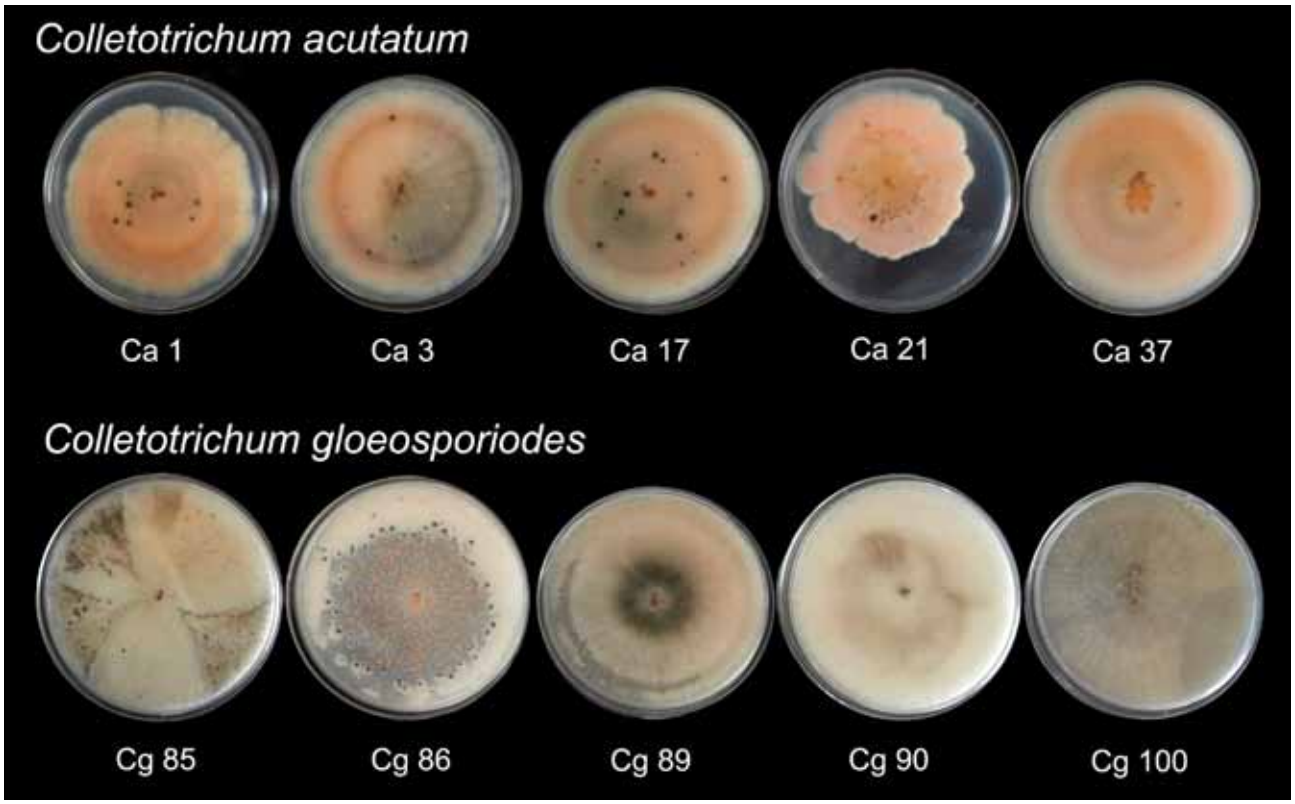


Figure 2.3 Representative *Colletotrichum* spp. isolates grown on PDA showing morphological differences in colouration and spore development. Underside of plate. Ca 1=DAR 75547, Ca 3=77282, Ca 17=77283, Ca 21=77284, Ca 37=77285) and five isolates of *C. gloeosporioides* (Cg 85=DAR 77292, Cg 86=77293, Cg 89=77296, Cg 90=77297, Cg 100=80218

examined in experiments involving fungicide sprays at flowering. The three strobilurin fungicides inhibited fungal growth to different degrees. Pyraclostrobin (Cabrio) inhibition of *C. acutatum* and *G. uvicola* on PDA is shown on *Figure 2.4b*. Pyraclostrobin was more effective than either azoxystrobin or trifluoroxystrobin, inhibiting growth of all isolates by more than 50% at a concentration of 1 µM. The concentration of pyraclostrobin required to inhibit radial growth by 50% for individual fungal isolates ranged from 0.02–0.11 µM for *C. acutatum* and 0.11–1.8 µM for *G. uvicola* (data not shown). Isolates of *G. uvicola* were more sensitive to trifluoroxystrobin than were isolates of *C. acutatum*. Boscalid did not inhibit the radial growth of any of the *G. uvicola* isolates at 1 µM and only weakly inhibited the growth of the *C. acutatum* isolates.

Although boscalid inhibited growth at higher concentrations (data not shown), EC50 values were in excess of 100 µM for all isolates tested.

Experiment 2.3
Fungicide field-trials

Materials and methods

Fungicide trials were conducted at commercial sites with histories of bunch rot occurrence. Site 1 located in the Hastings Valley 450 km north of Sydney planted with 10-year old Shiraz grapevines was used in the 2004–05 growing season. Sites 2 and 3, used in the 2005–06 growing season were both located in the Hunter Valley, 200 km north of Sydney and planted to 15 year-old Chardonnay and Shiraz vines respectively. Grapevines at all sites were 1.5 m apart with 2 m between the rows and trained to a Vertical Shoot Positioning (VSP) canopy. Each treatment plot comprised 3 rows × 8 grapevines that were replicated five times in a random block design. There was one guard row between plots and two guard grapevines between treatments.

In addition to the test treatments, all grapevines were sprayed with Delan 700WG (a.i. diathianon) and Topas EC50 (a.i. penconazole) when the shoots were 5 cm in length to control downy mildew (*Plasmopara viticola*) and powdery mildew (*Erysiphe necator*) respectively and with Bravo (a.i. chlorothalonil) when the shoots were 10 cm in length for grey mould (*Botrytis cinerea*) control.

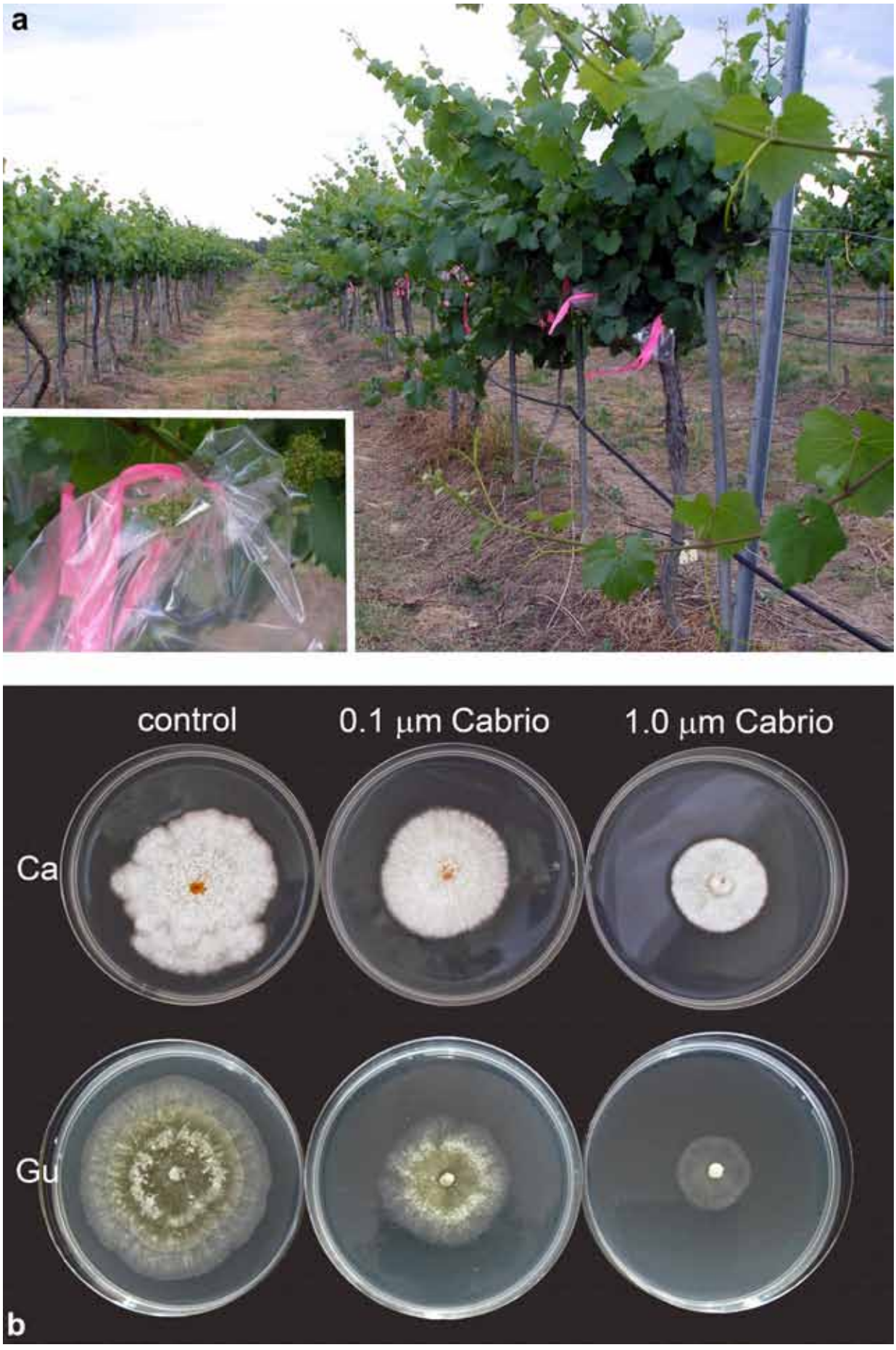


Figure 2.4 (a) Field trial of Cabrio fungicide treatment with inflorescences contained in plastic bags for 24 hours to maintain high humidity. (b) in vitro fungicide screening for Cabrio sensitivity of *Colletotrichum acutatum* (Ca) and *Greeneria uvicola* (Gu).

Cabrio and Filan were applied at one or more of the following growth stages, 10% cap fall, 80% cap fall or when the berries were pea size, (corresponding to the E-L phenological growth stages of 20, 25 and 31 respectively as described by Coombe (1995).

Grape bunches were scored for visible signs of bunch rot incidence (i.e. disease presence or absence on a single bunch) and severity by randomly selecting 100 bunches per treatment per replicate using a four point scale viz. 0=no rot symptoms; 1=one or two berries in a bunch with rot symptoms; 2=approximately one tenth to one quarter of the berries in a bunch with rot symptoms; 3=approximately one third to a half of the berries in a bunch with rot symptoms; 4=greater than half of the berries in the bunch with rot symptoms.

Results and discussion

A single spray of Cabrio at 10% cap fall led to a significant reduction in incidence and severity of bunch rot at berry maturity at Site 1 in 2004-05 (*Table 2.2*). A second application when the berries were pea size led to a further reduction in bunch rot, although this was not significantly different from the level of disease reduction achieved with a single spray at flowering.

At Site 2 in 2005–06, Cabrio applied when the berries were pea size reduced bunch rot severity but not incidence at harvest (*Table 2.3*). There was a further reduction when Filan was also used in the spray program at flowering however; this reduction in disease severity was not significantly greater than when Cabrio was applied in the absence of a Filan spray. The level of bunch rot incidence ranged from 72–84% at Site 2 in the 2005–06 growing season.

At Site 3 in 2005–06 all treatments with the exception of a single Filan application at 10% flowering led to a significant reduction in bunch rot severity, but not incidence at harvest (*Table 2.4*).

Table 2.2 Effect of Cabrio on the incidence and severity of bunch rot at vineyard Site 1 in 2004-05 at harvest. Letters in columns indicate significant differences (P<0.05).

Treatment		Bunch rot	
Cabrio (10% capfall)[a]	Cabrio (pea size) [a]	% Incidence	Severity
–	–	39 a	0.5 a
+	–	23 b	0.27 b
+	+	15 c	0.20 b

[a] + spray applied at the indicated phenological stage,
[a] – spray omitted from the trial at that phenological stage

Table 2.3 Effect of Cabrio and Filan on the incidence and severity of bunch rot at vineyard Site 2 in 2005-06 at harvest. Letters in columns indicate significant differences (P<0.05). There was no significant difference in the percentage of incidence between treatments.

Treatment[a]		Bunch rot	
Filan (10% capfall)	Cabrio (pea size)	% Incidence	Severity
–	–	84	1.74 a
–	+	75	1.35 b
+	–	72	1.24 b

[a] + spray applied at the indicated phenological stage,
[a] – spray omitted from the trial at that phenological stage

Table 2.4 Effect of Cabrio and Filan on the incidence and severity of bunch rot at vineyard site 3 in 2005-06. Letters in columns indicate significant differences (P<0.05). There was no significant difference in the percentage of incidence between treatments.

Treatmenta			Bunch rot at harvest	
Filan (10% cap fall)	Cabrio (80% cap fall)	Cabrio (pea size)	% Incidence	Severity
–	–	–	25.2	0.34 a
+	–	–	19.5	0.28 ab
–	–	+	20.3	0.27 bc
–	+	–	22.5	0.26 bc
+	+	–	20.7	0.24 bc
+	–	+	16.8	0.21 c

a + spray applied at the indicated phenological stage,
a – spray omitted from the trial at that phenological stage

Experiment 2.4
Flower infection studies

Materials and methods

Impact of temperature on flower infection

Grapevine inflorescences (cv. Chardonnay) were collected from a vineyard (100 km south of Wagga Wagga, NSW) by cutting the peduncle at mid-flowering. Inflorescences were surface sterilised using sodium hypochlorite (1% v/v) plus Tween 80 (0.05% v/v) for 2 min and successively rinsed three times with sterile deionised water before immersion in a spore suspension of either a mixture of *C. acutatum* (DAR 75574, DAR 77283 and DAR 77284) or *G. uvicola* (DAR 77258, DAR 77261 and DAR 77271) isolates in equal proportions to give a final spore concentration of 105 spores per mL. Control inflorescences were immersed in water only and each treatment was replicated 15 times. The peduncle of each inflorescence was inserted into Oasis floral foam (thickness 3 cm) saturated with water. The Oasis blocks containing the inflorescences were then incubated in sealed plastic bags in the dark at a range of temperatures from 15 to 30°C for 24 hours. Ten individual flowers were then removed from each cluster and surface sterilised in sodium hypochlorite (1% v/v) plus Tween 80 (0.05% v/v) for 2 min and successively rinsed three times with sterile deionised water before plating onto Dichloran Rose Bengal Chloramphenicol agar (DRBC agar base Chloramphenicol media supplement) (Oxoid) and incubating in the dark at 27°C for 7 days. The number of flowers per cluster infected with either organism was expressed as a percentage of the control treatments.

Flower infection of potted glasshouse-grown grapevines

Two-year old grapevines (cv. Chardonnay) growing in 330 mm diameter pots at 25°C (±5°C) in a glasshouse were used to determine the susceptibility of inflorescences to *C. acutatum* and *G. uvicola*. At mid-flowering, clusters were dipped in Cabrio (a.i. pyraclostrobin) (prepared according to label instructions for field applications) for 10 s. After five days the clusters were dipped in a 10^5 spore ml^{-1} suspension of either *C. acutatum* (DAR 75574, DAR 77283 and DAR 77284) or *G. uvicola* (DAR 77258, DAR 77261 and DAR 77271) prepared as described above while control grapevines were dipped in water. There were 15 replicates per treatment. Each cluster was sealed in a plastic bag to maintain high humidity. After 24 hours the bags were removed and the entire cluster was detached from the plant. Ten individual flowers were removed from each cluster and surface sterilised in sodium hypochlorite (1% v/v) plus Tween 80 (0.05% v/v) for 2 min and successively rinsed three times with sterile deionised water. The flowers were transferred to DRBC agar and subsequent fungal growth was transferred to PDA plates and incubated at 27°C. The number of flowers per cluster infected with either *C. acutatum* or *G. uvicola* was expressed as the mean percentage of infected flowers per cluster.

Flower infection studies in vineyard-grown grapevines

Twenty-year old grapevines (cv. Shiraz) vines located in the Hunter Valley, NSW (200 km north of Sydney) were sprayed with Cabrio (a.i. pyraclostrobin 250 g L^{-1}) at mid-flowering on 17 October, 2008 using a hand-lance attached to a Silvan Selecta 50 L "Spot Pak" sprayer (ATR18 hollow cone delivering nozzle, delivery rate 1.18 L min^{-1}, application pressure 3 bar). Control vines were unsprayed. After five days inflorescences were sprayed with an atomised spore suspension (10^5 spores mL^{-1}) of either *C. acutatum* (DAR 75574, DAR 77283 and DAR 77284) or *G. uvicola* (DAR 77258, DAR 77261 and DAR 77271) isolates mixed in equal proportions. Inflorescences (40 per treatment) were tagged with marker-ribbon for subsequent identification and sealed in a plastic bag (*Figure 2.4a*). All bags were removed after 24 hours. Twenty clusters were detached and collected 24 hours after inoculation and a further twenty clusters collected at véraison on 4 January, 2009. Depending on the phenological stage, either 10 flowers or five berries were detached from each cluster; surface sterilised and transferred to DRBC and PDA plates as described above. The number of flowers or berries per cluster infected with either organism was expressed as the mean percentage of flowers or berries infected per cluster.

Results and discussion

Susceptibility of grapevine inflorescences to C. acutatum and G. uvicola infection

Grapevine flowers were susceptible to infection by both *C. acutatum* and *G. uvicola* at a range of temperatures from 15–30°C. Flower susceptibility was particularly temperature dependant for *G. uvicola* with a marked difference in infection rate observed between 25 and 30°C and no significant difference in the number of flowers infected at 15 and 25°C (*Figure 2.5*). Overall flowers appeared to be more

susceptible to *C. acutatum* than to *G. uvicola* at the temperature range examined.

Having confirmed the susceptibility of detached grapevine flowers to *C. acutatum* and *G. uvicola* we then examined the susceptibility of flowers to fungal infection using glasshouse-grown and field-grown vines.

The percentage of flowers infected in glasshouse-grown vines held at 25±5°C (*Table 2.5*) was comparable with the results obtained with the detached flowers (*Figure 2.5*) at these temperatures. The percentage of flowers infected in the field was lower than that encountered in the glasshouse presumably because of the lower field-temperatures (approximately 15°C) at the time of the experiment.

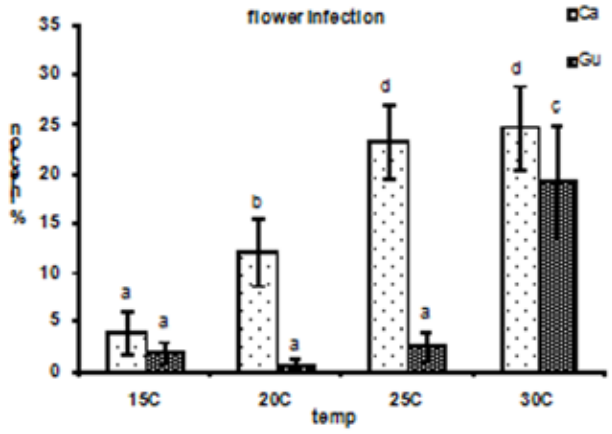


Figure 2.5 Effect of incubation temperature on percentage of detached *Vitis vinifera* (cv Chardonnay) flowers per cluster infected with either *Colletotrichum acutatum* (Ca) or *Greeneria uvicola* (Gu) 24 hours after inoculation. Controls showed no infection and therefore are not presented. Letters indicate significant differences between means. Bars represent standard errors of means.

Table 2.5 Mean percentage of glasshouse-grown *Vitis vinifera* (cv. Chardonnay) flowers per cluster infected with *Colletotrichum acutatum* (Ca) or *Greeneria uvicola* (Gu) following pre-treatment with Cabrio and assessed 24 hours post-inoculation. Letters in columns indicate significant differences (P<0.05)

	Mean percentage of flowers per cluster infected	
Treatment *	***C. acutatum***	***G. uvicola***
Water	0.0 a	0.0 a
Water + Ca	28.7 b	-
Water + Gu	-	15.3 c
Cabrio	0.0 a	0.0 a
Cabrio + Ca	5.3 c	-
Cabrio + Gu	-	2.0 ab

Effect of Cabrio on C. acutatum and G. uvicola infection of grapevine inflorescences

Flowers treated with Cabrio five days prior to inoculation, either under glasshouse or field conditions, resisted infection. Under glasshouse conditions Cabrio reduced the percentage of individual flowers infected with *C. acutatum* from 28.7 to 5.3% and from 15.3 to 2.0% for *G. uvicola* (*Table 2.5*). Neither pathogen was recovered from the non-inoculated flowers.

Cabrio applied mid-flowering to field-grown vines reduced *C. acutatum* infection from 11 to 6% at flowering and from 88 to 0% at véraison (*Table 2.6*). Similar reductions in *G. uvicola* infection were also observed, from 7 to 0% at flowering and from 86 to 2% at véraison (*Table 2.7*). Low levels of *G. uvicola* infection were present at the berry stage in non-inoculated flowers but this was not significantly different from the unsprayed and un-inoculated water control (*Table 2.7*).

Table 2.6 Mean percentage of flowers or immature berries per cluster infected with *Colletotrichum acutatum* (Ca) 24 hours post-inoculation (i.e. flowers) or immediately after véraison (i.e. berries). Letters in columns indicate significant differences (P<0.05).

	% flowers/berries infected with *C. acutatum*	
Treatment *	**Flowers**	**Berries**
Water	0 a	0 a
Water + Ca	11 c	88 b
Cabrio	0 a	0 a
Cabrio + Ca	6 b	0 a

* Cabrio applied five days prior to fungal inoculations.

Table 2.7 Mean percentage of field-grown *Vitis vinifera* (cv. Shiraz) flowers or immature berries per cluster infected with *Greeneria uvicola* (Gu) 24 hours post-inoculation (i.e. flowers) or immediately after véraison (i.e. berries). Letters in columns indicate significant differences (P<0.05)

	% flowers/berries infected with *G. uvicola*	
Treatment*	**Flowers**	**Berries**
Water	0 a	0 a
Water + Gu	7 b	86 b
Cabrio	0 a	1 a
Cabrio + Gu	0 a	2 a

* Cabrio applied five days prior to fungal inoculations, water treatments are controls. Gu indicates flowers were inoculated with *G. uvicola*.

Summary of flower infection studies

Detached inflorescences and inflorescences on pot-grown and field-grown vines were all susceptible to infection by *C. acutatum* and *G. uvicola*. Ripe rot and bitter rot of grapes tend to occur in relatively warm vineyard regions (e.g. sub-tropical Australia, south eastern states of the USA). Consistent with this observation, infection of detached inflorescences was greatest at 30°C for both organisms, although for *C. acutatum* this was not significantly different to the percentage infection at 25°C. The susceptibility of flowers at these temperatures is in contrast to observations on B. cinerea, a pathogen associated with cooler climates (Nair and Allen 1993; Broome *et al*. 1995). Our observation reported here on the susceptibility of grapevine inflorescences to fungal attack at different temperatures correlates with our earlier observations on the susceptibility grapevine berries (cv Cabernet Sauvignon) at different temperatures to Botrytis bunch rot, ripe rot and bitter rot infection (Steel *et al*. 2011). Our studies confirm that infection of inflorescences by either *C. acutatum* or *G. uvicola* may subsequently lead to ripe rot or bitter rot respectively in field-grown grapevines when assessed at véraison.

Although the life cycle of *G. uvicola* on grapevines is believed to involve infection via the pedicel in spring (Longland and Sutton 2008), the importance of flower infections and implications for disease management have not been studied previously. *C. acutatum* infects both vegetative and reproductive tissues, including the flowers of a wide range of host plants (Freeman 2008; Peres *et al*. 2008), the susceptibility of *V. vinifera* inflorescences to *C. acutatum* however has not been documented previously. We demonstrate here that glasshouse-grown (cv Chardonnay) and field-grown (cv Shiraz) grapevine inflorescences are susceptible to infection by *C. acutatum* and *G. uvicola*. It is therefore likely that flower infections may be important in the pathogenesis of bitter rot and ripe rot of grapes, in which case flowering may be an important growth stage for disease management practices to be implemented. Our observations on the incidence of bitter rot and ripe rot in open canopies coupled with heat stress damage (Steel and Greer 2008) suggests that canopy management practices for Botrytis bunch rot may be ineffective for bitter rot and ripe rot management in situations where fruit is exposed to high temperatures favouring the growth of bitter rot and ripe rot.

Cabrio applied at flowering reduced the level of infection of both pathogens and although there were differences between the glasshouse and field-grown grapevines in terms of the percentage flowers infected, this could be attributed at least partially to differences in temperature. Grapevines were maintained at 25°C in the glasshouse while temperatures in the field at mid flowering were closer to 15°C.

Recommendations for the control of ripe rot and bitter rot of both *Vitis vinifera* L. (Fukaya and Takahashi 1999; Sutton and Burrack 2011) and Muscadinia grapevines (Cline and Kennedy 2011) in countries other than Australia involve application of strobilurin and other fungicide sprays up to 14 days prior to harvest. These management options are not available to wine grape growers in Australia because of restrictions on the use of pesticide on grapes that are to be processed into wine for export markets. In Australia Cabrio cannot be used after bunch closure while Amistar (active ingredient azoxystrobin, also a strobilurin fungicide) can only be applied up to flowering (Essling *et al*. 2010). Despite these restrictions we have demonstrated that a single application of a strobilurin fungicide at flowering has the potential to limit the degree of ripe rot and bitter of grapes following berry set. A single fungicide application is unlikely to provide complete management of bitter rot and ripe rot, particularly in high-disease pressure seasons. However our results suggest that an application of a strobilurin fungicide at flowering should be incorporated into an integrated management strategy for bitter rot and ripe rot of grapes.

Experiment 2.5
Berry infection studies

Materials and methods

All berry infection analyses were prepared in the following way. Disease-free grape bunches (mean 13° Baumé) were collected from Chardonnay and Cabernet Sauvignon vines from the Charles Sturt University vineyard at Wagga Wagga (35.05°S, 147.35°E), a region not naturally prone to ripe rot infection. The vineyard was sprayed with Thiovit Jet (a.i. sulphur) after bud burst (EL growth stages 4–13), and with Topas (a.i. penconazole), Legend (a.i. quinoxyfen) and Flint (a.i. trifloxystrobin) at EL growth stages 15, 26 and 31 respectively, for powdery mildew control as needed. Berries were surface sterilised in sodium hypochlorite (1% v/v) plus Tween 80 (0.05% v/v) for 2 min, then successively rinsed three times with sterile deionised water. Single berries were placed into the wells of microtitre plates plus lids (24 well, flat-bottom, Iwaki Microplates) with 20 mL of water surrounding the wells to create a relative humidity (RH) of 100%, as shown in *Figure 2.6*.

Berries were inoculated as previously described (Steel *et al*. 2007). Fungal spore suspensions were prepared by dislodging spores from the surface of cultures growing on potato dextrose agar (PDA) into 3 mL of sterile deionised water using a sterile glass rod. Spore concentration was quantified using a haemocytometer and adjusted to 2×10^6 spores mL^{-1} by diluting with sterile deionised water. Berries were inoculated with a 10 µL droplet of spore suspension (i.e. 10^4 spores) placed near the distal apex of the berry. Control berries were treated with 10 µL of sterile distilled water. Each treatment was replicated three times with 24 berries per replicate. Berries were incubated for 5 days at either 20°C or 27°C under a 12 hour light/12 hour dark photoperiod and examined for fungal infection with a dissecting microscope and. Symptoms of fungal infection, including skin cracks, oozing, splitting, browning, bleaching, mycelial growth and presence of black pycnidia (*G. uvicola*) and acervuli with orange spores (*Colletotrichum* spp.), were noted (*Figure 2.6* and *2.7*). Results were expressed as the mean percentage of berries infected and analysed by ANOVA using GenStat (VSN International Ltd, Hemel Hempstead, UK).

Effect of temperature on Botrytis cinerea, Colletotrichum acutatum and Greeneria uvicola mixed fungal infection of Vitis vinifera grape berries

Cabernet Sauvignon berries (12.5° Baumé) were inoculated with *B. cinerea* (TN 030), *C. acutatum* (DAR 75574) and *G. uvicola* (DAR 77258) isolated from *V. vinifera* (Cabernet Sauvignon) as described above. Berries were inoculated with either single or combined isolates of the three fungi.

The three fungal genera could be differentiated from each other on the berry surface according to spore colour and morphology (Sutton and Gibson 1977; Dyko and Mordue 1979) and identification confirmed by aseptically removing spores or small areas of infected berry skin and sub-culturing onto DRBC agar with subsequent sub-culture to PDA.

Results and discussion

Temperature significantly influenced the infection of grapes by each fungal species. More berries were infected with *B. cinerea* at 20°C (92%) than at 27°C (65%) (*Figure 2.8a*). While *G. uvicola* infected berries at both temperatures, infection was significantly greater at 27°C (99% infection) than at 20°C (57% infection) (*Figure 2.8b*). In contrast, *C. acutatum* was equally and highly infective at both temperatures (*Figure 2.8c*).

Colonisation of grape berries by *B. cinerea* was reduced when co-inoculated with *C. acutatum* at either temperature but was only reduced with *G. uvicola* at 27°C (*Figure 2.8a*). Conversely, the percentage of berries infected with *C. acutatum* (*Figure 2.8c*) and *G. uvicola* (*Figure 2.8b*) was reduced by co-inoculation with *B. cinerea* at 20°C but not at 27°C. *G. uvicola* did not colonise berries at 20°C when co-inoculated with *B. cinerea* despite its ability to infect berries at this temperature when it was the sole inoculum. Co-inoculation with *C. acutatum* and *G. uvicola* led to a significant reduction ($P<0.01$) in the percentage of berries infected with *G. uvicola* at both temperatures but had no effect on infection by *C. acutatum*. These observations were confirmed by inoculating berries with all three bunch rot pathogens together. *B. cinerea* was the predominant pathogen at 20°C, whereas at 27°C, *C. acutatum* predominated.

B. cinerea is frequently associated with bunch rot of grapes in cool, wet climates and our results support previous findings regarding the optimum temperature of 20.8°C for berry infection (Nair and Allen 1993). Our observations on mixed berry inoculations at

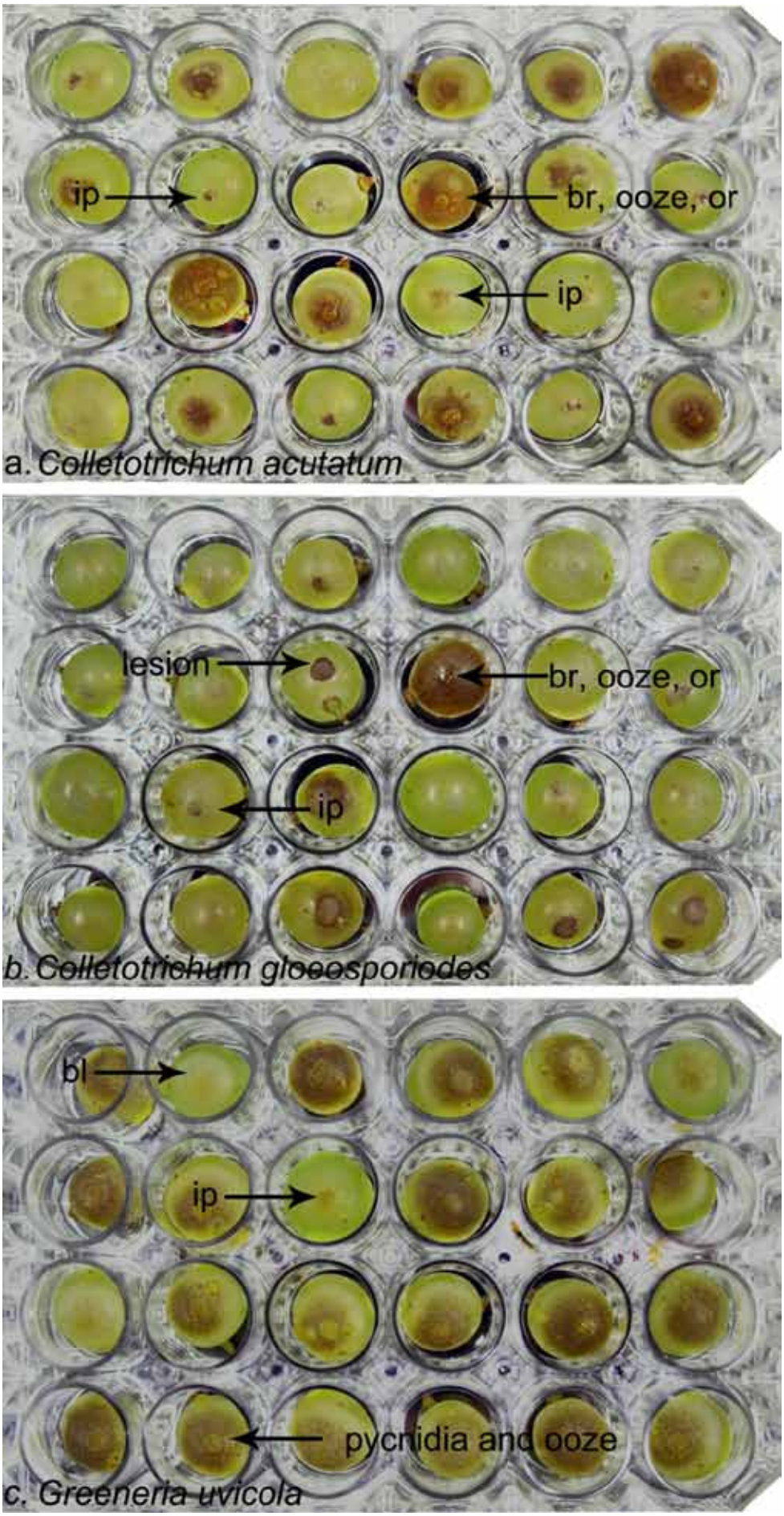


Figure 2.6 *In vitro* infection of berries with three fungal species and observation of disease development. ip - inoculation point, br – brown, ooze – oozing, or – orange spores, bl – bleaching.

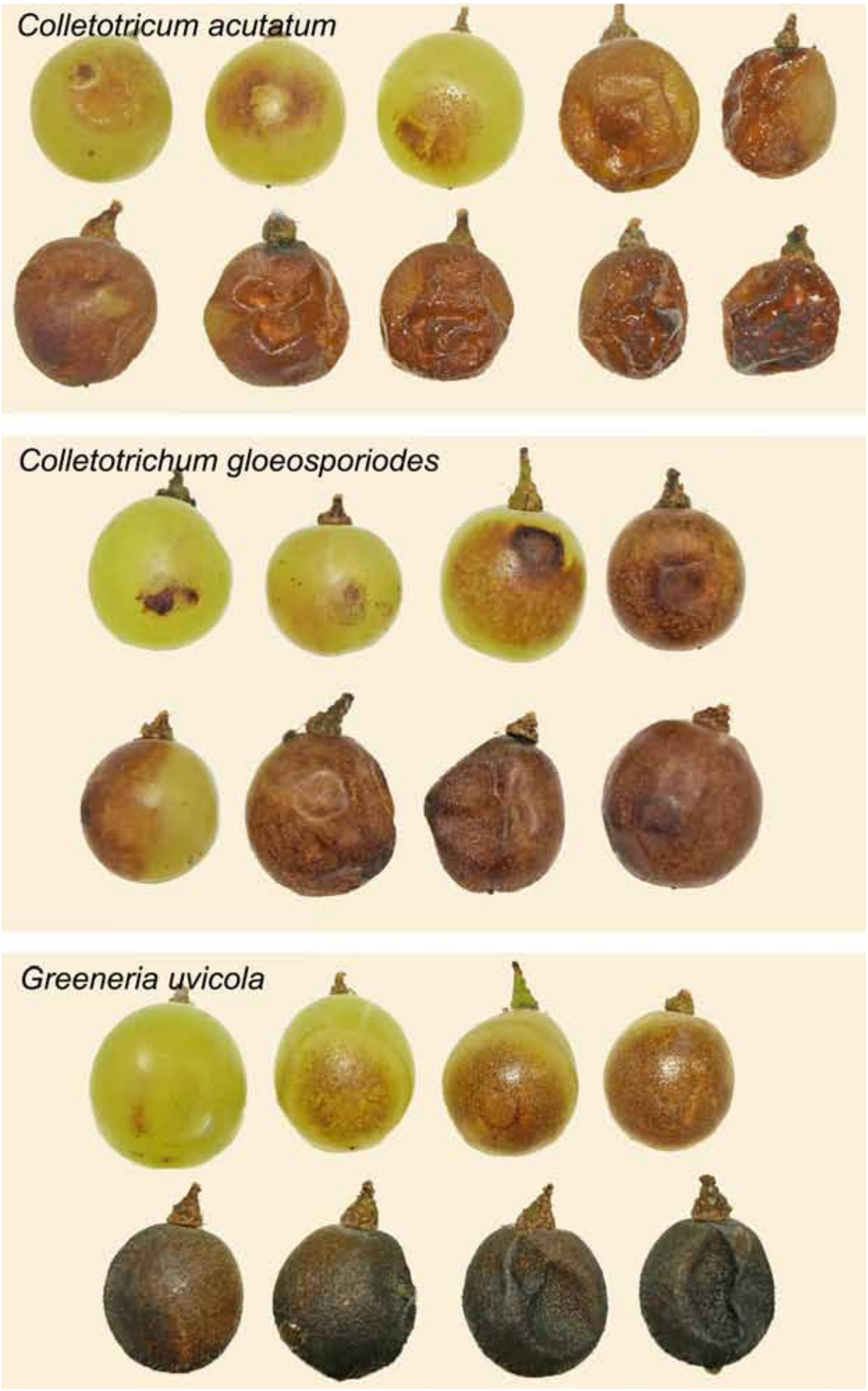


Figure 2.7 Stages of disease development of three fungal pathogens on Chardonnay berries.

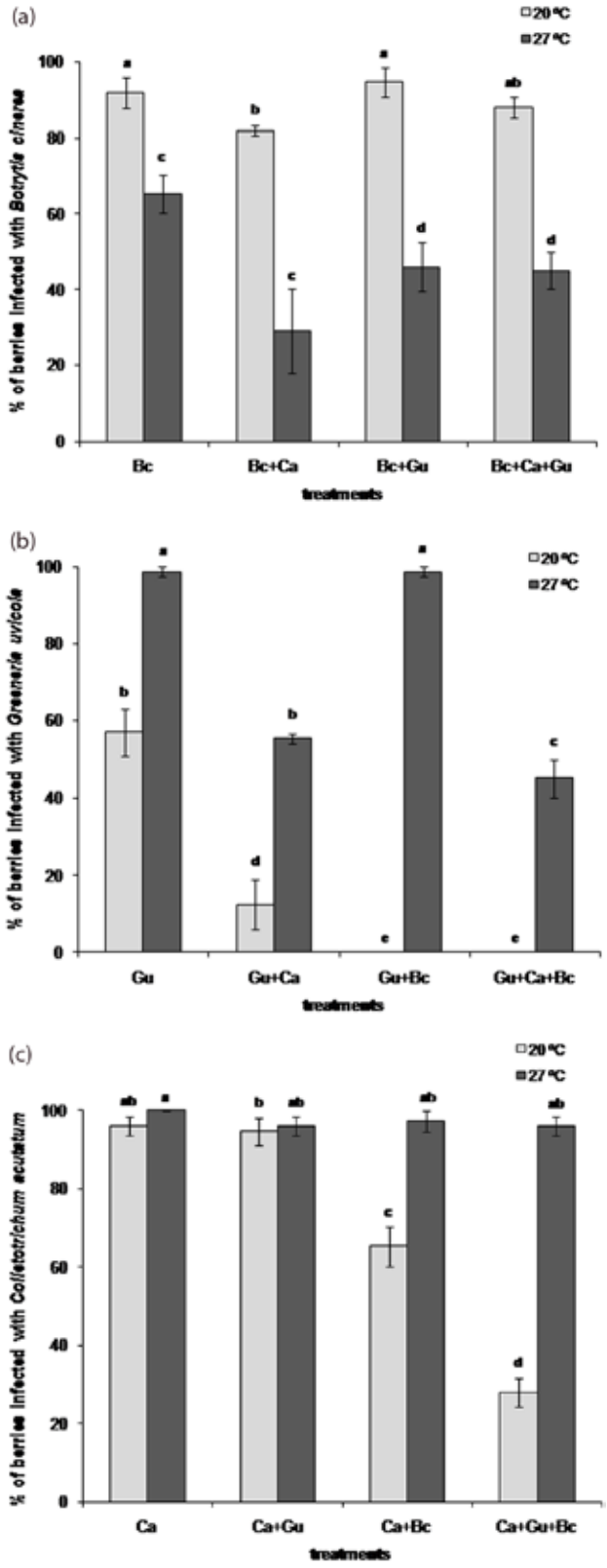


Figure 2.8 Effect of co-inoculating *Vitis vinifera* 'Cabernet Sauvignon' berries (12.5° Baumé) on (a) *Botrytis cinerea* (b) *Greeneria uvicola* and (c) *Colletotrichum acutatum* infection. Treatments relate to inoculation with each of the three organisms; Bc=*B. cinerea*; Ca=*C. acutatum*; Gu=*G. uvicola* either singularly or in combination at 20 or 27°C. Letters indicate significant differences ($P<0.01$). Bars represent standard errors of the mean.

27°C and our previous observations on the studies of hyphal growth of *C. acutatum* and *G. uvicola* on PDA at a range of temperatures (Steel *et al.* 2007), support our hypothesis that temperature influences the prevailing mix of bunch rotting pathogens in humid conditions.

C. acutatum and *G. uvicola* were first reported as bunch rot pathogens in Australia only recently (Steel *et al.* 2007). Long term historical records for bunch rot incidence in the Hunter Valley are lacking although earlier grape ripening patterns coupled with a recent increase in the frequency of high temperature seasons have been documented (Hall and Jones 2009). Rainfall events coupled with higher temperatures may explain observed changes in the bunch rot pathogen profile in the Hunter Valley, particularly in situations where rotting of the grape berries is due to a complex of different fungal organisms. Aside from the three bunch rot organisms described in this work, there are a number of other fungi that are largely opportunistic pathogens of grape berries (e.g. *Alternaria, Aspergillus, Penicillium*) (Pearson and Goheen 1988) and the interactions between each of these organisms, *B. cinerea* and the environment is unknown.

Our findings on the infection of grape berries by these three major fungal pathogens found in sub-tropical vineyards have broader ramifications for bunch rot management. *B. cinerea* is likely to become less of a problem in warmer, wetter regions, whereas other bunch rotting organisms such as *G. uvicola* and *C. acutatum* may predominate. However, proving this hypothesis conclusively would require extensive monitoring of field sites in conjunction with collection of micro climate data from the fruiting zone over several growing seasons. If predicted increases in temperature occur, adaptations to current control strategies are likely to be required. New strategies in fungicide selection and foliar canopy management will need to be considered, particularly as the well ventilated canopies used for *B. cinerea* control can expose fruit to temperatures favouring both bitter rot and ripe rot infection (Steel and Greer 2008).

Experiment 2.6
Comparison of the biology of the ripe rot fungi, *C. acutatum* and *C. gloeosporioides*

Materials and methods

Frequency of Colletotrichum isolation

A series of vineyards in the Hastings Valley, within a 30 km radius of Wauchope, NSW (152.7°E, 31.5°S) were sampled at berry maturity in February 2007 and February 2009 to determine the relative incidence of *Colletotrichum* species associated with ripe rot of grapes. In 2007 Shiraz vines were sampled from Vineyard 1 and Cabernet Sauvignon from vineyard 2. In 2009 sampling included the same Shiraz vines at Vineyard 1 and also Chardonnay from Vineyard 2 and Cabernet Sauvignon from a third vineyard. Sampling involved collecting 50 bunches at random from five rows of grapes at each site. Ten bunches were arbitrarily selected from each row. Five berries were selected from each bunch and fungal isolations made from surface sterilised berries as previously described (Steel *et al*. 2007). Frequency of *Colletotrichum* isolation was expressed as a percentage of the 50 bunches sampled. Identification was based on classical morphological techniques (Mordue 1971; Dyko and Mordue 1979; Barnett and Hunter 1998). The identity of the two species of *Colletotrichum* was further confirmed using a commercially available ELISA Diagnostic kit, (Colletotrichum acutatum IDENTIKIT™, ADGEN Phytodiagnostics, NEOGEN Europe LTD Ayr, Scotland) and sequencing techniques as previously described (Whitelaw-Weckert *et al*. 2007).

Fungal isolates

Studies on the growth rates and biology of *Colletotrichum* spp. were carried out using five isolates of *C. acutatum* (DAR 75547, 77282, 77283, 77284, 77285) and five isolates of *C. gloeosporioides* (DAR 77292, 77293, 77296, 77297, 80218) (*Figure 2.3*). DAR 77292 and DAR 77293 were homothallic while the other three *C. gloeosporioides* isolates were heterothallic. These isolates were obtained from grapevines located in sub-tropical regions of Australia using the methods of isolation as previously described (Steel *et al*. 2007) and cultures deposited with the Agricultural Scientific Collection Unit at NSW Department of Primary Industries, Orange, NSW, Australia.

Growth rates and fungicide sensitivity

Radial growth rates of the five isolates of each of the *Colletotrichum* species were determined by inoculating the centre of a PDA plate with a 6 mm plug of mycelium, obtained from the growing edge of a culture on PDA. Isolates were grown at 15, 20, 25, 30 and 35°C, in the dark, with the radial growth measured daily across 4 radii. Isolates of each species were screened for sensitivity to a number of fungicides belonging to the anilide (boscalid), benzimidazole (benomyl), phthalimide (captan), strobilurin (azoxystrobin, pyraclostrobin, trifloxystrobin) and triazole (triadimenol) chemical groups by determining radial growth rates on fungicide-amended PDA using a logarithmic range of concentrations from 0.1–100 µM at 25°C (Steel and Nair 1993). There were three replications for each fungicide/isolate combination and four measurements were taken for each and the experiment repeated three times. Active ingredients were obtained from Riedel de Haën (Sigma-Aldrich, Sydney, Australia), diluted in acetone, and results expressed with respect to the percentage inhibition of radial growth at 10 µM. Results from experiments on growth rates at different temperatures and inhibition in the presence of fungicides were expressed as the mean of three plates. Data were subjected to analysis of variance (ANOVA) using the GenStat statistical package (VSN International Ltd, Hemel Hempstead, UK). The Least Significant Difference (LSD) test was used for comparison of means of fungal growth at P=0.05.

Spore germination on 'cellulosic microscope slides'

Percentage spore germination of the five *C. acutatum* and five *C. gloeosporioides* isolates described above was determined at 25°C, in the dark, using 'cellulosic microscope slides' (Sammon and Harrower 2006). Two squares of sterile cellophane (2 cm^2) were attached with gelatine onto each microscope slide (Liberty HealthCare, Australia). Spore suspensions were prepared by dislodging spores in sterile distilled water from the surface of a culture growing on PDA. Spore concentration was determined using a haemocytometer and the concentration adjusted to 107 spores per mL, using sterile distilled water. Spore suspensions were sprayed onto the cellophane squares, to wetness, using a small atomiser. Inoculated slides were placed back to back in 50 mL screw-capped plastic Falcon tubes. Each tube contained moistened tissue packed in its base to achieve high humidity

for hydration and germination of the spores. Isolates were incubated for 8 hours with tubes being held upright to prevent wicking of moisture onto the slides. Spore germination was determined for each isolate in triplicate and germination was deemed to have occurred if a germ tube was present that was equal or greater in length than the spore. The slides were stained with lactophenol cotton blue (0.05% w/v) to arrest germination and stain the spores. Germinating and non-germinating spore counts plus hyphal length measurements were determined by light microscopy. Five photographs were taken over different fields of each slide, with a total of 100 spores counted per isolate. Spore viability was determined by staining spores with the LIVE/DEAD® BacLight™ Bacterial Viability Kit, (Invitrogen, Thornton, NSW 2322, Australia) and total spores counted under Brightfield illumination (Olympus U-MWIG2 Ex 520-550 nm, Em 580IF nm, Olympus AX70 True Research System Microscope). Non-viable spores fluoresced red under green excitation (wideband) and were counted and subtracted from the total spores to give the proportion of viable to non-viable conidia.

Infection studies

Spore suspensions of the 10 isolates of *Colletotrichum* spp. were prepared as described above. The microtitre plates containing the inoculated berries were placed in zip lock plastic bags and incubated at 27°C with 12 hours of light. Berries were examined daily for seven days post-inoculation, using a dissecting microscope. Fungi were re-isolated from a subset of berries and identification of *Colletotrichum* was confirmed satisfying Koch's postulates. Final results were expressed as the mean of the five isolates of each species.

Histology of the infection process

The infection of grape berries by *C. acutatum* (DAR 75547) and *C. gloeosporioides* (DAR 77293) was investigated using detached Chardonnay berries sourced from the Charles Sturt University vineyard. Berries were surface sterilised and placed into the wells of a microtitre plate as described above for the infection studies. A 10 µL drop of inoculum (10^5 spores per mL) was placed within a circle (4–5 mm diameter) drawn on the berry surface. Samples were taken at 4, 8, 12 and 18 hours post-inoculation by peeling off the inoculated area of epidermis with a razor blade. The skins were cleared in saturated chloral hydrate at 60°C overnight, rinsed in water, stained in lactophenol cotton blue (0.05% w/v) and mounted in 50% v/v glycerol. Fifty conidia per berry were assessed from five berries, i.e. 250 conidia were counted at each time point. Un-germinated conidia, germ tubes, non-melanised appressoria and melanised appressoria were counted and measured with results expressed as a mean.

Representative Nomarski Differential Interference Contrast Observation images, from the two *Colletotrichum* species at different stages of infection of the Chardonnay berries described above, were captured using Olympus ColorView analySIS® LS Research software (Soft Imaging System) and an Olympus AX70 microscope. Photographic plates were created using Adobe Photoshop CS4 Extended.

Results and discussion

Frequency of Colletotrichum isolation

Both *C. acutatum* and *C. gloeosporioides* were isolated from each of the three vineyards examined in this study. *C. acutatum* was the predominant species isolated from Cabernet Sauvignon and Chardonnay vineyards whilst the predominant species found at the Shiraz vineyard was *C. gloeosporioides* (*Table 2.8*). *C. gloeosporioides* isolations were further divided into those that produced perithecia (i.e. homothallic or *Glomerella cingulata*) and those that did not (i.e. heterothallic). The Cabernet Sauvignon vineyards had the highest overall frequency of infection out of the three vineyards examined in the two years of the study.

Table 2.8 Relative frequency of *Colletotrichum acutatum* and *C. gloeosporioides* isolations from grape berries at harvest at from three vineyards in 2007 and 2009. *C. gloeosporioides* isolates were further sub-divided in to those that produced perithecia (i.e. homothallic or *Glomerella cingulata*) and those that did not (i.e. heterothallic).

			Percentage frequency of isolation		
				C. gloeosporioides	
Year	Vineyard	Variety	*C. acutatum*	Homothallic	Heterothallic
2007	1	Shiraz	4	8.0	12.0
2007	2	Cabernet Sauvignon	84	15.7	4.3
2009	1	Shiraz	6	16.0	0.0
2009	2	Chardonnay	64	10.2	1.8
2009	3	Cabernet Sauvignon	82	7.5	2.5

Growth rates and fungicide sensitivity

All *C. acutatum* isolates produced fine, moderately compact, salmon orange mycelium on PDA which developed a grey hue, with orange spore masses. The underside of cultures were also salmon orange in appearance and dense in texture. *C. gloeosporioides* isolates were more variable but distinguishable from *C. acutatum* ranging from a dull grey-white to dark-grey appearance and producing more aerial mycelium. Some isolates produced black perithecia and frequently produced orange spore masses. Both had temperature optima for growth on PDA around 25°C although the overall rate of growth was greater for *C. gloeosporioides* (*Figure 2.9*). There was no significant difference in the sensitivity of the two species to azoxystrobin, pyraclostrobin, trifloxystrobin or boscalid. *C. acutatum* was significantly more sensitive than *C. gloeosporioides* to captan and triadimenol. Conversely *C. gloeosporioides* was more sensitive to benomyl than was *C. acutatum* (*Table 2.9*).

Differences in growth were also observed when spore germination and hyphal elongation were compared on cellulose coated microscope slides after 8 hours of incubation at 25°C (*Table 2.10*). A greater proportion of *C. gloeosporioides* spores germinated after 8 hours and the germ tube lengths were longer than those recorded for *C. acutatum*, consistent with the faster growth rates observed on PDA. The spores of the two species were easily distinguishable, *C. acutatum* (*Figure 2.10a*) were smaller (6.3–16.1 µM × 3.4–6.8 µM), smooth and clavate whereas *C. gloeosporioides* had larger spores (14.6–20.7 µM × 5.5–10.1 µM), cylindrical with obtuse ends containing a greater number of spherical bodies (*Figure 2.10b*).

Berry infection

The pathogenicity of both species was confirmed on detached Chardonnay and Cabernet Sauvignon berries at 27°C at a relative humidity of ~100%.

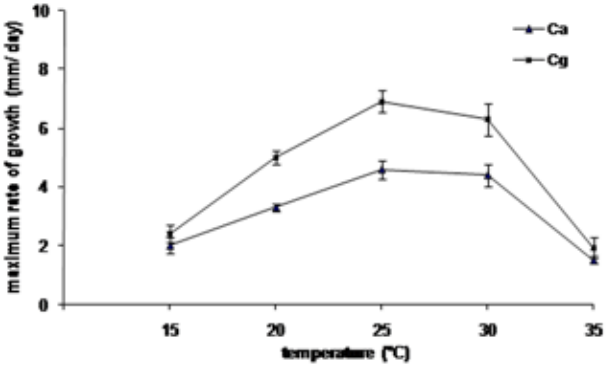


Figure 2.9 Radial growth rates of *Colletotrichum acutatum* (Ca) and *C. gloeosporioides* (Cg) on potato dextrose agar. Results are the mean of five isolates of each species

Table 2.9 Fungicide sensitivity of *Colletotrichum acutatum* and *C. gloeosporioides* as determined by growth on fungicide amended media. Results are the mean percentage inhibition of five isolates of each species when examined at a fungicide concentration of 10 µM. Letters in columns indicate significant differences (P<0.05).

Fungicide	*C. acutatum*	*C. gloeosporioides*
Azoxystrobin	29.1 a	26.90 a
Benomyl	53.1 a	97.02 b
Boscalid	9.2 a	4.80 a
Captan	36.8 a	0.06 b
Pyraclostrobin	82.1 a	81.30 a
Triadimenol	58.8 a	26.90 b
Trifloxystrobin	47.8 a	43.10 a

Table 2.10 Spore germination of *Colletotrichum acutatum* (Ca) and *C. gloeosporioides* (Cg) isolates following 8 hours incubation at 25°C on 'cellulosic microscope slides'.

Species	Isolate DAR#	% Germination	Mean germ tube length (µM)[a]	
C. acutatum	75547	60	41	(13–137)
	77282	26	38	(18–81)
	77283	52	48	(13–132)
	77284	31	59	(20–141)
	77285	56	32	(10 – 92)
Average for Ca isolates		45.0	43.6	(14.8–116.6)
C. gloeosporioides	77292	92	141	(19–352)
	77293	91	93	(23–194)
	77296	71	45	(17–153)
	77297	14	36	(19–71)
	80218	81	78	(16–185)
Average for Cg isolates		69.8	78.6	(18.8–191.0)

[a] Minimum and maximum germ tube length (µM) are presented in parentheses.

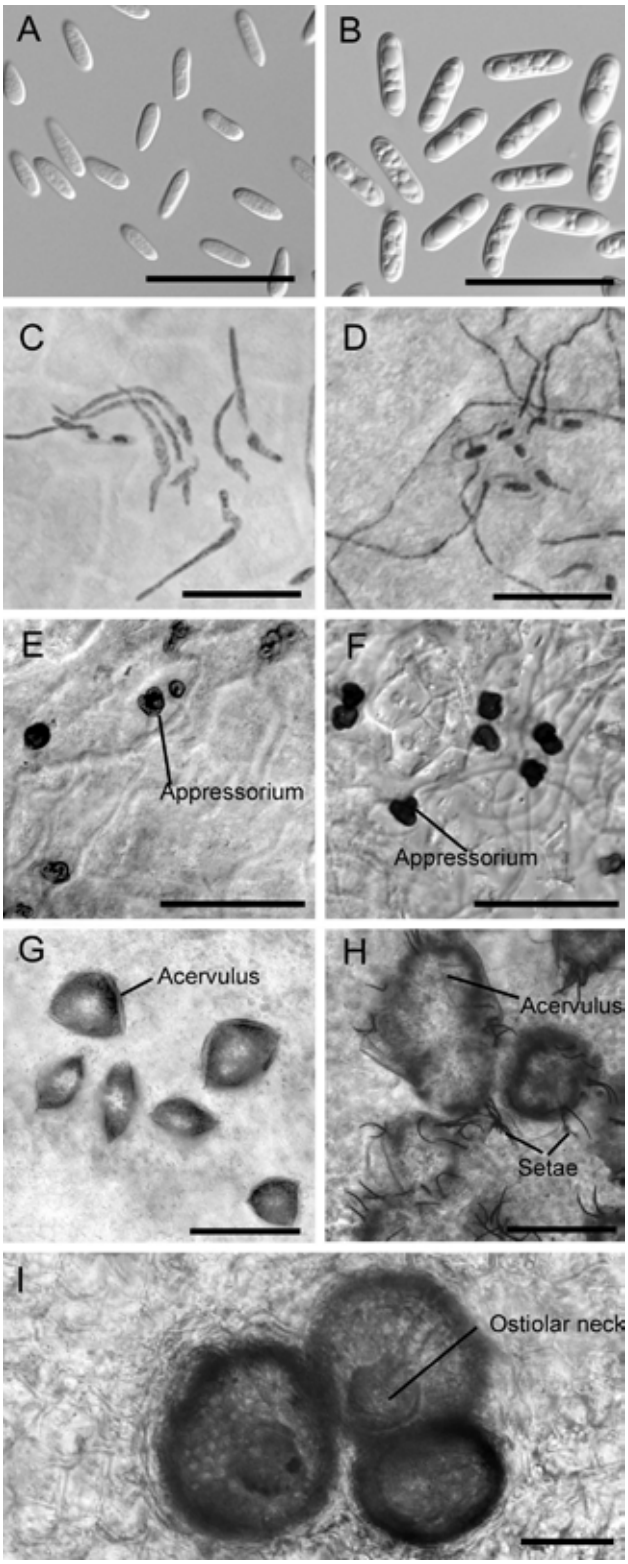


Figure 2.10 Comparison of spores, hyphae, appressoria and acervuli of *Colletotrichum acutatum* (A, C, E, G) and *C. gloeosporioides* (B, D, F, H, I). Images C to I are various time points following inoculation of *Vitis vinifera* var. Chardonnay berries. Scale bars in images A to D=30 µM; images E, F and I=50 µM and G and H=200 µM. A and B=spore appearance on a cellulose-coated glass microscope slide; C and D=hyphal growth 12 hours post-inoculation; E and F=appressoria formation 18 hours post-inoculation; G and H=acervuli production 48 hours post-inoculation; I =perithecia with ostiolar neck visible 10 days post-inoculation.

Despite *C. gloeosporioides* having faster growth rates on artificial media both grape varieties were infected more rapidly by *C. acutatum* (*Figure 2.11*).

Histological examination of infected grape berry skins (cv. Chardonnay) revealed that conidia of both *C. acutatum* and *C. gloeosporioides* germinated 8 hours after inoculation and that there were no differences in the time for spore germination. However, after 12 hours *C. gloeosporioides* had produced longer hyphae (mean=54.1 µM) than *C. acutatum* (mean=19.2 µM) (*Figure 2.10e* and *2.10f*). Appressoria began forming between 12 and 18 hours post-inoculation (*Figure 2.10c* to *2.10f*). Appressoria produced by *C. acutatum* were dark brown and obovate while those produced by *C. gloeosporioides* were a dark sepia-brown, also obovate and frequently lobed. Appressoria produced by the two species were a comparable size, approximately 8 µM in diameter. While *C. acutatum* tended to form more appressoria (*Figure 2.10e*) than *C. gloeosporioides* (*Figure 2.10f*), this was variable.

Both species produced acervuli 48 hours after inoculation, those produced by *C. acutatum* were

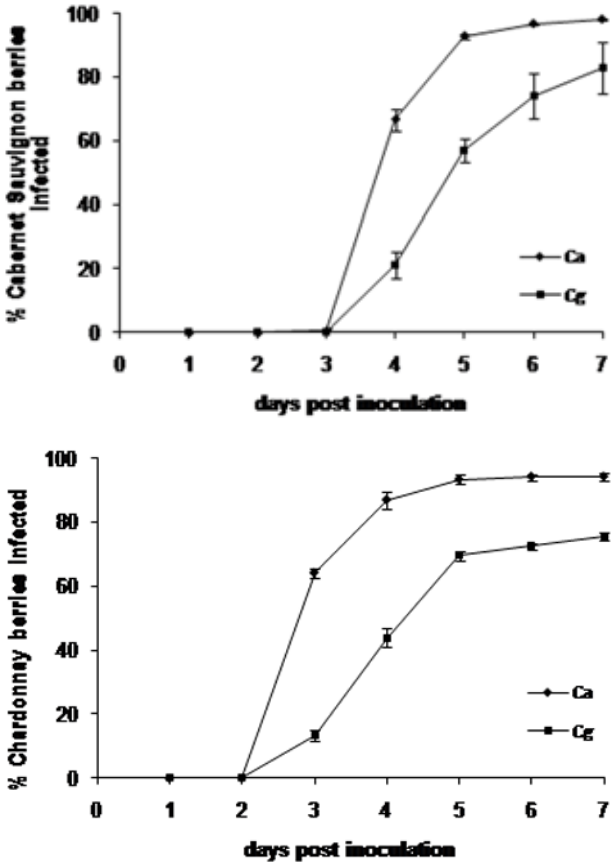


Figure 2.11 Percentage of (A) Cabernet Sauvignon and (B) Chardonnay grape berries with visible symptoms of ripe rot following inoculation with either *Colletotrichum acutatum* (Ca) or *C. gloeosporioides* (Cg). Results are the means of five isolates of each species.

typically 150–200 µM in length (*Figure 2.10g*) while those produced by *C. gloeosporioides* varied in size but were generally larger, typically 250–600 µM. Acervuli produced by *C. gloeosporioides* had melanised setae that were not present in *C. acutatum* (*Figure 2.10h*). Furthermore, perithecia were visible on berries inoculated with *C. gloeosporioides*, 7 to 10 days after inoculation (*Figure 2.10i*).

Summary of findings on the two species of Colletotrichum associated with ripe rot

Ripe rot of grapes was reported in Australia for the first time relatively recently (Melksham *et al.* 2002) and until now the disease was believed to be caused solely by C. acutatum (Whitelaw-Weckert *et al.* 2007). Another species, *C. gloeosporioides* is associated with ripe rot in countries other than Australia (Yamamoto *et al.* 1999; Whitten-Buxton and Sutton 2008). While *C. gloeosporioides* has been reported previously as causing ripe rot of grapes in Australia, (Emmett *et al.* 1992) the described symptoms appear to relate to another bunch rot organism, *Greeneria uvicola* (Pearson and Goheen 1988). Our further examination of three vineyards in the Hastings Valley, NSW coupled with molecular methods for fungal identification (Samuelian *et al.* 2011) confirms that both *Colletotrichum* species do in fact exist in Australia on *V. vinifera*.

C. acutatum and *C. gloeosporioides* isolates differed in their growth rates and sensitivity to a range of fungicides when assessed on PDA. A difference in the fungicide sensitivities of these two species is a trait that has been widely used for species differentiation in the past. Our observation that *C. acutatum* is less sensitive to benomyl and has slower growth rates than *C. gloeosporioides* is consistent with findings for isolates originating from rubber (Jayasinghe and Fernando 1998), peach and almond (Adaskaveg and Hartin 1997) but is in contrast to the benomyl sensitivity of *C. gloeosporioides* from citrus which appears to be more variable (Peres *et al.* 2004). Similar variability in benomyl sensitivity has been observed for apple isolates depending on whether they produce leaf spot and bitter rot or just bitter rot of the fruit alone (González and Sutton 2005). It is possible that some isolates of *C. gloeosporioides* originating from citrus have developed benzimidazole resistance following exposure to the fungicide. The grape isolates of *C. acutatum* used in this study were more sensitive than isolates of *C. gloeosporioides* to captan and the DMI fungicide, triadimenol.

Aside from the citrus isolates described above, *C. gloeosporioides* on a range of host plants can generally be controlled with benzimidazole sprays while use of benomyl as part of a disease management strategy is likely to be ineffective against *C. acutatum* (Bernstein *et al.* 1995; Freeman *et al.* 1998; Adaskaveg and Forster 2000). Benzimidazole sprays have been used extensively in the past to manage an unrelated bunch rot pathogen of grapevines, Botrytis cinerea (Leroux and Clerjeau 1985; Smith 1988). Although now rarely used in Australian viticulture, benzimidazole sprays have been used previously in regions such as the Hastings Valley. It is possible that in some vineyards, this has resulted in selection of *C. acutatum* as a bunch rot pathogen since it is naturally less sensitive to benomyl.

There was no significant difference in sensitivity to the three strobilurin fungicides, products which are registered for ripe rot management in countries other than Australia (Cline and Kennedy 2009), suggesting that this chemical grouping may also be effective for ripe rot management in Australia.

All five isolates of each species infected both Chardonnay and Cabernet Sauvignon and there was no variability in the rate of infection amongst isolates within a species. *C. acutatum* however infected grape berries at a faster rate than *C. gloeosporioides*. This work was repeated using just one isolate of *C. acutatum* (DAR 75547) and one isolate of *C. gloeosporioides* (DAR 77293) on Merlot, Riesling and Shiraz grape berries and a similar difference in the rate of infection between the two fungal species was observed. A 24 hour period at a RH~100% with subsequent transfer to an environment with a lower humidity (e.g. 40%) was sufficient for both species to infect berries, confirming earlier observations on *C. acutatum* (Steel *et al.* 2007). Since there was little difference in the relative susceptibility of the grape cultivars tested to the fungal isolates, subsequent histological observations were conducted on only one grape variety, Chardonnay.

While other studies have examined the histopathology of *C. gloeosporioides* on muscadine grapes, (Daykin and Milholand 1984*a*), we believe this to be the only study where the infection of *C. acutatum* and *C. gloeosporioides* have both been compared at the histological level on *V. vinifera*. At the cellular level no differences were observed in germination rates although *C. gloeosporioides* produced longer hyphae before producing appressoria. Appressoria were produced within 18 hours of berry inoculation,

faster than the three days reported for infection of *Muscadinia rotundifolia* (syn. *V. rotundifolia*) (Daykin and Milholland 1984*a*) by *C. gloeosporioides* but comparable for *C. gloeosporioides* infection of mango (Estrada *et al*. 2000). This suggests that *V. vinifera* is more susceptible to infection by *Colletotrichum* spp than is *M. rotundifolia*, an assumption consistent with observations on the screening of grape germplasm for resistance to *C. acutatum* (Shiraishi *et al*. 2007). Our observation that *C. acutatum* produces more appressoria than *C. gloeosporioides* on *V. vinifera* may explain the greater prevalence of the former species in vineyards. This may also explain why symptoms appear more rapidly on inoculated berries despite the fact that *C. gloeosporioides* has a faster growth rate in vitro on PDA and on microscope slides coated with cellulose.

The variability between isolates of *C. gloeosporioides* in terms of growth on PDA and observations at the cellular level on infected berries may be partially explained by the presence of the teleomorph or sexual stage, *Glomerella cingulata*, which we observed. In contrast, the sexual stage of *C. acutatum* is unknown.

This work confirms that both *C. acutatum* and *C. gloeosporioides* are responsible for ripe rot of wine grapes in Australia. Subtle differences in the infection process may explain the prevalence of each species isolated from vineyards. Differences in fungicide sensitivity may explain why *C. acutatum* was isolated with greater frequency from the vineyards examined. This information has wider implications in the management of bunch rot of grapes grown under sub-tropical conditions. Further examination of ripe rot susceptible vineyards over a greater time period is warranted.

Experiment 2.7
Development of a real time PCR method for the rapid detection of bitter rot and ripe rot

Materials and methods

Thirty-two *G. uvicola*, fifteen *C. acutatum* and nine isolates of other fungal species were isolated from vineyards in eastern Australia (*Table 2.11*). Pure cultures of single spore isolates were established according to Steel *et al*. (2007) and representative isolates maintained in a collection at the National Wine and Grape Industry Centre (NWGIC, Wagga Wagga, NSW, Australia), and deposited in the Australian Scientific Collection Unit (DPI NSW, Orange, Australia) (*Table 2.11*).

For the in vitro and field inoculation studies, mixtures of either *G. uvicola* DAR77258, DAR77260 and DAR77270 or *C. acutatum* DAR75574, DAR77283 and DAR77284 (*Table 2.11*) isolates were utilized. Mycelia and conidia were collected from 2-week-old cultures by adding 20 mL sterile water to the Petri dish and gently scraping the surface of the colony with a scalpel blade. Mycelial fragments were removed by filtration through a double layer of sterile cheesecloth. Conidia were counted using a haemocytometer and the concentration adjusted to 10^5 mL^{-1} conidia. Uninoculated controls were treated with an equivalent volume of sterile water.

Greenhouse inoculations

Inflorescences of disease-free three year old greenhouse-grown *Vitis vinifera* (cv. Chardonnay) grapevines, maintained in natural daylight with daily irrigation and temperatures ranging between 15 and 30°C, were inoculated by dipping them in 10^5 mL^{-1} conidia suspensions of either *G. uvicola* or *C. acutatum*. Two inflorescences were collected at 0, 4, 8, 16, 24, 48, 72, 96, 120, 144, 168 and 192 hours post inoculation. Ten flowers per inflorescence were surface-sterilized in 1% bleach (active ingredient 1.0% w/v sodium hypochlorite, Biolab) and 0.05% Tween 80 for 2 min, rinsed 3 times with sterile water and the presence (or absence) of the bunch rot pathogens was verified on DRBC followed by subsequent subculturing onto PDA and microscopic analyses. Results were expressed as mean percentage of infected flowers. The remaining parts of the inflorescences were analyzed by real-time PCR as each whole individual inflorescence was treated as an independent sample.

Table 2.11 Fungal isolates included in this study.

Species	Isolate	Geographic origin	Date of isolation	Cultivar	Host tissue
Greeneria uvicola	DAR77258	Upper Hunter Valley, NSW	2002	Chardonnay	Berry
	DAR77259	Hastings Valley, NSW	2004	Chambourcin	Berry
	DAR77260	Hastings Valley, NSW	2004	Chardonnay	Berry
	DAR77261	Hastings Valley, NSW	2004	Chambourcin	Berry
	DAR77262	Hastings Valley, NSW	2004	Cabernet Sauvignon	Berry
	DAR77263	Upper Hunter Valley, NSW	2004	Chardonnay	Berry
	DAR77264	Upper Hunter Valley, NSW	2004	Chardonnay	Berry
	DAR77265	Upper Hunter Valley, NSW	2004	Chardonnay	Berry
	DAR77266	Upper Hunter Valley, NSW	2004	Chardonnay	Berry
	DAR77267	Upper Hunter Valley, NSW	2004	Chardonnay	Berry
	DAR77268	Upper Hunter Valley, NSW	2004	Chardonnay	Berry
	DAR77269	Upper Hunter Valley, NSW	2004	Chardonnay	Berry
	DAR77270	Upper Hunter Valley, NSW	2004	Chardonnay	Berry
	DAR77271	Upper Hunter Valley, NSW	2004	Cabernet Sauvignon	Berry
	DAR77272	Upper Hunter Valley, NSW	2004	Cabernet Sauvignon	Berry
	DAR77273	Upper Hunter Valley, NSW	2004	Cabernet Sauvignon	Berry
	DAR77274	Upper Hunter Valley, NSW	2004	Cabernet Sauvignon	Berry
	DAR77275	Upper Hunter Valley, NSW	2004	Chardonnay	Berry
	DAR77276	Upper Hunter Valley, NSW	2004	Chardonnay	Berry
	DAR77277	Upper Hunter Valley, NSW	2004	Chardonnay	Berry
	DAR77278	Upper Hunter Valley, NSW	2004	Chardonnay	Berry
	DAR77279	Upper Hunter Valley, NSW	2004	Chardonnay	Berry
	DAR77280	Upper Hunter Valley, NSW	2004	Chardonnay	Berry
	DAR77281	Upper Hunter Valley, NSW	2004	Chardonnay	Berry
	MA231a	Lower Hunter Valley, NSW	2006	Shiraz	Wood
	MAC11a	Lower Hunter Valley, NSW	2006	Chardonnay	Wood
	MD25a	Lower Hunter Valley, NSW	2006	Semillon	Wood
	MD53a	Lower Hunter Valley, NSW	2006	Semillon	Wood
	MF322a	Upper Hunter Valley, NSW	2006	Chardonnay	Wood
	MF522a	Upper Hunter Valley, NSW	2006	Chardonnay	Wood
	MG53a	Upper Hunter Valley, NSW	2006	Cabernet Sauvignon	Wood
	MG55a	Upper Hunter Valley, NSW	2006	Cabernet Sauvignon	Wood
Colletotrichum acutatum	DAR75574II	Hastings Valley, NSW	2000	Cabernet Sauvignon	Berry
	DAR77283I	Upper Hunter Valley, NSW	2002	Cabernet Sauvignon	Berry
	DAR77284III	Murgon, QLD	2002	Semillon	Berry
	B004II	Hastings Valley, NSW	2009	Chardonnay	Berry
	B019II	Hastings Valley, NSW	2009	Chardonnay	Berry
	B024I	Hastings Valley, NSW	2009	Chardonnay	Berry
	B030III	Hastings Valley, NSW	2009	Chardonnay	Berry
	B033II	Hastings Valley, NSW	2009	Chardonnay	Berry
	B069II	Hastings Valley, NSW	2009	Shiraz	Berry
	B111III	Hastings Valley, NSW	2009	Cabernet Sauvignon	Berry
	B138I	Hastings Valley, NSW	2009	Cabernet Sauvignon	Berry
	B140I	Hastings Valley, NSW	2009	Cabernet Sauvignon	Berry
	B143II	Hastings Valley, NSW	2009	Cabernet Sauvignon	Berry
	B146III	Hastings Valley, NSW	2009	Cabernet Sauvignon	Berry
	L038II	Hastings Valley, NSW	2009	Chardonnay	Leaves
Colletotrichum gloeosporioides	B015	Hastings Valley, NSW	2009	Chardonnay	Berry
	B022	Hastings Valley, NSW	2009	Chardonnay	Berry
	B045	Hastings Valley, NSW	2009	Chardonnay	Berry
	B084	Hastings Valley, NSW	2009	Shiraz	Berry
	B090	Hastings Valley, NSW	2009	Shiraz	Berry
	B148	Hastings Valley, NSW	2009	Cabernet Sauvignon	Berry
Botrytis cinerea	TN50	Hunter Valley, NSW	1988	Chardonnay	Unknown
Phomopsis spp.		Upper Hunter Valley, NSW	2009	Unknown	Berry
Erysiphe necator		Wagga Wagga, NSW	2009	Semillon	Leaves

a Kindly provided by Dr. Wayne Pitt and Yu Qiu (NWGIC, CSU). The fungi were isolated and cultured as described by Pitt *et al*. (2010).
I, II, III The isolates were divided into three subgroups based on their sequence. See Figure 2.7

Field material and inoculations

A vineyard in the Hunter Valley, NSW, situated 200 km north of Sydney, and prone to bunch rot diseases, was chosen to perform the field trials. The experimental site, elevation 56 m, consisted of approximately 20-year-old drip-irrigated own rooted *V. vinifera* (cv. Chardonnay). The vines were grown on Pokolbin series Chocolate tending to Red Podzolic soil (as per description in 'Soil Landscape of the Singleton 1:250 000 sheet' published by the Soil Conservation Service of NSW) at a row spacing of 3.0 m and vine spacing of 1.4 m. The row alignment was north/south. Ten inflorescences were inoculated by applying approximately 800 µL inoculum (i.e. $0.8x10^5$ conidia) with a hand sprayer and collected 24 hours later. Ten flowers per inflorescence were plated on DRBC agar and analysed as described in the section 'Greenhouse inoculations'. Mature berries were inoculated in the Hunter Valley as per the inoculation of inflorescences and 10 bunches collected 24 hours later. Ten berries per bunch were surface sterilized, plated on DRBC and analysed as described earlier. Asymptomatic inflorescences and berries were also collected and analysed in the same way as the artificially infected material. Results were expressed as mean percentage of infected flowers or berries. Real-time PCR studies were performed on two inflorescences or two berries.

In vitro inoculations

Mature berries without visible disease symptoms were collected from greenhouse facilities and from the Charles Sturt University vineyard (Wagga Wagga, NSW, Australia), a region free of *G. uvicola* and *C. acutatum*. Berries were surface-sterilized and placed in sterile 24-well microplates (IWAKI brand, Asahi Glass Co., Japan) containing 20 mL water surrounding the wells to maintain high humidity. Each berry was inoculated with 10^3 mL^{-1} conidia and incubated at 25°C in the dark. Two berries were analysed by real-time PCR at 0, 4, 8, 16, 24, 48, 72, 96, 120, 144, 168 and 192 hours post inoculation.

DNA extraction, PCR amplification and sequencing

Fungal DNA was extracted as described by Pitt *et al.* (2010) with minor modifications. In brief, colonised agar plugs of each isolate were transferred to 50 mL Falcon tubes containing 20 mL potato dextrose broth (Oxoid). Broth cultures were incubated on a Sartorius orbital shaker at 90 rpm for 6 days at 25°C in the dark. Mycelia were harvested by gradient filtration on a 110 mm filter paper, lyophilized and DNA extracted with the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. DNA was extracted directly from inflorescences and skin peels of mature berries without prior surface sterilization. Each inflorescence or berry was treated as an individual sample. Approximately 100 mg of each sample was ground to a powder in liquid nitrogen and the DNA extracted using the DNeasy Plant Mini Kit. A protocol for DNA extraction from spores was optimized. An average of $3x10^7$ spores per mL was collected from fungal isolates grown on PDA agar into either AP1 buffer from the Qiagen DNEasy Plant Mini Kit or water. Spore suspensions were adjusted to 10^5 mL^{-1} with either AP1 buffer or water according to method for harvesting conidia. DNA was extracted from 100 µL (10^4 mL^{-1}) of individual spore suspension with the DNEasy Plant Mini Kit. Four different approaches were investigated to find the optimal procedure for DNA extraction from spores: the spores were (i) heated at 95°C for 10 min; or (ii) microwaved for 20 s three times at maximum power (1000 W); or (iii) frozen at –80°C for 20 min and thawed, 3 times; or (iv) 1 µL spore suspension used directly for real-time PCR. DNA was dissolved in 100 µL sterile water and 1 µL used for real-time PCR.

Molecular identifications of the pure cultures on PDA were achieved by amplification and comparison of the rDNA internal transcribed spacer (ITS) regions (ITS1, 5·8S and ITS2) using the standard oligonucleotides ITS1 and ITS4 (White *et al.* 1990) (*Figure 2.12*). Amplification was performed in 50 µL reactions containing 10 ng template DNA, 1 U Taq polymerase (New England BioLabs), 1 × New England BioLabs standard buffer, 250 µM dNTPs, and 10 pmol of each primer. PCR amplification was conducted in a C1000 Thermocycler (Bio-Rad) with an initial step of 2 min at 94°C, followed by 40 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 90 s. DNA samples and PCR products were visualized on 1% agarose gels stained with ethidium bromide. PCR products were purified using the QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions to remove excess primers and nucleotides. ITS regions were sequenced in both directions with the ITS1 and ITS4 primers by the Australian Genome Research Facility (University of Queensland). Multiple sequence alignments, contig assembly, comparison of nucleotide sequences and primer design were performed with DNASTAR software (DNASTAR Inc., Madison, WI; www.dnastar.com). Confirmation of *G. uvicola* and *C. acutatum* was achieved by

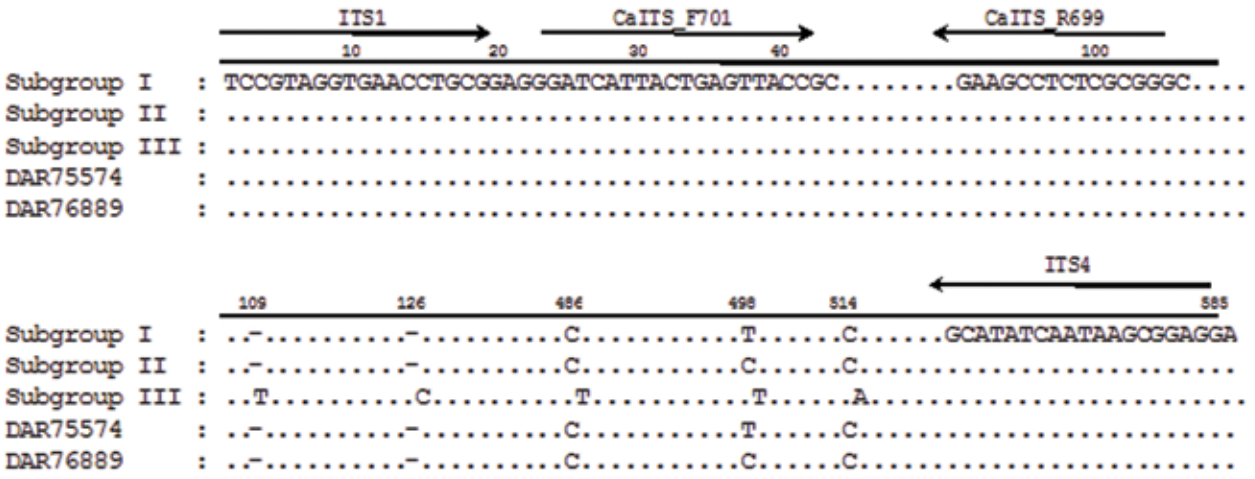


Figure 2.12 Multiple sequence alignment of *Colletotrichum acutatum* isolates from three subgroups and the primers used to amplify them for sequencing (ITS1 and ITS4) and RT-PCR (CaITS_F701 and CaITS_R699). The three subgroups differ at positions 109, 126, 486, 498 and 514. Subgroup I sequence was identical to DAR75574 and Subgroup II to DAR76889; subgroup III is >99% homologous to Subgroups I and II.
'.' represent identical nucleotides, '-' indicate nucleotides not present.

comparison of the identified ITS sequences to those available in GenBank. Nucleotide homology searches were performed with the BLAST nucleotide program (http://www.ncbi.nlm.nih.gov).

Real-time PCR

Specificity of the PCR primers used in this study was tested on all of the fungi listed in *Table 2.11*. Real-time PCR amplification and cycling parameters were performed according to the SYBR Green JumpStart Taq ReadyMix Kit (Sigma-Aldrich) using a Corbett Rotor-Gene™ 6000 5-plex Thermocycler (Qiagen). The PCR reaction contained either 1 µL of spore DNA or appropriate amount of template DNA, 5 pmol of each primer and 1× SYBR Green JumpStart Taq ReadyMix in a final volume of 25 µL. PCR conditions were optimized for annealing temperature and DNA dilutions. The final profile was: initial denaturation for 2 min at 94°C, followed by 40 cycles of 94°C for 30 s, annealing temperature of 60oC for 30 s, and extension at 72°C for 60 s. Visualization of the SYBR Green was achieved by a melting ramp from 62°C to 94°C, rising 1°C each step, hold for 90 s for pre-melt on the first step and 5 s for each step afterwards. All real-time PCRs were repeated twice for the duplicate fungal or plant samples and each inoculation time point. Control reactions contained water instead of fungal or plant DNA. The data was normalised as previously described (Samuelian *et al.* 2009) using the primer pair UbiF: 5'-ACTCTCACCGGAAAGACCATC-3' (forward) and UbiR: 5'-TCACGTTGTCAATGGTGTCAG-3' (reverse) derived from the Arabidopsis thaliana polyubiquitin 10 (NM_178969.2; ubq10). Comparison of relative DNA concentrations was determined using the $2^{\Delta\Delta C_T}$ method (Livak and Schmittgen 2001). $\Delta\Delta C_T=(C_{T,target} - C_{T,ubq10})$, where CT is the threshold cycle which represents the PCR cycle at which the copy number passes the fixed threshold and can be first detected.

To check if an excess of plant DNA could inhibit the amplification of fungal DNA, serial dilutions were performed as follows: the amount of plant DNA was maintained constant (15 ng) while fungal DNA was decreased to achieve 10-fold dilutions.

Results and discussion

Extraction of genomic DNA from fungi, spores and infected material

The highest level of DNA detection was achieved when spores were frozen at –80°C and thawed repeatedly and this approach was used further in the study. DNA extracted from pure fungal isolates as well as from all artificially and naturally infected inflorescences, mature berries and healthy plant tissue yielded approximately 50 ng DNA per 1 mg dried fungal tissue or 5 mg wet plant sample.

ITS sequencing, design and validation of specific primers for G. uvicola and C. acutatum.

The ribosomal DNA polymorphic internal transcribed spacer 1 region (rDNA 5·8S ITS) was amplified using the universal primers ITS1 and ITS4. A single PCR fragment of approximately 600 bp was produced for all *G. uvicola* isolates, which after

sequencing was established to be 599 bp in length. Multiple alignment of all amplicons revealed that all isolates were 100% identical at their ITS region. A DNA sequence from this study was submitted to the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov, Ref No. GU907101), and currently represents the only sequence of the ITS region of this fungus in NCBI. Amplification of *C. acutatum* isolates generated two fragments of 583 and 585 bp. Three variations of *C. acutatum* were identified as two had the same size of 583 bp and one of 585 bp. The *C. acutatum* isolates were placed in three subgroups (*Figure 2.12*). A comparison of homology to the *C. acutatum* sequences in GeneBank revealed that the highest nucleotide sequence identity was shared with DAR75574 and DAR76889 described by Whitelaw-Weckert *et al.* (2007).

Three pairs of primers were designed for *G. uvicola* and two for *C. acutatum* using the DNASTAR software; the primers were compared to sequences available at NCBI to insure that they would not amplify DNA from other organisms. The CaITS_F701/CaITS_R699 and CaITS_F701/CaITS_R815 primer pairs developed by Debode and co-workers (2009) to study *C. acutatum* on strawberries were also tested in this study as they did not coincide with the region of nuclear mismatches between the different isolates (*Figure 2.12*). Based on melting curve analyses the primer pairs GuF2b (5'-TCTGAACGTATCTCTTCTGAG-3') and GuR2 (5'-TAAGTCAACCTAAGCGAGAAG-3') designed in this study, and CaITS_F701/CaITS_R699 did not result in the production of non-specific bands when tested against genomic fungal DNA of pure cultures of all isolates presented in *Table 2.11* and therefore were chosen for further analyses.

RT-PCR assay

Real-time PCR was first performed on fungal genomic DNA to establish the procedure. Serial 1:10 DNA dilutions of 2 ng of DNA were used to determine the sensitivity of the method. The standard curves obtained showed a linear correlation between input DNA and C_t with r^2 of 0.9916 and 0.9964 for *G. uvicola* and *C. acutatum*, respectively (*Figure 2.13*). Fluorescence remained below threshold values for the water controls and non-target species. Agarose gel electrophoresis of the real-time PCR products showed

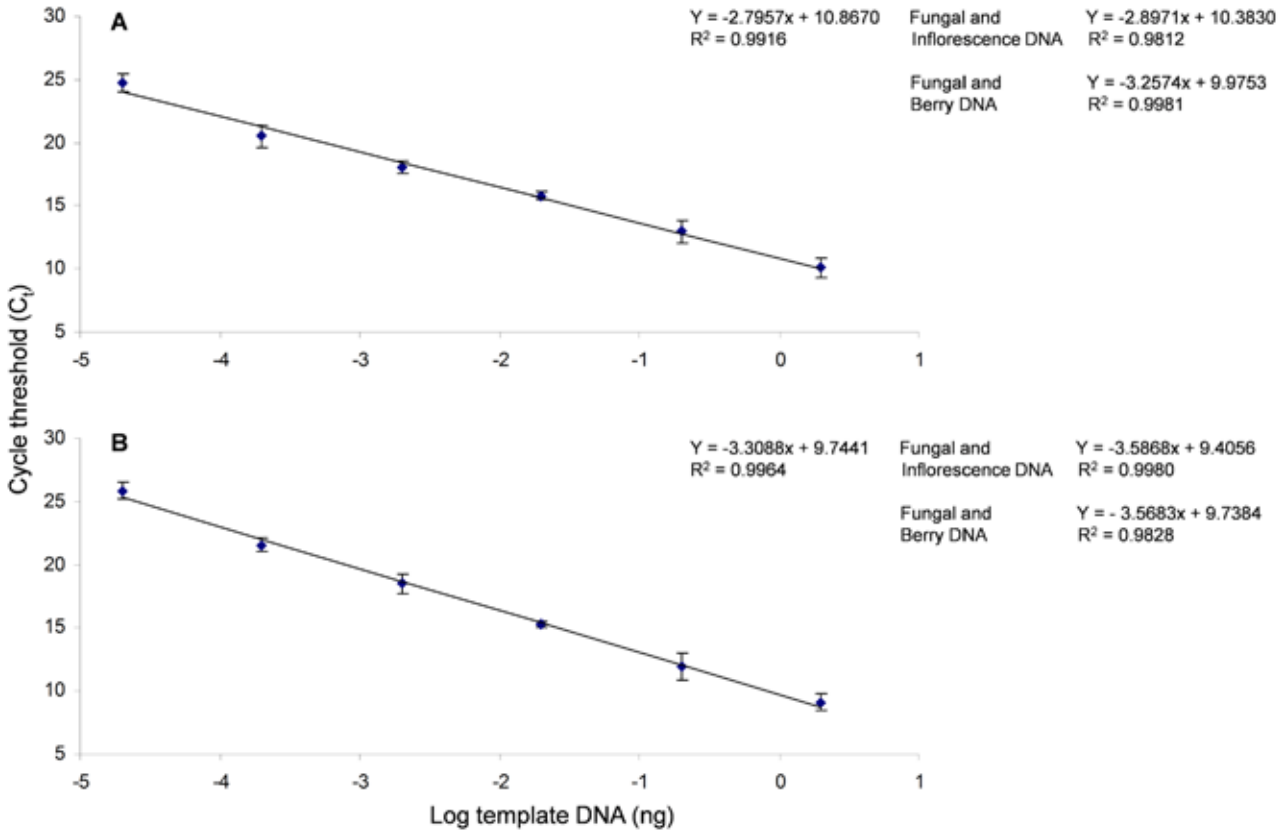


Figure 2.13 Linear relationship between cycle threshold (C_t) and fungal DNA concentrations of (A) *Greeneria uvicola* and (B) *Colletotrichum acutatum* amplified using RT-PCR. Cycle thresholds were plotted against the log of 10-fold serial dilutions of known genomic DNA from 20 fg to 2 ng. C_t values shown are the mean of three replications; error bars represent standard deviations. The same dilutions were tested in the presence of flower and berry DNA (15 ng). Since the lines for the three sets of data are overlapping, the R^2 equations when the sensitivity of RT-PCR was tested in the presence of flower and berry DNA are presented separately.

a positive correlation between the fluorescence and amplification of the expected DNA fragments. In the dilution series of both *G. uvicola* and *C. acutatum* the practical detection limit of the real-time PCR method was 20 fg μL^{-1} fungal genomic DNA. The same experiment was performed to study whether grape DNA inhibits the sensitivity of the real-time PCR. It was found that the same fungal DNA quantities were detected in the presence of both flower and berry DNA (*Figure 2.13*). The standard curves obtained revealed high precision and reproducibility between the different assays as indicated by correlation coefficients ranging from R^2=0.9912 to 0.9981 for *G. uvicola* and from R^2=0.9828 to 0.9980 for *C. acutatum*. Serial dilution of spore DNA in water allowed detection of *c.* 10 conidia with both GuF2b/GuR2 (R^2=0.9855) and CaITS_F701/CaITS_R699 (R^2=0.9971). No amplification was obtained from DNA extracted from healthy grape tissue or from the other fungal species examined (*Table 2.11*).

Monitoring fungal development on plant tissues.

First visible symptoms of Bitter Rot and Ripe Rot development on berries in vitro included discharge of juice combined with cracking of the skin, typically observed 2 to 4 days after inoculation. Black or orange spore masses were visible 3 to 5 days post inoculation (*G. uvicola* and *C. acutatum*, respectively). Conventional plating detected *G. uvicola* and *C. acutatum* in 40% and 72% of all artificially infected flowers, respectively, and 100% on mature berries. Real-time PCR was able to detect both fungi on all artificially inoculated plant material (inflorescences and berries). The two pathogens were not detected by either method on non-inoculated inflorescences from the Hunter Valley collected in 2009, a viticultural region prone to bunch rot diseases, but were detected on asymptomatic mature berries later in the season.

Fungal growth was quantified by real-time PCR at different time points post inoculation. The relative quantities of grape genomic DNA were analysed as template DNA samples were equalized and calculations were performed based on amplification with the UbiF and UbiR primers. Differences in amplification intensity were not observed for flowers. In contrast, a gradual increase in the amplification intensity was detected from 0 to 8 days post inoculation on mature berries in vitro (*Figure 2.14*). Real-time PCR was used to detect the presence or absence of *G. uvicola* and/or *C. acutatum* DNA from 50 randomly sampled and asymptomatic mature berries from the Hunter Valley. Conventional plating methods detected *G. uvicola* and *C. acutatum* from 5% and 55% of mature berries, respectively, while real-time PCR detected *G. uvicola* and *C. acutatum* from 20% and 76% of mature berries, respectively. Real-time PCR was able to simultaneously detect both pathogens on one berry in 13% of the berries.

Summary of development of real time PCR methods for bunch rot detection

A molecular method was evaluated for the fast and precise detection and quantification of *G. uvicola* and *C. acutatum* in artificially and naturally infected plant material from grapevine. Comparison of the ITS sequences successfully confirmed the accuracy of morphological fungal identification and provided information for phylogenetic analyses as well as design of species-specific primers. To the best of our knowledge there is no literature concerning the genetic variability of *G. uvicola*. This study revealed uniformity among all the Australian isolates of *G. uvicola* analyzed in this study and a high similarity

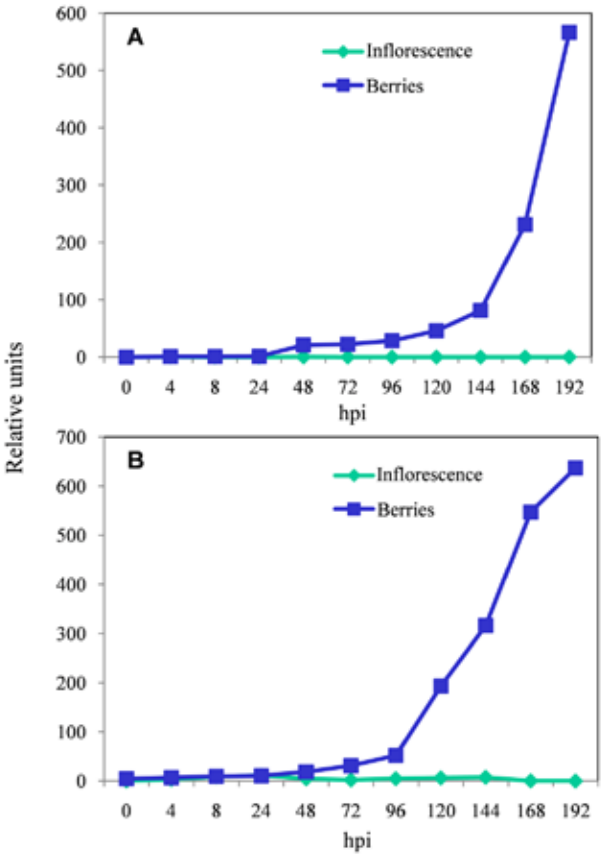


Figure 2.14 Development of (A) *Greeneria uvicola* and (B) *Colletotrichum acutatum* on inflorescences and physiologically mature berries at different time points/hours post inoculation (hpi). Standard deviations were negligibly small and are not presented on the chart for better visualisation.

between *C. acutatum* isolates based on their ITS sequence. The *C. acutatum* isolates analyzed in this study were positioned in three subgroups: 1 and 2 which were identical to isolates DAR75574 and DAR76889, respectively, and group 3 which was >99% homologous to DAR75574 and DAR76889 placing them in group A9 containing all Australian *C. acutatum* isolates from subtropical wine grape growing regions (Whitelaw-Weckert *et al.* 2007). It might be speculated that the lack of genetic diversity within the different *G. uvicola* and *C. acutatum* isolates is due to both the absence of sexual reproduction of these species and to the relatively small geographical area from which the isolates were collected.

ITS regions of the rDNA have been widely used for phylogenetic studies and diagnostic assay development (Schena *et al.* 2004; Mumford *et al.* 2006; Cooke *et al.* 2007). These regions are highly stable, can be easily amplified and sequenced, occur in multiple copies and possess conserved as well as variable sequences (White *et al.* 1990; Campanile *et al.* 2008). ITS-based sequencing and ITS-based primers have been reported and successfully used to identify and analyse a number of fungal plant pathogens including *C. acutatum* (Daykin and Milholland 1984b; Parikka *et al.* 2004; Nam *et al.* 2007). However, the standard sequencing method is time consuming and tedious while real-time PCR offers a rapid and simple method with increased sensitivity. No post-PCR processing and sequencing is necessary as quantification of DNA is achieved based on the cycle threshold (C_t) value measured during the PCR amplification. The real-time PCR method allowed the detection of as little as 20 fg genomic DNA and 10 spores for *G. uvicola* and *C. acutatum*. These thresholds are lower than those detected for *C. acutatum* by nested PCR (Nam *et al.* 2007) and TaqMan real-time PCR (Debode *et al.* 2009). In addition, the SYBRGreen technology was employed in this study because it is less labor-intensive, has comparatively low operational costs, has higher sensitivity and provides reliable quantitative results compared to Nested PCR and TaqMan (Schena *et al.* 2004; Debode *et al.* 2009).

One of the limitations when using PCR techniques is the preparation of good quality nucleic acid that is free of PCR inhibitors. There are a number of PCR inhibitors in grape tissue, particularly in the flesh of mature berries that affect DNA amplification in conventional PCR (Schaad *et al.* 2002). The DNeasy Plant Kit (Qiagen) was used to extract grape DNA as it is considered the best method for DNA extraction for amplification of pathogens from 'difficult' samples (Schaad *et al.* 2002; Li *et al.* 2006; Li *et al.* 2008; Debode *et al.* 2009). Parikka and Lemmetty (2004) have added polyvinylpolypyrrolidone (PVPP) to the extraction buffer of the kit but this was not used in the present study because there was no difference between the sensitivity of the method when the target DNA was quantified based on serial dilutions in water, in the presence of both flower and berry DNA, and also because DNA was extracted from berry skins rather than from the whole berries. Another reason for the omission of PVPP was the fact that the fungi develop on the surface of the berry.

In the current study, modified extraction methods were utilized that allowed rapid and simple DNA extraction from up to 30 samples of spores and grape tissues within 2–3 hours, without the use of toxic reagents such as phenol, chloroform or β-mercaptoethanol. Although the simple commercially-available extraction method used was found to be successful for flowers and berries, it is likely that the method will have to be adapted for extraction of the fungal DNA from wood and other tissues.

Grape berries do not always show clear symptoms of either Bitter Rot or Ripe Rot. While *G. uvicola* frequently presents itself as concentric black rings of sporulation on mature grapes, these symptoms might be confused with those produced by other bunch rotting fungi that produce black spores (e.g. *Alternaria, Phomopsis viticola* etc.). Although *C. acutatum* produces orange masses of spores on the berries these are only visible in the latter stages of infection. Furthermore Ripe Rot of grape can also be caused by other *Colletotrichum* species (Daykin and Milholland 1984b). Similar symptoms are observed on grape berries, making morphological differentiation difficult. In a bunch rot complex, infection levels may be low or a mixture of organisms can be present and clear symptoms may not be observed (Steel *et al.* 2007). In these cases, a small amount of plant tissue or pure sample of spores can be collected for direct PCR analyzes. If a more precise detection (determination) of species belonging to the *Colletotrichum* complex is desired, primers from other genes (Hughes *et al.* 2006; Cai *et al.* 2009) can be designed and used following the protocol demonstrated in this study.

Conventional isolation of *G. uvicola* and *C. acutatum* from plant material on artificial media was less sensitive than detection with real-time PCR. *G. uvicola* and *C. acutatum* could not be isolated from

more than half of the artificially inoculated flowers and mature berries y*et al*l these samples showed positive PCR amplification of fungal DNA. It might be speculated that some PCR products could have been amplified from non-viable pathogen propagules (Parikka and Lemmetty 2004).

In conclusion, a real-time PCR method was developed to reliably identify and quantify two fungal species, *G. uvicola* and *C. acutatum*, associated with bunch rot of grapes, including three *C. acutatum* intraspecific subgroups, under controlled and natural conditions. The results demonstrate that this method is highly specific and sensitive. Amplification was achieved immediately after inoculation with relatively small amounts of DNA template present in the conidial and mycelial inoculum. Application of the method demonstrated that both *G. uvicola* and *C. acutatum* infect flowers but remain quiescent. Their infection progress could be monitored through real-time PCR during berry ripening. The method can now be used in epidemiological studies to advance the understanding of the dynamics of these and other bunch rot pathogens in the vineyard. In future, real-time PCR, applied as a diagnostic tool, may also be used to inform grape producers in regard to the timing of fungicides and/or harvest to avoid fruit losses.

Experiment 2.8
The overwintering of ripe rot

Materials and methods

This study was conducted in a commercial vineyard situated in the Hunter Valley, with a known history of ripe rot. Samples were collected from 20 year old, own rooted Chardonnay vines, planted in 3.0 m wide rows, with 1.4 m distance between vines. Twenty bunches were collected randomly at maturity (11.5° Baumé) in the summer of 2009–10 and 2010–11. Ten berries were cut from each bunch and surface-sterilised with 1% w/v hypochlorite (Biolab) and 0.05% Tween 80 for 2 min, rinsed three times in sterile water and placed onto DRBC agar. Subsequent fungal growth was transferred to PDA and identified after 5–7 days of incubation at 25°C.

In August 2010 18 spurs, approximately 5–10 cm in length, were collected randomly from 18 vines; 33 mummified bunches and 33 canes (approximately 1 m long) were collected randomly from the ground in the middle of the rows. Ten mummified berries per bunch and 10 peduncles were analysed as described for mature berries while canes and spurs were cut into 1 cm long sections, surface sterilised as described above and plated onto DRBC agar.

Additionally, 10 developing shoots (50–120 cm lengths) were collected randomly from 10 vines when the berries were pea size. For this study, the 5 cm section of shoot attached to the cane represents a spur at pea size berry stage. Each shoot had a bunch at pea size berry stage. Shoots, leaves, and tendrils were cut

Table 2.12 Detection and monitoring of *Colletotrichum acutatum* on *V. vinifera* tissues at different time points and phenological stages. Number of samples where *C. acutatum* was identified is presented in percentage.

			***C. acutatum* detected**	
Time of collection	**Growth stage ***	**Plant tissue**	**on DRBC**	**by real-time PCR**
Sumer 2009-10	E-L 38, harvest, berries ripe	berries	50%	70%
Winter 2010	E-L 1, dormancy	mummified bunches (ground)	15%	85%
		dormant canes (ground)	15%	85%
		Spurs (vine)	17%	67%
Summer 2010-11	E-L 31 berries pea size	developing shoots:		
		• green tissue (excluding spurs)	n.d.	n.d.
		• berries	n.d.	n.d.
		• leaves	n.d.	n.d.
		• spurs	60%	80%
	E-L 38, harvest, berries ripe	berries	40%	50%

* Grapevine growth stages are presented according to Coombe (1995).
n.d. – not detected

into 2.5–3 cm long sections and plated onto DRBC together with 10 berries per shoot. Results were expressed as mean percentage of infected samples. Morphological identification was confirmed by real-time PCR (Samuelian *et al.* 2011). From each shoot five samples were taken from leaves, five from stems, three from tendrils (2.5–3 cm long sections) and the material from each tissue was pooled for subsequent PCR analyses; five berries per bunch were treated independently for DNA extraction and consequent PCR analyses.

Results and discussion

Characteristic salmon-coloured spore masses are produced on ripe berries during the life cycle of *C. acutatum*. Plating of mature berries onto artificial media and subsequent identification revealed the presence of viable *C. acutatum* spores in 50 and 40% of the analysed bunches during the summers of 2009–10 and 2010–11, respectively, while real-time PCR detected the pathogen in 70% and 50% of the bunches during the studied period (*Table 2.12*). *C. acutatum* was revived from 15% of mummified berries, peduncles and winter canes, 17% of winter spurs, 60% of spurs at pea size but not on the remaining parts of developing shoots when plated on DRBC. In contrast, real-time PCR detecting *C. acutatum* in 85% of mummified berries, peduncles and winter canes, 67% of winter spurs and 80% of spurs at pea size berry stage. This result is consistent with other findings (Parikka and Lemmetty 2004; Samuelian *et al.* 2011) as molecular biology techniques tend to detect non-viable spores.

C. acutatum has been reported to overwinter in infected strawberry crowns and fruit (Freeman *et al.* 2002) and on living leaves of Valencia orange (*Citrus sinensis*) (Zulfiqar *et al.* 1996). On sweet cherry buds, it was observed often at high frequency (Børve and Stensvand 2006); superficially or slightly internally on detached shoots and particularly on winter buds and wounds on the bark of mulberry tree (Yoshida and Shirata 1999); in dormant buds and twigs but not in dropped leaves or fruit mummies of apple fruit (Crusius *et al.* 2002). The pathogen was recovered at a high rate on almond mummies in California (Føster and Adaskaveg 1999), probably due to the protection provided by the mummified fruits. On blueberry bushes, *C. acutatum* survives the winter in or on blighted twigs, spent fruit trusses and live buds, as well as in other symptomless tissues (Yoshida *et al.* 2007). The fungus appears to prefer bud scales and bark as general overwintering sites in woody plants.

Even though dormant plant tissues have been identified as the main source of inoculum for several tree species, the contribution of *C. acutatum* conidia in the soil to disease outbreaks should also be taken into consideration. Conidia have been found to survive for nine months in soil attached to strawberry plants (Freeman *et al.* 2002). Yoshida and Shirata (1999) reported that conidia remain viable in the soil for up to six months while Norman and Strandberg (1997) reported that survival of conidia on leatherleaf fern in diseased debris buried in the soil declined rapidly under moist conditions but some remained viable for up to 12 months. The role of the conidia that survive in the soil remains unknown for grapevine.

To the best of our knowledge there are no studies published concerning the overwintering of *C. acutatum* on *V. vinifer*a. Our attempts to isolate the pathogen from mummified berries, winter wood samples and spurs was successful for approximately 17% of the samples investigated even though it was detected in significantly larger portion of the material by real-time PCR. This is due to the amplification of non-viable pathogen propagules (Parikka and Lemmetty 2004), suggesting that a large proportion of the pathogen population cannot survive winter conditions. However, the remaining portion of the population, especially on spurs, is a sufficient inoculum source to cause infections during the next growing season (*Table 2.11*). Furthermore, secondary conidia were reported to be produced from the conidial and hyphal phialides within six hours after infection of symptomless strawberry leaves and were responsible for up to threefold increase in the total number of conidia within seven days (Leandro *et al.* 2001) which could explain the three times higher number of *C. acutatum* isolates detected on spurs at pea size berry stage. Detection of *C. acutatum* on different plant tissues indicates that the pathogen may overwinter on different parts of the plant. At present it is not clear when spurs and other plant parts are infected and what fungicides should be applied to reduce or prevent such infection. Fungicide applications during winter months were rather inefficient in eradication of the *Colletotrichum* complex on apple (Crusius *et al.* 2002). Future research is necessary to address this issue and to provide improved management strategies for ripe rot of grapevine.

Experiment 2.9
Comparison of bitter rot isolates from Australia and USA

Materials and methods

Sixty-seven Australian and 27 American *G. uvicola* isolates were compared as part of a collaborative study with North Carolina State University. Morphology on culture media, fungicide sensitivity, molecular profiling and pathogenicity to *Vitis vinifera* and *Muscadinia rotundifolia* vines were compared using the techniques as described above.

Results and discussion

Morphological comparison

In total 67 *G. uvicola* isolates were collected from subtropical grape growing regions in eastern Australia and 27 from south eastern USA (*Table 2.13*). Of those, 83 isolates were identified on *Vitis vinifera* cultivars and 11 on *Muscadinia rotundifolia* cultivars. Single spore pure cultures were established as described under Section 5.7. Representative isolates are maintained at the National Wine and Grape Industry Centre (Wagga Wagga, NSW, Australia), North Carolina State University (NC, USA) and the Australian Scientific Collection Unit (Department of Primary Industry, NSW, Australia).

Mycelium of all *G. uvicola* isolates grown on PDA was creamy white at the outside edge of the petri plates to greyish brown at the centre (*Table 2.14*). Exceptions were isolates AU13, AU32, AU35, AU55 and AU65 which were brownish black throughout the plate and US17 and US26 which were black (*Figure 2.15*). The texture of the mycelium of all US isolates were ropey to slightly ropey while all Australian isolates had flat mycelium.

Molecular characterisation

Three gene regions–the ribosomal DNA (rRNA) internal transcribed spacer 1 (ITS), 28S large subunit (LSU) nuclear rDNA, and β-tubulin 2–were amplified and sequenced using standard primers (*Table 2.15*), PCR amplification procedures and sequencing as already described under Section 5.7.4.

Genetic distances were calculated and phylogenetic trees constructed for the three genes analysed with MEGA 5.0 (*Figure 2.16*). Nucleotide sequences obtained during this study were deposited at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). Where several isolates had the same DNA sequence, only one accession number was generated to represent identical isolates.

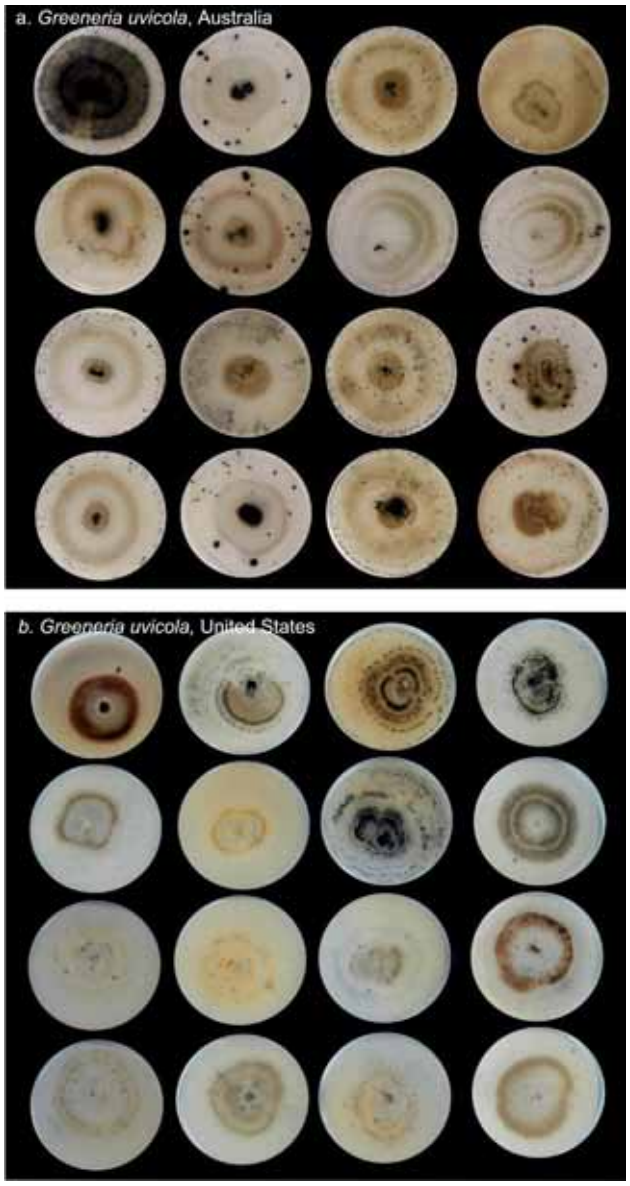


Figure 2.15 Representative *Greeneria uvicola* isolates grown on PDA showing morphological differences in colouration and spore development. Underside of plate. Isolates of Australian origin (a) and America origin (b).

BLAST analyses revealed homology to several *G. uvicola* isolates from Uruguay and one from Ohio (AF362570; Farr *et al.* 2011) at the 5·8-ITS and 28S rDNA regions. Phylogenetic analyses of β-tubulin-2 and 5·8-ITS regions differentiated *G. uvicola* isolates in more details than the 28S rDNA sequences (*Figure 2.16*). All isolates identified on muscadine grapes had the same 28S rDNA sequence as well as all Australian ones (*Figure 2.16b*). Most of the North American isolates identified on vinifera grapes in this study revealed identical sequences in that region with two exceptions–US12 and US13. Only one 28S rDNA sequence was identified from *G. uvicola* isolates

Table 2.13 Complete list of *Greeneria uvicola* isolates analysed in this project. Cultures submitted to the Herbarium Collection at DPI NSW, Orange, have received DAR numbers. Isolates from the USA did not receive such numbers as they will not be maintained in the collection. Sequence information for three genes has been submitted to NCBI.

№	Name	Herbarium accession	Geographic origin	Date of isolation	Cultivar	Host tissue	GenBank accession		
							ITS	b-Tub	LR
Isolates from Australia									
1	Gu1	DAR77258	Upper Hunter Valley, NSW	2002	Chardonnay	Berry	JN381031	JN561305	JN547720
2	Gu2	DAR77260	Hastings Valley, NSW	2004	Chardonnay	Berry	JN381031	JN561305	JN547720
3	Gu3	DAR77261	Hastings Valley, NSW	2004	Chambourcin	Berry	JN381031	JN561305	JN547720
4	Gu4	DAR77262	Hastings Valley, NSW	2004	Cabernet sauvignon	Berry	JN381031	JN561305	JN547720
5	Gu5	DAR77263	Upper Hunter Valley, NSW	2004	Chardonnay	Berry	JN381031	JN561305	JN547720
6	Gu6	DAR77264	Upper Hunter Valley, NSW	2004	Chardonnay	Berry	GU907101	JN561304	JN547720
7	Gu7	DAR77265	Upper Hunter Valley, NSW	2004	Chardonnay	Berry	GU907101	JN561304	JN547720
8	Gu8	DAR77266	Upper Hunter Valley, NSW	2004	Chardonnay	Berry	GU907101	JN561304	JN547720
9	Gu9	DAR77267	Upper Hunter Valley, NSW	2004	Chardonnay	Berry	JN381031	JN561305	JN547720
10	Gu10	DAR77268	Upper Hunter Valley, NSW	2004	Chardonnay	Berry	JN381031	JN561305	JN547720
11	Gu11	DAR77269	Upper Hunter Valley, NSW	2004	Chardonnay	Berry	JN381031	JN561305	JN547720
12	Gu12	DAR77270	Upper Hunter Valley, NSW	2004	Chardonnay	Berry	JN381031	JN561305	JN547720
13	Gu13	DAR77271	Upper Hunter Valley, NSW	2004	Cabernet sauvignon	Berry	GU907101	JN561304	JN547720
14	Gu14	DAR77272	Upper Hunter Valley, NSW	2004	Cabernet sauvignon	Berry	GU907101	JN561304	JN547720
15	Gu15	DAR77273	Upper Hunter Valley, NSW	2004	Cabernet sauvignon	Berry	JN381031	JN561305	JN547720
16	Gu16	DAR77274	Upper Hunter Valley, NSW	2004	Cabernet sauvignon	Berry	JN381031	JN561305	JN547720
17	Gu17	DAR77275	Upper Hunter Valley, NSW	2004	Chardonnay	Berry	JN381031	JN561305	JN547720
18	Gu18	DAR77276	Upper Hunter Valley, NSW	2004	Chardonnay	Berry	JN381031	JN561305	JN547720
19	Gu19	DAR77277	Upper Hunter Valley, NSW	2004	Chardonnay	Berry	JN381031	JN561305	JN547720
20	Gu20	DAR77278	Upper Hunter Valley, NSW	2004	Chardonnay	Berry	JN381031	JN561305	JN547720
21	Gu21	DAR77279	Upper Hunter Valley, NSW	2004	Chardonnay	Berry	JN381031	JN561305	JN547720
22	Gu22	DAR77280	Upper Hunter Valley, NSW	2004	Chardonnay	Berry	GU907101	JN561304	JN547720
23	Gu23	DAR77281	Upper Hunter Valley, NSW	2004	Chardonnay	Berry	JN381031	JN561305	JN547720
24	Gu24	DAR77259	Hastings Valley, NSW	2004	Chambourcin	Berry	JN381031	JN561305	JN547720
25	MA231	DAR 81469	Lower Hunter Valley, NSW	2006	Shiraz	Wood	JN381031	JN561305	JN547720
26	MAC11	DAR 81470	Lower Hunter Valley, NSW	2006	Chardonnay	Wood	JN381031	JN561305	JN547720
27	MD25	DAR 81471	Lower Hunter Valley, NSW	2006	Semillon	Wood	JN381031	JN561305	JN547720
28	MF522	DAR 81472	Upper Hunter Valley, NSW	2006	Chardonnay	Wood	JN381031	JN561305	JN547720
29	MG53	DAR 81473	Upper Hunter Valley, NSW	2006	Cabernet sauvignon	Wood	JN381031	JN561305	JN547720
30	MG55	DAR 81474	Upper Hunter Valley, NSW	2006	Cabernet sauvignon	Wood	JN381031	JN561305	JN547720
31	B13	DAR 81475	Hastings Valley, NSW	2009	Chardonnay	Berry	JN381031	JN561305	JN547720

№	Name	Herbarium accession	Geographic origin	Date of isolation	Cultivar	Host tissue	GenBank accession		
							ITS	b-Tub	LR
32	B18	DAR 81476	Hastings Valley, NSW	2009	Chardonnay	Berry	JN381031	JN561305	JN547720
33	B24	DAR 81477	Hastings Valley, NSW	2009	Chardonnay	Berry	JN381031	JN561305	JN547720
34	B25	DAR 81478	Hastings Valley, NSW	2009	Chardonnay	Berry	JN381031	JN561305	JN547720
35	B26	DAR 81479	Hastings Valley, NSW	2009	Chardonnay	Berry	JN381031	JN561305	JN547720
36	B27	DAR 81480	Hastings Valley, NSW	2009	Chardonnay	Berry	JN381031	JN561305	JN547720
37	B28	DAR 81481	Hastings Valley, NSW	2009	Chardonnay	Berry	JN381031	JN561305	JN547720
38	B33	DAR 81482	Hastings Valley, NSW	2009	Chardonnay	Berry	JN381031	JN561305	JN547720
39	B34	DAR 81483	Hastings Valley, NSW	2009	Chardonnay	Berry	JN381031	JN561305	JN547720
40	B38	DAR 81484	Hastings Valley, NSW	2009	Chardonnay	Berry	JN381031	JN561305	JN547720
41	B39	DAR 81485	Hastings Valley, NSW	2009	Chardonnay	Berry	JN381031	JN561305	JN547720
42	B41	DAR 81486	Hastings Valley, NSW	2009	Chardonnay	Berry	JN381031	JN561305	JN547720
43	B42	DAR 81487	Hastings Valley, NSW	2009	Chardonnay	Berry	JN381031	JN561305	JN547720
44	B43	DAR 81488	Hastings Valley, NSW	2009	Chardonnay	Berry	JN381031	JN561305	JN547720
45	B50	DAR 81489	Hastings Valley, NSW	2009	Chardonnay	Berry	JN381031	JN561305	JN547720
46	QLD101	DAR80940	Shire of Balonne, QLD	2009	Chardonnay	Berry	JN381031	JN561305	JN547720
47	QLD106	DAR80941	South Burnett Region, QLD	2010	Chardonnay	Berry	JN381031	JN561305	JN547720
48	QLD120	DAR80942	South Burnett Region, QLD	2010	Chardonnay	Berry	JN381031	JN561305	JN547720
49	QLD151	DAR80943	South Burnett Region, QLD	2010	Semillon	Berry	GU907101	JN561304	JN547720
50	QLD152	DAR80944	South Burnett Region, QLD	2010	Semillon	Berry	GU907101	JN561304	JN547720
51	QLD411	DAR80945	South Burnett Region, QLD	2010	Semillon	Berry	JN381031	JN561305	JN547720
52	QLD412	DAR80946	South Burnett Region, QLD	2010	Semillon	Berry	JN381031	JN561305	JN547720
53	QLD413	DAR80947	South Burnett Region, QLD	2010	Semillon	Berry	JN381031	JN561305	JN547720
54	QLD414	DAR80948	South Burnett Region, QLD	2010	Semillon	Berry	JN381031	JN561305	JN547720
55	QLD421	DAR80949	Southern Downs Region, QLD	2010	Pinot gris	Berry	GU907101	JN561304	JN547720
56	QLD422	DAR80950	Southern Downs Region, QLD	2010	Pinot gris	Berry	GU907101	JN561304	JN547720
57	QLD511	DAR80951	Southern Downs Region, QLD	2010	Chardonnay	Berry	GU907101	JN561304	JN547720
58	QLD512	DAR80952	Southern Downs Region, QLD	2010	Chardonnay	Berry	GU907101	JN561304	JN547720
59	QLD611	DAR80953	Southern Downs Region, QLD	2010	Semillon	Berry	JN381031	JN561305	JN547720
60	QLD612	DAR80954	Southern Downs Region, QLD	2010	Semillon	Berry	JN381031	JN561305	JN547720
61	QLD621	DAR80955	South Burnett Region, QLD	2010	Chardonnay	Berry	GU907101	JN561304	JN547720
62	QLD622	DAR80956	Southern Downs Region, QLD	2010	Viognier	Berry	GU907101	JN561304	JN547720
63	QLD711	DAR80957	Southern Downs Region, QLD	2010	Semillon	Berry	JN381031	JN561305	JN547720
64	QLD712	DAR80958	Southern Downs Region, QLD	2010	Semillon	Berry	JN381031	JN561305	JN547720
65	QLD722	DAR80959	Southern Downs Region, QLD	2010	Viognier	Berry	JN381031	JN561305	JN547720

№	Name	Herbarium accession	Geographic origin	Date of isolation	Cultivar	Host tissue	GenBank accession		
							ITS	b-Tub	LR
66	QLD723	DAR80960	Southern Downs Region, QLD	2010	Viognier	Berry	JN381031	JN561305	JN547720
67	QLD724	DAR80961	Southern Downs Region, QLD	2010	Chardonnay	Berry	JN381031	JN561305	JN547720
Isolates from USA									
1	IG1		Forsyth County, NC	2010	Sauvignon blanc	Berry	JN547707	JN561306	JN547723
2	IG2		Forsyth County, NC	2010	Sauvignon blanc	Berry	JN547708	JN561307	JN547723
3	IG5		Forsyth County, NC	2010	Sauvignon blanc	Berry	JN547709	JN561308	JN547723
4	IG6		Forsyth County, NC	2010	Sauvignon blanc	Berry	JN547708	JN561307	JN547723
5	IG8		Forsyth County, NC	2010	Sauvignon blanc	Berry	JN547708	JN561309	JN547723
6	IG17		Forsyth County, NC	2010	Sauvignon blanc	Berry	JN547707	JN561306	JN547723
7	IG10		Forsyth County, NC	2010	Sauvignon blanc	Berry	JN547710	JN561310	JN547723
8	IG4		Forsyth County, NC	2010	Sauvignon blanc	Berry	JN547707	JN561306	JN547723
9	G1.1		Forsyth County, NC	2010	Chardonnay	Berry	JN547711	JN561311	JN547723
10	G2.6		Forsyth County, NC	2010	Chardonnay	Berry	JN547712	JN561311	JN547723
11	G3.1		Forsyth County, NC	2010	Chardonnay	Berry	JN547712	JN561311	JN547723
12	G7.2		Forsyth County, NC	2010	Chardonnay	Berry	JN547713	JN561312	JN547722
13	G8.1		Forsyth County, NC	2010	Chardonnay	Berry	JN547714	JN561313	JN547722
14	G9.1		Forsyth County, NC	2010	Chardonnay	Berry	JN547714	JN561314	JN547723
15	G10.2		Forsyth County, NC	2010	Chardonnay	Berry	JN547714	JN561314	JN547723
16	G11.1		Forsyth County, NC	2010	Chardonnay	Berry	JN547714	JN561315	JN547723
17	CH6.1		New Hanover County, NC	2010	Carlos	Berry	JN547715	JN561316	JN547721
19	CH10.1		New Hanover County, NC	2010	Carlos	Berry	JN547716	JN561317	JN547721
20	CH1.2		New Hanover County, NC	2010	Triumph	Berry	JN547716	JN561317	JN547721
22	CH7.2		New Hanover County, NC	2010	Triumph	Berry	JN547716	JN561318	JN547721
23	CH2.1		New Hanover County, NC	2010	Sweet Jenny	Berry	JN547716	JN561317	JN547721
24	CH8.2		New Hanover County, NC	2010	Sweet Jenny	Berry	JN547717	JN561317	JN547721
25	CH4.2		New Hanover County, NC	2010	Sweet Jenny	Berry	JN547716	JN561317	JN547721
26	CH7.1		New Hanover County, NC	2010	Sweet Jenny	Berry	JN547718	JN561319	JN547721
27	HCRS2		New Hanover County, NC	2010	Fry	Berry	JN547716	JN561320	JN547721
28	BC2		Bladen County, NC	2002/03	Carlos	Berry	JN547719	JN561321	JN547721
29	WC5		Wake County, NC	2002/03	Nesbitt	Berry	JN547716	JN561322	JN547721

Table 2.14 Morphological characteristics of *G. uvicola* isolates.

		Reverse		Surface		
№	**Name**	**Edge**	**Centre**	**Edge**	**Centre**	**Texture**
Isolates from Australia						
1	Gu1	creamy white	greyish brown	creamy white	pale grey	flat
2	Gu2	creamy white	greyish brown	creamy white	pale grey	flat
3	Gu3	creamy white	greyish brown	creamy white	pale grey	flat
4	Gu4	creamy white	greyish brown	creamy white	pale grey	flat
5	Gu5	creamy white	greyish brown	creamy white	pale grey	flat
6	Gu6	creamy white	greyish brown	creamy white	pale grey	flat
7	Gu7	creamy white	greyish brown	creamy white	pale grey	flat
8	Gu8	creamy white	greyish brown	creamy white	pale grey	flat
9	Gu9	creamy white	greyish brown	creamy white	pale grey	flat
10	Gu10	creamy white	greyish brown	creamy white	pale grey	flat
11	Gu11	creamy white	greyish brown	creamy white	pale grey	flat
12	Gu12	creamy white	greyish brown	creamy white	pale grey	flat
13	Gu13	brownish black	brownish black	brownish black	brownish black	flat
14	Gu14	creamy white	greyish brown	creamy white	pale grey	flat
15	Gu15	creamy white	greyish brown	creamy white	pale grey	flat
16	Gu16	creamy white	greyish brown	creamy white	pale grey	flat
17	Gu17	creamy white	greyish brown	creamy white	pale grey	flat
18	Gu18	creamy white	greyish brown	creamy white	pale grey	flat
19	Gu19	creamy white	greyish brown	creamy white	pale grey	flat
20	Gu20	creamy white	greyish brown	creamy white	pale grey	flat
21	Gu21	creamy white	greyish brown	creamy white	pale grey	flat
22	Gu22	creamy white	greyish brown	creamy white	pale grey	flat
23	Gu23	creamy white	greyish brown	creamy white	pale grey	flat
24	Gu24	creamy white	greyish brown	creamy white	pale grey	flat
25	MA231	creamy white	greyish brown	creamy white	pale grey	flat
26	MAC11	creamy white	greyish brown	creamy white	pale grey	flat
27	MD25	creamy white	greyish brown	creamy white	pale grey	flat
28	MF522	creamy white	greyish brown	creamy white	pale grey	flat
29	MG53	creamy white	greyish brown	creamy white	pale grey	flat
30	MG55	creamy white	greyish brown	creamy white	pale grey	flat
31	B13	creamy white	greyish brown	creamy white	pale grey	flat
32	B18	brownish black	brownish black	brownish black	brownish black	flat
33	B24	brownish black	brownish black	brownish black	brownish black	flat
34	B25	brownish black	brownish black	brownish black	brownish black	flat
35	B26	brownish black	brownish black	brownish black	brownish black	flat
36	B27	brownish black	brownish black	brownish black	brownish black	flat
37	B28	brownish black	brownish black	brownish black	brownish black	flat
38	B33	brownish black	brownish black	brownish black	brownish black	flat
39	B34	brownish black	brownish black	brownish black	brownish black	flat
40	B38	brownish black	brownish black	brownish black	brownish black	flat
41	B39	brownish black	brownish black	brownish black	brownish black	flat
42	B41	brownish black	brownish black	brownish black	brownish black	flat
43	B42	brownish black	brownish black	brownish black	brownish black	flat
44	B43	brownish black	brownish black	brownish black	brownish black	flat
45	B50	brownish black	brownish black	brownish black	brownish black	flat
46	QLD101	brownish black	brownish black	brownish black	brownish black	flat
47	QLD106	brownish black	brownish black	brownish black	brownish black	flat
48	QLD120	brownish black	brownish black	brownish black	brownish black	flat
49	QLD151	brownish black	brownish black	brownish black	brownish black	flat
50	QLD152	brownish black	brownish black	brownish black	brownish black	flat

		Reverse		Surface		
№	**Name**	**Edge**	**Centre**	**Edge**	**Centre**	**Texture**
51	QLD411	brownish black	brownish black	brownish black	brownish black	flat
52	QLD412	brownish black	brownish black	brownish black	brownish black	flat
53	QLD413	brownish black	brownish black	brownish black	brownish black	flat
54	QLD414	brownish black	brownish black	brownish black	brownish black	flat
55	QLD421	brownish black	brownish black	brownish black	brownish black	flat
56	QLD422	brownish black	brownish black	brownish black	brownish black	flat
57	QLD511	brownish black	brownish black	brownish black	brownish black	flat
58	QLD512	brownish black	brownish black	brownish black	brownish black	flat
59	QLD611	brownish black	brownish black	brownish black	brownish black	flat
60	QLD612	brownish black	brownish black	brownish black	brownish black	flat
61	QLD621	brownish black	brownish black	brownish black	brownish black	flat
62	QLD622	brownish black	brownish black	brownish black	brownish black	flat
63	QLD711	brownish black	brownish black	brownish black	brownish black	flat
64	QLD712	brownish black	brownish black	brownish black	brownish black	flat
65	QLD722	brownish black	brownish black	brownish black	brownish black	flat
66	QLD723	brownish black	brownish black	brownish black	brownish black	flat
67	QLD724	brownish black	brownish black	brownish black	brownish black	flat
Isolates from USA						
1	IG1	creamy white	greyish brown	creamy white	pale grey	flat to slightly ropey
2	IG2	creamy white	greyish brown	creamy white	pale grey	flat to slightly ropey
3	IG5	creamy white	greyish brown	creamy white	pale grey	flat to slightly ropey
4	IG6	creamy white	greyish brown	creamy white	pale grey	flat to slightly ropey
5	IG8	creamy white	greyish brown	creamy white	pale grey	ropey
6	IG17	creamy white	greyish brown	creamy white	pale grey	ropey
7	IG10	creamy white	greyish brown	creamy white	pale grey	ropey
8	IG4	creamy white	greyish brown	creamy white	pale grey	flat to slightly ropey
9	G1.1	creamy white	greyish brown	creamy white	pale grey	flat to slightly ropey
10	G2.6	creamy white	greyish brown	creamy white	pale grey	flat to slightly ropey
11	G3.1	creamy white	greyish brown	creamy white	pale grey	flat to slightly ropey
12	G7.2	creamy white	greyish brown	creamy white	pale grey	ropey
13	G8.1	creamy white	greyish brown	creamy white	pale grey	ropey
14	G9.1	creamy white	greyish brown	creamy white	pale grey	flat to slightly ropey
15	G10.2	creamy white	greyish brown	creamy white	pale grey	flat to slightly ropey
16	G11.1	creamy white	greyish brown	creamy white	pale grey	flat to slightly ropey, scalloped edge
17	CH6.1	black	black	black	black	ropey, scalloped edge
19	CH10.1	creamy white	greyish brown	creamy white	pale grey	ropey, scalloped edge
20	CH1.2	creamy white	greyish brown	creamy white	pale grey	ropey, scalloped edge
22	CH7.2	creamy white	greyish brown	creamy white	pale grey	ropey
23	CH2.1	creamy white	greyish brown	creamy white	pale grey	ropey
24	CH8.2	creamy white	greyish brown	creamy white	pale grey	ropey
25	CH4.2	creamy white	greyish brown	creamy white	pale grey	ropey
26	CH7.1	black	black	black	black	ropey
27	HCRS2					
28	BC2					
29	WC5	creamy white	greyish brown	creamy white	pale grey	ropey

Table 2.15 Oligonucleotide primer sequences used to amplify three conserved DNA regions from *Greeneria uvicola*

	Primers		
Gene	**Forward**	**Reverse**	**Reference**
5·8-ITS rDNA	ITS1: TCCGTAGGTGAACCTGCGG	ITS4: TCCTCCGCTTATTGATATGC	White *et al.*, 1990
28S rDNA (large subunit)	LR0R: ACCCGCTGAACTTAAGC	LR7: TACTACCACCAAGATCT	Navarrete *et al.*, 2009
β-tubulin 2	Bt1a: AACATGCGTGAGATTGTAAGT	Bt1b: ACCCTCAGTGTAGTGACCCTTGGC	Glass and Donaldson, 1995

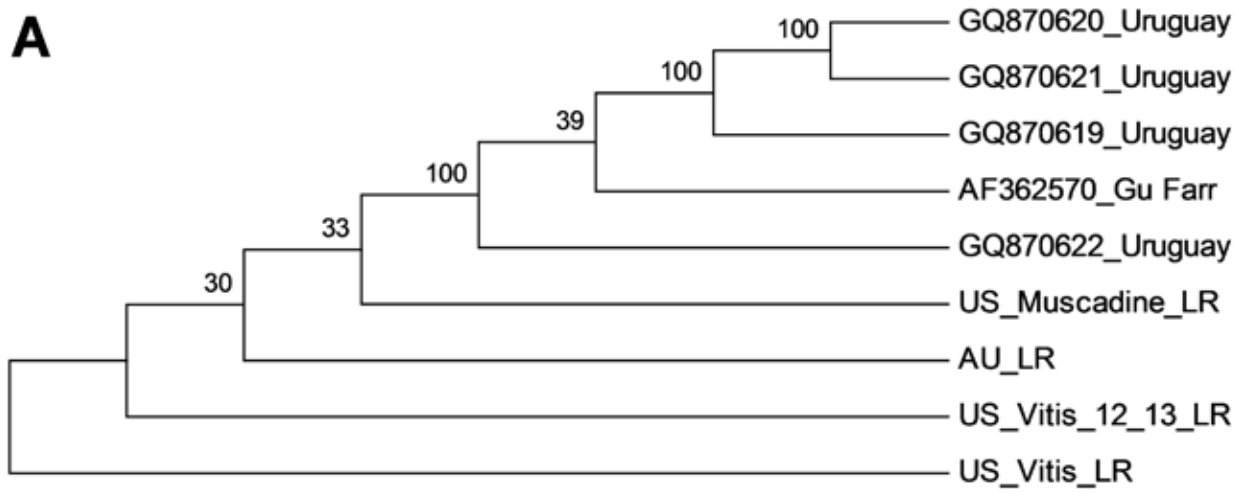
in the USA by Farr *et al.* (2011) which had higher homology with the sequences from South America compared with the other samples. The isolates from the muscadine grapes and the North American isolates were grouped together based on the 5·8-ITS and β-tubulin-2 regions (*Figure 2.16b* and *2.16c,* respectively). The differences in the 5·8-ITS and β-tubulin-2 regions between the Australian isolates were only one and two nucleotides, respectively. Differentiation between the Australian isolates from the different geographic regions and host tissues based on the analysed genes was not found. The isolates from Uruguay were also grouped together for both 28S rDNA and 5·8-ITS, however, we will restrain from further conclusions as the sequences available at GenBank are incomplete with a number of unknown 'N" nucleotides. Homology to β-tubulin-2 sequences from other studies was not found.

Pathogenicity of G. uvicola isolates on V. vinifera

Three *G. uvicola* isolates from Australia and 13 isolates from North Carolina were inoculated on to detached chardonnay berries and the percentage of berries with symptoms of bitter rot recorded after seven days. All isolates were pathogenic, although those isolates that originated from *Muscadinia rotundifolia* were not as virulent as those that had originated from *V. vinifera* (*Table 2.16*).

Table 2.16 Pathogenicity of *G. uvicola* isolates on chardonnay berries.

Isolate ID	Country of origin	Host of origin	% berries infected
GR1	Australia	*V. vinifera*	70.8
GR49	Australia	*V. vinifera*	83.3
GR67	Australia	*V. vinifera*	79.2
US1	USA	*V. vinifera*	79.2
US4	USA	*V. vinifera*	80.6
US6	USA	*V. vinifera*	84.7
US 9	USA	*V. vinifera*	83.3
US12	USA	*V. vinifera*	70.8
US16	USA	*V. vinifera*	77.8
US17	USA	*M. rotundifolia*	40.3
US19	USA	*M. rotundifolia*	59.7
US20	USA	*M. rotundifolia*	55.6
US24	USA	*M. rotundifolia*	43.1
US25	USA	*M. rotundifolia*	59.7
US26	USA	*M. rotundifolia*	47.2



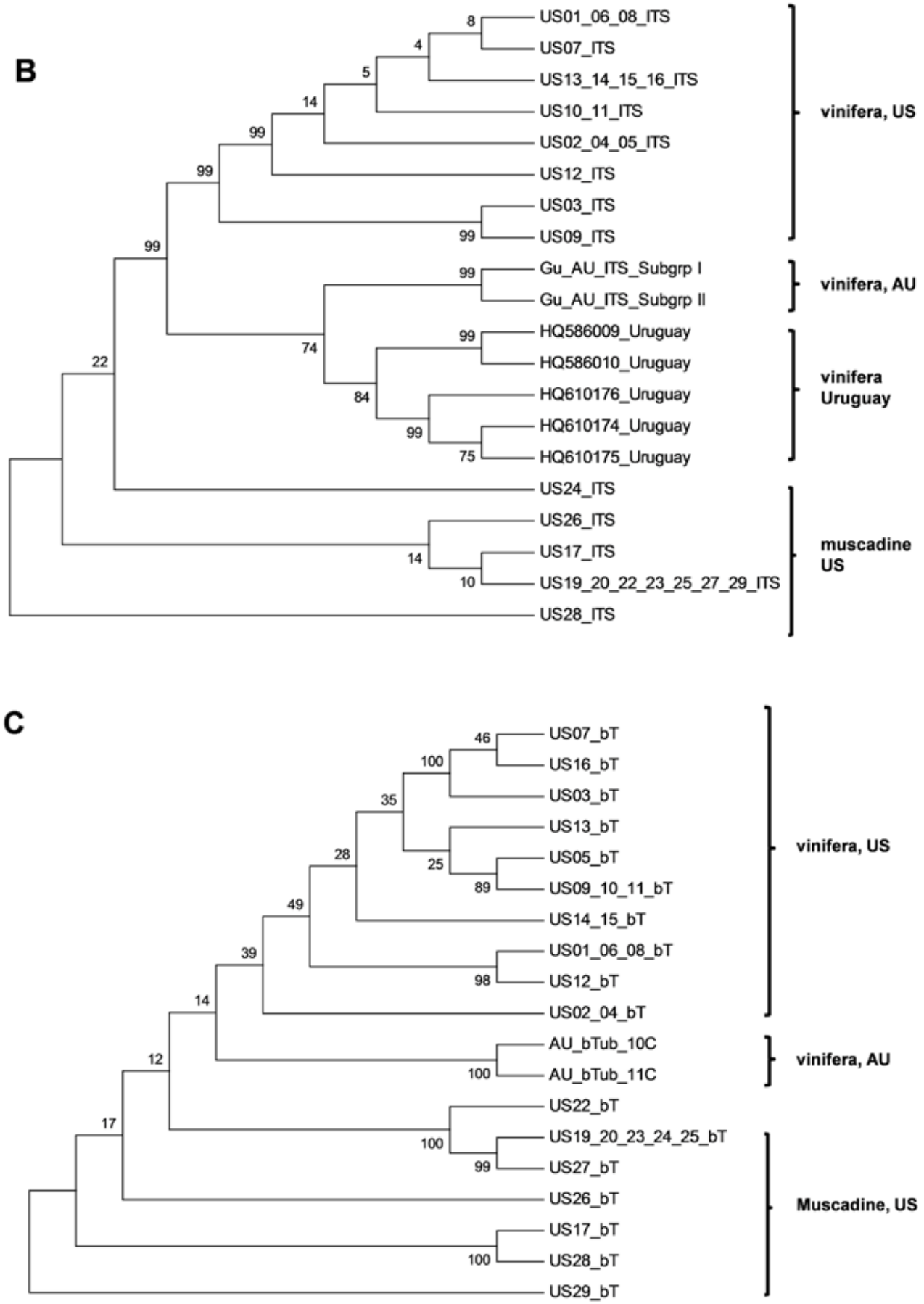


Figure 2.16 Phylogenetic relationship between *G. uvicola* isolates based on: (A) internal transcribed spacer 1 (ITS); (B) 28S large subunit (LSU) nuclear rDNA; and (C) β-tubulin 2. Numbers at nodes represent 1000 bootstrap replications.

Trunk diseases

At the commencement of the Winegrowing Futures Program trunk diseases such as Eutypa dieback, Petri disease and Esca had already been identified in Australian vineyards and were known to affect productivity (Creaser and Wicks 2001; Edwards and Pascoe 2004; Lardner *et al.* 2005). Species of Botryosphaeriaceae fungi were also implicated in grapevine decline (Bot Canker) and research had begun to identify which strains were present in Australian vineyards and which of these are responsible for the decline and dieback symptoms observed (Savocchia *et al.* 2007; Qiu *et al.* 2011). Symptoms of Bot Canker are similar to those caused by Eutypa dieback and include the distinct wedge-shaped lesion however no foliar symptoms have been recorded in Australia. Stunted shoots and loss of spur positions have also been identified.

Bot canker appeared to be predominately a problem of white varieties such as Chardonnay and Semillon whereas Eutypa dieback was known to occur mainly in red varieties. It is difficult to estimate losses caused by trunk diseases in Australia however a recent study has suggested that these diseases result in a mean national economic impact of $6 million per annum (Scholefield and Morison 2010). Bot canker alone can result in 10–50% crop loss.

This project involved an integrated management system to restore the productivity of vines infected with *Botryosphaeria* canker. A survey of vineyards provided information on the extent of the disease and which varieties are mostly at risk. Field trials incorporating various control methods were conducted and abiotic environmental factors on disease progression were examined.

The majority of Botryosphaeriaceae found on grapevines are usually considered as secondary pathogens (Phillips 1998). However, it was not known whether this was the case for species found on grapevines in Australia, and whether certain stresses lead to these secondary pathogens becoming primary pathogens. The characterisation and identification of Botryosphaeriaceae species and the management of Bot canker was a major focus of this project.

This final report also includes data gathered from a PhD study where the association and pathogenicity of Botryosphaeriaceae fungi was examined on different parts of the grapevine. The genetic diversity of the species isolated was also studied. This project was funded by a CSU PhD scholarship with additional funds provided to the student under the Winegrowing Futures program.

This project involved both field trials and laboratory based studies to elucidate the biology, genetic diversity and management of Bot canker of grapevines.

Experiment 2.10
Distribution of Botryosphaeriaceae species associated with grapevine decline in New South Wales and South Australia

Materials and methods

The methods detailed below are adapted from Pitt *et al.* (2010a).

Collection, isolation and culturing of isolates

Between November 2006 and April 2008, 73 vineyards throughout the major winegrowing regions of NSW (Big Rivers, Central Ranges, Northern Rivers, Northern Slopes, Southern NSW and South Coast), and 18 vineyards throughout SA (Adelaide Hills, Clare Valley, Eden Valley, Barossa Valley and Loxton) were surveyed for grapevines displaying symptoms of *Botryosphaeria* canker, including dead canes and cordons, cankers and bleached or discoloured wood tissue. Wood samples were taken from 2239 declining grapevines of various cultivars, viz. Cabernet Sauvignon, Cabernet Franc, Chambourcin, Chardonnay, Doradillo, Gamay, Grenache, Harslevelu, Italia, Malbec, Mataro, Merlot, Muscat, Pinot Grigio, Pinot Noir, Ruby Cabernet, Riesling, Sauvignon Blanc, Shiraz, Semillon, Saint Macaire, Sylvana, Siegerrebe, Tinta Cão, Trebbiano, Traminer and Verdello.

Fungal isolations from wood samples were carried out as previously described by Savocchia *et al.* (2007). Briefly, diseased wood samples were surface sterilised with 2% sodium hypochlorite for 2 min and rinsed twice in successive volumes of sterile distilled water, and 0.5 cm^2 wood pieces from the interface of dead and healthy tissue were transferred to 90 mm diameter petri dishes containing potato dextrose agar (PDA) supplemented with 50 $\mu g\ mL^{-1}$ of streptomycin sulphate (PDA-Strep) (Oxoid Ltd.; Sigma-Aldrich). Samples were incubated at room temperature (25 ±1°C) until fungal colonies were observed. Pure cultures of Botryosphaeriaceae species were obtained by transferring onto fresh PDA-Strep, a hyphal tip

from colony margins emerging from wood tissue pieces.

Morphological characterisation

Botryosphaeriaceae species isolated from declining grapevines were initially separated from other fungal species collected during the survey based on gross colony morphology, as reported by Urbez-Torres *et al.* (2006*a*). The characteristics of conidial morphology were then observed for each isolate, by inducing sporulation through transfer of pure cultures to 1.5% water agar containing triple autoclaved *Pinus radiata* needles. Isolates were incubated at room temperature (25 ±1°C) for four to six weeks under diurnal light consisting of 12 hour dark and near ultraviolet (UVB, 315–280 nm) light cycles. Isolates were examined and grouped according to conidial size, shape, colour and the presence or absence of septation. Several isolates from each group were selected for detailed identification to species level (*Table 2.17*).

DNA extraction, amplification and sequencing

Prior to DNA extraction, selected isolates of Botryosphaeriaceae were single-spored before being transferred to PDA for three to four days at 25°C. Colonised agar plugs were used to inoculate 50 mL Falcon tubes containing 20 mL of potato-dextrose broth (Oxoid). Tubes were incubated on an orbital shaker at 90 rpm for up to seven days at 25°C. Mycelia were harvested by filtration, lyophilised and DNA extracted using the Qiagen Plant Mini Kit according to the manufacturer's instructions (Qiagen Pty. Ltd.). Molecular identification of Botryosphaeriaceae species was achieved via amplification and comparison of ribosomal DNA internal transcribed spacer (ITS) regions, and if necessary, partial sequencing of the translation elongation factor 1-alpha (EF1-α) gene.

For ITS PCR each reaction contained 0.1 volume of 10× buffer (containing 15 mM $MgCl_2$, Qiagen), 200 µM each of dNTPs, 0.15 µM each of primers ITS1 and ITS4 (White *et al.* 1990), 1 unit of HotStar *Taq* DNA polymerase (Qiagen), approximately 50 ng of DNA template, and were adjusted with sterile nanopure water to a total volume of 50 µL. PCR reactions were performed using an Eppendorf Master Thermocylcer. Amplification was achieved by an initial step of 15 min at 95°C, followed by 40 cycles of 30 s at 94°C, 45 s at 55°C, and 1.5 min at 72°C, with a final extension of 5 min at 72°C. For EF1-α PCR, reaction components were as described above however; reactions were made up to a total volume of 40 µL and comprised instead 0.5 µM each of primers, EF1-728F and EF1-986R (Carbone and Kohn 1999). After an initial step of 15 min at 95°C, amplification comprised 35 cycles of 30 s at 95°C, 40 s at 58°C, and 1 min at 72°C, with a final extension of 5 min at 72°C. PCR products were separated by electrophoresis on 1% agarose containing 0.5 × Tris-acetate-EDTA (TAE) buffer. ITS and EF1-α PCR products were purified using the QIA quick PCR purification kit (Qiagen), and sequenced in both directions by the Australian Genome Research Facility (University of Queensland, St Lucia, Queensland, Australia).

Phylogenetic analysis

Molecular identification of Botryosphaeriaceae species isolated during the survey was confirmed by comparison of gene sequences of our isolates with those available in GenBank (*Table 2.18*). With the exception of *Dothiorella iberica* and *Neofusicoccum australe* for which definitive identification required, in addition to ITS, partial sequencing and comparison of the EF1-α gene, all species were identified to species level based on ITS sequence analysis. Individual sequences were compiled in BioEdit sequence alignment editor (Hall 1999) and aligned for comparison using ClustalX (Thompson *et al.* 1997). A phylogenetic tree was produced according to the neighbour-joining method of Saitou and Nei (1987) based on Kimura's (1980) two-parameter method for estimating evolutionary distances. Statistical support for inferred groups was estimated by bootstrap analysis (Felsenstein 1985) using 1000 replications. Phylogenies were displayed using NJ Plot (Perriere and Guoy 1996).

Representative isolates of each species reported in this paper are maintained in the collection at the NWGIC (Charles Sturt University, Wagga Wagga, NSW, Australia), and were deposited in the Australian Scientific Collections Unit (DAR, NSW Department of Primary Industries, Orange, NSW, Australia). DNA sequences from the studied region of each isolate were deposited in GenBank (*Table 2.17*).

Table 2.17 Morphological and molecular details of select Botryosphaeriaceae species isolated from diseased grapevines (*Vitis vinifera*) from New South Wales and South Australia, and used in this study (Pitt *et al.* 2010a).

			Conidial dimensions			Herbarium	GenBank accession	
Species	**Origin**	**Host/cultivar**	**Conidial size (μm)[a]**	**Mean ±SD (μm)[b]**	**L/W (μm)[c]**	**accession[d]**	**ITS[e]**	**EF1-α[f]**
Botryosphaeria dothidea								
CSU-07-WP-BMV14	Mittagong, NSW	Sauvignon Blanc	(21.0–)24.7–25.3(–28.1) X (5.6–)6.7–6.9(–8.0)	25.0 ±1.2 X 6.8 ± 0.5	3.69 ± 0.29	DAR79239	FJ176567	-
CSU-07-WP-BMV15	Mittagong, NSW	Sauvignon Blanc	(21.9–)24.5–25.2(–28.9) X (5.7–)6.9–7.2(–8.3)	24.9 ± 1.2 X 7.1 ± 0.6	3.53 ± 0.27	DAR79240	FJ176568	-
CSU-07-WP-GE14	Young, NSW	Chardonnay	(20.9–)25.9–27.2(–31.4) X (5.3–)6.4–6.6(–7.4)	26.5 ± 2.3 X 6.5 ± 0.5	4.10 ± 0.43	DAR79241	FJ176569	-
CSU-07-WP-TS16	Hunter Valley, NSW	Chardonnay	(24.1–)25.4–25.9(–27.8) X (6.7–)7.6–7.9(–8.9)	25.7 ± 0.9 X 7.8 ± 0.5	3.32 ± 0.21	DAR79242	FJ176570	-
CSU-07-WP-TS18	Hunter Valley, NSW	Chardonnay	(23.2–)26.1–26.8(–28.7) X (5.9–)6.8–7.0(–8.0)	26.5 ± 1.2 X 6.9 ± 0.4	3.83 ± 0.23	DAR79243	FJ176571	-
CSU-07-WP-VT1	Canowindra, NSW	Chardonnay	(24.5–)27.5–28.3(–31.4) X (6.2–)6.8–7.0(–7.9)	27.9 ± 1.5 X 6.9 ± 0.4	4.05 ± 0.24	DAR79244	FJ176572	-
Diplodia mutila								
CSU-07-WP-TS23	Hunter Valley, NSW	Chardonnay	(21.0–)23.8–24.7(–27.9) X (9.3–)10.7–11.2(–12.8)	24.3 ± 1.7 X 11.0 ± 0.9	2.21 ± 0.03	DAR79001	EU768879	-
CSU-07-WP-CG15	Tumbarumba, NSW	Pinot Noir	(15.6–)22.5–23.7(–30.1) X (8.0–)9.9–10.4(–12.4)	23.1 ± 2.2 X 10.2 ± 0.9	2.27 ± 0.07	DAR79135	EU919691	-
CSU-07-WP-CV5	Orange, NSW	Sauvignon Blanc	(19.7–)23.0–24.1(–28.2) X (8.6–)9.9–10.3(–12.0)	23.6 ± 1.9 X 10.1 ± 0.7	2.33 ± 0.23	DAR79129	EU919689	-
CSU-07-WP-DO7	Murrumbateman, NSW	Shiraz	(20.0–)24.4–25.6(–28.3) X (10.5–)11.9–12.5(–14.6)	25.0 ± 2.2 X 12.2 ± 1.1	2.05 ± 0.06	DAR79136	EU919692	-
CSU-07-WP-FF18	Adelaide Hills, SA	Shiraz	(19.1–)23.7–24.9(–28.3) X (9.0–)11.0–11.5(–13.3)	24.3 ± 2.3 X 11.2 ± 1.0	2.16 ± 0.04	DAR79137	EU919686	-
CSU-07-WP-GE24	Young, NSW	Chardonnay	(20.0–)24.4–25.6(–29.5) X (9.4–)10.8–11.4(–12.6)	25.0 ± 2.1 X 11.1 ± 0.9	2.26 ± 0.04	DAR79130	EU919693	-
CSU-07-WP-PG15	Wagga Wagga, NSW	Tinta Coa	(20.0–)22.9–23.8(–27.2) X (8.6–)11.2–11.8(–14.2)	23.3 ± 1.6 X 11.5 ± 1.1	2.03 ± 0.06	DAR79131	EU919687	-

[a] Data presented as the lower and upper 95% confidence limits of 50 conidia, with minimum and maximum dimensions in parenthesis; [b] Mean and SD=standard deviation of 50 conidia; [c] L/W=length/width ratio, mean and standard deviation of 50 conidia; [d] DAR, Australian Scientific Collections Unit, Orange, Australia; [e] ITS, internal transcribed spacer; [f] EF1-α, translation elongation factor 1-α.

Species	Origin	Host/cultivar	Conidial dimensions			Herbarium accession[d]	GenBank accession	
			Conidial size (μm)[a]	Mean ±SD (μm)[b]	L/W (μm)[c]		ITS[e]	EF1-α[f]
CSU-07-WP-TSE17	Jaspers Brush, NSW	Malbec/Shiraz	(20.1–)24.0–24.9(–27.0) X (8.9–)10.9–11.3(–12.3)	24.4 ± 1.6 X 11.1 ± 0.8	2.20 ± 0.02	DAR79138	EU919690	-
CSU-07-WP-VP11	Cowra, NSW	Chardonnay	(17.5–)21.8–23.1(–29.9) X (8.4–)9.7–10.3(–12.3)	22.4 ± 2.3 X 10.0 ± 1.0	2.24 ± 0.06	DAR79132	EU919688	-
Diplodia seriata								
CSU-07-WP-DO4	Murrumbateman, NSW	Shiraz	(18.2–)22.5–23.5(–25.9) X (8.4–)10.0–10.4(–12.0)	23.0 ± 1.9 X 10.2 ± 0.8	2.27 ± 0.25	DAR79133	FJ176562	-
CSU-07-WP-J10	Eden Valley, SA	Chardonnay	(20.1–)24.0–25.0(–28.0) X (8.1–)10.1–10.7(–12.6)	24.5 ± 1.8 X 10.4 ± 1.0	2.01 ± 0.08	DAR79140	FJ176561	-
CSU-07-WP-VQ14	Cowra, NSW	Chardonnay	(17.8–)22.6–23.7(–27.2) X (9.0–)11.1–11.9(–15.1)	23.1 ± 2.0 X 11.5 ± 1.4	2.38 ± 0.28	DAR79134	FJ176565	-
CSU-07-WP-YVW2	Murrumbateman, NSW	Chardonnay	(22.1–)23.7–24.5(–28.3) X (9.4–)10.8–11.3(–13.3)	24.1 ± 1.4 X 11.0 ± 1.0	2.19 ± 0.07	DAR79142	FJ176566	-
CSU-07-WP-ME10	Mittagong, NSW	Sauvignon Blanc	(20.7–)22.7–23.4(–25.7) X (9.6–)10.7–11.1(–12.4)	23.1 ± 1.3 X 10.9 ± 0.8	2.12 ± 0.04	DAR79236	FJ176563	-
CSU-07-WP-ME21	Mittagong, NSW	Sauvignon Blanc	(18.6–)23.6–24.8(–28.7) X (9.0–)10.3–10.9(–12.7)	24.2 ± 2.3 X 10.6 ± 1.0	2.28 ± 0.05	DAR79237	FJ176564	-
CSU-07-WP-II12	Barossa Valley, SA	Shiraz	(17.4–)22.3–23.7(–29.3) X (8.9–)11.3–12.0(–14.7)	23.0 ± 2.5 X 11.7 ± 1.3	1.97 ± 0.03	DAR79238	FJ176560	-
Lasiodiplodia theobromae								
CSU-07-WP-C2	Port Macquarie, NSW	Pinot Noir	(22.5–)27.3–28.3(–32.9) X (13.1–)14.5–15.0(–16.5)	2`7.8 ± 1.9 X 14.7 ± 0.8	1.89 ± 0.16	DAR79507	FJ422895	-
Neofusicoccum australe								
CSU-07-WP-DNW8	Tenterfield, NSW	Matteo	(20.4–)24.7–25.8(–29.7) X (5.0–)7.3–8.0(–10.1)	25.2 ± 2.1 X 7.7 ± 1.3	3.33 ± 0.29	DAR79501	FJ422897	FJ422905
CSU-07-WP-FF10	Adelaide Hills, SA	Shiraz	(23.2–)25.8–26.6(–28.6) X (5.4–)6.4–6.8(–8.1)	26.2 ± 1.4 X 6.6 ± 0.6	3.97 ± 0.19	DAR79502	FJ422904	FJ422906
CSU-07-WP-FF24	Adelaide Hills, SA	Shiraz	(22.1–)24.9–25.9(–28.8) X (6.1–)7.3–7.7(–8.8)	25.4 ± 1.8 X 7.5 ± 0.7	3.40 ± 0.12	DAR79503	FJ422898	FJ422907

[a] Data presented as the lower and upper 95% confidence limits of 50 conidia, with minimum and maximum dimensions in parenthesis; [b] Mean and SD=standard deviation of 50 conidia; [c] L/W=length/width ratio, mean and standard deviation of 50 conidia; [d] DAR, Australian Scientific Collections Unit, Orange, Australia; [e] ITS, internal transcribed spacer; [f] EF1-α, translation elongation factor 1-α.

Species	Origin	Host/cultivar	Conidial dimensions			Herbarium accession[d]	GenBank accession	
			Conidial size (μm)[a]	Mean ±SD (μm)[b]	L/W (μm)[c]		ITS[e]	EF1-α[f]
CSU-07-WP-ME10	Mittagong, NSW	Sauvignon Blanc	(19.8–)24.8–25.9(–28.6) X (5.7–)6.8–7.2(–7.9)	25.3 ± 2.0 X 7.0 ± 0.6	3.62 ± 0.09	DAR79504	FJ422899	FJ422909
CSU-07-WP-SDW4	Mittagong, NSW	Shiraz	(19.3–)24.4–25.6(–29.2) X (5.5–)7.1–7.7(–9.6)	25.0 ± 2.2 X 7.4 ± 1.0	3.41 ± 0.21	DAR79505	FJ422903	FJ422911
CSU-07-WP-VP13	Cowra, NSW	Chardonnay	(21.1–)23.3–24.1(–27.3) X (5.1–)6.7–7.3(–9.2)	23.7 ± 1.6 X 7.0 ± 1.0	3.42 ± 0.34	DAR79506	FJ422902	FJ422913
Neofusicoccum parvum								
CSU-07-WP-B12	Port Macquarie, NSW	Cabernet Sauvignon	(11.4–)15.7–16.8(–20.7) X (5.0–)7.4–8.2(–10.6)	16.2 ± 2.1 X 7.8 ± 1.5	2.10 ± 0.14	DAR78997	EU919694	-
CSU-07-WP-B19A	Port Macquarie, NSW	Cabernet Sauvignon	(14.2–)16.8–17.6(–19.6) X (5.4–)6.5–7.0(–8.7)	17.2 ± 1.4 X 6.8 ± 0.8	2.55 ± 0.10	DAR78998	EU919695	-
CSU-07-WP-B7A	Port Macquarie, NSW	Cabernet Sauvignon	(13.1–)15.9–16.9(–19.8) X (6.1–)7.4–7.9(–9.3)	16.4 ± 1.8 X 7.6 ± 0.8	2.15 ± 0.03	DAR78999	EU919700	-
CSU-07-WP-B7B	Port Macquarie, NSW	Cabernet Sauvignon	(10.8–)15.8–16.9(–19.6) X (5.7–)6.9–7.3(–8.4)	16.3 ± 2.0 X 7.1 ± 0.7	2.28 ± 0.08	DAR79000	EU919701	-
Dothiorella iberica								
CSU-07-WP-J24	Eden Valley, SA	Chardonnay	(17.9–)21.3–22.1(–25.2) X (7.9–)9.3–9.6(–11.3)	21.7 ± 1.4 X 9.4 ± 0.6	2.31 ± 0.22	DAR78991	EU768875	EU768880
CSU-07-WP-J4	Eden Valley, SA	Chardonnay	(17.9–)21.2–21.9(–24.2) X (8.3–)9.6–9.8(–10.6)	21.6 ± 1.2 X 9.7 ± 0.5	2.23 ± 0.13	DAR78992	EU768874	EU768881
CSU-07-WP-L5	Loxton, SA	Cabernet Sauvignon	(18.4–)21.0–21.6(–23.8) X (7.9–)9.2–9.6(–11.8)	21.3 ± 1.2 X 9.4 ± 0.6	2.27 ± 0.17	DAR78993	EU768876	EU768882
CSU-07-WP-M21	Barossa Valley, SA	Gamay	(19.1–)21.8–22.5(–24.8) X (8.7–)10.0–10.3(–11.6)	22.2 ± 1.2 X 10.2 ± 0.6	2.19 ± 0.16	DAR78994	EU768877	EU768883
CSU-07-WP-N19	Adelaide Hills, SA	Chardonnay	(18.0–)20.7–21.5(–23.4) X (8.0–)9.5–9.9(–10.9)	21.1 ± 1.3 X 9.7 ± 0.7	2.18 ± 0.15	DAR78995	EU768878	EU768884
Dothiorella viticola								
CSU-07-WP-J7	Eden Valley, SA	Chardonnay	(15.7–)19.6–20.4(–23.1) X (8.8–)10.3–10.8(–12.5)	20.0 ± 1.4 X 10.6 ± 0.8	1.90 ± 0.17	DAR78869	EU603287	-

[a] Data presented as the lower and upper 95% confidence limits of 50 conidia, with minimum and maximum dimensions in parenthesis; [b] Mean and SD=standard deviation of 50 conidia; [c] L/W=length/width ratio, mean and standard deviation of 50 conidia; [d] DAR, Australian Scientific Collections Unit, Orange, Australia; [e] ITS, internal transcribed spacer; [f] EF1-α, translation elongation factor 1-α.

Species	Origin	Host/cultivar	Conidial dimensions			Herbarium accession[d]	GenBank accession	
			Conidial size (μm)[a]	Mean ±SD (μm)[b]	L/W (μm)[c]		ITS[e]	EF1-α[f]
CSU-07-WP-CV11	Orange, NSW	Sauvignon Blanc	(20.3–)23.6–24.3(–26.1) X (8.4–)9.3–9.5(–10.3)	23.9 ± 1.2 X 9.4 ± 0.5	2.56 ± 0.17	DAR78873	EU603288	-
CSU-07-WP-J3	Eden Valley, SA	Chardonnay	(17.1–)19.3–19.9(–21.7) X (7.1–)9.5–9.9(–11.1)	19.6 ± 1.0 X 9.7 ± 0.7	2.02 ± 0.15	DAR78871	EU603289	-
CSU-07-WP-J8	Eden Valley, SA	Chardonnay	(16.8–)18.8–19.4(–21.2) X (8.6–)9.7–10.0(–11.2)	19.1 ± 1.0 X 9.8 ± 0.6	1.95 ± 0.14	DAR78867	EU603290	-
CSU-07-WP-K16	Eden valley, SA	Riesling	(16.7–)20.5–21.3(–23.6) X (8.9–)10.6–11.0(–12.7)	20.9 ± 1.4 X 10.8 ± 0.7	1.95 ± 0.14	DAR78866	EU603291	-
CSU-07-WP-N23	Adelaide Hills, SA	Chardonnay	(17.9–)20.5–21.1(–23.4) X (9.1–)10.3–10.7(–12.2)	20.8 ± 1.1 X 10.5 ± 0.7	1.98 ± 0.16	DAR78872	EU603292	-
CSU-07-WP-L19	Loxton, SA	Cabernet Sauvignon	(18.1–)19.9–20.5(–22.9) X (8.3–)9.3–9.6(–10.4)	20.2 ± 1.1 X 9.5 ± 0.6	2.14 ± 0.15	DAR78870	EU603293	-
CSU-07-WP-M11	Barossa Valley, SA	Gamay	(17.7–)20.4–21.0(–22.6) X (8.2–)9.3–9.6(–10.4)	20.7 ± 1.1 X 9.4 ± 0.6	2.20 ± 0.15	DAR78868	EU603294	-

[a] Data presented as the lower and upper 95% confidence limits of 50 conidia, with minimum and maximum dimensions in parenthesis; [b] Mean and SD=standard deviation of 50 conidia; [c] L/W=length/width ratio, mean and standard deviation of 50 conidia; [d] DAR, Australian Scientific Collections Unit, Orange, Australia; [e] ITS, internal transcribed spacer; [f] EF1-α, translation elongation factor 1-α.

Results and discussion

The results and discussion presented below are adapted from Pitt *et al.* (2010*a*)

Identification

Recent studies have shown that many species within the Botryosphaeriaceae are singularly or collectively responsible for grapevine decline in Australia (Taylor *et al*. 2005; Wood and Wood 2005; Savocchia *et al*. 2007; Qiu *et al*. 2011). Colony and conidial morphology (*Table 2.17*), along with phylogenetic analysis both of rDNA internal transcribed spacer regions (ITSI-5.8S-ITS2) and partial sequences of the EF1-α gene (*Figures 2.17* and *2.18*) showed that at least eight Botryosphaeriaceae species (*Figure 2.19*) occur on grapevines throughout Australia (*Figure 2.20*). In the phylogeny, these species; *Fusicoccum aesculi* (*Botryosphaeria dothidea*), *Neofusicoccum parvum*, *N. australe*, *Diplodia seriata*, *D. mutila*, *Lasiodiplodia theobromae*, *Dothiorella viticola* and *D. iberica*, fell into four highly supported clades (*Figure 2.17*), each separated and aligned with the distinct morphological characters typical of their respective genera and species.

Table 2.18 Botryosphaeriaceae and associated sequences from GenBank (Pitt *et al.* 2010).

Species	Host	Origin	Herbarium accession[b]	GenBank accession	
				ITS	EF1-α
Botryosphaeria dothidea					
CBS110302	*Vitis vinifera*	Portugal	CBS110302	AY259092	AY573218
CMW8000	*Prunus* sp.	Switzerland	CMW8000	AY236949	AY236898
Diplodia mutila					
CMW7060	*Fraxinus excelsior*	The Netherlands	CMW7060	AY236955	AY236904
CBS112553	*Vitis vinifera*	Portugal	CBS112553	AY259093	AY573219
Diplodia seriata					
CMW7775	*Ribes* sp.	United States	CMW7775	AY236954	AY236903
CBS112555	*Vitis vinifera*	Portugal	CBS112555	AY259094	AY573220
Lasiodiplodia theobromae					
CBS164.96	nk[a]	Papua New Guinea	CBS164.96	AY640255	AY640258
CMW9074	*Pinus* sp.	Mexico	CMW9074	AY236952	AY236901
Neofusicoccum australe					
CMW6853	*Sequiadendron giganteum*	Australia	CMW6853	AY339263	AY339271
CMW6837	*Acacia* sp.	Australia	CMW6837	AY339262	AY339270
Neofusicoccum luteum					
CBS110299	*Vitis vinifera*	Portugal	CBS110299	AY259091	AY573217
CBS110497	nk	Portugal	CBS110497	EU673311	EU673277
Neofusicoccum parvum					
ATCC58159	*Malus* sp.	New Zealand	ATCC58159	AF243395	AY236883
CBS110301	*Vitis vinifera*	Portugal	CBS110301	AF259098	AY573221
Dothiorella iberica					
CBS115041	*Quercus ilex*	Spain	CBS115041	AY573202	AY573222
CBS115035	*Quercus ilex*	Spain	CBS115035	AY573213	AY573228
Dothiorella sarmentorum					
IMI63581b	*Ulmus* sp.	United Kingdom	IMI63581b	AY573212	AY573235
CBS120.41	*Pyrus communis*	Norway	CBS120.41	AY573207	AY573224
Dothiorella viticola					
CBS117009	*Garnatxa negra*	Spain	CBS117009	AY905554	AY905559
CBS117006	*Garnatxa negra*	Spain	CSB117006	AY905555	AY905562
Mycosphaerella pini					
CMW11372	*Pinus radiata*	South Africa	CMW11372	AY808277	AY808242
Bionectria sp.					
CMW7063	nk	South Africa	CMW7063	AY236956	AY236905

[a] nk= not known; [b] CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; ATCC, American Type Culture Collection, Manassas, Virginia, USA; IMI, CABI Bioscience (formerly, International Mycological Institute), Oxfordshire, United Kingdom; CMW, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Clade one comprised species with *Lasiodiplodia* and *Diplodia* anamorphs, genera broadly characterised by the production of pigmented, thick-walled conidia (Denman *et al*. 2000). Clades two and three comprised species with *Fusicoccum* and *Neofusicoccum* anamorphs, for which conidia are generally hyaline and thin-walled (Denman *et al*. 2000), with some species such as *N. parvum* becoming pigmented and septate with age (Crous *et al*. 2006), and clade four comprised species of *Dothiorella*, which have *Diplodia*-like conidia and *Botryosphaeria*-like pigmented, septate ascospores (Phillips *et al*. 2005). While a range of other fungal species were isolated during these surveys, including

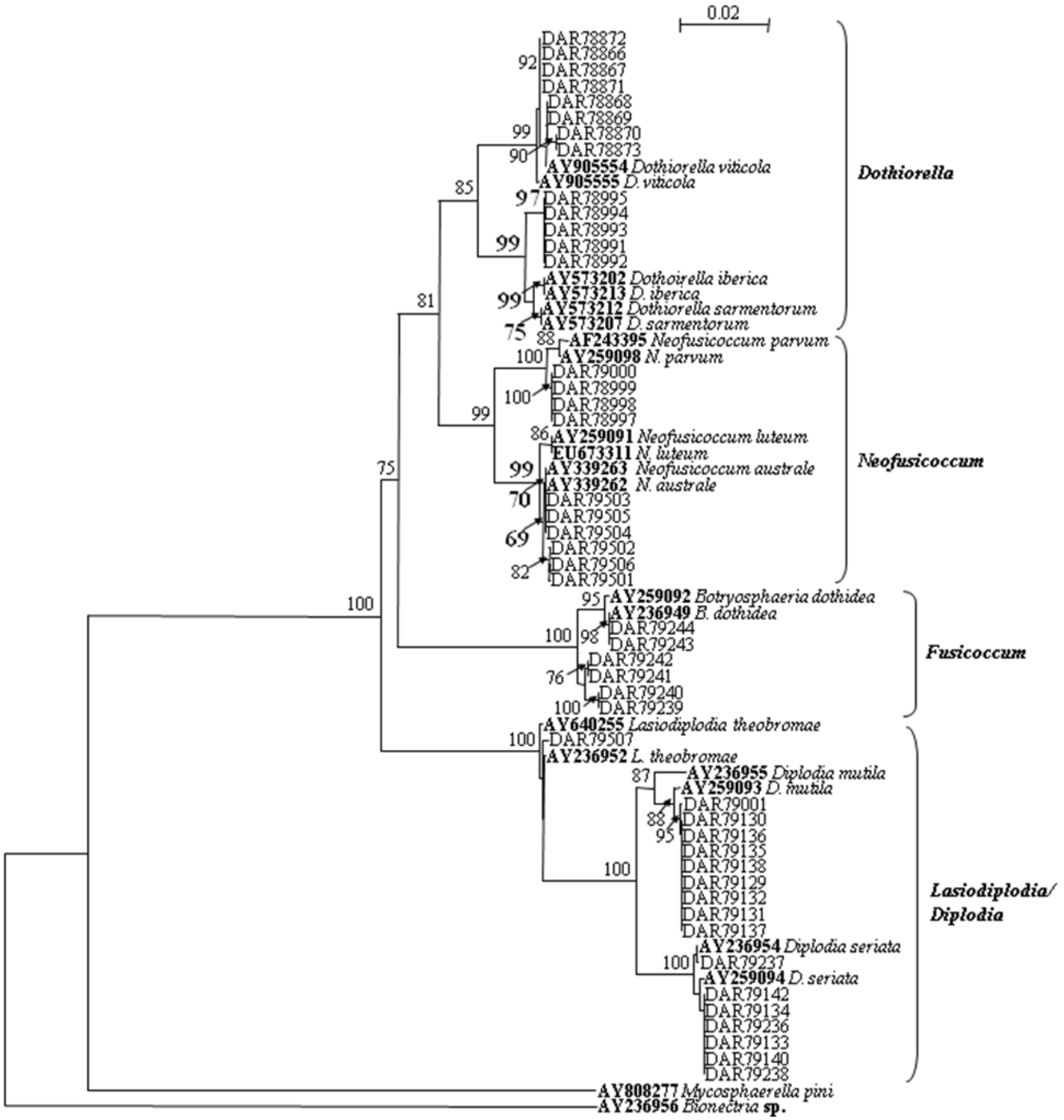


Figure 2.17 Neighbour-joining tree of ribosomal DNA internal transcribed spacer sequences from Botryosphaeriaceae species isolated during the field survey. Bootstrap values of 70* or greater (percentage of 1000 replications) are indicated, rounded to the nearest integer. Branch lengths are proportional to genetic distance, which is indicated by the bar at the upper right. The anamorph of each Botryosphaeriaceae lineage is shown. *Mycosphaerella* and *Bionectria* sp. (originally submitted to Genbank as *Guignardia philoprina*) were used as outgroups for the analysis, as demonstrated by Urbez-Torres *et al*. (2006*a*). GenBank reference sequences for each individual species are shown in bold.

* Bootstrap values at critical nodes are shown in large bold face. Critical values less than 70 are shown when significant.

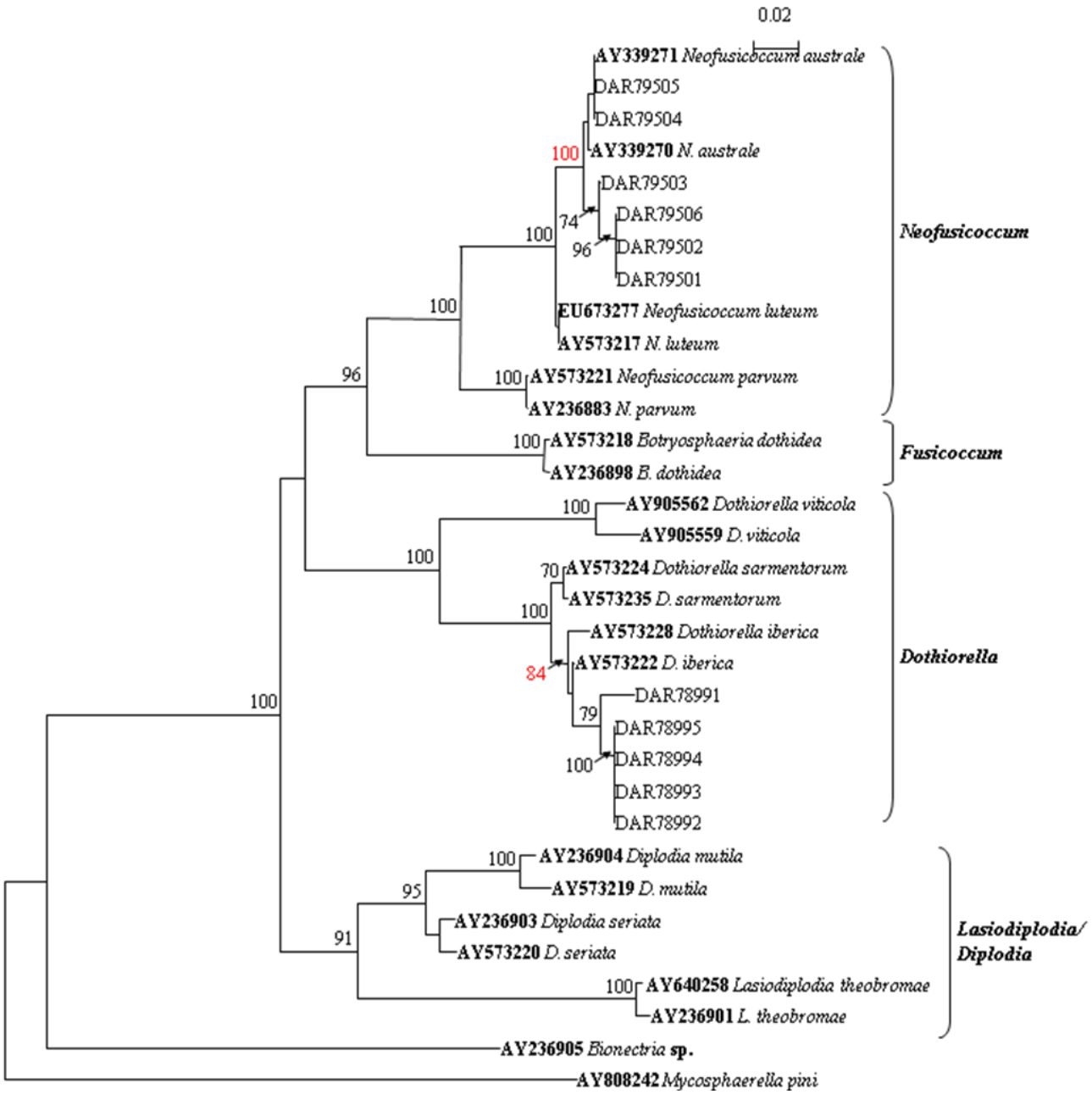


Figure 2.18 Neighbour-joining tree of partial translation elongation factor 1-a sequences from Botryosphaeriaceae species isolated during the field survey. Bootstrap values of 70* or greater (percentage of 1000 replications) are indicated, rounded to the nearest integer. Branch lengths are proportional to genetic distance, which is indicated by the bar at the upper right. The anamorph of each Botryosphaeriaceae lineage is shown. *Mycosphaerella* and *Bionectria* sp. were used as outgroups for the analysis, as demonstrated by Urbez-Torres *et al.* (2006*a*). GenBank reference sequences for each individual species are shown in bold.
* Bootstrap values at critical nodes are shown in large bold face.

other grapevine trunk disease pathogens such us *Phomopsis viticola*, *Phaeomoniella chlamydospora* and *E. lata*, *Cryptovalsa ampelina* and other diatrypaceous species (*Table 2.19*, Pitt *et al.* 2010*b*), discussions in this paper are confined to the Botryosphaeriaceae, species causal of 'bot' canker.

Lasiodiplodia and Diplodia

Within this clade, *L. theobromae* was highly supported (100%), with this species occupying a branch separate to that of *Diplodia*, which was itself then split into two at the inter-specific level, with each resulting node highly supported by bootstrap replications and comprising only isolates of a single species (*Figure 2.17*). Our results are in agreement with Pavlic *et al.* (2004) who considered *Lasiodiplodia* and *Diplodia* separate lineages on the basis of morphological differences, specifically the paraphyses and longitudinal striations characteristic of *L. theobromae*. However, both species were once synonymous with *Sphaeropsis* (Denman *et al.* 2000). *Diplodia*, as typified by *D. mutila*, is

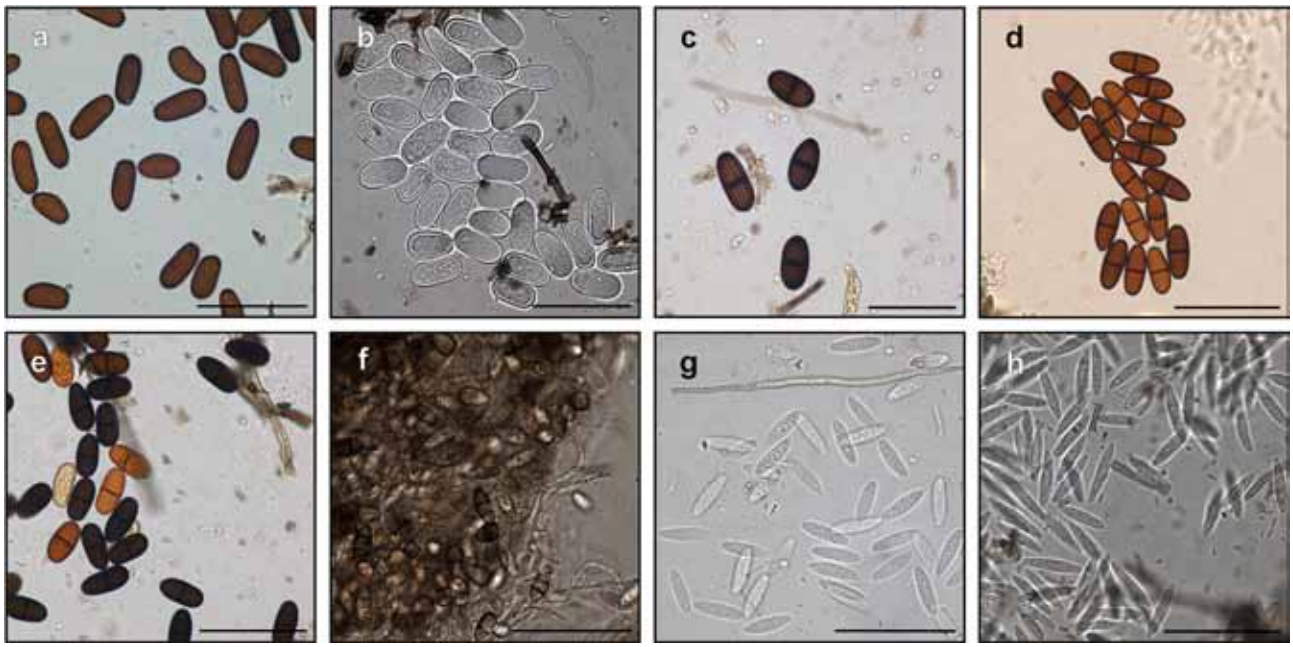


Figure 2.19 Morphology of Botryosphaeriaceae species isolated from grapevines in New South Wales and South Australia, a) *Diplodia seriata*, b) *Diplodia mutila*, c) Lasiodiplodia theobromae, d) *Dothiorella iberica*, e) *Dothiorella viticola*, f) *Neofusicoccum parvum*, g) *Neofusicoccum australe*, h) *Botryosphaeria dothidea*. Bar=50 μm.

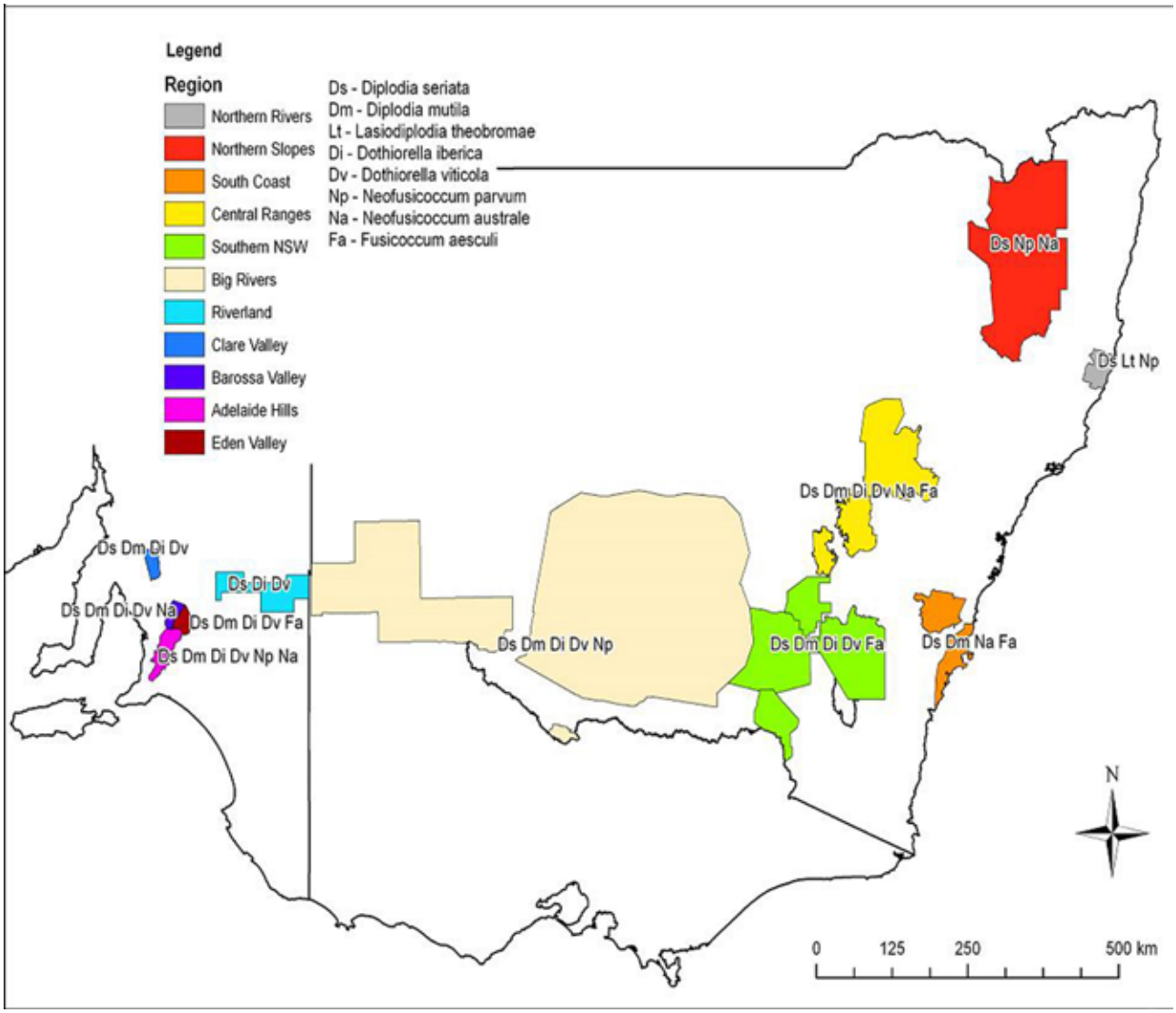


Figure 2.20 Geographical distribution of the Botryosphaeriaceae species isolated from grapevines in New South Wales and South Australia. Footnote; Hunter Valley and Mudgee regions previously surveyed by Qiu *et al.* (2011).

characterised by the production of hyaline aseptate conidia that occasionally become brown and septate with age (Sutton 1980; Alves *et al*. 2004). The distinction between *D. mutila* and *D. seriata*, as determined morphologically by the onset of conidial pigmentation, is well supported by obvious genetic differences between the species (*Figure 2.17*).

Neofusicoccum and Fusicoccum

While the positions of *Dothiorella* and *Neofusicoccum* on separate branches (*Figure 2.17*), both highly supported, duly reflect the differing morphological characters that make them distinct, *Neofusicoccum* and *Dothiorella* are related with a common ancestor, as demonstrated previously by Phillips *et al*. (2005) and Crous *et al*. (2006). Interestingly, this finding supports Petrak's (1922) decision to transfer *F. aesculi* to *Dothiorella* prior to the introduction of *Neofusicoccum* at a time when many of these species were still accommodated in *Fusicoccum*. However, that decision was found to be invalid (Phillips *et al*. 2005), and *Neofusicoccum* was introduced by Crous *et al*. (2006) to accommodate *Fusicoccum*-like species with *Botryosphaeria*-like teleomorphs and *Dichomera*-like synanamorphs possessing brown globose to pyriform conidia. Currently this genus accommodates many of the species previously described in *Fusicoccum*, including both *N. parvum* and *N. australe*, which were isolated in this study. Phylogeny reconstruction placed these two species together within the clade, but on separate branches, the later species grouping closely with two representative isolates of *N. luteum*, the ITS sequences of which were downloaded from GenBank. Like *Diplodia*, ITS sequence analysis clearly differentiated *Neofusicoccum* at the inter-specific level. However, while the segregation of *N. australe* and *N. luteum* was highly supported (99%), clustering of our *N. australe* isolates with those of the reference cultures, was less robust with only 69% of bootstrap replications supporting the group (*Figure 2.17*).

Morphologically, the most distinctive feature of *N. luteum* is a transient yellow pigmentation that appears early in the growth cycle of the fungus (Witcher and Clayton 1963; Pennycook and Samuels 1985). The production of a similar yellow pigmentation in cultures of *N. australe* (Slippers *et al*. 2004*b*), although useful, inadvertently reduced the taxonomic utility of this feature, making morphological differentiation of *N. luteum* from *N. australe* impossible. Hence, definitive segregation of *N. australe* and *N. luteum* was achieved through alignment of partial sequences of the EF1-α gene (*Figure 2.18*). Sequences from four of our isolates of *N. australe* differed from those of the two representative strains of *N. luteum* by seven base pairs, with a further two isolates comprising, in addition to this, an 11 base pair deletion in this region. Evident in alignments conducted against sequences both from *N. australe* and *N. luteum*, these additional substitutions were considered significant. Nevertheless, these isolates were still most closely related to *N. australe*, and along with the other four *N. australe* isolates from our study, formed a group together with the respective reference cultures. Both this group and its segregation from the branch comprising *N. luteum* were supported by 100% of bootstrap replications in the resulting EF1-α phylogram (*Figure 2.18*).

Fusicoccum aesculi, the anamorph of *Botryosphaeria dothidea*, the type species of the genus, was shown to cluster separately from *Neofusicoccum*, both in this study and in that of Crous *et al*. (2006). Hence, despite having conidia with some *Dichomera*-like characteristics, phylogeny reconstruction based on sequence compositions of the ITS region, clearly differentiate this species from those recently transferred to *Neofusicoccum*.

Dothiorella

ITS sequences from Australian isolates of *Dothiorella* successfully differentiated *D. iberica* and *D. viticola*. However, sequences for isolates DAR78991-78995 formed a sub-clade apart from the known species of *D. iberica* and *D. sarmentorum*. Since morphological differentiation between *D. iberica* and *D. sarmentorum*, a closely related plurivorous species possessing slightly longer, less clavate asci and shorter ascospores (Phillips *et al*. 2005), is difficult, partial sequencing and comparison of the EF1-α gene was used to resolve the identification of these isolates. Sequences of *D. iberica* contained a nine base pair deletion in this region sufficient to provide clear discrimination from *D. sarmentorum* when aligned and placed in phylogeny. *Figure 2.18* illustrates the grouping of these isolates with reference cultures of *D. iberica*, the union of which was supported by 84% of bootstrap replications. In culture however, the two species are indistinguishable as differences in conidial size, shape, and presence or absence of septation are insufficient to differentiate *D. iberica* from formal descriptions of *D. sarmentorum*.

Table 2.19 Diatrypaceous species isolated from grapevines throughout New South Wales (Pitt *et al.* 2010b)

Region (# vines sampled), location	Cultivar	Age	Species (# isolated)	Accession numbersa
Central Ranges (246)				
Orange	Chardonnay	9	*Eutypella* sp. (1)	-
	Grenache	10	*Diatrypella* sp. (3)	-
			*Cryptovalsa ampelin*a (1)	-
	Sauvignon blanc	14	*Eutypa lata* (2)	EU835160, DAR79045
			Cryptovalsa ampelina (2)	-
	Cabernet sauvignon	14	*Diatrypella* sp. (2)	-
			Cryptovalsa ampelina (1)	-
Canowindra	Verdello	14	*Diatrypella* sp. (1)	-
			Cryptovalsa ampelina (4)	-
	Chardonnay	13	*Eutypa lata* (2)	EU835166, DAR79048 EU835167, DAR79049
Cowra	Chardonnay	15	*Cryptovalsa ampelina* (2)	-
Mudgee	Chardonnay	8	*Cryptovalsa ampelina* (1)	-
Northern Slopes (300)				
Bendemeer	Shiraz	12	*Eutypella* sp. (1)	-
Armidale	Pinot grigio	8	*Eutypella* sp. (1)	-
Deepwater	Semillon	12	*Eutypella* sp. (3)	-
			Diatrypella sp. (2)	-
Inverell	Shiraz	13	*Diatrypella* sp. (2)	-
Southern New South Wales (525)				
Murrumbateman	Shiraz	37	*Eutypa lata* (2)	EU835162, DAR79046 EU835163, DAR79047
			Cryptovalsa ampelina (4)	EU835150, DAR79050 EU835151, DAR79051 EU835152, DAR79052
	Cabernet sauvignon	36	*Eutypella* sp. (1)	-
		29	*Eutypa lata* (1)	-
Young	Chardonnay	19	*Cryptovalsa ampelina* (2)	-
			Diatrypella sp. (1)	-
			Eutypella sp. (1)	-
Berridale	Riesling	24	*Diatrypella* sp. (1)	-
Tumbarumba	Pinot noir	28	*Diatrypella* sp. (2)	-
		16	*Diatrypella* sp. (3)	-
		20	*Diatrypella* sp. (1)	-
	Chardonnay	-	*Diatrypella* sp. (2)	-
			Eutypa lata (1)	EU835164, DAR79128
	Sauvignon blanc	-	*Cryptovalsa ampelina* (2)	-
South Coast (250)				
	-	-	-	-
Big Rivers (427)				
Wagga Wagga	Shiraz	11	*Diatrypella* sp. (1)	-
Book Book	Shiraz	12	*Cryptovalsa ampelina* (1)	EU835154, DAR79054
Griffith	Shiraz	39	*Eutypa lata* (3)	EU835157, DAR79040
	Traminer	27	*Cryptovalsa ampelina* (1)	-
	Ruby Cabernet	33	*Eutypa lata* (1)	EU835156, DAR79043
	Chardonnay	41	*Eutypella* sp. (6)	-
Northern Rivers (62)				
Port Macquarie	Pinot noir	22	*Cryptovalsa ampelina* (1)	-
Hunter Valley (36)				
Pokolbin	Semillon	22	*Diatrypella* sp. (2)	-
			Cryptovalsa ampelina (1)	FJ800509, DAR79966
			Eutypella sp. (4)	FJ800513, DAR79970 FJ800514, DAR79971 FJ800515, DAR79972 FJ800520, DAR79977

[a] Genbank number and DAR herbarium number, respectively.

Incidence and distribution

The diversity and distribution of species isolated in this study was not unexpected considering that nine species were previously isolated and identified from vineyards in California (Urbez-Torres *et al.* 2006*a*; Urbez-Torres *et al.* 2007), where climatic conditions are similar to those found in Australia (*Table 2.20*, *Figure 2.20*).

Lasiodiplodia and Diplodia

Of the eight Botryosphaeriaceae species collected in NSW and SA, *L. theobromae* was the least prevalent; the species being isolated only once from grapevines in the Northern Rivers region of NSW, the state's most northern viticultural area. Regarded as a pathogen of the tropics (Punithalingam 1976), *L. theobromae* has an optimum growth temperature of 30.8°C; the highest of eight Botryosphaeriaceae species tested by Urbez-Torres *et al.* (2006a). It appears well suited to tropical regions characterised by high temperatures and low precipitation. This was most evident by the predominance of this species throughout Mexico (Urbez-Torres *et al.* 2008), the southern parts of California, and the desert regions of the Coachella Valley (Urbez-Torres *et al.* 2006*a*; Leavitt and Munnecke 1987), where such temperatures are common. Isolations of the fungus in Western Australia (WA) arose only from climatically similar locations in the most northerly of surveyed regions (Taylor *et al.* 2005). In all of these studies, *L. theobromae* was absent in cooler locations, and the fungus seems to occupy a rather limited range.

The absence of *L. theobromae* in many parts of Australia suggests that the fungus poses less of a threat to grapevines in this country than in parts of the USA. While Cunnington *et al.* (2007) noted that many specimens of *L. theobromae* were in the databases of the Victorian Plant Disease Herbarium (VPRI), and the Australian Scientific Collections Unit (DAR), only six records of this species exist, one from SA and the remainder from NSW. Of these, only two were isolated from *Vitis*, with the remainder collected from avocado (*Persea americana*), date (*Phoenix dactylifera*) and sweet potato (*Ipomoea batatas*).

Species of *Diplodia* were by far the most prevalent, particularly *D. seriata*, which accounted for almost 80% of the total number of Botryosphaeriaceae isolates collected during the survey. This species was also most commonly isolated by Savocchia *et al.* (2007) from collections in the Hunter Valley and Mudgee regions of NSW, and by Taylor *et al.* (2005) from grapevines in WA, where it accounted for more than 50% of the total number of isolates collected during a regional survey of the five major grape growing districts. In both studies *D. seriata* was the only species to be found in all of the regions surveyed; its distribution being considerable, not only from a geographic standpoint, but also in terms of its apparent tolerance of a broad range of environmental conditions.

Diplodia seriata and *D. mutila* have rather low optimal growth temperatures; 26.8°C and 24.8°C, respectively (Urbez-Torres *et al.* 2006*a*). In our studies, *D. mutila* was almost as widely distributed throughout NSW and SA as *D. seriata*, but at a much lower incidence. *Diplodia* mutila, the second-most frequently isolated species, was restricted to the southern parts of NSW, and was absent in the Northern Slopes and Northern Rivers areas, the warmest and most humid areas surveyed. In WA, *D. mutila* has been isolated only in the cooler climate Pemberton/Manjimup region in the south-west of the state (Taylor *et al.* 2005).

Neofusicoccum and Fusicoccum

Three species with hyaline thin-walled conidia, including *B. dothidea*, *N. parvum*, and *N. australe* were found during the surveys. However, the incidence of these species was less than 2.5% of the total number of specimens collected. Of the three species, *N. parvum* was the most abundant, but also the most restricted geographically, with all but two isolates of the fungus being collected from the Northern Slopes and Northern Rivers regions of NSW. Cunnington *et al.* (2007) found *N. parvum* and *B. dothidea* in roughly equal numbers. However, we found that *N. parvum* was significantly more abundant. In contrast *B. dothidea* was isolated on only seven occasions in this study, and was only recently reported for the first time in Australia (Qiu *et al.* 2008). Neither *N. parvum* nor *B. dothidea* were found during surveys of WA vineyards (Taylor *et al.* 2005).

The similar distribution of *B. dothidea* and *N. parvum* in Australia suggests that these species are able to tolerate a similar range of environmental conditions. In previous studies, both species have been shown to have similar optimum growth temperatures, namely 30.8°C and 28.2°C, respectively (Urbez-Torres *et al.* 2006*a*). Why these species are found in such limited numbers remains unknown, but perhaps spore pigmentation offers some measure of environmental protection, as demonstrated in *Diplodia* and *Dothiorella* which are both more abundant and more widely distributed. *Neofusicoccum*

Table 2.20 Incidence and distribution of Botryosphaeriaceae species and other grapevine trunk disease pathogens isolated from diseased grapevines (Vitis vinifera) from New South Wales and South Australia.

				Number (%) of wood samples yielding[a]															
				Botryosphaeriaceae species													Others		
State/region	Vine-yards[b]	Nº (%) Bot.[c]	Total[d]	A[e]	B[f]	C[g]	A+B+C[h]	*N. parvum*	*N. australe*	*B. dothidea*	*L. theo*[i]	*D. mutila*	*D. seriata*	*D. viticola*	*D. iberica*	Total No. Bot.[j]	*P. viticola*	*P. chlami*	*E. lata*
South Australia																			
Adelaide Hills	4	4 (100)	100	33 (33)	3 (3)	1 (1)	37 (37)	1 (1)	2 (2)	0	0	14 (14)	16 (16)	6 (6)	2 (2)	41	0	0	0
Clare Valley	5	5 (100)	125	49 (39)	7 (6)	2 (2)	58 (46)	0	0	0	0	5 (4)	29 (23)	19 (15)	13 (10)	66	0	0	2 (2)
Eden Valley	2	2 (100)	50	19 (38)	27 (54)	5 (10)	49 (98)	0	0	2 (4)	0	22 (44)	36 (72)	13 (26)	8 (16)	81	0	0	0
Barossa Valley	6	6 (100)	150	84 (56)	9 (6)	4 (3)	97 (65)	0	1 (<1)	0	0	15 (10)	70 (47)	15 (10)	5 (3)	106	0	0	0
Riverland	1	1 (100)	25	8 (32)	1 (4)	1 (4)	10 (40)	0	0	0	0	0	6 (24)	3 (12)	2 (8)	11	0	0	0
New South Wales																			
Big Rivers	18	17 (94)	427	154 (36)	7 (2)	14 (3)	175 (41)	1 (<1)	0	0	0	3 (<1)	166 (39)	11 (3)	1 (<1)	182	62 (15)	0	4 (<1)
Central Ranges	9	9 (100)	225	130 (58)	6 (3)	8 (4)	144 (64)	0	1 (<1)	2 (<1)	0	13 (6)	128 (57)	5 (2)	1 (<1)	150	9 (4)	0	4 (2)
Northern Rivers	3	3 (100)	62	28 (45)	5 (8)	3 (5)	35 (56)	28 (45)	0	0	1 (2)	0	11 (18)	0	0	40	0	10 (16)	0
Northern Slopes	12	12 (100)	300	145 (48)	3 (1)	1 (<1)	148 (49)	8 (3)	6 (2)	0	0	0	138 (46)	0	0	152	0	1 (<1)	0
Southern NSW	21	20 (95)	525	300 (57)	4 (<1)	25 (5)	328 (62)	0	0	1 (<1)	0	17 (3)	306 (58)	6 (1)	2 (<1)	332	43 (8)	17 (3)	4 (<1)
South Coast	10	10 (100)	250	85 (34)	2 (<1)	8 (3)	95 (38)	0	4 (2)	2 (<1)	0	5 (2)	86 (34)	0	0	97	18 (7)	0	0
Totals	91	89 (98)	2239	1035 (46)	74 (3)	72 (3)	1176 (53)	38 (2)	14 (<1)	7 (<1)	1 (<1)	94 (4)	992 (44)	78 (4)	34 (2)	1258	132 (6)	28 (1)	14 (<1)

[a] Percentage of the total number of wood samples per region, rounded to the nearest integer
[b] Number of vineyards sampled
[c] Number of vineyards (and percentage of total number of vineyards sampled per region) yielding Botryosphaeriaceae species
[d] Total number of wood samples collected
[e] A=number of wood samples from which a single *Botryosphaeriaceae* species only isolated
[f] B=number of wood samples from which multiple *Botryosphaeriaceae* species isolated
[g] C=number of wood samples from which *Botryosphaeriaceae* species isolated in conjunction with other grapevine trunk disease pathogens such as *P. viticola*, *P. chlamydospora*, *E. lata*, *C. ampelina* and species of *Diatrypella* and *Eutypella*
[h] Total number of wood samples from which *Botryosphaeriaceae* species isolated
[i] Abbreviations; *P. chlam=Phaeomoniella chlamydospora*, *L. theo=Lasiodiplodia theobromae*
[j] Total number of *Botryosphaeriaceae* isolates collected per region

australe, which is thought to be native to the southern hemisphere and endemic to Australia (Slippers *et al*. 2004), has previously been isolated from grapevines in WA (Taylor *et al*. 2005) and parts of Victoria (Cunnington *et al*. 2007), and now from NSW and SA. This species was most abundant in NSW, particularly in the Northern Slopes region where close to half of the isolates were collected. However, a number of isolates were also obtained from vineyards in the coastal regions. *Neofusicoccum australe* tended to have a narrow distribution, somewhat analogous to that observed in the USA where this species was also restricted to a handful of vineyards (Urbez-Torres *et al*. 2006*a*).

Interestingly, *N. australe* was not isolated in prior surveys of the Hunter Valley and Mudgee regions of NSW (Savocchia *et al*. 2007), which are close to the Northern Slopes and South Coast districts. Prevalent species reported from these areas included *D. seriata*, *N. parvum*, *B. dothidea*, and *N. luteum* (Qiu *et al*. 2011, Savocchia *et al*. 2007), although the identities of isolates of the latter species were not confirmed via DNA sequence analysis. Instead isolates were identified based on morphological characters and the production of a taxonomically-informative yellow pigmentation, also produced by cultures of *N. australe*. To date, *N. luteum* has not been found elsewhere in Australia. Of 14 isolates identified as *N. luteum*, only one produced microconidia (Savocchia *et al*. 2007), a feature characteristic of *N. luteum*, but not known to occur in *N. australe* (Urbez-Torres *et al*. 2006*a*). Hence, the identity of some of these isolates may be questionable. While several records of this species from willow (*Salix* sp.) and bitou bush (*Chrysanthemoides monilifera*) are recorded from the South Coast of NSW (Cunnington *et al*. 2007), neither definitive identification, nor evidence of the role of *N. luteum* in grapevine decline in Australia are available.

Dothiorella

Dothiorella viticola and *D. iberica* were collected from all of the regions surveyed in SA and inland regions of southern NSW, including districts surrounding Wagga Wagga, Orange, Murrumbateman and Cooma. These species have only recently been discovered in Australia, where they were isolated for the first time from dormant buds and grapevine cankers (Wunderlich *et al*. 2008, Pitt *et al*. 2008*a*). Like *D. mutila*, the highest incidences of these species tended to be associated with the cooler climatic conditions.

Dothiorella species were second in abundance following *Diplodia*. While the anamorphs of these two genera are easily separated, with spores of *Diplodia* being infrequently septate (Phillips *et al*. 2007), the placement of species within *Dothiorella* has been more problematic (Phillips *et al*. 2008). For example, the teleomorphs of *Dothiorella* were purported to be placed in *Dothidotthia* Hohn. (Crous *et al*. 2006), a genus previously recognised as a member of the Botryosphaeriaceae with *Diplodia*-like anamorphs now accommodated in *Dothiorella* (Barr 1989). However, *Dothidotthia aspera* (Ellis. and Everh.) M.E. Barr was shown to possess a hyphomycetous anamorph in *Thyrostroma* Hohn., unlike members of the Botryosphaeriaceae that possess pycnidial anamorphs (Ramaley 2005). Recent studies of the Dothideomycetes have also shown that this genus is unrelated to the Botryosphaeriaceae (Schoch *et al*. 2006). Instead, *Dothidotthia* belongs in the Pleosporales, albeit outside any of the recognised families (Phillips *et al*. 2008). A new family, Dothidotthiaceae Crous and A.J.L. Phillips, was formerly introduced to accommodate these species (Phillips *et al*. 2008).

Concurrently Phillips *et al*. (2008) transferred *D. viticola* to the new genus *Spencermartinsia* A.J.L. Phillips, A. Alves and Crous, which now accommodates species of *Dothiorella* with brown 1-septate ascospores possessing apiculi at both ends. *Dothiorella iberica* and *D. sarmentorum* remain distinct from *Spencermartinsia viticola* (A.J.L. Phillips and J. Luque) A.J.L. Phillips, A. Alves and Crous with a teleomorph connection in '*Botryosphaeria*', the distinction being that ascospores of these species differ from *S. viticola* in the absence of ascospore apiculi (Phillips *et al*. 2008). Since *Dothidotthia* is no longer available for the teleomorph of *Dothiorella*, Phillips *et al*. (2008) have also recently proposed that the anamorph name *Dothiorella*, also be used for the teleomorphs of this genus. The epidemiology, pathogenicity and the role of species of *Dothiorella* and *Spencermartinsia* in grapevine decline in Australia is currently unknown.

Experiment 2.11
Evidence that *Eutypa lata* and other diatrypaceous species occur in New South Wales vineyards

The methods detailed below are adapted from Pitt *et al.* (2010b).

Materials and methods

Isolation and morphological identification

Between November 2006 and April 2008 field surveys were conducted from 77 vineyards across NSW, encompassing seven major grapegrowing regions (*Table 2.19*), viz. Big Rivers (growing season spatial mean average temperature 1 October 1971–30 April 2000, ~21.1°C), Central Ranges (~18.9°C), Hunter Valley (~20.7°C), Southern NSW (~18.3°C), South Coast (~17.8°C), Northern Rivers (~20.8°C), Northern Slopes (~18.6°C). A total of 1846 wood samples were collected from the cordons or trunks of grapevines with evidence of dieback, including dead spurs or cordons, cankers or bleached and discoloured tissue. Five 2 mm^2 portions of wood from each sample, comprising portions of both dead and live tissue, were excised, surface sterilised in 1.0% w/v sodium hypochlorite for 2 min and transferred to PDA. Cultures were incubated in the dark at 25°C for 5–7 days before being pure cultured on fresh PDA. Foliar symptoms typical of Eutypa dieback were encountered infrequently during the surveys, but in such cases wood samples were extracted and prepared for fungal isolation as described previously. Diatrypaceous species were tentatively identified based on gross cultural morphology (Carter 1991; Mostert *et al.* 2004; Glawe and Rogers 1984; Glawe and Jacobs 1987). In December 2008, additional surveys and collections were conducted from grapevines both in the Hunter Valley and Tumbarumba (NSW). Foliage symptoms of Eutypa dieback were not observed, at either location, and in contrast to the previous collections, isolations were made from fruiting bodies collected on dead wood from aged vines or debris from the vineyard floor. Isolations of diatrypaceous fungi from these samples were made directly from ascospores as described by Trouillas and Gubler (2004), with species tentatively identified based on morphology of the teleomorph (Glawe and Rogers 1984).

DNA extraction, amplification and sequencing

Prior to DNA extraction, selected Diatrypaceae isolates were pure cultured by hyphal tip, before being transferred by colonised agar plug, to 50 mL Falcon tubes containing 20 mL of potato dextrose broth (Oxoid). Broth cultures were incubated on an orbital shaker at 90 rpm for 7 days at 25°C. Mycelia were harvested by filtration, lyophilised and DNA extracted using the Qiagen Plant Mini Kit according to the manufacturer's instructions (Qiagen). Molecular identification of *E. lata* and other diatrypaceous species was achieved via amplification and comparison of ribosomal DNA internal transcribed spacer (ITS) regions (ITS1, 5.8S and ITS2) using the oligonucleotide primers ITS1 and ITS4 (White *et al.* 1990).

Each PCR reaction contained 0.1 volume of 10× buffer (containing 15 mM $MgCl_2$, Qiagen), 200 µM each of dNTPs, 0.15 µM of each primer, 1 unit of HotStart *Taq* DNA polymerase (Qiagen), approximately 50 ng of DNA template, and were adjusted with sterile nanopure water to a total volume of 50 µL. PCR reactions were performed using an Eppendorf Master Thermocylcer. Amplification was achieved by an initial step of 15 min at 95°C, followed by 40 cycles of 30 s at 94°C, 45 s at 55°C, and 1.5 min at 72°C, with a final extension of 5 min at 72°C. PCR products were separated by electrophoresis on 1% agarose containing 0.5 × Tris-acetate-EDTA (TAE) buffer, and photographed under UV light after staining with ethidium bromide. For sequencing, PCR products were purified using the QIA quick PCR purification kit (Qiagen). ITS regions were sequenced in both directions by the AGRF and identification of diatrypaceous species confirmed by comparison of ITS sequences of our isolates with those available in GenBank. Individual sequences were compiled in BioEdit sequence alignment editor (Hall 1999) and aligned for comparison using ClustalX (Thompson *et al.* 1997).

Isolates of *E. lata*, *Cryptovalsa ampelina*, *Eutypella* and *Diatrypella* used in this study are maintained on PDA agar slopes at 4°C in the collection at the NWGIC and representative isolates of each species were deposited in the Australian Scientific Collections Unit. DNA sequences of representative isolates used in this study were submitted to GenBank (*Table 2.19*).

Results and discussion

The results and discussion that follow are adapted from Pitt *et al.* (2010*b*). Representative photos of *Eutypella*, *Diatrypella* and *Cryptovalsa* are presented in Pitt *et al.* (2010*b*).

A total of 73 isolates developing white, cottony mycelium and conforming to gross morphological

descriptions of the Diatrypaceae (Glawe and Rogers 1987; Carter 1991; Luque *et al*. 2006) were isolated from diseased grapevines. *E. lata* was isolated from approximately 0.65% of grapevines surveyed (12 isolates), with the fungus being reported for the first time both in the Central Ranges and southern NSW regions. In each case, foliar symptoms typical of Eutypa dieback were present including shortened internodes, cupped shaped leaves and stunted shoots, but the teleomorph was not observed and identification of the anamorph was accomplished via sequencing and comparison of ribosomal DNA ITS sequences. Other diatrypaceous species isolated from initial field collections included 14 isolates of *Eutypella* (0.76% of samples), 23 isolates of *Diatrypella* (1.25%), and 21 isolates of *C. ampelina* (1.14%), which were tentatively identified according to morphological descriptions (Glawe and Rogers 1984). In all cases specimens were isolated from cankers from diseased trunks or cordons. Further isolations from fruiting bodies collected in the Hunter Valley tentatively identified an additional four isolates of *Eutypella* and one isolate of *C. ampelina* based on features of the stromata and teleomorph, but no foliar symptoms of Eutypa dieback were observed, nor were cultures of *E. lata* isolated. Molecular identification of isolates from this latter survey subsequently confirmed the identity of *C. ampelina* and revealed the presence of several different species of *Eutypella*, but neither these, nor isolates of *Diatrypella* could be identified at the species level based on available data from GenBank. No diatrypaceous species were isolated from the South Coast region of NSW.

The generic concept in the Diatrypaceae is principally based on stromatic characters, such as the degree of stromatal development, configuration of perithecial necks and type of host tissue in which stromata occur (Glawe and Rogers 1984). In general, diatrypaceous asci are clavate to spindle-shaped, long stipitate with a truncate or blunt apex, often containing cytoplasmatic strands in the apex and frequently possess a thicker-walled region above the ascospores (Carmaran *et al*. 2006). Differentiation of the various genera and species within the Diatrypaceae is difficult, as many are indistinguishable, possessing few if any unique taxonomic features. Apart from *Cryptovalsa* and *Diatrypella* which can be clearly separated on the basis of their polysporous asci, the number of ascospores being the only aspect of the ascus regularly used for diagnostic purposes (Carmaran *et al*. 2006), the other common species, viz. *Cryptosphaeria*, *Diatrype*, *Eutypa* and *Eutypella* are indistinguishable, and all comprise octosporous asci, with differentiation instead relying on the use of molecular tools (Rolshausen *et al*. 2004).

Several genera of the Diatrypaceae are known to occur on grapevines throughout the world (Farr *et al*. 1989). Until recently *E. lata* was thought to be responsible for dead and declining vines routinely observed in the Hunter Valley region of NSW. However, foliar symptoms of the disease have not been observed, nor has the fungus been isolated from material collected during extensive surveys of the region (Castillo-Pando *et al*. 2001; Creaser *et al*. 2003; Savocchia *et al*. 2007; Qiu *et al*. 2011;). Recently, *E. lata* was reported in the Riverina region of NSW for the first time (Pitt *et al*. 2007). Although prominent in SA vineyards, Eutypa dieback had not been reported north of Wentworth (latitude, 34°2'S) in NSW, which is on the Victorian border (T. Wicks *pers. comm.*), and until now, no definitive records of the fungus exist from the winegrowing regions of south-eastern NSW. Extensive surveys conducted throughout NSW have now shown that *E. lata* is more widespread than first thought, with new isolations from Canowindra and Orange in the Central Ranges and Murrumbateman and Tumbarumba in southern NSW. While Edwards and Pascoe (2004) reported the presence of *Eutypa* from grapevine samples received from Tumbarumba during a routine diagnostic survey, cultures appeared not to have been identified to species, nor characterised at a molecular level. Regardless, these results suggest that *E. lata* may be well suited to the cooler climate regions of NSW where low temperatures and high rainfall favour the growth of the fungus. Certainly, the isolation of *E. lata* from diseased grapevines in Orange (latitude, 33°15'S) represents a small but measurable expansion in the geographic range of the pathogen and the most northerly occurrence of E. lata reported in Australia.

While the epidemiology (Petzoldt *et al*. 1981; Carter 1991; Munkvold and Marois 1995) and management (Munkvold and Marois 1993a; Munkvold and Marois 1993b; Weber *et al*. 2007; Sosnowski *et al*. 2008) of *E. lata*, has been studied extensively, little is known about the other members of the Diatrypaceae, many of which are known to occur not only on grapevines but on other hosts including, apples, cherries, pears, olives and poplars (Farr *et al*. 1989). Several species of *Eutypella* and *Diatrypella*, as well as *C. ampelina*, a pathogen in its own right (Mostert *et al*. 2004; Luque *et al*. 2006), were isolated from grapevines from many regions throughout NSW. Interestingly, all three

of these genera were present in the Hunter Valley region, and clearly possess a far greater geographic range than *E. lata*. In contrast, *E. lata* was present only in the cooler climate regions of southern NSW, and once again the fungus was not recovered from samples collected from the Hunter Valley, nor was it or any other diatrypaceous species isolated from the south coast region of NSW. In the latter case, the reasons for this are unknown; however, our failure to isolate *E. lata* from the Hunter Valley and other regions throughout NSW in no way suggests that these regions are free from the disease. But, it is possible that other genera within the Diatrypaceae are contributing to the dieback phenomenon generally attributed to *E. lata* (Trouillas *et al*. 2001).

Why species such as *C. ampelina*, *Eutypella* and *Diatrypella* are prominent in the Hunter Valley, seemingly in the absence of *E. lata* remains a mystery. Perhaps the poorly developed stroma of *C. ampelina*, which is embedded in the bark or decorticated wood, as opposed to well developed and erumpent through bark as with *E. lata* (Glawe and Rogers 1984), affords the fungus some measure of protection from the elements, thereby enabling the fungus to survive under a greater range of environmental conditions. In all diatrypaceous genera, stromata are thought to aid in conserving moisture, but in some genera, their function is to aid discharge of ascospores by rupturing the host's bark, thereby exposing perithecial ostioles (Glawe and Rogers 1984). If the reduced stroma composed of both fungal and host tissue preserves less moisture or fails to expose ostioles to the environment, effectively limiting ascospore discharge, fruiting bodies may be better preserved, their survival enhanced, their ability to withstand adverse conditions heightened and geography improved as a function of age. Equally the reverse could be true, or maybe the host ranges of *C. ampelina* and the other more prominent diatrypaceous species are simply larger than that of E. lata; as species with larger host ranges often have greater geographic distributions (Glawe and Rogers 1984).

Other explanations for the geographic disparity among the different members of Diatrypaceae are speculative at best, although clearly the environmental conditions in the northern regions of NSW, including the Hunter Valley, are less favourable to *E. lata* than to some of the other species in the Diatrypaceae, likely due to stricter rainfall and temperature requirements of *E. lata* (Sosnowski *et al*. 2005; Sosnowski *et al*. 2007*b*). While the Mediterranean climate common in SA where Eutypa dieback is extensive contrasts well with the cooler climate regions of NSW, the subtropical climate of the Hunter Valley region, with higher average daily temperatures appears unfavourable for the establishment of Eutypa dieback, despite the fact that rainfall in the region is suitable for the disease. Notably, in a recent report concerning the seasonal variation in Eutypa dieback symptoms, Sosnowski *et al*. (2005) reported a positive relationship between disease incidence and spring temperatures; the higher the temperature, the lower the incidence of disease, as determined by expression of foliage symptoms. Glawe and Rogers (1984) reported that the teleomorph of the fungus fails to form in regions receiving less than 330 mm of annual rainfall. However, plants infected with *E. lata* have been found in such locations, with long-distance and airborne dispersal of ascospores known to accompany the onset of rainfall (Carter 1957; Ramos *et al*. 1975; Glawe and Rogers 1984). With an average annual rainfall of greater than 600 mm (Australian Government Bureau of Meteorology 2008, data for Cessnock), understandably, researchers continue to be baffled by the absence of Eutypa dieback in the Hunter Valley, a disease that for all intensive purposes should be present there. Incidentally, US researchers have also noted large geographical differences in the distribution of *E. lata* with respect to climate (Urbez-Torres *et al*. 2006*a*).

To date, the pathogenicity of *E. lata* (Carter 1991) and *C. ampelina* (Mostert *et al*. 2004, Luque *et al*. 2006) to grapevines has been confirmed, but investigations on the virulence of many of the other diatrypaceous species towards grapevines are limited (Trouillas *et al*. 2001). While a small number of industry fact sheets currently circulating on the internet report that *Eutypella vitis* can cause xylem necrosis and foliar symptoms similar to those caused by *E. lata* (Wolf 2006), for which it was suggested as an ulterior cause of Eutypa dieback (Myers 2008), neither of these reports contain detailed or replicated scientific research. In fact, with the exception of work conducted on *C. ampelina* and the abstract published by Trouillas *et al*. (2001), only two other reports are available on this topic. Incidentally, both are major works published by Trouillas and colleagues on related topics, but contain sufficient data to show that *E. leptoplaca* and *Diatrypella* are regularly isolated from diseased grapevines in California, and that inoculation of fresh pruning wounds with these species can cause disease (Rolshausen *et al*. 2004, Trouillas and Gubler 2004).

As cultures of diatrypaceous fungi are often indistinguishable from one another, the co-occurrence of multiple diatrypaceous fungi in diseased wood of grapevines could also lead to misidentification of the correct agents of disease, especially where multiple species are isolated from the same infection. Whilst this occurred in only two instances in the present study, whereby *C. ampelina* and *Diatrypella* were cultured from the same piece of wood (Central Ranges), more than half of the *C. ampelina* isolates collected in the survey co-occurred with species of the Botryosphaeriaceae, which are also well known trunk disease pathogens of grapevines (van Niekerk *et al*. 2004, Savocchia *et al*. 2007). The frequent occurrence of *C. ampelina* in conjunction with other known grapevine pathogens was thought by Luque *et al*. (2006) to represent a synergistic association by a facultative pathogen, which he then used as an explanation for the moderate virulence of the fungus. However, in recent years, *C. ampelina* has been isolated repeatedly from grapevines both in Australia and overseas, and under suitable conditions has been shown to be a pathogen in its own right (Mostert *et al*. 2004; Luque *et al*. 2006). Nevertheless, competition from other trunk disease pathogens like the Botryosphaeriaceae may play a role in reducing the incidence of the Diatrypaceae. While unlikely with respect to *C. ampelina*, interactions of this nature may contribute to the low incidence of *E. lata* throughout the state, or alternatively to its absence in some regions. To date, no reports of this nature have been published.

This study has shown that many of the other diatrypaceous species are more widespread and abundant than *E. lata* in NSW. While the exact species causing trunk diseases in the vineyard may be inconsequential to many grapegrowers, due to increasing knowledge that control strategies are the same for all of the grapevine canker causing fungi, correct diagnoses of the causal agent is essential to predict the severity of the disease and hence the urgency of management. In a major study of the pathogenicity of nine Botryosphaeriaceae species isolated from grapevines in California, Urbez-Torres and Gubler (2009) showed that four species within this family were equivalent to, or greater in virulence than *E. lata*. Furthermore, the most pathogenic species, *Lasiodiplodia theobromae* was almost four times more virulent, based on mean lesion length, than *Dothiorella viticola*, the least pathogenic species. Other researchers have reported considerable variation in the pathogenicity of isolates of *E. lata* both to grapevines and other hosts, suggesting the existence of two pathotypes, vastly different in virulence (Carter *et al*. 1985). This variation in pathogenicity of individual isolates of *E. lata*, as well as the influence of grapevine cultivar (Sosnowski *et al*. 2007*a*), and the effect of climate on seasonal disease expression (Petzoldt *et al*. 1991; Sosnowski *et al*. 2007*b*), can greatly influence the severity and perceived importance of such canker diseases. Hence, depending on the presence and virulence of individual isolates or species, more or less immediate management may be warranted in order to maintain vineyard longevity in the longer term. Because it is impossible to distinguish wood symptoms caused by the Botryosphaeriaceae from those of *E. lata* or other diatrypaceous species, the aggressiveness of the pathogen and hence its identification is of the utmost importance.

As few of the diatrypaceous species have been studied in detail in Australia, their incidence, distribution and pathogenicity towards grapevines and other cultivated crops requires further research. In the interim, vigilant monitoring, avoiding pruning during and directly after rainfall, protection of pruning wounds, and removal and incineration of dead infected wood from the vineyard remain the best methods of managing Eutypa dieback and other infections that may be caused by the Diatrypaceae.

Experiment 2.12
Association of Botryosphaeriaceae grapevine trunk disease fungi with the reproductive structures of *Vitis vinifera*

Materials and methods

The methods below are adapted from Wunderlich *et al.* (2011a).

Survey

Over the growing seasons of 2007–08 and 2008–09, a total of 200 grapevines (50 vines of Chardonnay and Shiraz each per vineyard) were sampled for the presence of Botryosphaeriaceae in two vineyards. The vines were established approximately 25 years ago in the Lower Hunter Valley, NSW, Australia, located at approximately 32°47'39.56"S 151°20'35.79"E (Vineyard A) and 32°48'24.77"S 151°16'30.03"E (Vineyard B). The vineyards and individual vines were selected based on an existing history of trunk diseases due to Bot canker.

Both sampling sites were commercial vineyards following routine fungicide programs. Throughout the two sampling seasons Vineyard A was sprayed with Thiovit Jet (active ingredient (a.i.) sulphur), Captan (a.i. Captan), Cabrio (a.i. pyraclostrobin), Switch (a.i. cyprodinil and fludioxonil), Medley Plus (a.i. metalaxyl + copper oxychloride), Dithane Rainshield (a.i. mancozeb), Liquicop (a.i. copper), Kocide Xtra (a.i. copper hydroxide), Prosper (a.i. clothianidin), Scala (a.i. pyrimethanil) and Rovral L (a.i. iprodione) for the control of Downy Mildew, Powdery Mildew, Phomopsis and Botrytis between the phenological growth stages of leaf emergence (E-L stage 7) and veraison (E-L stage 35). Vineyard B had a similar spray program for the control of Downy and Powdery Mildew and Botrytis with Cabrio, Captan, Kocide Blue (a.i. copper), Delan (a.i. Dithianon), Topas (a.i. penconazole), Thiovit Jet and Switch applications. For both vineyards the most frequent spraying occurred between flowering at 50% cap fall (E-L stage 23) and pea-sized berry stage (E-L stage 31).

Wood samples were taken from the trunk and cordons of each vine before commencing the survey of other reproductive tissues. During both seasons, samples were taken at the growth stages of dormant bud, flowering, pea-sized berry and harvest, corresponding to the E-L phenological stages of 1, 21, 31 and 35, respectively as described by Coombe (1995). At each sampling time five dormant buds, inflorescences or bunches per vine were collected at random and sub-sampled to florets and berries. This intensive method of sampling was chosen to increase the likelihood of isolating rarer species such as *B. dothidea* and *N. parvum* previously isolated from the Hunter Valley (Qiu *et al.* 2011).

Isolation

Vine samples were surface-sterilised in 0.5% sodium hypochlorite for 2 min followed by two rinses in sterile distilled water, placed on PDA amended with 50 µL mL^{-1} streptomycin sulphate and incubated at room temperature in the dark. Fungal colonies showing characteristics of Botryosphaeriaceae species were subcultured onto fresh PDA and single spore or hyphal tip cultures prepared using standard techniques.

Isolates of Botryosphaeriaceae were allowed to sporulate at room temperature in the dark for up to eight weeks prior to identification based on conidial morphology. Isolates which did not sporulate were subcultured onto triple autoclaved pine needles on 1.5% water agar in Petri dishes and stored for a further six weeks at room temperature under a light regime of 12 hours dark and 12 hours near UV light to encourage the formation of conidia. Preliminary morphological identification to species level was based on the length, shape, pigmentation and presence or absence of septa in conidia.

DNA extraction and molecular identification

A representative group of each morphologically identified species and a subset of those isolates failing to sporulate were chosen for further analysis. Three agar plugs per isolate were transferred from actively growing cultures to 125 mL conical flasks containing 50 mL DifcoTM PDB and were incubated at 25°C and 90 rpm in an orbital shaker (Sartorius Certomat BS-1). Mycelia were harvested after seven days, initially dried by filtration, freeze-dried in a Christ Gamma 1-16LSC freeze-dryer (Christ, Osterode, Germany) for 24 hours and then homogenised with a tissue lyser (Qiagen, Australia). DNA was extracted using the DNeasy Plant Maxi Kit (Qiagen) according to the manufacturer's handbook. This was followed by amplification of the rDNA internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2) with primers ITS1 and ITS4 (White *et al.* 1990). Each 50 µL polymerase chain reaction (PCR) contained a total of ~50 ng DNA template, 1 unit of HotStar *Taq* DNA polymerase (Qiagen), 0.1 volumes of 10× buffer (Qiagen), containing 15 mM $MgCl_2$, 200 µM each of dNTPs (Promega, Australia), and 0.15 µM each

of primers ITS1 and ITS4. PCRs were performed in a Master Thermocycler (Eppendorf, Germany) according to Slippers *et al.* (2004) with an amended initial denaturation step of 95°C for 15 min.

PCR products were submitted to the AGRF for dual direction sequencing. Isolates were identified to species level by comparing the resulting sequences with those of other Botryosphaeriaceae available in GenBank. Species identities for *D. viticola*, *N. australe*, *N. ribis* and *N. luteum* were confirmed using partial sequencing of the β-tubulin and the translation elongation factor 1-alpha (EF1-α) genes. β-tubulin gene analysis was carried out in 50 µL reactions containing ~50 ng DNA template, 0.2 µM of each primer Bt2a and Bt2b (Glass and Donaldson 1995), 1.25 units of HotStar *Taq* (Qiagen), 1× PCR buffer (Qiagen), 15 mM MgCl2, 200 µM each of dNTPs (Promega). The PCR cycling protocol consisted of an initial denaturation at 95°C for 15 min, followed by 40 cycles of 94°C for 20 s, 55°C for 45 s and 72°C for 1 min and 30 s and a final extension of 72°C for 5 min.

Each 40 µL EF1-α PCR contained ~50 ng DNA template, 0.5 µM of each primer EF1-728F and EF1-986R (Carbone and Kohn, 1999), 1 unit of HotStar *Taq* DNA polymerase (Qiagen), 0.1 volume of 10× buffer (Qiagen), containing 15 mM $MgCl_2$, 200 µM each of dNTPs. An initial denaturation at 95°C for 15 min was followed by 35 amplification cycles of 30 s at 95°C, 40 s at 58°C and 1 min at 72°C and a final extension of 5 min at 72°C.

A selection of isolates from each species reported here were submitted as live cultures to the Agricultural Scientific Collection Unit and corresponding DNA sequences of the regions used for identification were deposited in GenBank. GenBank and Herbarium Accession numbers are listed in *Table 2.21*.

Results and discussion

The following results and discussion are adapted from Wunderlich *et al.* (2011*a*).

Fungi belonging to the Botryosphaeriaceae are well known as trunk disease pathogens in grapevines however less is known of their incidence an importance on other parts of the vine. This aspect of the project investigated the association of Botryosphaeriaceae grapevine trunk disease fungi with the reproductive structures of *Vitis vinifera*.

Trunk disease pathogens belonging to Botryosphaeriaceae were isolated from all tissue types sampled in this survey. While dormant bud, flower and pea-sized berry samples appeared asymptomatic, the majority of bunches sampled prior to harvest showed symptoms of bunch rot including darkening of berry skins, softening and oozing of berries, mycelial growth and formation of black pycnidia on berry surfaces as well as berry collapse and drying out of berries.

The initial isolation of cultures characteristic of Botryosphaeriaceae resulted in a total of 330 isolates (Vineyard A: n=150; Vineyard B: n=180). Further identification to species level via ITS sequencing, and partial sequencing of EF1-α and β-tubulin genes combined with conidial morphology resulted in 9 different species of *Diplodia*, *Dothiorella* and *Neofusicoccum* anamorphs: *D. seriata*, *D. mutila*, *L. theobromae*, *D. viticola*, *N. australe*, *N. parvum*, *N. ribis*, *N. luteum* and *B. dothidea* (*Table 2.21*).

The number of isolations for each species, their vineyard of origin and host cultivar are shown in *Table 2.22*. *D. seriata*, *N. parvum*, *B. dothidea* and *N. luteum* were the most frequently isolated species, occurring in both vineyards and on both 'Chardonnay' and 'Shiraz'. *D. mutila*, *L. theobromae* and *D. viticola* were isolated only from Vineyard B with *D. viticola* being isolated from both cultivars, *D. mutila* on 'Chardonnay' only and *L. theobromae* on 'Shiraz' only. In contrast, *N. australe* and *N. ribis* were isolated only from Vineyard A with *N. ribis* occurring on both cultivars and *N. australe* occurring only on 'Shiraz'.

The greatest number of Botryosphaeriaceae isolations occurred from dormant buds and wood followed by berries at harvest, while isolations from flowers and pea-sized berries were scarce (*Table 2.23*). With the exception of *D. mutila*, *L. theobromae* and *N. ribis* all species mentioned were found on dormant buds and all species except *N. luteum* were isolated from wood (*Table 2.23*). *D. mutila*, *L. theobromae*, *N. australe* and *D. viticola* were not isolated from berries at harvest. Along with two unidentified Botryosphaeriaceae species, *D. seriata*, *N. parvum* and *N. luteum* were the only species isolated from flowers and *D. seriata* was the only species occurring on pea-sized berries.

Summary

The Botryosphaeriaceae family is species-rich, containing common trunk disease pathogens, frequently isolated from grapevine wood in vineyards worldwide including the Hunter Valley. Until now, *D. seriata*, *N. luteum* (Savocchia *et al.* 2007), *N. ribis* (Castillo-Pando *et al.* 2001), *N. parvum*, *B. dothidea*

Table 2.21 Identities and origin of Botryosphaeriaceae isolated from *Vitis vinifera* from the Lower Hunter Valley (Wunderlich *et al.* 2011a)

Identity (ID)						Origin		
			GeneBank accession					
Species	Isolate ID	Herbarium accession number	ITS	EF1-α	β-tubulin	Vineyard	Host cultivar	Host tissue
Botryosphaeria dothidea	H171-1	DAR 80994	HQ392689	HQ392757	HQ392756	B	Shiraz	Berries at harvest
B. dothidea	H171-2	DAR 80995	HQ392690	HQ392759	HQ392758	B	Shiraz	Berries at harvest
B. dothidea	BB56-2	DAR 81005	HQ392691	HQ392739	HQ392738	A	Shiraz	Dormant bud
B. dothidea	W64-5	DAR 81006	HQ392692	-	-	A	Shiraz	Wood
B. dothidea	W96-3	DAR 81007	HQ392693	-	-	A	Shiraz	Wood
B. dothidea	W126-5	DAR 81008	HQ392694	-	-	B	Chardonnay	Wood
B. dothidea	BB152-1	DAR 81009	HQ392695	HQ392742	HQ392743	B	Shiraz	Dormant bud
B. dothidea	BB158-4	DAR 81010	HQ392696	HQ392744	HQ392745	B	Shiraz	Dormant bud
B. dothidea	BB163-1	DAR 81011	HQ392697	-	-	B	Shiraz	Dormant bud
B. dothidea	BB174-1	DAR 80994	HQ392698	-	-	B	Shiraz	Dormant bud
B. dothidea	BB178-1	DAR 81029-	-	HQ392749	HQ392748	B	Shiraz	Dormant bud
Diplodia seriata	BB9-1	DAR 80978	HQ392699	-	-	A	Chardonnay	Dormant bud
D. seriata	W86-2	DAR 80979	HQ392700	-	-	A	Shiraz	Wood
D. seriata	W196-2	DAR 80980	HQ392701	-	-	B	Shiraz	Wood
D. seriata	H17-1	DAR 80984	HQ392702	-	-	A	Chardonnay	Berries at harvest
D. seriata	H64-1	DAR 80985	HQ392703	-	-	A	Shiraz	Berries at harvest
D. seriata	B98-3	DAR 80986	HQ392704	-	-	A	Shiraz	Berries at harvest
D. seriata	B106	DAR 80987	HQ932705	-	-	B	Chardonnay	Dormant bud
D. seriata	B178-1	DAR 80988	HQ392706	-	-	B	Shiraz	Dormant bud
D. seriata	H33-1	DAR 80996	HQ392707	-	-	A	Chardonnay	Berries at harvest
D. seriata	H118-1	DAR 80997	HQ392708	-	-	B	Chardonnay	Berries at harvest
D. seriata	FF197-1	DAR 80998	HQ392709	-	-	B	Shiraz	Flowers
D. seriata	PP136	DAR 80999	HQ392710	-	-	B	Chardonnay	Pea-sized berries
D. seriata	FF151-1	DAR 81000	HQ392711	-	-	B	Shiraz	Flowers
D. seriata	PP118	DAR 81002	-	-	-	B	Chardonnay	Pea-sized berries
D. seriata	PP119-1	DAR 81003	-	-	-	B	Chardonnay	Pea-sized berries
Dothiorella viticola	B146-3	DAR 80992	HQ932712	-	HQ392736	B	Chardonnay	Berries at harvest
D. viticola	B116-3	DAR 81012	HQ392713	HQ392735	HQ932734	B	Chardonnay	Dormant bud
Lasiodiplodia theobromae	W200	DAR 81024	HQ392714	-	-	B	Shiraz	Wood
Neofusicoccum australe	BB59-3	DAR 81004	HQ392715	-	-	A	Shiraz	Dormant bud
Neofusicoccum luteum	H12-1	DAR 80983	HQ392716	HQ392753	HQ392752	A	Chardonnay	Berries at harvest

Identity (ID)						Origin		
		Herbarium accession number	GeneBank accession					
Species	Isolate ID		ITS	EF1-α	β-tubulin	Vineyard	Host cultivar	Host tissue
N. luteum	HH119-1	DAR 81013	HQ392717	HQ392760	HQ392761	B	Chardonnay	Dormant bud
N. luteum	BB127-1	DAR 81014	-	HQ392740	HQ392741	B	Chardonnay	Dormant bud
N. luteum	BB161-2	DAR 81015	HQ393718	-	HQ392746	B	Shiraz	Dormant bud
N. luteum	BB175-2	DAR 81016	HQ392719	HQ392768	HQ392747	B	Shiraz	Dormant bud
N. luteum	BB192-1	DAR 81017	HQ392720	HQ392769	HQ392750	B	Shiraz	Dormant bud
N. luteum	HH197-1	DAR 81018	HQ392721	HQ392763	HQ392762	B	Shiraz	Berries at harvest
N. luteum	FF23-1	DAR 81019	HQ392722	HQ392770	HQ392751	A	Chardonnay	Dormant bud
N. luteum	BB29-1	DAR 81020	HQ392723	-	HQ392737	A	Chardonnay	Dormant bud
Neofusicoccum parvum	W27-5	DAR 80981	HQ392724	-	-	A	Chardonnay	Wood
N. parvum	W45-3-1	DAR 80982	HQ392725	-	HQ392764	A	Chardonnay	Wood
N. parvum	H77-1	DAR 80989	HQ392726	-	-	A	Shiraz	Berries at harvest
N. parvum	B114-2	DAR 80990	HQ392727	-	-	B	Chardonnay	Dormant bud
N. parvum	H162-1	DAR 80991	HQ392728	-	-	B	Shiraz	Berries at harvest
N. parvum	B8-3	DAR 80993	HQ392729	-	-	A	Chardonnay	Berries at harvest
N. parvum	FF194-5	DAR 81001	HQ392730	-	-	B	Shiraz	Flowers
N. parvum	W176-1	DAR 81021	HQ392731	HQ392767	HQ392766	B	Shiraz	Wood
Neofusicoccum ribis	W45-3-2	DAR 81022	HQ392732	HQ392765	-	A	Chardonnay	Wood
N. ribis	H73-1	DAR 81023	HQ392733	HQ392755	HQ392754	A	Shiraz	Berries at harvest

(Qiu *et al*. 2008) and *L. theobromae* (Qiu *et al*. 2011) were the only species of Botryosphaeriaceae reported from the Hunter Valley. The additional findings of *D. viticola*, *N. australe*, and *D. mutila* at relatively low frequencies compared to most of the previously recorded species except *L. theobromae* reflect the species distribution seen in other regions in eastern Australia, which largely seems to depend on climatic variations (Pitt *et al*. 2010*a*). This has also been observed in California (USA) and Mexico (Urbez-Torres and Gubler 2006*a*; Urbez-Torres *et al*. 2008). However, the findings of nine different species in two vineyards in the Lower Hunter Valley stands in contrast to the results of Pitt *et al*. (2010*a*) declaring a larger number of species distributed in the southern wine regions of NSW compared to those in the north-east. The isolations of the rarer species in our survey might be explained by the more intensive sampling method used (Qiu *et al*. 2011).

The isolation of one single isolate belonging to *L. theobromae*, a species favouring hot climatic conditions (Urbez-Torres *et al*. 2006), is also consistent with the findings of Qiu *et al*. (2011), who predict to see a greater abundance of this species in the Hunter Valley in the future due to increased temperatures caused by climate change.

Botryosphaeriaceae species reported in this survey have previously been reported on grapevines in Australia, however, isolations were limited to the analysis of grapevine wood (Castillo-Pando *et al*. 2001; Taylor *et al*. 2005; Savocchia *et al*. 2007; Pitt *et al*. 2008*a*; Qiu *et al*. 2008; Pitt *et al*. 2010a).

In previous reports that dealt with the infection of fruit in Australian vineyards the fungi were not identified to species level (Steel *et al*. 2007; Taylor 2007) other than Cunnington *et al*. (2007) who reported *N. australe* from grapevine berries in Victoria, Australia. To our knowledge our survey is

Table 2.22 Number of Botryosphaeriaceae species isolated from two vineyards planted to *Vitis vinifera* cultivars 'Chardonnay' and 'Shiraz'

	Vineyard A		Vineyard B	
Species	**Chardonnay**	**Shiraz**	**Chardonnay**	**Shiraz**
Diplodia seriata	65	26	48	39
Diplodia mutila	-	-	1	-
Lasiodiplodia theobromae	-	-	-	1
Dothiorella viticola	-	-	2	1
Neofusicoccum parvum	9	6	14	18
Neofusicoccum luteum	3	-	2	4
Neofusicoccum ribis	1	1	-	-
Neofusicoccum australe	-	1	-	-
Botryosphaeria dothidea	4	6	1	12
Botryosphaeria spp.*	14	14	15	22
Total	150		180	

*Isolates identified to genus level only

Table 2.23 Number of Botryosphaeriaceae isolated from different reproductive tissues of *Vitis vinifera*

	Origin tissue				
Species	**Dormant buds**	**Flowers**	**Pea-sized berries**	**Berries at harvest**	**Wood**
Diplodia seriata	100	3	3	13	59
Diplodia mutila	-	-	-	-	1
Lasiodiplodia theobromae	-	-	-	-	1
Dothiorella viticola	2	-	-	-	1
Neofusicoccum parvum	17	1	-	7	23
Neofusicoccum luteum	5	1	-	3	-
Neofusicoccum ribis	-	-	-	1	1
Neofusicoccum australe	1	-	-	-	-
Botryosphaeria dothidea	8	-	-	2	11
Botryosphaeria spp.*	14	2	-	4	46
Total	147	7	3	30	143

*Isolates identified to genus level only

the first report of *D. seriata*, *D. viticola*, *N. parvum*, *N. ribis*, *N. luteum* and *B. dothidea* in *V. vinifera* tissue other than wood.

D. seriata and *N. parvum* were most abundant in dormant buds, berries at harvest and in the wood. This is consistent with the findings of previous surveys of wood conducted in the Hunter Valley (Castillo-Pando *et al*. 2001; Savocchia *et al*. 2007; Qiu *et al*. 2008; Pitt *et al*. 2010a) and suggests that the species distribution on grapevine reproductive tissue is not different from that on wood.

The findings of Botryosphaeriaceae species in dormant buds, flowers, pea-sized berries and berries at harvest confirm that the presence and possible infection by these fungi is not limited to the wood. Many of the species found here occur on several different tissue types, confirming that the Botryosphaeriaceae are not tissue-specific. The relative pathogenicity or aggressiveness of the individual species is still unknown. Further studies including pathogenicity testing of individual isolates across various tissue types are necessary to confirm Botryosphaeriaceae as pathogens not limited to wood.

Comparing the large number of isolations from wood with those from reproductive tissues suggests that Botryosphaeriaceae occurring on wood may act as inoculum sources for infection in other tissues, in a similar way to Botryosphaeria fruit rot in other hosts. In apples the primary source of apple rot infection is the dispersal of Botryosphaeriaceae conidia from pycnidia found on the outside of infected branches and twigs (Drake 1971; Sutton 1981). This source is available throughout the whole season (Sutton 1981) and infection of apples has been reported to begin as early as petal fall (Parker and Sutton 1993).

No fungicides are currently registered for the control of Botryosphaeriaceae in Australian vineyards however, it is known that the management of other bunch rots, such as grey mould caused by *Botrytis cinerea*, relies extensively on fungicide sprays at flowering to reduce the infection rate of inflorescences and subsequent berry infection at harvest (Nair *et al*. 1987; Nair and Allen 1993; Nair *et al*. 1995; Elad *et al*. 2007). In addition Pitt *et al*. (2008*b*) highlighted commercially available products containing fludioxonil, penconazole, and iprodione as some of the most effective fungicides tested in vitro for the inhibition of Botryosphaeriaceae. All three products were applied to the vineyards surveyed between flowering and veraison possibly contributing to the low numbers of Botryosphaeriaceae isolated from flowers and pea-sized berries. Prior to bud burst spray, fungicide applications in both vineyards were low and post-veraison, applications were halted completely due to fungicide withholding period regulations. This may explain the more frequent isolations of Botryosphaeriaceae at the early and later stages of the growing season and would suggest that successful infection of dormant buds early in the season may lead to bunch infection towards the end of the season, with infections carried internally and unaffected by further fungicide applications throughout the season. However, the relatively high number of isolates from dormant buds compared to those from berries at harvest of the same vines could be explained by the hypothesis that infected buds will remain viable and produce bunches. Future research is therefore necessary to investigate the vitality/mortality of Botryosphaeriaceae infected buds. If infection leads to bud mortality we hypothesise that the isolates from berries at harvest may be a result of later infections from the dispersal of conidia onto the outside of the berries rather than from internal bud infection.

Experiment 2.13
Biological factors affecting virulence of Botryosphaeriaceae on grapevines

Materials and methods

The methods below are adapted from Wunderlich *et al*. (2012, *Annals of Applied Biology*).

Fungal isolates for pathogenicity studies

A total of 19 isolates were obtained from different vegetative tissues and wood of *V. vinifera* cultivars Chardonnay and Shiraz from the Hunter Valley and were stored at the Agricultural Scientific Collection Unit (*Table 2.24*). Isolate identities were confirmed in a previous study by Wunderlich *et al*. (2011a).

Pathogenicity on canes

Disease-free, certified detached one year old canes of Chardonnay and Shiraz (Murrumbidgee Irrigation Area Vine Improvement Society) were surface sterilised with 70% ethanol and cut into 80 mm lengths. Each piece was wounded by drilling a hole in the middle of the cane and into the pith with a 4 mm diameter surface-sterilised drill bit. Canes were inoculated by inserting 4 mm diameter mycelium plugs of 14 single spore Botryosphaeriaceae isolates

grown for three days as pure cultures on PDA into each hole (*Table 2.24*). All holes were covered with Parafilm and each cane was placed into a Petri dish on moist filter paper. For each isolate there were five replicate canes per cultivar. A control set of canes for each cultivar consisted of canes inoculated with sterile PDA plugs. Petri dishes were placed in a completely randomised design and incubated at 25°C in the dark. After 21 days the bark of each cane was removed carefully with a sterile dissecting blade and lengths of visible lesions originating from the inoculation point were recorded. Small tissue samples were removed from the margin of healthy wood and lesion on each cane and surface sterilised in 0.5% sodium hypochlorite for 1 min followed by three rinses in sterile distilled water for 1 min each. Samples were placed on PDA and incubated at 25°C in the dark to promote mycelial growth. To satisfy Koch's postulates fungi originating from the samples were identified to species level by their conidia morphology.

All statistical analyses associated with the pathogenicity studies were performed with Genstat® (2010, Thirteenth edition, VSN International Ltd., Hemel Hempstead, UK) and for the results presented all non significant interactions were removed from final models. The statistical terminology for all combination of effects is written as capitalised name of the effects separated by a full stop, such as 'Cultivar. Species.Isolate'.

Analysis of variance (ANOVA) was performed to first assess the effect of the interaction between cultivar and the fungus/no fungus (i.e. treated versus control) contrast on lesion length. This required the square root transformation of lesion lengths prior to analysis. Due to large comparative difference in occurrence of lesions between inoculated and control canes (see results), data from controls were removed from subsequent analysis.

Further ANOVAs were used to assess the effect of isolate (or species or tissue of origin) in combination with cultivar on lesion lengths. In view of the highly significant effects of both species and isolates (see results), to address the question of whether isolate effects exist separately from species effects we removed the two species (*D. viticola* and *N. luteum*) with only one isolate and did a further analysis of the effect of isolates nested within species interacting with cultivars.

Pathogenicity on berries

Conidial suspensions of 18 single spore Botryosphaeriaceae isolates (*Table 2.24*) were prepared by transferring 4 mm^2 mycelium plugs of three day old actively growing PDA cultures onto Petri dishes containing six triple autoclaved pine needles on sterile 1% water agar. Pine needle cultures were incubated for up to 6 weeks under a light regime of 12 hours dark and 12 hours near UV light

Table 2.24 Identities and origins of Botryosphaeriaceae isolates used in the pathogenicity tests.

Isolate ID	Accession number	Species	Origin		Pathogenicity test[a]
			Tissue	Cultivar	
H171-1	DAR 80994	*Botryosphaeria dothidea*	Berries at harvest	Shiraz	W, H
H171-2	DAR 80995	*B. dothidea*	Berries at harvest	Shiraz	W, H
B106	DAR 80987	*Diplodia seriata*	Dormant bud	Chardonnay	W, B
B178-1	DAR 80988	*D. seriata*	Dormant bud	Shiraz	W, H
B98-3	DAR 80986	*D. seriata*	Dormant bud	Shiraz	W, H
H118-1	DAR 80997	*D. seriata*	Berries at harvest	Chardonnay	W, H
H17-1	DAR 80984	*D. seriata*	Berries at harvest	Chardonnay	W, H
H33-1	DAR 80996	*D. seriata*	Berries at harvest	Chardonnay	W, H
H64-1	DAR 80985	*D. seriata*	Berries at harvest	Shiraz	W, H
W86-2	DAR 80979	*D. seriata*	Wood	Shiraz	H
B146-3	DAR 81012	*Dothiorella viticola*	Dormant bud	Chardonnay	W, H
W200	DAR 81024	*Lasiodiplodia theobromae*	Wood	Shiraz	H
H12-1	DAR 80983	*Neofusicoccum luteum*	Berries at harvest	Chardonnay	W, H
B114-2	DAR 80990	*Neofusicoccum parvum*	Dormant bud	Chardonnay	H
B8-3	DAR 80993	*N. parvum*	Dormant bud	Chardonnay	W, B, H
H162-1	DAR 80991	*N. parvum*	Berries at harvest	Shiraz	W, H
H77-1	DAR 80989	*N. parvum*	Berries at harvest	Shiraz	W, H
W27-5	DAR 80981	*N. parvum*	Wood	Chardonnay	H
W45-3-1	DAR 80982	*N. parvum*	Wood	Chardonnay	H

[a] 'Pathogenicity test' relates to the tissue type each isolate was tested on (W= wood, B=dormant bud, H= berry at harvest)

to encourage the formation of conidia. Following incubation, pine needles containing pycnidia were stored in McCartney bottles with sterile water for 6 hours before being crushed with a micro-pestle to encourage the release of conidia. Conidia suspensions were adjusted to concentrations of approximately $1x10^6$ conidia mL^{-1}.

Visually disease-free berries of Chardonnay and Shiraz were picked 1–2 days prior to harvest and at sugar concentrations of 13 and 14° Baume, respectively. Berries were cut from the bunches at the pedicel, leaving the brush of each berry intact and surface sterilised in 1% sodium hypochlorite and Tween 20 (0.05%) for 2 min followed by three rinses in sterile distilled water (Steel *et al*. 2007). Single unwounded berries were placed into individual wells of Microplates (24 well, flat-bottom, Iwaki Microplates) with each plate assigned to a separate treatment. Approximately 20 mL of sterile water was placed in each plate in the space surrounding the wells to maintain humidity during incubation.

Berries were inoculated by pipetting 10 µL conidia suspensions of each isolate onto the surface of each berry of three replicate plates of each treatment (isolate/cultivar combination). Sterile distilled water was used for control inoculation. After inoculation the plates were closed with lids, arranged in a totally randomised design and incubated at 27°C for 15 days.

At the time of inoculation disease incidence for each berry was given a score of zero. Twenty four hours after inoculation and on every second day thereafter until day 15, disease incidence was assessed and recorded for each berry. In addition at day 15 each berry was inspected for disease severity indicated by the presence or absence of visible pycnidia/conidia formation on the berry surface. To satisfy Koch's postulates, a subset of berries from each treatment were placed onto PDA, incubated at 25°C and examined for the presence of Botryosphaeriaceae corresponding to the isolate used for inoculation.

Disease incidence increase over time was analysed by fitting binomial generalised linear mixed models (GLMM, Schall's method 1991) with isolate (or species or origin), cultivar and time as fixed effects and plate as the random effect. The response variable was the number of diseased berries out of the 24 berries on each plate. Rates of disease incidence increase over time- were examined and the isolates ranked based on these rates. Further analysis was conducted to examine any remaining differences in isolate means, after removing species or origin portion.

Disease severity on day 15 was analysed for the effects of host cultivar and either isolate, species or origin tissue and their interactions using binomial GLMs. The response variable was presence or absence of sporulation on each berry. As in the analysis of disease incidence, isolates were ranked based on these probabilities and we assessed whether significant differences in isolates were due to differences in species or tissue of origin.

Pathogenicity on dormant buds (glasshouse)

Conidial suspensions of *N. parvum* isolate B8-3 and *D. seriata* isolate B106 were prepared as described for the berry pathogenicity test. The first three buds of 30 potted dormant cuttings (nursery supplied material as per methods for cane pathogenicity) each of Chardonnay and Shiraz were inoculated by pipetting either 10 µL of each conidia suspension or sterile water onto the bud surface. These six treatments, each replicated 10 times, were applied over three adjacent trays of 5x4 cuttings in a row-column design.

Immediately after inoculation each cutting was covered with a plastic bag for 48 hour to maintain humidity. Over the length of the experiment the cuttings were watered daily and kept at glasshouse temperatures of 22 ±2°C and under natural light conditions. Bud development stages (1–5) were recorded every second day. The scale of assessment consisted of the five stages: winter bud (1); bud swell (2); woolly bud–brown wool visible (3); green tip–first leaf tissue visible (4); and rosette of leaf tips visible (5) as described by Lorenz *et al*. (1995).

After 58 days the experiment was terminated with the assessment of visible disease symptoms, bud survival shown as successful shoot growth, lengths of shoots for each bud, the number of days to reach bud development stage 5 for the first bud of each cutting and root abundance (ranging from 1=low abundance to 4=high abundance) of each cutting. The survival of buds was analysed using binomial GLMMs and linear mixed models (LMMs, restricted maximum likelihood (REML), Patterson and Thompson 1971) were used to analyse shoot lengths and number of days to reach bud development stage 5, while root abundance was analysed by ordinal regression. In all cases the fixed effects were cultivar (2 levels) and inoculum (3 levels) and their interactions and the random effects were row and column.

Following assessment, Koch's postulates were completed by surface sterilising and placing the bud and shoot tissue onto PDA, followed by culturing and

identification of *N. parvum* or *D. seriata* cultures as per previous experiments.

Pathogenicity on dormant buds (field)

Conidia suspensions of *D. seriata* isolate B106 were produced as for the inoculations described above and six dormant buds were randomly selected on 30 randomly selected 12 year old Chardonnay grapevines in a vineyard with no history of *Botryosphaeria* canker. The buds were inoculated by pipetting either 50 µL of the conidia suspension or 50 µL sterile water onto the bud surfaces of 15 replicate vines per treatment. Dormant buds were covered with plastic bags for 48 hours to maintain humidity.

The experimental vines were assessed for bud-burst when the remaining vines in the same vineyard block had reached 100% bud-burst (i.e. E-L stage 4) (Coombe 1995). Further assessments included the counting of shoots developed from each bud and number of bunches per shoot as well as shoot length measurements at pea-sized berry stage (E-L stage 31) (Coombe 1995). Re-isolations were attempted from berries as well as rachis at pea-sized berry and at harvest stage (E-L stage 38) (Coombe 1995) with isolation and identification methods as described above for all previous pathogenicity tests.

The effect of inoculation on numbers of burst buds was analysed using binomial GLMMs, number of shoots and bunches per shoot were assessed with Poisson GLMMs, and LMMs were used to analyse the inoculation effect on shoot length. In all cases row and vine were used as random effects.

Results and discussion

The following results and discussion will be also be presented in Wunderlich *et al.* (2012, *Annals of Applied Biology*).

Pathogenicity on canes

The resulting lesions on the canes inoculated with Botryosphaeriaceae isolates were significantly longer than those on control canes (F=25.39; df=1, 143; P<0.001). There was no interaction between cultivar and treatment contrast. For the control inoculated canes (n=5), the mean lesion lengths were 0 and 0.4 mm and the standard deviations were 0 and 0.894 (four observations were zero) for Shiraz and Chardonnay, respectively. After removing the controls from the analysis, now known to be different from the isolates and largely zero, there was a significant interaction between isolate and cultivar (F=2.64; df= 13, 109; P=0.003). Mean square root values of lesion lengths were plotted with the least significant difference (*Figure 2.21*). While in general the mean

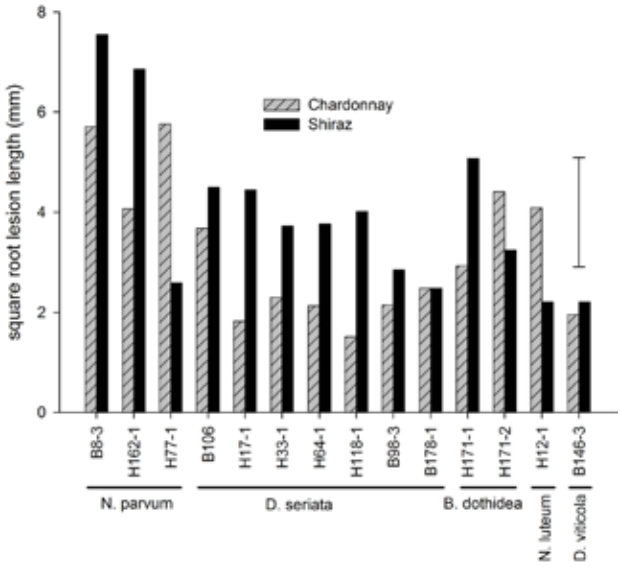


Figure 2.21 Mean square roots of lesion lengths caused by each isolate on canes of both host cultivars grouped by species. Least significant difference=2.191 at 5% level; degrees of freedom=109.

lesion lengths for the isolates in our study were longer for Shiraz (15.8 mm) than Chardonnay (10.3 mm), the opposite was observed for three isolates (H77-1, H171 2 and H12-1) which had longer lesions lengths on Chardonnay than Shiraz (*Figure 2.21*).

When analysing the species effect on lesion lengths, a highly significant effect of species (F=10.46; df=4, 127; P<0.001) and the expected significant difference between cultivars (F=6.28, df=1, 127; P=0.013) but no interaction between species and cultivar was shown. The species producing the highest mean lesion length was *N. parvum* (29.4 mm) and lowest mean lesion length was produced by *D. viticola* (4.3 mm). There was no effect of tissue of origin or its interaction with cultivar. The nested analysis showed a highly significant species effect (F=18.39; df=2, 93; P<0.001), the expected cultivar effect (F=9.07; df=1, 93; P=0.003) and a significant Species.Isolate.Cultivar interaction (F=2.58; df=9, 93; P=0.011). Thus while the majority of the difference between isolates is due to differences between species, there remain further differences in the behaviour of the isolates on the different cultivars.

Pathogenicity on berries

All isolates produced bunch rot symptoms on berries in vitro. Symptoms ranged from darkening of the berry skin, oozing of berries and the appearance of mycelia to pycnidia formation and berry collapse.

While the Species x Cultivar interaction explained a large proportion of the total variation between rates of disease incidence increase (F=12.79; df=11, 779;

P<0.001), there was still a highly statistically significant remainder of the variation explained by the Cultivar. Isolate interaction.Cultivar (F=2.69; df=24, 778.9; P<0.001). The same applies to Cultivar. Origin tissue interaction (F=11.53; df=5, 779.6, P<0.001) where a highly significant proportion of the remaining variation was explained by Cultivar.Isolate (F=4.92; df=30, 778.8, P<0.001).

For analysis of disease severity on day 15 the interaction of isolate, species and origin with cultivar and cultivar main effects were not statistically significant. The main effects of isolate, species and origin were all highly statistically significant (P<0.001). Nested analyses were therefore used to assess whether differences in isolate are due to a) differences in species or b) differences in origin.

The differences in disease severity between isolates was partially explained by species (F=17.14, df=5, 107.3, P<0.001). The remaining portion of variability explained by isolate was highly statistically significant (F=3.45, df=12, 79.3, P<0.001). Tissue of origin of isolates partially explained the differences between isolates (F=9.87, df=2, 82.4, P<0.001), however, the remaining differences between isolates were again statistically significant (F=7.16, df=15, 86.7, P<0.001).

In the presentation of results, both disease incidence increase over time and disease severity on day 15 were presented by isolates grouped by species (*Table 2.25* and *2.27*) and tissue of origin (*Table 2.26* and *2.28*), as well as ranked within those groups to show the difference in disease incidence increase and severity within species.

Disease incidence

There were statistically significant differences in rates of disease incidence increase between isolate and cultivar combinations (F=6.28; df=37, 820.9; P<0.001). The increase in disease incidence was higher on Shiraz than Chardonnay for all isolates except for *L. theobromae* isolate W200 where the opposite was observed. *Tables 2.25* and *2.26* present the rate of disease incidence increase for each isolate and host cultivar combination on the logit scale. The highest rates of disease incidence increase were observed for *N. parvum* isolate W27-5 on Shiraz followed by *L. theobromae* isolate W200 on Chardonnay (*Table 2.25*). Lowest rates of disease incidence increase were associated with *Dothiorella viticola* A.J.L. Philips and J. Luque isolate B 146-3 on both cultivars and were not statistically different from the controls (*Table 2.25*).

In terms of species, highest rankings for rate of disease incidence increase were attained by isolates

Table 2.25 Rates of disease incidence increase over time on berries inoculated with Botryosphaeriaceae isolates (logit scale). Isolates are ranked by rates off disease incidence increase and grouped by species.

		Chardonnay		Shiraz	
Species	**Isolate**	**Rate of increase[a]**	**Rank**	**Rate of increase[a]**	**Rank**
Lasiodiplodia theobromae	W200	0.95	1	0.93	2
Neofusicoccum parvum	H77-1	0.44	2	0.67	4
N. parvum	W45-3-1	0.38	3	0.53	7
N. parvum	W27-5	0.37	4	1.09	1
N. parvum	B114-2	0.34	5	0.55	5
N. parvum	H162-1	0.30	8	0.86	3
N. parvum	B8-3	0.25	15	0.37	13
Neofusicoccum luteum	H12-1	0.33	6	0.47	8
Diplodia seriata	H118-1	0.31	7	0.54	6
D. seriata	H17-1	0.30	9	0.39	12
D. seriata	B178-1	0.28	11	0.28	16
D. seriata	W86-2	0.27	13	0.31	15
D. seriata	B98-3	0.26	14	0.41	11
D. seriata	H33-1	0.23	16	0.36	14
D. seriata	H64-1	0.22	17	0.28	17
Botryosphaeria dothidea	H171-1	0.30	10	0.44	10
B. dothidea	H171-2	0.268	12	0.45	9
Dothiorella viticola	B146-3	0.20	18	0.21	18
-	Control	0.16	19	0.17	19

[a] Standard error of differences: 0.08 (average), 0.21 (maximum), 0.05 (minimum), degrees of freedom 820.9.

of the species *L. theobromae* and *N. parvum* for both cultivars. However, *N. parvum* isolate B8-3 fell outside this group and ranked for both cultivars within the isolates of the *D. seriata* group, which generally caused lower rates of disease incidence increase than those of *N. parvum* isolates.

For both host cultivars the isolates belonging to *N. luteum* and *B. dothidea* ranked lower than *N. parvum* isolates with the exception of B8-3. The lowest rates of disease incidence increase were caused by inoculation with *D. viticola* for both host cultivars.

The two highest ranked isolates on Shiraz, W27-5 and W200 as well as the first and third ranked isolated on Chardonnay W200 and W45-3-1 all originated from wood, however the wood-derived isolate W86-2 ranked significantly lower on both Chardonnay and Shiraz (*Table 2.26*). Large variations were also observed for some of the isolates derived from berries and dormant buds, which did not rank according to their tissue of origin grouping. The lowest rate of increase was produced by dormant bud-derived isolate B146-3 for both cultivars, on the other hand dormant bud-derived isolate B114-2 ranked 5th in terms of rate of disease incidence increase for both cultivars (*Table 2.26*).

Disease severity

The probability of sporulation on day 15 varied significantly for isolates ($F=6.85$; $df=18, 70.2$; $P<0.001$). Neither the interaction with cultivar nor cultivar effect alone was significant. *Tables 2.27* and *2.28* shows the ranked means for probability of sporulation on the berry surface at day 15 for different isolates, grouped by their species and tissue of origin, respectively. The ranked isolates do not fall into groups according to species or tissue of origin.

Highest probabilities to sporulate on berry surfaces at day 15 existed for the two wood-derived isolates of W200 (*L. theobromae*) and W27-5 (*N. parvum*) and lowest probabilities were associated with *D. seriata* isolates H64-1, H33-1 and B178-1 (*Table 2.27*). Three out of the four wood-derived isolates in this study ranked within the five most virulent, however, isolate W86-2 (*D. seriata*) fell outside this origin tissue group and ranked 14th (*Table 2.28*). Isolates from berries at harvest and dormant buds did not form groups in terms of disease severity on day 15 and both tissue of origin types appeared to include highly virulent as well as weakly pathogenic isolates (*Table 2.28*).

Pathogenicity on dormant buds (glasshouse)

There was no significant effect of any treatment on survival of buds, shoot lengths, number of days to stage 5 bud development or root abundance. In only

Table 2.26 Rates of disease incidence increase over time on berries obtained by Botryosphaeriaceae isolates (logit scale). Isolates are ranked by rates of disease incidence increase and grouped by tissue of origin.

		Chardonnay		Shiraz	
Origin tissue	**Isolate**	**Rate of increase[a]**	**Rank**	**Rate of increase[a]**	**Rank**
Wood	W200	0.95	1	0.93	2
	W45-3-1	0.38	3	0.53	7
	W27-5	0.37	4	1.09	1
	W86-2	0.27	13	0.31	15
Berries	H77-1	0.44	2	0.67	4
	H12-1	0.33	6	0.47	8
	H118-1	0.31	7	0.54	6
	H162-1	0.30	8	0.86	3
	H17-1	0.30	9	0.39	12
	H171-1	0.30	10	0.44	10
	H171-2	0.27	12	0.45	9
	H33-1	0.23	16	0.36	14
	H64-1	0.22	17	0.278	17
Dormant bud	B114-2	0.34	5	0.55	5
	B178-1	0.28	11	0.28	16
	B98-3	0.26	14	0.41	11
	B8-3	0.25	15	0.37	13
	B146-3	0.20	18	0.21	18
-	Control	0.16	19	0.17	19

aStandard error of differences: 0.08 (average), 0.21 (maximum), 0.05 (minimum), degrees of freedom 820.9.

17 instances could Botryosphaeriaceae be re-isolated from the plant tissue (*N. parvum* n=6 and *D. seriata* n=11).

Pathogenicity on dormant buds (field)

Inoculation with *D. seriata* isolate B106 did not have a significant effect on bud survival, number of shoots grown from each successfully sprouted bud, shoot length or number of bunches produced from each shoot. At pea-sized berry stage *D. seriata* isolate B106 was re-isolated from as few as three out of 90 bunch samples (twice from berries and once from the rachis). Similar figures were observed at harvest stage, where the fungus was re-isolated from two bunch samples and three times from the rachis of inoculated vines.

Summary

All of the Botryosphaeriaceae isolates examined were pathogenic on one year old canes and mature berries irrespective of their tissue of origin. Isolates originating from berries at harvest as well as dormant buds were able to infect the wood and cause symptoms typical of Botryosphaeria canker. In addition, isolates originating from wood and dormant buds were able to cause bunch rot symptoms on berries. Infection frequency and disease severity on berries caused by isolates from these tissue types was not less than for isolates which had originated from berries. We therefore conclude that Botryosphaeriaceae isolates are not tissue specific in *V. vinifera* and the tissue of origin type does not determine their pathogenicity or virulence on the same or other tissue types. This contrasts with the findings of Botryosphaeriaceae in other crops such as olives where a pathogenic specialisation for different species exists in regards to pathogenicity on different tissue types (Moral *et al.* 2010).

In contrast to Botryosphaeriaceae infection of wood, no previous wounding was required for conidia to infect berries and to produce various degrees of berry rot symptoms. In olives the state of maturity affects the susceptibility of fruit to Botryosphaeriaceae (Lazzizera *et al.* 2008). If this is also the case for grapevines it could explain why the disease incidence over 15 days after inoculation of mature berries in vitro was relatively high compared to incidence data of Botryosphaeriaceae isolations in field situations and might also explain the low level of isolations at pea-sized berry stage (Wunderlich *et al.* 2011a).

The two species with a relatively high number of isolates *D. seriata* (n=7 and 7) and *N. parvum* (n=3 and 6), in one year old cane and berry pathogenicity tests, respectively, showed large differences in disease severity within species. This shows that the discrepancies in pathogenicity within species detected by other authors in wood pathogenicity tests (Taylor *et al.* 2005; Larignon *et al.* 2001; van Niekerk *et al.* 2004) also exist for the isolates used in the current study and further confirms that similar within-species variations exist for virulence of berry infection.

Isolates in this study came from various tissues of origin and belonged to different species. It is therefore possible that the differences seen in their pathogenicity is explained by a combination of these and could explain why not all isolates group within their species in terms of pathogenicity.

Some of the isolates that were used in both tests such as H162-1, B146-3 and H33-1, ranked equally virulent for either one or both cultivars on both tissue types. In contrast some isolates such as B8-3 and were highly virulent on one tissue type only, and much less virulent on the other tissue type. *D. viticola* isolate B146-3 was consistently weakly virulent in both tests. This coincides with the findings of Urbez-Torres and Gubler (2009) who labelled *D. viticola* as a weakly pathogenic trunk disease pathogen.

In our wood pathogenicity studies isolates of *D. seriata* caused significantly shorter lesions than isolates of *N. parvum*. However, out of the 14 isolates tested on one year old canes, *D. seriata* B106 (on Chardonnay) and H17-2, H118-1 and H64-1 (on Shiraz) were within the 50% most virulent isolates. In the berry pathogenicity test the probability of sporulation on day 15 and the rate of disease incidence increase was significantly less for isolates of *D. seriata* than for isolates of *N. parvum*, however, *D. seriata* isolate H118-1 was the 6th highest out of 14 isolates in probability of sporulation and had the 7th highest rate of disease incidence increase on Chardonnay. The within species differences observed in our study for *D. seriata* and *N. parvum* highlight the importance of including a large number of isolates for each species when determining species pathogenicity and explains that the data for *L. theobromae*, *N. luteum* and *D. viticola* are less reliable and cannot be used to judge these species differences because only one isolate was available for the tests.

While the question about true pathogenicity of *D. seriata* still remains, it can be concluded from the pathogenicity tests on one year old canes and berries that *D. seriata* is a primary pathogen which can cause

wood and berry symptoms in the absence of other pathogens. This addresses the question of Qiu *et al.* (2011) concerning the pathogenicity of *D. seriata* and contrasts with Taylor *et al.* (2005) and Phillips (1998) who labelled *D. seriata* as a secondary pathogen after it was seldom isolated in the absence of other pathogens.

In our study, higher rates of disease incidence increase on berries were generally obtained on Shiraz than Chardonnay and in one year old canes Shiraz was generally also more susceptible than Chardonnay, which is in contrast to the observations of Savocchia *et al.* (2007). However, in terms of interactions between isolate and cultivar our results concur with Savocchia *et al.* (2007). This shows that there might

Table 2.27 Mean probability of isolates to sporulate on berry surfaces at day 15 grouped by species

Isolate	Species	Probability (logit scale)[a]	Probability (back-transformed)	Rank
W200	*Lasiodiplodia theobromae*	8.77	1.00	1
W27-5	*Neofusicoccum parvum*	6.61	1.00	2
H77-1	*N. parvum*	1.11	0.75	3
B114-2	*N. parvum*	0.38	0.59	4
W45-3-1	*N. parvum*	0.18	0.54	5
H162-1	*N. parvum*	-0.85	0.30	8
B8-3	*N. parvum*	-0.97	0.27	9
H118-1	*Diplodia seriata*	-0.01	0.50	6
H17-1	*D. seriata*	-1.45	0.19	11
B98-3	*D. seriata*	-1.67	0.16	12
W86-2	*D. seriata*	-2.08	0.11	14
B178-1	*D. seriata*	-2.22	0.10	16
H33-1	*D. seriata*	-2.31	0.09	17
H64-1	*D. seriata*	-2.44	0.08	18
H171-2	*Botryosphaeria dothidea*	-0.39	0.40	7
H171-1	*B. dothidea*	-1.65	0.16	13
H12-1	*Neofusicoccum luteum*	-1.36	0.20	10
B146-3	*Dothiorella viticola*	-2.23	0.10	15
Control	-	-9.45	0.00	19

[a] Standard error of differences on logit scale: 10.32 (average), 53.70 (maximum) and 0.58 (minimum; degrees of freedom = 70.2

Table 2.28 Mean probability of isolates to sporulate on berry surfaces at day 15 grouped by tissue of origin

Isolate	Origin tissue	Probability (logit scale)[a]	Probability (back-transformed)	Rank
W200	Wood	8.77	1.00	1
W27-5	Wood	6.61	1.00	2
W45-3-1	Wood	0.18	0.54	5
W86-2	Wood	-2.08	0.11	14
H77-1	Berries at harvest	1.11	0.75	3
H118-1	Berries at harvest	-0.01	0.50	6
H171-2	Berries at harvest	-0.39	0.40	7
H162-1	Berries at harvest	-0.85	0.30	8
H12-1	Berries at harvest	-1.36	0.20	10
H17-1	Berries at harvest	-1.45	0.19	11
H171-1	Berries at harvest	-1.65	0.16	13
H33-1	Berries at harvest	-2.31	0.09	17
H64-1	Berries at harvest	-2.44	0.08	18
B114-2	Dormant bud	0.38	0.59	4
B8-3	Dormant bud	-0.97	0.27	9
B98-3	Dormant bud	-1.67	0.16	12
B146-3	Dormant bud	-2.23	0.10	15
B178-1	Dormant bud	-2.22	0.10	16
Control	-	-9.45	0.00	19

[a] Standard error of differences on logit scale: 10.32 (average), 53.70 (maximum) and 0.58 (minimum; degrees of freedom=70.2.

also be a difference in virulence for different host cultivars between isolates.

It was important for the current study to keep the extent of isolates used in the pathogenicity tests limited to the same origin vineyards, vines and tissue to avoid introducing extra factors which could contribute to differences in pathogenicity, such as climatic origin differences if including isolates from other sources. This however, limited the number of isolates available per species for some of the less frequently found species in this region. This is often the case in small collections derived from newly established research areas such as the current study and has been a limitation to previous Botryosphaeriaceae pathogenicity studies (Lazzizera *et al*. 2008; Inderbitzin *et al*. 2010; Moral *et al*. 2010).

We agree with Larignon (2001) and van Niekerk *et al*.'s (2004) suggestion to homogenise pathogenicity test for Botryosphaeriaceae and suggest from our current observations that further pathogenicity tests should include not only a large number of isolates per species but also a variety of host cultivars and a number of grapevine tissues.

Since the differences in virulence data for different isolates may be due to more than just the species component, we agree with van Niekerk *et al*. (2004) and Larignon *et al*. (2001) that a better way to express the pathogenicity of Botryosphaeriaceae in grapevine would be to establish virulence groups for isolates within species. Future research could examine the genotypes responsible for the groupings. We recommend caution for any further pathogenicity studies on Botryosphaeriaceae due to the large differences within species that exists for this family.

Inoculation of dormant buds in the glasshouse and vineyard resulted in some successful re-isolations of the Botryosphaeriaceae at later growing stages, however, did not affect bud burst and survival and shoot and bunch development. This suggests that Botryosphaeriaceae conidia landing on the surfaces of dormant buds can germinate and infect the bud and spread internally into shoots and bunches, however, the success rate of infection is very low and that of survival and internal upward spread even lower. This contrasts with observations in apples where an infection during early bud development can lead to fruit rot (Beisel *et al*. 1984; Taylor, 1955).

The results of dormant bud inoculations in glasshouse and vineyard are unexpected after Phillips (1998) showed that *B. dothidea* caused bud mortality in *V. vinifera* inoculation studies. The different outcomes of both studies might be explained by different species of the inoculum; however, it is more likely that the difference in inoculation technique caused this discrepancy. While Phillips (1998) opened up the dormant bud to apply the inoculum between bud scale and stipule, our study followed less damaging techniques of inoculating onto the bud surface. The discrepancies between the two studies therefore provide evidence to suggest that a wound increases the likelihood of infection of buds. One would therefore expect to see Botryosphaeriaceae infection of buds more frequently in vineyards where bud damage has occurred, possibly due to insects and other environmental factors. The reports of reduced bud burst by Qiu *et al* (2011) associated with Botryosphaeria canker in vineyards also stands in contrast with the current findings of our bud inoculations. The cuttings and grapevines in our study were young, healthy and free of Botryosphaeriaceae, while the reduced bud burst reported by Qiu *et al*. (2011) occurred in a vineyard of relatively older vines with established Botryosphaeria canker disease. This may have contributed to an overall decline of the infected vines including reduced bud burst.

Future work should therefore include direct comparison of inoculations on damaged and undamaged dormant buds on grapevines of different ages as well as with and without Botryosphaeriaceae infection in the wood.

The results of our study provide evidence that Botryosphaeriaceae are not tissue specific in *V. vinifera* and can infect wood, mature berries and dormant buds. It suggests that pycnidia formation as it is seen on infected wood and the fruit of other hosts is likely to appear on infected berries within a couple of weeks after conidia landing on the berry surface, thus making berries inoculum sources for the wood and for other bunches under favourable weather conditions. This highlights the importance of monitoring weather and berry symptoms particularly after veraison.

Although the effect of infected flower and unripe berries on further bunch development and spread throughout the vines still have to be ascertained there is enough evidence to suggest Botryosphaeriaceae needs to be controlled throughout the entire growing season. We therefore recommend considering Botryosphaeriaceae in Australian vineyards as both trunk disease pathogens and bunch rot pathogens and adjust control methods accordingly. The implications

differ from many other bunch rot pathogens because under the right conditions infected wood provides a constant inoculum source and these infections cannot currently be controlled by fungicides generally used to control other bunch rot pathogens.

Experiment 2.14
AFLP analysis of Botryosphaeriaceae

Materials and methods

Fungal isolates and DNA extractions for AFLP analysis

Botryosphaeriaceae isolates (n=178) (Wunderlich *et al*. 2011a) from two vineyards in the lower Hunter Valley which are approximately 6 km apart from each other were assessed (*Table 2.29*). Three PDA plugs per isolate were transferred from actively growing cultures to 125 mL conical flasks containing 50 mL DifcoTM PDB and were incubated at 25°C and 90 rpm in a Sartorius Certomat BS-1 orbital shaker. Mycelia were harvested after 7 days, initially dried by filtration, freeze-dried in a Christ Gamma 1-16LSC freeze-dryer for 24 hours and then homogenised with a tissue lyser (Qiagen). DNA was extracted using the DNeasy Plant Maxi Kit (Qiagen) according to the manufacturer's instructions. Concentrations of the genomic DNA was checked for each sample using a NanoDrop 2000 (Thermo Scientific, Wilmington, USA) and adjusted to yield 250 ng DNA in ≤18 µL.

AFLP analysis

This was performed according to the protocol described by Vos *et al*. (1995) with some modifications, using the AFLP® analysis system for microorganism (Invitrogen, Mulgrave Australia). Genomic DNA from each isolate was digested with the restriction enzymes *Eco*RI and *Mse*I. Following ligation of the genomic DNA fragments to *Eco*RI and *Mse*I adapters, pre-amplification of a 1:10 dilution of each template was carried out using non-selective AFLP primers E+0 and M+0. Each 12.75 µL reaction contained 1.25 µL template, 1-25 µL 10× PCR buffer (Invitrogen), pre-amp primer mix containing dNTPs and primers, 1.25 U *Taq* DNA polymerase (Taq Platinum, Invitrogen), and 15 mM $MgCl_2$. Amplifications were conducted in a GeneAmp PCR system 2700 thermocycler (Applied Biosystems, Foster City, USA) with 20 cycles at 94°C for 30 s, 56°C for 1 min and 72°C for 1 min. The amplified products were diluted in 10 mM Tris-HCL buffer (pH 8.0) containing 0.1 mM EDTA.

This optional pre-amplification step was followed by a selective amplification step using a series of different primer combinations containing one un-labelled *Mse*I primer and a 5' fluorescently labelled *Eco*R1 primer. Primer combinations for the final selective amplifications step were chosen after an initial screening of *Eco*RI/*Mse*I primer combinations on a subset of the eight phenotypically most diverse isolates (data not shown). Polymorphism for this subset with all 24 primer pairs were estimated using GenAlX (Peakall and Smouse 2006). The four sets of AFLP primer pairs which showed highest polymorphism (data not shown) were *Mse*I (M) primer MC in combinations with each of the *Eco*RI (E) -primers EA, EAA, EAC and EG. The E-primers were 5' fluorescently labelled with D4 WellRED™ dye (Beckman Coulter, Gladsville, Australia) by the supplier (Sigma-Aldrich). Each 10 µL selective amplification reactions contained 2.5 µL of a 1:50 dilution of the pre-amplification template, 1× PCR buffer (Invitrogen), pre-amp primer mix containing dNTPs and primers,, 0.5 U *Taq* DNA polymerase (Taq Platinum, Invitrogen), 2.25 µL of *Mse1*-T primer and 0.25 µL E-primer. PCR reaction was one initial cycle at 94°C for 30 s, 65°C for 30 s and 72°C for 1 min, followed by 12 cycles of the same conditions but with a 0.7°C decrease in annealing temperature in each consecutive cycle. After this 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s were performed. PCR products were stored at 4°C until used for capillary electrophoresis. One microlitre of each selective amplification product was added to 28.6 µL sample loading solution and 0.4 µL of 600 base pair (bp) DNA size standard labelled with D1 WellRED™ dye. Sample plates were loaded into the CEQ Genetic Analysis System 8000 (Beckmann Coulter) and samples denatured and separated at 4.8 kV over 60 min. AFLP fragments were separated and polymorphic banding patterns detected using a CEQ Genetic Analysis System 8000.

AFLP fragment bands between 60–600 bp lengths were scored using the program GenomeLab™ GeXP Version 10.2, as present (1) or absent (0) in each accession creating a binary matrix for each isolate/primer combination. Binary data was χ-square transformed and isolates analysed for their genetic distances with the software Primer v6 (Clarke and Gorley 2006) by first calculating the Eucledian distances between each of the isolates. All isolates were

Table 2.29 Species identities, origin vineyard, origin plant and origin host tissue types of isolates used in the AFLP analysis.

Origin			
Plant no.	Tissue type	Isolate	Species
Origin vineyard A			
3	Wood	W3-2	*Diplodia seriata*
6	Berries at harvest	H6	*Neofusicoccum parvum*
6	Dormant buds	BB6	unidentified
8	Dormant buds	B8-8	*N. parvum*
8	Dormant buds	B8	*D. seriata*
9	Dormant buds	BB9	*D. seriata*
11	Dormant buds	BB11-2	*N. parvum*
11	Dormant buds	BB11	*D. seriata*
12	Wood	W12	*D. seriata*
12	Berries at harvest	H12	*N. luteum*
12	Dormant buds	BB12	*D. seriata*
13	Berries at harvest	H13	*D. seriata*
13	Dormant buds	BB13-2	*D. seriata*
13	Dormant buds	BB131	*D. seriata*
14	Dormant buds	BB14	*D. seriata*
15	Dormant buds	BB15-2	*D. seriata*
16	Dormant buds	BB16-2	*D. seriata*
16	Dormant buds	BB16	*D. seriata*
16	Dormant buds	B16-3	*D. seriata*
17	Berries at harvest	H17	*D. seriata*
18	Wood	W18-2	unidentified
18	Berries at harvest	H18-2	*D. seriata*
18	Berries at harvest	H18	*D. seriata*
18	Dormant buds	BB18	*D. seriata*
23	Dormant buds	BB23	*D. seriata*
26	Dormant buds	BB26	*D. seriata*
26	Wood	W26	*Botryospaheria dothidea*
27	Wood	W27-5	*N. parvum*
28	Dormant buds	BB28-1	*D. seriata*
29	Dormant buds	BB29	*N. luteum*
32	Wood	W32-4	unidentified
33	Berries at harvest	H33	*D. seriata*
33	Dormant buds	BB33-2	*D. seriata*
36	Wood	W36-5	unidentified
36	Wood	W36	unidentified
36	Dormant buds	BB36-3	*D. seriata*
36	Dormant buds	BB36-2	*D. seriata*
42	Wood	W42-3	*D. seriata*
42	Wood	W42-5	unidentified
43	Dormant buds	BB43-2	*D. seriata*
43	Dormant buds	BB43	*D. seriata*
44	Dormant buds	BB44-2	*D. seriata*
45	Wood	W45-4	unidentified
45	Wood	W45-3-2	*Neofusicoccum ribis*
45	Wood	W45-3	*N. parvum*
45	Wood	W45	*N. parvum*
47	Wood	W47-5	*D. seriata*
47	Wood	W47-3	*D. seriata*
47	Wood	W47	*D. seriata*
54	Wood	W54-4	unidentified
55	Wood	W55-4	*D. seriata*
55	Wood	W55-3-2	*D. seriata*
57	Dormant buds	BB57	*D. seriata*
59	Dormant buds	BB59-3	*Neofusicoccum australe*
60	Wood	W60-2	unidentified
60	Dormant buds	BB60-2	*D. seriata*
61	Wood	W61-2	*D. seriata*
63	Wood	W63-3	*D. seriata*
63	Wood	W63	*N. parvum*
64	Wood	W64-3	unidentified
64	Berries at harvest	H64	*D. seriata*
70	Berries at harvest	HH70	*D. seriata*
72	Wood	W72-2	*D. seriata*
73	Berries at harvest	H73	*N. ribis*
74	Dormant buds	BB74	*D. seriata*
76	Dormant buds	BB76	*D. seriata*
77	Berries at harvest	HH77	unidentified
77	Berries at harvest	H77	*N. parvum*
82	Berries at harvest	HH82	*D. seriata*
83	Wood	W83-6	*D. seriata*
87	Wood	W87-2	*D. seriata*
88	Wood	W88-4	*N. parvum*
88	Wood	W88-3	*D. seriata*
90	Wood	W90	*D. seriata*
91	Dormant buds	BB91	*D. seriata*
93	Wood	W93-2	*N. parvum*
94	Dormant buds	BB94	*D. seriata*
95	Wood	W95-2	unidentified
95	Wood	W95	unidentified
95	Berries at harvest	HH95	*D. seriata*
96	Wood	W96-3	*B. dothidea*
98	Dormant buds	B98-3	*D. seriata*
Origin vineyard B			
103	Wood	W103	*N. parvum*
103	Dormant buds	BB103	*N. parvum*
105	Dormant buds	BB105	*D. seriata*
112	Wood	W112-4	*D. seriata*
112	Berries at harvest	H112	*N. parvum*
112	Dormant buds	BB112	*D. seriata*
113	Wood	W113-3	*D. seriata*
114	Dormant buds	B114-3	*N. parvum*
114	Dormant buds	B114-2	*N. parvum*
114	Dormant buds	B114	*N. parvum*
117	Wood	W117	*D. seriata*
118	Pea size berries	PP118	*D. seriata*

Origin			
Plant no.	Tissue type	Isolate	Species
118	Berries at harvest	H118-2	unidentified
118	Berries at harvest	H118	*D. seriata*
118	Dormant buds	BB118	*D. seriata*
119	Berries at harvest	HH119	*N. luteum*
119	Dormant buds	BB119-2	*D. seriata*
119	Dormant buds	BB119	*D. seriata*
122	Dormant buds	BB122	*D. seriata*
123	Dormant buds	B123-3	*N. parvum*
125	Wood	W125-2	*D. seriata*
125	Dormant buds	BB125	*D. seriata*
126	Wood	W126-5	*B. dothidea*
126	Wood	W126	*D. seriata*
126	Berries at harvest	H126	*D. seriata*
126	Dormant buds	BB126	*D. seriata*
127	Dormant buds	BB127	*N. luteum*
130	Dormant buds	BB130	*D. seriata*
131	Wood	W131-3-2	*D. seriata*
132	Wood	W132	*D. seriata*
132	Wood	W132	*D. seriata*
133	Wood	W133-3	*D. seriata*
133	Dormant buds	BB133	*D. seriata*
134	Wood	W134-4	*D. seriata*
134	Dormant buds	BB134	*D. seriata*
136	Pea size berries	PP136	*D. seriata*
136	Dormant buds	BB136	*D. seriata*
137	Wood	W137	*D. seriata*
137	Dormant buds	B137	unidentified
138	Berries at harvest	H138	*N. parvum*
140	Dormant buds	BB140-2	*N. parvum*
141	Dormant buds	BB141	*D. seriata*
141	Wood	W141	*D. seriata*
146	Dormant buds	BB146-2	*D. seriata*
146	Dormant buds	B146-3	*Dothiorella viticola*
151	Berries at harvest	HH151	*N. parvum*
151	Flowers	FF151	*D. seriata*
151	Dormant buds	BB151	*N. parvum*
152	Wood	W152-4	*D. seriata*
152	Dormant buds	BB152	*B. dothidea*
153	Dormant buds	BB153-3	*D. seriata*
153	Dormant buds	BB153	*D. seriata*
157	Wood	W157	unidentified
158	Dormant buds	BB158-4	*B. dothidea*
161	Dormant buds	BB161	*D. seriata*
162	Berries at harvest	H162	*N. parvum*
163	Dormant buds	BB163	*B. dothidea*
164	Wood	W164-2	*D. seriata*
164	Wood	W164-4	*D. seriata*
164	Dormant buds	BB164-2	*D. seriata*
165	Wood	W165-4	*N. parvum*
165	Dormant buds	BB165-2	*N. parvum*

Origin			
Plant no.	Tissue type	Isolate	Species
166	Wood	W166	*N. parvum*
166	Wood	W166-5	*N. parvum*
171	Berries at harvest	H171-2	*B. dothidea*
173	Wood	W173	unidentified
173	Dormant buds	B173	*D. seriata*
175	Dormant buds	BB175-2	*N. luteum*
176	Wood	W176	*N. parvum*
177	Dormant buds	BB177	*D. seriata*
178	Dormant buds	BB178-3	*D. seriata*
178	Dormant buds	BB178	*B. dothidea*
178	Dormant buds	B178	*B. dothidea*
179	Dormant buds	BB179-3	*D. seriata*
182	Berries at harvest	HH182	*D. seriata*
182	Dormant buds	BB182	*N. parvum*
183	Berries at harvest	HH183	*N. parvum*
183	Wood	W183-4	*N. parvum*
184	Wood	W184-2	*D. seriata*
184	Wood	W184-4	unidentified
184	Wood	W184-3	*D. seriata*
186	Dormant buds	BB186-3	*D. seriata*
186	Dormant buds	BB186-2	unidentified
187	Berries at harvest	HH187	*N. parvum*
187	Dormant buds	BB187- 2	unidentified
192	Dormant buds	BB192	*N. luteum*
194	Flowers	FF194-5	*N. parvum*
195	Wood	W195-3	*N. parvum*
196	Wood	W196-2	*D. seriata*
196	Dormant buds	BB196-2	*D. seriata*
197	Wood	W197	unidentified
197	Berries at harvest	HH197-2	*D. seriata*
197	Berries at harvest	HH197	*N. luteum*
197	Flowers	FF197-2	*D. seriata*
197	Dormant buds	BB197-2	*D. seriata*
200	Wood	W200	*D. seriata*

grouped in a dendogram based on complete linkage of these distances. Principal Coordinate Analysis (PCA) was performed using Primer v6 (Clarke and Gorley 2006) to evaluate the genetic diversity between all isolates belonging to species *D. seriata* the most abundant species in our Botryosphaeriaceae population and for all those *D. seriata* isolates with origin plants which hosted more than one *D. seriata* isolate.

Results and discussion

The four polymorphic primer pairs amplified 1203 fragments within 178 accessions. On the basis of cluster analysis, we could not identify any genetic diversity separating the isolates according to species. While there were four groups within the isolates, each of these groups contained accessions from a variety of different species, except for group three, which contained five out of the nine *B. dothidea* isolates from our study. However, this group also contained one isolate of the *N. parvum*, the second most abundant species, which also has isolates scattered throughout each of the four groups.

We therefore focussed our genetic diversity investigation on isolates from the most abundant species group, *D. seriata*, and excluded isolates of all other species. PCA confirmed that while there appears to be patterns within the distribution of *D. seriata* isolates, none of the groups show any obvious relationship to isolate origin. *D. seriata* isolates neither grouped according to their origin tissue types or vineyard location, nor did they necessarily group according to their spatial distance in the vineyard indicated by origin plant. Some *D. seriata* isolated from the same grapevines appear to be closely related to each other. For example, all three *D. seriata* isolates (W47, W47-3 and W47-5) from grapevine number 47, which all originated from the same plant as well as same tissue type, the two isolates (BB119-2 and BB119) both originating from grapevine 119 and dormant buds and two out of the three *D. seriata* isolates of grapevine 126 (W126, H126 and BB126), isolated from three different tissue types (*Table 2.29*). However, the majority of our *D. seriata* isolates from grapevines yielding multiple isolates were more genetically diverse than isolates from different origin plants. For example this is the case for grapevine 118 with *D. seriata* isolates from dormant buds, pea-sized berries and berries at harvest as well as grapevine 197 with isolates from dormant buds, flowers and berries at harvest and grapevine 126 with isolates from wood, dormant buds and berries at harvest (*Table 2.29*). Grapevine 16 is an example of three *D. seriata* isolates (BB16, BB16-2, BB16-3) originating from the same plant and same origin tissue (*Table 2.29*), which are however more different to each other than to isolates from other plants.

Our study representing an extensive marker analysis of 1203 alleles has shown that our isolates are all diverse and cluster distinctly into four groups, however the groups were neither conforming according to any of the three origins: vineyard, grapevine or tissue types nor according to species identity. While our AFLP study in terms of species separation is limited to showing the Euclidean distance of our isolates, belonging to seven different species, the non-conformity according to species could indicate that the species concept within the Botryosphaeriaceae fungi is relatively homogenous and that there may be genetic exchange between isolates which have been classified as different species. This would be in agreement with other studies which have indicated that some of the Botryosphaeriaceae species, such as those belonging to the species with *Neofusicoccum* and *Dothiorella* anamorphs, have common ancestors and have led to phylogenetic restructuring of this genus (Crous *et al.* 2006; Phillips *et al.* 2005). Future research to resolve the genetic diversity of our isolates in terms of species indication would therefore require a more in depth study such as whole-genome sequencing or screening of additional markers. However, the aim was to study the population not for species identification but rather from an isolate origin point of view in order to identify how Botryosphaeriaceae isolates move between and within vineyards and grapevines.

The lack of grouping according to these origins indicates a constant influx into the vineyards from the outside environment and reveals some important information about the spread of Botryosphaeriaceae in *V. vinifera*. There was no obvious division in the genetic structure of the isolates from vineyard A compared to vineyard B, which indicates that both populations are relatively new and have a uniform infection originating from a constant, common source of inoculum. This must be a common source for both vineyards coming from outside the vineyards, such as the Botryosphaeriaceae population in surrounding native vegetations in the Hunter Valley, such as eucalyptus trees or other crops such as stone fruit trees. These plant species have shown to be hosts for Botryosphaeriaceae trunk disease pathogens (Burgess *et al.* 2006; Cunnington *et al.* 2007; Slippers *et al.*

2004*c*) in other parts of the country and gene flow from native vegetation to crops has been identified (Burgess *et al*. 2006).

The lack of grouping of isolates according to their origin vine supports the theory of external rather than internal vineyard infection further by showing that infection of a vine from isolates already on the plant (i.e. in the wood) is not more likely than the inoculum source originating from another grapevine. We therefore need to stress the idea of considering native vegetation as well as other woody crops surrounding vineyards, as potential inoculum sources for grapevines and therefore agree with Qiu *et al*. (2011) on the importance to extend vineyard surveys for trunk disease pathogens to the surrounding vegetation. While such vegetation if not a commercial crop cannot be controlled for trunk disease pathogens, it can be incorporated into preventative vineyard management strategies such as when choosing a location for new plantings. However, the constant influx of new inoculum from outside the vineyard can also be seen as an advantage, such as the unlikelihood of fungicide resistance build-up in an ever-changing population. Research on Botryosphaeriaceae spread within vineyards has been conducted considering the conditions favourable for conidia release and transport. However, there have not been any studies so far looking at the distance of spread of these pathogens. This could be a useful topic for future research.

We can further conclude from our results that Botryosphaeriaceae infection in the wood and vegetative/reproductive parts of our vines have not originated from an initial infection in the planting material/rootstock or cuttings. There appears to be a mixture of inoculum from outside and inside the vineyard, indicating that the majority of reproduction in this population is asexual. We have also indicated that within our populations horizontal gene transfer exists, with more than one different Botryosphaeriaceae isolate (same or different species) being able to co-exist on the same plant.

The results of this study complement those of our previous work (Wunderlich *et al*. 2011*a*; Wunderlich *et al*. 2011*b*) investigating the tissue specificity of Botryosphaeriaceae in *Vitis vinifera*. In these two studies, it was shown that Botryosphaeriaceae have the potential to infect a variety of *Vitis vinifera* tissue other than wood and independent from their origin grapevine tissue type. We can further conclude from our results showing the lack of grouping of isolates conforming to origin tissue types that Botryosphaeriaceae trunk disease pathogens appear to be not specialised to tissue types. Often a plant is infected in more than one location and various tissue types (Wunderlich *et al*. 2011*a*), however, we can conclude from the results of our genetic diversity study of multiple *D. seriata* isolates per grapevine compared to isolates of the same species from other grapevine plants, that the infection pathway into the reproductive tissues of the plant is not likely to have happened systemically and as a result of spread from the infected wood of these plants. *D. seriata* isolates from the same origin tissue originating from the same plant are also expected to be closer related to each other than to isolates from other plants, if they were the result of systemic infection. However, the opposite was observed with many isolates originating from one plant and the same tissue types showing greater similarity to isolates originating from a different plant than to each other. This could indicate that multiple infection events have occurred within the one plant rather than one infection event and a systemic spread.

Our study has shown some important aspects for the understanding of Botryosphaeriaceae infection pathways into the reproductive structures of grapevines and confirmed the lack of tissue specificity of these pathogens indicated through previous studies. Intra-and inter- vineyard movements of Botryosphaeriaceae isolates have been investigated and highlighted the need to study the role of the surrounding vegetation as an inoculum source for Botryosphaeriaceae infection of grapevines.

Experiment 2.15
Evaluation of fungicides for the management of Bot canker of grapevines

Materials and methods

Fungal isolates, fungicides and in-vitro studies

Nineteen technical grade fungicides currently registered for use on grapevines (*Table 2.30*), and a preparation of boric acid previously shown to inhibit mycelial growth of *E. lata* (Rolshausen and Gubler 2005), were selected via an exhaustive search of the literature and screened in-vitro for their capacity to inhibit mycelial proliferation of four species of '*Botryosphaeria*', previously isolated from declining grapevines in the Hunter Valley and Mudgee regions of northern NSW (Qiu *et al.* 2011). Three isolates each of *Diplodia seriata* ('*Botryosphaeria*' *obtusa* isolates A142a, F411a and J25a) and *Neofusicoccum parvum* ('*B*'. *parva* isolates A142La, E126 and H451a), and one each of *Lasiodiplodia theobromae* (*B. dothidea* isolate B31c) and *Fusicoccum aesculi* (*B. rhodina* isolate G31a) were cultured on PDA for 3–4 days at 25°C, before being transferred to fungicide-amended-agar. Fungicides (technical grade) were suspended in 1 mL of acetone before being added to molten PDA (50°C). With the exception of fludioxonil, carbendazim, fluazinam and flusilazole, which were tested at concentrations; 0 (control), 0.01, 0.05, 0.1, 0.5, 1.0 and 2.5 mg L^{-1}, all fungicides were assessed at concentrations; 0 (control), 0.1, 0.5, 1.0, 2.5, 5.0, 10.0 mg L^{-1}. Boric acid (~17.5% boron) was dissolved in water prior to be applied to PDA at concentrations; 0 (control), 100, 200, 300, 400, 500 and 1000 mg L^{-1}, while pyrimethanil was tested on Czapex-Dox (Oxoid)-amended-agar. Fifteen millilitre aliquots of fungicide-amended-agar were then dispensed into 90 mm petri-dishes, and 5 mm diameter plugs removed from the centres of each plate. Five millimetre-diameter agar plugs from the margins of actively growing 3–4 day-old fungal cultures were then transferred to each of three (3) replicate plates per isolate×fungicide×concentration combination and the plates sealed and incubated for 2–3 days at 25°C. Controls consisted of PDA without the addition of fungicide, and mycelial growth was recorded by measuring the radial growth of fungal colonies along two perpendicular axes at a point when colony diameters of control plates approached 75% colonisation. The experiment was completely randomised with a 20×8×7 factorial and three replications. The factors were 20 fungicides, 8 isolates, and seven concentrations, and the experiment was repeated twice.

The mean colony diameters for each isolate and fungicide concentration were calculated and the percent growth rate inhibition relative to the control determined. Percentage inhibition data were fitted over fungicide concentrations for each isolate and fungicide separately using log-probit regression. Data were normalised by logarithmic transformation and EC_{50} values (concentration of fungicide at which 50% of mycelial growth is inhibited) calculated. Differences in treatment effects (fungicides) were then investigated by analysis of variance (ANOVA), and means separated using Duncan's multiple range test (DMRT; P=0.01) (GenStat, Rothamsted Experimental Station, UK). Means were back-transformed to the original scale. A Bartlett's test was conducted to test for homogeneity of variance between the two trials.

Field trials

Between 2007 and 2010, field trials were established in the Hunter Valley and in the Barossa Valley to assess a range of fungicides (*Table 2.30*) as potential pruning wound protectants against *Diplodia seriata*, the most abundant species in this region (Qiu *et al.* 2011) and *D. mutila*. Fungicides were tested at label rate (1×) and 10× label rate, whilst Vinevax™ (spray-on formulation 10 g L^{-1}) and BacSeal were used according to the manufacturer's recommendations.

Pruning wounds (10 per vine) were made in mid-July on one-year-old canes of 20 year old Semillon and treatments applied liberally with a paintbrush within two hours of wounding. Controls; inoculated (I) and non-inoculated (NI), were treated with sterile distilled water (SDW). Wounds were then inoculated with 10 µL of a 1.0×10^{6} mL^{-1} spore suspension of *D. seriata* (isolate A142) or *D. mutila* using a pipette. The experiment was conducted as a random complete block (RCB) with 20 treatments (fungicides) and 10 replicates (blocks). Each treatment occurred once in each block and consisted of a single vine comprising 10 wound/spurs. To determine the incidence of natural inoculum in the trials, some vines were treated with water but not inoculated.

Treated canes were removed after one year and the presence or absence of the inoculated fungi determined by isolating the fungus from detached spurs. Bark was removed from each spur using a sharp knife, and the exposed wood surface sterilised by flaming. Wood chips (2×2×3 mm) were then

Table 2.30 Treatments evaluated to control Bot canker in-vitro and on grapevines in the field.

Treatment	Active ingredient	Application rate[a] (per L)	Contact/ systemic	Chemical group	Manufacturer
ATCS tree wound dressing[c]	Acrylic paint	n/a	n/a	n/a	Hortitape
BacSeal Super[c]	Tebuconazole (10 g/L)	n/a	S	Sterol biosynthesis inhibitors (C)	Bayer New Zealand Ltd.
Bavistin[bc]	Carbendazim (500 g/L)	1 mL	S	Benzimidazoles (A)	BASF Australia Ltd.
Bayfidan[b]	Triadimenol (250 g/L)	n/a	S	Sterol biosynthesis Inhibitors (C)	Bayer Crop Science Pty. Ltd.
Boric acid[b]	Boron (99.5%)	n/a	n/a	n/a	Sigma-Aldrich
Bravo[b]	Chlorothalonil (500 g/L)	n/a	C	Non-Specific inhibitors (Y)	Syngenta Crop Protection Ltd.
Cabrio[b]	Pyraclostrobin (250 g/L)	n/a	C	Strobilurins (K)	Nufarm Australia Ltd.
Domark[c]	Tetraconazole (40 g/L)	0.3 mL	S	Sterol biosynthesis Inhibitors (C)	Sipcam Pacific Australia
Filan[b]	Boscalid (500 g/kg)	n/a	S	Anilide (G)	Nufarm Australia Ltd.
Folicur[bc]	Tebuconazole (430 g/L)	0.3 mL	S	Sterol biosynthesis Inhibitors (C)	Bayer Crop Science Pty. Ltd.
Garrison[c]	Cyproconazole (2.5 g/L)/ Iodocarb (1 g/L)	n/a	S	Sterol biosynthesis Inhibitors (C)/ Benzimidazoles (A)	Chemcolour Industries Ltd.
Legend[b]	Quinoxyfen (250 g/L)	n/a	C	Phenoxy quinolines (M)	Dow AgroSciences Australia Ltd.
Mycloss™ Xtra[bc]	Myclobutanil (200 g/L)	0.16 mL	S	Sterol biosynthesis Inhibitors (C)	Dow AgroSciences Australia Ltd.
Nustar[bc]	Flusilazole (200 g/kg)	0.1 g	S	Sterol biosynthesis Inhibitors (C)	DuPont Australia Ltd.
Prosper[b]	Spiroxamine (500 g/L)	n/a	S	Morpholines (E)	Bayer Crop Science Pty. Ltd.
Rovral Liquid[bc]	Iprodione (250 g/L)	2 mL	C	Dicarboximides (B)	Bayer Crop Science Pty. Ltd.
Rubigan[b]	Fenarimol (120 g/L)	n/a	S	Sterol biosynthesis Inhibitors (C)	DuPont Australia Ltd.
Scala[b]	Pyrimethanil (400 g/L)	n/a	C	Anilino-pyrimidine (I)	Bayer Crop Science Pty. Ltd.
Shirlan[bc]	Fluazinam (500 g/L)	1 mL	C	Non-specific inhibitors (Y)	Crop Care Australasia Pty. Ltd.
Sumisclex[b]	Procymidone (500 g/L)	n/a	C	Dicarboximides (B)	Sumitomo Chemical Australia Pty. Ltd.
Switch[bc]	Cyprodinil (375 g/kg)/ Fludioxonil (250 g/kg)	0.8 g	S/C	Anilinopyrimidines (I)/ Phenyl pyrroles (L)	Syngenta Crop Protection Ltd.
Teldor[b]	Fenhexamid (500 g/L)	n/a	C	Hydroxyanilides (J)	Bayer Crop Science Pty. Ltd.
Topas[bc]	Penconazole (100 g/L)	0.125 mL	S	Sterol biosynthesis inhibitors (C)	Syngenta Crop Protection Ltd.
Vinevax prune wound dressing[c]	Trichoderma spp. (5.0 × 108 cfu/g)	10 g/100 g	n/a	n/a	Agrimm Technologies Ltd.

[a] Application rates specified for grapevines in Australia
[b] Treatments assessed in-vitro
[c] Treatments assessed in the field
n/a not applicable

cut from each side of the margin between live and dead wood tissue, and five plated onto each of two PDA plates per spur. Plates were incubated at 25°C for 3–5 days and then assessed morphologically for the presence or absence of *Botryosphaeria*. Efficacy was based on the mean percent recovery (MPR) of Botryosphaeriaceae spp. from the treated canes. Mean percent disease control (MPDC) was calculated as the reduction in MPR as a proportion of the inoculated control.

Results and discussion

In-vitro studies

Fludioxonil, carbendazim, fluazinam, tebuconazole, flusilazole, penconazole, procymidone and iprodione were most effective fungicides tested in this study, with EC50 values of 0.007, 0.017, 0.036, 0.054, 0.068, 0.127, 0.230 and 0.304 mg L^{-1}, respectively. Myclobutanil, pyraclostrobin, fenarimol and chlorothalonil were intermediately effective. However, the anilino-pyrimidine, anilide, and morpholine fungicides, pyrimethanil and cyprodonil, boscalid and spiroxamine were largely ineffective at the range of concentrations tested. Boric acid, fenhexamid and quinoxyfen failed to inhibit mycelial growth of *Botryosphaeria* spp. and were subsequently omitted from the analysis. The analysis of variance of EC50 values revealed similar results for the two in-vitro trails (P=0.294), and the variances between the two trials were homogeneous according to Bartlett's test for homogeneity of variances (P=0.851), hence data were pooled. EC50 values for in-vitro inhibition of mycelial growth were significantly affected by both fungicide and isolate (P=0.01), and there was a significant interaction between the two (P=0.01). However, differences in mean EC50 values of individual isolates were significant between species, but not within species, wherein each of the three isolates both of *B. parva* and *B. obtusa* behaved similarly. On average *Botryosphaeria parva* and *B. dothidea* were the most sensitive to the fungicides, whilst *B. obtusa* and *B. rhodina* were the least sensitive. Nonetheless, individual isolates reacted differently to different fungicides regardless of species. Of the eight most effective agents trialled in this study, more than half were group B or C fungicides, which suggested that *Botryosphaeria* spp. are relatively sensitive to the dicarboximides and sterol-biosynthesis inhibitors, both of which function at the membrane level. Also, half of the most effective chemicals were contact or protective fungicides, indicating that the contact fungicides were equally effective at inhibiting mycelial proliferation of *Botryosphaeria* spp. *in-vitro* as their systemic counterparts.

Field trials

In the Hunter Valley, Bavistin, Folicur, Garrison, Rovral, Shirlan, Switch and Vinevax significantly reduced the MPR of *D. seriata* from inoculated canes, providing between 12-43% control of the pathogen (*Table 2.31*). However, Folicur, Shirlan and Switch were only effective when applied at rates exceeding that recommended by the manufacturer.

In the Barossa Valley, ATCS tree wound dressing, Bacseal, Bavistin, Folicur, Garrison, Nustar, Shirlan and Switch applied at label rates, reduced the MPR of *D. mutila*, providing between 32-65% control (*Table 2.31*).

In contrast to *D. mutila*, recovered from 33% of un-inoculated controls, *D. seriata* arose from 63% of un-inoculated canes. Low levels of control encountered in the Hunter Valley may be due to the level of background infection at this location. The greater prevalence of *D. seriata* in the environment (Pitt *et al.* 2010*a*), proximity of alternate hosts and differences in climate between the regions may also influence the results.

Fungicides containing the sterol-biosynthesis inhibitors tebuconazole and cyproconazole, and the benzimidazole fungicide carbendazim, proved to be the most effective protectants of pruning wounds. Consequently, these products were also the most effective when evaluated for control of eutypa dieback (Sosnowski *et al*. 2008).

Experiment 2.16
Spore trapping of wood-inhabiting fungi

Materials and methods

Spore trapping using glass slides

The method was adapted from Eskalen and Gubler (2001).

The trial commenced in August 2009 and was terminated in August in 2011. Shiraz vines in a vineyard in the Hunter Valley were used for this study. Glass microscope slides were coated with petroleum jelly and placed onto the cordons of five randomly selected vines displaying symptoms of Bot canker. At each time point six glass slides per vine (three on each cordon) were attached with wire onto five randomly selected vines displaying symptoms of Bot canker (*Figure 2.22*). Slides were placed at least 1–2 cm away from the surface of the cordon. Of the slides, three had the petroleum jelly on the top surface (facing the sky) and three had the petroleum jelly on the bottom surface (facing the cordon). Slides were collected weekly by placing them directly into 50 ml Falcon tubes. To each tube 10 ml of sterile water was added and gently agitated by hand to wash the spores off the slides. The water was passed through a 5 µm filter followed by a 0.45 µm filter. The filter was then washed with 1 ml of sterile distilled water and 200 µl was pipette onto PDA amended with 25 µg mL^{-1} streptomycin. The water was spread evenly over the plate using a sterile plastic spreader. Each plate was incubated at 25°C and monitored for the growth of

Table 2.31 Efficacy of fungicides and physical barriers when applied 1 day before inoculation with Botryosphaeriaceae in the Hunter Valley and Barossa Valley.

			Hunter Valley		Barossa Valley	
Treatment	**Active ingredient**	**Field application rate† (/L)**	**MPR$^{\Phi}$**	**MPDC**	**MPR$^{\Phi}$**	**MPDC**
ATCS tree wound dressing	Acrylic paint	paint n/a§	-	-	37.2 bcd	46.5
BacSeal Super	tebuconazole (10 g/L)	paint n/a	75.4 bcde	6.5	43.0 bc	38.2
Bavistin	carbendazim (500 g/L)	1 mL	58.8 hi	27.1	41.0 bcd	41.1
		10 mL¶	54.4 ij	32.6	-	-
Domark	tetraconazole (40 g/L)	0.3 mL	-	-	64.2 a	7.7
Folicur	tebuconazole (430 g/L)	0.3 mL	71.6 cdefgh	11.2	32.1 cde	53.9
		3 mL¶	59.1 hi	26.7	-	-
Garrison	cyproconazole (2.5 g/L)/ lodocarb (1 g/L)	paint n/a	46.4 j	42.5	24.4 e	64.9
MyclossXtra	myclobutanil (200 g/L)	0.16 mL	85.9 a	0	-	-
		1.6 mL	82.8 ab	0	-	-
Nustar	flusilazole (200 g/kg)	0.1g	84.7 ab	0	47.2 bc	32.2
		1 g¶	75.7 bcde	6.1	-	-
Rovral Liquid	iprodione (250 g/L)	2 mL	70.7 defgh	12.3	-	-
		20 mL	65.4 fgh	18.9	-	-
Shirlan	fluazinam (500 g/L)	1 mL	83.4 ab	0	30.8 de	55.7
		10 mL¶	69.6 defg	13.7	-	-
Switch	cyprodinil (375 g/kg)/ fludioxonil (250 g/kg)	0.8 g	74.9 bcdef	7.1	47.6 bc	31.6
		8 g¶	65.8 efgh	18.4	-	-
Topas	penconazole (100 g/L)	0.125 mL	78.9 abcd	2.1	65.9 a	5.3
		1.25 mL¶	80.8 abc	0	-	-
Vinevax prune wound dressing	*Trichoderma* sp. (5.0×10^8 cfu/g)	spray 10g/L	69.8 defg	13.4	-	-
		paste 100g/L	63.2 ghi	21.7	-	-
Non-inoculated control	-	n/a	63.0 ghi	-	32.8 cde	-
Inoculated control	-	n/a	80.6 abc	-	69.6 a	-

† Efficacy based on the MPR of Botryosphaeriaceae from treated canes by isolation on PDA. MPDC was calculated as the reduction in MPR as a proportion of the inoculated control. MPDC, mean percent disease control; MPR, mean percent recovery.

¶ 10 times label rate

§ n/a, not applicable

$^{\Phi}$ Values within a column with the same letter are not significantly different (P=0.05).



Figure 2.22 Spore trapping methods using glass microscope slides (top) and gutters (bottom).

fungi for 10 days. Fungi of interest were subcultured onto fresh PDA plates when necessary. The number of colonies produced by each organism were recorded after 10 days incubation. Daily temperatures, humidity and rainfall were downloaded from the Australian Government Bureau of Meterology website for Cessnock Airport.

Spore trapping using PVC guttering

This method was adapted from Sutton (1981).

The trial site was as described above for the glass slide trapping method. A 1 m length of gutter with a funnel and collection bottle attached at one end was placed under a cordon at a slight incline to facilitate water runoff into the bottle (Figure 2.22). The gutter was attached to the cordon or wire with plastic ties. At weekly intervals a 1 or 5 ml sample was filtered through a 5 µm filter and then a 0.45 µm filter. If too much water was collected the spores were concentrated by centrifuging the water in 50 ml falcon tubes and then carefully decanting the water. This was repeated until 1–5 ml of water remained. The filter was washed with 1 ml of sterile water and analysed as described above for the glass slide method.

Results and discussion

The spore trapping trials were completed in August 2011 whilst the chief investigator, Dr Sandra Savocchia was on maternity leave, therefore the data collected is yet to be fully analysed and will not be presented in this report. In brief, Botryosphaeriaceae, *Greeneria uvicola*, *Phaeomoniella* spp., Diatrypaceae and *Phomopsis* spp. were detected using the glass slide method. However, only Botryosphaeriaceae and *Phomopsis* spp. were detected using the gutter method. The gutter trap method was no longer used in 2010–11 due to the low detection of organisms. In addition, a large amount of debris was collected in the gutter traps making filtration difficult. Only the glass slide method was used in 2010-11. There appears to be a correlation with the detection of Botryosphaeriaceae and other organisms following rainfall. We hypothesise that humidity and temperature also play a role in the release of Botryosphaeriaceae. In a South African vineyard, spores of *E. lata*, *Phomopsis* and Botryosphaeriaceae species were trapped during or after periods of rainfall and/or high relative humidity (van Niekerk *et al.* 2010). Similarly, release of Botryosphaeriaceae spores was highly correlated with precipitation and irrigation events in California vineyards (Úrbez-Torres at al. 2010).The authors of this study suggest delaying the time of pruning in California to when spore release is lowest.

Following analysis of the data we hope to make some preliminary recommendations on when spores of Botryosphaeriaceae and other trunk disease pathogens are likely to be dispersed. This information will then be used to inform timing of pruning and application of fungicides to manage these diseases.

Experiment 2.17
Spatial variability of soil salinity and grapevine dieback in Chardonnay and Shiraz

Materials and methods

A survey of soil salinity and grapevine dieback was conducted in a vineyard planted to non-grafted Chardonnay and Shiraz in the Hunter Valley. Individual vines were scored according to the extent of dieback in the trunk and arms. A range of fungi were isolated from vines showing dieback symptoms. Soil electrical conductivity (EC) was assessed using EM38. Lateral variability of soil salinity in the vineyard was mapped using an EM38 electromagnetic sensor.

Results and discussion

Stress due to biotic or abiotic factors often play a crucial role in the manifestation of disease symptoms. An endophytic pathogen in healthy tissue can induce symptoms of the disease when its host is physiologically weakened by stress. Soil salinity is a major factor limiting vineyard performance and it could be a contributing factor in the dieback of grapevines. We hypothesized that spatial soil variability might influence vine health and predispose them to trunk disease pathogens. The objectives were to assess the spatial variability of soil, vine health and grapevine dieback based on spatial correlations. A range of fungi were isolated from vines showing dieback symptoms. Of the 119 wood samples collected, the genera isolated were *Botryosphaeria*, *Epicoccum*, *Pestalotia*, *Alternaria*, *Greeneria*, *Phaeomoniella*, *Phaeoacremonium*, *Aureobasidium*, *Phellinus* and *Phomopsis*. The dominant genera isolated were *Alternaria* (43%), *Epicoccum* (41%), *Botryosphaeria* (36%) and *Phaeoacremonium* (14%) with others being isolated infrequently (1-10%). Grapevine dieback was not strongly associated with soil salinity levels. This aspect of the project was not pursued further because of the lack of correlation between vineyard soil type and grapevine trunk disease incidence.

Young vine decline

Experiment 2.18
Co-infection by *Botryosphaeria* and *Cylindrocarpon* spp. at different stages during propagation

Many grapegrowers in Australia's Riverina wine region have been reporting disease in their newly planted vines. The buds of many do not burst and others die soon after planting. Surviving plants are developmentally retarded during the first five seasons after planting and often die within a few years. Typically, declining plants had been grafted. Their shoot growth is retarded and fruiting is low in comparison with healthy neighbouring plants. They have low root density, few feeder roots and black lesions on the roots, and tend to develop new shoots from the trunk above the graft union. The syndrome occurs in the absence of other common debilitating conditions of young grapevines such as water stress, water logging, nutrient deficiency and parasitic nematodes. Significantly, it has not been confined to particular soil types.

This syndrome was described as young vine decline (YVD). Similar symptoms have been reported, from many regions, worldwide, and fungal pathogens have been shown to be responsible, although the causative species vary considerably between regions. The pathogens reported to be responsible are: in Argentina *Phaeoacremonium* spp. and '*Botryodiplodia*' (Gatica *et al.* 2001); in Australia, *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* (Edwards and Pascoe 2004); in Brazil *Cylindrocarpon destructans* (Garrido *et al.* 2004); in California, *Cylindrocarpon* spp. (Scheck *et al.* 1998; Petit and Gubler 2005, 2007); in Chile, *Cylindrocarpon* spp. (Auger *et al.* 2007); in France, *Cylindrocarpon destructans*, (Maluta and Larignon, 1991); in Greece, *P. chlamydospora*, *Phaeoacremonium* spp., *Botryosphaeria* spp. and *Cylindrocarpon* spp. (Rumbos and Rumbou 2001); in Italy Cylindrocarpon destructans (Grasso 1984; Grasso and Magnano di San Lio, 1975); in Lebanon, *Cylindrocarpon* sp. (Choueiri *et al.* 2009); in New Zealand, *Cylindrocarpon* spp. (Halleen *et al.* 2004); in North Eastern USA and South Eastern Canada, *Cylindrocarpon* spp. (Petit *et al.* 2011); in Portugal, *Phaeoacremonium* spp. and *C. destructans* (Rego *et al.* 2000); in South Africa *P. chlamydospora*, *Phaeoacremonium* spp.,

Botryosphaeria spp. and *Cylindrocarpon* spp. (Halleen *et al.* 2003); in Spain *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp., *Botryosphaeria* spp., *Cylindrocarpon* spp. and *Phomopsis* spp. (Aroca *et al.* 2006; Giménez-Jaime *et al.* 2006; Martin and Cobos, 2007; Gramaje *et al*. 2009; Alaniz *et al.* 2011); and in Uruguay *Cylindrocarpon* spp. (Abreo *et al*. 2010).

We have been able to confirm that YVD syndrome in the Riverina, in grafted grapevines, is caused by the infection by two different wound and root-invading fungal organisms, namely *Botryosphaeria* and *Cylindrocarpon* spp. at different stages of the propagation process.

Co-infection between *Botryosphaeria* and *Cylindrocarpon* at different stages during propagation

Riverina vineyards and sources of planting stock survey

A survey for incidence of wood and root fungal pathogens was conducted in 20 Riverina vineyards with young vine decline (YVD) symptoms in grafted plants. There were a variety of scion and rootstock cultivars but Chardonnay grafted to Ramsey was the predominant scion rootstock combination. Plant and samples were obtained from both newly planted (vines from 1 to 6 months old), or older vines (2 to 8 years old) reported to have been affected with YVD symptoms from the time they were planted.

Three diseased and three apparently healthy grapevines were removed intact from each vineyard. Segments of roots, stems and shoots from which exfoliating bark had been removed, were cut longitudinally and transversely to expose any internal symptoms of dark-brown vascular staining. Other segments of each plant tissue were surface-sterilised for 3 min with sodium hypochlorite (1% active chlorine), and rinsed three times with sterile deionised water (SDW). Bark was removed from the surface, and thin disks cut from whole cross sections were placed on Dichloran Rose Bengal Chloramphenicol agar (DRBC; Oxoid) and incubated at 25°C for three to 14 days in darkness. All developing fungal colonies were transferred to potato dextrose agar (PDA; Oxoid). In order to enhance sporulation of Botryosphaeriaceae fungi, cultures were incubated in daylight with sterilized pine needles on 2% water agar and isolates were examined weekly for formation of pycnidia and conidia. The growth rates and colour of all isolates on PDA at 25°C in darkness were measured using colonies generated from 5 mm-diameter mycelial plugs obtained from the margins of 5-day-old single-spore PDA colonies. Species of *Cylindrocarpon* and *Botryosphaeria* were identified by colony and conidial morphology (Halleen *et al*. 2004; Halleen *et al*. 2006b; Phillips, 2007) and comparison of the β-tubulin sequences of typical Riverina isolates with sequences from Genbank.

DNA extraction, PCR amplification and sequencing of the partial β-tubulin 2 gene were performed as described by Whitelaw-Weckert *et al*. (2007). Once identified, *C. macrodidymum*, *C. liriodendri* and *B. obtusa* isolates from Riverina grapevines, plus a known pathogenic *C. macrodidymum* isolate from Tasmanian grapevine, were selected for pathogenicity studies. The isolates utilised in this study were deposited in the Plant Disease Herbarium Collection of the Agricultural Scientific Collection Unit at NSW DPI in Orange, New South Wales.

Spatial distribution of YVD affected grapevines

Normalized difference vegetation index (NVDI), a common indicator of photosynthetically active biomass used in remote sensing, was used to map the grapevine canopies in two typical YVD affected vineyards using high-spatial-resolution aerial multispectral images (Hall *et al*. 2010).

Epidemiological case study

In 2008, 6200 grafted vines (Pinot Noir on Ramsey rootstock) were planted in a Riverina vineyard but 1075 failed to shoot. The most visibly affected plants were replaced immediately but many others grew with typical YVD symptoms in the following years. The supplier nursery had propagated the plants from dormant rootstock cuttings obtained from a single rootstock source block.

We investigated a possible connection between the grapevine wood/root pathogens isolated from vineyard, the supplier nursery and the rootstock source block. First soil from a depth of 0–10 cm depth was sampled from both the vineyard and the supplying nursery. The vineyard soil was collected from within 2 m from YVD-affected vines. The nursery field soil was collected from two locations: the first which had been used to propagate the affected grapevines; and the second which had been used in the following season with no adverse plant effects. A sub-sample of the soil from the first nursery site was sterilised by autoclaving (121°C, 20 min, three times over 6 days). Dormant 1-year-old cv. Chardonnay rootlings, previously tested free of fungal pathogens (by surface sterilising and plating sub-samples as

described previously), were planted in 1.5 L pots containing soil (1.2 kg dw) from each source. The pots were arranged in randomised complete blocks in a glasshouse at 15–25°C and watered daily to field capacity daily. The plants were destructively sampled 8 months later. Root and shoot dry weights (50°C until constant weight) were determined. Roots and stem segments were surface sterilised and incubated first on DRBC and then PDA as described earlier to detect fungal species that were transmitted from the soil.

In the following year we sampled ten 1-year old dormant field propagated grafted vines (Colombard on Ramsey rootstock) from the same nursery; and 2 lignified canes from each of 4 Ramsey vines in the rootstock source block. To detect fungal species, roots and stem segments were surface sterilised and incubated first on DRBC and then on PDA as described previously.

Pathogenicity studies

Rapid pathogenicity assay on Chardonnay grapevine seedlings

The visible disease symptoms on rooted grapevine cuttings after *Cylindrocarpon* inoculation can be very slow to appear, so we decided to investigate a more rapid test for pathogenicity using grapevine seedlings. Grapevine seeds (Chardonnay) were surface sterilised in 0.5 M H_2O_2 for 24 hour. The seeds were then washed with SDW three times and blotted dry on sterile tissue paper before being soaked for 5 min in 2% hydrogen cyanamide (H_3NCN, Sigma) with 0.03% surfactant Triton-100 in SDW to break dormancy. The seeds were planted in sterile potting mix (Hortico Premium Blend, autoclaved 2 times in 4 days as above) until the seedlings were 4 cm high. Seven unwounded seedling roots were, in bright sunny conditions, dipped for 30 min into a suspension of conidia and mycelium of *C. macrodidymum* DAR1461 ($1x10^5$ spores mL^{-1}). Seven control plants were dipped in water. The plants were then pruned and planted in 12 cm diameter, 1.2 L pots containing sterile potting mix (as above). The pots were arranged in randomised complete blocks in a glasshouse maintained at 15–25°C and watered to field capacity. Observations on seedling growth were recorded weekly and after six weeks the seedlings were destructively sampled. The roots were scored for root health: where 0=no roots remaining; 1=75–100% blackened; 2=50% blackened; 3=33% blackened; and 4=100% healthy roots. Roots and stems were surface sterilised and incubated first on DRBC and then PDA as above and root and shoot dry weight (50°C until constant weight) were determined.

Effects of co-infection by C. macrodidymum and B. obtusa

To investigate the effect of co-infection by *C. macrodidymum* and *B. obtusa* on the growth of young grapevines, the roots of nine one-year old Chardonnay rootlings, each with one leafy shoot (1 m long), were pruned to 15 cm length. Immediately afterwards, in bright sunny conditions, the roots of six plants were submerged for 90 min in a suspension of conidia and mycelium of *C. macrodidymum* DAR81461 ($1x10^6$ spores mL^{-1}). Three control plants were submerged in water. The plants were then pruned to three basal buds and planted in 28 cm diameter pots (8.1 L) containing steam pasteurised (65°C for 45 min) coarse river sand:loam:Canadian peat moss (2:2:1). The pots were arranged in randomised complete blocks in a glasshouse maintained at 15–25°C and watered daily to field capacity. After seven weeks, 10 mL aliquots of *B. obtusa* DAR81462 suspension were applied to the surface of three of the pots previously inoculated with *C. macrodidymum* and washed in with a further 100 mL water. Equivalent volumes of water were added to the remaining pots. The plants were destructively sampled after 16 weeks. The roots were scored for root health, where: 0=no roots remaining; 1=75–100% blackened; 2=50% blackened; 3=33% blackened; and 4=100% healthy roots. Root and shoot dry weight (maintained at 50°C until constant weight) were determined. Recovery of the inoculum from roots and stems was used to satisfy Koch's Postulates. This was tested by surface sterilising and incubating sub-samples of roots, stems and shoots on DRBC and PDA as described previously.

Longer term impacts of YVD on grapevine yield

The growth and fruiting of 30 YVD-affected Chardonnay grapevines on Ramsey rootstock was compared with 30 asymptomatic plants over 3 consecutive seasons (seasons 5 to 8) in a Riverina vineyard. The YVD-affected plants displayed typical YVD symptoms from the time they were planted. Total fruit weight and bunch number were determined at harvest. Shoot elongation was determined from soon after budburst and the total weight of prunings was determined after leaf-fall.

Results and discussion

Identity of fungal isolates

Overall, 60 diseased and 60 apparently healthy (asymptomatic) vines were sampled from 20 Riverina vineyards affected by YVD. *Cylindrocarpon macrodidymum* and *B obtusa* were the predominant species isolated from these vineyards. *C. macrodidymum* or *C. liriodendri* were also isolated from the rootstocks of every vineyard sampled and *B. obtusa*, *B. stevensii* or *B. rhodina* were isolated from rootstocks in 89% of those vineyards. *Cylindrocarpon* spp. and *Botryosphaeria* spp. were less commonly isolated from the scions (*Table 2.32*).

Relationship between YVD symptoms and fungal isolates

Genus-level identification of the isolates, based on spore and colony morphology, showed that the causative organisms were isolated from the YVD-affected grapevines in the surveyed vineyards but not from the asymptomatic plants. *C. macrodidymum* was isolated from the rootstocks of all YVD-affected vines tested in every vineyard. It was associated with dark stained patches of wood from the oldest xylem rings and was isolated from rootstock roots, rootstock trunks and graft unions in 77%, 62% and 31% of the vineyards respectively. *C. macrodidymum* was also isolated from scion cordons in 8% of vineyards (*Table 2.32*).

Botryosphaeria obtusa was isolated from the rootstocks of YVD-affected grapevines in 77% of the surveyed vineyards. It was present in the roots of diseased vines in 15% of vineyards but was more often isolated from the rootstock trunk and graft unions (50% and 28% respectively) where it was associated with dark wedge shaped discolouration. *B. obtusa* was also found colonising the scion cordons in 22% of vineyards. In the few vineyards where *B. obtusa* did not colonise the rootstocks, *Botryosphaeria stevensii* or *Botryosphaeria rhodina* was present (*Table 2.32*).

Spatial distribution of YVD affected grapevines within vineyards

Distribution of low vigour vines within two of Riverina vineyards was determined by remote sensing. Using the normalized difference vegetation index, an indicator of photosynthetically active biomass, large patches of low vigour vines were identified in positions corresponding to the location of diseased vines within the vineyard. The spatial distributions indicated that the diseased grapevines were not located together along the vine rows but were randomly distributed over the entire planted area (*Figure 2.23*).

Epidemiological case study: vineyard, supplier nursery and rootstock source block

Fungal isolates from vineyard

C. macrodidymum was isolated from every rootstock stem and root sample from the YVD-affected plants. *B. obtusa* was also isolated from every rootstock stem and from 33% of graft unions of symptomatic grapevines (*Table 2.33*). Dark discoloured patches and wedges were seen in cross section on the rootstock stems and the graft union of every YVD affected plant. Light microscopy of thin sections of the infected roots and stems showed that the xylem was heavily colonised by brown fungal hyphae 1.5–2 µm thick. No pathogens were isolated from stems or graft unions of the healthy vines or from the scions. Microscopy showed the scions to be free of fungal hyphae (data not shown).

Fungal isolates from the supplier nursery plants

Dark discoloured patches, wedges or spots in cross section were seen in every nursery rootstock stem. We isolated *C. macrodidymum* from the thick (4 mm) roots of every rootstock sample and *B. obtusa* from

Table 2.32 Survey results showing relevant fungal pathogens isolated from three diseased grafted vines in each of 20 Riverina vineyards affected with young vine decline (YVD) (i.e. 60 grapevines). The pathogens were not isolated from the apparently healthy vines selected from each vineyard.

	Proportion of diseased grapevine samples from which fungi were isolated (%)						
	Rootstock				Scion		
Pathogenic fungi	Total	Roots	Trunk below graft	Graft union	Total	Trunk	Cordon
Cylindrocarpon macrodidymum	100	77	62	31	8	0	8
Botryosphaeria obtusa	77	15	50	28	22	0	22
Botryosphaeria stevensii	6	8	6	6	6	6	0
Cylindrocarpon liriodendri	6	8	0	0	0	0	0
Botryosphaeria rhodina	6	0	0	6	0	0	0

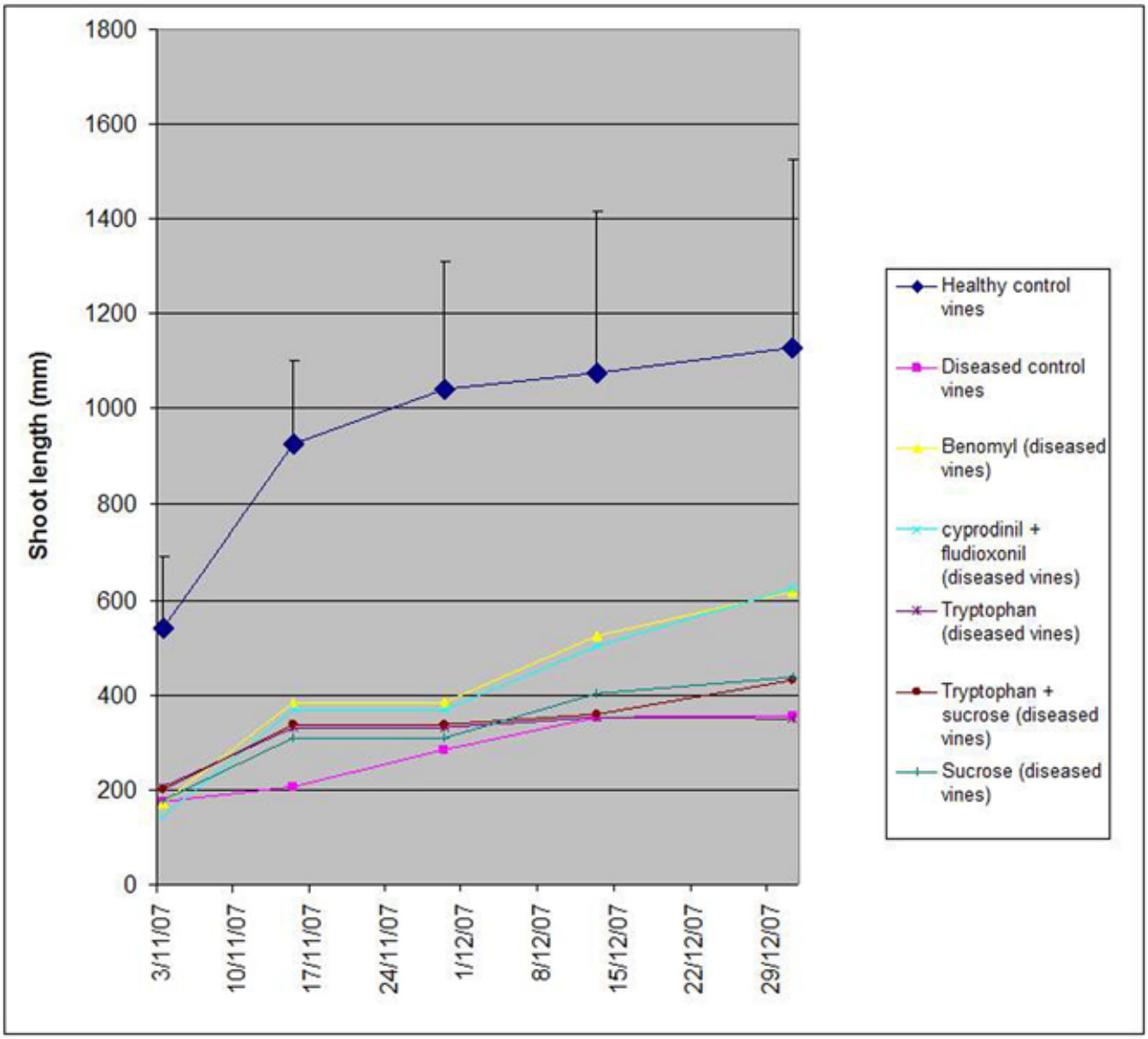


Figure 2.23 Impact of YVD, fungicides, and tryptophan, sucrose or sucrose + tryptophan on the length of five selected shoots over the growing season in a typical Riverina YVD-affected vineyard B. Each data point represents the mean of three vines. Error bars for each time point, l.s.d at P<0.05.

Table 2.33 Epidemiological case study: fungal pathogens isolated from Riverina vineyard grafted vines (Pinot Noir on Ramsey) 2008; Collombard on Ramsey from the supplying nursery in 2009; and canes from the Ramsey rootstock mother vines in 2009.

	Young vines from vineyard (2008)				Young vines from propagation nursery (2009)				Rootstock mother vine source block (2009)
Fungal organisms	**Roots (%)**	**Rootstock trunk below graft (%)**	**Graft union (%)**	**Scion (%)**	**Thick roots (4 mm) (%)**	**Rootstock trunk below graft (%)**	**Graft union (%)**	**Scion (%)**	**Canes from mother vines (%)**
Cylindrocarpon macrodidymum	100	100	0	0	100	0	0	0	0
Botryosphaeria obtusa	0	100	33	0	0	0	10	0	25

10% of rootstock graft unions. No pathogens were isolated from the scions which were clean in cross section (*Table 2.33*).

Fungal isolates from the rootstock source vines

Although the canes from rootstock source vines appeared macroscopically uninfected in cross section, *B .obtusa* was isolated from 35% of the canes (*Table 2.33*).

Fungal isolates from the supplier nursery soil

After eight months, Chardonnay potted vines grown in the soil from the original nursery site had lower root and shoot mass compared to those planted in the sterilised soil from the same site. This was also the case for vines planted in apparently pathogen free soil from the second site. This indicates that soil from the nursery site must have contained some biotic factor limiting vine growth (*Table 2.34*).

Pathogenicity studies

Rapid pathogenicity assay on Chardonnay grapevine seedlings

After 21 days, the growth of the inoculated seedling was visibly inferior to that of the control seedlings but the leaf area and number of dead leaves were not yet significantly different. However, after ten weeks the root health score, root dry weight and shoot dry weights were significantly lower (*Figure 2.24, Table 2.35*).

Co-infection by C. macrodidymum and B. obtusa

Vines that had been exposed to root inoculation with *C. macrodidymum* alone had significantly decreased shoot length, shoot dw, total leaf dw, dw per leaf, number of leaves and root dw after 16 weeks growth. Dual root inoculation with *C. macrodidymum* and *B .obtusa* caused a further decrease in total leaf dw, total number of leaves, root health rating and fine root rating compared to inoculation with *C. macrodidymum* alone. As mass per leaf was unchanged, the decrease in total leaf mass by *B. obtusa* came not from smaller leaf sizes but from less leaves (*Table 2.36*). The inoculated fungi were isolated from the roots of every inoculated diseased plant but not from the control plants; thus satisfying Koch's postulates.

Impacts of YVD on grapevine yields.

Three years of yield data recorded for a typical Riverina YVD-affected vineyard showed that diseased vines consistently produced lower yields and lower bunch numbers per vine (*Figure 2.25*). The YVD

Table 2.34 Case study: root and shoot growth of Chardonnay potted vines after eight months growth in soil from the Riverina vineyard and the supplying nursery.

	Root dry weight (g)	Shoot dry weight (g)	Pathogenic fungi isolated from surface sterilised pot experiment roots
Vineyard soil	7.1 a,b	4.1 a	0
Nursery soil from original ('diseased') site	4.0 b	2.3 b	*Cylindrocarpon macrodidymum*
Sterilised nursery soil from original ('diseased') site	9.7 a	3.5 a	0
Nursery soil from second ('healthy') site	12.5 a	4.7 a	0
P	0.033	0.05	
l.s.d.	5.4	1.7	

Values within a column followed by the same letter are not significantly different based on l.s.d. (P=0.05)

Table 2.35 Rapid pathogenicity assay. Riverina *Cylindrocarpon macrodidymum* isolate on Chardonnay grapevine seedlings.

	Leaf surface area 21days (mm^2)	Dead leaves 21 days	Root health score[1]	Root dry weight (g)	Shoot dry weight (g)
Uninoculated control	7217	1	4	0.283	0.337
Intact root inoculated with *C. macrodidymum* DAR81461	944	0	1		0.110
P	0.162	0.225	0.027	0.027	0.046
l.s.d.	1240	2.484	2.151	0.1526	0.2166

Values within a column followed by the same letter are not significantly different based on l.s.d. (P=0.05)
[1] Root health score: 0=no roots remaining, 1=75–100% blackened, 2=50% blackened, 3=33% blackened and 4=100% healthy roots

affected vines also had shorter shoots throughout the season and lower pruning weights (data not shown).

We have identified the cause of the young vine decline syndrome (YVD) in the Riverina as the co-infection by two different wound or root-invading fungal genera: *Cylindrocarpon* and *Botryosphaeria*. Our surveys have shown that those fungi were involved in all cases of Riverina YVD investigated, but they were not found in healthy vines in the same vineyards. We also demonstrated grapevine yields in the field were significantly decreased and that remote sensing using normalized difference vegetation index could identify areas within a vineyard that contain the diseased vines. As newly planted diseased vines had internal trunk discolouration from the oldest xylem below the graft union; and as both *Cylindrocarpon* spp. and *Botryosphaeria* spp. were isolated in our case study from grapevines in the vineyard, the supplying propagation nursery and rootstock mother vine source block, it is probable that the disease has been initiated in the source block and propagation nursery.

Table 2.36 Effects of co-infection of Chardonnay by *C. macrodidymum* and *B. obtusa*: 16 weeks after inoculation.

	Total shoot length (cm)	Shoot dry weight (g)	Total leaf dry weight (g)	Dry weight per leaf (mg)	Number of leaves	Root dry weight (g)	Root health rating	Fine root rating
Uninoculated control	844 a	77.6 a	54.0 a	45 a	120 a	116.9 a	4.70 a	7.29 a
Trimmed roots inoculated with *Cylindrocaron macrodidymum* DAR81461	478 b	38.8 b	20.8 b	30 b	70 b	39.6 b	4.32 a	6.62 a
Trimmed roots inoculated with *C . macrodidymum* DAR81461plus *Botryosphaeria obtusa* DAR81462	337 b	19.0 b	7.6 c	30 b	55 c	27.1 b	3.07 c	3.36 c
P	<0.001	0.001	<0.001	0.007	<0.001	<0.001	0.002	<0.001
lsd	171	29.7	12.6	13.7	24.9	25.8	1.16	2.19

Values within a column followed by the same letter are not significantly different based on l.s.d. (P=0.05)

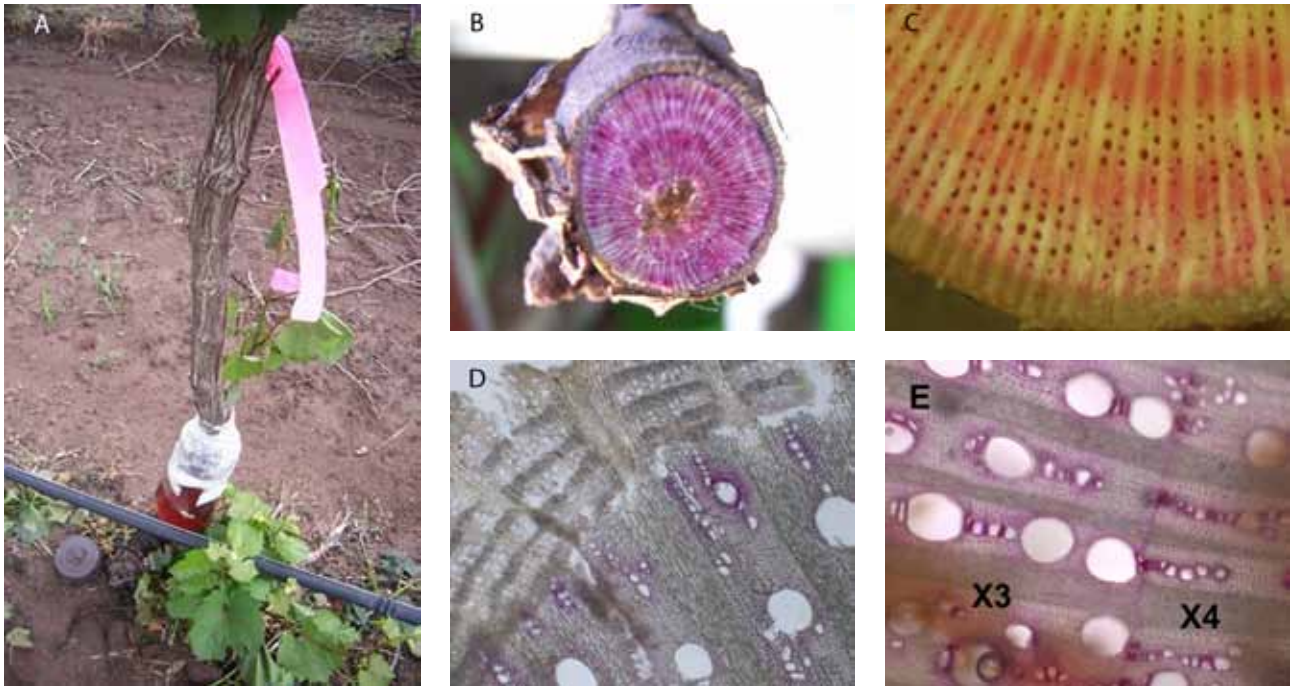


Figure 2.24 Acid fuchsin experimental set-up (A); colouration in diseased vine trunks, 3cm above cut (B) and 60cm above the cut (C); Microscopy 22 cm above cut showing conducting xylem vessels and non-conducting phloem (D); 48 cm above cut showing third year xylem (X3), fourth year xylem (X4) conducting the dye (E).

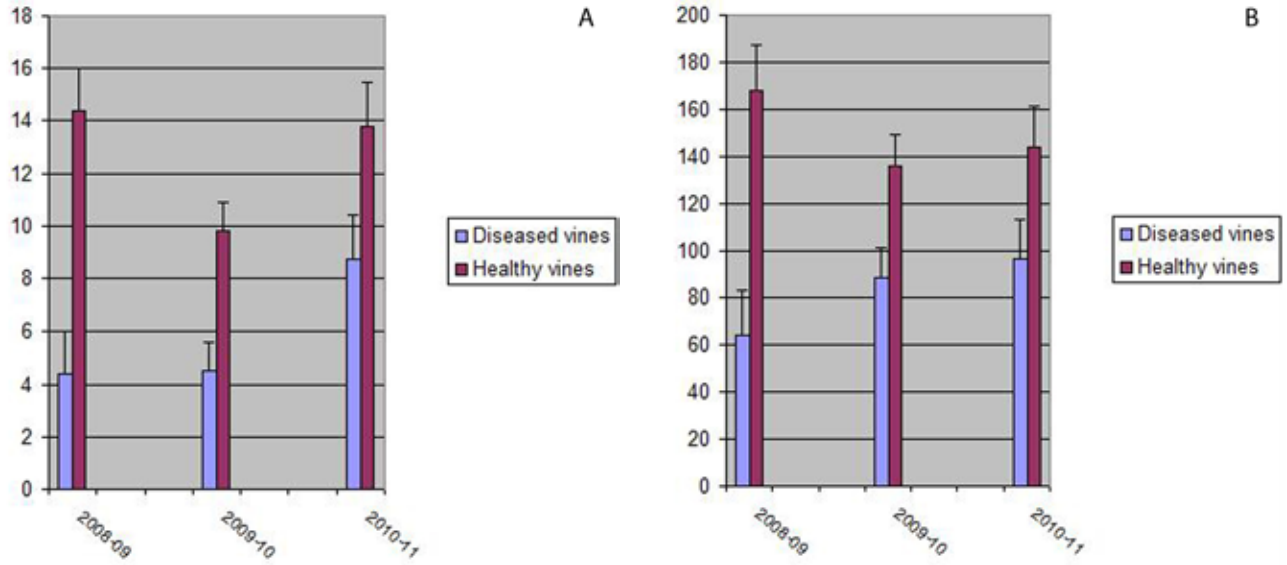


Figure 2.25 Impact of YVD on grapevines yields. Grapevine performance in typical Riverina YVD-affected vineyard showing three years of yield data from 30 diseased vines and 30 healthy vines (5-year old Chardonnay on Ramsey rootstock). (A) Yield per vine; (B) bunches per vine. Error bars, l.s.d at P <0.001.

Cylindrocarpon spp. from young grapevines in Riverina vineyards

The Riverina vineyard survey showed that *Cylindrocarpon macrodidymum* was the predominant pathogen isolated from the roots, the rootstock wood and graft union of most young diseased vines in the surveyed YVD vineyards. Wherever *C. macrodidymum* was not isolated, *C. liriodendri* was recovered. *C. macrodidymum* was also consistently isolated from the roots of young grafted grapevines located in a nursery implicated in supply of diseased planting material. *Cylindrocarpon* spp. have been isolated from roots and basal ends of grafted cuttings in young vineyards and nurseries in many other viticultural regions world wide (as shown in Introduction). In Australia, Sweetingham (1983) described a serious disease of five-year old or older *V. vinifera* cv. Cabernet Sauvignon in Bream Creek, Tasmania, consistently associated with the incidence of *C. destructans* (later identified as *Cylindrocarpon macrodidymum* by Halleen *et al.* 2004) in the roots and trunk near ground level. In accord with symptoms for young vine decline in the Riverina, symptoms included very poor shoot growth early in the growing season and dark brown discolouration of wood in the trunk at ground level. Microscopy and fungal isolation studies indicated that the buried portion of the trunk, 2–12 cm below the ground surface, was the region where discolouration and fungal hyphae first became evident.

In support of the nursery connection, Davoren *et al.* (1999) reported that *Cylindrocarpon* spp. were commonly found in stunted vines in grapevine nurseries in South Australia and Victoria and showed that *C. destructans* was pathogenic to some non-V. vinifera rootstocks. Whitelaw-Weckert *et al.* (2007) established the pathogenicity of an Australian isolate of *C. liriodendri* from declining grapevines in the Hunter Valley, NSW. From 1998 to 2002, Victorian diagnostic services isolated *Cylindrocarpon* spp. from 12 grapevines showing Petri disease symptoms in New South Wales (NSW), SA and Vic. and it was stated that *Cylindrocarpon* spp. were always associated with decline symptoms in young grapevines less than five years old (Edwards and Pascoe 2004).

Botryosphaeria from young grapevines in Riverina vineyards

B. obtusa was the most commonly isolated *Botryosphaeria* species in the surveyed YVD-affected Riverina vineyards. The high levels of infection in roots, rootstock trunk and graft union in the vineyards, plus the isolations of *B. obtusa* from the young nursery vines and mother vine canes indicate that the fungus was introduced with the planting material and moved up through the graft union into the scion.

B. obtusa was also the most commonly isolated *Botryosphaeria* species in three Australian studies investigating older vines, where these fungi have caused the disease, 'Bot canker' by infecting pruning wounds. Castillo-Pando *et al.* (2001) was the first to report that *Botryosphaeria* spp. were causing grapevine dieback in Australia. Pitt *et al.* (2010) surveyed 91 vineyards across New South Wales and South Australia and reported that *B .obtusa* was consistently isolated from declining older grapevines,

accounting for almost 80% of the total number of *Botryosphaeria* spp. isolates collected. Taylor *et al.* (2005) and Savocchia *et al.* (2007) reported that *B. obtusa* was the most commonly isolated member of the Botryosphaeriaceae in Western Australia; and Hunter Valley/Mudgee regions of NSW respectively.

Botryosphaeria stevensii was recovered from roots, rootstocks, graft unions and trunk scions in 6% of the Riverina vineyards surveyed, predominantly from plant organs free of *B. obtusa*. Whitelaw-Weckert *et al.* (2006) reported severe failure (3% strike rate) of newly planted young Shiraz vines in the NSW Canberra region, and showed this to be caused mainly by *B. stevensii*. Whitelaw-Weckert *et al.* (2006) showed that *B. stevensii*, which usually infects grapevines through wounds in aerial parts of the grapevine, could also initiate infection by planting hole inoculum (from diseased roots in replanting situation) in agreement with the result from our Riverina pathogenicity testing for *B. obtusa*.

Botryosphaeria rhodina was isolated from graft unions in 6% of the Riverina vineyards surveyed. Pitt *et al.* (2010) recovered only one isolate of *B. rhodina* from their survey of 91 vineyards in NSW and South Australia. Interestingly, that isolate was from a vineyard in a sub-tropical region of NSW. As the optimum growth temperature of *B. rhodina* is 30.8°C, it has been suggested that it is more suited to regions such as Mexico, southern California and the desert regions of the Coachella Valley with high temperatures and low rainfall (Urbez-Torres *et al.* 2006; Leavitt and Munnecke, 1987). Prior to the Riverina isolations, there were only three Australian grapevine records of *B. rhodina* (Cunnington *et al.* 2007; Pitt *et al.* 2010). The fact that we have isolated this serious pathogen in the graft union of young vines in a warm region of the Riverina is of concern.

Cylindrocarpon/Botryosphaeria co-infection process in the nursery

Our evidence points to the initial *Cylindrocarpon* root infection occurring in nursery soil. The decomposition of cortical cells was evident in cryo-SEM micrographs (not shown) which showed that *C. macrodidymum* hyphae were present in decomposed empty cells. The destruction of the grapevine root cortex would eventually restrict the uptake of water and nutrients from the soil, prevent movement of the photosynthate to the roots, thus decreasing the root starch reserves and leading to continual decline in capacity of the grapevine. The root starch reserves are very important for shoot growth and development (Smith and Holzapfel, 2009). However, further work is required to demonstrate this disease mechanism in the field.

In this study, when Chardonnay vine roots were co-infected with *C. macrodidymum* and *B .obtusa*, the effect on root health and leaves was greater than when infected with *C. macrodidymum* alone. *B. obtusa* was isolated from surface sterilised roots and wood, indicating that root and stem colonisation had been successful (*Table 2.36*). An earlier soil inoculation study (Whitelaw-Weckert *et al.* 2006) also produced upward translocation of *B. stevensii* to grapevine shoots.

Our inability to isolate *C. macrodidymum* from a limited number of Ramsey rootstock mother vine canes is in agreement with Rego *et al.* (2001) and Halleen *et al.* (2003) who reported that *Cylindrocarpon* spp. were rarely isolated from canes of rootstock mother vines in Portugal and South Africa respectively. It is likely that the fungus is more likely to infect young vines from the nursery soil. Our case study showed that *C. macrodidymum* was the most commonly isolated pathogen from the nursery that supplied diseased grapevines to a Riverina vineyard. Our findings are in agreement with a study showing that *Cylindrocarpon* spp. were the most common pathogens associated with young nursery vines in California (Dubrovsky and Fabritius, 2007). *Cylindrocarpon* spp. were also reported to be isolated from young nursery vines in Uraguay (Abreo *et al.* 2009); Spain (Aroca *et al.* 2006; Giménez-Jaime, 2006); New Zealand (Bleach *et al.* 2007) and Portugal (Rego *et al.* 2009).

It is well known that the *Botryosphaeria* spp. can invade rootstock mother plants (Rego *et al.* 2009, Portugal). Our study isolated *B. obtusa* from asymptomatic rootstock mother vine canes. In South Africa *Botryosphaeria* spp. were recovered from basal internodes of 1-year-old rootstock canes, indicating that the fungi probably originated in the trunk of the mother vine (Fourie and Halleen, 2004). Similarly, the middle and basal parts of rootstock canes from New Zealand were infected with *Botryosphaeria* spp. (Billones *et al.* 2010). The high rate of root colonisation by both *B. obtusa* and *B. stevensii* in the surveyed Riverina vineyards can probably be explained by the downward growth of fungal hyphae from infected rootstock canes to the roots, although, based on evidence from the pot experiments described above, the upwards growth from soil cannot yet be discounted. Amponsah (2010) reported that hyphae

in xylem of grapevine stems inoculated with *B. lutea* moved faster upwards (75 mm in 4 months) than downwards (47 mm in 4 months), probably helped by the upwards direction of the xylem stream.

Botryosphaeria spp. are also commonly recovered from young vines in nurseries. In this study, *B. obtusa* was isolated from young grafted plants in nurseries that accessed rootstock canes from infected mother vines. *Botryosphaeria* spp. were also isolated from young nursery plants in Spain (Aroca *et al*. 2006; Giménez-Jaime *et al*. 2006); France (Vigues *et al*. 2009); Hungary (Lehoczky 1974), South Africa (van Niekerk *et al*. 2006); New Zealand (Billones *et al*. 2010); and Portugal (Rego *et al*. 2009). The co-existence of *Cylindrocarpon* and *Botryosphaeria* spp. in nursery plants reported in this study is in accord with similar reports from Portugal (Oliviera *et al*. 2004); Greece (Rumbos and Rumbou 2001); and Spain (Aroca *et al*. 2006; Giménez-Jaime *et al*. 2006).

Alternate sources of *Cylindrocarpon* and *Botryosphaeria* spp. inoculum may be important in the spread of disease in mother vine blocks and nurseries. *Cylindrocarpon* spp. infect many different plant species. Agustí-Brisach *et al*. (2011) isolated *C. macrodidymum* from the roots of weeds in 17 out of the 32 Spanish field sites including grapevine rootstock mother fields, open-root field nurseries and commercial vineyards, indicating that weeds may act as sources of inoculum for grapevine infection in nursery fields. *Cylindrocarpon* spp. are also important components of the peach root rot complex (Manici and Caputo 2010) and the apple replant disease complex in South Africa (Tewoldemedhin *et al*. 2011).

Botryosphaeria spp. also infect many woody host plant species, including Prunus (plum, peach, nectarine and apricot) in South Africa which are often situated in close proximity to vineyards (Damm *et al*. 2007). *B. obtusa* was also isolated from wood from willow, apple and pear in Australia (Cunnington *et al*. 2007). Genetic similarities between grape and non-grape isolates, and the fact that non-grape hosts may be located in wine-growing regions, suggest the potential for cross-infection between grape and other horticultural crops.

This study has shown that the *Botryosphaeria* spp. infecting young Riverina grapevines are likely to have originated in rootstock mother vine canes. Most rootstock mother vines are allowed to sprawl on the ground, thus allowing soil surface contamination (Gramaje and Armengol 2011). Alternatively, spores, from pycnidia on diseased wood and pruning debris, may be released during wet weather and enter unprotected wounds on the mother vine head. After budburst, hyphae can move up the xylem into the current season's canes.

Hot water treatment (HWT) will kill *Botryosphaeria* infected canes (Crous *et al*. 2001) but unfortunately most nurseries supplying grapevines to the Riverina do not currently use HWT, so the canes remain infected. One of the earliest stages of infection spread to non-infected canes is during the post harvest hydration process prior to cold storage. Afterwards, both *Botryosphaeria* spp. and *Cylindrocarpon* spp. nursery contaminants can enter the many wounds produced as part of the grapevine preparation process (disbudding, grafting, improperly matched or healed graft unions, and rooting) (Waite and Morton 2007).

In contrast to *Botryosphaeria*, South African studies have shown that *Cylindrocarpon* infections establish in nurseries predominantly after callused cuttings are planted (Halleen *et al*. 2003, 2006*a*). The rooting phase is a susceptible stage because susceptible basal ends, especially the pith, can be exposed and vulnerable to infection. In addition, the callus is fragile and can break during planting, resulting in wounds susceptible to infection by *Cylindrocarpon* spp. (Halleen *et al*. 2003). The high percentage of isolations of *Cylindrocarpon* spp. from the roots, rootstock trunk and grafting unions of YVD diseased vines in the Riverina vineyards (100%) and young nursery vines (100%) strongly indicate that the nursery soils probably harbour pathogenic *Cylindrocarpon* spp.

Experiment 2.19
Root infection of *Vitis vinifera* by *Cylindrocarpon liriodendri*

Black-foot disease, caused by *Cylindrocarpon* spp. is a serious disease of *Vitis vinifera* and non-*V. vinifera* grapevine rootstocks in most viticultural regions, including California (Scheck *et al*. 1998), France (Maluta and Larignon 1991), Italy (Grasso and Magnano Di San Lio 1975; Grasso 1984), New Zealand (Halleen *et al*. 2004*a*), Portugal (Rego *et al*. 2000) and South Africa (Fourie *et al*. 2000). The symptoms include a black discolouration of the wood and black streaks in the vascular system, with internal necrosis extending from the bark to the pith. The xylem vessels become occluded with fungal tissue, gums and tyloses and the infected roots show black, sunken, necrotic lesions (Grasso 1984). Abnormal development of roots, such as necrotic root crowns and development with growth parallel to the soil surface, may also occur (Halleen *et al*. 2004b).

Above ground symptoms include weak or absent shoot growth in spring and drying and dying of shoots in summer. When pathogenic *Cylindrocarpon* spp. attack young vines they die quickly, but older vines may take more than a year to die. Heavy, wet and poorly drained soils contribute to disease severity and significant crop losses (Gubler *et al*. 2004). In Australia, Davoren *et al*. (1999) reported that *Cylindrocarpon* spp. were commonly found in stunted vines in grapevine nurseries in South Australia (SA) and Victoria (Vic.) and showed that an isolate of *C. destructans* increased the death of some non-V. vinifera rootstocks. *Cylindrocarpon* spp. were isolated from 12 grapevines showing Petri disease but not black-foot symptoms in New South Wales (NSW), SA and Vic. from 1998 to 2002 (Edwards and Pascoe 2004). Sweetingham (1983) described a serious disease of *V. vinifera* cv. Cabernet Sauvignon in Bream Creek, Tasmania, consistently associated with the incidence of *C. destructans* (later identified as *Cylindrocarpon macrodidymum* by Halleen *et al*. 2004*b*). Symptoms included dark brown discolouration of wood in the trunk at ground level, sometimes extending into the larger roots. Halleen *et al*. (2006) recently reported that grapevine black-foot isolates from Europe and South Africa were morphologically and genetically identical and had identical morphology and 5.8 S rDNA/ITS and β-tubulin 2 gene sequences to *C. liriodendri*, and so were re-identified from *C. destructans* to *C. liriodendri*.

Materials and methods

The materials and methods detailed below are adapted from Whitelaw-Weckert *et al*. 2007.

In 1989, roots were sampled from own-rooted *V. vinifera* cv. Pinot Noir grapevines in a vineyard in the Hunter Valley, NSW showing symptoms of general decline in growth, weak shoot growth, reduced foliage and reduced leaf size and root growth. The roots were dark brown and showed rotting of the epidermal tissues, necrosis of the cortical tissue and browning of the xylem. A *Cylindrocarpon*-like fungus was found to be the only pathogenic fungus isolated. The fungal isolate (DAR77726) was deposited in the Herbarium, Orange, NSW. Single spore isolates of the fungus (three replicates) were grown in darkness at 25°C on potato dextrose agar (PDA) on 9 cm Petri dishes. After 14 days, colony growth rates were determined and the macroscopic characters of colonies described. Colony colours were recorded using the nomenclature of Kornerup and Wanscher (1989). After 14–21 days, the reproductive structures were mounted in lactic acid for microscopic observation, and the size and shape of conidia, phialides and chlamydospores (n=30) were determined.

DNA extraction, PCR amplification and sequencing of the partial β-tubulin 2 gene was performed as described by Whitelaw-Weckert *et al*. (2006) except that one U *Taq* DNA polymerase was used and T1 and T2 (O'Donnell and Cigelnik 1997) primers were used. The sequence has been deposited in GenBank (accession number EF176600).

Six 1-year-old *V. vinifera* cv. Chardonnay vines were grown in 28 cm diameter, 8.1 L pots containing coarse river sand:loam:Canadian peat moss (2:2:1). A 1.5 cm auger was used to aseptically remove five cores of potting mixture from the pots and 1.9 g sterilised wheat germ was aseptically placed into each hole. Inoculum, containing macroconidia and microconidia (105 spores mL^{-1}), was prepared in sterile deionised water from a 21-day-old colony of the *Cylindrocarpon* isolate grown on PDA at 25°C in a 12 hour light/dark cycle. Aliquots (1 mL) of suspension were applied to the wheat germ core of three pots and the holes were back filled with the extracted potting mixture. Three control uninoculated pots were inoculated in the same way with sterile deionised water. The pots were arranged in randomised complete blocks in a glasshouse maintained at 15–25°C and watered to field capacity twice weekly.

At 18 months after inoculation (a.i.) root samples were collected aseptically from 2.5 cm soil cores

removed from each pot. The roots were surface sterilised for 3 min with calcium hypochlorite (1% active chlorine) and rinsed three times with sterile deionised water. Twenty pieces (3 mm) from each pot were incubated on Dichloran Rose Bengal Chloramphenicol agar (DRBC) (Oxoid Australia, Adelaide). Fungi growing from the roots were transferred to PDA and incubated as above.

Shoot length measurements were taken at 27 months a.i. and the pots were destructively sampled at 31 months a.i. The roots were scored for 'root health', where: 0=no roots remaining; 1=75–100% blackened; 2=50% blackened; 3=33% blackened; and 4=100% healthy roots. Roots and bark were surface sterilised with calcium hypochlorite. The trunks were cut into 4 cm pieces. The bottom 1 cm of these pieces was surface-sterilised by brief flaming then aseptically cut lengthwise to expose the centre. Surface-sterilised vine tissue was incubated first on DRBC and then PDA as above. Hand cross sections were prepared from the grapevine trunks and stained with aniline blue.

Statistical analysis

Fungal morphological data and vine pathological data were subjected to ANOVA and least significant differences were calculated at P<0.05 and P<0.01 using GENSTAT for Windows, 8th edition.

Results and discussion

The results and discussion below are adapted from Whitelaw-Weckert *et al.* 2007.

Colony colours on PDA were cinnamon to sepia (6B3–6D6) both on the surface and reverse. Colony diameter was 40 mm after 7 days at 25°C in darkness. No soluble pigment was produced. Brown hyphae, singly or in dense aggregates, and simple or complex monophialidic conidiophores formed in the aerial mycelium. Phialides were cylindrical (20–40 by 3–4 µm; 2–2.5 µm wide near the aperture). Macroconidia predominated; these were brown and cylindrical, 1–3 septate, straight or slightly curved, (24–)35–40(–55)×(4.5–)5.5–6(–6.5) µm. Microconidia were hyaline and ellipsoidal (5–15× 2.5–4 µm) or ovoid (3–5×3–4 µm). Chlamydospores were brown, ovoid (10–20×10–17 µm) and occurred mostly in short chains.

The β-tubulin 2 gene alignment for the fungal isolate contained 553 characters including alignment gaps. The alignment had 99.6% similarity with that of a *C. liriodendri* grapevine black-foot isolate from Portugal (CBS117640, GenBank accession number DQ178173). On this evidence, isolate DAR77726 was identified as *C. liriodendri* (GenBank accession number EF176600).

At 18 months a.i., *C. liriodendri* was isolated from root subsamples from all the inoculated vines but from no control vines (*Table 2.37*), although there were no obvious disease symptoms and shoot length did not differ significantly (data not shown). Thirty-one months a.i., the Chardonnay vines inoculated with *C. liriodendri* exhibited the following disease symptoms: black bark above ground level; vascular discolouration and black discolouration of the wood in the basal area of the trunk (below ground level) with most roots black and rotted off (*Figure 2.26*). None of the control vines had these symptoms.

Confirming Koch's postulates, *C. liriodendri* was isolated from the roots and bark of the diseased vines but not from control vines. Inoculation caused 58% decrease in mean vine shoot length at 27 months, 98% decrease in mean root biomass at 31 months and a decrease in root health rating from 2.33–0 at 31 months a.i (P<0.05, *Table 2.37*).

Microscopy of trunk hand cross sections showed that the trunks 2–3 cm above the potting mix surface were colonised by a fungus with brown coloured septate mycelium, brown chlamydospores

Table 2.37 Effects of *Cylindrocarpon liriodendri* inoculation on potted *Vitis vinifera* cv. Chardonnay. Assessment times were 18 months for pathogen incidence (root cores taken), 27 months for shoot length and 31 months for black-foot symptoms and root biomass. Values within a column followed by the same letter are not significantly different based on l.s.d. (P=0.05). NA, not applicable

	Vines with roots infected by *C. liriodendri*	Shoot length (mm)	Root Biomass[A]	Root health rating[B]	Diseased plants
Inoculated	100%	1100 a	1.0 a	0.00 a	100
Control	0%	2610 b	53.9 b	2.33 b	0
P-value	NA	0.037	0.050	0.020	NA
l.s.d.	NA	1288	52.9	1.43	NA

[A] Root biomass dry weight measured at final destructive sampling, 31 months after inoculation (a.i.).
[B] Root health rating 31 months a.i. (0=no roots remaining, 1=75–100% blackened, 2=50% blackened, 3=33% blackened, 4=100% healthy roots).

and microconidia similar to those of *C. liriodendri* (*Figure 2.27*).

This study has established the pathogenicity of an Australian *C. liriodendri*, isolated from own-rooted *V. vinifera* cv. Pinot Noir grown at Hunter Valley, NSW, towards own-rooted *V. vinifera* cv. Chardonnay. However, disease development was relatively slow. The roots of the inoculated vines were colonised by the fungus after 18 months but there were no above ground disease symptoms apparent at that stage. After three years the inoculated vines showed severe black-foot symptoms, with the roots almost completely rotted away. These results differed from those of Sweetingham (1983) who was able to demonstrate the pathogenicity of Tasmanian grape *C. macrodidymum* on potted

V. vinifera but only in severely waterlogged conditions achieved by placing potted vines into containers of water so that the water level rose to the surface of the pot. Other studies have shown that black-foot disease symptoms can appear in potted non-*V. vinifera* rootstock cultivars within two months (Rego *et al*. 2000) and 4 months (Petit and Gubler 2005).



Figure 2.26 Black-foot symptoms on Chardonnay potted grapevine, 31 months after inoculation with *Cylindrocarpon liriodendri*. Symptoms included black bark above ground level; vascular discolouration and black discolouration of the wood in the basal area of the trunk (below ground level) with most roots being black and rotted away.

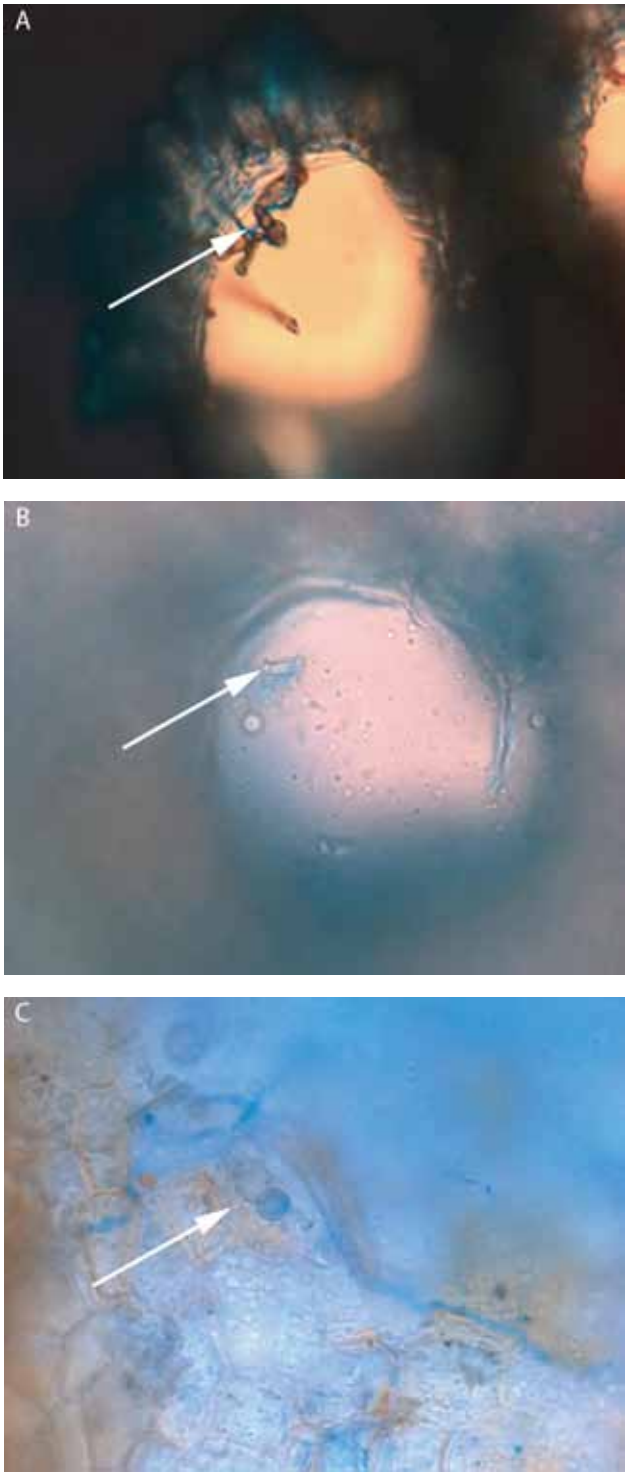


Figure 2.27 Stem hand cross sections, stained with aniline blue, from Chardonnay potted grapevines with black-foot symptoms 31 months after inoculation with *Cylindrocarpon liriodendri*. (A) Hyphae in xylem; (B) microconidia in xylem; (C) chlamydospores in bark. Scale bars=10 µm

Experiment 2.20
Chemical and biological control of young vine decline

Increasing vineyard soil organic matter increases the size of soil microbial communities (Whitelaw-Weckert *et al*. 2007), many of which are known to be important in soil suppression of pathogenic fungi such as *Cylindrocarpon* (Whitelaw-Weckert, 2004). We reasoned that addition of organic amendments may be a strategy for increasing the naturally occurring 'suppressive' soil bacteria and actinomycetes to improve vine health. Edwards (2006) reported to the GWRDC that an organic soil amendment had shown promise for ameliorating Petri Disease, caused by *Phaeomoniella chlamydospora*, and that "vineyards can recover from Petri disease over time with management practices that reduce stress e.g. mulch, bunch-thinning."

The severity of *Cylindrocarpon* infection is reported to be more severe with abiotic stresses such as inadequate nutrition, water, aeration or soil structure; and the severity of *Botryosphaeria* infection is exacerbated by water stress. These stressors can also be ameliorated by increased soil organic matter. We trialled four organic amendments in four Riverina vineyards (Chardonnay on Ramsey) affected by *Cylindocarpon*/*Botryosphaeria* YVD. We also trialled a potential biological control actinomycete (MW555, *Streptomyces violaceoruber*), extending our earlier GWRDC funded research on Vineyard Floor Management.

As we were interested in the beneficial effects of suppressive and growth promoting soil microbes, soil carbon (sucrose) and L-tryptophan were also applied under the grapevines. Sucrose soil amendments increase soil copiotrophic (K-strategist) populations of β-Proteobacteria such as Pseudomonas (Fierer *et al*. 2007). Auxin production by soil bacteria, including Pseudomonas, promotes plant growth and L-tryptophan increases this auxin production (Karnwal, 2009).

Our earlier soil study investigated the influence of soil organic matter on beneficial nematodes, which are an important part of the soil nutrient cycle. The results of this study are directly relevant to the YVD project because as soil organic matter rises, beneficial nematode numbers rise and cause a decrease in the parasitic nematode populations (Rahman *et al*. 2009). Root knot (*Meloidogyne javanica*), root lesion (*Pratylenchus* spp.), spiral (*Helicotylenchus* spp.) and ring (*Crconemoid* spp.) nematodes were found on the roots of some YVD vines, suggesting that they may exacerbate the disease process by injuring the roots and creating favourable water stress conditions for *Botryosphaeria*.

The effect of *Cylindrocarpon* on the root starch reserves through dormancy was also of concern because of the impact of low reserves on spring vegetative growth and bunch initiation (Holzapfel *et al*. 2007). Our nematode studies have also shown that root infection decreases carbohydrate levels (Rahman *et al*. 2011).

Finally, application of fungicides Benomyl or cyprodinil+fludioxonil to the diseased soil was tested in the vineyard. Nursery trials have established the effectiveness of combining fungicides cyprodinil+fludioxonil in the prevention or reduction of natural infections caused by *Cylindrocarpon* spp. and *Botryosphaeria* spp. (Rego *et al*. 2009). Benomyl, applied to the soil, was affective against *Cylindrocarpon* spp. on apple tree roots (Mazzola *et al*. 1998) and was also successfully trialled as a grapevine wound treatment against *Botryosphaeria* (Bester *et al*. 2007).

The objectives of this study were to ascertain the effect on YVD of organic soil amendments and fungicides, and to determine the role of the soil microbial communities associated with diseased vines.

Materials and methods

Organic amendments

In September 2008, four types of high carbon soil amendment; rice hulls, Biochar, composted cow manure and composted green waste; were applied to selected diseased and asymptomatic grapevines in two Riverina YVD affected vineyard: one flood irrigated Chardonnay on Ramsey; and the other drip irrigated Chardonnay (own rooted). All amendments were analysed by NSW DPI Diagnostic and Analytical Services, Wollongbar, NSW for pH ($CaCl_2$); electric conductivity (EC); KCl extractable ammonium-nitrogen; KCl extractable nitrate-nitrogen; available phosphorus (Colwell); organic C; total nitrogen; total carbon; and total elements aluminium, arsenic, boron, calcium, cadmium, cobalt, chromium, copper, iron, potassium, magnesium, manganese, molybdenum, sodium, nickel, phosphorus, lead, sulphur, selenium and zinc; plus exchangeable cations: aluminium, calcium, potassium, magnesium, sodium, total CEC (*Table 2.38*).

Table 2.38 Basic chemical properties of the biochar, composted green waste, composted cow manure and rice hulls used in the field trials.

	units	Biochar	Composted green waste	Composted cow manure	Rice husks
pH	-	9.1	5.8	7.5	6.3
EC	ds/m	4.0	5.2	9.9	-
NH^{4+} -N	mg/kg	6.1	69	348	11
NO^{3-} -N	mg/kg	0.32	1133	360	4.1
Available P	mg/kg	2600	860	4900	150
Total organic C	%	15	17	12	31
Total N	%	2.2	2.2	1.8	0.44
Total C	%	23	20	13	34
C/N	-	10.5	9.1	7.2	77.3
Total Al	mg/kg	10,000	22,000	25,000	83
Total arsenic	mg/kg	81	7.1	3.3	<3
Total boron	mg/kg	33	<1.9	3.6	<1.9
Total calcium	%	2.4	2.0	2.8	0.074
Total cadmium	mg/kg	<0.9	<0.9	<0.9	<0.9
Total cobalt	mg/kg	2.7	8.3	5.1	<1.2
Total chromium	mg/kg	110	27	24	4.5
Total copper	mg/kg	200	59	28	1.0
Total iron	mg/kg	5,600	12,000	12,000	120
Total potassium	%	1.6	0.85	2.1	0.46
Total magnesium	%	0.38	0.33	0.71	0.037
Total manganese	mg/kg	300	330	200	300
Total molybdenum	mg/kg	3.9	3.2	3.3	<1.2
Total sodium	%	0.34	0.18	0.34	0.015
Total nickel	mg/kg	8.8	13	13	3.5
Total phosphorus	%	1.3	0.57	0.94	0.031
Total lead	mg/kg	<1.7	15	<1.7	<1.7
Total sulfur	%	0.26	0.50	0.58	0.033
Total selenium	mg/kg	<6.6	<6.6	<6.6	<6.6
Total zinc	mg/kg	360	230	160	21
CEC aluminium	Cmol(+)/kg	<0.03	<0.03	0.2	<0.03
CEC calcium	Cmol(+)/kg	3.1	34	11	1.2
CEC potassium	Cmol(+)/kg	24	11	42	13
CEC magnesium	Cmol(+)/kg	1.2	13	18	2.4
CEC sodium	Cmol(+)/kg	6.4	7	14	0.65
Total CEC	Cmol(+)/kg	34	65	86	17
Calcium/magnesium ratio		2.6	2.7	0.59	0.49
Exchangeable calcium	%	9	53	13	7
Exchangeable potassium	%	69	17	49	75
Exchangeable magnesium	%	3.5	20	21	14
Exchangeable sodium	%	18	11	17	3.9

The trial design consisted of treatments completely randomised to healthy/diseased paired vines. Paired vines (healthy and diseased), in close proximity but not adjacent, were given one of six treatments (Rice hulls, Biochar, composted cow manure, composted green waste and potential biocontrol agent MW555 (*Streptomyces violaceoruber*). The latter treatment was applied a year later, 22 October, 2009. The rice hulls were obtained from the CopRice Leeton Feedmill, Leeton, NSW. The biochar was produced from poultry litter produced in the BEST Energies Pty Ltd continuous slow pyrolysis pilot unit, Somersby, NSW (Chan *et al.* 2008). It was manufactured at a temperature of 550°C and was activated using high temperature steam. Composted cow manure was obtained from Rivcow Environmental Pty Ltd, Yanco, NSW. The composted green waste was obtained from Riverina Compost Mulching, Gregadoo Waste Management Centre, Wagga Wagga, NSW. Volumes of soil amendment, equivalent to 1.2 kg C, were

placed 5 cm deep (by mattock) 30 cm either side of vines within the vine row. Equivalent tillage was performed for the control, nil organic amendment, vines. The mattock was washed and sterilised after every vine. Ammonium-N was applied to the soil to produce a C/N ratio of 10 for all treatments except composted green waste and composted cow manure which already had C/N ratio of 10.

S. violaceoruber MW555 was grown in Tryptone Yeast Extract (TYE) broth at 25°C until the concentration was 106 cfu mL^{-1}. The cultures were centrifuged (4,000 rpm for 10 min), washed twice in sterile deionised water (SDW) and resuspended in SDW. 500 mL spore suspension was poured into two depressions (40 cm×10 cm, 5 cm deep) on either side of the trunk.

Yields were determined by hand-harvesting all vines just before the commercial harvest. The number of bunches per vine were counted at harvest.

Soil and root sampling and testing

Prior to applying the treatments (T_0), a thermometer was inserted into the soil 20 cm from the trunk along the vine row, and the temperature was noted. At T_0 and thereafter in November and June of each season, three soil cores (0–20 cm deep) were obtained from each vine at positions 25 cm from either side of the trunk. The cores were cut in half and three shallow (0–10 cm deep) and three deeper (10–20 cm deep) half cores were bulked and placed in a zip-lock plastic bag on ice. Gravimetric soil moisture (oven dried at 105°C for 24 hour) was determined for each composite soil sample. Soil chemical analyses were performed by Pivot Limited, Werribee, Victoria from bulk soil collected November 2008 (T_0). Roots were separated from bulk soil analysed for starch. Both roots and bulk soil were analysed for beneficial and parasitic nematodes at each sampling. A sub-sample of 1 kg $vine^{-1}$ was taken to the laboratory for nematode assessment and soil moisture determination (oven dried at 105°C for 48 hours). Samples were stored in a cool room at ca. 4° until processing.

Soil baiting experiments

Forty soil cores (diameter 6 cm, 0–25cm depth) were obtained from each field trial (20 diseased and 20 asymptomatic vines) for a plant root baiting study. The cores were placed in the centre of 8 cm diameter pots and sterile (autoclaved three times in three days) potting mix was used to fill in the edges. Single node Chardonnay rooted cuttings (tested disease free by plating of surface sterilisation of roots as previously described) were planted in the cores by cutting a large slit with a knife, with minimal root disturbance. The plants were watered to 80% field capacity for twelve months and then destructively sampled. The roots were scored for root health, where: 0=no roots remaining; 1=75–100% blackened; 2=50% blackened; 3=33% blackened; and 4=100% healthy roots. Root dry weight (50°C until constant weight) was determined and root sub-samples were surface sterilised and incubated on DRBC and PDA as described previously for isolation of root pathogenic fungi.

In June, July 2010, weighed grapevine debris were enclosed in 250×350 mm bags, constructed from 7 mm-aperture plastic mesh. Each bag to contain weighed sub-samples (three items each) of cane (25 cm long), whole rachis and tendril (5–8 cm long), collected on same day from the vineyard floor. The vine debris bags were placed 5 cm under the soil surface under 20 asymptomatic and 20 diseased vines. After five months, the bags were retrieved and the contents were washed, surface sterilised and plated as previously described.

Nematode extraction, count and identification

From each sample, two 200 g soil sub-samples were processed for nematode extraction using the Whitehead tray method (Whitehead and Hemming, 1965) with a 5-day incubation period. The resulting suspension was passed twice through a 15 µm nylon sieve. The sieve was back washed to collect nematodes in a 75 ml plastic container. Then nematodes in the supernatant were counted using a Doncaster counting dish (Doncaster, 1962) under a stereo-microscope at 50×. Nematodes were identified either to family, genus or species levels. Nematode populations from two duplicate samples from each replication were averaged and numbers were calculated to population density kg^{-1} dry soil by using the soil moisture percentage (oven dried at 105°C for two days) recorded from each sample in each season.

Soil and root fungi and bacteria

Soil and root bacteria and fungi were analysed in November 2008 (T_0) and two years later. Bulk soil was sieved (0.5 cm) and 10 g (moist weight) representative samples vortex mixed with 90 mL phosphate-buffered saline (pH 7.2) (PBS) for 10 s, sonicated at 260 W cm^{-2} for 15 s and orbitally shaken at 290 rpm for 30 min on ice. The grapevine roots retrieved from each soil sample were washed under tap water, blotted dry and 1 g (fw) was vortex mixed with 9 ml PBS

for 10 s, sonicated at 260 W cm^{-2} for 15 s and shaken at 290 rpm for 30 min on ice. The suspensions were serially diluted and aliquots (0.1 ml) were spread onto solid media and incubated in darkness at 25°C.

Copiotrophic pseudomonads were selectively cultured on Pseudomonas agar CCF (Oxoid). Cellulolytic bacteria were cultured on cellulose bacterial agar (CBA) (Tuitert *et al.* 1998)containing carboxymethyl cellulose as the sole source of C plus the antibiotic cycloheximide. Colonies were counted after 4 days (fast growing cellulolytic bacteria) and also after 84 days (total cellulolytic bacteria). Nutrient Benomyl Agar (NBA) (Oxoid Nutrient agar 30 mg ml^{-1} benomyl fungicide) was used to isolate copiotrophic bacteria which are able to grow quickly (4 days) on a high C medium containing high salt concentrations. Dilute Nutrient Benomyl Agar (DNBA) (100-fold diluted Oxoid Nutrient Agar with 30 mg mL^{-1} benomyl) was used to isolate bacteria able to grow on a low C medium (Hattori and Hattori 2000). Colonies were counted after 4 days (fast growing low nutrient bacteria) and those that grew from 5 days to 84 days (slow growing oligotrophic bacteria). General fungi were isolated on dichloran rose bengal chloramphenicol (DRBC) agar. Cellulolytic fungi were isolated on cellulose Czapek chloramphenicol agar which was modified from cellulose Czapek agar (Omar and Abdel-Sater 2000) with chloramphenicol (100 mg ml^{-1}) instead of ampicillin.

Fungal root pathogens were isolated from grapevine roots by surface sterilisation and plating on solid media. Root segments were surface-sterilised for 3 min with sodium hypochlorite (1% active chlorine), and rinsed three times with sterile deionised water (SDW). Thin disks cut from whole cross sections were placed on Dichloran Rose Bengal Chloramphenicol agar (DRBC; Oxoid) and incubated at 25°C for three to 14 days in darkness. All developing fungal colonies were transferred to potato dextrose agar (PDA; Oxoid) and identified to genus level. The data were used to compute 'General ratio fungi:bacteria" (ratio DRBC fungi to total oligotrophic bacteria) and 'Cellulolytic ratio fungi:bacteria" (ratio cellulolytic fungi to cellulolytic bacteria).

Root structure

Washed roots were placed into a Perspex tray for scanning using an Epson scanner. Images were saved and analysed using WinRHIZO Pro 2007c software. The root-scanning software determined (i) total root length; (ii) total root surface area; and (iii) the percentage of each of total root length, total root surface area and total number of root tips in each of the following classes of root diameter (0–0.5 mm, 0.6–1.0 mm, 1.1-1.5 mm, 1.6-2.0 mm, 2.1-2.5 mm, 2.6-3.0 mm, 3.1-3.5 mm, 3.6-4.0 mm, >4.1 mm).

Root starch

For root starch content, root samples were oven dried at 60°C, and ground through a heavy-duty cutting mill (Restch SM2000, Hann, Germany) to 5 mm and then a subsample was ground to 0.12 mm in a centrifugal mill (Retsch ZM200). A 20 mg subsample was suspended in 200 mL of dimethylsulfoxide and heated at 98°C for 10 min. The remainder of the analysis was then performed using commercial starch and glucose assay kits (Megazyme International). Briefly, 300 mL of thermostable α-amylase in 3-(N-orpholino) propanesulfonic acid buffer was added, mixed and incubated for 15 min in a 98°C water bath. After cooling, 400 mL of amyloglucosidase in sodium acetate buffer was added and incubated at 50°C for 60 min. The samples were mixed at 20 min intervals and then centrifuged at 8000 g for 2 min. Supernatant from root samples was diluted 1:11, and trunk and shoot samples were diluted 1:6 in ultra pure water. Glucose concentration of the diluted samples was then determined colorimetrically at 510 nm against glucose standards and the amount of starch in the original 20 mg sample calculated.

Juice and wine quality

The effect of YVD (both *Cylindrocarpon* and *Botryosphaeria*) on juice and wine quality was also of concern, because berries from the diseased vines with sparse canopies were commonly observed to be sun-burned. In February 2009 mini-ferments were produced. Juice (3 L) was prepared from manually crushed berries from diseased or asymptomatic vines (five replicates). 100 mg L^{-1} PMS was added and the juice was allowed to cold settle at 2°C for two days. Settled juice (900 mL) was siphoned off into sterile 1 L Schott bottles and warmed to 16°C. A 50 mL subsample was collected and checked for pH, TA, YAN (Foss Wine Scan), temperature and Baumé. Juice pH was adjusted to pH 3.4 with tartaric acid and fermentation was initiated with GoFerm and yeast (DV10). The 1 L flasks were weighed twice daily and sugar consumption vs time was graphed. When the ferment was completed, the wine was allowed to cold settle for three days at 2°C and was racked off the lees into clean 1 L Schott bottles, PMS was added and the head space was filled with nitrogen gas. Difference

testing was conducted between wines from the 'healthy' and diseased vines.

Fungicides -L-tryptophan and sucrose

A well was formed under every vine (5 cm deep by sterilised mattock, 30 cm either side of vines within the vine row). Equivalent tillage was performed for the control, nil organic amendment, vines. Solutions of Benomyl (5.5 mg a.i. per vine); cyprodinil/fludioxinil mixture (375 g kg^{-1} and 250 g kg^{-1} respectively; 1 mg $vine^{-1}$); L-tryptophan (400 mg $vine^{-1}$); sucrose (67 g $vine^{-1}$); or L-tryptophan plus sucrose (500 mg and 67 g $vine^{-1}$, respectively) were poured into the wells with 10 L water. For the first year, three shoots per cordon of every vine were tagged and their length measured weekly. Pruning weights, yields and bunch numbers were also determined.

Xylem function

In November 2007 and just before harvest February 2010, five vines were cut at the base of the trunk underwater (to prevent embolisms) and placed in beakers containing acid fuchsin (0.5%) and left in the field for eight hours in bright sunny conditions. The trunks were hand sectioned and observed under light microscopy.

Data analysis.

Statistical analysis was conducted using GenStat 14th Edition (VSN, Herts, UK). An analysis of variance was applied separately for each site to detect differences among the treatments. Differences between means were detected using a least significant difference (LSD) test (p=0.05).

Results and discussion

The diseased vines within both vineyards had significantly lower yields and bunch numbers every year (*Table 2.39*). Over the three seasons average annual yields ranged from 37–69% lower, and numbers of bunches per vine ranged from 33–62% lower for the diseased vines.

Chemical soil properties

In both vineyards, there were few differences in soil chemical and physical characteristics between the diseased and asymptomatic vines. Compared to asymptomatic vines, diseased vines had lower CEC-potassium in vineyard A but higher in vineyard B; lower available phosphorus in vineyard A but higher in vineyard B; equivalent pH in vineyard A but lower at vineyard B; lower organic carbon in vineyard A but equivalent in vineyard B; lower zinc in vineyard A but equivalent in vineyard B; a non-significant trend to higher CEC-sodium in both vineyards; and lower boron at vineyard A but equivalent at vineyard B. Both vineyards had high soil dispersion indexes, indicating poor soil structure but there was no evidence for higher dispersion index associated with YVD disease. In both vineyards soil CEC-potassium, nitrate-N, iron and manganese were higher than recommended for wine grapes. Total organic carbon and sulphate-S were lower than recommended in vineyard A. Soil pH under diseased vines in vineyard B was also lower than recommended (*Table 2.40*).

Isolation of Cylindrocarpon

Cylindrocarpon was consistently isolated from roots of diseased grapevines. *Cylindrocarpon* was also isolated from the roots of all bate Chardonnay vines

Table 2.39 Effect of YVD disease on vine and berry measurements over time.

	Yield February 2009 (kg/vine)	Bunches per vine February 2009	Yield February 2010 (kg/vine)	Bunches per vine February 2010	Yield February 2011 (kg/vine)	Bunches per vine February 2011
Vineyard A (flood irrigated, Chardonnay on Ramsey)						
Diseased vines	4.40 b	64.3 b	4.49 b	88.4 b	8.74 b	96.2 b
Asymptomatic vines	14.37 a	167.7 a	9.79 a	135.9 a	13.77 a	144.1 a
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
lsd	2.512	19.19	1.126	13.39	1.699	16.85
Vineyard B (drip irrigated, Chardonnay)						
Diseased vines	9.00	136.0	11.25	161.4	8.97	89.1
Asymptomatic vines	14.96	216.6	15.37	207.0	11.67	118.8
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
LSD	0.894	18.84	1.502	22.90	1.26	14.39

Values within a column followed by the same letter are not significantly different based on l.s.d. (P=0.05). Means of 25 replicate determinations are shown.

planted in the 'diseased' soil, indicating that the soil must have been contaminated with this pathogen (*Table 2.41*). The Chardonnay bait plants in soil from the diseased field trial vines had either lower root dry weight (flood irrigated vineyard A) or visual root health (drip irrigated vineyard B).

Grapevine pruning material buried under diseased vines, but not those buried under asymptomatic vines, were found to be colonised by *Cylindrocarpon* (data not shown) indicating a strong correlation between this fungus and the disease.

Soil and root nematode populations

Shallow soil

In vineyard A (flood irrigated) in November 2008 (T_0) before the application of soil organic amendments, there were no significant differences in soil parasitic nematode populations between the diseased and asymptomatic vines (*Tables 2.42a* and *2.42b*) although there was a non-significant trend towards lower free-living nematode populations under diseased vines (*Table 2.42a*, P=0.08). In vineyard B (drip irrigated) there were no significant differences

Table 2.40 Chemical soil properties for the field trials before treatments were applied.

	Asymptomatic vines	Diseased vines	P	l.s.d.
Vineyard A (flood irrigated)				
CEC-potassium	2.453[1] a	2.087[1] b	0.006	0.2438
Available phosphorus (Colwell)	164.70[1] a	148.00[1] b	0.007	11.21
pH ($CaCl_2$)	6.373	6.307	0.478	0.1960
Total organic carbon (%)	0.991[2] a	0.960[2] b	0.011	0.0299
Zinc (mg/kg)	1.747 a	1.620 b	0.042	0.1211
CEC-sodium	0.126	0.165	0.056	0.0399
Boron (mg/kg)	1.453	1.320	0.017	0.1060
EC (ds/m)	0.1160	0.1060	0.200	0.01594
Chloride (mg/kg)	2.27	2.00	0.858	3.136
Sulfate-S (MCP) (mg/kg)	7.54[2]	6.35[2]	0.108	1.484
Nitrate-N (mg/kg)	15.8[1]	17.2[1]	0.567	5.12
Aluminium	0	0	-	-
CEC-Calcium	9.80	9.97	0.494	0.509
CEC-Magnesium	4.393	4.440	0.536	0.1578
Iron (DTPA) (mg/kg)	23.00[1]	21.07[1]	0.199	3.072
Manganese (DTPA)(mg/kg)	13.64[1]	12.97[1]	0.498	2.078
Ammonium-N	2.09	1.71	0.228	0.656
Dispersion index	10.10[1]	6.30[1]	0.308	7.71
Vineyard B (drip irrigated)				
CEC-potassium	1.20[1]	1.43[1]	0.055	0.2409
Available phosphorus (Colwell)	46.20 a	68.30 b	0.009	15.76
pH ($CaCl_2$)	5.61	5.24[2]	0.050	0.3659
Total organic carbon (%)	1.51	1.42	0.166	0.1271
Zinc (mg/kg)	2.66	2.19	0.331	1.001
CEC-sodium	0.19	0.26	0.053	0.0730
Boron (mg/kg)	0.64	0.75	0.125	0.693
EC (ds/m)	0.09	0.14	0.061	0.0520
Chloride (mg/kg)	15.0[1]	31.0[1]	0.241	28.03
Sulfate-S (MCP) (mg/kg)	15.7	27.0	0.117	21.30
Nitrate-N (mg/kg)	19.0[1]	29.1[1]	0.183	15.49
Aluminium (mg/kg)	10.5[1]	7.7[1]	0.175	0.0488
CEC-Calcium	6.25	6.91	0.307	1.349
CEC-Magnesium	3.92	3.88	0.754	0.2679
Iron (DTPA) (mg/kg)	141.0[1]	164.0[1]	0.103	28.63
Manganese (mg/kg)	12.4[1]	13.0[1]	0.757	4.121
Ammonium-N	3.73	2.74	0.211	1.622
Dispersion index	22.3[1]	5.5[1]	0.163	24.59

Values within a row followed by the same letter are not significantly different, based on l.s.d<0.05. Means of 25 replicate determinations are shown. [1] considered too high for wine grapes; and [2] too low. (Lanyon, Cass and Hansen, 2004)

Table 2.41 Soil baiting experiment

	Asymptomatic vines	Diseased vines	P	l.s.d.
Vineyard A (flood irrigated)				
Root dry weight (g)	3.14	1.84	0.017	0.938
Visual root health	3.68	3.93	0.286	0.500
Cylindrocarpon isolated from roots	No	Yes	-	-
Vineyard B (drip irrigated)				
Root dry weight (g)	3.11	2.72	0.291	0.748
Visual root health	3.50	3.00	0.015	0.38
Cylindrocarpon isolated from roots	No	Yes	-	-

Values within a row followed by the same letter are not significantly different, based on l.s.d<0.05. Means of 20 replicate determinations are shown.

Table 2.42a Soil nematodes[1] –shallow soil in vineyard A (flood irrigated), November 2008 (T_0).

Disease status of vines	Dorylaimidae (omnivorous)	*Rhabditis* (bacterial feeder)	Total free-living nematodes (TFN)	Total plant parasitic nematodes (TPPN)	Ratio TFN:TPPN
Diseased	6.78	7.01	7.67	1.75	1.909
Healthy	7.17	7.40	8.07	1.89	1.880
P	0.136	0.139	0.080	0.824	0.829
LSD	0.901	0.520	0.455	1.289	0.3451

[1] Units: loge(nematode density+1)m^{-2}. Means of 25 replicate determinations are shown.

Table 2.42b Soil nematodes[1] –deep soil in vineyard A (flood irrigated), November 2008 (T_0).

Disease status of vines	Dorylaimidae (omnivorous)	Rhabditis (bacterial feeder)	Total free-living nematodes (TFN)	Total plant parasitic nematodes (TPPN)	Ratio TFN:TPPN
Diseased	5.46	5.38	6.20	6.98	0.903
Healthy	5.64	5.26	6.20	6.79	0.949
P	0.521	0.574	0.988	0.545	0.374
LSD	0.556	0.435	0.412	0.657	0.1053

[1] Units: loge(nematode density+1)m^{-2}. Means of 25 replicate determinations are shown.

Table 2.42c Soil nematodes[1] –shallow soil in vineyard B (drip irrigated), November 2008 (T_0).

Disease status of vines	Dorylaimidae (omnivorous)	Rhabditis (bacterial feeder)	Total free-living nematodes (TFN)	Total plant parasitic nematodes (TPPN)	Ratio TFN:TPPN
Diseased	5.45	4.77	6.00	5.51	1.108
Healthy	5.51	3.92	5.93	5.40	1.134
P	0.800	0.127	0.801	0.708	0.686
LSD	0.515	1.105	0.561	0.598	0.1259

[1] Units: loge(nematode density+1)m^{-2}. Means of 25 replicate determinations are shown.

Table 2.42d Soil nematodes[1] –deep soil in vineyard B (drip irrigated), November 2008 (T_0).

Disease status of vines	Aporcelaimellus (predatory)	Dorylaimidae (omnivorous)	Rhabditis (bacterial feeder)	Total free-living nematodes (TFN)	Total plant parasitic nematodes (TPPN)	Ratio TFN:TPPN
Diseased	0.00 b	6.10	4.14	6.33	6.69	0.947 b
Healthy	0.61 a	5.52	3.04	5.74	6.94	0.833 a
P	0.029	0.102	0.089	0.119	0.320	0.015
LSD	0.570	0.701	1.272	0.741	0.500	0.0906

[1] Units: loge(nematode density+1)m^{-2}. Values within a column followed by the same letter are not significantly different, based on l.s.d < 0.05. Means of 25 replicate determinations are shown.

in soil parasitic nematode populations between the diseased and asymptomatic vines (*Tables 2.42c* and *2.42d*). Diseased vine soil had lower soil predatory nematodes (*Aporcelaimellus*) (*Table 2.42d*).

Two years later (November 2010) composted green waste had increased the numbers of bacterial feeder Rhabditis and total free-living nematodes in shallow soil (0–10 cm depth) in vineyard A (flood), (*Table 2.43b* and *2.43c*) but there were no significant differences between diseased and asymptomatic vines in shallow soil in either vineyard (*Table 2.43a*, *2.43d*, *2.43e* and *2.43f*).

Table 2.43a Soil nematodes[1] showing data for shallow soil in vineyard A (flood irrigated), combined treatments in November 2010.

Treatment	Dorylaimidae (omnivorous)	Rhabditis (bacterial feeder)	Total free-living nematodes (TFN)	Total plant parasitic nematodes (TPPN)	Ratio TFN:TPPN
Diseased	6.95	8.60	8.79	4.72	1.895
Healthy	6.85	8.75	8.90	4.77	1.923
P	0.526	0.242	0.395	0.821	0.739
lsd	0.308	0.255	0.250	0.416	0.1674

[1] Units: loge(nematode density+1)m^{-2}. Means of 25 replicate determinations are shown.

Table 2.43b Soil nematodes[1] showing data for shallow soil in vineyard A (flood irrigated), combined diseased and asymptomatic vines, in November 2010.

Treatment	Dorylaimidae (omnivorous)	Rhabditis (bacterial feeder)	Total free-living nematodes (TFN)	Total plant parasitic nematodes (TPPN)	Ratio TFN:TPPN
Control	6.95	8.69 b	8.86 b	4.70	1.926
Biochar	6.81	8.36 b	8.55 b	4.47	1.964
Composted cow manure	7.71	8.77 ab	8.90 ab	5.24	1.749
Rice Hulls	6.89	8.48 b	8.67 b	4.59	1.950
Composted green waste	6.96	9.20 a	9.31 a	4.79	1.958
P	0.780	0.008	0.024	0.372	0.668
lsd	0.534	0.442	0.433	0.721	0.290

1 Units: loge(nematode density+1)m^{-2}. Values within a column followed by the same letter are not significantly different, based on l.s.d < 0.05. Means of 10 replicate determinations are shown.

Table 2.43c Soil nematodes[1] showing data for shallow soil in vineyard A (flood irrigated), all treatments for diseased or asymptomatic vines, in November 2010.

Treatment	Disease status	Dorylaimidae (omnivorous)	Rhabditis (bacterial feeder)	Total free-living nematodes (TFN)	Total plant parasitic nematodes (TPPN)	Ratio TFN:TPPN
Control	Diseased	7.166	8.607 b	8.823 ab	4.55	1.941
	Healthy	6.731	8.769 ab	8.898 ab	4.85	1.911
Biochar	Diseased	6.513	7.996 b	8.210 b	4.18	2.008
	Healthy	7.102	8.713 ab	8.898 ab	4.77	1.919
Composted cow manure	Diseased	6.950	9.087 ab	9.201 ab	5.26	1.820
	Healthy	6.475	8.458 b	8.592 b	5.22	1.677
Rice Hulls	Diseased	7.086	8.506 b	8.723 b	4.36	2.015
	Healthy	6.700	8.455 b	8.624 b	4.82	1.884
Composted green waste	Diseased	6.968	9.241 a	9.360 a	4.88	1.927
	Healthy	6.957	9.153 ab	9.263 ab	4.70	1.990
P		0.305	0.022	0.032	0.382	0.240
lsd		0.7552	0.6254	0.6125	1.019	0.4100
Grand mean		6.902	8.675	8.847	4.75	1.909

1 Units: loge(nematode density +1)m-2. Values within a column followed by the same letter are not significantly different, based on l.s.d < 0.05. Means of 5 replicate determinations are shown.

Table 2.43d Soil nematodes[1] showing data for shallow soil in vineyard B, combined treatments in November 2010.

Treatment	Dorylaimidae (omnivorous)	Rhabditis (bacterial feeder)	Total free-living nematodes (TFN)	Total plant parasitic nematodes (TPPN)	Ratio TFN:TPPN
Diseased	6.99	6.72	7.72	5.60	1.415
Healthy	6.89	6.56	7.53	5.38	1.443
P	0.642	0.555	0.368	0.461	0.655
lsd	0.464	0.561	0.421	0.583	0.1250

[1] Units: loge(nematode density +1)m^{-2}. Means of 25 replicate determinations are shown.

Table 2.43e Soil nematodes[1] –shallow soil in vineyard B (drip irrigated), combined diseased and asymptomatic vines, in November 2010.

Treatment	Dorylaimidae (omnivorous)	Rhabditis (bacterial feeder)	Total free-living nematodes (TFN)	Total plant parasitic nematodes (TPPN)	Ratio TFN:TPPN
Control	7.46	6.75	8.05	5.05	1.621
Biochar	6.85	7.06	7.68	5.96	1.308
Composted cow manure	6.49	6.63	7.37	5.53	1.359
MW555	6.98	6.30	7.48	5.05	1.495
Rice Hulls	7.07	6.67	7.76	5.99	1.365
Composted green waste	6.79	6.42	7.40	5.36	1.425
P	0.278	0.692	0.411	0.240	0.066
lsd	0.803	0.972	0.730	1.010	0.2166

[1] Units: loge(nematode density +1)m^{-2}. Means of 10 replicate determinations are shown.

Table 2.43f Soil nematodes[1] – shallow soil in vineyard B (drip irrigated), all treatments for diseased or asymptomatic vines, in November 2010.

Treatment	Disease status	Dorylaimidae (omnivorous)	Rhabditis	Total free-living nematodes (TFN)	Total plant parasitic nematodes (TPPN)	Ratio TFN:TPPN
Control	Diseased	7.55	7.08	8.23	4.98	1.676
	Healthy	7.36	6.41	7.87	5.12	1.566
Biochar	Diseased	6.63	7.21	7.65	6.25	1.247
	Healthy	7.07	6.92	7.71	5.68	1.368
Composted cow manure	Diseased	6.70	6.88	7.60	6.05	1.265
	Healthy	6.28	6.37	7.13	5.01	1.453
MW555	Diseased	7.13	6.67	7.76	5.41	1.445
	Healthy	6.84	5.94	7.21	4.68	1.545
Rice hulls	Diseased	7.31	6.56	7.93	5.93	1.400
	Healthy	6.82	6.78	7.59	6.05	1.330
Composted green waste	Diseased	6.63	5.93	7.14	4.97	1.456
	Healthy	6.94	6.92	7.67	5.75	1.394
P		0.790	0.459	0.669	0.478	0.653
lsd		1.136	1.375	1.032	1.428	0.3063
Grand mean		6.94	6.64	7.62	5.49	1.429

[1] Units: loge(nematode density +1)m^{-2}. Means of 5 replicate determinations are shown.

Deeper soil

Vineyard A (flood irrigated)

In contrast to results from shallow soil, in deeper soil (10–20 cm depth) of vineyard A (flood irrigated) total parasitic nematode populations were higher in soil under diseased vines and the ratio of free living to parasitic nematodes (TFN:TPPN) was significantly greater under the asymptomatic vines (*Table 2.44a*). Rice hulls and *Streptomyces violaceoruber* MW555 had significantly increased the soil Dorylaimidae (omnivorous) nematode populations but these were decreased with Biochar. Rice hulls and composted green waste increased the bacterial feeding Rhabditis populations while Biochar, composted cow manure and rice hulls decreased the total soil parasitic nematode numbers (*Table 2.44b*). Parasitic

Table 2.44a Soil nematodes[1] –deep soil in vineyard A (flood irrigated), combined treatments in November 2010.

Treatment	Dorylaimidae (omnivorous)	Rhabditis (bacterial feeder)	Total free-living nematodes (TFN)	Total plant parasitic nematodes (TPPN)	Ratio TFN:TPPN
Diseased	6.183	7.543	7.843	6.15 a	1.35 b
Healthy	6.114	7.619	7.866	5.53 b	1.50 a
P	0.647	0.662	0.878	0.006	0.041
lsd	0.299	0.349	0.303	0.427	0.145

[1] Units: loge(nematode density +1)m^{-2}. Values within a column followed by the same letter are not significantly different, based on l.s.d<0.05. Means of 25 replicate determinations are shown.

Table 2.44b Soil nematodes 1 – deep soil in vineyard A (flood irrigated), combined diseased and asymptomatic vines in November 2010.

Treatment	Dorylaimidae (omnivorous)	Rhabditis (bacterial feeder)	Total free-living nematodes (TFN)	Total plant parasitic nematodes (TPPN)	Ratio TFN:TPPN
Control	6.272 ab	7.121 c	7.508 cd	6.64 a	1.140 c
Biochar	5.613 c	6.701 c	7.076 d	4.18 d	1.726 a
Composted cow manure	5.786 b	7.403 bc	7.601 cd	5.37 c	1.485 ab
S. violaceoruber MW555	6.593 a	7.344 bc	7.763 bc	6.55 a	1.191 c
Rice Hulls	6.661 a	8.516 a	8.695 a	5.40 c	1.690 a
Composted green waste	5.965 b	8.399 a	8.484 a	6.89 a	1.298 bc
P	<0.001	<0.001	<0.001	<0.001	<0.001
lsd	0.5186	0.6051	0.5250	0.740	0.2502

[1] Units: loge(nematode density +1)m^{-2}. Values within a column followed by the same letter are not significantly different, based on l.s.d<0.05. Means of 10 replicate determinations are shown.

Table 2.44c Soil and root nematodes[1] –deep soil in vineyard A (flood irrigated), all treatments for diseased or asymptomatic vines in November 2010.

Treatment	Disease status	Dorylaimidae (omnivorous)	Rhabditis (bacterial feeder)	Total free-living nematodes (TFN)	Total plant parasitic nematodes (TPPN)	Ratio TFN:TPPN
Control	Diseased	6.276	7.018	7.428	6.40 bc	1.160 bc
	Healthy	6.267	7.225	7.587	6.87 bc	1.121 bc
Biochar	Diseased	5.642	6.494	6.964	4.18 e	1.681 a
	Healthy	5.584	6.908	7.189	4.18 e	1.771 a
Composted cow manure	Diseased	5.650	7.228	7.413	6.11 c	1.224 bc
	Healthy	5.923	7.578	7.788	4.64 d	1.746 a
MW555	Diseased	6.706	7.157	7.684	6.81 bc	1.128 bc
	Healthy	6.480	7.532	7.841	6.29 cd	1.254 bc
Rice hulls	Diseased	6.736	8.588	8.741	4.96 d	1.828 a
	Healthy	6.586	8.44	8.650	5.85 cd	1.552 ab
Composted green waste	Diseased	6.084	8.771	8.827	8.41 a	1.057 c
	Healthy	5.845	8.027	8.141	5.38 cd	1.538 ab
P		0.924	0.358	0.395	<0.001	0.020
lsd		0.7335	0.8557	0.7425	1.047	0.3539
Grand mean		6.148	7.581	7.854	5.84	1.422

[1] Units: loge(nematode density +1)m^{-2}. Values within a column followed by the same letter are not significantly different, based on l.s.d<0.05. Means of 5 replicate determinations are shown.

Table 2.44d Soil nematodes[1] –deep soil in vineyard B, combined treatments in November 2010.

Treatment	Dorylaimidae (omnivorous)	Rhabditis (bacterial feeder)	Total free-living nematodes (TFN)	Total plant parasitic nematodes (TPPN)	Ratio TFN:TPPN
Diseased	5.56	6.31	6.77	6.71	1.026
Healthy	5.98	6.29	6.95	7.21	0.980
P	0.163	0.958	0.567	0.125	0.234
lsd	0.608	0.703	0.633	1.667	0.0768

1 Units: loge(nematode density +1)m-2. Means of 25 replicate determinations are shown.

Table 2.44e Soil nematodes[1] – deep soil in vineyard B (drip irrigated), combined diseased and asymptomatic vines in November 2010.

Treatment	Dorylaimidae (omnivorous)	Rhabditis (bacterial feeder)	Total free-living nematodes (TFN)	Total plant parasitic nematodes (TPPN)	Ratio TFN:TPPN
Control	5.52	6.04	6.56	7.20	0.924 b
Biochar	5.87	6.60	7.13	7.43	0.964 b
Composted cow manure	5.63	6.77	7.10	6.40	1.125 a
MW555	5.30	5.84	6.41	7.04	0.914 b
Rice Hulls	5.52	5.69	6.24	6.55	0.979 b
Composted green waste	6.73	6.85	7.69	7.15	1.113 a
P	0.110	0.250	0.094	0.470	0.005
lsd	1.053	1.217	1.096	1.179	0.1329

[1] Units: loge(nematode density +1)m^{-2}. Values within a column followed by the same letter are not significantly different, based on l.s.d < 0.05. Means of 10 replicate determinations are shown.

Table 2.44f Soil nematodes[1] –deep soil in vineyard B (drip irrigated), showing data for all treatments for both diseased or asymptomatic vines in November 2010.

Treatment	Disease status	Dorylaimidae (omnivorous)	Rhabditis (bacterial feeder)	Total free-living nematodes (TFN)	Total plant parasitic nematodes (TPPN)	Ratio TFN:TPPN
Control	Diseased	5.34	5.85	6.42	6.88	0.951
	Healthy	5.70	6.23	6.70	7.52	0.896
Biochar	Diseased	5.84	6.59	7.17	7.25	0.992
	Healthy	5.90	6.61	7.09	7.60	0.937
Composted cow manure	Diseased	5.61	6.77	7.08	6.17	1.173
	Healthy	5.66	6.78	7.11	6.63	1.077
MW555	Diseased	5.33	6.18	6.71	7.34	0.917
	Healthy	5.27	5.51	6.12	6.73	0.910
Rice hulls	Diseased	4.83	5.18	5.55	5.28	1.076
	Healthy	6.20	6.21	6.93	7.83	0.883
Composted green waste	Diseased	6.34	7.28	7.66	7.35	1.048
	Healthy	7.13	6.41	7.73	6.96	1.179
P		0.725	0.651	0.606	0.125	0.276
lsd		1.489	1.721	1.549	1.667	0.1880
Grand mean		5.76	6.30	6.86	6.96	1.003

[1] Units: loge(nematode density +1)m^{-2}. Means of 5 replicate determinations are shown.

nematode populations in healthy vine soil were reduced by Biochar and composted cow manure. Parasitic nematode populations under diseased vines were decreased by Biochar and rice hulls, but were increased by composted green waste. Biochar increased the ratio of beneficial to parasitic nematodes (TFN:TPPN) on both healthy and diseased vines; whereas composted cow manure increased it mainly on healthy vines, and rice hulls increased it only on diseased vines (*Table 2.44c*).

Vineyard B (drip irrigated)

In deep soil (10–20 cm depth) of vineyard B (drip) there were no significant differences in soil parasitic nematode populations between the diseased and asymptomatic vines (*Table 2.43d*, *2.43f*). However, Composted cow manure and composted green waste had both increased the ratio of beneficial to parasitic nematodes (TFN:TPPPN) (*Table 2.44e*).

Soil and root fungi and bacteria

In comparison with 'healthy' soil, diseased soil was less fungal dominated. Vineyard A diseased soil had lower fungi:bacteria ratios (both general and cellulolytic) and lower cellulolytic fungal populations. Vineyard B diseased soil also had a lower general fungal population. Diseased soil in vineyard A also had less oligotrophic bacteria (*Table 2.45a*).

Two years of soil organic amendment caused changes in the soil bacterial and fungal composition, but the effect was much greater in vineyard A where biochar increased the fungi to bacteria ratios (general and cellulolytic) and cellulolytic fungal population. Biochar, composted cow manure and rice hulls also increased the numbers of oligotrophic bacteria. However, in vineyard B none of the organic amendments had yet made any impact on the soil microbial community (*Table 2.45b*).

Root structure

In comparison with 'healthy' soil, diseased soil had a significantly lower percentage of length of very fine roots (0–0.5 mm diameter) and there was also a trend towards a lower percentage 0.6–1 mm diameter. There was a trend towards the percentage of root length 1.1–1.5 mm diameter to be greater in the diseased soil, caused by the loss of finer roots (*Table 2.46a*). For roots 1.6–2.0 mm diameter (*Table 2.46a*) and above (data not shown), disease status did not influence the root diameter. These alterations indicate that *Cylindrocarpon* may have caused a loss of the finest roots, especially those of 0–0.5 mm diameter. *Cylindrocarpon* also appears to have caused a 29% decrease in the number of root forks, a measure of root branching (*Table 2.46a*).

Two years of soil organic amendment caused changes in the root diameter. Composted cow manure increased the length of 0–0.5 mm diameter roots and the root length density. Both composted cow manure and composted green waste decreased the proportion of 1.6–2.0 mm diameter roots. Every organic amendment increased the root average diameter, although this was not statistically significant (P=0.073). Biochar, composted green waste and rice hulls increased the soil moisture content and biochar increased the root length density by 233% (*Table 2.46b*).

Root starch

The spring root starch levels were significantly lower for the diseased vines. The asymptomatic vines had longer shoots (495 mm compared to 272 mm long) and yet still had more than double the root starch content (2.69% compared to 1.23%) (*Table 2.47*), indicating that starch reserves were depleted in the diseased vines.

Juice and wine quality

The juice from the diseased and asymptomatic vines had equivalent Baumé, pH and titratable acidity.

The juice from the diseased vines had significantly lower yeast available nitrogen, resulting in slower fermentation and significantly lower mass loss from sugar consumption (*Table 2.48*). However wine sensory evaluation by duo-trio difference testing showed that there were no significant differences in wine quality between the diseased and asymptomatic vines (data not shown).

Fungicides, L-tryptophan and sucrose

In Vineyard B (Chardonnay, drip irrigated) shoot lengths were significantly decreased, throughout the season, in diseased vines. There were insignificant trends towards increased shoot growth caused by Benomyl and cyprodinil/fludioxinil on 29 January, 2008 and the cyprodinil/fludioxinil mixture significantly increased the shoot length growth rate for diseased vines early in the season (*Figure 2.26*). However pruning weights were not increased by these fungicides (*Table 2.49a*). Tryptophan and sucrose had no effect on shoot lengths (*Figure 2.26*) but there were some non-significant indications that soil tryptophan application may have increased pruning weight (*Table 2.49a*).

Table 2.45a Effect of YVD disease on bacteria and fungi isolated from soil under diseased and asymptomatic vines.

	General ratio fungi to bacteria	Cellulolytic ratio fungi to bacteria	Copiotrophic bacteria (10^4 cfu/g)	Slow growing oligotrophic bacteria (10^4 cfu/g)	Copiotrophic pseudomonads (10^3 cfu/g)	Cellulolytic fungi (10^2 cfu/g)	DRBC (general) fungi (10^2 cfu/g)
Vineyard A (flood irrigated)							
Diseased vines	0.74	1.3	7.72	1.51	0.16	0	1.09
Asymptomatic vines	2.88	7.0	8.89	2.85	0.19	4.49	2.13
P	<0.001	<0.001	0.473	0.003	0.554	0.002	0.191
lsd	1.410	1.48	3.63	0.727	0.10	2.34	1.69
Vineyard B (drip irrigated)							
Diseased vines	1.02	0.75	3.22	4.11	1.22	1.00	2.61
Asymptomatic vines	3.05	0.86	7.94	5.17	0.11	1.67	3.70
P	0.124	0.488	0.520	0.403	0.520	0.763	0.042
lsd	2.385	7.037	9.149	3.353	1.92	6.311	1.042

Values within a column followed by the same letter are not significantly different based on l.s.d. (P=0.05). Means of 25 replicate determinations are shown.

Table 2.45b Effect of organic amendments on soil bacteria and fungi, two years after application.

	General ratio fungi to bacteria	Cellulolytic ratio fungi to bacteria	Copiotrophic bacteria (10^4 cfu/g)	Slow growing oligotrophic bacteria (10^4 cfu/g)	Pseudomonads (10^3 cfu/g)	Cellulolytic fungi (10^2 cfu/g)	DRBC (general) fungi (10^2 cfu/g)
Vineyard A (flood irrigated)							
Control	0.42 b	1.30 b	5.60	0.52 c	1.12	0.10 b	0.70
Biochar	3.59 a	12.04 a	4.89	1.25 b	1.07	6.06 a	1.02
Composted cow manure	0.84 b	0.97 b	9.41	3.50 a	2.66	0.68 b	0.99
Rice Hulls	1.24 b	2.07 b	8.46	4.48 a	1.41	1.32 b	1.52
Composted green waste	2.97 ab	1.75 b	13.17	1.16 bc	0.76	2.58 ab	3.81
P	0.045	<0.001	0.062	<0.001	0.149	0.029	0.137
lsd	2.229	1.90	5.742	1.149	1.606	3.700	2.675
Vineyard B (drip irrigated)							
Control	2.03	0.81	5.58	4.64	0.67	1.33	3.08
Biochar	2.72	0.98	8.14	2.11	0.92	1.72	3.08
Composted cow manure	2.50	1.15	7.64	2.72	0.51	2.36	3.14
Rice Hulls	3.24	0.75	8.36	3.92	0.92	1.58	2.36
Composted green waste	1.42	5.75	4.69	3.17	0.19	6.19	4.11
P	0.231	0.188	0.619	0.215	0.716	0.163	0.291
lsd	1.686	4.976	6.469	2.371	1.357	4.462	1.648

Values within a column followed by the same letter are not significantly different, based on l.s.d<0.05. Means of 10 replicate determinations are shown.

Table 2.46a Structure for roots collected from 0–20 cm depth under Chardonnay on Ramsey in flood irrigated Vineyard A (November 2010).

	Root length (cm)				Root average diameter (mm)	Root forks per cm root length	Root dry weight (g/cm³ soil)	Root length density (cm/cm³ soil)	Soil moisture (%)
	Diameter 0–0.5 mm	Diameter 0.6–1.0 mm	Diameter 1.1–1.5 mm	Diameter 1.6–2.0 mm					
Diseased vines	13.4 b	10.1	17.9	15.5	1.90	0.805	0.94	75	11.79
Asymptomatic vines	31.3 a	15.6	10.2	16.5	1.58	1.130	1.41	69	12.20
P	0.003	0.056	0.085	0.766	0.457	0.034	0.084	0.785	0.131
LSD	11.21	5.67	8.79	7.40	0.864	0.2975	0.536	45.7	0.538

Values within a column followed by the same letter are not significantly different based on l.s.d. (P=0.05). Means of 25 replicate determinations are shown.

Table 2.46b Effect of organic amendments on root diameters and branching, two years after application. Roots collected from 0-20cm depth under Chardonnay on Ramsey in flood irrigated Vineyard A (November 2010).

	Root length (cm)				Root average diameter (mm)	Root forks per cm root length	Root dry weight (g/cm³ soil)	Root length density (cm/cm³ soil)	Soil moisture (%)
	Diameter 0–0.5 mm	Diameter 0.6–1.0 mm	Diameter 1.1–1.5 mm	Diameter 1.6–2.0 mm					
Control	22.4 b	15.3	13.0	21.4 a	0.32	1.205 ab	0.79 b	34 b	11.04 c
Biochar	23.4 ab	9.8	12.7	26.4 a	2.02	0.971 ab	2.63 a	50 b	13.07 a
Composted cow manure	42.1 a	12.3	7.4	8.2 b	2.04	1.473 a	0.19 b	156 a	11.32 bc
Composted green waste	22.4 b	16.2	18.1	6.7 b	2.18	0.843 b	1.08 b	84 b	12.03 b
Rice Hulls	20.1 b	12.1	8.1	14.8 ab	2.46	0.853 b	1.35 ab	62 b	12.51 ab
MW555							2.40 a	46 b	12.01 b
P	0.018	0.775	0.192	0.027	0.073	0.009	0.020	0.041	0.003
LSD	19.42	9.82	15.23	12.82	1.497	0.5154	1.31	79.1	0.932

Values within a column followed by the same letter are not significantly different based on l.s.d. (P=0.05). Means of 10 replicate determinations are shown.

Table 2.47 Root starch and average shoot length for diseased and asymptomatic Chardonnay on Ramsey vines, Vineyard A, 28th October 2008.

	Average shoot length (mm)	Root starch
Diseased vines	272 b	1.23 b
Asymptomatic vines	495 a	2.69 a
P	<0.001	0.004
l.s.d	57.2	0.964

Values within a row followed by the same letter are not significantly different, based on l.s.d <0.05. Means of five replicate determinations are shown.

Diseased vines in Vineyard C (Chardonnay on Ramsey, drip irrigated) also had significantly decreased shoot lengths throughout the season. Tryptophan and sucrose soil drench caused significantly increased shoot length in early December and Benomyl and cyprodinil/fludioxinil caused significantly increased shoot growth rates from 17 December, 2007 to 22 January, 2008. The cyprodinil/fludioxinil mixture also increased the shoot length at pruning. However, yields were not affected by the treatments. (*Table 2.49b*).

Xylem function

After four hours in the acid fuchsin solution, all leaves had become bright red. Microscopy showed that there was little xylem blockage and most xylem vessels, especially the newest (third and fourth year) xylem, conducted the dye (*Figure 2.27*).

Cylindrocarpon was isolated from roots of the diseased field trial vines in the vineyards, indicating that this pathogen was probably a major cause of YVD. The soil baiting experiment showed that the soil under the diseased vines was also contaminated with *Cylindrocarpon*. Sweetingham (1983) reported that diseased Tasmanian grapevines appeared to die as a result of occlusion of xylem vessels and phloem cells with tyloses and brown gum. However we did not observe this sort of vascular failure in our acid fuchsin uptake assay. Loss of root function, causing observed desiccation of trunk and thus increased severity of *Botryosphaeria* infection may have been the major cause of the disease in the Riverina. There may also have been xylem blockage at the base of the vine below the position at which the trunk was severed.

The diseased grapevines had much poorer vegetative growth, especially early in the season, and yields were lower than those of the healthy vines. Over the three seasons average annual yields ranged from 37–69% lower, and numbers of bunches per vine ranged from 33–62% lower for the diseased vines. Shoot growth was from 39–49% lower and root length was 65% lower for the diseased vines. Detailed analysis of the root structure showed that diseased vines had significantly less fine roots (<0.5 mm diameter), indicating that *Cylindrocarpon* probably destroys the finest roots which are most important for water and nutrient uptake.

The carbon soil amendments had some immediate positive effects, increasing soil moisture and improving the root structure. Within the first year, Biochar, composted cow manure and composted green waste had increased root density for diseased vines to the level of healthy vines, although after two years this effect had disappeared (data not shown). In the healthy vines Biochar, composted green waste and rice hulls were still causing significantly increased soil moisture and Biochar was still increasing root length density (233% greater) two years after application. The biocontrol agent MW555 (*Streptomyces violaceoruber*) also increased root density and soil moisture. However, these improvements in root structure and soil moisture did not translate into increased yields or vegetative growth.

As diminished root function is such an important factor in the severity of YVD, we were also interested

Table 2.48 Juice and wine quality for grapes from diseased and asymptomatic Chardonnay on Ramsey vines, Vineyard A 2008-09.

	Juice Baumé	Yeast available nitrogen (YAN) (mh/L)	Juice pH	Juice titratable acidity (g/L)	Juice mass loss during fermentation[a] (%)
Diseased vines	13.18	363	3.83	71.2	5.83
Asymptomatic vines	12.88	422	3.74	70.93	6.37
P	0.248	0.049	0.134	0.368	<0.001
l.s.d	0.688	58.6	0.127	0.819	0.522

[a] sugar consumed and CO_2 released in 13 days

Values within a row followed by the same letter are not significantly different, based on l.s.d<0.05. Means of five replicate determinations are shown.

Table 2.49a Effect of fungicides, tryptophan and sucrose on shoot growth and yield (Vineyard B, Chardonnay).

	Shoot length (mm)						Increased shoot length 15/11/07 to 29/11/07 (%)	Pruning dry weight per vine (g)
	3/11/07	15/11/07	29/11/07	13/12/07	31/12/07	29/1/08		
Control healthy	540 a	926 a	1043 a	1077 a	1129 a	1135	11.3 b	805
Control diseased	174 b	206 c	283 b	354 b	356 b	382	37.3 b	586
Benomyl in soil	172 b	259 bc	384 b	525 b	617 b	655	46.6 b	982
cyprodinil/ fludioxinil in soil	142 b	180 c	367 b	503 b	623 b	656	108.3 a	829
Tryptophan in soil	207 b	202 c	331 b	351 b	350 b	401	75.0 ab	855
Tryptophan and sucrose in soil	200 b	227 c	337 b	360 b	432 b	444	57.8 b	709
Sucrose in soil	178 b	255 bc	310 b	402 b	436 b	493	25.5 b	558
P	0.005	<0.001	<0.001	0.005	0.028	0.119	0.025	0.188
LSD	148.6	173.7	266.2	339.5	397.3	480.7	55.91	320.1

Values within a column followed by the same letter are not significantly different, based on l.s.d<0.05. Means of three replicate determinations are shown.

Table 2.49b Effect of fungicides, tryptophan and sucrose on shoot growth and yield (Vineyard C, Chardonnay on Ramsey).

	Shoot length (mm)			Shoot length increase 17/12/07 to 22/1/08 (%)	Shoot length at pruning August 2008 (mm)	Pruning dry weight per vine August 2008 (g)	Yield per vine (kg)	Bunches per vine
	7/12/07	16/1/08	22/1/08					
Control healthy	875 a	921 a	888 a	4 c	992 a	742 a	11.42 a	146.2 a
Control diseased	408 bc	436 b	520 b	24 c	689 bc	397 bc	6.55 ab	81.7 b
Benomyl in soil	76 c	421 b	441 b	696 a	747 bc	504 b	4.08 b	48.2 b
cyprodinil/ fludioxinil in soil	289 c	545 b	544 ab	298 b	815 a	338 bc	6.03 b	71.3 b
Tryptophan in soil	261 c	319 b	306 b	8 c	256 d	112 c	2.02 b	38.7 b
Tryptophan and sucrose in soil	658 a	772 ab	757 ab	13 c	724 bc	485 bc	6.06 b	59.7 b
Sucrose in soil	439 bc	518 b	532 b	16 c	590 c	278 c	7.38 ab	101.3 ab
P	<0.001	0.005	0.025	<0.001	<0.001	<0.001	0.018	0.012
LSD	290.1	333.4	350.1	201.4	196.7	212.7	5.218	61.25

in the soil nematode population, reasoning that parasitic nematodes were likely to exacerbate the *Cylindrocarpon* root damage. Two years after application, green waste compost and rice hulls had increased the populations of beneficial nematodes by 57%. The total parasitic nematode populations decreased by 91%, 72% and 71% with biochar, composted cow manure and rice hulls respectively. There was no evidence that parasitic nematodes were a cause of yield loss, but the ratio of beneficial nematodes to parasitic nematodes was 116% higher in the healthy vines than in the diseased vines.

We found that root starch reserves were diminished in YVD diseased vines. Similarly, phylloxera infestation (Ryan *et al*. 2000) and root-knot nematode (Rahman *et al*. 2011) caused decreased starch in grapevines. Root starch reserves are important for new shoot growth and development (Smith and Holzapfel, 2009). Reductions in root and trunk starch reserves are reported to be closely associated with decreased numbers of bunches per shoot (Bennett *et al*. 2005), possibly explaining the lower bunch numbers, and thus yields, found for YVD diseased grapevines in our study.

The lower microbial population in the diseased plots indicates that, in both the vineyards studied, *Cylindrocarpon* from the grapevine roots may have been deleterious to the soil microbial community. The diseased soils were generally less fungal dominated. Diseased soil in Vineyard A also contained less oligotrophic bacteria. Oligotrophic bacteria are thought to contribute to the decomposition of organic matter and nutrient dynamics, because they bring the soil glucose concentration down to the threshold level for catabolite repression of hydrolytic enzymes, and so contribute to the activity of other bacteria (Senechkin *et al*. 2010).

The organic soil amendments increased soil microbial populations in vineyard A but not in vineyard B. Vineyard A is a regularly cultivated flood irrigated vineyard with low soil organic carbon (0.99%; *Table 2.40*) and low ratio of fungi to bacteria ratio (0.42). In contrast, vineyard B is drip irrigated, has a higher total organic carbon (1.51%) and composted cow manure had been regularly applied for a number of years before this trial began. As a result, the original fungi to bacteria ratio and levels of oligotrophic bacteria, cellulolytic fungi and general fungi were all much higher in vineyard B than in vineyard A (*Table 2.44b*). As a result, adding organic amendments to vineyard B soil was less likely to make a significant impact on the soil microbial communities within two years. Our earlier experience shows that from three to five years are usually required before added soil organic carbon can improve soil microbial communities.

The increased soil microbial populations induced by organic soil amendment did not decrease the effect of YVD on yield, and so presumably did not suppress *Cylindrocarpon* in the grapevine root. *Cylindrocarpon* can be sensitive to antagonism and to competition by other saprophytic fungi, but in our case it had already colonised the grapevine roots before other soil microbes and so was relatively immune to antagonism (Unestam *et al*. 1989). It appears that the YVD root infections were not amenable to amelioration by fungicides or by increased soil organic carbon from the carbon amendments. Repeated applications of organic amendments may improve their efficacy, but this practice would probably not be economically viable.

Outcomes/conclusions

Elucidate the organisms occurring in bunch rot complexes

Data gathered indicates that the predominant bunch rot pathogen occurring in the Hunter Valley is bitter rot caused by *Greeneria uvicola* followed by grey mould caused by *Botrytis cinerea* with other bunch rots such as *Colletotrichum* (ripe rot) occurring occasionally. Vineyards in dry inland regions encounter a greater incidence of *Aspergillus* and *Rhizopus*. Sour rot appears to occur wherever bunch rots are a problem.

The project has confirmed that both *C. acutatum* and *C. gloeosporioides* are responsible for ripe rot of wine grapes in Australia. Subtle differences in the infection process may explain the prevalence of each species isolated from vineyards. Differences in fungicide sensitivity may explain why *C. acutatum* was isolated with greater frequency from the vineyards examined. This information has wider implications in the management of bunch rot of grapes grown under sub-tropical conditions.

Bunch rot type is influenced by both local and seasonal changes in climate, an assumption confirmed by laboratory based studies using detached berries inoculated under controlled environmental conditions. Changes in the pathogen profile with climate have implications for models to predict

disease incidence in response to changes in global climate.

The susceptibility of grapevine flowers to bitter rot and ripe rot colonisation has been demonstrated and methods of rapid detection using molecular techniques have been developed. This work has now been published, and follow-up work will investigate the application of this technology to study the epidemiology of these pathogens. Work on how these pathogens overwinter in the vineyard is nearing completion as is the data collection on spore dispersal in vineyards.

Develop rapid field based tests for identification of bunch rot diseases

A real-time PCR method has been developed for bunch rot detection. The molecular techniques developed in this project can now be used in survey work and to advance the understanding of the dynamics of these and other bunch rot pathogens in the vineyard. We have applied this technology to examine sources of bunch rot inoculum in the vineyard.

Sources of bunch rot inoculum include dead wood and mummified bunch prunings from the previous year as well as wood tissues on the vines. Conidia from the ground environment can be distributed through wind and rain water splashes. *C. acutatum* conidia that remain on spurs are a likely source of inoculum under appropriate climatic conditions during the next growing season as are conidia from the debris. Removal of this material during pruning will reduce the amount of pathogen present in the vineyard.

As a result of this study the wine industry will be able to manage bunch rot plant disease epidemics in a more effective way, particularly with regards to the timing of both chemical sprays and decisions relating to harvest dates. This will help growers to avoid potential downgrading of fruit due to fungal diseases.

Improved bunch rot management through the use of fungicides and canopy management

Our results demonstrate for the first time that grapevine flowers are susceptible to *C. acutatum* and *G. uvicola* and that flower infections can remain latent in the developing berry. Furthermore our work demonstrates the utility of an application of the strobilurin fungicide, Cabrio at flowering to reduce the impact of ripe rot and bitter rot occurrence at harvest. Despite this partial control, the severity of bunch rot recorded in these field trials is likely to have a noticeable impact on wine quality (Meunier and Steel 2009). Additional work is required to better manage both ripe rot and bitter rot of grapes in situations where there are restrictions on pesticide applications close to harvest. While sprays of Cabrio at flowering and when the berries are pea-size offer some control of bitter rot, the degree of effectiveness is not as great as an application post-véraison.

Overall this project has benefited the wine industry by providing growers with better methods of bunch rot identification and improved options for disease management.

Trunk disease

This project achieved the objectives set out in the original application and in some cases exceeded the planned outputs. Furthermore findings from the work have been presented widely at both international scientific conferences and grower extension workshops. A brief summary of some of the important achievements of this project with reference to the original planned outputs are presented below.

This study has identified nine species of Botryosphaeriaceae associated with decline and dieback of grapevines in the major viticultural regions of NSW and SA. Phylogenetically, eight of these species fall into four main groups. The prevalence of individual species and their distributions appear to be influenced by climate, as demonstrated in the USA and Mexico (Urbez-Torres *et al*. 2006*a*, Urbez-Torres *et al*. 2008). However, other factors such as the robustness of infective propagules, and environmental stress are likely to play a role in dissemination and disease occurrence. Current management strategies for these fungi rely almost entirely on the removal of infected wood, retraining of vines, and prevention of infection of fresh pruning wounds.

Consideration of the incidence, distribution, and pathogenicity of individual species is likely to play an important role in determining improved control strategies. Further research is required, especially on *Dothiorella*, for which few studies have thus far been undertaken.

This study also revealed other fungi that have the potential to contribute to the decline and dieback of grapevines. These fungi belong to the family Diatrypaceae and include *Eutypa lata*. Collaborative efforts with researchers at UC Davis have assisted in the identification of these fungi. Glasshouse studies are currently being conducted to determine the pathogenicity of these fungi toward grapevine.

Spore trapping studies have isolated various wood inhabiting fungi, some pathogenic toward grapevines. The release of Botryosphaeriaceae spores in Australia appears to be correlated with precipitation and perhaps with other climatic parameters. The results of this data were to be used to inform disease models, however time constraints have not allowed this to occur. Further spore trapping studies would be required to provide sufficient data for such models to be developed.

The isolation of most Botryosphaeriaceae species from all tissue types sampled confirms that Botryosphaeriaceae species can infect different *V. vinifera* tissue types throughout all stages of the growing season. This is important information for the management of Botryosphaeriaceae in vineyards, which is currently limited to remedial surgery of infected wood and the protection of pruning wounds.

In contrast to many other bunch rot pathogens, Botryosphaeriaceae infection of the wood presents a constant inoculum source with pycnidia on the surface of trunks and cordons. It is therefore insufficient to only protect pruning wounds in winter, risking infection of the grapevine reproductive tissue throughout the growing season which could lead to infection of the fruit. As described previously, pycnidia may form on infected berries acting as another primary source of inoculum for the wood. In addition it is unknown if the infection pathway includes a downward movement into the wood which could lead to wood infection through infected buds or shoots. Further research is required to investigate the

Theme outcomes, outputs and milestones

Number	Description	Achieved
Outcome		
1	Determine distribution and epidemiology of wood and bunch diseases	Y
2	Improved detection and identification of bunch rot and trunk diseases	Y
3	Reduction in the incidence of bunch rot organism in vineyards	N*
4	Improved management and control of wood and bunch diseases	Y
5	A benchmark for the incidence of young vine decline in NSW and identification of principal mode of infection	Y
6	Revision of grapevine nursery hot water treatment protocols for production of clean, healthy planting material free of fungi involved in young vine decline	Y
7	Understanding of the influence of abiotic and biotic stress on disease development	Y
Output		
1	Industry Presentations at AWITC in 2007 and 2010	Achieved
2	Presentations to industry associations	Achieved
3	Five industry technical publications	Exceeded: 19 publications
4	Six or more refereed publications in internationally peer reviewed journals.	Exceeded: 21 publications plus 5 publications under review
5	One PhD Completion	Achieved
6	Presentation of research findings at one or more major international conferences	Exceeded (see below)
Milestone		
1	15 vineyards in MIA and 15 in Sunraysia surveyed for incidence of young vine decline–Sept 2007	Y
2	Impact of soil type and vine vigour on Bot Canker incidence known–Sept 2008	Y
3	Growers provided with improved management options for non-Botrytis bunch rot control–March 2009	Y
4	Identification of pathogens involved in young vine decline–Dec 2009	Y
5	Impact of abiotic and biotic factors on trunk and bunch diseases established–June 2010	Y
6	Field-based molecular based tools evaluated for vine disease detection–Dec 2010	Y

* too early to assess this outcome

pathways of Botryosphaeriaceae infection in various grapevine tissues.

We suggest considering Botryosphaeriaceae species as more than trunk disease pathogens and incorporating control strategies, other than the current ones, throughout the entire growing season for the management of Botryosphaeriaceae spread in Australian vineyards. Future research is needed to confirm the aggressiveness of the various species isolated from this survey to determine their role as bunch rot pathogens and provide information for control strategies. This should include an investigation into the pathway of Botryosphaeriaceae infection for each tissue.

All species of Botryosphaeriaceae are able to infect berries in vitro and should be considered in management of bunch rots. Some species are more virulent than others. In addition, all species are pathogenic on one year old canes. Botryosphaeriaceae are not tissue specific therefore those species appearing on the wood are also capable of infecting the berries. Although Botryosphaeriaceae were isolated from buds in the field, glasshouse and field inoculations of buds with *D. seriata* revealed these isolates to be non-pathogenic towards buds of grapevines. Both Shiraz and Chardonnay were susceptible to infection by Botryosphaeriaceae.

The Botryosphaeriaceae species isolated in this study appear to be genetically similar and do not group according to vineyard origin, grapevine origin or tissue origin. These results were obtained using a small set of AFLP markers. Additional screening of markers may provide more information on the genetic status of Botryosphaeriaceae in Australia.

No fungicides were able to provide complete control of Botryosphaeriaceae. In the Hunter Valley, the application of Bavistin, Folicur, Garrison, Rovral, Shirlan, Switch and Vinevax to wounds significantly reduced the MPR of *D. seriata*. Out of the fungicides tested, Folicur, Shirlan and Switch were only effective when applied at rates exceeding that recommended by the manufacturer. In the Barossa valley, these fungicides were also effective at reducing the MPR of *D. mutila*. In addition, ATCS tree wound dressing, Bacseal and Nustar applied at label rates also reduced the MPR of *D. mutila*. The products containing tebuconazole (Folicur, Bacseal), cyproconazole (Garrison) and carbendazim (Bavistan) show some promise in the management of trunk diseases in general as they were also the most effective at controlling Eutypa dieback (Sosnowski *et al*. 2008).

The field trials in the Hunter Valley were complicated by an already existing background level of *D. seriata*. Perhaps future trials in this region should include the inoculation of rarer species of Botryosphaeriaceae.

Finally, hands-on workshops conducted in various regions have provided growers and other industry personnel with the information to identify and manage Bot canker in vineyards.

Young vine decline

We have shown that the cause of the YVD syndrome in the Riverina for grafted vines is the co-infection by two different wound or root-invading fungi, namely *Botryosphaeria* spp. and *Cylindrocarpon* spp., that infect grapevines at different stages of the propagation process. The general epidemiology of YVD is proposed: first, dormant cuttings, some of which are *Botryosphearia*-infected, are taken from source rootstock plants; next, infected cuttings from those plants contaminate uninfected cuttings during hydration soaking, callusing and/or storage; next, *Botryosphearia*-infected cuttings are invaded by *Cylindrocarpon* and possibly also by *Botrysphaeria* spp. when they are rooted in soil inhabited by those fungi. Consequently the rootstock stem below the graft and the graft union become internally infected with both *Cylindrocarpon* and *Botryosphaeria* while usually the scion, taken from less infection-prone source plants (due to higher above-ground, training and pruning practices) and subsequently subjected to less wounding and hydration soaking, is initially uninfected. As a result, *Cylindrocarpon* disrupts root function, and consequently retards early plant development while *Botyrosphaeria* spp. gradually invades the xylem vessels of the stem, both basipetally and acropetally, and contributes to the plant decline and eventual death. Although both *Botryosphaeria* and *Cylindrocarpon* alone can cause the decline and death of young grapevines, co-infection leads to more severe disease symptoms It is well known that symptoms from *Botryosphaeria* infections are especially severe in cases where the host plant has been subjected to water stress, so the reduced root function caused by *Cylindrocarpon*, which induces severe water stress even under non-water-logged vineyard conditions, probably acts as a stressor for the *Botryosphaeria* infection.

Recommendations

Bunch rots

Bunch rot incidence and bunch rot type varies from season to season. For example, in the Hunter Valley *B. cinerea* is a problem in those years when the climate of the growing season is close to the long term average. In hotter and drier years bitter rot caused by *G. uvicola* is likely to be a greater problem. There are also geographical variations in bunch rot incidence. *Aspergillus* and *Rhizopus* are likely to occur in warm dry inland regions while bitter rot and ripe rot are more likely to occur in vineyards classified as sub-tropical. A greater understanding of climate variability and climate change will help growers to predict bunch rot occurrence with greater accuracy and may be an avenue for future research. While canopy management is used widely for *B. cinerea* control, care must be excised to avoid heat stress and sunburn since this appears to pre-dispose fruit to some of the other non-Botrytis bunch rots. Avoiding late maturing varieties (e.g. Cabernet Sauvignon) which mature during the periods of high summer rainfall in the Hunter Valley is a sensible management strategy.

Chemical sprays for bunch rots remain limited, despite the fact that this project has demonstrated the efficacy of strobilurin fungicides for bitter rot and ripe rot management. This is due to restrictions on when fungicides can be applied particularly for wine grapes destined for the export market. Future research directions will have to examine alternatives to chemical control.

Aside from yield losses bunch rots can impact on wine quality by producing off flavours and taints. One approach in the absence of suitable disease management measures in the vineyard might be to determine what can be achieved in the wine production stage to minimise the impacts of bunch rots on wine quality. A greater understanding of the nature of the taints produced by fungal pathogens and how they can be corrected in wine has been identified as an area for further investigation.

Trunk disease

Bot canker and Eutypa dieback are two diseases confirmed to cause decline and dieback of grapevines in Australia. The occurrence of these diseases in the winegrowing regions appears to be dependent on climate. Growers and vineyard managers concerned about vines exhibiting decline and dieback symptoms due to fungal diseases should confirm what organisms are causing these symptoms. Informed decisions can then be made on the prevention and management of these diseases. Remedial surgery remains the best method for removing infected tissue from the vine and for extending the longevity of infected vines. All visibly infected wood and 10–20 cm of healthy wood should be removed. All wood and prunings should be removed from the vineyard and burnt. If cordons are removed, new canes can be laid down in the next season to replace these. If vines are cut at ground level, water shoots produced may be used to replace the vine. Double pruning is a method that has been tested in the USA for the prevention of Bot canker and Eutypa dieback (Weber *et al.* 2007). Future research should consider trialling this method in Australia for the prevention of trunk diseases.

Bot canker may be prevented by protecting pruning wounds and any large cuts or damage made to the vine. This will depend on the age of the vine, time of pruning, time required for the wound to heal and the amount of sap flow. Spore trapping studies such as those tested in this project will need to continue to ensure pruning is conducted during climatic conditions and times that are not conducive to spore release. Spore trapping studies will also need to be conducted in different climatic regions if disease models are to be developed. Trials conducted in collaboration with Eutypa dieback researchers at SARDI have revealed that Shirlan, Bavistin and Folicur may provide protection against both Eutypa dieback and Bot canker. Although none of the chemicals tested provide complete control we believe that any measures that reduce Bot canker in the vineyard will facilitate disease control. Since 2010 Bavistin has been banned for use in Australian vineyards therefore disease prevention options are limited. Further research is required to test new fungicides including biological and alternatives for the control of Bot canker and other grapevine trunk diseases. In addition, strategies for applying these compounds onto pruning wounds will need to be evaluated. Registration and label changes will also need to be considered for any new control options.

For many years it was believed that Eutypa dieback was the predominant disease associated with the dieback of grapevine in Australia. Current research in Australia and overseas has shown that other fungi such as the Botryosphaeriaceae also play an equally important role in the dieback of grapevines. The collaboration with researchers at UC Davis highlighted the diversity of organisms associated with

the decline and dieback of grapevines in Australia. Glasshouse trials to determine the pathogenicity of these other organisms are currently being conducted. In addition, a large field trial assessing the pathogenicity of Botryosphaeriaceae is currently in place and results will be available in the near future. Furthermore, future studies should involve the careful identification of these fungi and their role in the decline of grapevines in Australia.

This study has also highlighted that Botryosphaeriaceae should not only be considered as wood pathogens but also pathogens of the berries. The isolation of these fungi from the vegetative tissues suggests that these may also be sources of inoculum. This will need to be taken into account when developing control strategies for Bot canker and bunch rot. Any fungicide trials will need to be assessed for their ability to prevent Botyospaheriaceae from infecting the wood and other vegetative tissues. Further research is required to investigate the epidemiology and life cycle of Botryosphaeriace in grapevines.

Anecdotal evidence suggests that stresses such as extreme temperature and drought may play a role in Bot canker. Future studies should consider assessment of these stresses under controlled environment conditions on the infection of Botryosphaeriaceae.

The workshops conducted in various winegrowing regions were well received by growers and other industry personnel. Continued education is required to ensure that these personnel remain vigilant in identifying and managing Bot canker and other trunk diseases. Extension packages similar to those developed for Eutypa dieback should also be developed for Bot canker. Efforts should also be made to investigate better and more novel ways of delivering extension information to growers.

Young vine decline

Young vine decline (YVD) in the Riverina was found to be caused by pathogenic fungi *Botryosphaeria* and *Cylindrocarpon* from rootstock source blocks and grapevine propagation nurseries. As it is better to prevent YVD in the first case, than to try to manage the disease for many years for minimal return, it is important to establish the efficacy of hot water treatment against these fungi. Although rootstock mother vines have been found to be contaminated with *Botryosphaeria*, it is likely that HWT of the cuttings before nursery propagation would kill this fungus. However, nursery hydration tanks and soil also carry the YVD pathogens so work is needed to assist the nurseries in establishing protocols to prevent contamination within the propagation system.

A rapid real-time PCR protocol for *Botryosphaeria* and *Cylindrocarpon* would to allow faster detection of the pathogenic fungi in roots and stems of diseased grapevines. We envisage passing this protocol on to various diagnostic services, many of which currently find it difficult to isolate *Cylindrocarpon*.

It is essential to continue to monitor the yields for the vineyard field trial for three more years because the yields for the diseased vines appear to be increasing with time over the first three years, and it is not yet clear whether this may simply be a short term effect after the drought.

Susceptibilities to *Cylindrocarpon* and *Botryosphaeria* infection of different rootstocks should also be a priority.

More work on the effect of the mycotoxin produced by *Cylindrocarpon* (brefeldin A) should also be a priority. Brefeldin A production may be the key to understanding how Cylindrocarpon and Botryosphaeria interact in the grapevine. We need to investigate treatment of planting holes after infected plants have been removed to avoid replant problems (e.g. bio-fumigation with mustard meal; application of Trichoderma etc.)

Biochar has had a significant positive effect on soil water content, soil microbial activity and the soil nutrient cycle. More work needs to be done on its effect on soil pathogens and mycorrhizal fungi.

Finally, in light of our results, regions currently experiencing large 'Bot canker' outbreaks should be surveyed for presence of root disease (*Cylindrocarpon*, parasitic nematodes etc) that would exacerbate the severity of *Botryosphaeria* wood infection from pruning wounds.

Appendix 1
Communications

Scientific publications (refereed journals)

Greer, L.A., Harper, J.D.I., Savocchia, S., Samuelian, S.K. and Steel, C.C. (2011). Ripe rot of south eastern Australian wine grapes is caused by two species of *Colletotrichum*: *C. acutatum* and *C. gloeosporioides* with differences in infection and fungicide sensitivity. *Australian Journal of Grape and Wine Research* **17**: 123–128.

Meunier, M.A. and Steel, C.C. (2009). Effect of *Colletotrichum acutatum* ripe rot on the composition and sensory attributes of Cabernet Sauvignon grapes and wine. *Australian Journal of Grape and Wine Research* **15**: 223–227.

Pitt, W.M., Huang, R., Steel, C.C. and Savocchia, S. (2010). Identification and distribution of Botryosphaeriaceae species associated with grapevine decline and dieback in New South Wales and South Australia. *Australian Journal of Grape and Wine Research* **16**: 258–271.

Pitt, W.M., Huang, R., Trouillas, F.P., Steel, C.C. and Savocchia, S. (2010). Evidence that *Eutypa lata* and other diatrypaceous species occur in New South Wales vineyards. *Australasian Plant Pathology* **39**: 97–106.

Qiu, Y., Steel, C.C., Ash, G.J. and Savocchia, S. (2011). Survey of Botryosphaeriaceae associated with grapevine decline in the Hunter Valley and Mudgee grape growing regions of New South Wales. *Australasian Plant Pathology* **40**: 1–11.

Rahman, L., Creecy, H. and Orchard, B. (2008). Impact of citrus nematode (*Tylenchulus semipenetrans*) densities in soil on yield of grapevines (*Vitis vinifera* 'Shiraz') in south-eastern New South Wales. *Vitis* **47**: 175–180.

Rahman, L. and Somers, T. (2005). Suppression of root knot nematode (*Meloidogyne javanica*) after incorporation of Indian mustard cv. Nemfix as green manure and seed meal in vineyards. *Australasian Plant Pathology* **4**: 77–83.

Rahman, L., Weckert, M.A., Hutton, R.J. and Orchard, B. (2009). Impact of floor vegetation on the abundance of nematode trophic groups in vineyards. *Applied Soil Ecology* **42**: 96–106.

Rahman, L., Whitelaw-Weckert, M.A. and Orchard, B. (2011). Consecutive applications of brassica green manures and seed meal enhances suppression of *Meloidogyne javanica* and increases yield of *Vitis vinifera* cv Semillon. *Applied Soil Ecology* **47**: 195–203.

Samuelian, S.K., Greer, L.G., Savocchia, S. and Steel, C.C. (2011). Detection and monitoring of *Greeneria uvicola* and *Colletotrichum acutatum* development on grapevines by real-time PCR. *Plant Disease* **95**: 298–303.

Samuelian, S.K., Greer, L.G., Savocchia, S. and Steel, C.C. (2012) Overwintering and presence of *Colletotrichum acutatum* (ripe rot) on mummified berries, dormant wood, developing berries and mature berries of *Vitis vinifera*. *Vitis* **51**: 33–37.

Savocchia, S., Greer, L.A. and Steel, C.C. (2007). First report of *Phomopsis viticola* causing bunch rot of grapes in Australia. *Plant Pathology* **56**: 725.

Savocchia, S.,Steel, C.C., Stodart, B.J. and Somers, A. (2007). Pathogenicity of Botryosphaeria species isolated from declining grapevines in subtropical regions of eastern Australia. *Vitis* **46**: 27–32.

Steel, C.C. and Greer, D.H. (2008). Effect of climate on vine and bunch characteristics: Bunch rot disease susceptibility. *Acta Horticulturae* **785**: 253–262.

Steel, C.C., Greer, L.A. and Savocchia, S. (2007). Studies on *Colletotrichum acutatum* and *Greeneria uvicola*: two fungi associated with bunch rot of grapes in sub-tropical Australia. *Australian Journal of Grape and Wine Research* **13**: 23–29.

Steel, C.C., Greer, L.A. and Savocchia, S. (2010). Influence of climate on the susceptibility of grape berries to bunch rot fungi. *Australian Journal of Grape and Wine Research* **16**: 63–80.

Steel, C.C., Greer, L.A. and Savocchia, S. (2012) Grapevine inflorescences are susceptible to the bunch rot pathogens, *Greeneria uvicola* (bitter rot) and *Colletotrichum acutatum* (ripe rot). *European Journal of Plant Pathology* (in press).

Steel, C.C., Greer, L.A., Savocchia, S. and Samuelian, S.K. (2011). Effect of temperature on *Botrytis cinerea, Colletotrichum acutatum* and *Greeneria uvicola* mixed fungal infection of *Vitis vinifera* grape berries. *Vitis* **50**: 69–71.

Trouillas, F.P., Pitt, W.M., Sosnowski, M., Huang, R., Peduto, F., Loschiavo, A., Savocchia, S., Scott, E. and Gubler, W.D. (2011). Taxonomy and DNA phylogeny of Diatrypaceae associated with *Vitis vinifera* and other woody plants in Australia. *Fungal Diversity* **49**: 203–223.

Weckert, M.A., Nair, N.G., Lamont, R., Alonso, M., Priest, M.J. and Huang, R. (2007). Root infection of *Vitis vinifera* by *Cylindrocarpon liriodendri* in Australia. *Australasian Plant Pathology* **36**: 403–406.

Weckert, M.A., Rahman, L., Hutton, R. and Coombes, N. (2007). Permanent swards increase soil microbial counts in two Australian vineyards. *Applied Soil Ecology* **36**: 224–232.

Weckert, M.A., Sergeeva, V. and Priest, M.J. (2006). *Botryosphaeria stevensii* infection of Pinot Noir grapevines by soil/root transmission. *Australasian Plant Pathology* **35**: 369–371.

Whitelaw-Weckert, M.A., Curtin, S., Huang, R., Steel, C.C., Blanchard, C.L. and Roffey, P. (2007). Phylogenetic relationships and pathogenicity of grape isolates of *Colletotrichum acutatum* in sub-tropical Australia. *Plant Pathology* **56**: 448–463.

Whitelaw-Weckert, M.A., Rahman, L., Appleby, L. and Hardie, W.J. Co-infection by *Botryosphaeria* and *Cylindrocarpon* at different stages during propagation causes 'Young Vine Decline' of grapevines. Plant Pathology (submitted).

Wunderlich, N., Ash, G.J., Steel, C.C., Raman, H., Cowling, A. and Savocchia, S. (2012) Refining the biological factors affecting virulence of Botryosphaeriaceae on grapevines. *Annals of Applied Biology* **159**: 467–477.

Wunderlich, N., Ash, G.J., Steel, C.C., Raman, H. and Savocchia, S. (2011). Association of Botryosphaeriaceae grapevine trunk disease fungi with the reproductive structures of *Vitis vinifera*. *Vitis* **50**: 89–96.

Technical reports and industry journal articles

Alonso, M., Whitelaw-Weckert, M.A., Harden, T., Blanchard, C., Greer, D. and Hutton, R. (2005) Grapevine disease resistance and compost. *The Australian and New Zealand Grapegrower and Winemaker*. .**500**: 51–53.

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Industry conference and workshop presentations

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Pitt, W.M., Huang, R., Steel, C.C. and Savocchia, S. (2009). Botryosphaeria canker – distribution and management. Western Australia Grapevine Trunk Disease Workshop, Mount Barker, WA, 6 November.

Pitt, W.M., Huang, R., Steel, C.C. and Savocchia, S. (2009). Botryosphaeria canker – distribution and management. Western Australia Grapevine Trunk Disease Workshop, Margaret River, WA, 4 November.

Pitt, W.M., Huang, R., Steel, C.C.and Savocchia, S. (2009). Botryosphaeria canker – distribution and management. Western Australia Grapevine Trunk Disease Workshop, Swan Valley, WA, 2 November.

Pitt, W.M., Huang, R., Steel, C.C. and Savocchia, S. (2009). Grapevine trunk diseases – an overview. Winegrowing Futures Spring Vine Health Workshop. Young, NSW, 10 July.

Pitt, W.M., Huang, R., Steel, C.C. and Savocchia, S. (2009). Grapevine trunk diseases – an overview. Winegrowing Futures Spring Vine Health Workshop. Mudgee, NSW, 8 July.

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Pitt, W.M., Steel, C.C., Huang, R. and Savocchia, S. (2007). Survey and management of Botryosphaeria canker. Hunter Valley Workshop, Hunter Valley, NSW, 16–19 July.

Pitt, W.M., Steel, C.C., Huang, R. and Savocchia, S. (2007). Survey and management of Botryosphaeria canker; progress and early results. NWGIC Symposium, Wagga Wagga, NSW, 20–21 June.

Pitt, W.M., Steel, C.C., Huang, R. and Savocchia, S. (2008). Bot canker – distribution and management. Theme 2 Meeting, Wagga Wagga, NSW, 17 December.

Pitt, W.M., Steel, C.C., Huang, R. and Savocchia, S. (2008). Bot canker – distribution and management. Yarra Valley Wine Growers Association Healesville, Victoria, 26–27 November.

Pitt, W.M., Steel, C.C., Huang, R. and Savocchia, S. (2008). Bot canker and Eutypa dieback – symptoms and management. Hanwood Growers Association, Hanwood, NSW, 21 October.

Pitt, W.M., Steel, C.C., Huang, R. and Savocchia, S. (2008). Grapevine trunk diseases – an overview – symptoms and management. Orange Regional Vignerons Association, Orange, NSW, 13 June.

Pitt, W.M., Steel, C.C., Huang, R. and Savocchia, S. (2008). Grapevine trunk diseases – an overview – symptoms and management. Wine Grape Growers Australia, Cowra, NSW, 12 June.

Pitt, W.M., Steel, C.C., Huang, R. and Savocchia, S. (2008). Grapevine trunk diseases – an overview – symptoms and management. IHD Vitilink Technical Conference, Mt Evelyn, Victoria, 11–13 May.

Samuelian, S. (2011) Resistance of rootstocks and nematodes in your vineyard. ASVO: Below Ground Management for Quality and Productivity Seminar. 28–29 July 2011, Mildura, Victoria.

Savocchia, S., Pitt, W.M., Huang, R. and Steel, C.C. (2009). Botryosphaeria canker: distribution and management. Trunk Disease Workshop, Griffith, NSW, 24 November.

Savocchia, S., Sosnowski, Pitt, W.M., Huang, R. and Steel, C.C. (2009). Botryosphaeria canker and Eutypa Dieback. Trunk Disease Workshop, Rutherglen Victoria, 25 November.

Savocchia, S., Weckert, M., Pitt, W.M., Huang, R. and Steel, C.C. (2009). Young Vine Decline, Botryosphaeria canker and Eutypa Dieback. Trunk Disease Workshop, Griffith, NSW, 24 November.

Steel, C.C. (2005). Advances in the management and biology of bitter rot and ripe rot of grapes McLaren Valley, SA, 8 November.

Steel, C.C. (2006). Current best management practices for the control of bitter rot and ripe rot of gapes. Hunter Valley, 3 May.

Steel, C.C. (2007) Recent advances in our understanding of non-Botrytis bunch rot of grapes. Swan Valley, WA, 1 June.

Steel, C.C. (2007). Recent advances in our understanding of non-Botrytis bunch rot of grapes. Margaret River, WA, 29 May.

Steel, C.C. (2007). Vine Health and the Environment – Theme 2 overview. NSW Wine Industry Association, Wagga Wagga, 2 November.

Steel, C.C. (2008). Biology and management of bunch rot of grapes. Albury, July.

Steel, C.C. (2008). Biology and management of bunch rot of grapes. Mudgee, 28 April.

Steel, C.C. (2010). Fungicide resistance management strategies. Spring Vine Health Field Day; Lilydale, Yarra Valley, 20 October.

Steel, C.C., Greer, L.A., Haywood, C., Samuelian, S. and Savocchia, S. (2009). Use of fungicides sprays applied at flowering to reduce the severity of bitter rot and ripe rot in the Hunter Valley. Hunter Valley, 30 September.

Steel, C.C., Greer, L.A., Haywood, C., Samuelian, S.K. and Savocchia, S. (2009). Use of fungicide sprays applied at flowering to reduce the severity of bitter rot (*Greeneria uvicola*) and ripe rot (*Colletotrichum acutatum*) of grapes (*Vitis vinifera* L.) in the Hunter Valley. Pokolbin, NSW, 2 October.

Steel, C.C., Samuelian, S.K., Savocchia, S., Greer, L.A. and Harwood, C. (2009) Bunch rot research update. Hunter Valley, 12 August.

Weckert, M. (2010). Cover crops and compost in vineyards – opportunities for improving soil health. Spring Vine Health Day; Mudgee, 29 July.

Weckert, M. (2010). Cover crops and compost in vineyards – opportunities for improving soil health. Spring Vine Health Day; Hunter Valley, 28 July.

Weckert, M. (2010). Herbicide vs cultivation: the better of two evils? Organic Viticulture Workshop. 14th Australian Wine Technical Conference, Adelaide Convention Centre, Adelaide, 3–8 July.

Weckert, M. (2010). How important is vineyard soil for good Riesling wine? Riesling Challenge; Canberra, 14 October.

Weckert, M. (2010). Soil biology; cover crops; and how important is soil for good wine? Oral presentation: Spring Vine Field Day; Stanthorpe, Granite Belt, 16 October.

Weckert, M., Rahman, L. and Alonso, M. (2009). Cover crops and composts in vineyards – opportunities for improving soil health. ASVO Annual Seminar: Vineyard soil health: unearthing fact from fiction. Mildura, 30 August.

Weckert, M.A. (2011). Plant pathology in viticulture: cooperative scientific detective work. Invited speaker: National Primary Industry Centre for Science Education, SEO Planning Forum, Wagga Wagga, 1 June.

Weckert, M.A. (2011). The grapevine as a model system to study rhizosphere interactions – a perennial system. E.H. Graham Centre Workshop on Soil Rhizosphere Interactions in Australian Cropping Systems; Characterization of soil/rhizosphere interactions using novel research techniques. Wagga Wagga, 4 August.

Weckert, M.A. (2011). Young Vine Decline in the Riverina: Causal organisms, origin, remedies and carbohydrate relations. NWGIC Industry Showcase Day: Winegrowing Futures and More. NWGIC, Wagga Wagga, NSW, Australia, 16 June

Whitelaw-Weckert, M.A. (2005). Managing soil microflora for healthy vineyard soil. Mudgee Growers Group, June.

Whitelaw-Weckert, M.A. (2005). Pest and disease control in the vineyard. Yenda Growers Group, August.

Whitelaw-Weckert, M.A. (2005). Soil microbial activity and water retention. Young Growers Group, September.

Whitelaw-Weckert, M.A. (2005). Vineyard microbial soil health. Orange Growers Group.

Whitelaw-Weckert, M.A. (2006). Vine Health – projects in Griffith. Wine Growers Marketing Board, Griffith, 23 April.

Whitelaw-Weckert, M.A. (2006). Young vine decline. Wine Growers Marketing Board, Griffith, 20 June.

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Industry conference and workshop posters

Appleby, L. and Lamont, R. (2011). Young Vine Decline. NWGIC Industry Showcase Day: Winegrowing Futures and More. Wagga Wagga, NSW, Australia, 16 June.

Greer, L.A., Savocchia, S., Samuelian, S.K. and Steel, C.C. (2011). A plague of pimples. A guide to identification of some common rots in grapes. NWGIC Industry Showcase Day: Winegrowing Futures and More. Wagga Wagga, NSW, Australia, 16 June.

Greer, L.A., Savocchia, S., Samuelian, S.K. and Steel, C.C. (2011). What rot: Exposing the bitter taint of destruction. A guide to identification of bitter rot and ripe rots in grapes. NWGIC Industry Showcase Day: Winegrowing Futures and More. Wagga Wagga, NSW, Australia, 16 June.

Greer, L.A., Steel, C.C. and Savocchia, S. (2007). Prevalence of bunch rots in the Hunter and Hastings Valleys. 13th Australian Wine Industry Technical Conference, Adelaide.

Pitt, W.M., Huang, R., Steel, C.C. and Savocchia, S. (2010). *Diatrypaceae* species associated with grapevines and other hosts in New South Wales. Riverina Field Days 7–8 May.

Pitt, W.M., Huang, R., Trouillas, F.P., Steel, C.C., Weckert, M. and Savocchia, S. (2010). Identification and distribution of *Botryosphaeriaceae* species associated with grapevine decline in south-eastern Australia. Riverina Field Days 7–8 May.

Pitt, W.M., Qiu, Y., Savocchia, S. and Steel, C.C. (2007). *In vitro* evaluation of fungicides for the management of Botryosphaeria canker. 13th Australian Wine Industry Technical Conference, Adelaide, 28 July – 2 August.

Rahman, L., Whitelaw-Weckert, M. and Hutton, R. (2007). Floor vegetation increases beneficial, and reduces pest-nematodes in vineyards. 13th Australian Wine Industry Technical Conference, Adelaide, 28 July – 2 August.

Samuelian, S.K., Greer, L.A., Savocchia, S. and Steel, C.C., (2011). Improved detection and identification of grape bunch rots. NWGIC Industry Showcase Day: Winegrowing Futures and More. Wagga Wagga, NSW, Australia, 16 June.

Whitelaw-Weckert, M.A., Rahman, L., Appleby, L., Rogiers, S., Lamont, R. and Waite, H. (2011). Young Vine Decline in the Riverina: Causal organisms, Origin, Remedies and Carbohydrate relations. NWGIC Industry Showcase Day: Winegrowing Futures and More. Wagga Wagga, NSW, Australia, 16 June.

Whitelaw-Weckert, M.A., Rahman, L. and Hutton, R.J. (2007). Permanent swards increase soil microbial counts in two Australian vineyards. 13th Australian Wine Industry Technical Conference, Adelaide, 28 July – 2 August.

Whitelaw-Weckert, M.A., Whitelaw, E., Rogiers, S., Quirk, L. and Clark, A. (2011). Bacterial inflorescence rot (BIR) caused by *Pseudomonas syringae* pv. *syringae*. NWGIC Industry Showcase Day: Winegrowing Futures and More. Wagga Wagga, NSW, Australia, 16 June.

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Appendix 2
Intellectual property

Not applicable

Appendix 3
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Appendix 4
Staff

Dr Melanie Weckert

Professor Chris C Steel

Dr Sandra Savocchia

Dr Loothfar Rahman

Dr Suren K Samuelian

Dr M Priest

Mrs K Cowan

Dr J D I Harper

Mrs Lindsay A Greer

Ms Lynne Appleby

Ms Nicola Wunderlich

Mr Chris Haywood

Ms Hoa Truong

Ms Lisa Greer

Ms Stacey Greer

Dr Andrew Hall

Lorraine Spohr

Dr Anne Cowling

Mr Jason Cappello

Mr Shayne Hackett

Ms Belinda Taylor

Ms Rosy Raman

Ms Barbara Dyer

Collaborators

Dr Mark Sosnowski

Dr Adrian Loschiavo

Mr Mark Sims

Appendix 5
Other relevant material

Not applicable

Appendix 6
Budget reconciliation

See *Winegrowing Futures Final Report, Theme 1*