ASSOCIATION AND PATHOGENICITY OF BOTRYOSPHAERIACEAE FUNGI ON DIFFERENT VITIS VINIFERA TISSUE

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Research is to see what everybody else has seen, and to think what nobody else has thought.

~ Albert Szent-Györgyi ~
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I, Nicola Wunderlich, hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of another degree or diploma at Charles Sturt University or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by colleagues with whom I have worked at Charles Sturt University or elsewhere during my candidature is fully acknowledged.

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ABSTRACT

Botryosphaeriaceae fungi causing Botryosphaeria canker, an internal infection of the wood, which leads to grapevine decline and dieback, are a great concern for the viticulture industry. Primarily regarded as wood pathogens of grapevines in Australia, some Botrysphaeriaceae fungi have been associated with bunch rots in table grapes and wine grapes in the United States and Europe. More recently, species of Botryosphaeriaceae have been isolated from rotten bunches of grapevines in subtropical wine regions of Australia. The role of Botrysphaeriaceae in the infection of the fruit of Australian wine grapes remains unclear. The overall aim of this thesis was to investigate the pathogenicity of Botryosphaeriaceae in grape bunches and whether infection with these fungi could result in bunch rot.

A major part of this study was to survey several different grapevine tissues at various phenological stages for the occurrence of Botrysphaeriaceae. A two year survey of two vineyards in the Hunter Valley (New South Wales, Australia) planted with Chardonnay and Shiraz resulted in a total of 188 isolates conforming to nine different species with Diplodia, Dothiorella and Neofusicoccum anamorphs. These were isolated from dormant buds, flowers, pea-sized berries and mature berries prior to harvest. A further 142 isolates were obtained from the trunks of the same vines. Dothiorella viticola, Diplodia mutila and Neofusicoccum australe were reported for the first time from grapevines in the Hunter Valley. These results provide evidence that grapevine tissue other than wood may act as potential inoculum sources for Botrysphaeriaceae in vineyards.

The second aim was to determine whether species of Botrysphaeriaceae are tissue specific or can infect various tissues, independently from their host tissue origin. A subset of isolates obtained in the survey was assessed for their potential to cross-infect other grapevine tissues. Tests showed that all isolates were able to cause symptoms on one year old canes and mature berries, independently from their tissue of origin and with virulence varying within species. No adverse effects were recorded in dormant buds inoculated with Botrysphaeriaceae.

These findings demonstrate that grapevine wood infected with Botrysphaeriaceae may act as inoculum sources for infection of vegetative tissue and vice versa and therefore
Botryosphaeriaceae, which have been considered exclusively as trunk disease pathogens in *Vitis vinifera*, should be considered as important pathogens of the vegetative tissues of grapevines as well.

The final aim of this study was to determine the genetic diversity of the collection of isolates using amplified fragment length polymorphism (AFLP) analysis. After an initial screening of 24 *EcoRI/MseI* primer pairs on a subset of the eight most phenotypically diverse isolates, the four primer pairs which showed highest heterozygosity were chosen for the final selective amplifications of 180 isolates from the collection. Capillary electrophoresis was performed to detect AFLP fragments and resulting chromatograms scored for presence and absence of polymorphic peaks, providing individual identity and population structure for isolates.

Isolates showed no meta-population structure according to vineyards, origin tissues or origin plants. The population appeared to be panmictic indicating that asexual reproduction is likely to be the predominant reproductive strategy for these organisms. Population structures also showed that inoculum sources from outside each vineyard were equally important than from within the vineyards, suggesting that there have been multiple introductions of Botryosphaeriaceae and that the infection pathway into reproductive tissues of grapevines is not systemic. The genetic diversity study of these Botryosphaeriaceae populations identified possible gene flow within and between vineyards. Results of this study have provided information on the spread of Botryosphaeriaceae within the vineyards surveyed, as well as within individual plants.

This thesis has developed knowledge on the spread of Botryosphaeriaceae in vineyards; has expanded the known tissue tropism of these fungi; and highlights the importance for management of Botryosphaeriaceae in vineyards beyond the existing control approaches which are limited to their role as trunk disease pathogens.
LITERATURE REVIEW

Introduction

Since the first introduction of grapevines to Australia from Europe on the first fleet in 1788 (Clark 2004) Australia has become one of the major wine producers in the world, exporting over 400 million litres annually. The total production for the season 2009/10 was 1.9 million tonnes, which were grown over an area of 160,000 ha (Australian Bureau of Statistics 2010) covering a variety of climatic regions.

One of the oldest wine regions in Australia is the Hunter Valley, comprised of the Upper and Lower Hunter, which are situated in north east New South Wales (NSW). Main cultivars grown in the region to date are Shiraz and Chardonnay. The climate in the Hunter Valley is moderate, mediterranean-like, with an annual rainfall of approximately 760mm (Bureau of Meterology, 2009) and with the highest rainfall periods occurring in summer and immediately before the beginning of winter. During the growing season (September to March), the weather is hot with mean daily maximum temperatures of 33 ºC and mean relative humidity ranging from 43 to 81 % throughout the day (Bureau of Meterology, 2009).

As an intensively grown crop, grapevines are subject to a number of diseases including trunk and bunch rot diseases. Trunk diseases can contribute to an overall loss of grapevine vigour, yield and quality, often referred to as grapevine decline (Morton 2000, Castillo-Pando et al. 2001, Taylor et al. 2005), whereas bunch rot diseases directly impact on grape yield and quality. There are many causal agents for grapevine decline including bacteria, viruses, or fungi, which exert a range of different symptoms. Grapevine decline is sometimes considered a complex rather than a disease (Bumbieris 1972).

The most common grapevine trunk and bunch rot diseases are caused by fungi. Disease pressure from fungi is usually higher in warm climate regions with high relatively humidity, the prevailing conditions of many wine growing regions in Australia, such as the Hunter Valley. Several species of the ascomycete family Botryosphaeriaceae Theiss & P. Syd., typified by the genus Botryosphaeria Ces. & De Not. have been associated worldwide and in Australia with grapevine decline diseases referred to as excorioses, black dead arm, diplodia die back, bot canker, macrophoma rot and bunch rot (Phillips...
1998, Larignon et al. 2001, van Niekerk et al. 2002, Savocchia & Laurent 2005, van Niekerk et al. 2006). Disease symptoms include stunted growth, cankers, wood necrosis, bud necrosis, delayed bud burst, dead arms, canes and shoots, bleached canes and fruit rot (Phillips 1998, Taylor et al. 2005, van Niekerk et al. 2006). While most Botryosphaeriaceae species found in vineyards have been associated with trunk diseases the botryosphaeriaceous fungus *Guignardia bidwellii* has been identified as a fruit rot pathogen in Asia, Europe and South America (Jermini and Gessler 1996, Wilcox 2003), however, this has not been reported from Australian vineyards. While several research directions have been taken to discover the role of Botryosphaeriaceae as wood pathogens in grapevines in Australia, little is known about these species in regards to their role as as bunch rot pathogens. Botryosphaeriaceae have been isolated from symptomatic grapevine bunches along with other known bunch rot pathogens. However, it is unknown whether these fungi are actually pathogenic towards the non-woody plant tissue.

This review provides an overview of the most common grapevine trunk and bunch rot diseases in Australia. Taxonomy of the Botryosphaeriaceae will be discussed, and a summary of significance to the wine industry, epidemiology, current control methods and history in regards to grapevine decline in Australian vineyards will be given. Information of Botryosphaeriaceae as fruit rot pathogens of other hosts and their possible role as grapevine bunch rot pathogens will be discussed. In addition a variety of techniques and their practical application for the analysis of genetic diversity of plant pathogens such as the Botryosphaeriaceae will be reviewed.

**Bunch rot diseases of subtropical viticulture in Australia**

**Sour rot**

Sour rot, also referred to as summer bunch rot, is one of the most common berry rots in subtropical vineyards. It is often caused by various acetic acid bacteria, yeasts and species of a range of fungi commonly present in vineyards such as *Aspergillus*, *Cladosporium* and *Penicillium*, which cause a ‘wet rot’ within grape clusters and produce a vinegar smell (Marois et al. 1992, Schilder 2011). Sour rot infection is often predisposed through primary infection of other fungi or physical damage to the grape berry surface such as hail, sunburn, or insect damage and can be spread by fruit flies.
Wine quality is directly affected by a change in aroma and flavour (Meneguzzi et al. 2008).

**Ripe rot**

*Colletotrichum acutatum* and *Colletotrichum gloeosporioides* are the causal organism of ripe rot (Wu & Chang 1993, Kummuang et al. 1996a) in grapevines. While no data has been published on the significance of ripe rot to the Australian wine grape industry it can be said that this fungal disease causing off-flavoured compounds has a direct effect on wine quality (Meunier & Steel 2009). In Australia, *C. acutatum* was first reported as a pathogen causing ripe rot on grapevines from the Hunter Valley in 2002 (Melksham et al.). Symptoms are described as loss of berry turgor and orange coloured discharge from conidia on the berries (Kummuang et al. 1996b, Steel et al. 2007). As shown by field isolations by Steel et al. (2007) ripe rot is a latent infection possibly developing as early as flowering, with symptoms only developing post veraison. This is supported by a study of infection by *C. gloeosporioides* on muscadine grapes which showed that green fruit could be successfully infected, however, fungal growth did not start to develop until ripening of the berries (Daykin & Milholland 1984).

**Bitter rot**

Bitter rot or Greeneria fruit rot is a bunch rot disease common to vine growing regions with warm-wet weather conditions during the ripening season (Kummuang et al. 1996b, Farr et al. 2001). *Greeneria uvicola* (syn. *Melanconium fuligineum*), an asexual ascomycete (Farr et al. 2001), is the causal organism of bitter rot. There are no economic data on the significance of this disease to the Australian wine industry (Steel et al. 2007). In Australia *G. uvicola* has been isolated from grapevine wood (Castillo-Pando et al. 2001, Sergeeva 2004, Qiu et al. 2011), however, it was not recognised as a major wood pathogen (Ullasa & Rawal 1986, Farr et al. 2001). Only recently has *G. uvicola* been associated with bunch rots in Australian vineyards (Steel et al. 2007). Symptoms of bitter rot include brown necrotic lesions covering half or more of the berry surface. This is followed by the development of dark brown acervuli inside the lesions before the berries shrink and become mummified. These mummified berries often remain on the bunch (Reddy & Reddy 1980) acting as a source of overwintering for the fungus (Farr et al. 2001). Although *G. uvicola* can infect leaves, tendrils and
stems and occasionally cause shoot die-back (Reddy & Reddy 1980), it is primarily considered a fruit pathogen (Farr et al. 2001).

**Grey mould**

Grey mould or grey rot caused by *Botrytis cinerea* has been considered as one of the most important diseases of grapevine (Goetz et al. 1999). Nair and Nadtotchei (1987) confirmed that sclerotia on canes are the primary source of inoculum for Botrytis bunch rot. Flowering is considered as a critical time for infection with *B. cinerea* (Keller et al. 2003). Post flowering the berries are resistant to infection and the pathogen remains latent in the fruit until veraison (Goetz et al. 1999, Keller et al. 2003), when disease expression begins to show as grey mould forming on the outside skin of berries, developing into bunch rot and fruit loss (Nair 1985, Keller et al. 2003). *B. cinerea* can also infect berries post veraison. However, being an opportunistic pathogen it requires mechanical damage to the berry, often in forms of insect or hail damage or splitting of berry skins due to pressure inside the berry.

Maintaining an open canopy is often recommended for the control of *B. cinerea* infection, however this has shown to increase infections with other bunch rot pathogens following sunburn and increased heat-stress (Steel & Greer 2008).

**Trunk diseases**

**Phomopsis cane and leaf spot**

*Phomopsis viticola* is the causal organism for Phomopsis cane and leaf spot (Pearson and Goheen 1988). There are two taxa of *Phomopsis* commonly associated with grapevines referred to as Phomopsis Type 1 (*Diaporthe australafricana*) and Type 2 (*Phomopsis viticola*). Both types have a similar life cycle but vary in symptoms. *Phomopsis viticola* overwinters as mycelium in pycnidia on the bark of the vines and in dormant buds (Hewitt and Pearson 1988). During spring spores are released from the erupting pycnidia, resulting in yellow ooze, which is washed off or rain-splashed onto other plant or plant parts (van Niekerk et al. 2010). Another form of spread occurs via the mycelium. Spore production favours cool temperatures and at least 10 hours of rainfall (Emmett et al. 2001). The fungus is therefore more active during winter and virtually inactive during hotter months.
Phomopsis Type 1 does not cause any foliar symptoms, however vines may show symptoms of dotted appearance and bleaching of canes and spurs caused by the pycnidia and lifting of bark when pycnidia erupt (Hewitt and Pearson 1988). The symptoms caused by Type 2 include small necrotic lesions with yellow margins on leaves, which in severe cases appear distorted, die and become detached (Gubler and Leavitt 1992). Cane bleaching is also associated with infection by Type 2 making it difficult to distinguish infections caused by Type 1. The most significant symptoms of Type 2, however, are the lesions on shoots that develop into black elongated, sunken marks, often mistaken for hail damage, which in severe cases can lead to cracking of the cane (Gubler and Leavitt 1992). Occasionally pycnidia can be observed on fruit leading to fruit rot symptoms (Hewitt and Pearson 1988).

**Petri disease**

Petri disease is one of the two grapevine trunk diseases caused by *Phaeoacremonium* spp. of which sixteen species have been described (Crous *et al*. 1996). Eleven of these were found on grapevines (Mostert *et al*. 2006). The second fungus associated with Petri disease is *Phaeomoniella chlamydospora* (Scheck *et al*. 1998, Crous & Gams 2000, Mostert *et al*. 2006). Petri disease contributes to grapevine decline causing graft failure, stunted and weak growth and shoot dieback leading to gradual death of the vine (Edwards & Pascoe 2004, Mostert *et al*. 2006). Internal symptoms of diseased vines in the wood of trunk and cordons are seen as black spots when transverse wood sections are taken. If trunks and cordons are cut longitudinally the infection shows up as dark brown to black streaking of the wood (Mostert *et al*. 2006). Another distinct symptom of Petri disease is the oozing of black xylem sap from infected xylem vessels. The disease is therefore sometimes also known as black goo (Mostert *et al*. 2006)

Petri disease mainly occurs on young vines (Mugnai *et al*. 1999, Sidoti *et al*. 2000, Edwards & Pascoe 2004, Mostert *et al*. 2006) and has been reported to cause major damage in newly planted vineyards (Edwards & Pascoe 2004). This might be explained by the findings of Edwards and Pascoe (2004) and Eskalen *et al*. (2001), who isolated *P. chlamydospora* from rooted cuttings. This is also supported by Retief *et al*. (2006) who identified root stock and grafting material in nurseries, as well as soil, as inoculum sources of *P. clamydospora*. 
**Esca**


There are two forms of Esca, a chronic and an acute form. Symptoms of the chronic form include interveinal chlorosis and necrosis of the leaves, often referred to as ‘tiger-stripes’. Fruit may become shriveled, discoloured and covered with black spots (Edwards & Pascoe 2004), the reason why Esca disease is sometimes referred to as ‘black measles’ (Mugnai *et al.* 1999). The acute form of Esca is also called ‘apoplexy’ and appears as sudden collapse of the whole plant, mainly during hot weather and late in the growing season (Edwards & Pascoe 2004). Internal wood symptoms are white rotted wood surrounded by a dark border line and dark brown to black spots (Mugnai *et al.* 1999). Esca has generally been associated with older vines, however over the last decade Esca symptoms have also been observed from younger vines from two years onwards (Edwards *et al.* 2001).

**Eutypa dieback**

Eutypa dieback has been found in vineyards worldwide (Emmett *et al.* 2001) and has the potential to effect vineyard productivity and longevity. This disease is also known as dying arm (Carter *et al.* 1983, Bolay & Carter 1985, Emmett *et al.* 2001) and is caused by *Eutypa lata* (Pers.: Fr,) Tul et C. Tul. (syn. *E. armeniacae* Hansf. & Carter; anamorph, *Libertella blepharis* A.L.Smith (syn. *Cytosporina* sp.), an ascomycete fungus with a wide host range (Highet & Wicks 1998). Creaser and Wicks (2001) performed surveys in South Australia (SA), which estimated that 40% of the vines were infected with *E. lata*, however, up to 60% of vines were affected in the older vineyards. Yield reductions in two Shiraz vineyards in the Coonawarra region ranged from 15-32 % (Moller & Carter 1965, Carter *et al.* 1983).

*E. lata* is spread across vineyards via wind-dissemination of ascospores and thus the pathogen can be transported over long distances of over 100 miles (Ramos *et al.* 1975). This and the ability of *E. lata* isolates from alternative hosts to infect grapevines has led
to claim that surrounding vegetation of vineyards can be a primary inoculum source of grapevines (Trouillas & Gubler 2010). *E. lata* infection in *V. vinifera* occurs via pruning wounds where, if infected by ascospores, germination occurs and the mycelium grows into the vascular tissue inhibiting xylem and phloem function. This is a slow process taking between 3 and 8 years (Emmett *et al.* 2001) and explains why Eutypa dieback symptoms are mainly observed in older vines.

Typical eutypa dieback symptoms include stunted shoots with shortened internodes (Creaser & Wicks 2001), discoloured wedge-shaped lesions in the cross-sections of internal wood and canker formation around infected wounds. Entire plants can die after 10 years as a result of infection by *E. lata*. The only symptoms associated with *E. lata* on grape bunches, have been uneven berry size on clusters of affected shoots (Carter 1988) and foliar symptoms of small distorted chlorotic leaves have been observed. Their incidence seems to vary with climatic and seasonal variations (Chapuis *et al.* 1998), however an increase in foliar symptoms observed at the beginning of the season seems to be directly related to increased yield loss at the end of the season and thus foliar symptoms can be used as an indicator of seasonal Eutypa dieback severity. In addition Chapuis (1981) showed variations in susceptibility of grapevine pruning wounds to *E. lata* in France due to seasonal conditions. This supports Petzoldt’s (Creaser & Wicks 1981) theory that early pruning in the season increases the chance of inoculum density and leads to a prolonged susceptibility period of the wounds.

Eutypa dieback can be controlled through protection of pruning wounds immediately after pruning. For this a variety of pruning wound pastes are available either functioning as a mechanical barrier to the wound or containing fungicides that reduce the germination of ascopores (Creaser & Wicks 2002a). Among these, boron was found to be a successful active ingredient of pruning wound protective pastes (Rolshausen & Gubler 2005).

As an alternative to chemical applications, remedial surgery incorporating the removal of infected wood from the plant and retraining of these vines has been suggested as another management option (Sosnowski *et al.* 2005). Preventative cultural control methods are also available and should be used in order to reduce fungicide use and the risk of developing resistance. In areas where dry springs are experienced, double pruning including pre-pruning during the dormant period followed by pruning to two
bud spurs in late winter when Eutypa infections are less likely to happen has been proven to be an efficient preventative method (Weber et al. 2007).

**Botryosphaeria canker and bunch rot**

**Taxonomy of Botryosphaeriaceae**

The family Botryosphaeriaceae Theiss. & P. Syd., typified by the genus Botryosphaeria Ces. & de Not is a cosmopolitan, species-rich family, commonly associated with wood diseases and fruit rot with a wide host range (Smith 1934, von Arx & Mueller 1975). The genus *Botryosphaeria* was introduced by Cesati and De Notaris (1863). It was originally based on one of its type species *Botryosphaeria dothidea* (Moug.: Fr.) Ces & De Not.(Slippers et al. 2004b) and belongs to the ascomycete fungi and represents the teleomorph (sexual form) of which the anamorph (asexual form) is represented by different genera. The Botryosphaeriaceae family has had multiple revisions since its introduction and more recent phylogenetic studies (Crous et al. 2006, Phillips et al. 2008) have reduced this genus to two true “Botryosphaeria” species: *B. dothidea* and *B. cortices*. However, the remaining species were grouped into different as well as new genera within the Botryosphaeriaceae family. Species associated with grapevine generally belong to the genera *Botryosphaeria, Diplodia, Lasiodiplodia, Dothiorella, Spencermartinsia and Neofusicoccum* with anamorphs in the genera *Diplodia* and *Fusicoccum* (Phillips 2002, Slippers et al. 2004a, Urbez-Torres & Gubler 2007). This involved renaming the species based on their anamorph genera. For convenience and when first mentioned both new and old names will be used in this chapter, stating the old names in inverted commas.

All species formerly belonging to the genus Botryosphaeria can be divided into two groups, those with *Fusicoccum*-like and *Diplodia*-like anamorphs (Denman et al. 2000, Zhou & Stanosz 2001a, Phillips 2002, Slippers et al. 2004b). Other authors refer to these groups as the Hyala group and Brunnea group (Zhou & Stanosz 2001b). Groups differ in their conidia morphology with hyaline thin-walled conidia and pigmented thick-walled conidia for *Fusicoccum* - and *Diplodia*-like anamorphs, respectively. Further restructuring of the genus occurred when Van Niekerk et al. (2004) suggested inclusion of the genera with septate ascospores and *Diplodia* anamorphs, such as *Diplodia sarmentorum*, confirmed to be the anamorph of *Othhia spiraeae*, to the genera concept of *Botryosphaeria*. 
The teleomorph stage of the different species of the Botryosphaeriaceae is seldomly found in field situations and has only rarely been produced under laboratory conditions. Identification is therefore largely based on characteristics of the anamorph (Denman et al. 2000, Phillips 2002). This is problematic as often conidia morphology does not differ much or overlaps between different species. The use of molecular identification techniques has improved this situation significantly, with the analysis of the former genus ‘Botryosphaeria’ through phylogenetic studies, indicating the relatedness of members of the Botryosphaeriaceae to each other with results often implicating the restructuring of this fungi family. However, even the investigation into DNA phylogeny does not always provide consistent results for the taxonomy of Botryosphaeriaceae. Denman et al. (2000) suggested that the genus ‘Botryosphaeria’ is monophyletic, based on results of an ITS rDNA phylogenetic study. This conflicts with Zhou and Stanosz (2001b) who suggest that this genus is not monophyletic but rather originated from two separate genera Botryosphaeria and Guignardia. In addition, low variation of ITS sequences within groups such as the three subgroups containing 1. ‘B. ribis’, ‘B. parva’ and ‘B. lutem’; 2. ‘B. dothidea’, ‘B. mamane’ and ‘B. cortices’ and 3. ‘B. obtusa’, ‘B. stevensii’ and Sphaeropsis sapinea have been suggested to be caused by the slow rate of evolution of the preserved ITS region (Zhou et al. 2001). This indicates the need for more comprehensive and sensitive methods to analyse the very closely related fungal taxa.

**Host range**

Species of the Botryosphaeriaceae are responsible for diebacks and fruit rots in a wide range of woody hosts (Phillips 2002, Denman et al. 2003, Phillips et al. 2005, Mohali et al. 2006, van Niekerk et al. 2006), which has been estimated to be more than 100 plant genera (Buckley & Gould 2000). While some species appear to be more host specific such as ‘B. protearum’, which is only found on protea plants (Denman et al. 2003), others such as the grapevine pathogens D. seriata (‘B.’ obtusa), N. parvum (‘B.’ parva), N. ribis (‘B. ribis’), ‘B.’ dothidea and L. theobromae (‘B.’ rhodina) are more diverse (van Niekerk et al. 2004) and include several hosts such as blueberries (Creswell & Milholland 1987, Creswell & Milholland 1988), peaches (Pusey 1989a), apples (Brown-Rytlewski & McManus 2000), almonds (English et al. 1975), olives (Lazzizera et al. 2008), pomegranates (Liu et al. 2009), eucalyptus (Davidson & Tay 1983) and oak trees (Linaldeddu et al. 2009). Susceptibility to Botryosphaeriaceae of these hosts seems to
be directly related with stress, as shown by the increase in severity of disease symptoms with increasing water stress in peach trees (Pusey 1989b) and pistachios (Ma et al. 2001b).

**Fruit rots caused by Botryosphaeriaceae**


The disease cycle of Botryosphaeria fruit rot has been most intensively studied for apple. Initially *D. seriata* (‘*B. obtusa*’) was found to be the cause of black rot disease in apples (Beisel-Smith & Hendrix 1984). Later *N. ribis* (‘*B. ribis*’) and *B. dothidea* were also recognised as fruit pathogens of apple (Sutton 1981). A study by Brown-Rytlewski and Mc Mannus (2000) showed that pycnidia on dead twigs of apple are the major source of inoculum for apple fruit rot. In addition rotten apples left on the trees or fallen to the ground are considered a potential source of conidia release during periods of rain (Brown & Britton 1986). Taylor (1955) showed that prior wounding of apple fruits was not necessary for the infection and that infection could start as early as bud burst. Beisel et al. (1984) supported this theory with a field study showing that infection already occurs during the silver tip stage, a very early stage of bud development and thus could be controlled with early applications of fungicides.

The association of Botryosphaeriaceae with fruit in vineyards has been known for many years; however, their recognition as bunch rot pathogens of *V. vinifera* in Australia still remains low. Earlier literature identifies the botryosphaeriaceous fungal species *Guignardia bidwellii* as a fruit rot pathogen of *V. vinifera* causing black rot in Asia, Europe and North- and South America (Scribner & Viala 1888, Jermini & Gessler 1996, Wilcox 2003), where it presents a great threat to the wine industry. In Australia studies of Botryosphaeriaceae in bunches have been limited to incidence data, however, there is no evidence of *G. bidwellii*. In addition observational reports of bud failure from
vineyards with Botryosphaeriaceae wood infection exist (Castillo-Pando et al. 2001, Qiu et al. 2011).

**Spread and significance to the wine industry**

The Botryosphaeriaceae are responsible for several grapevine diseases leading to grapevine decline, also referred to as Botryosphaeria canker, ‘bot’ canker, black dead arm, excoriose and Botryosphaeria bunch rot, however, a recent review summarises these diseases under the name grapevine decline (Urbez-Torres 2011). Under the current revision of the genus there are 21 species of the anamorph (Urbez-Torres 2011) of which 10 have been reported from grapevines in Australia. These are:

- *Botryosphaeria dothidea* (Moug. Ex Fr.) Ces. & De Not.
- *Diplodia seriata* (‘B. obtusa’) De Not.
- *Diplodia mutila* (‘B. stevensii’) (Fr.) Mont.
- *Lasiodiplodia theobromae* (‘B. rhodina’) (pat.) Griffon & Maubl.
- *Neofusicoccum parvum* (‘B. parva’) (Pennycook & Samuels) Crous, Slippers & A.J.L. Philips
- *Neofusicoccum ribis* (‘B. ribis’) (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Philips
- *Neofusicoccum luteum* (‘B. lutea’) (Pennycook & Samuels) Crous, Slippers & A.J.L. Philips
- *Neofusicoccum australae* (‘B. australis’) (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Philips
- *Dothiorella viticola* (‘B. viticola’) A.J.L. Philips & J. Luque

The grapevine trunk disease, black dead arm, was first reported from grapevines in Hungary in 1974 and was associated with infection by Botryosphaeriaceae species *D. mutila* (‘B. stevensii’) (Lehoczky). Further species of the fungal family Botryosphaeriaceae have since been reported from most countries where grapevines are grown and from the wood of several different *Vitis vinifera* cultivars showing symptoms of grapevine decline (van Niekerk et al. 2006). Vineyard surveys reported the isolation of Botryosphaeriaceae in South Africa (Fourie & Halleen 2004, van Niekerk et al. 2004), Portugal (Phillips 1998, Phillips 2002), Spain (Luque et al. 2005, Aroca et al. 2006, Urbez-Torres & Gubler 2006, Aroca et al. 2008), Italy (Burrano et al. 2008)
Literature Review


From wood surveys conducted in Australia, species of Botryosphaeriaceae have been found in the wine grape growing regions of Western Australia (WA) (Taylor et al. 2005, Wood & Wood 2005), SA (Pitt et al. 2010) and in the subtropical wine grape growing regions of eastern Australia (Phillips 1998, Dunne et al. 2006, Savocchia et al. 2007, Pitt et al. 2010, Qiu et al. 2011). The species found in the Hunter Valley include D. seriata, N. luteum (Savocchia et al. 2007), N. ribis (Castillo-Pando et al. 2001), N. parvum (Cunnington et al. 2007), B. dothidea (Qiu et al. 2008) and L. theobromae (Qiu et al. 2011).

It is difficult to estimate the economic impact of Botryosphaeriaceae species on grapevine production, primarily because vines are often infected simultaneously by several pathogens all contributing to grapevine decline, particularly with bunch infections (Buchanan & Beever 1986). In New Zealand, Buchanan and Beever (1986), reported a low recognition of Botryosphaeriaceae as bunch rot pathogens despite their high incidences of berry infection. According to these authors this is due to the similarity of Botryosphaeria bunch rot disease symptoms with those caused by P. viticola and B. cinerea.

Following disease assessment in wood and canes, Wood and Wood (2005) estimated the potential loss to the wine industry in a region planted to a very susceptible cultivar to be 11% of the industry’s value. The economic impact of bunch rots caused by Botryosphaeriaceae in ‘Muscadine’ table grapes (Vitis rotundifolia) has been estimated to be accountable for 20-30% fruit losses in south eastern USA (Pearson & Goheen 1988) and 5 to 80 % in V. vinifera in parts of Europe (Jermini and Gessler 1996). No records exist on the production loss in V. vinifera caused by Botryosphaeria bunch rot in Australia, with the exception of an industry survey which estimated yield losses to be between 0.5% in hot dry conditions to 20% in hot and wet climatic zones (Scholefield & Morison 2010). Economic losses and cost associated with Botryosphaeriaceae infection were estimated as one of the most concerning due to high additional operating costs and reduced yields. In their report representatives of the hot and wet climatic wine
regions state the need to recognise Botryosphaeriaceae as both a fruit rot and trunk
disease pathogens (Scholefield & Morison 2010).

**Botryosphaeriaceae trunk disease and bunch rot symptoms**

Various grapevine decline symptoms have been associated with the isolations of
Botryosphaeriaceae species from various parts of the grapevine. Visible symptoms in
the wood and shoots include cankers, wood necrosis, spur dieback and retarded growth
2006, Urbez-Torres *et al.* 2008). Internal symptoms have been described as wood
streaking and internal necrotic lesions (Lehoczky 1974, Phillips 1998, Phillips 2000,
Castillo-Pando *et al.* 2001). In addition, grapevine bud mortality and in some cases
bunch rots have been observed worldwide (Luttrell 1948, Lehoczky 1974, Milholland
Castillo-Pando *et al.* 2001, Larignon *et al.* 2001). In Australia, incidence reports of
Botryosphaeriaceae exist from bunch rot surveys in WA (Taylor 2007) and the Hunter
Valley (Steel *et al.* 2007), however, neither of the authors isolated Botryosphaeriaceae
from symptomatic bunches in the absence of other pathogens, nor were the isolates
identified to species level.

Botryosphaeriaceae infected berries have a water-soaked appearance, white mycelium
growth and occasional cracking of the skin. In more severe cases, berries dry out and
turn black before being mummified and developing black pycnidia on the surface
(Luttrell 1948). This is not commonly observed as dried out berries often become
invaded by secondary pathogens.

Foliar symptoms such as leaf necrosis and chlorosis have also been recorded from
grapevines in association with Botryosphaeriaceae (Lehoczky 1974, Larignon *et al.*
2001, Auger *et al.* 2004), while Denman (2000) observed that leaf spots and fruit rots
are more common on other hosts.

The identification of Botryosphaeriaceae species based on die back and internal wood
symptoms is very difficult because these symptoms are very similar to those caused by
*E. lata* and sometimes *P. viticola* and often all three pathogens are found together on the
same plant sample (Savocchia *et al.* 2007, Qiu *et al.* 2008). This led to the
misconception in the Hunter Valley, where grapevine decline symptoms associated with Botryosphaeriaceae were thought to be due to *E. lata*. To date, *E. lata* has never been isolated from that region (Castillo-Pando *et al.* 2001).

As for Eutypa dieback, the symptoms of grapevine decline caused by Botryosphaeriaceae develop slowly eventually causing reduced vigour and decline in yield (Phillips 1998). The symptoms are therefore often only seen on grapevines eight years and older (Larignon *et al.* 2001).

**Diagnosis**

Correct diagnosis of Botryosphaeriaceae infection in grapevines is difficult because of the range of possible symptoms that are not unique to the Botryosphaeriaceae (van Niekerk *et al.* 2006). Conventionally, Botryosphaeriaceae identification to species level involves the isolation of the fungus from diseased tissue by culturing on artificial media and categorisation based on conidia morphology. This is a time consuming and often complex task due to the many species and overlap in conidial morphology in their asexual state (van Niekerk *et al.* 2006). Morphological identification has therefore been complimented by molecular identification involving DNA sequencing (van Niekerk *et al.* 2006). This not only allows the identification process to be a more accurate task, it also requires less time between sampling from a diseased plant and pathogen identification by eliminating the time consuming tasks of culturing the pathogen, often over several weeks until conidia form.

**Pathogenicity**


Results of virulence for individual species have often been conflicting, indicating that symptom severity varies between different geographical and environmental conditions (Slippers & Wingfield 2007). Some of this variation was thought to be due to
differences in the susceptibility of different host cultivars (Larignon et al. 2001, van Niekerk et al. 2004). It is therefore extremely difficult to classify the different species of Botryosphaeriaceae in terms of their pathogenicity or virulence and thus identify which species presents the greatest threat to the wine grape industries.

**Life cycle of Botryosphaeriaceae in vineyards**

A complete disease cycle has not been reported for Botryosphaeriaceae and bunch rots in *V. vinifera*, however, bud mortality has been reported as one of the symptoms (van Niekerk et al. 2004), which suggests that infection may occur very early in the season. From there the infection may travel through the young shoots causing black lesions on internodes which may lead to shoot dieback and bleaching appearance of canes at the end of the season. (van Niekerk et al. 2004). For the occurrence of black rot of *V. vinifera* in other countries it is known that infection of berries start with an infection of the lenticels or lesions on pedicels from where they spread into the berries (Wilcox 2003).

Studies of Botryosphaeriaceae on apple and peach have shown that ascospores of ‘*B.* dothidea’ and conidia of ‘*B.* obtusa’ are released from pycnidia on the bark of diseased wood and rotten fruit during periods of prolonged wetness (Sutton & Boyne 1983, Pusey 1989a). The spores then spread onto pruning wounds and fruit. A similar situation may also occur in grapevines and this may explain why infected buds have been found.

Botryosphaeriaceae enter the wood of grapevines via pruning wounds (Urbez Torres and Gubler 2009) however, no information exists on the pathway of infection into berries. Although, grapevines appear to be most susceptible to Botryosphaeria bunch rot at the mature berry stage, indicated by increasing abundance of infections towards the harvest stage (Kummuang et al. 1996a).

Milholland (1988) suggested that Diplodia cane dieback and bunch rot is favoured by high humidity and rain and that conidia overwinter in the dead wood and pruning debris on the vineyard floor. Others isolated Botryosphaeriaceae from mummified berries (Milholland 1991, Kummuang et al. 1996a) and suggest that these add to the inoculum source on the vineyard floor (Halleen & Fourie 2005). From these, propagules are
released during rain periods and conidia are splash dispersed onto pruning wounds (Amponsah et al. 2010). Using spore traps in vineyards, Van Niekerk et al. (2010) correlated spore release with weather data and showed that rain events as little as 0.25 to 1 mm in combination with high relative humidity facilitate the release of conidia. Furthermore, single rainfall events have been reported to cause spore movements of up to two metres from the inoculum source (Baskarathevan et al. 2010). The life cycle may vary for different Botryosphaeriaceae species, which have been shown to require different climatic conditions to produce fruiting structures (van Niekerk et al. 2006). This is in contrast to E. lata, which spreads over vast distances and infects grapevines via ascospore wind-dissemination. The role of wind for the dispersal of Botryosphaeriaceae, mainly seen as conidia in vineyards, remains unresolved and thus further research into the role of surrounding vegetation as inoculums sources for vineyards is needed (Urbez-Torres 2011). In addition to the potential sources of inoculum discussed, Whitelaw-Weckert et al. (2006) established that species of the Botryosphaeriaceae have the potential to infect roots of young grapevines eventually moving through the trunk into the shoots. Furthermore, high frequencies of Botryosphaeriaceae infection have been found on propagation material and rootstock mother vines from nurseries in New Zealand and Spain (Aroca et al. 2010, Billones et al. 2010).

Control

Cultural control

There are several cultural control approaches to assist in preventing or reducing the risk of Botryosphaeriaceae infection in the vineyard. These should be included in an integrated management approach in order to reduce the reliance on the use of fungicides and bio control agents.

Cultural control methods include the reduction of inoculum sources by removing plant material from the vineyard floor after pruning (van Niekerk et al. 2004), the prevention of unnecessary wounding of the vines (Lehoczky 1974, Milholland 1988), avoidance of stress (Larignon et al. 2001) such as water stress or heavy crop loads (Halleen & Fourie 2005) and avoiding pruning during periods of high inoculum availability (van Niekerk et al. 2010).
Remedial surgery and the practice of double pruning have been used for many years to manage the spread of trunk diseases including *E. lata* (Creaser & Wicks 2002b, Weber *et al.* 2007, Urbez-Torres & Gubler 2008). Both practices have also been suggested for the control of Bot canker (Savocchia *et al.* 2005, Sosnowski *et al.* 2005, Urbez-Torres & Gubler 2008) and have since become the most common practiced control strategy for this disease. However, these methods are limited to controlling the spread of Botryosphaeriaceae from wood tissue only during the dormant season and do not control spread from other inoculum sources such as the reproductive tissue.

A commonly recommended management strategy for botrytis bunch rot is canopy management, where thinning of dense canopies is said to increase airflow into the canopy and therefore reduce the temperature and humidity inside the canopy and around the bunches. However, this management strategy is often limited by an increase of susceptibility to non-botrytis bunch rots explained by the increase in sunburned fruit (Steel *et al.* 2007). It is yet to be established whether canopy management would be a feasible strategy to limit the occurrence of Botryosphaeriaceae bunch infections or whether similar limitations than with canopy management associated with *Botrytis* exist.

**Biological control**

Biological control is the use of living organism to control pests. Commonly used bio control agents in plant pathology include species of the fungus *Trichoderma*. Fourie *et al.* (2001) established that *Trichoderma harzianum* reduces infection of *Cylindrocarpon, Phaeomoniella* and *Phaeoacremonium* species in roots and rootstock material in nurseries and it has been successfully used to protect pruning wounds from *E. lata* (John *et al.* 2005). Biocontrol field trials showed that application of spore suspensions from two *Trichoderma* species, *T. harzianum* and *T. atroviride* effectively controlled apple ring rot in shoots and stems and apple fruit rot caused by *B. berengeriana*. *In vitro* studies showed that mycelium growth was inhibited through the modes of hyphal cell penetration and disruption and cytoplasm thinning, which also reduced sporulation of *B. berengeriana* (Kexiang *et al.* 2002). So far there have been no studies reported on the potential of *Trichoderma* spp. as biocontrol agents for Botryosphaeriaceae in grapevines.
Literature Review

**Chemical control**

There are currently no products registered for the control of Botryosphaeriaceae in Australian vineyards. The effectiveness of available fungicides overseas has been shown to vary with regional and climatic conditions as well as for each individual species (van Niekerk et al. 2006). *In vitro* tests have been conducted to study the suitability of fungicides as control agents for Botryosphaeriaceae species. In South Africa products with the active ingredients benomyl, tebuconazole, prochloraz manganese chloride, flusilazole and fenarimol were considered to be the most effective inhibitors of mycelium growth of *N. australe*, *D. seriata*, *N. parvum* and *L. theobromae* (Bester et al. 2007). Four of these five fungicides are registered for use in vineyards in South Africa. Field trials by Denman et al. (2004) found the same fungicides to be effective for the control of ‘*B. protearum*’ in Proteaceae. *In vitro* fungicide trials in Australia showed that the active ingredients tebuconazole, fenarimol, spiroxamine, fluazinam, fludioxonil, carbendazim, penconazole and iprodione (Savocchia et al. 2005, Pitt et al. 2008) have the potential to control some Botryosphaeriaceae commonly found in grapevines in Australia. Some of these fungicides are routinely used to control *B. cinerea* and may also have some efficacy against Botryosphaeria bunch rot. In addition studies have shown that infection risk could be reduced by preventative application of fungicides as pruning wound protectants (Brown-Rytlewski & McManus 2000, Leavitt 2003).

Further fungicide studies in the Hunter Valley to establish the efficacy of fungicides as pruning wound protectants in the field have shown that some of the products were able to reduce infection by up to 43%, however some of these required application rates exceeding the recommended rates (Pitt et al. 2011). As for fungicide spray applications, this approach also requires a rotation between different fungicides in order to prevent resistance build-up (van Niekerk et al. 2006). Limited availability of efficient fungicides brings the risk of overusing one product and promoting fungicide resistance in Botryosphaeriaceae species. For example in California *B. dothidea* in pistachio has developed resistance to iprodione, a fungicide registered for use in vineyards and particularly popular because it is registered for the use after 80% capfall (Ma et al. 2001a) and can be used 14-21 days before harvest in Australia. A choice of fungicides with different active ingredients and the use of integrated pest management is therefore important for the successful control of Botryosphaeria canker and bunch rot.
Genetic diversity of plant pathogens

DNA profiling, or the use of molecular markers to determine the genotype of organisms has brought great improvement to plant pathology, simplifying the often laborious and time consuming process of plant pathogen identification. Analysis of genetic diversity and linking this to the phenotypic traits has often led to a re-grouping of species complexes, originally based on morphological differences such as those of the Botryosphaeriaceae (Denman et al. 2000, Crous et al. 2006, Phillips et al. 2008, Pavlic et al. 2009). In addition, population genetics, the study of change of allele frequency distribution over time, has provided an aid to answering biological questions such as the origin of plant pathogens and helps to predict likely development of populations. This genetic change within a population can be the cause of mutation, mating systems, gene flow or migration, population size and selection (McDonald 1997). To detect this variability, several different DNA profiling techniques can be used in plant pathology, combining efficient examination techniques of informative segments of DNA, with user-friendly statistics packages to analyse DNA related data. Some of the different methods to analyse the genetic diversity of populations are explained here with examples of their use within fungal plant pathogens.

Genetic markers of specific targeted sequences

Selective genetic markers are commonly used to answer questions relating to the effects of selection (McDonald 1997) within populations. Examples of techniques commonly used for fungal plant pathogens are Simple or Short Sequence Repeats (SSRs), Inter Simple or Short Sequence Repeats (ISSRs) and Restriction Fragment Length Polymorphisms (RFLPs).

Simple Sequence Repeat Markers (SSRs) and Inter Simple Sequence Repeat Markers (ISSRs)

SSR markers, also referred to as Microsatellites or Short Tandem Repeats (STRs) are tandem repeat loci of simple, short (1-10bp) nucleotide sequences which are highly abundant in genomic DNA (Levinson & Gutman 1987). This technique uses polymerase chain reaction (PCR) primers to amplify the fragments between SSR primer sequences from genomic DNA. Multiple locus genetic markers that are amplified can be used for genome fingerprinting. This technique has the simplicity of other DNA fingerprinting approaches, is reliable and derived from known heritable DNA sequences (Zhou et al. 2001). In the Botryosphaeriaceae, SSRs were recently used to identify a
lack of host specificity for the tree pathogen *L. theobromae* with the detection of a very high gene flow between isolate populations from different hosts (Mohali *et al.* 2005).

Similar to SSR, in ISSR the complementary sequences to two neighbouring micro satellites are the PCR primers between which a variable region, either conserved or non-conserved, is amplified resulting in DNA strands of varying lengths. This technique allowed Zhou *et al.* (2001) to differentiate between species of Botryosphaeraceae with very similar morphology, for which sequencing of the highly conserved internal transcribed spacer (ITS) region had failed to identify to species level, and to divide within the species complex of *N. parvum, N. ribis* and *N. luteum* (Zhou *et al.* 2001).

**Restriction Fragment Length Polymorphism (RFLP)**

This technique involves the digestion of genomic DNA with restriction enzymes followed by separation of the fragments, and can detect heterozygosity at a single base pair level. This change, which alters the restriction endonuclease recognition site and prevents the enzyme from cutting the DNA causing a new restriction site to be formed (Barnum 2005), causes the DNA fragments obtained from this digestion to differ in size. This difference, also referred to as polymorphism can either be visualised with hybridisation methods to a labelled probe such as southern blotting or by PCR amplification of the enzyme digest and separation of the amplified fragments by agarose gel electrophoresis (Barnum 2005).

RFLP analysis produces co-dominant markers exhibiting multiple alleles per locus, making it a powerful and much more suitable tool for genetic analysis of populations than RAPDs (Rapid Amplified Polymorphic DNA) (McDonald 1997, Burgess *et al.* 2001).

RFLP results are easy to interpret, however, being based on DNA hybridisation this method is technically more difficult than RAPDs and not as cost efficient when analysing small numbers of loci (McDonald 1997). Another disadvantage of RFLPs is the requirement of large amounts of DNA (5 to 10 µg) as starting material (Antoni Rafalski & Tingey 1993, McDonald 1997, Burgess *et al.* 2001), however, it has been described as a technique more reproducible between laboratories than RAPDs and probes and enzymes can be shared (McDonald 1997).
Rolshausen et al (2004) identified *E. lata* from other grapevine trunk disease pathogens using PCR-RFLP after in situ PCR. They also amplified DNA from other *Diatrypaceae* grapevine trunk disease pathogens but were unable to distinguish those from *E. lata*.

**Genetic markers of anonymous genomic sequences**

These type of DNA markers are generally used in genetic diversity studies to answer questions regarding the effect of population size, mating systems and gene flow. However, in fungi, which mainly reproduce asexually, it is appropriate to use a neutral marker to answer selection related questions (McDonald 1997).

**Random Amplified Polymorphic DNA (RAPD)**

RAPD-PCR is a technique which produces banding patterns due to mutations at the primer banding sites with the use of short primers (~10bp) and low annealing temperatures (Burgess et al. 2001). It involves PCR amplification of random DNA sequences (Barnum 2005). While RAPD is a powerful tool in terms of molecular analysis of fungi, it has the disadvantage of requiring low annealing temperatures which limit reproducibility between laboratories compared to other techniques (Burgess et al. 2001). With the use of RAPDs Borie et al. (2002) identified infection events and origins for Esca decline caused by *P. chlamydospora* and *Phaeoacremonium aleophilum* in vineyards in France and allowed recognition of *P. aleophilum* as a recombining species. RAPDs were used by Smith and Stanosz (2001) to distinguish between three groups of Botryosphaeriaceae with *Fusicoccum* anamorphs which are difficult to separate morphologically.

**Single nucleotide polymorphism (SNP) genotyping**

Single nucleotide polymorphism genotyping uses the ability to detect a single base pair difference at one locus. Position and number of such point mutations varies between different species but also strains or plant pathogenic races. Knowledge of SNPs within species of plant pathogens allows to develop markers for PCR based identification of the pathogens to the sub-species levels (Gherbawy & Voigt 2010). This technique has the advantage of requiring a low amount of DNA starting material, however, the DNA needs to be of high quality due to sensitivity of the methods (Benali et al. 2011).

**Amplified Fragment Length Polymorphism (AFLP)**

This DNA fingerprinting technique, first described by Vos et al.(1995) involves the digestion of genomic DNA with two restriction enzymes (Barnum 2005). The pair of enzymes has to contain a frequent and infrequent cutter, such as *MseI* and *EcoRI*
(Barnum 2005). Oligonucleotide double stranded adaptors are then ligated to the sticky ends of the restriction sites and act as primer binding sites. This is the template for DNA amplification by PCR (Vos et al. 1995). There are two PCR amplifications; the first one is a pre-selective PCR with primers complementary to the two end adaptor sequences including up to three extra nucleotides at the 3’end of each primer. This is followed by a second PCR, referred to as selective amplification of a subset of the previously amplified fragments (Barnum 2005).

The second PCR reduces the number of fragments to be analysed further by using selective primers containing two additional nucleotides. The banding patterns generated after the selective amplification contain only a 1/4000th of the original EcoR1/Mse1 fragments, however, the entire genome is randomly sampled as opposed to the previously described techniques which focused on selected sequences of DNA only (Barnum 2005). Thus AFLP generates a large number of bands (100-200) with a small number of primers and detects more polymorphism per reaction than RAPDs (Vos et al. 1995). The primer annealing to the EcoR1 adaptor sequence can be labeled either fluorescently or radioactively for visualization with gel electrophoresis or left unlabelled if polymorphism in fragments is to be visualized through silver-staining techniques (Polanco et al. 2005). Figure 1 summarises the steps involved in AFLP from cutting of the genomic DNA to visualization of fragments through gel electrophoresis.

![Figure 1: Flow diagram of the different steps of AFLP based on an example of one selective primer pair (Vuylsteke et al. 2007).](image-url)
Polymorphism in populations is analysed with AFLP by detecting common bands as well as different bands between samples, where the differences are typically inherited in a Mendelian fashion, thus it can be used for typing, identification of molecular markers as well as mapping of genetic loci, to determine kinship, reproductive mode and genetic isolation (Vos et al. 1995, Burgess et al. 2001). Another advantage of AFLP over RAPDs is that it is less sensitive to variations in the reaction conditions and is therefore more reproducible and robust than other techniques (Barnum 2005).

Disadvantages of AFLPs are the labor intensity of several steps involved in the analysis, making it not a practical approach to be used by a diagnostics lab (Vincelli & Tisserat 2008). This problem is overcome in smaller scale operations through the availability of commercial AFLP analysis kits with standardised protocols and a range of primers available for different types of organisms, such as the Invitrogen AFLP Analysis System for Microorganisms and AFLP Microorganism Primer Kit (Invitrogen, USA), making it a reliable and solid tool for the analysis of genetic diversity of plant pathogen populations. Vos et al. (1995) describe AFLP as a combination of the reliability of classical hybridisation based fingerprinting such as RFLPs and the power of PCR based fingerprinting such as RAPDs, making it a very sensitive DNA profiling method and an improved version of other techniques (Barnum 2005).

The above mentioned advantages of AFLPs over other techniques to analyse genetic diversity in populations are reflected in a comparative study between AFLPs, RAPDs, ISSRs and universally primed PCR methods. Here, AFLP was the most informative method, showing greatest polymorphism at inter-vineyard, intra-vineyard and intra-vine level for *P. chlamydospora*, the causal agent of Petri diseases. From the detected low genetic variation it could be concluded that this fungus reproduces mainly asexually in New Zealand vineyards (Pottinger et al. 2002).

The most recent example of practical implication for the use of AFLP technique in Botryosphaeriaceae is a study by Piškur et al (2010) identifying *B. dothidea* from European hop hornbeam (*Ostrya carpinifolia*) and linking its appearance to a change in climatic conditions.
SUMMARY AND PROJECT AIMS
In this review, the role and epidemiology of Botryosphaeriaceae species as wood pathogens of V. vinifera, identified so far, has been described, along with reports of their occurrence in bunches and other hosts surrounding vineyards. In addition, the role of Botryosphaeriaceae fungi as fruit rot pathogens of other hosts has been discussed. Despite the current reports and their well-studied role as wood pathogens, little is known about Botryosphaeriaceae as potential pathogens of other tissues of V. vinifera. However, their ability to infect various tissue types and cause fruit rot in a variety of hosts indicate the importance of understanding the role of Botryosphaeriaceae fungi as potential bunch rot pathogens. This importance is further highlighted by recent incidence reports of Botryosphaeriaceae from V. vinifera bunches and the current industry survey requesting recognition of Botryosphaeriaceae as both trunk and bunch rot pathogens in a range of grape growing regions. If fungi belonging to the Botryosphaeriaceae can also affect tissue other than wood of grapevines, then these could be a source of inoculum throughout the growing season. A rapid increase of the number of reports of new species and wine regions displaying Botryosphaeria canker, suggests that this will also be the case for association of Botryosphaeriaceae with bunch and fruit rot. For the effective control of this grapevine pathogen it is important that epidemiological knowledge of Botryosphaeriaceae infection in Australian vineyards also considers the role of the whole plant.

Botryosphaeriaceae are haploid fungi with often very small differences between species of the commonly sequenced regions and genes and morphologically. In addition, no member of this family of fungi has been completely sequenced yet. Therefore AFLP provides a useful tool for the investigation of genetic diversity of Botryosphaeriaceae isolate collections. This information will be useful to generate new knowledge on the distribution of Botryosphaeriaceae isolates within and between vineyards and could help to identify possible infection pathways to Botryosphaeriaceae bunch rot.

A more detailed and holistic knowledge of Botryosphaeriaceae infection in grapevines and their infection pathway throughout the vine will not only help elucidate the role of these fungi in the vineyard but may also help in understanding and managing this grapevine pathogen in the future.

The aims of this project were to:
• determine the incidence of Botryosphaeriaceae infection of grapevine reproductive tissue at different phenological stages in order to establish if species commonly found in grapevine wood can also infect other tissues and to gain knowledge about their abundance in the different tissues at different growing stages;
• inoculate different grapevine tissues with isolates of Botryosphaeriaceae to determine their ability to infect these tissue types and whether they are tissue specific;
• assess the genetic diversity of the isolates from the survey in order to gain information about their origin and movement within the vineyard and/or vines and information about the role of Botryosphaeriaceae in bunch rots.

PROJECT OUTPUTS

The work conducted in this project resulted in three peer-reviewed journal articles. Preliminary results leading to these publications were presented at five different conferences and workshops and in an industry article. These publications, the conference abstracts and the industry article are presented here. A unifying discussion forms the concluding chapter of this thesis.
Introduction

Several species belonging to the family Botryosphaeriaceae, pose a great threat to the viticulture industry worldwide by causing the grapevine decline disease commonly known as Botryosphaeria (‘Bot’) canker, and other diseases such as excorirose, black dead arm, and diplodia dieback (Largnon et al. 2001, Van Niekerk et al. 2002 and 2006, Savocchia et al. 2007). Disease symptoms include stunted growth, cankers, wood necrosis, dead arms, canes and shoots and bleached canes (Phillips 1998, Van Niekerk et al. 2006, Savocchia et al. 2007). The importance and wide distribution of these ascomycete fungi has become evident over recent years guided by the results of a large number of vineyard surveys worldwide (Castillo-Pando et al. 2001, Phillips 2002, Taylor et al. 2005, Wood and Wood 2005, Qui et al. 2010, Urbez-Torres and Gubler 2006, Urbez-Torres et al. 2006, Van Niekerk et al. 2006, Savocchia et al. 2007, Urbez-Torres and Gubler 2007, Urbez-Torres et al. 2008, Pitt et al. 2010). To date, ten species of Botryosphaeriaceae have been reported from Vitis vinifera in Australia. These are Diplodia seriata, Diplodia mutila, Lasiodiplodia theobromae, Neofusicoccum parvum, Neofusicoccum australiae, Neofusicoccum luteum, Neofusicoccum ribis, Botryosphaeria dothidea, Dothiorella viticola and Dothiorella iberica (Castillo-Pando et al. 2001, Taylor et al. 2005, Wood and Wood 2005, Savocchia et al. 2007, Qui et al. 2008, Pitt et al. 2010). These surveys have been limited to the wood of V. vinifera, however, other vineyard surveys such as those in Western Australia (WA) and the Hunter Valley (Steel et al. 2007, Taylor 2007) studying the occurrence of bunch rot pathogens have revealed that Botryosphaeriaceae can also be isolated from grapevine bunches. A survey of wine regions in WA during the season of 2006/07 found 20 % of vineyards to contain Botryosphaeriaceae species in symptomatic bunches, despite this being a season of relatively low rainfall and unfavourable conditions for bunch rot (Taylor and Wood 2007). More recently Cunnington and Priest (2007) reported the identification of two N. australiae isolates from grapevine berries in Victoria, Australia. Several studies have identified these fungi as important fruit rot pathogens of various hosts including apple (Fen-Ner 1925, Fulkerson 1960), pear (Al-Haq et al. 2002), peach (Rittenburg and Hendrix 1983, Brown and Britton 1986), olive (Romero et al., 2005, Phillips et al. 2005, Laziziera et al. 2008, Sergeeva et al. 2009)) and table grape (Luttrell 1948, Kummuang et al. 1996 a and b). As far as we are aware, other than the incidence data presented by Kummuang et al. (1996 b) and Luttrell (1948), there has been no research conducted on Botryosphaeriaceae as bunch rot pathogens of grapevines. The economic impact of Botryosphaeriaceae on V. vinifera fruit is therefore unknown; however, Pearson and Goheen (1988) claim a 20-30 % loss of ripening in Muscadine (Vitis rotundifolia) fruit due to B. dothidea. Research into the lifecycle of Botryosphaeria fruit rot of apples showed that pycnidia on dead apple twigs and rotten apples left on the tree after harvest or fallen to the

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

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Summary

Several species belonging to the Botryosphaeriaceae were isolated from grapevine (Vitis vinifera) tissue other than wood during a survey of two vineyards planted to cultivars ‘Chardonnay’ and ‘Shiraz’ in the Hunter Valley, New South Wales, Australia over the 2007/08 and 2008/09 growing seasons. A total of 188 isolates corresponding to nine different species of Diplodia, Dothiorella and Neofusicoccum anamorphs were isolated from dormant buds, flowers, pea-sized berries and mature berries prior to harvest in addition to 142 isolates from the trunks of the same vines. Furthermore, the occurrence of Dothiorella viticola, Diplodia mutila and Neofusicoccum australiae is reported here for the first time from grapevines in the Hunter Valley. These findings may provide important information for the management and spread of Botryosphaeriaceae in vineyards where they are considered serious wood-invading pathogens. Botryosphaeriaceae are occasionally found on bunches, however, until now they have not directly been related to bunch rots. Control strategies for trunk diseases caused by Botryosphaeriaceae are currently limited to remedial surgery and wound protection. These strategies do not consider other grapevine tissue as potential inoculum sources for infection of Botryosphaeriaceae in the vineyard.

Key words: Botryosphaeria, Bot canker, bunch rot.
ground are the major source of inoculum in orchards. During rain periods, conidia are released from the pycnidia and dispersed onto the plants by wind and rain (Brown and Britton 1986, Brown-Rytlewski and McManus 2000). Infection occurs as early as bud burst and apples do not require prior wounding for Botryosphaeria fruit rot infections (Beisel et al. 1984, Taylor 1955).

In contrast to studies dealing with hosts other than V. vinifera, little information exists on the infection pathway of Botryosphaeriaceae as bunch rot pathogens of grapes. Symptoms of Muscadine table grapes infected with N. ribis and B. dothidea have been described as water-soaked in appearance with occasional berry skin cracking, berries covered in white mycelium and in severe cases the drying and blackening of berries which eventually mum-mify and develop black pycnidia on the surface (Luttrell et al. 1948, Kummuang et al. 1996 a). Similar to the Botryosphaeriaceae life cycle of apples, pycnidia on the surface of Bot canker diseased grapevines and pruning debris on the vineyard floor have been identified as inoculum sources for Botryosphaeriaceae infection of wounded grapevine wood (Urbeiz-Torres and Gübler 2008, Rolshausen et al. 2010).

This information together with the knowledge that Botryosphaeriaceae can grow and form pycnidia on table grapes and infect dormant buds in apples raises the questions of how do Botryosphaeriaceae infect grapevine bunches in Australian vineyards and do species found in grapevine wood infect reproductive tissues leading to bunch rot development? Therefore the aim of this research was to isolate Botryosphaeriaceae from the reproductive structures of grapevine over different phenological stages and to compare these isolations with those from grapevine wood.

**Material and Methods**

**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. The climate in the Lower Hunter Valley is moderate, Mediterranean-like, with an annual rainfall of 766 mm (Bureau of Meteorology 2009) and with the highest rainfall periods occurring in summer and just before the start of winter. During the growing season of September to March, the region is hot with mean daily maximum temperatures of 33 °C and mean relative humidity ranging from 43 to 81 % throughout the day (Bureau of Meteorology 2009). Both sampling sites were commercial vineyards fol-lowing 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 & dioxonil), Medley Plus (a.i. metalaxyl + copper oxychloride), Dithane Rainshield (a.i. mancozeb), Liquiopac (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 cordon 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 (Qi et al. 2010).

**Isolation**: Vine samples were surface-sterilised in 0.5 % sodium hypochlorite for 2 min followed by two rinses in sterile distilled water, placed on potato dextrose agar (PDA, Oxoid Ltd., Basingstoke, Hampshire, England) amended with 50 µL/mL streptomycin sulphate (Sigma-Aldrich, Castle Hill, NSW, Australia) 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 technique.

Isolates of Botryosphaeriaceae were allowed to sporulate at room temperature in the dark for up to 8 weeks prior to identification based on conidial morphology. Isolates that did not sporulate were subcultured onto triple auto-claved pine needles on 1.5 % water agar in Petri dishes and stored for a further 6 weeks at room temperature under a light regime of 12 h dark and 12 h 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 Difco™ potato dextrose broth (Bacto Laboratories, Liverpool, NSW, Australia) and were incubated at 25 °C and 90 rpm in an orbital shaker (Sartorius Certosat...
BS-1). Mycelia were harvested after 7 d, initially dried by filtration, freeze-dried in a Christ Gamma 1-16SC freeze-dryer (Christ, Osterode, Germany) for 24 h 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 MgCl₂, 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 S. LIPPERS (et al., 2004) with an amended initial denaturation step of 95 °C for 15 min. PCR products were submitted to the Australian Genome Research Facility (Brisbane, Australia) for dual di-resection sequencing. Isolates were identified to species level by comparing the resulting sequences with those of other Botryosphaeriaceae available in GenBank. Species identifications for D. viticola, N. australis, 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 Bt2α 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 an 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 MgCl₂, 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, Industry and Investment NSW, Orange, NSW, Australia (Herbarium code: DAR) and corresponding DNA sequences of the regions used for identification were deposited in GenBank. GenBank and Herbarium Accession numbers are listed in Tab. 1.

Results

Trunk disease pathogens belonging to the 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 juice from 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 different species of Diplodia, Dothiorella and Neofusicoccum: D. seriata, D. mutila, L. theobromae, D. viticola, N. australis, N. parvum, N. ribis, N. luteum and B. dothisidea (Tab. 1). The number of isolations for each species, their vineyard of origin and host cultivar are shown in Tab. 2. D. se-riata, N. parvum, B. dothisidea 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. muti- lla on ‘Chardonnay’ only and L. theobromae on ‘Shiraz’ only. In contrast, N. australis and N. ribis were isolated only from Vineyard A with N. ribis occurring on both cultivars and N. australis 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 (Tab. 3). 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 (Tab. 3). D. mutila, L. theobromae, N. australis 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.

Discussion

The Botryosphaeriaceae family is species-rich, contain-ing common trunk disease pathogens, frequently isolated from grapevine wood in vineyards worldwide including the Hunter Valley. Until now, D. seriata, N. luteum (SANOCCHIA et al. 2007), N. ribis (CASTILLO-PANDO et al. 2001), N. par- vum, B. dothisidea (QU et al. 2008) and L. theobromae (QU et al. 2010) were the only species of Botryosphaeriaceae reported from the Hunter Valley. The additional findings of D. viticola, N. australis, 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). This has also been observed in California (USA) and Mexico (URBEZ-TORRES and GUBLER 2006, 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) declaring a larger number of species distributed in the southern wine regions of NSW compared to those in the north-east. The isolations
Table 1

Identifies and origin of Botryosphaeriaceae isolated from *H. sinifera* from the Lower Hunter Valley

<table>
<thead>
<tr>
<th>Identity (ID)</th>
<th>Species</th>
<th>Isolate ID</th>
<th>Herbarium accession number</th>
<th>GeneBank accession</th>
<th>Vineyard</th>
<th>Host cultivar</th>
<th>Host tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>H17-1</td>
<td><em>Botryosphaeria dothidea</em></td>
<td>DAR 80994</td>
<td>HQ392689</td>
<td>HQ392757</td>
<td>B</td>
<td>Shiraz</td>
<td>Berries at harvest</td>
</tr>
<tr>
<td>H17-2</td>
<td><em>Botryosphaeria dothidea</em></td>
<td>DAR 80995</td>
<td>HQ392690</td>
<td>HQ392758</td>
<td>B</td>
<td>Shiraz</td>
<td>Berries at harvest</td>
</tr>
<tr>
<td>BB56-2</td>
<td><em>Botryosphaeria dothidea</em></td>
<td>DAR 81005</td>
<td>HQ392691</td>
<td>HQ392739</td>
<td>A</td>
<td>Shiraz</td>
<td>Domant bud</td>
</tr>
<tr>
<td>W64-5</td>
<td><em>Botryosphaeria dothidea</em></td>
<td>DAR 81006</td>
<td>HQ392692</td>
<td>-</td>
<td>A</td>
<td>Shiraz</td>
<td>Wood</td>
</tr>
<tr>
<td>W96-3</td>
<td><em>Botryosphaeria dothidea</em></td>
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<td>HQ392693</td>
<td>-</td>
<td>A</td>
<td>Shiraz</td>
<td>Wood</td>
</tr>
<tr>
<td>W126-5</td>
<td><em>Botryosphaeria dothidea</em></td>
<td>DAR 81008</td>
<td>HQ392694</td>
<td>-</td>
<td>B</td>
<td>Chardonnay</td>
<td>Domant bud</td>
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<td>Domant bud</td>
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<td>Shiraz</td>
<td>Domant bud</td>
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<td>Shiraz</td>
<td>Domant bud</td>
</tr>
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<td>Wood</td>
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<td>Wood</td>
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<td>DAR 80980</td>
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<td>Shiraz</td>
<td>Berries at harvest</td>
</tr>
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<td>HQ392702</td>
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<td>Berries at harvest</td>
</tr>
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<td>Berries at harvest</td>
</tr>
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<td>Berries at harvest</td>
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<td>Berries at harvest</td>
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</tr>
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<td>Pea-sized berries</td>
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<td>Chardonnay</td>
<td>Flowers</td>
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<td>Pea-sized berries</td>
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<td>-</td>
<td>B</td>
<td>Chardonnay</td>
<td>Pea-sized berries</td>
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<td>Berries at harvest</td>
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<td>HQ392741</td>
<td>-</td>
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</tr>
</tbody>
</table>

*Note: The isolation of one single isolate belonging to *L. theobromae* of Qu et al., 2010*, predicted to see a greater abundance increased temperatures caused by climate change of this species in the Hunter Valley in the future due to increased temperatures caused by climate change. The isolation of one single isolate belonging to *L. theobromae* of Qu et al., 2010*, predicted to see a greater abundance increased temperatures caused by climate change.
The isolation of most Botryosphaeriaceae species from all tissue types sampled confirmed that Botryosphaeriaceae species can infect different *Vitis 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 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 and 1995, Nair and Allen 1993, Elad et al. 2007). In addition Pitt et al. (2008) 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.

The isolation of most Botryosphaeriaceae species from all tissue types sampled confirmed that Botryosphaeriaceae species 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).

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Association of Botryosphaeriaceae grapevine trunk disease fungi with *Vitis vinifera*

### Table 2

Number of Botryosphaeriaceae species isolated from two vineyards planted to *Vitis vinifera* cultivars ‘Chardonnay’ and ‘Shiraz’

<table>
<thead>
<tr>
<th>Species</th>
<th>Vineyard A</th>
<th>Vineyard B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chardonnay</td>
<td>Shiraz</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Diplodia seriata</em></td>
<td>65</td>
<td>26</td>
</tr>
<tr>
<td><em>Diplodia mutila</em></td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td><em>Lasiodiplodia theobromae</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Dothiorella viticola</em></td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td><em>Neofusicoccum parvum</em></td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td><em>Neofusicoccum luteum</em></td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><em>Neofusicoccum ribis</em></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>Neofusicoccum australis</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Botryosphaeria dothidea</em></td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td><em>Botryosphaeria spp.</em></td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>150</td>
<td>180</td>
</tr>
</tbody>
</table>

*Isolates identified to genus level only.

### Table 3

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Origin tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dormant buds</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Diplodia seriata</em></td>
<td>100</td>
</tr>
<tr>
<td><em>Diplodia mutila</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Lasiodiplodia theobromae</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Dothiorella viticola</em></td>
<td>2</td>
</tr>
<tr>
<td><em>Neofusicoccum parvum</em></td>
<td>17</td>
</tr>
<tr>
<td><em>Neofusicoccum luteum</em></td>
<td>5</td>
</tr>
<tr>
<td><em>Neofusicoccum ribis</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Neofusicoccum australis</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Botryosphaeria dothidea</em></td>
<td>8</td>
</tr>
<tr>
<td><em>Botryosphaeria spp.</em></td>
<td>14</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>147</td>
</tr>
</tbody>
</table>

*Isolates identified to genus level only.

Botryosphaeriaceae infection of the wood presents a constant inoculum source with pycnidia on the surface of trunks and cordon. 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 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.

Acknowledgements

This work was conducted within the Winegrowing Futures Program – a joint initiative of the Grape and Wine Research and Development Corporation and the National Wine and Grape Industry Centre (NWGIC) and a Charles Sturt University PhD scholarship. The authors wish to acknowledge the support of the E-H Graham Centre in the preparation of this manuscript. We thank C. Haywood (I & I NSW) for assistance in the collection of samples, the grape growers for allowing access to their vineyards, Rujian Huang (CSU) for assistance with sample processing and Prof. J. Hardie (National Wine and Grape Industry Centre) for comments on the manuscript.
Association of Botryosphaeriaceae grapevine trunk disease fungi with Vitis vinifera

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Refining the biological factors affecting virulence of Botryosphaeriaceae on grapevines

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Keywords
Botryosphaeria; Botryosphaeria canker; bunch rot; grapevine pathogen; Vitis vinifera.

Abstract
Botryosphaeriaceae isolates of six species were assessed for their potential to infect grapevine tissues other than their tissues of isolation, primarily to determine sources of inocula that could contribute to bunch rot. Pathogenicity tests were conducted in vitro on berries and wood and in vivo on dormant buds of cultivars Chardonnay and Shiraz in glasshouse and field experiments. Tissue specificity and variation in virulence for different isolates was assessed. All isolates were able to infect and cause symptoms on detached 1-year-old canes and mature berries. Virulence was not affected by origin tissue and varied between isolates and within species. Inoculation of dormant buds did not affect bud burst or further development of shoots and fruit, however, a small number of Botryosphaeriaceae were reisolated from bunches at later growth stages. We conclude that Botryosphaeriaceae species are 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. Equally, the vegetative and reproductive tissues can also act as inoculum sources for wood infection. Therefore, all sources of inocula should be taken into consideration when developing management strategies for Botryosphaeria bunch rot and Botryosphaeria canker diseases.

Introduction
Members of the ascomycete family Botryosphaeriaceae are species rich, cosmopolitan plant pathogens with a wide host range primarily infecting woody hosts (von Arx & Mueller, 1975; Denman et al., 2000). Various species of the Botryosphaeriaceae have been associated with symptoms in grapevine wood such as internal wood streaking, necrotic lesions and cankers, as well as cordon and cane dieback (Phillips, 1998; van Niekerk et al., 2006). In some instances reduced bud burst has been reported from vineyards with wood infection (Castillo-Pando et al., 2001; Qiu et al., 2011). Occasionally, the fungi have been isolated from diseased berries together with other bunch rot pathogens as components of bunch rot complexes in Australian bunch rot surveys (Steel et al., 2007; Taylor & Wood, 2007).

While the Botryosphaeriaceae are prominently known to cause trunk diseases in Vitis vinifera L., some species are confirmed fruit rot pathogens in other hosts such as apple (Fenner, 1925; Fulkerson, 1960), pear (Al-Haq et al., 2002), peach (Rittenburg & Hendrix, 1983; Brown & Britton, 1986), olive (Phillips et al., 2005; Romero et al., 2005; Lazzizera et al., 2008) and Muscadinia rotundifolia (Michx.) Small (Luttrel, 1948; Kummuang et al., 1996).

The epidemiology of Botryosphaeriaceae as wood and fruit rot pathogens has been most intensively studied on apples. Brown & Britton (1986) and Brown-Rytlewski & McManus (2000) described pycnidia on dead twigs and rotten apples in the orchard as the major source of inoculum, releasing and dispersing conidia during rain periods. These spores have the ability to germinate and infect healthy branches and twigs, buds, shoots and...
wounded and unwounded fruit (Taylor, 1955; Beisel et al., 1984; Beisel-Smith & Hendrix, 1984).

In vineyards, pycnidia on the surface of wood and pruning debris of infected vines release conidia during rain and high humidity (Lehoczky, 1974). van Niekerk et al. (2010) found that 0.25–1 mm of rainfall led to spore release. This knowledge is important for wine regions with favourable conditions for the development of bunch rots, particularly in vineyards with a history of Botryosphaeriaceae in the wood.

Although well known to be associated with fruit in vineyards, the role of Botryosphaeriaceae as bunch rot pathogens has been rarely recognised. Studies of Botryosphaeriaceae in bunches have been limited to incidence data and symptom descriptions on table grapes. The economic impact of bunch rots caused by Botryosphaeriaceae in ‘Muscadine’ grapes (M. rotundifolia) has been estimated to account for 20–30% fruit loss in southeastern USA (Pearson & Goheen, 1988). In New Zealand, Buchanan & Beever (1987) reported a low recognition of Botryosphaeriaceae as bunch rot pathogens despite the high incidence of berry infection. According to these authors, the reason for this is the similarity of Botryosphaeria bunch rot disease symptoms with those caused by Phomopsis viticola (Berk & Curt) and Botrytis cinerea (De Bary) Whetzel.

More recently, pathogenicity has been confirmed for many of the Botryosphaeriaceae found on V. vinifera wood. Although Botryosphaeriaceae have been associated with buds and berries of V. vinifera (Castillo-Pando et al., 2001; Taylor & Wood, 2007; Qiu et al., 2011; Wunderlich et al., 2011) the majority of pathogenicity tests have been conducted on the wood of grapevines. The work of Phillips (1998) which demonstrated bud mortality caused by inoculations with Botryosphaeria dothidea (Moug. Ex Fr.) Ces. & De Not. is an exception to this.

There are conflicting reports in the literature with regards to the pathogenicity of the Botryosphaeriaceae on grapevine wood. Authors of different pathogenicity tests of Botryosphaeriaceae to grapevine wood do not always report the same level of virulence for the same species, including those used in this study, making it difficult to label a species as pathogenic to grapevines. Neofusicoccum australae Slippers, Crous & M.I. Wingf., frequently isolated as a wood pathogen from V. vinifera in Western Australia (WA) and South Africa but less frequently in eastern Australia, has been reported as the most virulent species in pathogenicity tests conducted in WA (Taylor et al., 2005) and South Africa (van Niekerk et al., 2004), where its isolates were more virulent than those of the Diplodia species used in the same tests. However, both Taylor et al. (2005) and van Niekerk et al. (2004) reported large differences in virulence between individual isolates within the species N. australae while virulence data for Diplodia seriata (Larignon et al., 2001) and Lasiodiplodia theobromae (Pat.) Griffon & Maubl. (Taylor et al., 2005) show a similar within-species difference.

Major discrepancies also exist between reports of the virulence of D. seriata, the most frequently isolated Botryosphaeriaceae species in Australian vineyards (Taylor et al., 2005; Savocchia et al., 2007; Pitt et al., 2010; Qiu et al., 2011; Wunderlich et al., 2011). While Taylor et al. (2005) described D. seriata (teleomorph ‘Botryosphaeria obtusa’) as non-pathogenic to healthy cuttings of the cultivar ‘Red Globe’, they also called for its role as an endophyte to be elucidated. Qiu et al. (2011) questioned the pathogenicity of D. seriata further after it was seldom found in the absence of other wood pathogens. In contrast, Castillo-Pando et al. (2001) confirmed D. seriata as pathogenic using in vivo pathogenicity tests on Chardonnay, in agreement with pathogenicity tests conducted by Auger et al. (2004) and Savocchia et al. (2007) who found D. seriata to be pathogenic and even more virulent than Neofusicoccum luteum Pennycook & Samuels on 1-year-old canes.

Some of the observed variation in virulence may be explained by differences in environmental factors (Slippers & Wingfield, 2007). L. theobromae, which has high optimum growth rate temperatures (Úrbez-Torres & Gubler, 2009), is considered the most virulent species in in vivo pathogenicity tests on cuttings in WA (Taylor et al., 2005) and on green shoots and rooted cuttings in Mexico (Úrbez-Torres et al., 2008). In contrast in South Africa, this species was considered weakly pathogenic on green shoots in vivo but highly virulent on mature canes in vitro (van Niekerk et al., 2004).

These discrepancies make it difficult to classify a species as pathogenic or endophytic (Slippers & Wingfield, 2007). As a consequence, Larignon et al. (2001) and van Niekerk et al. (2004) suggested more standardised pathogenicity testing techniques should be used and proposed the idea of virulence groups within species.

Most surveys of Botryosphaeriaceae in vineyards have also been concerned with the sampling of only wood tissue. However, in a recent survey of Botryosphaeriaceae on Chardonnay and Shiraz several species were found on different types of reproductive tissue at different phenological stages (Wunderlich et al., 2011). In this survey, Botryosphaeriaceae were isolated five times more frequently from dormant buds than from mature berries and rarely from flowers and pea-sized berries.

The aim of this study was to confirm the pathogenicity of Botryosphaeriaceae isolated from Australian vineyards to grapevine wood and reproductive tissues and to determine whether they could infect more than one tissue type. In addition, this study aimed to determine...
if tissue origin played a role in the virulence towards different tissue types and to gain information on possible infection pathways of Botryosphaeriaceae into bunches.

**Materials and methods**

**Fungal isolates**

All 19 isolates used in the following pathogenicity studies were obtained from different vegetative tissues and wood of *V. vinifera* cultivars Chardonnay and Shiraz from the Hunter Valley, New South Wales, Australia and were stored at the Agricultural Scientific Collection Unit, NSW Department of Primary Industries, Orange, NSW, Australia (Herbarium code: DAR) (Table 1). Isolate identities were confirmed in a previous study by Wunderlich *et al.* (2011).

**Pathogenicity on canes**

Certified, commercially disease free, detached 1-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 of cane was wounded by drilling a hole in the middle of the cane into the pith with a 4 mm diameter surface-sterilised drill bit. Canes were inoculated by inserting into each hole a 4 mm diameter mycelium plug from one of the 14 single spore Botryosphaeriaceae isolates (Table 1), grown for 3 days as pure cultures on potato dextrose agar (PDA; Oxoid, Hampshire, UK). 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 conidial morphology.

All statistical analyses associated with the pathogenicity studies were performed with Genstat® (2010, 13th 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 combinations 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) on potato dextrose agar (PDA; Oxoid, Hampshire, UK).

**Table 1** Identities and origins of Botryosphaeriaceae isolates used in the pathogenicity tests

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Accession number</th>
<th>Species</th>
<th>Tissue</th>
<th>Cultivar</th>
<th>Pathogenicity test*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H171-1</td>
<td>DAR 80994</td>
<td><em>Botryosphaeria dothidea</em></td>
<td>Berries at harvest</td>
<td>Shiraz</td>
<td>W, H</td>
</tr>
<tr>
<td>H171-2</td>
<td>DAR 80995</td>
<td>B. dothidea</td>
<td>Berries at harvest</td>
<td>Shiraz</td>
<td>W, H</td>
</tr>
<tr>
<td>B106</td>
<td>DAR 80987</td>
<td><em>Diplodia seriata</em></td>
<td>Dormant bud</td>
<td>Chardonnay</td>
<td>W, B</td>
</tr>
<tr>
<td>B178-1</td>
<td>DAR 80988</td>
<td>D. seriata</td>
<td>Dormant bud</td>
<td>Shiraz</td>
<td>W, H</td>
</tr>
<tr>
<td>B98-3</td>
<td>DAR 80986</td>
<td>D. seriata</td>
<td>Dormant bud</td>
<td>Shiraz</td>
<td>W, H</td>
</tr>
<tr>
<td>H118-1</td>
<td>DAR 80997</td>
<td>D. seriata</td>
<td>Berries at harvest</td>
<td>Chardonnay</td>
<td>W, H</td>
</tr>
<tr>
<td>H17-1</td>
<td>DAR 80994</td>
<td>D. seriata</td>
<td>Berries at harvest</td>
<td>Shiraz</td>
<td>W, H</td>
</tr>
<tr>
<td>H33-1</td>
<td>DAR 80996</td>
<td>D. seriata</td>
<td>Berries at harvest</td>
<td>Shiraz</td>
<td>W, H</td>
</tr>
<tr>
<td>H64-1</td>
<td>DAR 80985</td>
<td>D. seriata</td>
<td>Berries at harvest</td>
<td>Shiraz</td>
<td>W, H</td>
</tr>
<tr>
<td>W86-2</td>
<td>DAR 80979</td>
<td>D. seriata</td>
<td>Wood</td>
<td>Shiraz</td>
<td>H</td>
</tr>
<tr>
<td>B188-3</td>
<td>DAR 81012</td>
<td><em>Dothiorella viticola</em></td>
<td>Dormant bud</td>
<td>Chardonnay</td>
<td>W, H</td>
</tr>
<tr>
<td>W200</td>
<td>DAR 81024</td>
<td><em>Lasiodiplodia theobromae</em></td>
<td>Wood</td>
<td>Shiraz</td>
<td>H</td>
</tr>
<tr>
<td>H12-1</td>
<td>DAR 80993</td>
<td><em>Neofusicoccum luteum</em></td>
<td>Berries at harvest</td>
<td>Chardonnay</td>
<td>W, H</td>
</tr>
<tr>
<td>B114-2</td>
<td>DAR 80990</td>
<td><em>Neofusicoccum parvum</em></td>
<td>Dormant bud</td>
<td>Chardonnay</td>
<td>H</td>
</tr>
<tr>
<td>B18-3</td>
<td>DAR 80993</td>
<td>N. parvum</td>
<td>Dormant bud</td>
<td>Chardonnay</td>
<td>W, B, H</td>
</tr>
<tr>
<td>H162-1</td>
<td>DAR 80991</td>
<td>N. parvum</td>
<td>Berries at harvest</td>
<td>Shiraz</td>
<td>W, H</td>
</tr>
<tr>
<td>H77-1</td>
<td>DAR 80899</td>
<td>N. parvum</td>
<td>Berries at harvest</td>
<td>Shiraz</td>
<td>W, H</td>
</tr>
<tr>
<td>W27-5</td>
<td>DAR 80981</td>
<td>N. parvum</td>
<td>Wood</td>
<td>Chardonnay</td>
<td>H</td>
</tr>
<tr>
<td>W45-3-1</td>
<td>DAR 80982</td>
<td>N. parvum</td>
<td>Wood</td>
<td>Chardonnay</td>
<td>H</td>
</tr>
</tbody>
</table>

* ‘Pathogenicity test’ relates to the tissue type each isolate was tested on. W = wood, B = dormant bud and H = berry at harvest.
contrast on lesion length. This required the square root transformation of lesion lengths prior to analysis. As a result of a large comparative difference in occurrence of lesions between inoculated and control canes (see Section '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 Section 'Results'), to address the question of whether isolate effects exist separately from species effects we removed the two species (Dothiorella 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 1) were prepared by transferring 4 mm² mycelium plugs of 3-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 h dark and 12 h near ultraviolet light to encourage the formation of conidia. Following incubation, pine needles containing pycnidia were stored in McCartney bottles with sterile water for 6 h before being crushed with a micropipette to encourage the release of conidia. Conidial suspensions were adjusted to concentrations of approximately $1 \times 10^6$ 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° Baumé, 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 conidial 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. Disease severity, indicated by the presence or absence of visible pycnidia/conidia formation on the berry surface, was inspected on day 15.

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 (1991) method] 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 of the 24 berries on each plate. Predicted 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 because of differences in species or tissue of origin.

Pathogenicity on dormant buds (glasshouse)

Conidial suspensions of Neofusicoccum parvum Pennycook & Samuels 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 conidial suspension or sterile water onto the bud surface. These six treatments, each replicated 10 times, were applied over three adjacent trays of 5 × 4 cuttings in a row–column design.

Immediately after inoculation each cutting was covered with a plastic bag for 48 h 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: (1) winter bud, (2) bud swell, (3) woolly bud – brown wool visible, (4) green tip – first leaf tissue visible and (5) rosette of leaf tips visible 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 & 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 (two levels) and inoculum (three 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)

Conidial 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 conidial 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 h 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). Reisolations 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

Pathogenicity on canes

The resulting lesions on the canes inoculated with Botryosphaeriaceae isolates were significantly longer than those on control canes \( F = 25.39 \); degrees of freedom (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 control data from the analysis, now known to be different from the isolate effects and largely composed of zero values, further analysis showed that there was a significant interaction between isolate and cultivar \( F = 2.64; \text{df} = 13, 109; P = 0.003 \). Mean square root values of lesion lengths were plotted with the least significant difference (Fig. 1). While for the majority of isolates the mean lesion length was longer for Shiraz than Chardonnay, the opposite was observed for three isolates (H77-1, H171-2 and H12-1) which caused longer lesions on Chardonnay than Shiraz (Fig. 1).

When analysing the species effect on lesion lengths, a highly significant effect of species \( F = 10.46; \text{df} = 4, 127; P < 0.001 \) and the expected significant difference between cultivars \( F = 6.28; \text{df} = 1, 127; P = 0.013 \) 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 \( F = 18.39; \text{df} = 2, 93; P < 0.001 \), the expected cultivar effect \( F = 9.07; \text{df} = 1, 93; P = 0.003 \) 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 was due to differences between species, there remained 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 as well as the appearance of mycelia or pycnidia to berry collapse.

While the ‘Species.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 ‘Species’ interaction ($F = 2.28; df = 24, 778.9; P < 0.001$). The same applies to the ‘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 the ‘Cultivar.Origin tissue’ interaction ($F = 4.92; df = 30, 778.8; P < 0.001$). For analysis of disease severity on day 15 the interaction of isolate, species and origin with cultivar and cultivar main effects was 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 test whether differences in isolate were because of (a) differences in species or (b) differences in origin.

The differences in disease severity between isolates were partially explained by species ($F = 17.14; df = 5, 107.3; P < 0.001$). The remaining proportion 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 (Tables 2 and 4) and tissue of origin (Tables 3 and 5), 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 ($F = 6.28; df = 37, 820.9; P < 0.001$). For

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Rates of disease incidence increase over time on berries inoculated with Botryosphaeriaceae isolates (logit scale)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>Chardonnay</td>
</tr>
<tr>
<td>Isolate</td>
<td>Rate of increase</td>
</tr>
<tr>
<td>Lasiodiplodia theobromae</td>
<td>W200</td>
</tr>
<tr>
<td>Neofusicoccum parvum</td>
<td>H77-1</td>
</tr>
<tr>
<td>P. n. parvum</td>
<td>W45-3-1</td>
</tr>
<tr>
<td>N. parvum</td>
<td>W27-5</td>
</tr>
<tr>
<td>N. parvum</td>
<td>B114-2</td>
</tr>
<tr>
<td>N. parvum</td>
<td>H162-1</td>
</tr>
<tr>
<td>N. parvum</td>
<td>B8-3</td>
</tr>
<tr>
<td>Neofusicoccum luteum</td>
<td>H12-1</td>
</tr>
<tr>
<td>Diplodia seriata</td>
<td>H118-1</td>
</tr>
<tr>
<td>D. seriata</td>
<td>H17-1</td>
</tr>
<tr>
<td>D. seriata</td>
<td>B178-1</td>
</tr>
<tr>
<td>D. seriata</td>
<td>W86-2</td>
</tr>
<tr>
<td>D. seriata</td>
<td>B98-3</td>
</tr>
<tr>
<td>D. seriata</td>
<td>H33-1</td>
</tr>
<tr>
<td>D. seriata</td>
<td>H64-1</td>
</tr>
<tr>
<td>Botryosphaeria dothidea</td>
<td>H171-1</td>
</tr>
<tr>
<td>B. dothidea</td>
<td>H171-2</td>
</tr>
<tr>
<td>Dothiorella viticola</td>
<td>B146-3</td>
</tr>
</tbody>
</table>

- Isolates are ranked by rates of disease incidence increase and grouped by species.
- Standard error of differences: 0.08 (average), 0.21 (maximum) and 0.05 (minimum); degrees of freedom = 820.9.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Rates of disease incidence increase over time on berries obtained by Botryosphaeriaceae isolates (logit scale)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin tissue</td>
<td>Chardonnay</td>
</tr>
<tr>
<td>Isolate</td>
<td>Rate of increase</td>
</tr>
<tr>
<td>Wood</td>
<td>W200</td>
</tr>
<tr>
<td>Wood</td>
<td>W45-3-1</td>
</tr>
<tr>
<td>Wood</td>
<td>W27-5</td>
</tr>
<tr>
<td>Wood</td>
<td>W86-2</td>
</tr>
<tr>
<td>Berries</td>
<td>H77-1</td>
</tr>
<tr>
<td>Berries</td>
<td>H12-1</td>
</tr>
<tr>
<td>Berries</td>
<td>H118-1</td>
</tr>
<tr>
<td>Berries</td>
<td>H162-1</td>
</tr>
<tr>
<td>Berries</td>
<td>H17-1</td>
</tr>
<tr>
<td>Berries</td>
<td>H171-1</td>
</tr>
<tr>
<td>Berries</td>
<td>H171-2</td>
</tr>
<tr>
<td>Berries</td>
<td>H33-1</td>
</tr>
<tr>
<td>Berries</td>
<td>H64-1</td>
</tr>
<tr>
<td>Dormant bud</td>
<td>B114-2</td>
</tr>
<tr>
<td>Dormant bud</td>
<td>B178-1</td>
</tr>
<tr>
<td>Dormant bud</td>
<td>B98-3</td>
</tr>
<tr>
<td>Dormant bud</td>
<td>B8-3</td>
</tr>
<tr>
<td>Dormant bud</td>
<td>B146-3</td>
</tr>
<tr>
<td>—</td>
<td>Control</td>
</tr>
</tbody>
</table>

- Isolates are ranked by rates of disease incidence increase and grouped by species.
- Standard error of differences: 0.08 (average), 0.21 (maximum) and 0.05 (minimum); degrees of freedom = 820.9.
all isolates except the \textit{L. theobromae} isolate W200 the increase in disease incidence was higher on Shiraz than Chardonnay (Tables 2 and 3).

Tables 2 and 3 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 \textit{N. parvum} isolate W27-5 on Shiraz followed by \textit{L. theobromae} isolate W200 on Chardonnay (Table 2). Lowest rates of disease incidence increase were associated with \textit{D. viticola} A.J.L. Philips & J. Luque isolate B 146-3 on both cultivars and were not statistically different from the controls (Table 2).

In terms of species, highest rankings for rate of disease incidence increase were attained by isolates of the species \textit{L. theobromae} and \textit{N. parvum} for both cultivars. However, \textit{N. parvum} isolate B8-3 fell outside this group and ranked for both cultivars within the isolates of the \textit{D. seriata} group, which generally caused lower rates of disease incidence increase than those of \textit{N. parvum} isolates.

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

### Table 4 Mean probability of isolates to sporulate on berry surfaces at day 15 grouped by species

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Species</th>
<th>Probability (logit scale)</th>
<th>Probability (back transformed)</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>W200</td>
<td>Lasiodiplodia theobromae</td>
<td>8.78</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>W27-5</td>
<td>Neofusicoccum parvum</td>
<td>6.61</td>
<td>1.0</td>
<td>2</td>
</tr>
<tr>
<td>H77-1</td>
<td>N. parvum</td>
<td>1.11</td>
<td>0.75</td>
<td>3</td>
</tr>
<tr>
<td>B114-2</td>
<td>N. parvum</td>
<td>0.38</td>
<td>0.59</td>
<td>4</td>
</tr>
<tr>
<td>W45-3-1</td>
<td>N. parvum</td>
<td>0.18</td>
<td>0.54</td>
<td>5</td>
</tr>
<tr>
<td>H162-1</td>
<td>N. parvum</td>
<td>−0.85</td>
<td>0.30</td>
<td>8</td>
</tr>
<tr>
<td>B8-3</td>
<td>N. parvum</td>
<td>−0.97</td>
<td>0.27</td>
<td>9</td>
</tr>
<tr>
<td>H118-1</td>
<td>Diplodia seriata</td>
<td>−0.01</td>
<td>0.50</td>
<td>6</td>
</tr>
<tr>
<td>H17-1</td>
<td>D. seriata</td>
<td>−1.45</td>
<td>0.19</td>
<td>11</td>
</tr>
<tr>
<td>B98-3</td>
<td>D. seriata</td>
<td>−1.67</td>
<td>0.16</td>
<td>12</td>
</tr>
<tr>
<td>W86-2</td>
<td>D. seriata</td>
<td>−2.08</td>
<td>0.11</td>
<td>14</td>
</tr>
<tr>
<td>B178-1</td>
<td>D. seriata</td>
<td>−2.22</td>
<td>0.10</td>
<td>16</td>
</tr>
<tr>
<td>H33-1</td>
<td>D. seriata</td>
<td>−3.24</td>
<td>0.00</td>
<td>17</td>
</tr>
<tr>
<td>H64-1</td>
<td>D. seriata</td>
<td>−3.44</td>
<td>0.00</td>
<td>18</td>
</tr>
<tr>
<td>H171-2</td>
<td>Botryosphaeria dothidea</td>
<td>−0.39</td>
<td>0.40</td>
<td>7</td>
</tr>
<tr>
<td>H171-1</td>
<td>B. dothidea</td>
<td>−1.65</td>
<td>0.16</td>
<td>13</td>
</tr>
<tr>
<td>H12-1</td>
<td>Neofusicoccum luteum</td>
<td>−1.36</td>
<td>0.20</td>
<td>10</td>
</tr>
<tr>
<td>B146-3</td>
<td>Dothiorella viticola</td>
<td>−2.23</td>
<td>0.10</td>
<td>15</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>−9.45</td>
<td>0</td>
<td>19</td>
</tr>
</tbody>
</table>

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

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 3). 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 the dormant bud-derived isolate B146-3 for both cultivars, whereas the dormant bud-derived isolate B114-2 ranked fifth in terms of rate of disease incidence increase for both cultivars (Table 3).

### Disease severity

The probability of sporulation on day 15 varied significantly for isolates \((P = 6.85; \text{df} = 18, 70.2; P < 0.001)\). Neither the interaction with cultivar nor cultivar effect alone was statistically significant \((P < 0.05)\).
Tables 4 and 5 show 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 did 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 4). 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 5). 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 5).

Pathogenicity on dormant buds (glasshouse)

There was no statistically significant ($P < 0.05$) effect of any treatment on survival of buds, shoot lengths, number of days to stage 5 bud development or root abundance. In only 17 instances could Botryosphaeriaceae be reisolated 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 ($P < 0.05$) 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 reisolated 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 reisolated from two bunch samples and three times from the rachis of inoculated vines.

Discussion

All of the Botryosphaeriaceae isolates examined were pathogenic on 1-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., 2011).

The two species with a relatively high number of isolates $D. seriata (n = 7$ and 7) and $N. parvum (n = 3$ and 6), in 1-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 (Larignon et al., 2001; van Niekerk et al., 2004; Taylor et al., 2005) also exist for the isolates used in this 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 were highly virulent on one tissue type only, and much less virulent on the other tissue type. Dothiorella viticola isolate B146-3 was consistently weakly virulent in both tests. This coincides with the findings of Úrbez-Torres & 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 1-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 sixth highest out of 14 isolates in probability of sporulation and had the seventh 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 1-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 queries raised by 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 1-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 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 this 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 *et al.* (2001) and van Nierkerk *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.

As the differences in virulence data for different isolates may be due to more than just the species component, we agree with van Nierkerk *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 because of the large differences within species that exists for this family.

Inoculation of dormant buds in the glasshouse and vineyard resulted in some successful reisolations 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 (Taylor, 1955; Beisel *et al.*, 1984).

The results of dormant bud inoculations in glasshouse and vineyard are unexpected because Phillips (1998) showed that *B. dothidea* caused bud mortality in *V. vinifera*. The different outcomes of both studies might be explained by different species; 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 need to be controlled throughout the entire growing season.

Botryosphaeriaceae in Australian vineyards need to be regarded as pathogens of both the wood and the berries. Current disease management practices for the Botryosphaeriaceae, which concentrates on the woody tissues of the vine, needs to be modified as infected wood has the potential to infect the fruit.

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Short title: AFLP analysis of Botryosphaeriaceae in vineyards

Title: Amplified fragment length polymorphism analysis suggests non-systemic development of Botryosphaeriaceae bunch rot in Botryosphaeria dieback diseased grapevines

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Abstract: Botryosphaeriaceae fungi, causing trunk disease in Australian vineyards have recently been associated with the reproductive structures of grapevines leading to bunch rots in the Hunter Valley, New South Wales. However, little information exists on the sources and spread of Botryosphaeriaceae into grapevine tissue other than wood. The DNA fingerprinting technique based upon amplified fragment length polymorphism (AFLP™) was used to analyse a population of 178 isolates of Botryosphaeriaceae belonging to seven different species. Isolates were obtained from 103 grapevines in two different vineyards and from five tissue types. A set of four EcoRI / MseI primer combinations generated a total of 1203
fragments with polymorphism rates ranging from 0.08 to 0.14. Genetic distance matrices generated from these fragments showed no meta-population structure according to vineyard, tissue or origin grapevine. The population appeared to be panmictic indicating that asexual reproduction is likely to be the predominant reproductive strategy for these organisms. Population structures also showed that inoculum sources from outside each vineyard were equally prevalent than from within the vineyards, suggesting that there have been multiple introduction of Botryosphaeriaceae into each vineyard from a common inoculum source and that inoculum may have been dispersed over further distances than so far recorded for the spread of Botryosphaeriaceae conidia by splash dispersal. This study provided insights into pathogen population structure and provided important information for the management of Botryosphaeriaceae fungi as a pathogen of wood and reproductive tissues in grapevines.

**Key words:** AFLP, genetic diversity, trunk disease pathogen, *Vitis vinifera*

**Introduction**

Several species in the Botryosphaeriaceae Theiss. & P. Syd. are economically important fungal pathogens of many woody hosts including grapevine (*Vitis vinifera* L) (Urbez-Torres 2011). Several members of this species-rich family contribute to grapevine decline and loss of vigour and yield by causing the trunk diseases formerly known as ‘Botryosphaeria canker’, ‘black dead arm’, ‘excoriosis’ or ‘Diplodia Dieback’ (Larignon et al. 2001; van Niekerk et al. 2002; van Niekerk et al. 2006; Savocchia et al. 2007), collectively referred to nowadays as Botryosphaeria dieback (Urbez-Torres 2011). In addition some Botryosphaeriaceae species have been associated with bud necrosis (Phillips 2002).
Botryosphaeriaceae fungi are known as fruit rot pathogens of many hosts including apple (Fenner 1925; Fulkerson 1960; Rittenburg and Hendrix 1983; Al-Haq et al. 2002), peach (Rittenburg and Hendrix 1983), pear (Al-Haq et al. 2002), mango (Costa et al. 2010), olive (Romero et al. 2005; Phillips et al. 2005a; Lazzizera et al. 2008) and table grape (Luttrell 1948; Kummuang et al. 1996a; Kummuang et al. 1996b). In V. vinifera the botryosphaeriaceous fungal species Guignardia bidwellii has been identified as a fruit rot pathogen in Asia, Europe and South America (Jermini and Gessler 1996; Wilcox 2003), however, this has not been reported from Australian vineyards. In Australian vineyards Botryosphaeriaceae species have mainly been considered of economic importance as wood pathogens (Taylor et al. 2005; Pitt et al. 2008; Qiu et al. 2011). A recent survey of vineyards with a history of Botryosphaeria dieback, however, reported relatively large numbers of isolates of several Botryosphaeriaceae species from the reproductive structures of V. vinifera, including dormant buds and berries at harvest stage, and less frequently from flowers and pea-sized berries (Wunderlich et al. 2011b).

While Botryosphaeriaceae are known to require wounding such as pruning-wounds, for the conidia to infect the lignified wood of grapevines (Halleen and Fourie 2005; Urbez-Torres and Gubler 2008; Rolshausen et al. 2010), Phillips (1998) showed that some species were able to infect unwounded shoots. With respect of bunches and other reproductive structures of grapevines, there is limited information on infection with the exception of the records for Guignardia bidwellii, which seems to originate from initial infection of lenticels and pedicels and spread from there into bunches (Wilcox 2003).

Pycnidia over-wintering on the outside of grapevine trunks, cordons and canes as well as pruning debris and mummified berries on the vineyard floor have been identified as primary inoculum sources for Botryosphaeria dieback (Lehoczky 1974; van Niekerk et al. 2004;
Urbez-Torres *et al.* 2006). However, van Niekerk *et al.* (2004) have shown that isolates from an alternative host plant *Ribes* sp. showed pathogenicity towards grapevines and thus suggested that the surrounding vegetation can act as additional inoculum sources for vineyards.

During rain periods, conidia are released from pycnidia and splash dispersed over short distances onto the vines (Amponsah *et al.* 2010; van Niekerk *et al.* 2010). Baskarathévan *et al.* (2010) quantified the distance of splash dispersal in one single rainfall event to a maximum of 2 m from the inoculum source.

Studies on other grapevine trunk disease pathogens such as *Eutypa lata* have shown that the surrounding vegetation of vineyards can act as inoculum sources for grapevines, which are infected through the wind dispersed ascospores, which can travel distances of more than 100 miles (Ramos *et al.* 1975). However, with pycnidia being the main over-wintering structures of Botryosphaeriaceae, the dispersal of these fungi has so far only been studied in the form of splash dispersed conidia. The role of wind for the release of conidia from pycnidia and consequently long distance dispersal of conidia is yet unknown for the botryosphaeriaceous fungi (Urbez-Torres 2011).

Additionally, Whitelaw-Weckert *et al.* (2006) reported that species of the Botryosphaeriaceae have the potential to infect the roots of young freshly planted grapevines from where they move upward, infecting the trunk and shoots. Qiu *et al.* (2011) investigated the distribution of Botryosphaeriaceae within and between vineyards of the Hunter Valley and Mudgee regions of New South Wales and revealed a high diversity between isolates from grapevines within this study. These isolates were more closely related to reference isolates from other woody hosts suggesting a gene transfer from Botryosphaeriaceae fungi of other hosts into those isolated from vineyards and supported findings from a simple sequence repeat based genetic
diversity study by Burgess et al. (2006) showing gene exchange between pathogen populations of native and plantation eucalypts.

A recent study elucidated the lack of tissue specificity of Botryosphaeriaceae spp. infecting grapevines and has indicated that these fungi have the potential to spread from the wood onto the reproductive structures of the grapevine and *vice versa* (Wunderlich et al. 2011a). It is not known if Botryosphaeriaceae spread systemically from wood into other grapevine tissue or if infections of dormant buds and fruit occur externally.

Molecular markers such as amplified fragments length polymorphisms (AFLP) can be used to establish DNA fingerprinting profiles and determine gene flow levels between and within fungal pathogen populations in order to elucidate the origin and spread of pathogens. While the majority of genetic diversity studies on Botryosphaeriaceae from grapevines have focused on species identification and taxonomy (Crous et al. 2006; Urbez-Torres and Gubler 2006; Urbez-Torres et al. 2006; Urbez-Torres and Gubler 2007; Urbez-Torres et al. 2008; Pitt et al. 2010), genetic diversity studies on Botryosphaeriaceae in other hosts such as eucalypts and pistachios using micro-satellites, and random amplified polymorphic DNA markers assessing geneflow between Botryosphaeriaceae populations have indicated surrounding vegetations as inoculum sources for these hosts (Ma et al. 2001b; Burgess et al. 2005). For a different grapevine trunk disease pathogen *Phaeomoniella chlamydospora*, AFLP analysis has been successfully used to identify sexual reproductive modes and pathogen spread through transportation of diseased planting material in vineyards (Comont et al. 2010).

The objectives of this study were to investigate the population structure of Botryosphaeriaceae of grapevines originating from five different tissue types from two different vineyards in the Hunter Valley, NSW, with the aim of determining the movement of this grapevine pathogen between vineyards, within vineyards and within vines.
Materials and methods

Fungal isolates and DNA extractions

Botryosphaeriaceae isolates (n=178) used in this study were collected from two different vineyards in the lower Hunter Valley, NSW, Australia, approximately 6km apart. Sampling procedures included the isolation of Botryosphaeriaceae from dormant buds, flowers, peasized berries and berries at harvest of a total of 200 approximately 25 year old vines over two consecutive growing seasons. Pure cultures were obtained for each isolate through subculturing onto potato dextrose agar (PDA) and isolates were identified to species level using conidia morphology and sequencing of the ITS region followed by sequence comparison with reference isolates deposited in Genbank, using the Basic Local Alignment Search Tool (BLAST). Sampling procedures, isolate collection and identification were as described previously (Wunderlich et al. 2011b). The isolates used in this study and their origins (vineyard, plant and host tissue type) are listed in TABLE I.

For each isolate three 4 mm² PDA plugs were transferred from actively growing cultures to 125 mL conical flasks containing 50 mL Difco™ potato dextrose broth (Bacto Laboratories, Liverpool, Australia) and incubated at 25 °C and 90 rpm in a Sartorius Certomat BS-1 orbital shaker (Sartorius Mechatronics, Goettingen, Germany). Mycelia were harvested after 7 days, 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, Doncaster, Australia). DNA was extracted using the DNeasy Plant Maxi Kit (Qiagen) according to the manufacturer’s instructions. Genomic DNA quality and concentration for each sample were determined by electrophoresis and a NanoDrop 2000 (Thermo Scientific, Wilmington, USA) and adjusted to yield 250 ng DNA in ≤18 µL.
AFLP analysis

AFLP analysis was performed according to the protocol described by Vos et al. (1995) with some modifications, using the AFLP® analysis system for microorganisms (Invitrogen, Mulgrave Australia). Genomic DNA samples were digested with the restriction enzymes EcoRI and MseI in 12.5 µL. Following ligation of the digested genomic DNA fragments to EcoRI and MseI 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, 1.275 µL 10× PCR buffer (Invitrogen), 3.675 µL pre-amp primer mix containing dNTPs and primers, 1.25 U Taq DNA polymerase (Taq Platinum, Invitrogen), and 15 mM MgCl. Amplifications were conducted in a GeneAmp PCR system 2700 thermocycler (Applied Biosystems, Foster City, USA) with 20 cycles at 94 °C for 30 sec, 56 °C for 1 min and 72 °C for 1 min. The amplified product was diluted 50 fold using 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 MseI primer and a 5' fluorescently labelled EcoRI primer. Markers for the final selective amplification step were chosen after an initial screening of 24 EcoRI/MseI primer combinations on a subset of eight phenotypically diverse isolates. Polymorphism for this subset for each of the 24 primer pairs was estimated by generating average polymorphic information contents (PIC) using GenAlX (Peakall and Smouse 2006). Each of the E primers of these pairs were 5’ fluorescently labelled with D4 WellRED™ dye (Beckman Coulter, Gladesville, Australia) by the supplier (Sigma-Aldrich, Sydney, Australia). Each 10 µL selective amplification reaction contained 2.5 µL of a 1:50 dilution of the pre-amplification template, 1× PCR buffer (Invitrogen), 0.5 U Taq DNA polymerase (Taq Platinum, Invitrogen), 2.25 µL of selective-amplification primer mix containing MseI-T primer and dNTPs and 0.05 µL E primer. The PCR was one initial cycle at 94 °C for 30 sec, 65 °C for 30
sec 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 sec, 56 °C for 30 sec, and 72 °C for 60 sec were performed. PCR products were stored at 4 °C until used for capillary electrophoresis. From each selective amplification product 1µL 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 the CEQ Genetic Analysis System 8000. AFLP fragment bands between 60 and 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.

Data analysis

Binary data was square-root transformed in order to normalise the variables and isolates analysed for their genetic distances with the software Primer v6 (Clarke and Gorley 2006) by first calculating the Euclidean distances between each of the isolates. All isolates were grouped in a dendogram based on complete linkage of these distances (supplementary Fig 3). Principal Coordinate Analysis (PCA) was performed using Primer v6 (Clarke and Gorley 2006) to evaluate the genetic diversity between all isolates belonging to the species Diplodia seriata, the most abundant species in our Botryosphaeriaceae population and for all those D. seriata isolates originating from plants which hosted more than one D. seriata isolate.

Results

Highest polymorphism rates on the 8 isolates chosen for primer screening were shown by MseI (M) primer MT in combinations with each of the EcoRI(E) primers EA, EAA, EAC and EG (supplementary Fig 1). These four polymorphic primer pairs amplified a total of 1203
fragments ranging from 60 to 600bp in size and with average PIC values of 0.082 to 0.14 for the 178 accessions (supplementary Fig 2).

On the basis of cluster analysis, the markers did not separate the isolates according to species (supplementary Fig 3). 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 this study (supplementary Fig 3.). However, this group also contained one isolate of *N. parvum*, the second most abundant species of our study, which had isolates scattered throughout each of the four groups. We therefore focussed our genetic diversity investigation using *D. seriata* isolates, the most abundant species in our sample set (Wunderlich et al. 2011b), and excluded isolates of all other species. PCA confirmed that while there appears to be patterns within the distribution of *D. seriata* isolates (Supplementary Fig 3), none of the groups show any obvious relationship to the origin of the isolates. *D. seriata* isolates neither grouped according to their origin tissue types or vineyards (Fig 1.), nor did they necessarily group according to their spatial distance in the vineyard indicated by origin plant numbers, as shown in Fig 2. Some *D. seriata* isolates from grapevines, harbouring more than one *D. seriata* isolate, were closely related to each other. This is the case for 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 the same tissue type (TABLE 1), the two isolates (BB 119-2 and BB119) both originating from grapevine 119 and dormant buds (TABLE 1) and two out of the three *D. seriata* isolates from grapevine 126 (W126, H126 and BB126), isolated from three different tissue types (TABLE 1).

However, the majority of our *D. seriata* isolates from grapevines with multiple *D. seriata* isolates were more genetically diverse than isolates from different origin plants, which are more spatially distanced in the vineyard. This is for example 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 1). 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 1), which are however more different to each other than to isolates from other plants.

**Discussion**

Our study, which represents an extensive marker analysis with 1203 fragments has revealed that these isolates are all diverse and distinctly cluster into 4 groups. However, the groups did not conform according to isolate origin: vineyard, grapevine or tissue type, 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 recent studies which have indicated that some of the Botryosphaeriaceae species such as those belonging to the *Neofusicoccum* and *Dothiorella* anamorphs have common ancestors and has therefore led to phylogenetic restructuring of this genus (Phillips *et al.* 2005b; Crous *et al.* 2006). Future research to resolve the genetic diversity of our isolates in terms of species would therefore require a more in depth study such as whole-genome sequencing.

However, this study was aimed at looking at the populations not from a species identification point of view 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 an extensive gene flow into the vineyards from outside the vineyards 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 could 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, plant species which are also hosts for Botryosphaeriaceae pathogens (Slippers *et al.* 2004c; Burgess *et al.* 2006; Cunnington *et al.* 2007) in other regions of Australia.

Furthermore gene flow from native vegetation to commercial plants has been identified (Burgess *et al.* 2006). Alternatively the constant influx shown from our data as gene flow between the two vineyards could also be a caused by direct isolate transfer between the two vineyards rather than an intermediate area such as the surrounding vegetation. This could potentially be through contaminated machinery such as pruning sheers, however, this is highly unlikely, knowing the different ownerships and operational management of the two vineyards. Transfer of conidia directly between the two vineyards is another potential reason for the extensive gene flow between both spatially separated populations, however this would infer a conidia dispersal over a long distance, possibly by wind, which conflicts with the current literature, which has yet only identified water as the main dispersal mechanism for Botryosphaeriaceae conidia and hence a much shorter distance of dispersal (Urbez-Torres 2011). Since the isolates in our study do not behave like splash dispersed organisms and knowing that strong sub-tropical winds are common throughout the area of our study (Bureau of Meterology 2009), we agree with Urbez-Torres (2011) on the need to further investigate the role wind has for Botryosphaeriaceae conidia dispersal.
The lack of grouping of isolates according to their origin vines 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. The native vegetation as well as other woody crops surrounding vineyards need to be considered as potential inoculum sources for grapevines. Extending vineyard surveys for trunk disease pathogens to the surrounding vegetation, as suggested by Qiu et al. (2011) therefore appears important.

We further conclude from our results that the development of Botryosphaeriaceae in the wood and vegetative/reproductive parts of our vines have not been exclusively restricted to an initial origin infection in the planting materials such as rootstocks 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 and has originated from a common inoculum source for both vineyards or that there still is a constant common inoculum source for them.

The results of this study complement those of our previous work (Wunderlich et al. 2011a; Wunderlich et al. 2011b), which showed that Botryosphaeriaceae have the potential to infect a variety of V. vinifera tissue other than wood and pathogenicity to each tissue type is independent from the origin tissue type. We can further conclude from our AFLP analysis results that Botryosphaeriaceae trunk disease pathogens appear to be not specialised to tissue types. Often a grapevine is infected in more than one location and various tissue types (Wunderlich et al. 2011b) and we 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 is not likely to have happened systemically and as a result of spread from the infected wood of these plants. This
conflicts with the findings by Whitelaw-Weckert et al. (2006) describing the systemic infection of Botryosphaeriaceae from roots to shoots suggesting that infection of one grapevine tissue such as the root will lead to an upward spread into other vegetative tissues such as shoots. If such upward movement was the case in the grapevines of our study, we would expect to see less genetic diversity within the *D. seriata* isolates originating from different tissue types of the same plant than within isolates from different plants and therefore spatially more separated in their origin. *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 occurring in 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.

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*Isolates stored as live cultures at the Agricultural Scientific Collection Unit, Industry and Investment NSW, Orange, NSW, Australia (Herbarium code: DAR). Refer to Wunderlich et al. (2011b) for DAR numbers.
FIGURE 1

A scatter plot showing two principal components (PC1 and PC2). The plot includes various symbols and colors to represent different data points.
**Fig. 1** Principal components analysis data analysed in Primer v6, showing the relationship between *Diplodia seriata* isolates from vineyards A (red) and B (black) and five different origin tissue types: wood ▲, dormant bud ◆, flowers *, pea-sized berries ■ and berries at harvest ○).
**Fig. 2** Principal component analysis data analysed in Primer v6, showing the genetic similarity of *Diplodia seriata* isolates from different origin grapevines which harbour multiple *D. seriata* isolates. Numbers present the identity of the origin vine.
Supplementary Figure 1

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**Supplementary Fig. 1** Primer screening results for 24 AFLP primer pairs on a subset of eight phenotypically most diverse Botryosphaeriaceae isolates. Polymorphism for each primer pair is shown as average polymorphic information content (PIC) value, generated in GenAlX (Peakall and Smouse 2006). The 4 primer pairs chosen for AFLP analysis are highlighted.
Supplementary Figure 2

<table>
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**Supplementary Fig. 2** Number of fragments, range of fragment sizes and average PIC for the four AFLP primer pairs consisting of *Mse*I-T primer in combinations with each of the *EcoRI*-A/ EAA/ EAC and EG primers based on 178 Botryosphaeriaceae isolates.
**Supplementary Fig. 3** (to be viewed in A3): Dendogram from data of 178 Botryosphaeriaceae isolates from 2 different vineyards and 5 different tissue types conforming to 7 different species (*D. seriata*, *N. parvum*, *N. luteum*, *N. ribis*, *N. australe*, *B. dothidea*, and *D. viticola*). Unidentified Botryosphaeriaceae species.

Cluster analysis on binary data from 4 AFLP primer pairs (MT/EA, EAA, EAC, EG) was done on Euclidian distance.

Abstract to the 9th International Congress of Plant Pathology, Torino, Italy, 25th-29th August 2008 (oral presentation and poster).

Species of the fungus *Botryosphaeria* are responsible for cankers in the wood and bunch rot of grapevines. Pathogenicity tests have been conducted on grapevine wood for several species, but it is unknown which of these infect bunches. *Botryosphaeria* spp. have been isolated from bunches in Australian vineyards, but again little is known about the infection pathway into bunches. This project aims to identify *Botryosphaeria* spp. isolated from grapevines at different phenological stages (dormant buds, flowers, pea-sized berries, berries at veraison and berries at harvest) and from the wood. Samples will be collected over 3 seasons from *Vitis vinifera* cv. Chardonnay and Shiraz from two vineyards in the Lower Hunter Valley in south-eastern Australia. Pathogenicity tests will be conducted on berries using the species isolated from the survey. Species variation within a plant will be analyzed and compared to the species found in diseased wood of the same plant. To date, *Botryosphaeria* spp. have been isolated from 6% (3% from Chardonnay and 3% from Shiraz) of bud samples in one vineyard, and 17% (12% from Chardonnay and 5% from Shiraz) in a second vineyard. *Botryosphaeria* spp. have also been isolated from approximately 50% of the wood samples collected from these vineyards. The results from this study will contribute to understanding the epidemiology of *Botryosphaeria* bunch rot and will lead to better management strategies for this disease.

Abstract to the 6th International Workshop on Grapevine Trunk Diseases, Florence, Italy, 1\textsuperscript{st}-3\textsuperscript{rd} September 2008 (poster).

http://ejour-fup.unifi.it/index.php/pm/issue/view/270
Identification of **Botryosphaeria** spp. and first report of **Dothiorella viticola** (**Botryosphaeria viticola**) associated with bunch rot in Australia. N. WUNDERLICH¹, S. SAVOCCHIA¹, G.C. STEEL¹, G.A. ASH² and H. RAMAN³. National Wine and Grape Industry Centre, School of Agricultural and Wine Sciences, Charles Sturt University, Wagga Wagga, NSW 2678, Australia. E-mail: nwunderlich@csu.edu.au

*Botryosphaeria* spp. are commonly associated with trunk disease of grapevines but in some situations can cause berry rot. Two hundred vines symptomatic of *Botryosphaeria* canker at two vineyards in the lower Hunter Valley, south eastern Australia, a region with a known history of *Botryosphaeria* canker, were sampled for species of *Botryosphaeria*. Samples were collected from grapevine tissues at different phenological stages: dormant buds, flowers, pea-sized berries and berries at harvest. Fungi isolated from these samples included **Alternaria** spp., **Penicillium** spp., **Epichloë** spp., **Cladosporium** spp., **Phomopsis** spp. and **Botrytis** sp. In addition, 26 isolates were suspected to be species of **Botryosphaeria**. These were identified according to spore morphology and sequencing of the rDNA internal transcribed spacer (ITS) region. **Dipodina serata**, **Nefusiococcus parvum** and **Dothiorella viticola** were isolated from dormant buds and **D. serata**, **N. parvum**, **N. luteum** and **N. austral** and **C. dothidea** were isolated from berries at harvest. Species of **Botryosphaeria** were not isolated from flowers or pea-sized berries. This suggests that species of **Botryosphaeria** associated with bunch rot infections do not arise from infection of the vegetative tissues earlier in the season. To our knowledge, this is also the first report of **D. viticola** on grapevines in Australia. Further sequencing of additional gene regions (β-tubulin and EF1-α) will be conducted to confirm the identity of the putative **N. luteum** and **N. austral** isolates. Future work will utilise genetic methods to investigate the homology of isolates originating from the wood and other reproductive tissues. Confirmation of isolate pathogenicity towards different tissues of **Vitis vinifera** is currently in progress.

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Trunk disease pathogens within the *Botryosphaeriaceae* are associated with bunch rot disease in the Hunter Valley

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Researchers in the Hunter Valley looked for the presence of *Botryosphaeriaceae* species on Shiraz and Chardonnay vines. A survey found the greatest number of *Botryosphaeriaceae* were isolated from dormant buds and all species from dormant buds were able to infect berries, resulting in bunch rot symptoms.

**Introduction**

Species of fungi belonging to the *Botryosphaeriaceae* are responsible for grapevine trunk diseases such as Botryosphaeria (Bot) canker, Escoríose, Black Dead Arm or Diplodia Cane Dieback (Larignon *et al.* 2001; Savocchia *et al.* 2007; Urbez-Torres and Gubler, 2009; Van Niekerk *et al.* 2006). Recently some of these species have also been isolated from Chardonnay and Shiraz bunches with and without bunch rot symptoms in the lower Hunter Valley, New South Wales. While the epidemiology and distribution of Bot canker in the wood of grapevines and other hosts has been widely researched, it is unknown if and how *Botryosphaeriaceae* species invade grape berries and contribute to bunch rot diseases. *Botrytis cinerea, Colletotrichum spp.*, Greenberg riparia, and *Phomopsis viticola* are considered as the most common bunch rot pathogens in the Hunter Valley (Melksham *et al.* 2002; Nair, 1985; Steel *et al.* 2007), whilst *Botryosphaeriaceae* species have been mainly considered as secondary bunch rot pathogens, relying on wounding or prior infection by other fungi to colonise the berry tissue. However, studies in other regions of Australia have reported frequent isolations of *Botryosphaeriaceae* species from bunches, resulting in questions being raised about their role as primary bunch rot pathogens (Taylor, 2007).

The aims of this PhD project are to: (i) survey various parts of the grapevine at different phenological stages in the Hunter Valley for *Botryosphaeriaceae* species; (ii) assess the pathogenicity of the species on various parts of the grapevine, (iii) determine the level of genetic variation that exists within and between species isolated from different grapevines and tissue origin. This article reports on...
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A survey conducted over two consecutive seasons in two vineyards of the lower Hunter Valley for the presence of Botryosphaeriaceae species on various parts of the grapevine. Preliminary results from in vitro pathogenicity tests of the species collected during the survey are also presented.

Methods

Survey and identification

The survey was conducted in the lower Hunter Valley over two seasons between May 2007 and March 2009 and in two vineyards with a known history of Bot canker. From each vineyard, 50 Chardonnay and 50 Shiraz vines were selected for sampling of dormant buds, flowers, pea-sized berries and berries at harvest. In addition, five wood samples were taken from the trunks of each of the vines surveyed. At each sampling time, five dormant buds or clusters were collected from each grapevine and surface sterilised with 0.5% bleach followed by three rinses with sterile water.

Samples were transferred to Potato Dextrose Agar (PDA) amended with Streptomycin and incubated at 25°C in the dark for 2 to 4 days. The culture morphologies of the fungi isolated were examined and used to separate Botryosphaeriaceae species from other fungi such as Alternaria, Epicoccum, Cladosporium, Penicillium, Fusarium, Pestalotiosis and Botsrytis cinerea. All Botryosphaeriaceae were identified and grouped based on conidial morphology. The identities of a selection of isolates from each morphological group were confirmed via partial DNA sequence analysis of the ITS region.

In vitro Pathogenicity tests

Berries

Berries were inoculated by applying 10µL spore suspensions (10^7 spores/mL) from 10 single spore isolates of Botryosphaeriaceae onto three replicates of 24 surface sterilised Chardonnay berries at 12 Baumé. Identities, species and origin tissue of each isolate are displayed in Table 1. Control berries were inoculated with sterile water. The berries were incubated at 27°C and 100% humidity for 15 days. The disease incidence (%) and severity (1-10 scale) was recorded every second day. Disease index (DI) and disease severity score (DSS) were calculated for all replicates of each of the isolates using the following formulas:

\[ DI = \frac{\sum \text{diseased berries} \times \sum \text{total berries}}{24} \times \frac{\sum \text{disease index for each isolate}}{240} \]

A regression analysis was performed to determine the rate of disease index increase for each treatment. Differences in these rates and disease index data for each isolate on each individual day were analysed using ANOVA. To satisfy Koch’s postulates, berries from each treatment were placed onto PDA, incubated at 25°C and examined for the presence of spores corresponding to the inoculated isolate.

Flowers

At 100% cap fall, the flower clusters from glasshouse grown Chardonnay and Cabernet Sauvignon were dipped in a spore suspension (10^7 spores/mL) of a mixture of three isolates of Diplodia seriata or three isolates of Neofusicoccum parvum. Control clusters were dipped in sterile water and covered in plastic bags for 48 hours to maintain high humidity. Visual symptoms were recorded at the pea-sized berry stage. Koch’s postulates were satisfied according to the procedure described for berry inoculations.

Results

Survey and identification

A total of 246 isolates belonging to the Botryosphaeriaceae were collected over two seasons and to date, 181 isolates have been identified (Table 2). The isolates conform to seven species of the Botryosphaeriaceae: D. seriata (n=118), N. parvum (n=16), N. luteum (n=8), Dothiorella viticola (n=5), Lasiodiplodia theobromae (n=3), Diplodia mullia (n=4) and Botryosphaeria dothidea (n=27). All species but L. theobromae and D. mullia were isolated from dormant buds. Only D. seriata and N. parvum were isolated from flowers and only D. seriata was isolated from pea-sized berries of Chardonnay. All species but D. viticola, L. theobromae and D. mullia were isolated from berries at harvest. In addition, all species were isolated from the wood of Shiraz however N. luteum and D. viticola were not isolated from the wood of Chardonnay.

Pathogenicity tests

Berries

All isolates produced bunch rot symptoms on berries in vitro. Symptoms included the appearance of mycelia and pycnidia, darkening of the berry skin, cooting and berry collapse (Figure 1). Koch’s postulates were fulfilled by re-isolating each isolate from infected berries. The rate of disease development over 15 days varied between isolates and appears to be independent from the isolate species or origin. Figure 2 shows the mean disease index for each treatment at day 15.

Flowers

At pea-size berries stage a reduction in bunch size was observed for the inoculated clusters compared to the uninoculated clusters. Berry splitting and browning of the skin was observed on occasion for berries of the inoculated clusters. Koch’s postulates were fulfilled by re-isolation of the inoculated isolate from infected tissue.

Discussion

A survey of different grapevine tissues at different phenological stages showed that the greatest number of Botryosphaeriaceae was isolated from dormant buds, followed by the wood and berries at harvest. The number of isolates derived from flowers and pea-sized berries was very low compared to isolates derived from the other grapevine tissue types and only included D. seriata and N. parvum. The low percentage isolation of Botryosphaeriaceae from flowers and pea-sized berries may suggest that these fungi invade bunches externally later in the season. While most species were found on all tissue types, D. mullia and L. theobromae were only isolated from the wood. In addition, D. viticola was isolated from the wood of Shiraz but not Chardonnay. Further studies are in progress to identify the remaining isolates collected during the survey.

Pathogenicity tests on berries and flowers showed symptoms similar to those observed in the field. All species of Botryosphaeriaceae collected from dormant buds and the wood were able to infect berries, resulting in bunch rot symptoms. Rate of disease development was independent from species and the tissue origin of the isolate. Further studies will be conducted using isolates collected from flowers, pea-size berries and berries at harvest to determine if these are tissue specific with regards to pathogenicity.

The limitation of L. theobromae and D. mullia to the wood may suggest that these species are unable to infect the other tissue types, however, the pathogenicity tests on berries showed that an isolate of L. theobromae was able to produce bunch rot symptoms under laboratory conditions. To confirm this, further pathogenicity tests including L. theobromae and D. mullia will be conducted.

The results of the in vitro pathogenicity tests on berries and flowers suggest that Botryosphaeriaceae species have the potential to contribute to the complex of bunch rot diseases observed in the Hunter Valley. Field inoculation trials will be conducted to determine whether the results of the in vitro pathogenicity tests can be reproduced under field conditions and whether the infection of dormant buds leads to infection of bunches at harvest. These results will contribute to the epidemiological understanding of the Botryosphaeriaceae as primary pathogens of grapevines, which in turn will allow the development of better management strategies for Bot canker and bunch rot diseases.
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Acknowledgements

This project is supported by a National Wine and Grape Industry Centre (NWGIC) scholarship and through the Wine Growing Futures program, a joint initiative of the Grape and Wine Research and Development Corporation and the NWGIC. We thank the growers for allowing access to their vineyards, Chris Haywood (NSW DPI) for assistance with sampling, Ruijun Huang, Lindsay Greer, Wayne Pitt and Michael Qiu (CSU) for laboratory-based assistance.

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Table 2. Total number of Botryosphaeriaceae species isolated from Chardonnay and Shiro. Significance levels were determined using the Fisher’s exact test. 

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<td>W</td>
<td>11</td>
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*G = dormant bud; F = flowers; P = pea-sized berries; H = harvest; W = wood.

Wood samples were only collected in the first season.

References


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Figure 1. Chardonnay berries showing the development of bunch rot symptoms 15 days after inoculation with D. seriata isolate B114-2 and control treatment. Disease indices are 73.87, and 7.87, respectively.

Figure 2. Pathogenicity of Botryosphaeriaceae species on Chardonnay berries 15 days after inoculation. Error bars represent the standard error of the mean.
**PUBLICATION 7:** Wunderlich N, Steel CC, Ash GJ, Raman H and Savocchia S 2009. Trunk disease pathogens within the Botryosphaeriaceae are associated with bunch rot disease in the Hunter Valley.

Species of fungi belonging to the Botryosphaeriaceae are responsible for grapevine trunk disease Botryosphaeria (Bot) canker. Recently some of these species have also been isolated from Chardonnay and Shiraz bunches with and without bunch rot symptoms in the lower Hunter Valley, New South Wales.

While the epidemiology and distribution of Bot canker in the wood of grapevines and other hosts has been widely researched, it is unknown if and how Botryosphaeriaceae species invade grape berries. Botryosphaeriaceae species have mainly been considered as secondary bunch rot pathogens, relying on wounding or prior infection by other fungi to colonise the berry. Frequent isolations of Botryosphaeriaceae species have recently been reported from bunches, raising the question as to whether these fungi play a role as primary bunch rot pathogen.

The aims of this study are to: (i) survey various parts of the grapevine at different phenological stages in the Hunter Valley for Botryosphaeriaceae species; ii) assess the pathogenicity of the species on various parts of the grapevine, (iii) determine the level of genetic variation that exists within and between species isolated from different grapevines and tissue origin.

A survey was conducted over two consecutive seasons (2007-2009) in two vineyards of the lower Hunter Valley with a known history of Bot canker. Samples were taken from various parts of the grapevine at different phenological stages as well as from the trunk of each vine. A total of 246 isolates conforming to seven species of the Botryosphaeriaceae: Diplodia seriata, Neofusicoccum parvum, Neofusicoccum luteum, Dothiorella viticola, Lasiodiplodia theobromae, Diplodia mutila and Botryosphaeria dothidea were isolated. The highest number of isolates originated from dormant buds and wood followed by berries at harvest. A small number of isolates was found on
flowers and pea-sized berries. While most species were found on all tissue types, *D. mutila*, *L. theobromae* and *D. viticola* were only isolated from the wood.

Botryosphaeriaceae species originating from different tissues were tested in *in vitro* for pathogenicity on berries at harvest and one-year-old canes of Shiraz and Chardonnay. Pathogenicity towards flowers of glasshouse-grown Chardonnay was also assessed. All isolates produced bunch rot symptoms on berries including the appearance of mycelia and pycnidia, darkening of the berry skin, oozing and berry collapse. All isolates produced lesions on one-year-old canes. At pea-size berries stage a reduction in berry-set including a reduction in bunch size was observed for the inoculated flowers compared to the un-inoculated flowers. Berry splitting and browning of the skin was also observed.

Pathogenicity tests suggest that Botryosphaeriaceae species have the potential to cause bunch rot disease. The low percentage isolation of Botryosphaeriaceae from flowers and pea-sized berries suggests that these fungi may invade bunches closer to harvest. Further investigations using molecular techniques have commenced to determine the genetic diversity of Botryosphaeriaceae species originating from each vine. In addition, field inoculations of dormant buds have been established to determine if these fungi can be recovered at harvest.

Abstract to the 17th Biennial Australasian Plant Pathology Society Conference, Newcastle, Australia, 29th September-2nd October (oral presentation).

Botryosphaeria spp. associated with bunch rot of grapevines in south-eastern Australia

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INTRODUCTION
Species of Botryosphaeria are common wood pathogens of grapevines and are responsible for the disease known as ‘Bot
canker’ [1]. Recently some species have also been implicated in
bunch rot of Vitis vinifera in Australia [2]. While pathogenicity
tests have been conducted on grapevine wood for several
species, it is unknown which of these infect bunches and how
they enter the berry.

MATERIALS AND METHODS
Plant survey. Between 2007 and 2009 two vineyards in the
lower Hunter Valley, NSW, were sampled for species of
Botryosphaeria. Samples were collected from 200 each of
Chardonnay and Shiraz grapevines symptomatic of Bot canker at
different phenological stages: dormant buds (B), flowers (F), pea-
sized berries (P) and berries at harvest (H). Samples were also
collected from the margin of healthy and discoloured internal
wood (W) from the trunks of each plant.

Fungal isolation and identification. Samples were surface
sterilised, placed onto Potato Dextrose Agar (PDA) amended
with Streptomycin Sulfate and incubated at 25°C in the dark.
Fungal cultures characteristic of Botryosphaeria spp. were sub-
cultured onto PDA and/or triple autoclaved pine needles on 1%
water agar maintained under near UV light (12hr light/dark) to
curcourage sporulation. Botryosphaeria spp. were identified
according to spor morphology and sequencing of the rDNA
internal transcribed spacer region.

Pathogenicity tests on berries. Disease-free, surface sterilised
Shiraz and Chardonnay berries at harvest were inoculated with
10 μl spor suspensions from 19 isolates belonging to the
species listed in table 1. Conidial suspensions at concentrations
of 103 and 106 spores/ml were used in trial 1 and 2, respectively.
Control berries were inoculated with 10 μl of sterile distilled
water. The berries were incubated in 24 well plates at 27°C in
the dark for 15 days. A constant relative humidity was
maintained by the addition of 20 ml of sterile water to each
plate. Disease incidence (%) and severity (1–10) was recorded
for each treatment. A disease index (DI) was calculated for each
treatment replicate at each time of recording:

\[ DI = \frac{\sum \text{diseased berries} \times \text{total berries} \times \text{disease severity scores}}{\sum \text{max disease severity scores}} \]

Pathogenicity tests on canes. Detached one year old canes were
inoculated with 4 mm diameter mycelium plugs of 14
Botryosphaeria isolates, previously tested on berries, by
inserting the plugs into the wood. Canes were incubated on
moist filter paper in Petri dishes at 27°C in the dark. After 15
days, lesion lengths were measured for each isolate.

RESULTS
Survey and fungal identification. To date, a collection of 177
isolates of Botryosphaeria spp. has been established. The species
isolated included Diplodia seriata, Botryosphaeria dothidea,
Neofusicoccum parvum, N. luteum, Dothiorella viticola, Diplodia
mutia and Lasiodiplodia theobromae. Abundance and origin of
each species is listed in Table 1.

<table>
<thead>
<tr>
<th>Species</th>
<th>Origin tissue</th>
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<tr>
<td>D. seriata</td>
<td>83</td>
</tr>
<tr>
<td>B. dothidea</td>
<td>15</td>
</tr>
<tr>
<td>N. parvum</td>
<td>7</td>
</tr>
<tr>
<td>N. luteum</td>
<td>3</td>
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<tr>
<td>D. viticola</td>
<td>0</td>
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<tr>
<td>D. mutia</td>
<td>0</td>
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<tr>
<td>L. theobromae</td>
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</table>

Pathogenicity tests All isolates produced bunch rot symptoms
on berries including the formation of mycelia and pycnidia,
darkening of the berry skin, oozing and berry collapse. Disease
indices for each replicate treatment varied significantly from
control berries. Variation in the rate of increase of the disease
index was detected within and between species. Inoculation of
canes showed lesion development and pycnidia formation on
the cane surface. There were significant variations in lesion
lengths between species. In both berry and cane pathogenicity
tests, virulence appeared to be independent from the origin of
the isolate.

DISCUSSION
Results suggest that Botryosphaeria spp. have the potential to
contribute to grapevine bunch rots and infect grapevine canes.
Botryosphaeria spp. appear to be non-tissue specific in their
pathogenicity toward grapevine. Trials are in progress to
establish if these results can be reproduced under field
conditions and whether the infection of buds or flowers by
Botryosphaeria results in bunch rot at harvest.

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Abstract to the 7th International Workshop on Grapevine Trunk Diseases, Santa Cruz, Chile, 17th-21st January 2010 (oral presentation).

http://ejour-fup.unifi.it/index.php/pm/issue/view/304
Botryosphaeriaceae associated with bunch rot of grapes in South Eastern Australia. N. Wunderlich, G. Asl, C. Steyer, H. Rahmani2 and S. Savocauchy. 1National Wine and Grape Industry Centre, School of Agricultural and Wine Sciences, Charles Sturt University, Locked Bag 586, Wagga Wagga, NSW 2678, Australia. 2Industry & Investment New South Wales, Private Mail Bag, Wagga Wagga, NSW 2650, Australia. E-mail: nwunderlich@csu.edu.au

Species of fungi belonging to the Botryosphaeriaceae are responsible for the grapevine trunk disease Botryosphaeria canker. While the epidemiology and distribution of Botryosphaeria canker in the wood of grapevines and other hosts has been widely researched, little is known about the epidemiology of these pathogens on grape berries. Frequent isolations of Botryosphaeriaceae have recently been reported from grape bunches, raising the question as to whether these fungi can be considered as primary bunch rot pathogens. A survey was conducted over two consecutive seasons (2007–2009) in two vineyards of the lower Hunter Valley with a known history of Botryosphaeria canker. Samples were taken from various parts of the grapevine at different phenological stages, as well as from the trunk of each grapevine. Fungi conforming to seven species of the Botryosphaeriaceae: Diplodia seriata, Neofusicoccum parvum, N. luteum, Dothiorrilla viticola, Lasiodiplodia theobromae, D. matlta and Botryosphaeria dothidea were isolated. The largest number of isolates originated from dormant buds and wood followed by berries at harvest. A small number of isolates was found on flowers and pea-sized berries. Isolates originating from different tissues were found to be pathogenic on berries at harvest and one-year-old canes of Shiraz and Chardonnay. In addition, dormant buds of glasshouse and field grown Chardonnay were inoculated with various Botryosphaeriaceae to investigate into the affect of Botryosphaeria infection on budburst and shoot development.
General discussion

Botryosphaeriaceae fungi are well-known pathogens invading the wood and fruit of many woody hosts. In grapevine these fungi are also well-studied, however, in Australia so far these studies have only concerned the role of Botryosphaeriaceae fungi as wood pathogens. There have been incidental reports of Botryosphaeriaceae fungi on grapevine fruit (Steel et al. 2007, Taylor & Wood 2007) and industry reports such as Scholefield and Morison (2010), that assert the anecdotal association of Botryosphaeriaceae with fruit rot of grapevines, support the need to investigate their recognition as bunch rot pathogens of *V. vinifera*, particularly in hot and wet grape growing regions. The current studies further highlight that this is necessary by confirming that Botryosphaeriaceae fungi are neither tissue specific (Wunderlich et al. 2011b, Wunderlich et al. under revision), nor is their pathogenicity or virulence affected by their origin tissue (Wunderlich et al. 2011a). This indicates that grapevines infected with Botryosphaeriaceae fungi in the wood can act as inoculum sources for other grapevine tissue and further supports the argument that Botryosphaeriaceae fungi should be considered as more than just trunk disease pathogens within vineyards. This has implications for the management of these pathogens in vineyards. Currently, management practices are limited to the protection of pruning- and other wounds and remedial surgery of Botryosphaeriaceae infected wood. In addition, it can be suggested that other tissues infected with Botryosphaeriaceae, such as rotten bunches, shoots, buds or mummified berries on the vineyard floor, should also be removed from the vineyard to reduce the sources of inoculums, as it is practiced for other bunch rot pathogens.

The survey of Botryosphaeriaceae in different grapevine tissues revealed that similar species exist in grapevine wood and in the other tissue types (Wunderlich et al. 2011b). The same species as reported from the wood of grapevines in Australia were isolated from dormant buds, flowers, pea-sized berries and berries at harvest stage. In a previous survey by Qiu et al. (2011) the distribution of *B. dothidea* in the Hunter Valley remained inconclusive. This has now been clarified with the finding of 23 *B. dothidea* isolates from two vineyards in the lower Hunter Valley identified via ITS sequencing (Wunderlich et al. 2011b). The survey is also a first report of *D. viticola* isolation from grapevine in Australia (Wunderlich et al. 2008). This species has so far only been isolated from grapevine in other parts of the world (Luque et al. 2005, Urbez-Torres & Gubler 2007).
A large number of isolates belonging to the species *D. seriata* from both vineyards and five different tissue types were isolated and therefore the genetic diversity of these two populations was assessed (Wunderlich *et al.* under revision).

In terms of the infection process of Botryosphaeriaceae fungi into grapevine tissue other than wood, this study showed that internal systemic infection from the wood into the reproductive tissues is less likely than an external infection of these tissues. Considering that bud inoculation did not lead to significant numbers of re-isolation from the reproductive tissues of the experimental vines at later growing stages (Wunderlich *et al.* 2011a) those numbers of isolates obtained in the survey (Wunderlich *et al.* 2011b) from berries at harvest, flowers and pea-sized berries may have come from the environment into the bunches. Frequency of isolations coincided with the rain patterns in the lower Hunter Valley, which are greater during winter when the grapevines are dormant than in summer. The least rain in this region occurs during the stages of flowering and pea-sized berries.

The genetic diversity study (Wunderlich *et al.* under revision) complements the information that isolates originating from various tissue types have been the cause of external infection rather than a systemic internal infection originating in the wood. This is the first time Botryosphaeriaceae infection on grapevine tissue other than wood has been investigated and it can be concluded that while internal movement in the wood occurs, it is not likely that wood infection will necessarily lead to a systemic infection of the buds and fruit of the same plant.

In terms of pathogenicity, discrepancies were detected between isolates within a species (Wunderlich *et al.* 2011a). While these results are in contrast with the idea that individual species of Botryosphaeriaceae fungi can be labeled either pathogenic or endophytic, such as *N. australe* and *D. seriata*, respectively (Taylor *et al.* 2005), the current results coincide with those of other authors such as Larignon *et al.* (2001) and van Niekerk *et al.* (2004) who conducted pathogenicity tests, showing that there is more to an isolates’ virulence than its species identity. Outcomes of the pathogenicity studies (Wunderlich *et al.* 2011a) have also shown that caution is necessary when investigating the pathogenicity of pathogens in an area so far unstudied, with limited available isolates.
However, this study has clearly shown even with a small number of isolates that an isolate effect occurs which may overrule the species and origin tissue effect (Wunderlich et al. 2011a). Therefore an attempt to partition the components of variability in pathogenicity was made and it was found that ‘isolate’ explains the highest variability on wood and berries. Further research to what determines the pathogenicity of Botryosphaeriaceae is necessary and it can be concurred with van Niekerk et al. (2004), that grouping individual Botryosphaeriaceae isolates according to their virulence could be done in the future, incorporating a very large number of isolates from various different collections shown to be endophytic or pathogenic, into one homogenised pathogenicity test on different *V. vinifera* tissue types. The lack of grouping of the isolate collection according to their virulence could be done in the future, incorporating a very large number of isolates from various different collections shown to be endophytic or pathogenic, into one homogenised pathogenicity test on different *V. vinifera* tissue types. The lack of grouping of the isolate collection according to their virulence may result in an association between the genetics of isolates and their virulence. This could lead to further studies to investigate the gene(s) involved in the pathogenicity of Botryosphaeriaceae fungi and development of markers to detect pathogenic from non-pathogenic isolates. This knowledge would allow management strategies to be based on the level of virulence of the existing isolates rather than on their species identity.

To further confirm the pathway of Botryosphaeriaceae infection in berries it is important to continue epidemiology studies in the field. It is yet to be established what happens to the Botryosphaeriaceae after being transferred externally onto dormant buds. While inoculation studies in the field did not result in infection of the buds and further reproductive tissues (Wunderlich et al. 2011a), it can be speculated that the inoculum was controlled by the routine fungicide applications occurring in the experimental vineyard. Future studies should include field inoculation in an unregulated environment without fungicide application to see if infection of buds follows through to other reproductive tissues at later stages. Studies into inter-vineyard infections should also include the spread of Botryosphaeriaceae into vineyards from populations of surrounding vegetation. Botryosphaeriaceae isolates from other host species, such as native vegetation commonly surrounding vineyards should be considered in future pathogenicity tests.

The genetic diversity study also showed that there is extensive geneflow between the two Botryosphaeriaceae populations in the two vineyards (Wunderlich et al. under
revision) indicating that infection of grapevines occurred from a common external source for both vineyards or directly from one vineyard into the other, suggesting larger dispersal distances than recorded in the current literature (Baskarathevan et al. 2010). Isolates thus do not behave like splash dispersed organisms and in agreement with Urbez-Torres (2011) it can be concluded that the role of wind-dissemination and alternative hosts as inoculum sources for Botryosphaeriaceae infection of grapevines needs to be investigated further.

Results from this study have contributed knowledge on the infection pathways leading to Botryosphaeriaceae canker and bunch rot and suggest that current management techniques for Botryosphaeriaceae in vineyards should be amended to consider infections occurring in tissues other than wood.

Understanding the likelihood of external infection of grapevine tissue other than wood not only increases the depth of knowledge of Botryosphaeriaceae infection within vineyards and between vineyards, it also provides background information for future research into the lifecycle of Botryosphaeriaceae as bunch rot pathogens. The outcomes of the studies presented here have contributed to defining the role of Botryosphaeriaceae as pathogens of *V. vinifera* further and beyond its role as wood pathogens. This may form the foundation for continued research into the epidemiology of Botryosphaeriaceae fungi in vineyards and their role as bunch rot pathogens.
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