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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 australe* 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.

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 excoriose, black dead arm, and diplodia dieback (Larignon *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 australe*, *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, Qiu *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 rots (Taylor and Wood 2007). More recently Cunnington and Priest (2007) reported the identification of two *N. australe* isolates from grapevine berries in Victoria, Australia.

Several studies have identified these fungi as important fruit rot pathogens of various hosts including apple (Fenner 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, Lazzizera *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

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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 mummify and develop black pycnidia on the surface (Luttrell 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 (Urbez-Torres and Gubler 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

S u r v e y : 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 32º47'39.56''S 151º20'35.79''E (Vineyard A) and 32º48'24.77''S 151º16'30.03''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 Meterology 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 Meterology 2009).

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

Wood samples were taken from the trunk and cordons of each vine before commencing the survey of other reproductive tissues. During both seasons, samples were taken at the growth stages of dormant bud, flowering, pea-sized berry and harvest, corresponding to the E-L phenological stages of 1, 21, 31 and 35, respectively as described by Coombe (1995). At each sampling time five dormant buds, inflorescences or bunches per vine were collected at random and sub-sampled to florets and berries. This intensive method of sampling was chosen to increase the likelihood of isolating rarer species such as *B. dothidea* and *N. parvum* previously isolated from the Hunter Valley (Qiu *et al*. 2010).

I s o l a t i o n : 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 autoclaved 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.

D N A e x t r a c t i o n a n d m o l e c u l a r i d e n t i f i c a t i o n : 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^{TM} potato dextrose broth (Bacto Laboratories, Liverpool, NSW, Australia) and were incubated at 25 °C and 90 rpm in an orbital shaker (Sartorius Certomat

BS-1). Mycelia were harvested after 7 d, initially dried by filtration, freeze-dried in a Christ Gamma 1-16LSC 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 $MgCl_2$, 200 µM each of dNTPs (Promega, Australia), and 0.15 µM each of primers ITS1 and ITS4. PCRs were performed in a Master Thermocycler (Eppendorf, Germany) according to Slippers *et al*. (2004) with an amended initial denaturation step of 95 ºC for 15 min.

PCR products were submitted to the Australian Genome Research Facility (Brisbane, Australia) for dual direction sequencing. Isolates were identified to species level by comparing the resulting sequences with those of other Botryosphaeriaceae available in GenBank. Species identities for *D. viticola*, *N. australe*, *N. ribis* and *N. luteum* were confirmed using partial sequencing of the β-tubulin and the translation elongation factor 1-alpha (EF1-α) genes.

β-tubulin gene analysis was carried out in 50 µL reactions containing ~50 ng DNA template, 0.2 µM of each primer Bt2a and Bt2b (Glass and Donaldson, 1995), 1.25 units of HotStar *Taq* (Qiagen), 1 × PCR buffer (Qiagen), 15 mM $MgCl_2$, 200 µM each of dNTPs (Promega). The PCR cycling protocol consisted of an initial denaturation at 95 °C for 15 min, followed by 40 cycles of 94 °C for 20 s, 55 °C for 45 s and 72 °C for 1 min and 30 s and a final extension of 72 °C for 5 min.

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

A selection of isolates from each species reported here were submitted as live cultures to the Agricultural Scientific Collection Unit, 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 9 different species of *Diplodia*, *Dothiorella* and *Neofusicoccum*: *D. seriata*, *D. mutila*, *L. theobromae*, *D. viticola*, *N. australe*, *N. parvum*, *N. ribis*, *N. luteum* and *B. dothidea* (Tab. 1).

The number of isolations for each species, their vineyard of origin and host cultivar are shown in Tab. 2. *D. seriata*, *N. parvum*, *B. dothidea* and *N. luteum* were the most frequently isolated species, occurring in both vineyards and on both 'Chardonnay' and 'Shiraz'. *D. mutila*, *L. theobromae* and *D. viticola* were isolated only from Vineyard B with *D. viticola* being isolated from both cultivars, *D. mutila* on 'Chardonnay' only and *L. theobromae* on 'Shiraz' only. In contrast, *N. australe* and *N. ribis* were isolated only from Vineyard A with *N. ribis* occurring on both cultivars and *N. australe* occurring only on 'Shiraz'.

The greatest number of Botryosphaeriaceae isolations occurred from dormant buds and wood followed by berries at harvest, while isolations from flowers and pea-sized berries were scarce (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. australe* and *D. viticola* were not isolated from berries at harvest. Along with two unidentified Botryosphaeriaceae species, *D. seriata*, *N. parvum* and *N. luteum* were the only species isolated from flowers and *D. seriata* was the only species occurring on pea-sized berries.

Discussion

The Botryosphaeriaceae family is species-rich, containing common trunk disease pathogens, frequently isolated from grapevine wood in vineyards worldwide including the Hunter Valley. Until now, *D. seriata*, *N. luteum* (Savocchia *et al*. 2007), *N. ribis* (Castillo-Pando *et al*. 2001), *N. parvum, B. dothidea* (Qiu *et al*. 2008) and *L. theobromae* (Qiu *et al*. 2010) were the only species of Botryosphaeriaceae reported from the Hunter Valley. The additional findings of *D. viticola*, *N. australe*, and *D. mutila* at relatively low frequencies compared to most of the previously recorded species except *L. theobromae* reflect the species distribution seen in other regions in eastern Australia, which largely seems to depend on climatic variations (Pitt *et al*. 2010). 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

Identities and origin of Botryosphaeriaceae isolated from *Vitis vinifera* from the Lower Hunter Valley

Identity (ID)						Origin		
Species	Isolate ID	Herbarium accession number	GeneBank accession			Vineyard	Host cultivar	Host tissue
			ITS	EF1-α	β-tubulin			
Botryosphaeria dothidea	H171-1	DAR 80994	HQ392689	HQ392757	HQ392756	B	Shiraz	Berries at harvest
B. dothidea	H171-2	DAR 80995	HQ392690		HQ392758	B	Shiraz	Berries at harvest
B. dothidea	BB56-2	DAR 81005	HQ392691	HQ392739	HQ392738	A	Shiraz	Dormant bud
B. dothidea	W64-5	DAR 81006	HQ392692	-	-	A	Shiraz	Wood
B. dothidea	W96-3	DAR 81007	HQ392693	-	-	A	Shiraz	Wood
B. dothidea	W126-5	DAR 81008	HQ392694	-	-	B	Chardonnay	Wood
B. dothidea	BB152-1	DAR 81009	HQ392695	HQ392742	HQ392743	B	Shiraz	Dormant bud
B. dothidea	BB158-4	DAR 81010	HQ392696	HQ392744	HQ392745	B	Shiraz	Dormant bud
B. dothidea	BB163-1	DAR 81011	HQ392697	-	-	B	Shiraz	Dormant bud
B. dothidea	BB174-1	DAR 80994	HQ392698	-	-	B	Shiraz	Dormant bud
B. dothidea	BB178-1	DAR 81029-	-	HQ392749	HQ392748	B	Shiraz	Dormant bud
Diplodia seriata	BB9-1	DAR 80978	HQ392699	-	-	A	Chardonnay	Dormant bud
D. seriata	W86-2	DAR 80979	HQ392700	-	-	A	Shiraz	Wood
D. seriata	W196-2	DAR 80980	HQ392701	-	-	B	Shiraz	Wood
D. seriata	H17-1	DAR 80984	HQ392702	-	-	A	Chardonnay	Berries at harvest
D. seriata	H64-1	DAR 80985	HQ392703	-	-	A	Shiraz	Berries at harvest
D. seriata	B98-3	DAR 80986	HQ392704	-	-	A	Shiraz	Berries at harvest
D. seriata	B106	DAR 80987	HQ932705	-	-	B	Chardonnay	Dormant bud
D. seriata	B178-1	DAR 80988	HQ392706	-	-	B	Shiraz	Dormant bud
D. seriata	H33-1	DAR 80996	HQ392707	-	-	A	Chardonnay	Berries at harvest
D. seriata	H118-1	DAR 80997	HQ392708	-	-	B	Chardonnay	Berries at harvest
D. seriata	FF197-1	DAR 80998	HQ392709	-	-	B	Shiraz	Flowers
D. seriata	PP136	DAR 80999	HQ392710	-	-	B	Chardonnay	Pea-sized berries
D. seriata	FF151-1	DAR 81000	HQ392711	-	-	B	Shiraz	Flowers
D. seriata	PP118	DAR 81002	-	-	-	B	Chardonnay	Pea-sized berries
D. seriata	PP119-1	DAR 81003	-	-	-	B	Chardonnay	Pea-sized berries
Dothiorella viticola	B146-3	DAR 80992	HQ932712	-	HQ392736	B	Chardonnay	Berries at harvest
D. viticola	B116-3	DAR 81012	HQ392713	HQ392735	HQ932734	B	Chardonnay	Dormant bud
Lasiodiplodia theobromae	W200	DAR 81024	HQ392714	-	-	B	Shiraz	Wood
Neofusicoccum australe	BB59-3	DAR 81004	HQ392715	-	-	A	Shiraz	Dormant bud
Neofusicoccum luteum	H12-1	DAR 80983	HQ392716	HQ392753	HQ392752	A	Chardonnay	Berries at harvest
N. luteum	HH119-1	DAR 81013	HQ392717	HQ392760	HQ392761	B	Chardonnay	Dormant bud
N. luteum	BB127-1	DAR 81014	-	HQ392740	HQ392741	B	Chardonnay	Dormant bud
N. luteum	BB161-2	DAR 81015	HQ393718	-	HQ392746	B	Shiraz	Dormant bud

of the rarer species in our survey might be explained by the more intensive sampling method used (Qiu *et al*. 2010).

The isolation of one single isolate belonging to *L. theobromae*, a species favouring hot climatic conditions (Urbez-Torres *et al*. 2006), is also consistent with the findings of Qiu *et al.* 2010, who predict to see a greater abundance of this species in the Hunter Valley in the future due to increased temperatures caused by climate change.

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

In previous reports that dealt with the infection of fruit in Australian vineyards the fungi were not identified to species level (Steel *et al*. 2007, Taylor 2007) other than Cunnington *et al*. (2007) who reported *N. australe* from grapevine berries in Victoria, Australia. To our knowledge our survey is the first report of *D. seriata*, *D. viticola*, *N. parvum*, *N. ribis*, *N. luteum* and *B. dothidea* in *V. vinifera* tissue other than wood.

Tab. 1 continued

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

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

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

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

No fungicides are currently registered for the control of Botryosphaeriaceae in Australian vineyards however, it is known that the management of other bunch rots, such as grey mould caused by *Botrytis cinerea*, relies extensively on fungicide sprays at flowering to reduce the infection rate of inflorescences and subsequent berry infection at harvest (Nair *et al.* 1987 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 confirms that Botryosphaeriaceae species can infect different *V. vinifera* tissue types throughout all stages of the growing season. This is important information for the management of Botryosphaeriaceae in vineyards, which is currently limited to remedial surgery of infected wood and the protection of pruning wounds. In contrast to many other bunch rot pathogens, Botryosphaer-

Table 2

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

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

*Isolates identified to genus level only.

Table 3

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

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

*Isolates identified to genus level only.

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

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