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Identification, potential inoculum sources and pathogenicity of botryosphaeriaceous species associated with grapevine dieback disease in New Zealand

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Abstract This study investigated the prevalence and identity of botryosphaeriaceous dieback pathogens in necrotic grapevines tissues in New Zealand vineyards, and other woody hosts growing nearby. The presumptive identities of the isolates by conidial and cultural morphology were confirmed with ITS sequence data as *Neofusicoccum australe*, *N. luteum*, *N. parvum* and *Diplodia seriata.* They were isolated predominantly from necrotic stems of grapevine and other hosts, but also from leaves, flowers and wood debris of grapevines. Inoculation with conidia and mycelium of multiple isolates of each species onto excised and attached green shoots and trunks of five grapevine varieties, Cabernet sauvignon, Chardonnay, Pinot noir, Riesling, and Sauvignon blanc, showed that all varieties became infected to a similar extent. All species except *D. seriata* were pathogenic, irrespective of the host source, with *N. luteum* being the most and *D. mutila* the least pathogenic ($P < 0.05$). On trunks, *N. parvum* caused cankers and the other pathogenic species caused die-back when the inoculated vines became winter-dormant. Conidia were produced from green shoot lesions and die-back wood, which indicates potential inoculum sources for vineyard infection.

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Introduction

The botryosphaeriaceous fungi have only recently been recognised as significant pathogens of grapevines worldwide. In earlier studies their presence in woody grapevine tissue was often overlooked because of their reputation as saprophytic or endophytic organisms (Castillo-Pando et al. 2001; Phillips 2002). However, they have now been shown to cause dieback of shoots, spurs and arms, severe internal wood necrosis (Larignon et al. 2001; van Niekerk et al. 2004), canker and general loss of vigour, termed 'grapevine decline syndrome' (Denman et al. 2000; Castillo-Pando et al. 2001; Taylor et al. 2005). They have also been associated with stunted growth, bud necrosis and mortality, delayed budburst, bleached canes, incomplete graft unions, and bunch rot (Phillips 2000; Castillo-Pando et al. 2001; van Niekerk et al. 2004; Taylor et al. 2005). However, the species of these pathogens reported from grapevines show some differences between countries (Phillips 2002; Taylor et al. 2005) but the reports have not always contained detailed morphological descriptions of the recorded species so comparisons have been difficult to make (Slippers et al. 2004).

The identification of the botryosphaeriaceous species by conventional means using morphological characters

has been reported to be difficult. The teleomorphs of most species have not been found in nature, and few have been produced in culture (Denman et al. 2000; Crous et al. 2006). Their identification has therefore relied heavily on the morphological characters of conidia; however, production of pycnidia and conidia in culture is unreliable in many botryosphaeriaceous species. The sole use of conidia for identification is also problematic because there is variation in the conidial characters of any one species and considerable similarity between conidia of some different species (van Niekerk et al. 2004). For example, the conidia of *N. ribis* and *N. parvum* are morphologically similar, as are those of *N. vitifusiforme*, *N. australe* and *N. luteum* (Slippers et al. 2004; Crous et al. 2006). Furthermore, colony characteristics of these fungi can be strongly influenced by the substrate on which they are grown (Sutton 1990) and may differ with temperature (Pennycook and Samuels 1985; Slippers et al. 2004). These difficulties have led to confusion and the potential for errors, and have contributed to the taxonomy of the botryosphaeriaceous genera and species being confused for a long time (Slippers et al. 2004).

While some members of the *Botryosphaeriaceae* are difficult to identify using morphology, some of the anamorph genera can be distinguished by colony morphology or characters of conidial morphology such as size, colour, septation and surface texture. However, it is increasingly accepted that molecular sequence analysis of the internally transcribed spacers (ITS) of the rRNA gene region should be used to confirm or clarify the taxonomy of the botryosphaeriaceous species (Denman et al. 2000; Alves et al. 2004). Based on a combination of morphological and molecular characteristics, Denman et al. (2000) and Alves et al. (2004) recognised two anamorph genera. These genera are *Diplodia* (dark, mostly >10 μm wide, thick-walled conidia) and *Neofusicoccum* (hyaline, mostly <10 μm wide, thin-walled conidia). Phylogenetic re-evaluation of the botryosphaeriaceous anamorphs has also confirmed that they represent two separate groups, those with dark conidia and *Diplodia*-like anamorphs and those with hyaline conidia with *Neofusicoccum*-like anamorphs (Denman et al. 2000). Slippers et al. (2004) showed that a combined analysis of sequences from different gene regions (i.e., ß-tubulin, translation elongation factor 1-α [EF1- α] and the rRNA ITS1, 5.8S, and ITS2 regions), can effectively differentiate closely related species such as in the *N. ribis* and *N. parvum* group, and the *N. luteum* and *N. australe* group. The genus *Dothiorella*, which differs from *Diplodia* by having conidia that are brown and 1-septate early in their development while they are still attached to the conidiogenous cells, was also re-introduced as a distinct botryosphaeriaceous anamorph (Phillips et al. 2005). The anamorph species that occur on grapevines are currently placed in the genera *Diplodia*, *Dothiorella*, *Fusicoccum*, *Neofusicoccum*, and *Lasiodiplodia* (Denman et al. 2000; Phillips et al. 2005; Crous et al. 2006).

Most pathogenicity studies with these species have used mycelium colonised agar plugs for inoculum (Taylor et al. 2005), probably because of the difficulties associated with producing sufficient conidia in culture. However, infection and spread of these species is likely to be *via* conidia or ascospores, so the research results from mycelial inoculations may not be completely relevant to the natural situation. More recently a reliable method was developed for producing copious amounts of conidia (Amponsah et al. 2008), which will now allow for relevant investigations into the disease development processes in vineyards.

The aims of this study were to: (1) determine the prevalence of the botryosphaeriaceous species in woody hosts in and around New Zealand vineyards; (2) identify those species associated with dieback symptoms, by morphological characteristics and molecular techniques; and (3) to determine their pathogenicity, using conidia and mycelium plugs as inoculum.

Materials and methods

Vineyard sampling

Samples were collected during summer from 20 New Zealand vineyards, in Canterbury (6), Marlborough (6), Nelson (4) and Auckland (4). In each vineyard, woody vine debris was collected and 10 grapevines that exhibited characteristic symptoms (e.g., stunted growth, trunk, shoot or cane dieback, and bleached canes) were selected for tissue isolation. Samples of necrotic woody tissues, weak buds, shrivelled flowers and leaves with necrotic spots were collected from these vines. Other woody hosts growing around the vineyards were also inspected for symptoms of dieback and necrotic wood lesions, and when found

samples were collected. In addition, necrotic tissues on stumps and cordons of 10 nursery rootstock mother vines, were sampled from a grapevine propagation nursery in Gisborne.

Fungal isolations

Collected plant samples were washed under tap water and surface sterilized by immersion in 70% ethanol for 30 s, followed by immersion in 1% sodium hypochlorite (NaOCl) for 1 min, and 70% ethanol for another 30 s (van Niekerk et al. 2004) before being rinsed twice in sterile tap water and dried under sterile air in a laminar flow hood (Airpure™ Westinghouse Pty Ltd Inc. NSW). From each of these samples, 10 pieces (3–5 mm^2) of tissue were aseptically excised from the edges of necrosis, placed cut surface down onto half strength potato dextrose agar plates (PDA; Difco™ Becton, Dickinson and Company, Maryland, USA) amended with 50 μg ml^{-1} chloramphenicol (Sigma-Aldrich™ Co. St. Louis, MO USA; ½ PDA-Cph), which were incubated at room temperature (18–24°C) for 3 to 4 d. Any fungal colonies emerging from plant pieces that were characteristic of botryosphaeriaceous species as described on the *Botryosphaeria* website (http://www.crem.fct.unl.pt/botryosphaeria_site/) were subcultured onto PDA plates and incubated at 25°C in the dark, with observation at 3, 7 and 30 d.

Morphological characterization of fungi by colony and conidial characteristics

The cultures were allocated to groups according to colony colour, growth rate and landscape at 3 d and the production of pycnidia after 30 d. Plates containing pycnidia were flooded with 10 ml sterile distilled water (SDW) containing 0.01% of polyoxyethylene (20) sorbitan mono-oleate (Tween 80; BDH Chemicals Ltd, Poole England), and the pycnidia dislodged from the agar with a sterile scalpel, causing them to float in the water. The pycnidia and wash water were placed into a sterile mortar, where the pycnidia were crushed with a pestle to release conidia. The conidial suspension was centrifuged for 5 min at 2,000 × *g*, some of the supernatant discarded and the remaining 4–5 ml vortexed briefly to resuspend the conidia. Isolates that did not sporulate on PDA, were inoculated onto grapevine green shoots as described below and the subsequent lesions induced to form pycnidia and conidia by placing them under high relative humidity as described by Amponsah et al. (2008). The exuded conidia were washed from the lesions in 10 ml SDW containing 0.01% Tween 80 and the suspensions were centrifuged and vortexed as above. The conidia of each isolate were examined by light microscope at 100× magnification for characteristic shape, colour, and other distinguishing features. Digital images were also used to measure 50 conidia of each isolate using AnalySIS® imaging software, which calculated mean measurements, including mean length to width (L/W) ratios. These features were used to further modify the groups assigned by colony characteristics, using all characteristics in the descriptions of the species published on the *Botryosphaeria* website and by Taylor et al. (2005), and with the assistance of Dr A. J. L. Phillips (Centro de Recursos Microbiológicos, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829–516 Caparica, Portugal).

Confirmation of identifications using ITS sequence analysis

For confirmation of the presumptive morphological identifications, 7, 6, 3 and 4 isolates, from Groups 1, 2, 3 and 4, respectively, a total of 20 isolates were randomly selected for verification using molecular techniques. The numbers of isolates selected per group was related to the total number of isolates assigned to each group, according to their colony and conidium characteristics. The isolates were subcultured on 2% water agar and after 5 d growth at 25°C in continuous darkness a tuft of mycelium was plucked from the growing edge of each culture for inoculation into 20 ml potato dextrose broth (PDB; Difco™ Becton, Dickinson and Company, Maryland, USA). After 7 d incubation at 25°C in continuous darkness, the mycelia were harvested, pressed between two layers of sterile Calbiochem Miracloth (Merck, Darmstadt, Germany) to remove the broth, wrapped in aluminium foil and snap frozen in liquid nitrogen before storage at −80°C. The frozen mycelium was finely ground in liquid nitrogen with a heat-sterilised mortar and pestle and approximately 100 mg of it was used for genomic DNA extraction using a Puregene genomic isolation kit (Gentra systems, Minneapolis, MN). The extracted DNA was quantified using spectrophotometry (NanoDrop Technologies Inc., Delaware, USA) and diluted to a concentration of 10–20 ng μl^{-1}.

PCR was carried out using a Bio-Rad iCycler Thermal cycler (Hercules, California, USA) and each reaction contained 1 × buffer (40 mM Tris HCl pH 8.5, 2 mM $MgCl_2$ and 25 mM KCl), 200 μM of each dNTP, 5 μM of each primer, 1.25 U of Hotstart Taq polymerase (MBI Fermentas, Vilnius, Lithuania) and 20 ng of template DNA in a total volume of 25 μl per isolate sample. The ITS1, 5.8S and ITS2 regions of the ribosomal RNA gene region were amplified using universal primers ITS1 and ITS4 (White et al. 1990) (Invitrogen Technologies, New Zealand) as follows: an initial 2 min hot start at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 68°C, with a final elongation period of 7 min at 68°C. The resulting PCR products were sequenced by the Lincoln University DNA Sequencing Facility using an ABI PRISM® 310 Genetic Analyzer (PE Applied Biosystems, Foster City, California).

All DNA sequences were analysed using Sequencher™ (Gene Codes Corporation, Michigan, USA) and compared with those of known origin using the Basic Local Alignment Search Tool (BLAST) and the GenBank database (http://www.ncbi.nlm.nih.gov). These known sequences, together with those determined in this study were subjected to distance-based phylogenetic analyses in MEGA 4.0.2 [Molecular Evolutionary Genetics Analysis; Tamura, et al. (2007)]. A neighbour joining tree was inferred and branch support was estimated using a bootstrap analysis based on 1,000 replicates.

Pathogenicity studies on green shoots

Excised green shoots In this study, the excised green shoots of Pinot noir were used to investigate the pathogenicity of the five common botryosphaeriaceous species found on grapevines in New Zealand. These included three isolates each of *N. australe*, *N. luteum*, *N. parvum*, *D. seriata* and *D. mutila*, and 11 isolates from non-grapevine hosts (Tables 1 and 2). Since there was only one isolate of *Dothoriella* sp. it was not included in the pathogenicity test. The soft green shoots (20–25 cm long) were cut in early summer from Pinot noir vines in the Lincoln University vineyard from which botryosphaeriaceous species where not detected following repeated sampling and isolation.

Table 1 Mean lesion lengths caused by the botryosphaeriaceous species isolated from grapevines or non-grapevine hosts, 10 d after inoculation with mycelium-colonised agar plugs onto wounded detached green grapevine shoots of Pinot noir

Grapevine isolates			Non-grapevine isolates			
Species name	Isolate	Lesion lengths (mm)	Species name	Isolate	Source host	Lesion lengths (mm)
N. luteum	N(12)2	78.6 a[a]	*N. luteum*	MJ3	Blueberry	69.2 g
N. luteum	G(s)-1	69.5 b	*N. australe*	J-3	Broom	65.1 g
N. luteum	M(13)2	67.2 bc	*N. parvum*	O-1	Willow	43.4 i
N. australe	K(18)1	68.4 bc	*N. parvum*	O-3-1	Cherry	43.1 ij
N. australe	Kat-1	67.9 bc	*D. mutila*	Iso-2	Native ngaio	58.6 h
N. australe	Mel-2	63.9 bcd	*D. mutila*	J-4	Oak	51.1 h
N. parvum	I(15)3	61.6 cd	*D. mutila*	C-4	Plum	44.0 i
N. parvum	Q-2	59.3 d	*D. mutila*	A-3	Apple	43.2 i
N. parvum	I(15)2	59.0 d	*D. mutila*	F-1	Pine	42.9 j
D. mutila	M(30)3	57.8 d	*D. mutila*	A-2	Olive	37.4 j
D. mutila	Q	31.9 e	*D. seriata*	I-1	Lemon wood	5.2 k
D. mutila	F(12)2	27.0 e				
D. seriata	L-1	5.9 f				
D. seriata	L(17)4	5.6 f				
D. seriata	L(16)2	5.2 f				
LSD ($P \leq 0.05$)		6.80				5.77

[a] Values within columns followed by the same letter do not differ significantly at $P \leq 0.05$ according to Fisher's protected LSD. *Controls inoculated with sterile agar had no lesions and were excluded from ANOVA

Table 2 Mean lesion lengths caused by isolates of four botryosphaeriaceous species 10 d after inoculation with mycelium colonised agar plugs onto wounded detached green shoots of five grapevine varieties

Species	Isolates	Lesion lengths (mm) on grapevine varieties					
		Cabernet sauvignon	Chardonnay	Pinot noir	Riesling	Sauvignon blanc	Isolate means (mm)
N. luteum	M (13)2	28.3	25.9	34.9	22.1	25.9	27.4 a[a]
N. australe	Kat-1	25.7	23.5	27.4	21.9	24.8	24.7 a
N. parvum	I (15)2	18.9	20.3	16.3	17.6	21.5	18.9 b
D. mutila	F (12)2	14.8	11.9	14.2	13.4	16.0	14.0 c
Variety means (mm)		21.9	20.4	23.1	18.7	22.1	

[a] Values within columns followed by the same letters are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. The main effects of isolates was significant ($P<0.001$, LSD=3.24), but not significant for grapevine varieties ($P=0.141$) or a cultivar and isolate interaction ($P=0.378$). Controls inoculated with sterile agar had no lesions and were excluded from ANOVA

The shoots were immediately placed into Universal bottles filled with sterile water and the top of each bottle was wrapped with Parafilm® to support the shoot. In the centre of each shoot a superficial wound (~4 mm in diameter) was made using a sterilised scalpel, and it was inoculated with a 3 mm disc of mycelium cut from the growing edge of a 3 day old PDA culture or sterile agar (control). For non-wounded shoots, the mycelium discs were placed directly onto the centre of each shoot. The six replicates of each treatment were arranged in a completely randomised design in a transparent humid chamber.

Lesion lengths were measured 10 d later with a digital calliper (Mitutoyo, Kanagawa, Japan) and then these sections were air-dried for 6 h in a laminar flow cabinet and moist -incubated for 36 h to induce production of pycnidia and conidia as described in Amponsah et al. (2008). To confirm Koch's postulates, isolations were made from infected tissues and the isolates' identities confirmed by colony and conidial characteristics as described above. Two identical experiments were conducted for the grapevine and non-grapevine isolates.

Attached green shoots The susceptibility of attached green shoots growing on 18 month old potted Pinot noir vines to infection by conidia of *N. australe*, *N. luteum*, *N. parvum* and *D. mutila*, were also tested. The potted vines were grown from mature 40 cm canes cut from dormant vines in the Lincoln University vineyard. The canes were induced to form roots by placing the bases in pumice granules set on a heat pad at 25°C for 5–6 weeks and then grown in potting mix (80% composted bark, 20% pumice and 2 kg/m^3 Osmocote® Exact® Standard) in a shade house. Plants were grown for 18 months, during which time each pot was top-dressed with 10 g of Triabon® (BASF New Zealand Ltd). The conidia of three isolates per species, using the three same isolates as before (Table 2), were produced from lesions on infected green shoots which were washed in sterile water with 0.01% Tween 80 and used to make a mixed isolate conidium suspension (10^5 ml^{-1}) for each botryosphaeriaceous species. In early summer, one green shoot per plant (~6–8 mm diameter) was wounded at 2 cm from the base by cutting out a core of the tissue (1–2 mm deep and ~2.5 mm wide) with the tip of a sterile scalpel. Each hole was immediately inoculated with a 50 µl drop of a conidium suspension (10^5 ml^{-1}) or sterile water (controls). Each plant was covered with a new transparent plastic bag for 24 h to prevent the immediate evaporation of the conidium suspensions. The 35 replicate plants per treatment were arranged in a completely randomised block design (CRBD) in an open area similar to field conditions and left to grow. At 10, 20, 30, 40, 50 and 60 d after inoculation, five replicate plants of each treatment were assessed for lesion development. At each assessment time, the shoots were sliced through lengthwise and the internal lesions visually observed, except that at 60 d lesion lengths were measured using a digital calliper. Pathogen isolations were made from the lesion edges of the final five replicate plants as before.

Varietal susceptibility

Excised green shoots Five scion varieties (Cabernet sauvignon, Chardonnay, Pinot noir, Riesling, and Sauvignon blanc) and their excised green shoots,

were evaluated for their susceptibility to infection by one isolate each of *N. australe* (Kat-1), *N. luteum* [M (13)2], *N. parvum* [I (15)2] and *D. mutila* [F (12)2] as described above, using the same design, incubation conditions and assessment.

Trunks For the same five varieties, plant trunks were also tested for susceptibility on wounded or non-wounded tissues using the same three isolates of *N. australe*, *N. luteum*, *N. parvum* and *D. mutila* as above (Table 2). The 18 month-old potted vines were grown from dormant canes cut from the appropriate vines in the Lincoln University vineyard as before. In early summer, the trunks of the 18 months old potted grapevines were wounded by drilling a hole in the centre of each vine trunk, 2–3 mm deep and 3.5 mm wide, which was immediately inoculated. For each species, the inoculum applied consisted of either 100 μl of a mixed isolate conidium suspensions (10^5 ml^{-1}), made as above, or a mashed mixture of the isolates' 3 day-old PDA colonies, using sufficient to fill the wound. For non-wounded treatments, the mycelium mash and conidia were placed directly onto the trunk tissue and control plants were inoculated with sterile water or sterile agar, respectively. The inoculated areas were wrapped with Parafilm® for 3 d. The 10 replicates of each treatment combination were arranged in a completely randomised block design and grown in an open area that simulated field conditions.

In late summer, at 4 months after inoculation, five plants per treatment and variety were randomly selected and assessed for lesion development by scraping off the bark and measuring the lesion lengths with a digital calliper. However, lesions were indistinct and so isolation was carried out on the surface-sterilised vines by cutting slices across each trunk at 10 mm intervals above and below the inoculation point, and placing the tissue slices onto PDA for incubation as before. The mean overall distance moved by each species was used as a measure of its pathogenicity. The five remaining replicate plants for each treatment combination were left to grow until they became completely dormant in winter, after which each plant was pruned to two canes and 4–5 buds per cane. After a further 6 weeks, dormancy was broken by putting plants into a greenhouse (12–20°C) under high pressure sodium lights (400 W SON-T-AGRO, Phillips, Belgium) which were turned on from 4 am to 12 pm and 4 pm to 8 pm each day. After 3–4 weeks, plants were assessed for bud development, shoot growth and dieback.

Statistical analysis

Lesion length data and pathogen isolation distances were subjected to analyses of variance using GenStat 11 (Lawes Agricultural Trust, Rothamsted Experimental Station) to determine the significance of treatment effects, and means were separated using Fisher's protected LSD at $P \leq 0.05$. Since non-wounded and control treatments caused no lesions and provided no isolations of botryosphaeriaceous fungi, these data were excluded from analyses.

Results

Vineyard sampling

Botryosphaeriaceous fungi were found to be associated with cane, trunk and shoot dieback, growth decline, and weak buds in 90% of vineyards from which samples were collected, but in shrivelled or dead flowers and grapevine leaf spots from only 5% of vineyards (Fig. 1a and b). Cross sections of symptomatic and infected grapevine trunks showed wedge-shaped lesions and internal trunk necrosis (Fig. 1c and d). Dieback and internal necrotic lesions were almost always associated with old pruning wounds and/or obvious wounds caused by mechanical damage. The botryosphaeriaceous isolates from non-grapevine woody plants, such as olive trees, broom and pine trees were associated with trunk or stem cankers, and dieback of trunks and stems with pruning cuts.

Fungal isolation and morphological identification

Isolations from the margins of the dead and healthy tissues examined resulted in the recovery of 63 isolates from grapevine and 14 isolates from other woody hosts. Overall, six botryosphaeriaceous fungi were identified by their culture and conidium morphology. These fungi were recovered from grapevines 2 to 33 old, of varieties commonly grown in New Zealand, including scions Chardonnay, Reisling, Sauvignon blanc, Pinot noir and Cabernet sauvignon,

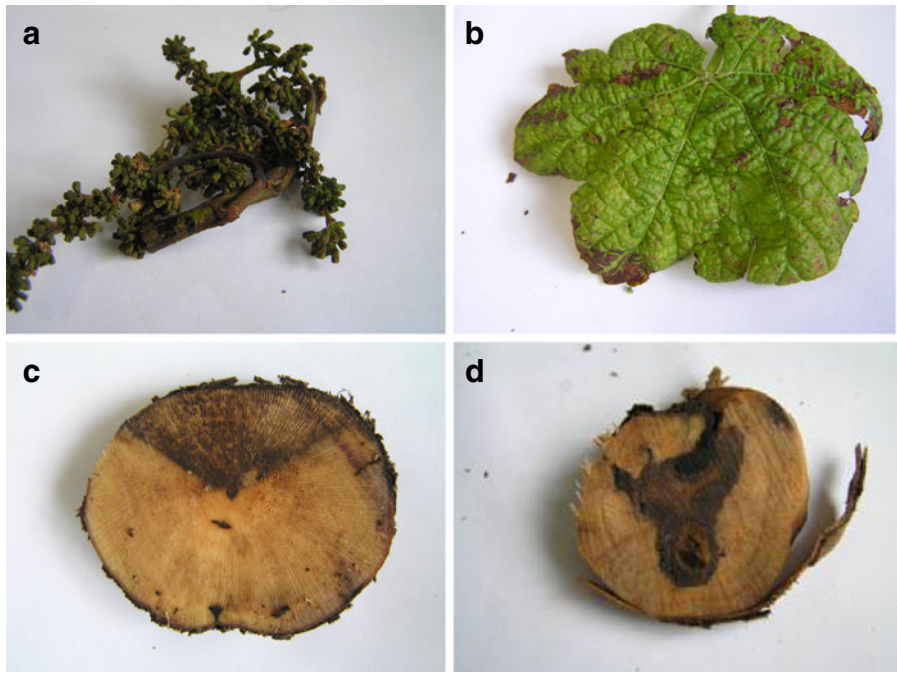

Fig. 1 Necrotic tissues from which botryosphaeriaceous species were isolated. (**a**) Necrotic flower (**b**) Leaf necrosis (**c**) Wedge-shaped trunk necrosis and (**d**) Internal necrosis in trunk

and rootstocks SO4, 101–14, 3309, Schwarzmann and Richter. The greatest prevalence of these species were in trunks (42%), followed by canes (17%), green shoots (19%), plant debris (8%), weak buds (8%), leaf spots (3%) and shrivelled flowers (3%). Multiple botryosphaeriaceous species were isolated from most vineyards and in all the major grapevine growing regions (data not shown).

Morphological characteristics of the isolates

The characteristics of the isolate groups that allowed for their presumptive identification are described below, with 3 d-old colonies on PDA shown in Fig. 2 and conidia shown in Fig. 3.

Group 1 colonies were deep yellow after 3 d, especially on the undersides, with appressed mycelium in the centre and dense, fluffy, white mycelium at the colony margins. Colony undersides became violaceous by 7 d and black by 30 d. The few conidia produced in pycnidia were hyaline, thin walled, and aseptate (becoming septate when germinating), fusiform and with subtruncate bases. Conidial size ranges were 18.9–26.0×4.0–8.1 μm, with a mean L/W ratio of 3.6 (Fig. 3). The colony and conidium characteristics were typical of *Neofusicoccum luteum* (Taylor et al. 2005).

Group 2 colonies were yellow on the undersides by 3 d with dense, fluffy, white mycelium, being more pronounced at the colony margin. Colony undersides became brown by 7 d, with appressed mycelium in the centres, and black after 30 d. No pycnidia were produced on PDA but they were produced on green shoots. The conidia were hyaline, thin walled, and aseptate (becoming septate when germinating), fusiform with subtruncated bases. Conidial size ranges were 22.1–32.0×5.0–8.5 μm, with a mean L/W ratio of 3.9. The colony and conidium characteristics were typical of *Neofusicoccum australe* (Slippers et al. 2004).

Group 3 colonies were characterized by white fluffy mycelium, and became slightly yellow or brownish on the undersides by 3 d. By 7 d, the centres became light brown and appressed with fluffy white mycelium at the margins, and by 30 d the entire colony was dark grey/brown. No pycnidia were produced. Conidia produced on green shoots were initially hyaline and aseptate, becoming light brown and 1-or 2-septate with age. They were ellipsoidal with round apices and truncate bases and 13.2–20.9×3.9–7.3 μm, with a mean L/W ratio of 3.1. The colony and conidium characteristics were typical of *N. parvum*, as described on the Botryosphaeria website: http://www.crem.fct.unl.pt/botryosphaeria_site/ and confirmed by Alan Phillips (pers. comm. 2006).

Group 4 colonies grew more slowly than colonies of Groups 1, 2, 3 and 5, were white by 3 d, with short smooth aerial mycelium and smoother, more distinct

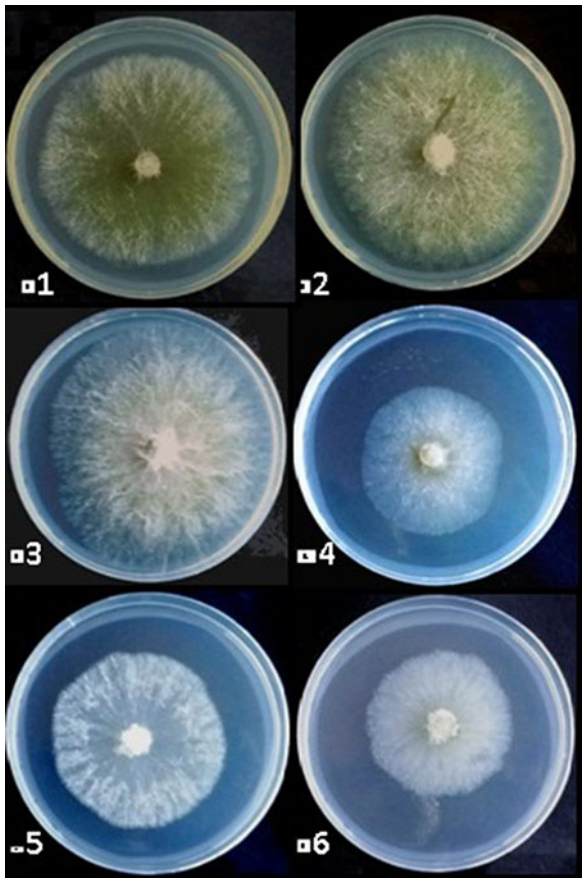

Fig. 2 Culture morphology of (1) *N. luteum,* (2) *N. australe,* (3) *N. parvum* (4) *D. mutila,* (5) *D. seriata,* and (6) *Dothiorella* sp. isolates on PDA after 3 d incubation at 25°C in continuous darkness

margins than Groups 5 and 6 colonies. By 7 d, the colonies were grey to dark grey, with the colour change starting from the centre, and by 30 d colonies were black with many pycnidia. The conidia were hyaline and aseptate with thick walls, occasionally becoming pale brown and 1-septate, with broadly rounded apices and bases. Conidial size ranges were 20.5–28.4×12.3–16.1 μm with a mean L/W ratio of 1.8. The conidium and colony characteristics were typical of *Diplodia mutila* as described on the Botryosphaeria website and confirmed by Alan Phillips (pers. comm. 2006).

Group 5 colonies were characterized by white, fluffy mycelium, being similar to Group 4 colonies except that they were less fluffy and had undulating colony edges by 3 d. By 7 d, colonies were greyish brown with little to no aerial mycelium, and became black with no aerial mycelium by 30 d. Numerous pycnidia, which had white tufts of mycelium emerging from them, were visible after 30 d. Pycnidia oozed conidia that were usually hyaline and aseptate but occasionally turned light brown and 1-septate. The conidia were ovoid, with obtuse apices and truncated or rounded bases, and their size ranges were 21.2–26.2×10.5–13.4 μm, with a mean L/W ratio of 2.1. The colony and conidium characteristics were typical of *D. seriata* as described on the Botryosphaeria website and confirmed by Alan Phillips (pers. comm. 2006).

Group 6 comprised only one isolate. Its white colonies after 3 d turned grey by 7 d and dark grey to black by 30 d. By 30 d, there was little or no aerial mycelium and a few pycnidia. The conidia were ovoid and hyaline when young, becoming dark brown with rounded, apices and truncate bases, and 1-septate by 30 d, while they were still attached to the conidiogenous cells. The size ranges of the conidia were 18.2.–28.1×6.6–11.7 μm, with a mean L/W ratio of 2.5. The colony and conidium characteristics were typical of *Dothiorella* species, as described on the Botryosphaeria website.

Confirmation of identifications using ITS sequence analysis

Molecular identification confirmed Group 1 isolates as being *N. luteum*, Group 2 as *N. australe*, Group 3 as *N. parvum* and Group 4 as *D. mutila*. BLAST searches showed that DNA sequences of the New Zealand botryosphaeriaceous isolates had 99 to 100% homology with the same species previously identified and deposited in GenBank. Groups 5 and 6 were identified as *D. seriata* and *Dothiorella* sp., respectively, based solely on conidial characteristics. *Neofusicoccum parvum*, *N. luteum* and *D. mutila* were the most common on grapevines (27, 17 and 14%, respectively) and non-grapevine woody hosts (3, 6 and 10%, respectively), followed by *N. australe* (10 and 2% on grapevines and non-grapevines, respectively) and *D. seriata* (8 and 2%, on grapevines and non-grapevines, respectively). The identification of these species was confirmed by the phylogenetic analysis (Fig. 4).

Pathogenicity

Excised shoots No lesions were produced on the inoculated non-wounded green shoots. All botryos-

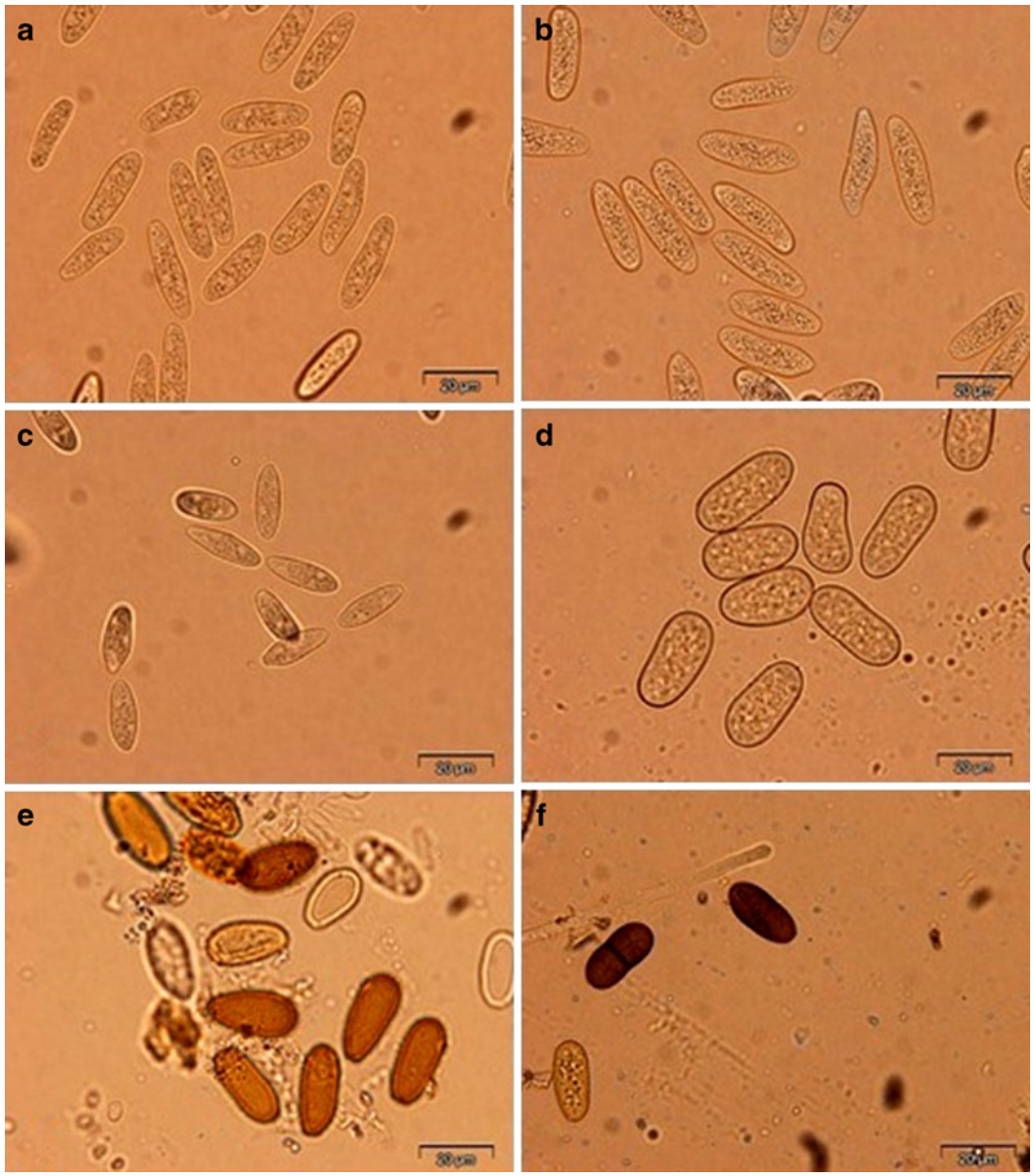

Fig. 3 Conidium characteristics of (**a**) *N. luteum,* (**b**) *N. australe,* (**c**) *N. parvum* (**d**) *D. mutila,* (**e**) *D. seriata,* and (**f**) *Dothiorella* sp. isolates. Bars show 50 μm

phaeriaceous species from grapevines produced brown to dark brown lesions on wounded green shoots, which extended upward and downward from the point of inoculation (Fig. 5a). There were significant differences ($P<0.001$) in the lesion lengths produced by the different Botryosphaeriaceae isolates, but lengths were generally similar for isolates of the same species, except for *D. mutila* which caused long lesions (57.8 mm) with isolate M (30)3 and significantly shorter ($P\leq0.05$) lesions with isolates Q (31.9 mm) and F(12)2 (27.0 mm). The longest lesions were produced by *N. luteum* isolate N(12)2 (78.6 mm) followed by the other two isolates of *N. luteum*, all *N. australe* isolates, *N. parvum* isolates and the less pathogenic *D. mutila* isolates. *Diplodia seriata* isolates produced very small lesions that were similar to the controls (Table 1).

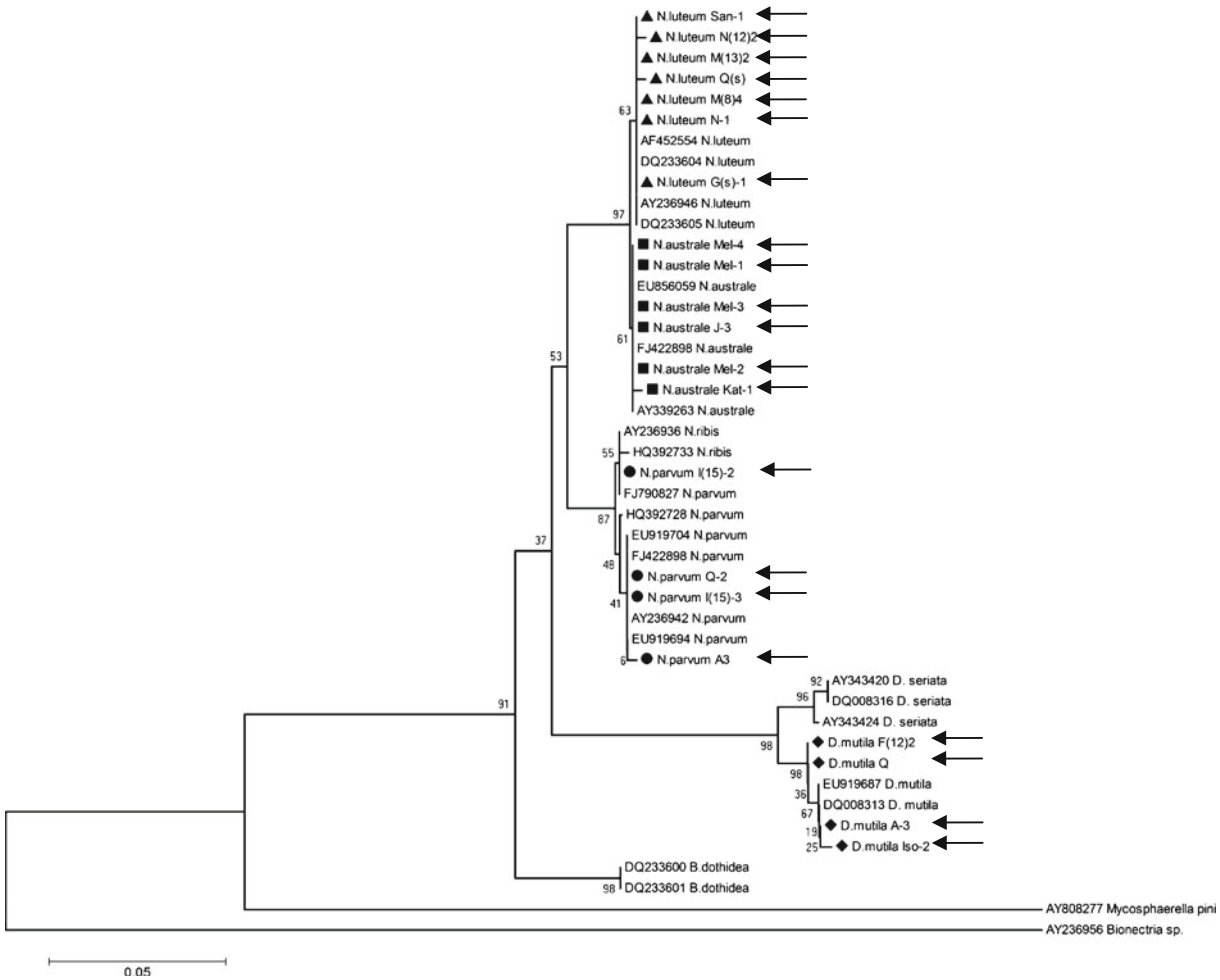

Fig. 4 The neighbour-joining tree with bootstrap value using 1,000 replicates generated in MEGA 4.0.2 using internal transcribed spacer sequences of the ribosomal RNA gene region of the botryosphaeriaceous species from the GenBank database and those isolated from New Zealand vineyards shown by arrows: *N. luteum* (*black triangle*), *N. australe* (*black square*), *N. parvum* (*black circle*) and *D. mutila* (*black diamond*)

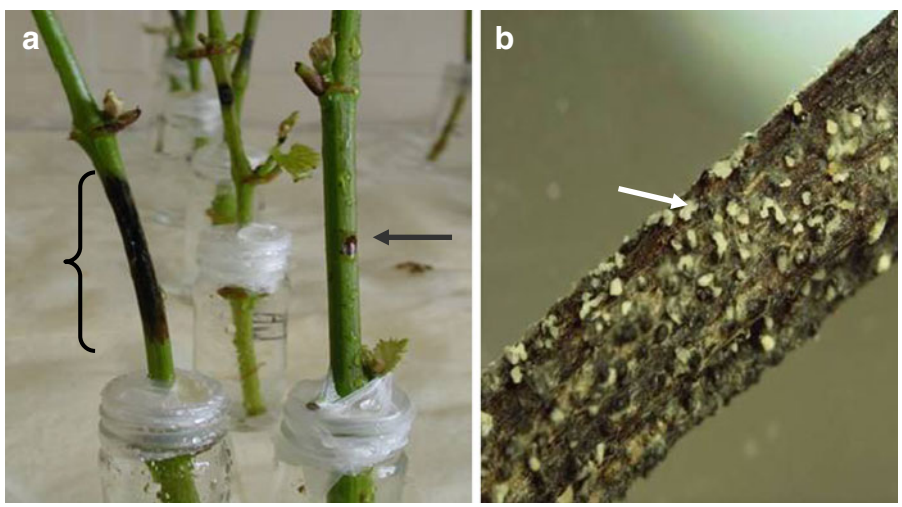

Fig. 5 Development of lesions on green shoots after wound inoculation by botryosphaeriaceous species. (**a**) lesion shown by bracket and no lesion at agar inoculation point on control shoot shown by arrow. (**b**) conidium tendril oozing from pycnidia of a lesion (*arrow*) after incubation under high relative humidity

Fig. 6 Typical internal lesions that developed 60 d after inoculating the green shoots of 1 year old Pinot noir potted grapevines with conidia of (**a**) *N. luteum*, (**b**) *N. parvum*, (**c**) *N. australe*, and (**d**) *D. mutila* that were left to grow in an open area similar to field conditions

Isolates from non-grapevine hosts also produced no lesions on the inoculated non-wounded green shoots. The lengths of lesions on wounded green shoots differed between botryosphaeriaceous isolates ($P<0.001$; Table 1), generally following the species trends shown by the grapevine isolates and again varying considerably between *D. mutila* isolates. The longest lesions were produced by *N. luteum* isolate MJ3 and *N. australe* isolate J-3 and the shortest by the single *D. seriata* isolate which was similar to the control. The lesions caused by all botryosphaeriaceous isolates in both experiments produced pycnidia that oozed conidia (Fig. 5b), and Koch's postulates were confirmed for all isolates.

Attached green shoots At 10 d after inoculation with conidial suspensions, the attached green shoots showed internal lesions, although the lesion lengths were much smaller than the lesions which developed over the same period on detached shoots inoculated with mycelium. However by 60 d after inoculations, lengths differed significantly between inoculating species ($P<0.001$). The mean lesion lengths were 73.2, 57.9, 56.5 and 40.3 mm for *N. luteum*, *N. australe*, *N. parvum* and *D. mutila,* respectively, which differed between species ($P\leq0.05$) except for *N. australe* and *N. parvum* which were similar ($P>0.05$). The lesion colours differed for the species, being light brown for *N. luteum* and dark brown or black for *N. parvum, N. australe* and *D. mutila* (Fig. 6). Fungal colonies characteristic of the inoculated isolates were recovered from the lesion edges of all the wounded inoculated shoots but not from the control shoots.

Varietal susceptibility

Excised green shoots The excised green shoots of the five grapevine varieties tested were susceptible to infection by the four botryosphaeriaceous species tested. Mean lesion lengths differed significantly ($P<0.001$)

Table 3 Mean distances (mm) of recovery of four botryosphaeriaceous species, from above and below trunk inoculation points on 18-month-old potted grapevines of five varieties, 4 months after inoculating them with mycelium (M) or conidia (C)

Species		Isolation distances (mm) on grapevine varieties					
		Cabernet sauvignon	Chardonnay	Pinot noir	Riesling	Sauvignon blanc	Inoculum means (mm)
N. luteum	*(C)*	98	102	106	102	102	102.0 c[a]
	(M)	118	122	126	122	120	121.6 a
N. parvum	*(C)*	84	90	88	88	90	88.0 d
	(M)	110	110	108	108	110	109.2 b
N. australe	*(C)*	86	86	90	84	86	86.4 d
	(M)	106	106	114	104	106	107.2 b
D. mutila	*(C)*	54	54	52	54	60	54.8 f
	(M)	74	76	74	76	80	76.0 e
Variety means		91.3	93.3	94.8	93.2	94.3	

[a] Values within a column followed by the same letters are not significantly different according to Fisher's protected LSD at $P\leq0.05$ (LSD =2.50). Main effects of species and inoculum type were significant ($P<0.001$), but there were no significant effects for grapevine varieties ($P=0.62$) nor for any interactions between variety, pathogen species or inoculum type ($P>0.05$). Controls were not inoculated and were excluded from ANOVA

between the species (Table 3), also reflecting the species trend shown in Table 2. However mean lengths of lesions were not significantly affected by grapevine varieties ($P = 0.141$) or a cultivar and isolate interaction ($P = 0.378$) (Table 2). Fungal colonies characteristic of the inoculating species were recovered from the lesion edges of all the inoculated excised shoots but not the control shoots.

Trunks No visible symptoms were observed on the trunks of the five grapevine varieties 4 months after inoculation with any of *N. luteum N. australe, N. parvum* or *D. seriata*. However, isolation distances, which indicated pathogen movement differed between inoculum types ($P < 0.001$), all species having moved further with mycelial than conidial inoculum (Table 3). The isolation distances differed for the four pathogenic botryosphaeriaceous species ($P < 0.001$) and generally reflected similar species effects as on green shoots, with *N. luteum* having longest and *D. mutila* the least movement. Distances moved by the pathogens were not affected by grapevine varieties ($P = 0.62$) nor by any interactions between variety, pathogen species or inoculum type). No botryosphaeriaceous fungi were isolated from non-wounded or control trunks.

After 7 months growth at the onset of dormancy in early winter, the trunks previously inoculated with *N. parvum* were observed to have depressed elliptical necrotic cankers. When the bark was removed dark brown lesions were visible, that extended along one side of the trunk by the inoculation point and about 5–10 mm above and below the inoculation sites. Microscope examination of the lesions showed raised points like pycnidia but they produced no conidia when the wood segments were later placed under high relative humidity. Cross sections cut through the trunks of the vines revealed wedge-shaped symptoms, which were similar to some of the symptoms found in the field sampling.

At the same time, the vines inoculated with *N. luteum, N. australe* and *D. mutila* showed bud death and dieback of 44–86 mm from the shoot tips, while the dieback lesions were usually smaller for *N. parvum* (37–68 mm). Samples of the dead tip tissues observed under a stereo microscope at 400x magnification showed numerous pycnidia that oozed conidia when placed under high relative humidity for 36 h. When dormancy was broken and shoots began to develop, the dieback extension stopped. The shoots continued to develop normally for 3–4 weeks, when plants were harvested and isolations made from the symptomatic tissues. Fungal colonies characteristic of the relevant inoculated isolates were recovered from the lesion edges on all the inoculated wounded trunks. No fungi characteristic of Botryosphaeriaceae species were isolated from the control shoots.

Discussion

In this study the six Botryosphaeriaceous species isolated from grapevine and non-grapevine woody hosts were identified as *N. parvum*, *N. luteum*, *N. australe*, *D. mutila, D. seriata* and a *Dothiorella* sp. Identification of the isolates was challenging as most did not produce spore-bearing structures on PDA and their colonies initially appeared similar. However, careful observation of 3-day-old colonies showed differences between some isolates, which allowed them to be divided into morphological groups. The characteristic yellow colonies of Group 1 and 2, identified as *N. luteum* and *N. australe,* were also reported by Pennycook and Samuels (1985) and Phillips (2000). However, some isolates of Group 3 (*N. parvum*) also showed a slightly yellowish to brownish colour. Other authors also have reported variations in colony morphology between isolates of a species. For example, Slippers et al. (2004) noted that isolates of *N. luteum* from South Africa produced a much brighter yellow pigment in culture than the Australian isolates. Therefore, colony characteristics were clearly not reliable indicators of identity.

Reproductive structures of most isolates were not produced on PDA, and even when pycnidia were produced by some isolates they often did not contain enough conidia for reliable observation of shapes, sizes and colours, which is consistent with what was found by Denman et al. (2000). The novel green shoot inoculation method developed by Amponsah et al. (2008) provided sufficient conidia for description of conidium characteristics, and for the subsequent pathogenicity experiments. However as also reported by Denman et al. (2000), the overlap in conidium characteristics of *Neofusicoccum* species made them difficult to differentiate, and so DNA sequence analysis of the ITS regions of the rRNA gene region was used to discriminate among them and also to

identify the *D. mutila* isolates. Nevertheless, the careful colony descriptions initially made were considered sufficient to allow confirmation of isolates from tissues inoculated with a known species.

In New Zealand vineyards, *N. parvum* was the species found most frequently, followed by *N. luteum, D. mutila,* and *N. australe,* with the least common being *D. seriata*. More samples taken from a wider range of vineyards are needed to confirm apparent prevalence of this species. In contrast, *D. seriata* was the most frequently isolated species in France (Larignon et al. 2001), New South Wales (Castillo-Pando et al. 2001), Western Australian (Taylor et al. 2005) and California (Úrbez-Torres et al. 2006). The symptoms associated with the botryosphaeriaceous isolates were trunk cankers, cane and shoot dieback, internal trunk necrosis, shoot lesions, leaf spots, shrivelled flowers, poor bud development and bud necrosis, some of which were also reported in Portugal (Phillips 2002), Western Australia (Taylor et al. 2005), USA (Úrbez-Torres et al. 2006), South Africa (van Niekerk et al. 2004), France (Larignon and Dubos 2001) and Spain (Luque et al. 2005). In this study, botryosphaeriaceous species were isolated from grapevines between 2 and 33 years old, although most of the severe trunk symptoms were observed in older vines. Larignon and Dubos (2001) reported that in a survey of French vineyards, the 'excoriose' disease caused by botryosphaeriaceous species usually developed slowly and so severe symptoms became visible only in grapevines that were eight or more years old. However, the ease with which relatively young tissues of potted vines became infected and developed lesions indicated the potential for these pathogens to also cause field symptoms on younger vines and non-woody tissues, which are not commonly reported.

The isolation of five botryosphaeriaceous species from 11 non-grapevine woody hosts, agrees with reports from the United States, where Stanosz et al. (1998) isolated *N. luteum, N. parvum* and *N. australe* from avocado branches and McDonald et al. (2009) *D. mutila* from *Juniperus* species. In Victoria and New South Wales, Cunnington et al. (2007) also isolated *N. australe, D. seriata* and *N. parvum* from some horticultural plants such as *Vaccinium corymbosum, Phoenix canariensis, Olea africana,* and *Persea americana*. In New Zealand, Pennycook and Samuels (1985) isolated *N. luteum* and *N. parvum* from kiwifruit (*Actinidia deliciosa*) and apple (*Malus x domestica*) whilst Sammonds et al. (2009) isolated *N. luteum, N. parvum* and *D. seriata* from blueberries (*Vaccinium* spp.).

Inoculation with conidia from the botryosphaeriaceous fungi demonstrated that they were as pathogenic as when mycelium plugs were used for inoculations onto wounded woody trunks of five different grapevine varieties. However, the infections appeared to develop faster when conidial suspensions were used, which was true for all the botryosphaeriaceous species and the grapevine varieties. This was probably due to the time required for conidia to penetrate and develop mycelia within the internal tissues. Prusky and Plumbley (1992) also observed that the rate of conidium germination of some fungi, including botryosphaeriaceous species, has been found to be influenced by the presence of nutrients and the chemical nature of the host surfaces. The faster symptom expression with mycelial inoculations could also be due to sap nutrients released by wounding being available for absorption by the mycelium, leading to rapid growth into the plant tissue.

Conidia of *Neofusicoccum* and *Diplodia* species have been found in spore-trapping experiments carried out in vineyards in New Zealand (Amponsah et al. 2009) and in California (Úrbez-Torres et al. 2010). Although conidial ooze was not observed in the field in the current study, all species isolated from grapevines and non-grapevine hosts were able to infect green grapevine shoots, ultimately producing pycnidia and conidia from the lesions during moist conditions. The conidia were able to infect 100% of wounded green shoots and trunks, which clearly indicates their infection capacity in vineyards where shoot trimming and winter pruning would provide entry points at 3–4 times a year. Since the lesions seem to require only moderate temperatures and high relative humidity for conidium production, the limiting factors may well be the presence and suitability of wound sites for infection. However, Munkvold and Marois (1995) reported that infection incidence of grapevine wounds by *Eutypa lata* ascospores reduced with the age of the wound and during warmer days. Further research is thus required to determine the conditions that maintain wounds in a condition suitable for penetration by botryosphaeriaceous species conidia and the time scales.

All the isolates used in the pathogenicity studies on detached green shoots were pathogenic except the *D. seriata* isolates, which produced minor lesions that

did not extend beyond the inoculation point. This finding was in agreement with Taylor *et al.* (2005) who reported that although *D. seriata* was shown to be a wound pathogen, the lesions produced were small and caused very little damage to the host. In California, *D. seriata* was also found to be the least virulent of all nine botryosphaeriaceous species tested (Urbez-Torres et al. 2006). However in contrast to these findings, other authors have reported this species to be very pathogenic on several scion varieties of excised mature canes (Larignon et al. 2001; van Niekerk *et al.* 2004). Epstein et al. (2008) also reported that conidia of *D. seriata* were the most abundant species collected in rain water traps in grapevines, in the non-coastal areas of California. They demonstrated its pathogenicity in greenhouse studies, conducted by inoculating wounded stems of own-rooted Cabernet sauvignon vines with mycelium discs.

The types of symptoms that developed differed between species for excised and attached green shoots and also for woody trunks. On the excised grapevine green shoots, dark external lesions were quickly caused by *N. australe, N. luteum, N. parvum* and *D. mutila* inoculation and they spread along the shoot reaching 30–70 mm within 10 d. However, on the green shoots still growing on young vines, it took 2 months for lesions of similar size to develop. These lesions could only be observed within the shoots and their colour intensities and lengths differed with respect to the inoculated species. Lesions were dark brown for *N. parvum*, *N. australe* and *D. mutila* infected shoots, while for *N. luteum* they were light brown. In contrast, the lesions produced on the *in vitro* inoculated shoots were dark brown for all species and were visible on shoot surfaces. The greater lesion development on excised shoots was probably due to the stress of excision and supply of only water, with associated reduction in natural resistance by the plant tissue.

Symptoms that developed on the woody trunks of 2-year-old grapevines 7 months after inoculations differed between species. Inoculations with *N. australe*, *N. luteum* and *D. mutila* caused only minor external lesions at the inoculation point where wounding had occurred. However, inoculation with *N. parvum* caused external wood necrosis visible as cankers, which were usually restricted to one-half of the stem. When the plants infected by botryosphaeriaceous species were pruned after 7 months in winter and put under lights to break bud dormancy, similar lengths of dieback were observed in those plants inoculated with *N. luteum*, *N. australe* and *D. mutila*, although the dieback lengths were shorter with *N. parvum*. From this observation it seems that all species are dieback pathogens and that *N. parvum* is a canker pathogen in New Zealand. The wedge-shaped lesions visible in cross sections of the *N. parvum* trunk cankers were similar to symptoms reported in California by Úrbez-Torres and Gubler (2009) who found that *L. theobromae* was associated with wedge shaped wood staining and canker symptoms in grapevines, a disease usually referred to as "Bot canker".

In the current study, *N. luteum*, *N. australe* and *N. parvum* were the most virulent while *D. seriata* was the least virulent. Similar findings have been reported for *N. luteum*, *N. parvum* (van Niekerk et al. 2004; Úrbez-Torres and Gubler 2009; Úrbez-Torres et al. 2009) and *D. seriata* (Taylor et al. 2005; Úrbez-Torres et al. 2006). However, other research has found varying levels of virulence for *N. luteum* (van Niekerk et al. 2004), *N. parvum* (Urbez-Torres et al. 2006) and *D. seriata* (Castillo-Pando et al. 2001; Larignon et al. 2001; van Niekerk et al. 2004). In a study of phytotoxic metabolites, Martos et al. (2008) found that they were produced by five botryosphaeriaceous species, with variation among isolates that could explain their observed variability in virulence. In particular, *N. luteum* and *N. parvum* consistently produced metabolites with high phytotoxic activity and corresponding high virulence when inoculated onto grapevines, whereas *D. seriata* showed lower phytotoxicity. From this work, a relationship was established between phytotoxic activities of *N. luteum* and *N. parvum* and previously reported virulence (Martos et al. 2008). The greater virulence observed for *N. luteum* and *N. parvum* compared to *D. seriata* in the current study could thus be due to the two former species producing phytotoxic metabolites with higher activity compared to those produced by *D. seriata*. Future research should therefore address issues such as toxin production, comparisons of virulence and phylogeny to explain some of the development of the disease symptoms caused by botryosphaeriaceous isolates and species.

The field sampling, fungal identification and pathogenicity studies reported here have shown that the botryosphaeriaceous species, *N. luteum, N. parvum, N. australe*, *D. mutila* and *D. seriata,* are common in New

Zealand vineyards and also present in non-grapevine hosts. The pathogenicity studies indicated that all species except *D. seriata* were able to infect green shoots and mature woody tissues, causing cankers, vascular discolouration of internal tissues or dieback. Conidia were readily produced from the dead infected tissues, and their presence in a vineyard year-round (Amponsah et al. 2009) has clearly shown that once these pathogens have established in a vineyard, they are likely to spread and infect pruning and trimming wounds, with potential for significant losses.

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