Pathogenicity of a New Zealand grapevine isolate of *Neofusicoccum macroclavatum* on *Eucalyptus globulus*

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Abstract *Neofusicoccum macroclavatum*, a recognised pathogen of *Eucalyptus globulus* in Western Australia, was recently isolated for the first time from grapevines in New Zealand. Its identity was confirmed by analysis of ribosomal DNA (rDNA), β-tubulin gene and elongation factor α1 sequence analyses. Previous pathogenicity studies of nursery isolates showed that this isolate (M353) was pathogenic to Sauvignon blanc 1-year-old rooted canes and green shoots similar to other *Botryosphaeriaceae* pathogens of grapevines. To further investigate its pathogenicity on eucalypts, M353 was inoculated onto 1-year-old seedlings of *E. globulus* and shown to produce external stem lesions with a mean length of 29 mm that was significantly longer ($P<0.001$) than those caused by *N. australe* (17 mm), another common *Botryosphaeriaceae* pathogen of *Eucalyptus* in Australia. This research demonstrates that the *N. macroclavatum* isolated from grapevine is pathogenic to eucalypts.

Keywords *Neofusicoccum macroclavatum*, *Botryosphaeriaceae*, grapevines, *Eucalyptus globulus.*

INTRODUCTION

Botryosphaeriaceae species are cosmopolitan fungi commonly associated with cankers and dieback of woody hosts (Crous et al. 2006) and are considered to be important pathogens of grapevines worldwide (Urbez-Torres et al. 2006). Nine *Botryosphaeriaceae* species (*Neofusicoccum parvum, N. luteum, N. australe, N. ribis, Diplodia mutila, D. seriata, Botryosphaeria dothidea, Dothiorella iberica* and *Do. sarmentorum*) were reported to infect grapevines in New Zealand (Baskarathevan et al. 2012). Amponsah et al. (2011) also found some of these species infecting non-grapevine woody hosts such as shelter, fruit and ornamental species.

Botryosphaeriaceae species were shown to be prevalent in grapevine material in New Zealand grapevine propagation nurseries, being found in eight out of nine nurseries and in 5 to 63% of samples submitted (Billones-Baaijens 2011). From the 311 young grafted plants and propagation materials assessed, 23% were infected by *Botryosphaeriaceae* fungi. The 114 isolates were identified using the amplified ribosomal DNA restriction analysis (ARDRA) described by Alves et al. (2005) and Baskarathevan et al. (2012) as *N. luteum* (56%), *N. parvum* (18%), *N. australe* (8%), *B. dothidea* (6%), *D. seriata* (3%) and *D. mutila* (8%), using the above method, while one isolate (M353) could not be identified. The sequence analyses of ribosomal DNA (rDNA), β-tubulin and elongation factor α1 gene identified the novel isolate as *N. macroclavatum* (Billones et al. 2010). This species was first isolated from *Eucalyptus globulus*

and *E. saligna* in Western Australia in 2005 by Burgess et al. (2005). They named it *Fusicoccum macroclavatum* but it was subsequently renamed *Neofusicoccum macroclavatum* after a complete review of the taxonomy of the *Botryosphaeriaceae* by Crous et al. (2006). Other *Botryosphaeriaceae* species considered as pathogens of eucalypts include *B. dothidea*, *N. ribis*, *N. parvum*, *N. luteum*, *N. australe*, *N. eucalyptorum*, *N. eucalypticola*, *N. mangiferae* and *Lasiodiplodia theobromae* (Burgess et al. 2005). In New Zealand, *Eucalyptus* spp. are commonly used as shelter belts for orchards and other agricultural systems because they grow very quickly (Sale 1978).

Pathogenicity studies by Billones-Baaijens (2011) provided evidence that all 114 *Botryosphaeriaceae* nursery isolates from New Zealand including *N. macroclavatum* were pathogenic to detached green shoots and 1-year-old Sauvignon blanc vines. This study further showed that the pathogenicity of *N. macroclavatum* (isolate M353) was similar to the pathogenicity of *N. luteum* and *N. australe*, which are considered important species both in vineyards (Amponsah et al. 2011) and nurseries (Billones-Baaijens 2011). Since this species was isolated from an asymptomatic young grapevine, but was shown to cause cankers on both young and mature tissues of this host (Billones et al. 2010), there is a need to further investigate its pathogenicity on *Eucalyptus*. The present study investigated the pathogenicity of the single isolate of *N. macroclavatum* (M353) on *E. globulus* to determine if this New Zealand isolate is pathogenic to eucalypts, which may then provide an inoculum source for vineyards.

METHODS

To investigate the pathogenicity of *N. macroclavatum* (M353) on *E. globulus*, 1-year-old seedlings of *E. globulus* were purchased from a commercial nursery in May 2010 and transplanted into 2-litre plastic pots containing potting mix (80% composted bark, 20% pumice and 2 kg/m^3 Osmocote® Extract Standard). Seedlings were allowed to grow inside a greenhouse for 2 weeks until inoculation. Wounds (~4 mm diameter) were made on one side of the main stem between the middle internodes of the *E. globulus* seedlings using a sterile scalpel. Mycelial plugs (4 mm diameter) cut from the edges of the 4-day-old potato dextrose agar (PDA, Difco™, New Jersey, USA) cultures of the fungal isolates were placed onto the wounds with the mycelial surface facing the wound, and the area was wrapped with Parafilm™ (Pechiney Plastic Packaging, Chicago, USA). One isolate of *N. australe* (A143), a reported pathogen of *Eucalyptus* spp. in Australia (Burgess et al. 2005) and grapevines in New Zealand (Amponsah et al. 2009) was used as positive control while sterile agar was used as negative control. The six replicate seedlings per treatment were laid out in a randomised block design and kept in a greenhouse at 22±2°C and external lesions were measured using a digital caliper after 7 days.

To confirm the identity of the pathogen growing on inoculated plants, stems were surface sterilized by dipping in 70% ethanol for 30 s and passed through a Bunsen flame to dry off the alcohol (Billones-Baaijens 2011). Tissue segments (5 mm) were excised from both edges of the lesions or wounds and plated onto PDA supplemented with 0.05 g/litre streptomycin sulphate (PDAS). Plates were incubated on a laboratory bench at room temperature (20 ±2°C) for 4-10 days and assessed for characteristic growth of the inoculated pathogen, as described by Billones et al. (2010) and Amponsah et al. (2011).

Data analysis

Data were analysed by one-way ANOVA ($P \leq 0.05$) using GenStat© 14th Edition and differences between means was determined with least significant difference test (LSD) at $P \leq 0.05$.

RESULTS

By 7 days after inoculation, seedlings inoculated with *N. macroclavatum* and *N. australe* exhibited dark brown external lesions, which developed both upward and downward from the inoculation point (Figures 1a & b, respectively), while negative control plants had only a narrow (2-4 mm) necrotic area around the inoculation point (Figure

1c). Mean lesion lengths differed significantly between inoculation treatments (P<0.001; Table 1), with M353 (*N. macroclavatum*) causing the longest (P≤0.05) mean lesion of 29.0 mm that was significantly longer than the mean lesion length caused by A153 (*N. australe*; 17.1 mm) and those observed in the negative control plants (3.3 mm).

Re-isolation from plants inoculated with M353 (*N. macroclavatum*) produced white colonies that turned olivaceous brown within 10 days, which was characteristic of *N. macroclavatum* (Billones et al. 2010). Re-isolation from plants inoculated with *N. australe* on the other hand, produced colonies that were yellow on the undersides by 3 days as described by Amponsah et al. (2011). No pathogens were re-isolated from any of the negative control plants.

DISCUSSION

This study showed that the New Zealand isolate of *N. macroclavatum* from grapevines was also highly pathogenic on *E. globulus* seedlings. This result was consistent with the report by Burgess et al. (2005) that *N. macroclavatum* caused longer lesions on excised woody stems of *E. globulus,* than the other four Botryosphaeriaceae species found infecting *Eucalyptus* spp. They further reported that *N. macroclavatum* was only found on *Eucalyptus* spp. after the trees had been introduced from eastern areas of Australia, which may account for

Table 1 Mean lesion lengths (n=6) caused by *Neofusicoccum macroclavatum* and *N. australe* on 1-year old *Eucalyptus globulus* seedlings.

Species	Mean length (mm)	Range (mm)
N. macroclavatun (M353)	29.0 a[1]	17.1-42.0
N. australe (A143)	17.1 b	12.0-25.5
Negative control	3.3 c	2.0-4.0
LSD (P<0.05)	8.19	

[1]Means with different letters within the column are significantly different.

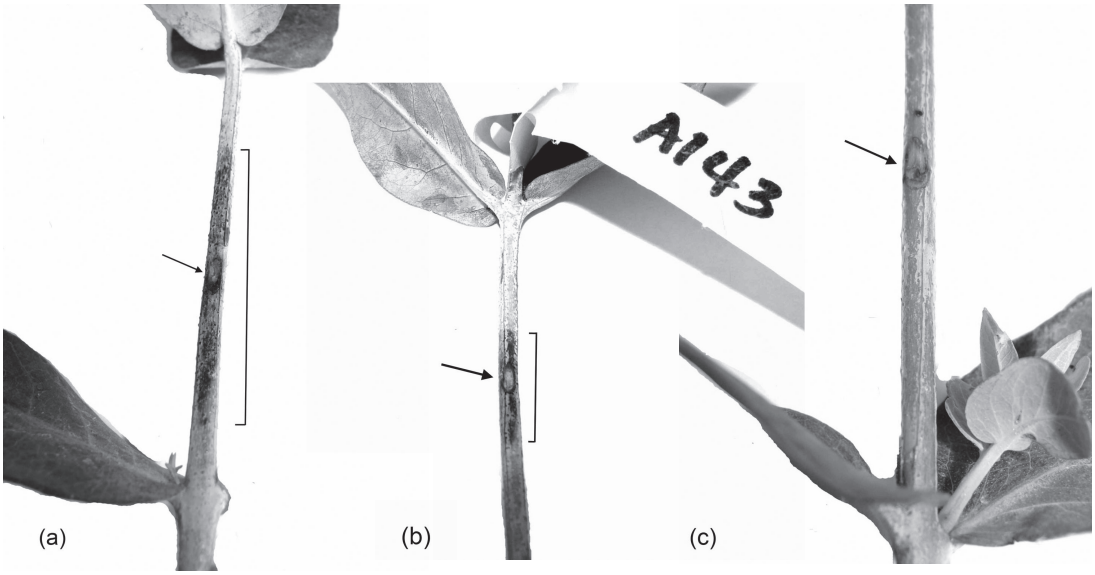

Figure 1 Stems of 1-year-old seedlings of *Eucalyptus globulus* at 7 days after inoculation with: (a) *Neofusicoccum macroclavatum* (M353), (b) *N. australe* (A143) and (c) sterile agar. Arrows indicate inoculation points and brackets indicate external lesion lengths.

the low incidence of *N. macroclavatum* in Western Australia. Therefore, the pathogenicity study presented here and the one reported by Billones-Baaijens (2011) demonstrated the cosmopolitan nature of this fungal group. *Botryosphaeriaceae* species have a broad geographical distribution and occur on a wide range of woody hosts including eucalypts (Burgess et al. 2005), apples, (Sutton & Arauz 1991), pistachios (Ahimera et al. 2003) and pine trees (Flowers et al. 2001). In addition, Amponsah et al. (2011) showed that non-grapevine *Botryosphaeriaceae* isolates were also pathogenic to grapevines, further demonstrating that these species are non-host specific.

The nursery study by Billones et al. (2010) represented the first report of *N. macroclavatum* in New Zealand and as canker pathogen of grapevines. Prior to this research, this species was only known to infect *Eucalyptus* spp. in Australia (Burgess et al. 2005). The genus *Eucalyptus* is endemic to Australia and was introduced to New Zealand approximately 170 years ago (Withers 2001). In New Zealand, eucalypts are commonly planted as shelter belts for agricultural systems (Sale 1978) as well as in commercial forests for processed timber (Treeby 1999). Importation of untreated *Eucalyptus* foliage was allowed into New Zealand until 1999 when it was banned after wide infestations of blackbutt leaf miner from Australia were discovered in Auckland (Treeby 1999). Fungal pathogens also represented 79% of newly recorded organisms associated with exotic and indigenous woody hosts in New Zealand between 1988-1997 (Ridley et al. 2000). Therefore, the origin of *N. macroclavatum* is most likely through the movement of *Eucalyptus* spp. from Australia. However, since only one *N. macroclavatum* isolate has been obtained from the nursery studies by Billones-Baaijens (2011) and none from vineyards by Baskarathevan et al. (2012) and Amponsah et al. (2011), it is possible that it is not yet widespread on grapevines, and that it may have originated from *Eucalyptus* spp. planted near the nursery from which the infected sample was collected. Therefore, there is a need to further investigate the prevalence of this species in grapevines and in *Eucalyptus* spp. in nearby vineyards to determine its potential threat to the New Zealand viticulture industry.

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