PRODUCTION OF *BOTRYOSPHAERIA* SPECIES CONIDIA USING GRAPEVINE GREEN SHOOTS

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ABSTRACT

Methods for producing large numbers of conidia were developed to allow investigation of the pathogenicity of five *Botryosphaeria* species (*B. lutea*, *B. australis*, *B. obtusa*, *B. parva* and *B. stevensii*), previously isolated from symptomatic grapevines. Pycnidium formation of all five species was low on five mycological media tested. Further, any pycnidia produced on the media contained few conidia. An alternative method was developed, whereby grapevine green shoots were infected with *Botryosphaeria* species, the lesions air dried and then induced to sporulate under moist conditions. *Botryosphaeria australis*, *B. lutea* and *B. stevensii* produced abundant pycnidia oozing conidia within 36 h. The air dried stem pieces could still produce pycnidia that oozed conidia after 3 months of storage at room temperature. *Botryosphaeria parva* produced few pycnidia that did not release any conidia. *Botryosphaeria obtusa* lesions were small and restricted to the inoculation point, with only a few pycnidia and conidia being produced.

Keywords: *Neofusicoccum australe*, *Neofusicoccum luteum*, *Diplodia seriata*, *Neofusicoccum parvum*, *Diplodia mutila*, pycnidia, conidia, grape.

INTRODUCTION

Botryosphaeria species have recently gained recognition as major pathogens of grapevines worldwide (Taylor et al. 2005). They are cosmopolitan and have wide geographical distribution, being saprophytes and endophytes on a range of woody angiosperm and gymnosperm hosts (Denman et al. 2000). On grapevines, these pathogens are frequently found associated with trunk dieback, incomplete graft unions, internal necrotic vine tissues, bud mortality, brown streaking inside the wood, as well as a general loss of vigour, termed 'grapevine decline' (Castillo-Pando et al. 2001; Larignon et al. 2001). Brown-Rytlewski & McManus (1999) reported that pycnidia and perithecia of *Botryosphaeria* species produced large numbers of conidia and ascospores, respectively, which are dispersed during spring rains. Sutton (1981) observed that the ascospores and conidia of *Botryosphaeria dothidea* can infect through natural openings and wounds on a range of apple tissues, causing lesions on which pycnidia are subsequently produced. Most researchers working on *Botryosphaeria* diseases have used mycelium-colonised agar plugs for pathogenicity studies (Taylor et al. 2005; Maloney et al. 2004; Sanchez-Hernandez et al. 2002). However, in nature conidia and ascospores are more likely to play a major role in infection. Some researchers have reported the use of various types of agar media to induce sporulation, with the most common ones being half-strength potato dextrose agar (½PDA), prune agar (PA), oat meal agar (OMA), malt extract agar (MEA) and water agar (WA) embedded with sterile pine needles. The aim of this study was to develop a method for producing sufficient conidia for subsequent infection studies.

MATERIALS AND METHODS

Fungal isolates

All the experiments used three isolates each of five *Botryosphaeria* species [*B. australis* (Anamorph: *Neofusicoccum australe*), *B. lutea* (Anamorph: *Neofusicoccum luteum*), *B. obtusa* (Anamorph: *Diplodia seriata*), *B. parva* (Anamorph: *Neofusicoccum parvum*) and *B. stevensii* (Anamorph: *Diplodia mutila*)], which had been isolated from grapevine and non grapevine hosts in New Zealand. The isolates were stored on potato dextrose agar in Petri dishes at 4°C. Species identity of all isolates was confirmed using sequence comparison of rDNA with those reported on GenBank and/or morphological characteristics (Phillips 2004). Since the species *B. australis* was isolated for the first time from grapevines and other plants in New Zealand, the isolates used here (J-3, Mel-2 and Kat-1) were positively identified and stored in the MAF Biosecurity culture collection. The other isolates used in this experiment were G-1, N(12)2 and M(13)2 for *B. lutea*; L-1, L-5 and L(16)2 for *B. obtusa*; I(15)2, I(15)3 and Q-2 for *B. parva* and Q, F(12)2 and Iso-2 for *B. stevensii*.

Inducing sporulation on artificial media

Mycelium discs (3 mm) were cut from the growing edges of three day old PDA cultures of the 15 isolates and placed on Petri plates of MEA, OA, ½PDA, PA (prune extract 100 ml, sucrose 6 g, yeast extract 1.25 g, agar 16 g and water 900 ml) and WA (2%) embedded with three pieces of autoclaved sterilised pine (*Pinus* sp.) needles (each 30 mm). Duplicate plates of each isolate and each medium were incubated at 25°C with alternating 12 h cycles of near ultraviolet (NUV) light and darkness for 4-5 weeks, and then the numbers of pycnidia within the plates were assessed. On the 2% WA with pine needles, numbers of pycnidia produced across each 30 mm length of pine needle were recorded. To harvest pycnidia and conidia each plate was flooded with 10 ml of sterile water containing 0.01% Tween 80 and all pycnidia dislodged from the media using a sterile scalpel, thus causing them to float. The pycnidia in suspension were crushed with a mortar and a pestle, the resulting slurry filtered through a double layer of cheesecloth and the filtrate centrifuged at 9.7× g for 2 min. The supernatant was discarded to leave 5 ml remaining suspension, which was vortexed to re-suspend the conidia and their concentration determined using a haemocytometer.

Inducing sporulation on grapevine shoots

Grapevine shoots tips of 20-25 cm long were cut from field-grown Pinot noir vines during summer 2006. Mycelium discs (3 mm) cut from the growing edges of 3 day old PDA cultures of each isolate were inoculated mycelium side into wounds (4 mm core) in the centres of the grapevine shoots, six shoots per isolate. The base of each shoot was inserted into a Universal bottle containing sterile distilled water and they were placed in a completely randomised design within an enclosed transparent chamber for 10 days at room temperature (15-23°C), with frequent misting for the first 3 days. Control plants with wounds were inoculated with sterile agar. Ten days after inoculation, the stem pieces containing the lesions were surface sterilised in 1% sodium hypochlorite for 1 min, then in 70% alcohol for 30 s and rinsed twice in sterile distilled water before air drying under a laminar flow hood for 6 h. The dried stem lesions were then placed on moist filter paper in a Petri dish and sprayed with a fine mist of sterile water to ensure high humidity. After a 36 h incubation at 23-24°C, the numbers of pycnidia that oozed conidia within the central 15 mm of stem lesions were recorded. To harvest the conidia, each 15 mm stem piece was shaken vigorously in a 1.5 ml Eppendorf tube containing 1 ml sterile tap water for 1 min to disperse the conidia into the water, and then the conidium concentration determined with a haemocytometer. Since Alan Phillips has shown (pers. comm.) that water agar embedded with *Populus* shoots can be used for inducing sporulation, the experiment was repeated using shoot tips of a *Populus* species that is grown as shelterbelts in many areas.

Statistical analyses

The data were analysed using ANOVA (GenStat 9) to determine the significance of differences between treatments. Means were separated according to Fisher's protected least significance difference (LSD) test.

RESULTS AND DISCUSSION

After 4 weeks of growth on ½PDA, OMA, MEA, PA and WA isolates of *B. australis*, *B. lutea*, *B. obtusa* and *B. stevensii* yielded pycnidia, but none were produced by *B. parva* on all media. More ($P<0.05$) pycnidia were produced by *B. obtusa* than the other species, which had similar numbers (Table 1). The effect of medium type was statistically significant, with OMA and PA having higher ($P<0.05$) numbers of pycnidia than ½PDA and MEA for all species (Table 1). After a further 5 weeks incubation, *B. parva* had still not produced any pycnidia on any of the media and hence was not included in the analyses. The interaction between species and media was not significant ($P>0.43$).

Conidium production differed between species, being significantly higher ($P<0.001$) for *B. obtusa* than the other species (Table 2). There was no significant difference between *B. lutea* and *B. australis* though both were significantly lower than *B. stevensii* in conidium on all media (Table 2). There was no significant effect of media on conidium production ($P>0.37$) and the interaction between species and media was not significant ($P>0.38$).

The production of *B. obtusa* conidia on agar was efficient and reliable, with most of the pycnidia oozing conidia, thus making harvesting more simple than with pycnidia produced on agar by the other species. Many researchers have reported different incubating conditions and media types for inducing sporulation of *Botryosphaeria* species, with the most commonly used media being OMA, ½PDA and MEA incubated at 23–25°C with alternating cycles of 12 h near ultraviolet (NUV) light and darkness for a period of 3–4 weeks (Phillips 2002; Swart & Botes 1995; Pusey 1989; Taylor et al. 2005). These published studies had primarily focused on identification of the isolates, and the degree of sporulation was probably sufficient for their purposes. However, further investigations of these media showed that they also yielded many pycnidia but few conidia (W. Pitt & M.A. Whitelaw-Weckert, pers. comm.), which is consistent with the present findings (Table 2). During the present study it was generally observed that the pathogens lost their ability to sporulate after repeated subculturing, irrespective of medium type and incubation conditions, an observation that has been found by other researchers (A. Philips, pers. comm.).

For the 2% WA embedded with pine needles, *B. lutea* had significantly highest ($P<0.05$) numbers of pycnidia, followed by *B. obtusa* and *B. stevensii*, with the least produced by *B. australis* (Table 3; $P<0.05$). Conidia extracted from these pycnidia yielded the highest ($P<0.05$) number for *B. lutea* with the rest being similar (Table 3). Taylor et al. (2005) used this method for inducing sporulation to allow identification of species by shape, colour and size of *Botryosphaeria* pycnidia and conidia.

Pycnidium production on the green grapevine shoots was highest ($P<0.05$) for *B. lutea*, followed by *B. australis* and *B. stevensii*, which were similar, and *B. obtusa* which produced the least ($P<0.05$). After 36 h under moist incubation at room temperature, the air-dried lesions exuded numerous conidium paste tendrils. *Botryosphaeria lutea* produced highest ($P<0.05$) numbers of conidia, greater than *B. australis* and *B. stevensii*, which were similar, with the least produced by *B. obtusa* ($P<0.05$; Table 3). Fresh shoots of *Populus* species also yielded lots of pycnidia but these did not contain any conidia (data not shown).

TABLE 1: Mean numbers of pycnidia produced per plate after 4 weeks of incubation for four *Botryosphaeria* spp. on four different agar media.

Media type	<i>B. australis</i>	<i>B. lutea</i>	<i>B. obtusa</i>	<i>B. stevensii</i>	Media main effect
MEA	18.7	48.0	186.7	32.0	71.3 b ¹
OMA	186.7	157.3	360.0	208.0	228.0 a
PA	152.0	266.7	328.0	98.7	211.3 a
½PDA	18.7	93.3	226.7	80.0	104.7 b
Species main effect	94.0 b ¹	104.7 b	275.3 a	141.3 b	

¹For the main effects of media ($P<0.001$) and species ($P<0.001$), values within the row or column followed by the same letters are not significantly different; LSD=57.5 for both.

TABLE 2: Mean numbers of conidia/ml ($\times 10^4$) produced per plate after 4 weeks of incubation for four *Botryosphaeria* spp. on four different agar media.

Media type	<i>B. australis</i>	<i>B. lutea</i>	<i>B. obtusa</i>	<i>B. stevensii</i>	Media main effect
MEA	1.3	3.0	16.0	4.3	6.2
OMA	1.7	1.7	18.3	7.3	7.3
PA	1.3	3.7	21.0	6.0	8.0
½PDA	1.3	1.7	23.3	5.7	8.0
Species main effect	1.4 c ¹	2.5 c	19.7 a	5.8 b	

¹For the main effect of species ($P<0.001$), values within the row followed by the same letters are not significantly different; LSD=2.4.

TABLE 3: Mean number of *Botryosphaeria* spp. pycnidia and conidia (no./ml $\times 10^4$) produced per 30 mm length of autoclaved pine needle embedded in water agar after 3 weeks incubation and on 15 mm lesion length of green Pinot noir grapevine shoots at 12 days after inoculation.

<i>Botryosphaeria</i> species	Pine needle in water agar		Green grapevine shoot	
	Pycnidia	Conidia	Pycnidia	Conidia
<i>B. australis</i>	6.7 c ¹	15.0 b	143.7 b	161.7 b
<i>B. lutea</i>	21.3 a	20.0 a	165.7 a	186.0 a
<i>B. obtusa</i>	10.0 b	13.3 b	5.0 c	14.7 c
<i>B. stevensii</i>	11.7 b	15.7 b	137.3 b	147.0 b
LSD	2.49	3.35	16.46	15.86

¹Means followed by the same letters within each column are not significantly different.

Although *B. parva* is pathogenic to the grapevine (Phillips 2000; Van Niekerk et al. 2004), and the present studies showed that it had a mean lesion length of 53.4 mm on the green grapevine shoot, it did not produce any pycnidia after incubating the air dried

stem lesion. These lesions were covered with white fluffy mycelium in contrast to what was observed on lesions produced by the other species.

With the grapevine shoot method, sufficient conidia for pathogenicity experiments could be obtained within 12-14 days from the onset of the experiment, as opposed to 4 or more weeks on agar with fewer numbers. Harvesting of conidia produced on the lesion shoots was also easier and less time consuming than the agar method. However, if the conidia that oozed from the pycnidia on grapevine shoots remained in the high humidity chamber they germinated within 24 h. To prevent this, lesion stem pieces when air dried could be stored at room temperature until required. Initial trials showed that they were still effective at producing pycnidia and oozing conidia after 3 months when incubated under moist conditions for 36 h. Future studies on the pathogenicity of *Botryosphaeria australis*, *B. lutea* and *B. stevensii* on grapevines will be conducted using these methods to provide conidial inoculum.

ACKNOWLEDGEMENT

The authors wish to thank the New Zealand Winegrowers association for funding this project and also individual grape growers who allowed sample collection from their vineyards.

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