Comparison of *in vitro* and *in planta* sporogenesis in *Neofusicoccum* species from blueberry

Y.L.Yang[1,2], J. Turner[2], J. Stephens[2], R.E. Campbell[2] and M. Walter[2]

[1]*Institute of Alpine Economic Plant, Yunnan Academy of Agricultural Science, Lijiang 674199, Yunnan, China*
[2]*The New Zealand Institute for Plant & Food Research Ltd, Old Mill Road, RD3, Motueka, 7198, New Zealand*
Corresponding author: monika.walter@plantandfood.co.nz

Abstract Stem diseases are an economically important problem in the production of blueberry (*Vaccinium* spp.). The diseases cause significant crop loss, including the death of entire bushes. Resistance phenotyping assays require large numbers of conidia. A fast and reliable *in vitro* method to mass produce viable and pathogenic *Neofusicoccum* spores would be more suitable than the current *in planta* methods. Two strains each of *N. parvum* and *N. ribis* were used to develop a new *in vitro* method, which involved interrupting mycelial mats produced on agar plates, with wet and dry cycles. Spores were generated at room temperature after 7–12 days' incubation. Spore production varied among replicate tests and the four isolates used. *Neofusicoccum ribis* isolate Nr175 (LUPP1348) generated the most spores in the shortest time. Pathogenicity assays using spores generated *in vitro* suggest that these spores are more virulent and pathogenic than those produced *in planta*.

Keywords *Neofusicoccum parvum*, *Neofusicoccum ribis*, conidia, blueberry.

INTRODUCTION

Species in the family Botryosphaeriaceae typically have a wide host range and geographical distribution (Wright et al. 2010). *Neofusicoccum parvum* and *N. ribis* species are the most widespread and have been reported on many hosts, such as *Eucalyptus* sp., *Malus sylvestris*, *Populus nigra*, *Araucaria* sp., *Actinidia deliciosa* and others (Sakalidis et al. 2013). Both species have been associated with stem canker, stem dieback, twig blight and fruit-rot symptoms in a range of *Vaccinium* spp. (blueberry) cultivars. Their worldwide distribution and importance as stem canker-causing organisms have been extensively reported (Espinoza et al. 2009; Sammonds et al. 2009; Wright et al. 2009; Wright et al. 2010; Choi et al. 2012; Castillo et al. 2013; Koike et al. 2014; Xu et al. 2015; Boyzo-Marin et al. 2016).

Phenotyping assays to assess resistance can require large amounts of pathogen inoculum. *Neofusicoccum* spp. do not produce conidia easily so researchers often use mycelial plugs for inoculation studies (Espinoza et al. 2009). However, this is a fairly artificial and aggressive method of inoculation and a fast, reliable method to mass produce viable and pathogenic *Neofusicoccum* spores *in vitro* would be advantageous. For conidia production, researchers have reported

the use of various types of agar and plant-based media to induce sporulation. The most common ones are half-strength potato dextrose agar, prune agar, oat meal agar, malt extract agar as well as water agar embedded with sterile pine needles, blueberry stem chips or young apple fruit and generally incubated at 25°C for 1–4 weeks under continuous UV light. These conditions have often resulted in low numbers of spores being produced (Amponsah et al. 2008; Wright et al. 2009; Yao et al. 2011; Xu et al. 2015). However, a detached-stem assay has been developed for spore production of *Botryosphaeria* spp. in grapes (Amponsah et al. 2008) and blueberry (Tennakoon et al. 2017) that generates high numbers of spores with stable virulence. For any inoculum production method, isolate virulence is the one of the most critical factors in the accurate and reliable determination of host resistance. It is important to minimise the likelihood of using isolates that might give false resistance readings due to lack of virulence. Virulence may be affected by strain, age of the culture, number of subcultures taken and spore production method (Cline 1993; Smith 2004; Polashock 2006; Polashock et al 2006). The objective of this study was to develop a rapid, *in vitro* method for producing virulent conidia of *Neofusicoccum* spp. in sufficient quantity for subsequent identification and host resistance/susceptibility studies.

MATERIALS AND METHODS

Fungal isolates and cultivars

Two strains each of *N. parvum* (isolates Np98 [= LUPP1288] and Np173 [= LUPP1363]) and *N. ribis* (isolates Nr175 [= LUPP1348] and Nr158 [= LUPP1365]) were used, which had been isolated from blueberry bushes in New Zealand (Tennakoon 2017). The isolates were provided by Lincoln University, New Zealand and stored on potato dextrose agar (PDA, Difco™) in sealed Petri dishes at 4°C at the New Zealand Institute for Plant & Food Research Limited (PFR), Motueka. Shoots from two *Vaccinium virgatum* (rabbiteye blueberry) cultivars 'Powderblue' and 'Dolce Blue' were collected from mature plants in the Motueka PFR site and used as inoculation hosts.

Inducing sporulation using interrupting mycelial mat

Fungal isolates were grown and maintained on PDA plates. At first, mycelium plugs were cut aseptically from the growing edge of the plates using a cork borer (3-mm diameter) and transferred to new PDA plates. These were incubated for 3–4 days on the laboratory bench (20°C) until the plates were covered with mycelium. Sterile cotton swabs were used to disrupt the mycelial mat gently by working in a grid pattern. Plates then were rinsed with sterile distilled water and allowed to dry in a laminar-flow cabinet for 1 hour, followed by continued incubation on the bench covered with filter papers. Plates were monitored in 2–3 day intervals until sporogenesis was observed. To harvest the conidia, each plate was flooded with 10 mL of sterile water, rubbed with a glass rod and decanted into a sterile jar. Spore concentration was determined using a haemocytometer (Neubauer). This method of spore production was repeated four times with modifications as described below. *Neofusicoccum parvum* isolate Np98 was chosen for further studies because Amponsah et al. (2008) had been unable to produce spores of other isolates from this species *in vitro* but spores were produced readily on detached grape shoots (Sammonds et al. 2016; Billones-Baaijens et al. 2015; Amponsah et al. 2012). Four different experiments were conducted to assess the effect of different variables.

Experiment 1: The effect of the number of mycelial inoculation plugs per plate on sporulation. One, three or six mycelia plugs were transferred onto each PDA plate at the same time. There were three replicates for each subculture for isolate Np98.

Experiment 2: The effect of temperature/light combinations. Plates with three mycelial plugs from Experiment 1 were used. After the mycelial disruption, rinsing and drying, plates were incubated at 4°C (dark), 16°C (bench, natural light) or 22°C (bench, natural light). There were three replicates for each treatment combination for isolate Np98.

Experiment 3: The effect of cover type (after disruption, washing and drying). Plates

were inoculated with three mycelial plugs, and incubated at 22°C (bench, natural light). The covers trialled were filter paper, gauze and Petri dish lids. There were three replicates for each treatment combination for isolate Np98.

Experiment 4: Use of isolates Np98, Np173, Nr175 and Nr158 for method validation. Each plate was inoculated with three mycelial plugs, incubated at 22°C and covered with filter paper. There were three replicate plates for each isolate. Spores were harvested and enumerated as described above. Spore concentrations were counted and then adjusted to $1x10^4$ spores/mL for use in pathogenicity tests.

Inducing sporulation using detached blueberry shoots

All four *Neofusicoccum* strains were used in this study. For spore production, young green shoots (semi-hardwood cuttings) approximately 20 cm in length were harvested from blueberry cultivar 'Powderblue'. These were wounded and inoculated with a mycelial plug and incubated in humidity boxes at 22°C as described by Tennakoon et al. (2017) and Campbell et al. (2017). Within 10 days, all shoots showed clear signs of the disease. Infected shoot segments were dried in a laminar-flow cabinet and stored at 4°C in Petri dishes. Shoots were rehydrated and incubated in Petri dishes on moist filter paper for 2 days and washed individually in 10 mL sterile water. Spores were counted using a haemocytometer (Neubauer). The freshly produced spores were also used in pathogenicity tests.

Testing conidium virulence

Forty young green shoots roughly 20 cm in length were harvested from blueberry cultivar 'Dolce blue' and separated into replicates of five to be inoculated with spores from the four isolates used in the current study. Shoots were prepared, wounded and inoculated with the spore concentrations adjusted to 1×10^4 spores/mL by applying a 10 µL droplet and incubated (22°C) in humidity chambers (Tennakoon et al. 2017; Campbell et al. 2017). Lesion length was measured after 3, 6 and 9 days of incubation.

Statistical analyses

The data were analysed using Analysis of Variance General Linear Model (ANOVA GLM) to determine the significance of differences between treatments. Means were separated according to Tukey's least significance difference (LSD) test on Minitab 16.1.

RESULTS

Conidium production was affected by the number of agar plugs per plate ($P<0.05$). The period of incubation post mycelial disruption was shortest for inoculations with three plugs (7–8 days) compared with inoculations involving either one or six plugs (11–12 days). However, the effects of temperature/light and cover type were much greater, with no spores produced at the other two temperature/light combinations tested (4°C/dark and 16°C/light). Similarly, no spores were produced when plates were covered with either a Petri dish lid or gauze. Spore production potential varied amongst isolates ($P<0.05$). A comparison of spore production potential using either the detached shoot or the final mycelial disruption method (plate inoculated with three mycelial plugs, incubated at 22°C/light and covered with filter paper) is shown in Table 1. For the *in planta* produced spores, there was no significant difference of conidia production between isolates ($P>0.27$, Table 1).

Comparison of conidium virulence

Shoots inoculated with conidia from the *in vitro* production method had larger lesions than those inoculated with conidia from *in planta* production (Figure 1). This was particularly noticeable for isolate Np173. However, isolate Nr158 produced the largest and the Np98 the smallest lesions overall (Figure 2). Lesion length continuously increased during the assessment period for all isolates.

DISCUSSION

In this study, the mycelial disruption method of PDA plate inoculated with three mycelial plugs, incubated at 22°C/light and covered with filter paper, yielded conidia for all four of the *Neofusicoccum* isolates

Table 1 Time taken for *Neofusicoccum parvum* (Np) and *N. ribis* (Nr) isolates to produce spores using semi-hardwood blueberry shoots (*in planta*) or the final mycelial disruption method (*in vitro*) and the spore production potential for each method. Mean number of conidia are shown based on 2–3 repeat experiments.

	In planta (2 experiments, n=10 shoots)		In vitro (3 experiments, n=3 plates)	
Isolate	Time (days)	Concentration conidia/mL (10^4)	Time (days)	Concentration conidia/mL (10^4)
Nr175	12	1.19 a[1]	7	11.30 a *
Nr158	12	1.94 a	11	1.78 c
Np173	12	1.76 a	12	6.74 b *
Np98	12	1.50 a	7	2.00 c

[1]Different letters within a column indicate significant difference at P<0.05
*Indicates *in vitro* spore production is significantly higher than *in planta* spore production (P<0.05).

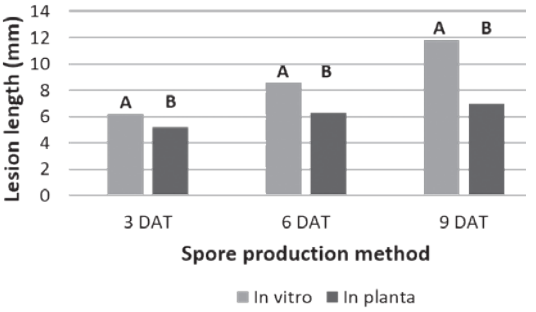

Figure 1 Lesion length (mm) data pooled for *Neofusicoccum parvum* and *N. ribis* isolates on detached *Vaccinium virgatum* (rabbiteye) blueberry shoots ('Dolce Blue') inoculated with conidia (1×10^4 conidia/mL) from *in planta* and *in vitro* production methods incubated in the laboratory at 22°C, 3, 6 and 9 days after treatment (DAT). Different letters at each DAT indicate significant difference at P<0.05.

tested (all sourced from blueberry). In contrast, Amponsah et al. (2008) could not produce spores *in vitro* for *N. parvum* isolated from grapes. However, these authors did not disrupt the mycelial mat on the agar plate. Our findings suggest that agitation is required to generate spores. Temperature/light and humidity were also found to be important factors since sporulation was not induced by mycelial disruption alone at the lower temperatures tested, nor when humidity was high (Petri dish lid cover) or low (gauze cover). Humidity under the filter paper cover was not determined, but assumed to be intermediate; playing an important role in spore production. Further studies are required to determine and optimise the abiotic conditions of post-disruption incubation.

While spores could be readily produced using both the *in planta* and *in vitro* methods, the latter method facilitates laboratory-based inoculum production all year round and is not dependent on the availability of semi-hardwood shoot cuttings. More spores could be produced in a shorter time with similar or greater pathogenicity using the *in vitro* method than the *in planta*-produced conidia. It is noteworthy that the blueberry isolates of *N. parvum* examined here readily produced pycnidia and spores on blueberry shoots, but a previous study using grape isolates of *N. parvum* on grape shoots was unsuccessful initially (Amponsah et al. 2008). Subsequent modification of temperature and relative humidity (Amponsah et al. 2012; Billones-Baaijens et al. 2015; Sammonds et al. 2016) led successful spore release from grape isolates of *N. parvum* on grape

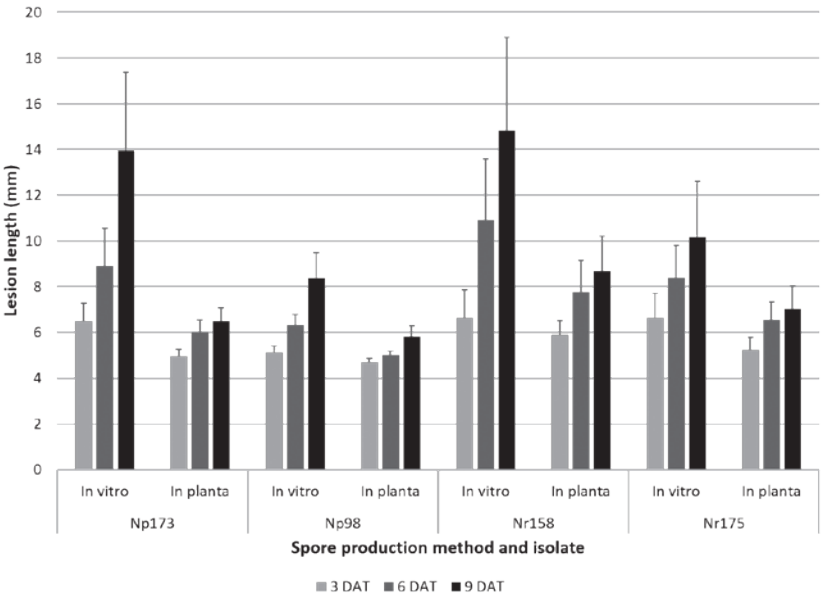

Figure 2 Lesion length (mm) for *Neofusicoccum parvum* (Np173, Np98) and *N. ribis* (Nr158, Nr175) on detached *Vaccinium virgatum* (rabbiteye) blueberry shoots ('Dolce Blue') inoculated with conidia (1×10^4 conidia/mL) from *in planta* and *in vitro* production methods incubated in the laboratory at 22°C, 3, 6 and 9 days after treatment (DAT). Standard error bars of the means are shown.

shoots. Spore production potential has been reported to vary amongst host and isolates (Yao et al. 2011).

Traditional *in vitro* methods are generally designed for taxonomic purposes and are also based on media, light and/or temperature manipulations. These take up to 4 weeks to produce conidia and generally produce few conidia (Wright et al. 2010; Choi et al. 2012; Xu et al. 2015). The extent of sporulation using traditional methods is probably sufficient for identification purposes. The *in vitro* method presented here for *Neofusicoccum* isolates is simple and fast, and produced high numbers of conidia suitable for biological and resistance/susceptibility screening studies.

ACKNOWLEDGEMENTS

Thanks to the Yunnan Academy of Agricultural Science, China and The New Zealand Institute for Plant & Food Research Limited for funding the research collaboration. We thank Lincoln University for providing the isolates.

REFERENCES

Amponsah NT, Jones EE, Ridgway HJ, Jaspers MV 2008. Production of *Botryosphaeria* species conidia using grapevine green shoots. New Zealand Plant Protection 61: 310–305.

Amponsah NT. Jones EE, Ridgway HJ, Jaspers MV 2012. Susceptibility of grapevine tissues to *Neofusicoccum luteum* conidial infection. Plant Pathology 61: 719–729.

Billones-Baaijens R, Jaspers MV, Allard A, Hong Y, Ridgeway HJ, Jones EE 2015. Management of Botryosphaeriaceae species infection in grapevine propagation materials. Phytopathologia Mediterranea 54: 355–367.

Boyzo-Marin J, Rebollar-Alviter A, Silva-Rojas HV Ramirez-Maldonaldo G 2016. First report of *Neofusicoccum parvum* causing stem blight and dieback of blueberry in Mexico. Plant Disease 100: 2524.

Campbell RE, Walters H, Tennakoon KMS, Jones EE, Walter M 2017. Development of phenotyping assays for resistance screening

of blueberry plants against *Neofusicoccum* spp. New Zealand Plant Protection 70: 215-223.

Castillo S, Borrero C, and Castaño R 2013. First report of canker disease caused by *Neofusicoccum parvum* and *N. australe* on blueberry bushes in Spain. Plant and Disease 97: 1112.

Choi IY, Shama, PK, Cheong SS 2012. First report of bark dieback on blueberry caused by *Neofusicoccum parvum* in Korea. Plant Pathology Journal 28: 217.

Cline WO, Miholland RD, Rooks SD, Ballington JR 1993. Techniques in breeding for resistance to blueberry stem blight caused by *Botryosphaeria dothidea*. Acta Hortculturae 346: 107–110.

Espinoza, JG, Briceño EX, Chávez ER, Úrbez-Torres JR, Latorre BA 2009. *Neofusicoccum* spp. associated with stem canker and dieback of blueberry in Chile. Plant Disease 93: 1187–1194.

Polashock JJ 2006. Screening for resistance to Botryosphaeria stem blight and Phomopsis twig blight in blueberry. Acta Horticulturae 715: 493–495.

Polashock JJ, Matthew K 2006. Resistance of blueberry cultivars to Botryosphaeria stem blight and Phomopsis twig blight. HortScience 41: 1457–1461.

Koike ST, Rooney-Latham S, Wright AF 2014. First report of stem blight of blueberry in California caused by *Neofusicoccum parvum*. Plant Disease 98(9): 1280.

Sammonds J, Jaspers MV, Jones EE 2016. Pre-infection processes of Botryosphaeriaceae spp.: adhesion of conidia to different substrata. Plant Pathology 65: 1142–1152.

Sammonds J, Billones R, Rocchetti M, Ridgway HJ, Walter M, Jaspers MV 2009. Survey of blueberry farms for Botryosphaeria dieback and crown rot pathogens. New Zealand Plant Protection 62: 238–432.

Sakalidis ML, Slippers B, Wingfield BD, Hardy GEStJ, Burgess TI 2013. The challenge of understanding the origin, pathways and extent of fungal invasions: global populations of the *Neofusicoccum parvum–N. ribis* species complex. Diversity and Distributions 19: 873–883.

Smith BJ 2004. Susceptibility of southern highbush blueberry cultivars to Botryosphaeria stem blight. Small Fruits Review 3: 193–201.

Tennakoon KMS 2017. Botryosphaeria disease in New Zealand blueberry gardens: identification of pathogens, inoculum sources and factors affecting disease development. Lincoln University, Lincoln, New Zealand, PhD thesis.

Tennakoon KMS, Ridgway HJ, Jaspers MV, Jones EE 2017. Production of *Neofusicoccum* species conidia and their pathogenicity on wounded and non-wounded attached blueberry shoots. New Zealand Plant Protection 70: 209-214.

Wright AF, Harmon PF 2009. First report of *Lasiodiplodia theobromae* associated with stem blight of southern highbush blueberries in Florida. Plant and Disease 93: 962.

Wright AF, Harmon PF 2010. Identification of species in the Botryosphaeriaceae family causing stem blight Southern Highbush Blueberry in Florida. Plant and Disease 94: 966–971.

Xu CN, Zhang HJ, Zhou ZS, Hu TL, Wang ST, Wang YN, Cao KQ 2015. Identification and distribution of Botryosphaeriaceae species associated with blueberry stem blight in China. European Journal of Plant Pathology 143: 737–752.

Yao SW, Xie Y, Li XH 2011. Method evaluation of inducing sporogenesis of *Botryosphaeria dothidea* causing grape canker. Sino-Overseas Grapevine & Wine. 9: 4–7.