Phytopathologia Mediterranea (2015) 54, 2, 355–367
DOI: 10.14601/Phytopathol_Mediterr-16159

RESEARCH PAPERS - 9TH SPECIAL ISSUE ON GRAPEVINE TRUNK DISEASES

Management of Botryosphaeriaceae species infection in grapevine propagation materials

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Summary. In New Zealand grapevine propagation nurseries, Botryosphaeriaceae species have been reported to infect the source blocks of the nursery propagators leading to infection of the propagation materials. This research investigated the efficacy of different control methods which could prevent infection or eradicate the pathogen from harvested canes prior to plant propagation. In the source blocks, attempts to reduce infection of shoots by protecting trimming wounds were partially successful (P=0.036), with 19.5% incidence in fungicide-treated shoots and 24.3% infection in the control shoots. Further sampling showed that overall 19.9% of these infections were in the bark and 9.6% in the wood. Hot water treatment (HWT) of dormant rootstock 5C canes, previously infected with *Neofusicoccum luteum* and *N. parvum*, at 50°C for 30 min resulted in internal infection incidences of 55 and 100%, respectively. HWT at 53°C reduced infection incidence to 0 and 8.5%, respectively, but killed the buds. In naturally infected canes, HWT of 50°C for 30 min reduced infection incidence from 35% in controls, to 0–15% over all Botryosphaeriaceae species. Shorter periods of HWT, at 55°C for 10 min, designed to kill bark infections, were effective in Sauvignon blanc but killed the buds of Pinot noir. Sauvignon blanc canes superficially infected with *N. luteum* were soaked for 30 min in the fungicides carbendazim, tebuconazole, thiophanate methyl and flusilazole, with and without a polyether-modified trisiloxane adjuvant. Results showed that carbendazim with no adjuvant and tebuconazole with 0.5 mL L^{-1} adjuvant eliminated 100% of bark infections. A further experiment that soaked 2,000 canes (Sauvignon blanc and Pinot noir) in a carbendazim solution prior to rooting found that all canes were free of Botryosphaeriaceae species infection, compared to 17% natural incidence. These results have indicated the potential efficacy of several methods for preventing or reducing infection Botryosphaeriaceae species in grapevine propagating materials.

Key words: hot water treatment, fungicide treatments, latent infections, *Neofusicoccum luteum*, *N. parvum*.

Introduction

Botryosphaeriaceae species cause trunk cankers and dieback on many woody hosts including grapevines and they can also exist as endophytes or latent pathogens of some woody hosts (Slippers and Wingfield, 2007). Disease surveys conducted in vineyards of many viticultural regions have reported the presence of Botryosphaeriaceae species (Taylor *et al.*, 2005; Úrbez-Torres *et al.*, 2006a; 2006b; van Niekerk *et al.*, 2006; Pitt *et al.*, 2010). In New Zealand, a survey of symptomatic plant material in vineyards from the six main grape growing regions identified nine species of the Botryosphaeriaceae, namely *Diplodia seriata*, *D. mutila*, *Dothiorella sarmentorum*, *Do. iberica, Neofusicoccum parvum, N. luteum, N. australe*, *N. ribis* and *Botryosphaeria dothidea* (Baskarathevan *et al.*, 2012a). These pathogens were found to be widespread, oc-

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ISSN (print): 0031-9465
ISSN (online): 1593-2095

curring in 80% of the 43 vineyards and in 68% of 238 symptomatic vines sampled.

Inoculum within vineyards is believed to originate largely from the diseased wood, both on the vines and the pruning debris, on which pycnidia exude conidia in the presence of high moisture (van Niekerk *et al.*, 2010). Dispersal of conidia is mainly by rain splash and occurs over relatively short distances (Úrbez-Torres *et al.*, 2010; Baskarathevan *et al.*, 2012b). Pruning wounds are widely believed to be the main points of entry for Botryosphaeriaceae species (van Niekerk *et al.*, 2010; Úrbez-Torres and Gubler, 2011). However, pathogenicity studies performed on other woody hosts such as peach, pistachio and apple have demonstrated that these pathogens can also infect through natural openings such as stomata and lenticels, or even penetrate host tissue directly (Michailides, 1991; Kim *et al.*, 2001). More recently, infection was shown to occur on non-wounded buds and excised berries of grapevines (Wunderlich *et al.*, 2011; Amponsah *et al.*, 2012a).

Propagation material has also been found to be a likely inoculum source for grapevines, although the infected plants are unlikely to show symptoms until they have developed into mature productive vines. Surveys of source blocks for propagation materials found Botryosphaeriaceae species in both the pruning wound ends and the basal ends of two year old stubs in South Africa (Fourie and Halleen, 2004) and from the basal ends of the shoots in Spain (Aroca *et al.*, 2010). In New Zealand, a survey of nine commercial grapevine nurseries confirmed the presence of the six most common Botryosphaeriaceae species, with an overall incidence of 23% in mostly asymptomatic samples of rootstock and scion canes, and grafted plants (Billones-Baaijens *et al.*, 2013a). In a further investigation to determine inoculum sources in New Zealand source blocks, Billones-Baaijens *et al.* (2013b) detected Botryosphaeriaceae propagules on the surfaces of 33 to 100% of cane samples and 33% of grapevine detritus using microscopy, plating assays and PCR with Botryosphaeriaceae multi-species primers (Bot100F/Bot472R) (Ridgway *et al.*, 2011). Botryosphaeriaceae DNA was also found on grafting tools, in callusing media and samples from hydration tanks, although the main sources of inoculum were the canes grown in the source blocks.

To investigate the potential infection pathways of Botryosphaeriaceae species within a rootstock mother vine, Billones-Baaijens *et al.* (2015) conducted three genotyping studies with *N. luteum* and *N. parvum* using a universally-primed polymerase chain reaction (UP-PCR) method which identified individual isolates. Results indicated that the multiple Botryosphaeriaceae infections within a vine or shoot were likely to be from different external inoculum sources. Trunk and shoot isolates from the same vine were often of different genotypes. Further, when numerous isolates were recovered from the surfaces and internal tissues of shoots, genotyping studies showed that the surface isolates were frequently of different genotypes to those from adjacent internal tissues, which were much fewer in number. The presence of the same genotypes on a few adjacent wood and bark positions indicated that wood infections may have originated from the bark. It therefore seems likely that conidia dispersed throughout a source block are able to cause latent infections in the bark of the shoots, which can be harvested as dormant canes for plant propagation. The infected grafted plants may then act as a primary infection source for new vineyards.

Nursery management practices which focus on fungal trunk pathogens of grapevines, have reported some successes. Hot water treatment (HWT) of nursery canes at 50°C for 30 min has been shown to be relatively effective for Petri disease in Spain (Gramaje *et al.*, 2009), South Africa (Fourie and Halleen, 2004) and for black foot (Halleen *et al.* 2007). Rego *et al.* (2009) also demonstrated that when dormant grapevine canes were soaked for 50 min in a range of fungicides prior to grafting, the field-grown plants harvested 9 months later had reduced numbers of woody trunk pathogens, including those caused by the Botryosphaeriacea. However, to-date no comprehensive investigations have been conducted into management of the Botryosphaeriaceae fungi in grapevine propagation material. This research project investigated the efficacy of fungicides for preventing infection of grapevine shoots and for eliminating these pathogens from grapevine canes, as well as different hot water treatments for eliminating the pathogens.

Materials and methods

Experiment 1. Use of fungicide to protect trimming wounds in a source block

This experiment was conducted in a source block of rootstock variety 101-14 in a commercial grape-

vine nursery in New Zealand. The vines which provided the canes for propagation (mother vines) were trained in an upright trellis which usually reached 3–5 m by the end of each growing season. The 20 randomly selected vines each had at least 12 canes, of which four canes were allocated to each of the different inoculation treatments*: N. luteum*, *N. parvum* or a non-inoculated control. The selected mother vines were randomly assigned to be fungicide treated or non-treated controls, 10 of each.

The conidial suspensions of *N. luteum* and *N. parvum* isolates were produced from infected detached green grapevine shoots as described by Amponsah *et al.* (2012a). Suspensions of conidia were produced by washing the shoots in sterile water and the conidial concentrations determined with a hemacytometer. After dilution to give 1×10^4 mL^{-1} conidial suspensions, the viability of conidia in each suspension was confirmed by spreading 50 µL aliquots of a 10^3 mL^{-1} dilution onto three replicate plates of potato dextrose agar (PDA; Difco). After incubating the plates at 25°C for 1–2 days they were observed with a stereo microscope (× 40 magnification) for presence of germinating conidia.

For each inoculation date, all lateral shoots of 2–30 cm (that had developed within the last month) were removed using sterile secateurs, leaving a 3–5 mm stub, and marked with cable ties of different colours for each inoculation period. The designated shoot sections of the vines were then sprayed with flusilazole at a rate of 1 mg a.i. L^{-1} using a knapsack sprayer, while negative control vines were sprayed with tap water. Flusilazole was selected because it was effective *in vitro* against *N. luteum, N. australe* and *Diplodia mutila,* and in New Zealand vineyard experiments it prevented infection of grapevine pruning wounds by *N. luteum* (Amponsah *et al.*, 2012b). Sprayed vines were allowed to dry for 1–2 h prior to inoculation on each wound with 20 µL of the designated conidial suspension or sterile water (control). The same treatments were repeated on the new growth of the same shoots, monthly in December 2009, January and February 2010.

All treated shoots were harvested when dormant (then called 'canes') in July 2010 and stored at 2°C until processed within 6 weeks. For each fungicide-time combination, two canes per vine were randomly selected for assessment (a total of 40 canes per treatment). From each shoot, 8–9 cm sections were cut from 4 cm above and 4 cm below each inoculation point, surface-sterilised by dipping in 70% ethanol for 30 s and flamed to dry off the ethanol. Each cane was then transversely dissected into eight segments of 1 cm (with the inoculation point being discarded) plated onto PDA supplemented with chloramphenicol (0.05 g L^{-1}; PDAC) and incubated at 25°C for 5–7 days. Plates were assessed daily after 3 days for colony growth from the segments which was characteristic of the inoculated species. Another three to five canes were randomly selected from each treatment and inoculation period, and isolation carried out using the same method as above except that the bark and wood of each were separated before plating onto PDAC.

Experiment 2. Hot-water treatment of inoculated canes

Dormant one-year-old canes of rootstock variety 5C, which were ~40 cm long and contained 3-4 nodes were obtained from a commercial nursery in winter. They were stored at 2°C for 4 months prior to the experiment and then removed from cold storage and placed at room temperature (~20°C) overnight (12–16 h) prior to experimentation. The isolates of *N. luteum* (L106, L114 and L228) and *N. parvum* (P178, P94, and P116) used in these experiments had previously been identified by amplified ribosomal DNA restriction analysis (ARDRA) and sequence analysis of ribosomal RNA genes as described by Billones-Baaijens *et al*. (2013a). Mycelium plugs of these isolates, which were stored at -80°C, were sub-cultured onto PDA and incubated at room temperature for 4 days. Immediately prior to inoculation, the colonised PDA plates for three isolates of each species were mixed by chopping and light grinding with a sterile mortar and pestle. A 2–3 mm deep wound that exposed the pith was made with a 4 mm cork borer into the middle of an internode of each cane. The hole was immediately filled with the mycelial mixture of the designated species and covered with a single layer of Parafilm® (Pechiney Plastic Packaging Co., Chicago). All inoculated canes were incubated for 1 week at ambient temperature to allow for mycelial growth within the cane.

The inoculated canes were tied into bundles of five using a cable tie, with four replicate bundles for each species and hot water treatment (HWT). The HWTs were: (1) 50°C for 30 min; (2) 53°C for 30 min; (3) 53°C for 60 min; (4) control with cold water for

30 min. The randomly selected bundles were treated separately by immersing them in a hot water bath followed by cooling through 1 h immersion in cold tap water. To ensure that cross-contamination could not occur, the water in the water bath and cooling bath were replaced between species and treatments. The bundles of canes were air-dried on paper towels in a laminar flow cabinet for 2 h and then placed into individual plastic bags and held at room temperature (20-25°) for ~24 h. The treated canes were surface-sterilised by dipping in 70% ethanol for 30 sec and flaming to dry off the ethanol. For each cane, a section was removed spanning six cm from above and below the ~1 cm inoculation site (~13 cm long) which was then transversely dissected into 13 segments of 1 cm. The segments were placed onto PDA plates with their original positions being labelled. The plates were sealed with food-grade clingfilm and allocated to positions on a laboratory bench in a randomised complete block design (RCBD) for incubation at room temperature for 7 days. After 3–5 days, they were observed daily for characteristic mycelial growth of the inoculating species. Presence of the inoculating fungi in any of the 13 segments indicated incidence of the pathogen, and the number of pieces infected with the inoculating pathogen indicated the degree of pathogen development (infection severity).

Experiment 3. Hot-water treatment of naturally-infected canes

Dormant one-year-old canes (~40 cm) of rootstock variety 5C from the same source as in the previous experiment were used. They were held in cold storage, acclimatised as before and tied into eight bundles of five canes each and then treated as in Experiment 2, with 1 cm segments cut from the centre of each cane used for 13 isolations per cane, as described in Experiment 2. The isolates from the naturally infected canes were identified by ARDRA (Billones-Baaijens *et al.*, 2013a).

Experiment 4. Effects of hot-water-treatment on plant growth

The same batch of dormant one-year-old 5C canes was acclimatised after cold storage as before. There were four replicate bundles of five canes each for each of the HWT treatments of 53°C for 30 min and 60 min, and a control with cold water for 30 min, which were carried out as described in Experiment 2. After cooling, air-drying and holding for 24 h at room temperature, the canes were planted in plastic trays filled with 1–3 mm pumice and arranged in a randomised complete block design (RCBD) with each tray representing a replicate block. The trays were randomly allocated to places on a heat pad (27°C) inside a shade house and after 8 weeks, plants were uprooted and the number of plants with developing shoots and roots was recorded.

Experiment 5. Brief hot water-treatment to eliminate superficial infections

Sauvignon blanc dormant one-year-old canes, which were ~20 cm and contained 2 nodes, were obtained from the Lincoln University Vineyard. They were washed under tap water and air-dried on a laboratory bench at room temperature for 2 h and then stored at 2°C for 4 months. The canes were removed from storage and allowed to acclimatise at room temperature overnight. The canes were then surface-sterilised by soaking in 0.5% sodium hypochlorite for 1 minute and rinsing twice with sterile tap water, and allowed to air-dry for 2 h in a laminar flow cabinet.

Conidial suspensions of *N. luteum* isolates L106 and L228 were produced from infected detached green grapevine shoots as before. The conidial suspensions (1×10^4 mL^{-1}) were combined to provide a mixed isolate inoculum. The tubes of conidia were kept in 4°C water until used for inoculation (1–3 h). Each cane was marked for inoculation using a permanent marker pen; two circles of ~1 cm diameter were drawn on the bark, at 1 cm from the upper end and 1 cm below the first node. A 10 uL drop of the conidial suspension (100 conidia) was placed in each circle and spread gently around the circle area using the tip of a pipette. The canes were air-dried in a laminar flow cabinet for 10–20 min and then incubated in individual plastic bags at room temperature for 24 h. There were four replicate canes for each of the seven treatments which were randomly selected for the designated HWTs, being 55 or 60°C, for 5, 8 or 10 min, and an inoculated, cold water treated control. The canes were cooled and air-dried as before, then held at room temperature in a sterile, closed container for 24 h prior to assessment.

For isolation, ~3 cm segments were excised, each segment contained the 1 cm inoculated circle plus 1

cm above and 1 cm below it. The segments were surface sterilised with 0.5% sodium hypochlorite for 1 minute, rinsed twice in sterile water then air-dried for 30 min inside a laminar flow cabinet. The bark was removed from each segment and then the bark and wood segments were each dissected into three ~1 cm pieces which were plated onto PDA, followed by incubation as described in Experiment 2. The remaining sections of the canes which each contained one node were encouraged to produce roots and shoots in the laboratory. The canes were inserted into 1 cm holes cut ~4 cm apart in a 1.5 cm thick polystyrene sheet (20 × 30 cm). Each polystyrene sheet was placed in a plastic tray containing ~2 cm depth of tap water, with one tray representing each treatment. The trays were placed on laboratory shelves at room temperature with a 12:12 h diurnal regime which comprised fluorescent light and darkness. After 4 weeks, the number of plants with developing shoots was recorded. The experiment was repeated with Pinot noir canes from Lincoln University Vineyard. The HWT treatments were 55°C for 10 min, 57°C for 8 and 10 min and an inoculated untreated control. All other methods were the same as in the previous experiment.

Experiment 6. Fungicide dips to eliminate superficial infections

Winter-dormant canes of Sauvignon blanc were collected from the Lincoln University Vineyard. They were washed under tap water and air-dried on a laboratory bench at room temperature for 2 h and then stored at 2°C for 2 weeks. The canes were removed from storage, allowed to acclimatise at room temperature overnight and then cut into one node sections of ~10 cm. The cane sections were surface sterilised in 0.5% sodium hypochlorite and rinsed twice in sterile water. After air-drying, they were marked and inoculated as described in Experiment 5. The circles of ~1 cm diameter drawn at 2 cm from the upper ends were inoculated with conidia of *N. luteum* (mixed isolates L106 and L228), then air-dried for 30 min and incubated in separate plastic bags for 24 h as described in Experiment 5. They were then soaked for 30 min, with occasional agitation, in one of the following systemic fungicides: carbendazim (MBC 500 Flo; 0.25 g a.i. L^{-1}), tebuconazole (Hornet™ 430 SC; 0.08 g a.i. L^{-1}), thiophanate methyl (Topsin M-4A; 0.2 g a.i. L^{-1}) and flusilazole (Nustar® 0.05 g a.i. L^{-1}). There was also a non-inoculated and inoculated control, both of which were soaked in sterile water for 30 min. A polyether-modified trisiloxane adjuvant (Engulf™) was also added to each fungicide at the alternative rates, 0, 0.5 and 1 mL L^{-1}. The canes were tied in bundles of four for each of the four replicates for each treatment. After treatment, the canes were allowed to air-dry on paper towels in a laminar flow cabinet for 2–3 h, then wrapped in paper towels and held at room temperature overnight. For isolations, the canes were surface sterilised with 0.5% sodium hypochlorite and rinsed twice in sterile water as previously described. From each cane, three 1 cm tissue pieces were cut at and next to each inoculation point, the bark and wood separated and plated onto PDA as before.

To prepare plants for a separate field experiment, dormant, three node canes of 30–40 cm, comprising varieties Sauvignon blanc (1,500) and Pinot noir (500), were collected from the Lincoln University Research Vineyard. They were washed as described in the previous experiment and stored for 1–2 weeks at 2°C. The vineyard which had previously been free of Botryosphaeriaceae species had become infected the previous year due to planting of new vines; 13% of canes were recently found to be infected in another experiment in which cane samples were subjected to separate isolations from the bark and wood. Results showed that most (~95%) of the infections were found in the bark, with only ~5% within the wood (unpublished data). These canes were therefore considered suitable for treating with fungicides to attempt elimination of the pathogen. The canes were removed from cold storage and allowed to acclimatise at room temperature overnight. They were then soaked for 30 min in carbendazim (0.5 mL L^{-1}). All treated canes were then placed upright in trays containing 20 cm of 1–3 mm vermiculite and placed on a heat pad (27°C) for 6 weeks for root development. To check the efficacy of the treatment, 1 cm samples were trimmed from the tops of all canes 4 weeks after they were treated with fungicide and set up for rooting. These samples were surface sterilised in 0.5% sodium hypochlorite, followed by two rinses in sterile water and placed onto PDA for isolation. They were incubated at 25°C and inspected after 4 and 7 days for presence of mycelia characteristic of Botryosphaeriaceae species The rooted canes were transplanted into pots of a commercial potting mix and grown in a non-heated tunnel house. The number of plants that developed healthy shoots was

recorded. A further 100 Sauvignon blanc canes were left untreated and used for immediate isolation with segments cut at 2-3 cm from each end and in the middle of each cane.

Data analyses

All data were initially tested for homogeneity of variance using Levene's test at $P<0.05$. The significance of treatments was determined using two-way ANOVA conducted with Genstat for all experiments. Means from treatments that were significant at $P\leq0.05$ were separated by pair-wise comparisons using Fisher's least significant differences (LSD) at $P\leq0.05$. The relevant data tables presented were generated by ANOVA. In Experiment 1, differences in disease incidence between bark and wood were determined using a t-test at $P\leq0.05$. In Experiment 2, the relationship between inoculation site and isolation positions was tested with Pearson's chi-square test.

Results

Experiment 1. Use of fungicide to protect trimming wounds in a source block

There was a significant effect of inoculation time ($P=0.001$), with the lowest ($P\leq0.05$) proportion of infected cane segments from the December inoculation (15.0%) but similar proportions from the January (22.9%) and February (19.4%) inoculations. Disease incidence differed between inoculation treatments ($P=0.051$; Table 1), with significant ($P\leq0.05$) differences between *N. parvum* (24.0%), *N. luteum* (19.7%) and non-inoculated canes (12.3%). The incidence of Botryosphaeriaceae species in cane segments differed significantly between bark and wood ($P<0.001$), with bark having 19.9% incidence and wood having 9.6% incidence. Overall incidence of these species in the cane segments was significantly affected by fungicide treatment ($P=0.036$), with fungicide-treated vines having 19.5% of segments infected while vines without fungicide treatments had 24.3% infection in the cane segments. There was no significant interaction between fungicide and inoculation treatments ($P>0.05$). Viability of the conidia used for this experiment was 93-97% across the replicate plates.

Table 1. Mean incidence (%) of infection in dormant grapevine canes for which the trimming wounds of the shoots had been treated during the growing season with the fungicide flusilazole prior to inoculation with *Neofusicoccum luteum* and *N. parvum*.

Pathogen inoculum	Treatments		
	Fungicide	No fungicide	Means
N. luteum	21.0 a[a]	18.4 b	19.7 b
N. parvum	20.2 a	27.8 a	24.0 a
Non-inoculated control	15.4 b	9.2 c	12.3 c
Treatment means	19.5 A[b]	24.3 B	

[a] Means within a column.
[b] Means within the final row which have different letters are significantly different according to Fisher's LSD at $P\leq0.05$

Experiment 2. Hot-water treatment of inoculated canes

There were significant effects of the treatments ($P<0.001$) on infection incidence, with the control canes having significantly greater mean levels of infection incidence and severity than the HWT canes. There was also an effect of species

Table 2. Mean pathogen incidence and severity (extent of infection) in canes treated with different temperatures and times of hot-water treatments 1 week after being inoculated with *Neofusicoccum luteum* and *N. parvum*

Species	Hot-water-treatment	% Incidence	% Severity
N. luteum	50°C – 30 min	55 b[a]	13.5 cd
	53°C – 30 min	0 c	0 e
	53°C – 60 min	0 c	0 e
	No HWT	100 a	87.5 b
N. parvum	50°C – 30 min	100 a	17.3 c
	53°C – 30 min	50 b	8.1 d
	53°C – 60 min	85 b	11.5 cd
	No HWT	100 a	100 a

[a] Means within a column with different letters are significantly different according to Fisher's LSD at $P\leq0.05$

Table 3. Infection incidence (out of 20 replicates) of *Neofusicoccum luteum* and *N. parvum* in continuous positions along cane segments, with 0 indicating the inoculation point, negative numbers 1 cm sections below and positive numbers 1 cm sections above position 0. The different hot-water treatments (HWT) of the canes were applied 1 week after inoculation.

Species	HWT	Tissue sections at 1 cm distances from inoculation points (0)												
		-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6
N. luteum	50°C 30 min[a]	2	4	2	1	3	4	7	4	1	2	0	2	3
	53°C 30 min[a]	0	0	0	0	0	0	0	0	0	0	0	0	0
	53°C 60 min[a]	0	0	0	0	0	0	0	0	0	0	0	0	0
	Control means	15.3	17.3	19.0	19.3	19.7	20	20	20	19.7	18.3	17.7	16.7	15.3
N. parvum	50°C 30 min[b]	0	0	0	0	0	19	18	8	0	0	0	0	0
	53°C 30 min[b]	0	0	0	0	1	5	7	4	3	1	0	0	0
	53°C 60 min[b]	0	0	0	0	0	3	13	10	0	1	0	1	0
	Control means	20	19.7	20	20	20	20	20	20	20	20	20	19.7	20

[a] Association between tissue infections and distances from the inoculation point were not significant (P=0.322) using Pearson Chi-square test

[b] Association between tissue infections and distances from the inoculation point were significant (P<0.001) using Pearson Chi-square test

(P<0.001), with *N. parvum* being more resistant to the heat treatments than *N. luteum* (Table 2). The inoculated control canes (no HWT) had 100% infection incidence. Treatment of 50°C for 30 min significantly reduced (P≤0.05) mean incidence of *N. luteum* (55%) but not *N. parvum* (100%). The higher temperature of 53°C completely eliminated *N. luteum* infection incidence and reduced *N. parvum* incidences (P≤0.05) similarly for the 30 and 60 min treatments (Table 2).

Treatment effects on infection severity (extent of cane colonisation) were also significant for both species (P<0.001) (Table 3). The isolations from the 13 sections of 1 cm showed that in the control canes inoculated with *N. luteum*, most segments from all positions were infected with *N. luteum* (Table 3). After treatment at 50°C for 30 min, there were significantly fewer (P≤0.05) isolations along the 13 sections of the canes used, but some segments were infected with *N. luteum* even to the end of the cane sections. There was no significant correlation between infected segment positions and initial inoculation site (P=0.322). No segments were infected with *N. luteum* after 53°C treatments for 30 and 60 min. For *N. parvum*, the inoculated control canes were infected in almost all segments along the 13 positions. The reduced severity after treatment at 50°C for 30 min seemed to be associated with the zero incidences further than 1 cm from the inoculated positions. Treatment at 53°C for 30 and 60 min did not reduce severity further (P>0.05) (Table 3). For *N. parvum* there was a significant correlation between positions of infected segments and the initial inoculation site (P<0.001).

Experiment 3. Hot-water treatment of naturally-infected canes

There was a significant effect of the treatments on incidence of infection (P<0.001), with 35% incidence in canes without hot water treatments, comprising species *N. luteum* (17%), *N. parvum* (4%), *Diplodia seriata* (10%), and *D. mutila* (4%). Incidence was 0% for all other treatments except 53°C for 60 min, for which there was a single isolation (5%) of each species, *N. luteum, N. parvum* and *Botryosphaeria dothidea.*

Experiment 4. Effects of hot-water-treatment on plant growth

There was no bud burst or root development on canes treated at 53°C for 30 and 60 min. In contrast, the non-treated canes had well-developed roots and shoots after 8 weeks (Figure 1).

Figure 1. Effects of hot-water treatment on root development and bud burst of rootstock canes (variety 5C). Treatments were: (A) 53°C-30 min; (B) 53°C-60 min; (C) control canes with no HWT.

Experiment 5. Brief hot-water-treatment to eliminate superficial infections

There was a significant effect (P=0.028) of the treatments on incidence of the pathogens. For Sauvignon blanc, all heat treatments reduced incidence of *N. luteum* infection to 0% in comparison to the non-treated control, for which the infection incidence was 50% (Table 4). There was also a significant effect of the heat treatments on the proportion of canes that developed shoots from the buds which were dormant at the time of the treatment (P≤0.001). Treatment at 55°C had similar budburst to the non-treated control for all three treatment times which was significantly greater (P≤0.05) than budburst after treatment at 60°C, which was 0 for all treatment times. For Pinot noir, treatments at 55°C (10 min) and 57°C (8 and 10 min) reduced incidence of *N. luteum* infection to 0% in comparison to the non-treated control, for which the infection incidence was 25%. However, none of the treatments exhibited any budburst, in comparison to the control for which there was 100% budburst.

Experiment 6. Fungicide dips to eliminate superficial infections

There was a significant effect of the fungicide treatments on disease incidence (P <0.001), with significantly lower incidence (P≤0.05) in the carbendazim and tebuconazole treatments than the other treatments. The adjuvant concentrations had no affect (P=0.482), but there was a significant fungicide by adjuvant interaction (P=0.009) which was associated with the differential effects of the adjuvant with carbendazim and tebuconazole; carbendazim with no adjuvant and tebuconazole with 0.5 mL/L adjuvant were the most effective treatments as they completely eliminated the cane infections (Figure 2).

The isolations from 2000 canes soaked for 30 min in a carbendazim solution prior to rooting and

Table 4. Mean pathogen incidence in Sauvignon blanc and Pinot noir canes treated with different temperatures and times of hot-water treatments being superficially inoculated with *Neofusicoccum luteum* conidia and percent budburst of treated canes after 4 weeks in growth conditions

HWT Temp & Time (min)		Incidence	Bud burst (%)
Sauvignon blanc			
55°C	5	0 b [a]	100 a
	8	0 b	75 a
	10	0 b	100 a
60°C	5	0 b	0 b
	8	0 b	0 b
	10	0 b	0 b
Control	0	50.0 a	100 a
Pinot Noir			
55°C	10	0 b [a]	0 b
57°C	8	0 b	0 b
57°C	10	0 b	0 b
Control	0	25 a	100 a

[a] Means within a column with different letters are significantly different according to Fisher's LSD at $P \leq 0.05$.

planting for another experiment found no Botryosphaeriaceae-like fungi, although those from the untreated canes from the same source showed 17% infection by Botryosphaeriaceae species Of the 2000 canes treated, two Sauvignon blanc canes failed to break bud. The remainder were vigorous, producing shoots of ~40 cm within 8 weeks. Three Pinot noir canes failed to break bud although the remainder produced shoots of~ 25 cm within 8 weeks.

Discussion

The fungicide applications to trimming wounds in the source blocks did reduce rates of infection overall, however they did not give complete protection against the inoculated fungi or natural inoculum. Since 12.3% of segments in control shoots were infected through natural means, this showed that ongoing natural infection could have caused further infections between times of fungicide application. Further, the greater number of natural bark infections than wood infections indicated that trimming wounds were not the main routes of infection for these fungi. Clearly, further investigations are needed to find how fungicides might be used to prevent bark infection in source blocks. Potentially

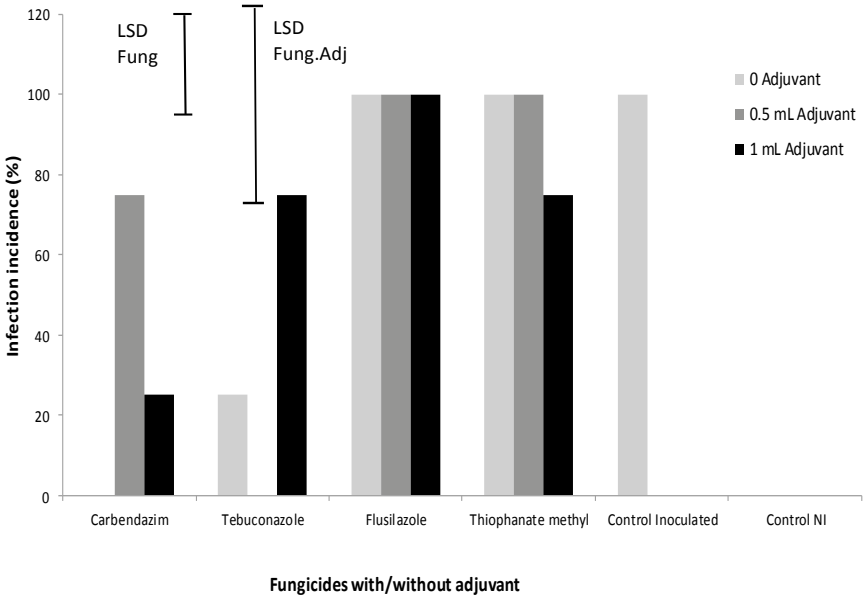

Figure 2. Effects of soaking grapevine canes superficially infected with *Neofusicoccum luteum* conidia in fungicide solutions, with or without a polyether-modified trisiloxane adjuvant (Engulf™), on incidence of the pathogen. Error bars are the 5% Fishers least significant differences (LSDs) for the fungicides (27.8%) and the fungicide.adjuvant interaction (48.1%).

these could include a range of fungicides, such as carbendazim and tebuconazole which were found to eradicate bark infections in the final experiment of this research project. Use of adjuvants, to improve retention of the fungicides, and multiple fungicide applications may also provide greater efficacy than single applications.

The HWT protocols tested in the current study demonstrated that the standard HWT protocol of 50°C for 30 min significantly reduced the incidence of *N. luteum* to 55% but did not affect incidence of *N. parvum* (100%). Since the most stringent treatment (53°C for 60 min) did not eliminate the infection by *N. parvum* in canes with wood infection, this method alone cannot be considered to provide effective control for nursery propagators. However, Luque *et al.* (2014) who reported significant reductions in incidence of *D. seriata*, *Dothoriella viticola*, *N. luteum* and *N. parvum* after treatment of the infected canes for 30 min at 51°C considered that HWT could be used to control Botryosphaeriacea species in grapevine propagation. Further, Crous *et al.* (2001) reported that relatively few naturally-occurring Botryosphaeriaceae species were found in dormant canes which had been treated at 50°C for 30 min. The different tolerances of these fungi to HWT are unlikely to be associated with their optimum growth temperatures which were similar for New Zealand isolates of *N. luteum* and *N. parvum* (26.3 and 26.1°C, respectively) (Baskarathevan *et al.*, 2012a), and Californian isolates (29.4 and 28.2°C, respectively) (Úrbez-torres *et al.*, 2006a).

The pathogens that cause Petri disease have also been reported to have different tolerances to a range of HWT temperatures. In South Africa, Crous *et al.* (2001) reported that HWT at 50°C for 30 min was completely effective against *Phaeomoniella chlamydospora* although Fourie and Halleen (2004) reported that it was not completely effective against *Phaeoacremonium* spp. Similar results were reported from Australia by Edwards *et al.* (2004) who reported that HWT at 50°C for 30 min eliminated 88% of the *Pa. chlamydospora* but was ineffective against *Pm. aleophilum*. However, they also reported that the same temperatures were ineffective for dormant rooted plants infected with *Pa. chlamydospora*, which they concluded was due to the well-established infections being difficult to eradicate. A similar result was demonstrated in the current study, in which the newly developed mycelia of both pathogens were largely eradicated although the mycelia at ~1 cm from the inoculated area were much less affected by the treatments. In Spain, Gramaje *et al.* (2009) found similar effects, with *Pa. chlamydospora* being almost completely eliminated from the canes after 60 min at 50°C, but that *Pm. aleophilum* was more resistant, with 37.5% of canes remaining infected.

In the current study, HWT at 53°C was clearly damaging to the canes since none of them were able to grow roots or shoots in contrast to the standard 50°C for 30 min, which was shown to have no effect on subsequent growth of dormant plants in New Zealand (Bleach *et al.*, 2013) or southern Australia (Edwards *et al.*, 2004). In contrast, the experiments conducted by Gramaje *et al.* (2009) in Spain found that 73–96% of canes were able to sprout after 53°C treatments, with no differences between the 30 and 60 min treatments. Further, Gramaje *et al.* (2014) reported that HWT at 53°C for 30 min did not affect plant viability and yield parameters during the following 4 years growth in the field. The differences between the effects of cool and warm regions were discussed by Waite and Morton (2007), who concluded that the physiological features of canes and plants (and possibly their pathogens) grown in cool climates caused greater susceptibility to HWT than those grown in warmer climates. The efficacy of some short duration HWT treatments against superficial infections shown in the current study indicated that further research could provide for control of these pathogens in some grapevine varieties. Waite and May (2005) reported that there was a range of nursery factors, including variety, size of canes, harvest time, duration of hydration and cooling, as well as storage conditions, which significantly affected the ability of canes to withstand HWT, and these should be incorporated into such research trials.

The experiments which soaked dormant canes in fungicide suspensions showed promising results, with the carbendazim and tebuconazole treatments significantly reducing incidence of infection during the first experiment, and carbendazim completely eliminating natural infection from dormant canes. However, thiophanate-methyl (a benzimidazole) was ineffective, although it provided some control with the adjuvant at the highest rate. In addition, flusilazole was ineffective although the other systemic triazole, tebuconazole, was relatively effective. All these fungicides were described by MacBean (2012) as being absorbed by roots and leaves, and being systemic with protective and curative activ-

ity, with both fungicides in each group being used to control similar pathogens. The fungicides tested in the current research have all been reported to be effective against isolates of the Botryosphaeriaceae species. In New Zealand, Amponsah *et al.* (2012b) reported that flusilazole, carbendazim, tebuconazole, thiophanate-methyl and mancozeb were effective at reducing mycelial growth and/or conidial germination of three isolates each of *N. australe*, *N. luteum* and *D. mutila*, with *N. luteum* generally showing the greatest resistance. Further, these fungicides effectively prevented infection of grapevine pruning wounds by *N. luteum* conidia in 100, 93, 87, 83 and 80% of field vines, respectively. In Australian field trials, Pitt *et al*. (2012) also reported that flusilazole, carbendazim and tebuconazole reduced infection of pruning wounds by *D. seriata* and *D. mutila* by 15, 29 and 35%, respectively. However, these fields had abundant natural inoculum, with 48% incidence in the non-inoculated control, and so single applications of the fungicides were not as effective as the New Zealand trial where the experimental vines were not exposed to natural inoculum. Luque *et al.* (2008) also reported that carbendazim and thiophanate-methyl were effective at preventing Botryosphaeria canker of cork oak, caused by *Botryosphaeria corticola*, if applied to the exposed wood after cork removal from the trees. It therefore seems likely that the efficacy or lack thereof in the current research may have been associated with the abilities of the fungicides to be absorbed into the cane tissues. MacBean (2012) reported that tebuconazole was rapidly absorbed in vegetative tissues, which by definition includes bark and wood, and Barak *et al.* (1983) demonstrated that carbendazim was strongly adsorbed onto lignin, which indicated its uptake into woody stems and tissues within the apoplastic pathway of stems. In general, uptake of fungicides into plants is known to be improved by specific adjuvants, however, there appeared to be little value from addition of the adjuvant Engulf in the current research, despite its reported abilities as providing for superior penetration of plant tissues (Smith *et al.*, 2014). However, this study showed no clear trends of greater efficacy with increasing concentration which could be reliably interpreted to indicate potential efficacy. There is clearly a need for further research which should be conducted with different adjuvant and fungicide treatments and perhaps longer soaking times, as used by Rego *et al.* (2009). When they soaked the canes in a range of fungicides for 50 min prior to grafting, the plants lifted 9 months later from the field nurseries demonstrated reduced rates of infection to a range of pathogens including the Botryosphaeriaceae.

For preventing Botryosphaeriaceae infection of nursery propagation material, an integrated disease management programme may be needed which targets the pathogens throughout the propagation process. The comprehensive studies of Billones-Baaijens *et al.* (2013a; 2013b; 2015) have indicated likely Botryosphaeriaceae inoculum sources and infection pathways, including a latent phase represented by bark infection. The control strategies within an integrated programme should aim to target these sources and pathways during all phases of propagation, including shoot growth, harvesting and storage, rather than relying solely on methods of pathogen eradication such as those investigated in this research. For stem end rot of mango, which is caused by *Diplodia* species that infect the inflorescences and remain latent until fruit ripening, Akem *et al.* (2013) reported that an integrated programme provided good control and extended the shelf life of the ripe fruit. This programme included seasonal fungicide applications which targeted the susceptible stages of fruit development and so reduced stem end rot incidence by harvest, as well as careful handling and sanitation after harvest. HWT of the fruit at 52–55°C for 10 minutes further reduced incidence of stem end rot pathogens and an additional treatment of fungicide soaks for 5 min using tebuconazole (125–156 ppm) or carbendazim (312.5 ppm) further extended the shelf life of the fruit, possibly by preventing reinfection by these pathogens. The additional tebuconazole and carbendazim treatments reduced incidence to 20.0 and 11.7% compared to non-treated control fruit (70% incidence) after cold storage for 28 d followed by 6 days at room temperature. They further suggested that the fungicides could be incorporated into a HWT with potential for improved penetration.

This research represented a first step in developing an integrated management programme for preventing Botryosphaeria disease of grapevine propagation materials. It has indicated that further research should be conducted in source blocks with more fungicide and adjuvant treatments; multiple fungicide application could significantly reduce incidence of infection in the harvested canes. HWT at 50°C for 30 min or longer may further reduce infection incidence and should be investigated in a range

of grape-growing regions with all the relevant varieties and Botryosphaeriaceae species. Further, this research programme has indicated that soaking the harvested canes in fungicide suspensions may eradicate their latent bark infections. However, further research using a range of fungicides and adjuvants with longer soak periods should be conducted to confirm these results and provide for greater efficacy against a wide range of Botryosphaeriaceae species.

Acknowledgements

The authors acknowledge Winegrowers New Zealand for funding this research and New Zealand grapevine propagators for access to source blocks and plant materials.

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Accepted for publication: June 16, 2015
Published online: September 15, 2015