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RESEARCH PAPERS - 9TH SPECIAL ISSUE ON GRAPEVINE TRUNK DISEASES

Viability of Botryosphaeriaceae species pathogenic to grapevine after hot water treatment

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Summary. The viability of eight species of Botryosphaeriaceae pathogenic to grapevine was studied after a hot water treatment (HWT) in order to evaluate the feasibility of this technique as a potential tool to control these species during the grapevine propagation process. In a first trial (*in vitro*), mycelial plugs contained in Eppendorf tubes with sterile distilled water were subjected to different combinations of temperature (50–54°C) and exposure time (15, 30 and 45 min) in a hot water bath. Growth rates of treated mycelia were compared to untreated controls. Significant differences in survival and growth for all factors (species, temperature and time) and their 2-way interactions were observed. Fungal survival and growth generally decreased with increasing temperatures and exposure times. *Diplodia seriata*, *Neofusicoccum luteum*, *N. parvum* and *Spencermartinsia viticola* were the most susceptible species to temperature, while *Lasiodiplodia theobromae* and *N. vitifusiforme* were the most tolerant. In a second trial (*in planta*), the fungi were inoculated into grapevine canes (Richter 110 rootstock). Inoculated canes were incubated at 25°C for 3 weeks to allow the fungi to colonize the wood and then subjected to HWT in the range of 50–53°C for 30 min, and survival of fungi after HWT was assessed. Survival of all species was sharply reduced after HWT of 30 min at 51°C and higher temperatures. At 50°C, *Lasiodiplodia theobromae* was the most tolerant species whereas *N. luteum* was the most susceptible. Results obtained in this study demonstrate the feasibility of controlling these pathogens by HWT in the nursery grapevine propagation process.

Key words: Botryosphaeria dieback, control, grapevine trunk diseases, nursery, propagation.

Introduction

Several species included in the Botryosphaeriaceae Theiss. & Syd. are causal agents of Botryosphaeria dieback of grapevine (*Vitis vinifera* L.), and have been reported as major trunk pathogens of grapevines worldwide (Úrbez-Torres, 2011). These pathogens cause wood necrosis in trunks and arms of infected vines, which decrease the productivity and longevity of vineyards (Úrbez-Torres, 2011; Bertsch *et al*., 2013). Although the incidence of Botryosphaeria dieback is common in 8-year and older vineyards (Larignon and Dubos, 2001), Botryosphaeriaceae species have also been reported from grapevine nurseries. These pathogens have been isolated from grapevine rootstock mother plants, canes of scion and rootstock varieties, nursery cuttings and young grafted grapevines in nurseries (Fourie and Halleen, 2002; 2004a; Halleen *et al*., 2003; Giménez-Jaime *et al*., 2006; Aroca *et al*., 2010; Spagnolo *et al*., 2011; Billones-Baaijens *et al*., 2013a; 2013b). These findings demonstrate that Botryosphaeriaceae species are present in grapevine propagation material and in the grafted plants supplied by nurseries, indicating that some of the current vineyard infections may have originated in the propagation process (Billones-Baaijens *et al*., 2013b). However, in some cases the presence of Botryosphaeriaceae species in grapevine propagation material

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was not related to the observed external symptoms (Fourie and Halleen, 2004a; Aroca *et al*., 2010; Billones-Baaijens *et al*., 2013b). Presence of these pathogens in symptomless grapevines could be explained by published reports that indicate that Botryosphaeriaceae species can behave as endophytes or latent pathogens in asymptomatic wood of symptomless plants (Slippers and Wingfield, 2007; Phillips *et al*., 2013). Therefore these pathogens could be carried into vineyards in apparently healthy plants used for vine establishment or replacement (Billones-Baaijens *et al*., 2013b).

Control of endogenous fungal pathogens in grapevines is difficult and there is an urgent need to revise the management strategies applied in nurseries to improve the phytosanitary quality of planting material (Gramaje and Armengol, 2011). Several research studies have evaluated the *in vitro* and *in vivo* effectiveness of different fungicides to control Botryosphaeriaceae spp. in vineyards, mainly when applied as pruning wound protectants (Bester *et al*., 2007; Pitt *et al*., 2010; 2012; Rolshausen *et al*., 2010; Amponsah *et al*., 2012). Nevertheless, the application of fungicides to control fungal trunk pathogens in the nursery process is difficult, because traditional techniques, such as chemical sprays and dips used for the control of surface pathogens, do not penetrate sufficiently into dormant grapevine cuttings to control internal infections (Gramaje and Armengol, 2011). In addition, the range of authorized products for this purpose is becoming limited in most countries.

The use of hot water treatment (HWT) at different temperature-time combinations has been reported as a promising management strategy for the control of black-foot and Petri disease pathogens in grapevine propagation material (Fourie and Halleen, 2004b; Gramaje *et al*., 2008; 2009; 2010; 2014; Bleach *et al*., 2013). One of the main advantages of a HWT protocol is that the heat is able to completely penetrate the wood, killing internal pathogens, without any detrimental effects on the vine tissue (Waite and Morton, 2007). Recently, Billones-Baaijens *et al*. (2014) reported that HWT can effectively reduce the infection incidence of *Neofusicoccum luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips and *N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips in artificially infected 5C rootstock cuttings, as well as any existing Botryosphaeriaceae species in naturally infected cuttings. To the best of our knowledge, no additional information on the susceptibility of Botryosphaeriaceae species to HWT is available to date. Therefore, the objective of this study was to assess the susceptibility of eight pathogenic Botryosphaeriaceae species to HWT and to evaluate the efficacy of HWT as a control strategy for Botryosphaeriaceae species in grapevine cuttings. The sensitivity of Botryosphaeriaceae species to HWT was assessed in two complementary tests, by 1) evaluating the survival and growth of fungal mycelia after HWT *in vitro*, and 2) assessing fungal survival in artificially inoculated grapevine cuttings that were subjected to HWT.

Materials and methods

Fungal isolates

Eight Botryosphaeriaceae taxa were tested for survival and growth after HWT, namely *Botryosphaeria dothidea* (Moug.:Fr.) Ces. & De Not., *Diplodia seriata* De Not., *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *N. luteum*, *N. mediterraneum* Crous, M.J. Wingf. & A.J.L. Phillips, *N. parvum*, *N. vitifusiforme* (Niekerk & Crous) Crous, Slippers & A.J.L. Phillips and *Spencermartinsia viticola* (A.J.L. Phillips & J. Luque) A.J.L.Phillips, A. Alves & Crous. Two isolates per taxon were used in this study except for *N. vitifusiforme*, with only one representative isolate (Table 1). All isolates used in the experiments were previously hyphal tip subcultured. Isolates were stored at 4°C in sterile distilled water tubes with mycelial plugs previously grown on potato dextrose agar (PDA; Difco). Fungi were grown for 1 week on PDA plates at 25°C to obtain sufficient quantities of mycelium for the experiments.

Effect of hot water treatment on mycelial survival and growth *in vitro*

An experiment was carried out to check for survival and growth of isolates after treating fungal mycelia at different combinations of temperature (50 to 54°C at 1°C interval) and exposure time (15, 30 or 45 min) in a waterbath (JP SELECTA model Unitronic Vaiven; Abrera, Spain; homogeneity 0.1°C, resolution 0.1°C). All three exposure times were tested initially at 50°C, and temperature was increased in 1°C intervals as fungal growth after HWT was observed in at least 10% of cases. Agar plugs with mycelium (5 mm diam.) were obtained from the actively growing

Table 1. Botryosphaeriaceae species and representative isolates obtained from grapevine and used in this study. CBS[a] accession numbers are given for some isolates.

Species	Culture No.	CBS Accession No.	Geographic origin	Collector / Isolator
Botryosphaeria dothidea	JL353	CBS 121484	Caldes de Montbui, Spain	J. Luque
B. dothidea	JL380	CBS 110302	Montemor-o-Novo, Portugal	A.J.L. Phillips
Diplodia seriata	JL354		Caldes de Montbui, Spain	J. Luque
D. seriata	JL398	CBS 121485	Pacs del Penedès, Spain	J. Luque
Lasiodiplodia theobromae	JL664		Fontanars, Spain	R. Raposo
L. theobromae	JL819	CBS 124060	Marsala, Sicily, Italy	S. Burruano
Neofusicoccum luteum	JL381		Sintra, Portugal	A.J.L. Phillips
N. luteum	JL519	CBS 121482	Gandesa, Spain	J. Luque
Neofusicoccum mediterraneum	JL562		Ginestar, Spain	J. Luque
N. mediterraneum	JL763		Spain	R. Raposo
Neofusicoccum parvum	JL396	CBS 121486	L'Arboç del Penedès, Spain	J. Luque
N. parvum	JL445		Piera, Spain	J. Luque
Neofusicoccum vitifusiforme	JL563	CBS 121481	Gandesa, Spain	J. Luque
Spencermartinsia viticola	JL525	CBS 117007	Gandesa, Spain	J. Luque
S. viticola	JL571	CBS 117009	Vimbodí, Spain	J. Luque

[a] Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

edges of 1-week-old colonies growing on PDA. Four agar plugs per isolate were placed into a 1.5 mL Eppendorf tube containing 1 mL of sterile distilled water, and treated at the above temperature-time combinations in the waterbath. Four Eppendorf tubes per isolate were used as pseudoreplicates in each temperature-time combination. After HWT, Eppendorf tubes were cooled under running tap water for 5 min. The agar plugs were removed from the tubes and placed in the centre of PDA plates, one plug per plate, with the mycelium facing the medium surface. Plates were incubated at 22°C in darkness for 3 d. In addition, six non-treated mycelial plugs were plated onto PDA and incubated under the same conditions as experimental controls. After the incubation period, survival of each mycelial plug was recorded and four colony radii were measured from the colony center at perpendicular angles. Radial growth data were averaged (0.25 cm subtracted from the mean value to account for the original agar plug size), and the relative growth compared to the controls was calculated for each isolate as

$$100 \times (T_{ijk}/C_k)$$

where T_{ij} is the mean of the four PDA plates per Eppendorf tube in each temperature-time combination i, pseudoreplicate j (j = 1–4), and experiment k (k = 1–2), and C_k is the mean of the six control PDA plates per experiment k.

Mean mycelium survival in each temperature-time combination was expressed as a percentage (0, 25, 50, 75 or 100%) thus corresponding to the proportion of living mycelial plugs within the same tube. The experiment was repeated once.

Effect of hot water treatment on mycelial survival *in planta*

The survival of Botryosphaeriaceae species colonizing grapevine wood was evaluated after the *in vitro* test was completed. Extreme exposure times to HWT in the *in planta* assay were not tested because 30 min is the most common duration time used for HWT in grapevine nurseries. This allows temperature to

uniformly equilibrate in the internal wood tissues of rootstock or scion cuttings (Gramaje and Armengol, 2011). The *in planta* test was therefore conducted at different temperatures (50 to 53°C) and with a single 30 min treatment period. Dormant grapevine cane segments (20 cm long) of Richter 110 rootstock obtained from a commercial nursery located in Valencia province (Spain) were surface sterilized for 10 min in a sodium hypochlorite solution (1.5% available chlorine), washed twice with sterile distilled water, and allowed to dry on sterile filter paper in a laminar flow cabinet for 1 h. Two holes (4 mm diam.) were drilled on a cane internode at 10 cm from each other. The drill was sterilized by flaming before each use. A mycelial plug (4 mm diam.) obtained from the growing edge of a 1-week-old fungal PDA colony was placed on the hole and the inoculation site was sealed with Parafilm®. For each isolate and temperature combination, four cane segments were used (eight holes in total for each pseudoreplicate). After inoculation, the ends of each cane segment were sealed with paraffin wax to prevent water loss. Cane controls were inoculated with the fungi as explained above but were not hot water treated. Cane segments corresponding to each time and temperature combination were incubated separately in closed plastic boxes (50 × 35 × 15 cm; 26 L capacity) at 25°C for 3 weeks in darkness, to allow for fungal colonization of the wood. The boxes were opened twice each week to prevent the onset of fermentative respiration that could affect the physiology of the cuttings and the pathogens. After the incubation period, cane segments were treated in a waterbath (JP SELECTA Unitronic 320OR, standard error ± 0.1°C) filled with distilled water and following the above described schedule. After HWT, canes were cooled for 30 min in a bath of tap water at room temperature, and fungal recovery was performed as follows. Two equidistant points (15 mm) were set at each side from the inoculation site. Bark at each reisolation site was removed with a sterile scalpel and a wood chip (5 mm) was cut with a sterile secateur. Each wood chip was then subdivided into two pieces, which were surface sterilized (70% ethanol, 4 min) and plated on malt extract agar (MEA) supplemented with 0.5 g·L^{-1} streptomycin sulphate (Sigma-Aldrich). Fungi were reisolated and identified after 4 d incubation at 25°C. Fungal survival was annotated for each individual wood chip and the percentage of recovery was calculated for each inoculation site (hole). The experiment was repeated once.

Statistical analyses

Mycelium survival in the *in vitro* and *in planta* experiments, and relative colony growth rates in the *in vitro* experiment were logit transformed prior to analyses. The experiments used a multifactorial design with species, temperature and exposure time (when applicable) considered as fixed factors. Experiment repetition and isolates (when applicable) were used as random factors. The *in vitro* experiments used a split plot design with temperature as the whole plot factor and time as the subplot factor. The *in planta* experiments used a completely randomized design. Analyses of variance for the main factors and their 2-way interaction effects on the dependent variables were performed using SAS Enterprise Guide v4.2 running on SAS v9.2 (SAS Institute Inc.), using the MIXED procedure. The Residual Maximum Likelihood (REML) method was used to estimate the variance components. Transformed data means were compared using a least significant difference test ($P = 0.05$).

Results

Effect of hot water treatment on mycelial survival and growth *in vitro*

A preliminary statistical analysis of the whole data sets of both mycelial survival and growth showed that all fixed factors and their 2-way interactions were significant ($P<0.05$), with the Species factor and all 2-way interactions being highly significant ($P<0.001$). Therefore the whole data set was split into different species groups and the data were re-analysed. A summary of the statistical analyses results is shown in Table 2. Regarding mycelium survival, Temperature was significant ($P<0.05$) for *B. dothidea*, *L. theobromae* and *N. mediterraneum*. Exposure time to hot water was significant only for *N. vitifusiforme*. However, the interaction Temperature × Time was significant for all species except *N. vitifusiforme* and *S. viticola* (Table 2).

The mean values of mycelium survival for all the species at different temperature and time combinations are shown in Figure 1. *Spencermartinsia viticola* was the most susceptible fungus to HWT since survival at 50°C for 15 min was only 1.6% and no growth was recorded at higher temperatures and longer exposure times (Figure 1). In a second group, survival of *D. seriata*, *N. luteum* and *N. parvum* at 50°C, 15 min ranged between 26.6 and 56.3 %, but was less than 15%

Table 2. Summary of the statistical analyses of data of survival and growth of mycelia of eight Botryosphaeriaceae species after hot water treatment in the *in vitro* experiment. Variance components estimated through the Residual Maximum Likelihood (REML) method not shown.

Species	Factor	Mycelium survival				Mycelium growth			
		Num DF[a]	Den DF[a]	F value	P > F	Num DF[a]	Den DF[a]	F value	P > F
Botryosphaeria dothidea	Temperature (A)	3	3	11.46	0.0377	3	3	12.97	0.0318
	Time (B)	2	2	14.64	0.0640	2	2	11.68	0.0788
	A × B	6	167	13.78	<.0001	6	167	31.95	<0.0001
Diplodia seriata	Temperature (A)	1	1	1.67	0.4197	1	1	1.39	0.4480
	Time (B)	2	2	4.92	0.1690	2	2	1.81	0.3555
	A × B	2	81	27.95	<.0001	2	81	40.13	<0.0001
Lasiodiplodia theobromae	Temperature (A)	4	4	14.69	0.0117	4	4	8.00	0.0343
	Time (B)	2	2	7.07	0.1469	2	2	21.87	0.0437
	A × B	8	210	5.91	<.0001	8	210	10.78	<0.0001
Neofusicoccum luteum	Temperature (A)	1	1	1.43	0.4430	1	1	0.76	0.5436
	Time (B)	2	2	1.22	0.4508	2	2	0.84	0.5444
	A × B	2	66	23.16	<.0001	2	66	13.20	0.0005
Neofusicoccum mediterraneum	Temperature (A)	3	3	9.72	0.0470	3	3	5.81	0.0911
	Time (B)	2	2	9.39	0.0962	2	2	23.41	0.0410
	A × B	6	167	5.63	<.0001	6	167	23.65	<0.0001
Neofusicoccum parvum	Temperature (A)	1	1	1.44	0.4422	1	1	1.43	0.4438
	Time (B)	2	2	1.72	0.3670	2	2	1.39	0.4190
	A × B	2	81	26.62	<.0001	2	81	35.15	<0.0001
Neofusicoccum vitifusiforme	Temperature (A)	4	4	0.25	0.8976	4	4	0.31	0.8585
	Time (B)	2	2	25.93	0.0371	2	2	8.77	0.1024
	A × B	8	98	1.64	0.1227	8	98	4.75	<0.0001
Spencermartinsia viticola	Temperature (A)	1	1	1.25	0.4646	1	1	1.25	0.4646
	Time (B)	2	2	1.04	0.4898	2	2	1.04	0.4898
	A × B	2	66	1.25	0.2676	2	66	1.25	0.2676

[a] Num DF, numerator degrees of freedom; Den DF, denominator degrees of freedom.

for *D. seriata* and *N. parvum* when the exposure time was increased to 30 min, and no growth was observed for *N. luteum*. These three species were not recovered over 4% at any time at 51°C, and were therefore not tested at 52°C and higher temperatures (Figure 1). In a third group, survival of *B. dothidea* and *N. mediterraneum* from HWT after 15 min was more than 67.2 and 90.6% respectively for temperatures equal to or lower than 52°C. A HWT at 52°C for 45 min reduced the viability of these two fungi down to 6.3% for *B. dothidea* and 1.6% for *N. mediterraneum*. However, a large reduction in mycelium survival was observed for *B.*

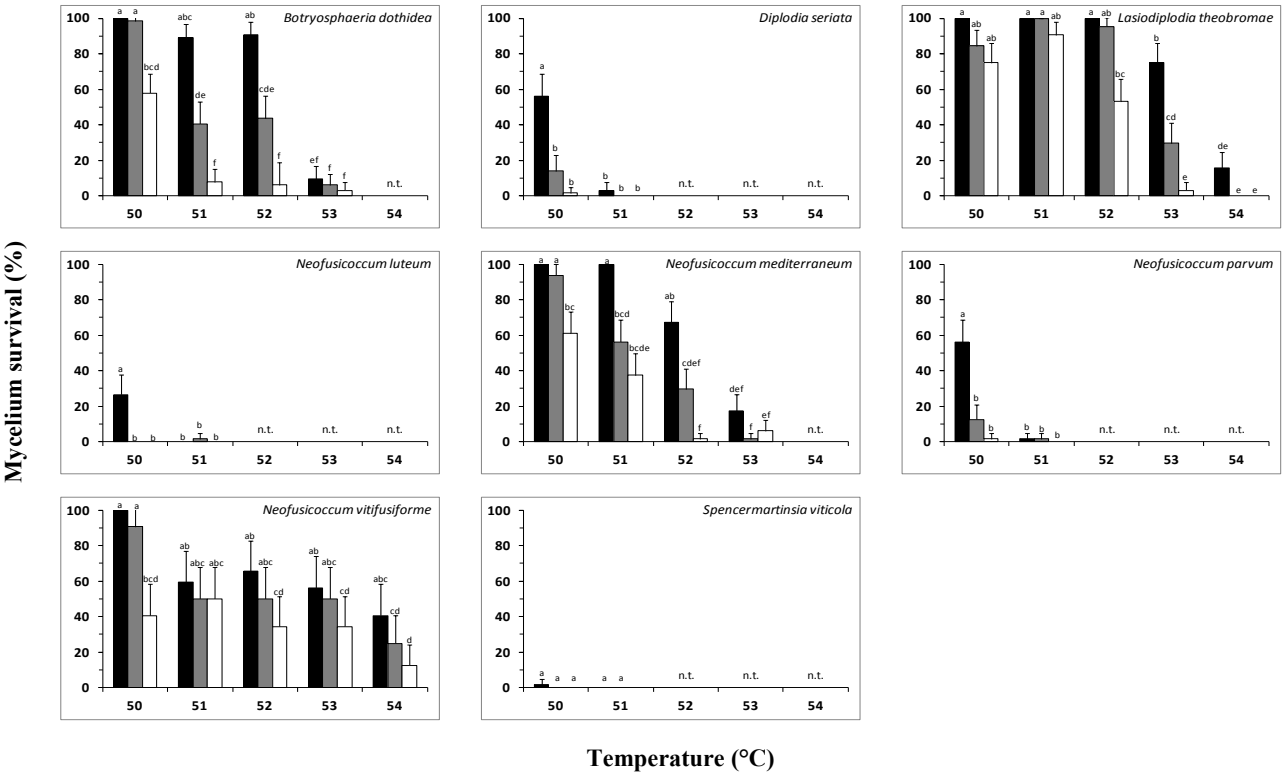

Figure 1. Mean mycelium survival of eight Botryosphaeriaceae species after hot water treatment in the *in vitro* experiment. Survival is expressed as the percentage of living mycelial plugs after treatments (N = 16 in each temperature by time combination for all species, except *N. vitifusiforme*, where N = 8). Means for different exposure times are expressed in black (15 min), grey (30 min) and white (45 min). Mean values significantly different ($P < 0.05$) according to LSD tests are indicated by different letters. n.t. = not tested.

dothidea and *N. mediterraneum* at 53°C, with percentages lower than 7% for both species at exposure times of 30 and 45 min (Figure 1). *Lasiodiplodia theobromae* and *N. vitifusiforme* were included in a fourth group that was the most tolerant to HWT (Figure 1). Survival of *L. theobromae* was significantly reduced at 53°C, with survival percentages of 75.0% after 15 min, 29.7% after 30 min and 3.1% after 45 min, compared with the corresponding values at lower temperatures. Survival of this fungus at 54°C was below 16% after the 15 min treatment. No mycelium survival was observed at exposure times longer than 15 min. In contrast, survival of *N. vitifusiforme* progressively decreased with temperature and time, and mean percentages at the maximum tested temperature ranged from 40.6% (15 min) to 12.5% (45 min), as shown in Figure 1.

Regarding the effects of HWT on mycelial growth rates, Temperature was significant ($P<0.05$) only for *B. dothidea* and *L. theobromae* (Table 2). Exposure time to hot water was significant ($P<0.05$) for *L. theobromae* and *N. mediterraneum*. In addition, the interaction Temperature × Time was highly significant ($P<0.001$) for all species except *S. viticola* (Table 2).

The mean values of the relative mycelium growth for all the species at different temperature and time combinations are shown in Figure 2. *Neofusicoccum luteum* and *S. viticola* were the most susceptible fungi to HWT, since growth rates of both fungi were never greater than 1.0% of the corresponding controls regardless of the treatment (Figure 2). In a second group, *D. seriata* and *N. parvum* showed relative growth rates of approx. 20% of the controls at 50°C for 15 min, but the growth rates at other Temperature × Time combinations were never greater than 2% (Figure 2). In a third group, mycelial growth rates of *B. dothidea* and *N. mediterraneum* after HWT de-

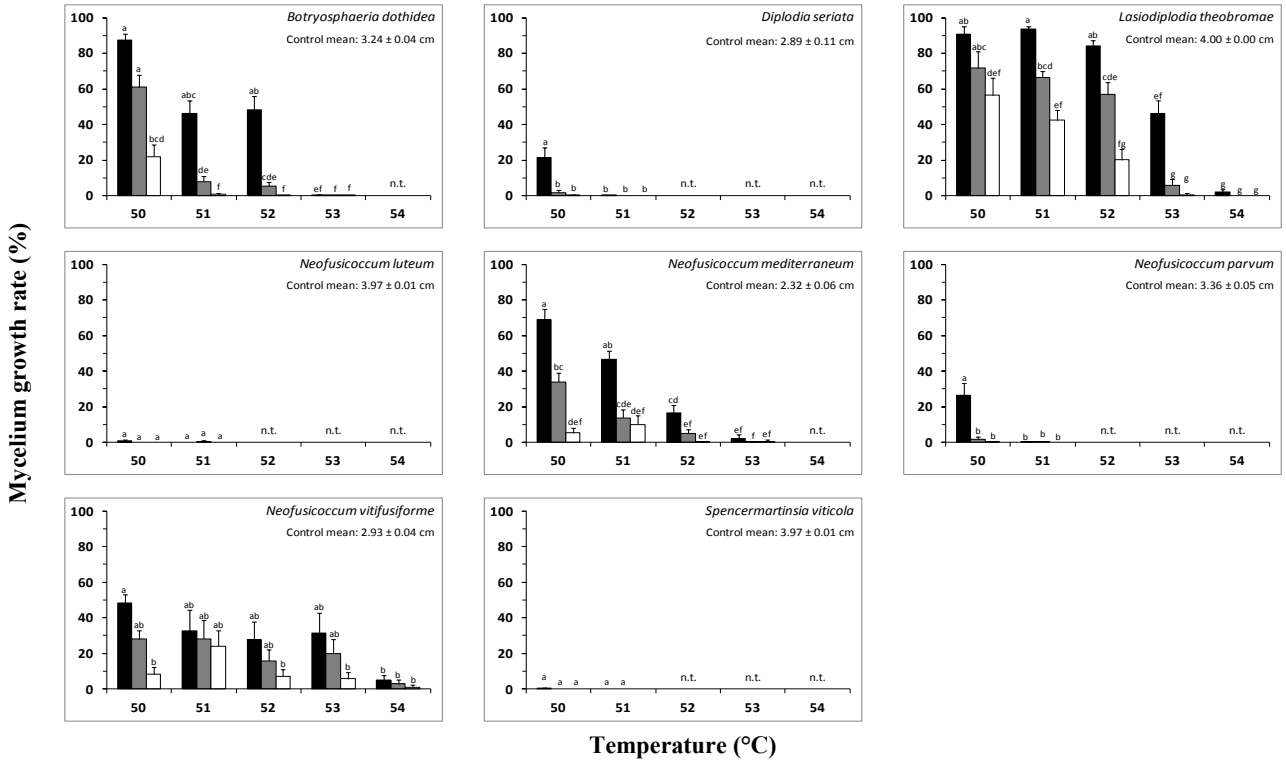

Figure 2. Mean relative mycelium growth of eight Botryosphaeriaceae species after hot water treatment in the *in vitro* experiment. Growth rates are expressed as the relative percentage of the radial colony growth of treated mycelial plugs (N = 16 in each temperature by time combination for all species, except *N. vitifusiforme*, where N = 8) compared to an untreated control (N = 6). Means for different exposure times are expressed in black (15 min), grey (30 min) and white (45 min). Mean values significantly different ($P < 0.05$) according to LSD tests are indicated by different letters. The absolute mean values +/- standard errors of the mean for the control group is shown at the top right corner of each graphic. n.t. = not tested.

creased progressively with increasing temperatures and exposure times, and reached negligible values (< 2.5%) at 53°C (Figure 2). *Lasiodiplodia theobromae* and *N. vitifusiforme* were the species most tolerant to higher temperatures (Figure 2). Mycelial growth of both species was severely inhibited only at 54°C, with mean relative growth rates below 6% at any exposure time within this temperature. However, growth rates of *N. vitifusiforme* were never greater than 50% whereas rates of *L. theobromae* were greater than those for *N. vitifusiforme* especially at 52°C and lower temperatures.

Effect of hot water treatment on mycelial survival *in planta*

No signs of fermentation were noticed in the incubation period prior to HWT in the experiments. No botryosphaeriaceous fungi other than the inoculated species were reisolated from the untreated controls. Mean mycelial survival in control, untreated canes ranged between 39.8% (*D. seriata*) and 72.7% (*L. theobromae*) (Table 3). Survival of fungi after HWT was significantly dependent on Species (P=0.004), Temperature (P=0.005) and their interaction (P<0.001). Therefore, the whole data set was re-analysed in two separate 1-way analyses according to each main factor.

Neofusicoccum luteum was not recovered from the treated canes at any tested temperature (Table 3), showing this to be the species most susceptible to HWT in colonised grapevine wood. In general, viability of fungi was reduced as temperature increased, with nil to very low recovery frequencies (< 4%) for all species at 51°C and higher temperatures. However, *L. theobromae* and *N. vitifusiforme* were recovered oc-

Table 3. Fungal recovery of different Botryosphaeriaceae species from inoculated grapevine wood treated with hot water for 30 min at different temperatures. Data are expressed in percentages as mean values +/- standard errors (SE) of the means (N = 16 for all species, except *N. vitifusiforme*, where N = 8). Mean values in the same column followed by different capital letters were statistically different among species. Mean values across the same row followed by different lower case letters were statistically different among temperature treatments.

Species	Untreated		50°C		51°C		52°C		53°C	
	Mean ± SE	Grouping	Mean ± SE	Grouping	Mean ± SE	Grouping	Mean ± SE	Grouping	Mean ± SE	Grouping
B. dothidea	49.22 ± 7.32	B a	17.97 ± 4.79	BC ab	0.00 ± 0.00	B b	0.00 ± 0.00	A b	0.00 ± 0.00	A b
D. seriata	39.84 ± 6.13	B a	25.00 ± 5.83	B ab	0.00 ± 0.00	B b	0.00 ± 0.00	A b	0.00 ± 0.00	A b
L. theobromae	72.66 ± 6.58	A a	57.81 ± 5.88	A a	0.78 ± 0.78	B b	0.00 ± 0.00	A b	0.00 ± 0.00	A b
N. luteum	42.19 ± 6.29	B a	0.00 ± 0.00	C b	0.00 ± 0.00	B b	0.00 ± 0.00	A b	0.00 ± 0.00	A b
N. mediterraneum	50.00 ± 6.83	B a	27.34 ± 5.43	B ab	0.00 ± 0.00	B b	0.00 ± 0.00	A b	0.00 ± 0.00	A b
N. parvum	56.25 ± 6.55	AB a	28.91 ± 5.84	B ab	0.00 ± 0.00	B b	0.00 ± 0.00	A b	0.00 ± 0.00	A b
N. vitifusiforme	64.06 ± 9.67	AB a	25.00 ± 7.91	B b	3.13 ± 2.13	A b	0.00 ± 0.00	A b	3.13 ± 2.13	A b
S. viticola	46.09 ± 6.16	B a	10.94 ± 3.54	BC ab	0.00 ± 0.00	B b	0.00 ± 0.00	A b	0.00 ± 0.00	A b

casionally from treated canes under these conditions (Table 3). At 50°C, all fungi were recovered at lower frequencies than in the control treatments, but differences were statistically significant only for *N. luteum* and *N. vitifusiforme* (Table 3). Reduction in recovery percentages for each species at 50°C were as follows: *B. dothidea* 63.5%, *D. seriata* 37.3%, *L. theobromae* 20.4%, *N. luteum* 100%, *N. mediterraneum* 45.3%, *N. parvum* 48.6%, *N. vitifusiforme* 61.0% and *S. viticola* 76.3%. Reduction in recovery percentages at 51°C for *L. theobromae* was 98.9%, and for *N. vitifusiforme* was 95.1%.

Discussion

The results obtained in this study show that, depending on time and temperature, HWT can reduce the viability and mycelial growth of Botryosphaeriaceae pathogens of grapevines. In the *in vitro* experiment, responses of fungal viability and growth to different temperature and exposure time combinations were variable and highly dependent on all the experimental factors, including species, temperature and exposure time. *Diplodia seriata*, *N. luteum*, *N. parvum* and *S. viticola* were the most susceptible species to temperature, while *L. theobromae* and *N. vitifusiforme* were the most tolerant. In general, the viability ranges of the eight Botryosphaeriaceae species included in the *in vitro* experiments were in accordance with previous published data on the range of cardinal temperatures for mycelial growth of these species, so that the higher are the optimal temperatures for growth the greater is the tolerance to HWT. Van Niekerk *et al*. (2004) reported optimal temperature for growth for *N. vitifusiforme* to be 30°C. Úrbez-Torres *et al*. (2006) studied the optimum temperature for *in vitro* colony growth for seven Botryosphaeriaceae species and estimated that the optimum temperature was 30.8°C for *L. theobromae* and *B. dothidea*, 29.4°C for *N. luteum*, 28.2°C for *N. parvum*, and 26.8°C for *D. seriata*. Martos (2008) observed that minimum temperature for growth for several Botryosphaeriaceae species (*B. dothidea*, *D. seriata*, *N. luteum*, *N. parvum* and *N. vitifusiforme*) in different culture media was about 15°C, whereas maximum temperature was about 35°C. She also observed that the minimum temperature for growth for *S. viticola* was 10°C, and this species also showed higher growth rates than any other species at 10 and 15°C. However, growth of this species was reduced at temperatures above 20°C. These data suggest that *S. viticola* is well

adapted for growth at cooler temperatures. In addition, Martos (2008) reported that growth rates for *B. dothidea* and *N. vitifusiforme* at 35°C were greater than those of any other Botryosphaeriaceae species, thus showing their capability for growth at high temperatures. In a study on colony growth of eight Botryosphaeriaceae species associated with grapevine, Pitt *et al*. (2013) reported comparable results to those cited above, as they observed that *S. viticola* showed high growth rates at low temperatures (10°C), and that optimal growth for *L. theobromae* and *B. dothidea* was at close to 30°C. In addition, the two latter species were still able to grow at 40°C, whereas all other species did not. Pitt *et al*. (2013) also reported optimal temperatures for *D. seriata* (26.6°C) and *N. parvum* (26.8°C), which were similar to those reported by Úrbez-Torres *et al*. (2006).

In the *in planta* experiment, responses of fungal viability were not as variable as those observed in the *in vitro* experiment. With the exception of *N. luteum*, which was completely inhibited at 50°C, mycelium recovery of all the other species was practically suppressed at 51°C and higher temperatures. Effects of HWT on the viability of *N. luteum* and *N. parvum* have been recently studied by Billones-Baaijens *et al*. (2014). They found that 5C rootstock cuttings artificially infected with either pathogen and hot water treated at 50°C for 30 min resulted in internal infections indices of 100% for *N. luteum* and 50% for *N. parvum*. While the reduction in viability of *N. parvum* was comparable to the results obtained in the present study, 100% survival of *N. luteum* observed by Billones-Baaijens *et al*. (2014) is clearly in conflict with our results. However, both pathogens were effectively controlled by treatment at 53°C (Billones-Baaijens *et al*., 2014), in agreement with our results.

Diplodia seriata, *N. luteum*, *N. parvum* and *S. viticola*, showed similar responses to HWT both *in vitro* and *in planta*, as they were clearly inhibited at 51°C in both types of experiments. Conversely, the other four species tested, *B. dothidea*, *L. theobromae*, *N. mediterraneum* and *N. vitifusiforme*, showed different viability responses among the experiments, with a higher tolerance to temperature in the *in vitro* experiments than *in planta*. We have no explanation for this differential behaviour, but it is clear that the conditions in the *in planta* experiment, i.e. the use of infected grapevine canes to check for fungal viability after HWT, are similar to the conditions in nurseries, where infected wood coming from infected mother vines is hot water treated to control latent wood pathogens.

Results obtained in this study provide further evidence supporting the potential of HWT as an effective control method for grapevine fungal trunk pathogens in propagation material. In Spain, Gramaje *et al*. (2008; 2009; 2010) determined that HWT at 53°C for 30 min is needed to effectively control Petri disease pathogens, although the treatments above 51–52°C drastically reduced conidial germination and mycelial growth of the pathogens, while HWT protocols at 50°C for 30 min may be sufficient to control black-foot pathogens. However, further research is required to enhance the development of effective HWT protocols to reduce the incidence of fungal infections and ensure healthy propagation material is released from grapevine nurseries.

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