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RESEARCH PAPER

Hot water treatment to reduce incidence of black foot pathogens in young grapevines grown in cool climates

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Summary. Black foot disease causes death of infected grapevines but management of this soil-borne disease by preventative measures such as pre-planting fungicide dips has not been totally effective. Hot water treatment (HWT; 50°C for 30 min) of young dormant grapevine plants has been shown to significantly reduce infection. However, it has been reported to cause unacceptable damage to young vines in cooler climate countries like New Zealand, so this study examined the effects of different HWT protocols on the New Zealand black foot isolates. *In vitro* testing of different HWT protocols was conducted on conidia, mycelium and detached, inoculated grapevine canes using three isolates each of the species *I. liriodendri* (*"C". liriodendri*) and the complexes, *I. radicicola* (*"C". destructans)* and *I. macrodidyma* (*"C". macrodidymum)*. Heat treatments greater than 40°C for 5 min killed all conidia ($P<0.001$), and treatments greater than 47°C for 30 min inhibited ($P\leq0.003$) further growth of treated mycelium plugs for all but one isolate. Within cane pieces, infection by *Ilyonectria* ("*Cylindrocarpon*") isolates was significantly reduced ($P<0.001$) by 30 min at 48.5 and 50°C. Additionally, these studies showed different responses to the different treatments for the three isolates of each species complex and differences between species. In field trials, HWT of 48.5 and 50°C for 30 min significantly reduced disease incidence in dormant plants to 0% ($P\leq0.001$). This study confirmed that HWT of 48.5°C for 30 min could be used to eliminate black foot disease in dormant nursery grapevines grown in New Zealand prior to their use for establishing new vineyards.

Key words: "Cylindrocarpon", New Zealand, *in vitro*, field experiments, *Ilyonectria.*

Introduction

Black foot is a serious disease of grapevines in nurseries and vineyards world-wide which causes stunting, chlorosis, late bud break and often death of vines. Infected vines often show small necrotic lesions on roots and the butts of affected vines show dark purplish or reddish brown necrotic streaks which spread across the whole trunk eventually causing death of the vine. The disease has been identified in all major viticulture regions throughout the world, including Italy (Grasso and Magnano Di San Lio, 1975; Grasso, 1984), California (Scheck *et al.*, 1998; Petit and Gubler, 2005), Portugal (Rego *et al.*, 2000), South Africa (Fourie *et al.*, 2000), New Zealand (Halleen *et al.*, 2004c), Australia (Whitelaw-Weckert *et al.*, 2007), Chile (Auger *et al.*, 2007) and Spain (Alaniz *et al.*, 2009) and in less well known viticultural regions such as Lebanon (Choueiri *et al.*, 2009), Iran (Mohammadi *et al.*, 2009), Uruguay (Abreo *et al.*, 2010) and Canada (Petit *et al.*, 2011).

The pathogens reported to cause black foot include *"Cylindrocarpon" liriodendri, "C". macrodidymum* and *"C". destructans* and in New Zealand were isolated from 121 of 141 symptomatic vines, (86%) contributed by 49 growers (Bleach *et al.*, 2006). These were later identified in a study by Mostert *et al*. (2006) by molecular analyses of the internal transcribed spacers 1 and 2 (ITS1 and ITS2), the 5.8S ribosomal RNA gene and the partial β-tubulin genes. However, recent publications used new molecular techniques that reclassified these species into mul-

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tiple new species. Chaverri *et al*. (2011) divided the *Neonectria* into four groups: 1) *Neonectria/Cylindrocarpon* sensu stricto, 2) *Rugonectria,* 3) *Thelonectria* and 4) *Ilyonectria.* Cabral *et al.* (2012a) introduced 12 new taxa within the *Ilyonectria radicicola* (*"C". destructans*) species complex and more recently Cabral *et al*. (2012b) demonstrated that there are six monophyletic species within the *Ilyonectria macrodidyma* complex. They used a combination of morphology, culture characteristics and multi gene analysis which included the β-tubulin, nuclear ribosomal RNA internal transcribed spacer (ITS), translation elongation factor 1-α genes and the histone H3 sequences, which were most useful. However the purpose of this research was to develop control strategies which were effective against a broad range of "*Cylindrocarpon"* species, and so the precise taxonomy of the isolates within the three originally designated species was considered of relatively low importance for the overall outcome.

Ilyonectria macrodidyma species complex and *Ilyonectria radicicola* species complex have been most commonly reported (Rego *et al*., 2000; Armengol *et al*., 2001; Halleen *et al*., 2003; Gubler *et al*., 2004; Oliveira *et al*., 2004) and in South Africa, *Campylocarpon fasciculare* and *Campyl. pseudofasciculare* were also frequently associated with black foot of grapevines (Halleen *et al*., 2004b). In New Zealand vineyards, *Ilyonectria liriodendri*, *Ilyonectria macrodidyma* complex and *Ilyonectria radicicola* complex, have been found associated with black foot, were widespread and of similar importance (Bleach *et al*., 2007). More recently *Cylindrocladiella parva* (Jones *et al*., 2012) has been reported as a pathogen responsible for black foot of grapevines in New Zealand.

Black foot is a soil-borne disease, thus the pathogen propagules and infected host debris that remain in the soil after infected host plants have been removed can infect subsequent plantings. Severe levels of disease were reported in replanted vineyards in Portugal (Oliveira *et al*., 2004) and in New Zealand vineyards which replaced apple orchards (Bonfiglioli, 2005). Nursery field sites were also reported to harbour the pathogens, with more than 50% of the grafted vines becoming infected after 7 months growth in previously used nursery soils in South Africa (Halleen *et al*., 2003). Although the disease cycle of "*Cylindrocarpon"* spp. in vineyards has not been well studied, the behaviours of these pathogens on other hosts (Booth, 1966; Brayford, 1993) has indicated that conidia and chlamydospores are likely to be produced on the diseased roots and stem bases of infected vines. Agustí-Brisach *et al*. (2011) also reported isolation of *"C" macrodidymum* from 26 of 52 weed species growing in propagation nurseries and vineyards, with these being pathogenic to vineyard weeds and therefore may provide sources of spores. Previous research reports (Rego *et al*., 2001; Halleen *et al*., 2003; Probst, 2011) have shown that contact between these spores and the grapevine roots or callused stem bases results in high rates of infection.

Few specific recommendations are currently available for the control of back foot disease. Research reports on the use of pre-planting fungicide treatments have provided varying results. In South Africa, Halleen *et al*. (2007) tested the efficacy of fungicides in reducing mycelium growth of four black foot pathogens, *"C". liriodendri*, *"C". macrodidymum*, *Campyl. fasciculare* and *Campyl. pseudofasciculare.* They found that prochloraz manganese chloride was the most effective at reducing mycelium growth of all four pathogens, while benomyl, flusilazole and imazalil were only effective in reducing mycelium growth of *"C". liriodendri* and *"C". macrodidymum.* In contrast, their nursery field trials showed that these four fungicides as pre-planting soak treatments were not able to prevent infection by *"C". liriodendri* and *"C". macrodidymum*. In a field experiment in 2002–2003, Halleen *et al*. (2007) showed that these fungicides were not totally effective, with incidence of black foot pathogens in bases of uprooted plants being 53.3, 40.7, 54 and 31.3%, respectively, compared to the untreated controls (45.3%). However, other more recent trials have demonstrated that these fungicides can reduce incidence of infection when applied before planting into inoculated pots or naturally infested nursery fields. Nascimento *et al*. (2007) reported significantly reduced incidence of *"C". liriodendri* for potted grapevines treated with cyprodinil + fludioxonil (21.7%) and chitosan (32.0%) than for the control (80.8%), but the carbendazim + flusilazole treatment caused similar incidence to the control (40.8%). Rego *et al*. (2009) reported that in a field site with a previous grapevine history, plants treated with cyprodinil + fludioxonil and fludioxonil alone had significantly less disease incidence (40 and 25%, respectively) and severity (4.2 and 2.9%, respectively) than the water treated control plants (75 and 10.4%, respectively). Alaniz *et al*. (2011) found cuttings dipped into the fungicides captan, carbendazim, copper oxychloride, prochlo-

raz, didecyldimethyl-ammonium chloride and hydroxyquinoline sulphate, significantly decreased the severity of root disease for both "*Cylindrocarpon*" species compared with the control treatment. However, disease incidence for cuttings inoculated with "*C*". *liriodendri* was significantly reduced by only captan, carbendazim and didecyldimethyl-ammonium chloride, while for cuttings inoculated with "*C*". *macrodidymum* only prochloraz reduced disease incidence (Alaniz *et al*., 2011).

Hot water treatment (HWT) at 50°C for 30 min has been reported to effectively eliminate the black foot pathogens and some other grapevine pests and pathogens from grapevine materials in Spain (Gramaje *et al*., 2009), South Africa (Halleen *et al*., 2004a), California (Rooney and Gubler, 2001) and Australia (Waite and Morton, 2007). The industry standard HWT (50°C for 30 min) has been advocated for Petri disease (Ferreira, 1999a; Pascoe *et al*., 2000), however, there are contradictory reports about the efficacy of the treatment. In experiments with dormant cutting material, both Laukart *et al*. (2001) and Rooney and Gubler (2001) did not observe a significant reduction in infection and concluded that HWT was ineffective. However, Edwards *et al*. (2003b) and Fourie and Halleen (2003) observed an average reduction of 76% in the incidence of infected vines and concluded that while the treatment did not eliminate the pathogen, this level of reduction suggested it may be of use in disease management. However, HWT has sometimes been reported to cause damage to propagation material (Crocker and Waite, 2004; Waite and May, 2005) and "negative side effects on vine development" (Habib *et al*., 2009) especially to grapevines grown in cooler climates like New Zealand (Graham, 2007b; Waite and Morton, 2007). The damage seems to be most common in cool climates and is thought to be related to the lower heat tolerance within these grapevines (Crocker *et al*., 2002; Crocker and Waite, 2004). In grapevine cuttings grown in New Zealand, HWT at 47°C for 30 min reduced "known pathogens and endophytes" in grafted rootstock to 3% compared to the untreated controls (15%) and reduced vine mortality to less than 10% in vines that were treated at lower temperatures (45–47°C for 30 min) compared to vines that were HWT using the industry standard (60%) (Graham, 2007b). A field experiment which hot water treated 2-year-old rootstock plants at 47°C for 30 min and then grew them on for 8 months in a greenhouse found no adverse effects on shoot development (Bleach *et al*., 2009). The aim of this experiment was to examine the effects of different HWT temperature and time combinations on nine New Zealand *Ilyonectria* isolates in laboratory and field experiments, as well as to evaluate the differential sensitivity of the isolates to heat treatments.

Materials and methods

Isolates

Nine *Ilyonectria* isolates, three each of *I. liriodendri*, *I. macrodidyma* complex and *I. radicicola* complex that had been isolated from the trunks and roots of symptomatic grapevines, collected throughout New Zealand (Bleach *et al*., 2006) were used for this study (Table 1). These isolates were maintained on Spezieller Nährstoffarmer Agar (SNA) slants (Brayford, 1993) at 4°C until required, when they were subcultured to potato dextrose agar (PDA; Oxoid Ltd, Basingstoke, Hampshire, England,) plates and incubated at 20°C for 2–4 weeks.

Effect of different HWT treatments on viability of mycelium and conidia

Treatments at 40, 45, 47, 50 and 55°C for 5, 15 and 30 min or 40, 45, 47 and 50°C for 5, 15 and 30 min were applied to mycelia and conidia, respectively, of the nine *Ilyonectria* isolates (Table 1), using a PCR temperature cycler (Eppendorf Mastercycler®, Biorad iCyclerTM, California, USA). For each temperature/time treatment there were three replicate Eppendorf plastic tubes (0.6 mL) per isolate, comprising 27 tubes of agar plugs of mycelium or of conidium suspensions in total. The 27 tubes were randomly arranged in the PCR temperature cycler and hot water treated together. After HWT, the Eppendorf Mastercycler® automatically dropped the temperature to 4°C for a holding period of 3–5 min, after which the tubes were removed and their viability assessed. The controls comprised untreated tubes that were randomly positioned on a laboratory bench and held at ambient room temperature of 20°C ± 2°C for 30 min.

Mycelium

For each of the nine isolates, three replicate 3 mm plugs of agar with mycelium and conidia, were cut from the growing edges of 2–4 week old agar colonies. Each agar plug was placed into a separate tube

Table 1. Source of *Ilyonectria* isolates obtained from characteristic necrotic lesions on grapevine roots and trunks in New Zealand.

Strain Code	Species (isolate)	LUPP [a] No.	Geographic origin
1R	*I. radicicola* complex	1071	Marlborough
2R	*I. radicicola* complex	1022	Central Otago
3R	*I. radicicola* complex	989	Waipara
1L	*I. liriodendri* (1L)	1000	Central Otago
2L	*I. liriodendri* (2L)	1102	Marlborough
3L	*I. liriodendri* (3L)	953	Hawkes Bay
1M	*I. macrodidyma* complex (1M)	974	Hawkes Bay
2M	*I. macrodidyma* complex (2M)	1039	Gisborne
3M	*I. macrodidyma* complex (3M)	1120	Marlborough

[a] LUPP, Lincoln University Plant Pathology.

containing 100 μL of sterile distilled water (SDW) and the lid closed. Immediately after the designated treatments, each mycelium plug was removed from its tube and placed onto malt extract agar (MEA; 2% agar, 2% Maltexo). The plates were sealed with cling film and randomly allocated to positions in a 20°C incubator for 12 days under a diurnal light schedule (12 h light, 12 h dark). Growth was assessed on each plate after 7 and 12 days by measuring the perpendicular diameters with a digital calliper (Mitutoyo, Mitutoyo Corp, Kanogawa, Japan), and the mean percent mycelium growth was determined in relation to the growth on untreated control plates.

Conidium suspension

For each of the nine isolates, a 2–4 week-old PDA colony was flooded with 5 mL of SDW and the colony surface was rubbed with a sterile hockey stick to release the conidia. Each resulting suspension was poured into a sterile Universal bottle, the conidium concentration assessed using a haemocytometer and then the final concentration was adjusted to 1×10^4 conidia mL^{-1} with SDW. Three replicate 120 μL aliquots of each conidium suspension (for each isolate) were placed into separate tubes and the lids closed. After the designated HWT, three 40 μL droplets were pipetted from each tube onto a glass slide, 20 mm apart. The three slides for each isolate were then placed into separate 85 mm diameter Petri dishes; each Petri dish contained three slides that contained conidium suspensions of different isolates. Each Petri dish was placed inside a larger square Petri dish (100 × 100 mm) that contained 5 mL of water and covered with the lid to act as a humidity chamber. The dishes were randomly allocated to positions in a 25°C incubator and incubated for 5 h in the dark. To maintain the 5 h germination rate and prevent further development during the assessment period, individual glass cover slips were placed onto each conidium droplet on removal from the incubator and the slides were then held in a 7–8°C temperature controlled room until counting was completed (up to 4 h). Germination of 100 randomly selected conidia in each droplet was assessed. A conidium was considered germinated if the length of the germ tube exceeded half the length of the conidium. The mean percent germinated conidia was determined for each slide relative to the untreated controls. To confirm that lack of germination reflected non-viability of the conidia, the coverslips were removed from the slides which were replaced in their Petri dishes and humid chambers. These were randomly replaced in the 25°C incubator for a further 24 h and conidium germination assessed again.

In vitro trunk inoculation

One year old dormant cuttings of rootstock 101-14 were cut into 40 cm pieces, surface sterilised (Halleen *et al.*, 2003) and left to air dry in a laminar flow cabinet on paper towels for 1 h. After drying, the

canes were clamped into a bench vice ('P&B, England') and an electric drill with a 2 mm bit (Makita™ New Zealand Limited), which had been surface sterilised with 70% ethanol and flamed, was used to drill three 5 mm deep holes into the pith of each cane approximately 80 mm apart. These holes provided inoculation ports for the 3 mm diameter mycelium plugs cut from the colony edges of the nine *Ilyonectria* isolates as described. Each hole in a cane was inoculated with a different *Ilyonectria* sp. and isolate using aseptic techniques and the remaining cavity was filled with sterilised sawdust, which was previously collected from drill holes in surplus canes and autoclaved at 121°C for 15 min. The mycelium plug and sterilised sawdust were held in place by wrapping the inoculation area with a single layer of waterproof grafting tape (Aglis & Co. Ltd, Fukuoka, Japan). There were three replicate canes for each isolate and HWT protocol. The inoculated canes were placed into separate, new plastic bags and randomly allocated to positions in a 25°C incubator for 7 d in the dark to allow colonisation of the wood by the pathogen. The canes were then removed from their bags and HWT, by placing them into HWT baths set at 47, 48.5 or 50°C for 30 min, followed by immediately plunging them into cold water for 30 min. The control canes were inoculated in a similar manner but they were not HWT. The canes were air dried in a laminar flow cabinet and when dry the grafting tape was removed. Isolations were made from the inoculation point (0 cm) and ~1 cm above and below that point; a 1–2 mm section was sliced across the cane and divided into four pieces which were placed equidistantly around the perimeter of a PDA plate amended with 250 mg L^{-1} chloramphenicol (PDAC) (Sigma-Aldrich® Inc., St. Louis, MO, USA). The data available for analysis were the presence or absence of the inoculating *Ilyonectria* spp. (incidence) in each plate.

HWT of dormant grapevine rootstock plants from a field nursery

The experiment was conducted in a commercial field nursery located in Auckland, New Zealand from September 2007 to May 2008. Conidium suspensions were prepared by first dislodging conidia from ten replicate plates for each isolate as described for the *in vitro* experiment. The contents of the plates (agar and conidial suspensions) for each isolate were then placed in a new plastic bag and emulsified with 100 mL of SDW using a Colworth Stomacher 400, (A.J. Seward & Co., Blackfriars Rd, London, UK) set at 50 Hz for 10 min. A further 500 mL of SDW was mixed into the resulting mash and it was strained through a series of sterilised sieves (pore sizes of 710, 500 and 150 μm), with the filtrate from the 150 μm sieve being collected into a sterile 2 L bottle. The mash that remained on the first sieve (710 μm) was replaced into the plastic bag with a further 100 mL of SDW and the emulsifying process repeated. The resulting mash was again mixed with 500 mL of SDW and strained through two sieves (500 μm and 150 μm), combining the final filtrates from the 150 μm sieves. The conidial concentration for each isolate was adjusted to 1×10^8 conidia mL^{-1} using a haemocytometer to ensure equivalent numbers of conidia for each isolate before mixing them. The bottle containing the mixed conidial suspension was placed into an insulated container filled with crushed ice for overnight transport to the field site. On site, the suspension was diluted with enough water to give 1×10^4 conidia mL^{-1} immediately prior to soil inoculation.

Preparation of the field site was carried out in line with standard nursery practices; the soil was cultivated, then mounded and the mounds covered in black polythene. Planting holes were made through the polythene to a depth of 15 cm, in double rows 100 mm apart. They were inoculated immediately prior to planting using a drench pack and gun suitable for dosing farm animals (N J Phillips Pty Limited, NSW, Somersby, Australia). The 20 mL of mixed conidium suspension (1×10^4 conidia mL^{-1} of the nine isolates, three each of *I. liriodendri*, *I. macrodidyma* and *I. radicicola* groups used in the *in vitro* experiment) was injected at the base of each planting hole. Apparently healthy, two node cuttings of callused rootstocks varieties: 101-14 MGT (101-14; *Vitis riparia* × *V. rupestris*) and Teleki 5C (5C; *V. berlandieri* × *V. riparia*) supplied by the commercial nursery, were inserted into the planting holes. The experiment was laid out in a completely randomised split plot (rootstock varieties, 101-14 and 5C) design with six blocks, each containing 14 plots (seven treatments by two rootstock varieties) that were 600 mm long, and separated by 200 mm buffer zones. The experimental site was managed by the on-site nursery staff according to standard nursery practices, which included a calendar spray program every 14 days of sulphur (3 kg

ha^{-1} Kumulus®; BASF Canada Inc. Ontario, Canada), Dithane® (2 kg ha^{-1}; Dow AgroSciences, Indianapolis, USA), and Tracel Plus (5 kg ha^{-1}; Fruitfed Supplies, PGG Wrightson Limited, New Zealand). The vines were trimmed after 4 months growth to reduce canopy density and thus plant susceptibility to mildew diseases. After 8 months, the dormant plants were harvested, then washed under running tap water. Control plants were set aside and those allocated to HWT of 47, 48.5 and 50°C for 15 and 30 min were treated according to standard practice in the nursery HWT plant (7,500 L with accuracy of ± 0.5°C) followed by 30 min immersion in cold water.

Assessment

The roots and shoots were removed from the plants and the bare trunks that remained were washed in cold water, air dried and then surface-sterilised (Halleen *et al*., 2003) one plot at a time. From each trunk, the ~1 cm root crown was removed and discarded. A 1–2 mm section was sliced from across the basal end of the trunk (0 cm) and divided into four pieces which were placed equidistantly around the perimeter of a PDAC plate and another 1–2 mm slice cut from further up the trunk (5 cm) to assess the progression of the pathogen was transferred to the centre of the same PDAC plate. Plates were incubated as described above.

Statistical analysis

The *in vitro* experimental design was a randomised factorial design with nine isolates (three isolates from the *I. liriodendri*, *I. macrodidyma* and *I. radicicola* groups), and three replications for each of the experimental combinations. The percent growth and germination data and incidence data (trunk inoculation) were analysed by general analysis of variance using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) to determine HWT temperature/time and isolate/species effects. The field experiment data of disease incidence (from 0 cm and 5 cm isolations) and severity (the mean proportion of tissues infected at 0 cm) were analysed by general linear model (GenStat Release 14.1, VSN International Ltd, Hemel Hempstead, UK) with terms appropriate to the design and the two-way interactions amongst the factors of interest. Where significant, main effects or two-way interactions were identified and the significance of differences between individual treatments was further explored using Fisher's protected LSD tests at $P \leq 0.05$.

Results

Mycelium

There was a significant effect of species (P=0.034) on mycelium growth, with temperature treatments causing significantly less inhibition for isolates of *I. liriodendri* than for *I. macrodidyma* complex but not for *I. radicicola* complex (46.3, 40.4 and 42.0%, respectively, of the untreated control growths, LSD = 4.60). The HWT temperatures significantly affected mycelium growth of isolates overall (P<0.001), means as proportions of the control growth being 99.7, 56.6, 35.7, 20.5 and 1.8%, respectively for 40, 45, 47, 50 and 55°C (LSD = 5.08). Similarly, HWT time had a significant effect on mycelium growth (P<0.001), with means as proportions of the control growth being 69.1, 37.8 and 21.8% for 5, 15 and 30 min, respectively, (LSD = 3.94; Figure 1). There was also a significant effect for isolates within individual species which sometimes conflicted with the apparent species effects (P<0.003; Figure 1).

The significant interaction of HWT temperature × time × isolates (P<0.001, LSD = 26.41) was particularly evident with the 45°C treatment for 30 min and 47°C treatment for 15 and 30 min. The 30 min treatment at 45 and 47°C treatment for 30 min totally inhibited growth for all isolates except 3L (30.8%) and 1L (16.1%), respectively (Figure 1) and the 15 min treatment at 47°C totally inhibited growth of all isolates except 1R, 2M and 3M.

However, these different isolate effects for the 30 min HWT were due to the different results from the three replicates, with mycelium growth on only one of the three replicate plates, being 48% (1L) and 93% (3L) growth, respectively, which accounted for the 16.1 and 30.8% mean growths, respectively. Treatment at 50°C for 15 and 30 min provided complete inhibition of all isolates except 3L and treatment at 55°C for 15 and 30 min completely inhibited growth of all isolates (Figure 1).

Conidia

The HWT temperature and time combinations were significant for all isolates (P<0.001), however all treatments completely inhibited germination of

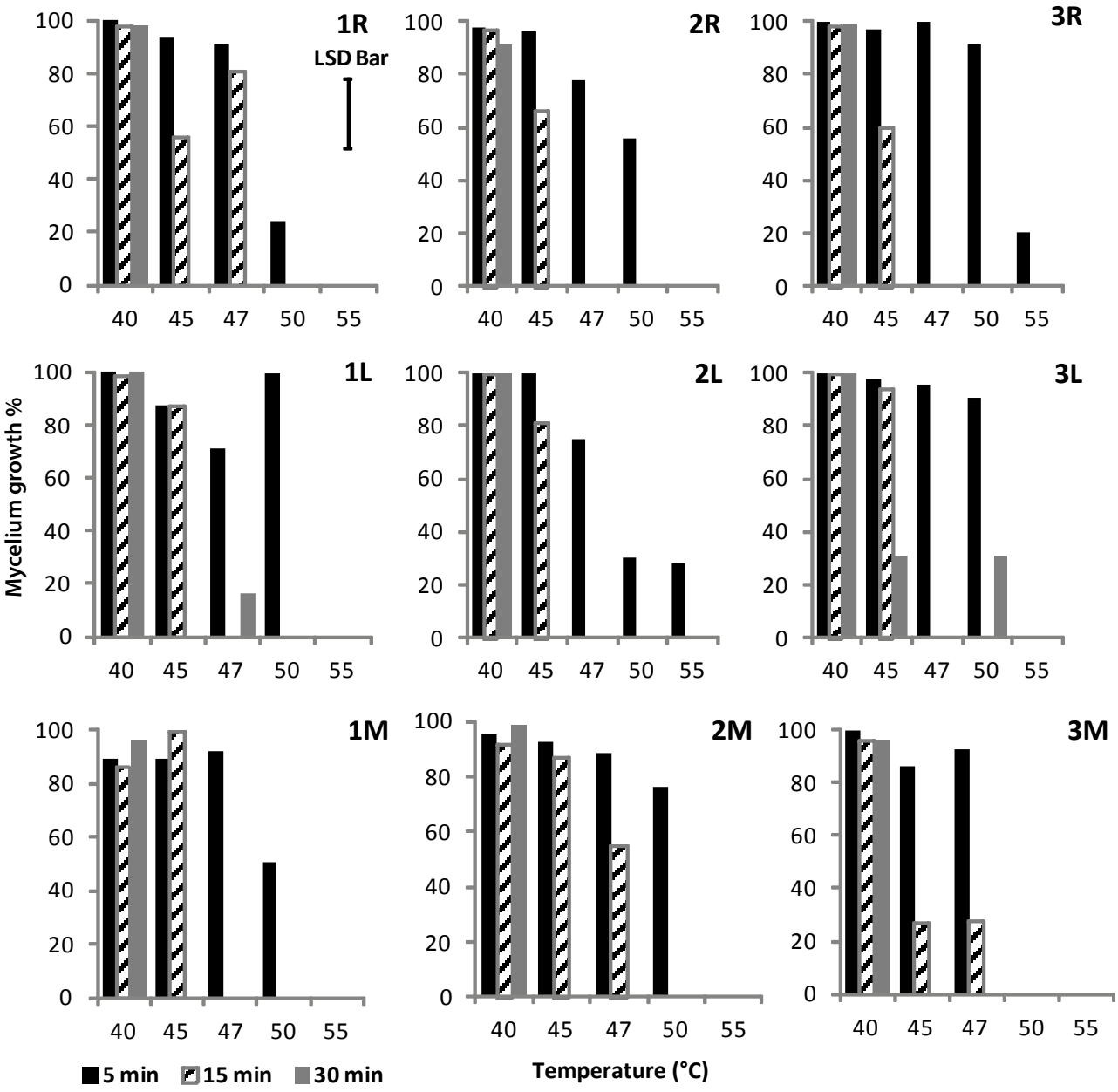

Figure 1. Percent mycelium growth of *Ilyonectria* isolates for *I. radicicola* (1R, 2R and 3R), *I. liriodendri* (1L, 2L and 3L) and *I. macrodidyma* (1M, 2M and 3M) compared to the untreated controls after hot water treatment at 40, 45, 47, 50 or 55°C for 5, 15 or 30 min. Results are the means of three replicates for each isolate for each temperature and time combination ($P<0.001$, LSD = 26.41).

conidia of the three *Ilyonectria* species except 40°C for 5 min. Germination was also assessed after a further 24 h incubation to allow more time for inhibitory effects to be overcome but there was no further germ tube development for any of the nine *Ilyonectria* isolates. After HWT at 40°C for 5 min, germination of *I. radicicola* conidia was more than for *I. liriodendri* and *I. macrodidyma* (20.2, 11.2 and 10.3%, respec-

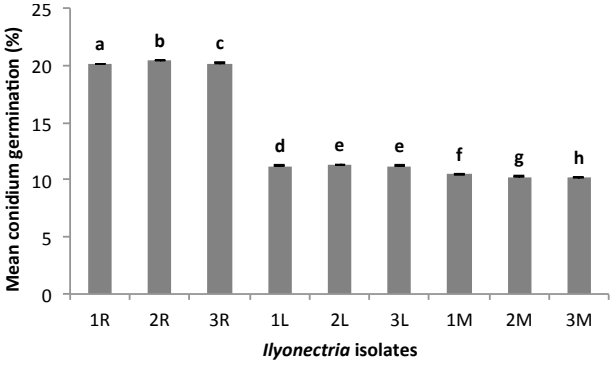

Figure 2. Mean conidium germination compared to the untreated control for nine *Ilyonectria* isolates, after HWT at 40°C for 5 min. Bars with the same letter are not significantly different according to Fisher's protected LSD ($P \leq 0.001$, LSD = 0.06).

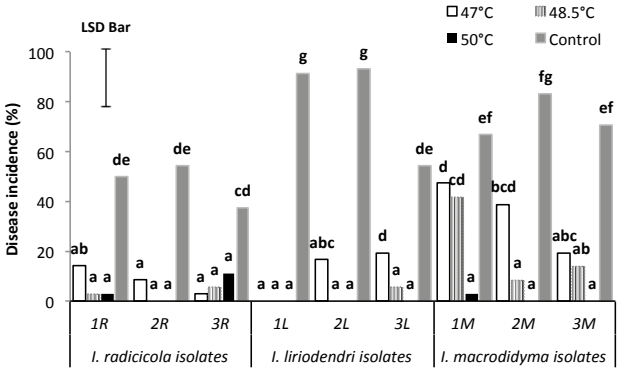

Figure 3. Mean pathogen incidence of three isolates for each of the *Ilyonectria* groups after the nine isolates were allowed to grow for 7 d inside grapevine canes and then HWT at 47, 48.5 and 50°C for 30 min. Bars with the same letter are not significantly different according to Fisher's protected LSD ($P \leq 0.001$, LSD = 22.68).

tively, LSD = 0.06), with some differences between isolates ($P<0.001$; Figure 2). However, these minor differences were not considered to have biological significance.

In vitro trunk inoculation

Incidence of infection within trunks was significantly affected by HWT temperature, *Ilyonectria* species, isolates and interactions for species × temperature (all $P<0.001$) and species × isolate × temperature ($P=0.029$). Treated canes inoculated with *I. macrodidyma* isolates had greater disease incidence than those inoculated with *I. radicicola* and *I. liriodendri* isolates (19.1, 5.3 and 4.6 %, respectively, LSD = 8.62). The species × HWT temperature interaction was associated with the significantly greater disease incidence for *I. macrodidyma* than *I. radicicola* and *I. liriodendri* after treatment at 47°C (35.2, 8.3 and 12.0%, respectively) and 48.5°C (21.3, 2.8 and 1.9%, respectively) but not 50°C when incidences were similar (0.9, 4.6 and 0%, respectively, LSD = 15.12). The significant isolate effects and their interactions (Figure 3) show that some isolates were generally more resistant than others and these differences diminished as the HWT temperature increased. Incidence of infection in wood pieces from the treated canes differed between isolation positions ($P<0.001$), being significantly greater for those from the inoculation site (0 cm) than 1 cm above or 1 cm below that site (47.7, 27.9 and 20.5%, respectively, LSD = 9.13).

Dormant grapevine rootstock plants from a field nursery

There were significant effects of the HWT treatments on disease severity and incidence (both $P<0.001$), which reduced with increasing temperature and time (Figure 4). There was zero disease incidence in plants HWT at 48.5 and 50°C for 30 min. However, these zero incidences did not differ significantly from the disease incidences after HWT at 48.5 and 50°C for 15 min and 47°C for 30 min (3.1, 1.0 and 2.1%, respectively LSD = 7.26). The least effective treatment (47°C for 15 min) caused significantly less disease incidence than in control plants (44.8%).

Disease severity was similarly low for all HWT plants and significantly less than in the inoculated control plants ($P \leq 0.05$), reflecting a similar trend as observed for disease incidences (Figure 4). Zero disease severity occurred in plants that were HWT at 48.5 and 50°C for 30 min, and the remaining treatments had significantly reduced severities (2.9–0.3%) compared to the control plants (20.8%, LSD = 4.74). There was no significant difference in disease incidence and severity between rootstock varieties ($P=0.225$) nor any interaction between HWT treatment and rootstock varieties ($P=0.540$) although mean disease incidence and severity were less in rootstock variety 5C (7.1 and 3.4%, respectively) than rootstock variety 101-14 (9.5 and 4.1%, respectively).

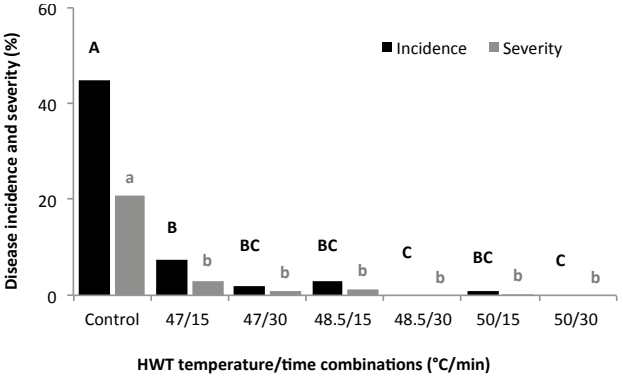

Figure 4. Mean percent incidence and severity of isolates of *I. radicicola*, *I. macrodidyma* and *I. liriodendri* spp. in grapevine plants after hot water treatment. Columns which are the same colour, black for disease incidence LSD = 7.26 and grey for disease severity LSD = 4.74, with the same letters above are not significantly different (Fishers protected LSD $P<0.05$).

Discussion

This study showed that some of the different HWT temperature and time combinations tested inhibited mycelium growth and conidium germination but that effects differed between isolates of *I. radicicola* and *I. macrodidyma* complexes and *I. liriodendri,* the pathogens commonly associated with black foot disease in grapevines. The increasing HWT temperatures and times used for treating the mycelium plugs, caused the subsequent mycelium growth to be progressively more inhibited. Comparable HWT effects were reported by Gramaje *et al.* (2010) who investigated the effects of 41–55°C for 30-45 min for single isolates of three black foot pathogens and eight *Phaeoacremonium* species. They reported complete inhibition after 30 min at different temperatures for *I. liriodendri* (48°C) and *I. macrodidyma* (49°C). However in this study, growth of all *I. macrodidyma* isolates was completely inhibited if the mycelium plugs were treated for 30 min at 45°C while the effects on *I. liriodendri* isolates were generally similar, although one replicate each of 1L and 3L had greater resistance to the treatments. These uneven trends may have been caused by presence of occasional chlamydospores on the treated plugs. Chlamydospores are more heat resistant than conidia and mycelium (Smith *et al.*, 2009), and Halleen *et al.* (2004b) reported that *I. liriodendri* and *I. radicicola* readily produced chlamydospores when growing on agar, unlike *I. macrodidyma* which rarely produced them. The Petri disease pathogen, *Cadophora luteo-olivacea* (Gramaje *et al*., 2010) was reported as more resistant to HWT requiring 55°C for 30 min to completely inhibit growth however, this pathogen has been reported once only in New Zealand (Manning and Mundy, 2009) and was not found in the New Zealand survey conducted by these authors (Bleach *et al*., 2006). Comparisons could not be made regarding *I. radicicola* isolates as they were not investigated in the study by Gramaje *et al.* (2010).

Conidial germination in this study was completely inhibited after very little heat treatment. Germination occurred only after 5 min at 40°C when mean germination rates differed slightly between species, being greater for *I. radicicola* than *I. liriodendri* and *I. macrodidyma* (20.2, 11.3 and 10.3%, respectively). In contrast, Gramaje *et al.* (2010) found that much greater heat treatments were required to provide 100% inhibition of germination. They found that 41°C for 30 min reduced conidium germination of "*C". liriodendri* and "*C". macrodidymum* isolates to 38.8% and 65.7% respectively, and complete inhibition occurred after 30 min at 45°C for "*C". liriodendri*, 46°C for "*C". macrodidymum* and 51°C for *Ca. luteo-olivacea.* This study clearly showed that conidia of the New Zealand "*Cylindrocarpon"* isolates were more sensitive to HWT than those of the Spanish isolates. The significant differences between isolates from a species, with respect to mycelium growth after different HWT treatments in this study, clearly indicates that more isolates should be tested from a range of climatic regions before a final conclusion can be reached about the effects of climate on plant tolerances to heat treatment, as suggested by Crocker *et al*. (2002).

When the inoculum of the isolates was inserted into canes, and mycelium allowed to grow in each cane sample prior to HWT, 48.5 and 50°C were similarly effective, with 50°C treatment reducing incidence to 4.6, 0.0 and 0.9% for *I. radicicola, I. liriodendri* and *I. macrodidyma,* respectively. In this experiment *I. macrodidyma* appeared to be more resistant to HWT than *I. liriodendri* and *I. radicicola*, as mean incidences at 47°C were 35.2, 12.1 and 8.3%, respectively, whereas in the *in vitro* experiments, HWT of 45°C for 30 min completely inhibited mycelium growth of the three *I. macrodidyma* isolates. Two isolates, (mycelium of isolate 3L of *I. liriodendri* in the *in vitro* HWT experiment and mycelium of isolate 1M of *I. macrodidyma*,

in the cane inoculation experiment) appeared to be more tolerant to HWT than the other seven isolates since temperatures which had inhibited their growth did not completely inhibit growth of isolates 3L and 1M. Interestingly, both isolates 3L and 1M originated from grapevines grown in the warmer Hawke's Bay region of New Zealand.

Although Gramaje *et al.* (2010) suggested that the industry standard HWT (50°C for 30 min) was the minimum required to eliminate *Ilyonectria* spp. of Spanish origin from grapevines, this *in vitro* study indicated that a reduced HWT may be effective for New Zealand *Ilyonectria* isolates and the cane inoculation experiment showed that HWT temperatures at 48.5 and 50°C, which were similarly and significantly more effective than 47°C for inhibiting mycelium growth, could be used in New Zealand. However, other HWT protocols may be more suitable for grapevines and isolates from other countries, which should be determined by similar investigations.

The field experiment demonstrated the efficacy of a less stringent HWT protocol (48.5°C for 30 min) than the industry standard treatment of 50°C for 30 min to completely eliminate *Ilyonectria* spp. from grapevine rootstocks plants, which had become naturally infected while growing in soil infested with the black foot pathogens. In addition, the 15 min HWT at 48.5 and 50°C significantly reduced the levels of disease incidence (3.1 and 1%) and could be considered by grapevine propagators who feared that HWT could reduce viability in their plants. Although plant viability was not tested in the current experiment, it was tested in a later and similar field experiment undertaken by the authors (data not provided); none of the grafted grapevines that had been HWT at 48.5°C for 30 min died or displayed retarded growth. In other HWT experiments carried out in New Zealand, Graham (2007a) reported that vines which had been HWT at 45 and 47°C for 30 min and grown in a field nursery for three weeks had reduced mortality (<10%) compared to vines treated at 50°C (60%), and reduced 'pathogen' incidence (11 and 3%, respectively) compared to untreated controls (15%). In earlier experiments (Graham, 2007b), the mortality of rootstock cuttings 5C and 101-14 six weeks after HWT at 50°C was 60 and 95%, respectively. These reports concurred with the current study in showing that in a cooler climate, lower HWT temperatures were effective against grapevine pathogens and reduced the physiological damage to the grapevine tissues. In this study, the rates of mortality were considerably less than those of Graham (2007a; 2007b). Clearly, further investigations should be conducted with more grapevine varieties and in conjunction with cold storage, which commonly occurs before HWT of dormant plants and after HWT of grapevine cuttings.

HWT injury has been reported as less common for grapevines grown in warmer regions than in cool regions (Crocker *et al.*, 2002), which was concluded to be due to the higher levels of thermotolerance reported for grapevines grown in warmer regions than those from cooler regions (Crocker and Waite, 2004). This was attributed to the heat shock proteins synthesised by plants during hot weather, which persist into dormancy and provide protection to the plant during HWT (Crocker and Waite, 2004). Because of the increased tolerance of grapevines to HWT in regions warmer than New Zealand, those research programs have mainly used HWT protocols with temperatures equal to or above 50°C, for example, 50°C in South Africa (Crous *et al.*, 2001; Fourie and Halleen, 2004) and Australia (Edwards *et al.*, 2004; Waite and May, 2005), 51°C in California (Rooney and Gubler, 2001; Whiting *et al.*, 2001) and 50 to 53°C in Spain (Gramaje *et al.*, 2008; Gramaje *et al.*, 2009; Gramaje *et al.*, 2010) and Italy (Mannini, 2007). In the latter study, which was conducted over three years, HWT at 52°C for 45 min was found to reduce vine losses after planting to an acceptable level (zero to 20%) compared to the untreated controls (Mannini, 2007).

The HWT protocol used by grapevine propagators in any region must be effective for many of the prevalent trunk diseases, such as Petri disease, caused by *Phaeomoniella chlamydospora* and *Phaeoacremonium* species. The HWT protocols published for these pathogens have indicated that they are also affected by climatic factors. In Spain, *Pa. chlamydospora* and *P. aleophilum* in grapevine cuttings were reported to require HWT of 51–53°C for 30 min to eliminate them (Gramaje *et al.*, 2009). In contrast, in South African field trials conducted over two growing seasons, Halleen *et al.* (2007) reported that HWT at 50°C for 30 min effectively eliminated the Petri disease pathogens, *Pa. chlamydospora and Phaeoacremonium* spp. and the black foot pathogens "*C". liriodendri* and "*C". macrodidymum* as well as the *Campylocarpon* species, *Campyl. fasciculare* and *Campyl. pseudofasciculare*, from dormant grapevine plants grown in naturally infested soil. These results showed pathogen, isolate and country variability to HWT and concur with the current study.

Since HWT temperatures equal to or above 50°C have not reduced viability of grapevine cuttings grown in warmer regions and were required to control the Petri disease pathogens (Rooney and Gubler, 2001; Gramaje *et al*., 2008; Gramaje *et al*., 2009), the warm climate grapevine industries have not needed to modify the standard HWT. Hence little *in vivo* research has been conducted on the effectiveness of HWT temperatures below 50°C for control of grapevine pathogens until this study, which has indicated the efficacy of reduced temperatures against New Zealand *Ilyonectria* isolates, and the study into *Pa. chlamydospora* infection of grapevines by (Graham, 2007a).

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