OENO One vine & wine

DOI: https://doi.org/10.20870/oeno-one.2025.59.2.8385

OPEN ACCESS

VITICULTURE ORIGINAL RESEARCH ARTICLES

Hot water treatment combined with *Trichoderma* inoculation in the nursery protects planting material against grapevine trunk disease

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Article number: 8385

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Associate editor:
Astrid Forneck

Received:
11 November 2024

Accepted:
30 April 2025

Published:
20 May 2025

ABSTRACT

Grapevine trunk diseases (GTDs) cause significant losses in viticulture. To enhance the phytosanitary quality of planting material, a method was developed to control the common GTD pathogens *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*, and *Diplodia seriata* using hot water treatment (HWT) and inoculation with the biocontrol agent *Trichoderma atroviride* SC1 (Vintec®, Certis Belchim B. V.) (*Ta* SC1). Sensitivity of *P. minimum* and *P. chlamydospora* isolates to HWT was tested *in vitro* and in autoclaved wood at conidial and mycelium stages and at various time/temperature combinations (30 and 40 min at 40 °C, 45 °C, 50 °C and 55 °C). The results showed that *P. minimum* had greater tolerance to HWT compared to *P. chlamydospora*. Spore germination of all *P. chlamydospora* isolates was completely inhibited at 45 °C, while *P. minimum* isolate 117607 tolerated 50 °C. Mycelium growth of both pathogens was completely inhibited at 55 °C. In autoclaved wood, *P. chlamydospora* growth was inhibited after 30 min at 50 °C, while treatments for 45 min were necessary to inhibit the growth of *P. minimum*. In nursery experiments, cuttings were artificially inoculated and incubated with the pathogens and treated with HWT at 50 °C for 45 min under conditions of common viticultural practice. In these conditions, no recovery was recorded for *P. chlamydospora* and *D. seriata* seven days post-inoculation (dpi), but the survival rate of *P. minimum* was 5 %. In 2021, *P. minimum* recovered in 40 % of the samples six months post-inoculation, *P. chlamydospora* in 7 % twelve months post-inoculation, and *D. seriata* was eliminated. In 2022, *D. seriata* was detected in 10 % of samples six months post-inoculation and *P. chlamydospora* in 25 % of the samples twelve months post-inoculation. *P. minimum* recovered at all sampling time points during the assessment period. Combined applications of HWT and *Ta* SC1 provided protection of the planting material over the twelve months assessment period, with molecular analysis confirming 90 % recovery of *Ta* SC1. Field experiments carried out with naturally infected scions showed that HWT at 50 °C for 45 min significantly reduced the incidence of *Diplodia* spp. Overall, HWT effectively reduced GTD pathogens in grapevine planting material, and the combined treatment with *Ta* SC1 enhanced long-lasting protection in the nursery.

KEYWORDS: *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*, *Diplodia seriata*, biological control, phytosanitary treatment

INTRODUCTION

Grapevine trunk diseases (GTDs) are present in nearly all wine-growing areas worldwide and are among the most destructive diseases of grapevine (Bertsch *et al.,* 2013; Fischer & Peighami, 2019; Gramaje *et al.,* 2018; Larignon & Dubos, 1997). GTDs comprise a complex of diseases, such as Esca, Petri disease, *Eutypa* dieback, and *Botryosphaeria* dieback (Úrbez-Torres *et al.,* 2012; Fischer, 2006). The causal agents of these diseases are fungal pathogens that colonise the xylem tissue; these pathogens belong to 34 genera and more than 130 species of ascomycetes and basidiomycetes (Fischer & Kassemeyer, 2003; Gramaje *et al.,* 2018). The most common organisms isolated from symptomatic grapevine trunks in Germany are *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*, *Diplodia seriata*, *Eutypa lata*, *Phomopsis viticola*, *Fomitiporia mediterranea*, and *Cylindrocarpon destructans* (Fischer & Kassemeyer, 2003). Pathogen infection of the woody tissue occurs primarily through wounds resulting from the annual pruning of established grapevine (Larignon & Dubos, 2000) or through the grafting of propagation material in the nursery (Gramaje *et al.,* 2011; Gramaje *et al.,* 2018). Infections in the nursery are commonly caused by Petri disease pathogens, *P. chlamydospora* (Crous & Gams, 2000) *P. minimum* (Crous *et al.,* 1996), and species of the *Botryosphaeriaceae* (Fischer, 2006; Halleen *et al.,* 2007; Gramaje & Armengol, 2011; Bertsch *et al.,* 2013). Early infection of the planting material leads to significant losses in newly established vineyards due to stunted growth, wilting or dieback, and can serve as a source of areal inoculum for neighbouring vineyards (Aroca *et al.,* 2010; Fourie & Halleen, 2004; Mugnai *et al.,* 1999). Currently, no curative treatment is available for the control of these diseases (Bertsch *et al.,* 2013; Mutawila *et al.,* 2011). The elimination of fungal organisms from the xylem tissue is difficult, since standard chemical treatment methods do not access the inner tissue of grapevine cuttings (Gramaje & Armengol, 2011; Waite & May, 2005).

An alternative method for controlling GTD pathogens in dormant woody plant material is hot water treatment (HWT) (Crous *et al.,* 2001; Laukart *et al.,* 2001; Waite & May, 2005). However, contradictory results have been obtained in several studies on the effect of HWT against individual GTD pathogens conducted in different wine-growing regions worldwide: HWT at 50 °C for 30 min was effective in controlling *Cylindrocarpon* spp., (Bleach *et al.,* 2013; Gramaje *et al.,* 2010) and *Neofusicoccum luteum* (Elena *et al.,* 2015); meanwhile, *Lasiodiplodia theobromae*, *Phaeoacremonium inflatipes*, and *Neofusicoccum vitifusiforme* tolerated treatments at 51 °C for 30 min (Elena *et al.,* 2015; Rooney & Gubler, 2001). Additionally, the tolerance of *P. chlamydospora* and *P. minimum* to temperatures of 53 °C has been found in southern wine regions (Gramaje *et al.,* 2008). The sensitivity of planting material to HWT has been shown to depend on the climate of the growing region and on grapevine variety: an increased loss of vitality and higher graft failure have been observed in cuttings treated with HWT grown in cooler wine-producing areas compared to treatment carried out on planting material grown in southern wine regions (Bleach *et al.,* 2013; Waite & Morton, 2007).

Several biological control agents (BCAs) have been tested for their antagonistic activity against GTD pathogens. The *Trichoderma* spp. are among the most studied BCAs due to their wide-ranging modes of action; for example, direct antagonism, the release of volatile and non-volatile metabolites, and plant-growth-promoting abilities (Del Pilar Martínez-Diz *et al.,* 2021; Di Marco *et al.,* 2021; Fourie *et al.,* 2001; Harman, 2006; Kotze *et al.,* 2011; Leal *et al.,* 2023; Leal *et al.,* 2021). Several *Trichoderma* spp. are used in commercial products as suspension for dipping propagation material during propagation, as well as wound protectants that are applied after the pruning of established grapevines. The most common active ingredients of commercial bio-fungicides contain strains of *Trichoderma koningii* (TK7, Condor Shield®), *Trichoderma atroviride* (SC1, Vintec®) (Berbegal *et al.,* 2020; Del Pilar Martínez-Diz *et al.,* 2021; Leal *et al.,* 2023; Leal *et al.,* 2021), *Trichoderma harzianum* (T39, Trichodex®) (Di Marco *et al.,* 2004), *Trichoderma asperellum* and *Trichoderma gamsii* (strain ICC 012 and ICC 080, Remedier®) (Di Marco *et al.,* 2021).

HWT has not been found to provide long-lasting effects against targeted pathogens (Crous *et al.,* 2001). Therefore, the aim of this study was to assess the efficacy of HWT against the most commonly occurring GTD pathogens in viticulture, and to test a combined treatment with Vintec® (Certis Belchim B. V.) containing *Trichoderma atroviride* SC1 (*Ta* SC1) to provide a durable protection of the planting material in the nursery against *P. chlamydospora*, *P. minimum*, and *D. seriata*.

MATERIALS AND METHODS

1. Experimental setup

In order to develop an effective HWT protocol, experiments were conducted both *in vitro* and in artificially inoculated autoclaved wood under sterile environmental conditions to determine the heat tolerance of the target fungal isolates (Figure 1). Thereafter, a nursery experiment was carried out under practical conditions to test the long-term effectiveness of a treatment alone and in combination with BCA application on artificially inoculated scion cuttings. Finally, the HWT protocol was implemented on naturally infected scion cuttings, where the pathogen presence was assessed *via* real-time polymerase chain reaction (PCR).

2. Fungal organisms

The effect of HWT was tested on *P. chlamydospora*, *P. minimum* and *D. seriata*. Three isolates per species were used in this study, except for *D. seriata* for which one isolate was tested. Commercially available isolates, obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, and isolates from the Institute of Plant Protection, Dienstleistungszentrum Ländlicher

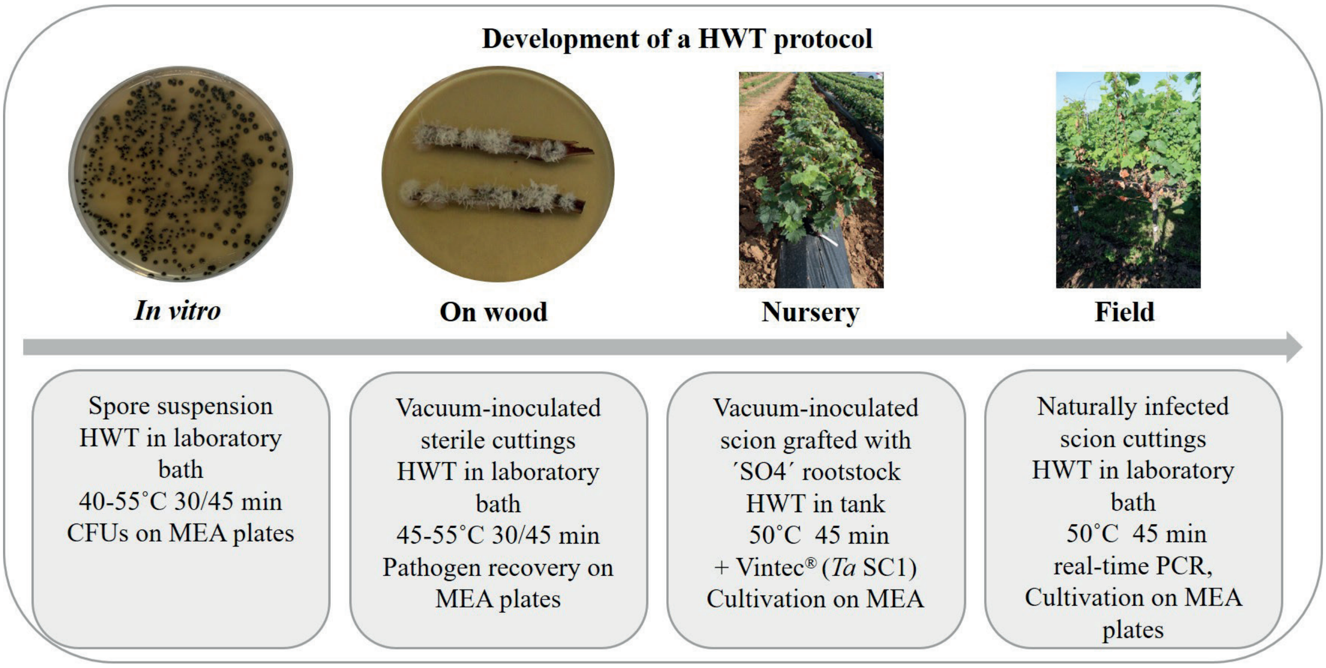

FIGURE 1. Diagram showing the workflow of the protocol development for hot water treatment (HWT) applied to eliminate GTD pathogens during the grapevine propagation process (CFU = colony forming unit, MEA = malt extract agar, *Ta* SC1 = *Trichoderma atroviride* SC1).

TABLE 1. Fungal isolates used in the experiments, comprising naturally occurring and commercially available isolates.

Species	Strain	Abbreviation	Strain collection	Geographic origin
		Botryosphaeria		
Diplodia seriata	41	Dse41	DLR	Italy
		Phaeomoniella		
P. chlamydospora	CBS 117 179	Pch117179	DSMZ	South Africa
P. chlamydospora	CBS 101 571	Pch101571	DSMZ	USA
P. chlamydospora	9886	Pch9886	DLR	Germany
		Phaeoacremonium		
P. minimum	CBS 117 607	Pmi117607	DSMZ	Italy
P. minimum	CBS 631.94	Pmi63194	DSMZ	Italy
P. minimum	9896	Pmi9896	DLR	Germany

Raum (DLR) Rheinpfalz were included (Table 1). All the fungal isolates were cultivated on 2 % malt extract agar (MEA; 2 % agar, 2 % malt extract) plates, except for *D. seriata*, which was grown on 4 % oatmeal agar (2 % agar, 4 % oatmeal). Fungal colonies were sub-cultured monthly on agar plates and incubated in the dark at 21 °C in a cultivation chamber.

3. Preparation of spore suspensions

Liquid spore suspensions of *P. chlamydospora* and *P. minimum* were prepared by flooding 2-3-week-old colonies grown on agar plates with sterile distilled water. In the case of *D. seriata*, a spore suspension was prepared according to Úrbez-Torres *et al.* (2010). The suspensions were filtered through a 30 µm pore diameter sterile gauze and the densities were adjusted to 10^4 spores/mL using a hemocytometer (Fuchs Rosenthal Counting Chamber, NanoEnTek Inc., Waltham, USA).

4. *In vitro* experiment on the sensitivity of fungal spores and mycelium to HWT

The sensitivity to HWT of the ungerminated spores and mycelium development of *P. chlamydospora* and *P. minimum* was determined in a laboratory experiment (Figure 1).

For each fungal pathogen, three isolates listed in Table 1 were tested individually. Therefore, a 50 mL spore suspension of the isolates was prepared and suspended in distilled water (spore suspension) or in 2 % liquid malt extract (ME; mycelium suspension), obtaining a final volume of 100 mL in a 200 mL Erlenmeyer flask. The spore suspension was directly treated with HW in a laboratory hot water bath for 30 min at 50 °C and 55 °C, and for 45 min at 40 °C, 45 °C, 50 °C, and 55 °C. The mycelium suspension was prepared by incubating the spore suspension on a shaker at 100 rpm for three days. The control suspension was untreated but remained on the laboratory bench at room temperature (~21 °C) for the duration of the treatment. Following treatment, 10 µL of spore or mycelial suspensions were plated onto 2 % MEA, and the plates were incubated for seven days in the dark at ~21 °C in a cultivation chamber. The number of colony-forming unit (CFU) of the isolates was counted and the treatment efficacy was calculated relative to the control. The experiment was repeated three times with ten replicates/treatments with time-temperature combinations and isolates.

5. Testing pathogen sensitivity to HWT on wood

The sensitivity of *P. chlamydospora* and *P. minimum* to HWT at several temperature-time combinations was tested on artificially inoculated sterile wood (Figure 1). Therefore, one-year-old ‘Müller-Thurgau’ dormant cane segments were autoclaved at 121 °C for 30 min. The most virulent isolates (Pch117179, Pmi63194) of each pathogen were selected for testing based on internal laboratory results. The sterile cuttings were vacuum inoculated for 15 min under 2×10^{-3} mbar pressure in a table vacuum apparatus with 700 mL spore suspension/isolate of each species. Following inoculation, the surface of the canes was rinsed with distilled water and the cuttings were treated in a laboratory hot water bath for 30 min at 50 °C and 55 °C, and for 45 min at 40 °C, 45 °C, 50 °C and 55 °C. Treatment was carried out on the day of inoculation or four days after incubation in the dark at ~21 °C to enable colonisation and mycelium development in the cane segments. Control canes were inoculated but not exposed to HWT. Following the incubation period, the cane segments were HWT at the same time-temperature combinations as previously described. The treated cuttings were surface sterilised by flaming, subdivided longitudinally, and plated on 2 % MEA plates. The presence or absence of the fungal pathogens was assessed after seven days of incubation in the dark at ~ 21°C in a cultivation chamber, and the growth rate was calculated relative to the untreated control. The experiment was conducted three times with five replicates per treatment for each tested isolate.

6. Nursery experiment on the effect of HWT on GTD pathogens and *T. atroviride* SC1

Nursery experiments were carried out to assess the long-term effect of HWT and its influence on the efficacy of *Trichoderma* (Figure 1). For this purpose, a 600 mL spore suspension of each of the following species was prepared: two combined isolates of *P. chlamydospora* (Pch117179 and Pch101571) and *P. minimum* (Pmi117607 and Pmi63194), and one isolate of *D. seriata* (Dse41). Similarly, a suspension of Vintec® containing *T. atroviride* SC1 (*Ta* SC1) was obtained by suspending the formulated granulate in 600 mL tap water (2 g/L; 10^{10} conidia per gram formulated product) according to the manufacturer’s recommendations. One-year-old ‘Dornfelder’ shoots were collected from the experimental vineyards of DLR Rheinpfalz. One-internode cuttings underwent vacuum inoculation as described above using spore suspensions of the individual GTD pathogens, and in combination with *Ta* SC1 at a ratio of 1:1. The inoculated cuttings were stored in a cooler at ~2 °C for seven days. HWT and the handling of propagation material was carried out under practical conditions seven days post-inoculation (dpi) at 50 °C for 45 min in a 6,000 L professional hot water tank in March of each experimental year. The material was stored at ~2 °C for four weeks until the grafting process took place. The cuttings were grafted with an Omega-cut on healthy ‘SO4’ rootstock and sealed with paraffin. The grafted plant material was incubated in callusing boxes for two weeks, then planted in the nursery in a randomised block design in May in both experimental years.

Plant foliage protection against powdery and downy mildew infestation and trimming was carried out based on common practice. The planting material was uprooted in November in each experimental year and stored in cold storage wrapped in plastic bags until the last sampling time point. Wood samples were taken directly after HWT (7 dpi), six months post inoculation (mpi) from plants in the nursery, and 12 mpi during the cold storage phase. The bark of the scion cuttings was removed, and samples were taken from three different parts of each cutting. A 0.5 cm wood chip was removed every centimetre starting 1 cm below the top of the scion. The wood chips were surface sterilised by flaming, then cut into four equal-sized fragments. Twelve wood fragments originating from the same cane were plated on the same agar plate containing 2 % MEA supplemented with 0.1 % tetracycline hydrochloride (MEA+TE). The presence of the pathogens was visually assessed after three weeks of incubation in the dark at ~21 °C in a cultivation chamber. The incidence of pathogen infections was calculated from the mean of three sections for each cane. The experiment was carried out in each of two independent experimental years, with a total of 60 replicates per treatment per year, and 20 replicates per sampling time point.

7. DNA extraction from wood samples and real-time PCR

DNA was extracted from ~100 mg wood material using the method described by Pouzoulet *et al.* (2013). DNA quality was verified *via* gel electrophoresis and final DNA concentration was measured *via* NanoDrop 2000 (Thermo Fisher Scientific GmbH, Osterode, Germany). Real-time PCR was performed to detect the fungal pathogens and *Ta* SC1 in triplicate using reaction mixtures at a total volume of 25 µL and containing 12.5 µL 2 × iTag Universal SYBR Green Supermix Buffer (Bio-Rad Laboratories, Inc., Hercules, USA), and forward and reverse primers at a concentration of 0.3 µM, 10 µL nuclease-free H_2O with 1 µL DNA template at a dilution of 1:10 in a Rotor-Gene Q cycler (Qiagen, Hilden, Germany). PCR products were purified using GenepHlow™ Gel/PCR Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan) according to the user manual. Sequencing of PCR products was conducted by Eurofins Genomics GmbH (Ebersberg, Germany). Sequences were edited using the BioEdit Sequence Alignment Editor (version 7.0.5.3) and alignments were processed *via* Clone Manager 9.2 software.

8. *Ta* SC1 isolation and identification at the end of the nursery experiment

Identification of *Ta* SC1 was carried out at the end of each experimental year. Three samples were randomly selected from each *Ta* SC1 treatment group for molecular analysis. A 0.5 cm segment was removed from the scion 2 cm below the top cut and chopped into thin slices. Amplification of the endochitinase 42 gene was carried out by the forward (Ech42 Fw; 5´-GTTCTGAGGCTGGAAGTTGC-3´) and reverse primer (Ech42 Rv; 5´- ACGCCGTCTACTTCACCAAC -3´) pair designed by Savazzini *et al.* (2008) and applying the cycling programme described by the authors. Sequence analysis of the amplicons was carried out to identify the presence of the probe (Ech42 P; TACCCCTTCAATCACCAATTGTTAG) specific to the target isolate.

9. Selection of material to evaluate the efficacy of HWT on naturally infected scions

Detection of *P. minimum* and *P. chlamydospora* was carried out by the forward and reverse primers according to Pouzoulet *et al.* (2013), with reaction mixtures containing the same ingredients as listed above. The primer set was designed to target the ß-tubulin gene of *D. seriata.* Sequence alignment was carried out with *Diplodia* spp. isolates KARE1632 (NCBI accession no. MN318125.1), BoF99.7 (NCBI accession no. KY701766.1), AKBA8 (NCBI accession no. KX259170.1), and MRHf12 (NCBI accession no. MK388682.1) obtained from the NCBI database. Primer specificity tests were carried out on species that commonly occur in grapevine scion. Therefore, fungal colonies from the strain collection of the Institute of Plant Protection at DLR Rheinpfalz were grown on 2 % MEA; namely, *Trichoderma koningiopsis*, *Trichoderma gamsii*, *Phomopsis ampelina*, *Penicillium expansum*, *Fomitiporia mediterranea*, *Trichothecium roseum*, *Botrytis cinerea*, *Cladosporium herbarum,* and *Cylindrocarpon destructans* (Table S1). DNA extraction from fresh fungal colonies was carried out by scraping off fungal mycelium from two-week-old colonies grown on agar plates using a sterile scalpel. The samples were freeze-dried in liquid nitrogen and homogenised as described previously. DNA extraction was performed by DNeasy plant mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Real-time PCR reactions were prepared as mentioned above using the target-specific primers (Table 2).

The PCR programme was run as follows: initial denaturation at 95 °C for 5 min, 35 cycles of 10 s at 95 °C for denaturation, and the extension step at 61 °C for 30 s, followed by the melt curve analysis from 65 °C to 95 °C with 0.5 °C increments at 5 seconds/step. A standard curve was prepared by plotting the average threshold cycle value (Ct values) against the log concentration of the target DNA to estimate the primer efficiency calculated by the software. A dilution series of 5 ng, 500 pg, 50 pg, 5 pg, and 500 fg total fungal DNA extracted from fresh mycelium was thus amplified. Melting curve analysis was carried out by the Rotor-Gene Q Series Software (version 2.0.2.4) with threshold settings at 0.16 dF/dT. Real-time PCR product clean-up and sequencing were carried out as described earlier. NCBI Nucleotide BLAST was used to identify the species of the obtained sequences.

In order to select naturally-infected scion material for the laboratory experiment, an experimental field in DLR Rheinpfalz containing 15 years old 'Riesling' plants was tested for the presence of *P. minimum*, *P. chlamydospora*, and *Diplodia* spp. *via* molecular analysis and re-isolation. Twenty plants were selected for the experiment, of which 10 plants had shown symptoms during the previous vegetative phase. Wood powder samples were taken from the trunk heads by drilling a hole with a hand drill equipped with a 5 mm drill bit in December 2023. DNA extraction

TABLE 2. Primer sequences used in this study to detect *D. seriata*, *P. minimum*, and *P. chlamydospora*.

Target organism	Target gene	Primer name	Primer sequence (5´-3´)	Product length (bp)	MC* temperature (°C)	Efficiency (%)	R² Value	Reference
Diplodia spp.	ß-tub	Dse_Tub_41F	GCGAGCACGGCCTG	~70 bp	84.6 ± 0.03	97.4	0.948	This study
		Dse_Tub_111R	AGACGTTCATGCGCTCC					
P. chlamydospora	ß-tub	PchQF	CTCTGGTGTGTAAGTTCAATCGACTC	72 bp				Pouzoulet *et al.* (2013)
		PchQR	CCATTGTAGCTGTTCCAAGATCAG					
P. minimum	ß-tub	PalQF	CGGTGGGGTTTTTACGTCTACAG	96 bp				Pouzoulet *et al.* (2013)
		PalQR	CGTCATCCAAGATGCCGAATAAAG					

* Melting curve peak temperature

was conducted as described above. Additionally, 10 mg of the powder was plated on 2 % MEA + TE for re-isolation in parallel to the molecular analysis. Powder samples plated on agar were incubated for 14 days in the dark at ~21 °C, and the presence of the target pathogen was visually assessed based on morphological traits using a stereomicroscope.

The effect of HWT on *Diplodia* spp. on naturally infected scion cuttings was assessed (Figure 1). Therefore, four dormant one-year-old shoots were collected from each of the previously tested plants and analysed for pathogen presence. Fifteen positive and 15 negative shoots were used in the experiment, and samples were taken from the first four internodes at the basal and top sections. Approximately 100 mg of drill powder samples were taken from each section. In each trial, five sections per treatment were randomly selected as replications. The experimental setup included both healthy and diseased groups of the non-HWT control and the HWT material. Assessment was carried out *via* real-time PCR and *via* re-isolation on 2 % MEA + TE prior to treatment. HWT was carried out in a laboratory water bath at 50 °C for 45 min. Drill samples were taken 24 hours post-HWT directly next to the previous sampling location, and the presence of the pathogen was assessed *via* real-time PCR and re-isolation.

10. Statistical analysis

A statistical analysis was performed using RStudio software (version 4.3.2, R. Posit Software, PBC, Boston, MA). CFU count data of the *in vitro* HWT assay was analysed by fitting the data to a negative binomial generalised linear model (GLM). *Post hoc* pairwise comparisons were conducted to assess the differences between groups using the Šídák method ($P \leq 0.05$). Binomial GLM was used to analyse the results of the wood sensitivity test and incidence in the nursery study and field study. To examine the differences in mean values between groups, the 'emmeans' package version 1.10.0 was applied using the Šídák or Tukey methods ($P \leq 0.05$). To estimate the marginal means of non-zero groups, a linear model was fitted to the data using the 'lm' function in RStudio, and the resulting model was then analysed using the 'emmeans' function. Contrasts were calculated to assess whether the estimated means significantly differed from zero. Model validation was conducted using the DHARMa package (version 0.4.6).

RESULTS

1. *In vitro* experiment on the sensitivity of fungal spores and mycelium to HWT

The results of HWT on conidial germination of three isolates of *P. minimum* and *P. chlamydospora* showed that, *in vitro*, the tested pathogens survived higher treatment temperatures in their mycelium stage compared to the ungerminated conidia stage (Figure 2). The treatment duration of 30 min did not influence the treatment efficacy at temperatures of 50 °C and 55 °C; all treatments effectively inhibited spore germination of both tested fungal pathogens (Figures 2A and 2B). *P. minimum* revealed a higher tolerance to HWT compared to *P. chlamydospora* when subjected to lower temperatures (40 °C and 45 °C). Treatments on *P. minimum* at the lowest temperature (45 min, 40 °C) did not significantly decrease the number of CFUs of the tested isolates compared to the non-treated control (Figure 2A). For isolates Pmi117607 and Pmi63194, this treatment reached an efficacy of only 5.6 % and 9.0 %, respectively. While this temperature/duration combination had no effect on isolate Pmi9896 (Table 3), the treatment at 45 °C for 45 min reduced the number of CFUs of Pmi117607 significantly (by 60.0 %) when compared to the non-treated control. This treatment showed an efficacy of 32.6 % against Pmi63194 and 53.8 % against Pmi9896. Spore germination of Pmi63194 and Pmi9896 was inhibited at 50 °C. Pmi117607 tolerated 50 °C, but conidial survival at this temperature was negligible (Figure 2A). In the case of *P. chlamydospora*, treatment at 40 °C of ungerminated spores decreased the conidial survival of Pch101571 by 93.8 %, by 89.5 % of the wild isolate Pch9886 (Figure 2B) and by 62.6 % of Pch117179 compared relative to the non-treated control. The temperatures of 45 °C completely inhibited the spore germination of all the tested *P. chlamydospora* isolates (Table 3).

Regarding the mycelium developmental stage of *P. minimum*, Pmi117607 was observed to survive treatment for 30 min at 50 °C, with an efficacy of 98.2 % against the isolate (Figure 2C). The results from the longer treatment durations showed that *P. minimum* tolerated treatments at 40 °C with a treatment efficacy ranging between 4.9 % and 54.5 % (Table 3). Pmi117607 showed the highest tolerance to HWT at the mycelium stage, since it survived treatments of 50 °C regardless of the duration of the treatment (Figure 2C). A high HWT treatment efficacy was observed against *P. chlamydospora* mycelium, the lowest being 93.4 % at the lowest tested temperature against Pch101571. With further temperature increase, the number of CFUs of the tested isolates was found to decrease (Figure 2D). Efficacy at 50 °C reached more than 99 % in case of Pch101571 and Pch9886. Temperatures at 55 °C inhibited the growth of all the isolates.

2. Pathogen sensitivity to HWT on wood

In order to determine the sensitivity of the fungal pathogens to HWT in a more natural matrix – as opposed to in the artificial *in vitro* laboratory conditions – conidia and mycelium were tested on autoclaved woody cuttings that had undergone vacuum inoculation with a spore suspension of the individual pathogens. HWT was carried out on the day of inoculation or three days later to allow spore germination and mycelium development in the inoculated canes. Because the results of the *in vitro* study had shown the survival of the two test pathogens at 40 °C, this temperature was excluded from the HWT assays on the detached canes. *P. chlamydospora* was found to be more sensitive to HWT than *P. minimum* at all temperature/time combinations (Figure 3). *P. minimum* conidia germination was not completely inhibited after treatment durations of 30 min. Treatments lasting 30 min at 50 °C showed a 17 % growth rate at the ungerminated spore stage, whereas a 7 % growth rate was detected at the

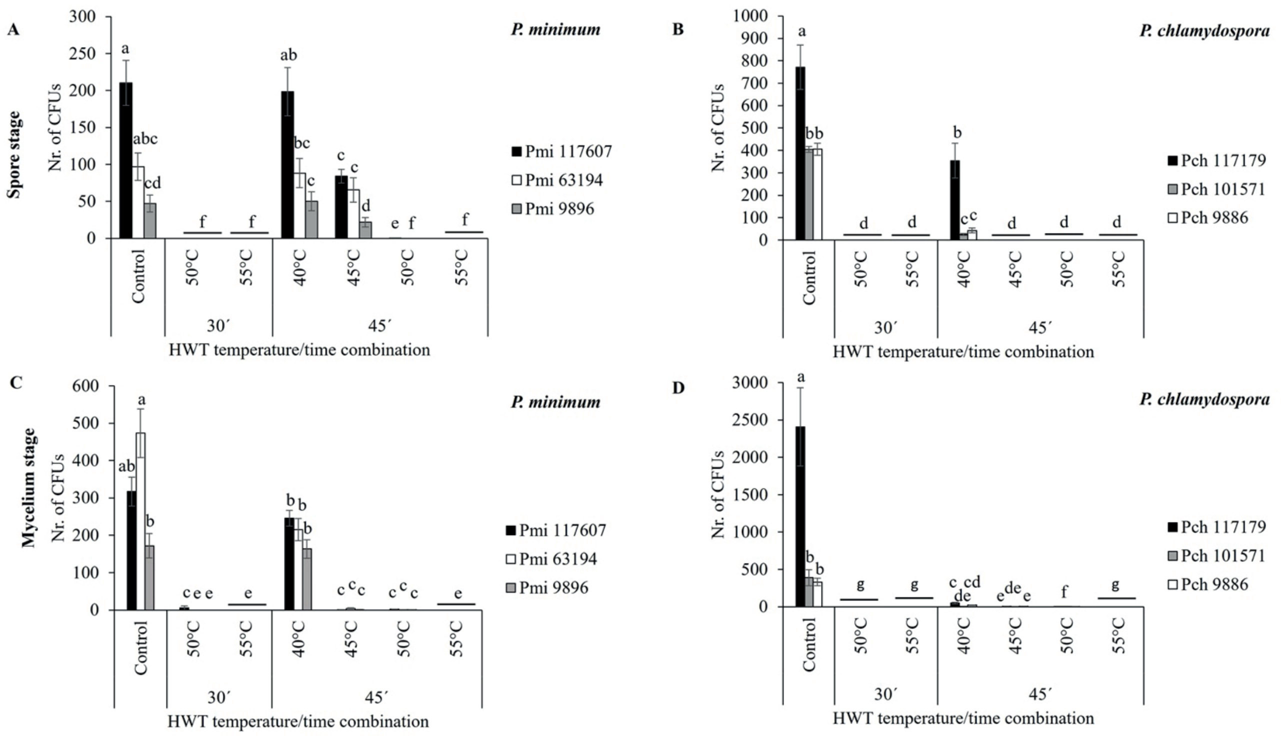

FIGURE 2. Effects of hot water treatments (HWT) comprising different combinations of temperature and exposure time on the survival of conidia (A, B) and mycelium (C, D) of three isolates of *P. minimum* and *P. chlamydospora* (CFU = colony forming unit).

Groups sharing the same letter are considered statistically indistinguishable. Significant differences between treatments were analysed by fitting a negative binomial GLM followed by a Sidak's *post hoc* test. Different letters indicate significant differences at $P \leq 0.05$ ($n = 30$).

TABLE 3. Efficacy (%) of the tested time-temperature combinations of hot water treatment (HWT) against spore and mycelium stages of three isolates of *P. minimum* and *P. chlamydospora*.

Treatment			**HWT efficacy (%)**					
			P. minimum			***P. chlamydospora***		
	Duration (min)	Temperature (°C)	Pmi117607	Pmi63194	Pmi9896	Pch117179	Pch101571	Pch9886
Spore stage	30′	50 °C	100	100	100	100	100	100
		55 °C	100	100	100	100	100	100
		40 °C	5.6	9.0	0.0	62.6	93.8	89.5
	45′	45 °C	60.0	32.6	53.8	100	100	100
		50 °C	99.9	100	100	100	100	100
		55 °C	100	100	100	100	100	100
Mycelium stage	30′	50 °C	98.2	100	100	100	100	100
		55 °C	100	100	100	100	100	100
		40 °C	22.6	54.5	4.9	98.0	93.4	98.6
	45′	45 °C	99.8	99.1	99.5	99.7	98.6	99.3
		50 °C	99.5	99.9	99.9	100	99.9	99.9
		55 °C	100	100	100	100	100	100

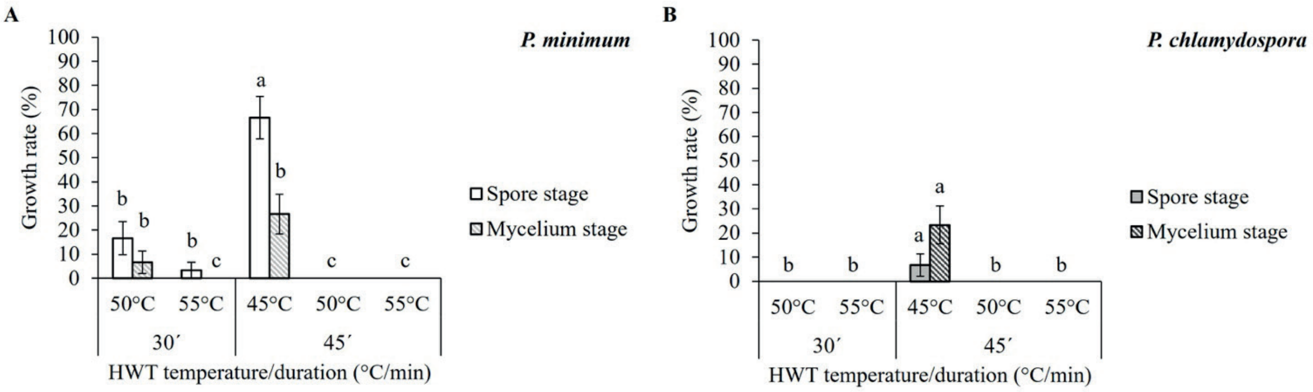

FIGURE 3. Growth rate of *P. minimum* (A) and *P. chlamydospore* (B) following hot water treatment (HWT) at different time-temperature combinations. Sensitivity was tested at the spore (HWT at 0 dpi) and mycelium stages (HWT at 3 dpi after incubation at ~21 °C in the dark).

Different letter above bars indicate significant differences between the treatments according to binomial GLM with *post hoc* comparison with Sidak's *post hoc* test ($P \leq 0.05$, $n = 30$).

mycelium developmental stage (Figure 3A). Temperatures of 55 °C for 30 min were still not sufficient for the inhibition of spore germination, the growth rate being 3 %. Nonetheless, no pathogen growth was detected when treatment was carried out at the mycelium stage of *P. minimum*. HWT at 45 °C for 45 min did not prevent spore germination, the growth rate being more than 66 %, and it reduced the growth rate of mycelium development to 27 %. Nonetheless, treatments with a duration of 45 min were effective in completely eliminating *P. minimum* at 50 °C at each of the different developmental stages. Regarding the results of *P. chlamydospora*, a shorter treatment duration of 30 min at 50 °C and 55 °C prevented the growth of the pathogen at both developmental stages (Figure 3B). *P. chlamydospora* showed tolerance to HWT for 45 min at 45 °C, with a 7 % survival rate at the ungerminated spore stage, whereas the mycelium stage showed a higher tolerance to the treatment at that temperature, with a 23 % growth rate. Temperatures at 50 °C for 45 min completely inhibited the growth of *P. chlamydospora* in detached scion cuttings.

3. Nursery experiments

As the previous tests had indicated high HWT efficacy at 50 °C for 45 min, this temperature-treatment combination was selected for further tests in the nursery experiments. None of the tested pathogens had been isolated prior to planting in the experimental year of 2021, whereas contamination of the non-inoculated samples had reached 45 % incidence prior to planting in 2022 (Table 4). Based on morphological characterisation, *Diplodia* spp. was identified in the contaminated control samples. Over the growing season, the presence of *Diplodia* spp. in non-inoculated control samples reached 10 % in 2021 and 40 % in 2022. Still, the incidence of the artificially inoculated samples was significantly higher than in non-inoculated control samples. All tested GTD pathogens successfully colonised the wooden cuttings and these pathogens were detectable one-year post-inoculation after all the usual nursery procedures associated with grapevine propagation had been carried out. *P. chlamydospora* was re-isolated from non-treated cane samples at 7 dpi in more than 70 % of the samples in both experimental years. At the first sampling time point, *P. minimum* incidence reached 90 % in 2021 and 40 % in 2022. The highest *D. seriata* incidence on the non-treated control canes was observed at 7 dpi, with 100 % in 2021 and 90 % in 2022. *P. chlamydospora* incidence throughout the experimental years reached more than 65 %, of *P. minimum* it exceeded 60 % and *D. seriata* remained above 35 % at all sampling time points. The application of *Ta* SC1 resulted in long-lasting inhibition of pathogen growth – as determined by the visual assessment – after cultivation on media. However, in the case of a cane sample inoculated with *P. minimum* and treated with *Ta* SC1, an incidence of 5 % was recorded. While no *Ta* SC1 growth could be observed on the agar plate for this sample, the inoculated pathogen was re-isolated. Following HWT, the incidence of the individual pathogens decreased significantly at the first sampling time point. No pathogen recovery was detectable at 7 dpi in the case of *P. chlamydospora* and *D. seriata*, and *P minimum* incidence reached only 5 %. In general, the treatment was not capable of fully eliminating the targeted pathogens. Throughout the two experimental years, *P. chlamydospora* was recovered at the last sampling time points. By contrast, *D. seriata* was entirely eliminated in 2021, and in the following experimental year, it was detected on 10 % of the samples at 6 mpi.

It is worth noting that the incidence of *D. seriata* in the inoculated HWT samples might have originated from natural infection, as the incidence of non-inoculated samples also increased at this time point (6 mpi in 2022). At the second sampling time point, *P. minimum* incidence reached 40 %, but no pathogen was detected at 12 mpi in 2021. In 2022, *P. minimum* incidence after HWT increased from 20 % to 30 % at the last sampling time points of the observation period.

A complete, long-term eradication of all tested pathogens was achieved through the combined treatment of HWT and *Trichoderma*. *Trichoderma* growth was not influenced by HWT, and, at the end of the two experimental years, the

TABLE 4. Pathogen incidence (%) of scion cuttings inoculated with the individual pathogens and *Ta* SC1 and exposed to hot water treatment (HWT) based on visual assessment (data merged from two independent experiments). Different letters indicate significant differences between groups based on binomial GLM followed by Tukey's *post hoc* test ($P \leq 0.05$, n = 20).

	Treatment	Pathogen incidence (%)					
		1st sampling		2nd sampling		3rd sampling	
		2021	2022	2021	2022	2021	2022
Non-HWT	Control	0 ± 0a	45 ± 11.4bc	10 ± 6.9b	40 ± 11.2bcd	10 ± 6.9b	40 ± 11.2bcd
	Pch	80 ± 9.2c	70 ± 10.5cd	80 ± 9.2c	65 ± 10.9cd	67 ± 16.7bc*	70 ± 10.5cd
	Pmi	90 ± 6.9c	40 ± 11.2bc	60 ± 11.2bc	60 ± 11.2cd	75 ± 9.9c	75 ± 9.9d
	Dse	100 ± 0c	90 ± 66.9d	65 ± 10.9c	70 ± 10.5d	35 ± 10.9bc	65 ± 10.9cd
	Pch + *Ta* SC1	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
	Pmi + *Ta* SC1	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	5 ± 5b	0 ± 0a
	Dse + *Ta* SC1	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
HWT	Control	0 ± 0a	0 ± 0	10 ± 6.9b	25 ± 9.9bcd	14 ± 9.7b**	15 ± 8.2b
	Pch	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	6.7 ± 6.7b	25 ± 9.9bc
	Pmi	5 ± 5b	5 ± 5b	40 ± 11.2bc	20 ± 9.2bc	0 ± 0a*	30 ± 10.5bcd
	Dse	0 ± 0a	0 ± 0a	0 ± 0a	10 ± 6.9b	0 ± 0a	0 ± 0a
	Pch + *Ta* SC1	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
	Pmi + *Ta* SC1	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a***	0 ± 0a
	Dse + *Ta* SC1	0 ± 0c	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a****	0 ± 0a

*n = 9, **n = 14, ***n = 6, ****n = 10

recovery of the *Trichoderma*-based BCA product from the wood sections exceeded 95 % (Table S2). The presence of the SC1 strain was confirmed through sequencing, indicating 90 % recovery of *Ta* SC1 in the treated samples (Table S3).

4. HWT of naturally infected shoot material

In order to obtain naturally infected scion material, a 15-year-old Riesling vineyard was tested for the presence of *P. minimum*, *P. chlamydospora* and *Diplodia* spp. According to the results of the real-time PCR assessment, *P. minimum* was present on the trunks of 14 % of the plants, but pathogen re-isolation was only successful in one sample. In general, *P. chlamydospora* was present in 73 % of the samples according to the molecular analysis (Table S4), while *Diplodia* spp. were present in 32 % of the tested plant trunks, which was confirmed by re-isolation on agar plates. Seventeen out of 88 shoots were tested positive for possessing *Diplodia* spp., which partially originated from mother plants that had not shown any symptoms in the previous vegetative season. The pathogen was detected in the basal first three internodes of the shoots (Table S5). Due to the high incidence of *Diplodia* spp. in the shoot material, this species was selected as the model pathogen for testing the molecular analysis method for the evaluation of HWT efficacy.

According to the visual assessment of the wood fragments, the percentage of plants infected with *Diplodia* spp. significantly decreased following HWT at 50 °C for 45 min (Figure 4): pathogen incidence was 20 % in treated plants compared to 93 % in untreated infected plants. The sequence analysis confirmed that it was *D. seriata* which had been isolated and cultivated from these infected samples. No pathogen presence was detected in the non-treated negative control group, whereas an incidence of 7 % was detected in the HWT group *via* visual assessment of the wood fragments.

According to the results of the molecular analysis, pathogen incidence in the untreated infected material reached 80 %, with two samples showing an amplification signal, but the melt curve remained below the threshold limit. *Diplodia* spp. was detected in 53 % of the infected HWT samples one-day post-treatment, with no significant impact of the treatment compared to the non-treated infected group. The incidence of the non-infected control samples treated with HWT was 7 % according to the real-time PCR detection, confirming the observations made on the agar plates. This incidence was

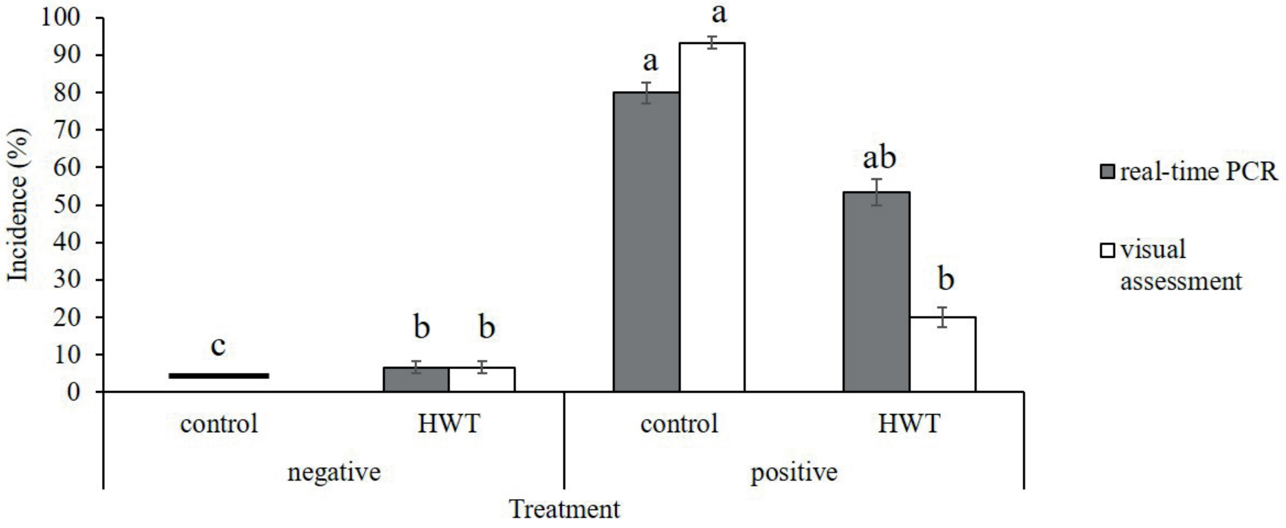

FIGURE 4. Incidence (%) of *Diplodia* spp. in one-year-old cane samples non-treated or exposed to hot water treatment (HWT) grouped as infected (positive) and non-infected (negative) according to visual and molecular (real-time PCR) analysis.

Significant differences are indicated with different letters above the bars based on binomial GLM followed by Tukey's *post hoc* test ($P \leq 0.05$, $n = 15$).

reached in one sample colonised by *Diplodia mutila,* which was verified by sequence analysis.

DISCUSSION

This study investigated the efficacy of using HWT against several GTD pathogens, and the influence of HWT on the antagonistic ability of *Ta* SC1. In order to assess the sensitivity of different isolates to HWT, pathogenic isolates of diverse origin (from German grapevine growing areas, as well as from Italy and South Africa) were selected and tested *in vitro*. The survival of *P. minimum* conidia was observed following HWT at 50 °C for 45 min, whereas *P. chlamydospora* conidia had already been killed by 45 °C. Gramaje *et al.* (2008) had similarly reported the higher sensitivity of *P. chlamydospora* to HWT compared to *P. minimum,* confirming the present findings. In the present study, no significant differences between individual isolates of one fungal species were observed with regard to HWT efficacy. However, a previous study has shown that in the case of isolates originating from southern vine-growing regions a higher temperature was required to inhibit pathogen growth. In that study, the inhibition of conidial germination of *P. chlamydospora* required temperatures of 53 °C, whereas complete elimination of *P. minimum* was achieved following treatments at 54 °C (Gramaje *et al.,* 2008). In the present study, the sensitivity results of the two distinct developmental stages indicate that both pathogens have a high temperature tolerance at the mycelium stage. HWT at 40 °C showed no or only poor efficacy against isolates of *P. minimum*, and colony formation of the pathogen was detected following treatment at 50 °C regardless of the treatment duration. In the case of *P. chlamydospora*, HWT on the mycelium reached an efficacy of more than 90 % at 40 °C and 45 °C, and survival of the pathogen was still observed at 50 °C.

The survival of *P. minimum* and *P. chlamydospora* observed after treatment at 50 °C are consistent with the findings of Gramaje *et al.* (2008) and Whiting *et al.* (2001), who reported that HWT at 50 °C is not sufficient for the elimination of these GTD pathogens. It is important to mention that HWT above 50 °C for grapevine varieties grown in cooler wine-producing areas is limited due to the higher sensitivity of the planting material to heat stress, leading to increased graft failure and loss of vitality (Waite & Morton, 2007). Additionally, the present findings indicate that the duration of a treatment has less impact on its efficacy than temperature, since treatments lasting 30 min at 50 °C were already able to inhibit germination of *P. chlamydospora* and *P. minimum* conidia *in vitro*. Similarly, Whiting *et al.* (2001) found no conidia germination of *P. chlamydospora* following treatment at 51 °C for 15 min. In contrast, the present study indicated that in detached canes artificially inoculated with *P. chlamydospora* and *P. minimum,* 30-min treatments at 50 °C did not inhibit germination of *P. minimum*, but 45 min at 50 °C was sufficient for the elimination of both tested pathogens in scion cuttings.

Based on these results, no initial infection of *P. minimum* was expected in the nursery experiment, but survival of the pathogen was nonetheless recorded at the first sampling time point in both experimental years. This difference is most likely due to the different grapevine varieties tested in the two experiments, since the diameter of 'Dornfelder' cuttings is, on average, larger than that of 'Müller-Thurgau', which may have reduced the ability of heat to penetrate the wood tissue, thereby better protecting fungal structures. However, further studies should be undertaken to confirm whether the efficacy of HWT depends on the diameter of the wood material of diverse grapevine cultivars. The results of the nursery experiment showed that HWT on scion cuttings inoculated with *P. chlamydospora* and *P. minimum* was effective in significantly reducing pathogen incidence. Nonetheless, re-isolation of the target pathogens occurred at several time points in the two experimental years, suggesting a partial survival of the pathogens after HWT. This points to the inability of HWT to provide long-term protection for planting material, which is consistent with previous findings (Crous *et al.,* 2001; Gramaje *et al.,* 2009).

Similarly, *Diploda* spp. recovered after six months in the second experimental year. It is worth noting that a high abundance of *Diploda* spp. occurred in non-inoculated control samples in 2022, indicating its presence in the planting material before the propagation process had begun. Recent studies have investigated potential inoculum sources of the *Botryosphaeriaceae* species in commercial nurseries, finding high infection rates by *Neofusicoccum* luteum, *Neofusicoccum* parvum, *D. mutila*, and *D. seriata* in mother plants (Billones-Baaijens *et al.,* 2013; Spagnolo *et al.,* 2011), as well as in rootstock tissues (Gramaje *et al.,* 2022). This may explain the high contamination level of planting material with *Diploda* spp. in the present study.

A study by Fischer and Kassemeyer (2003) on trunk samples collected from grapevines from established vineyards confirmed that *D. seriata* is one of the most commonly occurring GTD pathogens in Germany (Fischer & Kassemeyer, 2003). Thirty-two percent of the tested plants were infected with the pathogen, and more than 5 % of the tested one-year-old shoots were infected with *Diplodia* spp., which, moreover, colonised the base of the shoots in an asymptomatic manner. Kraus *et al.* (2019) studied the endophytic fungal community of healthy grapevine shoots and found *D. seriata* to be the most frequently isolated GTD pathogen in young shoots of up to two months old, whereas *P. chlamydospora* was only detected in perennial grapevine branches of up to one-year-old.

The DNA analysis of HWT wood material revealed that viable and non-viable pathogens were not distinguishable by real-time PCR when samples were taken 24 hours post-treatment. To identify viable pathogens by molecular methods, RNA extraction with subsequent RT-qPCR was performed. However, both the amount and quality of RNA extracted from woody material was unsatisfactory and pathogen detection unreliable (data not shown), hence this method was not further applied. Therefore, the most reliable method for disease assessment after HWT remains the isolation of pathogens on agar plates followed by visual assessment.

The present results revealed that HWT significantly reduced the incidence of scion cuttings naturally infected with *D. seriata*. Based on the results of the nursery experiment, treatment at 50 °C for 45 min of *D. seriata* inoculated cuttings showed complete eradication of the pathogen at 7 dpi in both experimental years. Previous studies from New Zealand showed less than 4 % survival of *D. seriata* mycelium plugs when treated *in vitro* for 45 min at 50 °C. Moreover, a high reduction in the vitality of several *Botryosphaeriaceae* pathogens was observed following HWT of artificially inoculated canes at 50 °C for 30 min (Elena *et al.,* 2015), thus indicating a high sensitivity of the pathogen to HWT, which is similar to the present *Diplodia* spp. observations.

Overall, these findings confirm that HWT efficacy is strongly dependent on the targeted pathogens, and that when applied alone it does not provide the treated planting material with sufficient long-term protection from re-infection. However, the results from the nursery experiment indicate that a combined treatment of HWT and *Ta* SC1 application may successfully prevent recovery of the pathogens at any of the tested time points. Previous studies have already shown the high biological activity of *Trichoderma* spp. against GTD pathogens (Del Pilar Martínez-Diz *et al.,* 2021; Di Marco & Osti, 2007; Di Marco *et al.,* 2004; Úrbez-Torres *et al.,* 2020; van Jaarsveld *et al.,* 2020). While the commercial product containing *T. harzianum* (TrichoFlow-T™) tested in nursery experiments reduced the incidence of several GTD pathogens (Fourie & Halleen, 2006), the same product tested again by Halleen and Fourie (2016) displayed only poor efficacy. It is worth noting that, in the latter study, the re-isolation rate of *T. harzianum* varied between three and nine percent after drench application. By contrast, in the present study, the biocontrol agent containing *Ta* SC1 was re-isolated from nearly every sample following vacuum inoculation. Together with the results of the sequence analysis, which confirmed the presence of *Ta* SC1, our results verify the high potential of the strain to colonise grapevine canes. These findings are in line with those of Berbegal *et al.* (2020), who reported an 80 % re-isolation rate of *Ta* SC1 from rootstock planting material at the end of the growing season following soaking treatments. This all suggests the higher ability of *Ta* SC1 to colonise the wood tissue of grapevine compared to *T. harzianum*. In the present study, this ability might have been promoted by the applied vacuum inoculation method, allowing conidia to enter the vessels of the planting material. Moreover, HWT had no adverse effects on the antagonist ability of *Ta* SC1 when it colonised the wood of the planting material prior to the heat treatment. This indicates that flexible BCA application can be combined with HWT in an integrated management approach during the propagation process.

CONCLUSION

The results of the present study indicate that HWT at 50 °C for 45 min or *Ta* SC1 application alone were insufficient for full elimination of GTD pathogens. However, the combined treatment significantly reduced pathogen incidence and provided long-lasting protection by *Ta* SC1 of the graft unions in the nurseries during the 12-month observation period. Additionally, the low sensitivity of *Ta* SC1 to HWT was shown to enable a flexible sequence of treatments during the propagation process. This study highlights the potential of applying combined HWT and *Ta* SC1 treatments in the production of grapevine planting material to improve its phytosanitary quality.

ACKNOWLEDGEMENTS

This research was financially supported by the Forschungsring des Deutschen Weinbaus (FDW) at the Deutsche Landwirtschafts-Gesellschaft (DLG e.V.). The authors thank Maja Kube, Helena Becker and Jens Holzhauser for the technical support.

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