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VITICULTURE ORIGINAL RESEARCH ARTICLES

Enhancing grapevine viability and pathogen control through optimised hot water treatment

Margarida Lourenço[1], Cláudia Neto[2,3,*], Justino Sobreiro[2,3], and Jorge Sofia[4,5]

[1] VITICERT, Rua Cândido dos Reis, 1, Polígono IVV, 2560-312 Torres Vedras, Portugal
[2] Polytechnic University of Beja, Escola Superior Agrária, Rua Pedro Soares, 7800-295 Beja, Portugal
[3] Centre for Sci-Tech Research in Earth System and Energy (CREATE), Pólo de Beja, Rua Pedro Soares, 7800-295 Beja, Portugal
[4] Instituto Nacional de Investigação Agrária e Veterinária, I.P., Polo de Inovação de Dois Portos/Estação Vitivinícola Nacional, Quinta d'Almoinha, 2565-191 Dois Portos, Portugal
[5] Centre for Functional Ecology, Associate Laboratory TERRA, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, Portugal

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*correspondence:
claudia.neto@ipbeja.pt

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ABSTRACT

Petri disease is a Grapevine Trunk Disease (GTD) primarily associated with the ascomycete species *Phaeomoniella chlamydospora*, which leads to substantial losses in young vineyards, particularly during the first years after planting. Contamination of plant material before and during nursery procedures with this species represents a potential risk for future vineyards. Nurseries need tailor-made and effective methods for guaranteeing the supply of GTD-free material. This study evaluated Hot Water Treatment (HWT), a widely used method to reduce or eliminate GTD-associated fungi in grapevine propagation materials, in one of the most common scion-rootstock combinations in Portuguese grapevines. The assessment focused on the elimination of *P. chlamydospora* and plant viability in plants of cv. Touriga Nacional grafted onto 1103 P rootstock. Treatments included non-HWT and HWT plant parts at 46 °C, 50 °C and 54 °C for 45 min, applied to both non-inoculated and *P. chlamydospora*-inoculated scions and rootstocks. Fungal presence was assessed before and after HWT, and growth parameters of grafted plants were measured after stratification and three months in a nursery. HWT effectively eliminated *P. chlamydospora*; however, inoculated plants exhibited reduced quality traits compared to non-inoculated plants. The 46 °C treatment produced the highest-quality plants, enhancing the callogenesis process of the grafting union. Mathematical modelling of the pathosystem comprising the pathogen *P. chlamydospora* and hosts *Vitis vinifera* as scion and *V. rupestris* × *V. berlandieri* as rootstock suggests a better resilience of the symbiont plants to higher temperatures when compared to *P. chlamydospora*. Optimised HWT protocols are recommended to reduce GTD pathogens while maintaining plant viability.

KEYWORDS: hot water treatment, petri disease, *Phaeomoniella chlamydospora*, *Vitis vinifera*

INTRODUCTION

Portugal's vineyards and wine heritage are significant. The Portuguese vineyard mosaic spreads across the country, distributed by 14 wine regions encompassing the insular wine regions of Azores and Madeira, with well-defined specific cultivars, mostly of Iberian or national origin (Cunha *et al.*, 2016; Cunha *et al.*, 2020). In 2023, Portugal had 177,945 ha of planted vineyards (Amaro, 2023), and the wine production represented 10.1 % of national agricultural production (Duarte *et al.,* 2022). The autochthonous grape cultivar Touriga Nacional represented 7.1 % of Portugal's grapevine area in 2023, being the third most important variety, spread by all demarcated wine regions, except for 'Vinhos Verdes' (Amaro, 2023). Touriga Nacional grafted onto 1103 Paulsen rootstock is one of the top-selling grapevine propagation materials sold in Portugal (VITICERT, personal communication).

Grapevine trunk diseases (GTDs) are increasingly present in Portuguese vineyards, leading to yield losses over the years (del Pilar Martínez-Diz *et al.*, 2021; Guerin-Dubrana *et al.*, 2019; Sofia *et al.*, 2013). Petri disease, a young grapevine decline, is a GTD causing the premature death of young grapevine plants within the nursery or in the first five years after planting (Gramaje & Armengol, 2011). It is characterised by the manifestation of typical decline symptoms as stunting, chlorotic leaves with necrotic margins, decreased general vigour and the wilting of the entire plant. When the trunk of diseased Petri plants is transversely sectioned, it evidences black punctuations in xylem vessels corresponding to discolouration extending several centimetres in longitudinal sections. *Phaeomoniella chlamydospora* a xylem-inhabiting fungus is commonly isolated from the previous lesions and is considered the main species associated with Petri disease (Leal *et al.*, 2024), although the species *Phaeoacremonium minimum* and *Cadophora luteo-olivacea* are also frequently associated with the disease (Gramaje *et al.*, 2018; Mondello *et al.*, 2018b). Eventually this disease leads to the death of the young grapevines with consequent heterogeneity in the vineyards, diminishing its production and life span and it is accounted as responsible for reducing the quality of plants in nurseries (Mondello *et al.*, 2018a). *Phaeomoniella chlamydospora* might enter the nursery mainly through infected propagation materials being its dispersal favoured not only by typical nursery conditions, such as a wet environment, high root density or close spacing between plants but also by common nursery practices as disbudding and grafting, that expose inner tissues to the pathogen (Gramaje & Armengol, 2011; Waite & Morton, 2007; Waite *et al.*, 2018). Managing *P. chlamydospora* is challenging as the fungus is embedded in woody tissues, and there is no effective chemical control (del Pilar Martínez-Diz *et al.*, 2021). Hot water treatment (HWT), an economic and environmentally friendly solution for pathogen control, is based on the application of heat to the material to denature pathogens and nematodes or pests like insects. It has been adopted by the European and Mediterranean Plant Protection Organization (EPPO) as a standard for the elimination of the flavescence dorée phytoplasma from grapevine plant parts (EPPO, 2012). The same standard refers to the elimination, from the treated materials, of several fungi associated with GTDs as *P. chlamydospora*. Several studies evaluated the effect of HWT on GTD-associated fungi. Gubler and Rooney (2001) concluded that HWT was ineffective in eliminating *P. chlamydospora* from dormant wood. Gramaje *et al.* (2008) observed the survival of *P. chlamydospora* after HWT with temperature below 53 °C, for periods of 30 to 60 min, suggesting that HWT at 53 °C is necessary to control the pathogen in Spanish nurseries. It is also known that higher temperatures can negatively impact plant material, resulting in losses of propagation material (Goussard, 1977). Post-HTW procedures are equally important in preventing plant stress, reducing its survival or treatment efficiency (Waite & May, 2005). Additionally, HWT can reduce plant biomass accumulation in the short term (Lade *et al.*, 2022). This aspect requires more attention to fully understand the contradictory results of many studies.

The effect of thermal treatments on the fungus metabolism is linked to its specific Thermal Performance Curve (TPC), since fungi are considered ectotherm organisms. Therefore, TPC models are very useful to define the boundaries of the ecological niches. There are many models of TPC; nevertheless, up to now, none have been considered universal for all living organisms (Kontopoulos *et al.*, 2024).

The Sobreiro model for *Venturia pyrina* only uses three parameters to simulate organism growth rate, being based on a second-order differential equation (Sobreiro, 2003). Due to its simplicity and strong mathematical theoretical background, it can be used in a wide range of living organisms, from bacteria and fungi to plants. Moreover, this model can calculate TPC based on the organism's optimal temperature and one of its cardinal temperatures.

There is a specific thermal performance curve for *P. chlamydospora* metabolism, where each temperature influences the organism in distinct proportions. The highest influence on the fungus growth rate occurs at an optimal temperature (T_0), whereas below the minimum temperature (T_{min}) or above the maximum temperature (T_{max}), the fungus growth is suppressed. The *P. chlamydospora* ecological niche has an optimal temperature of 20-25 °C and a metabolic range of 5 to 35 °C (Valtaud *et al.*, 2009; Tello *et al.*, 2010). When maintained for more than two weeks at 35 °C or 40 °C and then moved to the optimal range, this fungus is unable to resume growth. Low temperatures (5 °C or below) also inhibit the fungus's growth, although it is possible to resume its activity (Valtaud *et al.*, 2009).

The tolerance to HWT by plants and GTDs associated pathogens could be affected by the conditions in which cultivar and rootstock are grown (Waite & Morton, 2007; Lade *et al.*, 2022). Effectively, there is a high variability in the responses of *P. chlamydospora* elimination by HWT. On the other hand, the sustainability of the vine and wine sector is highly dependent on the supply of *P. chlamydospora*-free

plant propagation material. Therefore, the objectives of the present work were to understand the minimum HWT temperature, at fixed interval time, that provides the elimination of *P. chlamydospora* in Touriga Nacional grafted on 1103 P, without having major implications in the viability of the grafted plants.

MATERIALS AND METHODS

1. Experimental layout and operationalisation

The present experiment was designed to analyse the effect of three levels of Hot Water Treatment (HWT) temperature, 46 °C, 50 °C, and 54 °C, in the elimination of *P. chlamydospora*. The experiment was conducted in *P. chlamydospora*-inoculated canes and rootstock cuttings, as in Figure 1.

1.1. Plant material

Rootstock cuttings (1103 P cl. 4 JBP PT) and cv. Touriga Nacional cl. 16 JBP PT canes (enough to provide a minimum of 400 one-bud scions) were collected from certified rootstock and scion mother grapevines from the Lisboa and Vale do Tejo wine regions. All plant materials were stored at 5 °C until processed. Before storage, all rootstock cuttings underwent disbudding of all buds except for the basal one, a standard nursery procedure.

1.2. Inoculum preparation

An isolate Pch-1DP of *P. chlamydospora*, maintained at INIAV/Polo de Dois Portos, was used (Lourenço, 2024). For the inoculum preparation, four Petri dishes were inoculated with Pch-1DP and placed in complete obscurity in an incubator with a temperature of 24 °C ± 1 °C for approximately four weeks. The conidial suspension was prepared one day before the inoculation procedures according to Gramaje *et al.* (2009) and stored at room temperature until use. A total of 10 mL of sterile distilled water (SDW) was distributed with a 5 mL micropipette over each of the four-week-old *P. chlamydospora* colonies. After allowing SDW to soak the colonies for approximately 5 min, the colonies' surfaces were softly rubbed with a sterile glass spreading rod to liberate spores in the water. The resulting spore suspension was then filtered through two layers of sterile cheesecloth into a 250 mL Erlenmeyer flask. The filtrate was diluted with SDW, and the conidial concentration was adjusted to 10^6 conidia/mL using a hemacytometer.

1.3. Inoculation

Canes and rootstock cuttings were removed from cold storage 24 hours before the inoculation procedures (Figure 1) and kept at room temperature. Before inoculation, all plant parts were washed under running water, disinfected by immersion for 4 min in a 3 % sodium hypochlorite solution (a common household bleach with known NaOCl content was used), rinsed three times with distilled water, and allowed to dry off at room temperature for 30 min. The inoculation was performed according to Gramaje *et al.* (2009). Half of the cuttings and canes were inoculated by aspiration of the spore solution, while the other half of the material suffered a simulation of the inoculation with SDW (Figure 1). Just before inoculation, thin sections of upper and bottom portions of the cuttings and canes were cut off with a disinfected pruning scissor to expose fresh tissue, a rubber tubing was fitted around the upper portion of each cutting or cane and connected to a Millipore Millivac Mini pump while the basal portion of the plant materials were immersed in the spore suspension. Inoculation was performed for each sample during 7-10 s, ensuring an even distribution of the suspension. The inoculation time was previously determined by simulation with a solution stained with ink. After inoculation, all plant parts remained for 24 hours at room temperature before being packed and stored at 5 °C until HWT.

1.4. Hot water treatment

The HWT was performed at VITICERT (Portuguese national association of grapevine nurseries) facilities in an HWT Unit (Thermotherapia RG Termo T-110, RG-Projects, Spain), duly calibrated and certified by DGAV (General Directorate of Food and Veterinary, the Portuguese national phytosanitary authority). Bundles of *P. chlamydospora*-inoculated and non-inoculated plant parts were treated separately according to treatment (46 °C, 50 °C or 54 °C) for 45 min (Figure 1), using the normal procedures defined by DGAV before, during and after the HWT (DGAV, 2024).

1.5. Grafting, stratification and nursery

Grafted plant production followed the standard procedures of Portuguese nurseries. Before grafting, all plant parts were hydrated for two hours in tap water. Grafting (omega bench grafting) took place, separately for inoculated and

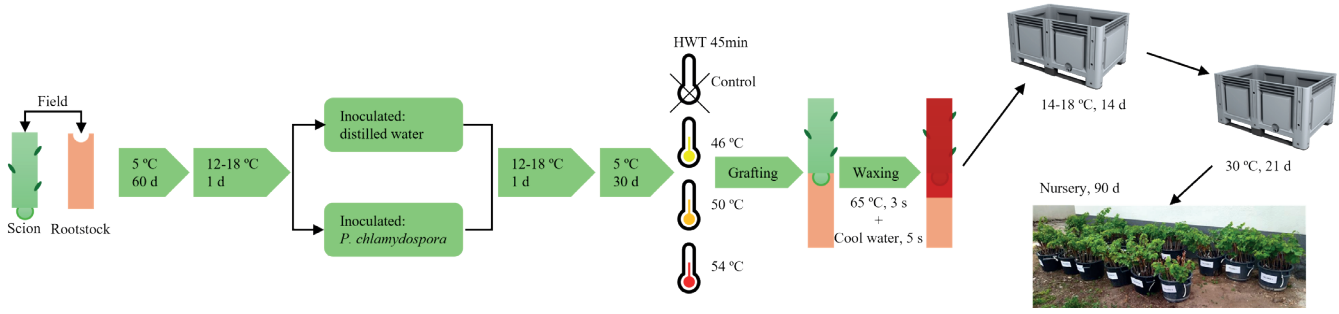


FIGURE 1. Simplified diagram of the experimental design for hot water treatments, field and warehouse procedures.

non-inoculated cuttings and scions and for each treatment, non-HWT, 46 °C, 50 °C, and 54 °C, in a certified commercial nursery (Figure 1), followed by dipping the graft in liquid paraffin at 65 °C and cooling in water at room temperature. Waxed grafted plants were then placed vertically, side by side, in layers in callusing boxes with their basal portion standing in 5 cm of water. The bins were transferred to a stratification chamber at 30 °C and a relative humidity of 90 % until callus formation was noticeable and considered finished (Figure 1). After this period, containers were removed from the stratification room and stored in a warehouse at room temperature in the dark for approximately one week before planting.

After stratification, all plants were assessed for viability, namely, callus formation and adventitious root formation. All viable grafted plants were planted in 20-litre pots containing organic substrate (Plantobalt SUB 80/20 05) and placed outside in an open field, simulating conditions similar to the ones found on local nurseries. The plants from the different treatments were planted randomly in five blocks, with 80 plants each (10 from each HWT temperature/inoculation combination). The plants were irrigated for 90 days, after which they were assessed for several indicators of plant viability.

2. Observations and measurements

2.1. *Phaeomoniella chlamydospora* presence

The presence of *P. chlamydospora* in plant tissue was assessed in several stages of the experiment, namely before inoculation, after inoculation and before HWT, after HWT, and after 90 days in the nursery. The assessment of the presence of *P. chlamydospora* was performed on 5 cm segments of wood taken from the basis of each cutting and cane, while for grafted plants, the samples were collected 2 cm below the graft union. Isolation took place as described in Sofia *et al.* (2018); bark was peeled off the samples, and the exposed tissue was disinfected with 70 % alcohol. Transversal disks (2 mm thick) were taken from the tip of the samples and disinfected with a 1.5 % sodium hypochlorite solution for 1 min, rinsed twice in SDW and then blotted dry on sterile filter paper. Each disk was split into four fragments that were placed in Petri dishes containing malt extract agar (2 % *w/v*) (MEA, VWR International, Leuven, Belgium) amended with 0.25 gL^{-1} chloramphenicol (VWR International, Sanborn, USA). The Petri dishes were sealed with Parafilm® and incubated in the dark at 24 °C, being visually monitored regularly for *P. chlamydospora* colonies.

2.2. Plant measurements

To quantify the commercial viability of the grafted vines, several growth characteristics were assessed after stratification and at the end of 90 days in the nursery. After the stratification period and before going to the open-air nursery, all the plants from the several HWT temperature/inoculation treatments were observed individually. The callogenesis was assessed through a numerical scale of callus formation according to Fourie and Halleen (2004). Through visual observation, the 0 value in the scale represents the absence of callus formation in the grafting region circumference; class 1 represents 1 % to 25 % of callus coverage; class 2 between 26 % and 50 % of callus coverage; class 3 from 51 % to 75 % of callus coverage; class 4 from 76 % to 99 % of callus coverage and finally in class 5 all the grafting region is covered with callus tissue (100 % callus coverage).

After a period of 90 days in the open-air nursery, all the vines were removed from the pots, washed to remove the substrate and analysed as to the quality of the roots, according to the number, length and distribution of the roots. According to the legal assumptions defined for commercialisation (DL 194/2006), a good root system was considered when a minimum of three independent roots was observed, and insufficient roots showed fewer than three roots. The resistance of the grafting zone to the thumb test (Carrere *et al.*, 2022) was also performed. Combining the two abovementioned assessments, the commercial viability of the grafted vines was graded (commercially acceptable or not acceptable).

3. Statistical analysis

The experimental design used for grafted vines' assessments consisted of five randomised blocks with 10 plants each, for a total of 50 plants per treatment. Each treatment considered two factors: inoculation with *P. chlamydospora* (two levels) and temperature of HWT (four levels). The nominal variables (root quality, thumb resistance, and commercial viability) and the ordinal variable (callogenesis scale) were analysed through the Kruskal–Wallis non-parametric test, and contingency tables. Whenever significant differences occurred ($p < 0.05$), Dwass–Steel–Critchlow–Fligner (DSCF) multiple comparisons were performed.

Statistical analyses were performed using IBM SPSS Statistics v. 29.0.0.0 (241) and Jamovi v. 2.6.

4. Modelling temperature performance curves

The *P. chlamydospora* ecological niche was established and compared with the hosts (*Vitis vinifera* and *V. rupestris* × *V. berlandieri*) using Equation 1 developed by Sobreiro for pear scab (*Venturia pyrina*) (Sobreiro, 2003). The minimum (T_{min}) and maximum (T_{max}) temperatures were calculated to correspond to 0.05 of the normalised maximum metabolic rates ($G_0 = 1$).

Equation 1:

$$G = G_o \frac{(\pi+1)(T_o+1)}{T_o(\pi+1)+\pi e^{-\kappa(T-T_o)}+e^{\kappa\pi(T-T_o)}} \qquad (1)$$

Where:

- G – Metabolic growth rate
- G_0 – Maximum metabolic rate
- T – Air or soil temperature (°C)
- T_0 – Optimal temperature (°C)
- κ – Ecosystem curvature or thermal compensation ($°C^{-1}$)

The model was adjusted based on available data of *P. chlamydospora* colony growth in a range of temperatures (Valtaud *et al.*, 2009; Tello *et al.*, 2010) and reported cardinal temperatures of *V. vinifera* from air measurement (Ferrante & Mariani, 2018; Kriedemann, 1968; Meggio & Pitacco, 2019). In the case of the rootstock *V. rupestris* × *V. berlandieri,* soil temperatures were used (Mahmud, 2016; Mahmud *et al.*, 2023).

The model was fitted using the Maplesoft Maple 2024.2 procedure NLPSolve *(SSE, method = sqp, assume = nonnegative, optimalitytolerance = 0.01, iterationlimit = 30)*, and using the Microsoft Excel add-in solver.

RESULTS

The presence of *P. chlamydospora* was detected in the samples originating from the plants inoculated with the fungus, confirming the effectiveness of the inoculation process, whereas in the control plants, *P. chlamydospora* colonies were not detected. Identical results were obtained in the plant material (canes and rootstocks) after cold storage and before HWT (Table 1).

After performing the HWT, the presence of *P. chlamydospora* was only observed in the inoculated cane and rootstock cuttings that were not treated, whereas no observations led to the fungus identification in any of the treated plants (Table 1). Similar results were obtained in the grafted plants, after 90 days in the open-air nursery, which means that possibly no re-contamination with *P. chlamydospora* occurred during this period in the HWT inoculated plants. None of the control plants (non-inoculated) showed any presence of *P. chlamydospora* after 90 days in the nursery (Table 1). In summary, in the present experiment, all the temperatures used in the HWT (46 °C, 50 °C and 56 °C) during 45 min seem to have led to the elimination of *P. chlamydospora* from the inoculated plant material.

Inoculation of the canes and rootstocks with *P. chlamydospora* significantly influenced ($p < 0.001$) the callogenesis process after grafting, measured by the percentage of callus formation after the stratification process. Inoculated plants presented higher frequencies in the lower callogenesis classes, namely class 0 (0 % of callus formation) and class 1 (1-24 % of callus formation) for all the HWT (Figure 2). In contrast, in non-inoculated plants, the highest ranking, class 5 (100 % callus formation), was predominant (Figure 2).

The highest HWT temperature (54 °C) presented a negative effect in callus formation in both inoculated and non-inoculated plants, inhibiting callogenesis in more than 50 % of the plants (0 % callus formation) (Figure 2). Conversely, the lowest HWT temperature used in this study (46 °C) showed the highest frequency of plants classified as 100 % callus formation (class 5) in both inoculated and non-inoculated plants (Figure 2). In the HWT at 50 °C during 45 min, the quality of callus formation was lower than in the 46 °C HWT (considering more than 75 % of callus formation), but higher than in the non-inoculated plants without HWT (Figure 2). In inoculated plants, plants exposed to 46 °C or without HWT showed similar callus formation.

TABLE 1. Presence of *Phaeomoniella chlamydospora* in 1103 P rootstock and Touriga Nacional scion with or without inoculation (control) before Hot Water Treatment (HWT), after HWT and after 90 days in the nursery.

Sampling date	Inoculated	HWT temperature	*Phaeomoniella chlamydospora* presence
After inoculation and before HWT	Yes	-	100 %
	No (Control)	-	0 %
After HWT	Yes	Without HWT	100 %
		46 °C	0 %
		50 °C	0 %
		54 °C	0 %
	No (Control)	Without HWT	0 %
		46 °C	0 %
		50 °C	0 %
		54 °C	0 %
After 90 days in nursery	Yes	Without HWT	100 %
		46 °C	0 %
		50 °C	0 %
		54 °C	0 %
	No	Without HWT	0 %
		46 °C	0 %
		50 °C	0 %
		54 °C	0 %

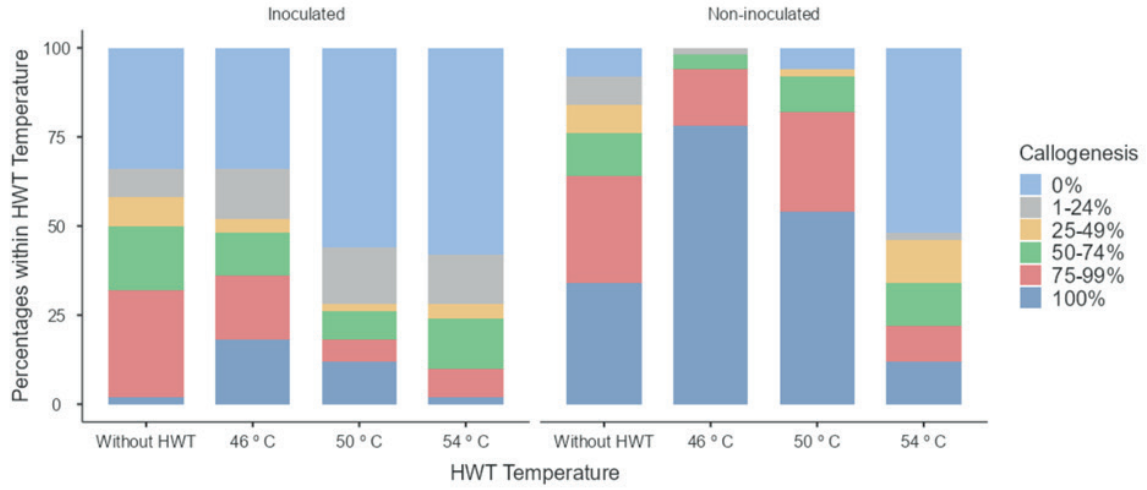


FIGURE 2. Frequencies of the several classes of callus formation (%) in vines of Touriga Nacional grafted on 1103 P inoculated with *Phaeomoniella chlamydospora* (left) and non-inoculated (right), after stratification, in response to different Hot Water Treatment (HWT) temperatures.

The visual quality of the root system of the vines after 90 days in the nursery presented significant differences ($p < 0.001$) between the *P. chlamydospora* inoculated and non-inoculated plants. There was a significant percentage of plants without a good quality root system (< 3 independent roots), which supports the future development of the vines. Hence, whereas 27.5 % of the non-inoculated plants presented good-quality roots (≥ 3 independent roots), only 9 % of the inoculated plants could guarantee this characteristic at this stage.

In both inoculated and non-inoculated vines, the HWT at 54 °C during 45 min resulted in the lowest frequency of plants with good roots (2 %) (Figure 3). In inoculated plants, less than 20 % of good quality rooted plants were found for each of the remaining HWT, although 46 °C showed the best result (Figure 3). In non-inoculated plants without HWT, more than 50 % showed good roots, followed by the HWT at 46 °C, with slightly less than 40 %.

The effectiveness of the graft union was assessed by the thumb test after 90 days in the open-air nursery, and it was significantly different ($p < 0.001$) in plants inoculated with *P. chlamydospora* and non-inoculated. Although in both cases the frequency of plants that showed thumb-resistance was below 50 % (data not shown), non-inoculated plants passed the thumb test more times than inoculated plants (Figure 4).

Considering the different HWT temperatures, in both inoculated and non-inoculated plants, a positive thumb test was more frequent in vines without HWT or with HWT at 46 °C during 45 min (Figure 4). In the higher HWT temperature (54 °C), zero to very few plants (less than 5.6 %) could resist the thumb pressure in the grafting union in non-inoculated and inoculated plants, respectively (Figure 4).

After 90 days in the nursery, a low number of plants succeeded in resisting the thumb test, which means that the grafting union did not develop positively into new vascular tissues, restoring the functionality of the xylem and phloem vessels. This was especially the case in the *P. chlamydospora* inoculated plants, where a positive thumb test was always below 10 % of the plant in all HWT except for HWT at 46 °C (20 %) (Figure 4). These results show some correspondence with those obtained for the callogenesis, since HWT at 46 °C in inoculated plants showed the highest frequency of class 5 (100 % callus formation in the grafting union). In the non-inoculated plants, the HWT at 54 °C during 45 min severely impacted the grafting process, since there was no plant that resisted the thumb test (Figure 4). In this case, not treated plants or HWT at 46 °C showed similar thumb test results, with half the plants slightly above the non-resistant grafting unions (Figure 4).

The final decision as to the viability of the grafted plants collected from the nursery considered both root quality and the thumb test. There was a significant difference ($p < 0.001$) between inoculated and non-inoculated vines in terms of commercially viable plants. Overall results showed that less than 60 % of non-inoculated plants were commercially viable after 90 days in the nursery, three times more than inoculated plants (around 20 %).

In the plants treated with the highest HWT temperature (54 °C) for 45 min, few commercially viable plants were obtained (Figure 5). The highest percentage of commercially viable plants in *P. chlamydospora* inoculated plants was observed in those with HWT at 46 °C, although slightly less than 20 % of the total batch (Figure 5). In non-inoculated plants, the percentage of potentially commercial plants decreased with increasing HWT temperature, and only the control plants presented a positive balance (more than 50 %) between commercially viable and non-viable plants (Figure 5).

The canes and rootstocks inoculation with *P. chlamydospora* was a significant factor for the unsuccessful results obtained in the commercial viability of the plants of Touriga Nacional grafted on 1103 P after 90 days in an open-air nursery (Figure 5).

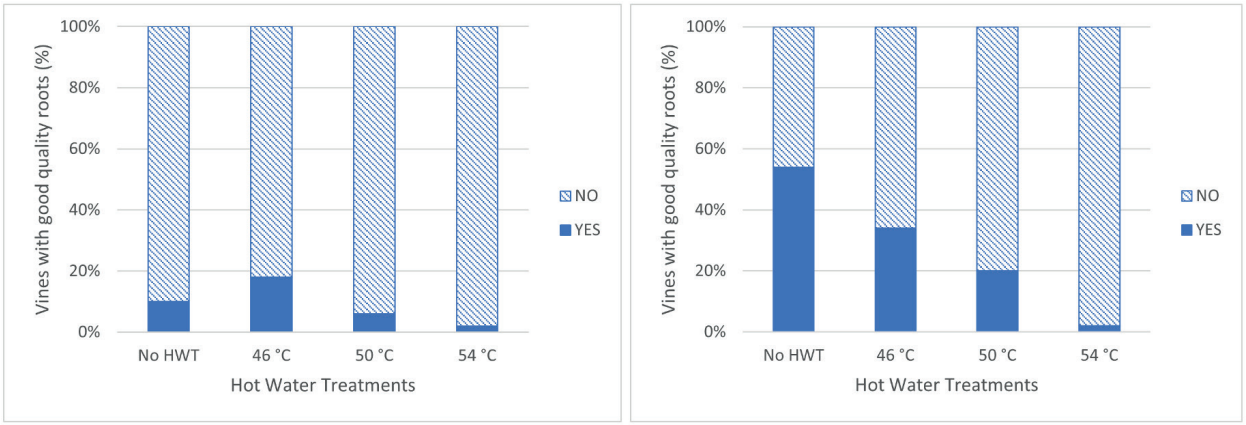


FIGURE 3. Average percentages of vines of Touriga Nacional grafted on 1103 P inoculated with *Phaeomoniella chlamydospora* (left) and non-inoculated (right), presenting a good quality root system (≥ 3 independent roots) after 90 days in nursery, in response to different Hot Water Treatment (HWT) temperatures.

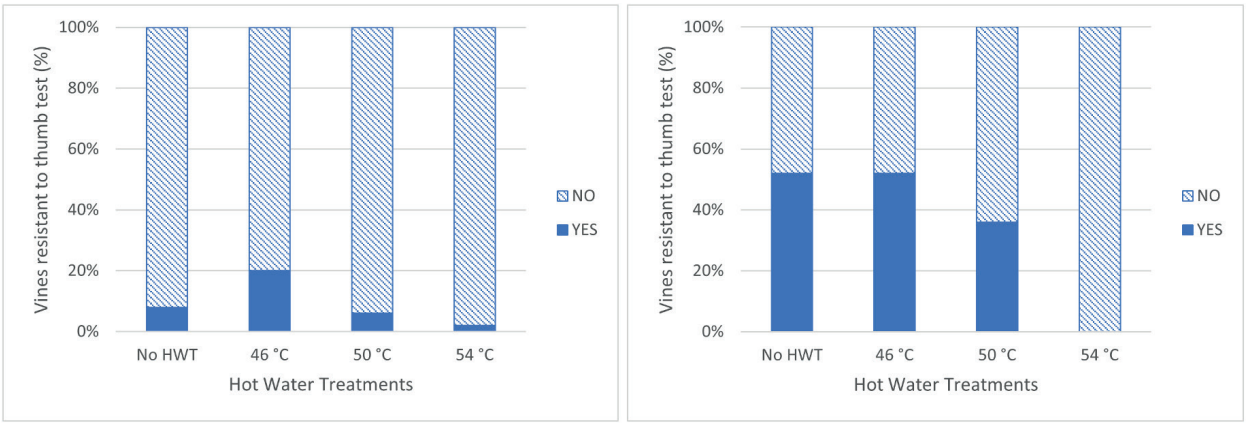


FIGURE 4. Average percentages of plants of Touriga Nacional grafted on 1103 P inoculated with *Phaeomoniella chlamydospora* (left) and non-inoculated (right), showing thumb-resistance after 90 days in the nursery, in response to different Hot Water Treatment (HWT) temperatures.

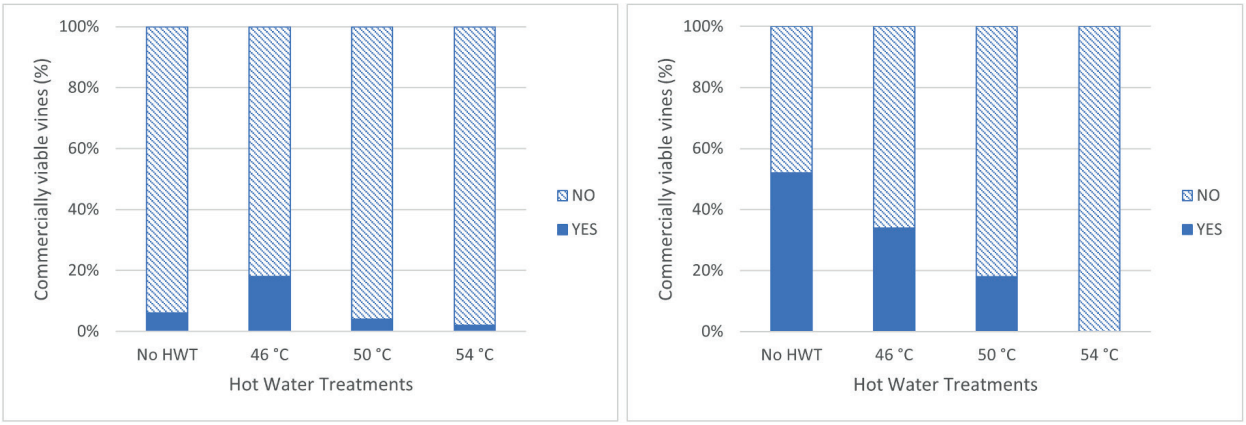


FIGURE 5. Average percentages of commercially viable plants of Touriga Nacional grafted on 1103 P inoculated with *Phaeomoniella chlamydospora* (left) and non-inoculated (right), after 90 days in the nursery, in response to different Hot Water Treatment (HWT) temperatures.

The relation between *P. chlamydospora* pathosystem and the environment is described in Figures 6 and 7, and the parameters estimated from the fitted models obtained from cultured *P. chlamydospora* reported in the literature (Valtaud *et al.*, 2009; Tello *et al.*, 2010) are in Table 2. Changes in optimal temperature shift the ecological niche towards lower or higher temperatures. The parameter κ is the only one necessary to explain the temperature ecosystem's plasticity. Nevertheless, for optimal temperatures under 20 °C, this model compensates for the lack of temperature for kinetic reactions, narrowing the simulated ecological niche.

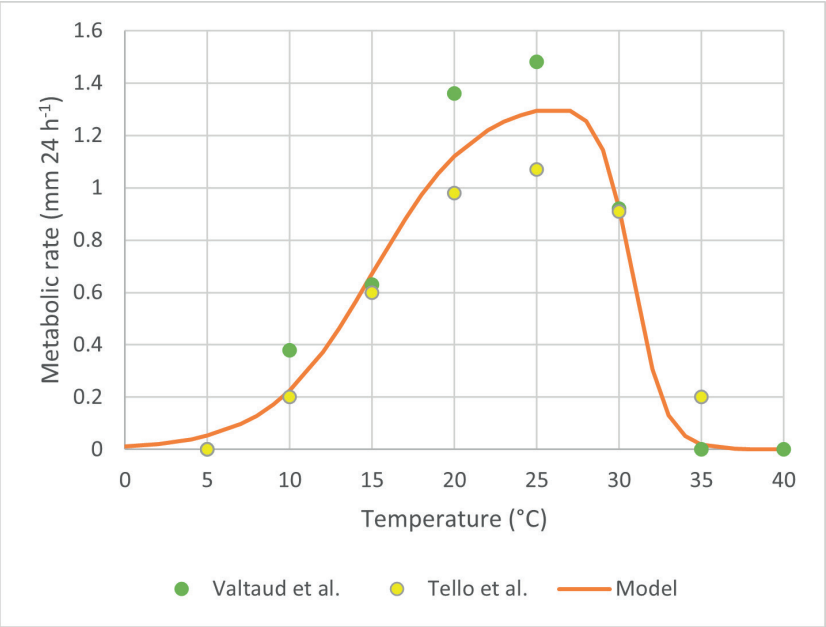


FIGURE 6. Metabolic growth rate (G) for *Phaeomoniella chlamydospora* growing in culture medium, data from Valtaud *et al.* (2009); Tello *et al.* (2010). The adjusted model ($p < 0.001$, $R^2 = 0.938$) is Equation 1 (Sobreiro, 2003).

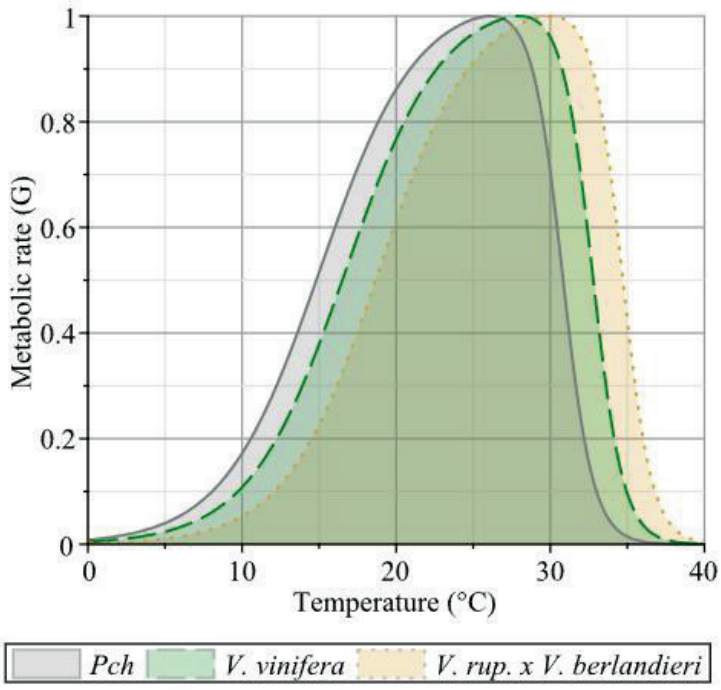


FIGURE 7. Metabolic growth rate (G) for *Phaeomoniella chlamydospora* (Pch) growing in culture medium (data from Valtaud *et al.*, 2009; Tello *et al.*, 2010), *Vitis vinifera*, and rootstock (*Vitis rupestris* × *Vitis berlandieri*). The adjusted normalised model is Equation 1 (Sobreiro, 2003).

P. chlamydospora ecological niche model ($p < 0.001$, $R^2 = 0.938$) shows that fungus infection, colonisation and growth occur from 6-34 °C with an estimated optimal temperature of 26 °C (Figure 6). Reduced fungus biological activity is expected outside this temperature interval. Higher temperatures than T_{max} could promote the fungus suppression and limit its pathogenicity heavily. The estimated environmental temperature range (ΔT, $T_{max} - T_{min}$) for *P. chlamydospora* is about 28 °C.

When compared with its hosts, *P. chlamydospora*'s ecological niche has a lower optimal temperature (Figure 7). The estimated *V. vinifera* optimal air temperature promoting better growth and development was 28 °C, and in the case of *V. rupestris* × *V. berlandieri*, it was 30 °C for optimal soil temperature. The *V. vinifera* growth occurs from 8-36 °C, and the rootstock thrives from 10-38 °C, defining the ecological niches (Figure 7). The ΔT was 28 °C for *P. chlamydospora*, vine and rootstock (Table 2).

TABLE 2. Estimated (G_0, T_0, and κ) and calculated (T_{min}, T_{max}, ΔT) parameters for *Phaeomoniella chlamydospora* pathosystem (pathogen, scion, and rootstock) based on the Sobreiro model (Sobreiro, 2003).

Model parameters[a]	G_0	T_0	κ	T_{min}	T_{max}	ΔT
Phaeomoniella chlamydospora[b]	1.30	26.11	0.32	5.85	33.70	27.85
Vitis vinifera[c]	1.00	28.00	0.32	7.52	35.67	28.15
V. rupestris × *V. berlandieri*[d]	1.00	30.00	0.33	9.93	37.49	27.56

[a] G_0—maximum metabolic rate, T_0—optimum temperature (°C), κ—ecosystem curvature ($°C^{-1}$), T_{min}—minimum temperature, T_{max}—maximum temperature, ΔT—ecosystem range.
[b] Valtaud *et al.*, 2009; Tello *et al.*, 2010
[c] Ferrante and Mariani (2018); Kriedemann (1968); Meggio and Pitacco (2019)
[d] Clarke *et al.* (2015); Mahmud (2016); Mahmud *et al.* (2023); Richards (1983)

The parameter κ estimated for the pathosystem was between 0.32 to 0.33, basically the same value. According to the model estimation, *P. chlamydospora* activity starts at lower temperatures than the vine and rootstock. Moreover, *V. rupestris* × *V. berlandieri* root growth and development seem to occur even at higher soil temperatures in the vineyard ecosystem.

DISCUSSION

The results obtained in this study are in accordance with some previous studies but also provide new insights into the HWT efficacy in *P. chlamydospora* control and its impact on plant physiological development.

Vascular connectivity implies the complete alignment of the vessels between scion and rootstock during the grafting process (Cookson *et al.,* 2019; Marín *et al.*, 2022). This improves both water and carbohydrate flow between below and above ground and is quite an important target of the grafting process. The development of callus tissue is a result of metabolic changes, namely, the increase of auxins and the decrease of phenolic compounds and peroxidases (Fayek *et al.*, 2022). Auxins are well known as key hormones for root development and promoting cell proliferation (Han *et al.*, 2025). Cell division in the contact area between the rootstock and the scion is essential for establishing a successful symbiont.

Hydration and hot water treatments prior to grafting have been reported to enhance grafting as they promote callus formation at the graft interface (Goussard *et al.*, 1977; Waite & May, 2005), a critical stage to establish a functional vascular hydraulic connection between scion and rootstock. Soaking Flame Seedless or Early Sweet vines scions and Freedom rootstock in ambient temperature tap water for 24 hours before grafting improved the success rate of both scion × rootstock combinations (Fayek *et al.*, 2022). The authors have shown that dipping the ungrafted material in Benziladenine at 250 mgL^{-1} for 30 s also improved the callus formation. HWT may contribute to better hydraulic flow by promoting callus formation through oxidative stress reduction (Lima & Dias, 2012; Lima *et al.*, 2012; Fayek *et al.*, 2022), which is critical for water and nutrient transport in grafted plants.

Some authors have observed that ideal callus formation occurred with HWT at 50 °C for 30 min, better than in control plants without HWT (Goussard, 1977). In the present study, an improvement in callogenesis after stratification was observed after HWT at 46 °C for 45 min, with better results than non-treated plants. This was especially true in the non-inoculated plants, which could indicate a potential improvement in nursery practices, with a potential benefit in fungus elimination. Even in the case of infected material, simulated by the *P. chlamydospora* inoculated plants, the same positive response of HWT at 46 °C during 45 min was observed. However, the HWT at 54 °C for 45 min in Touriga Nacional canes and 1103 P rootstocks greatly reduced the callogenesis process, especially in *P. chlamydospora* inoculated plants. Besides limited callogenesis, weak symbiont union and rooting inhibition were observed for HWT at 54 °C during 45 min. Goussard (1977) reported bud break inhibition at HWT temperatures above 55 °C. The HWT at 50 °C during 45 min proved to be adequate for a good callogenesis in non-inoculated plants, but in inoculated plants led to only 25 % of the plants with more than 50 % of callus formation.

Phaeomoniella chlamydospora spreads slowly through grapevine woody tissues, progressing through xylem upwards when initiating colonisation from the roots. This slow dispersion is attributed to host defence mechanisms, such as tylose production and accumulation of phenolic compounds in the infected areas, as trans-resveratrol and ε-viniferin (Martin *et al.*, 2009). Cultivars as Chardonnay and Touriga Nacional exhibited higher accumulation of infection-induced phenols when compared to cv. Aragonez (Martin *et al.*, 2009). These mechanisms delay, but do not fully prevent *P. chlamydospora* colonisation (Mori *et al.*, 2001).

A significant difference was observed between *P. chlamydospora*-inoculated and non-inoculated plants throughout the study, as to the physiological development after grafting. Even though the response to HWT temperatures showed a similar trend, and no pathogen was detected after the HWT, *P. chlamydospora*-inoculated plants always presented lower callogenesis, root development, resistance to the thumb-test and commercially viable plants than the control batch. This could be explained by the host defence reaction to *P. chlamydospora* infection,

due to accumulation of phenols and free hydroxyl radicals (Perez-Gonzalez *et al.*, 2021) that negatively affect the callogenesis process (Fayek *et al.*, 2022).

HWT has been shown to inhibit the growth of *P. chlamydospora* and reduce its incidence in grapevine propagation materials (Brambatti *et al.*, 2023; Gramaje *et al.*, 2008; Gramaje *et al.*, 2010). Usually, higher temperatures are more effective in fungus inactivation. HWT at 55 to 70 °C for 30 min fully inactivated *P. chlamydospora*, while temperatures lower than 50 °C were less effective (Brambatti *et al.*, 2023; Gramaje *et al.*, 2008). The good efficacy in *P. chlamydospora* elimination shown by HWT at 54 °C was counteracted by some negative impact on plant development, namely in bud break and shoot weight (Gramaje *et al.*, 2008). Gramaje *et al.* observed that HWTs above 51 °C for 30 min were necessary to reduce the incidence of *P. chlamydospora* in infected cuttings, also impacting negatively the fungus's future colonisation capacity (Gramaje *et al.*, 2008; Gramaje *et al.*, 2010). HWT temperatures up to 53 °C did not hinder plant development, as to sprouting and shoot weight (Gramaje *et al.*, 2009). Fourie and Halleen (2004) also did not find any significant differences in plant growth parameters between plants treated with HWT at 50 °C for 30 min and not treated (Fourie & Halleen, 2004). The authors refer to the fact that HWT at 50 °C for 30 min in plants severely infected by *P. chlamydospora* increased 10.8 % the number of certifiable plants. This could probably be due to the elimination of the fungus, which is considered to have an inhibiting action in callogenesis (Wallace *et al.*, 2003).

The efficacy of HWT can, however, be influenced by the length of the treatment. For instance, a 30-min HWT at 51 °C was insufficient to eradicate *P. chlamydospora* from dormant vine wood, since the fungus survived and was recovered from the treated cuttings (Gubler & Rooney, 2001). In a wide range of GTD species, the HWT at 50 °C and 53 °C for 30 min reduced the infection by GTD pathogens but was not completely effective in eliminating their growth (Eichmeier *et al.*, 2018). This highlights the need to optimise temperature and duration combinations to ensure effective control. In the present study, *P. chlamydospora* was not detected after HWT at 46 °C, 50 °C or 54 °C for 45 min, leading to the unlikely survival of the fungus. We hypothesise that it may be possible to reduce the HWT temperature while extending the HWT duration to guarantee the same efficacy level on the fungus elimination from the scion and rootstock cuttings, but this lacks experimental validation.

All the HWT temperatures tested, for 45 min, impacted negatively on the rooting process of the Touriga Nacional × 1103 P symbiont. Hence, the best rooting development was observed in the control plants not subjected to HWT. In non-inoculated plants, the percentage of plants with good roots decreased with an increment of HWT temperature, and only the control plants showed a satisfactory result, with most plants showing good roots, which seems to indicate some sensitivity of the rootstock root formation to HWT. Ferreira *et al.* (2018) obtained different results, with HWT at 51 °C for 30 min not affecting the root formation of IAC 766 rootstock cuttings, nor eliminating the fungus from the plants. When a biofumigation treatment of 37 °C was applied for 21 days, with or without HWT (51 °C, 30 min), it hampered the rooting of IAC 766 rootstock cuttings by 75-100 % (Ferreira *et al.,* 2018).

The development of roots in grafted plants in the nursery was assessed after 90 days (end of August), two months before vine dormancy, which might be one of the reasons that underscore the percentage of plants with good root development (less than 70 %). Nevertheless, there was a clear negative impact of *P. chlamydospora* inoculation on the root development of the grafted plants.

The results obtained in the final assessment of the plant quality seem to indicate an inverse relation with the increase of HWT temperature, especially in non-inoculated plants.

The order and duration of the technical operations in the nursery, such as storage and hydration, can also influence the efficacy of HWT (Waite & May, 2005). Studies have shown that HWT can affect callus and root development in grapevine cuttings, with variations between distinct cultivars (Waite & May, 2005). This stresses the importance of optimising HWT protocols for a specific combination of cultivar and rootstock.

Considering that the pathosystem is influenced by temperature, mathematical modelling suggested that the *P. chlamydospora* ecological niche prefers slightly lower temperatures for development than *V. vinifera,* or the rootstocks based on *V. rupestris* × *V. berlandieri* hybrids. The last ones seem to have the highest temperature of the ecological niche, based on soil measurements. The T_{min} for *P. chlamydospora* is around 6 °C (Valtaud *et al.*, 2009; Tello *et al.*, 2010; Whiting *et al.*, 2001), for V. *vinifera* is from 7 to 10 °C (Kriedemann, 1968; Meggio & Pitacco, 2019), and it is 10 to 12 °C for *V. rupestris* × *V. berlandieri* (Mahmud, 2016). Based on the pathosystem ecological niches, long-term survival is not possible for continuous temperatures over 34 °C (Valtaud *et al.*, 2009; Tello *et al.*, 2010; Whiting *et al.*, 2001), 36 °C (Ferrante & Mariani, 2018; Kriedemann, 1968; Meggio & Pitacco, 2019) and 38 °C for *P. chlamydospora*, *V. vinifera* and, *V. rupestris* × *V. berlandieri*, respectively. Other studies report the response of *P. chlamydospora* to temperature, taking into account fungi germination (Ji *et al.*, 2023a; Ji *et al.*, 2023b) or the development of fruiting bodies (Berbegal *et al.*, 2024).

This framework could be used for future adjustment of ecological conditions for HWT treatments and other temperature studies. For instance, if the ecological niche of the pathogen fully overlaps the ecological niche of the host, it could be difficult or impossible to implement the HWT, since eliminating the fungus also means suppressing the host's survival ability. It must be stressed that the temperature data *versus* growth to define the ecological niches for vine-specific rootstocks and cultivars based on controlled conditions is not completely known.

In summary, HWT influenced vine propagated materials in two important ways:

1. Improving callogenesis of rootstock-scion combination up to 50 °C (for 45 min), probably by auxin hormonal stimulus (Han *et al.*, 2025). Temperatures below 50 °C were required to develop three or more roots per rootstock. This is a mechanism independent of *P. chlamydospora* infection.
2. Potentially eliminating *P. chlamydospora* in vine symbionts, at temperatures between 46 °C to 54 °C (for 45 min), reducing the negative impact of phenolic and highly reactive hydroxyl radicals' accumulation on callogenesis. The presence of *P. chlamydospora* prevents a good grafting bond and rooting process.

CONCLUSION

Hot water treatments have been reported to significantly influence the physiological responses of grapevine rootstocks and scion interaction during the grafting process in nurseries, with implications on long-term plant performance. By promoting callus formation, decreasing oxidative stresses and enhancing vascular connectivity, HWT can improve the grafting success and contribute to water stress tolerance of the future vine plants, improving the overall plant's performance in the long term. Nevertheless, the water temperature and treatment duration play a key role in the final results. The HWT temperature of 46 °C for 45 min showed the lowest impact on the quality traits of Touriga Nacional × 1103 P. Additionally, by promoting the rootstock-scion vascularisation, the HWT may enhance the ability of grafted plants to cope with water stress through the improvement of symbiont water balance.

The infection of vines with *P. chlamydospora* may promote vascular blockage, water stress and oxidative stress that prevents the development of a strong bond between rootstock and scion tissues, and future root development. As a result, in scions and rootstocks with heavy fungal contamination, the suppression of *P. chlamydospora* by HWT is not enough to guarantee plant viability, due to the chemical or other "memory" effect from previous infection. In the present study, HWT at 46 °C for 45 min showed the best result for Touriga Nacional and 1103 P symbiont, without *P. chlamydospora* posterior colonisation of the tissues, keeping the plant's survival rate over 40 %.

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