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Effect of hot-water treatments above 50ºC on grapevine viability and survival of Petri disease pathogens

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Abstract

Rootstocks (41 B Mgt., 140 Ruggeri, 161-49 Couderc, 1103 Paulsen and 110 Richter) and scion/rootstock combinations (Bobal/1103 P, Merlot/110 R, Tempranillo/110 R and Tempranillo/161-49 C) were hot-water treated (HWTed) at 50, 51, 52, 53, or 54ºC for three periods: 30, 45 or 60 min. Four groups of 10 cuttings were treated for each temperature and time, and four additional groups of 10 untreated cuttings were prepared as controls. At the end of the growing season, cutting sprouting and shoot weight were evaluated. In a second

experiment, healthy cuttings of R-110 rootstock were vacuum-inoculated with conidial suspensions (10^6 conidia ml^{-1}) of one isolate of either *Phaeomoniella chlamydospora* or *Phaeoacremonium aleophilum*. These cuttings were subjected to the treatments indicated above. Four groups of 10 cuttings were treated for each temperature, time and isolate, with their respective controls. Isolations were made immediately after the treatments and at the end of the growing season, when cutting sprouting and shoot weight were evaluated. Results demonstrated that it is possible to hot-water treat grapevine planting material in Spanish nurseries using protocols with temperatures of up to 50ºC. HWTs at 53ºC are able to eliminate *Pa. chlamydospora* completely and strongly reduce the reisolation of *Pm. aleophilum* from grapevine wood. These findings will contribute to the development of an effective control for Petri disease in grapevine propagating material in Spanish grapevine nurseries.

1. Introduction

Petri disease of grapevines has been increasingly reported in wine-growing areas worldwide (Whiteman *et al*., 2007). External symptoms of this disease include stunted growth, shorter internodes, small leaves, interveinal chlorosis, and a general decline of young vines resulting in plant death (Mostert *et al*., 2006). When viewed in cross-section, vessels in the xylem of the root crown and basal rootstock of declining vines appear as dark-brown to black spots. In longitudinal view, vessel elements appear as dark streaks (Whiting *et al*., 2001). The causal agents of Petri disease have been identified as *Phaeomoniella chlamydospora* (W. Gams, Crous, M. J. Wingf. & L. Mugnai) Crous & W. Gams and numerous species of *Phaeoacremonium* (Fourie and Halleen, 2004a), mainly *Pm. aleophilum* (W. Gams, Crous, M. J. Wingf. & L. Mugnai), the most commonly isolated and studied species of this genus (Mostert *et al*., 2006).

It is widely held that contaminated nursery stock is a primary cause of the dissemination of Petri disease in many regions of the world (Bertelli *et al*., 1998; Feliciano and Gubler, 2001; Ridgway *et al*., 2002; Halleen *et al*., 2003; Edwards *et al*., 2004; Aroca *et al*., 2006; Giménez-Jaime *et al*., 2006; Retief *et al*., 2006; Fourie and Halleen, 2002, 2004a, 2006; Whiteman *et al*., 2004, 2007; Zanzotto *et al*., 2007). *Pa. chlamydospora* and *Phaeoacremonium* spp. can infect rootstock mother vines and spread from these infections by means of conidia (Pascoe and Cottral, 2000; Feliciano and Gubler, 2001) or hyphal fragments via xylem vessels into rootstock canes (Edwards *et al*., 2003). Furthermore, wounds made during the preparation and grafting processes of the propagating material may be infected by these pathogens (Fourie and Halleen, 2004b). Sanitary and protective measures to limit such infections are therefore crucial.

In recent years, there has been a frantic search for possible ways of eradication and control of these pathogens. Nevertheless, control methods for Petri disease are still limited in effectiveness. To date, only Sporekill® (120 gL^{-1} didecyldimethylammonium chloride) has been recently registered in South Africa to reduce infection by these pathogens in grapevine, but no other chemical and biological products are registered for this use, despite promising results both *in vitro* and *in vivo* (Whiteman *et al*., 2007). However, hot-water treatment (HWT) is known to be an effective, practical and relatively inexpensive method for the control of a number of endogenous and exogenous grapevine pests and diseases in dormant grapevine cuttings and young rooted vines (Lear and Lider, 1959; Goheen *et al*., 1973; Von Broembsen and Marais, 1978; Suatmadji, 1982; Ophel *et al*., 1990; Burr *et al*., 1996; Stonerod and Strick, 1996; Caudwell *et al*., 1997; Haviland *et al*., 2005; Halleen *et al*., 2007).

The use of HWT as an eradication measure for Petri disease pathogens in contaminated nursery stock has been investigated (Crous *et al*., 2001; Laukart *et al*., 2001; Rooney and Gubler, 2001; Whiting *et al*., 2001; Fourie and Halleen, 2004b; Edwards *et al*.,

2004; Halleen *et al*., 2007), but no clear consensus about the efficiency of such treatments has emerged. Crous *et al*. (2001) demonstrated that HWT at 50ºC for 30 min to rootstock cuttings before grafting was effective in eliminating the most well-known fungal pathogens and endophytes from grapevine tissue. Laukart *et al*. (2001) reported that HWT was not very effective as a curative treatment, in agreement with the results of Rooney and Gubler (2001), who found that the immersion of *Pa. chlamydospora*-inoculated cuttings in a hot water bath at 51ºC for 30 min was not effective against subsequent disease development. Whiting *et al*. (2001) observed that HWT of fungal mycelium in agar plugs caused a slight reduction in the growth rate of *Pa. chlamydospora* after 30 min but did not affect growth of *Pm. inflatipes* W. Gams, Crous & M. J. Wingf. Fourie and Halleen (2004b) reported that HWT of rootstock cuttings prior to grafting or after uprooting proved to be the only treatment that significantly reduced the levels of *Pa. chlamydospora* and *Phaeoacremonium* spp. Similar results were obtained by Edwards *et al.* (2004), who considered the reductions obtained in severity and incidence of infection warranted the use of HWT, even though *Pa. chlamydospora* was not completely eliminated. Halleen *et al*. (2007) indicated that the reduction of Petri disease pathogens caused by HWT at 50ºC for 30 min clearly demonstrated the potential of this control measure to eradicate pathogen infections from dormant nursery vines.

There is a mounting body of evidence to suggest that tolerance of plants and their accompanying pathogens to HWT are affected by the climate in which the cuttings are grown (Waite and Morton, 2007). Cuttings grown in cool climate in New Zealand and their pathogens were more susceptible to HWT, and treatments at 47ºC reduced the incidence of *Pa. chlamydospora* rather than the standard treatment (50ºC for 30 min) that resulted in unacceptable losses of cuttings (Graham, 2007a). Conversely, studies of HWT *in vitro* carried out by Gramaje *et al*. (2008), demonstrated that Petri disease pathogens in Spain were able to tolerate HWTs at temperatures of up to 52ºC, although these treatments drastically reduced

conidial germination and mycelial growth. These latter findings suggest that further research is needed on developing HWTs using temperatures above 50ºC.

The study of higher temperatures applied to HWT in grapevine propagating material could provide an optimum treatment which would reduce more effectively the incidence of fungal infections without detrimental effects on grapevine cuttings. Nevertheless, very little is known about the effects of HWT protocols on the viability of grapevine cuttings. The objectives of this study were i) to evaluate conditions suitable for HWT of dormant rooted rootstocks and grafted cuttings using temperatures of treatment above 50ºC and variable periods of treatment and ii) to determine if these conditions for HWT are effective in eliminating *Pa. chlamydospora* and *Pm. aleophilum* from inoculated grapevine wood. A preliminary report of this study was presented in the 5th International Workshop on Grapevine Trunk Diseases (Armengol *et al*., 2007).

2. Materials and methods

2.1 Hot-water treatment and grapevine viability

Two experiments were simultaneously carried out in 2005 to examine the tolerance of dormant rootstocks and grafted cuttings to high temperatures and different periods of treatment. Dormant grapevine materials were obtained from a commercial nursery.

Five sample rooted rootstocks (41 B Mgt., 140 Ruggeri, 161-49 Couderc, 1103 Paulsen and 110 Richter) and four 1-year-old scion/rootstock combinations (Bobal/1103 P, Merlot/110 R, Tempranillo/110 R and Tempranillo/161-49 C) were used in this experiment. These planting materials were placed in a hydrating bath for 1 h in order to pre-soak material before treatment. Following hydration, plants were placed in mesh polyethylene bags and

immersed in a temperature controlled water bath at 50, 51, 52, 53, or 54ºC for three periods of time: 30, 45 or 60 min. Four groups of 10 cuttings were treated for each temperature and time, and four additional groups of 10 untreated cuttings were prepared as controls.

On removal from the HWT bath, the cuttings were immediately plunged into a cool bath of clean potable water at ambient temperature for 30 min in order to stop the heating process. The cuttings were then removed from the bath and allowed to drain until there was no free moisture on the surface of the cuttings. Rootstocks and grafted cuttings were planted in May 2005 immediately after the treatment in a field nursery, where grapevine had never been grown, located in Ayelo de Malferit (Valencia, Spain). Cultural practices were carried out according to the common nursery guidelines. The experimental design consisted of four randomized blocks in which 40 cuttings (10 per block) of each temperature and time of treatment and planting material were used. In December 2005, at the end of the growing season, cutting sprouting and shoot weights were evaluated. The results were expressed as percentages of cutting sprouting or shoot weight compared to the untreated control.

2.2 Hot-water treatment of inoculated dormant grapevine wood

Experiments were conducted in two fields located in Navarrés (Valencia) in which grapevines had never been grown from May 2005 to December 2005 (Field 1) and from May 2006 to December 2006 (Field 2). Healthy dormant grapevine material was obtained from a commercial nursery.

Cuttings of 110 R rootstock (50 cm long) were vacuum-inoculated with either *Pa. chlamydospora* (isolate Pch-23) or *Pm. aleophilum* (isolate Pal-28). Fungal isolates were grown on PDA and incubated for 3-4 weeks at 25ºC in the dark. A conidial suspension was prepared for each isolate by flooding the agar surface with 10 ml of sterile distilled water

(SDW) and scraping with a sterile spatula. The resulting spore suspension was filtered through two layers of cheese cloth into a 250 ml erlenmeyer flask. The filtrate was diluted with SDW and the conidial concentration was adjusted with a hemacytometer to 10^6 conidia mL^{-1}. Cuttings were inoculated using a lab bench vacuum apparatus (COMECTA S.A, Cod: 5900621, Barcelona, Spain) as described by Rooney and Gubler (2001). Rubber tubing was fitted around each cutting and connected to the vacuum apparatus. End of cuttings were immersed in spore suspensions for approximately 7-10 s. This allowed ample time for uniform inoculation throughout the cuttings' vascular system. These cuttings were subjected to the HWTs indicated above 24 h after inoculation. Two different controls were prepared as follows: cuttings were vacuum-inoculated with fungal pathogens and not hot-water treated (HWTed) (Control A); cuttings were also vacuum-infiltrated with sterile water and not HWTed (Control B). Four groups of 10 cuttings were treated for each temperature, time and isolate, with their respective controls.

Isolations were made immediately after the treatments from sections (10 cm long) that were cut from the base end of the cuttings (Isolation 1). These sections were then washed under running tap water, surface-disinfested for 1 min in a 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water. Seven internal xylem fragments per cutting were placed on malt extract agar (MEA) supplemented with 0.5 gL^{-1} of streptomycin sulphate (MEAS). Plates were incubated for 10-15 days at 25ºC in the dark. Emerging colonies were transferred to potato dextrose agar (PDA) for further identification.

The remaining fragments (40 cm long) of the rootstock cuttings were immediately planted in the field. Cultural practices were done according to the common nursery guidelines. The experimental design consisted of four randomized blocks in which 40 cuttings (10 per block) of each temperature, time of treatment and isolate were used. In December, at the end of the growing season, cutting sprouting and shoot weight were evaluated. Plants were

also uprooted and taken to the laboratory for isolations performed as described before (Isolation 2).

2.3 Statistical analysis

In the HWT and grapevine viability experiment, the effects of rooted rootstocks, scion/rootstock combinations, temperature and time of treatment on grapevine viability were assessed by analysis of variance. Student's t-Least Significant Difference was calculated at the 5% significance level to compare treatment means. In the HWT of inoculated dormant grapevine wood experiment, the effects of pathogens, temperature and time of treatment on grapevine viability were assessed by analysis of variance. Student's t-Least Significant Difference was calculated at the 5% significance level to compare treatment means. All the analysis described before were performed using the Statistical Analysis System (version 9.0, SAS Institute Inc., Cary, NC, USA).

3. Results

3.1 Hot-water treatment and grapevine viability

Significant rooted rootstock x temperature x time and scion/rootstock combination x temperature x time interactions were observed for the sprouting percentages ($P<0.05$) (Table 1). Although there was little variability in the sprouting percentages obtained in all grapevine propagating materials, these significant interactions were attributed to the low values of sprouting reached at the highest temperatures and times of treatment (Table 2 and 3). The three-factor interaction can also be attributed to the higher sensitivity of some rootstocks or

scion/rootstock combinations to HWT than others: 48.30% or 69.90% (54ºC for 30 min and 60 min on 140 Ru rootstock, respectively) and 20.45% or 34.37% (54ºC for 60 min or 45 min on Tempranillo/161-49 C, respectively) (Table 2 and 3).

For shoot weight data, significant effects of rootstock cultivars tested were observed (P<0.05) (Table 1). The influence of rootstocks on shoot weight indicates that some rootstock cultivars are more sensitive to HWT than others. A significant effect of temperature was also observed on shoot weight of scion/rootstock combinations. The influence of the temperature on shoot weight can be attributed to the low values reached at the highest temperatures of treatment (Table 2 and 3).

3.2 Hot-water treatment of inoculated dormant grapevine wood

Analysis of variance revealed no significant effect of pathogens, temperature, time or their interactions on sprouting (%) and shoot weight (gr) in field 1 (Table 4). However, significant effects of temperature, time and pathogen x temperature interaction were observed for the sprouting percentages in field 2 (P<0.05) (Table 4). In this field, these influences can be attributed to the low values of sprouting reached at the highest temperatures and times of treatment. Moreover, in general, sprouting percentages were lower in the case of cuttings inoculated with *Pa. chlamydospora* (Table 5). In field 2, significant effects of temperature were also observed for the shoot weight (gr) (P<0.05) (Table 4). This was largely attributed to the low values reached at the highest temperatures (Table 5). In both fields, the values of sprouting and shoot weight obtained for cuttings vacuum-infiltrated with sterile water (Control B) were greater than the values of sprouting and shoot weight obtained for cuttings vacuum-infiltrated with fungal pathogens and not HWTed (Control A).

The percentages of isolation of *Pa. chlamydospora or Pm. aleophilum* immediately after the HWT (Isolation 1) and at the end of the growing season (Isolation 2) are shown in Table 5. For *Pa. chlamydospora*, the percentage of isolation after the HWT and at the end of the growing season was very low in both fields. Moreover, in all cases the percentage of isolation of this pathogen in Control A was higher than the percentage of isolation of this pathogen for each temperature and time of treatment. In contrast, *Pm. aleophilum* was isolated more frequently at the end of the growing season than after the HWT in both fields. The percentage of isolation of this pathogen in Control A was always the highest, with the exception of the Isolation 2 in Field 1. The percentage of isolation of *Pm. aleophilum* at the end of the growing season decreased with increased temperature and time combinations, and it was almost negligible at the highest temperatures of treatment (53-54ºC).

4. Discussion

The effect of HWT on the viability of grapevine planting material has been investigated in this study. In general, there was little variability in the percentages of sprouting and shoot weight for all rooted rootstocks and scion/rootstock combinations after the HWT, with the exception of the HWT at 54ºC in which the highest reduction was obtained. These results demonstrated that it is possible to hot-water treat grapevine planting material in Spanish nurseries using protocols with temperatures of up to 50ºC.

Similar findings were obtained in the evaluation of inoculated grapevine wood, where data means of sprouting (%) and shoot weight (gr) for 110 R cuttings previously vacuum-inoculated with *Pa. chlamydospora* or *Pm. aleophilum*, also reached the lowest values at the highest temperature of the HWT (54ºC). In this case, the percentages of sprouting and shoot weight for the cuttings vacuum-infiltrated with sterile water and not HWTed (Control B), was

higher than the cuttings vacuum-inoculated with fungal pathogens and not HWTed (Control A), indicating that Petri disease pathogens can negatively affect sprouting and subsequent plant growth. However, there was not a significant reduction on sprouting and shoot weight data for Control A with respect to the HWTed cuttings, indicating some effect of the HWT on plant development in spite of the reduction on fungal wood colonization. This agrees with the findings of Waite and May (2005) indicating that HWTed cuttings, particularly sensitive varieties, are generally slower to establish than cuttings that have not been HWTed and suffer retarded growth in the early part of the growing season.

Very low isolation percentages of *Pa. chlamydospora* were obtained from 110 R cuttings after the HWT and at the end of the growing season. These results are in accordance with the findings obtained by Fourie and Halleen (2004b) and Halleen *et al*. (2007), who concluded that HWT has been shown as the most effective control measure to reduce *Pa. chlamydospora* in naturally infected rootstock cuttings. In contrast, *Pm. aleophilum* was isolated more frequently at the end of the growing season in both fields, although the number of fungal colonies obtained was strongly reduced at the highest temperatures of treatment (53-54ºC). Crous *et al*. (2001) reported drastic reductions in the pathogen population occurring in the stems of the HWTed cuttings in isolations made directly after treatment. However, Rooney and Gubler (2001), in studies conducted with *Pa. chlamydospora* and *Pm. inflatipes*, indicated that although there is an initial shock to these pathogens by HWT at 51ºC for 30 min they could recover from this treatment and survive.

Our findings and those obtained by Gramaje *et al*. (2008), who showed that conidial germination and mycelial growth of Petri disease pathogens in Spain are drastically reduced at temperatures of up to 52ºC, demonstrate that HWT at 53ºC can be used as an effective control method for Petri disease since grapevine propagating material is able to grow without detrimental effects on sprouting and just some reduction on shoot weight. This treatment was

able to eliminate *Pa. chlamydospora* completely, the most important fungal organism associated with Petri disease (Ridgway *et al*., 2005), and strongly reduced the reisolation of *Pm. aleophilum* on grapevine wood. It is important to note, that these HWT protocols at high temperatures cannot be applied in all regions where grapevines are cultivated. There is a need to evaluate the sensitivity of Petri disease pathogens and grapevine planting material to HWT in each country, due to the possibility of the existence of a great variability in the tolerance of these pathogens, rootstocks and grapevine cultivars to temperatures in different viticulture regions worldwide (Gramaje *et al*., 2008). In this regard, Crocker *et al*. (2002) indicated that grapevine cuttings taken from vines grown in warm climates are of better quality to cuttings taken from vines grown in cool climates and better able to withstand HWT. Recent studies carried out by Graham (2007a) showed that cuttings grown in cool climates in New Zealand were susceptible to damage at 50ºC for 30 min, and there was also parallel evidence indicating that pathogens, particularly *Pa. chlamydospora*, were similarly affected and were controlled at lower temperatures (45-47ºC). Additionally, reports that some *Vitis vinifera* varieties are more sensitive to HWT than others began to surface in the mid-1990s when HWT was integrated into standard nursery practice (Waite and Morton, 2007). Waite *et al*. (2001) studied the sensitivity of different grapevine varieties to HWT in Australia and concluded that Pinot Noir was the most sensitive variety, Chardonnay, Reisling and Merlot were moderately sensitive and Cabernet Sauvignon the least sensitive. Very few problems have been reported with rootstock cuttings, but there have been some recent reports from Australian nurseries that 1103 P is somewhat sensitive to HWT (Waite and Morton, 2007).

It is evident that the treated cuttings can be re-infected in the field once planted out, and that the advantage of being ‘fungal free’ may be short lived. For this reason, this does raise interesting possibilities of combining chemical and biological control measures with

HWT to provide high-quality grapevines with low levels of infection (Crous *et al*., 2001; Laukart *et al*., 2001; Fourie and Halleen, 2004b, 2006; Graham, 2007b; Halleen *et al*., 2007).

Despite the benefits of this control measure, HWT of dormant nursery plants has not been embraced as a standard treatment in Spanish nurseries yet. This is largely attributed to the confusion in industry about its efficacy and safety. There is an urgent need to improve the quality and consistency of planting material for growers. For this reason, the results of this work reinforce the recommendations to nurseries that this strategy is clearly one of the most practical methods to ensure the availability of healthy planting material.

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Table 1
Analysis of variance for the effects of five representative rooted rootstocks (Rootstock), four representative scion/rootstock combinations (Scion/rootstock), temperature and time of treatments on the percentages of sprouting and shoot weight

		Sprouting (%)		Shoot weight (%)	
	df[a]	MS[b]	*P*<F[c]	MS	*P*<F
Rootstock (A)	4	206.21	0.3135	9923.04	<0.001
Temperature (B)	4	801.73	0.0013	646.83	0.1362
Time (C)	2	683.47	0.0203	23.63	0.9374
A x B	16	365.41	0.0085	514.53	0.1397
A x C	8	272.38	0.1320	569.75	0.1388
B x C	8	241.63	0.1970	325.89	0.5248
A x B x C	32	312.82	0.0070	328.78	0.6277
Residual	225	172.45		365.80	
Scion/rootstock (A)	3	2692.91	<0.001	241.81	0.6350
Temperature (B)	4	4709.83	<0.001	3772.12	<0.001
Time (C)	2	1936.91	<0.001	1192.71	0.0626
A x B	12	981.34	<0.001	681.61	0.0925
A x C	6	350.95	0.0465	113.28	0.9515
B x C	8	265.17	0.1130	234.37	0.8150
A x B x C	24	271.5	0.0287	138.99	0.9989
Residual	180	160.46		423.74	

[a]Degrees of freedom.
[b]Mean square.
[c]Probabilities associated with individual F tests.

Table 2
Data means of sprouting (%) and shoot weight (%) for each rooted rootstock after HWT at different temperature and time combinations

		41 B M	140 Ru	161-49 C	1103 P	110 R
Sprouting (%) [a]						
Temperature (ºC)	Time (min)					
50	30	79.87[b]	97.71	78.03	80.52	81.27
	45	80.41	95.33	100	84.93	96.04
	60	84.78	93.10	90.11	84.70	83.33
51	30	86.63	93.31	95.93	88.98	80.50
	45	85.55	100	78.71	91.19	91.25
	60	77.01	88.70	83.45	93.04	83.05
52	30	79.80	97.78	86.60	94.44	82.82
	45	86.61	85.20	76.62	92.80	96.75
	60	84.95	95.67	76.62	90.49	92.09
53	30	81.12	63.91	86.11	68.94	77.78
	45	91.55	88.61	76.23	80.18	100
	60	72.89	76.70	93.30	96.04	78.31
54	30	88.89	48.30	86.78	85.73	96.75
	45	78.73	93.15	87.01	87.24	77.87
	60	72.80	69.90	84.11	71.25	75.67
	LSD (1)[c]	18.29				
	LSD (2)[c]	9.94				
	LSD (3)[c]	8.05				
Shoot weight (%) [a]						
Temperature (ºC)	Time (min)					
50	30	86.25	85.07	62.51	91.35	65.10
	45	87.46	96.89	70.63	80.51	57.06
	60	91.22	80.56	76.44	75.73	76.55
51	30	81.30	90.88	54.95	98.30	79.35
	45	89.83	82.78	65.16	85.11	77.43
	60	89.51	100	65.75	87.58	89.78
52	30	88.94	95.66	54.90	84.31	77.76
	45	86.82	96.89	76.63	88.09	70.79
	60	100	83.98	74.81	85.70	75.15
53	30	100	100	76.53	81.72	66.03
	45	100	91.99	60.38	92.62	45.59
	60	100	75.55	56.62	79.76	64.39
54	30	67.61	93.22	55.66	99.15	40.98
	45	70.17	94.19	55.73	91.36	72.52
	60	89.19	97.16	60.30	60.53	72.09
	LSD (4)[c]	6.87				

[a] Percentages were calculated with respect to the untreated control and measured at the end of the growing season

[b] Average of four groups of ten cuttings each

[c] Least significant difference: means followed by the same letter do not differ significantly ($P<0.05$). $LSD_{0.05}(1)$ is for comparison of all temperature-time combination means of all rooted rootstocks; $LSD_{0.05}(2)$ is for comparison of means between temperatures; $LSD_{0.05}(3)$ is for comparison of means between times; $LSD_{0.05}(4)$ is for comparison of means between rooted rootstocks.

Table 3

Data means of sprouting (%) and shoot weight (%) for each scion/rootstock combination after HWT at different temperature and time of treatment

		Bobal/1103 P	Merlot/110 R	Temp/110 R	Temp/161-49 C
Sprouting (%) [a]					
Temperature (ºC)	Time (min)				
50	30	95.56 [b]	100	81.42	96.87
	45	90.10	97.50	92.85	100
	60	97.50	95.56	95.56	90.62
51	30	100	95.56	97.85	93.75
	45	100	100	87.85	93.75
	60	87.56	100	100	69.98
52	30	95.01	100	100	96.87
	45	97.56	100	85.01	84.37
	60	82.56	92.50	87.85	93.75
53	30	97.50	100	95.56	90.62
	45	92.50	100	89.28	92.70
	60	86.94	95.56	71.42	67.71
54	30	62.56	100	87.14	68.75
	45	75.01	100	76.42	34.37
	60	72.50	77.50	76.42	20.45
	LSD (1)[c]	17.67			
	LSD (2)[c]	8.34			
	LSD (3)[c]	10.13			
Shoot weight (%) [a]					
Temperature (ºC)	Time (min)				
50	30	87.59	87.26	92.74	97.17
	45	92.59	82.38	97.09	96.87
	60	79.96	78.20	92.15	99.60
51	30	87.30	89.19	84.47	85.23
	45	80.95	89.19	72.96	73.51
	60	77.38	89.93	62.37	94.56
52	30	86.50	90.18	83.53	88.88
	45	74.01	86.12	86.86	91.40
	60	78.57	81.83	77.57	75.12
53	30	82.60	87.37	78.55	78.76
	45	82.40	83.19	72.85	69.25
	60	79.17	75.57	80.63	77.55
54	30	71.55	84.70	86.86	68.74
	45	62.01	72.76	63.82	43.74
	60	70.33	75.23	57.51	40.79
	LSD (4)[c]	8.27			

[a] Percentages were calculated with respect to the untreated control and measured at the end of the growing season

[b] Average of four groups of ten cuttings each

[c] Least significant difference: means followed by the same letter do not differ significantly ($P<0.05$). $LSD_{0.05}(1)$ is for comparison of all temperature-time combination means of all grafted plants; $LSD_{0.05}(2)$ is for comparison of all scion/rootstock-temperature combination means; $LSD_{0.05}(3)$ is for comparison of all scion/rootstock-time combination means; $LSD_{0.05}(4)$ is for comparison of means between temperatures.

Table 4
Analysis of variance for the effects of pathogens (*Pa. chlamydospora* or *Pm. aleophilum*), temperature and time of treatments on sprouting (%) and shoot weight (gr)

		Sprouting (%)		Shoot weight (gr)	
	df[a]	MS[b]	*P*<F[c]	MS	*P*<F
Field 1					
Pathogens (A)	1	520.83	0.0966	15.41	0.4880
Temperature (B)	4	454.17	0.0551	25.77	0.5215
Time (C)	2	400.83	0.1201	71.11	0.1127
A x B	4	333.33	0.1349	64.06	0.0990
A x C	2	65.83	0.7012	2.13	0.9352
B x C	8	218.54	0.3181	21.79	0.7030
A x B x C	8	237.71	0.2604	61.55	0.0640
Residual	90	184.72		31.78	
Field 2					
Pathogens (A)	1	367.5	0.1918	0.023	0.9880
Temperature (B)	4	9450.8	<0.001	2421.3	<0.001
Time (C)	2	2363.33	<0.001	141.75	0.2584
A x B	4	1046.67	0.0012	189.57	0.1286
A x C	2	10.0	0.9541	220.14	0.1244
B x C	8	372.71	0.0968	120.06	0.3299
A x B x C	8	361.05	0.1094	60.99	0.7827
Residual	90	212.5		103.15	

[a]Degrees of freedom.
[b]Mean square.
[c]Probabilities associated with individual F tests.

Table 5
Data means of sprouting (%) and shoot weight (gr) for cuttings previously vacuum-inoculated with *Pa. chlamydospora* or *Pm. aleophilum* and the percentage of isolation of these pathogens from artificially inoculated rootstock cuttings after HWT

		Sprouting (%) [a]		Shoot weight(gr) [a]		Isolation 1 (%) [b]		Isolation 2 (%) [c]	
		Pa. chlamydospora	*Pm. aleophillum*	*Pa. chlamydospora*	*Pm. aleophillum*	*Pa. chlamydospora*	*Pm. aleophillum*	*Pa. chlamydospora*	*Pm. aleophillum*
Field 1									
Temperature (ºC)	Time (min)								
50	30	80	77.5	15.03	13	0	2.5	7.5	2.5
	45	82.5	80	15.09	12.86	0	0	0	47.5
	60	75	80	14.06	12.95	0	0	0	37.5
51	30	85	72.5	15.11	15.47	2.5	0	0	20
	45	75	72.5	23.25	10.13	0	0	0	30
	60	77.5	70	15.04	14.89	5	0	2.5	42.5
52	30	90	77.5	17.59	19.24	0	0	0	2.5
	45	80	75	13.98	13.71	0	0	0	2.5
	60	85	85	13.20	12.64	0	0	0	0
53	30	72.5	72.5	13.06	12.91	0	0	0	0
	45	80	70	18.62	14.18	0	0	0	0
	60	77.5	55	14.82	17.50	0	0	0	2.5
54	30	87.5	67.5	15.35	11.95	2.5	0	2.5	0
	45	82.5	37.5	16.20	16.72	0	0	0	0
	60	57.5	42.5	16.38	16.85	0	0	0	0
Control A[d]		82.5	80	20.36	15.16	32.5	32.5	35	35
Control B[e]		97.5	97.5	26.05	25.06	0	0	0	0
Field 2									
Temperature (ºC)	Time (min)								
50	30	72.5	92.5	31.65	33.95	0	2.5	0	2.5
	45	60	100	33.05	24.17	5	0	0	5
	60	75.5	100	32.45	15.63	0	5	0	7.5
51	30	92.5	87.5	24.27	25.90	0	0	2.5	5
	45	72.5	100	33.32	36.75	0	5	0	20
	60	57	97.5	31.81	29.91	0	0	0	12.5
52	30	57.5	72.5	20.2b	22.44	0	0	2.5	25
	45	45	97.5	30.08	16.57	0	0	2.5	5
	60	35	75	34.14	10.23	0	0	0	2.5
53	30	65	77.5	22	19.94	0	0	0	2.5
	45	50	72.5	28.45	21.93	0	0	0	10
	60	60.5	70	30.29	37.29	0	0	0	7.5
54	30	25	10	2.66	6.15	0	0	0	10
	45	20	30	7.5	19.12	0	0	0	0
	60	0	0	0	0	0	0	0	0
Control A[d]		72.5	27.5	25.77	33.63	25	27.5	32.5	47.5
Control B[e]		85	85	44.23	44.23	0	0	0	0
LSD(1)[f]		6.98							
LSD(2)[f]		5.41							
LSD(3)[f]		9.87							
LSD(4)[f]				4.86					

[a] Values were measured at the end of the growing season
[b] Isolations were performed immediately after HWT. Data are the percentage of positive isolation of *Pa. chlamydospora* or *Pm. aleophilum* in four groups of ten cuttings each
[c] Isolations were performed at the end of the growing season Data are the percentage of positive isolation of *Pa. chlamydospora* or *Pm. aleophilum* in four groups of ten cuttings each
[d] Cuttings were vacuum-inoculated with *Pa. chlamydospora* or *Pm. aleophilum* and not hot-water treated
[e] Cuttings were vacuum-infiltrated with sterile water and not hot-water treated
[f] Least significant difference: means followed by the same letter do not differ significantly ($P<0.05$). $LSD_{0.05}(1)$ is for comparison of means between temperatures of treatment on sprouting in field 2; $LSD_{0.05}(2)$ is for comparison of means between times of treatment on sprouting in field 2; $LSD_{0.05}(3)$ is for comparison of all pathogen-temperature combination means on sprouting in field 2; $LSD_{0.05}(4)$ is for comparison of means between temperatures of treatment on shoot weight in field 2