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Effect of hot-water treatments *in vitro* on conidial germination and mycelial growth of grapevine trunk pathogens

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Abstract

In this study, the sensitivity of *Cadophora luteo-olivacea*, *Cylindrocarpon liriodendri, Cn. macrodidymum* and eight species of the genus *Phaeoacremonium* to hot-water treatments (HWTs) *in vitro* was evaluated. Conidial suspensions and plugs of agar with mycelia were placed in Eppendorf vials and incubated for 30, 45 or 60 min in a hot water bath at 41, 42, 43, 44, 45, 46, 47, 48 or 49ºC for *Cylindrocarpon* spp. and at 49, 50, 51, 52, 53, 54 or 55ºC for *Ca. luteo-olivacea* and *Phaeoacremonium* spp. In general, conidial germination and the

colony growth rate of all pathogens decreased with increased temperature and time combinations. *Cylindrocarpon* spp. were more sensitive than *Ca. luteo-olivacea* and *Phaeoacremonium* spp. to HWT temperatures. Conidial germination of *Ca. luteo-olivacea* was inhibited by treatments above 51ºC-30 min, while treatments up to 54ºC-60 min were necessary to inhibit the mycelial growth. For *Cylindrocarpon* spp., conidial germination was inhibited by treatments above 45ºC-45 min, while treatments above 48ºC-45 min were necessary to inhibit the mycelial growth. Regarding *Phaeoacremonium* spp., treatments up to 54ºC-60 min were necessary to completely inhibit both conidial germination and mycelial growth. These results suggest that current HWT protocols at 50ºC for 30 min may be sufficient to control *Cylindrocarpon* spp. However, it would be necessary to develop HWT using higher temperatures to reduce the incidence of *Ca. luteo-olivacea* and *Phaeoacremonium* spp. infections.

Introduction

The main fungal diseases associated with young grapevine decline are black-foot disease, caused by *Cylindrocarpon* spp., and Petri disease, caused by *Phaeomoniella chlamydospora* (W. Gams, Crous, M. J. Wingf. & L. Mugnai) Crous & W. Gams, as well as several species of *Phaeoacremonium* (Mugnai *et al*., 1999; Pascoe & Cottral, 2000; Groenewald *et al*., 2001; Halleen *et al.*, 2006). These diseases usually affect vines younger than 7 years old and are associated with infection and disease of grapevine rootstocks (Mugnai *et al*., 1999; Crous & Gams, 2000). Recently, several *Phialophora*-like and *Acremonium* species have also been involved in the decline of young vines, mainly species of the genus *Cadophora* Lagerb. & Melin (Overton *et al*., 2005a; Halleen *et al*., 2007b). Of those, *Cadophora luteo-olivacea* (van Beyma) Harrington & McNew has been shown to be

quite common on grapevines affected by esca and Petri-disease in California (Rooney-Latham, 2005), South Africa (Halleen *et al*., 2007b) and New Zealand (Manning & Munday, 2009).

To date, 25 species of *Phaeoacremonium* have been reported on grapevines, the most frequently isolated species being *Pm. aleophilum* W. Gams, Crous, M. J. Wingf. & Mugnai and *Pm. parasiticum* W. Gams, Crous & M. J. Wingf (Mostert *et al.*, 2006). In addition, other numerous species of the genus *Phaeoacremonium* have also been associated with grapevine decline in grape-growing regions throughout the world (Crous *et al.*, 1996; Mugnai *et al*., 1999; Dupont *et al*., 2000, 2002; Groenewald *et al*., 2001; Mostert *et al*., 2006; Essakhi *et al*., 2008; Graham *et al*., 2009; Gramaje *et al.*, 2009a).

Infected propagation material, particularly rootstock material, has been indicated as a major means of spread of pathogens causing young vine decline (Fourie & Halleen, 2004a). Hot-water treatment (HWT) at different temperature-time combinations has been successfully used for the proactive management of Petri and black-food diseases in grapevine nurseries in several countries, such as Australia (Laukart *et al*., 2001; Edwards *et al*., 2004; Waite & May, 2005; Waite & Morton, 2007), New Zealand (Graham, 2007), South Africa (Crous *et al*., 2001; Fourie & Halleen, 2004b) or Spain (Gramaje *et al*., 2009b). However, the efficiency of the standard HWT protocols (50ºC for 30 min) still remains controversial, as some authors have reported its ineffectiveness to eliminate the pathogens (Rooney & Gubler, 2001; Whiting *et al*., 2001; Habib *et al*., 2009; Serra *et al.,* 2009) or damage which the heat causes in young plants (Bleach *et al*., 2009; Habib *et al*., 2009). Waite & Morton (2007) suggested that tolerance of plants and their accompanying pathogens to HWT is affected by the climate in which the cuttings are grown. In this regard, Graham (2007) and Bleach *et al.* (2009) found that both cuttings grown in cool climate in New Zealand and their pathogens were more susceptible to HWT, and the use of temperatures below 50ºC reduced the incidence of *Pa.*

chlamydospora and *Cylindrocarpon* spp., respectively. Conversely, Gramaje *et al*. (2008, 2009b) demonstrated that treatments above 51-52ºC were necessary to drastically reduce conidial germination and mycelial growth of *Pa. chlamydospora*, *Pm. aleophilum* and *Pm. parasiticum* in Spain without detrimental effects to grapevine cuttings. These authors commented that a better knowledge of the sensitivity of these pathogens and grapevine planting material in each country would be necessary to develop more effective HWT.

To date, most of the HWT research has been conducted to evaluate its potential for the control of *Pa. chlamydospora* and *Pm. aleophilum*. However, the involvement of other trunk disease pathogens in the decline of young vines seems to be evident. Consequently, there is a need for expanding the information reported by Whiting *et al*. (2001) and Gramaje *et al*. (2008) about the tolerance of these pathogens to hot-water treatments *in vitro*. Therefore, the objective of this research was to evaluate the sensitivity of *Ca. luteo-olivacea* and several species of *Phaeoacremonium* associated with Petri-disease affected grapevines in Spain, and *Cylindrocarpon macrodidymum* and *Cn. liriodendri* isolates associated with black foot disease to hot-water treatments *in vitro*.

Materials and methods

Fungal isolates

In this study, 11 isolates were used (one isolate of the following species: *Cadophora luteo-olivacea*, *Cylindrocarpon macrodidymum*, *Cn. liriodendri*, *Phaeoacremonium cinereum*, *Pm. hispanicum*, *Pm. inflatipes*, *Pm. iranianum*, *Pm. mortoniae*, *Pm. scolyti*, *Pm. sicilianum* and *Pm. viticola*) obtained from different geographic locations and rootstock–scion combinations in Spain (Table 1). Isolates were recovered from roots, basal ends of rootstocks,

and rootstock wood of plants that showed symptoms of Petri or black foot diseases. Sections were cut from affected areas, washed under running tap water, surface disinfested for 1 min in a 1.5% sodium hypochlorite solution and washed twice with sterile distilled water (SDW). Small pieces of discoloured or decayed tissues were plated on malt extract agar (MEA) (Oxoid Ltd., Basingstoke, Hants, England) supplemented with 0.5 gL^{-1} of streptomycin sulphate (MEAS) (Sigma-Aldrich, St. Louis, MO, USA). Plates were incubated for 10–15 days at 25ºC in the dark. Isolates were transferred to potato dextrose agar (2% PDA; Biokar-Diagnostics, Zac de Ther, France) and were incubated using the same conditions.

Isolates were identified by morphological and molecular methods. *Ca. luteo-olivacea* was identified based on conidiophore morphology, size of phialides and conidia, and colony characters and pigment production on MEA, PDA and oatmeal agar (OA; 60 g oatmeal; 12.5 g agar; Difco, France) (Gams, 2000; Harrington & McNew, 2003). Species of *Cylindrocarpon* were identified based on macroscopic characters such as colony texture, color, and the type of growing margin on PDA after incubation at 25ºC in darkness for 10 days (Alaniz *et al.*, 2006). Colonies grown on PDA were further incubated for 20 days to determine the presence/absence of chlamydospores. Conidia size was also measured on Spezieller Nährstoffarmer agar (SNA) with the addition of 1 × 1 cm piece of filter paper to the colony surface (Alaniz *et al*., 2007). Morphological characters used in distinguishing species of *Phaeoacremonium* included conidiophore morphology, phialide type and shape, size of hyphal warts and colony characters and pigment production on MEA, PDA and OA (Mostert *et al.*, 2006).

Identification of *Ca. luteo-olivacea* was confirmed by the analysis of the ITS region of DNA amplified using fungal universal primers ITS1F and ITS4 (Gardes & Bruns, 1993). Identification of *Cylindrocarpon* species was confirmed by a multiplex PCR system using a set of three pairs of specific primers (Alaniz *et al*., 2009). Identification of *Phaeoacremonium*

species was confirmed by the sequence analysis of the β-tubulin gene using primers sets T1 (O'Donnell & Cigelnik, 1997) and Bt2b (Glass & Donaldson, 1995) followed by comparison to the polyphasic, online identification system for *Phaeoacremonium* species recognition (http://www.cbs.knaw.nl/phaeoacremonium/biolomics.aspx) developed by Mostert *et al.* (2006). PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Germany) and sequenced in both directions by the DNA Sequencing Service of the Universidad Politécnica de Valencia-CSIC.

The isolates were single spored by means of the serial dilution method (Dhingra & Sinclair, 1995) prior to their storage in 15% glycerol solution at -80ºC in 1.5 mL cryovials.

Effect of hot-water treatments on conidial germination

Prior to use, fungal isolates were grown from the cryovial cultures on PDA and incubated for 1 (*Cylindrocarpon* spp.), 3 (*Ca. luteo-olivacea*) or 4 (*Phaeoacremonium* spp.) weeks at 25ºC in the dark. A conidial suspension was prepared for each isolate by flooding the colony surface with 10 mL of SDW and scraping with a sterile spatula. The resulting spore suspension was filtered through two layers of cheesecloth into a 250 mL Erlenmeyer flask. The filtrate was diluted with SDW and conidial concentration was adjusted with a haemocytometer to 10^6 conidia mL^{-1}. Aliquots (1 mL) of each conidial suspension were pipetted into 1.5 mL Eppendorf vials and subjected to HWT. The vials were placed in a circulating hot water bath (PSELECTA Unitronic 320OR; Barcelona, Spain) and were kept at the following constant temperatures (± 0.1ºC): 41, 42, 43, 44, 45 or 46ºC for *Cylindrocarpon* spp. isolates and 49, 50, 51, 52, 53 or 54ºC for *Ca. luteo-olivacea* and *Phaeoacremonium* spp. isolates. The times of exposure at each temperature were 30, 45 or 60 min. On removal from the HWT bath, the Eppendorf were immediately plunged into a cool water bath at ambient

temperature for 5 min in order to stop the heating process. Treatments consisted of four pseudo-replicates randomly positioned in the water bath for each temperature–time combination. The hot-water bath was used independently for all treatment combinations. The experiment was repeated twice to give two biological replicates.

After HWT, 0.2 mL of each treated conidial suspension was pipetted onto each of four 1.5% water agar (WA) plates and spread with a sterile bent-glass rod (four plates per vial). Plates were incubated at 25ºC in temperature controlled chambers in darkness. To serve as controls, 0.2 mL aliquots of the untreated conidial suspensions were plated out on four 1.5% WA plates incubated under the same conditions as described before. Conidial germination was assessed after 24 h for *Cylindrocarpon* spp. isolates, 48 h for *Ca. luteo-olivacea* isolate and 72 h for *Phaeoacremonium* spp. isolates by counting the number that had germinated out of 500 randomly selected conidia per plate. Germination was considered to have occurred when the germ tube had exceeded one half the length of the conidium. The effect of the HWT on each isolate was determined as the mean percent germination of each isolate, relative to mean percent germination on the relevant control plates.

Effect of hot-water bath treatments on mycelial growth

Plugs of agar with mycelia and spores, 4 mm in diameter, were cut from the growing edge of colonies growing on PDA, culture ages being 1, 3 and 4 weeks old, respectively, for *Cylindrocarpon* spp., *Ca. luteo-olivacea* and *Phaeoacremonium* spp. Four agar plugs per isolate were placed into each 1.5 mL Eppendorf vial containing 1 mL of SDW. The vials were placed at random positions in the circulating hot water bath and were kept at the following constant temperatures: 43, 44, 45, 46, 47, 48 or 49ºC for *Cylindrocarpon* spp. isolates and 49, 50, 51, 52, 53, 54 or 55ºC for *Ca. luteo-olivacea* and *Phaeoacremonium* spp. isolates. The

times of exposure at each temperature were 30, 45 or 60 min. On removal from the HWT bath, the Eppendorf vials were immediately plunged into a cool water bath at ambient temperature for 5 min in order to stop the heating process. Treatments consisted of four pseudo-replicates in each temperature–time combination. The hot-water bath was used independently for all treatment combinations. The experiment was repeated twice to give two biological replicates.

After HWT, agar plugs were removed from heated vials and blotted briefly, agar side down, on sterile filter paper (Whatman no. 2). The treated plugs were each placed in the centre of a MEAS plate and incubated at 25ºC in temperature controlled chambers in darkness. In addition, four untreated plugs of each isolate were placed in the centre of four MEAS plates to serve as a control treatment. Control plates were incubated under the same conditions as described before. After 1 week of incubation for *Ca. luteo-olivacea* and *Cylindrocarpon* spp. isolates and 3 weeks incubation for *Phaeoacremonium* spp, growth was determined from the colony diameters (the 4 mm diameter of the plug was subtracted), which were measured across two perpendicular axes. The effect of HWT on each isolate was determined as the mean percent growth rate with respect to the mean growth rate on control plates.

Statistical analysis

Conidial germination and colony growth percentages were arcsin square root transformed prior to analysis to provide normality and homogeneity of variances. The analysis used a factorial design with the following factors: experiment, temperature, time and pseudo-replicates. Separate analyses of variance were performed for the *Ca. luteo-olivacea*,

Cylindrocarpon spp. and *Phaeoacremonium* spp. isolates using the Statistical Analysis System (version 9.0, SAS Institute Inc., Cary, NC, USA).

Results

Effect of hot-water treatments on conidial germination

The effect of HWT on conidial germination is shown in Figs. 1, 2 and 3. Analysis of variance indicated that the effects were similar in both experiments (Tables 2, 3 and 4), thus germination data were combined in a single analysis. For all isolates, there were significant effects of temperature and time, as well as their interaction, on conidial germination ($P < 0.05$) (Tables 2, 3 and 4), with conidial germination relative to the control being gradually decreased with increasing temperature and time of HWT.

Cylindrocarpon spp. were more sensitive than *Ca. luteo-olivacea* and *Phaeoacremonium* spp. to HWT temperatures. For *Cn. liriodendri* isolate Cy-59 and *Cn. macrodidymum* isolate Cy-14, conidial germination after treatment at the lowest temperature-time combination (41ºC for 30 min) was 38.8% and 65.7%, respectively, and no germination for temperatures above 44 and 45ºC, respectively.

For *Ca. luteo-olivacea* isolate Clo-54, conidial germination was 54.5% in the treatments with the lowest temperature–time combination (49ºC, 30 min) and no germination for temperatures above 51ºC.

Among the species of *Phaeoacremonium, Pm. hispanicum* isolate Phi-1 and *Pm. mortoniae* isolate Pmo-1 were the most sensitive to HWT with no germination for temperatures above 51ºC, while *Pm. cinereum* isolate Pci-7 and *Pm. sicilianum* isolate Psi-2 were the most tolerant species to HWT with no germination for temperatures above 53ºC.

Effect of hot-water bath treatments on mycelial growth

The effect of HWT on mycelial growth is shown in Figs.1, 2 and 3. Analysis of variance indicated that the effects were similar in both experiments (Tables 2, 3 and 4), thus growth rate data were combined in a single analysis. For all isolates, there were significant effects of temperature and time, as well as their interaction, on mycelial growth ($P < 0.05$) (Tables 2, 3 and 4), with mycelial growth relative to the control being gradually decreased with increasing temperature and time of HWT.

Cylindrocarpon spp. were more sensitive than *Ca. luteo-olivacea* and *Phaeoacremonium* spp. to HWT temperatures. For *Cn. liriodendri* isolate Cy-59 and *Cn. macrodidymum* isolate Cy-14, the growth rate after treatment at the lowest temperature-time combination (43ºC for 30 min) was 75.8% and 74.2%, respectively, and no mycelial growth for temperatures above 47ºC and 48ºC, respectively.

For *Ca. luteo-olivacea* isolate Clo-54, the growth rate was 100% in the treatments with the lowest temperature–time combination (49ºC, 30 min) and no mycelial growth for temperatures above 54ºC.

Among the species of *Phaeoacremonium*, *Pm. hispanicum* isolate Phi-1 and *Pm. cinereum* isolate Pci-7 were the most sensitive species to HWT with no mycelial growth for temperatures above 52ºC, while *Pm. inflatipes* isolate Pin-2, *Pm. mortoniae* isolate Pmo-1, *Pm. scolyti* isolate Psc-1, *Pm. sicilianum* isolate Psi-2 and *Pm. viticola* isolate Pvi-1 were the most tolerant species to HWT with no mycelial growth for temperatures above 54ºC.

Discussion

The results of this study complement the previous one by Gramaje *et al*. (2008) which investigated the sensitivity of *Pa. chlamydospora*, *Pm. aleophilum* and *Pm. parasiticum* to hot-water treatments *in vitro*. In this study, significant differences were found between different temperatures and times on the viability of conidia and mycelia for all fungal species tested. In general, conidial germination and the colony growth rate decreased with increased temperature and time combinations. Mycelium was generally less susceptible to heat treatments than conidia.

Species of *Cylindrocarpon* were more sensitive than *Ca. luteo-olivacea* and *Phaeoacremonium* spp. to HWT temperatures. Conidial germination for both *Cn. macrodidymum* and *Cn. liriodendri* isolates was inhibited at temperatures above 45ºC, while temperatures above 48ºC were necessary to inhibit the mycelial growth. Bleach *et al*. (2009) also found in a similar *in vitro* experiment that heat treatment at 40-50ºC for 15-30 min inhibited conidial germination of *Cn. liriodendri*, *Cn. macrodidymum* and *Cn. destructans*. However, they found that when the pathogen was introduced into the wood of 1-year cuttings, higher temperatures were required to totally eliminate infections. These results indicate that the standard HWT protocols at 50ºC for 30 min used in several grapevine nurseries worldwide (Crous *et al*., 2001; Edwards *et al*., 2004; Fourie & Halleen, 2004b; Waite & May, 2005; Halleen *et al*., 2007a) may be sufficient to control *Cylindrocarpon* spp.. The fact that black foot pathogens infect grafted grapevine plants from nursery soils (Halleen *et al*., 2003) clearly emphasizes the importance of suitable control measures to prevent or eradicate these infections (Halleen *et al.*, 2006). The results of this study and those of Halleen *et al*. (2007a) and Bleach *et al*. (2009) demonstrate the potential of this control method to eradicate black foot disease infections from dormant nursery vines.

Cadophora luteo-olivacea was found to be quite tolerant to the different temperature-time combinations, since temperatures above 52-53ºC were needed to significantly reduce the

mycelial growth and above 51ºC to kill all conidia of this species. The pathogenesis of *Ca. luteo-olivacea* was tested by Halleen *et al.* (2007b), who confirmed that after artificial inoculation this species was able to colonize grapevine pruning wounds and cause trunk lesions, in which it survived, and could therefore be regarded as a potential grapevine pathogen. The involvement of *Ca. luteo-olivacea* in young grapevine decline has not yet been extensively studied, with no reports about its distribution, epidemiology and control. Thus, this study is the first to investigate the development of an effective control strategy to reduce the level of *Ca. luteo-olivacea* infection in grapevines.

The effects of heat treatments on species of the genus *Phaeoacremonium* studied here were similar to the effects reported by Gramaje *et al*. (2008), for other *Phaeoacremonium* species, in which treatments above 51-53ºC were needed to kill conidia of *Pa. chlamydospora*, *Pm. aleophilum* and *Pm. parasiticum*. In the present study, conidial germination of *Phaeoacremonium* spp. was inhibited at treatments above 53ºC for 45 min, while treatments up to 54ºC for 60 min were necessary to inhibit the mycelial growth. However, treatments above 52ºC greatly reduced conidial germination and mycelial growth in all species as found for *Ca. luteo-olivacea*. The results obtained for *Pm. inflatipes* are similar to those obtained by Whiting *et al*. (2001), who demonstrated that a HWT *in vitro* at 51ºC for 30 min may not be effective in reducing or eliminating this species from infected vines.

This research clearly shows that HWT protocols at 50ºC for 30 min may be sufficient to control black foot disease pathogens, but it would be necessary to use treatments above 52-53ºC to control *Ca. luteo-olivacea* and *Phaeoacremonium* spp. Studies carried out by Gramaje *et al.* (2009b) also demonstrated that HWT at 53ºC can be used as an effective control method for Petri disease in Spain since grapevine propagating material is able to grow without statistically significant effects on sprouting and shoot weight. This treatment was able

to strongly reduce the re-isolation of *Pa. chlamydospora* and *Pm. aleophilum* on grapevine wood after one-growing season.

The information generated here is of great value since there has been increasing detection and identification of fungal trunk pathogens associated with young grapevine decline in recent years worldwide through the use of molecular characters (Tegli *et al*., 2000; Dupont *et al*., 2002; Overton *et al*., 2005b; Mostert *et al*., 2006; Aroca & Raposo, 2007; Aroca *et al*., 2008; Alaniz *et al*., 2009). An integrated nursery management program that includes HWT, chemical, biological or other control measures could be developed from the range of treatments shown to be effective by their reduction in infections by fungal trunk pathogens. Recent research raised interesting possibilities of combining rootstock drenches in didecyldimethylammonium chloride or carbendazim during the hydration stage (Gramaje *et al*., 2009c) with HWT at 53ºC for 30 min applied to dormant plants before planting out to limit infections by fungal trunk pathogens in Spanish grapevine nurseries (Gramaje *et al*., 2009b). A pruning wound protection using *Trichoderma* formulations could be also combined with other control measures for the sanitation of propagating material in grapevine nurseries (Fourie & Halleen, 2006).

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Table 1 Isolation record of *Ca. luteo-olivacea, Cylindrocarpon* spp. *and Phaeoacremonium* spp. used in this study

Species	Isolate	Year	Location	Scion/Rootstock
Ca. luteo-olivacea	Clo-54	2008	Cariñena (Zaragoza)	Syrah/110 Richter
Cn. liriodendri	Cy-59	2003	Tarazona de la Mancha (Albacete)	Tempranillo/1103 Paulsen
Cn. macrodidymum	Cy-14	2002	Burgos	Tempranillo/110 Richter
Pm. cinereum	Pci-7	2007	Olivenza (Badajoz)	Syrah/nd
Pm. hispanicum	Phi-1	2008	Yecla (Murcia)	Monastrell/nd
Pm. inflatipes	Pin-2	2007	Olivenza (Badajoz)	Syrah/nd
Pm. iranianum	Pir-5	2008	Cariñena (Zaragoza)	Syrah/110 Richter
Pm. mortoniae	Pmo-1	2006	Daimiel (Ciudad Real)	Cabernet Sauvignon/nd
Pm. scolyti	Psc-1	2007	Aielo de Malferit (Valencia)	Tempranillo/110 Richter
Pm. sicilianum	Psi-2	2008	Balearic Islands	Tempranillo/Rupestris de Lot
Pm. viticola	Pvi-1	2008	Cariñena (Zaragoza)	Tempranillo/110 Richter

nd, not determined.

Table 2 Analysis of variance for the effects of temperature and time on conidial germination and colony growth of *Cadophora luteo-olivacea* (Clo-54), *Cylindrocarpon liriodendri* (Cy-59) and *Cn. macrodidymum* (Cy-14) isolates

		Ca. luteo-olivacea		***Cn. liriodendri***		***Cn. macrodidymum***	
		Clo-54		**Cy-59**		**Cy-14**	
	df	**MS**	***P*>F[a]**	**MS**	***P*>F**	**MS**	***P*>F**
Conidial germination							
Experiment	1	50.34	0.7030	6.58	0.5676	0.35	0.9654
Temperature (A)	5	4926.31	<0.001	11221.03	<0.001	3546.33	<0.001
Time (B)	2	813.59	<0.001	2445.11	<0.001	929.23	<0.001
A X B	10	326.92	<0.001	201.33	0.0102	150.14	<0.001
Pseudo-replicates (C)	3	27.19	0.0701	2.13	0.1123	2.13	0.3326
A X C	15	13.47	0.1070	2.66	0.1556	6.76	0.1110
B X C	6	5.21	0.7257	4.80	0.1978	4.20	0.1324
A X B X C	30	5.13	0.9417	4.02	0.0946	3.88	0.2112
Residual	71	8.63		7.98		8.15	
Total	143						
Colony growth							
Experiment	1	1332.02	0.0790	6.75	0.7765	29.43	0.4930
Temperature (A)	6	24330.06	<0.001	6321.45	<0.001	9865.81	<0.001
Time (B)	2	896.47	0.0467	2167.21	<0.001	2381.41	0.0119
A X B	12	306.02	0.0477	119.39	<0.001	676.98	0.0023
Pseudo-replicates (C)	3	213.93	0.4937	124.54	0.4283	170.78	0.1678
A X C	18	342.98	0.2138	245.67	0.0928	60.57	0.6572
B X C	6	109.27	0.8691	56.77	0.1223	51.57	0.9273
A X B X C	36	306.79	0.2891	199.86	0.0787	129.88	0.8861
Residual	83	265.20		156.34		102.11	
Total	167						

df., degrees of freedom; MS, mean square

[a] Probabilities associated with individual F tests.

Table 3 Analysis of variance for the effects of temperature and time on conidial germination and colony growth of *Phaeoacremonium cinereum* (Pci-7), *Pm. hispanicum* (Phi-1), *Pm. inflatipes* (Pin-2) and *Pm. iranianum* (Pir-5) isolates

		Pci-7		Phi-1		Pin-2		Pir-5	
	df	**MS**	***P*>F[a]**	**MS**	***P*>F**	**MS**	***P*>F**	**MS**	***P*>F**
Conidial germination									
Experiment	1	46.80	0.6673	17.81	0.6739	0.18	0.9753	118.72	0.0878
Temperature (A)	5	6841.29	<0.001	1722.70	<0.001	7221.33	<0.001	1917.64	<0.001
Time (B)	2	1464.43	<0.001	236.98	<0.001	1449.23	<0.001	65.03	<0.001
A X B	10	238.62	<0.001	94.83	<0.001	182.84	<0.001	33.17	<0.001
Pseudo-replicates (C)	3	13.04	0.3490	2.33	0.7182	1.13	0.3276	2.03	0.9316
A X C	15	10.66	0.5562	1.40	0.9965	2.54	0.1889	2.84	0.9993
B X C	6	11.93	0.4200	1.13	0.9701	3.90	0.0982	4.53	0.9210
A X B X C	30	5.85	0.9813	1.03	0.9923	3.88	0.1102	6.92	0.9817
Residual	71	11.70		5.19		5.89		13.88	
Total	143								
Colony growth									
Experiment	1	181.02	0.3465	19.13	0.8585	349.25	0.3357	0.21	0.9828
Temperature (A)	6	7292.31	<0.001	18865.12	<0.001	28854.88	<0.001	9454.97	<0.001
Time (B)	2	800.92	<0.001	1181.01	<0.001	95.27	0.0113	790.47	<0.001
A X B	12	188.53	<0.001	576.92	<0.001	347.30	0.0053	438.93	<0.001
Pseudo-replicates (C)	3	10.22	0.7228	70.77	0.3722	173.80	0.0691	3.18	0.9918
A X C	18	9.41	0.9828	61.57	0.5582	123.30	0.0648	6.76	0.9233
B X C	6	3.70	0.9863	21.36	0.9254	94.35	0.2245	22.88	0.9628
A X B X C	36	10.04	0.9867	29.51	0.9963	115.84	0.6224	56.90	0.9594
Residual	83	23.07		66.99		67.41		96.15	
Total	167								

d,f., degrees of freedom; MS, mean square

[a]Probabilities associated with individual F tests.

Table 4 Analysis of variance for the effects of temperature and time on conidial germination and colony growth of *Phaeoacremonium mortoniae* (Pmo-1), *Pm. scolyti* (Psc-1), *Pm. sicilianum* (Psi-2) and *Pm. viticola* (Pvi-1) isolates

		Pmo-1		Psc-1		Psi-2		Pvi-1	
	df	**MS**	***P*>F[a]**	**MS**	***P*>F**	**MS**	***P*>F**	**MS**	***P*>F**
Conidial germination									
Experiment	1	10.82	0.8705	8.45	0.8938	1.73	0.9375	15.17	0.8025
Temperature (A)	5	2637.03	0.0052	12474.16	<0.001	11496.25	<0.001	8416.17	<0.001
Time (B)	2	1178.10	<0.001	2296.72	<0.001	2765.l3	<0.001	2109.38	<0.001
A X B	10	386.88	<0.001	450.78	<0.001	267.43	<0.001	229.81	<0.001
Pseudo-replicates (C)	3	15.27	0.3655	21.58	0.1168	4.06	0.8867	2.05	0.5588
A X C	15	39.32	0.4305	14.01	0.2132	22.75	0.2944	6.90	0.1229
B X C	6	1.22	0.9975	8.50	0.5716	2.48	0.9921	3.01	0.2451
A X B X C	30	15.96	0.3378	10.71	0.4701	11.40	0.9391	3.59	0.4212
Residual	71	14.21		10.60		19.06		2.95	
Total	143								
Colony growth									
Experiment	1	167.31	0.1580	1877.27	0.1176	1115.74	0.1481	3851.27	0.1283
Temperature (A)	6	15494.76	<0.001	28438.25	<0.001	24890.06	<0.001	12505.02	<0.001
Time (B)	2	414.54	<0.001	44.82	<0.001	540.53	0.0021	7676.59	<0.001
A X B	12	246.65	<0.001	343.82	<0.001	466.88	<0.001	620.29	0.0112
Pseudo-replicates (C)	3	13.20	0.8311	49.96	0.7205	102.99	0.3060	160.19	0.6144
A X C	18	22.56	0.9521	64.58	0.9065	127.32	0.1058	153.71	0.9050
B X C	6	4.75	0.9956	134.30	0.3145	33.02	0.8818	183.78	0.6562
A X B X C	36	23.05	0.9871	33.06	0.9999	73.47	0.6682	231.85	0.6681
Residual	83	45.31		111.91		84.10		265.36	
Total	167								

d,f., degrees of freedom; MS, mean square

[a]Probabilities associated with individual F tests.

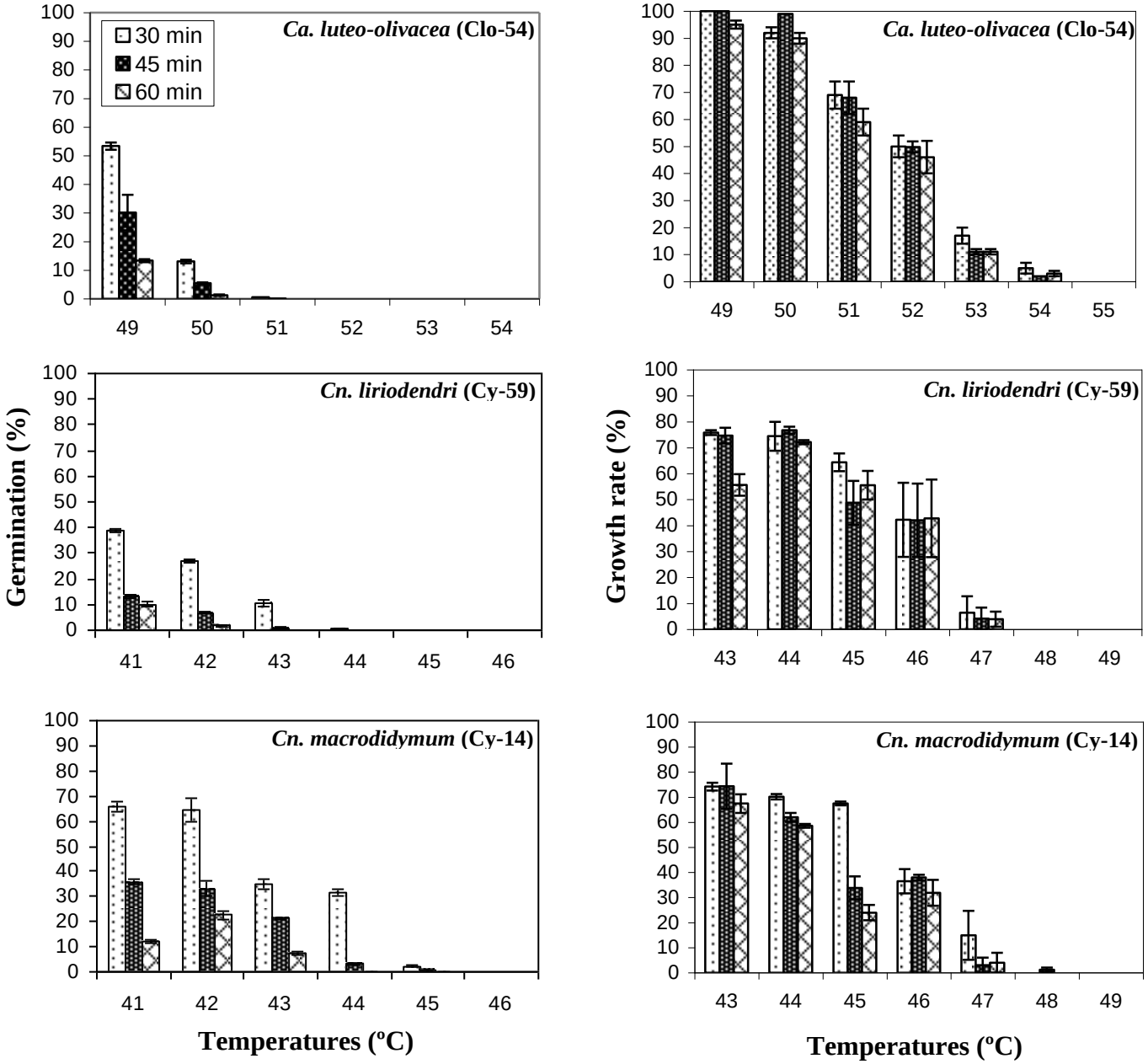
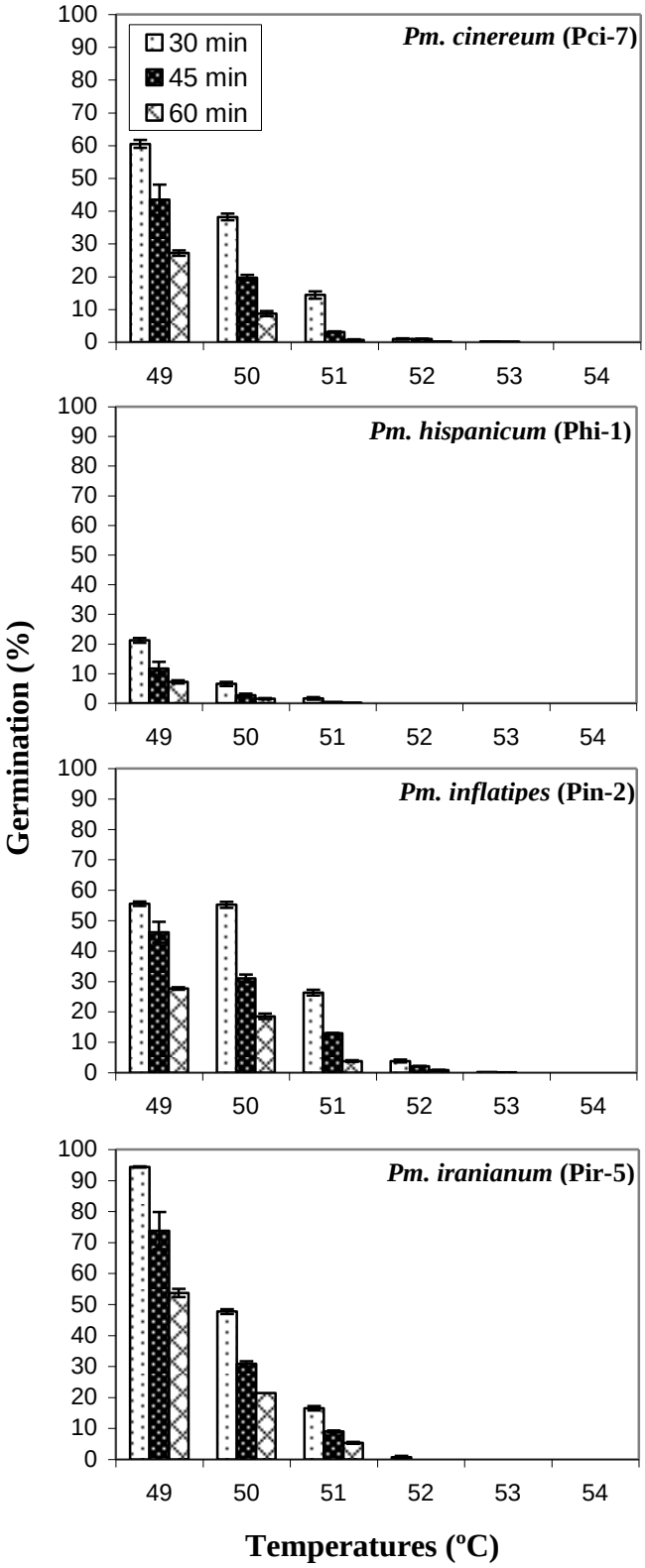

30 min
45 min
60 min
Ca. luteo-olivacea (Clo-54)
Cn. liriodendri (Cy-59)
Cn. macrodidymum (Cy-14)
Germination (%)
Growth rate (%)
Temperatures (ºC)
Temperatures (ºC)

Figure 1


30 min
45 min
60 min
Pm. cinereum (Pci-7)
Pm. hispanicum (Phi-1)
Pm. inflatipes (Pin-2)
Pm. iranianum (Pir-5)
Germination (%)
Temperatures (ºC)

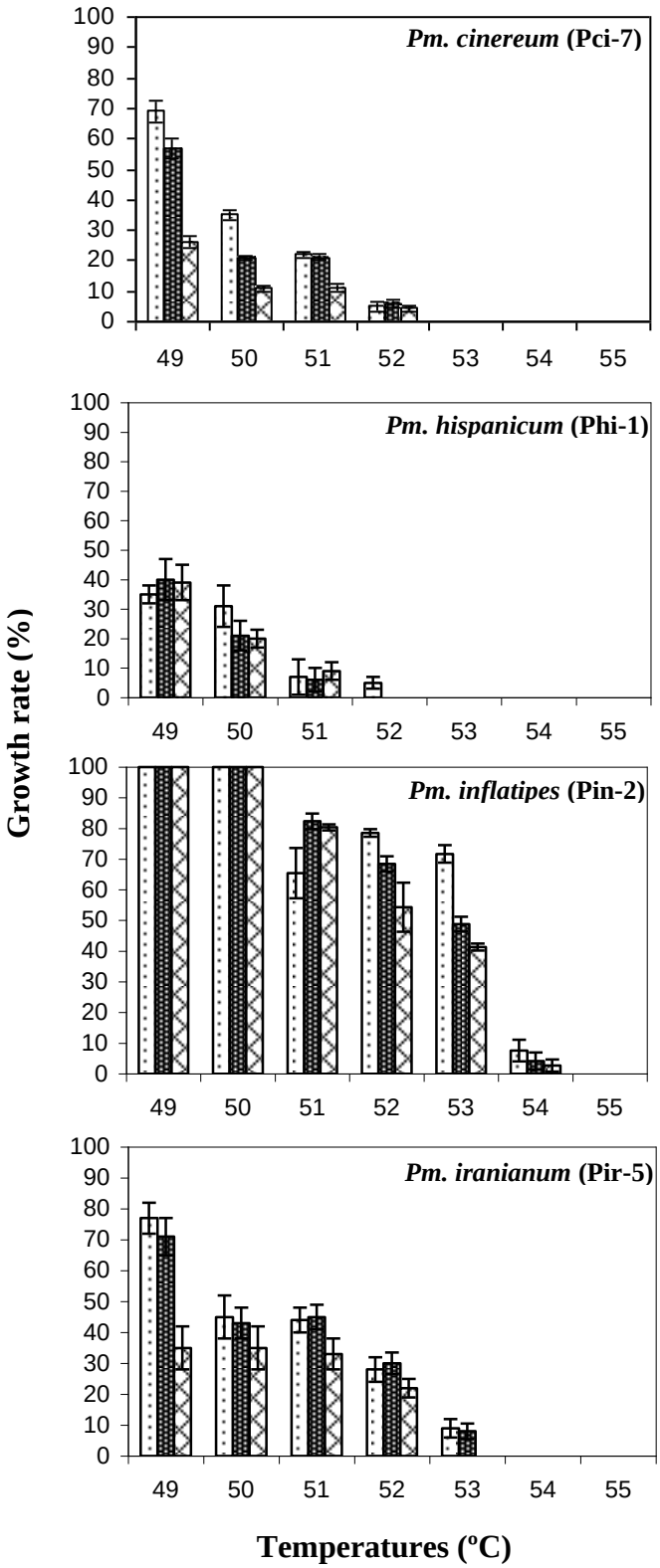

Pm. cinereum (Pci-7)
Pm. hispanicum (Phi-1)
Pm. inflatipes (Pin-2)
Pm. iranianum (Pir-5)
Growth rate (%)
Temperatures (ºC)

Figure 2

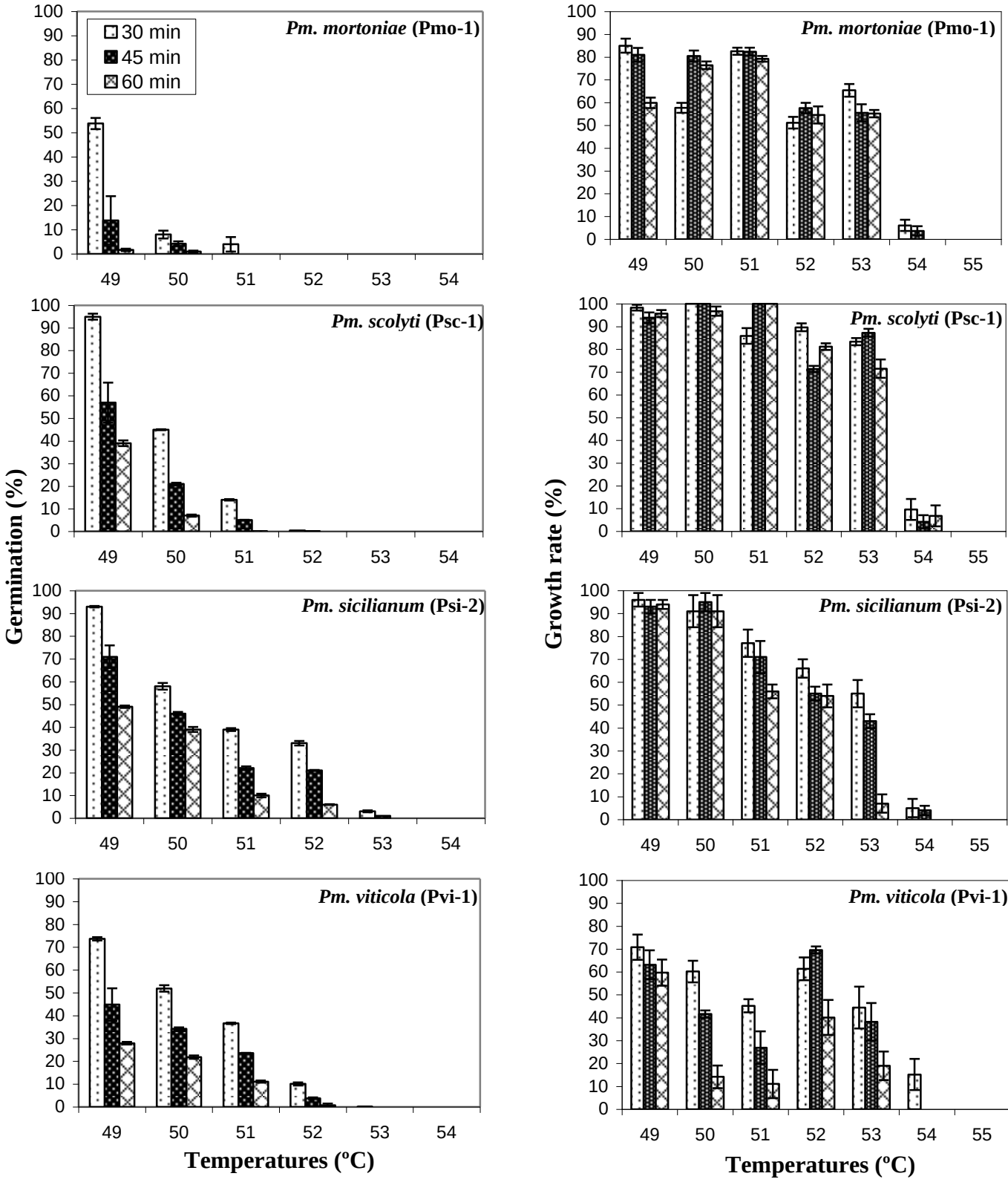

30 min
45 min
60 min
Pm. mortoniae (Pmo-1)
Pm. scolyti (Psc-1)
Pm. sicilianum (Psi-2)
Pm. viticola (Pvi-1)
Germination (%)
Growth rate (%)
Temperatures (ºC)
Temperatures (ºC)

Figure 3

Figure 1 Percent conidial germination and colony growth after hot water treatment for C*adophora luteo-olivacea* (Clo-54), *Cylindrocarpon liriodendri* (Cy-59) and *Cylindrocarpon macrodidymum* (Cy-14). Temperatures differed between isolates (as shown) but treatment times were the same (30, 45 or 60 min.). Error bars are the standard errors of the means from two independent experiments, each with four pseudoreplicates for each combination of treatments.

Figure 2 Percent conidial germination and colony growth after hot water treatment for *Phaeoacremonium cinereum* (Pci-7), *Pm. hispanicum* (Phi-1), *Pm. inflatipes* (Pin-2) and *Pm. iranianum* (Pir-5). Temperatures differed between isolates (as shown) but treatment times were the same (30, 45 or 60 min.). Error bars are the standard errors of the means from two independent experiments, each with four pseudoreplicates for each combination of treatments.

Figure 3 Percent conidial germination and colony growth after hot water treatment for *Phaeoacremonium mortoniae* (Pmo-1), *Pm. scolyti* (Psc-1), *Pm. sicilianum (*Psi-2) and *Pm. viticola* (Pvi-1). Temperatures differed between isolates (as shown) but treatment times were the same (30, 45 or 60 min.). Error bars are the standard errors of the means from two independent experiments, each with four pseudoreplicates for each combination of treatments.