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Research Papers

Effects of temperature, pH and water potential on mycelial growth, sporulation and chlamydospore production in culture of *Cylindrocarpon* spp. associated with black foot of grapevines

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Summary. The effects of temperature, pH and water potential (Ψ_s) on mycelial growth, sporulation and chlamydospore production of *Cylindrocarpon liriodendri, C. macrodidymum* and *C. pauciseptatum* isolated from grapevines was studied. Three isolates per species were incubated on potato dextrose agar (PDA) under different temperature, pH, and Ψ_s conditions. All isolates were able to grow over a range of temperatures from 5 to 30ºC, with an optimum temperature between 20 to 25ºC, but they did not grow at 35ºC. Active mycelial growth was observed over a range of pHs, from 4 to 8. Regarding the effect of Ψ_s, in general, mycelial growth was greater on amended media at -0.5, -1.0 or/and -2.0 MPa compared with that obtained on nonamended PDA (-0.3 MPa), and was reduced at Ψ_s values lower than -2.0 MPa. Most of the *Cylindrocarpon* spp. isolates were sporulated at all temperatures, pHs and water potentials tested. In all studied conditions, *C. liriodendri* had the greatest sporulation capacity compared with *C. macrodidymum* and *C. pauciseptatum.* In general, chlamydospore production was not much affected by temperature, pH and Ψ_s. Chlamydospores were observed in PDA cultures of all isolates at all pH values studied, while some isolates did not produce chlamydospores at 5 and 10**°C or** -4.0 and/or -5.0 MPa. These results improve understanding of the biology of these important grapevine pathogens.

Key words: *Cylindrocarpon liriodendri, Cylindrocarpon macrodidymum, Cylindrocarpon pauciseptatum,* soilborne diseases.

Introduction

Black foot disease of grapevines, caused by *Cylindrocarpon* spp., is a serious disease in most wine and grape-producing regions of the world, particularly in nurseries and young vineyards (Halleen *et al.*, 2006a). It was first described in 1961 (Grasso and Magnano Di San Lio, 1975), and over the last decade, black foot disease has been reported in most grapevine production areas of the world, including Portugal (Rego *et al*., 2000), Argentina (Gatica *et al*., 2001), Germany (Fischer and Kassemeyer, 2003), New Zealand and South Africa (Halleen *et al*., 2004), Brazil (Garrido *et al*., 2004), California (Petit and Gubler, 2005), Chile (Auger *et al*., 2007), Australia (Whitelaw-Weckert *et al*., 2007), Spain (Alaniz *et al*., 2007), Iran (Mohammadi *et al*., 2009), Uruguay (Abreo *et al*., 2010), northeastern United States and southeastern Canada (Petit *et al*., 2011).

Vines affected by *Cylindrocarpon* spp. often show sunken necrotic root lesions with a reduction in root biomass and root hairs. Removal of rootstock bark reveals black discoloration and necrosis of wood tissues which develops from the base of the rootstock. Moreover, affected vines show low vigour with small trunks and short internodes, a reduction in total foliage and leaf size, with leaves depicting interveinal chlorosis and necrosis, frequently leading to death of the plants (Halleen *et al*., 2006a; Alaniz *et al*., 2007, 2009; Abreo *et al.*, 2010).

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Black foot disease of grapevine is caused by *Cylindrocarpon* spp. (*Cylindrocarpon destructans* [Zinnsm.] Scholten, *C. liriodendri* J.D. MacDonald & E.E. Butler, *C. macrodidymum* Schroers, Halleen & Crous and *C. pauciseptatum* Schroers & Crous) and *Campylocarpon* spp. (*Campylocarpon fasciculare* Schroers, Halleen & Crous and *Campyl. pseudofasciculare* Halleen, Schroers & Crous) (Halleen *et al*., 2004; Halleen *et al*., 2006a, 2006b; Schroers *et al*., 2008).

In Spain, surveys carried out in recent years in grapevine nurseries and young vineyards have confirmed the importance of *Cylindrocarpon* spp. affecting this crop. These pathogens were found in grapevine nurseries very early in the planting material production process, in grapevine plants ready to be planted and in young vineyards showing decline symptoms (Aroca *et al*., 2006; Giménez-Jaime *et al*., 2006; Alaniz *et al*., 2007; Gramaje *et al*., 2010). In all cases, *Cylindrocarpon* spp. were mostly isolated from rootstocks, especially from the basal ends. To date, *C. liriodendri, C. macrodidymum* and *C. pauciseptatum* are the species which have been identified associated with young vines showing symptoms of black foot disease in Spain (Alaniz *et al*., 2007; Martin *et al*., 2011).

Species of *Cylindrocarpon* are common and may be isolated as soil inhabitants, saprobes on dead plant material, root colonizers or pathogens, or weak pathogens of various herbaceous and woody plants (Brayford, 1993). The production of chlamydospores may allow *Cylindrocarpon* spp. to survive for extended periods in soil (Halleen *et al*., 2004). Given these findings, it could be assumed that black foot disease pathogens could survive in the soil to infest grapevine plants. However, very little information is currently available regarding the basic biology of these pathogens, such as mycelial growth, sporulation and chlamydospore production under various environmental conditions, and the role that chlamydospores might play during the infection processes (Halleen *et al*., 2006a).

In common with all microorganisms, fungi are profoundly affected by physical and physicochemical factors, such as temperature, aeration, pH, water potential ($\Psi_{s)}$ and light. These factors not only affect the growth rate of fungi but can also act as triggers in developmental pathways (Deacon, 2006). These factors are known to influence host-pathogen interactions in *C. destructans* on Ginseng (Rahman and Punja, 2005) and in a number of other soilborne pathogens such as *Monosporascus cannonballus* Pollack & Uecker on muskmelon and watermelon (Ferrin and Stanghellini, 2006; Armengol *et al*., 2011), *Rhizoctonia solani* J.G. Kühn on lupin and potato (Kumar *et al*., 1999; Ritchie *et al*., 2006; Ritchie *et al*., 2009), and diseases caused by *Pythium* and *Phytophthora* spp. (Sommers *et al*., 1970; Abdelzaher *et al*., 1997).

In *Cylindrocarpon* spp. associated with black foot disease of grapevines, only growth temperature experiments have been conducted so far. These studies were performed when these pathogens were recently described and/or characterized (Halleen *et al*., 2004; Petit and Gubler, 2005; Halleen *et al*., 2006b; Alaniz *et al*., 2007; Schroers *et al*., 2008). The aim of the present study was to expand knowledge of the effects of temperature, pH and Ψ_s on mycelial growth, sporulation and chlamydospore production of *C. liriodendri, C. macrodidymum* and *C. pauciseptatum* isolated from grapevines.

Materials and methods

Fungal isolates

Three isolates of *C. liriodendri* (Cy59, Cy89 and Cy100), three isolates of *C. macrodidymum* (Cy47, Cy14 and Cy81) and one isolate of *C. pauciseptatum* (Cy593), obtained from roots or the basal ends of rootstocks from grapevines exhibiting symptoms of black foot in Spain were used in this study. Additionally, two isolates of *C. pauciseptatum* from roots of grapevines in Slovenia (CBS120171 and CBS120173) were obtained from the collection of the Centraalbureau voor Schimmelcultures (CBS, Utrecht, the Netherlands) (Table 1). Single spore isolates were stored in 15% glycerol solution at –80°C in cryovials (1.5 mL capacity). Prior to use, a small plug of the colonized agar from each cryovial was transferred to potato dextrose agar (PDA) (Biokar-Diagnostics, Zac de Ther, France) plates and allowed to grow at 25ºC in darkness for 14 d.

Effects of temperature on mycelial growth, sporulation and chlamydospore production of *Cylindrocarpon* spp.

To determine the effect of temperature on mycelial growth, all isolates were maintained and grown on PDA plates at 25°C. Agar plugs (8 mm diam.) were cut from the leading edges of 14-d-old colonies

Table 1. Sources of isolates of *Cylindrocarpon* spp. associated with black foot disease and their optimum growth temperatures.

Species/Isolate	Year	Geographical origin		Scion/rootstock	Optimum growth (°C)[b]
		Town	Province		
C. liriodendri					
Cy59	2003	Tarazona de la Mancha	Albacete	Cencibel/1103 P	21.9
Cy89	2004	Aielo de Malferit	Valencia	Garnacha/110R	23.7
Cy100	2004	Alesanco	La Rioja	Garnacha/110R	24.3
C. macrodidymum					
Cy14	2002	n.d.[c]	Burgos	Tempranillo/110R	24.5
Cy47	2003	Mollina	Málaga	Pedro Ximénez/1103 P	23.3
Cy81	2003	Beneixama	Alicante	Tempranillo/161-49C	24.1
C. pauciseptatum					
Cy593	2009	Tobarra	Albacete	Syrah/S04	24.4
CBS120171[a]	2005	Krsko (Eslovenia)	n.d.	n.d.	24.0
CBS120173[a]	2005	Doljenska (Eslovenia)	n.d.	n.d.	22.9

[a] Reference isolates of *C. pauciseptatum* (CBS 120171 and CBS 120173) were obtained from the collection of Centralbureau voor Schimmelcultures inUtrecht, the Netherlands (CBS).
[b] For each *Cylindrocarpon* spp. isolate, temperature average growth rates were adjusted to a regression curve to estimate the optimum growth temperature.
[c] n.d., Not determined.

and placed in the center of PDA plates (one plug per plate) that were then incubated in the dark at 5, 10, 15, 20, 25, 30 or 35°C. There were four pseudoreplicates for each isolate and temperature combination. After 10 d, colony diameter was measured along two perpendicular axes, and data were converted to radial growth (mm d^{-1}).

Colonies grown on PDA were further incubated over 20 days to evaluate sporulation and determine the presence/absence of chlamydospores. The number of conidia produced on mycelia from agar plugs was measured following the method described by Whiting *et al*. (2001) and Alaniz *et al*. (2007). After 30 d of incubation, two plugs of agar (4 mm diam.) with mycelia and spores, were cut from the growing edge of each colony, and each was placed in an Eppendorf vial (1.5 mL capacity) containing 1 mL of sterile water. Vials with plugs were vortexed for 5 s, and the number of conidia per mL was counted using a haemocytometer. To observe chlamydospores, a small quantity of fungal material was removed from each colony surface with a sterile needle taking care to minimize disturbance of the fungal structures, and placed in a drop of distilled water in the centre of a clean slide, and a cover slide was carefully lowered on to the drop. Two preparations per Petri dish were observed microscopically at ×100 and ×400 magnification. The experiment was repeated.

Effects of pH on mycelial growth, sporulation and chlamydospore production of *Cylindrocarpon* spp.

The effects of pH on mycelial growth and sporulation of *Cylindrocarpon* spp. in culture was determined on PDA. Mycelial plugs (8 mm diam.) obtained from the growing edges of colonies were transferred to the center of PDA plates (one plug per plate) which were adjusted to pH 4, 5, 6, 7 and 8 with the addition of 50 mM citrate phosphate buffer (pH 4–7) or 50 mM Tris-HCl buffer (pH 8) (Gomori, 1955). Plates were incubated in the dark at 25ºC. There were two replicates for each isolate and pH combination.

Mean mycelial growth rates, the number of conidia produced on mycelia and the presence/absence of chlamydospores were evaluated as described above. The experiment was repeated.

Effects of water potential (Ψs) on mycelial growth, sporulation and chlamydospore production of *Cylindrocarpon* spp.

The effect of Ψ_s on mycelial growth and sporulation of *Cylindrocarpon* spp. in culture was determined on PDA. Mycelial plugs (8 mm in diam.) obtained from the growing edges of colonies were transferred to the center of PDA plates (one plug per plate) amended with KCl or NaCl prior to sterilization to obtain six Ψ_s values: -0.5, -1.0, -2.0, -3.0, -4.0 and -5.0 MPa, according to Robinson and Stokes (1959). Non-amended PDA (-0.3 MPa) was used as and experimental control. Plates were incubated in the dark at 25ºC. There were two replicates for each isolate, type of solute and Ψ_s combination. Mean mycelia growth rates, the number of conidia produced on mycelia and the presence/absence of chlamydospores were evaluated as described above. The experiment was repeated.

Statistical analyses

Data from each *Cylindrocarpon* spp. were analyzed separately. Two way analyses of variance (ANOVA) were conducted with radial growth and sporulation data obtained from temperature and pH experiments, and a three way ANOVA was conducted for Ψ_s experiments using Statgraphics Plus 5.1 software (Manugistics Inc., Rockville, MD, USA). For all *Cylindrocarpon* spp., ANOVA analyses indicated that the radial growth and sporulation data between the two repetitions in temperature, pH and Ψ_s experiments were similar (P>0.05). Thus, in all cases, data from both experiments were combined.

For each *Cylindrocarpon* spp. isolate, temperature, pH or Ψ_s average mycelial growth rates were adjusted to a regression curve using Statgraphics Plus 5.1 software, and the best polynomial model was chosen based on parameter significance (P<0.05) and coefficient of determination (R^2). Previous to this analysis, data of mycelial growth from the Ψ_s experiment were converted to relative growth rate as a percentage of the experimental controls. Additionally, the polynomial models in the temperature experiments were used to estimate the optimum growth temperature for each isolate.

Results

Effects of temperature on mycelial growth, sporulation and chlamydospore production of *Cylindrocarpon* spp.

Statistically significant effects of the isolate on radial growth were observed for *C. macrodidymum* (P=0.0019) and *C. pauciseptatum* (P=0.0073), but not for *C. liriodendri* (P=0.4999). The effect of isolate × experiment was also not significant in all of the species studied (P>0.05) (Table 2).

The effects of temperature on mycelial radial growth and sporulation of the nine *Cylindrocarpon* spp. isolates are shown in Figure 1. All isolates were able to grow on PDA over a range of temperatures from 5 to 30ºC, and no growth was obtained at 35ºC. At 5ºC, *C. liriodendri* isolates showed growth rates between 0.038 cm day^{-1} for isolate Cy89 and 0.043 cm day^{-1} for isolate Cy59, while the growth rates of the *C. macrodidymum* and *C. pauciseptatum* isolates were almost negligible at this temperature. Optimum growth temperatures for all isolates ranged between 21.9**°C** for isolate Cy59 (*C. liriodendri*) and 24.5**°C** for isolate Cy14 (*C. macrodidymum*) (Table 1).

Regarding sporulation, significant effects of the isolate on sporulation were observed for *C. macrodidymum* (P<0.001) and *C. pauciseptatum* (P<0.001), but not for *C. liriodendri* (P=0.9328). The effect of isolate × experiment was also not significant in all species studied (P>0.05) (Table 2).

Most of the *Cylindrocarpon* spp. isolates produced conidia at all temperatures, showing broad variation. In general, the three *C. liriodendri* isolates sporulated more abundantly than *C. macrodidymum* and *C. pauciseptatum* isolates in all studied temperatures, with values greater than 10^4 conidia mm^{-2}. In *C. macrodidymum*, there was more variability among the isolates, isolate Cy14 being the only one for which values greater than 10^4 conidia mm^{-2} were obtained at 15, 20, 25 and 30ºC. Isolate Cy47 only sporulated at 15, 20 and 25ºC. Sporulation of *C. pauciseptatum* isolates was also variable among isolates, isolate Cy593 being the only one in which values greater than 10^4 conidia mm^{-2} were obtained at 5, 20, 25 and 30**°C.** Isolate CBS120173 did not sporulate at 5**°C.**

Chlamydospores were observed in PDA cultures of all isolates from 15 to 30ºC. No chlamydospores were observed at 5 or 10ºC.

Table 2. Analysis of variance for the effects of temperature, pH and Ψ_s on radial growth and sporulation of *Cylindrocarpon liriodendri, C. macrodidymum* and *C. pauciseptatum* isolates.

Parameter	*C. liriodendri*			*C. macrodidymum*			*C. pauciseptatum*		
	d.f.[a]	MS[b]	*P* < *F*[c]	d.f.	MS	*P* < *F*	d.f.	MS	*P* < *F*
Temperature									
Radial growth									
Experiment (A)	1	0.00012	0.8911	1	<0.0001	0.9894	1	0.00007	0.9199
Isolate (B)	2	0.00427	0.4999	2	0.04492	0.0019	2	0.03480	0.0073
A × B	2	0.00019	0.9688	2	0.00011	0.9839	2	0.00001	0.9980
Residual	326	0.00615		329	0.00703		330	0.00697	
Sporulation									
Experiment (A)	1	17.5208	0.1418	1	17.2798	0.0629	1	5.17642	0.3420
Isolate (B)	2	0.56193	0.9328	2	379.699	<0.001	2	195.172	<0.001
A × B	2	4.69997	0.5596	2	2.11654	0.6532	2	4.39406	0.4645
Residual	330	8.08063		330	4.96344		330	5.71707	
pH									
Radial growth									
Experiment (A)	1	<0.0001	0.9790	1	0.00005	0.8122	1	<0.0001	0.9793
Isolate (B)	2	0.00318	0.0895	2	0.04015	<0.001	2	0.00557	0.0006
A × B	2	0.00017	0.8787	2	0.00005	0.9459	2	<0.0001	0.9987
Residual	226	0.00131		221	0.00089		225	0.00073	
Sporulation									
Experiment (A)	1	0.02486	0.8548	1	<0.0001	0.9132	1	<0.0001	0.2186
Isolate (B)	2	2.26406	0.0492	2	<0.0001	<0.001	2	<0.0001	<0.001
A × B	2	0.09818	0.8759	2	<0.0001	0.9882	2	<0.0001	0.2346
Residual	212	0.74085		210	<0.0001		208	<0.0001	
Ψ_s									
Radial growth									
Experiment (A)	1	0.00004	0.8891	1	0.00002	0.9411	1	0.00002	0.9329
Isolate (B)	2	0.02469	<0.001	2	0.05107	<0.001	2	0.05301	<0.001
Salt type (C)	1	0.05410	<0.001	1	0.05925	<0.001	1	0.16224	<0.001
A × B	2	0.00020	0.8982	2	0.00016	0.9547	2	<0.0001	0.9992
A × C	2	0.00247	0.2742	2	0.02014	0.0026	2	0.00127	0.6735
B × C	1	0.00182	0.3290	1	0.00021	0.8009	1	0.00005	0.9011
A × B × C	2	0.00042	0.8029	2	0.00019	0.9449	2	0.00006	0.9817
Residual	639	0.00191		658	0.00335				

(*Continued*)

Table 2. *Continues.*

Parameter	*C. liriodendri*			*C. macrodidymum*			*C. pauciseptatum*		
	d.f.[a]	MS[b]	*P* < *F*[c]	d.f.	MS	*P* < *F*	d.f.	MS	*P* < *F*
Sporulation									
Experiment (A)	1	4.06421	0.1092	1	2.37550	0.5960	1	0.52946	0.8002
Isolate (B)	2	0.88494	0.5723	2	68.3923	0.0003	2	91.4964	<0.001
Salt type (C)	1	0.67427	0.0391	1	1.26907	0.6983	1	118.754	0.0002
A × B	2	1.70771	0.3409	2	1.70329	0.8175	2	3.05828	0.6908
A × C	2	10.0494	0.0019	2	297.890	<0.001	2	52.2602	0.0019
B × C	1	3.36179	0.1452	1	0.62988	0.7848	1	15.4059	0.1721
Residual	2	0.06835	0.9578	2	7.15412	0.4293	2	8.13652	0.3742
A × B × C	660	1.58416		660	8.44962		660	8.26433	

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

Effects of pH on mycelial growth, sporulation and chlamydospore production of *Cylindrocarpon* spp.

Statistically significant effects of the isolates on radial growth were observed for *C. macrodidymum* ($P<0.001$) and *C. pauciseptatum* ($P=0.0006$), but not for *C. liriodendri* ($P=0.0895$). The effect of isolate × experiment was also not significant in the three species studied ($P>0.05$) (Table 2).

The effects of pH on mycelial radial growth and sporulation of the nine *Cylindrocarpon* spp. isolates are shown in Figure 2. All isolates were able to grow on pH-adjusted PDA at all pH values studied. In general, for each *Cylindrocarpon* spp. all isolates showed similar growth rates from pH 4 to pH 8, although in *C. liriodendri* and *C. macrodidymum* radial growth increased slightly as pH increased. In *C. liriodendri*, mycelial radial growth was greatest at pH 8 for all isolates, and in *C. macrodidymum,* mycelial growth of Cy47 and Cy81 isolates was also greatest at pH 8, while for Cy14 this was greatest at pH 6. In *C. pauciseptatum*, mycelial growth of isolates CBS120171 and CBS120173 was greatest at pH 6, while for isolate Cy593 this was greatest at pH 8.

Regarding sporulation, significant effects of the isolate on sporulation were observed for *C. liriodendri* ($P=0.0492$), *C. macrodidymum* ($P<0.001$) and *C. pauciseptatum* ($P<0.001$). The effect of isolate × experiment was not significant for all *Cylindrocarpon* spp. ($P>0.05$) (Table 2).

Most of the isolates produced conidia at all pH values, showing a broad range of variation among *Cylindrocarpon* spp. In general, the three *C. liriodendri* isolates sporulated more abundantly than *C. macrodidymum* and *C. pauciseptatum* at all studied pH values, producing more than 10^5 conidia mm^{-2}. In *C. macrodidymum*, there was variability among the isolates, isolate Cy14 being the only one for which values greater than 10^5 conidia mm^{-2} were obtained at pH 6 and 8. Isolates Cy47 did not sporulate at pH 5 and Cy81 did not sporulate at pH 6. Sporulation of *C. pauciseptatum* isolates was also variable among isolates, isolate Cy593 being the only one for which values greater than 10^5 conidia mm^{-2} were recorded at pH 6, but this isolate did not sporulate at pH 4. Isolate CBS120171 only sporulated at pH 5, 6 and 7, and isolate CBS120173 did not sporulate at pH 5.

Chlamydospores were observed in PDA cultures of all isolates at all pH values studied.

Effects of water potential (Ψs) on mycelial growth, sporulation and chlamydospore production of *Cylindrocarpon* spp.

Statistically significant effects of the isolate and salt type on mycelial growth were observed for all three species ($P<0.001$). All the interactions were not

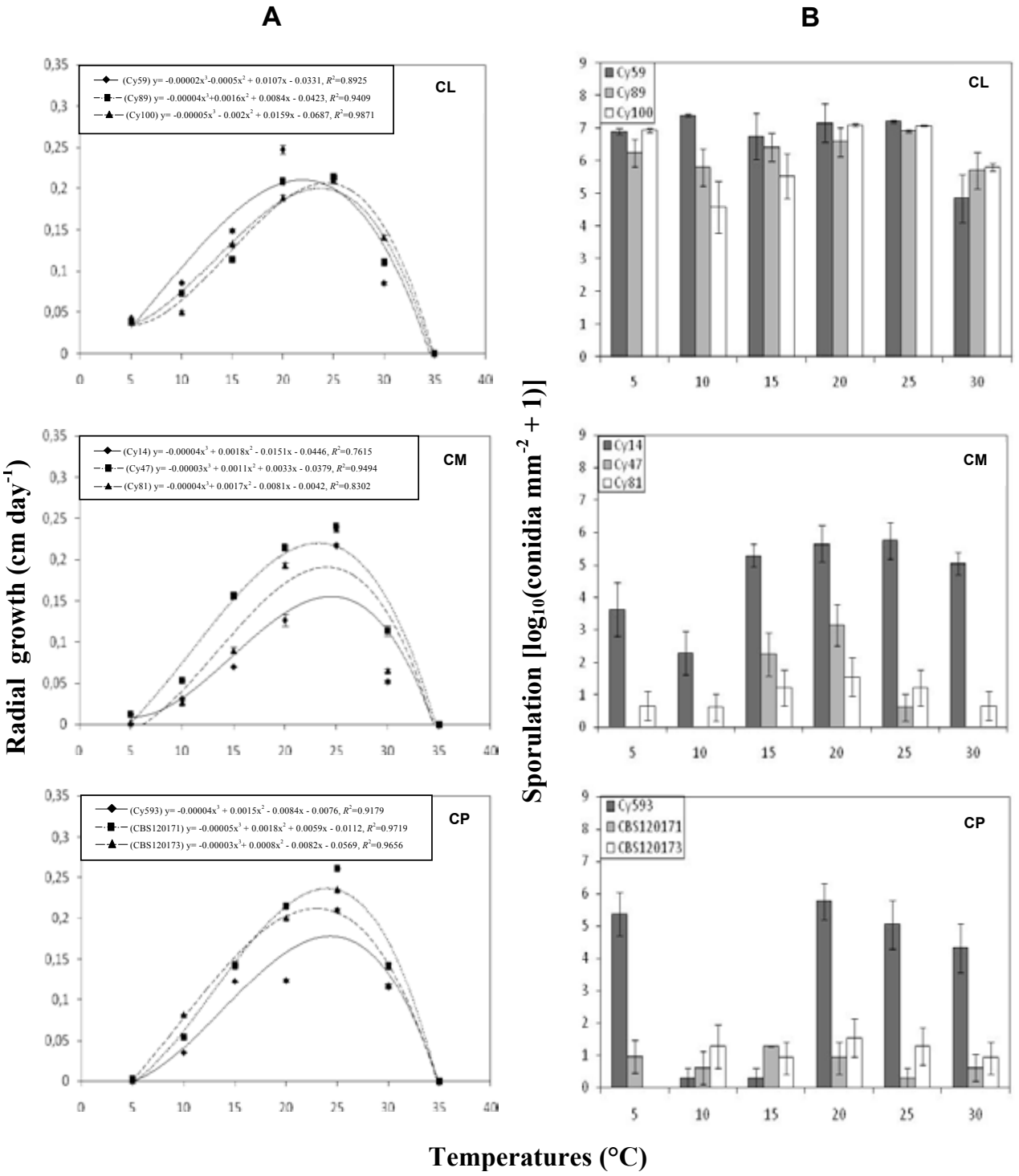

Figure 1. A, mean colony radial growth rates (cm day^{-1}) of three isolates of *C. liriodendi* (CL), *C. macrodidymum* (CM) and *C. pauciseptatum* (CP), after 10 days of incubation in darkness on PDA at 5, 10, 15, 20, 25, 30 or 35ºC; **B,** mean amounts of sporulation [$\log_{10}$(conidia mm^{-2} + 1)] of three isolates of *C. liriodendri* (CL), *C. macrodidymum* (CM) and *C. pauciseptatum* (CP), after 30 days of incubation in darkness on PDA at 5, 10, 15, 20, 25, 30 or 35ºC. Results are the mean of two independent sets of four pseudoreplicates for each temperature. Vertical bars are the standard error of the means.

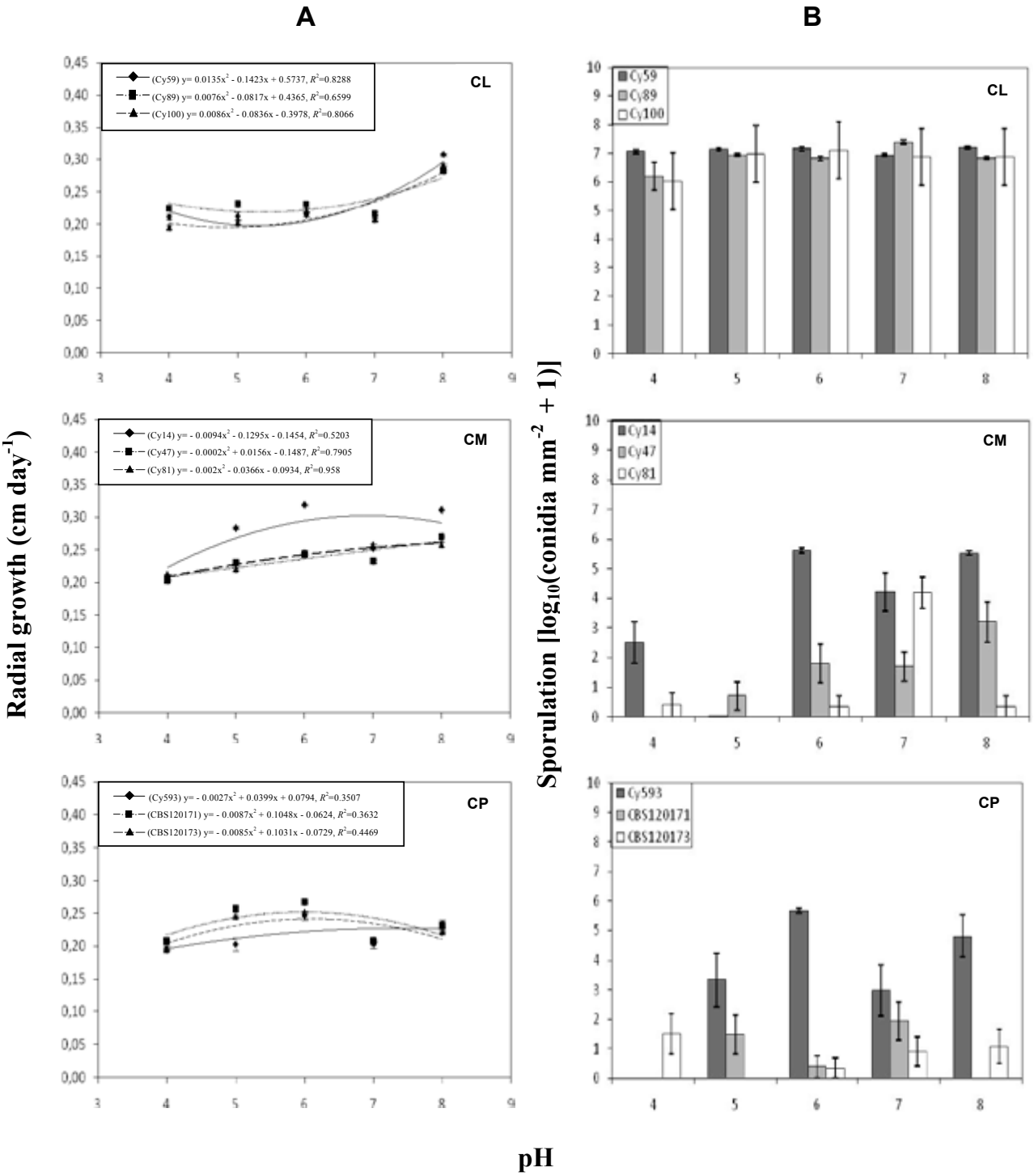

Figure 2. A, mean colony radial growth rates (cm day^{-1}) of three isolates of *C. liriodendi* (CL), *C. macrodidymum* (CM) and *C. pauciseptatum* (CP), after 10 days of incubation in darkness on PDA at pHs 4, 5, 6, 7 or 8; **B,** mean amounts of sporulation [$\log_{10}$(conidia mm^{-2} + 1)] of three isolates of *C. liriodendri* (CL), *C. macrodidymum* (CM) and *C. pauciseptatum* (CP), after 30 days of incubation in darkness on PDA at pHs 4, 5, 6, 7 or 8. Results are the mean of two independent sets of four pseudoreplicates in each pH.Vertical bars are the standard error of the means.

significant with the exception of experiment × salt type for *C. macrodidymum* ($P<0.0026$) (Table 2).

The effect of Ψ_s on mycelial growth of the nine *Cylindrocarpon* spp. isolates is shown in Figure 3, and on sporulation is shown in Figure 4. The patterns of the mycelial radial growth responses of the isolates to decreasing Ψ_s were similar for the two osmotica tested, but, in general, *Cylindrocarpon* spp. were more tolerant to NaCl than KCl. Mycelial growth generally increased compared with mycelial growth on nonamended PDA (-0.3 MPa) at -0.5 MPa and -1.0 Mpa by the addition of KCl and NaCl. The exception was isolate Cy89 which showed the greatest mycelial growth at -2.0 MPa. At lower water potentials, mycelial growth decreased as Ψ_s reduced, showing the lowest percentages at -5.0 MPa.

Regarding sporulation, a significant effect of the isolate on sporulation was observed for *C. macrodidymum* ($P=0.0003$) and *C. pauciseptatum* ($P<0.001$), but not for *C. liriodendri* ($P=0.5723$). The effect of salt type was significant for *C. liriodendri* ($P=0.0391$) and *C. pauciseptatum* ($P=0.0002$), but not for *C. macrodidymum* ($P=0.6983$). All the interactions were not significant with the exception of experiment × salt type for all *Cylindrocarpon* spp. (Table 2).

Most of the isolates were able to produce conidia at all Ψ_s values, showing a broad range of variation. In general, the three *C. liriodendri* isolates sporulated more abundantly than *C. macrodidymum* and *C. pauciseptatum* isolates in all studied Ψ_s values for each salt tested, with values greater than 10^5 conidia mm^{-2} in KCl and 10^4 conidia mm^{-2} in NaCl. In *C. macrodidymum*, there was more variability among the isolates, and isolates Cy47 and Cy81 did not sporulate at -5.0 MPa in NaCl. In *C. pauciseptatum*, sporulation was also variable among isolates. Isolate CBS120171 did not sporulate at -1.0 MPa in KCl and at -0.5 in NaCl, and isolate Cy593 did not sporulate at -5.0 MPa in NaCl.

Chlamydospores were observed in PDA cultures of all isolates at almost all Ψ_s values in both salts. No chlamydospores were observed in isolate Cy593 (*C. pauciseptatum*) at -4.0 MPa in NaCl and at -5.0 MPa both in NaCl and KCl, and in Cy59 (*C. liriodendri*) at -4.0 and -5.0 MPa in KCl.

Discussion

This study has identified differences in the effects of temperature, pH and Ψ_s on mycelial growth, sporulation and chlamydospore production of *C. liriodendri, C. macrodidymum* and *C. pauciseptatum*.

In general, these *Cylindrocarpon* spp. were able to grow over a range of temperatures from 5 to 30ºC, with optimum temperatures for growth between 20 to 25ºC, but they did not grow at 35ºC. These results are in agreement with those obtained in previous studies (Halleen *et al*., 2004; Petit and Gubler, 2005; Halleen *et al*., 2006b; Alaniz *et al*., 2007; Schroers *et al*., 2008), and indicate that *Cylindrocarpon* spp. associated to black foot disease of grapevine are mesophilic, as most fungi, which commonly grow within the range 10–40ºC (Deacon, 2006). In addition, our study showed differences among *Cylindrocarpon* spp. in the effect of low temperatures on mycelial growth. Growth of *C. macrodidymum* and *C. pauciseptatum* at 5 and 10ºC was almost negligible compared with that of *C. liriodendri*. This is in agreement with the results obtained by Alaniz *et al*. (2007), who demonstrated that *C. macrodidymum* can be differentiated from *C. liriodendri* by growing more slowly at 5 and 10ºC.

Previous research has suggested that species belonging to the genus *Cylindrocarpon* are calcicolous, with optimal pH around 7.0 and being poorly represented in acid soils (Matturi and Stenton, 1964a). Nevertheless, in our study *Cylindrocarpon* spp. associated with black foot of grapevine showed broad pH tolerance for mycelial growth, although with slight differences among isolates. All isolates were able to grow between pH 4.0 to 8.0. This corresponds with the optimum pH range indicated by Deacon (2006) for mycelial growth of most fungi.

Regarding the effect of Ψ_s, the response of *Cylindrocarpon* spp. isolates was reduced mycelial growth as Ψ_s decreased. Our results indicate that the isolates may have benefited from small to modest additions of solutes. In general, mycelial growth was greater on amended media at -0.5, -1.0 and/or -2.0 MPa compared with that on nonamended PDA (-0.3 MPa), and was reduced at Ψ_s values less than -2.0 MPa. Moreover, the effect of Ψ_s on mycelial growth was similar whether KCl or NaCl was used as the osmoticum, indicating that the observed responses were caused by changes in Ψ_s rather than by toxicity of the osmotic. These results, together with those obtained in the pH experiments, suggest that *Cylindrocarpon* spp. pathogenic to grapevine are likely to proliferate in most vineyard soils.

In all studied conditions, *C. liriodendri* was the species with the greatest capacity for sporulation

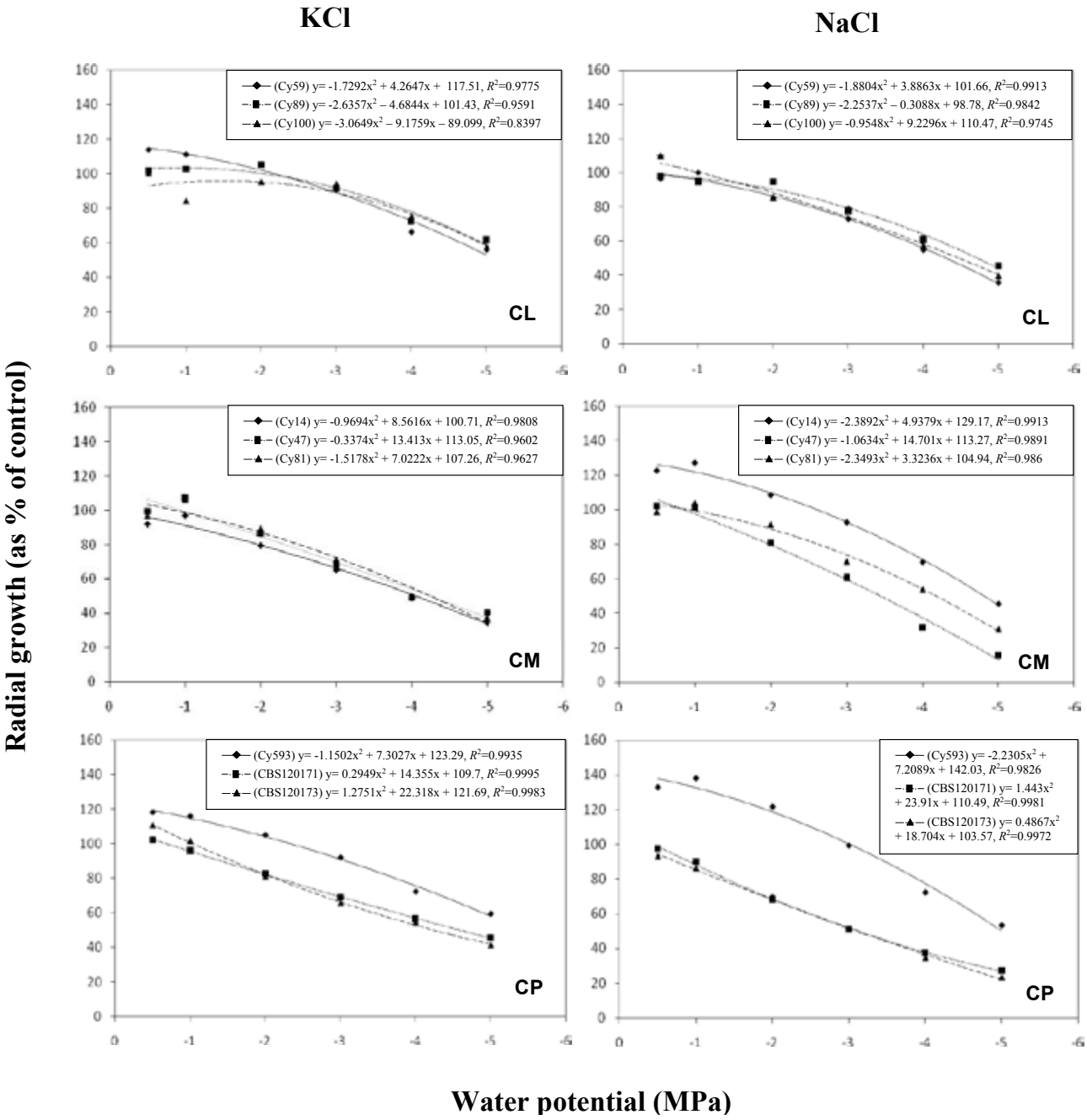

Figure 3. Mean relative colony radial growth (as a percentage of that on nonamended media) of three isolates of *C. liriodendri* (CL), *C. macrodidymum* (CM) and *C. pauciseptatum* (CP), after 10 days of incubation in darkness at 25ºC on PDA amended with KCl or NaCl to give the following water potentials -0.5, -1, -2, -3, -4 or -5 MPa. The water potential of nonamended PDA was -0.3MPa. Data points are the means of two independent sets of four pseudoreplicates in each experiment. Vertical bars are the standard error of the means.

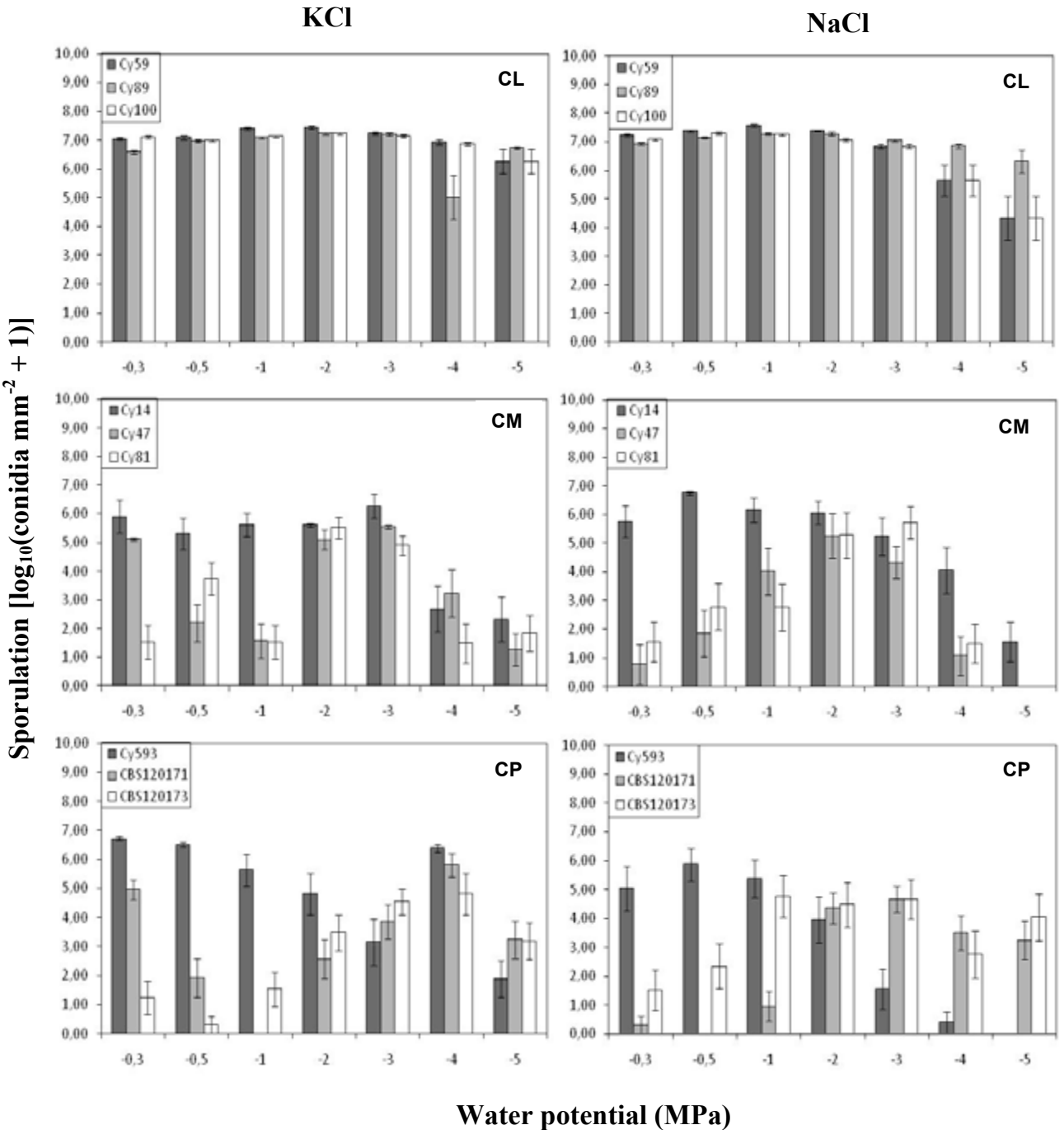

Figure 4. Mean amounts of sporulation [$\log_{10}$(conidia mm^{-2} + 1)] of three isolates of *C. liriodendri* (CL), *C. macrodidymum* (CM) and *C. pauciseptatum* (CP), after 30 days in darkness at 25ºC on PDA amended with KCl or NaCl to give the following water potentials -0.5, -1, -2, -3, -4 or -5 MPa. The water potential of nonamended PDA was -0.3MPa. Data points are the means of two independent sets of four pseudoreplicates in each experiment. Vertical bars are the standard error of the means.

compared with *C. macrodidymum* and *C. pauciseptatum.* Specifically, regarding the effect of temperature, Alaniz *et al*. (2007) indicated that *C. macrodidymum* can be differentiated from *C. liriodendri* by producing fewer conidia at 5 and 10ºC, which agrees with our results. It is also interesting to note that the *C. macrodidymum* isolates Cy47 and Cy81, which showed reduced mycelial growth and sporulation in the temperature, pH and Ψ_s experiments when compared with *C. liriodendri* isolates and *C. macrodidymum* isolate Cy14, belong to the genetic Groups G7 and G6, respectively, described by Alaniz *et al*. (2009) using inter-simple sequence repeats analysis. These authors demonstrated that isolates included in these groups were significantly more virulent to grapevine than other *C. macrodidymum* and *C. liriodendri* isolates. Thus, our results suggest a possible relationship between high virulence of the isolates and a low mycelial growth and sporulation.

Previous studies have shown that *C. liriodendri*, *C. macrodidymum* and *C. pauciseptatum* are able to produce chlamydospores in culture media such as carnation leaf agar (CLA), oatmeal agar (OA), PDA, Spezieller Nährstoffarmer Agar (SNA) or Diluted V8-juice Agar (V8) after 14–21 dys of incubation in darkness at 20–25ºC (Halleen *et al*., 2004; Halleen *et al*., 2006b; Schroers *et al*., 2008). Moreover, chlamydospores of *C. destructans*, which causes root rot of *Panax ginseng*, formed over a range of temperatures from 5 to 30ºC (Yoo *et al*., 1996). In general, our results showed that chlamydospore production was not much affect by temperature, pH and Ψ_s. Chlamydospores were observed in PDA cultures of all isolates at all pH values studied, while just some isolates did not produced them at 5 and 10**ºC** or -4.0 and/or -5.0 MPa in both osmotic media tested. Petit and Gubler (2005), indicated that *C. macrodidymum* isolates produced relatively more chlamydospores than *C. liriodendri*, although they considered this characteristic too inconsistent to be used for identification purposes. Chlamydospores allow *Cylindrocarpon* spp. to survive for extended periods in soil and remain dormant until they are stimulated to germinate by plants or are destroyed by other soil organisms (Matturi and Stenton, 1964b; Booth, 1966). However, further research is needed to determine the role of chlamydospores during subsequent infections on grapevines.

Our study provides further information on factors affecting growth, reproductive and survival potential of *C. liriodendri, C. macrodidymum* and *C. pauciseptatum*. From the results reported here, it can be concluded that these species have abilities to be active over a wide pH range, while temperature and Ψ_s changes can affect their growth and reproductive potential. In this sense, the results obtained here improve understanding of the biology of these important grapevine pathogens. This is likely to be important for developing *in vitro* assays for assessing pathogenicity of *Cylindrocarpon* spp. (Pathrose *et al*., 2010), or new methods for control of black foot disease of grapevines, such as the use of arbuscular mycorrhizae (Petit and Gubler, 2006; Bleach *et al*., 2008) or soil biofumigation (Bleach *et al*., 2009).

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