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 ($\Psi_s$) 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 $\Psi_s$ conditions. All isolates were able to grow over a range of temperatures from 5 to 30°C, with an optimum temperature between 20 to 25°C, but they did not grow at 35°C. Active mycelial growth was observed over a range of pHs, from 4 to 8. Regarding the effect of $\Psi_s$, in general, mycelial growth was greater on amended media at -0.5, -1.0 or -2.0 MPa compared with that obtained on nonamended PDA (-0.3 MPa), and was reduced at $\Psi_s$ 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 $\Psi_s$. 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).
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 Campylcarpon spp. (Campylcarpon fasciculare Schroers, Halleen & Crous and Campyl. pseudofasciculare Halleen, Schroers & Crous) (Halleen et al., 2006a, 2006b; 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 (Arroca et al., 2006; Giménez-Jaime et al., 2006; Alaniz et al., 2007; Gramajé 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 (Ψs) 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 Uecker on muskmelon 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, Ψs 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 25°C 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.
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 25°C. There were two replicates for each isolate and pH combination.

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

<table>
<thead>
<tr>
<th>Species/Isolate</th>
<th>Year</th>
<th>Geographical origin</th>
<th>Scion/rootstock</th>
<th>Optimum growth (°C)b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Town</td>
<td>Province</td>
<td></td>
</tr>
<tr>
<td>C. liriodendri</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cy59</td>
<td>2003</td>
<td>Tarazona de la Mancha</td>
<td>Albacete</td>
<td>Cencibel/1103 P</td>
</tr>
<tr>
<td>Cy89</td>
<td>2004</td>
<td>Aiello de Malferit</td>
<td>Valencia</td>
<td>Garnacha/110R</td>
</tr>
<tr>
<td>Cy100</td>
<td>2004</td>
<td>Alesanco</td>
<td>La Rioja</td>
<td>Garnacha/110R</td>
</tr>
<tr>
<td>C. macrodidymum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cy14</td>
<td>2002</td>
<td>n.d.c</td>
<td>Burgos</td>
<td>Tempranillo/110R</td>
</tr>
<tr>
<td>Cy47</td>
<td>2003</td>
<td>Mollina</td>
<td>Málaga</td>
<td>Pedro Ximénez/1103 P</td>
</tr>
<tr>
<td>Cy81</td>
<td>2003</td>
<td>Beneixama</td>
<td>Alicante</td>
<td>Tempranillo/161-49C</td>
</tr>
<tr>
<td>C. pauciseptatum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cy593</td>
<td>2009</td>
<td>Tobarra</td>
<td>Albacete</td>
<td>Syrah/S04</td>
</tr>
<tr>
<td>CBS120171a</td>
<td>2005</td>
<td>Krsko (Eslovenia)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CBS120173a</td>
<td>2005</td>
<td>Doljenska (Eslovenia)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

a Reference isolates of C. pauciseptatum (CBS 120171 and CBS 120173) were obtained from the collection of Centraalbureau voor Schimmelcultures in Utrecht, 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.
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 ($\Psi_s$) on mycelial growth, sporulation and chlamydospore production of Cylindrocarpon spp.

The effect of $\Psi_s$ 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 $\Psi_s$ 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 a control. Plates were incubated in the dark at 25ºC. There were two replicates for each isolate, type of solute and $\Psi_s$ 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.

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 $\Psi_s$ experiments using Statgraphics Plus 5.1 software. 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 observed at the three tested temperatures: 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 30ºC. Isolate CBS120173 did not sporulate at 5ºC.

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

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 $\times$ 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 observed 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 21.9ºC for isolate Cy59 (C. liriodendri) and 24.5ºC 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 $\times$ 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 observed at the three tested temperatures: 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 30ºC. Isolate CBS120173 did not sporulate at 5ºC.

Chlamydospores were observed in PDA cultures of all isolates from 15 to 30ºC. No chlamydospores were observed at 5 or 10ºC.
with values greater than 10
isolates in all studied temperatures, *C. pauciseptatum*
ulated more abundantly than
ation. In general, the three
*C. liriodendri*
isolates spor-
conidia at all temperatures, showing broad varia
-thesis × experiment was also not significant in all spe
but not for
**P** <0.001),
**P** <0.001) and
didymum (**P**
isolate on sporulation were observed for
isolate Cy14 (**P**
C. macrodidymum
C for isolate Cy59 (**P**
24.5
ºC
21.9
º
optimum
growth temperatures for all isolates ranged between
almost negligible at this temperature. Optimum
C. macrodidymum
-lates was also variable among isolates, isolate Cy593
15, 20 and 25ºC. Sporulation of
15, 20, 25 and 30ºC. Isolate Cy47 only sporulated at
between 0.038 cm day
At 5ºC,
from 5 to 30ºC, and no growth was obtained at 35ºC.
spp. isolates are shown in Figure 1. All isolates were
growth and sporulation of the nine
spp. *Cylindrocarpon*
Effects of temperature on mycelial growth,
Results

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th><em>C. liriodendri</em></th>
<th><em>C. macrodidymum</em></th>
<th><em>C. pauciseptatum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d.f.</td>
<td>MS</td>
<td>P &lt; F</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radial growth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment (A)</td>
<td>1</td>
<td>0.00012</td>
<td>0.8911</td>
</tr>
<tr>
<td>Isolate (B)</td>
<td>2</td>
<td>0.00427</td>
<td>0.4999</td>
</tr>
<tr>
<td>A × B</td>
<td>2</td>
<td>0.00019</td>
<td>0.9688</td>
</tr>
<tr>
<td>Residual</td>
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<td>0.00615</td>
<td></td>
</tr>
<tr>
<td>Sporulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment (A)</td>
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<td>17.5208</td>
<td>0.1418</td>
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<tr>
<td>Isolate (B)</td>
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<td>0.56193</td>
<td>0.9328</td>
</tr>
<tr>
<td>A × B</td>
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<td>4.69997</td>
<td>0.5596</td>
</tr>
<tr>
<td>Residual</td>
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<td>8.08063</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td></td>
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<tr>
<td>Radial growth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment (A)</td>
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<td>&lt;0.0001</td>
<td>0.9790</td>
</tr>
<tr>
<td>Isolate (B)</td>
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</tr>
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<td>A × B</td>
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<td>Residual</td>
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<td>Sporulation</td>
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<tr>
<td>Experiment (A)</td>
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<tr>
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<tr>
<td>Residual</td>
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<tr>
<td>Ψ</td>
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<td>Radial growth</td>
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<td></td>
<td></td>
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<tr>
<td>Experiment (A)</td>
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</tr>
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<td>Isolate (B)</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>Salt type (C)</td>
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<td>0.05410</td>
<td>&lt;0.001</td>
</tr>
<tr>
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</tr>
<tr>
<td>A × C</td>
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<td>0.2742</td>
</tr>
<tr>
<td>B × C</td>
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<td>0.3290</td>
</tr>
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<td>0.8029</td>
</tr>
<tr>
<td>Residual</td>
<td>639</td>
<td>0.00191</td>
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</table>

(Continued)
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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...
Growth, sporulation and chlamydospore production of Cylindrocarpon spp.

Figure 1. A, mean colony radial growth rates (cm day$^{-1}$) of three isolates of *C. liriodendri* (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 35°C; 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 35°C. 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. liriodendri* (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 \([\log_{10}(\text{conidia mm}^{-2} + 1)]\) 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 Ψs 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 Ψs 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 Ψs 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 Ψs 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 Ψs values for each salt tested, with values greater than 10⁵ conidia mm⁻² in KCl and 10⁴ 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 Ψs 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 Ψs, 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 30°C, with optimum temperatures for growth between 20 to 25°C, but they did not grow at 35°C. 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 10–40°C (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 10°C 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 10°C.

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 Ψs, the response of Cylindrocarpon spp. isolates was reduced mycelial growth as Ψs 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 Ψs values less than -2.0 MPa. Moreover, the effect of Ψs on mycelial growth was similar whether KCl or NaCl was used as the osmoticum, indicating that the observed responses were caused by changes in Ψs, 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.
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**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 25°C 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 $[\log_{10}(\text{conidia mm}^{-2} + 1)]$ of three isolates of *C. liriodendri* (CL), *C. macrodidymum* (CM) and *C. pauciseptatum* (CP), after 30 days in darkness at 25°C 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 -0.3 MPa. 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 10°C, 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	extsubscript{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 20–25°C (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 30°C (Yoo et al., 1996). In general, our results showed that chlamydospore production was not much affect by temperature, pH and Ψ	extsubscript{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°C 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	extsubscript{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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