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In vitro ecology of *Seiridium cardinale* and allied species: the effect of solute stress and water potential on fungal growth

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Summary. Defining the potential implications of global climate change on Mediterranean forest ecosystems requires a basic knowledge on the ecology of fungal pathogens under conditions that would stress host plants. The Mediterranean cypress (*Cupressus sempervirens*)-*Seiridium* spp. pathosystem represents an important case study. In the last century, epidemics of cypress canker have killed historical plantations and the decades-long host resistance will probably break down in the future as a result of both host and pathogen adaptation to increasing temperature and decreasing summer precipitation. In this study, the effect of osmotic water stress on mycelial growth of *Seiridium cardinale*, *S. unicorne* and *S. cupressi* in culture was examined and compared to that of *Diplodia cupressi*, which is a pathogen of cypress known to be favoured by host water stress. Growth responses were evaluated on potato sucrose agar amended with KCl or NaCl to give water potentials in the range of -0.34 to -15 MPa. Mycelial growth decreased with decreasing water potential and ceased at -15 MPa, although the mycelium remained alive. Histochemical analysis conducted on *S. cardinale* grown at -12 MPa revealed melanization and thickening of hyphal walls, in addition to abundance of lipid-rich organelles. These results suggest that the three *Seiridium* spp. might survive drying cycles in cypress wood, but their tolerance is different. Successful survival strategies may partly result from changes in mycelium structure. Furthermore, *S. unicorne* was positively stimulated by a water potential of -3 MPa, suggesting that it may have high adaptive potential for life in a drier Mediterranean ecosystem, which is predicted to occur under scenarios of global warming.

Key words: cypress canker, Mediterranean region, drought stress, mycelium survival.

Introduction

Climate change is projected to have significant impacts on landscapes everywhere, with severe effects on plant communities (Parmesan and Yohe, 2003; Tylianakis *et al.*, 2008). Forecasting climatic models predict decreasing summer precipitation and increasing temperature in many areas, which can result in drought stress. Agricultural and forest productivity and diversity will be reduced (Kuussaari *et al.*, 2009; Butchart *et al.,* 2010) and, as a part of an ecosystem, the role of parasites will be modified. In many cases climate change will alter stages and rates of pathogen development and changes in the physiology of host-pathogen interactions (Garret *et al.*, 2006). In this scenario, pathogens will expand their geographic boundaries and may be brought in contact with previously unencountered hosts (Baker *et al.*, 2000), and invasive pathogens might overcome natural barriers spreading into new ecosystems. Occasionally dramatic and uncontrolled epidemics may therefore result (Parker and Gilbert, 2004; Garrett *et al.*, 2006).

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The Mediterranean region has been recently identified as a climate change "hot spot zone", that is a zone where changes will be considerable in terms of mean temperature and rainfall. Risk of epidemic events or migration of invasive pathogens, with emergence of new pathosystems, are expected in the near future (Desprez-Loustau *et al*., 2007; La Porta *et al*,. 2008).

The anamorphic fungus *Seiridium cardinale* (Wag.) Sutton and Gibson, which causes cypress canker, has devastated thousands of planted and ornamental cypress (*Cupressus sempervirens* L.) trees in the last 30 years, with adverse effects on the Mediterranean environment and landscape (Graniti, 1998). Two other species are also involved in the disease. *Seiridium unicorne* (Cooke & Ellis) Sutton is considered a weak pathogen, while *S. cupressi* (Guba) Boesenwinkel is an aggressive pathogen from the second year following inoculation (Xenopoulos, 1991; Spanos *et al*., 2001). *Seiridium cardinale* is the most thermophilic and aggressive in regions were summer heat waves occur in association with low rainfall,and therefore this pathogen has the highest potential for spread in Mediterranean countries. Infections occur in spring and fall, which are the most rainy seasons (Raddi and Panconesi, 1984). Nevertheless, increasing temperature driven by climate change may speed up the establishment of *Megastigmus watchli* (Seitner) and *Orsillus maculates* (Fieber), secondary insect vectors involved in the dissemination of these pathogens (Zocca *et al*., 2008). As insect vectors are likely to expand their distribution range, so the vectored pathogens will follow suit.

The aim of the present study was to ascertain whether *S. cardinale, S. unicorne,* and *S. cupressi* have the potential to remain alive in a stressful (dry) environment. Here we simulated the effect of drought stress on the three pathogens by measuring their *in vitro* mycelial growth under conditions of decreasing osmotic potential. *Diplodia cupressi* (Phillips & Alves) was used as a reference species because it is a pathogen of cypress known to be favoured by host water stress (Madar *et al*., 1989; Angelopoulos *et al*., 2009).

Materials and methods

Fungal strains

Four isolates belonging to the genus *Seiridium* were chosen for this study: *S. cardinale* (designated card 167 and 170), *S. cupressi* (cup 186) and *S. unicorne* (uni 237). One isolate of *Diplodia cupressi* (dip 111) was included in the experiment as a reference strain. Isolates were all recovered from fresh stem cankers on *Cupressus sempervirens*. All isolates have been deposited in the mycological collection of the Istituto per la Protezione delle Piante, CNR, Sesto Fiorentino, Italy, maintained in 15% glycerol at -80°C.

Effect of solute stress and water potential on mycelial growth

The isolates were maintained on potato sucrose agar (PSA) medium, at 4°C, throughout the period of experimentation and were transferred to PSA adjusted medium as needed.

Five levels of water potential (Ψ_s -3, -6, -9, -12 and -15 MPa), were obtained osmotically by adding appropriate concentration of NaCl or KCl to the basal medium prior to sterilization, according to the tables of Liddell (1992). The two ionic solutes are widely used in mycological studies because the direct "salt" effect on fungal growth is less than that of water potential. A control, non-amended medium (-0.34 MPa), was included in each test. The respective media were added to Petri plates for experiments. A 6-mm agar plug taken from a 7-day-old mother colony of each isolate tested was placed in the centre of each Petri plate. Tests were conducted in the dark at 25°C, the optimal temperature for field infection by *S. cardinale* (Graniti and Frisullo, 1990). Colony growth was determined at 7-day intervals by measuring two orthogonal diameters on each Petri plate, until one strain covered the whole plate, for a maximum of 21 d. There were five replicates for each isolate × water potential × osmoticum combination. The experiment was conducted twice.

Histochemical observations

Histochemical observations were conducted on *S. cardinale* isolate 167 (card 167) grown in potato sucrose liquid medium and modified osmotically with KCl to a -12 MPa water potential. Experimental control was represented by mycelium of *S. cardinale* grown in non-amended medium (-0.34 MPa). Mycelium fragments were harvested from 21-day-old cultures grown on a reciprocal shaker at 25°C, and mounted directly on micro-

scope slides in the vital histological stain solutions described below. Binding fluorescent sites were visualized with a Leitz "Laborlux S" brightfield microscope with epi-fluorescence light. The other stained compartments were detected with a Carl Zeiss Axioskop microscope. Autofluorescence was controlled by omitting dye reagents from the incubation solution. It was used 0.1 μg mL^{-1} DAPI (4',6-diamidino-2-phenylindole; Sigma, St. Louis, MO, USA), a fluorescent stain that binds strongly to DNA, at pH 7.5 to stain nuclei and live cells of *S. cardinale* for 5 min. Incubation of mycelium in 0.02% Calcofluor White 2MR (Sigma) in water for 10 min was used to stain β-1,4 glycans and their derivatives in cell wall compartments. This stilbene dye produces an intense blue fluorescent mycelium. Neutral Red was used to differentiate membrane-bound acidic compartments and lipid granules. Mycelium was incubated for 20 min in 0.14 mg mL^{-1} Neutral Red 8 (VWR International Ltd., Poole, UK) in water. To manipulate pH of staining solution, a few drops of 0.15 M K_2HPO_4 buffer (pH 8.6) were added and lipid droplets were visualized under fluorescent microscope.

Statistical analysis

Analysis of variance (ANOVA) of all the collected data was performed to determine main effects and interactions among water potential, solute and isolate. Means were separated using the Tukey test (P<0.01). Mycelial growth, recorded every 7 days, was converted to relative colony growth (RCG) as a percentage of control (growth on nonamended PSA), and polynomial regressions were calculated to describe the relationships between rate of growth and water potentials per each solute. Statistical analyses were carried out using the GLM procedure of Systat v. 10 (SPSS Inc, Richmond, CA, USA).

Results

Effect of solute stress and water potential on mycelial growth

ANOVA showed an overall statistically significant effect (P<0.001) of all the main factors (isolate, water potential, ionic solute and time), and of their interactions, on mycelial growth (Table 1).

Mycelial growth responses to changes in water potential (Ψ_s) of the four isolates of *Seiridium* (card 167, card 170, cup 186 and uni 237) were variable among the species and between isolates within species. Mycelium growth decreased significantly with decreasing water potential (Figure 1a, b). Three out of four *Seiridium* isolates (card 167, card 170 and cup 186) had the greatest my-

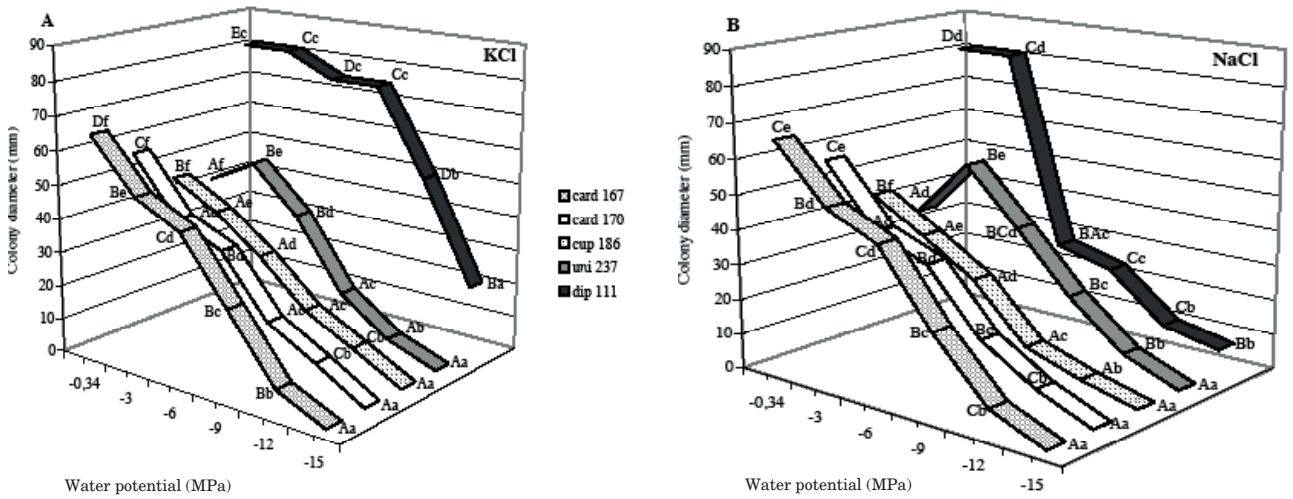

Figure 1. Mean colony diameter of *Seiridium cardinale* (card 167 and card 170), *S. cupressi* (cup 186), *S. unicorne* (uni 237) and *Diplodia cupressi* (dip 111) on PSA medium (A) amended with KCl or (B) amended with NaCl, after 21 days of incubation. The water potential of non-amended PSA was -0.34 MPa. Means accompanied by the same letter (uppercase: same treatment for different isolates; lowercase: same isolate at different treatments) are not significantly different based on Tukey test (P<0.001).

Table 1. Analysis of variance of all the main factors (isolate, water potential, ionic solute and time) and of their interactions on colony growth.

Source of variation	Df	Sum of Squares	F-ratio	*P*
Isolate	4	1649.5	10403.7	<0.001
Water potential	5	8739.4	44095.7	<0.001
Ionic solute	1	85.4	2153.9	<0.001
Time	2	1411.1	17799.8	<0.001
Isolate × Water potential	20	122.9	154.9	<0.001
Isolate × Ionic solute	4	356.4	2247.9	<0.001
Isolate × Time	8	14.0	44.2	<0.001
Water potential × Ionic solute	5	36.8	185.9	<0.001
Water potential × Time	10	155.3	391.7	<0.001
Ionic solute × Time	2	2.8	35.6	<0.001
Isolate × Water potential × Ionic solute	20	148.4	187.2	<0.001
Isolate × Water potential × Time	40	147.3	92.9	<0.001
Water potential × Ionic solute × Time	10	13.5	34.0	<0.001
Isolate × Water potential × Ionic solute × Time	40	47.2	46.8	<0.001

celial extension on unmodified PSA (control, -0.34 MPa), with significant difference among them both on KCl and NaCl ionic solutes (Figure 1a, b). Mycelial extension of all the four isolates was observed at water potentials down to -12 MPa, while complete inhibition of growth was recorded at the lowest water potential (-15 MPa). The smallest variation on growth occurred over the range -3 to -6 MPa of Ψ_s, regardless of the type of ionic solute (41.8 and 34.2 mm ± 0.2 SE, respectively), and growth declined markedly below -6 MPa; at -9 MPa mycelium extension was at least 50% less than that measured at -6 MPa.

Two isolates of *Seiridium cardinale* (card 167 and card 170) grew similarly (P>0.05) at -3 and -6 MPa on NaCl (Figure 1b). Although both isolates followed similar trends in response to variation in water potential, card 167 grew significantly more than card 170. The *S. cupressi* isolate (cup 186) showed the lowest radial growth at any water potential, although it tended to be more sensitive to NaCl (Figure 1b). The *S. unicorne* (uni 237) isolate was sharply stimulated by a small decrease in water potential; at Ψ_s of -3 MPa, colony diameter after 21 days of incubation was 18.5% and 48% greater than control (-0.34 MPa), on media modified osmotically with KCl and NaCl, respectively (Figure 1a, b). No differences in colony morphology were observed at any of the water potentials regardless of the two ionic solutes. All isolates resumed growth when transferred onto unamended PSA at 25°C from -15 MPa media after 21 days of incubation.

The *Diplodia cupressi* isolate (dip 111) covered whole plates in 21 days. Colony diameter did not change between -0.34 (control) and -9 MPa on KCl-amended PSA (P>0.4) (Figure 1a). However, when NaCl was used to modify the water potential of PSA medium, a large growth decrease was observed just below -3 MPa (Figure 1b). *Diplodia cupressi* was the isolate with the greatest growth rate, and was the only isolate showing any mycelial growth at the lowest Ψ_s tested (-15MPa) (Figure 1a, b).

The relative colony growth (RCG), averaged over three different times (7, 14 and 21 d), responded to different water potentials in a quadratic manner on both types of media (Figure 2a, b). ANOVA showed a significant effect (P<0.001) of all the main factors (isolate, water potential, ionic solute) and of their two- and three-way interactions on RCG, except for the water potential × ionic solute interaction (P=0.017). Comparison between the two isolates of *S. cardinale* showed

that card 167 had a significantly greater RCG than card 170 over the range -3 to -9 MPa, whereas they behaved similarly at -12 MPa on either KCl- or NaCl-amended PSA (Figure 2a, b). The behaviour of isolate cup 186 tracked most similarly that of isolate card 170 on both media (Figure 2a, b). Isolate uni 237 was the only one showing RCG greater than 100%, occurring at -3MPa on both KCl- (103%) and NaCl-amended medium (120%, Figure 2a, b). For this isolate, the RCG was significantly greater than the other two *Seiridium* species (*S. cardinale* and *S. cupressi*) between -3 and -9 MPa on NaCl (Figure 2b), whereas it was restricted to the -3 to -6 MPa range on KCl (Figure 2a). RCG was 0% for all four *Seiridium* isolates at the lowest Ψ_s (-15 MPa).

The relative colony growth of *D. cupressi* was 74–92% on KCl at Ψ_s above -9 MPa (Figure 2a); on NaCl the RCG reduced from 82 to 21.5% over the same Ψ_s range (Figure 2b). RCG was reduced to 13% at -15 MPa on KCl (Figure 2a), three-fold greater than that observed on NaCl at the same water potential.

All but one isolate (uni 237) grew better on KCl than NaCl.

Histochemical observations

After 21 days of growth on KCl at Ψ_s = -12 MPa, the mycelium of *S. cardinale* 167 appeared as a fluffy mass of dark-brown and melanized hyphae (Figure 3a). No pigmented hyphae were seen in the control.

DAPI staining revealed mono-, bi- or tri-nucleate hyphal compartments (Figure 3b) with yellow fluorescent and well organized cytoplasm on non-amended medium (Figure 3c). Conversely, in the presence of KCl the hyphae were granulose and dehydrated with nuclei at close range at -12 MPa (Figure 3d). Additional observations revealed clusters of very thin live hyphae (where nuclei were DAPI stained) forming bundles arising from each inoculation plug (Figure 3e–f).

Within 10 min of incubation in the Calcofluor staining solution, a blue fluorescence was observed on both control and KCl-stressed mycelium. The hyphal compartments appeared thinner and longer on non-amended medium than at -12 MPa (Figure 3g–h). Furthermore, the stressed mycelium appeared to be less fluorescent than non-stressed mycelium.

On non-amended medium, Neutral Red was taken up by hyphae of *S. cardinale* and accumulated in large vacuoles throughout all cell compartments (Figure 3i), but only when the pH of the staining solution was modified with K_2HPO_4 buffer. However, the vacuoles were clearly stained in the KCl-treated mycelium without any pH adjustments (Figure 3j). Lipids were visualized using the same procedure under the fluorescence microscope. The vacuoles were still visible in the

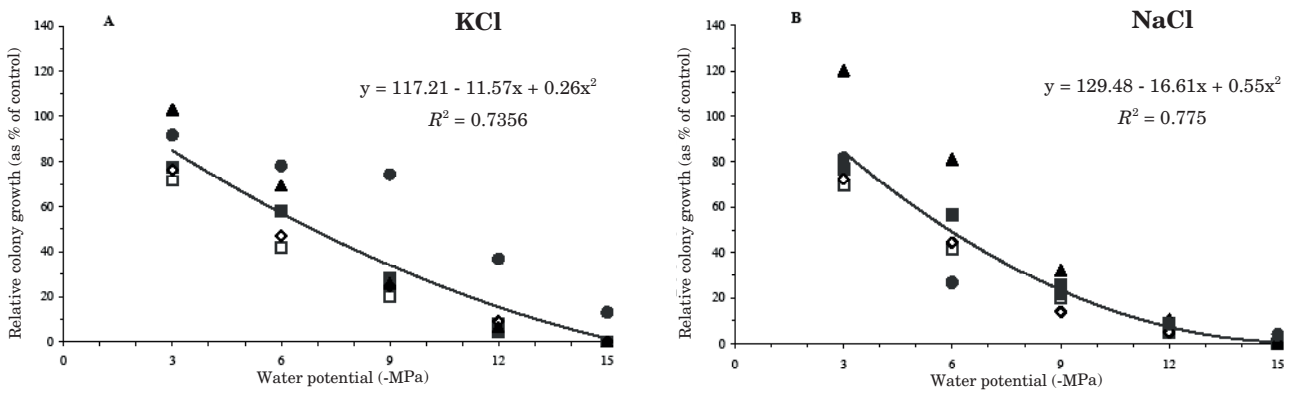

Figure 2. Relative colony growth (RCG), averaged over three different times (7, 14 and 21 d) and calculated as percentage of growth relative to that on nonamended medium (-0.34 MPa) of *Seiridium cardinale* (card 167, ■; card 170, □), *S. cupressi* (cup 186, ◊), *S. unicorne* (uni 237, ▲), and *Diplodia cupressi* (dip 111, ●) grown on PSA modified osmotically with KCl (A) or NaCl (B).

control along with fluorescent dots that were widely distributed within the hyphae, indicating high amounts of lipid droplets (Figure 3k). Lipid-enriched hyphae were more abundant in the treated mycelium (Figure 3l).

Discussion

This study demonstrates for the first time that *Seiridium* spp. can grow over a wide range of water potentials. Mycelium growth occurred at -12 MPa and viable hyphae were present at the lowest

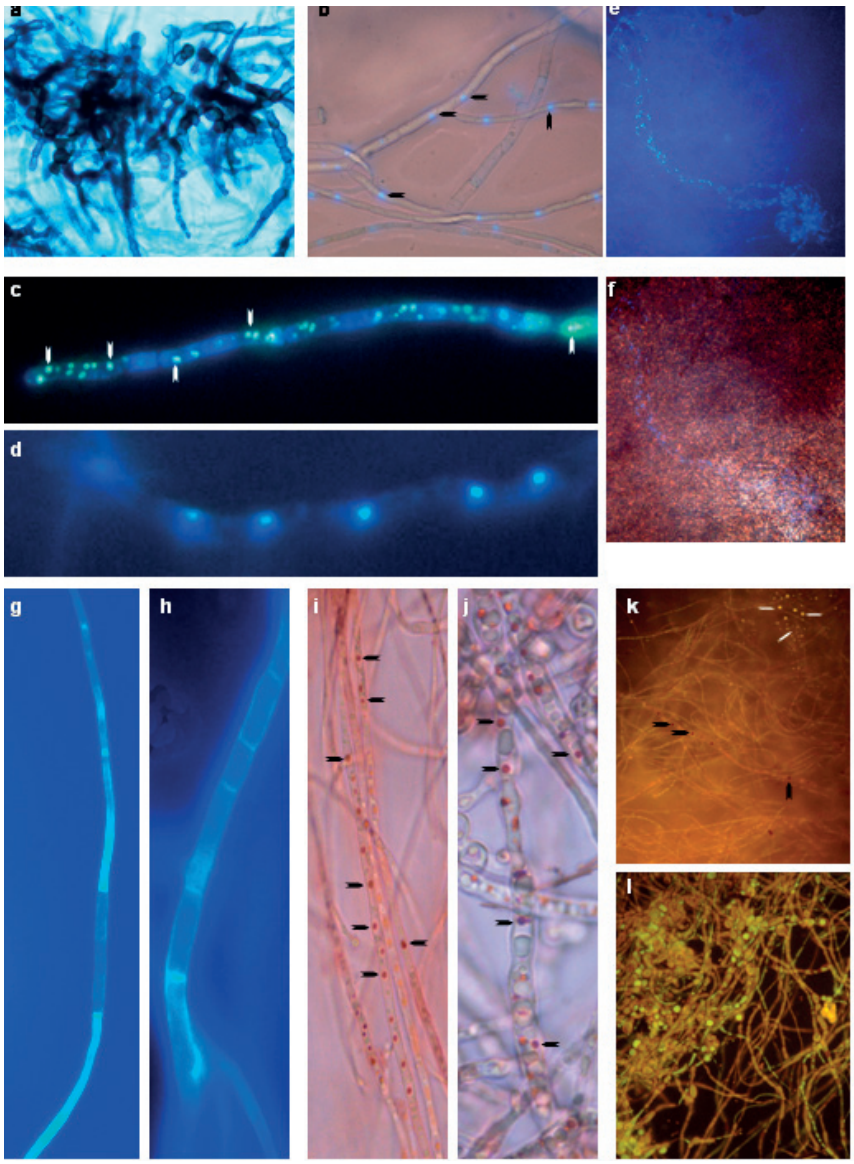

Figure 3. Histochemistry of live hyphae of *Seiridium cardinale* isolate 167. A. Dark-brown and melanized hyphae. B–F. DAPI staining. One–three separate nuclei (b) and yellow fluorescent cytoplasm (c) characterized hyphal compartments on non-amended medium, whereas nuclei appeared at close range (d), and live hyphae clustered in thin bundles at Ψ_s= -15MPa (e and f); reference points are indicated by arrowheads. G–H. Calcofluor uptake by hyphae in control (g) and KCl amended medium (h). I–L. Neutral Red staining for vacuoles (black arrowheads) and lipids (white arrowheads) in hyphae grown on control (i and k) and KCl (j and l). Magnification: Figure 3a, b, e, f, k and l, 20×; Figure 3c, d, g, h, i and j, 40×.

water potential (-15 MPa), as revealed by the resumed growth once the isolates were transferred onto unamended PSA. The relative lack of effects of the osmotic stress on mycelium growth suggests that the three *Seiridium* species are well adapted to stressful (dry) conditions and that they could be considered drought-tolerant fungal pathogens. This tolerance is well beyond the limits of their hosts, and is consistent with their status as pathogens of the semi-arid Mediterranean region (Suleman *et al.*, 2001).

The effect of water potential on mycelial growth was strictly species-dependent. *Seiridium cardinale* and *S. cupressi* had optimal growth on unamended medium (control, - 0.34 MPa); conversely, *S. unicorne* was positively stimulated at -3 MPa. For this species, RCGs of 103 and 120% were found on PSA modified osmotically with KCl and NaCl, respectively. Down to -6 MPa, the growth of all three *Seiridium* species declined in a similar way. These differential responses within the *Seiridium* species, in the face of a modest reduction in water potential (-3 MPa), could be related to different rates of solute uptake by the fungal cells, as mentioned by Griffin (1994). These discrepancies in physiological responses among allied species or isolates within the same species are not unusual, and they might simply reflect differences in their nutrient requirements and metabolism (Griffin, 1994). The ability of *S. unicorne* to benefit from water potential reduction might contribute to its wide distribution on *Cupressaceae* throughout the world, and it would indicate that this species may represent a future menace for the Mediterranean cypress ecosystem. Therefore, the potential for canker development and disease spread either on new hosts or in new ecosystems may be underestimated at present. The potential danger posed by *S. unicorne* may be exacerbated by the widespread use of cypress clones carrying an intermediate type of resistance against *S. cardinale*, and by a wider host range and variability in virulence of *S. unicorne* relative to *S. cardinale* and *S. cupressi* (Tisserat *et al.*, 1991; Graniti, 1998).

The ability of *Seiridium* spp. to grow at water potential as low as -6 MPa suggests it may be able to survive well in water-deprived cypress tissues. *Seiridium cardinale* dwells in the bark cortex and secondary phloem tissue of host trees (Mutto and Panconesi, 1987), where a continuous deposition of ligno-suberized boundary zones is observed in response to mycelium growth (Spanos *et al.*, 1999). Whether the host responds slowly or too late, the suberization process is often incomplete, leading to pathogen defeat of host defences in waves that keep the infection expanding. This is probably facilitated by stressful conditions for the host, in which defences are reduced and an exacerbation of competitive interactions is expected. New cypress canker epidemics will probably occur in the future in the Mediterranean region, which has experienced increasingly longer droughts during the last 50 years.

The black pigment exhibited by hyphae of *S. cardinale* growing at water potential of -12 MPa suggests that the synthesis of melanin-like pigments is favoured under such conditions, serving as a protective barrier. Melanisation has important roles in both pathology and ecology of fungal pathogens (Bell and Wheeler, 1986; Butler *et al.*, 2001). These findings are in agreement with the appearance of resting mycelium often found in dead cypress wood (Raddi and Panconesi, 1981), which along with chlamydospores, presumably aids in fungal survival and longevity under unfavourable conditions. Along with abundance of lipid-rich organelles (a form of stored chemical energy), all the observed histochemical modifications are part of known adaptation mechanisms for microorganisms when shifts in either osmotic or water potential occur as response to many different stressors (Gharieb and Gadd, 2004; Gangwar *et al.*, 2006; Magan, 2007). For example, overwintering *Plasmopora viticola* oospores were shown to accumulate large lipid globules, along with an increase in inner wall thickness (Vercesi *et al.*, 1999). Ramirez *et al.* (2004) found a marked increase in glycerol and arabitol content, in parallel with decreases in mannitol, in two *Fusarium graminearum* strains grown on NaCl-amended medium at potentials as low as -14 MPa.

KCl seemed to increase tolerance to osmotic stress because four out of five isolates grew better on this osmoticum than on NaCl. K ions are easily absorbed by fungal cells, and have a role in maintaining a suitable environment for all cellular processes in the face of unfavourable external water potential (Larsen, 1986). Since in this study K_2HPO_4 was added to Neutral Red to stain only the vacuoles of non-stressed mycelium, it is possible

that K ions from the medium may have been accumulated and utilized by the fungus to increase tolerance to osmotic stress, as reported for other filamentous fungi (Esteves *et al*., 2009).

The evidence from the present study suggests that *Seiridium* spp. may continue vegetative growth, i.e. in saprophytic form, resting mycelium, or latent pathogen state, under conditions of low moisture. This ability should be taken into consideration in the context of management of the Mediterranean cypress-*Seiridium* pathosystem, under scenarios of future climate change.

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