Mycorrhiza (2011) 21:247–253
DOI 10.1007/s00572-010-0329-z

ORIGINAL PAPER

Effect of water stress on *in vitro* mycelium cultures of two mycorrhizal desert truffles

Alfonso Navarro-Ródenas · M. Cecilia Lozano-Carrillo · Manuela Pérez-Gilabert · Asunción Morte

Received: 5 March 2010 / Accepted: 1 July 2010 / Published online: 20 July 2010

Abstract The ability of two species of desert truffle, *Terfezia claveryi* strain TcS2 and *Picoa lefebvrei* strain OL2, to tolerate water stress in pure culture has been investigated. Both *T. claveryi* and *P. lefebvrei* strains exhibited a mycelium growth pattern characteristic of drought tolerant species. However, they were only tolerant to moderate water stress, below −1.07 MPa, with the *P. lefebvrei* isolate being slightly more drought tolerant than the *T. claveryi* isolate. The increased alkaline phosphatase (ALP) activity observed in both fungi at moderate water stress with respect to the control indicated the functional adaptation of these mycelia to these drought conditions. ALP activity can be used as an indicator of the metabolic activity of these fungi. Slight water stress (−0.45 MPa) could improve mycelial inoculum production of these desert truffles. Moreover, *P. lefebvrei* could be a good candidate for further desert truffle mycorrhizal plant cultivation programmes in semiarid Mediterranean areas.

Keywords Mycelium drought tolerance · Alkaline phosphatase · *Terfezia* · *Picoa*

A. Navarro-Ródenas · M. C. Lozano-Carrillo · A. Morte (✉)
Dept. Biología Vegetal (Botánica), Facultad de Biología,
Universidad de Murcia,
Campus de Espinardo,
30100 Murcia, Spain
e-mail: amorte@um.es

M. Pérez-Gilabert
Dept. Bioquímica y Biología Molecular-A, Facultad de Biología,
Universidad de Murcia,
Campus de Espinardo,
30100 Murcia, Spain

Introduction

The term "desert truffles" includes species of different hypogeous Ascomycetes genera, such as *Terfezia, Picoa, Balsamia, Delastreopsis, Delastria, Leucangium, Mattirolomyces, Phaeangium* and *Tirmania,* and some *Tuber* species. The name "desert truffles" matches the nature of its distribution, which is typical of countries or territories with arid and semiarid conditions. All these fungi require localised outbreaks of rain to provide soil with sufficient water for them to develop and fructify. Although the mycorrhizal association is well adapted to arid and semiarid climates (Morte et al. 2000), desert truffle fruiting depends on rainfall distribution, which is one of the most important factors for successful cultivation (Morte et al. 2008, 2009).

In this study we assessed the ability of two species of desert truffles, *Terfezia claveryi* Chatin and *Picoa lefebvrei* (Pat.) Maire, to tolerate water stress in pure culture. Growth under low water potential conditions, induced using polyethylene glycol (PEG; Coleman et al. 1989; Mexal and Reid 1973), should, in theory, reflect the ability of the fungi to grow in dry soil and possibly to obtain water for the associated plant. Studies on the effect of water stress on in vitro mycelial growth have been carried out with non-mycorrhizal Basidiomycetes (Griffin 1977; Wilson and Griffin 1979; Boddy 1983; Dix 1984), mycorrhizal Basidiomycetes (Coleman et al. 1989; Boyle and Hellenbrand 1991; Sánchez et al. 2001; Duñabeitia et al. 2004), Deuteromycetes (Griffin 1972; Manandhar and Bruehl 1973; Brownell and Schneider 1985), non-mycorrhizal Ascomycetes (Elliott and Henson 2001; Pascual et al. 2003; Ramírez et al. 2004) and, recently, with the mycorrhizal Ascomycetes *Hymenoscyphus* sp.

and *Phialocephala* sp. and three Basidiomycetes species (*Laccaria bicolor*, *Hebeloma crustuliniforme* and *Suillus tomentosus*) (Bois et al. 2006). However, no such study has been carried out on desert truffle fungi until now.

T. claveryi and *P. lefebvrei* present very slow mycelium growth in vitro. Although these fungi are well adapted to semiarid conditions, severe drought negatively affects truffle formation (Morte et al. 2008). Both fungal species are able to establish an endo-, ectendo- or ecto-mycorrhiza with the same host plant, *Helianthemum almeriense* Pau, depending on the culture conditions and phosphorus availability in the medium (Gutiérrez et al. 2003). The importance of phosphorus in the biological cycle of *T. claveryi* led us to characterise and localise the enzymes involved in the metabolism of this macronutrient during the different stages of its life cycle (Navarro-Ródenas et al. 2009). Phosphatase activity has been used as a general biochemical indicator in the measurement of biological activity, since it is present in many soil organisms and responds to adverse conditions like pollution, soil degradation, drought and phosphorus limitation (e.g. Kuperman and Carreiro 1997; Trasar-Cepeda et al. 1998; Ming and Hui 1999; Van Aarle and Plassard 2010). In fungi, phosphatase may be used as a general indicator of metabolic activity and, as such, it has been used extensively in connection with vital staining of intraradical (e.g. Tisserant et al. 1993; Larsen et al. 1996) and extraradical (Zhao et al. 1997; Vosatka and Dodd 1998) arbuscular mycorrhizal hyphae.

Both species *T. claveryi* and *P. lefebvrei* produce ascocarps in spring and share ecology, although the fruiting period of *P. lefebvrei* precedes that of *T. claveryi,* by several weeks. The productivity of *P. lefebvrei* is greater than that of any other species of the *Terfezia* genus, which permits it to occupy a wider area. Nonetheless, the accumulation of soil in its peridium and its small size lowers the commercial value of this fungus although it is more tasteful than *T. claveryi* which makes it also much appreciated (Morte et al. 2009). Both fungi are good candidates for desert truffle production, however, only *T. claveryi* has been successfully cultivated since 1999 (Morte et al. 2008, 2009).

The objective of this work is to examine the ability of two strains corresponding to two species of desert truffle to grow in pure culture with imposed water stress. Moreover, we study whether the alkaline phosphatase activity of the mycelium responds to water stress and could be used as an indicator of its biological activity. Such information may be useful in selecting specific fungi for mycelium inoculum production and for the inoculation of seedlings destined for outplanting to dry sites in order to increase the numbers of areas where desert truffles could be cultivated.

Material and methods

Fungal growth in pure culture

Mycelia from both fungi were isolated from ascocarps collected in the southeast of Spain (Zarzadilla de Totana, Lorca, Murcia), under *H. almeriense* as host plant, in April 2007. Both mycelium cultures were maintained at 23 °C on modified Melin-Norkrans medium (MMN; Marx 1969) without malt extract, at pH 7.0 and solidified with 7 g/l Panreac agar. Isolates TcS2 (*T. claveryi*) and OL2 (*P. lefebvrei*) were selected from our fungal collection because they were the only strains which were able to grow after subculture. Uniform inoculum plugs (5 mm) were taken from the colony edge for the screening test.

MMN growth medium was modified to achieve the water stress conditions. Drought stress was induced using 0, 10, 15, 20, 25, 27 and 30% polyethylene glycol (PEG-6000, Fluka) to adjust the water potential of the medium (Coleman et al. 1989). The final water potentials determined with a Wescor 5500 osmometer were −0.16, −0.45, −0.72, −1.07, −1.52, −1.68 and −1.96 MPa, respectively. As PEG reduces agar solidification, fungal isolates were grown in liquid medium. To prevent anoxic conditions (Mexal et al. 1975), colonies were grown in Petri dishes (60 mm) on cellophane filters (previously treated with 1 mM EDTA for 1 h at 100 °C, washed with milliQ-water and autoclaved) saturated with 4 ml of liquid medium but placed on autoclaved 3 mm-glass beads (Sigma) to avoid submersion. After inoculation, the Petri dishes were sealed with Parafilm and maintained in the dark at 23 °C for 9 weeks. Each isolate in each treatment was replicated six times.

Growth determination

Colony diameters were measured weekly to the nearest millimetre under a stereomicroscope. The average of two diameter measurements along perpendicular axes was used to estimate colony size at weekly intervals. Diameter measurements were collected until growth in the control ceased; then, fungal fresh weight was measured and the mycelia were frozen at −20 °C. Average growth rates were determined as the average increase in diameter divided by the total number of days in culture (63 days). Further examination of the growth curve also allowed evaluation of the effect of water stress on growth initiation.

Determination of mycelium alkaline phosphatase (ALP) activity

Firstly, in order to select the best conditions to measure ALP activity in *T. claveryi* and *P. lefebvrei* mycelia, the effect of

pH and of substrate concentration on this activity were studied. These characterization assays were carried out using mycelium grown in the control treatment (without PEG) and on MMN agar medium with the cellophane filters for 9 weeks. At harvest, mycelial fresh weight was measured and the mycelia were frozen at −20 °C until use. For the enzyme extraction, mycelia were homogenized with a mortar and pestle at 4 °C after suspension in 50 mM Tris-HCl buffer pH 7.0 in a ratio of 1:10 (w/v). The homogenate was then centrifuged at 10000 g for 10 min (Navarro-Ródenas et al. 2009). This supernatant was called the soluble extract. The pellet was resuspended in the initial volume of 50 mM Tris-HCl pH 7.0 supplemented with 0.5 M NaCl and incubated overnight at 4 °C to extract the cell wall protein. The homogenate was then centrifuged at 10000 *g* for 10 min. This supernatant was called the cell wall extract.

The effect of pH on ALP activities was determined by adding 50 µl of enzymatic extract and 50 mM *p*-nitrophenyl phosphate (*p*-NPP, Fluka) to the following buffers: 0.1 M Tris-HCl (pH 7.0 to pH 10.0) and 0.1 M carbonate buffer (pH 10.0 to 11.0), final volume 1 ml. Because the absorbance of *p*-NP depends on the pH of the reaction medium, the molar extinction coefficients of *p*-NP at 410 nm, previously calculated by Dreyer et al. (2008) at different pHs, were used. The increase in absorbance was followed at 410 nm using a Shimadzu UV-1700 spectrophotometer. A blank was prepared for each measurement. The relative phosphatase activity was calculated considering the maximum activity to be 100%.

The effect of substrate concentration on ALP activity was studied at the pH optimum of each enzyme, changing *p*-NPP concentration while keeping constant the volume of enzyme. K_m and V_{max} were determined by nonlinear regression fitting of the experimental data to the following equation

$$v = \frac{V \max[S]}{Km + [S]}$$

Assays were performed in triplicate. One unit of enzyme activity (UE) is defined as the amount of enzyme that releases 1 µmol of *p*-NP per minute at room temperature in a reaction medium that consisted in 50 mM *p*-NPP at the optimum pH of each enzyme. The protein content was measured according to the bicinchoninic acid method (Smith et al. 1985), using BSA as standard.

Secondly, after the enzymatic characterization, the ALP activities from the mycelium of both fungal strains in the different water stress conditions were measured according to their optimum enzymatic parameters. ALP activities were only measured in the mycelia from −0.16, −0.45, −0.72, −1.07 MPa treatments, since there was insufficient mycelium biomass from the colonies grown at −1.52, −1.68 and −1.96 MPa to make the measurements.

Statistical analyses

The effects of treatments on growth parameters were assessed by an analysis of ANOVA and treatment means were compared by least significant difference ($P \leq 0.05$) using Tukey's test. The Student's t-test ($P \leq 0.05$) was applied for ALP activity data.

Results

Growth curves for the different water stress treatments, for both fungi, showed an initial lag phase followed by an exponential growth phase and a maximum rate phase, before growth slowed and the colony finally became inactive (Fig. 1). For both fungi, the initial colony diameter of the control treatment (−0.16 MPa) exceeded that of the stress treatments; however, as time progressed, the colony diameters in the −0.45 MPa treatment for *T. claveryi* (Fig. 1a) and −0.45, −0.72 and of the −1.07 MPa treatments for *P. lefebvrei* (Fig. 1b) were greater than the control. For *T. claveryi*, significant differences were found between two groups of treatments, that of [−0.45>−0.16>−1.07 MPa] treatments with that of [−1.96>−1.52, −1.68 MPa] treat-

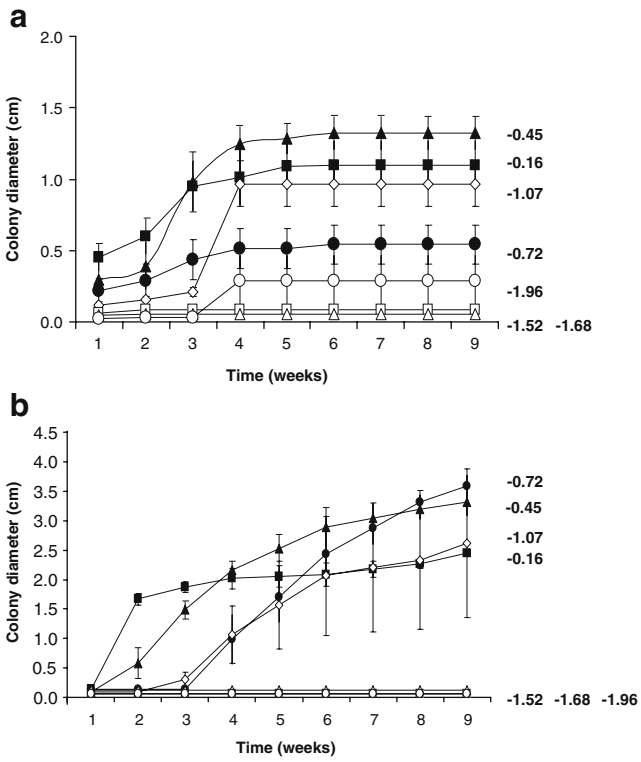

Fig. 1 Mycelial growth (cm) of *T. claveryi* (**a**) and *P. lefebvrei* (**b**) depending on time (weeks) at different water potentials (■ −0.16, ▲ −0.45, ● −0.72, ◊ −1.07, □ −1.52, Δ −1.68, ○ −1.96 MPa). Values are the mean of six replicates. Bars indicate standard error

ments (Fig. 2) and for *P. lefebvrei*, the significant differences were between [−0.45>−0.72>−1.07>−0.16 MPa] treatments and [−1.52, −1.68 and −1.96 MPa] treatments (Fig. 2).

T. claveryi mycelium grew more slowly and less than *P. lefebvrei* mycelium under the same water stress conditions (−0.45 MPa), so that, the maximum colony diameter for *T. claveryi* was 1.32 cm at week 7 while the maximum colony diameter for *P. lefebvrei* was 3.32 cm at week 9 (Fig. 1b). By the end of the stress period (week 9), *T. claveryi* at −0.45 MPa had grown 14% (1.33 cm) more than at the control treatment (1.10 cm), while *P. lefebvrei* at −0.72 MPa had grown 31% (3.58 cm) more than the control (2.45 cm), although these differences were not statistically significant (Fig. 1).

Moreover, *P. lefebvrei* mycelium grew faster than *T. claveryi* mycelium and both grew better with a moderate water stress than at the control level (Table 1).

For *T. claveryi*, growth inhibition was higher when expressed in terms of colony diameter than of colony fresh weight (Fig. 2). The same occurred in *P. lefebvrei*, except for the −0.16 and 0.45 MPa treatments (Fig. 2).

Alkaline phosphatase activity in the crude extract of *T. claveryi* mycelium pointed to a pH optimum of 11.0 for soluble alkaline phosphatase and 10.0 for cell wall-bound alkaline phosphatase, and 10.0 for both phosphatases in *P. lefebvrei* mycelium (Table 2). *T. claveryi* and *P. lefebvrei* ALP activities follow a Michaelis-Menten kinetics over wide range of substrate concentrations. The values obtained for K_m of each alkaline phosphatase are shown in Table 2.

Soluble and cell wall-bound ALP activities were higher when *T. claveryi* mycelium was grown under water stress (−0.45, −0.72, −1.07 MPa) and this increase was significantly different from to the control treatment (−0.16 MPa)

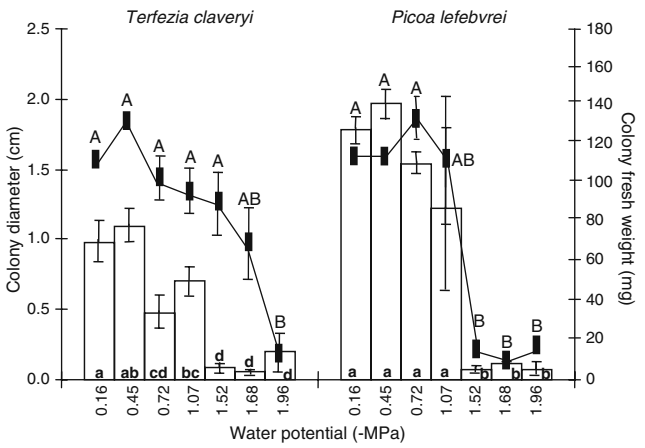

Fig. 2 Effect of water potential on the diameter (*bars, lower case*) and fresh weight (*lines, upper case*) of *T. claveryi* and *P. lefebvrei* colonies, grown on PEG-amended liquid MMN medium, after 9 weeks. Values followed by the same letter are not significantly different ($P \leq 0.05$) according to Tukey's test

Table 1 Average growth rates (μm/day) of the *T. claveryi* and *P. lefebvrei* mycelia grown on PEG-amended liquid MMN media with different water potentials, at 23 °C for 9 weeks. Values are the average of six replicates (± standard error)

Water potential (MPa)	Growth rate (μm/day)	
	Terfezia claveryi	*Picoa lefebvrei*
−0.16	174±26	389±89
−0.45	210±18	526±46
−0.72	86±21	568±78
−1.07	153±25	415±34
−1.52	13±5	10±5
−1.68	10±3	18±5
−1.96	46±27	10±6

at −1.07 MPa and −0.45 MPa, for soluble and cell wall-bound ALPs respectively (Table 3). The same occurred for the cell wall-bound ALP of *P. lefebvrei*, which showed a maximum and significant increase of 100% activity at −0.72 MPa with respect to the control treatment. However, the *P. lefebvrei* soluble ALP activity had the highest values at −0.16 MPa (control treatment) and −0.72 MPa, while this activity decreased significantly by 47% at −0.45 MPa and by 52% at −1.07 MPa (Table 3).

Discussion

According to Coleman et al. (1989), the mycelial growth response of ectomycorrhizal fungi to imposed water stress shows three different patterns, referred to as types I, II and III. In the type I pattern, growth occurred only in the control treatment level and no growth occurrs with increasing stress. In the type II pattern, growth rates decrease with increasing stress and the maximum growth rate always occur in the control. In the type III pattern, maximum growth rate do not occur in the control but at some greater stress level, followed by decreasing growth with further increases in stress. Both isolates of the two desert truffle species studied, *T. claveryi* and *P. lefebvrei*, exhibited the type III pattern characteristic of drought tolerant species like *Cenococcum geophylum* (Coleman et al. 1989) and *Rhizopogon roseolus* (Duñabeitia et al. 2004). However, *T. claveryi* and *P. lefebvrei* were only tolerant of moderate water stress below −1.07 MPa, similar to *Rhizopogon luteolus* (Duñabeitia et al. 2004) but they did not tolerate severe stress as other mycorrhizal ascomycetes fungi did (Bois et al. 2006). Coleman et al. (1989) defined drought tolerance when growth was possible at −2.3 MPa and if the growth rate as a percent of the control exceeded 30%. Using these criteria, the same authors selected one isolate of *Boletus edulis*, four isolates of *C. geophylum*, two isolates

Table 2 pH optimum of the soluble and cell wall-bound alkaline phosphatase of *T. claveryi* and *P. lefebvrei* mycelia. The Km for each enzyme was calculated at its pH optimum using *p*-NPP as substrate. Values are the average of six replicates (± standard error)

Enzyme parameters	*Terfezia claveryi*		*Picoa lefebvrei*	
	Soluble	Cell wall-bound	Soluble	Cell wall-bound
pH	11.0	10.0	10.0	10.0
Km (mM)	6.7±0.7	2.5±0.4	16.0±2.2	12.0±3.5

of *Suillus granulatus* and two isolates of *S. luteus*. In addition, six isolates of an anamorphic ericoid mycorrhizal fungal endophyte from an *Ericaceae* host have been reported as being drought tolerant at −2.24 MPa (Chen et al. 2003).

A relationship between fungal fresh weight and hyphal extension (diameter growth) could not be clearly established neither for *T. claveryi* nor *P. lefebvrei* (Fig. 2). This observation agrees with those of Coleman et al. (1989) who reported that the relationship between fungal dry weight and hyphal extension may not be consistent for each species and can vary depending upon growth conditions. In our case, the lack of a clear correlation between the colony fresh weight and the colony diameter in *T. claveryi* mycelium at −1.52, −1.68 and −1.96 MPa can be explained admitting that, under these conditions, the mycelium is growing more in thickness than in length (Fig. 2). This increase in the colony density with increased stress explains the type III pattern observed for *T. claveryi* (Coleman et al. 1989).

Alternatively, with the difficulty of removing PEG from the fungal tissue, inadequate rinsing could create an artificially high fresh value under stress (Mexal and Reid 1973; Griffin 1978; Coleman et al. 1989).

T. claveryi and *P. lefebvrei* grown under stress undergo an extended lag phase before entering the exponential growth phase (Fig. 1). Estimating growth rates over a shorter period (1–3 weeks) favoured the control, which rapidly entered exponential growth. Estimating growth rates over longer periods (6–7 weeks) favoured the stress treatments because fungi under stress have time to acclimate while growth in the control has ceased. These results agree with those obtained by Coleman et al. (1989) for other ectomycorrhizal fungi. Moreover, these authors suggested examining the growth curve and using the maximum growth rates rather than estimates of growth based on weight or diameter increases during a specific period. Tolerance to water stress may result from the ability of the fungus to adjust osmotically during stress. The extension of the lag phase with increasing water stress may represent a period of osmotic adjustment (Coleman et al. 1989).

Coleman et al. (1989) reported that drought tolerance depends more on fungal species than on annual precipitation at the site of collection. *P. lefebvrei* isolate was slightly more drought tolerant than *T. claveryi* isolate with the same annual precipitation (275 mm in Lorca, Murcia). This behaviour could explain why *P. lefebvrei* occupies a wider area and why its productivity is much greater than that of any of the other species of the *Terfezia* genus in Murcia province. Therefore, annual precipitation is very irregular and do not reflect the dry conditions supported in some locations during a long time to which a fungus from that location may be adapted (Coleman et al. 1989).

In general, except for soluble ALP activity of *P. lefebvrei*, the increased ALP activity observed in both fungi at moderate water stress with respect to the control (Table 3) indicated the functional adaptation of these mycelia to these drought conditions, in which they were able to use the phosphorus from the medium, which is more insoluble as

Table 3 Soluble and cell wall-bound alkaline phospahatase activity (expressed in U.E./ mg protein), of the mycelia of *T. claveryi* and *P. lefebvrei* grown with different water potentials (-MPa). Values are the average of six replicates (± standard error). Values followed by the same letter are not significantly different ($P \leq 0.05$) according to Student's t-test

Water potential (-MPa)	*Terfezia claveryi*		*Picoa lefebvrei*	
	Soluble	Cell wall-bound	Soluble	Cell wall-bound
0.16	0.036±0.01 a	0.062±0.02 a	0.095±0.04 a	0.150±0.03 a
0.45	0.059±0.01 ab	0.093±0.03 b	0.045±0.01 b	0.221±0.08 a
0.72	0.056±0.01 ab	0.072±0.01 a	0.088±0.02 a	0.314±0.13 b
1.07	0.061±0.01 b	0.091±0.00 ab	0.050±0.01 b	0.267±0.08 a

water stress increases. ALP activity has been used as an indicator of the metabolic activity of arbuscular mycorrhizal fungi (Tisserant et al. 1993, 1996; Guillemin et al. 1995). Furthermore, the hyphae of arbuscular mycorrhizal fungi with ALP activity had a positive and strong effect on plant host growth and its drought-resistance (Ming and Huy 1999). In arid and semiarid soils, the hydrolysis of organic phosphorus is predominantly mediated by the activity of fungal enzymes (Yadav and Tarafdar 2003). *T. claveryi* ascocarps have 2.8-times higher ALP activity than the acid phosphatase (ACP) activity (Navarro-Ródenas et al. 2009). As ACP activity was not detected in *T. claveryi* mycelium (unpublished data), the ALP activity can also be considered an indicator of the metabolic activity in these desert truffles. Moreover, the soluble ALP activity of *T. claveryi* ascocarp had the same magnitude (0.06 U.E. / mg protein) (Navarro-Ródenas et al. 2009) as the soluble ALP activity of *T. claveryi* mycelium under water stress.

Mycorrhizal symbiosis can increase drought resistance of the host, improving the plant water status as a result of an indirect effect of improved phosphorus nutrition in mycorrhizal plants (Augé et al. 1986; Fitter 1988) and/or an improvement of water uptake in mycorrhizal root systems by the extraradical phase (Duddridge et al. 1980; Ruiz-Lozano and Azcón 1995; Sánchez-Blanco et al. 2004) among other factors. It is not clear whether the drought tolerance of fungi in pure culture is transmitted to associated host plants. Parke et al. (1983) found no relationship between pure culture experiments and seedling experiments. The higher ALP activity in the water stressed *T. claveryi* mycelium, measured in this study, could be related with P accumulation in the drought-stressed mycorrhizal host plant (Morte et al. 2000). Reduced negative effects of drought stress on *H. almeriense* by the desert truffle *T. claveryi* were attributed to specific physiological and nutritional mechanisms, suggesting that this mycorrhizal symbiosis aids adaptation to arid climates (Morte et al. 2000). A positive relationship between pure culture experiments and seedling experiments was observed between radiate pine seedlings and *Rhizopogon roseolus* (Duñabeitia et al. 2004 and Ortega et al. 2004, respectively). In those studies, inoculation of radiate pine seedlings with *R. roseolus* increased dehydratation tolerance (Ortega et al. 2004). Of the three tested fungal species, *R. roseolus* was the most effective; this species was also the most adaptable and showed the greatest range of tolerance to adverse environmental conditions in pure culture (Duñabeitia et al. 2004).

The increased demand for these mycorrhizal plants for cultivation has created the need to improve mycelium inoculum production by liquid fermentation (Morte et al 2008) and to search for new strains of desert truffles which transmit their drought tolerance to their associated host plants. In this sense, to grow TcS2 and OL2 mycelia under a slight water stress (−0.45 MPa) could improve the production of mycelial inoculum in a bioreactor. Moreover, *P. lefebvrei* could be a good candidate for further desert truffle mycorrhizal plant cultivation programmes in semiarid Mediterranean areas, where the cultivation of desert truffle could contribute to improving the rural development (Morte et al. 2009).

Acknowledgements This work was supported by the projects CGL2007-61175/BOS and BIO2007-62510 (Ministerio de Educación y Ciencia, MEC, Spain) and 08812/PI/08 (Fundación Séneca, Región de Murcia, Spain). A. Navarro-Ródenas holds a FPU grant from the Spanish Ministry of Education (MEC).

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