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ORIGINAL PAPER

The role of phosphorus in the *ectendomycorrhiza continuum* of desert truffle mycorrhizal plants

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Abstract The influence of inorganic and organic phosphorus (P) and the absence of P in the culture medium on the type of mycorrhizal colonization formed (ecto-, ectendo-, or endomycorrhiza) during *Helianthemum almeriense x Terfezia claveryi* symbiosis in in vitro conditions was analyzed. This is the first time that the relative proportions of the different mycorrhizal types in mycorrhizal roots of *H. almeriense* have been quantified and statistically analyzed. The relative proportions of the mycorrhizal types depended on the P source in the medium, suggesting that it is the organic P form that induces the formation of intracellular colonization. The above association should be considered as a *continuum* between intra- and intercellular colonizations, the most appropriate term for defining it being ectendomycorrhiza. The influence of the endogenous concentration of P on plant growth was also analyzed. P translocation was observed from shoot to roots, especially in mycorrhizal plants because mycorrhizal roots showed higher growth than non-mycorrhizal roots and/or because of an extra P demand from mycelium inside the roots. Soluble and cell wall acid phosphatases activities from *H. almeriense* roots were kinetically characterized at optimum pH (5.0), using *p*-nitrophenyl phosphate as substrate, with K_m values of 3.4 and 1.8 mM, respectively. Moreover, the plant acid phosphatase and fungal alkaline phosphatases activities were histochemically localised in mycorrhizal *H. almeriense* roots by fluorescence with enzyme-labelled fluorescence substrate.

Keywords Ectendomycorrhiza · Inorganic phosphorous · Phytate · Acid phosphatase · Alkaline phosphatase · Desert truffle

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Introduction

The genus *Terfezia* belongs to the so-called "desert truffles" which are mycorrhizal hypogeous fungi mostly distributed in semiarid lands of Mediterranean countries. The most important desert truffles are those included in the genera *Terfezia* and *Picoa* because of their highly appreciated edible and commercial value (Murcia et al. 2002). Their ecological interest is derived from their position in semi-arid ecosystems as symbiotic mycorrhizal fungi associated with annual and perennial species of the genera *Cistus* and *Helianthemum*. The introduction of desert truffle cultivation into dry environments is a useful way of exploiting lands which have been regarded as unproductive (Morte et al. 2000). In addition, the host plants are xerophytic species characteristic of semiarid environments and their plantation could contribute to preserving lands from the ravages of erosion (Morte et al. 2000, 2008).

Alsheikh (1984) used the term "helianthemoid mycorrhiza" for the first time to describe the mycorrhiza formed by different *Helianthemum* species with desert truffle. This author distinguished this mycorrhiza from ecto-, ectendo-, and endomycorrhiza and its similarity with some features of ericoid mycorrhizae led him to propose the term "helianthemoid" mycorrhiza. In the mycorrhiza formed in *Helianthemum*

guttatum with different desert truffles Fortas and Chevalier (1992) showed, for the first time, that the type of mycorrhiza formed depended on the phosphorus (P) content of the medium in different culture conditions. These authors observed an endomycorrhiza, together with ectomycorrhiza in the same root, in a P-poor medium and only ectomycorrhiza in a P-rich medium. Roth-Bejerano et al. (1990) and Kagan-Zur et al. (1994) showed that mycorrhization between *Helianthemum sessifolium* and *Terfezia leonis* was inhibited at low phosphate concentration, and enhanced by low iron in the growth medium. Gutiérrez et al. (2003) described the association formed by *Terfezia claveryi* with *Helianthemum almeriense* as an endomycorrhiza in natural field conditions, an ecto- and ectendomycorrhiza without a sheath in pot cultures, and an ectomycorrhiza with a characteristic sheath and Hartig net in in vitro cultures. These authors proposed that the differences probably depended, not only on the culture conditions but also on the P content of the medium. Kovács et al. (2003) observed an increase in the fungal colonization of roots at high inorganic phosphate levels in the in vitro association of *Terfezia terfezioides* with two host plants, suggesting the term "terfezioid" to describe the presence of intra- and intercellular hyphae in the same root. Using transformed roots of *Cistus incanus* inoculated with mycelium of *Terfezia boudieri*, Zaretsky et al. (2006) proposed that the resulting mycorrhizal type is influenced not only by the fungal isolate but also by the differences in indolacetic sensitivity within the host plant, which may be related to interdependency between exogenous phosphate and auxin levels.

In the living cell, P is involved in important cellular functions such as the signalling pathways or energetic processes. Phosphorus is absorbed by plant roots mainly as inorganic orthophosphate, which is extremely insoluble in most soils and a limiting macronutrient for plants and microorganisms. Molecules containing the organic form of phosphorus, such as phytate, are abundant in the soil, but they must be mineralized to phosphate before they can be assimilated by plants (Schachtman et al. 1998). Different types of phosphatases are able to increase the rate of hydrolysis of organic P to inorganic P, thus making it available to the plant. Phosphatases can be synthesized by plant roots which mainly produce acid phosphatases (ACP) (Ridge and Rovira 1971; Shaykh and Roberts 1974; Dinkelaker and Marschner 1992) and by fungi and bacteria which produce acid and alkaline phosphatase (ALP) (Dighton 1983; Tarafdar and Claassen 1988; Joner et al. 2000; Navarro-Ródenas et al. 2009, 2011). In soil, the hydrolysis of organic P is predominantly mediated by the activity of fungal enzymes (Tarafdar and Marschner 1995; Tarafdar et al. 2001, 2002; Yadav and Tarafdar 2003; Pandey et al. 2008). Secretion of acid phosphatase to the rhizosphere has been observed in many plants grown under P-deficient conditions and is considered to be involved in the mineralization of organic P (Duff et al. 1994).

In this paper, the influence of inorganic or organic P and the absence of P on the type of colonization (endo-, ectendo, or ectomycorrhiza) formed in the *H. almeriense x T. claveryi* symbiosis is analyzed. The role of phosphatases and their origin (from plant or fungus) in the efficient use of P in this mycorrhizal symbiosis are also discussed.

Materials and methods

Reagents

Sodium orthovanadate, phytate acid sodium, ethylenediaminetetraacetic acid (EDTA), acid fuchsin and *p*-nitrophenol (*p*-NP) were purchased from Sigma (Madrid, Spain). 4-nitrophenyl phosphate (*p*-NPP) was from Fluka (Madrid, Spain). "ELF®-97 Endogenous Phosphatase Detection Kit" from Molecular Probes (Leiden, The Netherlands). Potato dextrose agar (PDA) was from Biokar Diagnostics. PELCO CryO-Z-T was from Ted Peya, Inc (CA, USA). The remaining reagents were of analytical grade.

Fungal material

T. claveryi mycelium, isolated from ascocarps collected in Zarzadilla de Totana (Murcia, Spain) under *H. almeriense* plants, was routinely grown on PDA medium at pH 7.0. For purposes of enzyme extraction, mycelium was grown on the same medium, covered with cellophane previously boiled for 1 h in 1 mM EDTA, washed in distilled water and autoclaved prior to use. Mycelium was grown for 2 months, frozen in liquid nitrogen and stored at −20°C.

Experimental design and plant material

Three treatments were carried out according to the P source and concentration:

- P_i treatment: MH medium with 42.5 mg L^{-1} PO_4H_2K as inorganic P source (Morte and Honrubia 1994, 1995) which is easily absorbed by plants. Plants from this treatment were called P_i plants.
- Phy treatment: MH medium with inositol hexaphosphate (phytate) instead of inorganic P. The molar concentration of P was the same as in the MH medium (34.4 mg L^{-1} phytate) but needs to be hydrolysed before being absorbed by plants. Plants from this treatment were called Phy plants.
- $P_Ø$ treatment: MH medium without any source of P. Plants from this treatment were called $P_Ø$ plants.

All treatments were made in 3 g of vermiculite saturated with 20-ml liquid medium, in individual tubes of 20 cm in length and 2.2 cm in diameter. The tubes were covered with their own lid and sterilized by steaming at 120°C for 20 min.

For each treatment, 48 plants were used, of which 24 were not inoculated and 24 were inoculated with mycelium of *T. claveryi*. Since *T. claveryi* has very slow mycelium growth in vitro, mycelium inoculation into the tube was made introducing two plug of agar bearing mycelium 2 months before introducing the plant. The tubes were sealed with Parafilm and incubated in the dark at 23°C, according to the method described by Morte et al. (2008). After these 2 months, micropropagated *H. almeriense* plantlets from MS medium (Murashige and Skoog 1962) were transplanted to inoculated and non-inoculated tubes. All plants were grown in a growth chamber at 25°C with a 16/8 h photoperiod at 100 μmol m^{-2} s^{-1}. After 12 weeks of treatment, the plants were harvested. The vermiculite was removed from the roots. The roots and shoots were weighed, frozen in liquid nitrogen and stored at −20°C. In addition, 15 initial plantlets from MS medium (MS plants) were also harvested and frozen as a control of the initial plantlet status. MS medium contains 170 mg L^{-1} PO_4H_2K. MS plants were included as control of the nutritional status of plantlets prior to transplanting.

Estimation of phosphorus content

Six plants randomly selected from each treatment and six MS plants (whole shoots and roots) were oven-dried overnight at 70°C and the dry weights were recorded. Samples were ground and dry-ashed in a muffle oven at 300°C for 3 h and at 550°C for 5 h (Feng et al. 2003). The ash was dissolved using 2 N HCl. The phosphorus content was determined by the molybdovanadophosphate method (Kitson and Mellon 1944) with PO_4H_2K as standard and expressed as micrograms of P per whole shoot, root or plant or in percent of dry weight.

Enzyme extraction from roots of *H. almeriense* and mycelium of *T. claveryi*

Nine whole roots of *H. almeriense* randomly selected from each treatment or mycelium of *T. claveryi* were homogenized in a mortar at 4°C after suspension in 50 mM Tris–HCl buffer pH 7.0 at a ratio of 1:10 (*w*/*v*). The homogenate was then centrifuged at 10,000×*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 enzyme (Saroop et al. 1999). The homogenate was then centrifuged at 10,000×*g* for 10 min. This supernatant was called the cell wall extract.

Determination of ALP and ACP activities

The standard reaction medium to measure ALP activity consisted of 50 μL of enzymatic extract and 50 mM *p*-NPP in 0.1 M Tris–HCl buffer pH 10.0, final volume 1 mL. The reaction was started by adding the substrate and the increase in absorbance at 410 nm was followed up for 3 min with a Shimadzu UV-1700 spectrophotometer. A blank without enzyme was prepared for each measurement.

ACP activity was determined using the end-point method, consisting of incubating 410 μL of enzymatic extract with 50 mM *p*-NPP in 0.1 M citric-citrate buffer pH 5.5, final volume 4.1 mL. At different times (1, 3, 5, and 7 min), 0.8 mL aliquots were withdraw from the reaction medium and placed in a tube with 200 μL of 2.5 M NaOH; the final pH of these assays was 13.1. The absorbance at 410 nm was measured and represented against time. The initial reaction rate was calculated from the linear part of these graphs using a molar extinction coefficient for *p*-NP of 17,750 M^{-1} cm^{-1} (Dreyer et al. 2008). A blank without enzyme was prepared following the same procedure.

The activity in each root sample was measured in triplicate. One unit of enzyme activity (U) is defined as the amount of enzyme that releases 1 μmol of *p*-NP per minute at room temperature in the standard reaction medium.

Effect of pH on phosphatase activity

The effect of pH on ACP and ALP activities was determined using the standard reaction medium previously described and the following buffers: for an acid range, 0.1 M citric-citrate buffer (pH 4.0 to 7.0) and for an alkaline range 0.1 M Tris–HCl (pH 7.0 to 10.0) and 0.1 M carbonate buffer (pH 10.0 to 11.0). 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 pH values, were used. The relative phosphatase activity was calculated considering the maximum activity to be 100%.

Mycorrhizal characterization

The whole frozen roots of five mycorrhizal plants randomly selected from each phosphorous treatment were included in PELCO CryO-Z-T, an embedding matrix for cryostat sectioning. Ten-micrometer-thick sections were obtained using a Cryostat (Leica CM 3050S) and stained with

an acid fuchsin solution (0.01% acid fuchsin in acetic acid, ethylene glycol and lactic acid, 1:1:1, *v*/*v*/*v*). All root sections were observed under an Olympus BH2 microscope and classified into one of the following mycorrhizal types: "ectomycorrhiza" when intercellular hyphae and/or a well-developed sheath were observed, "ectendomycorrhiza" when intracellular hyphae together with intercellular hypahe were observed, "endomycorrhiza" when only intracellular hyphae were observed (Gutiérrez et al. 2003). In this study, the term "endomycorrhiza" is referred solely to intracellular fungal colonization and no sheath or mantle but not to a specific type of mycorrhiza already described (arbuscular mycorrhizas or orchid mycorrhizas). Colonization percentage was calculated as the number of total colonized root sections divided by the number of total observed sections (around 650 per plant) and multiplied by 100. Colonization percentage of each mycorrhizal type was calculated as the number of colonized sections by each type divided by the total number of colonized roots sections and multiplied by 100.

Histochemical localisation

The detection of phosphatase activity within mycorrhizal root sections was carried out using the "ELF®-97 Endogenous Phosphatase Detection Kit". The ELF substrate was diluted 1:20 with the alkaline detection buffer (pH 8) to detect ALP activity, or with 0.1 M citric-citrate buffer pH 5.0 (acid detection buffer) to detect ACP activity. These ELF-solutions were shaken and then filtered through a 0.22-μm filter (Millipore, Bedford, USA). Root sections were incubated with 20 μL of acid-ELF-solution or alkaline-ELF-solution. Control sections were incubated only with alkaline or acid detection buffer only or with ELF-solution containing 100 mM orthovanadate as ALP inhibitor or 100 mM tartrate as ACP inhibitor (Wannet et al. 2000; Navarro-Ródenas et al. 2009). In both cases, sections were incubated for 30 min at room temperature in the dark and then washed with 30 mM Tris–HCl pH 8.0 (van Aarle et al. 2007). All root sections were observed using a Leica Leitz DMRD epifluorescence microscope fitted with a mercury lamp (Leica, Wetzlar, Germany) setting with a UV filter cube formed by Ex330-380, Di400, and Ba435.

Statistical analyses

Data were subjected to ANOVA or two-way ANOVA analyses in a factorial design of three applied P sources × two mycorrhizal colonization treatments. Natural logarithm transformation of the data was carried out to fulfil the requisites for parametric analyses when it was necessary. Data of the percentage of colonization and mycorrhizal type were subjected to Chi-square analysis. Statistical analyses were carried out using the software package SPSS (version 15).

Results

Mycorrhizal colonization

The lowest percentage of mycorrhizal colonization was observed when plants were provided with inorganic phosphorus (P_i; 19.3%) as P source, while plants that grew without P ($P_Ø$) presented the highest colonization percentage (30.3%). Plants grown with phytate (Phy) showed an intermediate colonization ratio (26.7%). All these percentages were significantly different among them (Table 1).

The three mycorrhizal types defined in Materials and Methods were found in the roots of the same plant for all the three P treatments, where a wide range of intra- and/or intercellular hyphal amount was observed. The predominant type of mycorrhiza in all P treatments was ectomycorrhizal, while the endomycorrhizal type was the least frequent. Plants from the $P_Ø$ treatment presented significantly both the lowest endo- and the largest ectomycorrhizal percentages. The significant lowest ectomycorrhizal percentage was found in plants from the Phy treatment, in which the highest ectendo- and endomycorrhizal percentages were quantified. Fungal colonization in plants from the P_i treatment showed intermediate values between that of the Phy and $P_Ø$ treatments (Table 1). In our assay, no special morphological features were observed in *H. almeriense* mycorrhizae.

Table 1 Percentages of total colonization and different types of mycorrhizal colonization in *Helianthemum almeriense* plants grown in vitro with different P treatments

P treatment	Colonization (%)	Endomycorrhiza (%)	Ectendomycorrhiza (%)	Ectomycorrhiza (%)
KH_2PO_4 (P_i)	19.3±1.2 a	19.6±2.9 a	23.8±3.1 a	56.6±3.6 a
Phytate (Phy)	26.7±1.7 b	20.2±3.0 a	33.6±3.5 b	46.2±3.7 b
Without P ($P_Ø$)	30.3±1.7 c	9.3±1.9 b	23.2±2.8 a	67.4±3.1 c

Different letters in the same column mean significant differences ($P<0.001$) by Chi-square analysis. Percentage ± confidence interval at 95%

Effect of *T. claveryi* inoculation on the in vitro growth of *H. almeriense* with different P sources

In all P treatment, total dry matter values were higher in mycorrhizal plants than in non-mycorrhizal plants. This effect was especially pronounced in roots, where mycorrhization induced an increase in growth regardless of the source of P (Table 2).

A significant effect of the P source on the shoot growth was also detected. Both Phy and P_i treatments reduced the shoot growth compared with $P_Ø$ (Table 2). No significant differences were found in the shoot/root ratio for any P treatments (Table 2). Moreover, compared with *MS* plants, all the P treatments plants not only increased biomass (Table 2) but led to taller plants and the development of new leaves (data not shown).

Plant P uptake

The P content of shoot was not affected by mycorrhizal status or by the P source significantly, while the shoots of MS plants contained around double the P of the treated plants (Table 3). However, root P content in all treatments increased compared with MS plants, especially in mycorrhizal plants with significant differences. The whole plant (shoot + root) P content in all treatments was not significantly different from that of MS plants, with the exception of non-mycorrhizal P_i plants (Table 3).

Phosphatase activity

The measurement of phosphatase activity both in the soluble and in the cell wall extracts from non-mycorrhizal roots revealed the presence of ACP activity with a similar pH optimum of around pH 5.0 (Fig. 1a, b).

Soluble and cell wall bound ACP follow a Michaelis–Menten kinetics. K_m was determined by non-linear regression fitting of the experimental data to the Michaelis–Menten equation. Using *p*-NPP as substrate at pH 5.0 the values obtained for soluble and cell wall bound ACP activities were 3.4 and 1.8 mM, respectively.

For *T. claveryi* mycelium, only an optimum of phosphatase activity was detected in soluble extract (around pH 11) (Fig. 1a) and in cell wall extract (around pH 10) (Fig. 1b).

Phosphatase activity was also assayed in mycorrhizal and non-mycorrhizal roots of *H. almeriense* (Table 4). ACP (soluble and cell wall bound) was the only activity detected in non-mycorrhizal roots. However, both, soluble and cell wall bound, ALP and ACP were present in mycorrhizal roots. The results presented in Table 4 indicate that, except in the case of cell wall bound ACP, the mycorrhizal status affects ALP and ACP in roots.

Histochemical localization of phosphatase activities

Firstly, the mycorrhizal root colonization was localized in the root sections under bright light (Fig. 2b). ALP activity, detected by ELF fluorescence in alkaline buffer, was localised only in the *T. claveryi* mycelium inside and around the roots (Fig. 2a). When mycorrhizal root sections were incubated with ELF in acid buffer, the fluorescence was detected only in root cells but never in the mycelium (Fig. 2c, d). Control sections did not show any fluorescence reaction (Figs. 2e, f). The specificity of the ELF method was demonstrated by adding orthovandate or tartrate, inhibitors of ALP and ACP activities, respectively. Incubation with

Table 2 Dry matter yield (g) of shoot, root, and plant (shoot + root) and shoot/root ratio of mycorrhizal and non-mycorrhizal *Helianthemum almeriense* plants with different in vitro P treatments

P treatment	Shoot		Root		Plant		Shoot/root	
	Non-mycorrhizal	Mycorrhizal	Non-mycorrhizal	Mycorrhizal	Non-mycorrhizal	Mycorrhizal	Non-mycorrhizal	Mycorrhizal
KH_2PO_4 (P_i)	0.022±0.005	0.028±0.005	0.029±0.003	0.038±0.008	0.050±0.007	0.065±0.012	0.8±0.1	0.8±0.1
Phytate (Phy)	0.032±0.004	0.044±0.009	0.028±0.005	0.039±0.007	0.060±0.007	0.083±0.011	1.4±0.5	1.2±0.4
Without P ($P_Ø$)	0.040±0.006	0.048±0.009	0.037±0.005	0.053±0.007	0.077±0.010	0.101±0.015	1.1±0.1	0.9±0.1
MS plants	0.019±0.002		0.0025±0.0004		0.022±0.002		9±2	
Significance due to:								
Mycorrhizal status	NS		*		*		NS	
P source	*		NS		*		NS	
Mycorrhizal status × P source	NS		NS		NS		NS	

Two-way ANOVA analysis. Values are means (n=6)±standard error. Data from MS are not subjected to statistical analysis

NS absence of significance

*$P \leq 0.05$, level of significance

Table 3 P content (in micrograms) per shoot, root, and plant (shoot + root) of MS plants and mycorrhizal and non-mycorrhizal plants with different in vitro P treatments

P treatment	Shoot		Root		Plant	
	Non-mycorrhizal	Mycorrhizal	Non-mycorrhizal	Mycorrhizal	Non-mycorrhizal	Mycorrhizal
KH_2PO_4 (P_i)	38±5 b	35±6 b	12±1 a	26±4 b	51±6 b	60±9 a, b
Phytate (Phy)	64±12 b	79±15 b	15±2 a	38±4 b	79±12 a, b	120±16 a, b
Without ($P_Ø$)	59±9 b	62±14 b	23±4 b	28±5 b	82±12 a, b	90±18 a, b
MS plants	117±8 a		11±2 a		128±7 a	

Different letters in the same tissue (shoot, root, or plant) mean significant differences ($P<0.05$) ANOVA analysis. Values are means ($n=6$)± standard error

orthovandate did not produce a fluorescence reaction for ALP activity (Fig. 2g) and only a weak fluorescence was observed with tartrate for ACP (Fig. 2h), indicating partial inhibition of this activity.

Discussion

Effect of P on plant growth

The benefits gained from mycorrhizae are traditionally recognized as improved access to limiting soil resources, most notably immobile nutrients such as P (Johnson et al. 1997). Growth responses to P are commonly assessed with regard to the element concentrations to which plants are exposed, disregarding the influence of the concentration of P accumulated in plants (Rotaru and Sinclair 2009). Thus, care should be taken to explain the effect of P in plants grown in vitro. In our conditions, plants were grown in MS medium prior to the treatments. The content of phosphate in this medium is extremely high (1.25 mM) compared with the amount detected in natural soils, which rarely exceeds 10 μM (Bieleski 1973; Abel et al. 2002). Figure 3 explains the high P concentration of our MS plants in which it makes up about 0.54% of plant dry weight compared with the average content (0.20%) observed in plants grown in soil (Bonilla et al. 2000; Stames et al. 2008) and around 3.5-fold higher than treated plants (Fig. 3).

Root development is remarkably sensitive to variations in the supply and distribution of P in the soil (Forde and Lorenzo 2001). Following its uptake, P is easily mobilised and distributed to sink tissues such as the growing roots (Bonilla et al. 2000; Bucher 2007). While there are no differences in the P content per shoot between mycorrhizal and non-mycorrhizal plants, the P content per root is significantly higher in mycorrhizal plants (Table 3). It seems that, in addition to increasing the growth of mycorrizal roots (Table 2), the presence of the fungi at this stage of development might represent an extra demand for P to the plant, which responds by translocating it from the shoot. P transport from plant roots to hyphae is not an uncommon process and a bi-directional flow of P between arbuscular mycelium and mycorrhizal roots was already described by Nielsen et al. (2002). In the conditions used in this assay, the whole plant P content in most of the treatments did not change with respect to MS plants (Table 3). This result suggests that

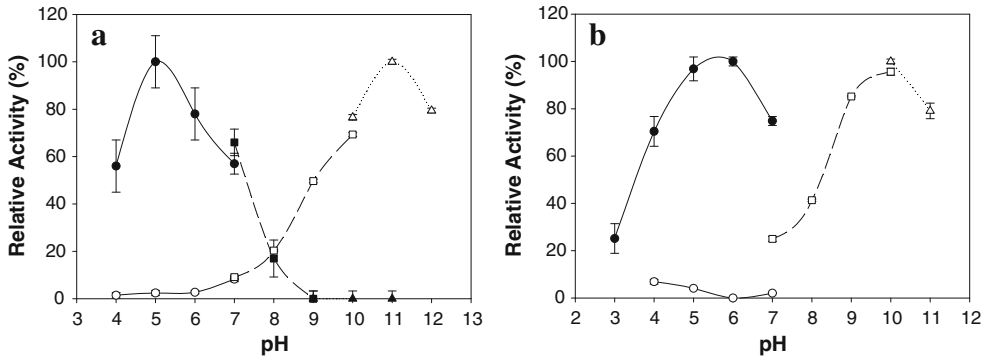

Fig. 1 Effects of pH on the relative phosphatase activity from the soluble extract (**a**) and from the cell wall bound extract (**b**). Relative activity was calculated considering, in each experiment, that the maximum activity was 100%. *Black symbols* are extracts from non-mycorrhizal roots and *empty symbols* are extracts from the fungal mycelium. *Circles* represent citrate buffer (pH 3.0–7.0), *squares* Tris–HCl buffer (pH 7.0–10.0) and *triangles* carbonate buffer (pH 10.0–12.0). Values are means ($n=3$)±standard error. Alkaline phosphatase from root cell wall-bound extract was not detected

Table 4 Phosphatase activities (U g^{-1} root fresh weight) in mycorrhizal and non-mycorrhizal *Helianthemum almeriense* plants with different in vitro P treatments

P treatment	Soluble ACP		Cell wall ACP		Soluble ALP		Cell wall ALP	
	Non-mycorrhizal	Mycorrhizal	Non-mycorrhizal	Mycorrhizal	Non-mycorrhizal	Mycorrhizal	Non-mycorrhizal	Mycorrhizal
KH_2PO_4 (P_i)	2.6±0.8	3.8±0.1	2.0±0.5	1.2±0.2	ND	0.46±0.05	ND	0.27±0.04
Phytate (Phy)	2.1±0.3	3.8±0.7	1.4±0.2	1.5±0.2	ND	0.9±0.2	ND	0.6±0.1
Without P ($P_Ø$)	1.9±0.2	3.5±0.9	1.2±0.1	1.5±0.4	ND	0.6±0.1	ND	0.4±0.1
Significance due to								
Mycorrhizal status	*		NS		–		–	
P source	NS		NS		NS		NS	
Mycorrhizal status × P source	NS		NS		–		–	

ALP data were subjected to ANOVA while ACP data, two-way ANOVA analysis. Values are means (n=9)±standard error

NS absence of significance, *ACP* acid phosphatase, *ALP* alkaline phosphatase, *ND* not detected

*$P \leq 0.01$, level of significance after natural logarithm transformation

there is no net uptake of P from the medium and plants simply redistribute the P accumulated while growing in the MS medium according to the needs of each tissue. This P redistribution become more evident if we observe that the P content in roots of non-mycorrhizal $P_Ø$ is significantly higher than that measured in MS, P_i, and Phy non-mycorrhizal plants (Table 3) and comparable to that measured in the roots of mycorrhizal plants. Since the $P_Ø$ plants did not take up P from the environment, it must have come from the shoot. It is not unexpected that the presence of a high content of P in roots in a medium without P favors their colonization by *T. claveryi*. The results in Table 1 confirm this hypothesis since the highest percentage of colonization is observed in $P_Ø$ plants. This result is in agreement with those obtained by Nielsen et al. (2002) and Harley and Smith (1983).

The influence of the P source on whole plant growth can be seen in Table 2, which indicates that the highest yields of shoot and whole plant dry matter (both mycorrhizal and non-mycorrhizal) were obtained in plants grown without inorganic P ($P_Ø$ and Phy plants). We concluded that these plants, while growing in the MS medium, stored more P than is required for normal physiological activity, and that at this stage of development (during the P treatment), their endogenous P concentration is adequate for sustaining the optimal growth of *H. almeriense* without the need to take it up from the medium. A similar effect was observed by Rotaru and Sinclair (2009) in soybean plants grown with a high P concentration. This accumulation of P also explains why a decrease in the shoot/root ratio, a specific response to P deficiency (Anghigoni and Barber 1980; Fredeen et al. 1989), was not observed even in $P_Ø$ plants (Table 2). Although P availability is rarely adequate for optimal growth, an excess of this macronutrient may cause micronutrient deficiencies, especially of iron or zinc (Bennett 1993; Rotaru and Sinclair 2009). In fact, a reduction in biomass with an excess of P in the soil has been reported (Stames et al. 2008).

Effect of P on phosphatase activity

Figure 1 clearly indicates that roots of *H. almeriense* present both soluble and cell wall bound ACP activities, but not ALP activity. Soluble ACP activity was affected by the mycorrhizal status of the plant, while cell wall bound ACP activity seemed to be constitutive and not affected by mycorrhization or by the source of P in the medium (Table 4).

Although the induction of ACP activity is a specific response for P deficiency (Baldwin et al. 2001), a decrease in ACP activity has also been observed under P deficiency (Stames et al. 2008). Neither of these responses was observed in our experiment (Table 4). The differences in ACP activity among the three P treatments were not statistically significant and this activity may also have been affected by the endogenous content of P (Stames et al. 2008). ELF labelling of mycorrhizal roots confirms that both soluble and cell wall ACP are produced only by the root cells of *H. almeriense* (Fig. 2).

When mycorrhizal root sections were incubated with ELF in alkaline buffer, soluble and cell-wall bound ALP activities were observed only in the mycelium (Fig. 2). Since ALP activity was not detected in extracts of non-mycorrhizal roots (Fig. 1), the ALP from mycorrhizal roots must correspond to the ALP from the mycelium colonizing these roots. In fact, *T. claveryi* mycelium only has ALP activity but not ACP activity. The ALP activity in arbuscular mycorrhizal fungi was used as a biochemical indicator of active hyphae in terms of P nutrition (Boddington and Dodd

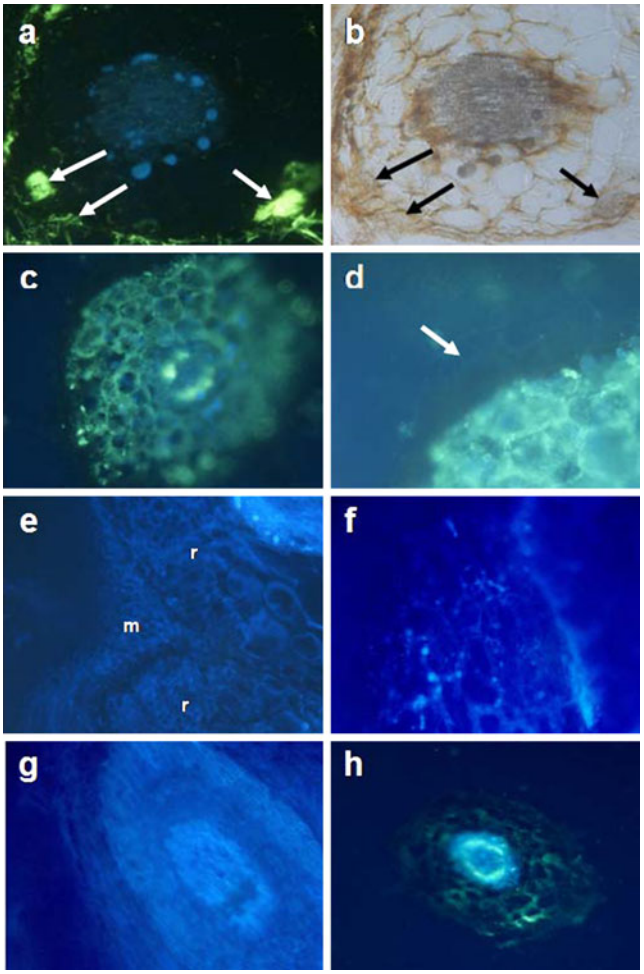

Fig. 2 Histochemical localization of phosphatase activities in *H. almeriense* mycorrhizal roots. **a** Cross section of root incubated with alkaline buffer and ELF; the fluorescence indicates the localization of ALP activity only in the mycelium inside (*arrows*) and outside the roots, ×200. **b** The same section as in a but observed under bright light, ×200. **c** Cross section of root incubated with acid buffer and ELF; the fluorescence localized the ACP activity only in root cells, ×200. **d** Detail of c showing the external mycelium (*arrow*) without fluorescence, ×400. **e** Cross section of root incubated with alkaline buffer; *r* root section, *m* mycelium, ×200. **f** Cross section of root incubated with acid buffer. **g** Cross section of root incubated with alkaline buffer, ELF, and orthovanadate, ×200. **h** Cross section of root incubated with acid buffer, ELF, and tartrate, ×200

1999). However, ALP activity has been also used as an indicator of the metabolic activity of arbuscular mycorrhizal fungi (Tisserant et al. 1993, 1996; Guillemin et al. 1995). Subsequent detailed characterization of the enzyme suggested that the ALP was involved in fungal carbon metabolism (Ezawa et al. 1999). Navarro-Ródenas et al. (2011) supposed that ALP activity can also be considered an indicator of the metabolic activity in desert truffles under stress condition such as drought. According to our results, the ALP activity in mycorrhizal roots might be involved in energetic processes since no P uptake was observed.

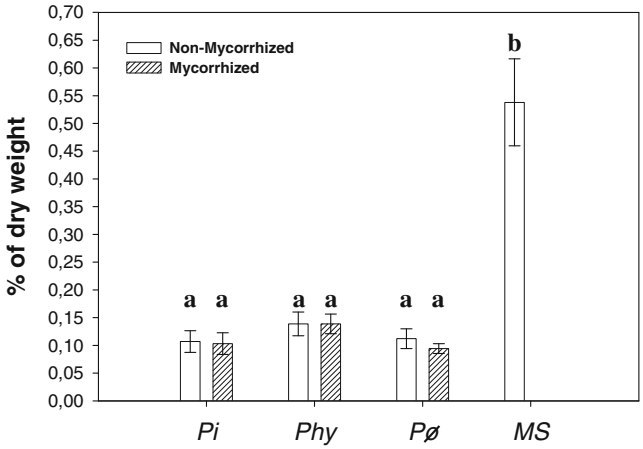

Fig. 3 P concentration (percent of dry weight) per plant (shoot + root) of *MS* plants and mycorrhizal and non-mycorrhizal plants with different in vitro P treatments. Data were subjected to ANOVA analysis. *Different letters* mean significant differences ($P<0.05$) according to Tukey's test. Values are means ($n=6$)±standard error

Influence of P in the type of mycorrhiza formed

Host plants from the genus *Helianthemum* establish mycorrhizal symbiosis with different species of dessert truffle (Dexheimer et al. 1985; Roth-Bejerano et al. 1990; Cano et al. 1991; Fortas and Chevalier 1992; Kagan-Zur et al. 1994; Gutiérrez et al. 2003; Kovács et al. 2003; Honrubia et al. 2007; Morte et al. 2008). Depending on the fertility of the substrate and, especially, on the P content (Fortas and Chevalier 1992; Gutiérrez et al. 2003), the symbiosis can be endo- or ectomycorrhizal.

Our results suggest that higher percentages of total colonization are achieved when de P source is less available for the plants since $P_{Ø}$ *plants* presented the highest percentage and P_i *plants* the lowest. Moreover, it has previously been shown that mycorrhizal formation is affected by the phosphate status of the medium (Smith and Read 2008).

In our experiment, the three mycorrhizal types were found in the same root, as previously observed by Gutiérrez et al. (2003) and Fortas and Chevalier (1992). The relative proportion of each mycorrhizal type, quantified and statistically analyzed in this paper for the first time, depended on the source of P. Plants grown in a medium with phytate, the most abundant P form in natural soils, presented a percentage of ectomycorrhizal colonization lower than plants grown with other sources of P. These results are unexpected since, according to Fortas and Chevalier (1992), the lowest percentage of ectomycorrhiza should appear in plants from the medium poorest in P ($P_{Ø}$ plants). In fact, $P_{Ø}$ plants presented the highest ectomycorrhizal percentage and the lowest endomycorrhizal percentage (Table 1). This result suggests that organic P is one of the factors that may induce the formation of intracellular colonization. Although the mycorrhizal profile in plants grown with Phy tended to be

more similar to those described in pot and field by Gutiérrez et al. (2003) (increasing their intracellular colonization), the predominant form among the three treatments remains the ectomycorrhizal type. We can conclude that although organic P may influence the main type of mycorrhiza formed, the general culture conditions (inoculation and growth) are probably more important than the P or other nutrient contents.

Alsheikh (1984) used for the first time the term "helianthemoid mycorrhiza" to describe the mycorrhiza formed by different *Helianthemum* species with desert truffle. Barea and Honrubia (2004) and Honrubia (2009) also maintained this term to describe the mycorrhizae in *Helianthemum* species. Kovács et al. (2003) suggested that the term "terfezioid" would probably more reasonable than "helianthemoid" because they found the same structures in *Robinia pseudoacacia*, *Helianthemum ovatun*, and in other *Terfezia* associations (Kagan-Zur et al. 1999) described previously. However, *Picoa lefebvrei*, another desert truffle, forms the same ectendomycorrhizal colonization as *T. claveryi* with the same host plant (Gutiérrez et al. 2003). These last authors also pointed out that there is no clear barrier between the three main types of mycorrhiza organization in *Helianthemum* species. Therefore, we think that it is more appropriate to describe the mycorrhiza formed by different *Helianthemum* species with desert truffle as an *ectendomycorrhiza*—defined by Smith and Read (2008) in the following terms: "the sheath may be reduced or absent, the Hartig net is usually well developed, but the hyphae penetrate into the cells of the plants". This definition matches the features observed in our assay where, although intra- and intercelullar hyphae are not always observed together in the same cross section, they are always present along the same root where they form an *ectendomycorrhiza continuum*.

Conclusions

The mycorrhizal association between *H. almeriense* roots and *T. claveryi* mycelium is an ectendomycorrhiza with a *continuum* range from intracellular to intercellular colonization, where the P source influences the total colonization and the relative proportion of the mycorrhizal types in in vitro conditions. Further studies in greenhouse or field conditions are needed to elucidate how these proportions change with the P source. In vitro *H. almeriense* plants did not take P from the medium but mobilized their P reserve toward the new organs, especially to mycorrhizal roots as an extra P demand from the mycelium. ACP and ALP activities were detected in mycorrhizal *H. almeriense* roots and their plant and fungal origin, respectively, were determined by ELF labelling. These activities, at least in our study conditions, might not be involved in P uptake since an increase in the same was not related to P acquisition in plants.

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