Chapter 21
Domestication: Preparation of Mycorrhizal Seedlings

Asunción Morte and Alberto Andrino

21.1 Introduction

There are many mycorrhizal fungi with a great potential as edible fungi, but only some species have been cultivated (Zambonelli and Bonito 2012). Among these fungal species, two species of desert truffle have been successfully cultivated and reported, *Terfezia claveryi* Chatin in Spain (Honrubia et al. 2001; Morte et al. 2008, 2009, 2010, 2012) and *Terfezia boudieri* Chatin in Tunisia (Slama et al. 2010) and Israel (Khagan-Zur, pers. com.).

Since the first plantation of *Terfezia* mycorrhizal plants was established in 1999 in Murcia (south-eastern Spain), the increasing demand for this crop, not only in Spain but also in other countries, has prompted research into new strategies to help the passage from experimental scale to medium-large-scale cultivation. The first step in this process is the selection and production of suitable mycorrhizal seedlings of quality and adapted to different cultivation sites. The present chapter describes our experience and the experiments carried out to improve this first step.

21.2 Host Plant Selection and Propagation

The election of a suitable host plant species is a very important factor in the production of mycorrhizal plants. The wide edaphic tolerance of *Helianthemum* as host allows them to share desert truffle symbionts (Díez et al. 2002), although bioclimatic conditions are probably the most important factor for choosing a host plant for a specific area.

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Table 21.1 Different mycorrhizal associations, obtained under controlled conditions, between species of desert truffles and different *Helianthemum* species

Fungal species	*Helianthemum* species	References
Terfezia claveryi	*H. salicifolium*	Awameh et al. (1979)
		Dexheimer et al. (1985)
	H. guttatum	Fortas and Chevalier (1992)
	H. almeriense	Morte et al. (1994, 2008)
	H. ledifolium	Gutiérrez (2001)
	H. violaceum	Morte et al. (2009)
	H. hirtum	Torrente et al. (2009)
	H. canariense	Andrino et al. (2012)
Terfezia boudieri	*H. salicifolium*	Awameh et al. (1979)
	H. sessiliflorum	Slama et al. (2010)
	H. lippii	Pers. com.
Terfezia leonis (redefined as *Terfezia boudieri)*	*H. sessiliflorum*	Roth-Bejerano et al. (1990)
Terfezia leptoderma	*H. salicifolium*	Dexheimer et al. (1985)
	H. guttatum	Fortas and Chevalier (1992)
Terfezia arenaria	*H. guttatum*	Fortas and Chevalier (1992)
Terfezia terfezioides (redefined as *Mattirolomyces terfezioides)*	*H. ovatum*	Kovács et al. (2003)
Tirmania nivea	*H. salicifolium*	Awameh et al. (1979)
	H. lippii	Pers. com.
Tirmania pinoyi	*H. salicifolium*	Awameh et al. (1979)

Among the plant families cited in the literature that contain some species which form mycorrhiza with desert truffles are *Cistaceae* (Alsheikh 1984; Awameh et al. 1979; Morte et al. 1994; Roth-Bejerano et al. 1990; Gutiérrez et al. 2003; Zaretsky et al. 2006), *Fagaceae* (Díez et al. 2002), *Pinaceae* (Díez et al. 2002; Honrubia et al. 2007), *Fabaceae* (Kovács et al. 2003) and even *Cyperaceae* (Ammarellou and Saremi 2008; Jamali and Banihashemi 2012). However, most of plant species reported as host plants for experimental desert truffle mycorrhization are perennial and annual species from *Helianthemum* genus, belonging to the Cistaceae (Table 21.1). We have chosen to employ *Helianthemum almeriense* in our experiments because we have a lot of previous information about its culture, which help us to continue improving its domestication.

21.2.1 Photoautotrophic (PA) Versus Photomixoautotrophic (PM) Cultivation Methods

Many *Helianthemum* species show erratic seed germination, and seed scarification is necessary to increase germination rates (Pérez-García and González-Benito 2006). Moreover, high mortality of the germinated seedlings is common during the first 2 months after germination in nursery conditions (Morte et al. 2012).

Micropropagation techniques have been used for plant production since they improve seed germination and plant survival (Morte and Honrubia 2009; Morte et al. 2008). The in vitro micropropagation protocols of the *Helianthemum* species studied are straightforward and rapid because plant multiplication, elongation and rooting occur in the same subculture. Consequently, they are also cheap because only small amounts of plant growth regulators and little labour are required (Morte and Honrubia 2009). However the plants that have been grown under in vitro conditions generally present non-functional stomata, weak root systems and poor development of waxes and cuticle (Mathur et al. 2010). The acclimation process helps to develop the necessary morphological and metabolic adaptations before plant goes to ex vitro conditions (Pospóšilová et al. 1999), although this does not always occur. To solve this problem, we have developed a photoautotrophic *Helianthemum* micropropagation system (Andrino et al. 2012) based on the methodology described by Kozai (1991). The photoautotrophic micropropagation technique which overcomes these problems can be defined as micropropagation without sugar in the culture medium, the growth or accumulation of carbohydrates by cultures being fully dependent on photosynthesis and inorganic nutrient uptake (Kozai 1991; Zobayed et al. 2004). Photoautotrophic (PA) micropropagation has many advantages over conventional or photomixoautotrophic (PM) micropropagation with including improved plantlet physiology (biological aspect) and operation/management of the production process (engineering aspect), although it also has some disadvantages (Xiao et al. 2011).

Helianthemum almeriense Pau has been successfully micropropagated by PM method (Morte and Honrubia 1992, 1997), and the same plant was used as a model to improve *Helianthemum* propagation by PA. When cultured in the absence of sucrose, this plant increased its survival rate during acclimation using a PA system (Andrino et al. 2009). At the same time, exchanging agar for perlite, as physical support, contributed to a significant reduction in plant losses during acclimation. In addition, the absence of sucrose reduced the presence of microbial contamination during the cultivation vessel phase. In the light of the above, our objectives were to ascertain why these differences in the survival rates existed between *H. almeriense* plants growth under PA and PM conditions. Also, we looked for any parameter that could help determine the quality of the seedling before the acclimation phase. For this purpose, *H. almeriense* was cultivated inside vessels in the following conditions: 21–23 °C, 60–70 % relative humidity, 350–360 ppm CO_2, 4,000–4,300 lux, 140–160 μmol/m^2 s, photoperiod of 16 h light under PM (salts and vitamins of Murashige and Skoog 1962) medium with 3 % sucrose and 8 g/l agar) or PA (salts and vitamins of MS medium without sucrose and with horticultural perlite). The plants were maintained for 60 days in these conditions, after which seedlings were randomly harvested for different physical (leaf area and stem and root lengths) and photosynthetic (photosynthetic pigments and the chlorophyll metre SPAD-502-Konica Minolta, Japan-value) parameters to be analysed.

The results showed significant differences ($p \leq 0.05$) between the PA and PM treatments (Fig. 21.1). Leaf area (Fig. 21.1a) was approximately 50 % greater in PA than in PM. The leaf area is a parameter that defines the quality of the crop

(Miyashita et al. 1996): greater leaf areas course with higher initial photosynthetic rates and, therefore, higher growth rates. Likewise, low relative humidity in the PA cultivation vessels enhances the development of leaf area and the length of the upper stems (Afreen 2005; Nguyen and Kozai 2005). According to Jackson et al. (1991), the ethylene content inside a PM cultivation container can play an important role, inhibiting stem growth, explant development and leaf expansion. This was reflected in the *H. almeriense* plants, which showed leaf areas and stem lengths that were significantly higher ($p \leq 0.05$) in the PA than in the PM treatment (Fig. 21.1a, b). Thanks to greater stem length and larger overall plant size, the cultivation period is shortened by 2 weeks in the PA treatment, a saving of time that is crucial for medium-large-scale mycorrhizal plant production.

H. almeriense plants showed significantly higher root development in PA than in PM conditions (Fig. 21.1c). Kozai and Kubota (2005) claimed that replacing conventional agar gel by porous materials, like perlite, significantly affects the environment of roots and, therefore, their anatomical characteristics. This improvement in the root environment in PA is based on experience gained from hydroponics, a technique that normally works without sugar in the culture medium and by irrigation with liquid medium on porous substrates. In our case, perlite was selected as substrate because it is widely used in the hydroponic cultivation of numerous horticultural species, due to its good aeration properties and water holding capacity (Urrestarazu-Gavilán 2004). A well-developed root system permits the plant to transport water and nutrients efficiently, promoting proper growth of the seedlings and improving their health. This translates into higher rates of survival during transplanting and in the final environmental conditions (Afreen et al. 1999). In other woody species, correct root development has been correlated with a high survival rate in ex vitro conditions (Kirdmanee et al. 1995).

The photosynthetic pigment concentrations (total chlorophyll, chlorophylls a and b, xanthophyll and carotene) were significantly ($p \leq 0.05$) higher in PA of *H. almeriense* plants (Fig. 21.1d–g), as has been observed in many other crops (Kozai 2005). In a PA cultivation system, photosynthesis is the only source for carbohydrate production and accumulation. In general, PM cultivation systems show poor photosynthetic function, non-functional stomata, poor development of epicuticular waxes and hyper-hydrated tissues, resulting in a low survival rate of the plant material in ex vitro conditions (Kozai 2005). Chlorophyll *a* is usually found to be 3 times more concentrated than chlorophyll *b* (Afreen 2005), as occurred in the *H. almeriense* plants (Fig. 21.1d, e). Moreover, it is well known that the air exchange rate is the key factor for increasing the photosynthetic pigment concentration, favouring suitable CO_2 levels for each phase and preventing the accumulation of harmful ethylene (Cui et al. 2000; Jo et al. 2002; Park et al. 2004; Righetti 1996; Zobayed et al. 2000). The PA cultivation system favoured gas exchange with the external atmosphere of cultivation, causing a significant increase in photosynthetic pigments in *H. almeriense* plants (Fig. 21.1d–h).

The quantity and quality of light and the absence of sucrose play an important role in the *H. almeriense* cultivation (Andrino et al. 2009, 2012). In the case of the xanthophylls and total carotenoids, there were significant differences ($p \leq 0.05$)

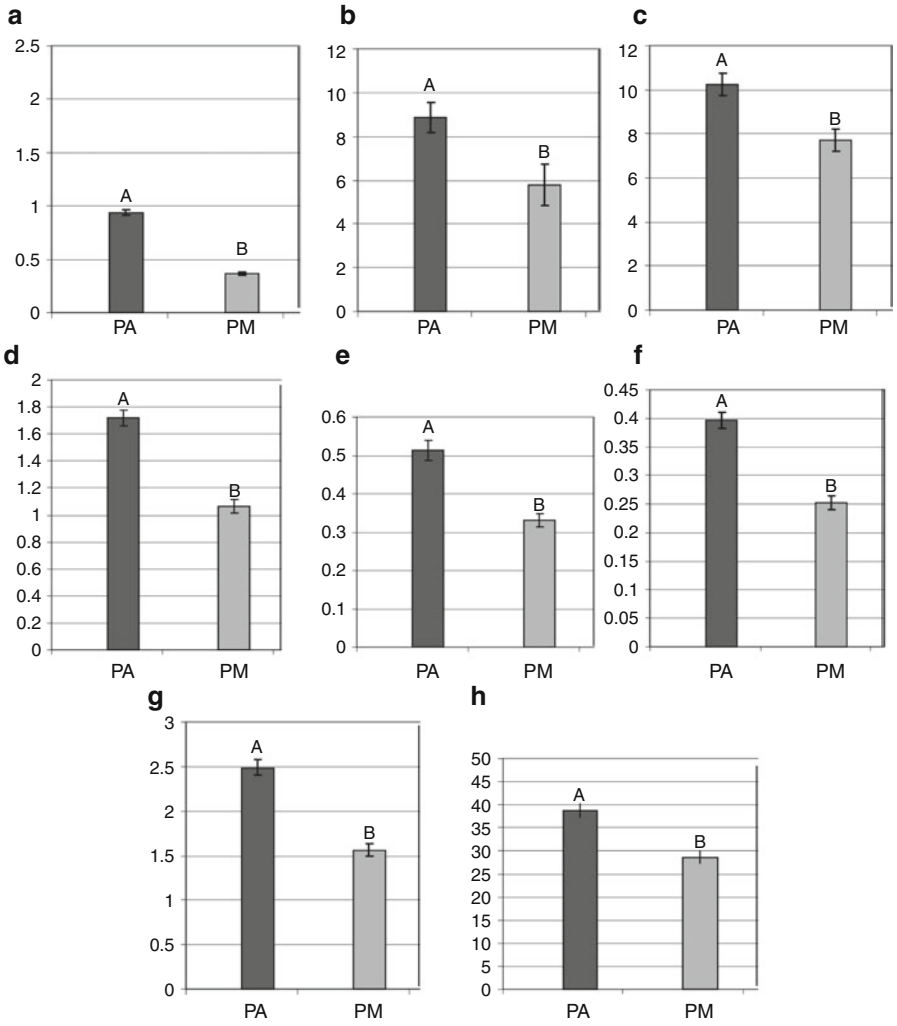

Fig. 21.1 Effect of PA and PM cultivation systems on leaf area (**a**, cm^2), stem (**b**, cm) and root (**c**, cm) lengths, chlorophyll a (**d**, mg/g leaf), chlorophyll b (**e**, mg/g leaf), xanthophylls and carotenoids (**f**, mg/g leaf), total chlorophylls (**g**, mg/g leaf), and SPAD-502 value (**h**) of *H. almeriense* seedlings. Each bar is the mean of fourteen measurements and its standard deviation value. The parameters, for each PA or PM treatment, that do not share the same letter are significantly different according to ANOVA test ($p = 0.05$)

between the PA and PM treatments (Fig. 21.1). These pigments provide mechanisms for the dissipation and extinction of light energy, as may also be antenna pigments that work between 450 and 500 nm (Rivas 2008). These protection pigments increase proportionally with the chlorophyll content of plants grown in vitro during the adaptation to a more intense light (ex vitro environment) (Hofman et al. 2002). The data obtained for *H. almeriense* indicated that the plants

grown under the PA cultivation system had higher values for these pigments, which could be related with better adaptation to higher light intensities following transplantation.

21.2.2 Improvement of the Culture Medium Composition

The composition of the medium is a determining factor for in vitro plant growth. Murashige and Skoog (MS) medium was used for *Helianthemum* species micropropagation (Morte and Honrubia 1992, 1997; Morte et al. 2009; Torrente et al. 2009). The medium contains the correct amounts and proportion of inorganic nutrients to satisfy the nutritional as well as the physiological needs of many plant cells in culture (Gamborg et al. 1976). A distinguishing feature of the MS medium is its high content of nitrate (NO_3^-), potassium and ammonium (NH_4^+) compared with other nutrient media, the relation between ammonium and nitrate being 1:2 (Gamborg et al. 1976). Ammonium and nitrate are the primary inorganic nitrogen (N) sources available for plants, and their uptake and assimilation have been well characterised (Li et al. 2006). On one hand, nitrate acts as a signal that regulates carbon metabolism, inducing the required genes for carbon absorption and reduction, the assimilation of ammonium and the synthesis of the carbonated skeletons necessary for amino acid synthesis. On the other hand, nitrate inhibits the genes involved in carbohydrate biosynthesis (Maldonado et al. 2008). The lack of sufficient quantity and quality of light, in combination with the presence of carbohydrates in the medium, produces low photosynthetic rates, high activity of the PEPC (phosphoenolpyruvate carboxylase) and low activity of RuBisCo (ribulose-1,5-bisphosphate carboxylase oxygenase), favouring the respiration of the plant tissue, instead of photosynthesis (Cristea et al. 1999; Genoud et al. 2001).

PA cultivation method uses the complete MS medium in the absence of a carbon source. This method permitted us to grow a large volume of *H. almeriense* seedlings with germination rates of around 80–90 % and very satisfactory results. However, some seedlings stopped growing and turned yellowish green during the first 2 months after germination before finally dying. The problem was attributed to the possible excess ammonium concentration in the MS medium. Theoretically ammonium should be the preferred N source because it consumes less energy than nitrate in the plant metabolism (Britto and Kronzucker 2002). However, excess ammonium nutrition usually has deleterious effects on growth and deprives cells of osmotic adjustment. The reported symptoms of NH_4^+ toxicity range widely and generally appear at external NH_4^+ concentrations above 0.1–0.5 mmol/L in sensitive species like barley (Britto and Kronzucker 2002). Two of the most dramatic of these symptoms are leaf chlorosis and the total suppression of growth. Yield reduction among sensitive species can range from 15 to 60 % and may even result in death. Other symptoms often include a decrease in the root:shoot ratio although the reverse effect has been observed for some species (Britto and Kronzucker 2002). Symptoms not so readily visible, but equally important, may include a

decline in mycorrhizal associations. Moreover, seed germination and seedling establishment can be inhibited by NH_4^+ toxicity, a feature of high ecological significance (Britto and Kronzucker 2002).

In aerated soils, the major form of inorganic N is nitrate; in flooded wetland or acidic soils, the major form is ammonium. In the rhizosphere, the root can release oxygen and exudates that greatly influence the local redox potential and the density and activity of microbial populations (Xu et al. 2012). The relative abundance of NH_4^+ compared with NO_3^- in the soil solution is determined by a number of factors, of which the accumulation of organic matter, soil pH, soil temperature, the presence of allelopathic chemicals and soil oxygenation status are the most important (Britto and Kronzucker 2002). Typically, low pH, low temperature, the accumulation of phenolic-based allelopathic compounds and poor oxygen supply inhibit many nitrifying microorganisms, resulting in higher rates of net ammonification than net nitrification. Soils exhibiting these conditions tend to be late successional, while NO_3^--rich soils tend to be early successional (Britto and Kronzucker 2002). The soils where *H. almeriense* mycorrhizal plants live are normally open places, dry rocky soils, limestone, loamy soils or gypsum soils; sometimes also in volcanic terrain and even in sandy soils, which are also rich in carbonates, with low/no organic matter and high soil temperature. The pH of the soils goes from slightly to moderately alkaline (7.5–8.5) (Honrubia et al. 2007). Carbonates influence the availability of plant nutrients such as phosphorus, molybdenum, iron, boron, zinc and manganese (Navarro-Blaya and Navarro-García 2003). Plant symbioses play an important role in the ability to take up these scarce nutrients. Our hypothesis is that the ecosystem in which *H. almeriense* develops does not present the necessary characteristics for ammonification, and it is assumed that the vegetation that grows naturally develops in its absence.

To assess this hypothesis, several culture media with different NH_4^+/NO_3^- ratios and different light conditions were tested. The complete formulation of MS medium was used as control treatment. This medium presents a $NH4^+/NO_3^-$ ratio of 0.52. The idea was to reduce the presence of ammonium ion but not the total concentration of nitrate ion. For the ratios in which the presence of ammonium was diminished, the lack of nitrogen ion was compensated by calcium nitrate salt. Moreover, for in vitro plant multiplication, plants are normally grown without daylight, and the light source selected has a higher proportion of blue to promote stem elongation and multiplication. The experimental design was made to study the effect of light source and NH_4^+/NO_3^- ratio on plant survival (Table 21.2). One month after sowing the seeds inside the vessels, the results pointed to the high toxicity of different ammonium/nitrate ratios, especially under high-intensity light conditions.

Statistically, under low-intensity light conditions (Fig. 21.2a), the 0 and 0.52 ratios (0 and 20.6 mM NH_4^+) presented the same low survival rates, the only difference being that the control treatment (ratio 0.52) showed a very high standard deviation value, which explains the erratic behaviour of some plant lots during seedling production. The best results were obtained in the 0.13–0.39 range (5.15–15.45 mM NH_4^+), in which *H. almeriense* seedlings were able to grow and showed very good survival rates of up to 80 %.

Table 21.2 Different treatments carried out to evaluate possible toxicity of NH_4^+/NO_3^- ratios

Treatment						
1. Different light sources	2. Different NH_4^+/NO_3^- ratios					
	NH_4NO_3 (mM)	KNO_3 (mM)	$Ca(NO_3)_2$ (mM)	Total NH_4^+ (mM)	Total NO_3^- (mM)	Ratio NH_4^+/NO_3^-
Fluorescent tube	0	18.8	10.3	0	39.4	0.00
Daylight 18 W (Sylvania)	5.15	18.8	7.7	5.15	39.4	0.13
PAR: 70–90 μmol/m^2 s	10.3	18.8	5.15	10.3	39.4	0.26
Sodium vapour bulb	15.45	18.8	2.6	15.45	39.4	0.39
Sodium vapour 400 W (Gavita)	Control: 20.6	18.8	0	20.6	39.4	0.52
PAR: 140–200 μmol/m^2 s						

The MS media composition has two nitrate forms, ammonium nitrate and potassium nitrate. The objective of this trial is to test the possible ammonium toxicity, for this purpose there has been established decreasing concentrations of ammonium nitrate. To restore MS initial nitrate concentration, but decreasing the total N, calcium nitrate has been used as substitute

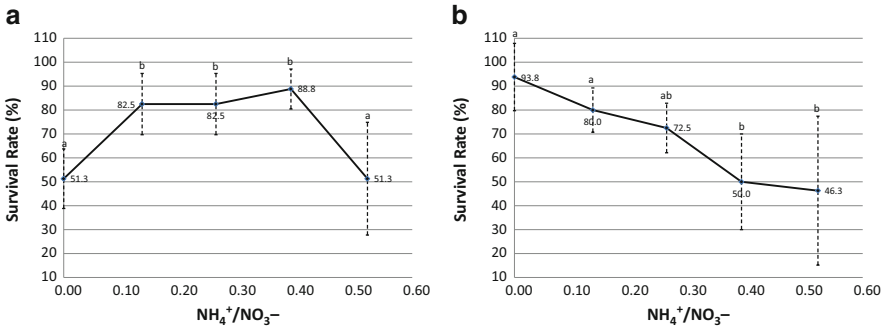

Fig. 21.2 Survival rates of *H. almeriense* in the tests carried out with different NH_4^+/NO_3^- ratios under fluorescent light (**a**) and sodium vapour light (**b**). Each point is the mean of eight measurements and its standard deviation value. The survival rates for each NH_4^+/NO_3^- ratio which do not share the same letter are significantly different in ANOVA test ($p = 0.05$)

Under high-intensity light conditions (Fig. 21.2b), seedlings did not show statistical differences in survival rates at NH_4^+/NO_3^- ratios ranging from 0 to 0.26 (0–10.3 mM NH_4^+), in which the survival of *H. almeriense* plants was not negatively affected. The 0 ratio (absence of ammonium) provided the best survival rate (up to 90 %) The survival rate changed as the ratio increased from 0.39 to 0.52 (15.4–20.6 mM NH_4^+) when *H. almeriense* started to suffer the deleterious effects of high ammonium concentrations, the survival rates being low and very variable (Fig. 21.2). Therefore, the presence of NH_4^+ starts to be harmful at a ratio of 0.26 (10.3 mM NH_4^+) (Fig. 21.2b). In the light of these findings, the nitrogen source of MS medium should be modified in order to improve *H. almeriense* seedling survival for commercial production.

Many studies have reported that ammonium toxicity is frequently more pronounced at high light intensity (Magalhaes and Wilcox 1984; Zhu et al. 2000; Britto and Kronzucker 2002), which agrees with the results obtained for *H. almeriense* (Fig. 21.2). It may be that the light optimum with NH_4^+ (relative to NO_3) nutrition is shifted to a higher intensity to compensate for increased carbon utilisation for respiration and amino acid production (Britto and Kronzucker 2002).

Plants susceptible to NH_4^+ toxicity are typically afflicted by reduced rates of net photosynthesis and enhanced photorespiratory rates (Blasco et al. 2010). Photorespiration is a possible means of alleviating light stress; it can produce 20-fold more NH_4^+ than is generated by the reduction of NO_3^- and is considered the major source of this cation, especially in C3 plants. Around 25 % of the fixed CO_2 is released during this metabolism; however, the suppression of photorespiration has negative effects on plants, producing a decrease in the CO_2 assimilation rate, poor vegetable growth and alterations in the chloroplast structure (Blasco et al. 2010). Further physiological analyses should be carried out in order to determine the importance of the photorespiration process under different light conditions with different NH_4^+ concentrations.

21.2.3 Plant Quality Control Before the Acclimation Phase

During seedling production, it is very important to know the most adequate moment for transplantation. The main objective is to know when the best moment is to start the transplantation from vessel to pot. For this purpose, a non-destructive determination of total chlorophyll was carried out using a SPAD-502. A linear relation was observed between SPAD-502 values and total chlorophyll in *H. almeriense* for each treatment (Fig. 21.3).

During the last decade, the use of this type of device for non-destructive measurements has increased in agricultural (Uddling et al. 2007) and forestry (Hawkins et al. 2009) field research, being associated with the estimation of chlorophyll and leaf nitrogen concentration with time. However, these measurements must take into account the different species cultivated, growth conditions, season of the year, stage of the crop, crop variety, light reflection, light dispersion and even the unequal distribution of leaf chloroplasts (Nauš et al. 2010). For these reasons, any measurement in *H. almeriense* should take into consideration its growth conditions.

Good linear correlations were observed between the total chlorophyll obtained by pigment extraction and the SPAD-502 measurement from the same leaves (Table 21.3), according to the Pearson correlation coefficient for both the PA and PM treatments. Once the linear correlation was established, the efforts were centred on modelling this relation. The desired relation is linear because it would permit to interpolate SPAD-502 values to total chlorophyll once as an indicator of plant health. Three different simulations were carried out, the first with only the PM data (total chlorophyll vs. SPAD-502 measurement, $n = 14$), the second with the PA data and the third with both PM and PA data ($n = 28$). For the first two simulations, the R^2 were very low, 0.693 for PA and 0.624 for PM, compared with the results of other studies, which ranged from 0.8 to 0.9 (Hawkins et al. 2009; Nauš et al. 2010; Uddling et al. 2007). The slope shown by the PA and PM linear functions was identical (0.04). However, the R^2 value reached 0.804 when PA and PM data were considered together, and the mathematical relation between total chlorophyll and SPAD-502 measurement could be expressed as a linear function (Fig. 21.3).

Once the linear relation had been described between both quality parameters, a logistic regression was carried out. The logistic regression is useful in those cases in which it is necessary to predict the presence or absence of a particular feature or result, according to the values of a set of predictor variables (independent), which may be quantitative or qualitative (Ferran-Aranaz 2001). A pseudo-R^2 value (Nagelkerke R^2) measures the strength of association of the model, whose values are between 0 and 1. Nagelkerke R^2 is considered a good indicator of the association strength (Dominguez-Rojas et al. 1993).

The dependent and dichotomous parameter is live (value = 1) or dead (value = 0), after the acclimation process. As co-variable, the set of physical parameters (stem and root lengths, leaf area) and photosynthetic parameters (SPAD-502, C_a, C_b, C_{x+c}, C_{total}) were used. This statistical test has two different purposes:

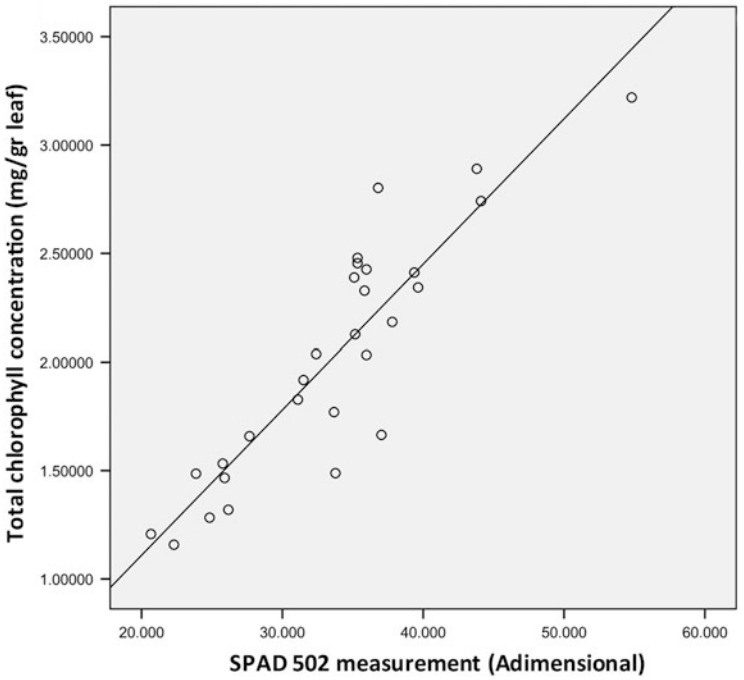

Fig. 21.3 Graphic representation of total chlorophyll and the SPAD-502 value ($R^2 = 0.804$) of *H. almeriense* seedlings before acclimation. The linear equation: total chlorophyll = −0.232 + (0.067 × SPAD 502). Data obtained from PM and PA treatments together ($n = 28$)

Table 21.3 Correlations between total chlorophyll and the SPAD-502 measurement

Treatment	Correlation coefficient	SPAD-502	C_{total}
PA	Pearson correlation test	1.000	0.832
	Significance		0.000
PM	Pearson correlation test	1.000	0.790
	Significance		0.001

The correlation was significant at $\alpha = 0.01$ level

(1) to identify which parameter (physical or photosynthetic) is able to determine whether a pre-acclimated plant is going to survive (or not) after the acclimation process and (2) to establish the interval value of the parameter between the live or dead *H. almeriense* seedlings.

The SPAD-502 parameter obtained the best Nagelkerke R^2 value, 0.71, which indicates a moderately strong relationship between prediction (live or dead) and the SPAD-502 measurement in 71 % of cases. Therefore, the SPAD-502 seems to be an easy and robust measurement to estimate the pre-acclimation condition in *H. almeriense*. The second objective was to identify the SPAD-502 interval value between live and dead plants. For this purpose, 68 acclimated plants were selected

to measure their SPAD values, and the number of dead plants was registered after the acclimation process. Then each dead plant was associated with its SPAD-502 value. After analysing the data, it was possible to estimate the probability (0.1) of plant survival before the acclimation process with only one SPAD-502 measurement. The maximum survival rate was established at 28 SPAD-502 units, or its equivalent total chlorophyll parameter, 1.6 mg/g leaf (Fig. 21.4), and, therefore, in vitro seedlings must be acclimated only when they reach this value to be able to survive during the acclimation phase.

21.3 Fungal Inoculum and Mycorrhizal Plant Productions

Both desert truffle spores and mycelia have been used successfully to produce mycorrhizal plants (Morte et al. 2008). However, mature spores are used more frequently than mycelium due to the slow growth of the latter in vitro.

Desert truffle mycelia have been grown successfully on MMN (modified Melin-Norkrans) medium and PDA (potato dextrose agar) medium. The pH should be adjusted to 7.0 if the ascocarps are from alkaline calcareous soils. Desert truffle mycelium can be used directly from the plates as inoculum for in vitro mycorrhizal synthesis (Morte et al. 1994; Morte and Honrubia 1995, 1997) and from liquid fermentation for both in vitro and in vivo inoculation trials (Morte and Honrubia 2009; Morte et al. 2008). However, only fungal strains well adapted to in vitro conditions should be used to produce mycelium by liquid fermentation in a bioreactor. A study on in vitro mycelium cultures of two mycorrhizal desert truffles in conditions of water stress demonstrated that *Terfezia* mycelium (strain TcS2) grows better under slight water stress (−0.45 MPa), which could improve the production of this mycelial inoculum in a bioreactor (Navarro-Ródenas et al. 2011).

The spore suspension is made taking into account the maturity of the spores. The spore suspension from mature ascocarps consists of 6 g of dried and crushed ascocarps per litre of distilled water. This spore solution is shaken overnight (12 h), and, then, instead of inoculating the plants directly, the spore solution was added to the perlite, allowing spore adhesion to the pores and cavities within (Andrino et al. 2012; Morte et al. 2012). Using such a technique allowed the quantity of spores per litre to be reduced from 10 (Morte et al. 2008) to 6 g (which means 3.5–4.5 $\times$ 10^5 mature spores/plant). The percentage of inoculum per plant represents 5 % of the final container volume.

For the production of desert truffle mycorrhizal plants, four ex vitro and two in vitro inoculation options were designed, the time required for each of them ranging between 4 and 9 months, depending on the type of plant propagation system and inoculum source used (Table 21.4). The photoautotrophic (PA) *Helianthemum* micropropagation system (7 months) allowed this time to be reduced to 3 months with respect to PM system (4 months) since fungal inoculation is carried out at the moment plants are transferred from in vitro to ex vitro conditions, so that plant acclimation and mycorrhization occur at the same time. Moreover, this last way has

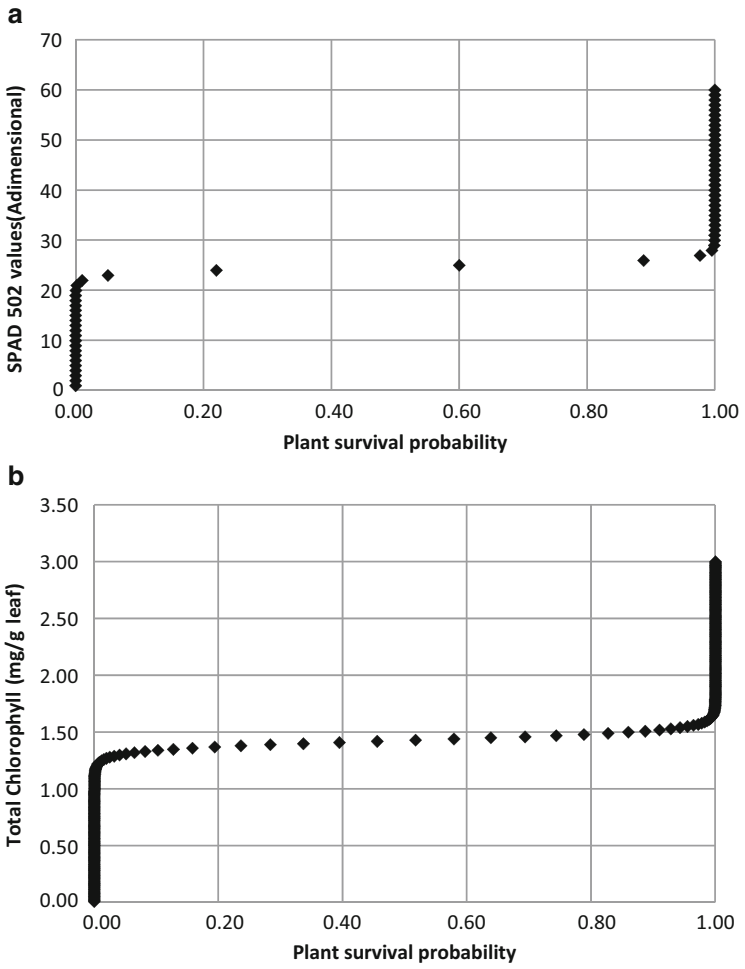

Fig. 21.4 Plant survival probability graphs, where 0 value means dead plant and 1 alive plant after acclimation process. (**a**) Representation of the survival interval value of SPAD-502 for pre-acclimated *H. almeriense* plant. (**b**) Representation of the survival interval value of total chlorophyll (mg/g leaf) for pre-acclimated *H. almeriense* plant

other advantages, including (1) a saving of fungal inoculum, (2) high survival percentage/smooth transition to ex vitro environment, (3) elimination of plant physiological disorders, (4) increased annual productivity per floor area, (5) reduced labour costs, (6) simplified micropropagation system and (7) no unwanted contamination due to the absence of sugar in the medium (Andrino et al. 2012; Morte et al. 2012).

After the acclimation phase, seedling irrigation is established to maintain the pot water potential between −15 and −30 kPa in nursery conditions. Approximately 30–40 days after transplanting, it is necessary to make a mycorrhization quality

Table 21.4 Options designed by in pot and in vitro methods to produce desert truffle mycorrhizal plants and the time required for each, depending on the type of plant propagation system and inoculum source used

Inoculation	Plant material (*Helianthemum*)	Fungal material	Time for plant production (months)	Time for plant mycorrhization (months)	Total time (months)
In pots	Seedlings directly germinated In pots	Mature spores	6	3	9
		Mycelial suspension	6	1–2	7.5
	Acclimated PM micropropagated plants	Mature spores	4	3	7
	Micropropagated PA plants	Mature spores or mycelium in perlite	3	1	4
In vitro	Micropropagated PM plants	Pieces of agar with mycelium	3	2	5
		Mycelial suspension	3	2	5

control. With all these production systems, mycorrhization rates range between 75 and 85 % after 2 months (Andrino et al. 2012; Morte et al. 2012).

21.3.1 Use of Fertilisation of Mycorrhizal Nursery Plants

Given that the objective is to obtain quality mycorrhizal plants, nursery maintenance tasks are crucial to ensure the quality of the final product. Using the desert truffle mycorrhizal plant production method described by Andrino et al. (2012), mycorrhization can reach values of around 70–80 %. Nine-month-old plants are normally taken to the field for plantations.

If the mycorrhizal plant is maintained longer than 12 months in the pot in nursery conditions, the mycorrhization percentage starts to decrease due to limited pot space and the absence of nutrients, which are periodically leached by the irrigation. For this reason, it is necessary to establish a viable fertilisation protocol of mycorrhizal *H. almeriense* plants that permit to maintain alive the symbiosis during potting.

One-year-old *H. almeriense* mycorrhizal plants were selected ($n = 4$ per treatment) to determine the amount of fertiliser necessary to support the desert truffle mycorrhizal plants (Table 21.5). Granular forms of fertiliser were spread over the soil surface around the potted plants. Two different fertilisers were used, a solid controlled release fertiliser and a solid soluble fertiliser. Irrigation was programmed at 16 mm/week and the experiment lasted 3 months, from14/02 to 14/05, when the average temperature was 16.4 °C (Fig. 21.5a). At the end of the experiment, all

Table 21.5 Fertilisation treatments carried out in 1-year-old desert truffle *H. almeriense* mycorrhizal plants in nursery conditions

TREATMENTS

Solid controlled release fertilizer: PLANTACOTE PLUSS 6M NPK-[Mg]

Brand: AGLUKON (Germany)
Composition: 14-9-15-[2]
Description and use: the nutrients are released as needed during the 6 months following deposition depending only on soil moisture and temperature:
15/16 °C -> longevity: 6-7 months
20/21 °C -> longevity: 5-6 months
26/27 °C -> longevity: 4-5 months

Dose:
AGLUKON recommended dose: 2 - 6.5 Kg/m^3.

Test	Fertilizer dose Kg/m^3	Fertilizer for 220 cc pot (g).
Control	0	0
L1	1	0.22
L2	2	0.44
L3	3	0.66
L4	4	0.88
L5	5	1.1
L6	6	1.32
L7	7	1.54

Solid soluble fertilizer: COMBIFERT NPK-[Mg]

Brand: FUENTES FERTILIZERS (Spain)
Composition: 12-12-17-[2]
Description and use: the nutrients are released after deposition and depending only on the irrigation.

Dose:
Fertilizer doses were calculated in terms of electrical conductivity measured from a solution made between irrigation water and solid fertilizer (Figure 21.5 b).

Test	Fertilizer conductivity (dS/m)	Fertilizer for 220 cc pot (g).
Control	1 (tap water)	0
R1	2	0.14
R2	3	0.42
R3	4	0.69
R4	5	0.97
R5	6	1.25
R6	7	1.53

roots from each fertiliser treatment were analysed, and the mycorrhization % was estimated following the methodology established by Phillips and Hayman (1970).

The controlled release fertiliser was applied following the recommended doses (from 2 to 6.5 kg/m^3). A total of seven fertilisation treatments were established (Table 21.5). For the solid soluble fertiliser, whose use depends on plant species, a calibration curve was made using the electrical conductivity (EC) values from 30 fertiliser solutions (Fig. 21.5b). The model curve describes a positive lineal regression ($R^2 = 0.98$) between EC and fertiliser weight. Six EC levels were established to apply different amounts of solid fertiliser.

After 3 months of fertiliser application, the best results were obtained with the controlled release fertiliser (Fig. 21.5c). The fertilisation levels from L1 to L4 (0.22–0.88 g) showed significant positive differences compared with the non-fertilised control samples. Mycorrhization values were three times higher for the L1–L4 interval (60.5–73.5 %) compared with the control (22.75 %). The

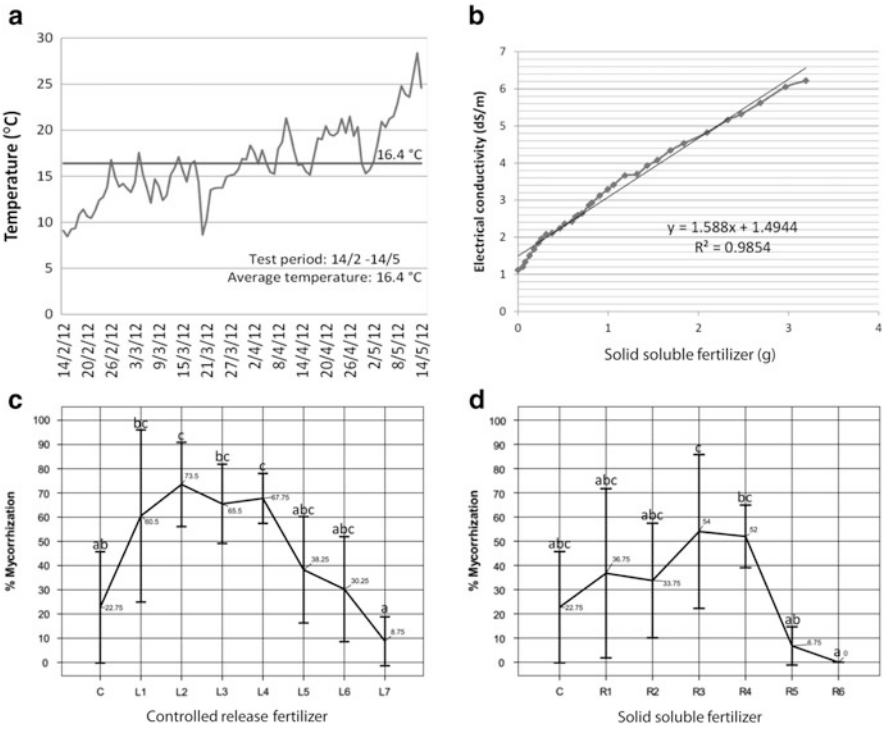

Fig. 21.5 Results of *H. almeriense* mycorrhizal plants under different fertilisation regimes. (**a**) Registry of cultivation temperatures (°C) period that goes from 14/02 to 14/05. (**b**) Electrical conductivity behaviour of increasing solid soluble fertiliser solutions in tap water. The graph shows a linear correlation explained by the equation: $y = 1.588x + 1.4944$ ($R^2 = 0.9854$). (**c**) Results for the controlled release fertiliser effects on *H. almeriense* mycorrhizal plants. Each point is the mean of four measurements and its standard deviation value. The mycorrhization % value belonging to each fertilisation step and that does not share the same letter is significantly different in ANOVA test ($p = 0.1$). (**d**) Results for the solid soluble fertiliser effects on *H. almeriense* mycorrhizal plants. Each point is the mean of four measurements and its standard deviation value. The mycorrhization % value belonging to each fertilisation step and that does not share the same letter is significantly different in ANOVA test ($p = 0.1$)

mycorrhization percentage fell significantly in the L5 to L6 interval (1.1–1.32 g) compared with the L1–L4 interval but was still significantly greater than in the control. The L7 (1.54 g) level shared the same mycorrhization value as the control (Fig. 21.5c).

The results were not better for the solid soluble fertiliser than for the controlled release fertiliser (Fig. 21.5d). Mycorrhization percentages from R1 to R2 (2–3 dS/m) did not show significant differences with regard to the control. The best results were obtained with the R3 and R4 values (4–5 dS/m) although these percentages showed very high standard deviations, especially R3 (±31.75 %). The results were statistically worse than the control for R5 and R6 (6–7 dS/m) treatments; moreover, all the plants died during the first month in R6 treatment.

Most studies on the fertilisation of nursery mycorrhizal plants have been made with ectomycorrhizal plants (Rudawska et al. 1994; Väre 1990; Danielson et al. 1984; Quoreshi 2003; Browning and Whitney 1992; Shaw Iii et al. 1982; Khasa et al. 2001; Gagnon et al. 1987, 1988, 1995; Quoreshi and Timmer 1998, 2000; Chakravarty and Chatarpaul 1990; Ek 1997) and only few studies look at ericoid mycorrhizal plants (Caporn et al. 1995; Johansson 2000). In general, fertiliser application has a positive response in these mycorrhizal associations, but it is necessary to establish the optimal values for each plant symbiosis. Neutral or negative results normally occur for high fertiliser concentrations. For mycorrhizal *H. almeriense* nursery seedlings, it has been possible to adjust the correct concentration using solid controlled release fertiliser. *H. almeriense* mycorrhizal seedlings respond to very low fertiliser concentrations (L1; 0.22 g.) and are able to withstand very high EC values (6 dS/m).

21.3.2 Certification of Desert Truffle Mycorrhizal Plants

Characterisation of the mycorrhiza formed in the *Helianthemum* root systems by the different desert truffle species is extremely important to ensure the high quality of mycorrhizal plants (Morte and Honrubia 2009). For this reason, a morphological and/or molecular analysis of the mycorrhiza should be carried out before planting. Such characterisation is also important to evaluate the permanence of the mycorrhiza under field conditions.

The morphological evaluation process consists of examining the entire root system by binocular microscope, observing the abundance and condition of mycorrhizal root morphotypes (Gutiérrez et al. 2003). The analyst should examine any root tips of doubtful identification by staining the roots (with 5 % blue ink in acetic acid or 0.01 % acid fuchsine solution). *T. claveryi* with *H. almeriense* forms an endomycorrhiza under natural field conditions, an ecto- and ectendomycorrhiza without a sheath in pot cultures, and an ectomycorrhiza with a characteristic sheath and Hartig net in vitro (Gutiérrez et al. 2003; Morte et al. 2008). Therefore, culture conditions can induce changes in mycorrhiza morphology, and there is no clear cut-off between these two main types of mycorrhiza organisation in *Helianthemum* species (Gutiérrez et al. 2003). A recent study has demonstrated that this colonisation varies from ecto- to endomycorrhiza according to the water available in the soil, the scarcer the water, the more intracellular the colonisation (Navarro-Ródenas et al. 2012). Therefore, this symbiosis should be considered as an *ectendomycorrhizal continuum* (Navarro-Ródenas et al. 2012), and water availability modifies the relative amount between intra- and intercellular hyphae in this *continuum*.

Certification of plant lots for colonisation by desert truffle mycorrhizae is a destructive and laborious process, but it is important to sample a minimum number of plants to statistically test the percentage of mycorrhizal plants. We suggest examining 12 plants for each lot of 1,000 plants and consider a mycorrhization

percentage exceeding 33 % of the root system as good (Morte et al. 2012). *Terfezia* mycorrhiza has no problems with other contaminant mycorrhizal fungi due to its host specificity.

Moreover, molecular identification of the desert truffle mycorrhiza is very useful for evaluating the permanence of the mycorrhiza in field conditions. Due to the number of ITS sequences from different desert truffles currently available in molecular data bases, it has been possible to design specific primers for this purpose (Kovács et al. 2008).

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References

Afreen F (2005) Physiological and anatomical characteristics of in vitro photoautotrophic plants. In: Kozai T, Afreen F, Zobayed SMA (eds) Photoautotrophic (sugar-free medium) micropropagation as a new micropropagation and transplant production system. Springer, Netherlands, pp 61–90. doi:10.1007/1-4020-3126-2_6

Afreen F, Zobayed SMA, Kubota C, Kozai T, Hasegawa O (1999) Supporting material affects the growth and development of in vitro sweet potato plantlets cultured photoautotrophically. Vitro Cell Dev Biol Plant 35(6):470–474

Alsheikh A (1984) Mycorrhizae of annual *Helianthemum* species formed with desert truffles. In: 6th North American Conference on Mycorrhizae, Bend, Oregon (USA), 25–29 Jun 1984. Oregon State University. Forest Research Laboratory

Ammarellou A, Saremi H (2008) Mycorrhiza between *Kobresia bellardii* (All) Degel and *Terfezia boudieri* Chatin. Turk J Bot 32:17–23

Andrino A, Morte A, Honrubia M (2009) Mejora en la aclimatación de dos especies del género Helianthemum sp. Paper presented at the Octava reunión de la Sociedad Española de Cultivo In Vitro de Tejidos Vegetales, Murcia, España

Andrino A, Morte A, Honrubia M (2012) Method for producing plants of the *Cistaceae* family that establish mycorrhiza with different desert truffle species. Spain Patent ES2386990

Awameh M, Alsheikh A, Al-Ghawas S (1979) Mycorrhizal synthesis between *Helianthemum ledifolium*, *H. salicifolium* and four species of the genera *Terfezia* and *Tirmania* using ascospores and mycelial cultures obtained from ascospore germination. In: Proceedings of the 4th North American Conference on Mycorrhizae, Colorado State University, Fort Collins, Colorado, pp 23–23

Blasco B, Rios JJ, Cervilla LM, Sánchez-Rodríguez E, Rubio-Wilhelmi MM, Rosales MA, Ruiz JM, Romero L (2010) Photorespiration process and nitrogen metabolism in lettuce plants (*Lactuca sativa* L.): induced changes in response to iodine biofortification. J Plant Growth Regul 29(4):477–486

Britto DT, Kronzucker HJ (2002) NH_4 toxicity in higher plants: a critical review. J Plant Physiol 159(6):567–584

Browning MHR, Whitney RD (1992) The influence of phosphorus concentration and frequency of fertilization on ectomycorrhizal development in containerized black spruce and jack pine seedlings. Can J Forest Res 22(9):1263–1270. doi:10.1139/x92-168

Caporn SJM, Song W, Read DJ, Lee JA (1995) The effect of repeated nitrogen fertilization on mycorrhizal infection in heather [*Calluna vulgaris* (L.) Hull]. New Phytologist 129 (4):605–609. doi:10.1111/j.1469-8137.1995.tb03028.x

Cristea V, Dalla Vecchia F, La Rocca N (1999) Developmental and photosynthetic characteristics of a photoautotrophic *Chrysanthemum* culture. Photosynthetica 37(1):53–59. doi:10.1023/a:1007066812957

Cui Y-Y, Hahn E-J, Kozai T, Paek K-Y (2000) Number of air exchanges, sucrose concentration, photosynthetic photon flux, and differences in photoperiod and dark period temperatures affect growth of *Rehmannia glutinosa* plantlets in vitro. Plant Cell Tissue Organ Cult 62(3):219–226. doi:10.1023/a:1006412321864

Chakravarty P, Chatarpaul L (1990) Effect of fertilization on seedling growth, ectomycorrhizal symbiosis, and nutrient uptake in *Larix laricina*. Can J Forest Res 20(2):245–248. doi:10.1139/x90-034

Danielson RM, Griffiths CL, Parkinson D (1984) Effects of fertilization on the growth and mycorrhizal development of container-grown Jack pine seedlings. Forest Sci 30(3):828–835

Dexheimer J, Gerard J, Leduc J-P, Chevalier G (1985) Étude ultrastructurale comparée des associations symbiotiques mycorhiziennes *Helianthemum salicifolium—Terfezia claveryi* et *Helianthemum salicifolium—Terfezia leptoderma*. Can J Bot 63(3):582–591. doi:10.1139/b85-073

Díez J, Manjón JL, Martin F (2002) Molecular phylogeny of the mycorrhizal desert truffles (*Terfezia* and *Tirmania*), host specificity and edaphic tolerance. Mycologia 94(2):247–259

Dominguez-Rojas V, Astasio-Arbiza P, Ortega-Molina P, Gordillo-Florencio E, Garcia-Nunez J, Bascones-Martinez A (1993) Analysis of several risks factors involved in dental caries through multiple logistic regression. Int Dent J 43(2):149

Ek H (1997) The influence of nitrogen fertilization on the carbon economy of *Paxillus involutus* in ectomycorrhizal association with *Betula pendula*. New Phytol 135(1):133–142. doi:10.1046/j.1469-8137.1997.00621.x

Ferrán-Aranaz M (2001) SPSS para Windows. Análisis estadístico. McGraw Hill, España

Fortas Z, Chevalier G (1992) Effects des conditions de culture sur la mycorrhization de l´ *Helianthemum guttatum* par trois espèces de terfez des genres *Terfezia* et *Tirmania* d´Algerie. Can J Bot 70:2453–2460

Gagnon J, Langlois CG, Bouchard D, Tacon FL (1995) Growth and ectomycorrhizal formation of container-grown Douglas-fir seedlings inoculated with *Laccaria bicolor* under four levels of nitrogen fertilization. Can J Forest Res 25(12):1953–1961. doi:10.1139/x95-210

Gagnon J, Langlois CG, Fortin JA (1987) Growth of containerized jack pine seedlings inoculated with different ectomycorrhizal fungi under a controlled fertilization schedule. Can J Forest Res 17(8):840–845. doi:10.1139/x87-133

Gagnon J, Langlois CG, Fortin JA (1988) Growth and ectomycorrhiza formation of containerized black spruce seedlings as affected by nitrogen fertilization, Inoculum type, and symbiont. Can J Forest Res 18(7):922–929. doi:10.1139/x88-140

Gamborg OL, Murashige T, Thorpe TA, Vasil IK (1976) Plant tissue culture media. Vitro Cell Dev Biol Plant 12(7):473–478. doi:10.1007/bf02796489

Genoud C, Sallanon H, Hitmi A, Maziere Y, Coudret A (2001) Growth, stomatal conductance, photosynthetic rate, ribulose-1,5-bisphosphate carboxylase/oxygenase and phosphoenolpyruvate carboxylase activities during rooting and acclimatisation of *Rosa hybrida* plantlets. Photosynthetica 38(4):629–634. doi:10.1023/a:1012425927121

Gutiérrez A (2001) Caracterización, identificación y cultivo en campo de las trufas de desierto. Murcia University, Murcia

Gutiérrez A, Morte A, Honrubia M (2003) Morphological characterization of the mycorrhiza formed by *Helianthemum almeriense* Pau with *Terfezia claveryi* Chatin and *Picoa lefebvrei* (Pat.) Maire. Mycorrhiza 13(6):299–307. doi:10.1007/s00572-003-0236-7

Hawkins TS, Gardiner ES, Comer GS (2009) Modeling the relationship between extractable chlorophyll and SPAD-502 readings for endangered plant species research. J Nat Conserv 17(2): 123–127. doi:10.1016/j.jnc.2008.12.007

Hofman P, Haisel D, Komenda J, Vágner M, Tichá I, Schäfer C, Čapková V (2002) Impact of in vitro cultivation conditions on stress responses and on changes in thylakoid membrane proteins and pigments of tobacco during ex vitro acclimation. Biol Plantarum 45(2):189–195. doi:10.1023/a:1015180219628

Honrubia M, Gutiérrez A, Morte A (2001) Desert truffle plantation from south-east Spain. In: Edible mycorrhizal mushrooms and their cultivation: proceedings of the second international conference on edible mycorrhizal mushrooms. Christchurch, New Zealand, pp 3–5

Honrubia M, Morte A, Gutiérrez A (2007) Las Terfezias. Un cultivo para el desarrollo rural en regiones áridas y semi-áridas. In: Reyna Domenech S (ed) Truficultura, fundamentos y técnicas, vol 10. Mundi-Prensa, España, pp 365–397

Jackson M, Abbott A, Belcher A, Hall K, Butler R, Cameron J (1991) Ventilation in plant tissue cultures and effects of poor aeration on ethylene and carbon dioxide accumulation, oxygen depletion and explant development. Ann Bot 67(3):229–237

Jamali S, Banihashemi Z (2012) Host and distribution of desert truffles in Iran, based on morphological and molecular criteria. J Agri Sci Tech 14:1379–1396

Jo MH, Ham I, Lee M, Lee E, Song N, Han G, Woo I (2002) Effects of sealing materials and photosynthetic photon flux of culture vessel on growth and vitrification in carnation plantlets in vitro. J Kor Soc Hort Sci 43(2):133–136

Johansson M (2000) The influence of ammonium nitrate on the root growth and ericoid mycorrhizal colonization of *Calluna vulgaris* (L.) Hull from a Danish heathland. Oecologia 123 (3):418–424. doi:10.1007/s004420051029

Khasa PD, Sigler L, Chakravarty P, Dancik BP, Erickson L, Mc Curdy D (2001) Effect of fertilization on growth and ectomycorrhizal development of container-grown and bare-root nursery conifer seedlings. New Forests 22(3):179–197. doi:10.1023/a:1015674921878

Kirdmanee C, Kitaya Y, Kozai T (1995) Effects of CO_2 enrichment and supporting material in vitro on photoautotrophic growth of *Eucalyptus* plantlets in vitro and ex vitro. Vitro Cell Dev Biol Plant 31(3):144–149. doi:10.1007/bf02632010

Kovács GM, Trappe JM, Alsheikh AM, Bóka K, Elliott TF (2008) *Imaia*, a new truffle genus to accommodate *Terfezia gigantea*. Mycologia 100(6):930–939

Kovács GM, Vágvölgyi C, Oberwinkler F (2003) In Vitro interaction of the truffle *Terfezia terfezioides* with *Robinia pseudoacacia* and *Helianthemum ovatum*. Folia Microbiol 48 (3):369–378. doi:10.1007/bf02931369

Kozai T (1991) Photoautotrophic micropropagation. Vitro Cell Dev Biol Plant 27(2):47–51. doi:10.1007/bf02632127

Kozai T (2005) Introduction. In: Kozai T, Afreen F, Zobayed SMA (eds) Photoautotrophic (sugar-free medium) micropropagation as a new micropropagation and transplant production system. Springer, The Netherlands, pp 1–5. doi:10.1007/1-4020-3126-2_6

Kozai T, Kubota C (2005) In vitro root zone environments and their effects on growth and development of plants. In: Kozai T, Afreen F, Zobayed SMA (eds) Photoautotrophic (sugar-free medium) micropropagation as a new micropropagation and transplant production system. Springer, The Netherlands, pp 53–60. doi:10.1007/1-4020-3126-2_5

Li B, Xin W, Sun S, Shen Q, Xu G (2006) Physiological and molecular responses of nitrogen-starved rice plants to re-supply of different nitrogen sources. Plant Soil 287(1):145–159

Magalhaes JR, Wilcox G (1984) Ammonium toxicity development in tomato plants relative to nitrogen form and light intensity. J Plant Nutr 7(10):1477–1496

Maldonado J, Agüera E, Pérez Vicente R (2008) Asimilación del nitrógeno y del azufre. In: Azcon-Bieto J, Talon M (eds) Fisiología y bioquímica vegetal. McGraw-Hill, Madrid, España, pp 215–236

Mathur A, Mathur AK, Verma P, Yadav S, Gupta ML, Darokar MP (2010) Biological hardening and genetic fidelity testing of micro-cloned progeny of *Chlorophytum borivilianum* Sant. et Fernand. Afr J Biotechnol 7(8):1046–1053

Miyashita Y, Kitaya Y, Kubota C, Kozai T (1996) Photoautotrophic growth of potato plantlets as affected by explant leaf area, fresh weight and stem length. Scientia Horticulturae 65 (2–3):199–202. doi:10.1016/0304-4238(96)00877-1

Morte A, Andrino A, Honrubia M, Navarro-Ródenas A (2012) *Terfezia* cultivation in arid and semiarid soils. In: Zambonelli A, Bonito GM (eds) Edible ectomycorrhizal mushrooms. Soil biology, vol 34. Springer, Heidelberg, Berlin. doi:10.1007/978-3-642-33823-6_14

Morte A, Cano A, Honrubia M, Torres P (1994) In vitro mycorrhization of micropropagated *Helianthemum almeriense* plantlets with *Terfezia claveryi* (desert truffle). Agr Food Sci Finland 3(3):309–314

Morte A, Honrubia M (1992) In vitro propagation of *Helianthemum almeriense* Pau (*Cistaceae*). Agronomie 12(10):807–809

Morte A, Honrubia M (1995) Improvement of mycorrhizal synthesis between micropropagated *Helianthemum almeriense* plantlets with *Terfezia claveryi* (desert truffle). In: Elliot TJ (ed) Science and Cultivation of Edible Fungi, vol 2, Balkema, Rotterdam pp 863–868

Morte A, Honrubia M (1997) Micropropagation of *Helianthemum almeriense*. In: Bajaj YPS (ed) High-tech and micropropagation VI, vol 40. Springer, India, p 163

Morte A, Honrubia M (2009) Biotechnology for the industrial production of ectomycorrhizal inoculum and mycorrhizal plants. In: Chauhan AK, Varma A (eds) A textbook of molecular biotechnology. IK International Pvt Ltd, India, pp 691–704

Morte A, Honrubia M, Gutiérrez A (2008) Biotechnology and cultivation of desert truffles. In: Varma A (ed) Mycorrhiza: state of the art genetics and molecular biology, eco-function, biotechnology, eco-physiology, structure and systematics. Springer, Heidelberg, Berlin, pp 467–483

Morte A, Navarro-Ródenas A, Nicolás E (2010) Physiological parameters of desert truffle mycorrhizal *Helianthemum almeriense* plants cultivated in orchards under water deficit conditions. Symbiosis 52(2–3):133–139. doi:10.1007/s13199-010-0080-4

Morte A, Zamora M, Gutiérrez A, Honrubia M (2009) Desert truffle cultivation in Semiarid Mediterranean areas. In: Azcón-Aguilar C, Barea JM, Gianinazzi S, Gianinazzi-Pearson V (eds) Mycorrhizas—functional processes and ecological impact. Springer, Heidelberg, Berlin, pp 221–233. doi:10.1007/978-3-540-87978-7_15

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plantarum 15(3):473–497

Nauš J, Prokopová J, Řebíček J, Špundová M (2010) SPAD chlorophyll meter reading can be pronouncedly affected by chloroplast movement. Photosynth Res 105(3):265–271. doi:10.1007/s11120-010-9587-z

Navarro-Blaya S, Navarro-García G (2003) Química agrícola: El suelo y los elementos químicos esenciales para la vida vegetal. Mundi-Prensa Libros Chapter 6, pp 99–118, ISBN 84-8476-155-X

Navarro-Ródenas A, Lozano-Carrillo MC, Pérez-Gilabert M, Morte A (2011) Effect of water stress on in vitro mycelium cultures of two mycorrhizal desert truffles. Mycorrhiza 21(4): 247–253. doi:10.1007/s00572-010-0329-z

Navarro-Ródenas A, Pérez-Gilabert M, Torrente P, Morte A (2012) The role of phosphorus in the ectendomycorrhiza *continuum* of desert truffle mycorrhizal plants. Mycorrhiza 22(7):565–575. doi:10.1007/s00572-012-0434-2

Nguyen QT, Kozai T (2005) Photoautotrophic micro-propagation of woody species. In: Kozai T, Afreen F, Zobayed SMA (eds) Photoautotrophic (sugar-free medium) micropropagation as a new micropropagation and transplant production system. Springer, The Netherlands, pp 123–146. doi:10.1007/1-4020-3126-2_8

Park SW, Jeon JH, Kim HS, Park YM, Aswath C, Joung H (2004) Effect of sealed and vented gaseous microenvironments on the hyperhydricity of potato shoots in vitro. Scientia Horticulturae 99(2):199–205. doi:10.1016/s0304-4238(03)00097-9

Pérez-García F, González-Benito ME (2006) Seed germination of five *Helianthemum* species: effect of temperature and pre-sowing treatments. J Arid Environ 65(4):688–693. doi:10.1016/j.jaridenv.2005.10.008

Phillips J, Hayman D (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55(1):158–161

Pospóšilová J, Tichá I, Kadleček P, Haisel D, Plzáková Š (1999) Acclimatization of micropropagated plants to ex vitro conditions. Biol Plantarum 42(4):481–497. doi:10.1023/a:1002688208758

Quoreshi AM (2003) Nutritional preconditioning and ectomycorrhizal formation of *Picea mariana* (Mill.) BSP seedlings. Euras J For Res-Hokkaido University 6:1–63

Quoreshi AM, Timmer VR (1998) Exponential fertilization increases nutrient uptake and ectomycorrhizal development of black spruce seedlings. Can J Forest Res 28(5):674–682. doi:10.1139/x98-024

Quoreshi M, Timmer VR (2000) Growth, nutrient dynamics, and ectomycorrhizal development of container-grown *Picea mariana* seedlings in response to exponential nutrient loading. Can J Forest Res 30(2):191–201. doi:10.1139/x99-208

Righetti B (1996) Chlorophyll, ethylene and biomass determination in *Prunus avium* cv. Victoria cultivated in vitro under different atmospheric conditions. J Hort Sci 71(2):249–255

Rivas J (2008) La luz y el aparato fotosintético. In: Azcon-Bieto J, Talon M (eds) Fisiología y bioquímica vegetal. McGraw Hill, Madrid, España, pp 165–190

Roth-Bejerano N, Livne D, Kagan-Zur V (1990) *Helianthemum-Terfezia* relations in different growth media. New Phytol 114(2):235–238. doi:10.1111/j.1469-8137.1990.tb00395.x

Rudawska M, Kieliszewska-Rokicka B, Debaud J-C, Lewandowski A, Gay G (1994) Enzymes of ammonium metabolism in ectendomycorrhizal and ectomycorrhizal symbionts of pine. Physiol Plantarum 92(2):279–285. doi:10.1111/j.1399-3054.1994.tb05338.x

Shaw Iii CG, Molina R, Walden J (1982) Development of ectomycorrhizae following inoculation of containerized *Sitka* and white spruce seedlings. Can J Forest Res 12(2):191–195. doi:10.1139/x82-029

Slama A, Fortas Z, Boudabous A, Neffati M (2010) Cultivation of an edible desert truffle (*Terfezia boudieri* Chatin). Afr J Microbiol Res 4(22):2350–2356

Torrente P, Navarro A, Gutiérrez A, Morte A (2009) Micropropagación de *Helianthemum hirtum* y micorrización in vitro con micelio de *Terfezia claveryi*. Paper presented at the Octava reunión de la Sociedad Española de Cultivo In Vitro de Tejidos Vegetales, Murcia, España

Uddling J, Gelang-Alfredsson J, Piikki K, Pleijel H (2007) Evaluating the relationship between leaf chlorophyll concentration and SPAD-502 chlorophyll meter readings. Photosynth Res 91(1):37–46. doi:10.1007/s11120-006-9077-5

Urrestarazu-Gavilán M (2004) Tratado de cultivo sin suelo, 3rd edn. Mundi-Prensa, Madrid

Väre H (1990) Effects of soil fertility on root colonization and plant growth of *Pinus sylvestris* nursery seedlings inoculated with different ectomycorrhizal fungi. Scand J Forest Res 5(1–4):493–499. doi:10.1080/02827589009382632

Xiao Y, Niu G, Kozai T (2011) Development and application of photoautotrophic micropropagation plant system. Plant Cell Tiss Organ Cult 105(2):149–158. doi:10.1007/s11240-010-9863-9

Xu G, Fan X, Miller AJ (2012) Plant nitrogen assimilation and use efficiency. Ann Rev Plant Biol 63:153–182

Zambonelli A, Bonito GM (2012) Edible ectomycorrhizal mushrooms. Soil biology, vol 34. Springer, Heidelberg, Berlin. doi:10.1007/978-3-642-33823-6_14

Zaretsky M, Kagan-Zur V, Mills D, Roth-Bejerano N (2006) Analysis of mycorrhizal associations formed by *Cistus incanus* transformed root clones with *Terfezia boudieri* isolates. Plant Cell Rep 25(1):62–70. doi:10.1007/s00299-005-0035-z

Zhu Z, Gerendás J, Bendixen R, Schinner K, Tabrizi H, Sattelmacher B, Hansen UP (2000) Different tolerance to light stress in NO_3^- and NH_4^+ grown *Phaseolus vulgaris* L. Plant Biol 2(5):558–570. doi:10.1055/s-2000-7498

Zobayed SMA, Afreen F, Kubota C, Kozai T (2000) Water control and survival of *Ipomoea batatas* grown photoautotrophically under forced ventilation and photomixotrophically under natural ventilation. Ann Bot 86(3):603–610. doi:10.1006/anbo.2000.1225

Zobayed SMA, Afreen F, Xiao Y, Kozai T (2004) Recent advancement in research on photoautotrophic micropropagation using large culture vessels with forced ventilation. Vitro Cell Dev Biol Plant 40(5):450–458. doi:10.1079/ivp2004558