Chapter 14
Terfezia Cultivation in Arid and Semiarid Soils

Asunción Morte, Alberto Andrino, Mario Honrubia, and Alfonso Navarro-Ródenas

14.1 Introduction

The genus *Terfezia* is formed of hypogeous fungi belonging to the family *Pezizaceae* within the order *Pezizales* (Norman and Egger 1999; Percudani et al. 1999; Laessøe and Hansen 2007). The hypogeus ascocarps of this genus are edible and are well known as desert truffles due to the nature of their distribution, which is typical of countries or territories with arid and semiarid ecosystems in the Mediterranean region, Middle East, and Southwestern Asia.

Terfezia species form mycorrhizal symbiosis with different annual and perennial rockrose species of the genus *Helianthemum*, including chamaephytes, hemicryptophytes, and therophytes (Table 14.1). Consequently, this species grows in open, sunny scrubland, or in the meadows of mountain plains, or sandy and rocky soils of arid deserts.

Since the first plantation of *Terfezia* mycorrhizal plants was established in 1999 in Murcia (Spain), most of the data related to the biotechnological aspects of the production of mycorrhizal plants and plantation management practices have been compiled in two publications of Springer (Morte et al. 2008, 2009). However, the increasing demand for this crop, not only in Spain but also in other countries, has prompted the research for new strategies to help pass from experimental scale to medium- to large-scale cultivation. One consequence of this leap, the creation of a spin-off company of the University of Murcia, called Thader Biotechnology S.L., has been necessary to satisfy the increasing demand and new data and processes, which are summarized in this chapter.

Since 1999, *Terfezia claveryi* Chatin has been cultivated throughout southeastern Spain and specific places in Israel, Abu Dhabi, and Argentina, and all plantations

A. Morte (✉) • A. Andrino • M. Honrubia • A. Navarro-Ródenas
Dpto. Biología Vegetal (Botánica), Facultad de Biología, Universidad de Murcia, Campus de Espinardo, 30100 Murcia, Spain
e-mail: amorte@um.es; honrubia@um.es

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Table 14.1 Different mycorrhizal associations, obtained under controlled conditions, between species from the *Terfezia* and *Helianthemum* genus

Terfezia species	*Helianthemum* species	References
T. boudieri	*H. salicifolium*	Awameh et al. (1979)
T. claveryi		
T. nivea		
T. pinoyi		
T. leptoderma	*H. salicifolium*	Dexheimer et al. (1985)
T. claveryi		
T. leonis	*H. sessiliflorum*	Roth-Bejerano et al. (1990)
T. arenaria	*H. guttatum*	Fortas and Chevalier (1992)
T. leptoderma		
T. claveryi		
T. claveryi	*H. almeriense*	Morte et al. (1994)
T. claveryi	*H. ledifolium*	Gutiérrez (2001)
T. terfezioides	*H. ovatum*	Kovács et al. (2003)
T. claveryi	*H. violaceum*	Morte et al. (2009)
T. claveryi	*H. hirtum*	Torrente et al. (2009)
T. boudieri	*H. sessiliflorum*	Slama et al. (2010)
T. claveryi	*H. canariense*	Andrino et al. (2011)

have been established with mycorrhizal plants produced by our technology (Morte et al. 2008, 2009). More recently, experimental results have been obtained in Tunisia with *Terfezia boudieri* Chatin, using *Helianthemum sessiliflorum* (Desf.) Pers as a host plant (Slama et al. 2010). Desert truffle fructification occurs 1 or 2 years after plantation, depending on seedling quality, site suitability, and management practices, which are a critical factor for the regularity of truffle production. This chapter describes all the experiments carried out to improve desert truffle production.

14.2 Production of Mycorrhizal Plants

14.2.1 Plant Propagation

The first step in the production of mycorrhizal plants is to choose a suitable host plant species. Among the plant families cited in the literature that contain some species which form mycorrhiza with desert truffles are the following: Cistaceae (Awameh et al. 1979; Alsheikh 1984; Roth-Bejerano et al. 1990; Morte et al. 1994; 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). However, only perennial and annual species from *Helianthemum* genus belonging to the Cistaceae have been reported as host plants for *Terfezia* mycorrhization (Table 14.1). For this purpose, we selected *Helianthemum almeriense* Pau, *Helianthemum violaceum* (Cav.) Pers., *Helianthemum canariense* (Jacq.)

Pers, and, more recently, *Helianthemum hirtum* (L.) Miller and *Helianthemum lippii* (L.) Dum.Cours. as host plants for large-scale production.

Most species of the host plant *Helianthemum* display 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 has been observed during the first 2 months after germination in nursery conditions. Therefore, micropropagation techniques have been used for plant production since seed germination and plant survival with these techniques are both around 90 % (Morte et al. 2008, 2009). The in vitro micropropagation protocols of the studied *Helianthemum* species are quite rapid (about 10 weeks) because plant multiplication, elongation, and rooting occur in the same subculture. Consequently, they are also cheap because only a small amount of plant growth regulators and labor are required (Morte et al. 2009).

However, the acclimatization stage from in vitro to ex vitro conditions is the step in which many plants, above all of *H. hirtum* and *H. lippii*, do not survive. To solve this problem, we have developed a photoautotrophic *Helianthemum* micropropagation system (Andrino et al. 2011) based on the methodology described by Kozai (1991). Until now, the conventional *Helianthemum* technique has been carried out, using culture vessels with agar containing nutrients and sucrose as a carbon source for the plantlets at a low photosynthetic photon flux (PPF) (Morte and Honrubia 1992, 1997; Morte et al. 2009; Torrente et al. 2009). The in vitro environment of this micropropagation system is characterized by a high relative humidity (RH), a high ethylene concentration, still air, and a low CO_2 concentration in the vessel during the photoperiod (Fujiwara and Kozai 1995). This in vitro environment is entirely different from the ex vitro environment of the greenhouse, and it often causes the malfunction of stomata, poor epicuticular wax development, elongated shoots, low chlorophyll concentration, the hyperhydration of plantlets, low growth rates, little rooting, callus formation at the base of explants, and a low survival percentage (Kozai 1991; Majada et al. 2002; Serret et al. 1996). The photoautotrophic micropropagation technique overcomes these problems and is defined as micropropagation without sugar in the culture medium, in which the growth or accumulation of carbohydrates of cultures is fully dependent upon photosynthesis and inorganic nutrient uptake (Kozai 1991; Zobayed et al. 2004).

The photoautotrophic *Helianthemum* micropropagation system mainly consists of replacing agar by autoclave-sterilized perlite that is watered with MS medium nutrient solution (Murashige and Skoog 1962) and without a sugar source. Simply removing sugar from the culture medium without increasing PPF and CO_2 concentration inside the vessel would not promote the growth of culture or plantlets (Xiao et al. 2011). For this reason, the lids of vessels are replaced by transparent holed plastic caps (Fig. 14.1a).

Plant culture growth takes place directly in the greenhouse, with the following environmental conditions: a temperature between 16 °C and 26 °C, relative humidity (RH) of 30–65 %, 350–400 ppm of CO_2, a light intensity range of 95–295 mol m^{-2} s^{-1}, and 0.1–0.7 m s^{-1} wind speed.

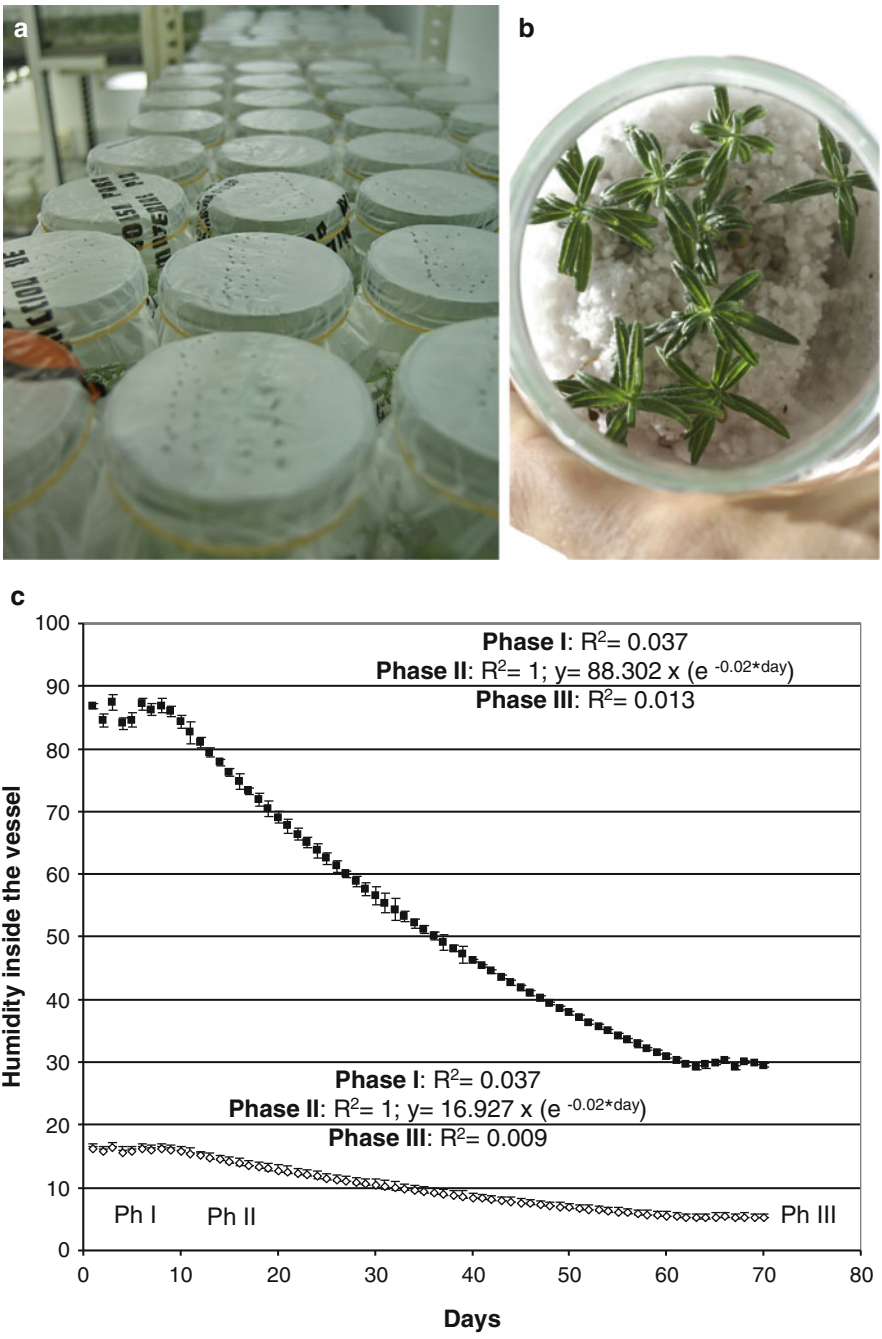

Fig. 14.1 (**a**) Photoautotrophic *Helianthemum* micropropagation system. (**b**) *H. almeriense* plants growing in perlite prior to the mycorrhization inoculation. (**c**) Phases of gradual hardening inside

After the rooting stage, the first holes are opened in the plastic cover (Fig. 14.1a). The size of the holes in the plastic ranges from 0.5 to 1 mm to facilitate the loss of RH inside the container. Their number will be determined by the vessel volume. The gas exchange balance is given by the internal vessel conditions (mainly CO_2, O_2, and RH) and external conditions (mainly CO_2, O_2, RH, and room wind speed) measured in the growing area in the greenhouse. The results show that RH loss with this method describes an exponential curve (Fig. 14.1c).

By opening holes in the plastic covers or using gas-permeable membrane disks, air diffusion or natural ventilation of the culture vessel can be improved and the CO_2 concentration inside the vessel increased during the photoperiod, resulting in enhanced photosynthesis, an increased growth rate, and hence a shorter production period (Cui et al. 2000; Kitaya et al. 2005). Meanwhile, relative humidity inside the vessel is reduced, which leads to increased transpiration and nutrient and water uptake by the plantlets. Numerous studies have shown the benefits of using gas-permeable membrane disks to enhance plantlet growth and quality as a result of increased vessel ventilation rates (Xiao et al. 2011). However, to minimize costs, we preferred to open holes in the plastic covers rather than to use gas-permeable membrane disks.

CO_2 can be a limiting factor for plant growth during the light phase. The use of perforated plastic caps versus normal in vitro culture containers avoids low CO_2 concentrations during the light phase and high concentrations during the dark one. Different gas concentrations (CO_2, O_2, and H_2O) are exchanged by passive diffusion between the perforated container and the outside atmosphere, until both are in balance. Thanks to a limited but necessary wind speed within the growing areas, renewal of the gaseous environment inside the container is favored. Due to a gradual adaptation to decreasing ambient RH, *Helianthemum* seedlings start to gradually control their stomas during the container cycle and not only during the acclimation phase. Coupled with the proper regulation of leaf transpiration, the roots are functional from the moment of emergence.

The rooting and photosynthetic ability of plantlets is usually affected by the physical and chemical nature of the supporting material (Zobayed et al. 2000). When agar was used as supporting material, the roots of *Helianthemum* species were usually thin and fragile. These roots were often damaged during transplanting, resulting in low growth or death of the plantlets. The use of porous supporting materials, such as perlite, improves the root zone environment and increases the

Fig. 14.1 (continued) the container for *H. almeriense*, *H. violaceum*, and *H. canariense* species. Humidity inside the vessel was registered as absolute humidity (*open diamonds* g/cm^3) and relative humidity (*filled square* RH %). These moisture values belong to the culture conditions 18–22 °C, 30–35 % outside RH, 360 ppm of CO_2, 150–170 µmol m^{-2} s^{-1}, and 0.3–0.5 m s^{-1} room wind speed. During phase II (when first holes have been opened), absolute humidity (g cm^{-3}) describes an exponential equation $y = 16.927 \times (e^{-0.02 * day})$. In the case of relative humidity (RH %), an exponential equation is described during phase II: $y = 88.302 \times (e^{-0.02 * day})$. Humidity values (RH %, g cm^{-3}) are the mean of 180 diary data taken every 8 min. *Bars* indicate standard error (SE)

oxygen concentration around the root system, which improves root development and enhances water and nutrient absorption by the plantlets. Moreover, the extensive root system produced in vitro appears to contribute to the higher survival percentage of plants during acclimatization to greenhouse or field conditions (Xiao et al. 2011). In addition, the use of perlite in this stage will facilitate the subsequent mycorrhizal inoculation (Fig. 14.1b).

This production system is not aseptic but fairly clean and insect-free. Moreover, photoautotrophic plantlets grown at high light intensities would be better suited to the intense irradiance found in sunlight, which also facilitates their mycorrhization.

14.2.2 Mycorrhizal Inoculation

Two types of *Terfezia* inocula have been used successfully to produce mycorrhizal plants: spores and mycelium (Morte et al. 2008). However, mature spores are used more frequently than mycelium due to the slow growth of the latter in vitro.

The best growth media for *Terfezia* mycelium growth are MMN (modified Melin-Norkrans) agar 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 (Morte et al. 2008, 2009). However, only *Terfezia* strains well adapted to in vitro conditions should be used to produce mycelium by liquid fermentation in a bioreactor. A recent 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).

Spore suspensions are made taking into account the maturation of the spores. A spore suspension from mature ascocarps consists of 6 g of dried and scratched ascocarps per liter of distilled water. This spore solution is shaken overnight (12 h). Instead of inoculating the plants directly with this spore solution, the spore solution is added to the perlite allowing spore adhesion to the pores and cavities within. Using such a mixture uses between 6 and 10 g of spores per liter of inoculum (Morte et al. 2008), which is approximately 3.5–4.5 $\times$ 10^5 mature spores per plant. The percentage of inoculum per plant is 5 % of the final container volume.

For the production of desert truffle mycorrhizal plants, five in vivo and in vitro options were designed, the time required for each of them ranging between 5 and 9 months, depending on the type of plant propagation system and inoculum source used (Morte et al. 2009) (Table 14.2). In addition, the new photoautotrophic *Helianthemum* micropropagation system proposed here (option 6, Table 14.2) allowed this time to be reduced to 3 months since fungal inoculation is carried out at the moment plants are transferred from in vitro to ex vitro conditions so that plant acclimatization and mycorrhization occur at the same time. Moreover, this

Table 14.2 Options by in vivo and in vitro methods to produce *Terfezia* mycorrhizal plants and the time required for each, depending on the type of plant propagation system and inoculum source used

Option nº	Plant material (*Helianthemum*)	Fungal material (*Terfezia*)	Time for plant production (months)	Time for plant mycorrhization (months)	Total time (months)
1	In vivo germinated seedlings	Mature spore solution	6	3	9
2	In vivo germinated seedlings	Mycelial suspension	6	1–2	7.5
3	Acclimatized micropropagated plants	Mature spore solution	4	3	7
4	Micropropagated plants	Pieces of agar with mycelium	3	2	5
5	Micropropagated plants	Mycelial suspension	3	2	5
6	Photoautotrophic micropropagated plants	Mature spores or mycelium in perlite	2	1	3

last option 6 has other advantages like (1) reduced 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) reduction in labor costs, (6) simplification of the micropropagation system, and (7) no unwanted contamination due to the absence of sugar in the medium.

After the acclimation phase, seedling irrigation is established to maintain the pot water potential between −15 and −30 kPa in nursery conditions. Irrigation management is vital to prevent pathogens that could compete with the mycorrhizal symbiosis. Approximately 30–40 days after transplanting, it is necessary to make a mycorrhization quality control. With this production system, mycorrhization rates range between 75 % and 85 % after 2 months.

14.2.3 Certification of Desert Truffle Mycorrhizal Plants

Characterization of the mycorrhiza formed in the *Helianthemum* root systems by the different *Terfezia* species is extremely important to ensure the high quality of mycorrhizal plants (Morte et al. 2009). For this reason, a morphological and/or molecular analysis of the mycorrhiza should be carried out before planting. Such characterization is also important to evaluate the permanence of the mycorrhiza in 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). At this stage, 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 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 vitro (Gutiérrez et al. 2003; Morte et al. 2008). Therefore, culture conditions can induce changes in mycorrhiza morphology, and there is no clear barrier between these two main types of mycorrhiza organization in *Helianthemum* species (Gutiérrez et al. 2003).

Certification of plant lots for colonization by *Terfezia* 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 as good a mycorrhization percentage of over 33 % of root system. *Terfezia* mycorrhiza has no problems with other contaminant mycorrhizal fungi due to its host specificity.

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

14.3 Plantations and Desert Truffle Production

Since the establishment of the first *Terfezia* orchard in 1999 in the province of Murcia (Spain), more than 20 plantations have been established, not only in Spain but also in Israel, Argentina, and UAE (Abu Dhabi), for which around 30,000 mycorrhizal plants have been produced by our group.

In the last 10 years, carpophores have fructified yearly, and production has increased because of suitable land management techniques and irrigation (Morte et al. 2008, 2009). The application of such plantation management is necessary to maintain desert truffle production because without it, plantations could lose their productivity after 2 and 3 years (Morte et al. 2008). Even so, desert truffle production fluctuates from one year to another in the same orchard. These fluctuations could be due to other environmental or soil conditions, such as temperature and relative humidity, which influence any crop production in the field.

Among the factors that most influence desert truffle production are water availability (irrigation), weed management, season of planting, soil characteristics, and the frame of the plantation.

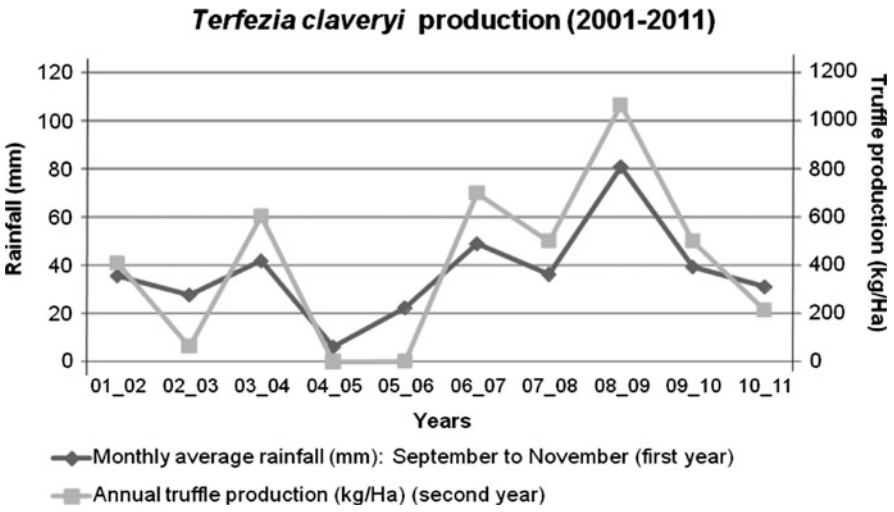

Fig. 14.2 Relation between *T. claveryi* ascocarp production and rainfall over a period of 10 years, in an orchard established in 1999 with 60 *H. almeriense* mycorrhizal plants

14.3.1 Water Availability

Irrigation is one of the most important factors for maintaining successful cultivation since desert truffle fruiting depends on rainfall (Morte et al. 2008).

The estimated desert truffle production in natural areas varies between 50 and 170 kg ha^{-1} in the province of Murcia, after years with a rainfall of between 350 and 400 mm (Honrubia et al. 2003). An irrigation system in the plantation is not necessary when the rainfall is available because the mycorrhizal association is well adapted to arid and semiarid climates (Morte et al. 2000). However, this natural desert truffle production dramatically decreases or even disappears when the rainfall is less than 150 mm, and so irrigation should be applied in dry years.

After following *T. claveryi* production for 10 years in an orchard established in 1999, we observed a statistical correlation, according to Pearson's test (coefficient value of 0.940), between the amount of precipitations during autumn (September, October, and November) of a year and the *T. claveryi* truffle production the following year (Fig. 14.2). This correlation fits a lineal equation with $R^2 = 0.87$.

This new and important finding will help maintain desert truffle production after dry years, by enabling us to adjust soil water potential to the plant physiological parameters necessary to keep the mycorrhizal symbiosis productive.

The main question is whether these fungi, known as "desert truffles," are able to resist dry conditions by themselves or due to association with the host plants. To answer this question, the effect of water deficit on the *T. claveryi* mycelium and on its symbiosis with *H. almeriense* was studied.

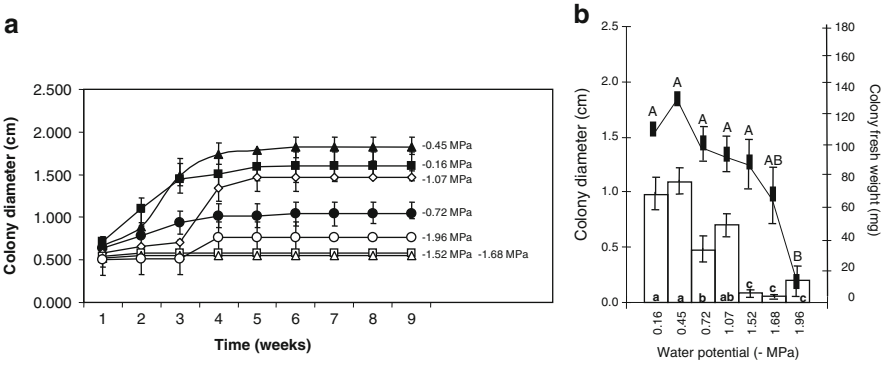

Fig. 14.3 (**a**) Mycelial growth (cm) of *T. claveryi* depending on time (weeks) at different water potentials (*filled square*–0.16, *filled triangle*–0.45, *filled circle*–0.72, *open diamond*–1.07, *open square*–1.52, *open triangle*–1.68, *open circle*–1.96 MPa). Values are the mean of six replicates. *Bars* indicate SE. (**b**) Effect of water potential on the diameter (*bars*, *lower case*) and fresh weight (*lines*, *upper case*) of *T. claveryi* colonies, grown on PEG-amended liquid MMN medium, after 9 weeks. Values followed by the same letter are not significantly different ($P \leq 0.05$) according to Tukey's test. (From Navarro-Ródenas et al. 2011, courtesy of Springer)

14.3.1.1 Effect of Water Deficit on In Vitro Mycelium Cultures of *T. claveryi*

The ability of *T. claveryi* to tolerate water stress was assessed in a pure culture (Navarro-Ródenas et al. 2011). Growth under low water potential conditions, induced using polyethylene glycol (Coleman et al. 1989; Mexal and Reid 1973), should, in theory, reflect the ability of the fungi to grow in dry soil and possibly to obtain water for the associated plant. Growth curves for the different water stress treatments showed an initial lag phase followed by an exponential growth phase and a period of maximum growth, before growth slowed and the colony finally became inactive (Fig. 14.3). The initial colony diameter of the control treatment (−0.16 MPa) exceeded that of the stress treatments; however, as time progressed, the colony diameters in the −0.45 MPa treatment (Fig. 14.3a) were greater than the control. Significant differences were found between two groups of both treatments [−0.45 > −0.16 > −1.07 MPa] and [−1.96 > −1.52 = −1.68 MPa] (Fig. 14.3a). For *T. claveryi*, growth inhibition was higher when expressed in terms of colony diameter than colony fresh weight (Fig.14.3b).

A relationship between fungal fresh weight and hyphal extension (diameter growth) could not be clearly established for *T. claveryi* (Fig. 14.3b). This observation agrees with those of Coleman et al. (1989) who reported that the relationship between fungal dry weight and hyphal extension may not be consistent for each species and can vary depending upon growth conditions. In our case, the lack of a clear correlation between the colony fresh weight and the colony diameter in

T. claveryi mycelium at −1.52, −1.68, and −1.96 MPa can be explained by the fact that, under these conditions, the mycelium grows more in thickness than in length (Fig. 14.3b). This increase in colony density with increased stress explains the type III pattern observed for *T. claveryi* (Coleman et al. 1989).

The isolate of *T. claveryi* exhibited a type III pattern characteristic of drought-tolerant species like *Cenococcum geophilum* Fr. (Coleman et al. 1989) and *Rhizopogon roseolus* (Corda) Th. Fr. (Duñabeitia et al. 2004). However, *T. claveryi* and *Picoa lefebvrei* (Pat.) Maire (Navarro-Ródenas et al. 2011) were only tolerant of moderate water stress below −1.07 MPa, similar to *Rhizopogon luteolus* (Duñabeitia et al. 2004), but they did not tolerate severe stress as other mycorrhizal ascomycete fungi could (Bois et al. 2006).

Terfezia claveryi and *P. lefebvrei* both undergo an extended lag phase before entering the exponential growth phase when grown under stress. Estimating growth rates over a shorter period (1–3 weeks) favored the control, which rapidly entered exponential growth. Estimating growth rates over longer periods (6–7 weeks) favored the stress treatments because fungi under stress have time to acclimate while growth in the control ceases (Navarro-Ródenas et al. 2011). Tolerance to water stress may result from the ability of the fungus to adjust osmotically during stress. The extension of the lag phase with increasing water stress may represent a period of osmotic adjustment (Coleman et al. 1989).

Moreover, soluble and cell wall-bound alkaline phosphatase (ALP) activities were higher when *T. claveryi* mycelium was grown under water stress (−0.45, −0.72, −1.07 MPa), and this increase was significantly different from to the control treatment (−0.16 MPa) at −1.07 MPa and −0.45 MPa for soluble and cell wall-bound ALPs, respectively (Navarro-Ródenas et al. 2011). The increased ALP activity observed in desert truffles at moderate water stress with respect to the control indicates the functional adaptation of these mycelia to drought conditions. Yet, they were able to use the phosphorus from the medium, which becomes more insoluble as water stress increases.

In arid and semiarid soils, the hydrolysis of organic phosphorus is predominantly mediated by the activity of fungal enzymes (Yadav and Tarafdar 2003). *T. claveryi* ascomata have a 2.8-times higher ALP activity than acid phosphatase (ACP) activity (Navarro-Ródenas et al. 2009). As ACP activity was not detected in *T. claveryi* mycelium (unpublished data), such activity can also be considered an indicator of the metabolic activity in these desert truffles.

It is not clear whether the drought tolerance of fungi in pure culture is transmitted to associated host plants. Parke et al. (1983) found no relationship between pure culture experiments and seedling experiments. A positive relationship between pure culture experiments and seedling experiments was observed between radiate pine seedlings and *R. roseolus* (Duñabeitia et al. 2004 and Ortega et al. 2004, respectively). The higher ALP activity in the water-stressed *T. claveryi* mycelium could be related with P accumulation in the drought-stressed mycorrhizal host plant (Morte et al. 2000).

14.3.1.2 Effect of Water Deficit on Desert Truffle Symbiosis

The effect of drought stress on the growth and water relations of the mycorrhizal association of *H. almeriense* with *T. claveryi* has been studied under nursery conditions with plants grown in pots (Morte et al. 2000). In that work, the greater tolerance of mycorrhizal plants to water stress was partially attributed to specific physiological mechanisms based on the chlorophyll content and gas exchange (Morte et al. 2000). Recently, a similar study of the association *Helianthemum sessiliflorum* (Desf.) Pers and *Terfezia boudieri* Chatin grown in pots demonstrated that mycorrhization alters plant physiology, increasing CO_2 assimilation rates and water use efficiency, which helps mycorrhizal plants to adapt to the harsh environmental conditions of deserts (Turgeman et al. 2011).

However, these studies on water deficit in mycorrhizal plants were conducted with potted plants under controlled greenhouse conditions, but recently, new work has been carried out in plots under field conditions (Morte et al. 2010). In this assay, 40 mycorrhizal plants and 40 non-mycorrhizal plants were transplanted to an experimental site in Espinardo (Murcia, Spain). Plant spacing was arranged in a 1×1-m square pattern. However, only mycorrhizal plants survived the experimental period, with a survival rate of 90 %; none of the non-inoculated plant survived to 9 months from planting. Apparently, *H. almeriense* plants are strongly dependent on the presence of a fungal symbiont in their roots for survival (Morte et al. 2010).

After acclimation, two irrigation treatments were applied to the 40 surviving mycorrhizal plants from March 2009 to May 2009. A control treatment (20 well-irrigated plants) maintained a soil matric potential (Ψ_m) of between -10 and -30 kPa (monitored with Watermark tensiometers placed at a depth of 30 cm). A water deficit irrigation condition was created (20 drought-stressed plants), in which irrigation was withheld during the study period, reaching a Ψ_m of around -120 kPa at the end of the experiment.

After the irrigation treatments, drought stress significantly affected the mycorrhizal colonization percentage, which was 70 % in nonirrigated mycorrhizal plants and 48 % in irrigated mycorrhizal plants. This effect was not observed in the potted *H. almeriense* plants where drought stress did not change the degree of root mycorrhizal colonization (Morte et al. 2000). However, no significant differences in plant growth were observed between nonirrigated and irrigated mycorrhizal plants before and after drought stress (Morte et al. 2010).

At the end of the experiment (May), most nonirrigated plants had lost all their leaves. Those watered during the dry season did not lose their leaves. Flowering for *H. almeriense* occurs in several waves (3–5) and lasts for 1 week. Flowering intensity was also similar at the end of the experimental period for both treatments (7.3 and 7.0 capsules/shoot for well-irrigated and drought-stressed mycorrhizal plants, respectively), suggesting that water deficit does not lead to more intense flowering. According to several authors, water stress in many ornamental plants may affect the flowering process, resulting in higher flowering intensity (Sánchez-Blanco et al. 1998; Nicolás et al. 2008), lower flowering intensity under severe

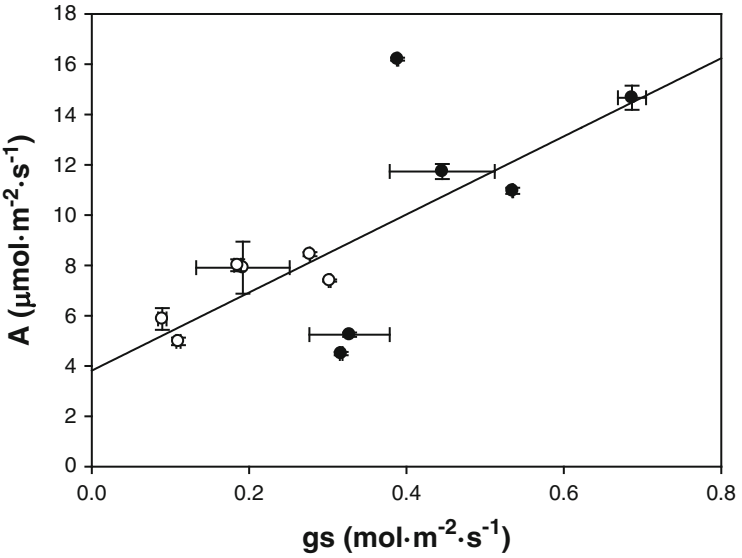

Fig. 14.4 Relationship between net photosynthesis (A) and stomatal conductance (g_s) of well-irrigated (*closed circles*) and drought-stressed (*open circles*) *H. almeriense* mycorrhizal plants at the end of the experimental period. Each point is the mean of three measurements per plant. *Bars* indicate SE. The data fitted a linear regression analysis ($A = 4 + 15g_s$, $R^2 = 0.51$, $P < 0.05$) (from Morte et al. 2010, courtesy of Springer)

drought conditions, or may be unaffected under moderate water deficit conditions (Sánchez-Blanco et al. 2009).

Stomatal conductance was more sensitive to water stress than photosynthesis. At 500 μmol m^{-2} s^{-1}, the irrigated M plants almost reached maximal stomatal conductance (0.41 mol m^{-2} s^{-1}). Nonetheless, the drought-stressed mycorrhizal plants showed a lineal response that reached less than half the maximum values (0.11 mol m^{-2} s^{-1}) (Morte et al. 2010). This stomatal conductance decreased twofold under the drought-stress conditions compared to the control mycorrhizal plants under irrigation and under light saturating conditions. What this finding indicates is the important stomatal closure that takes place under water deficit and low radiation conditions, which improved water use efficiency in the plants grown under drought conditions (Morte et al. 2010).

Moreover, a linear relationship was observed between leaf conductance and net photosynthesis, which suggests the absence of stomatal limitation in the levels of net photosynthesis under irrigated or drought conditions (Fig. 14.4).

It is well known that mycorrhizal fungi can influence water uptake ability and water use efficiency in host plants. On the one hand, the ability to maintain open stomata and photosynthesis during drought could increase the carbon supply for growth, particularly for new root growth, which requires current photosynthates (van den Driessche 1987). An extra supply of carbon is also required for truffle fruiting, and for this stage, most of the carbon derived from photosynthesis should

be allocated to mycorrhizal roots. Therefore, the inoculation of *H. almeriense* plants with *T. claveryi* provided the plant with a greater capacity to tolerate limited soil water availability.

No relationship was found between g_s and shoot water potential (ψ), which exhibited near-isohydric stomatal behavior in the face of a developing water deficit (Morte et al. 2010). The assumed primary role of this mechanism is the avoidance of a low water potential, which leads to xylem cavitation (Jones and Sutherland 1991). *H. almeriense* is a chamaephyte which loses its leaves to avoid drought and high temperatures in summer; however, if watered in the dry season, it is does not lose its leaves, which is probably due to the stomata being capable of preventing ψ from dropping below a critical threshold, such as the turgor loss point or the onset of xylem embolisms (Tyree and Sperry 1989; Franks et al. 2007).

H. almeriense, like many other Mediterranean perennials, shows a conservative water use strategy, based mainly on the avoidance of drought stress by reducing the transpiration rate, that is, by reducing stomatal conductance: the lower the soil water potential, the drier the atmospheric conditions. The results show that mycorrhizal *H. almeriense* plants are able to maintain good physiological parameters at low soil matric potentials (around −120 KPa) in field conditions, thus making them an alternative agricultural crop in arid and semiarid areas.

Two years after plantation, only the *H. almeriense* plants subjected to drought produced *T. claveryi* truffles (three in May 2011). Plants irrigated to water field capacity (−30 KPa) did not produce any ascocarps. This means that an excess of water irrigation would inhibit truffle production in the field, and a certain level of water deficit may actually stimulate the development of *T. claveryi* mycorrhizae, while elimination of the water deficit reduces that stimulus.

14.3.1.3 Molecular Base of Drought Tolerance

Finally, in an attempt to understand which molecular mechanisms are involved in the drought tolerance of desert truffles, we characterized cDNAs from *T. claveryi* and identified a sequence related to the aquaporin (AQP) gene family (Navarro-Ródenas et al. 2012). AQP are water channel proteins that facilitate and regulate the passive movement of water molecules along a water potential gradient. These proteins belong to the large major intrinsic protein family of transmembrane proteins and are represented in all kingdoms (Kruse et al. 2006; Zardoya 2005).

We cloned an AQP gene from *T. claveryi* (*TcAQP1*) (GenBank accession number JF491353) and made a functional analysis by heterologous expression in yeast, finding that this gene increases both water and CO_2 conductivity in biological membranes (Navarro-Ródenas et al. 2012).

The regulation of *TcAQP1* gene expression was tested by quantitative real-time PCR in free-living mycelium under different drought-stress conditions induced with polyethylene glycol (PEG) and in *H. almeriense* mycorrhizal roots cultivated under in vitro conditions (Navarro-Ródenas et al. 2012). When the regulation of *TcAQP1* expression in *T. claveryi* mycelium grown in in vitro culture media supplemented with PEG was evaluated, it was found that *TcAQP1* expression increased at moderate water stress (−0.45, −0.72 MPa) with respect to the control.

However, the *TcAQP1* expression decreased at higher water stress levels (−1.07, 1.52), when it showed similar expression values to the control treatment (Navarro-Ródenas et al. 2012). It seems likely that the active transporter mechanisms of *T. claveryi* mycelium contribute to maintaining cell turgor until an external water potential of −0.74 MPa is reached, at which point *TcAQP1* expression would help in turgor maintenance, facilitating water inflow. However, upon stronger water stress, ion pumping would be unable to maintain an adequate osmotic potential and *T. claveryi* mycelium would reduce its aquaporin expression to avoid loss of turgor. This bimodal behavior in *TcAQP1* expression with respect to external water stress demonstrates its role in improving the drought-stress tolerance of the mycelium.

In mycorrhizal roots of in vitro grown *H. almeriense* plants, the expression of three genes, *TcAQP1*, *EF1-alfaII* (JF491354), and *18S rRNA* (AF206926), was measured. The last two are constitutively expressed in the fungus and in the plant and can be used to normalize *TcAQP1* expression values. In this sense, the difference in the cycle threshold (Ct) values between *TcAQP1* and *EF1-alfaII* gave the *TcAQP1* expression rate, whereas the difference in the Ct values between *EF1-alfaII* and *18S rRNA* illustrated the amount of living mycelium colonizing the root and could be regarded as a measure of the degree of colonization. We observed that *TcAQP1* expression changes as a function of mycorrhizal root colonization. *TcAQP1* expression was seen to depend on the degree of colonization, with basal levels of expression when the colonization degree was low (about 16.2 %) and significantly higher levels when colonization increased (about 26.5 %). Therefore, *TcAQP1* expression was seen to be regulated to some extent during root colonization by the mycelium, which demonstrates the importance of this membrane channel in the mycorrhizal symbiosis. However, further studies are necessary to elucidate the precise role of *TcAQP1* in the symbiosis. One of the possible functions of mycorrhizal symbiosis could be related to its capacity to increase CO_2 permeability (Navarro-Ródenas et al. 2012).

It has been reported that the mycelium of many edible fungi can develop fruit bodies depending on the CO_2 concentration (Stamets 2000). Furthermore, in the cross talk between plant and fungus, the fungal responses to both root exudates and CO_2 concentration appear to define the transition from the asymbiotic to presymbiotic developmental stage (Bécard and Piché 1989). TcAQP1 is the first fungal MIP reported to have a CO_2 transport function. As in the case of other fungi, *T. claveryi* could use CO_2 as a signaling molecule in mycelium organization for ascoma formation or even for the growth and recognition of the root of its host plant, in which TcAQP1 could facilitate its passage through the membrane. This finding opens opportunities for research into the molecular mechanisms of recognition between symbionts (Bonfante and Genre 2010).

14.3.2 Weed Management

Competition from weeds has been shown to reduce desert truffle production (Morte et al. 2009). To reduce the impact of herbaceous competition, weeding is necessary,

Table 14.3 The ranges of the soil characteristics in which most of the *Terfezia* plantations have been made in Spain

Physical–chemical soil properties	
pH (1:2.5 water)	6.8–8.7
E.C. 1:5 (μS/cm)	123.1–302
Sodium (meq/100 g)	0.15–1.20
Potassium (meq/100 g)	0.28–1.5
Calcium (meq/100 g)	4.94–23.38
Magnesium (meq/100 g)	0.95–3.7
Organic matter (%)	0.58–3.92
Total organic carbon (%)	0.34–2.28
Total nitrogen (%)	0.058–0.267
C/N ratio	2.8–10.15
Total carbonate (%)	5.1–80.1
Active lime (%)	3.4–24.76
Phosphorus (ppm)	7.52–66.4
Chloride (meq/100 g)	0.05–0.09
Sulphates (meq/100 g)	0.01–0.32
Iron (ppm)	1.79–79.5
Copper (ppm)	0.31–2.73
Manganese (ppm)	3.03–57.12
Zinc (ppm)	0.3–3.12

at least once a year, in order to avoid plant competition for water and to maintain the open and sunny desert truffle ecosystem.

Two different weed control methods were used: a mechanical tilling between rows with cultivator tines set at 5–8 cm deep and the application of a commercial systemic glyphosate-based herbicide at half the recommended dose. In field conditions, the application of glyphosate has not been shown to produce any inhibition of short root formation or mycorrhizal colonization (Chakravarty and Chatarpaul 1990). However, we prefer the mechanical soil tilling to the herbicide application to keep *Terfezia* cultivation as an organic culture.

Due to the difficulty of controlling weeds in the La Garrobera plantation (Murcia, Spain), established in April 2008 with 3,000 *Helianthemum* mycorrhizal plants distributed in two hectares, the first *T. claveryi* truffles were obtained the third year after plantation (May 2011) rather than in the first or second year. A combination of the two different weed control methods, in early winter and late summer (never during or close to the fruiting season), allowed such weed control. However, it is advisable to study the long-term effects of weed control methods for desert truffle cultivation in field conditions.

14.3.3 Soil Characteristics

The ranges of the characteristics in which most of the plantations have been made in Spain are shown in Table 14.3.

In general, the soils used for plantations are poor and characterized by a clay loamy texture, basic pH (8.5), and low values of electrical conductivity (123 μs cm^{-1}), organic carbon (0.9–3.9%), and C/N ratio (7–10). These characteristics, especially pH and texture, change depending on the *Terfezia* species which grow in acid soils.

Soil texture can influence ascoma shape (Malençon 1973) but is not a determinant factor for truffle formation, since *T. claveryi* ascomata have been produced in clay loamy soils (plantations in Murcia) as well as in sandy soils (plantations in Lanzarote, Canary Islands). Similar results have been obtained for *T. boudieri* ascocarps in plantations in sand loamy and gypsy soils (Slama et al. 2010).

Soil fertilization has not been necessary until now. Fertilization has never been applied in the oldest plantation (12 years), and mycorrhizal *H. almeriense* plants are still producing *T. claveryi* truffles.

In Spain, *Terfezia* cultivation is generally associated with other crops like almond and olive trees to make the most of land use and irrigation facilities. This is possible because there is no competition for host plants between arbuscular mycorrhizal fungi from almond (Fig. 14.5a) and olive trees and the desert truffle species.

14.3.4 Season and Frame of Plantation

After testing in all seasons, spring was selected as the best time to set up a plantation owing to its moderate temperatures, the abundance of precipitations, and the long photoperiod (Morte et al. 2008). Moreover, planting in spring is essential to obtain the first ascocarps the following spring (11–12 months from plantation). When *Terfezia* orchards were established in autumn, the desert truffle production did not start in the following spring but the next one (19–20 months from plantation).

We tested different frames of plantation, 0.5 × 0.5, 1 × 1, 3 × 3, 3 × 2, and 4 × 2 m, distributed alternately in rows, ridges, and groups (9–12 plants). According to our experience, although a wide frame (4 × 2 m in rows) facilitates mechanical soil tilling and plant physiological measurements (Fig. 14.5d), a narrow frame (0.5 × 0.5 m in groups) is advisable to obtain desert truffles in the first year after plantation.

The distribution in ridges much facilitates mycorrhiza sampling (Fig. 14.5b) and ascoma collection (Fig. 14.5c, e), even for wild boars that usually eat them! In this sense, a fence surrounding the plants is necessary to avoid undesirable desert truffle hunting.

14.3.5 Conclusions

In order to obtain maximum *Terfezia* production in orchards, we recommend the following summarized management practices:

Fig. 14.5 (**a**) *T. claveryi* cultivation in *Helianthemum* species (*arrows*) associated with almond trees. (**b**) The distribution in ridges very much facilitates mycorrhiza sampling. (**c**) Ascoma of *T. claveryi* cultivated in orchard with *H. almeriense*. (**d**) Leaf gas exchange measurements (net photosynthetic rate and stomatal conductance were measured with a portable photosynthesis system LI-6400) in *H. almeriense* mycorrhizal plants in the orchard. (**e**) Ascoma of *T. claveryi* obtained from the orchard. (**f**) Desert truffles are food: a plateful of raw, sliced desert truffle as a salad, dressed in olive oil and salt

1. To use high-quality mycorrhizal plants.
2. To establish the plantation in spring.
3. To maintain a soil water potential between −50 MPa (during fruiting season) and −120 MPa (during summer) by means of an appropriate irrigation system.

This should take into account that the amount rainfall during autumn of one year directly influences *T. claveryi* truffle production the following year.
4. Weed elimination but never before and during the desert truffle fruiting season.
5. A narrow frame of plantation is advisable to obtain desert truffles the first year after plantation.
6. No fertilization.

14.4 Desert Truffle Market and Interest

Desert truffles have been known since ancient times and have been associated with Mediterranean cultures since their origins. We now know they were traded by the Greeks and Romans alike and were imported from Libya to be sold in various markets of the respective Empires (Honrubia et al. 2007). They have continued to be marketed and consumed and, even today, are linked with all three Mediterranean cultures: Christian, Jewish, and Muslim (Morte et al. 2009).

Sizable quantities of several species of wild *Terfezia* are collected and marketed in southern Europe, parts of North Africa, and other countries bordering the Mediterranean Sea. However, natural areas of desert truffles have been disappearing during the last 50 years. Large areas of the coastal desert in Egypt and Libya were mined in World War II. Certain aspects of the Gulf War seem to have ruined many truffle-gathering areas in Kuwait (Morte et al. 2008). In Spain as well as in Dubai and Abu Dhabi, this is due to the huge construction processes taking place in these "sunny" areas over the last 15 years. Moreover, climate change and global warming have reduced total rainfall and therefore the amount of naturally produced desert truffles.

The collection of desert truffles is a manual task accomplished well by those who are able to recognize the crack formed by the fungus in the soil near the host plant. Dogs or any olfactory animals are not necessary if the desert truffle hunter knows how to recognize the host plant and the crack in the soil.

Interest in desert truffles is not merely culinary and gastronomic, and not even purely commercial, since truffles also offer a high nutritional dietary value (Murcia et al. 2003). In addition, some studies have reported its antibiotic capacity (Janakat et al. 2004, 2005) and hepatoprotective activity against CCl_4 (Janakat and Nassar 2010). One of the most interesting food properties of desert truffles is their antioxidant activity with regard to their ability to inhibit lipid oxidation (Murcia et al. 2002), with higher percentages of oxidation inhibition than some common food antioxidants, even after being subjected to industrial freezing and canning processes (Murcia et al. 2002).

Moreover, they are a priori "ecological" given the ecosystem they are produced in and because of their potential for promoting rural development if we take into account the possibility of their being established as an alternative or complementary crop in a sustainable farming context (Morte et al. 2009).

The prices of the desert truffles vary between 20 € and 250 €. However, these prices are much lower than those of the *Tuber* species. One reason for this is that desert truffles are not as strongly flavored as *Tuber* species. However, marketing efforts could help promote desert truffles and increase their commercial value (Morte et al. 2008).

In Spain, *Terfezia* species are usually prepared as a plateful of raw, sliced desert truffles as a salad, dressed in olive oil and salt (Fig. 14.5f). However, the increasing interest of the general public in gastronomy has led many cooks to prepare more elaborate desert truffle dishes.

Most *Terfezia* and *Tirmania* species collected from Egypt and Morocco are sold in markets in Abu Dhabi, Doha, Kuwait, and Riyadh, which might explain why some of these countries are increasingly interested in desert truffle cultivation. Cultivation could avoid overexploitation of natural desert truffle areas that could otherwise lead to the disappearance of some desert truffle species.

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