Chapter 7
Advances in Desert Truffle Mycorrhization and Cultivation

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7.1 Introduction

Desert truffles include a group of edible hypogeous fungi that belong to different genera within the order Pezizales in the Ascomycota division. The most important genera are *Terfezia*, *Picoa* and *Tirmania* (Fig. 7.1).

These fungi are frequent in acid and alkaline soils of the Mediterranean Basin, and their fructification period ranges from February to May, depending on the quantity and distribution of precipitations that occurred along the year (Honrubia et al. 2007). Desert truffles have a great interest from an ecological point of view because the group of Cistaceae host plants with which they establish a symbiotic mutualism (Fig. 7.2) is well adapted to semiarid and arid environments. Desert truffles also have an important economic interest due to their great nutritional and gastronomic values (Murcia et al. 2003) and marketable fruiting bodies (Volpato et al. 2013).

7.2 Mycorrhizal Symbiosis

First descriptions of mycorrhizae of *Terfezia* with some species from the *Helianthemum* genus were made by Awameh et al. (1979), who, after obtaining axenic fungal culture, were able to achieve the first mycorrhizal synthesis with *Terfezia*. Later on, Chevalier et al. (1984) carried out the synthesis of *Terfezia leptoderma* mycorrhizae with different *Cistus* species. At that time, this type of mycorrhiza was considered to be intermediate between endotrophic and ectotrophic.

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Fig. 7.1 The most appreciated edible desert truffle species in the market: (**a**) *Terfezia claveryi*, (**b**) *Terfezia boudieri*, (**c**) *Picoa lefebvrei*, (**d**) *Tirmania nivea*, (**e**) *Tirmania pinoyi*, from alkaline soils, (**f**) *Terfezia arenaria*, (**g**) *Terfezia fanfani*, from acid soils

The first mycorrhizal synthesis of *T. claveryi* with *H. almeriense* was carried out by Cano et al. (1991). Then, Morte et al. (1994) obtained this symbiosis in vitro conditions and characterized the synthetized mycorrhizae as ectendomycorrhizae, both with intercellular and intracellular hyphae and with structures similar to coils. Furthermore, some hyphae growing around the roots were observed, but this was not considered a true mantle (Morte et al. 1994). Alsheikh (1984) differentiated this type of mycorrhiza from ecto-, endo- and ectendomycorrhizae and found similarities with some arbutoid mycorrhizae. As a result, the term "helianthemoid" was proposed, in order to describe the mycorrhiza formed between different species of *Helianthemum* and some desert truffles. Kovács et al. (2003) suggested that the term "terfezioid" was more reasonable than "helianthemoid" since they found this type of intermediate mycorrhiza in *Robinia pseudoacacia* and *Helianthemum ovatum*

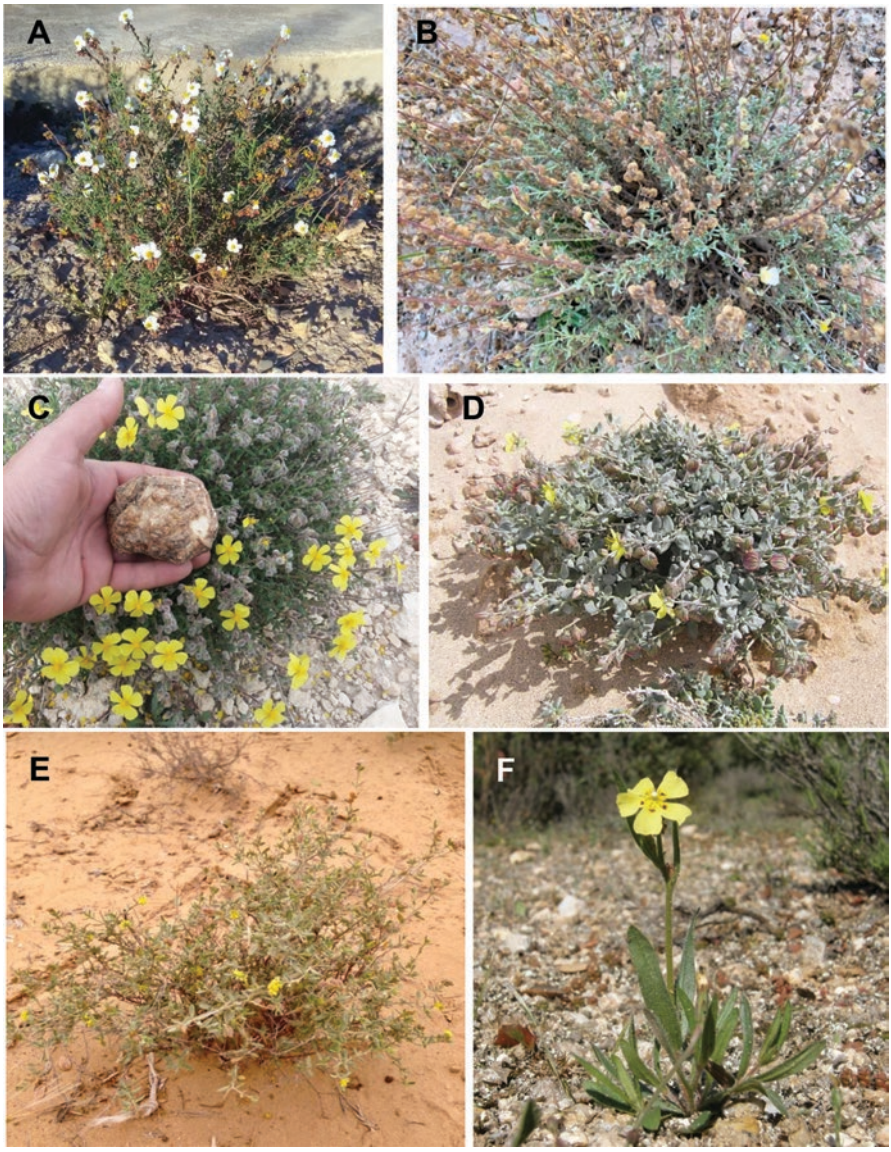

Fig. 7.2 Host plant species for desert truffles: (**a**) *Helianthemum almeriense*, (**b**) *Helianthemum violaceum*, (**c**) *Helianthemum hirtum*, (**d**) *Helianthemum canariense*, (**e**) *Helianthemum lippii*, from alkaline soils, (**f**) *Tuberaria guttata*, from acid soils

and considering other associations with *Terfezia* previously described (Kagan-Zur et al. 1999). Fortas and Chevalier (1992) showed for the first time, in different growing conditions, that the type of mycorrhizae established between *Helianthemum guttatum* (=*Xolantha guttata*) and different desert truffles depends on the phospho-

rus content of the medium. These authors observed, in the same root, structures of both an endomycorrhiza and an ectomycorrhiza in media with low phosphorus, while only ectomycorrhizal characteristics were observed in media with high phosphorus contents. Kagan-Zur et al. (1994) showed that, at the same time, low phosphorus concentrations in the medium culture may inhibit the mycorrhization between *Helianthemum sessiliflorum* and *Terfezia leonis*, but that the same mycorrhization could be stimulated by low iron concentration.

Gutiérrez et al. (2003) described the association formed by *T. claveryi* with *H. almeriense* as an endomycorrhiza in field conditions, an ecto- and ectendomycorrhiza in greenhouse conditions and an ectomycorrhiza with mantle and Hartig net in vitro conditions (Fig. 7.3). The authors proposed that these differences probably depended on the phosphorus concentration of the medium, since in nursery conditions the phosphorus concentration was intermediate between field and in vitro conditions. Furthermore, these authors described four mycorrhizal morphotypes in roots of *H. almeriense* mycorrhized by *T. claveryi*: "club-shaped", "capitated", "moniliform" and "branched" (Gutiérrez et al. 2003). Kovács et al. (2003) observed increased fungal colonization in the in vitro association between *T. terfezioides* (=*Mattirolomyces terfezioides*) and two host plants with high inorganic phosphorus concentration in the medium culture. On the other hand, Zaretsky et al. (2006), using transformed roots of *Cistus incanus* inoculated with *T. boudieri* mycelium, proposed that the type of mycorrhiza formed is not only influenced by the fungal

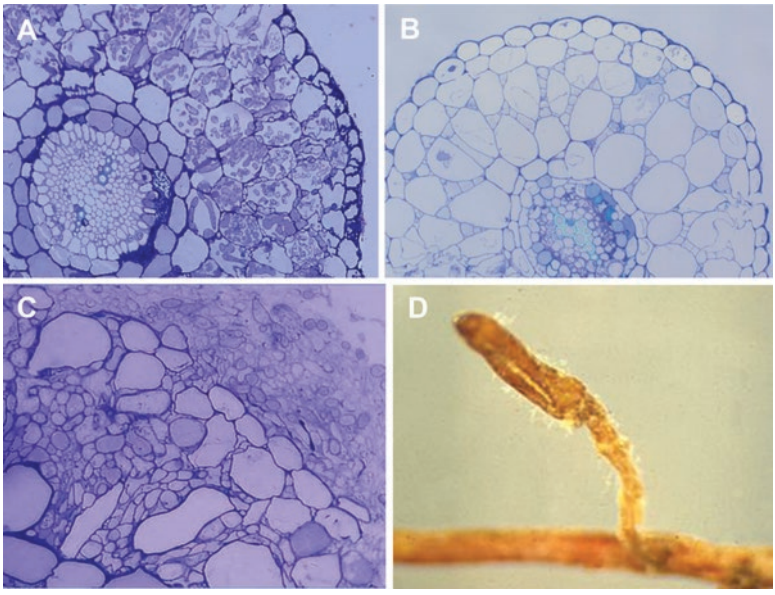

Fig. 7.3 Morphologies of the ectendomycorrhizal *continuum* between *H. almeriense* and *T. claveryi*: (**a**) inter- and intracellular hyphae, (**b**) Hartig net, (**c**) Hartig net with mantle, and (**d**) "club-shaped" root morphotype. (Photos A, C and D from Gutiérrez et al. (2003), with kind permission of Springer Science)

strain but also by the host plant's sensitivity to indoleacetic acid. This sensitivity may be related to the phosphorus and auxin exogenous levels.

Later on, Navarro-Ródenas et al. (2013) found that the mycorrhizal colonization between *H. almeriense* and *T. claveryi* increases as the availability of phosphorus and water decreases in the substrate. They found all types of colonization (intracellular, intercellular and both) in the same root, and they determined that the proportion of each one depended on the growing conditions. This led to propose the term "ectendomycorrhiza *continuum*", to define the morphology of this intermediate symbiosis (Navarro-Ródenas et al. 2013).

7.3 Mycorrhizal Plant Production

Having different in vivo and in vitro systems for plant and fungus propagation allowed to carry out several tests and combinations for the mycorrhizal synthesis between different species of *Helianthemum* with *T. claveryi* (Morte et al. 1994, 2008, 2009). In 1999, the first plantation of 60 plants of *H. almeriense* mycorrhized by *T. claveryi* was established in Zarzadilla de Totana (Murcia, Spain) (Gutiérrez 2001). The plantation produced the first truffles shortly before reaching 2 years after plantation (Honrubia et al. 2001). This plantation, the first one in the world, kept producing truffles until now, with an average of 250–400 kg/ha (Morte et al. 2008, 2009). During the last 15 years, more than 30 plantations have been established in Spain, with approximately 50,000 mycorrhizal plants of different *Helianthemum* species (*H. almeriense*, *H. violaceum* or *H. hirtum*) associated with *T. claveryi*.

The recent increasing demand for mycorrhizal plants has led to the search of new strategies in plant propagation, inoculum production (Morte et al. 2012) and the use of microorganisms present in the soil, such as plant growth-promoting rhizobacteria (PGPR) (Navarro-Ródenas et al. 2016), in order to increase the number and quality of desert truffle mycorrhizal plants.

Most of the bioassays for the production of ectomycorrhizal plants use seeds for seedling production and fungal spores as inoculum (Morte and Honrubia 2009). The same method is normally used to produce mycorrhizal seedlings of *Helianthemum x Terfezia*, but many *Helianthemum* species show erratic seed germination with high mortality of seedlings during the first 2 months after germination in nursery conditions (Morte et al. 2012). In vitro micropropagation techniques have solved this problem, getting up to 90% plant survival and a rapid micropropagation since plant multiplication, elongation and rooting occur in the same subculture, with no need of successive sub-cultivations nor addition of growth regulators (Morte et al. 2009, 2012). Moreover, a system of photoautotrophic micropropagation, based on the methodology described by Kozai (1991), has been designed for *H. almeriense* that involves a successful acclimatization from in vitro to ex vitro conditions (Andrino et al. 2012, Morte and Andrino 2014). In vitro conditions generally lead to a stomatal malfunction, poor development of epicuticular waxes, elongated and etiolated stems and poor root development (Kozai 1991, Majada et al. 2002). The methodology

consists in replacing agar with sterilized perlite moistened with MS culture medium (Murashige and Skoog 1962), without carbon source, increasing photosynthetic light intensity (140–160 μmol/m^2 s) and providing an atmosphere with low relative humidity (60–70%) and 350–400 ppm of CO_2, by using plastic covers that allow gas exchange (Morte and Andrino 2014).

In relation to the inoculum of desert truffles, spore solution is commonly used due to the erratic and slow growth of the pure culture mycelium. However, a great effort has been made to improve culture conditions and nutrients and understand factors or inhibitors that may limit *Terfezia* growth. Cano et al. (1991) carried out the first pure culture isolation of *T. claveryi*, establishing that the best growing medium for this fungus was MMN (Marx, 1969) with a pH adjusted to 7.0. Later, Navarro-Ródenas et al. (2011) proved that *T. claveryi* and *P. lefebvrei* mycelia require some water potential adjustment of the in vitro culture medium, growing better with a moderate water stress of −0.45 and −0.72 kPa for *T. claveryi* and *P. lefebvrei*, respectively. At the base of that tolerance to water stress, a greater expression of the *T. claveryi* aquaporin TcAQP1 gene was found, a membrane protein that acts as a water channel and other substances facilitating the transport of water between cells (Navarro-Ródenas et al. 2013). Moreover, the use of cyclodextrins (CD), especially β-CD, could stimulate mycelial growth of *T. claveryi* until achieving a final diameter and a growth rate five times greater than that of the control without CD (López-Nicolás et al. 2013).

Recently, we have found that the true limiting factor of in vitro growth of *T. claveryi* is not nutrients, nor growing conditions, but the deficiency in certain growth factors, such as vitamins involved in glucose catabolic pathways, that the fungus may not be able to synthesize. Thus, an assay involving response surface methodology was performed using Box-Behnken design to find the optimal parameters for the high production of *T. claveryi* mycelial biomass (Arenas et al. 2018). The best results were obtained with glucose as carbon source, buffering the pH at 5 during culture, adding a pool of vitamins and adjusting the optimal concentrations of carbon and nitrogen sources of the MMN medium to 15 and 0.6 g L^{-1}, respectively. Biomass production of strain T7 in the bioreactor increased from 0.3 to 3 g L^{-1} dry weight, and productivity increased from 10.7 to 95.8 mg L^{-1} day^{-1} dry weight, thus providing a suitable amount of mycelium for large-scale mycorrhizal inoculation (Arenas et al. 2018).

Generally, the mycorrhizal symbiosis involves other soil microorganisms that are in most cases beneficial or even necessary for a right development of mycorrhizal plants (Azcón 2014). Of these microorganisms, the most studied are PGPR bacteria, a heterogeneous group of soil bacteria that can stimulate plant growth, protect them from diseases or increase their production (Bhattacharyya and Jha 2012).

In order to identify native PGPR associated with desert truffles, Navarro-Ródenas et al. (2016) isolated bacteria from the mycorrhizosphere of *H. almeriense* roots and from the peridium of *T. claveryi*, which were characterized according to several PGPR traits (auxin and siderophore productions, phosphorus solubilization and ACC deaminase activity). Furthermore, the effect of some of these bacteria at different stages of the production of mycorrhizal plants with desert truffles was evaluated. After a phylogenetic analysis of the 16S rDNA, 64 bacterial colonies

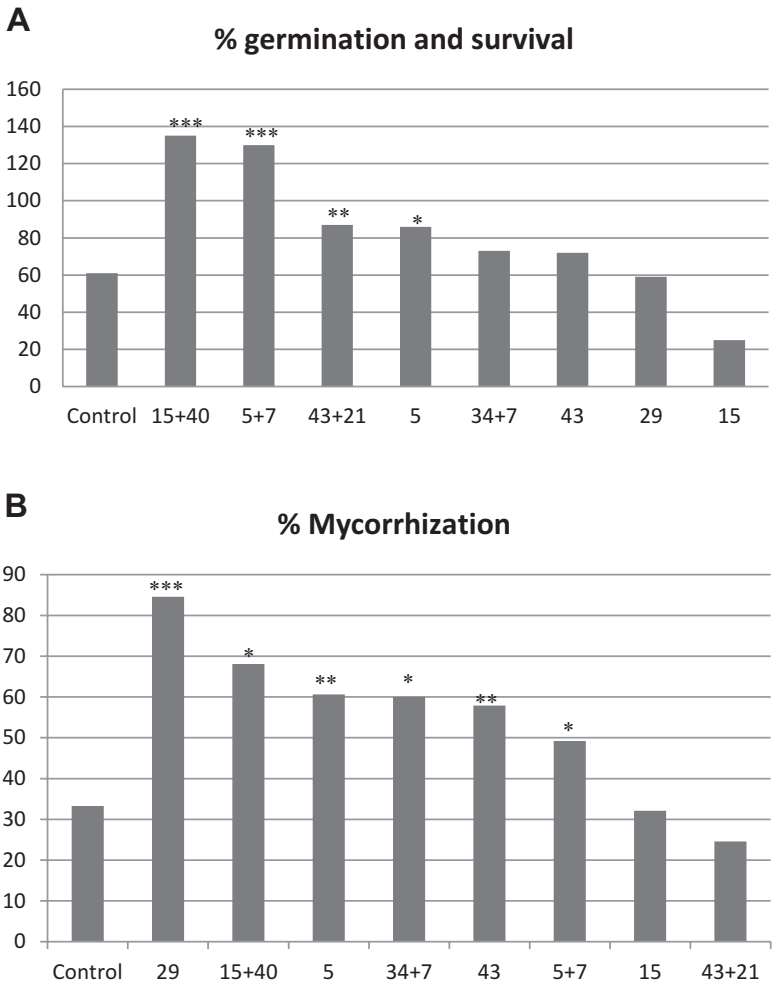

Fig. 7.4 Germination and survival (**a**) and mycorrhization (**b**) percentages of *H. almeriense* inoculated with different combinations of bacteria strains after 4 weeks in nursery conditions. 5 = *Pseudomonas fluorescens*; 15 = *Flavobacterium*; 29 = *Ps. mandelii*; 43 = *Arthrobacter* sp.; 5 + 7 = *Ps. fluorescens* + *Pseudomonas* sp.; 34 + 7 = *Ps. brenneri* + *Pseudomonas* sp.; 15 + 40 = *Flavobacterium* sp. + *Pseudomonas* sp.; 43 + 21 = *Arthrobacter* sp. + *Pseudomonas* sp. $*P \leq 0.05$ $**P \leq 0.01$ $***P \leq 0.001$; significant difference in comparison with control according to ANOVA test

were identified that were grouped in 45 strains. The 45 strains belong to 17 genera: *Achromobacter*, *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Flavobacterium*, *Microbacterium*, *Microvirga*, *Novosphingobium*, *Paenibacillus*, *Phyllobacterium*, *Pseudomonas*, *Rhizobium*, *Rhodococcus*, *Sinorhizobium*, *Sphingomonas*, *Stenotrophomonas* and *Variovorax.* The most abundant genera were *Pseudomonas* (40.8%), *Bacillus* (12.2%) and *Variovorax* (8.2%). The rest of the genera did not

exceed 5% of the total of strains. From the 45 strains, seven (15.6%) presented IAA (indoleacetic acid) production, ten (24.4%) produced siderophores, nine (20.0%) were able to solubilize phosphate, one strain (2.2%) showed ACC deaminase production and only one strain of *Pseudomonas fluorescens* showed three activities (P solubilizer, IAA producer and ACC deaminase activity).

Some of these bacteria had a positive effect on seed germination and plant survival, with an increase of 40–122% in comparison with the treatment without bacteria, depending on the bacterial treatment (Fig. 7.4a). The IAA-producing bacteria were particularly relevant during the mycorrhization stage increasing the root-stem ratio and colonization percentage by 47–154% in comparison with plants without bacterial inoculation, depending on the bacterial treatment (Fig. 7.4b).

Moreover, one strain of *Pseudomonas mandelii* was able to considerably increase mycorrhizal colonization but not the plant growth and could be considered as a mycorrhiza helper bacteria (MHB) (Navarro-Ródenas et al. 2016). Further studies have demonstrated that the mycorrhization percentage in plants inoculated with *Ps. mandelii* (40%) or in plants inoculated with bacteria in combination with a drought treatment (60%) was higher than control plants (20%) without bacteria inoculation or water stress (Fig. 7.5a) (Espinosa-Nicolás 2017). Moreover, this bacterium is able to mitigate the negative effect of water stress, maintaining both shoot water potentials of drought stressed and control plants at similar levels (Fig. 7.5b) (Espinosa-Nicolás 2017). *Pseudomonas mandelii* also improved the mycorrhization by *T. claveryi* in other *Helianthemum* species such as *H. violaceum* (Martínez-Ballesteros 2018).

7.4 Ecophysiological and Molecular Aspects of Desert Truffle Mycorrhizal Symbiosis Against Water Stress

In the case of desert truffles, it is essential to study the group of mechanisms that regulate the tolerance of mycorrhizal symbiosis to water stress. *Helianthemum almeriense* mycorrhizal plants with *T. claveryi* presented higher survival in drought conditions, as well as higher transpiration rates, stomatal conductance and photosynthesis than non-mycorrhizal plants, both in drought and irrigation conditions. Under water stress conditions, mycorrhizal plants accumulated higher N, P and K than non-mycorrhizal plants, which shows that the fungus is able to absorb more nutrients from the soil in drought conditions, helping the plant to keep better water and physiological levels (Morte et al. 2000). In another similar study, using desert truffle mycorrhizal plants in field conditions, it was observed that the water stress increased significantly the mycorrhizal colonization percentage up to 70%, while irrigated plants did not exceed 48% of mycorrhization (Morte et al. 2010). Furthermore, water stress induced a change in the mycorrhizal type formed, increasing intracellular colonization under drought stress (Navarro-Ródenas et al. 2013). Results obtained showed that *H. almeriense* mycorrhizal plants with *T. claveryi* maintain good physi-

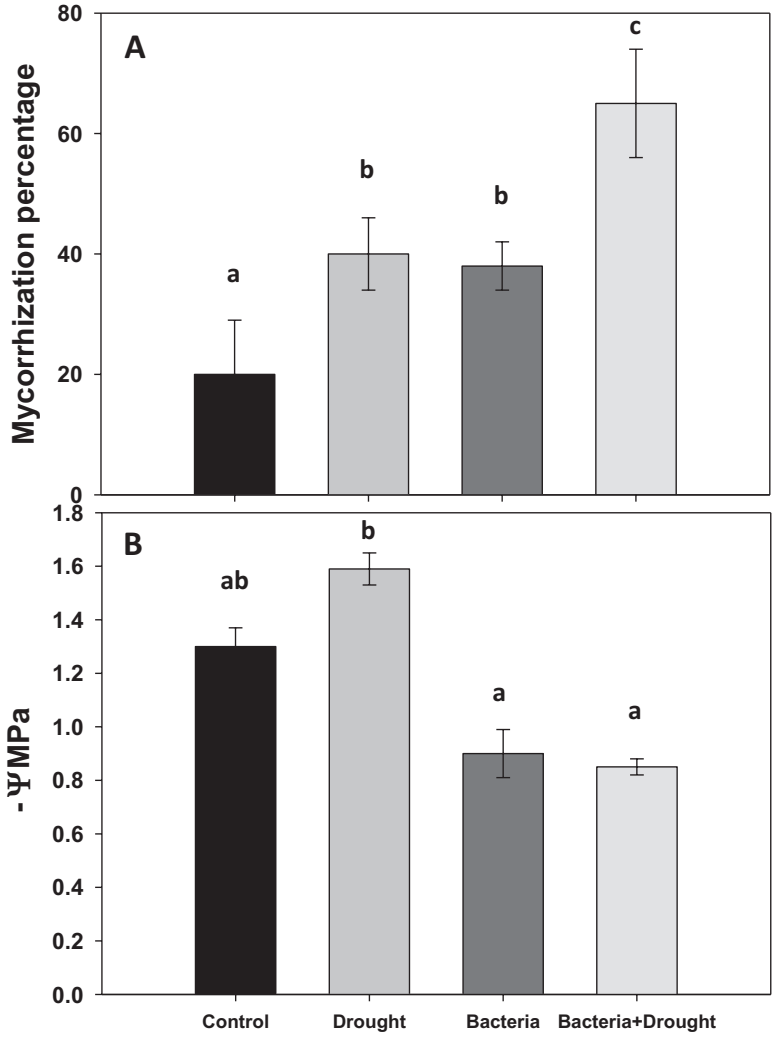

Fig. 7.5 Effect of MHB inoculation (*Pseudomonas mandelii*) and drought treatment on mycorrhization percentage (**a**) and plant water potential (**b**) of mycorrhizal *H. almeriense* plants with *T. claveryi* in nursery conditions

ological parameters with low soil matrix potential, which makes this symbiosis an alternative agricultural crop in arid and semiarid areas (Morte et al. 2010).

The molecular bases that regulate the efficient use of water in this symbiosis were analysed through the studies of aquaporin (Navarro-Ródenas et al. 2013). Aquaporins are membrane channels present in all biological kingdoms, which facilitate and regulate the passive movement of water and other small apolar molecules (Zardoya 2005). Depending on the biological group, the same organisms may present one or two aquaporin isoforms (fungi and bacteria) and up to 70 (in some plants) according

to Park et al. (2010). Based on their amino acid homology, subcellular location and selectivity of different substrates, aquaporins tend to divide in different types: PIP, TIP, NIP, SIP and XIP, and the latter in other sub-groups. Five aquaporin isoforms were identified in *H. almeriense*: two belonging to the PIP1 group (*HaPIP1;1* JF49134 and *HaPIP1;1* JF491350), two belonging to the PIP2 group (*HaPIP2;1* JF491351 and *HaPIP2;2* JF491352) and one belonging to the TIP group (*HaTIP1;1* HQ234609). Some *H. almeriense* aquaporins, from leaves and/or roots, present a fine adjustment of the expression, as a function of photosynthesis or stomatal conductance, where the number of isoforms, under photosynthesis control, is greater when the environmental conditions get more stressful (Navarro-Ródenas et al. 2013).

Recently, we have also described for the first time in the Fungi Kingdom the existence of a post-transcriptional maturation of the LSU rRNA, both in ascocarps and mycelia of some species of the genera *Terfezia* (*T. claveryi*, *T. arenaria*, *T. boudieri*, *T. extremadurensis*, *T. fanfani*) and *Tirmania* (*T. nivea*) (Navarro-Ródenas et al. 2018). This process, which results in two molecules of 1.6 and 1.8 kb, corresponding to the 5′ and 3′ ends, has never been observed in fungi, although it seems to be widespread in other kingdoms, including bacteria, protozoa, worms, several arthropods, fish and rodents. During the evolution of Pezizomycetes, the introduction of sequences susceptible of cleavage to form a hidden gap must have appeared relatively late, since, as far as we know, only these two genera share the hidden gap within the class Pezizomycetes (Navarro-Ródenas et al. 2018).

The biological significance of this non-canonical post-transcriptional maturation of the rRNA LSU is still unknown in the organisms in which it has been described. It seems to be related to a better adaptation and survival in extreme conditions, correlated with a higher fidelity of translation of ribosomes, as seen in the case of the naked mole rat (Azpurua et al. 2013). In addition, the chloroplast ribosomes of an *Arabidopsis thaliana* mutant, which exhibits a defective fragmentation of chloroplast 23S rRNA, have a drastically reduced level of ribulose-1,5-bisphosphate carboxylase/oxygenase and other photosynthetic proteins encoded by chloroplasts (Nishimura et al. 2010). Another interpretation proposes a ribosomal inactivation control mechanism in response to stress conditions by dividing the 60S subunits containing 28S rRNA with a hidden gap (Nomura et al. 2016). *Terfezia* species are adapted to extreme environments and show great tolerance to drought and high temperatures (Navarro-Ródenas et al. 2011, 2013, Zambonelli et al. 2014). It is still unknown if the hidden gap of the *Terfezia* 28S rRNA influences the efficiency of translation under stress conditions and what impact it may have on its biological cycle, so it is necessary to deepen the study of the processing of the hidden gap in these desert truffles and understand if it bears any relation with the slow and erratic in vitro growth of these fungi.

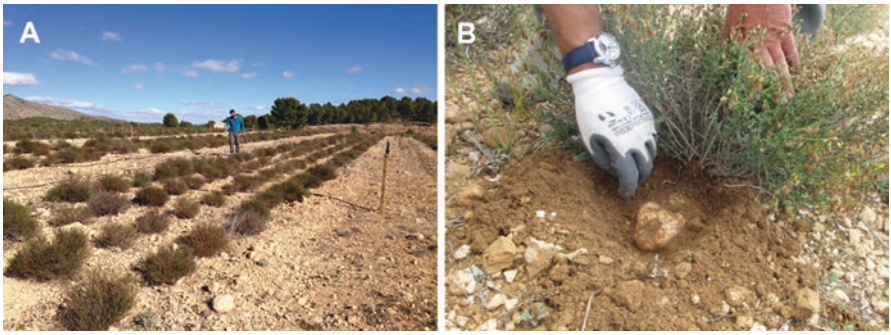

Fig. 7.6 (**a**) Plantation of *Helianthemum almeriense* mycorrhizal plants with *Terfezia claveryi* in Caravaca (Murcia, Spain), (**b**) *T. claveryi* ascocarp fruiting under *H. almeriense* in April

7.5 Desert Truffle Cultivation

The first step in the establishment of a desert truffle plot is to choose suitable host plants and fungal species that are well adapted to the environmental conditions and soil characteristics (Morte et al. 2017a). Moreover, high quality mycorrhizal plants, with certified mycorrhization levels, should be selected (Honrubia et al. 2014).

Field fructifications of *T. claveryi* occur from 1 to 3 years after plantation, depending on the quality of the mycorrhizal plants, the suitability of the site, the plantation season (spring or autumn), the plantation frame and, above all, the irrigation management and the weed elimination (Morte et al. 2012). A successful plantation frame has 1.5 × 1.5 m spacing in four to five rows forming a block, with 2–3 m separation between blocks. This design produced the first ascocarps after 2 years (Fig. 7.6). The small size of these shrubs allows to arrange them closer, thus optimizing the cultivated field. This means a plantation of around 6000 plants/ha, which, while very expensive to establish, could be amortized after 5 years of cultivation if production is adequate (200–450 kg/ha) (Morte et al. 2017a).

Honrubia et al. (2014) analysed and discussed the results of 12 years of experience on desert truffle plantations and the factors that may favour truffle formation in natural production areas through "desert truffle silviculture". A successful example of this mycosilviculture in natural production areas was done in Abu Dhabi (UAE), where the production of *T. boudieri* and *T. nivea* was stimulated by spore inoculation of areas with *Helianthemum lippii* plants and by applying sprinkler irrigation and placing a fence to avoid the consumption of truffles by animals (Gouws et al. 2014).

After following the *T. claveryi* ascocarp production in a plantation with appropriate management during 15 years, a statistical correlation between the precipitation volume during autumn (September, October and November) of 1 year and the truffle production of the following year was found (Morte et al. 2012). This finding has allowed us to maintain the truffle production in dry years, adapting soil water potential to the necessary parameters to keep the mycorrhizal symbiosis productive. The average production of desert truffles in this plantation (with a plantation frame of

0.5 × 0.5 m) was approximately 400 kg/ha, but this production varied from 1 year to another, from a minimum of 2 kg/ha to a maximum of 1050 kg/ha obtained over the course of these 15 years (Morte et al. 2012).

Among all the agro-climatic parameters studied, the cumulative rainfall between September the first and November the 15th of the year before the collection period showed the highest correlation with the production of desert truffle throughout 15 years of study in the plantation, with a Pearson coefficient of 0.926. The next climatic parameter that showed correlation was the cumulative rainfall during March of the year of the collection period, with a Pearson coefficient of 0.643 (Morte et al. 2017b). The model that involved both precipitation period and best adjusted R^2 was a multiple linear regression model, where kg/ha = autumn precipitation∗5.11 + spring precipitation∗2.83–33.7.

Even so, more eco-physiological studies on this symbiosis are needed to evaluate a continuous production over the years, regardless of the weather. It is also necessary to identify possible competitors that, fructifying or not, can displace or cohabit with desert truffles, as well as to quantify the soil fungal biomass necessary for ascocarp formation and analyse its relationship with the vegetation cover.

As it was previously remarked, there are several productive plantations in Spain of *H. almeriense*, *H. violaceum* and *H. hirtum* mycorrhizal plants with *T. claveryi* (Fig. 7.6). Positive cultivation results have also been obtained with *T. boudieri* in Tunisia (Slama et al. 2010) and Israel (Kagan-Zur, pers. comm.). So far, all these cultivated *Terfezia* species are typical of alkaline soils. The cultivation of desert truffle species from acid soils is hindered by the fact that the host plants are annual. Numerous tests are being done to adapt perennial host plants to these soils, with edible species like *T. arenaria*, trying to benefit from previous experiences obtained on alkaline soils (Morte et al. 2017b).

Definitely, desert truffle cultivation is relatively new, with almost 20 years of experience, and of extreme complexity due to inherent aspects of fungal and plant species. The weather of the different cultivation areas is also critical. It is therefore a challenge for research to make the cultivation of desert truffles sustainable and profitable.

7.6 Conclusions

The symbiosis formed by desert truffles with Cistaceae of the genus *Helianthemum* is defined as an *ectendomycorrhizal continuum*, where same roots may present intracellular or intercellular colonization, or both, at the same time. In addition, the presence of organic phosphorus in the medium and drought conditions trigger an increase of the fungal intracellular colonization of the root, making this symbiosis tighter.

The mycelia of *T. claveryi* and *P. lefebvrei* are able to deal with a moderate water stress. At the base of this stress tolerance, there is a greater expression of aquaporin genes, both in the fungus and in the host plant of the mycorrhizal symbiosis. A high expression of fungal aquaporin is observed as the plant's photosynthesis and stomatal conductance decrease in situations of water stress. The combination of intracellular colonization together with the expression of fungal aquaporin TcAQP1

produces a morpho-physiological adaptation that favours this mycorrhizal symbiosis in arid and semiarid conditions. The beneficial bacteria of the soil, isolated from *T. claveryi* plantations, are able to increase survival and mycorrhization of nursery plants and could possibly play an important role in the biological cycle of these fungi. It is crucial to keep studying these bacteria in order to improve the field cultivation of desert truffles.

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