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Beneficial native bacteria improve survival and mycorrhization of desert truffle mycorrhizal plants in nursery conditions

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Abstract

Sixty four native bacterial colonies were isolated from mycorrhizal roots of *Helianthemum almeriense* colonised by *Terfezia claveryi*, mycorrhizosphere soil and peridium of *T. claveryi* to evaluate their effect on mycorrhizal plant production. Based on the phylogenetic analysis of the 16S rDNA partial sequence, 45 different strains from 17 genera were gathered. The largest genera were *Pseudomonas* (40.8% of the isolated strains), *Bacillus* (12.2% of isolated strains) and *Varivorax* (8.2% of isolated strains). All the bacteria were characterized phenotypically and by their plant growth-promoting rhizobacteria (PGPR) traits (auxin and siderophore production, phosphate solubilization and ACC deaminase activity). Only bacterial combinations with several PGPR traits or *Pseudomonas* sp strain 5, which presents three different PGPR traits, had a positive effect on plant survival and growth. Particularly relevant were the bacterial treatments involving auxin release, which significantly increased the root-shoot ratio and mycorrhizal colonization. Moreover, *Pseudomonas mandelii* strain 29 was able to considerably increase mycorrhizal colonization but not plant growth, and could be considered as a mycorrhiza-helper bacteria. Therefore, the mycorrhizal roots, mycorrhizosphere soil and peridium of desert truffles are environments enriched in bacteria which may be used to increase the survival and mycorrhization in the desert truffle plant production system at a semi-industrial scale.

Key Words: *Terfezia*; desert truffle; Cistaceae; mycorrhiza; plant growth promoting rhizobacteria (PGPR); mycorrhiza-helper bacteria (MHB)

Introduction

Terfezia claveryi Chatin, a desert truffle that establishes ectendomycorrhizal associations with species of the genus *Helianthemum* (Morte et al. 2008), has become an alternative agricultural crop in semiarid areas because of the low water input required for its cultivation (Morte et al. 2009). The increasing demand for a large number of mycorrhizal plants, not only in Spain but also in other countries, has prompted a search for new strategies to increase the scale of production of mycorrhizal seedlings (Morte et al. 2012).

For mycorrhizal synthesis, both seedlings and micropropagated plants of *Helianthemum* species, together with *Terfezia* spores or mycelium, have been used (Morte and Andrino 2014; Morte et al. 2008, 2009). The mycorrhizal plant production system starting from *Helianthemum* seeds and *Terfezia* spores is the most widely used because it is less expensive than using micropropagated plants and mycelium as inoculum. The latter system can be subdivided into three different stages: i) seed germination, which includes seed germination itself and the development of true leaves for about 4 weeks, ii) plant growth, which includes shoot elongation, plant hardening and the development of secondary fine roots, and iii) inoculation and mycorrhization, which includes shoot and root developments and mycorrhization up to about 40%. However, each of the above stages presents its own problems. For example, in stage i) 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 4 weeks after germination in nursery conditions (Morte et al. 2008). In stage ii) there is high dependence on fertilization to stimulate plant growth (Morte and Andrino 2014) and in stage iii) the use of fungal spores as inoculum is expensive and their availability depends on annual fruiting variations in natural field production.

Stage i) is currently the bottleneck in *Terfezia* mycorrhizal plant production, and so micropropagation techniques have been used for plant production since plant survival with such techniques is around 90 % (Morte et al. 2008; Morte et al. 2009). However, many plants do not survive the acclimatization stage from *in vitro* to *ex vitro* conditions. Recently, the use of a photoautotrophic micropropagation technique has improved *Helianthemum almeriense* plantlet physiology and, therefore, the survival rate in *ex vitro* conditions (Morte and Andrino 2014). However, special controls of CO_2 concentration and the ventilation rate of the culture vessel are

needed, while keeping pathogen-free conditions, which are not always available in the greenhouse. In addition, the early inoculation of *Helianthemum* seedlings with *Terfezia* does not enhance the low survival rate in nursery conditions. That led us to hypothesize that the use of other microorganisms present in the rhizosphere of the mycorrhizal association *Helianthemum* x *Terfezia*, such as Plant Growth-Promoting Rhizobacteria (PGPR), could help in one or more stages of the mycorrhizal plant production system.

PGPR are a heterogeneous group of soil bacteria that can stimulate the growth of plants, protect them from diseases and increase their yield (Bhattacharyya and Jha 2012). In recent decades, a large array of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and *Serratia* have been reported to enhance plant growth. The direct promotion of growth by PGPR entails either providing the plant with plant growth promoting substances that are synthesized by the bacterium or facilitating the uptake of certain plant nutrients from the environment. The indirect promotion of plant growth occurs when PGPR prevent deleterious effects of one or more phytopathogenic microorganisms. The exact mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include (i) the ability to produce or change the concentration of endogenous plant growth regulators like indolacetic acid, giberellic acid, cytokinins and ethylene (Frankenberger Jr and Arshad 1995; Glick 1995), (ii) asymbiotic N_2 fixation (Boddey and Dobereiner 1995), (iii) antagonism against phytopathogenic microorganisms by production of siderophores (Prasad et al. 2015), antibiotics (Beneduzi et al. 2012) and cyanide (Kamei and Apou Kamei 2014), and (iv) solubilization of mineral phosphates and other nutrients (Jones and Darrah 1994; Mehta and Nautiyal 2001).

To date, no information is available regarding the rhizobacteria present in the micorrhizosphere of the mycorrhizal symbiosis *Helianthemum* x *Terfezia*. The aim of the present work is to isolate and identify the native bacteria present in the *Helianthemum almeriense* x *Terfezia claveryi* mycorrhizosphere to evaluate their ability to produce some of the most typical PGPR traits and to evaluate the use of some of these bacteria in desert truffle mycorrhizal plant production.

Material and Methods

Sample collection and PGPR isolation

H. almeriense x *T. claveryi* rhizosphere soil and roots samples were carefully collected from human-planted orchards and a natural area in Zarzadilla de Totana, Murcia (Spain) in autumn (November 2012) and spring (May 2013). Additionally, the peridium of *T. claveryi* ascocarps, collected in spring, was used to isolate bacteria. All samples (rhizosphere soil, roots, and ascocarps) were kept under refrigeration and processed within 24 hours. One gram of each type of sample (rhizosphere soil, 1-2 cm of root, and 0.5-1 cm of peridium) was transferred to individual 250 ml Erlenmeyer flasks containing 100 ml of sterile Ringer ¼ solution (Sigma-Aldrich, Madrid, Spain) and one drop of Tween-20, before shaking the flasks at 150 rpm for 60 min. Serial dilutions were prepared and 0.1 ml aliquots (10^{-6} to 10^{-3}) were spread on Nutrient Agar (NA) (Scharlau, Barcelona, Spain) solid medium plates. The plates were incubated for 5 days at 28 °C. Morphologically different colonies appearing in the medium were isolated and purified on plates with NA medium. The isolated strains were routinely subcultured every 4 weeks and stored in Nutrient Broth (NB) (Scharlau, Barcelona, Spain) amended with 25% glycerol at -80℃.

Phenotypic characterization

The physiological and biochemical characteristics of the bacterial isolates were examined according to the methods described in Bergey's Manual of Determinative Bacteriology Edition 9.0 (Holt et al. 1994). All colonies were characterized based on colony morphology (color shape, size), microscopy (Gram staining shape and size of bacteria, motility, endospore presence) and biochemical tests (catalase, oxidase, fluorescence).

Sequencing of 16S rDNA gene

All the isolates were identified based on 16S rDNA sequencing. The 16S rDNA amplification was carried out by colony PCR reaction, using 27F (5'AGAGTTTGATCMTGGCTCAG3') and 1492R (5'TACGGYTACCTTGTTACGACTT3') primers. PCR was performed using recombinant *Taq* DNA polymerase (Invitrogen) according to the manufacturer's instructions. A final concentration of 100 μg ml^{-1} BSA and 3% dimethyl sulfoxide (DMSO) as PCR additives were also added to the reaction mixture. Colonies were picked up with a sterilized toothpick and directly transferred to the PCR tube as DNA templates. The thermal cycle program consisted of

one cycle of 94°C for 10 min, 51°C for 2 min, 72°C for 2 min, and 35 cycles of 94°C for 20 s, 57°C for 45 s, 72°C for 1 min, and then incubation at 72°C for 5 min, and a final incubation at 4°C.

The PCR products were sequenced by the dideoxysequencing method (Sanger et al. 1977) using the ABI Prism 310 (Applied Biosystems, Foster City, CA, U.S.A.). The nucleotide sequences of the 16S rDNA were subjected to BLAST analysis with the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences with high similarity scores were downloaded from the NCBI database. A phylogenetic tree was constructed using MEGA 4.0 (Tamura et al. 2007). Sequences were deposited on GenBank (NCBI) under accession numbers indicated in Table 1.

Characterization of plant growth-promoting traits

Indole acetic acid (IAA) production was measured by the colorimetric method (Gordon and Weber 1951). For this, the isolates were cultivated in a NB medium supplemented with 3 g/L of Trytophan (Ahmad et al. 2005; Leveau and Lindow 2005) at 28°C for 2 d in a shaking incubator, at 100 rpm. Bacterial cells were removed from the culture broth by centrifugation (1.5 ml of bacterial suspension). Supernatants were vigorously mixed in a 1:4 ratio with Salkowski's reagent (Gordon and Weber 1951), and incubated in the dark for 30 min at 25°C. The absorbance of the final solution was measured at 530 nm. The concentration of IAA in the culture medium was determined using the standard curve of pure IAA (Sigma-Aldrich Madrid, Spain). For each strain, the IAA production value was calculated from six independent assays.

Siderophore production was determined using an Fe-deficient mineral salt medium (MM9) (Radzki et al. 2013). The strains were inoculated in MM9 and incubated in a shaking incubator at 28°C for 2 d at 100 rpm. The cell-free culture supernatants were assayed for siderophore production using a commercially available Chrome Azurol S (CAS) assay (SideroTec Assay™, www.emergenbio.com). For each strain, the siderophore production value was calculated from six independent assays.

Phosphorus solubilization by PGPR strains was quantified using insoluble tricalcium phosphate in modified National Botanical Research Institute's phosphate growth medium (NBRIP) (Nautiyal 1999). The strains were inoculated in NBRIP and incubated in a shaking incubator at 28°C for 2 d at 100 rpm. The culture medium was centrifuged to remove bacterial cells, and the

158 suspension was used to analyze phosphorus concentration. Solubilized phosphorus was
159 quantified according to the phospho-molybdate blue color method (Murphy and Riley 1962). For
160 each strain, the phosphorus solubilization value was calculated from six independent assays.

161 Six replicates of all the bacterial isolates were grown in 5 ml NB medium for 24 h at 100 rpm at -
162 28°C. The cell pellet, collected by centrifugation at 8,000 rpm for 5 min, was washed with sterile
163 Ringer ¼ solution and resuspended in 1 ml of Ringer ¼ solution and spot inoculated on Petri
164 plates containing DF salts (Penrose and Glick 2003) minimal medium supplemented with 6 mM
165 1-aminocyclopropane-1-carboxylate (ACC) or not (negative control), or supplemented with 0.4
166 g/L of $(NH_4)SO_4$ as positive control. The plates were incubated for 3-4 d at 28°C. The growth of
167 isolates on ACC-supplemented plates was compared with positive and negative control plates.

168 **Plant growth promotion**

169 Three bioassays were carried out in order to evaluate plant growth at different stages of the
170 mycorrhizal plant production system.

171 Bioassay 1: Strains 5, 7, 15, 21, 29, 34, 40 and 43 were selected based on their PGRP trait. All
172 the strains were cultured in NB medium for 48 h (200 rpm, 30ºC). From these cultures, 100-ml
173 dilutions of 10^8 cfu/ml were prepared. The solution was mixed with 600 ml of sterile perlite and
174 then added to a sterile peat:perlite mixture to reach a final proportion of 1:1. The bacteria were
175 applied alone or in combination. The different treatments were 5, 15, 29, 43, 5+7, 34+7, 15+40,
176 43+21. *H. almeriense* seeds were collected from wild plants in the Zarzadilla de Totana area
177 (Murcia, Spain). These seeds were scarified and surface-sterilized with 10% H_2O_2 (30 min) and
178 sown in 96 pots-tray (75-ml/pots) containing the above-described substrate. The final
179 concentration of bacteria was $\geq 1.0 \times 10^6$ cfu/g of substrate. Each bacteria-treatment tray was
180 introduced in an independent mini-greenhouse to maintain humidity during germination. The 96
181 seedlings were maintained in the greenhouse at 23ºC to 15ºC (day and night temperature), a
182 photoperiod of 16 h and a photosynthetic photon flux density maximum of 1,100 µmol m^{-2} s^{-1};
183 the substrate was moistened every 2 days. After 4-weeks, the survival rate was calculated. At the
184 same time, 1 g of substrate, from three pots per treatment, was sampled and the number of cfu/g
185 was calculated.

Bioassay 2: Four-week-old seedlings were re-inoculated with 1 ml of 10^8 cfu/ml of the corresponding bacteria-treatment. Half of the seedlings were fertilized with 2 kg/m^3 of a solid controlled release fertilizer [Plantacote Pluss 6M NPK-(Mg)]) and maintained in the same conditions as in Bioassay 1. After 6 weeks, the survival, shoot and root length, shoot and root weight and number of leaves were measured on six plants. At the same time, 1 g of substrate from three pots per treatment was sampled and the number of cfu/g were calculated.

Bioassay 3: Ten-week-old fertilized seedlings from bioassay 2 were transplanted to 200-ml pots with a substrate of soil:black peat:perlite [1:1:1 (vol/vol)] and sterilized by autoclaving at 100°C for 1 h in three non-consecutive days. The soil, which was collected from Zarzadilla de Totana, Murcia (Spain), had a calcareous clay-loamy texture, pH 8.1, 123 µS cm^{-1} electrical conductivity, 1.2% organic carbon, 10.2 C/N ratio, and nutrient concentrations (ppm) of P, 20.7; Fe, 4.4; Mn, 13.9; Cu, 1.0; and Zn, 1.5. The final pH of the mixture was 7.1. The plants were inoculated with approximately 10^7 mature spores each, obtained from *T. claveryi* ascocarps collected in the same area under *H. almeriense* plants (Morte et al. 2008) and reinoculated with 1 ml of 10^8 cfu/ml of the corresponding bacterial treatment. After three months of growth in the greenhouse, the survival, shoot and root lengths and shoot and root weights were measured on six plants. Frozen fine roots from three plants were randomly selected within each bacterial treatment and stained as previously described (Gutiérrez et al. 2003). All roots were observed under a light microscope and the mycorrhizal percentage was calculated. At the same time, 1 g of substrate from three pots per treatment was sampled and the number of cfu/g was calculated.

Statistical analyses

All data were subjected to ANOVA test and Dunnett's test regard to the non-bacteria treatment. Data of the percentage of mycorrhization and survival ratio were subjected to Chi-square analysis. Statistical analyses were carried out using the software package SPSS (version 15).

Results

Phenotypic characterization

Sixty four colonies were obtained from *H. almeriense x T. claveryi* mycorrhizosphere by non-selective media. 17% of the strains were Gram-negative motile rods, non-endospore forming, and oxidase negative; 22% were Gram-negative motile rods, non-endospore forming, and oxidase positive; 40% were Gram-negative fluorescent motile rods, non-endospore forming, and oxidase positive; 11% were Gram-positive, motile bacilli and endospore forming; 4% were Gram-positive, non-motile rods and non-endospore forming and 6% were Gram-positive, non-motile cocci and non-endospore forming (Supplementary Table 1).

Phylogenetic analysis

Based on the phylogenetic analysis of the 16S rDNA partial sequence, 64 colonies were identified. The 64 colonies were gathered in 45 different strains from the following 17 genera: *Achromobacter, Acinetobacter, Arthrobacter Bacillus, Flavobacterium, Microbacterium, Microvirga, Novosphingobium, Paenibacillus, Phyllobacterium, Pseudomonas, Rhizobium, Rhodococcus, Sinorhizobium, Sphingomonas, Stenotrophomonas* and *Variovorax*. The largest genera were *Pseudomonas* (40.8% of the isolated strains), *Bacillus* (12.2% of the isolated strains) and *Varivorax* (8.2% of the isolated strains). The other groups did not exceed 5% of the total number of strains (Table 1).

Of these 45 different strains, most were isolated only once, with the exception of strains 5, 10, 21, 40 (four isolates), 20 (three isolates), 17, 28, 32, 37 and 41 (two isolates). Strain 5 was the only strain isolated in all the different environments (root, rhizospheric soil and peridium), and in both sampled seasons (autumn and spring) (Table 1).

Plant growth-promoting traits characterization

Out of 45 tested strains, seven strains (15.6 %) were found to produce IAA, ranging from 37.6 to 111.5 µg/mL. Strain 43 (*Arthrobacter* sp) showed the highest IAA production. Ten strains (24.4%) produced siderophores, ranging from 9.1 to 61.1 µg/mL. Strain 42 (*Pseudomonas* sp) showed the highest siderophore production. Nine strains (20.0%) exhibited the ability to solubilize phosphate, ranging from 6.3 to 567.2 µg/mL. Strain 34 (*Pseudomonas brenneri*) showed the highest phosphate solubilization. One strain (2.2%) (strain 5, *Pseudomonas* sp) showed ACC desaminase production (Table 2). Of the isolated strains, twenty seven strains (60 %) did not exhibit any of the PGPR activities assayed in this study, eighteen strains (40 %)

exhibited at least one activity, eight strains (17.8%) exhibited at least two different activities and only strain 5 (2.2%) exhibited three activities (P-solubilizer, IAA and ACC) (Table 2).

Pseudomonas was the only genus isolated from peridium that exhibited some PGPR activity.

Bioassay 1: The plant survival of germinated seedlings (stage i) was significantly higher in treatments 15+40, 5+7, 43+21 and 5 than in the non-inoculated treatment (Table 3). Especially significant were treatments 15+40 and 5+7 with their 2.4-fold and 2.3-fold greater survival of seedlings, respectively, than the non-inoculated control treatment. No significant differences were observed in the rest of the bacterial treatments with respect to the non-inoculated treatment. In all treatments, the number of cfu/g of substrate was similar and around 10^7 cfu/g, including the non-inoculated treatment (Table 3). No thorough phylogenetic analysis of the most abundant colonies found in substrate was made, but these colonies corresponded to a Gram-negative bacteria with green fluorescence under UV light (fluorescent pseudomonads).

Bioassay 2: During the plant growth stage (stage ii), the height, weight and number of leaves were greater in fertilized plants than in non-fertilized plants in the case of most of bacterial treatments, but no differences were observed between the fertilization treatments with regard to the bacterial treatments (Table 3). No significant differences in survival rate were observed in response to the fertilization or bacterial treatments (data not shown). In all bacterial and fertilization treatments, the number of cfu/g in substrate was similar at around 10^7 cfu/g, including the non-inoculated treatment (Table 3). The most abundant microorganism in non-inoculated plants formed Gram negative rods and was oxidase positive and catalase positive with green fluorescence under UV light (fluorescent pseudomonads).

Bioassay 3: In all the bacterial treatments, 12 weeks following *T. claveryi* inoculation, 100% of the plants were mycorrhizal. In all bacterial treatments, with the exception of treatments 15 and 43+21, the mycorrhizal percentages were significantly higher than in the control treatment without bacteria inoculation (Table 3).

No significant differences in survival rates, root and shoot heights, axillary branching, and shoot weights were observed in response to the bacterial inoculation treatment in stage iii within mycorrhizal plants (data not shown). However, strain 43 significantly increased root weight, total plant weight and the root-shoot ratio in mycorrhizal plants. Also, strains 5, 15, 5+7 and 34+7

significantly increased the root-shoot ratio compared with the control treatment in mycorrhizal plants (Table 3). In all bacterial treatments, including the non-inoculated-bacterial treatment, the number of cfu/g of substrate had a similar value of about 10^7 cfu/g (Table 3). The bacterial community was complex with a high variety of colony morphology, color, size and shape. No colonies of fungi or actinomycetes were observed in any plate. In all treatments, the most abundant microorganism formed Gram negative rods, and was oxidase positive and catalase positive with green fluorescence under UV light (fluorescent pseudomonads).

Discussion

During the last twenty years, a considerable progress has been made in the mycorrhizal synthesis of desert truffle plants under controlled conditions (Gutiérrez et al. 2003; Morte et al. 1994; Morte and Honrubia 1995; Morte et al. 2000). However, both *in vitro* and *ex vitro* systems need to be refined before a semi-industrial mycorrhizal seedling production scale can be achieved. PGP Rhizobacteria are among the new and promising biotechnology tools (Bhattacharyya and Jha 2012) that may further enhance nursery plant production, the growth of mycorrhizal plants and sporocarp yield. Despite the extensive literature available regarding PGPR, very little has been reported on their effect on the desert truffle mycorrhizosphere (Dib-Bellahouel and Fortas 2014; Goudjal et al. 2016; Rougieux 1963) or that of other edible truffles (Antony-Babu et al. 2014; Barbieri et al. 2005, 2007; Gryndler et al. 2013; Sbrana et al. 2002).

On the basis of their color, size, shape and appearance, 64 different colonies were isolated in spring and autumn from three different environments (soil, roots and peridium). The 64 colonies were allocated to 45 different strains from 17 genera, of which the largest were *Pseudomonas* (40.8% of the isolated strains) and *Bacillus* (12.2% of the isolated strains). Among the soil microorganisms, both *Pseudomonas* and *Bacillus*, together with *Azospirillum*, have been the most reported as PGPR bacteria. These genera include species with several common traits, including indole-3-acetic acid (IAA) production (Asghar et al. 2002; Karnwal 2009; Mordukhova et al. 1991; Picard and Bosco 2005) siderophore production (Kloepper et al. 1980; Van der Hofstad et al. 1986), phosphate and potassium solubilization (Bhattacharyya and Jha 2012), and the presence of ACC deaminase (Glick et al. 1994; Glick 1995).

In this study, the *Pseudomonas* genus was the best represented PGPR since it was found in the highest number of isolated strains, some of which were isolated more than once (Strains 5, 10, 21, 28, 32, and 40). These bacteria were found in all examined environments (root, rhizospheric soil and peridium), and in both sampled seasons. In addition, out of eighteen strains that showed some PGPR traits, eleven strains (61.1%) belong to the *Pseudomonas* genus, which usually presented more than one PGPR trait per strain. Only one *Pseudomonas* strain (Strain 5) exhibited three activities (P-solubilizer, IAA and ACC deaminase) (Table 2). With the exception of strain 44 all the other pseudomonad strains exhibited fluorescence under U.V. light (Table S1). Within the *Pseudomonas sensu stricto*, which corresponds to the rRNA group I (Palleroni 2008), the fluorescent pseudomonads include all *Pseudomonas* species with the ability to produce fluorescent pyoverdine (Couillerot et al. 2009) that include *Pseudomonas fluorescens sensu stricto*, closely-related fluorescent pseudomonad species from the same '*Ps. fluorescens*' complex and few strains described as *Ps. fluorescens* belong to a separate fluorescent *Pseudomonas* lineage (Couillerot et al. 2009). These data point to the relevance of the *Pseudomonas* genus in the mycorrhizosphere of *T. claveryi-H. almeriense*, and agree with the previous reports where Pseudomonads were observed to be the dominant bacteria in mycorrhizal tips (Sbrana et al. 2002) and ascocarps (Barbieri et al. 2005, 2007; Dib-Bellahouel and Fortas 2014) of other truffles.

In the second part of this work, the role of particular bacteria or combinations of the same strains based on their high and/or complementary PGPR traits were analyzed, with emphasis placed on the bottlenecks at different stages of mycorrhizal plant production at semi-industrial scale. The survival rate of germinated seedlings (germination stage i) was significantly higher in treatments 15+41, 5+7, 43+21 and 5 with respect to the non-inoculated treatment (Table 3) ranging from 40% to 122% increases compared with the non-inoculated treatment. Only bacterial treatments with a combination of PGPR traits or strain 5 with its three different PGPR traits (including ACC), showed a positive effect. It seems that, at this stage, no particular PGPR trait can be correlated with increased germination and survival of *Helianthemum* plant. Probably a combination of several PGPR traits is simultaneously required to increase plant fitness.

No differences were detected for any of the assayed parameters at the second production stage (ii). However, regarding the third stage (*Terfezia* inoculation), a significant increase in root

biomass was observed when strain 43 was co-inoculated with *T. claveryi*. The increase in root weight led to a significant increase in the root-shoot ratio. In four additional treatments (5, 15, 5+7 and 34+7) a significant increase in the root-shoot ratio was also observed. This greater root development could be due to a greater branching by secondary roots triggered by the auxin released by these strains. In 4 out of 5 of these bacterial treatments, the supposed auxin release triggering extra root growth, a significant increase in the mycorrhizal rate was also observed. Roth-Bejerano et al. (2014) showed how auxin level or auxin-phosphate interaction can affect the type of *Terfezia* mycorrhizal colonization in the sense that when high levels of fungal auxin are coupled with high root sensitive to auxin under low phosphate, ectomycorrhizas are procuded. Recently, Turgeman et al. (2016) showed how auxin IAA induced negative taproot gravitropism, attenuated the taproot growth rate, and inhibited initial host development. The presence of bacteria that increase auxin levels in the rhizosphere of *H. almeriense* could be the reason for a stronger root system and higher mycorrhization. These traits also make these plants more likely to survive when they are transferred to field conditions, since correct mycorrhization has been shown to be a feature essential for survival under field conditions (Morte et al. 2010). Of the bacterial treatments that were assayed in this study, strain 29 (*Pseudomonas mandelii*) was of note since it did not improve any of the plant growth parameters measured during the three different stages. However plant inoculation with this strain dramatically increased root mycorrhization. In this sense, strain 29 may be considered as a mycorrhiza-helper bacteria (MHB) rather than a PGPR, having a similar effect to *P. fluorescens* CECT 844 in the symbiosis between *Pinus halepensis* and *Tuber melanosporum* (Dominguez et al. 2012).

In general, different bacteria or their combination seem to be important at different stages of desert truffle plant production (i.e. #15+41 in stage i or #5 in stage iii). However, the bacterial treatment that produced positive effects in several stages was 5+7 and also was the bacterial combination with the highest number of assayed PGPR traits.

Despite the positive results obtained by some native rhizobacteria, it should be borne in mind that the seedlings were produced in an open system with a wide range of bacterial inputs. This was evident from the presence of bacteria in the non-inoculated plants and from the complex bacterial community at the end of mycorrhization in all of the bacterial treatments. However, one pattern that was repeated in all three stages and treatments was the presence and dominance of

the fluorescent pseudomonads (Couillerot et al. 2009). Bacteria from *Ps. fluoresecens* complex are adapted to survival in soil and colonize plant roots (Kiely et al. 2006). They are effective at using seed and root exudates for growth and can intensively colonize the rhizosphere (Couillerot et al. 2009). They have been studied mostly for the protection of crop plants from oomycetes and fungi and to a lesser extent from other bacteria and nematodes (Couillerot et al. 2009) since they produce antimicrobial compounds, including 2,4-diacetylphloroglucinol (DAPG), phenazines, hydrogen cyanide and surfactants (Haas and Defago 2005). The abundance of bacteria from the *Ps fluorescens* complex could explain the absence of other saprophytic or parasitic fungi in the soil of *H. almeriense* plants during the plant production. Although, inoculation with certain PGPR bacteria resulted in an improvement in desert truffle plant production, the plant and/or the plant-fungi probably present some mechanisms to select and/or favor certain groups of bacteria in their rhizosphere. Even in the rhizosphere of non-inoculated plants, bacteria of the *Ps fluorescens* complex were the dominant bacteria, similarly to that obtained in natural and plantation desert truffle soils. Similar results concerning bacteria community selection by the plant have previously been reported by Sugiyama et al. (2012).

In conclusion, the mycorrhizal roots, mycorrhizosphere soil and peridium of desert truffles were seen to be enriched in bacteria with PGPR traits. Among these bacteria, the fluorescent pseudomonads complex was the most abundant and significant in terms of the effects on PGPR traits in the *Terfezia* x *Helianthemum* symbiosis. The use of some of these bacteria at different stages of nursery plant production may help overcome some of the current bottlenecks of desert truffle plant production at semi-industrial scale. The benefits include increased survival rates and mycorrhization, reduced production time and, ultimately, greater plant quality.

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Table 1. Identification of bacterial isolates based on 16S rDNA partial sequence analysis.

Strain	Genbank nº	Organisms identified	Origen	Season	Location
1	KX150802	*Pseudomonas sp*	R	Autumn	C
2	KX150803	*Paenibacillus sp*	R	Autumn	C
3	KX150804	*Bacillus thuringiensis*	R	Autumn	C
4	KX150805	*Achromobacter sp.*	R	Autumn	C
5	KX150806	*Pseudomonas fluorescens*	R/P/S	Autumn/Spring	C
6	KX150807	*Microbacterium paraoxydan*	R	Autumn	C
7	KX150808	*Pseudomonas sp.*	R	Autumn	C
8	KX150809	*Bacillus atrophaeus*	R	Autumn	C
9	KX150810	*Pseudomonas sp.*	R	Autumn	C
10	KX150811	*Pseudomonas sp.*	R/S	Autumn	C
11	KX150812	*Bacillus megaterium*	R	Autumn	C
12	KX150813	*Sphingomonas sp.*	S	Autumn	C
13	KX150814	*Rhizobium radiobacter*	S	Autumn	C
14	KX150815	*Acinetobacter lwoffii*	S	Autumn	C
15	KX150816	*Flavobacterium sp*	S	Autumn	C
16	KX150817	*Novosphingobium panipatense*	S	Autumn	C
17	KX150818	*Bacillus simplex*	S	Autumn	C
18	KX150819	*Stenotrophomonas rhizophila*	S	Autumn	C
19	KX150820	*Arthrobacter sp.*	S	Autumn	C
20	KX150821	*Sinorhizobium meliloti*	S	Autumn/Spring	C
21	KX150822	*Pseudomonas sp.*	S/P	Autumn	C/N
22	KX150823	*Variovorax paradoxus*	P	Spring	C
23	KX150824	*Variovorax paradoxus*	R	Spring	C
24	KX150825	*Phyllobacterium bourgognense*	S	Spring	C
25	KX150826	*Pseudomonas sp.*	P	Spring	C
26	KX150827	*Microvirga sp.*	S	Spring	C
27	KX150828	*Pseudomonas sp.*	P	Spring	C
28	KX150829	*Pseudomonas moraviensis*	R	Spring	C
29	KX150830	*Pseudomonas mandelii*	P	Spring	C
30	KX150831	*Pseudomonas sp.*	R	Spring	C
31	KX150832	*Pseudomonas sp.*	R	Spring	C
32	KX150833	*Pseudomonas sp.*	P	Spring	C
33	KX150834	*Pseudomonas sp.*	S	Spring	C
34	KX150835	*Pseudomonas brenneri*	P	Spring	C
35	KX150836	*Rhodococcus sp.*	P	Spring	C
36	KX150837	*Flavobacterium sp.*	R	Spring	C
37	KX150838	*Phyllobacterium ifriqiyense*	R/S	Spring	C
38	KX150839	*Variovorax paradoxus*	R	Spring	C
39	KX150840	*Rhizobium galegae*	R	Spring	C
40	KX150841	*Pseudomonas sp.*	S	Spring	C
41	KX150842	*Arthrobacter nitroguajacolicus*	S/P	Spring	C/N
42	KX150843	*Pseudomonas sp.*	S	Spring	C
43	KX150844	*Arthrobacter sp.*	P	Spring	C
44	KX150845	*Pseudomonas sp.*	P	Spring	N
45	KX150846	*Variovorax paradoxus*	P	Spring	N

[a] P, Peridium; R, Root; S, rhizosphere Soil
[b] C, Cultivation; N, Natural area

Table 2

Table 2. Characterization of plant growth-promoting traits. From the total of the 45 strains tested, only the 18 strains with some positive plant growth-promoting traits are showed.

		Plant growth-promoting traits			
Strain	Organisms identified	Phosphate solubilization (µg/ml)	IAA production (µg/ml)	Siderophore production (µg/ml)	ACC desaminase
1	*Pseudomonas sp.*	506.4	-	9.17	-
5	*Pseudomonas fluorescens*	532.1	37.8	-	+
6	*Microbacterium paraoxydan*	-	37.6	-	-
7	*Pseudomonas sp.*	-	84.5	41.3	-
10	*Pseudomonas sp.*	493.9	-	10.52	-
11	*Bacillus megaterium*	504.9	-	-	-
15	*Flavobacterium sp.*	-	-	20.08	-
16	*Novosphingobium panipatense*	-	46.7	-	-
17	*Bacillus simplex*	-	-	23.61	-
21	*Pseudomonas sp.*	411.7	-	50.4	-
26	*Microvirga sp.*	-	48.2	-	-
28	*Pseudomonas moraviensis*	-	-	12.65	-
29	*Pseudomonas mandelii*	541.2	-	-	-
33	*Pseudomonas sp.*	-	-	9.15	-
34	*Pseudomonas brenneri*	567.2	-	9.80	-
40	*Pseudomonas sp.*	519.4	46.1	-	-
42	*Pseudomonas sp.*	253.4	-	61.1	-
43	*Arthrobacter sp.*	-	111.5	-	-

Table 3. Plant growth data, survival, mycorrhization percentages and bacterial concentrations measured at different stages of mycorrhizal desert truffle plant production.

Treatment	Stage i		Stage ii						Stage iii		
	Survival (%)	Log cfu/g	Fert	Shoot (cm)	Roots (cm)	Shoot (g)	Roots (g)	Leaves	Log cfu/g	Root/shoot ratio	Myc (%)	Log cfu/g
Control	44±3	7.1	+	5.4±1.20	10.1±2.07	0.162±0.05	0.22±0.02	10.7±2.1	7.9	1.50±0.10	33±9	7.9
			-	1.7±0.18#	13.5±3.1	0.015±0.007#	0.04±0.02	4.5±1.1#	7.7			
5	62±4*	7.4	+	3.9±0.42	9.5±1.5	0.095±0.03	0.18±0.04	9.4±1.1	7.6	3.39±0.04**	60±9••	8.0
			-	1.5±0.38#	6.7±0.4	0.013±0.003	0.02±0.003#	4.9±0.5#	7.4			
15	18±2	8.0	+	2.9±0.35	9.8±1.4	0.068±0.009	0.14±0.02	10.3±0.6	7.0	2.37±007*	32±9	8.0
			-	2.8±1.20	6.8±3.8	0.023±0.01#	0.04±0.01	5.0±1.0#	7.1			
29	42±4	7.2	+	3.0±0.35	7.9±1.1	0.083±0.02	0.20±0.03	9.7±0.9	7.4	1.60±0.10	84±7•••	8.2
			-	1.8±0.20	7.3±0.8	0.007±0.001#	0.017±0.004#	5.2±0.6#	7.1			
43	52±4	7.6	+	5.7±0.60	10.2±1.1	0.158±0.04	0.25±0.04	12.8±1.8	8.1	4.16±0.03***	57±9••	7.7
			-	1.9±0.20#	8.0±0.5	0.011±0.002#	0.036±0.007	5.1±0.8#	7.1			
5+7	94±5***	7.2	+	3.5±0.39	10.7±1.6	0.111±0.01	0.18±0.01	11.2±1.2	7.5	2.53±0.05*	49±9•	7.9
			-	0.5±0.11#	7.5±1.3	0.008±0.001#	0.023±0.005#	4.2±0.4#	7.3			
34+7	53±4	7.0	+	2.6±0.55	7.4±0.8	0.038±0.02	0.059±0.008	6.0±1.5	7.3	2.42±0.07*	60±0.9•	7.8
			-	2.2±0.38	5.8±1.4#	0.016±0.004	0.056±0.009	6.2±1.1	7.1			
15+40	98±5***	7.9	+	2.8±0.58	10.5±0.7	0.105±0.05	0.16±0.02	11.0±1.4	7.9	1.83±0.04	68±9•	7.8
			-	0.7±0.19#	9.0±0.9	0.017±0.004	0.029±0.004	5.0±0.8#	8.0			
43+21	63±4**	7.0	+	3.8±0.60	9.6±0.7	0.185±0.09	0.36±0.09	12.0±1.4	7.4	2.10±0.10	24±8	7.5
			-	1.5±0.18#	8.8±1.2	0.012±0.002#	0.023±0.003#	5.0±0.4#	7.4			

*P≤0.05, ** P≤0.01,*** P≤0.001, level of significance with respect to the non-bacterial control according to Dunnett's test;

#P≤0.05, level of significance in non-fertilizer compare with fertilizer plants in the same bacterial treatment according to one way ANOVA;

•P≤0.05, •• P≤0.01,••• P≤0.001, level of significance with respect to the non-bacterial control according to Chi-square test.

Supplementary Table 1. Phenotypic characteristics of the isolated bacterial strains.

			Physiological and biochemical characteristics							
			Morphology and biochemical characteristics				Microscopy characteristics			
Strain	Colonies	Location[a]	Colony	Fluorescence	Catalase	Oxidase	Shape	Gram	Endospore	Mobility
1	1	C	Greenish yellow	+	+	+	Rods	-	-	+
2	1	C	Irregular waxy-whitish	-	+	-	Bacilli	+	+	+
3	1	C	Waxy-whitish	-	+	-	Bacilli	+	+	+
4	1	C	Whitish cream	-	+	-	Rods	-	-	+
5	4	C	Greenish yellow	+	+	+	Rods	-	-	+
6	1	C	Yellowish white	-	+	-	Rods	+	-	-
7	1	C	Greenish yellow	+	+	+	Rods	-	-	+
8	1	C	Brown	-	+	-	Bacilli	+	+	+
9	1	C	Greenish yellow	+	+	+	Rods	-	-	+
10	4	C	Greenish yellow	+	+	+	Rods	-	-	+
11	1	C	Waxy-whitish	-	+	-	Bacilli	+	+	+
12	1	C	Yellow	-	+	+	Rods	-	-	+
13	1	C	Translucent	-	+	-	Rods	-	-	+
14	1	C	Yellow	-	+	-	Rods	-	-	+
15	1	C	Yellow-mucilaginous	-	+	+	Rods	-	-	+
16	1	C	White	-	+	-	Rods	-	-	+
17	2	C	Waxy-whitish	-	+	-	Bacilli	+	+	+
18	1	C	Waxy-brownish	-	+	-	Rods	-	-	+
19	1	C	Brown	-	+	-	Cocci	+	-	-
20	3	C	Translucent	-	+	+	Rods	-	-	+
21	4	C/N	White	+	+	+	Rods	-	-	+
22	1	C	Whitish cream	-	+	+	Rods	-	-	+
23	1	C	Whitish cream	-	+	+	Rods	-	-	+
24	1	C	Translucent	-	+	-	Rods	-	-	+
25	1	C	Whitish cream	+	+	+	Rods	-	-	+
26	1	C	Translucent	-	+	-	Rods	-	-	+
27	1	C	Whitish cream	+	+	+	Rods	-	-	+
28	2	C	Whitish cream	+	+	+	Rods	-	-	+
29	1	C	Whitish cream	+	+	+	Rods	-	-	+
30	1	C	Whitish cream	+	+	+	Rods	-	-	+
31	1	C	Whitish cream	+	+	+	Rods	-	-	+
32	2	C	Yellowish white	+	+	+	Rods	-	-	+
33	1	C	Yellowish white	+	+	+	Rods	-	-	+
34	1	C	Greenish yellow	+	+	+	Rods	-	-	+
35	1	C	Translucent	-	+	-	Rods	+	-	-
36	2	C	Yellow mucilaginous	-	+	+	Rods	-	-	+
37	2	C	Translucent	-	+	-	Rods	-	-	+

38	1	C	Whitish cream	-	+	+	Rods	-	-	+
39	1	C	Translucent	-	+	+	Rods	-	-	+
40	4	C	White	+	+	+	Rods	-	-	+
41	2	C/N	Brown	-	+	-	Cocci	+	-	-
42	1	C	Whitish cream	+	+	+	Rods	-	-	+
43	1	C	Brown	-	+	-	Cocci	+	-	-
44	1	N	Whitish cream	-	+	+	Rods	-	-	+
45	1	N	Whitish cream	-	+	+	Rods	-	-	+

+, positive; -, negative

[a] C, Cultivation; N, Natural Area

Beneficial native bacteria improve survival and mycorrhization of desert truffle mycorrhizal plants in nursery conditions

MYCORRHIZA

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