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ORIGINAL ARTICLE

Mycorrhization between *Cistus ladanifer* L. and *Boletus edulis* Bull is enhanced by the mycorrhiza helper bacteria *Pseudomonas fluorescens* Migula

Olaya Mediavilla[1,2] · **Jaime Olaizola**[2] · **Luis Santos-del-Blanco**[1,3] · **Juan Andrés Oria-de-Rueda**[1] · **Pablo Martín-Pinto**[1]

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Abstract *Boletus edulis* Bull. is one of the most economically and gastronomically valuable fungi worldwide. Sporocarp production normally occurs when symbiotically associated with a number of tree species in stands over 40 years old, but it has also been reported in 3-year-old *Cistus ladanifer* L. shrubs. Efforts toward the domestication of *B. edulis* have thus focused on successfully generating *C. ladanifer* seedlings associated with *B. edulis* under controlled conditions. Microorganisms have an important role mediating mycorrhizal symbiosis, such as some bacteria species which enhance mycorrhiza formation (mycorrhiza helper bacteria). Thus, in this study, we explored the effect that mycorrhiza helper bacteria have on the efficiency and intensity of the ectomycorrhizal symbiosis between *C. ladanifer* and *B. edulis*. The aim of this work was to optimize an in vitro protocol for the mycorrhizal synthesis of *B. edulis* with *C. ladanifer* by testing the effects of fungal culture time and coinoculation with the helper bacteria *Pseudomonas fluorescens* Migula. The results confirmed successful mycorrhizal synthesis between *C. ladanifer* and *B. edulis*. Coinoculation of *B. edulis* with *P. fluorescens* doubled within-plant mycorrhization levels although it did not result in an increased number of seedlings colonized with *B. edulis* mycorrhizae. *B. edulis* mycelium culture time also increased mycorrhization levels but not the presence of mycorrhizae. These findings bring us closer to controlled *B. edulis* sporocarp production in plantations.

Keywords MHB · Mycelium culture · Mycorrhizal plants · Sporocarps · Ectomycorrhizal fungi cultivation

✉ Pablo Martín-Pinto
pmpinto@pvs.uva.es

Olaya Mediavilla
olayamediavillasantos@gmail.com

Jaime Olaizola
jaime@idforest.es

Luis Santos-del-Blanco
luis.santosdelblanco@unil.ch

Juan Andrés Oria-de-Rueda
oria@agro.uva.es

[1] Sustainable Forest Management Research Institute, Fire and Applied Mycology Laboratory, Departments of Agroforestry Sciences and Vegetal Production and Natural Resources, University of Valladolid (Palencia), Avda, Madrid 44, 34071 Palencia, Spain

[2] IDForest-Biotecnología Forestal Aplicada, Calle Tren Ter, s/n. Parcela 238, 34200 Venta de Baños, Palencia, Spain

[3] Department of Ecology and Evolution, Univeristy of Lausanne, CH-1015 Lausanne, Switzerland

Introduction

The species belonging to the *Boletus edulis* Bull. complex, commonly known as porcini, are currently among the most appreciated fungi worldwide (Hall et al. 1998). The different species in the complex occur frequently, and their combined distribution range spans both hemispheres, from Scandinavia to Southern Africa and Australia (Dentinger et al. 2010). As a result, global marketed productions are high (Boa 2004) with their annual world market value exceeding $250 million (Catcheside and Catcheside 2012). In Spain alone, annual harvested *B.* gr. *edulis* production widely ranges between 2000 and 20,000 Tm, depending on the year (Oria-de-Rueda et al. 2008). However, a drastic decrease in the presence and productivity of *B.* gr. *edulis* has been reported in several parts of Europe (Salerni and Perini 2004). Coupled by an ever-increasing demand of edible fungi (Sitta and Floriani 2008) and considering that currently *B.* gr. *edulis* are only collected

from the wild (Cannon and Kirk 2007), there is a high interest in achieving a controlled production of these fungi, similar to that of the mycorrhizal fungus *Tuber melanosporum* Vitt. (Bonet et al. 2009).

Most of the porcini only produce fruitbodies in association with *Pinus*, *Quercus*, or *Castanea* (Olivier et al. 1997), reaching high productions in relatively mature forests (40+ years old) (Díaz-Balteiro et al. 2003), thus limiting the feasibility of their domestication. Interestingly, *B. edulis* species have recently been reported to occur in *Cistus ladanifer* L. shrublands in Northwest Spain (Oria-de-Rueda et al. 2005; Martín-Pinto et al. 2006). This finding is very relevant because sporocarp production occurs as early as in 3-year-old *C. ladanifer* shrubs, and high yields have been reported in 8-year-old plants (Oria-de-Rueda et al. 2008).

The first steps toward *C. ladanifer* controlled mycorrhization with *B. edulis* have already been taken (Águeda et al. 2008). However, we poorly understand how to efficiently produce a large number of plants that harbor the targeted ectomycorrhizal (ECM) fungi in their root system. Several factors are known to influence the success of man-mediated ectomycorrhizal symbiosis, including fungal inoculum preparation, choice, and preparation of substrates, choice of nutrient solutions (Honrubia et al. 1994; Parladé et al. 2004; Pera and Parladé 2005), and the presence of other microorganisms in the rhizosphere (Walder et al. 2012).

Bacteria can enhance the ectomycorrhizal symbiosis in two ways (Frey-Klett et al. 2007), by helping the ectomycorrhizal symbiosis to take place, known as *mycorrhization* helper bacteria, or by enhancing within-plant mycorrhization rate, known as *mycorrhiza* helper bacteria. In either case, they are referred to as MHB (Garbaye 1994). The mechanisms by which bacteria interact with fungi involve the stimulation of mycelial growth, the increase of contact points between roots and fungi, and the reduction of environmental stress on the mycelium (Brulé et al. 2001; Kurth et al. 2013). Nonetheless, the activity of bacteria can also be detrimental to the mycorrhizal symbiosis in some cases (Kataoka et al. 2009).

Many different bacterial groups and genera have been reported as MHB in both endomycorrhizal and ectomycorrhizal symbiosis (Duponnois and Plenchette 2003). Among strains isolated from ectomycorrhizae, *Pseudomonas fluorescens* Migula is among the most frequent (Garbaye and Bowen 1989; Garbaye et al. 1990). In addition, various studies have confirmed that *P. fluorescens* generally improves the symbiotic relationship of ectomycorrhizae by increasing the within-plant ratio of mycorrhization, i.e., the percentage of mycorrhizal short roots to total short roots (Duponnois and Garbaye 1991; Duponnois 2006), and stimulating the ectomycorrhizal fungus growth under experimental conditions (Frey-Klett et al. 2007). Interestingly, in the context of ECM fungus domestication, a recent study reported a significant increase in colonization of *Pinus halepensis* Mill. roots by *Tuber melanosporum* Vitt. under the presence of *P. fluorescens* (Dominguez et al. 2012).

Also relevant to the feasibility of large-scale mycorrhized plant production is our ability to produce a large number of sterile plants ready to host ECM fungi (Diez et al. 2000). The micropropagation of plants is a fast method of propagation, which allows us to improve the quality of the plant by selecting optimum plant material and provide genetic uniformity (Diez et al. 2000). Despite the high cost of preparing vitroplants as the first step, it pays off with a higher number of mycorrhized plants. Once the in vitro production protocol with a suitable plant genotype(s) for mycorrhization is achieved, the cost per plant may be reduced, thus creating a profitable inoculation system. The positive results achieved with MHB and ECM fungus coinoculations, paired with improvements in plant propagation methods, open new expectations toward the domestication of valuable ECM fungus species. Thus, in this study, we aimed to optimize a protocol for the mycorrhizal synthesis of *B. edulis* with *C. ladanifer* vitroplants by assessing the qualitative and quantitative effects of coinoculation with MHB *P. fluorescens*. Our specific aims were (i) to assess the influence of *P. fluorescens* on the mycorrhizal presence (i.e., on the number of pots containing mycorrhized plants) and mycorrhizal colonization level of *C. ladanifer* vitroplants inoculated with *B. edulis* and (ii) to assess the influence of the culture time of fungal inoculum on mycorrhizal presence and colonization level alone and in interaction with *P. fluorescens*.

Material and methods

Mycorrhizal synthesis protocol

The mycorrhizal synthesis protocol consisted of a preliminary step for fungal strain isolation and growth, in parallel with propagation of *C. ladanifer* vitroplants. This step was followed by the preparation of bacterial inoculum and finally coinoculation of vitroplants with MHB into a substrate colonized by *B. edulis*. Below, we provide a detailed description of the different steps.

Fungal inoculum

Sporocarps of *B. edulis* were collected from pure *C. ladanifer* shrublands located in northwestern Spain. Immediately after collection, sporocarps were stored in closed polyethylene bags until their processing in the laboratory within 24 h after collection (Honrubia et al. 1994). Strain isolation was carried out under aseptic conditions in order to avoid contamination. A small piece of inner flesh from the cap was placed on MMN nutritive medium plates (Modified Melin-Norkrans) (Marx 1969) at pH 5.5 and kept at 22–24 °C in dark (Honrubia

et al. 1994). Mycelium was transferred to fresh MMN plates every 10 days to ensure its vitality and growth potential. Following Mello et al. (2006), we used ITS primers specific for *B. edulis* to confirm that the mycelium was the intended inoculated fungal species.

As a solid expanding culture substrate, we employed a mixture of vermiculite and peat (11/1; *v*/*v*) moistened with liquid MMN nutritive medium with a pH of 5.5 and 2.5 g/l of glucose. The substrate to liquid ratio was 2/1 (*v*/*v*). One hundred cubic centimeters of substrate were transferred to 300-cm^3 cylindrical glass pots (7.5-cm diameter) covered with a plastic lid which had a 1-cm^2 cellulose filter. This filter guaranteed an efficient gas exchange while precluding microbial contamination. Glass pots were sterilized in an autoclave at 121 °C during 20 min. The substrate was subsequently cooled, and the pots were inoculated under aseptic conditions with *B. edulis* by adding 20 plugs, 5-mm diameter, of active mycelium. One single fungal strain was used throughout the experiment. The inoculated glass pots were covered with the lid, sealed with Parafilm M®, and grown at 22 °C for different periods of time: 2, 3, or 4 months depending on the test, in order to obtain suitable development of the fungus in the substrate in a minimum time.

Bacterial inoculum

A single *P. fluorescens* strain was used in this experiment. The strain CECT 844 was selected based on its positive effect on the mycorrhizal symbiosis between *T. melanosporum* and *P. halepensis* (Dominguez et al. 2012). The bacterial strain was supplied by the CECT (Spanish Type Culture Collection), University of Valencia. The liquid bacterial inoculum was prepared by suspending *P. fluorescens* in a malt-glucose nutritive medium (3 g malt, 10 g glucose, and 1l distilled water). Inoculated nutrient medium was grown at 22 °C and shaken at 100 rpm. After a period of 48 h, bacterial concentration levels were measured with a Thoma camera, and bacterial inoculum was resuspended in sterile water to achieve a concentration of 5×10^8 bacteria per milliliter.

Plant material

Plant material was sampled from *C. ladanifer* shrubs hosting *B. edulis* fungi in northern Spain. Shoots tips from a single 5-year-old *C. ladanifer* shrub were collected to establish in vitro cultures. Fifty shoots were introduced during a 20-min period in an antioxidant solution (100 mg/l ascorbic acid and 150 mg/l citric acid) to prevent explants from browning (M'Kada et al. 1991). Then, the plant shoots were disinfected by soaking them in 100 % ethanol for 30 s, rinsing with sterile distilled water, and then further disinfecting for a 30-min period in 1.5 % sodium hypochlorite (NaClO) solution with a drop of Tween 20® detergent. Finally, they were rinsed three times with sterile distilled water (Madesis et al. 2011).

Propagation of the plant material was carried out by culturing shoots tips on Murashige and Skoog basal medium (MS basal medium) (Murashige and Skoog 1962) supplemented with 0.88 mg/l benzylaminopurine, 30 g/l sucrose, and 8 g/l agar. Shoot tips were subcultured every 4 weeks. Rooting was performed by transplanting microshoots from the third subculture into MS basal media supplemented with 0.49 μM of indolebutyric acid. Plants were grown for 2 months, until inoculation, at 25±1 °C under a 16-h photoperiod with a light intensity of 2000 lux provided by cool-white fluorescent lamps. Plants were approximately 7 cm tall by the end of the in vitro culture process.

Fungal and bacterial coinoculation

C. ladanifer vitroplants were transferred into glass pots containing the vermiculite-peat substrate where *B. edulis* (Be) had been growing during 2, 3, or 4 months or into noninoculated control (C) pots. By previously growing the fungus in the substrate, we intended to promote its development and expansion, avoiding the possible inhibitory action that the bacteria may have. Half of the Be plants were further inoculated with a bacterial dose of 5×10^8 bacteria/plant (Be×Pf). Plants were subsequently located randomly in a growth chamber and grown under a 16-h photoperiod with a light intensity of 2000 lux and at 25± 1 °C for 5 months. The plants were neither fertilized nor irrigated during that period.

Experimental design

The experiment followed a completely randomized factorial design, with the following treatments: (1) inoculation with *B. edulis* (*Be*) and (2) coinoculation with *B. edulis* × *Pseudomonas fluorescens* CECT 844 (*Be*×*Pf*). Each of these inoculation types were also tested for three different mycelium culture periods (2, 3, and 4 months). In addition, a control treatment without inoculation was used to detect potential mycorrhizal fungus contamination. As a result, a total of 7 treatments were established. Eighteen glass pots were used per treatment (126 glass pots), with four plants per pot. Hence, a total of 504 plants were tested.

Mycorrhizal colonization verification

Five months after (co)inoculation, plants were carefully extracted from the glass pots, and roots were cleaned according to the methodology established by Fischer and Colinas (1997). All plants looked healthy by the end of the experiment. Although growth measurement of plants was not carried out, differences between control and inoculated plants were not observed. The plant root system was examined with a

stereomicroscope to confirm *B. edulis* presence (Agerer 1991). Description of *B. edulis* ectomycorrhiza as described by Águeda et al. (2008) was taken as a reference to confirm the identification. Despite working under controlled conditions, a molecular analysis of fungal DNA was done to complement and certify with absolute certainty the visual results. Thus, the identification of *B. edulis* mycorrhizae was further confirmed by specific amplification of the ITS region (Mello et al. 2006) for one randomly chosen plant per test, i.e., seven in total.

We estimated mycorrhization levels as follows. Roots were cut into 1–2-cm pieces which were cleaned, rinsed with distilled water, and placed on a Petri dish in water. The total number of root tips per plant and the number of those that had been colonized by *B. edulis* were counted. The level of within plant mycorrhization was estimated as the percentage of total root tips which were colonized by *B. edulis*.

Data analysis

The pot was chosen as experimental unit due to its possible influence on the contained plants. Chi-square tests were used to compare mycorrhization among treatment frequencies. Average mycorrhization was used to assess the level of mycorrhization. A factorial analysis of variance was performed in order to test the influence of mycelium culture time, bacterial coinoculations as well as their interaction. Data were subjected to angular transformation prior to analysis, and the model was subsequently validated by checking normality and homoscedasticity of residuals. Mean values of levels within factors were compared by least significance difference (LSD) Fisher tests ($P<0.05$). Controls were only used to verify the absence of *B. edulis* or any other mycorrhizal fungi. All statistical analyses were done with R version 3.0.1 (2013).

Results

Mycorrhizal synthesis between *B. edulis* and *C. ladanifer* was successful. Mycorrhizae were formed in the two treatments where *B. edulis* inoculation was carried out: plants inoculated with *B. edulis* exclusively and plants inoculated with *B. edulis* and *P. fluorescens*, showing in this case a significant increase in the level of mycorrhization. Control plants remained nonmycorrhizal.

Mycorrhizal synthesis

Ectomycorrhizae of *B. edulis* in *C. ladanifer* plants were successfully synthetized in vitro. Ectomycorrhizae showed typical traits of *Boletales*, and fitted the description provided By Águeda et al. (2008). However, some differences in coloration (brownish) were noticed and the plectenchymatous mantle and rhizomorphs had highly differentiated hyphae. The inflated, smooth cystidia-like clavate end cells on the surface of the rhizomorphs and their slightly twisted external hyphae were additional characterizing features.

B. edulis mycorrhizal synthesis was further confirmed through molecular analysis. Every inoculated plant selected randomly from each treatment yielded positively for *B. edulis* presence while the plant selected from the control provided negative results.

Mycorrhizal presence

B. edulis formed mycorrhizae with *C. ladanifer* in both *B. edulis* (Be) and *B. edulis* × *P. fluorescens* (Be×Pf) treatments. Among the total 108 inoculated pots, 35 (32 %) pots were mycorrhized. However, coinoculation with bacteria did not significantly affect the presence of mycorrhizae ($P=0.836$). From the two groups of 54 pots per inoculation type, 18 (33 %) were mycorrhized in the Be treatment and 17 (31 %) in the Be×Pf treatment. Nor did mycelium culture time affect mycorrhizal presence ($P=0.743$). Amycelium culture time period of 2 months provided 28 % success in mycorrhization ininoculated pots, 36 % at 3 months, and 33 % at 4 months. No interaction between coinoculation with bacteria and mycelium culture time factors was detected ($P=0.742$) (Table 1).

Level of mycorrhization within mycorrhized plants

A positive influence of coinoculation ($P<0.001$) and extended mycelium culture time ($P=0.004$) on the level of mycorrhization was confirmed. No interaction between these two factors was found ($P=0.57$) (Table 2). Across all mycelium culture times, mycorrhization levels were doubled when coinoculated with *P. fluorescens* (Table 3). The best results

Table 1 Presence of mycorrhizae* (in percentage) as a function of inoculation type and mycelium culture time

Inoculation	Mycelium culture time			Total
	2 months	3 months	4 months	
Be	0.33±0.11aA	0.33±0.11aA	0.33±0.11aA	0.33±0.06A
BexPf	0.22±0.10aA	0.39±0.12aA	0.33±0.11aA	0.31±0.06A
Total	0.28±0.08a	0.36±0.08a	0.33±0.08a	0.32±0.05A

*Presence of mycorrhizae refers to the number of pots having mycorrhizae

Different lower case letters in the same rows and upper case letters in the same columns indicate significant differences according to Chi-square test at $P<0.05$ levels

Be plant inoculated only with *Boletus edulis, BexPf* plant inoculated with *Boletus edulis* and *Pseudomonas fluorescens*

Table 2 Analysis of variance (ANOVA) to test the influence of *Boletus edulis* mycelium culture time, with *Pseudomonas fluorescens* bacterial coinoculations as well as their interaction on the level of mycorrhization of *Cistus ladanifer* vitroplants

	df	Sum sq	Mean sq	F	Pr (>F)
Time	2	0.14587	0.07294	6.891	0.00356
Bacteria	1	0.13121	0.13121	12.396	0.00144
Time/bacteria	2	0.01204	0.00602	0.569	0.57236
Residuals	29	0.30695	0.01058		

(23.5 %) were achieved with plants cultivated for 4 months and coinoculated with *B. edulis* mycelium and *P. fluorescens* (Table 3). The level of mycorrhization at 4 months of mycelium cultivation was significantly different to those with 2 months (P=0.01) or 3 months of cultivation (P<0.001). However, the level of mycorrhization between 2 and 3 months of mycelium cultivation was not significantly different (P= 0.44).

Discussion

Mycorrhizae observed in plants were contrasted with those described by Águeda et al. (2008), and color differences were observed (i.e., our mycorrhizae were darker). We certified using molecular techniques that the mycorrhizae obtained were the result of the *B. edulis* isolate used and not produced by a contaminant.

Despite achieving in vitro mycorrhizal synthesis, several plants failed to form mycorrhizae, and regarding mycorrhizal presence, differences among treatments were not significant. Frey-Klett et al. (2007) clarified the difference between mycorrhization helper bacteria, i.e., when bacteria influence the presence of mycorrhizae, and mycorrhiza helper bacteria, i.e., when bacteria improves the level of mycorrhization once the plant is mycorrhized. Thus, in the context of our study, *P. fluorescens* may be regarded as mycorrhiza helper bacteria, increasing the level of mycorrhization.

The competition between fungus and bacteria in colonizing the substrate can affect the degree of mycorrhiza formation (Aspray et al. 2006). Accordingly, Brulé et al. (2001) reported the dominance of bacteria over fungi in rich growth media and stated that the success of the inoculation depends on the survival of the fungal inocula in the soil during the presymbiotic life of the fungus. Given that *B. edulis* is a comparatively slow growing ECM fungus in saprophytic conditions (Olaizola 2007), *P. fluorescens* and *B. edulis* may be competing for nutrients during the presymbiotic period, and consequently, *B. edulis* may have not been benefited by the bacteria before mycorrhization. Kurth et al. (2013) also suggested that the competition between fungus and bacteria for resources may inhibit mycorrhizal development, and Duponnois (1992) reported that high bacterial inoculum doses may also inhibit mycorrhizal development. Thus, the development of methods to quantify the abundance of bacteria and fungus in the presence of one another needs further investigation.

Our results showed that mycelium culture time did not substantially affect mycorrhizal presence. This result can be related to the slow growth of *B. edulis* mycelium. Olaizola (2007) tested the mycelium growth rate of 12 isolates of different mycorrhizal fungi under aseptic conditions. *B. edulis* was found to be one of the isolates with the lowest growth rate, significantly lower than *Lactarius deliciosus* (L.) Gray, a species whose mycelium colonizes the entire substrate after a minimum of 2 months under optimum conditions (Parladé et al. 2004). Thus, considering the slow growth of *B. edulis*, the pots may not have been uniformly colonized by the mycelium, lowering the odds of mycelium and root tip contact and thus mycorrhiza development.

Regarding the level of mycorrhization, both time and presence of bacteria had significant effects, but there was no interaction between them. In our experiment, the level of mycorrhization was doubled when coinoculating *B. edulis* and *P. fluorescens*. The importance of bacterial coinoculation has been reported in several studies where bacteria enhanced

Table 3 Level of mycorrhization *of Boletus edulis* on *Cistus ladanifer* vitroplants depending on the presence of *Pseudomonas fluorescens* and *B. edulis* mycelium culture times

Mycelium culture time	Inoculation		Total
	Be	BexPf	
2 months	6.98±2.73aA	18.55±3.48bAB	12.77±2.24A
3 months	6.32±2.83aA	11.91±2.63aA	9.11±1.93A
4 months	14.28±3.11aA	23.49±2.63bB	18.88±2.04B
Total	9.20±1.69a	17.98±1.70b	

Different lower case letters in the same rows and upper case letters in the same columns indicate significant differences according to LSD at P<0.05 levels

Be plant inoculated only with *B. edulis*, *BexPf* plant inoculated with *B. edulis* and *P. fluorescens*

mycorrhiza development (Garbaye and Bowen 1987; de Oliveira and Garbaye 1989; Duponnois and Plenchette 2003). Similar results were noted in previous studies by Dominguez et al. (2012) who coinoculated *Pinus halepensis* seedlings with *P. fluorescens* strain CECT 844 and *T. melanosporum*, resulting in a doubling of the level of mycorrhization from 15 to 28 % with respect to inoculation with *T. melanosporum* alone. Nonetheless, our results were below those reported by Wu et al. (2012) who studied the effects of coinoculating *B. edulis* and the bacteria *Bacillus cereus* Frankland and Frankland on *Pinus thunbergii* Parl. They also observed a positive effect of coinoculation with bacteria, increasing mycorrhization levels from 42 % in *B. edulis*-only plants up to 62 % in plant coinoculated with *B. edulis* and bacteria. However, the experiment was not conducted under in vitro conditions, and no information about baseline mycorrhization of the control plants was provided. Thus, even though the overall positive effect of coinoculation with MHB was clear, it remains unknown whether it was *B. edulis* or other unreported fungal species that benefited most from bacterial presence.

The relatively low percentages of *B. edulis* mycorrhizae obtained in our study (up to 23.5 %) may also be due to low levels of mycelium expansion, potentially related to several factors. Parladé et al. (2004) mentioned that although peat/vermiculite is the most suitable substrate for vegetative inoculum growth, the substrate can experience desiccation. In our study, the cellulose lid filter used to guarantee efficient gas exchange may have yielded loss of some moisture. This may have in turn negatively impacted mycelium expansion. Another important factor related to mycelium expansion is mycelium culture time, as poor fungal growth in the substrate may result in uneven quality of inocula in the pot (pers.obs.). Also, after long periods of mycelium cultivation, the availability of nutrients decreases. To the extent that low nutrient availability enhances the ability of fungi to form mycorrhizae (Requena et al. 1996), we would expect to see higher micorrhyzation rates at longer mycelium cultivation periods. In this sense, a positive overall correlation between culture time and mycorrization levels was observed in our study, with the highest level of mycorrhization occurring after 4 months of mycelium cultivation. In order to further improve mycorrhization levels, it might be interesting to explore mycelium culture times beyond 4 months.

Obtaining *B. edulis* mycorrhizae in *C. ladanifer* plants is a relevant first step toward achieving commercial plantations for early production of *B.edulis* sporocarps, particularly at a time when wild production of this species is likely to decline each year as a result of overexploitation and lack of regulation of this natural resource (Salerni and Perini 2004). Developing an efficient protocol of controlled mycorrhization is critical to enable the use of an ectomycorrhizal fungus species in forestry (Guerin-Laguette et al. 2000). Incorporating controlled mycorrhization procedures into forest nurseries implies the combination of up to three different organisms: plant, fungi, and also bacteria (Honrubia et al. 1997). Challenges associated with this increased level of complexity will need to be dealt with in order to obtain mycorrhized plants at a commercial scale (Pera and Parladé 2005). Once the production of mycorrhized plants is mastered, it will be also necessary to develop adequate management practices, i.e., mycosilviculture, to aid in the commercial production of sporocarps (Savoie and Largeteau 2011).

A relevant aspect of our study is the production and use of *C. ladanifer* vitroplants. Few studies on the micropropagation of *Cistaceae* have been conducted to date (M'Kada et al. 1991; Morte and Honrubia 1992; Iriondo et al. 1995; Pela et al. 2000; Madesis et al. 2011). Micropropagation techniques have not been previously applied to *C. ladanifer* because of its little economic value and great colonizing ability in its native habitat. However, given the difficulties in obtaining axenic seedlings from seeds due to frequent contamination (Talei et al. 2011), as well as the reduction in germination and seed viability when seeds are strongly disinfected (Sweet and Bolton 1979), production of vitroplants presents some advantages. Also, by using replicated genotypes, in vitro culture allows controlling for genetic variation of the host plant, which can also influence mycorrhization (Tagu et al. 2001).

Conclusions

Mycorrhizal synthesis between *C. ladanifer* and *B. edulis* was achieved successfully from a *B. edulis* strain collected in *C. ladanifer* shrubs. The results obtained confirmed the beneficial effects of *P. fluorescens* in enhancing the level of mycorrhization compared to inoculation with *B. edulis* alone. Longer mycelium culture times also improved the level of mycorrhization. The use of *C. ladanifer* vitroplants may allow for more efficient production of mycorrhized plants compared to use of inoculated seedlings. Theseresults bring us closer to producing mycorrhizal plants inoculated with economically valuable fungi for use in forestry.

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