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**Effects of the mycorrhiza helper bacteria
Pseudomonas fluorescens Migula on the
mycorrhizal synthesis between *Cistus ladanifer* L.
and *Boletus edulis* Bull.**

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0. ABSTRACT

Boletus edulis (L.) is one of the most valuable fungi worldwide, both for its edibility and economic importance. Sporocarps fruit associated symbiotically to different species of trees with more than 40 years of age. However, natural productions of *B. edulis* have been reported in 3 year old *Cistus ladanifer* shrubs. Several authors have found that the ectomycorrhizal symbiosis can be significantly improved by mycorrhiza helper bacteria (MHB). The aim of this work was to optimize the mycorrhizal protocol between *B. edulis* and *C. ladanifer* as a tool for future reforestation with early productions of *B. edulis* in this host. Effects of co-inoculating *Pseudomonas fluorescens* with *B. edulis* and mycelium culture time on the presence of mycorrhizas and on the level of mycorrhization were assessed. The results obtained were successful since it has been achieved the mycorrhizal synthesis at medium scale between *C. ladanifer* and *B. edulis* from an own isolate of *B. edulis*. Furthermore, results showed a significantly enhanced of the level of mycorrhization in the co-inoculated plants although the bacteria did not improve the number of mycorrhized plants. The same pattern followed the mycelium culture time. The obtained findings bring us closer to the controlled *B. edulis* sporocarps productions in plantations.

Key words: MHB, Mycelium culture, Mycorrhizal plants, Sporocarps.

0. RESUMEN

Boletus edulis es uno de los hongos más valiosos en todo el mundo, tanto por su comestibilidad como por su importancia económica. Fructifica asociado simbióticamente a diferentes especies de árboles más de 40 años. Sin embargo, producciones naturales de *B. edulis* se han observado en arbustos de *C. ladanifer* de 3 años de edad. Varios autores han encontrado que la simbiosis micorrícica puede ser mejorada por bacterias facilitadoras. El objetivo de este estudio fue optimizar el protocolo de micorrización entre *C. ladanifer* y *B. edulis* como una herramienta para futuras reforestaciones de *C. ladanifer* con producciones tempranas de *B. edulis*. Para ello evaluamos el efecto de la bacteria *Pseudomonas fluorescens* y del tiempo de cultivo del micelio en la presencia de micorrizas y el nivel de micorrización de la planta. Los resultados obtenidos fueron un éxito ya que se consiguió la micorrización entre *C. ladanifer* y *B. edulis* a media escala y a partir de un aislamiento propio. Además, los resultados mostraron una mejora del nivel de micorrización en las plantas co-inoculadas, aunque la bacteria no aumentó el número de plantas micorrizadas. El mismo patrón siguió el tiempo de cultivo de micelio. Los resultados obtenidos nos acercan a las producciones controladas de cuerpos de fructificación de *B. edulis*.

Palabras clave: MHB, Cultivo de micelio, Plantas micorrizadas, Cuerpos fructificación

1. INTRODUCTION

The species belonging to the *Boletus edulis* complex are currently among the most appreciated fungi, being a major commercial mushroom consumed worldwide (Alonso-Ponce *et al.*, 2011). They are distributed in both hemispheres, from Scandinavia to Southern Africa and Australia (Hall *et al.*, 1998). These valued edible mushrooms reach an annual world market around one billion Euros (Mello, 2012), harvesting in Spain an annual production of *Boletus* between 2,000 and 20,000 Tm, depending on the year (Oria-de-Rueda *et al.*, 2008). But a drastic decrease in the presence and productivity of *Boletus edulis* has been reported in several parts of Europe (Salerni and Perini, 2004). This phenomenon is accentuated by the increase in the demand of edible fungi (Sitta and Floriani, 2008). The fact that for the moment *Boletus edulis* is only collected from the wild (Cannon and Kirk, 2007), lead to search alternatives which be feasible for controlling the production of these fungi, as it happened with other mycorrhizal fungus like *Tuber melanosporum* (Bonet *et al.*, 2009).

Most of the Boletes fructify associated to *Pinus*, *Quercus* and/ or *Castanea* (Olivier *et al.*, 1997). Moreover, the production of *Boletus* sporocarps is linked to relatively mature forests, being observed mushroom productions in *Pinus* and *Quercus* stands with more than 40 years of age (Díaz-Balteiro *et al.*, 2003). *Boletus gr. edulis* species have been also reported under *Cistus* sp. shrubs in the northwest of Castile and Leon (Águeda *et al.*, 2006; Martín-Pinto *et al.*, 2006). Early natural productions of *B. edulis* have been reported in 3 year old *Cistus ladanifer* shrubs and high productions of these mushrooms in 8-year-old *Cistus* plants (Oria-de-Rueda *et al.*, 2008). The genus *Cistus* is widely distributed throughout the Mediterranean region, forming vast scrublands (Comandini *et al.*, 2006). *Cistus ladanifer* L. is, within this genus, the most abundant species, present in areas affected by forest wildfires. This natural association, along with sporocarps productions in early host ages allows being closer of producing artificial mycorrhized plants which then, may yield earlier *Boletus edulis* production.

In this sense, Águeda *et al.* (2008) synthesized ectomycorrhizas between *Boletus edulis* and *Cistus ladanifer* under laboratory conditions using synthesis tubes and obtaining plant material from seeds. After that, they identified and described the ectomycorrhizas through visual analysis based on standard morphological and anatomical characters in four replicates. The use of seeds in mycorrhizal synthesis causes some problems of contamination which can be avoided using other techniques like *in vitro* culture. Furthermore,

the use of vitro-plants may be an alternative method of commercial propagation, using it for the propagation of plants at medium or large scale (George and Sherrington, 1984).

Mycorrhizal identification through traditional methods is usually confusing and time consuming and high experience is required. For this reason, the development of techniques based on direct nucleic acid extraction coupled with polymerase chain reaction (PCR) amplification, as Real-time PCR, complement and confirm morphological studies, opening new possibilities to explore the cryptic phases of the symbiosis (mycorrhizas) (Mello *et al.*, 2006), resulting in prompt and reliable outcomes. The sensitivity of RT-PCR (greater than conventional PCR) allows detecting the presence of *B. edulis* DNA when small quantities are present in the sample (De la Varga *et al.*, 2011).

Also, it has been found that the formation of ectomycorrhizal symbiosis can be improved by mycorrhizosphere bacteria (Deveau *et al.*, 2007). The fungi-bacteria interaction brings ecological consequences, such as stimulating mycorrhization process (Kurth *et al.*, 2013). The group of bacteria which promotes this effect is called mycorrhiza helper bacteria (MHB) (Duponnois and Garbaye, 1991). For instance, they facilitate the mycorrhization from stimulating fungal mycelial extension, increasing root–fungus contacts and colonization, and reducing the impact of adverse environmental conditions on the mycelium of the mycorrhizal fungi (Brulé *et al.*, 2001; Frey-Klett *et al.*, 2007).

Several works isolated the bacteria located into the ectomycorrhizae formed between *Rhizopogon luteolus*- *Pinus radiata* and *Laccaria bicolor*- Douglas fir, and the most of the isolated strains belonged to the group *Pseudomonas fluorescens* (Garbaye *et al.*, 1989; Garbaye *et al.*, 1990). Various studies have confirmed that *P. fluorescens* improves the symbiotic relationship with some ectomycorrhizae, stimulating the ectomycorrhiza fungal growth (Frey-Klett *et al.*, 2007), and increasing the ratio of mycorrhization (Duponnois and Garbaye, 1991; Duponnois, 2006). Dominguez *et al.* (2012) reported the positive effect of *Pseudomonas fluorescens* in the mycorrhizal symbiosis between *Tuber melanosporum* and *Pinus halepensis*, where mycorrhization was significantly improved with the co-inoculation of *P. fluorescens*.

The integration of the new technologies concerning fungi and plant production, DNA detection applied to the mycology, as well as the positive results achieved with bacterial co-inoculations, allow us to have new expectations about *B. edulis* mycorrhizal plant production.

Despite the results have been limited worldwide to the control of *Boletus* sp. production, the possibility of obtaining productive plantations is a prospective aim.

Therefore, the general objective of this study was to optimize the mycorrhizal protocol between *B. edulis* and *C. ladanifer* as a tool for future reforestation with early productions of *B. edulis* in this host. In this sense, the following specific objectives have been addressed, i) analyze the effect of *P. fluorescens* bacteria in the presence and level of mycorrhization, ii / analyze the effect of the culture time of fungal inoculum in the presence and level of mycorrhization, and iii) guarantee the presence of mycorrhizae by using molecular techniques.

2. MATERIAL AND METHODS

2.1. Experimental design

The experiment included the following inoculation types: (1) Inoculation using *Boletus edulis* (*Be*), (2) inoculation using *B. edulis* × *Pseudomonas fluorescens* CECT 844 (*BexPf*) and (3) control without inoculation (*C*). Each of these inoculation types were established for three different mycelium culture times (two, three and four months). Eighteen *in vitro* pots were used per treatment and four plants were placed per pot. Thus, a total of 162 plants were tested.

Table 1. Experimental design

Inoculation	Mycelium culture time
<i>B. edulis</i>	Two months
	Three months
	Four months
<i>B. edulis</i> × <i>Pseudomonas fluorescens</i>	Two months
	Three months
	Four months
Control	Two months
	Three months
	Four months

2.2. Mycorrhizal syntesis protocol

2.2.1. Fungal inoculum

Sporocarps of *Boletus edulis* were collected from *Cistus ladanifer* shrubs located in northwest Spain. They were stored in closed polyethylene bags until the isolation in laboratory (Honrubia *et al.*, 1994).

Isolation was carried out under aseptic conditions in order to avoid possible contamination. A small piece of the fungus cap was placed on MMN nutritive medium plates (Modified Melin-Norkrans) (Marx, 1969) at pH 5.5, growing at 22-24°C in dark conditions (Honrubia *et al.*, 1994). Once the mycelium was large enough, it was transferred to fresh MMN plates to ensure its vitality and growth. A molecular analysis was carried out in order to verify the identification of the fungal species.

The solid expanding culture substrate was a mixture of vermiculite and peat (11/1; v/v) moistened with liquid MMN nutritive medium at pH 5.5 and the glucose content reduced at 2.5 g/l. The ratio of substrate to liquid was 2/1 (v/v). Substrate was transferred into glass pots and sterilized at 121°C for 20 min. Substrate was cooled down and the *in vitro* pots were inoculated with *B. edulis* adding 20 plugs, 5 mm diameter, of active mycelium and cultured at 22°C, for two, three or four months depending on the treatment.

2.2.2. Bacterial inoculum

The inoculum of *Pseudomonas fluorescens* CECT 844 was supplied by the CECT (Spanish Type Culture Collection), University of Valencia. The liquid 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 cultured and shaken at 100 rpm and 22°C. After 48h, bacterial concentration was recorded through the Thoma camera and bacterial inoculum was re-suspended in sterile water to get a concentration of 5×10^8 spores per milliliter.

2.2.3. Plant materials

Plant material was selected from *Cistus ladanifer* shrubs, which hosted *Boletus edulis* fungi located in the Western of Zamora province.

Shoots tips from five years old *Cistus ladanifer* shrubs were selected in order to establish *in vitro* cultures. Fifty shoots were introduced during 20 min in an antioxidant solution (100 mg/l ascorbic acid and 150 mg/l citric acid) to avoid explants browning (M'Kada *et al.*, 1991). Then shoots were surface sterilized by introduction in 100% ethanol for 30 s, rinsed with sterile distilled water and then further sterilized for 30 min in 1.5% sodium hypochloride (NaClO) solution with one drop of Tween 20[®] detergent. Finally, they were rinsed three times with sterile distilled water (Madesis *et al.*, 2011).

Proliferation was carried out culturing shoots tips on MS basal medium (Murashige and Skoog, 1962) supplemented with 0.88 mg l⁻¹ BAP, 30 g l⁻¹ sucrose and 8 g l⁻¹ agar. Shoot tips were sub-cultured every four weeks. Rooting was performed transplanting microshoots from the third subculture in MS basal media supplemented with 0.49 µm of IBA. Plants were grown, until the inoculation, at 25 ± 1 °C under a 16 h photoperiod with a light intensity of 2000 luxes provided by cool-white fluorescens lamps.

2.2.4. Mycorrhizal and bacterial inoculation

All of the inocula were applied at the same time (January 2014). *Cistus ladanifer* seedlings were placed into the *in vitro* pots which contained the vermiculite-peat substrate previously prepared. Depending on the treatment the plants were transferred into; substrate inoculated with *B. edulis* (*Be*); substrate inoculated with *B. edulis* and injected with a bacterial dose of 5×10⁸ spores/plant (*BexPf*); or substrate not inoculated (C). After performing inoculations, plants were stored in a culture chamber under a 16 h photoperiod with a light intensity of 2000 luxes and at 25 ± 1 °C for five months.

2.3. Mycorrhizal colonization verification using molecular technique

After five months from inoculation, plants were carefully extracted from the pots and roots were cleaning according to the methodology established by Fischer and Colinas, (1997). All the plants inoculated were visualized by stereo microscope to analyze the colonization of the mycorrhizal fungi in the roots by the morphological characterization and identification of the mycorrhizas (Agerer, 1991). Description of the *B. edulis* ectomycorrhiza performed by Águeda *et al.* (2008) was followed to ensure the identification.

The mycorrhization percentages were calculated. For that, roots were then chopped into 1–2 cm pieces that were cleaned, rinsed in distilled water and placed into a Petri dish with water for analysis. In the mycorrhized plants, the total roots as well as the *B. edulis* mycorrhized tips were counted.

The identification of *B. edulis* mycorrhiza was confirmed using molecular techniques. DNA extractions for the roots were performed with the PowerSoil™ DNA Isolation Kit and the presence by real-time polymerase chain reaction using specific primers and TaqMan® probe (De la Varga *et al.*, 2012). One plant were selected randomly and analyzed from each treatment, being a total of nine plants analyzed molecularly.

2.4. Data analysis

Pearson Chi-square Test was used to compare frequency tables for mycorrhization presence among treatments. For comparing the level of mycorrhization over the mycorrhized plants, a factorial ANOVA analysis was performed for the data and means were compared for the proposal factors by Least Significance Difference (LSD) Fisher Tests ($P < 0.05$). Statistical analysis was accomplished with STATISTICA '08 Edition software (StatSoft Inc., 1984–2008).

3. RESULTS

3.1. Mycorrhizal synthesis

The first noticeable result of this experiment is that ectomycorrhizas of *Boletus edulis* in *Cistus ladanifer* plants were synthesized *in vitro*, furthermore is the first time that this was possible at medium scale using *in vitro*-plants. To get this result, *in vitro* propagation of plant material was a relevant finding to optimize the inoculation protocol. The ectomycorrhizas visualized had typical traits of Boletales, and followed the description realized by Águeda *et al.* (2008) but with some differences in the color, showing brownish but with plectenchymatous mantle and rhizomorphs with highly differentiated hyphae. The inflated, smooth cystidia-like clavate end cells on the surface of the rhizomorphs and their slightly twisted external hyphae are additional characterizing features.

In addition, this was the first time that *B. edulis* mycorrhizal synthesis was verified through molecular analysis. Every plant selected randomly from each treatment was positive for *B. edulis* mycorrhization in Real-Time PCR.

3.2. Mycorrhizal infection presence

Both *Boletus edulis* and *B. edulis* × *Pseudomonas fluorescens* formed ectomycorrhizas with *Cistus ladanifer*. Among the total of 108 plants inoculated, 35 were mycorrhized, which corresponds with a mycorrhizal infection ratio of 32.41% over the whole experiment.

Among the 54 plants inoculated only with *B. edulis*, 18 were mycorrhized, which come into a mycorrhizal infection ratio of 33.33%. From the plants inoculated with *B. edulis* × *Pseudomonas fluorescens* 17 were mycorrhized, reaching a mycorrhizal infection ratio of 31.48%.

Using co-inoculation with bacteria did not affect significantly the presence of mycorrhized plants. In this sense, from the mycorrhized plants, 51.43% belonged to those inoculated with *B. edulis*, whereas 48.57% were inoculated with *B. edulis* × *Pseudomonas fluorescens*.

This same effect was observed analyzing the mycelium culture time. Thus, this variable did not affect significantly the percentage of mycorrhized plants. With a mycelium culture time of two months 27.78% of the inoculated plants were mycorrhized. The mycorrhizal infection ratio was 36.11% at three and 33.33% at four months of mycelium culture time.

Analyzing jointly the factors bacteria and mycelium culture time, no differences were evidenced among treatments (Table 2).

Table 2. Presence of mycorrhizas (in percentage) according to inoculation and mycelium culture time

Inoculation	Mycelium culture time			Total
	2 months	3 months	4 months	
<i>B.edulis</i>	33.33aA	33.33aA	33.33aA	33.33A
<i>B. edulis</i> +bacteria	22.22aA	38.89aA	33.33aA	31.48A
Total	27.78a	36.11a	33.33a	

Different minuscule letters in the same rows and capital letters in the same columns indicate significant differences according to Pearson Chi-square Test at P<0.05 levels.

3.3. Level of mycorrhization over the mycorrhized plants

Co-inoculation and mycelium culture time significantly affected the percentage of mycorrhized plants. However, no interaction between these two factors was found, thus bacteria affected increasing mycorrhization levels independently of the mycelium culture time (Table 3).

Table 3. Analysis of the variance (ANOVA) of the level of mycorrhization over the mycorrhized plants.

	SS	Degr. of freedom	MS	F	p
Intercept	6934,364	1	6934,364	119,8742	0,000000
Time	733,016	2	366,508	6,3358	0,005213
Bacteria	874,501	1	874,501	15,1175	0,000542
Time*Bacteria	74,915	2	37,457	0,6475	0,530740
Error	1677,563	29	57,847		

The most remarkable finding of this work was that co-inoculation of *Boletus edulis* and bacteria *Pseudomonas fluorescens* had a positive effect on the level of mycorrhization, being this the level significantly higher than in the case of plants mycorrhized exclusively with *Boletus edulis* ($P < 0.001$). Among the plants mycorrhized with *B. edulis* exclusively, the level of mycorrhization was 9.25% whilst for the plants inoculated also with *P. fluorescens*, the level reached 19.45% (Figure 1).

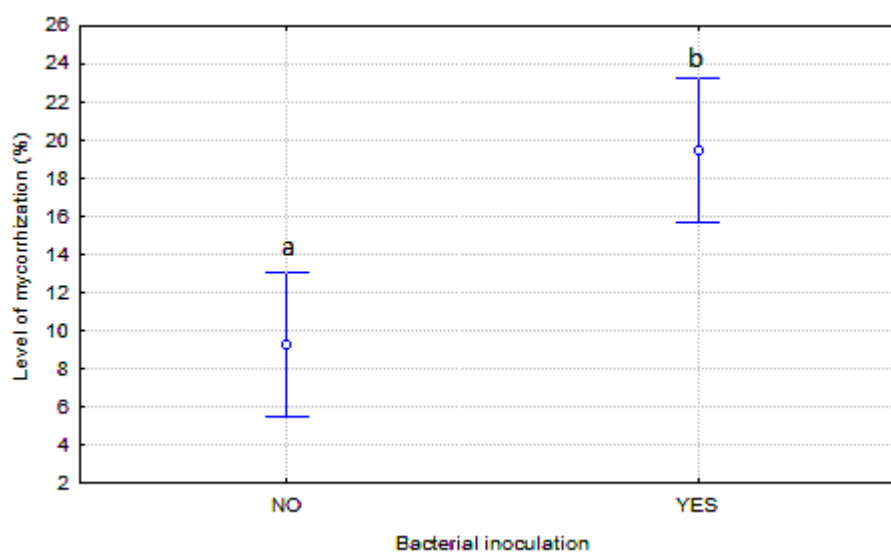


Figure 1. Effect of co-inoculation of *B. edulis* and *P. flourescens* on mycorrhization level. Different letters indicate significant differences according to LSD at $P < 0.05$ levels.

Mycelium culture time was favorable to the level of mycorrhization and, in this sense the percentage of tips mycorrhized was higher in the plants inoculated with mycelium cultured during four months, reaching 20.25% of tips mycorrhized. The level of mycorrhization at four months of mycelium cultivation was significantly different from the level

obtained at two ($P=0.009$) and three months of cultivation ($P<0.001$). However, differences between the level of mycorrhization at two and three months of mycelium cultivation were not significant ($P=0.43$).

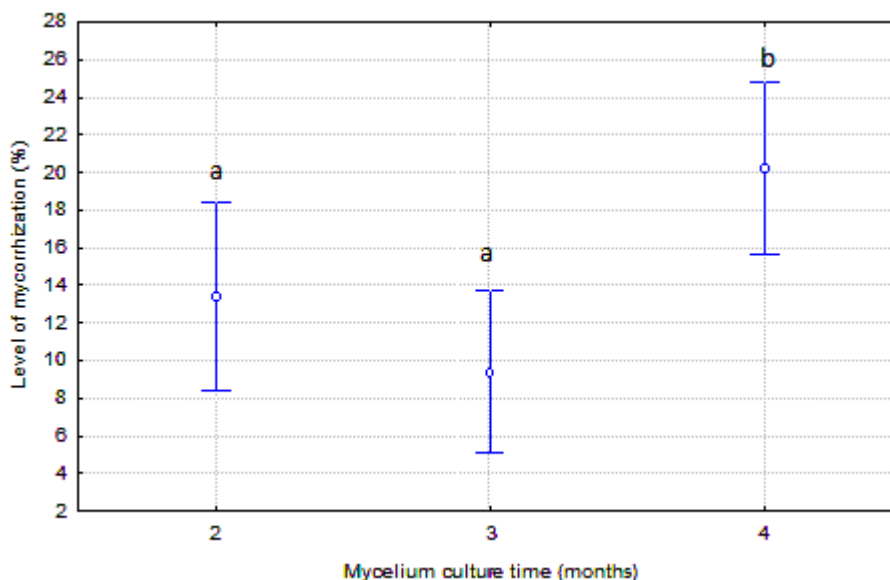


Figure 2. Effect of the mycelium culture time on mycorrhization level. Different letters indicate significant differences according to LSD at $P<0.05$ levels.

Analyzing together the use of bacteria and the mycelium culture time, some interesting results were appreciated. Higher levels of mycorrhizas were achieved when using the helper bacteria. For two and four months of mycelium culture time these differences were significant. Although not significant, the trend was similar for three months of mycelium cultivation. In all the cases, mycorrhization levels were duplicated when using co-inoculation with *B. edulis* x *P. fluorescens* (Table 5).

Table 5. Effects of inoculation of *B. edulis* inoculated alone and together with *Pseudomonas fluorescens* on *Cistus ladanifer* level of mycorrhization for the different mycelium culture times.

Mycelium culture time	Inoculation	
	Be	Be+bacteria
Two months	7.16 ± 2.70aA	19.69 ± 3.65bAB
Three months	6.32 ± 2.31aA	12.44 ± 2.81aA
Four months	14.28 ± 2.48aA	26.22 ± 2.58bB

Different minuscule letters in the same rows and capital letters in the same columns indicate significant differences according to LSD at $P<0.05$ levels. Be is the plant inoculated with *Boletus edulis*. Be+bacteria is the plant inoculated with *B.edulis* and *Pseudomonas fluorescens*.

The best results were achieved in the plants mycorrhized with *B. edulis* and bacteria, with four months of mycelium cultivation; in this case the level of mycorrhization was significantly higher 26.22% (Table 5).

If the mycelium culture time is analyzed separately for the two types of inoculation, a similar trend was observed. Thus, the highest mycorrhization levels were found when the culture time was four months in both cases, accordingly to the previous overall result. It was observed that inoculation with *B. edulis*, mycorrhization percentage reached doubles the ratios of two and three months. The higher value in the case of co-inoculation with the bacteria was also achieved when the culture time was four months (Table 5).

4. DISCUSSION

The results obtained are successful since it has been achieved the mycorrhizal synthesis between *C. ladanifer* and *B. edulis*, from own isolates of *B. edulis* at medium scale. In order to develop the use of an ectomycorrhizal species in forestry, it is crucial to establish an efficient protocol of controlled mycorrhization (Guerin-Laguette *et al.*, 2000). In this sense, an important improvement of the mycorrhizal protocol has been achieved with this work, showing the effect of the co-inoculation with mycorrhizal helper bacteria on the mycorrhization levels. Besides, different mycelium culture times have been analyzing to test the effect over the presence of mycorrhizas and the level of mycorrhization.

Obtaining *B. edulis* mycorrhizas in *C. ladanifer* plants is a very relevant fact as it is the first step which could enable to set future commercial plantations for early productions of *Boletus edulis* sporocarps, whose wild productions may decline each year by over exploitation and lack of regulation of this natural resource.

Mycorrhizas observed in plants were contrasted with those described by Águeda *et al.* (2008), but differences were observed in the color of our mycorrhizas that were darker. This difference in the color of the mycorrhizas may be due to the dryness of the substrate shown at the end of the experiment. When the mycorrhiza loss water it tends to get obscure because of the desiccation of the tissues. Furthermore, small differences evidenced in the mycorrhizas could be due to the fact that the *B. edulis* mycorrhized by Águeda *et al.* (2008) was associated to *Cistus albidus*. In spite of that, the rest of the traits were similar to those characterized, plectenchymatous mantle, monopodial ectomycorrhizal system and frequent hyphae and rhizomorphs.

In this point, molecular techniques play an important role when doubtful cases, as happened in the comparison among our mycorrhizas with the previously described. Molecular analysis will allow us to identify our sample quickly, accurate and reliable (Skena *et al.*, 2004). Our mycorrhizal identification was confirmed through molecular analysis, for both, the inoculum used and the mycorrhizas obtained. Every plant selected randomly from each treatment was positive for *B. edulis* mycorrhization. To our knowledge, this is the first time that a molecular verification is performed for *B. edulis* after mycorrhization to check the success of it. Some studies realized molecular analysis over the fungus previously to the inoculation (Águeda *et al.* 2008). This subsequent test certifies with total guarantee that the mycorrhiza obtained was caused by the isolated used and not by any contaminant.

Mycorrhizas were formed in the two treatments where there was *B. edulis* inoculation (plants inoculated with *B. edulis* exclusively and plants inoculated with *B. edulis* and *P. fluorescens*), whilst the control plants did not present any type of mycorrhizas, neither of *B. edulis* nor of possible contaminants. Despite the achievement in the mycorrhizal synthesis for both *B. edulis* inoculated alone and also co-inoculated with *P. fluorescens*, the presence of mycorrhizas was lower than expected. The relatively low percentages of *B. edulis* mycorrhizas obtained may be due to an insufficient mycelium expansion, which simultaneously may be promoted by various factors. An important factor to have into account is the mycelium culture time. As it happens with other mycorrhizal fungi as *Lactarius deliciosus* (Guerin-Laguette *et al.*, 2000; Parladé *et al.*, 2004), *B. edulis* grows slowly in pure culture and takes at least two months to complete the substrate colonization. In this sense, an insufficient cultivation of the substrate may result in an uneven quality inoculum. The slow colonization of *B. edulis*, as well as the uneven quality of the inoculums could be palliated by adding a larger quantity of fungal mycelium at the inoculation time.

Another relevant factor may be the intraespecific variation of ectomycorrhizal fungi. Thus, fungi of the same species may present different growing characteristics and also different colonization ratio in pure cultures (Pera and Alvarez, 1995; Parladé *et al.*, 2011). Several authors found diversity in the responses within the same species, in this sense, varying specificities among the fungal species has become apparent (Frey-Klett *et al.*, 2007; Aspray *et al.*, 2013). A future possibility for optimizing the protocol would be to study several *B. edulis* isolates in order to select the most productive ones.

A possible factor which may have affected negatively was the dryness that the substrate presented at the moment of the roots evaluation. Despite the inoculum type utilized is one of the most suitable for producing mycorrhizal seedlings (Marx and Kenney, 1982), some disadvantages related to the desiccation are probable and difficult to manage (Parladé *et al.*, 2004). In this way, a control of the humidity of the culture chamber would be a requisite for a successful mycelia expansion.

The relevance of the bacteria in the mycorrhiza formation was reported for first time by Bowen and Theodorou (1979). They tested that some bacterial strains promoted and others inhibited the colonization of *Pinus radiata* roots by *Rhizopogon luteolus*. Posterior studies were conducted in order to get more knowledge. Thus, Garbaye and Bowen (1987); de Oliveira and Garbaye (1989) confirmed that the co-inoculation with bacteria promoted the mycorrhiza formation. More recently, satisfactory results gained in previous works with *Pseudomonas* species. Duponnois and Plenchette (2003) co-inoculated *Pseudomonas monteilii* with several isolates of *Pisolithus* sp. and *Scleroderma* sp. in Australian *Acacia* species. The ectomycorrhizal colonization for all the *Acacia* species was significantly enhanced (from 45.8% to 70.3%). Deveau *et al.* (2007) tested the effect of various bacteria (*Collimonas fungivorans*, *Paenibacillus* sp., *Paenibacillus* sp., *Bacillus subtilis*, *Burkholderia* sp. and *Pseudomonas fluorescens*) through an *in vitro* confrontation with *Laccaria bicolor*. *Pseudomonas fluorescens* was the unique bacteria which stimulated the growth of *L. bicolor* mycelium, as well as the hypha density. Dominguez *et al.* (2012) stated the improvement of *P. fluorescens* in the establishment and mycorrhizal symbiosis of *Tuber melanosporum* in *Pinus halepensis*. They obtained the bacteria inoculum from the CECT (Spanish Type Culture Collection), University of Valencia. In this sense, regarding the good results obtained by Dominguez *et al.* (2012) with *P. fluorescens* CECT 844, this isolated was selected for our study.

According to the results acquired in our work when applying the bacteria, in the co-inoculated treatment (*B. edulis* x *P. fluorescens*) the presence of plants mycorrhized did not present differences respect to the inoculated with *B. edulis* exclusively. Specificity in MHB-mycorrhizal fungus interactions was already indicated in early studies, which described bacterial species that promote and others that were either neutral or inhibitory to mycorrhiza formation (Garbaye and Bowen, 1987, 1989).

The results obtained for the presence of mycorrhizas in the co-inoculation may also be explained because of the competition of *B. edulis* with the bacteria during the establishment into the substrate (Bowen and Theodorou, 1979). Various authors postulated that some interactions between MHB and the fungus may be beneficial but some others may be competitive to the mycorrhizal infection process (Garbaye, 1994; Frey-Klett *et al.*, 2007; Aspray *et al.*, 2013). Bearing in mind that *B. edulis* is a sensitive species of slow growing in saprophytic conditions (Olaizola, 2007), in a pre-symbiotic moment, i.e., before the association between the plant and *B. edulis*, *P. fluorescens* and *B. edulis* may compete for the nutrients and *B. edulis* may be particularly affected. This result agree with the observations of Brulé *et al.* (2001), who affirmed a situation of dominance of the bacteria over the fungus in rich growth media. Kurth *et al.* (2013) also suggested the inhibition of *Piloderma croceum* because the competition for resources with bacteria. Thus, considering the competition of *P. fluorescens* and *B. edulis* in the early stages, a reduction of the inoculated bacterial dosage could be beneficial for the growth of *B. edulis*. The development of methods for quantifying the abundance of bacteria and fungi in the presence of one-another is essential (Kurth *et al.*, 2013).

It was remarkable that our study showed that MHB increased the level of mycorrhization. In this way, once *B. edulis* was associated with the plant, bacteria was beneficial for its development which consequently was showed in a higher formation of mycorrhizas. This result was in accordance with the obtained in previous studies. Dominguez *et al.* (2012) co-inoculated *P. fluorescens* and *Tuber melanosporum* in *Pinus halepensis* seedlings. They achieved to increment significantly the level of mycorrhization from 15% to 28%. Regarding the interaction between *B. edulis* and MHB, Wu *et al.* (2012) analyzed the effects of co-inoculating the fungus and the bacteria *Bacillus cereus* in *Pinus thunbergii*. For that, they isolated *Bacillus cereus* strains from *B. edulis* sporocarps and inoculated the plants in nursery conditions. Interesting results were achieved since mycorrhizal infection ratio was significantly increased in the plants co-inoculated with the fungus and bacteria. However, they did not provide any molecular analysis to confirm the results which may lead to false positives, i.e., a possible mycorrhization with other mycorrhizal fungi of similar morphological appearance.

Although, the results obtained for the level of mycorrhization are apparently contradictory with the obtained in the mycorrhizal infection, this fact has been explained by other authors. Zhao *et al.* (2013) tested 140 bacterial isolates associated to poplar from soil

samples. From them 12 stimulated the growth of two fungal isolates and the four best were selected for further analysis. One of them increased the mycorrhizal presence significantly. However, the other three isolates promoted fungal growth *in vitro* experiment but not enhanced ectomycorrhiza formation. Zhao *et al.* (2013) suggested that the mechanism of MHB effect may be diverse, and stimulation of fungi growth by MHB is not necessarily correlated to effectiveness at promoting ectomycorrhiza formation. Brulé *et al.* (2001) studied the effect of *Pseudomonas fluorescens* on the Douglas fir-*Laccaria bicolor* symbiosis. They postulated that the success of the inoculation depends on the survival of the fungal inoculums in the soil during pre-symbiotic life of the fungus. Furthermore, they found a pattern of the bacteria in the fungus depending on the experimental conditions. Bacteria had a negative effect on the symbiosis under favorable conditions (rich growth media) and presented a positive effect under unfavorable conditions.

Considering the mycelium culture time, a positive overall correlation was showed for the level of mycorrhizas over the mycorrhized plants, reaching the highest level of mycorrhization at four months of mycelium cultivation. The maturity of the mycelium growing for four months could cause higher mycorrhization levels. At four months of culture time, the availability of nutrients is lower and the mycelium is becoming mature. Previous studies have shown the ability of the ectomycorrhizal fungi to cope with stress situations such as nutrient deficiency. Even in those poor conditions, fungi are able to increase their natural mycorrhizal potential to guarantee plant mycorrhization (Requena *et al.*, 1996).

However mycelium culture time did not affect mycorrhizal presence. Mycorrhization percentages are directly linked to mycelium presence in the soil. In this sense, the result can be explained because of the slow growth of *B. edulis* mycelium. Olaizola (2007) tested the mycelium growth of twelve isolates of different mycorrhizal fungi under axenic conditions. In that case, *B. edulis* was one of the isolates with lowest growth, with a growth significantly lower than that for *Lactarius deliciosus* which get the whole colonization of the substrate by the mycelium after two months as minimum under optimum conditions (Parladé *et al.*, 2004).

For explaining our results regarding to fungal culture time, we hypothesize the possibility that the quantity of mycelium achieved at two months was similar than that present in the soil after four months. However, the maturity of the mycelium growing for 4 months could cause higher mycorrhization levels.

Furthermore, we have to highlight the achievement in the proliferation of *Cistus ladanifer* plants through *in vitro* culture. To date only few studies on the micropropagation of *Cistaceae* have been conducted (M'Kada *et al.*, 1991; Morte and Honrubia, 1992; Iriundo *et al.*, 1995; Pela *et al.*, 2000; Madesis *et al.*, 2011), and *C. ladanifer* had not been cultured using *in vitro* propagation previously due to its scarce economic value and its great colonizing ability. Seeds of *Cistus* species have a form of dormancy due to their impermeable seed coat, which is broken when the seeds are exposed to heat (Madesis *et al.*, 2011). Furthermore, sometimes it is difficult obtaining axenic seedlings proceeding from seeds because of the contamination problems. When stronger disinfection treatments are performed, germination and seed viability are drastically reduced. De la Varga *et al.* (2011) confirmed the necessity of improving the production of *C. ladanifer* plants under axenic conditions pointing the difficulty of producing a large quantity of plants mycorrhized because of the high economic and time costs. Thus, plant tissue culture is an alternative method of commercial propagation, used widely for the propagation of a large number of plant species (George and Sherrington, 1984). Taking into account that our prospective objective is to place plantations of *C. ladanifer* mycorrhized with *B. edulis*, our work has gone a step further on the idea of making *B. edulis* fruiting plants a commercial product. This method would be more feasible for plant proliferation at medium and large scale.

5. CONCLUSIONS

Mycorrhizal synthesis between *C. ladanifer* and *B. edulis* was achieved from an own *B. edulis* isolate. Consecutive identification of *B. edulis* mycorrhizas was verified through molecular techniques. The obtained results confirmed the beneficial effects of *P. fluorescens* in an increment of the level of mycorrhization respect to the inoculation with *B. edulis* alone. A higher mycelium culture time also enhanced the level mycorrhization. Furthermore, the achievement in the production of *Cistus ladanifer* vitro-plants enable faster production, avoiding the high problems of contamination observed from seeds and facilitating the plant production at large scale. The accomplished results bring us closer to form controlled plantations of *C. ladanifer* producing *B. edulis*, where it will be possible to collect fruiting bodies. This will enable return a valuable resource to the rural areas.

6. REFERENCES

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