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Effects of Inoculation with Arbuscular Mycorrhizae and Ectomycorrhizae on Growth and Mycorrhizal Colonization of Cork Oak (*Quercus suber* L.)

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Abstract

The cork oak (*Quercus suber* L.) is a tree which forms important forests that play a vital socioeconomic and environmental value in the Mediterranean basin. The multiple factors of overgrazing, biological invasion by introducing acacia trees, and repeated fires weaken the cork oak ecosystem and affect its natural regeneration. The purpose of this study is to increase the production of cork oak. With this aim, a test of controlled mycorrhization was carried out on the nursery where two commercial inocula—the arbuscular mycorrhizal (Symbivit) (S) and ectomycorrhizal (Ectovit) (E)—were brought separately and combined on cork oak seedlings cultivated on sterilized or non-sterilized soil. Statistical models revealed that the controlled inoculation improved the growth of the plants inoculated by IE, which had significantly increased mycorrhizal root colonization levels and cork oak growth compared to the treatments of the Symbivit (IS) and the non-inoculated plants (NI); these rates were lower in both substrates: sterile and non-sterile. It is known that the best mycorrhizal partners of cork oak are ectomycorrhizae, however, in the presence of arbuscular mycorrhizae, the mycorrhizal root colonization levels and the growth parameters were considerably enhanced compared to previous treatments IE, IS and NI. The dual colonization had shown positive effects on the improvement of the mycorrhizal potential of the soil. Indeed, EM % colonization was the most strongly correlated with growth parameters compared to other mycorrhizal parameters. This research underlines that the use of controlled inoculation based on commercial inoculum can be an effective alternative in the case where the local inoculum is not available, and thus time saving.

Keywords: *Quercus suber*, Arbuscular mycorrhizae, Ectomycorrhizae, Dual colonization, Growth, Mycorrhizogenic potential

1. Introduction

Cork oak (*Q. suber*) woodlands are ecosystems of high environmental and socioeconomic values, characterized by a vegetation cover that supports high levels of biodiversity (Bugalho *et al*., 2011). Native cork oak forests occupy 1.3 million ha in southern Europe (Portugal, Spain, France and Italy) and 0.9 million ha in North Africa (Morocco, Algeria and Tunisia) (Lancellotti and Franceschini, 2013). The cork oak is threatened due to the combined effects of overexploitation of wood, overgrazing, deforestation, and the repeated fires (Lancellotti and Franceschini, 2013). However, the cork oak decline has multiple impacts at above ground and below ground levels, strongly affecting resilience and productivity of cork oak forests (Maghnia *et al*., 2017).

Natural regeneration from seed is not always successful, and the survival rate of transplanted seedlings is often low (Sebastiana *et al*., 2013). For this purpose, an exploration of the potential of the association between *Q. suber* and beneficial microbial symbionts can be the crucial solution to produce high-quality seedlings of cork oak in the nursery stage (Araújo *et al*., 2018).

Mycorrhizal fungi are ubiquitous components of most ecosystems throughout the world and are considered key ecological factors in biological processes (Schreiner *et al*., 1997), increasing plant tolerance to environmental stresses (Meddad-Hamza *et al*., 2010), promoting plant growth in soils with low water and mineral availability (Bingham and Simard, 2012), allowing seedlings survival (Wezowicz *et al*., 2017), reducing soil fertilization and irrigation requirements (Rillig *et al*., 2015), and contributing to the restoration of degraded soil (Asmelash *et al*., 2016).

Cork oak species form a dual symbiotic association with arbuscular (AMF) and ectomycorrhizal fungi (EMF) (Hamidi *et al*., 2017). Thus, in the objective to improve the quality and ecosystem resilience of nursery-produced *Q. suber* seedlings, the association with ectomycorrhizal and arbuscular fungi should be a forefront strategy (Araújo *et al*., 2018).

Thus, the main objective of this ecophysiological study is to highlight the beneficial effects that represent different AMF and EMF inoculations for the cork oak. For that, an arbuscular mycorrhizal and an ectomycorrhizal commercial inoculum (Symbivit and Ectovit respectively), were used separately or in combination. Concurrently, the objective of these root inoculations with different AMF and EMF strains is to determine the efficacy and infectivity of these both strains for cork oak.

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2. Materiels and Methods

2.1. Inoculation of Q. suber seedlings

Two inoculants were tested, a commercial arbuscular mycorrhizal inoculum Symbivit® and ectomycorrhizal fungi (EMF) Ectovit® **(**INOCULUM plus, France**)**.

Symbivit® granulated and full of 6 AMF species which are composed of natural clay and propagules (*Claroideoglomus etunicatum*, *C. claroideum*, *Glomus microaggregatum*, *Rhizophagus intraradices*, *Funneliformis mosseae*, *F. geosporum*) where we have used a number of 200 propagules per plantlets While Ectovit® contained propagules of 6 different species of EMF; 4 strains of mycorrhizal fungi on a liquid medium agar (*Amanita rubescens*, *Hebeloma velutipes*, *Laccaria proxima*, *Paxillus involutus*), and 2 strains of mycorrhizal fungi (*Pisolithus arrhizus*, *Scleroderma citrinum*) on a peat-based carrier with a minimum number of spores of 300 million per gram of dry matter.

The arbuscular mycorrhizal inoculum (Symbivit) is mixed with the substrate at the rate of 10 g of inoculum, while the ectomycorrhizal inoculum (Ectovit) was brought from the second month of growth as a paste prepared by mixing fungal mycelium with dry inoculum components (including fungal spores) and sufficient water. The mixture was subsequently distributed in six 10 cm deep holes, dug using a glass bar closest to the roots of each cork oak plant.

2.2. Experimental design

The experiment was conducted in station of the National Institute of Forest Research (NIFR) at the nursery of Guerbes (36°56'6.79'' N; 7°11'18.36" E), Skikda region, from 14 April 2014 to 14 January 2015, under natural daylight. The daily average temperature was 10.8-28.8 °C and relative humidity of 60-70%. This nursery is specialized in the production of forest plants, especially cork oak, intended for the reforestation of the Northeastern region of Algeria.

The culture substrate used in this experiment represents a mixture of 60% olive pomace and 40% forest humus. Olive pomace is the waste recovered from oil-mills that has undergone composting for three years in order to reduce the levels of acids and toxic compounds that may be present. The forest humus originates from the decomposition of the accumulated matter under a vegetation of cork oak forests of Guerbes. A portion of the substrate sample was sterilized (SS) twice in an autoclave, 20 min at 120°C with 24 h between each autoclave, and the rest was not sterilized (NS). *Q. suber* acorns were collected directly from one tree in November 2014 in the Brabtia nature reservation (36°52'09.27''N; 8°20'16.58''E), located in the commune of El Kala, in the Northeast of Algeria. The acorns were washed with tap water and a few drops of detergent disinfected by immersion (15 min) in a solution of 30% hydrogen peroxide. They were then rinsed several times with sterile water, later placed to germinate during ten days under aseptic conditions in wet soil previously autoclaved twice for 20 min at 120 °C and then stored in a culture chamber under strict conditions: temperature 20 °C, controlled humidity, and darkness.

The sterile (SS) and non-sterile (NS) substrates are dumped into WM containers at 900 g per container with a pregerminated acorn. The experimental setup was simple randomization with 8 treatments repeated 7 times for a total of 56 plants: cork oak seedlings inoculated (I) by Ectovit (E), Symbivit (S), the combination of both E and S that became (M) grown on sterile (SS) and non-sterile (NS) substrate. Two control non-inoculated (NI) grown on SS and NS were made. The plants were irrigated with an automatic system three times a week. This frequency starts daily in the summer and then decreases during the wet months.

2.3. Plant sampling and analyses

After 9 months of growth**,** plant height, shoot and root fresh weight and the mycorrhizal root colonization levels of the cork oak plants were estimated. Moreover, an evaluation of the content of the leaf chlorophyll was assessed with Chlorophyll Meter Konica Minolta SPAD-502 Plus. The SPAD values were taken at the top, the middle and the base of the leaf. The chlorophyll value obtained in the SPAD unit is the average of the three values read on the screen.

Growth percentage of all parameters was calculated by the use of the following formula: Growth increase (%) = [(growth of inoculated plants - growth of non-inoculated plants)/growth of non-inoculated plants)] × 100 (Plenchette *et al.*, 1983).

2.4. Assessment of EM root colonization

Ectomycorrhizal (EM) colonization assessment was determined by counting the presence or the absence of colonized root tips under a stereomicroscope according to the method of Brundrett *et al*., 1996. The percentage of root colonization was determined for each sample by examining 300 1 cm-long pieces of root, expressed with the following formula:

EM colonization rate (%) = [Number of mycorrhizal root pieces /Total number of observed root pieces] x100.

2.5. Determination of arbuscular mycorrhizal colonization

Arbuscular mycorrhizal (AM) root colonization was estimated on the basis of the Phillips and Hayman (1970) method. Samples were processed, undergoing the following stages: soil washing, cutting into segments of 1.5 cm, hot cleaning with KOH 10% (15 min), immersion into a solution of HCl 20% (10 min) and staining with 0.03% Trypan blue solution at 90 °C.

2.5.1. Estimation of root mycorrhization

Annotation was made according to the method described by Trouvelot and Kough (1986), which is a fast-technique reflecting as much as possible the potential and the state of activity of mycorrhizal symbiosis. Root observations were done for 5 repetitions of 30 root fragments of 1 cm, placed between slides and lamellae and observing them under a light microscope. The operation was repeated twice to calculate five mycorrhization parameters using the MycoCalc computer program (http://www2.dijon.inra.fr/mychintec/).

2.6. Mycorrhizogenic potential of soil

AMF propagules were assessed after inoculation using the most probable number (MPN) method (Porter, 1979), which is based on the use of a series of successive soil dilutions at the rate of 10 (1/10, 1/100, 1/ 1000, 1/10,000

and 1/100,000) for determining the limiting dilution at which no AMF propagules can be detected. The dilutions were prepared by mixing the original soil with the same soil autoclaved twice at 120 °C (Gianinazzi-Pearson *et al*., 1985). The soil mixture is divided into five replicates of 50 g per pot. Pregerminated clover (*Trifolium repens* L.) seeds were planted with one seedling per pot. The seedlings were transferred under controlled conditions in a greenhouse (average daily temperature 18–22 °C, with 60–70% relative humidity) and watered daily with distilled water. After six weeks, the entire root system per plant was stained according to the method of Philips and Hayman (1970) using acid lactic. Using mycorrhizal and non-mycorrhizal roots obtained for each level of dilutions and for the five repetitions, the number of propagules present in soil was evaluated with the help of the table of Cochran (1950). Furthermore, the mycorrhizal potential of soil was calculated for the following treatments: IS+SS, IM+SS, IS+NS, IM+NS and NI+NS.

2.7. Statistical analysis

Before analyses, all data were checked for normality and homogeneity of variance. We used analysis of variance (ANOVA) by a general linear model (GLM) to examine the effects of inoculation with AMF, EMF individually or in mixtures on the amelioration of the colonization levels and the growth of cork oak. The means were compared using Tukey's HSD test ($P < 0.05$). The Pearson correlation test is performed using an analysis of variance (ANOVA) and their plots were drawn using the package {ggplot2} (Chang, 2013). Statistical analyses and models were carried out using the software R.

3. Results

3.1. Plant growth

The inoculation by the ectomycorrhizal fungi (Ectovit), the arbuscular mycorrhizal fungi (Symbivit) and the dual inoculation of both had a significant impact on most of the measured cork oak plant growth parameters (Table 1). Overall, inoculated plants had better growth compared to the non-inoculated ones (i): height (F=88.08, P≤0.000), +73%, shoot fresh weight (F=169.75, P≤0.000), + 188%, root fresh weight (F=101.81, P≤0.000), + 147%, chlorophyll content (F=72.70, P 0.000), + 23%. (ii): height +14%, shoot fresh weight +35 %, root fresh weight + 38%, chlorophyll content + 10%. (iii): height +32%, shoot fresh weight + 111%, root fresh weight 86%, chlorophyll content + 35%. No significant difference (F=2.13, P≤0.109) was observed for the ratio root/shoot between the inoculated plants and the non-inoculated.

The treatment IE had the greater growth in comparison with IM and IS treatments. Significant differences were recorded. (i): IE compared to treatment IM +30% height + 36% shoot fresh weight, + 33% root fresh weight, (ii): IM compared to treatment IS, + 15% height, + 56% shoot fresh weight, + 34%, root fresh weight, +22% chlorophyll content. (iii): IE compared to treatment IS + 51% height, + 113% shoot fresh weight, + 79%, root fresh weight, + 12% chlorophyll content.

Furthermore, the effect substrate also showed significant differences on the shoot fresh weight (F=17.62, P≤0.000), the root fresh weight (F=28.36, P≤0.000), the ratio: root/shoot (F=16.56, P≤0.000) and the chlorophyll content (F=29.97, P 0.000). The interaction 'inoculation x soil treatment' was significant for the shoot fresh weight (F=9.15, P≤0.000), the root fresh weight (F=6.48, P≤0.001), the ratio: root/shoot (F=6.45, P≤0.001) and the chlorophyll content (F=5.02, P≤0.004).

3.2. Mycorrhizal root colonization

The mycorrhizal root colonization was observed in plants, both inoculated and non-inoculated with a commercial inoculum: IE, IS and IM (Table 1). However, the overall percentage of mycorrhizal colonization was significantly higher in the inoculated plants compared to the non-inoculated ones: (i): ectomycorrhizal root colonization (F=135.39, P≤0.000), + 279%, arbuscular mycorrhizal root colonization (F=69.18, P≤0.000), + 100%. (ii): ectomycorrhizal root colonization, + 141%, arbuscular mycorrhizal root colonization, + 300%. (iii): ectomycorrhizal root colonization, + 217 %, arbuscular mycorrhizal root colonization, +400 %.

Table 1. Height (H), shoot fresh weight (SFW), root fresh weight (RFW), ratio: root fresh weight/shoot fresh weight and chlorophyll content (CHC), ectomycorrhizal root colonization (EM%) and arbuscular mycorrhizal root colonization (AM%) of cork oak plants inoculated and non-inoculated with a commercial inocula EMF (IE), AMF (IS) and mixture of both inocula (EMF + AMF) (IM) grown on sterile substrate (SS) and non-sterile substrate (NS)

Parameters	Treatments								Significance 'Inoculation x soil treatment'
	Inoculation					Soil treatment			
	IE	IS	IM	NI	Significance	SS	NS	Significance	
H (cm plant-¹)	51.7 A	34.1 C	39.50 B	29.8 D	P<0.05	38.4 A	39.20 A	P=0.421	P=0.065 NS
SFW (g plant-¹)	4.9 A	2.3 C	3.6 B	1.7 D	P<0.05	3.3 A	2.9 B	P=0.000	P=0.000 ***
RFW (g plant-¹)	5.2 A	2.9 C	3.9 B	2.1 D	P<0.05	3.2 B	3.9 A	P=0.000	P=0.001***
Ratio root/shoot	1.1 A	1.3 A	1.1 A	1.5 A	P=0.109	1 B	1.5 A	P=0.000	P=0.001 ***
CHC(SPAD)	40.2 B 56.2	35.8 C 35.8	43.9 A 47.0	32.3 D	P<0.05	36.4 B	39.7 A	P=0.000	P=0.004 **
EM (%)	A 0.2 B	C	B	14.8 D	P=0.000	46.8 A	29.8 B	P=0.000	P=0.000***
AM (%)		0.4 A	0.5 A	0.1 C	P=0.000	0.3 A	0.3 B	P=0.021	P=0.001***

The means followed by the same letter within a column are not significantly different at $P < 0.05$ using Tukey's HSD test. *P <0.05, **P <0.01 and ***P <0.001**,** NS: no significance.

The ectomycorrhizal root colonization of cork oak plants inoculated with IE was significantly higher (56.2%) compared to IM and IS treatments (47% and 35.8%) presenting a gain of + 19% and + 56% respectively. The inoculation with IM also improved the EM root colonization which was greater by + 31% and + 19% compared to the previous treatments IS and IE. Conversely, no significant difference was detected for the

AM root colonization in the inoculated plants with the IM and IS treatments. The interaction 'inoculation x soil treatment' was significant (Table 1).

3.3. Mycorrhizogenic potential of soil

The number of infective propagules of indigenous AMF (MPN) in the soil before inoculation was 900 per 50 g of soil. The treatments IM and IS grown on the non-sterile substrate had a higher number of propagules: 2800 and 1800 respectively. In addition, the treatments IM and IS grown on the sterile substrate have shown MPN varied between 1500 and 1300 (Fig.1).

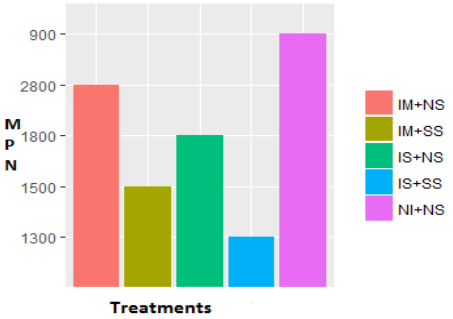

Figure 1. Number of propagules per kg of soil depending on the inoculum and the substrate used. (IS): arbuscular mycorrhizal inoculum, (IM): mixture inocula of ectomycorrhizal fungi and arbuscular mycorrhizal fungi, (SS): sterile substrate, (NS): non-sterile substrate

3.4. Correlations between mycorrhization and growth parameters

The relationships between the mycorrhization and the growth parameters revealed positive correlations between the ectomycorrhizal colonization rate (EM%) and the height (H), the fresh aerial biomass (SFW), the fresh root biomass (RFW), and the chlorophyll content (CHC), respectively 0.68, 0.66 and 0.80 and 0.76. Arbuscular mycorrhizal (AM%) showed a positive correlations with RFW, CHC and EM% by the coefficients of 0.23, 0.53 and 0.40. The CHC has recorded positive correlations with H, SFW, RFW, EM%, respectively 0.50, 0.52, 0.62, 0.76 and 0.53. Finally, the ratio root/shoot (R/S) is negatively correlated with SFW by a coefficient of -0.48 (Fig. 2).

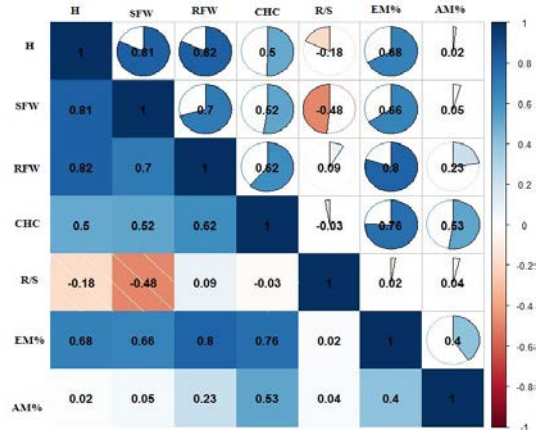

Figure 2. Pearson correlations between mycorrhization and growth parameters. Height (H), Shoot fresh weight (SFW), root fresh weight (RFW), ratio root/shoot (R/S), chlorophyll content (CHC) ectomycorrhizal root colonization (EM%) and arbuscular mycorrhizal (AM%). Numbers in the squares are correlation coefficients.

4. Discussion

The inoculation of cork oak plants with the arbuscular mycorrhizal fungi, ectomycorrhizal fungi and combination of these inoculum grown on both substrates (sterile and non-sterile) has significantly improved the mycorrhizal root colonization and the growth parameters. Whereas, the non-inoculated plants grown on the same substrates showed the low root colonization what has resulted in a low growth for all the measured parameters. Similarly, the number of propagules has been little compared to that obtained in the IM and IS treatments on the sterile substrate. The treatment IE recorded the highest root colonization levels, height and shoot and root fresh weight in comparison with the plants inoculated by IS and IM treatments. The results of this study are also in agreement with findings of Sebastiana *et al.* (2013) and Denis *et al.* (2015) who reported that the effect of inoculation with ectomycorrhizal fungi significantly increase the shoot and the root parts, the chlorophyll content and the performance of cork oak seedlings at nursery stage. The inoculation by the ectomycorrhizal fungi has also shown a positive effect on the growth of other forest trees as *Pinus tabulaeformis* Carr (Lu *et al*., 2016) and *Acacia mangnium* Willd (Diagnea *et al*., 2013). In the present experiment, the observed interaction between the substrate and the inoculation was significant indicating that the development of the seedlings is affected by these two factors. In parallel, similar studies revealed the significant effect between the substrate and the inoculation by ectomycorrhizal fungi on the growth of Pinus pinaster L. (Sousa *et al*., 2011) and *Picea abies* Karst. (Repac *et al*., 2015).

The treatment IS had less effect on the arbuscular mycorrhizal root colonization compared to the two previous treatments IM and IE, whereas it had a positive impact on the EMF root colonization. This may be due to the faster germination and growth of the arbuscular mycorrhizal propagules that can support hyphal growth in the direction of roots, or the quantity of existent propagules (Santos *et al*., 2001). The cork oak is a woody species, which has a strong symbiotic affinity to EMF at a more advanced stage of growth that would explain the low mycorrhizal intensity of the cork oak roots. Indeed, the eucalyptus forms AMF at the juvenile stage followed by EMF in adult age (Chen *et al*., 2000), Selosse *et al*. (2006) also reported that the AMF is generally the first to install and are joined by the EMF. The low AM colonization had also improved the growth parameters corroborating the results of other studies of *Salix repens* L. (Van der Heijden, 2001, Van der Heijden and Kuyper, 2003).

The dual inoculation (IM) had significantly improved the ectomycorrhizal colonization compared to treatments IE and IS. These observations suggest a succession in this dual system, which may be due to the rapid adaption of the AMF to primary infection within individual roots, and once the EMF is established the arbuscular mycorrhizal fungi might spread rapidly to new initials root, perhaps enabling the subsequent entrance of the AMF. The significant effect of the double inoculation on the root colonization may also be due to the competition and the interaction between the different inocula of the EMF and the AMF. This result is in the same line with the study of

(Chilvers *et al*., 1987) which proved that the differences observed in percent of roots colonized by AMF and EMF may be due to the competition between both of them. The influence of the dual inoculation on the arbuscular mycorrhizal root colonization is confirmed by the study of Michelsen *et al*. (1998) who concluded that the AMF and EMF were exploring separate pools of soil nutrients, thus suggesting a better resource exploration by tripartite symbiosis (plant, arbuscular and ectomycorrhizal fungi). Gagné *et al*. (2005) and Aggangan et al. (2010) have also demonstrated that the dual inoculation had enhanced the growth parameters of the plant species of the genus *Acacia* and *Eucalyptus*.

The low number of propagules was recorded in the non-inoculated plants while the highest number was obtained in the inoculated plants grown on the non-sterile substrate. The best mycorrhizal partners of cork oak are the ectomycorrhizae. However, despite the little presence of AMF in the roots of cork oak, the MPN is enriched by the synergistic intake of the dual inocula of (AMF+EMF) in the non-sterile substrate, even exceeding the intake of arbuscular mycorrhizae. This could be due to the composition of mycorrhizal fungi species of the inocula, Ectovit, Symbivit and their interactions with soil microflora. Dhillion and Gardsjord (2004) support this result while emphasizing that the specific composition, the productivity and the biodiversity of the epigeal flora were influenced by the composition and the specific richness of mycorrhizal communities. In the face of the AMF, which is not very specific to the host plant (Chagnon *et al*., 2012), the relative absence of specificity not only enables the fungi symbiotes to infect different plant species but also to form mycelial networks between plants. This could explain the increase in the number of propagules of plants inoculated by the AMF. Sanon *et al*. (2006) and Bilgo *et al*. (2011) have also shown that the AMF *Glomus intraradices* inoculated in sterilized soil could be maintained in a non-sterilized one (in the presence of native flora) and continue its beneficial activity to the host plant.

The positive significant relationships found between plant productivity and EMF infection parameter may be a consequence of the functional diversity of EMF (Hazard *et al*., 2017). This can be explained by the difference in mycorrhizal response within the same plant species for the same fungal strain. Indeed, it is difficult to determine the similarities, differences and the variance in the behavior of different plant species and even cultivars with the respect to the mycorrhizal symbiosis (Estaún *et al*., 2010).

5. Conclusion

This study showed that inoculation by the arbuscular mycorrhizae, the ectomycorrhizae and the dual inoculation by these two fungi, had positive effects on root colonization and growth of cork oak plants in the nursery. However, the mixture of inocula had significantly increased the mycorrhizal soil potential, enhanced by the interactions between the different populations of native mycorrhizae and the exogenous inputs of ectomycorrhizae and arbuscular mycorrhizae.

This research highlights that the controlled inoculation based on commercial mycorrhizal fungi can be a biotechnological technique in the improvement of the quality of seedling stock of *Q. Suber* and its performance after out-planting in the forests.

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