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Original article

Mycorrhization of *Pinus halepensis* Mill. and *Pinus pinaster* Aiton seedlings in two commercial nurseries

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Abstract – Two Mediterranean pine species (*Pinus halepensis* and *Pinus pinaster*) and seven fungi species (*Pisolithus tinctorius, Lactarius deliciosus, L. sanguifluus, Suillus mediterranensis, S. collinitus, S. bellinii*, and *Rhizopogon roseolus*) were selected for a mycorrhization study in two nurseries of SE Spain. Two types of inoculum (spore suspension and mycelium inoculum) were used on 3 700 pine seedlings. The aim of the study was to ascertain the influence of two different substrata (composted pine bark and fertilized-peat-vermiculite) on the mycorrhization rate and pine seedlings height. The results pointed out significantly differences between both substrata. In general, the best results were obtained with fertilized-peat-vermiculite-substratum although both the mycorrhization rate and seedlings height varied according to fungi species and the inoculum type used.

***Pinus halepensis* / *Pinus pinaster* / substrata / mycorrhization**

Résumé – **Mycorhization de semis de *Pinus halepensis* Mill. et *Pinus pinaster* Aiton dans deux pépinières forestières.** Deux espèces méditerranéennes de pin (*Pinus halepensis* et *Pinus pinaster*) et sept espèces de champignons (*Pisolithus tinctorius*, *Lactarius deliciosus, L. sanguifluus, Suillus mediterranensis, S. collinitus, S. bellinii* et *Rhizopogon roseolus*) ont été choisis pour une étude de mycorhization dans deux pépinières du Sud-est de l'Espagne. Deux types d'inoculum (suspension de spores et inoculum de mycélium) ont été employés sur 3 700 semis de pin. Le but de ce travail est d'étudier l'influence de deux substrats différents (écorce de pin compostée et tourbe-vermiculite fertilisée) sur le taux de mycorhization et la hauteur de semis de pin. Les résultats ont montré des différences significatives entre les deux substrats. En général, les meilleurs résultats ont été obtenus pour le substrat tourbe-vermiculite fertilisée, mais le taux de mycorhization et la hauteur de jeunes plants dépendait de l'espèce de champignon et du type d'inoculum.

***Pinus halpensis* / *Pinus pinaster* / substrat / mycorhization**

1. INTRODUCTION

During the last decades, the application of mycorrhizas in forestry has experienced a very important growth. Noteworthy studies are related to the selection of those fungi species and isolates which best adapt to the environmental conditions [4, 21, 29], the most effective inoculum production techniques [6, 14, 21, 22, 24, 26–28], and the inoculation and production of forestry species in the nursery [1, 2, 19]. These studies and many others have contributed to a considerable increase in profitability of the forest plant production [6, 19] and also the use of mycorrhized plants for afforestations purposes [7, 23].

Several studies have been carried out to facilitate the large-scale application and development of mycorrhization techniques. Initially, the spore inoculum method was the most widely used [17], while several authors tested the viability of mycelium inoculum at an industrial level [24, 25]. More recently, several new production methods for large quantities of mycelium have been developed including liquid fermentation using bio-reactors, and solid fermentation in vermiculite medium [9, 12].

In several Mediterranean countries and primarily in Spain, many studies have been carried out on two pine species, *Pinus halepensis* [8, 18] and *Pinus pinaster*. Some of these studies are based on morphological descriptions of mycorrhizas obtained under natural conditions [11, 13]. Other authors studied the utility of mature forest soil samples as an inoculum source [16]. As regards the studies involving controlled mycorrhization, those in which mycorrhizas were obtained under axenic conditions or even produced in vitro are of note. Furthermore, the formation of mycorrhizas between *Pinus halepensis* and *Amanita spissa*, *Lactarius deliciosus*, *Hebeloma edurum*, *Suillus luteus* and *Suillus variegates* was described [38]. Besides, spore inoculum has been successfully tested with *Pinus halepensis* and *Suillus collinitus*, *Rhizopogon roseolus* and *Pisolithus tinctorius* in containers [39]. Several mycelial inocula have

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also been studied using *Thelephora terrestris*, *Suillus collinitus*, *S. mediterranensis*, *Rhizopogon* sp. and *Pisolithus tinctorius* [6, 16, 34]. As regards *Pinus pinaster*, most studies have been carried out in Northern Spain, using *Pisolithus tinctorius*, *Rhizopogon* sp. and *Laccaria bicolour* as fungal species [29].

However, few papers written in Spanish consider the effects of nursery conditions on mycorrhization. In this sense, [16] studied the relation between several substrata and mycorrhization rates in various nurseries, obtaining significant differences in the process.

The purpose of this study was to ascertain the effects of two different substrata (composted pine bark and fertilized peat-vermiculite) on the mycorrhization rate and seedlings growth in the two most commonly used pines *Pinus halepensis* and *Pinus pinaster,* in two forestry nurseries of the Albacete province (SE Spain), using seven fungal species (*Pisolithus tinctorius*, *Lactarius deliciosus*, *L. sanguifluus*, *Suillus mediterranensis*, *S. collinitus*, *S. bellinii* and *Rhizopogon roseolus*). The main objective was to improve tree growth in the nurseries.

2. MATERIALS AND METHODS

Thermoformed plastic containers (220 mL capacity) were used after disinfections in water and bleach (1:1), before being filled with 2 types of substrate, one for each nursery: Dermont II substratum (composed bark pine), pH=5.5, and sterilized using thermal shock at 600 ºC in nursery A. A mixture of solid fertilized peat-vermiculite-(75%–24%) substratum, pH=5.7 (after sterilization) and sterilized 3 times at 100 ºC dry for 1 h once a week for 3 weeks in the nursery B.

Pinus halepensis and *P. pinaster* seeds were obtained from the Alcaraz-Segura mountain Range, located in SW Albacete province (SE Spain). No prior scarification or stratification treatments were applied. The seeds were rinsed in tap water and surface-sterilized with H_2O_2 (30%) for 20 min. After sterilization, the seeds were sown in the containers in March 2000 (3 seeds/cavity). Germination occurred 15–20 days after sowing and seedlings were thinned to one seedling per cavity.

Fruit bodies of all the fungi species (*S. bellinii, S. collinitus, S. mediterraneensis, L. deliciosus, L. sanguifluus, R. roseolus* and *P. tinctorius*) were collected in the Alcaraz-Segura Range in Autumn-Winter 1999. Isolates were obtained from the fruit bodies and some of them were preserved to obtain spore suspension.

Experimental design

Two types of inoculum were obtained: spore suspension and mycelium inoculum. Culture media were MMN for *Suillus* and *Rhizopogon* species and BAF for *Lactarius* species [37]. In all cases, mycelia were grown in 1-litre bottles with liquid medium previously sterilized and incubated at 23 ºC for 3 months. Spore suspensions were made using techniques described by [3]. This method consists of preparing spore slurries from fragments of hymenium, which are then triturated in sterile distilled water. Suspensions were frozen at –15 ºC until inoculation. In relation to the number of basidiospores per seedling used, [39] noted that 10^4–10^7 spores/seedling are sufficient to guarantee mycorrhization. In this study, 1.5×10^9 spores/seedling were used for *P. tinctorius*, 1.3×10^5 spores/seedling for *R. roseolus*, 1.4×10^4 spores/seedling for *Suillus* species and 0.7×10^5 spores/seedling for *Lactarius* species.

In June 2000, 1260 seedlings (630 of *P. halepensis* and 630 of *P. pinaster*) were inoculated in nursery A, and 1440 seedlings (720 from *P. halepensis* and 720 from *P. pinaster*) in nursery B. In all cases, 10 mL/seedling of mycelium inocula were applied on *Lactarius*, *Rhizopogon* and *Suillus* species. Inoculation was performed by injection of liquid inoculum using a micropipette. Three different containers (replicates) were used in each treatment. At the same time, the above mentioned dose of spore inoculum was used in the following combinations: *P. halepensis* × *S. collinitus*, *P. halepensis* × *S. mediterranensis*, *P. halepensis* × *L. deliciosus*, *P. halepensis* × *P. tinctorius*, *P. pinaster* × *L. deliciosus*, *P. pinaster* × *P. tinctorius*. Seedlings growth was evaluated six months after inoculation by measuring total height. Mycorrhization was evaluated considering the total number of inoculated seedlings of *P. halepensis* and *P. pinaster*. For this, each seedling was studied separately. The parameters used for quantitative estimation were: (1) Mycorrhization rate, relation between number of mycorrhized and the total number of seedlings for each pine-inoculum combination. (2) Percentage of ectomycorrhizas observed in each root system for each pine-inoculum combination. These values were grouped into six classes in accordance with [17]. 0 (0% mycorrhization); 1 (1–20%); 2 (21–40%); 3 (41–60%); 4 (61–80%) and 5 (81–100%). (3) Infection index, the average value obtained using the above classes for each treatment. Culture conditions were similar in nurseries A and B. In both of them plants were watered daily by using minidiffusers. All containers were cultured under shading condition. Besides, the culture parameters in both nurseries were similar: no weed-killer, fungi-killer nor fertilizers were applied. In addition, the same kind of thermoformed plastic container was used.

All data were subjected to analysis of variance and significant differences were determined using the Duncan's test ($P < 0.05$). In addition, simple regression models were calculated for height-percentage of ecomycorrhiza for those treatments in which average ectomycorrhizas exceeded 50%. Data shown as percentages were transformed using arcsin transformation to meet the assumptions of normality and homocedascity.

3. RESULTS

Tables I and *II* show a quantitative and qualitative evaluation of ectomycorrhizas obtained from all the seedlings in each treatment (an average of 105 seedlings per treatment due to seedlings death). In *tables I* and *II* infection index mean are noted. For all the fungal treatments more than one fungal species appears in *P. halepensis* as well as in *P. pinaster*. Apart from the inoculated species, different contaminant fungi were detected, mainly *P. tinctorius* and particularly *Thelephora terrestris*.

Class 1 (1–20% mycorrhization) was the most frequent class for both *P. halepensis* and *P. pinaster* (0 means no mycorrhization) followed by class 2 (20–40%). On the other hand, mycorrhization percentages were higher in nursery B than in A.

For *P. halepensis* in nursery A, the highest mycorrhization percentage (70–80%) referred to *T. terrestris*. In nursery B, *T. terrestris* also appeared but with lower mycorrhization percentages than in the nursery A.

In the case of *P. pinaster* the mycorrhization percentages of *T. terrestris* were higher than those obtained for *P. halepensis* in both nurseries, ranging from 80 to 100%.

3.1. *Pinus halepensis* mycorrhization percentage

From all the fungal species tested in nursery A, spore inoculum of *Suillus* and *Lactarius* showed the highest mycorrhization

Table I. Infection index and height of 6-month-old *P. halepensis* seedlings in 2 substrates inoculated with different fungi treatments. In each column, treatments with different letters have significant differences ($P < 0.05$). Sb: *Suillus bellini*; Sc: *Suillus collinitus*; Sm: *Suillus mediterranensis*; Ld: *Lactarius deliciosus*; Ls: *Lactarius sangluifluus*; Pt: *Pisolithus tinctorius*; Rr: *Rhizopogon roseolus*; m: mycelium; s: spore.

Fungi Treatment	Infection index		Height (cm)	
	Nursery A	Nursery B	Nursery A	Nursery B
Control	-	-	17.0^{c}	8.1^{cd}
Sb m	1.0^{a}	-	19.5^{e}	8.8^{e}
Sc m	1.0^{a}	1.0^{a}	18.5^{cde}	8.9^{e}
Sc s	1.3^{b}	1.3^{a}	15.4^{a}	7.4^{b}
Sm m	1.4^{b}	-	18.1^{c}	9.4^{f}
Sm s	1.0^{a}	1.0^{a}	15.0^{a}	7.6^{bc}
Ld s	1.1^{a}	1.0^{a}	18.5^{cde}	8.8^{e}
Ld m	1.0^{a}	1.0^{a}	18.4^{cd}	8.5^{de}
Ls m	1.0^{a}	1.0^{a}	19.1^{de}	8.5^{de}
Pt s	1.0^{a}	-	18.5^{cd}	7.8^{bc}
Rr m	-	1.1^{a}	18.3^{cd}	5.2^{a}

percentages (ranging from 40–65%). The same fungal species but with mycelium inoculum showed lower values (between 18–30%).

The best inoculations recorded in nursery B were obtained with the *L. sanguifluus* mycelial inoculum (64%) and with *L. deliciosus* spore inoculum (55%). For the remaining fungal species, the percentages of mycorrhized plants were lower or null.

3.2. Pinus pinaster mycorrhization percentage

The fungal treatments leading to the highest mycorrhization percentages were those with mycelium and spore inoculum of *Lactarius* in nursery A. Spore inoculum was the best with 93% of the seedlings micorrhized. In nursery B, *R. roseolus* showed a mycorrhization percentage of 80% with no other colonization species appearing in the trays treated with this mycelium species. On the contrary, *R. roseolus* in nursery A did not show a high mycorrhization percentage (24.8%) and it was infected by *T. terrestris*.

In nursery B the spore inoculum of *S. mediterranensis* showed 64% mycorrhization percentage, whereas this treatment in nursery A yielded only 7%.

Dealing with infection index and medium height (cm) of the seedlings (*tables I* and *II*), for *P. halepensis* in nursery B two homogeneous groups appeared, one including *S. mediterranensis* mycelial inoculum (with the highest infection index) and the second group with the remaining treatments. For this species only one group appeared in nursery B. For *P. pinaster* four different homogeneous groups appeared in nursery A, while three groups appeared in nursery B. In nursery A, *L. deliciosus* mycelium showed the highest infection index (1.9), while in nursery B, *R. roseolus* mycelium gave the highest colonization rate (1.3).

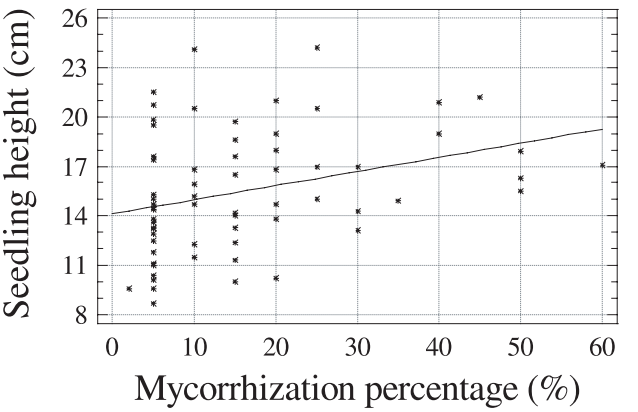

Figure 1. Regression model for *P. halepensis* seedlings inoculated with *S. collinitus* in nursery A. Y = 0.085656x + 14.1214; r = 0.32; $R^2 = 10.26\%$; $P < 0.05$.

Table II. Infection index and height of 6-month-old *P. pinaster* seedlings in 2 substrates. In each column, treatments with different letters have significant differences ($P < 0.05$). Sb: *Suillus bellini*; Sc: *Suillus collinitus*; Sm: *Suillus mediterranensis*; Ld: *Lactarius deliciosus*; Ls: *Lactarius sangluifluus;* Pt: *Pisolithus tinctorius*; Rr: *Rhizopogon roseolus*; m: mycelium; s: spore.

Fungi Treatment	Infection index		Height (cm)	
	Nursery A	Nursery B	Nursery A	Nursery B
Control	-	-	19.4^{abc}	9.0^{ed}
Sb m	1.4^{bc}	1.0^{ab}	18.1^{b}	9.9^{fg}
Sc m	1.1^{a}	1.0^{abc}	22.0^{d}	10.4^{h}
Sc s	-	1.0^{a}	-	8.4^{b}
Sm m	1.0^{ab}	1.5^{c}	18.3^{b}	10.3^{gh}
Sm s	-	1.0^{a}	-	8.9^{cd}
Ld s	1.0^{a}	1.0^{a}	19.8^{c}	10.0^{gh}
Ld m	1.9^{d}	1.0^{a}	23.0^{e}	9.2^{de}
Ls m	1.1^{a}	1.0^{a}	19.3^{bc}	8.7^{bc}
Pt s	1.2^{abc}	1.0^{ab}	15.9^{a}	9.5^{ef}
Rr m	1.5^{b}	1.3^{bc}	18.4^{bc}	7.7^{a}

In nursery A, 5 homogeneous groups appeared for seedling height. *P. tinctorius* showed the lowest value with 15.9 cm, while *L. deliciosus* mycelium showed the highest with 23 cm. In nursery B, 8 different groups were detected. *R. roseolus* mycelium was the fungal treatment producing the lowest seedling height (7.7 cm) while *S. collinitus* produced the highest with a value of 10.4 cm. Seedling height was always higher in nursery A than in nursery B for all treatments tested.

Regression analysis between height and mycorrhization percentage of inoculated fungi was significant ($P < 0.05$) for spore inoculum treatments of *S. collinitus* with *P. halepensis* in nursery A (*figure 1*) and for spore inoculum of *S. mediterranensis* with *P. pinaster* in nursery B (*figure 2*).

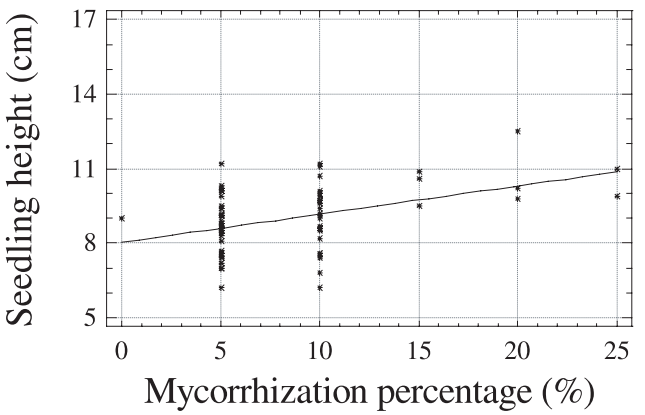

Figure 2. Regression model for *P. pinaster* seedlings inoculated with *S. mediterranensis* in nursery B. Y = 0.113282x + 8.03933; r = 0.42; R^2 = 18.09%; $P < 0.05$.

Table III. Mycorrhization percentage (%) for *P. halepensis* treatments in both nurseries. In each row, values with different letters show significant differences ($P < 0.05$).

P. halepensis	Nursery A	Nursery B
Ld s	11.09±7.62[a]	10.88±5.59[a]
Sm s	17.97±14.8[a]	10.38±7.7[a]
Ld m	7.01±5.08[a]	8.65±9.01[a]

Table IV. Mycorrhization percentage (%) for *P. pinaster* treatments in both nurseries. In each row, values with different letters show significant differences ($P < 0.05$).

P. pinaster	Nursery A	Nursery B
Ld s	30.25±17.7[a]	7.03±2.99[b]
Ld m	10.21±5.07[a]	8.0±3.68[a]
Ls m	9.35±6.04[a]	8.65±4.01[a]
Rr m	14.83±9.20[a]	15.45±7.43[a]

Table V. Mycorrhization percentage (%) for *P. halepensis* treatments in both spore and mycelium inoculum. In each row, values with different letters show significant differences ($P < 0.05$).

P. halepensis	Spore inoculum	Mycelium inoculum
Ld Ph nursery A	11.09±7.62[a]	7.01±5.08[b]
Sc Ph nursery A	16.23±13.75[a]	9.55±1.6[b]
Ld Ph nursery B	18.88±5.59[a]	8.65±9.01[a]

Table VI. Mycorrhization percentage (%) for *P. pinaster* treatments in both spore and mycelium inoculum. In each row, values with different letters show significant differences ($P < 0.05$).

P. pinaster	Spore inoculum	Mycelium inoculum
Ld Pp nursery A	30.25±17.7[a]	7.05±2.99[b]
Ld Pp nursery B	10.21±5.07[a]	8.0±3.68[a]

In the case of spore inoculum of *S. collinitus,* the R^2 value obtained means that the adjusted model explains 10.26% of the height variability. The correlation coefficient was 0.32. For *S. mediterranensis* and *P. pinaster* the mycorrhization percentage explained 18.09% of height variability. The correlation coefficient was 0.42. The positive slope in both regression models means that the higher the percentage of mycorrhization, the taller the saplings.

Comparisons between the same treatment and between the two nurseries were also done (*tables III* and *VI*). For this purpose only those treatments whose mycorrhization rate was higher than 10% were considered.

For *P. halepensis,* no significant differences appeared between the two kinds of substrate (both nurseries) in the case of *L. deliciosus* spore, *S. mediterranensis* spore and *L. deliciosus* mycelium (*table III*).

For *P. pinaster* (*table IV*), *L. deliciosus* spore was the only treatment where significant differences were recorded between the two nurseries (30.25±17.7 in nursery A and 7.03±2.99 in nursery B). No significant differences appeared between the remaining tested treatments (*L. deliciosus* mycelium, *L. sanguifluus* mycelium and *R. roseolus* mycelium).

For *P. halepensis* (*table V*), the *L. deliciosus* spore mycorrhization percentage was significantly higher than the observed for the *L. deliciosus* mycelium inoculum in nursery A. On the contrary, in nursery B there were not significant differences. *S. collinitus* spore showed a 16.23±13.75 mycorrhization percentage while *S. collinitus* mycelium showed a value of 9.55±1.6%, which meant there were significant differences between them.

For *P. pinaster* (*table VI*), there was a significant difference between the mycorrhization percentage obtained with *L. deliciosus* spore and mycelial inoculum in nursery A (30.25±17.7 and 7.03±2.99 respectively) while for the same treatments no significant differences appeared in nursery B (10.21±5.07 and 8.0±3.68).

4. DISCUSSION

Mycorrhization percentage results reveal the susceptibility for *P. halepensis* and *P. pinaster* to be colonized by mycorrhizas. This fact has been demonstrated by other authors, who described the mycorrhizal colonization process with this species using similar techniques [3, 15, 32, 37, 40]. However, the different mycorrhization rates and the different mycorrhization percentages emphasize the importance of the fungal species selected [41], the inoculation techniques [36] and the most useful substrate which can be used for each pine species.

Substrate showed as the principal factor influencing on mycorrhyzation results. Whatever substrate is used, it should present sufficient ventilation and drainage to allow the development of the fungi mycelium [16]. Such characteristics are also necessary for the correct development of the seedlings. The pH of substrate used in nursery B (4.5–5.5) was lower than that in nursery A (5.5–5.9) which could mean that a

moderately acid pH is better for ectomycorrhizal development. On the other hand, the fact that *Lactarius* is a genus only slightly affected by variations in pH [10] could explain the results obtained for that genus in nursery B, where the mycorrhization percentages obtained were higher than those in nursery A.

The better results obtained for mycorrhizal colonization in nursery A could be due to the greater ability of the peat-vermiculite mixture to encourage ectomycorrhizal development than the substrate of tritured bark pine. This could agree with the findings of other authors who demonstrated the compatibility of mixtures containing peat with the development of the ectomycorrhiza [3, 6].

The fungal treatments involving *Suillus* and *Lactarius* genus were in general successful both with mycelial and spore inoculum. Since spore inoculum is easily applied, this may be the most suitable method for inoculating seedlings in commercial nurseries, as demonstrated in other nurseries of SE Spain [17].

Comparing the results obtained for *P. halepensis* and *P. pinaster,* the former was found to be more receptive to colonization by ectomycorrhizal fungi than *P. pinaster* in both nurseries. This could be explained by the low number of seeds in *P. halepensis*, which needs to develop fine roots quickly, thus reducing the optimal time span for mycorrhization [10]. *P. pinaster* has larger seed stocks, and so, the time interval for inoculating the seedlings is longer.

T. terrestris is the other fungal species which appears in both nurseries in the majority of containers, demonstrating the natural adaptation of this species to the nursery conditions. This species is an important competitor in the development of other fungal species [39] which could explain the low mycorrhization rates and mycorrhization percentages obtained.

The significant differences in mean seedling height obtained for the different inocula is related to the presence of mycorrhiza. A significant relationship was observed for *S. collinitus* in *P. halepensis* in nursery A and for *S. mediterranensis* in *P. pinaster* in nursery B. However, the above correlations were not very high and indeed, other studies have concluded that the presence of ectomycorrhizas does not favour the growth of seedlings in containers. In such a situation, many processes which influence seedlings growth and which are due to the presence of mycorrhiza simply do not occur [31]. It is only later, when the seedling has been planted in the countryside, that the mycorrhiza has an effect on the survival and development of the seedlings [5, 25] . In some cases a negative influence of the mycorrhiza on height has been detected [35].

It is well known that the inclusion of a mycorrhization step in plant production has positive effects on the survival of seedlings when they are planted out [20, 30, 33]. However, the incorporation of a mycorrhization step in seedling productions in nurseries is not a common practice in Spain. This could be explained by the final economic value of the sapling, which although considerably higher than that of a non-mycorrhized plant, is not sufficient to recover any initial investment in the process. Besides, each nursery has its own particular production technique as shown in this study. As a consequence, the development of individual studies similar to this one is necessary for each nursery. The principal aim would be to identify the most effective inoculum technique from an economic and a biological point of view.

We can conclude that the effect of controlled inoculation in nurseries varies depending on the fungus species-seedling species combination, the substratum and production technique used. Despite this variation, a controlled mycorrhization process is always possible.

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