Ann. For. Sci. 64 (2007) 577–583
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DOI: 10.1051/forest:2007035

Available online at:
www.afs-journal.org

Original article

Influence of the fertilisation method in controlled ectomycorrhizal inoculation of two Mediterranean pines

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(Received 9 September 2006; accepted 20 December 2006)

Abstract – The influence of the fertilisation method: soluble (SF) vs. slow-release fertiliser (SRF) and of inoculation with *Laccaria laccata* (Scop.) Fr., *Pisolithus tinctorius* (Pers.) Coker & Couch and *Melanogaster ambiguus* (Vittad.) Tul & C. Tul. on ectomycorrhizal colonization and growth of *Pinus pinea* L. and *Pinus pinaster* Ait. was evaluated. For both pines, mycorrhization with *L. laccata* was not affected by the fertilisation method. Percentages of ectomycorrhizas (ECM) formed by *P. tinctorius* were dependent on the fertilisation method, the inoculum type (vegetative or spores) and the pine species involved. ECM formed by *M. ambiguus* were increased with fertilisation in both pines. Inoculation significantly improved *P. pinea* biomass when seedlings were fertilised with SRF whereas no effect was found in non-fertilised ones. For non-fertilised *P. pinaster*, inoculation with *L. laccata* and both inocula of *P. tinctorius* increased seedling biomass whereas fertilisation neutralised the fungal effect. Fertilisation increased *P. pinea* and *P. pinaster* biomass, independently of the inoculation treatment.

***Pinus pinea* / *Pinus pinaster* / controlled mycorrhization / ectomycorrhizal fungi / seedling nursery production / fertilisation**

Résumé – Influence de la méthode de fertilisation sur la mycorhization contrôlée de deux espèces de Pins méditerranéens. L'impact sur le degré de mycorhization et la croissance de jeunes plants de *Pinus pinea* L. et de *Pinus pinaster* Ait., de deux méthodes de fertilisation (fertilisant soluble (FS) et fertilisant à libération lente) et d'une inoculation contrôlée avec *Laccaria laccata* (Sco.) Fr., *Pisolithus tinctorius* (Pers.) Coker et Couch et *Melanogaster ambiguus* (Vittad.) Tul et C. Tul. Pour les deux pins, la mycorhization avec *Laccaria laccata* n'a pas été modifiée par la méthode de fertilisation. Le pourcentage d'ectomycorrhizes (ECM) formé by *P. tinctorius* dépendait de la méthode de fertilisation, su type d'inoculum (spores ou inoculum végétatif) et de l'espèce de pin. La fertilisation a augmenté les ECM produites par *Melanogaster ambiguus* chez les deux pins. L'inoculation a augmenté significativement la biomasse des semis de *Pinus pinea* lorsqu'ils ont été fertilisés avec SRF tandis qu'aucun effet n'a été trouvé pour les traitements non fertilisés. Pour les semis non fertilisés de *Pinus pinaster*, l'inoculation avec *Laccaria laccata* et avec les deux inoculums de *Pisolithus tinctorius* a augmenté la biomasse des semis tandis que la fertilisation a neutralisé l'effet de l'inoculation. La fertilisation a augmenté la biomasse de *Pinus pinaster* et de *Pinus pinea* indépendamment du traitement d'inoculation utilisé.

***Pinus pinea* / *Pinus pinaster* / mycorhization contrôlée / champignon ectomycorhizien / pépinière de production de semis / fertilisation**

1. INTRODUCTION

Fertilisation is a key factor for producing high quality nursery stock destined to reforestation [17]. An optimal fertilisation method adjusted to the tree species produced in the nursery will ensure the improvement of physiological traits such as growth, nutrient storage, photosynthetic rates and root growth potential [18]. The application of soluble fertilisers and the addition of slow-release fertilisers to the potting substrate are the two fertilisation methods most commonly used in nurseries [3, 37]. Soluble fertilisers can be more precisely adjusted than slow-release ones for each developmental stage of tree seedlings [28, 30] and they are commonly applied with the nursery irrigation system. On the other hand, slow-release fertilisers are easier to apply providing an important economical advantage for producing nursery tree seedlings at a commercial scale. Additionally, the effect of slow-release fertilisers can persist after outplanting [31].

Spontaneous mycorrhization of seedling commonly occurs in nursery although usually opportunistic fungi with low host specificity have been reported [11, 16, 19]. Inoculation with selected ectomycorrhizal fungi has been often signalled as a promising practise for improving the quality of nursery seedling stock [4, 11, 21]. Mycorrhization not only improves seedling growth and their photosynthetic capacity [12] but also notably extends the root surface allowing seedlings to a better exploration of soil after out-planting [36]. Obtaining a well-developed root system of seedlings in nursery is important since a vigorous root growth contributes to the ability of seedlings to overcome post transplanting stress [15]. Mycorrhization can be an important advantage for seedlings to surmount transplanting stress [7, 36] especially under unfavourable field conditions such as those imposed by the Mediterranean climate [25, 34]. When nursery production of mycorrhizal plants is desired, an adjustment of the fertilisation regime becomes essential, since high fertilisation inputs usually inhibit the formation of ectomycorrhizas [4, 14, 35].

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On the other hand, the method of fertilisation used can also alter the formation of ectomycorrhizas [4, 8]. Ectomycorrhizal fungi have variable nutrient demands for hyphal growth [23] and their response to fertilisation may be different. This fungal variability must be taken into account before undertaking a strategy for producing nursery mycorrhizal seedlings of a determined tree species [19, 38].

In this study, we have inoculated two Mediterranean pine species: *Pinus pinea* L. and *P. pinaster* Ait. commonly used for reforestation in Spain, with two wide-spread coloniser fungi: *Laccaria laccata* (Scop.) Fr. and *Pisolithus tinctorius* (Pers.) Coker & Couch, highly adapted to common nursery practices [21, 40], and with *Melanogaster ambiguus* (Vittad.) Tul & C. Tul., a good coloniser of *P. pinea* [32] with proven efficiency to increase seedling performance under field conditions [27]. We have evaluated the use of two fertilisation methods (soluble fertiliser and slow-release fertiliser) for producing mycorrhizal seedlings of both Mediterranean pines. The dosage of nutrients applied with both fertilisation methods was adjusted for avoiding the inhibition of ectomycorrhizas [25,26,33]. The objective of this work was to determine the effect of the fertilisation method and the inoculation with different fungi on mycorrhization and growth of containerized *P. pinea* and *P. pinaster* seedlings produced in nursery.

2. MATERIAL AND METHODS

2.1. Plant material

Pinus pinea seeds were collected from natural forests in the Montnegre and Montseny sierras in Catalonia (Spain). *Pinus pinaster* seeds were obtained from the "Centre National de Recherches Forestières" (France) origin Valdemoro sierra, Cuenca, Spain. Before use, seeds were soaked overnight in running tap-water, surface disinfected (30 min in 33% H_2O_2) and rinsed in distilled water.

2.2. Fungal material and production of fungal inoculum

Basidiomata of *P. tinctorius* and *M. ambiguus* were collected in mixed forest of *P. pinea* in different locations of Catalonia (Spain) and under *Pseudotsuga menziesii* (Mirb.) Franco plantations in Girona (Spain), respectively. Collected sporocarps were dried at 35 °C for 72 h and kept in paper bags until use. Pure cultures of *L. laccata* (strain 127, collected under *Quercus ilex* L.) and *P. tinctorius* (strain 93, collected under *Quercus suber* L.) were isolated from fresh sporocarp tissue as previously described [32]. Voucher dry basidiomata and pure cultures were deposited in the herbarium and culture collection of DPV-IRTA (Barcelona, Spain). Miceliar inocula of *L. laccata* and *P. tinctorius* and spore inocula of *P. tinctorius* and *M. ambiguus* were obtained as previously described [33, 34].

2.3. Inoculations and experimental set-up

A factorial experiment was carried out to test the effect of the factors: (a) inoculation with ectomycorrhizal fungi (*L. laccata*, *P. tinctorius*, *M. ambiguus*, non-inoculated), (b) application of different fertilisation methods (soluble fertiliser, slow-release fertiliser, not fertilised) and (c) tree species (*P. pinea* or *P. pinaster*), on seedling growth and ectomycorrhizal development.

Table I. Total amount of nutrients received per seedling with each fertilization method at the end of the experiment. SF = Soluble fertiliser; SRF = slow release fertiliser.

	Nutrient (total mg/seedling)									
	N	P	K	Fe	Mg	Mn	Zn	Cu	B	Mo
SF 20-7-19	43	15.1	41	4.2	0.8	0.8	0.2	0.2	0.1	0.1
SRF 15-8-11	42	22.5	32	1.2	5.7	0.2	0.04	0.15	0.5	0.5

A potting substrate containing equal volumes of peat (Floragard, Oldenburg, Germany) and grade 2 vermiculite (Asfaltex, Barcelona, Spain), autoclaved (60 min, 120 °C) and with a final pH 5.5 (in water) was used to fill Ray Leach C-10 "Cone-tainers"^{TM} (Stuewe & Sons, Inc., Oregon, USA) containers (165 mL capacity). Vegetative inoculum of either *L. laccata* or *P. tinctorius* were mixed with the potting substrate before filling the containers at the proportion of 1:20 (v:v, inoculum:substrate). Dried spores of *P. tinctorius* were mixed with vermiculite (0.12 g spores in 600 mL vermiculite) and incorporated into the potting substrate, before filling the containers, at the rate of 10^6 spores per plant. Two surface-disinfected seeds of either *P. pinea* or *P. pinaster* were sown in each container and thinned to one per container after emergence. Spores of *M. ambiguus* were inoculated to one-month-old seedlings as a water suspension to provide 10^6 spores per seedling.

The soluble fertiliser treatment (SF) consisted of the application every two weeks of 10 mL/seedling of a solution containing 20-7-19 Peter's fertiliser (Scott, Tarragona, Spain) at 1.8 g/L and the micronutrients preparations Fetrilon® and Hortrilon® (Basf, Barcelona, Spain) at 0.12 g/L and 0.28 g/L, respectively. The application of soluble fertiliser started one month after seedling germination (May) and it was stopped six months later at the end of the growing season (November).

Fertilisation with slow-release fertiliser (SRF) consisted of the application of 2.3 g/L substrate of Osmocote Plus® (Scotts, Marysville, USA) 15-8-11 (totally released after 12 months, at 21 °C). Both, SF and SRF applications were calculated to provide similar amount of nitrogen along the fertilization period. The total amount of nutrients received per plant in each fertilisation treatment is shown in Table I. The dosage of nutrients applied with both fertilisation methods was adjusted for allowing mycorrhizal development, as it has been proven in previous studies [25, 26, 33].

A total of 15 treatments per pine species were established with 20 replicates per treatment: (1) not inoculated/not fertilised (NF), (2) not inoculated/Soluble fertiliser (SF), (3) not inoculated/Slow Release Fertiliser (SRF), (4) *L. laccata*/NF, (5) *L. laccata*/SF, (6) *L. laccata*/SRF, (7) *P. tinctorius* vegetative inoculum/NF, (8) *P. tinctorius* vegetative inoculum/SF, (9) *P. tinctorius* vegetative inoculum/SRF, (10) *P. tinctorius* spores inoculum/NF, (11) *P. tinctorius* spores inoculum/SF, (12) *P. tinctorius* spores inoculum/SRF, (13) *M. ambiguus*/NF, (14) *M. ambiguus*/SF, (15) *M. ambiguus*/SRF. Seedlings were grown in a greenhouse with 16 h photoperiod (200 μmol s^{-1} m^{-2}) provided by high pressure sodium vapour lamps. Greenhouse temperature was between 15–25 °C and relative humidity was maintained over 40%.

Table II. Summary of the three-way ANOVA (*F* values) assessing the effects of inoculation (non-inoculated, *Laccaria laccata*, *Pisolithus tinctorius* miceliar or spores inoculum and *Melanogaster ambiguus*), fertilisation (non-fertilised, soluble fertiliser and slow-release fertiliser), pine species (*Pinus pinea* and *Pinus pinaster*) and their interactions on seedling growth parameters and ectomycorrhizal short roots. I = inoculation; F = fertilisation; P = pine species; ECM = ectomycorrhizas; SDW = Shoot dry weight; RDW = root dry weight; S/R = Shoot/Root ratio. Asterisks: * $0.05 \geqslant P > 0.01$; ** $P < 0.001$; ns = non-significant.

	ECM		Diameter		Height		SDW		RDW		S/R	
Inoculation	177.9	**	3.8	*	6.1	**	5.8	**	2.2	ns	1.1	ns
Fertilisation	8.1	**	153.8	**	268.5	**	188.4	**	43.9	**	137.3	**
Pine	9.3	*	194.2	**	179.7	**	205.4	**	1.7	ns	311.3	**
I × F	3.9	**	2.6	*	6.8	**	3.3	*	3.1	*	1.9	ns
I × P	7.9	**	2.3	ns	2.1	ns	5.5	**	11.0	**	8.1	**
F × P	3.7	*	111.2	**	50.3	**	33.0	**	37.6	**	8.2	**
I × F × P	1.7	ns	3.3	*	3.9	**	4.5	**	5.6	**	2.7	*
R^2	0.82		0.81		0.83		0.78		0.60		0.77	

2.4. Measured parameters and statistical analysis

Nine months after sowing, all the seedlings were harvested and their roots washed free of substrate. The percentage of mycorrhizal seedlings was determined in each treatment. Ectomycorrhizal short roots (ECM) were identified according to morphological criteria as previously described [32–34]. Each seedling root was cut in 2–3 cm segments, and the percentage of ECM assessed by counting at least 200 randomly selected short roots under the stereomicroscope. All plants were measured for stem height and root collar diameter. The seedlings shoots and roots were oven dried (60 °C, 72 h) to obtain the dry weights.

The proportion of mycorrhizal seedlings for each fertilisation treatment and tree species were analysed by contingency tables with the SPSS 11.0 Software Package. Mycorrhizal colonisation and growth data were analysed by multifactor-ANOVA. Percentages of ectomycorrhizas were arc-sin transformed before performing ANOVA. When interactions were detected, data were analysed separately for each factor by one-way ANOVA. Significant differences among treatments were detected by Tukey's test ($P < 0.05$).

3. RESULTS

3.1. Mycorrhization

The percentage of ectomycorrhizas was significantly affected by inoculation, fertilisation and the pine species (Tab. II). Interactions between all factors were found and the ANOVA was performed separately for each factor.

The inoculum of *L. laccata* was effective forming ectomycorrhizas (ECM) with almost all plants of both pine species (Figs. 1A and 1B). The percentage of ECM obtained with this fungus on *P. pinea* was under 50% in any treatment, while on *P. pinaster* it was slightly higher (Figs. 2A and 2B). For both pines, mycorrhization with *L. laccata* was not affected by the fertilisation method.

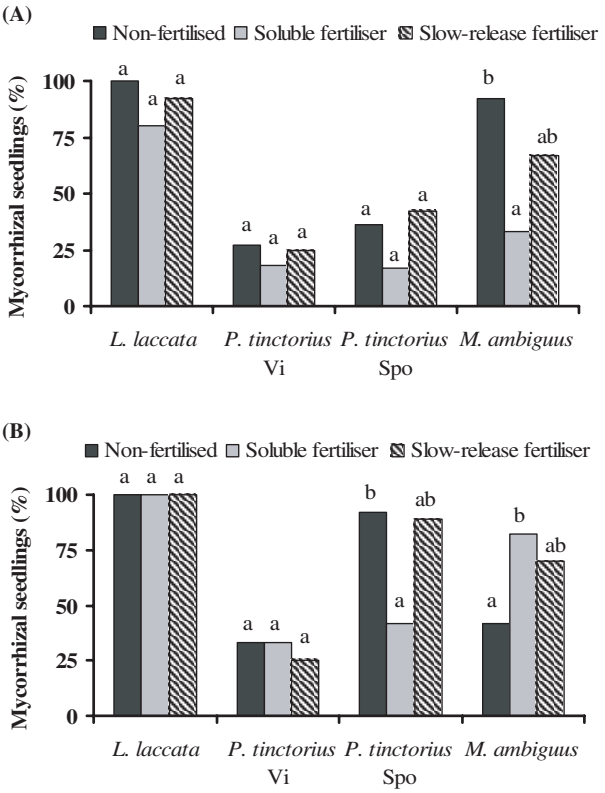

Figure 1. Percentages of mycorrhizal seedlings of *P. pinea* (A) and *P. pinaster* (B) inoculated with three ectomycorrhizal fungi under different fertilisation regimes. Different letters in each inoculation treatment denote significant differences among fertilisation treatments analysed by contingency tables ($P \leqslant 0.05$). Vi = vegetative inoculum; Spo = spores inoculum.

The application of vegetative inoculum of *P. tinctorius* produced less than 30% of colonised seedlings in both pine species (Figs. 1A and 1B). The percentage of ECM obtained with vegetative inoculum of this fungus on *P. pinea* seedlings was significantly increased by fertilisation with soluble fertiliser (SF) (Fig. 2A). Colonised *P. pinaster* seedlings showed more than 75% of ECM and mycorrhizal colonisation was independent of the fertilisation method used (Fig. 2B). *Pisolithus tinctorius* applied as spore inoculum was more effective for the mycorrhization of *P. pinaster* than for *P. pinea* (Figs. 1 and 2). For both conifers, rates of mycorrhizal seedlings obtained with this fungus were reduced when SF was applied (Figs. 1A and 1B). The percentage of ECM of *P. pinea* seedlings inoculated with spores of *P. tinctorius* was unaffected by the fertilisation method (Fig. 2A). On the other hand, a significant increase of ECM formed by this fungus was observed on not fertilised *P. pinaster* seedlings (Fig. 2B). The percentage of *P. pinea* seedlings mycorrhizal with *M. ambiguus* was reduced by fertilisation with SF (Fig. 1A). Contrarily, the proportion of *P. pinaster* seedlings colonized with this fungus was increased with both fertilisation methods (Fig. 1B). The percentage of ECM of *M. ambiguus* was slightly increased with fertilisation in both

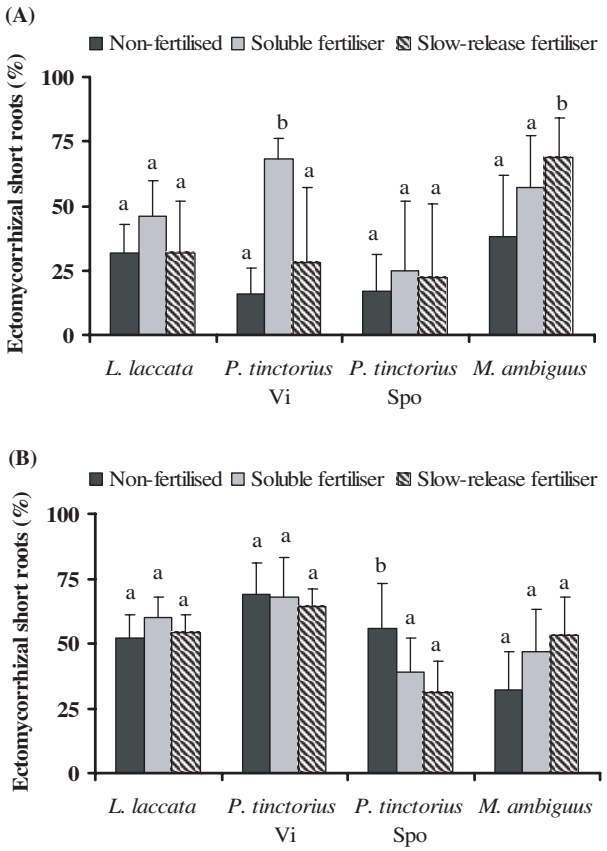

Figure 2. Percentages of ectomycorrhizas on *P. pinea* (A) and *P. pinaster* (B) seedlings inoculated with three ectomycorrhizal fungi under different fertilisation regimes. Different letters in each inoculation treatment denote significant differences among fertilisation treatments by ANOVA according to Tukey' test ($P \leqslant 0.05$). Vi = vegetative inoculum; Spo = spores inoculum.

pines (Figs. 2A and 2B), but significant differences were only found between *P. pinea* seedlings not fertilised and fertilised with SRF (Fig. 2A).

3.2. Seedling growth

In general, the studied factors significantly influenced the growth of seedlings (Tab. II). Significant interactions among factors were found for all parameters, and statistics were carried out separately for each factor by one-way ANOVA.

3.2.1. Effect of inoculation

Fungal inoculation did not affect the growth of *P. pinea* seedlings when they were not fertilised (NF), with exception of *L. laccata* which significantly stimulated the root dry weight (Tab. III). When soluble fertiliser (SF) was applied, only seedlings inoculated with vegetative inoculum of *P. tinctorius* had a significant increase in shoot dry weight compared with non inoculated seedlings. When *P. pinea* was grown with slow-release fertiliser (SRF), inoculation with both inocula of *P. tinctorius* significantly improved diameter and height of seedlings, and inoculation with all the fungi tested significantly increased seedling biomass (Tab. III). A variable effect of inoculation on seedlings growth was obtained for *P. pinaster* in all fertilisation treatments (Tab. IV). A growth enhancement was found in seedlings NF and inoculated with *L. laccata* (height and shoot dry weight) and with the two inocula of *P. tinctorius* (shoot and root dry weight) (Tab. IV). When each of SF and SRF was applied, inoculation did not significantly improved *P. pinaster* biomass (Tab. IV).

3.2.2. Effect of the fertilisation method

The height of *P. pinea* seedling was, in general, increased by fertilisation irrespectively of the method used, whereas for the diameter a variable effect was obtained (Tab. III). Fertilisation increased *P. pinea* shoot dry weight in any of the inoculation treatments except for non-inoculated seedlings and those inoculated with *L. laccata* and fertilised with SRF (Tab. III).

On the other hand, fertilisation with each method significantly increased the growth of *P. pinaster* seedlings, irrespective of the inoculation treatment (Tab. IV). For both pine species, the shoot/root ratio was significantly improved by fertilisation in all inoculation treatments, irrespectively of the fertilisation method used (Tabs. III and IV).

4. DISCUSSION

Laccaria laccata effectively colonised almost all seedlings and the formation of ECM was not affected by the fertilisation method. Similar results have been previously reported for this fungus in association with *P. pinea* and *P. pinaster* [26, 32]. It has been suggested that the mycorrhizal ability of this fungus is not affected by the fertilisation method used, but it mostly depends on the associated tree species [9].

Vegetative inoculum of *P. tinctorius* was poorly effective, producing low number of mycorrhizal seedlings of both pines. Percentage of ECM varied with the fertilisation method for *P. pinea*, whereas no differences were found for *P. pinaster*. Vegetative inoculum of *P. tinctorius* has been previously used for inoculating diverse conifer species in nursery in several countries [20, 21].The low number of mycorrhizal plants obtained in our experiment could be due to a poorly matured inoculum with not sufficient fungal active propagules. When *P. tinctorius* was applied as spores, it was more effective for the mycorrhization of *P. pinaster* than for *P. pinea*. Mycorrhization was reduced on fertilised pines, mainly when soluble fertiliser was applied, probably indicating this factor as affecting fungal spores viability. Spore inoculum of *P. tinctorius* has been previously tested in *P. pinea* and *P. pinaster* inoculations with higher mycorrhization rates than those obtained in our experiment [26, 32]. Our results suggest that the experimental conditions could have affected spore germination. A better knowledge of the factors regulating spore germination is required to improve both the speed and stability of mycorrhizal formation with this type of inoculum [5].

Table III. Effect of the inoculation with different ectomycorrhizal fungi and of the fertilisation method on growth of containerised *Pinus pinea* seedlings. For each fertilisation treatment, different minor letters in each column denote significant differences among inoculation treatments according to Tukey' test ($P < 0.05$). For an equal inoculation treatment, capital etters denote differences among fertilisation treatments according to Tukey' test ($P < 0.05$). Vi = vegetative inoculum; Spo = spores inoculum.

	Diameter (mm)		Height (cm)		Shoot dry weight (g)		Root dry weight (g)		Shoot/root ratio	
Non-fertilised										
Non-inoculated	3.6 ± 0.2 a	B	25.8 ± 3.0 a	A	1.9 ± 0.2 ab	A	0.7 ± 0.1 a	B	2.7 ± 0.3 b	A
L. laccata	3.6 ± 0.1 a	B	25.8 ± 3.0 a	A	2.1 ± 0.2 b	A	1.0 ± 0.1 b	A	2.1 ± 0.2 a	A
P. tinctorius (Vi)	3.5 ± 0.3 a	A	26.8 ± 4.3 a	A	1.6 ± 0.6 a	A	0.7 ± 0.1 a	A	2.2 ± 0.3 ab	A
P. tinctorius (Spo)	3.5 ± 0.3 a	A	26.6 ± 1.5 a	A	1.8 ± 0.3 ab	A	0.8 ± 0.1 a	A	2.3 ± 0.3 ab	A
M. ambiguus	3.3 ± 0.1 a	A	27.7 ± 1.7 a	A	1.7 ± 0.2 ab	A	0.7 ± 0.1 a	A	2.4 ± 0.4 ab	A
Soluble fertiliser (SF)										
Non-inoculated	3.7 ± 0.3 a	B	33.5 ± 0.9 ab	B	2.2 ± 0.4 a	B	0.7 ± 0.1 a	B	3.3 ± 0.3 a	B
L. laccata	4.1 ± 0.3 a	C	29.0 ± 2.6 a	A	2.9 ± 0.4 ab	B	0.9 ± 0.2 a	A	3.0 ± 0.3 a	B
P. tinctorius (Vi)	4.1 ± 0.2 a	B	34.5 ± 1.7 ab	B	3.2 ± 0.1 b	B	1.0 ± 0.1 a	A	3.3 ± 0.2 a	B
P. tinctorius (Spo)	4.0 ± 0.5 a	A	32.3 ± 4.6 ab	B	2.9 ± 0.6 ab	B	0.9 ± 0.2 a	A	3.3 ± 0.1 a	B
M. ambiguus	4.0 ± 0.2 a	B	35.6 ± 2.6 b	B	2.7 ± 0.2 ab	B	0.8 ± 0.1 a	A	3.1 ± 0.2 a	B
Slow-release fertiliser (SRF)										
Non-inoculated	3.2 ± 0.3 a	A	28.0 ± 4.3 a	A	1.7 ± 0.3 a	A	0.5 ± 0.1 a	A	3.2 ± 0.5 a	B
L. laccata	3.3 ± 0.1 ab	A	34.0 ± 2.8 ab	B	2.3 ± 0.3 b	A	0.8 ± 0.1 b	A	3.1 ± 0.2 a	B
P. tinctorius (Vi)	3.7 ± 0.1 b	AB	36.7 ± 0.8 b	B	2.6 ± 0.3 b	B	0.8 ± 0.2 b	A	3.4 ± 0.4 a	B
P. tinctorius (Spo)	3.7 ± 0.1 b	A	35.5 ± 2.1 b	B	2.8 ± 0.2 b	B	0.9 ± 0.1 b	A	3.2 ± 0.2 a	B
M. ambiguus	3.5 ± 0.1 ab	A	32.0 ± 4.5 ab	AB	2.4 ± 0.3 b	B	0.8 ± 0.1 b	A	2.8 ± 0.4 a	AB

Table IV. Effect of the inoculation with different ectomycorrhizal fungi and of the fertilisation method on growth of containerised *Pinus pinaster* seedlings. For each fertilisation treatment, different minor letters in each column denote significant differences among inoculation treatments according to Tukey' test ($P < 0.05$). For an equal inoculation treatment, capital letters denote differences among fertilisation treatments according to Tukey' test ($P < 0.05$). Vi = vegetative inoculum; Spo = spores inoculum;

	Diameter (mm)		Height (cm)		Shoot dry weight (g)		Root dry weight (g)		Shoot/root ratio	
Non-Fertilised										
Non-inoculated	1.8 ± 0.3 ab	A	11.3 ± 1.8 a	A	0.3 ± 0.1 a	A	0.3 ± 0.1 a	A	1.1 ± 0.2 a	A
L. laccata	2.0 ± 0.4 abc	A	16.8 ± 1.9 b	A	0.6 ± 0.1 c	A	0.4 ± 0.1 ab	A	1.3 ± 0.2 a	A
P. tinctorius (Vi)	2.3 ± 0.6 c	A	14.2 ± 1.9 ab	A	0.6 ± 0.1 c	A	0.5 ± 0.1 b	A	1.3 ± 0.2 a	A
P. tinctorius (Spo)	2.1 ± 0.2 bc	A	13.2 ± 1.5 a	A	0.5 ± 0.1 bc	A	0.5 ± 0.1 b	A	1.1 ± 0.2 a	A
M. ambiguus	1.6 ± 0.4 a	A	13.2 ± 3.1 a	A	0.4 ± 0.1 ab	A	0.4 ± 0.1 ab	A	1.2 ± 0.3 a	A
Soluble fertiliser (SF)										
Non-inoculated	3.5 ± 0.3 b	B	29.6 ± 3.0 b	B	1.9 ± 0.4 ab	B	1.0 ± 0.2 ab	B	2.0 ± 0.3 a	B
L. laccata	2.9 ± 0.4 a	B	24.9 ± 3.0 a	B	1.3 ± 0.3 a	B	0.6 ± 0.2 a	B	2.1 ± 0.3 a	B
P. tinctorius (Vi)	3.4 ± 0.3 ab	AB	23.5 ± 1.9 a	B	2.3 ± 0.3 b	B	1.1 ± 0.3 b	B	2.2 ± 0.3 a	B
P. tinctorius (Spo)	3.3 ± 0.2 ab	B	32.9 ± 2.2 b	B	2.0 ± 0.3 b	B	1.1 ± 0.3 b	B	1.9 ± 0.4 a	B
M. ambiguus	3.6 ± 0.4 b	B	32.3 ± 3.5 b	B	2.0 ± 0.5 b	B	1.0 ± 0.3 ab	B	2.0 ± 0.2 a	B
Slow-release fertiliser (SRF)										
Non-inoculated	3.6 ± 0.4 a	B	30.3 ± 3.9 a	B	2.2 ± 0.6 a	B	1.2 ± 0.4 b	B	1.8 ± 0.3 a	B
L. laccata	3.5 ± 0.4 a	C	29.7 ± 4.0 a	C	2.2 ± 0.6 a	C	0.9 ± 0.2 ab	C	2.5 ± 0.6 ab	B
P. tinctorius (Vi)	3.7 ± 0.9 a	B	31.2 ± 5.3 a	C	1.9 ± 0.6 a	B	0.7 ± 0.3 a	AB	2.7 ± 0.5 b	B
P. tinctorius (Spo)	3.9 ± 0.5 a	C	31.9 ± 4.5 a	B	2.3 ± 0.6 a	B	1.0 ± 0.2 ab	B	2.3 ± 0.7 ab	B
M. ambiguus	3.5 ± 0.2 a	B	33.0 ± 2.7 a	B	1.9 ± 0.6 a	B	0.6 ± 0.2 a	A	2.9 ± 0.6 b	B

Spore inoculum of *M. ambiguus* was effective for obtaining mycorrhizal seedlings of both pines. The highest ECM percentages were obtained in the fertilisation treatments, independently of the fertilisation method used. Spore inoculum of *M. ambiguus* has been previously used for inoculation of containerised conifers [24, 32]. The improvement of *M. ambiguus* mycorrhization ability by fertilisation could indicate a different foraging strategy of this fungus with higher nutrient demands for hyphal development compared with the rest of fungi tested in our study. In general, average mycorrhizal colonization levels were slightly higher on *P. pinaster* seedlings than on *P. pinea*. These differences agree with previous studies reporting a variable response of different tree species to fungal colonisation by a given fungus [22, 38].

Seedling growth was dependent on the three factors studied: (1) inoculation, (2) fertilisation and (3) pine species. It is remarkable that when *P. pinea* seedlings were fertilised with SRF, a significant increase on seedling biomass due to inoculation with the different fungi was observed. The nutrient levels supplied with this fertilisation method seemed to be optimal for impairing mycorrhizal inoculum function in the rhizosphere of *P. pinea*. Gradual and progressive nutrient enrichment of the substrate with slow-release fertiliser could enable the fungal mycelium to develop tolerance mechanisms to nutrient accumulation [41]. Contrary to *P. pinea*, a significant effect of inoculation on *P. pinaster* biomass was only achieved when seedlings were not fertilised, whereas fertilisation neutralised the fungal effect independently of the method used.

Different fungal species can differ in their tolerance to different fertilisation methods [2, 40]. Nevertheless, since the total inorganic nutrient demand for hyphal growth in pots relative to that of the plant is very small, the variable fungal effect under the same fertilisation treatments could be mostly dependent on the nutrient demand of the tree host.

Both, *P. pinea* and *P. pinaster*, grew better when fertilised, independently of the method used, and in general, *P. pinaster* was more dependent on fertilisation than *P. pinea*. Complexes environmental variables can be interconnected with seedling nutrient requirements, as demonstrated for *P. pinaster* and its demand of phosphorous being dependent on light availability [10]. The fertilisation methods (SF and SRF) at the dosage used were adequate for obtaining good morphological standards of seedling quality for both pine species.

The application of mycorrhizal inoculation in nursery, even if it does not necessarily mean an increase in seedling growth, has often demonstrated to allow a better survival of seedlings in the field [6, 7]. This is especially important under harsh climatic conditions such as those imposed by the Mediterranean climate [1, 25, 29]. Nursery mycorrhization can be an advantage for seedlings to surmount the transplanting stress, since it confers additional protection to roots against desiccation and aids seedlings to exploring a greater volume of soil for nutrient acquisition [13, 36]. On the other hand, a high fertilisation of seedlings in nursery does not necessarily offer a guarantee of survival after planting, since it usually causes an unbalanced shoot/root ratio [18, 39]. Thus, it would be highly desirable to conciliate both practises: fertilisation and inoculation with selected fungi, to minimise nursery fertilisation inputs and to assure physiological quality of seedlings and root protection by the formation of ectomycorrhizas with selected fungi.

In our work, the fertilisation method significantly affected the proportion and the colonisation extent of pine seedlings inoculated with different ectomycorrhizal fungi. Also, the growth effects were dependent on the fertilisation method and the fungal species inoculated. Therefore, matching selected fungi with the appropriate growth conditions is necessary for producing quality seedlings for commercial purposes [5].

Acknowledgements: This work was supported by the European Commission Contract AIR2-CT94-1149. The first author was granted (94/97 FPI programme) by the Ministerio de Educación y Ciencia, Spain.

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