Effects of *Lactarius deliciosus* and *Rhizopogon roseolus* ectomycorrhyzal fungi on seeds and seedlings of Scots and stone pines inoculated with *Fusarium oxysporum* and *Fusarium verticillioides*

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ABSTRACT

Two isolates of the edible ectomycorrhizal fungi (ECM), *Lactarius deliciosus* and *Rhizopogon roseolus*, were tested against *Fusarium oxysporum* and *F. verticillioides*, causal agents of damping-off on seeds and seedlings of Scots pine (*Pinus sylvestris*) and stone pine (*Pinus pinea*). The effects of ECM on *Fusarium* spp. in the rhizosphere of Scots and stone pines were evaluated by: (1) co-inoculating ECM and *Fusarium* when seeding (seed test) and (2) co-inoculating eight-week-old seedlings (seedling test). The seed tests showed significant reduction of Scots pine seed germination when treated with *F. verticillioides*, but this effect was absent when co-inoculated with *R. roseolus*. Higher germination rates were observed in stone pine when *F. oxysporum* was co-inoculated with *L. deliciosus* than the pathogen inoculation alone. In the seedling test, Scots and stone pines were not apparently affected by *Fusarium* spp. No obvious changes in plant growth-related variables were observed in either assay. Root colonization of Scots and stone pine seedlings by *R. roseolus* was 15.5% and 12% for the seed assay, as well as 21.6% and 11% for the seedling assay. *Lactarius deliciosus* mycorrhizal roots were found only in Scots pine seedlings (10%). *Rhizopogon roseolus* and *L. deliciosus* are two promising fungi for pine seedling protection against *Fusarium* damping-off at nurseries.

Keywords: biological control, ectomycorrhizal fungi, nursery diseases, *Pinus sylvestris*, *Pinus pinea.*

RÉSUMÉ

On a testé deux isolats des champignons ectomycorhiziens comestibles (ECM) *Lactarius deliciosus* et *Rhizopogon roseolus* pour lutter contre *Fusarium oxysporum* et *F. verticillioides*, qui provoquent la fonte des graines et des semis chez le pin sylvestre (*Pinus sylvestris*) et le pin parasol (*Pinus pinea*). L'expérience consistait à évaluer l'effet de l'ECM dans la rhizosphère des pins sylvestre et parasol en (1) inoculant à la fois l'ECM et *Fusarium* au moment de l'ensemencement (essai sur les semences) et (2) en les inoculant aussi à des semis âgés de huit semaines (essai sur des semis). Les essais sur les semences ont montré une réduction significative de la germination chez les semences de pin sylvestre traitées avec *F. verticillioides*, mais cet effet disparaissait lorsqu'on inoculait aussi *R. roseolus* aux semences. On a aussi obtenu un taux de germination plus élevé chez le pin parasol en inoculant à la fois *F. Oxysporum* et *L. deliciosus* qu'en n'inoculant que le pathogène. Dans l'essai sur les semis, le pin sylvestre et le pin parasol n'ont pas semblé affectés par *Fusarium* spp. On n'a pas observé non plus d'effet sur les paramètres de croissance de la plante dans les deux essais. Dans l'essai sur les semences, le pourcentage de colonisation des racines par *R. Roseolus* a atteint 15,5 % chez le pin sylvestre et 12 % chez le parasol ainsi que 21,6 % et 11 % respectivement dans l'essai sur les semis. Seules les racines du pin sylvestre ont été colonisées par *Lactarius deliciosus* (10 %). *Rhizopogon roseolus* et *L. deliciosus* sont deux champignons prometteurs pour la protection contre la fonte des semis de pin par *Fusarium* en pépinière.

Mots-clés : lutte biologique, champignons ectomycorhiziens, maladies en pépinière, *Pinus sylvestris*, *Pinus pinea.*

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Introduction

Forest nurseries are frequently affected by damping-off, caused by a fungal complex. *Fusarium* species are the main components of this complex, responsible for numerous losses of young trees (Sutherland *et al*. 1990). Specifically, *Fusarium oxysporum* Schlecht. and *F. verticillioides* (Sacc.) Nirenberg are the most frequent pathogens causing damage in forest nurseries (Martín-Pinto *et al*. 2006a). Numerous fungicides have been used to control this disease; however, some chemical products generate residual soil toxicity that affects nursery production and the environment in the short and long terms (Hwang *et al*. 1995).

Biological control of plant pathogens is currently receiving great attention (Whipps 2004). The control mechanism involves total or partial elimination of the pathogen population, or the reduction of damaging effects on plants (Cook and Baker 1996). Several authors have suggested that biological control with ectomycorrhizal fungi (ECM) may be feasible for controlling damping-off as an alternative or a complement to fungicides (Hwang *et al*. 1995; Pedersen *et al*. 1999; Machón *et al*. 2006, 2009; Zhang *et al*. 2011). However, the effectiveness of ECM in disease suppression may depend both on particular mycorrhizal and host species, as well as soil conditions (Sampagni *et al*. 1985).

Several species of ECM are known to protect conifer seedlings under *in vitro* conditions (Duchesne *et al*. 1987a ,b; Chakravarty *et al*. 1990; Chakravarty and Hwang 1991; Hwang *et al*. 1995; Pedersen *et al*. 1999; Martín-Pinto *et al*. 2006b; Zhang *et al*. 2017). A few *in vivo* experiments have been performed with environmental conditions simulating forest nurseries (reviewed in Okorski *et al*. 2014; Mateos *et al*. 2017). In addition, several ECM tested on *Fusarium* damping-off were poisonous to humans, for instance *Paxillus involutus* (Batsch ex Fr.) (Chakravarty *et al*. 1999; Pedersen *et al*. 1999). Based on previous results from *in vivo* tests (Olaizola *et al*. 2018), we selected two edible ectomycorrhizal species, *Lactarius deliciosus* (L.: Fr.) S.F. Gray. and *Rhizopogon roseolus* (Corda) T.M. Fries to study their protective effect under *in vivo* conditions.

Lactarius deliciosus is an edible ectomycorrhizal fungus commonly harvested in forests, where it naturally forms ectomycorrhizae with seedlings of many conifer species, particularly of the genus *Pinus*. This mushroom is consumed all over the world (Boa 2004), particularly in Spain where it is one of the most important wild mushroom species (Martínez de Azagra *et al*. 1997; de Román and Boa 2004). There is interest in the controlled production of this mushroom by means of mycorrhizal plants infected with either spores obtained from mature sporocarps or with mycelium grown *in vitro* (Guerin-Laguette et al. 2014). In this regard, sporocarp tissue of *L. deliciosus* can be easily isolated for *in vitro* cultivation, and will show a sufficiently fast growth rate for inoculum production (Olaizola *et al*. 2018). Particularly, it has been shown to form ectomycorrhizae with pine species like *P. sylvestris* L. (Guerin-Laguette *et al*. 2000), *P. pinaster* Ait. (Hortal et al. 2008) and *P. pinea* L. (Rincón *et al*. 2001) grown in containers.

Rhizopogon roseolus is a mid-quality mushroom traditionally consumed in several Spanish regions (Martínez de Azagra *et al*. 1997). However, within the genus there are other species of commercial interest, such as *R. rubescens* (Hall *et al*. 2001) which sells for up to 650 € per kg in Japan (Wang *et al*. 2002). *Rhizopogon roseolus* has a high potential in forest nursery mycorrhization programs because of its high capacity for colonizing young pine seedling roots (Ahonen-Jonnarth *et al*. 2000; Rincón *et al*. 2001). This ability and its proven antagonistic effect on *Fusarium* spp. *in vitro* (Martín-Pinto *et al*. 2006b) makes this species one of the most promising fungi for biological control of diseases in nursery seedling production.

There is limited research on the protective effects of ECM against fungal pathogens, with some reports using *Laccaria laccata* (Scop.) Cooke (Strobel and Sinclair 1991; Chakravarty and Hwang 1991; Branzanti et al. 1999; Machón *et al*. 2006, 2009), or the poisonous *Paxillus involutus* (Chakravarty *et al*. 1999; Pedersen *et al*. 1999). However, there is a lack of research about the effects of edible mushrooms with commercial cultivation interest against damping-off (but see Zhang *et al*. 2011). The aim of this study was to analyze the effects of *L. deliciosus* and *R. roseolus* on *Fusarium* damping-off with special significance on Eurasian Scots pine (*P. sylvestris* L.), and in southern European stone pine (*P. pinea* L.). We hypothesised a protective effect against damping-off provided by both ectomycorrhizal fungal species.

Materials and Methods

Organisms

Scots pine (Montaña Soriano-Burgalesa, Spain) and stone pine (Meseta Norte, Spain) seeds were obtained from a commercial forest nursery (Viveros Fuenteamarga S.L.) in Valladolid, Spain. The ectomycorrhizal fungi (ECM) *Lactarius deliciosus* (Ld-1 isolate) and *Rhizopogon roseolus* (Rr-1 isolate) were tested as biological control agents. These strains were previously tested for their *in vitro* antagonism against *Fusarium* spp. (Olaizola *et al*. 2018). Both strains were isolated from fresh sporocarps collected from *P. sylvestris* stands in Palencia (Ld-1) and Soria (Rr-1) provinces, Spain. Sporocarps were superficially abraded, and pieces of inner tissues were transferred to Petri dishes containing Modified Melin Norkrans (MMN) agar medium (Marx 1969) following Molina and Palmer (1982). ECM cultures were incubated in darkness for 30 days at 22.5 ºC. Sporocarps were then desiccated and stored at room temperature in sterile bags for further molecular analysis.

Thirty 5-mm agar plugs obtained from an ECM colony margin were transferred to each 1.8 L flask containing 1200 mL of autoclaved (121 ºC, 20 min) peat-vermiculite mixture (100:1100 mL) moistened with 600 mL of MMN liquid medium with glucose reduced to 2.5 g/l. Control flasks contained the same substrate without ECM. All flasks, including controls, were then incubated on a shaker in the dark at 22.5 ºC for two months.

Two pathogenic *Fusarium* strains, *F. oxysporum* (Fo-4P) and *F. verticillioides* (Fm-6P), isolated from symptomatic seedlings and verified for pathogenicity on Scots and stone pine (Olaizola 2007), were used to inoculate seeds and seedlings. To produce the inoculum, monoconidial isolates were subcultured on PDA (Potato Dextrose Agar – Sigma Aldrich) in 9-cm-diameter Petri dishes and incubated at 23 ºC in the dark. After seven days, 15 agar plugs from each colony margin were placed into each Erlenmeyer flask con-

taining 200 mL of PDB (Potato Dextrose Broth - Sigma Aldrich) and cultured at 23 ºC in the dark on a shaker at 200 rpm to stimulate sporulation. Five days later, spores were collected by filtration through a glass fibre filter. Spore concentration was estimated using a hemocytometer and adjusted through the addition of sterile water to obtain 10^6 spores/mL.

Seed test

Scots pine and stone pine seeds were surface sterilized with 30% H_2O_2 for 30 min and washed five times with sterile distilled water before sowing. Disposable containers (200 ml) were filled with three 60 ml-layers as follows: (1) sterilized peat-vermiculite (1:1), (2) ECM vegetative inoculum, (3) sterilized peat-vermiculite. Seeds were placed between layers two and three. Control containers were filled as described above but without ECM.

The experimental design integrated nine treatments for each pine species (*P. pinea* and *P. sylvestris*, P): 1. P+ Control, 2. P+ Ld, 3. P+ Rr, 4. P+ Fv, 5. P+ Fo, 6. P+ Ld+ Fv, 7. P+ Ld+ Fo, 8. P+ Rr+ Fv and 9. P+ Rr+ Fo. Fifteen days after seeding, 5 ml of *Fusarium* spore suspension (10^6 spore/ml) was added to the corresponding treatments. Five ml of sterile distilled water was added to treatments without *Fusarium*.

To evaluate the effect of ECM fungi on *Fusarium*, seed tests were set up with three replicates for each treatment where each replicate consisted of 12 pots, which were then randomly arranged on a greenhouse bench. Seed germination was scored 20 weeks after seeding. Fifteen seedlings per treatment were measured for shoot height, diameter, root length, shoot and root biomass and mycorrhizal percentage. Intensity of root colonization was expressed as a percentage of mycorrhizal apices over 250 observations. The identity of either *L. deliciosus* or *R. roseolus*, on the apices, was confirmed when conducting the mycorrhization evaluation.

Seedling test

Pine seeds were surface sterilized as described above, and sowed in plastic trays filled with sterile peat-vermiculite mixture (1:1) for eight weeks. Disposable containers (200 mL) were filled in three layers of equal volume as described in the seed assay, except for seeds being replaced by 8–week-old seedling transplants that were placed on the top layer. The same nine treatments for each pine species were applied for the seedling assay. Tests were set up with three replicates, each containing 12 pots, as described above.

Twenty weeks after transplanting, 15 plants per treatment were evaluated for seedling mortality, shoot height, diameter, root length, shoot and root biomass and mycorrhizal percentage. Seed and seedling tests were set up simultaneously. The identity of either *L. deliciosus* or *R. roseolus* on apices was confirmed when conducting the mycorrhizal evaluation.

Data analysis

Violations of ANOVA assumptions were first checked for all datasets. Data from each experiment were analyzed by two-way analysis of variance (ANOVA). Individual means were compared using HSD Tukey tests ($p = 0.05$) for multiple comparisons (STATISTICA 6.0 Software).

Results

Seed test

Fusarium oxysporum and *F. verticillioides* significantly reduced Scots pine seed germination (Fig. 1). *Fusarium verticillioides* showed higher effect on germination than *F. oxysporum* (13.9% vs. 28.5%, respectively, significantly different at $p<0.05$). This large decrease of *P. sylvestris* germination when treated with *F. verticillioides* was ameliorated by co-inoculation with *R. roseolus* (44.4% germination, significantly different than pathogen alone at $p<0.05$). When Scots pine seeds were co-inoculated with *F. oxysporum* and *L. deliciosus*, germination percentage was close to *F. oxysporum* treatment alone (34.0% vs. 28.5%, not significantly different).

On stone pine, *F. oxysporum* inhibited seed germination (37.4% germination, significantly different from no pathogen, $p<0.05$) whereas *F. verticillioides* did not cause any significant effect. Co-inoculation assay of *F. oxysporum* and *L. deliciosus*, resulted in increased germination (46.4% germination, significantly different from pathogen alone at $p<0.05$, Fig. 2), resembling *L. deliciosus* treatment alone (52.64 % no significant differences between *L. deliciosus* inoculations with or without *F. oxysporum*). Similarly, we observed no significant differences in treatments with *F. oxysporum* being or not co-inoculated with *R. roseolus*. Co-inoculation with *L. deliciosus* or *R. roseolus* did not affect germination of seeds inoculated with *F. verticillioides*.

No obvious changes in plant growth-related variables were observed (Tables 1 and 2), except a small reduction in shoot and root dry weight of stone pine seedlings co-inoculated with *R. roseolus* and *F. verticillioides*. Stone pine root length significantly decreased after inoculation with *F. verticillioides* alone or in combination with *R. roseolus* (12.26 cm and 12.00 cm respectively vs. 13.62 cm in control; $p<0.05$).

Root colonization of seedlings by *R. roseolus* was 15.5% (Rr) and 12% (Rr+Fv treatment) for Scots pine, and 12% (Rr) for stone pine, respectively. No mycorrhizal roots were observed in the *L. deliciosus* treatments. Root colonization was absent in the treatments without ECM inoculation.

Seedling test

The survival of Scots and stone pine seedlings showed no significant differences among treatments. Scots pines inoculated with *L. deliciosus* significantly decreased shoot height (cm), shoot dry weight (g) and root dry weight (g) compared to the control treatment (5.82 cm, 0.22 g and 0.39 g significantly different from the controls, 7.68 cm, 0.36 g and 0.70 g; $p<0.05$) (Table 3). Root collar diameter (cm) was significantly reduced in treatments with *R. roseolus* and *F. verticillioides* (0.13 cm vs. 0.15 cm in control; $p<0.05$). Stone pines treated with *F. oxysporum* or *L. deliciosus* showed a significant decrease in root collar diameter (0.25 cm in both treatments vs. 0.27 cm in control; $p<0.05$) (Table 4). Shoot dry weight (g) was significantly enhanced in the Ld+ Fv, Rr+ Fv and Rr+ Fo (2.22 g, 2.22 g, and 2.23 g vs. 1.86 g in control; $p<0.05$), and root dry weight in Rr treatment (1.55 g vs. 1.27 g; $p<0.05$).

Mycorrhizal roots were found on all Scots pine seedlings inoculated with *L. deliciosus* and *R. roseolus*. The proportion of colonised roots was apparently higher in seedlings inocu-

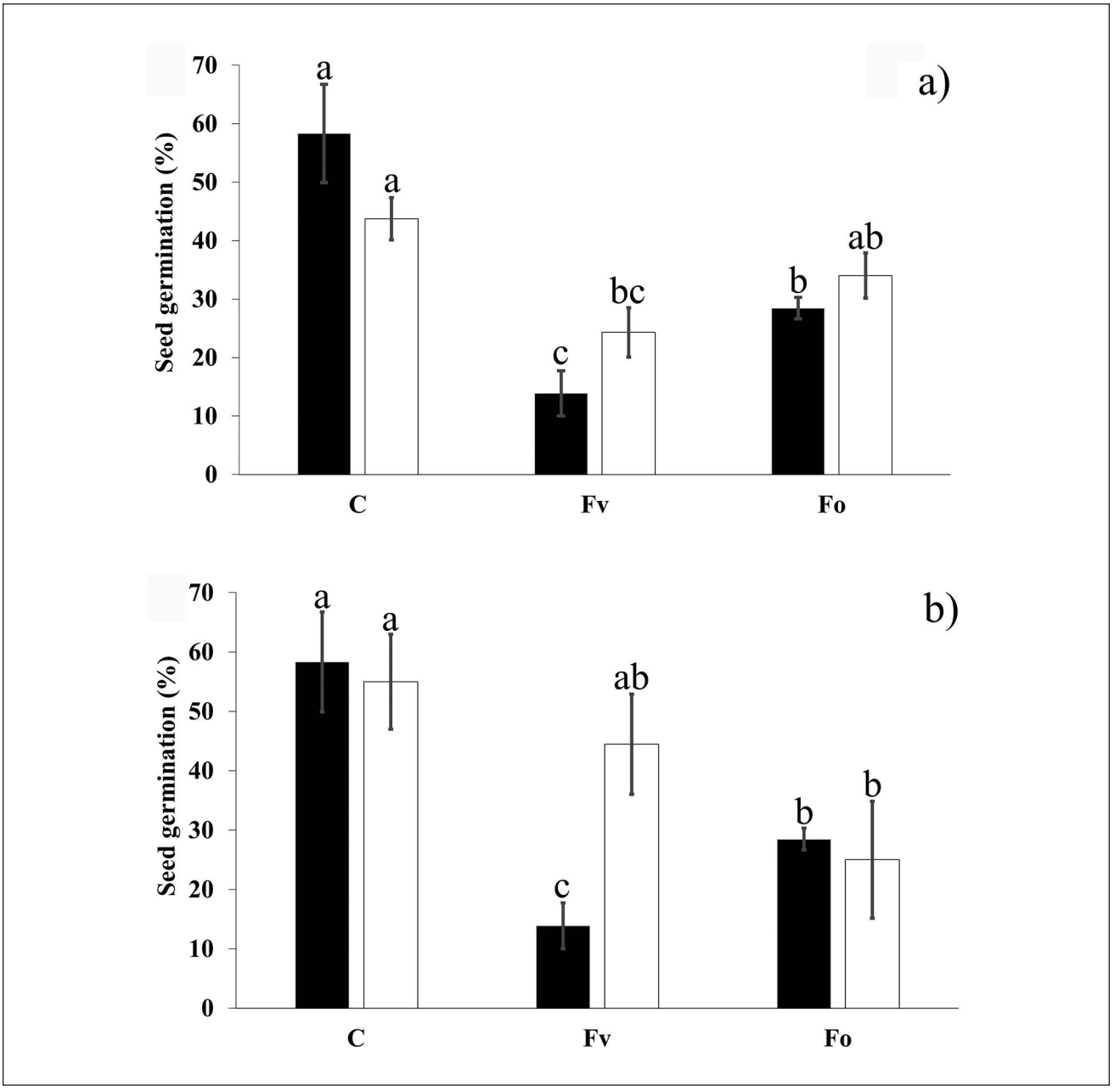

Fig. 1. Effect of *Lactarius deliciosus* (Panel A) and *Rhizopogon roseolus* (Panel B), together with *F. verticillioides* (Fv) and *F. oxysporum* (Fo) on germination of Scots pine seeds eight weeks after sowing. Filled bars represent control treatments, non-filled bars represent ECM treatments. Vertical bars followed by different letters are significantly different (HSD Tukey test, p = 0.05). Error bars represent standard error of the mean.

lated with *R. roseolus* than *L. deliciosus* (21.6 %, 10.0 % respectively; not significantly different); and significantly higher in Rr+Fv treatment (40.2 %; $p<0.05$). Eleven percent of stone pine seedlings were successfully colonized by *R. roseolus*, whereas no mycorrhizal structures were found in seedlings with *L. deliciosus* or control treatments.

Discussion

Zak (1964) described the use of ectomycorrhizal fungi in biological control of root pathogens and several authors (Hwang *et al.* 1995; Pedersen *et al.* 1999; Machón *et al.* 2006; Zhang et al. 2017) reported the ability of ECM fungi to suppress disease. Our study adds to the literature on the protective effect of edible ECM species (Zhang *et al.* 2011) and is the first to report on the effect of *L. deliciosus* and *R. roseolus*. Our study suggests that the inoculation of Scots pine seedlings with *R. roseolus* reduces the damage caused by *F. verticillioides*. Furthermore, inoculation of stone pine with *L. deliciosus* significantly reduced disease levels caused by *F. oxysporum* to levels observed on uninoculated control seedlings. Martín-Pinto *et al.* (2006b) obtained similar findings with *P. nigra* in an *in vitro* study. These results suggest that the extent of the protective effect can be related to host susceptibility, since *P. pinea* seedlings were less damaged than *P. sylvestris*.

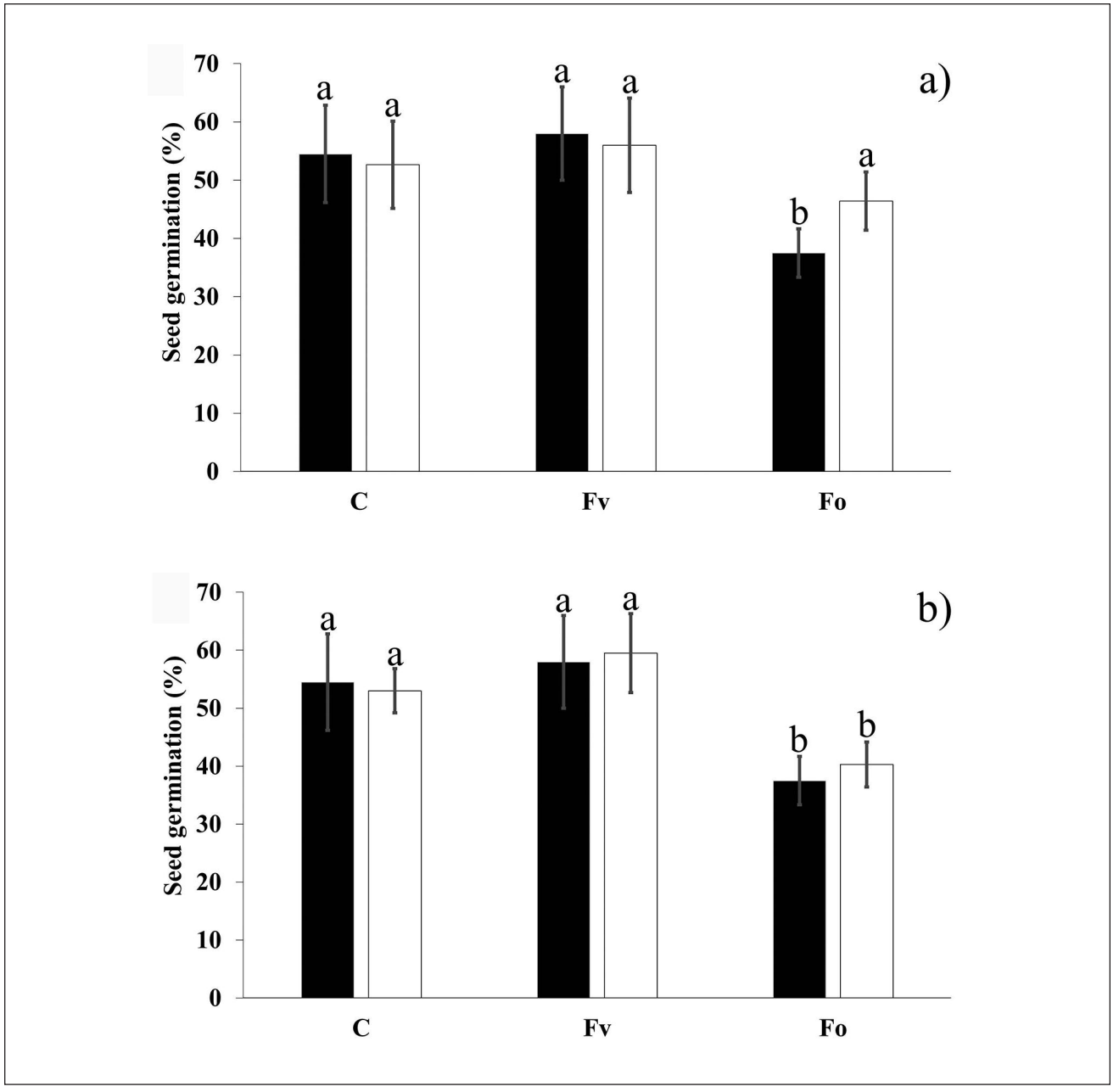

Fig. 2. Effect of *Lactarius deliciosus* (Panel A) and *Rhizopogon roseolus* (Panel B), together with *F. verticillioides* (Fv) and *F. oxysporum* (Fo) on germination of Stone pine seeds eight weeks after sowing. Filled bars represent control treatments, non-filled bars represent ECM treatments. Vertical bars followed by different letters are significantly different (HSD Tukey test, p = 0.05). Error bars represent standard error of the mean.

This is the first report demonstrating that ECM fungi with economic importance provided a protective effect against pine diseases under nursery conditions. This information can have practical implications for nursery owners with benefits attributed to a protective effect against diseases as well as to sales of mycorrhized plants. As the current price of one-year-old pine seedlings in Spain averages to 0.24 €, mycorrhizal seedlings with *L. deliciosus* can reach 7 €. In any case, we advocate for a conservative and rational use of fungal genetic material, and discourage the introduction of ECM species and isolates outside their natural distribution range.

The effects of *L. deliciosus* and *R. roseolus* on seed (pre-emergence) damping-off were tested to facilitate mechanization of the mycorrhization process in commercial nurseries with proper sowing equipment. Effects were studied on eight-week old seedlings (post-emergence damping-off) when mycorrhization is more probable but the mechanization process has more complications. Thus, the mechanization of mycorrhization during sowing would be easier and more effective than the current plant transplanting method used to obtain inoculated plants (Machón *et al.* 2006). Important differences were discovered between seed and seedling experi-

Table 1. Seed test: Effect of *L. deliciosus* and *R. roseolus* on shoot height, diameter, root length, shoot mass, root biomass and mycorrhizal development of Scots pine seedlings inoculated with *F. verticillioides* or *F. oxysporum*, 20 weeks after sowing

	Shoot height (cm)		Diameter (cm)		Root length (cm)		Shoot dry weight (g)		Root dry weight (g)		Mycorrhizal short roots (%)	
Control	4.92 a	(0.19)	0.10 b	(0.01)	14.04 a	(0.24)	0.15 ab	(0.02)	0.23 ab	(0.02)	0.00 b	(0.00)
Fv	5.08 a	(0.11)	0.10 b	(0.00)	14.60 a	(0.17)	0.11 b	(0.00)	0.15 b	(0.01)	0.00 b	(0.00)
Fo	4.42 a	(0.19)	0.16 a	(0.11)	13.56 a	(0.12)	0.18 ab	(0.01)	0.25 ab	(0.02)	0.00 b	(0.00)
Ld	5.50 a	(0.33)	0.10 b	(0.00)	13.56 a	(0.33)	0.21 a	(0.01)	0.27 a	(0.01)	0.00 b	(0.00)
Ld+Fv	5.16 a	(0.20)	0.11 ab	(0.01)	14.38 a	(0.28)	0.16 ab	(0.01)	0.23 ab	(0.02)	0.00 b	(0.00)
Ld+Fo	4.36 a	(0.17)	0.10 b	(0.00)	13.80 a	(0.33)	0.18 ab	(0.01)	0.24 ab	(0.02)	0.00 b	(0.00)
Rr	5.08 a	(0.44)	0.10 b	(0.00)	13.42 a	(0.17)	0.16 ab	(0.03)	0.21 ab	(0.03)	15.50 a	(10.33)
Rr+Fv	4.82 a	(0.31)	0.095 b	(0.00)	13.30 a	(0.27)	0.12 b	(0.03)	0.16 b	(0.03)	0.00 b	(0.00)
Rr+Fo	4.84 a	(0.33)	0.11 ab	(0.00)	13.56 a	(0.39)	0.15 b	(0.03)	0.21 ab	(0.03)	12.00 a	(0.00)

Values are the means of 15 seedlings (Standard Error of the mean). Means followed by the same letter within a column do not significantly differ ($p<0.05$) from each other by HSD Tukey test

Table 2. Seed test: Effect of *L. deliciosus* and *R. roseolus* on shoot height, diameter, root length, shoot mass, root biomass and mycorrhizal development of stone pine seedlings inoculated with *F. verticillioides* or *F. oxysporum*, 20 weeks after sowing

	Shoot height (cm)		Diameter (cm)		Root length (cm)		Shoot dry weight (g)		Root dry weight (g)		Mycorrhizal short roots (%)	
Control	14.02 a	(0.48)	0.22 a	(0.01)	13.62 a	(0.29)	0.97 b	(0.08)	0.54 ab	(0.05)	0.00 b	(0.00)
Fv	14.40 a	(0.50)	0.22 a	(0.01)	12.26 b	(0.10)	0.95 b	(0.06)	0.49 b	(0.04)	0.00 b	(0.00)
Fo	13.28 a	(0.77)	0.23 a	(0.01)	13.28 ab	(0.35)	0.88 b	(0.11)	0.5 b	(0.06)	0.00 b	(0.00)
Ld	12.80 a	(0.55)	0.22 a	(0.00)	13.84 a	(0.27)	0.75 b	(0.05)	0.37 b	(0.03)	0.00 b	(0.00)
Ld+Fv	13.94 a	(0.58)	0.22 a	(0.00)	12.68 ab	(0.15)	0.94 b	(0.03)	0.50 b	(0.03)	0.00 b	(0.00)
Ld+Fo	13.00 a	(0.80)	0.22 a	(0.01)	12.86 ab	(0.51)	1.02 b	(0.05)	0.50 b	(0.03)	0.00 b	(0.00)
Rr	14.82 a	(0.37)	0.22 a	(0.00)	13.70 a	(0.41)	0.82 b	(0.05)	0.40 b	(0.04)	12.00 a	(8.00)
Rr+Fv	13.94 a	(0.37)	0.21 a	(0.00)	12.00 b	(0.10)	1.15 a	(0.07)	0.70 a	(0.04)	0.00 b	(0.00)
Rr+Fo	12.58 a	(0.33)	0.22 a	(0.00)	13.24 ab	(0.28)	0.82 b	(0.07)	0.52 ab	(0.04)	0.00 b	(0.00)

Values are the means of 15 seedlings (Standard Error of the mean). Means followed by the same letter within a column do not significantly differ ($p<0.05$) from each other by HSD Tukey test.

ments. In seed tests, *L. deliciosus* improved germination of stone pine inoculated with *F. oxysporum*, and *R. roseolus* improved germination of Scots pine seeds inoculated with *F. verticillioides*. In post-emergence tests, ectomycorrhizal fungi did not affect seedling survival of both pines independently, and *Fusarium* inoculation did not produce significant damage on them. These results show that two-month-old seedlings are less susceptible to damping-off caused by *Fusarium* and corroborate the importance of this disease during the earliest plant stages, as revealed by other authors (Soldevilla 1995; Machón *et al.* 2006). Conversely, damage caused by both *Fusarium* species was higher on Scots pine than stone pine as previously reported by Machón *et al.* (2006, 2009) and Mateos *et al.* (2017). The larger seed size and testa thickness of stone pine as well as the plant size may be related to these differences.

There is limited research studying the interaction among mycorrhization, damping-off protection and seedlings age simultaneously (Machón *et al.* 2006, 2009). In our study, differences in germination between ECM were dependent on *Fusarium* spp., and differences between pines depended on ECM treatment (ECM x *Fusarium* x Pines interaction: $p<0.01$). Machón *et al.* (2006) obtained similar results in a study of Scots pine and *Laccaria laccata* co-inoculation with *F. oxysporum* or *F. verticilioides*. For example, germination of Scots pine seeds inoculated with *R. roseolus* was significantly reduced in treatments with *F. verticillioides*, while germination of stone pine seeds inoculated with *L. deliciosus* was significantly reduced in treatments with *F. oxysporum*. These interaction effects lead us to recommend the use of more than one ECM species for the control of damping-off.

Several causes of disease suppression by ectomycorrhizal fungi have been proposed and many factors are considered to be involved in the mechanism (Duchesne *et al.* 1987a). Marx and Davey (1969) suggested that disease resistance by ectomycorrhizal fungi may be associated with either the formation of a physical barrier by the fungal mantle, or the production of antibiotics. In addition, several authors (Chakravarty and Hwang 1991; Machón *et al.* 2009) reported that disease suppression by ectomycorrhizal fungi may also be associated with the production of antimicrobial substances by the plant.

Nutrient competition in the rhizosphere of the seedlings by mycorrhizal fungi could be another possible cause related to pathogen growth reduction (Li-Min *et al.* 2000). However,

Table 3. Seedling test: Effect of *L. deliciosus* and *R. roseolus* on shoot height, diameter, root length, shoot mass and root biomass and mycorrhizal development of Scots pine seedlings inoculated with *F. verticillioides* or *F. oxysporum*, 20 weeks after planting. n = 135

	Shoot height (cm)		Diameter (cm)		Root length (cm)		Shoot dry weight (g)		Root dry weight (g)		Mycorrhizal short roots (%)	
Control	7.68 a	(0.32)	0.15 a	(0.01)	15.66 ab	(0.49)	0.36 a	(0.02)	0.70 a	(0.03)	0.00 c	(0.00)
Fv	6.56 ab	(0.42)	0.15 a	(0.00)	16.32 a	(0.24)	0.33 ab	(0.01)	0.55 ab	(0.02)	0.00 c	(0.00)
Fo	7.20 ab	(0.45)	0.15 a	(0.00)	15.90 ab	(0.41)	0.28 ab	(0.02)	0.57 ab	(0.02)	0.00 c	(0.00)
Ld	5.82 b	(0.23)	0.14 a	(0.00)	14.26 b	(0.44)	0.22 b	(0.02)	0.39 b	(0.04)	10.00 b	(0.67)
Ld+Fv	7.33 ab	(0.43)	0.14 a	(0.01)	15.40 ab	(0.22)	0.36 a	(0.04)	0.70 a	(0.09)	11.50 b	(0.00)
Ld+Fo	6.32 ab	(0.58)	0.14 a	(0.01)	15.86 ab	(0.37)	0.32 ab	(0.01)	0.71 a	(0.04)	9.00 b	(0.00)
Rr	7.62 a	(0.37)	0.14 a	(0.01)	15.81 ab	(0.31)	0.36 a	(0.03)	0.67 a	(0.03)	21.60 b	(11.19)
Rr+Fv	7.44 a	(0.23)	0.16 a	(0.01)	16.50 ab	(0.24)	0.34 a	(0.03)	0.73 a	(0.11)	40.20 b	(10.00)
Rr+Fo	7.36 a	(0.26)	0.13 b	(0.00)	15.54 a	(0.23)	0.37 a	(0.03)	0.76 a	(0.06)	22.00 a	(11.37)

Values are the means of 15 seedlings (Standard Error of the mean). Means followed by the same letter within a column do not significantly differ ($p<0.05$) from each other by HSD Tukey test.

Table 4. Seedling test: Effect of *L. deliciosus* and *R. roseolus* on shoot height, diameter, root length, shoot mass and root biomass and mycorrhizal development of stone pine seedlings inoculated with *F. verticillioides* or *F. oxysporum*, 20 weeks after planting

	Shoot height (cm)		Diameter (cm)		Root length (cm)		Shoot dry weight (g)		Root dry weight (g)		Mycorrhizal short roots (%)	
Control	19.84 a	(0.91)	0.27 a	(0.01)	15.40 a	(0.66)	1.86 b	(0.10)	1.27 b	(0.05)	0.00 b	(0.00)
Fv	18.60 a	(1.12)	0.27 a	(0.01)	14.04 a	(0.33)	2.06 ab	(0.14)	1.38 b	(0.10)	0.00 b	(0.00)
Fo	20.70 a	(0.35)	0.25 b	(0.00)	14.00 a	(0.19)	1.96 ab	(0.05)	1.34 b	(0.04)	0.00 b	(0.00)
Ld	19.14 a	(0.49)	0.25 b	(0.00)	14.56 a	(0.30)	1.76 b	(0.06)	1.30 b	(0.07)	0.00 b	(0.00)
Ld+Fv	20.28 a	(0.45)	0.28 a	(0.01)	14.72 a	(0.24)	2.22 a	(0.04)	1.53 ab	(0.03)	0.00 b	(0.00)
Ld+Fo	20.72 a	(0.52)	0.26 ab	(0.00)	14.52 a	(0.13)	2.06 ab	(0.04)	1.51 ab	(0.05)	0.00 b	(0.00)
Rr	20.76 a	(0.70)	0.27 a	(0.01)	14.56 a	(0.31)	2.10 ab	(0.11)	1.55 a	(0.09)	11.00 a	(8.00)
Rr+Fv	21.86 a	(0.56)	0.27 a	(0.01)	13.92 a	(0.27)	2.22 a	(0.07)	1.49 ab	(0.03)	0.00 b	(0.00)
Rr+Fo	21.82 a	(0.12)	0.27 a	(0.01)	15.00 a	(0.32)	2.23 a	(0.02)	1.46 ab	(0.06)	0.00 b	(0.00)

Values are the means of 15 seedlings (Standard error of the mean). Means followed by the same letter within a column do not significantly differ ($p<0.05$) from each other by HSD Tukey test.

Smith and Read (1997) suggested more than one general mechanism of disease suppression may be present and according to Schisler and Linderman (1989 a, b), several mechanisms may act simultaneously or synergistically to suppress the disease.

Defense mechanisms of Scots and stone pines against damping-off are unknown. *Lactarius deliciosus* and *R. roseolus* inhibited *in vitro* growth and spore germination of *F. verticillioides* and *F. oxysporum* (Martín-Pinto *et al.* 2006b; Olaizola *et al.* 2018). Stone pine seeds and seedlings inoculated with *L. deliciosus* were protected against *F. oxysporum* attack prior to short root formation and in the absence of the mycorrhizal mantle as determined by microscopic observation. The same effect was observed with *R. roseolus* protecting Scots pine seeds and seedlings against *F. verticillioides*. Pre-emergence and post-emergence damping-off resistance by *L. deliciosus* and *R. roseolus* was not associated with the formation of a physical barrier, but may be related to the production of antifungal compounds by ECM, which inhibit the growth of several root pathogens (Schisler and Linderman 1989 a, b). Similar findings have been reported regarding mycorrhizal fungi such as *Paxillus involutus*, *Laccaria bicolor* (Maire) Orton and *L. laccata*. (Chakravarty and Hwang 1991; Chakravarty *et al.* 1999)

Our data corroborates that damping-off is more aggressive in the early seedling stages, mainly in pre-emergence and early post-emergence stages, with mortality rates reaching 100% (Soldevilla 1995; Machón *et al.* 2006). Conversely, the formation of mycorrhizal roots frequently occurs when the seedlings form secondary roots (Pera 1992; Torres and Honrubia 1993). If the protective effect by ECM depends exclusively of the presence of mycorrhiza, mycorrhization would be of no help as a biological control method against damping-off. Therefore, when secondary roots (suitable for mycorrhizal synthesis) are formed, damping-off effects become less important. However, our assay demonstrates that (1) mycorrhiza formation is possible by inoculating *R. roseolus* directly on the seeds, and (2) mycorrhiza formation is not mandatory for a protective effect of ECM against *Fusarium* damping-off, at least when referring to seeds. Similar results have also been obtained in similar case studies with *L. laccata* (Machón *et al.* 2006, 2009) and *Suillus luteus* (L. ex Fries) (Mateos *et al.* 2017).

No relevant changes in plant growth-related variables, shoot dry weight, root collar diameter, root length and root

dry weight, were observed among treatments. However, a clear enhancement of seedling growth after mycorrhizal colonization has been shown in other studies (Guerin-Laguette *et al.* 2000). This lack of relationship between mycorrhizal colonization and seedling growth may have been caused by low levels of mycorrhizae acquired for both ECM fungi (between 10% and 20%). However, levels of mycorrhization higher than 40% of Scots pine apexes for Rr+ Fv treatment were not related to enhancement of seedling growth.

Rhizopogon roseolus formed ectomycorrhizal roots in stone pine and Scots pine seed as well as seedling assays. In contrast, *L. deliciosus* only formed mycorrhizal roots in the Scots pine seedling assay. The occurrence of *R. roseolus* mycorrhizal short roots on very young seedlings may be related to its ability to form ectomycorrhizae on developmentally younger roots, or to an enhanced inoculum viability (the inoculum was added at the time of seeding). Furthermore, the lack of *L. deliciosus* mycorrhizal short roots on stone pines could be related to the fungal strains being isolated from Scots pines. However, *R. roseolus* did not show specificity for either pine species, as mycorrhizae were found on both species of pines. These findings concur with results of other reports, where *R. roseolus* colonized roots of both Scots pine and stone pine seedlings (Ahonen-Jonnarth *et al.* 2000; Rincón *et al.* 2001). According to this high potential for mycorrhization, *R. roseolus* has been recommended for its use in forest nursery mycorrhization programs.

When co-cultured with either *Fusarium* species, the number of *R. roseolus* ectomycorrhizal roots was low in both pine species, suggesting that the fungal pathogen inhibits ECM formation. These results are consistent with those on *Suillus tomentosus* (Kauffman) Singer and *F. moniliforme* J. Sheld (=*F. verticillioides* (Sacc.) Nirenberg) reported by Hwang *et al.* (1995). However, mycorrhizal suppression by *Fusarium* was not observed in Scots pine for *L. deliciosus*, in agreement with results obtained in other studies with *F. oxysporum* strains (García-Romera *et al.* 1998; Diedhiou *et al.* 2003).

Our results under greenhouse conditions, while less conclusive than those obtained *in vitro* with the same fungal strains (Martín-Pinto *et al.* 2006b; Olaizola *et al.* 2018), constitute a valuable proof of concept, showing interesting interactions with *Fusarium* spp. on Scots and stone pine seedling roots. *Rhizopogon roseolus* showed better results because of its protective effect and its enhanced mycorrhiza formation ability, developing mycorrhiza even when it was inoculated at the time of sowing. These data may have practical implications for the production of mycorrhizal plants in nurseries.

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