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Biological control of damping-off by *Fusarium oxysporum* and *F. verticillioides* on pine and oak seedlings using edible ectomycorrhizal fungi

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ABSTRACT

The Mediterranean forest has an important ecological and economic role, being holm oak (*Quercus ilex*), Pyrenean oak (*Quercus pyrenaica*), umbrella pine (*Pinus pinea*) and Scot pine (*Pinus sylvestris*) some of its main tree species. The fungal damping-off disease caused by *Fusarium* seriously threatens the establishment of these forest species in nurseries and reforestation, requiring the search for environmentally friendly alternatives to control the disease. We have used different species of ectomycorrhizal fungi (EMF) as potential biological control agents (BCAs) effective against the disease: *Lactarius sanguifluus*, *Tricholoma portentosum*, *Suillus luteus* and *Agaricus silvicola* from *Pinus*-species, and *Leccinum lepidum*, *Amanita rubescens* and *Xerocomus ferrugineus* from *Quercus*-species. A direct *in vitro* confrontation was performed and conidial germination of *Fusarium* in contact with cell-free filtrates produced by EMF was studied. *Le. lepidum* was the most effective *Quercus*-fungus *in vitro* against *F. oxysporum*, reducing its growth up to 32 % and its conidial germination up to 87 %. *S. luteus* was the most effective *Pinus*-fungus *in vitro* against *F. oxysporum* and *F. verticillioides*, reducing in direct confrontation, reducing its growth up to 30 %. However, *La. sanguifluus* was the *Pinus*-fungus that inhibited conidial germination of both pathogens, up to 55 %. *In planta* trials were carried out with seeds of the four forest species growing on substrate colonized by *Le. lepidum* (in *Q. ilex* and *Q. pyrenaica* seeds, infected by *F. oxysporum*) or by *La. sanguifluus* (*P. pinea* and *P. sylvestris* seeds, infected by *F. oxysporum* or *F. verticillioides*). Only *La. sanguifluus* was effective in reducing disease caused by *F. oxysporum* (strain Fo4) on *P. sylvestris* seeds. Therefore, EMF may be a potential tool in the control of damping-off in forest species, requiring further research.

1. Introduction

Forests fulfill important functions for life: climate regulation, biodiversity maintenance, ecosystem functioning or economic activities for humans (De Frenne et al., 2021). In particular, Mediterranean forests fulfill important ecosystem functions, such as prevention of soil erosion or water purification, as well as providing products to humans, such as timber, mushrooms or cork. However, the Mediterranean Basin is one of the points of greatest climate change in the world, with prolonged periods of drought and high temperatures that weaken trees and increase the spread of diseases, increasing the weakness of these ecosystems (Morán-Ordóñez et al., 2020).

Specifically, holm oak (*Quercus ilex*) is the most dominant tree species in the forest ecosystems of the western half of the Mediterranean Basin. This tree stands out especially for being the basis of the Spanish agrosilvopastoral system called "dehesa", of great local environmental and economic importance (Rey et al., 2019). An example of this is the large sector that exists around pork fed on *Q. ilex* acorns in the "dehesas" (Plieninger et al., 2021). Also, Pyrenean oak (*Quercus pyrenaica*) is a tree species of great ecological and economic importance in its area of maximum extension, the Iberian Peninsula. *Q. pyrenaica* plays an important role in biodiversity conservation, preservation of quality water resources, fire prevention due to its lower combustibility, or its uses in carpentry, charcoal and firewood (Carvalho, 2023). On the other hand, umbrella pine (*Pinus pinea*), mainly present in Spain, is one of the tree species with the greatest expansion in the Mediterranean Basin, due to its use in forest restoration and farmland afforestation. Moreover, due to the high cost in international markets, pine nuts from *P. pinea*

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represent the most important non-timber forest resource that can be obtained from Mediterranean forests (Freire et al., 2019; Jaouadi et al., 2021). Finally, Scot pine (*Pinus sylvestris*) is one of the most widely distributed forest species in the northern hemisphere, occurring in the mountains of the Mediterranean Basin (Pyhäjärvi et al., 2020). *P. sylvestris* plays an important role in the structure and functionality of the forest ecosystems in which it is found, and its wood represents an important resource for woodworking and for obtaining bioactive substances in the chemical industry (Vasilyeva et al., 2021). Although these four forest species can form pure stands, they also form mixed forests in the Mediterranean Basin; for example, the *Q. ilex-P. pinea* or the *P. sylvestris-Q. pyrenaica* mixtures (Zalloni et al., 2019; Pérez-Luque et al., 2020).

Damping-off is a fungal disease that affects the vascular system of the plant causing seed and root rot, being one of the most damaging diseases in nurseries and reforestation throughout the world (Gordon et al., 2015). It is a disease caused by multiple fungi that can act either individually or together, making it difficult to identify and characterize the disease. In the case of conifers, different species inside the genus *Fusarium* have been identified as the causal agents of damping-off, as well as hypocotyl and root rot (Gordon et al., 2015). Specifically, *Fusarium oxysporum* is the most widely distributed species associated with conifer damping-off, although other species, such as *F. verticillioides* (formerly: *F. moniliforme*), cause serious losses in countries such as Brazil or Spain (Machón et al., 2009; Gordon et al., 2015; Maciel et al., 2017). The main strategy to control the disease has been based on soil fumigation with chemical fungicides, such as methyl bromide, chloropicrin, metam-sodium and dazomet. However, these synthetic pesticides have been banned by different legislations worldwide, due to the serious environmental and health problems they cause (Gordon et al., 2015; Świecimska et al., 2020). For example, in the European Union, methyl bromide has been definitively banned since 2010 (Commission Decision 2010/273/EU), chloropicrin since 2022 (Commission Implementation Regulation 2022/751/EU), and metam-sodium and dazomet are currently under evaluation and have already been banned in several European countries. Therefore, it is necessary to search for new control strategies that respect the environment and health, such as biological control agents (BCAs). In this sense, the fungi *Trichoderma harzianum* and non-pathogenic strains of *F. oxysporum*, have been described as effective in the control of the disease (Gordon et al., 2015); and even secondary metabolites obtained from the artificial culture of common wood-destroying fungi (Waszczuk et al., 2022). However, bacteria of the genera *Bacillus* and *Streptomyces* and the fungus *Gliocladium virens* did not provided effective control against the disease (Gordon et al., 2015).

Ectomycorrhizal fungi (EMF) are a group of symbiotic fungi of plant roots characterized by nutrient and water supply through their extraradical mycelium and the formation of a fungus-plant cellular interaction structure between root cortical cells called Hartig net (Kaur and Reddy, 2019). This group of fungi represents a fundamental part of forest soil mycodiversity, forming important symbiotic relationships with trees (Vincent and Declerck, 2021). In addition to through the supply of nutrients (mainly N and P) and water (Liu et al., 2020), EMF benefit their host plant by improving its tolerance under abiotic stresses (e.g., by accumulating heavy metals from the soil) (Policelli et al., 2020) and its resistance against biotic stresses (e.g., by inducing plant defenses) (Dreischhoff et al., 2020). Moreover, EMF are a food of great importance in many regions of the world, such as the Mediterranean, where they are even cultivated in a directed way. This implies a sustainable food production, promoting forest sustainability, biodiversity conservation, economic development or conservation of biocultural heritages, in accordance with the United Nations global sustainable development goals 2030 and the European Green Deal strategies, such as the Farm to Fork and the EU Biodiversity strategies (Pérez-Moreno et al., 2021; Ferreira et al., 2023).

So far, several studies have addressed the use of EMF for the control of damping-off disease. Mainly against the pathogenic fungi *Fusarium solani* (Zhang et al., 2017; Yang and Zhang, 2023), *F. circinatum* (Chartier-FitzGerald et al., 2020), *F. oxysporum* (Machón et al., 2006, 2009; Mateos et al., 2017; Olaizola et al., 2018a, 2018b) and *F. verticillioides* (Machón et al., 2006, 2009; Mateos et al., 2017; Olaizola et al., 2018a, 2018b), and in the forest species *Pinus yunnanensis* (Yang and Zhang, 2023), *P. tabulaeformis* (Zhang et al., 2017), *P. patula* (Chartier-FitzGerald et al., 2020), *P. sylvestris* (Mateos et al., 2017; Olaizola et al., 2018a) and *P. pinea* (Machón et al., 2006, 2009; Mateos et al., 2017; Olaizola et al., 2018a).

In view of the problems raised and the great biotechnological potential of EMF, the present work aims to control damping-off caused by *F. oxysporum* and *F. verticillioides* in different forest species (*Q. ilex*, *Q. pyrenaica*, *P. pinea* and *P. sylvestris*) through the use of several EMF edible species. To achieve this, an *in vitro* confrontation of pathogenic fungi and possible antagonistic fungi was carried out, in addition to different *in planta* trials with seedlings of different ages of the four-forest species.

2. Materials and methods

2.1. Fungal material

Seven different species of mycorrhizal fungi were used and maintained in modified Melin Norkrans (MMN) medium:

- *Leccinum lepidum*: isolated from a fruiting body collected in a *Q. ilex* stand located at Villaviudas de Cerrato (Palencia, Spain).
- *Amanita rubescens*: isolated from a fruiting body collected in a *Q. ilex-Q. faginea* stand located at Perales (Palencia, Spain).
- *Xerocomus ferrugineus*: isolated from a fruiting body collected in a *Q. ilex-Q. faginea* stand located at Perales (Palencia, Spain).
- *Lactarius sanguifluus*: isolated from a fruiting body collected in a *P. pinaster* stand located at Osorno (Palencia, Spain).
- *Tricholoma portentosum*: isolated from a fruiting body collected in a *P. sylvestris* stand located at Celadilla (Palencia, Spain).
- *Suillus luteus*: isolated from a fruiting body collected in a *P. sylvestris* stand located at Celadilla (Palencia, Spain).
- *Agaricus silvicola*: isolated from a fruiting body collected in a *P. pinaster* stand located at Osorno (Palencia, Spain).

These EMF isolates were previously identified and characterized (Martín-Pinto et al., 2006; Olaizola et al., 2018a, 2018b, 2023).

F. oxysporum (Fo4 and Fo5 strains) and *F. verticillioides* (Fv5 and Fv6 strains) species were used as pathogenic fungi, both isolated from diseased seedlings growing in commercial nurseries located in the provinces of León (IMAVE nursery) and Soria (INDESFOR nursery) (both in Spain), respectively. These pathogenic fungi were previously identified and characterized (Martín-Pinto et al., 2006, 2008). The fungi were maintained by growth on Komada (K) medium.

2.2. Plant materials

Seeds from the four forest species of the study were used for the *in planta* assays: *P. pinea*, *P. sylvestris*, *Q. ilex* and *Q. pyrenaica*. *P. pinea* ("Meseta Castellana" provenance, ES.01) and *P. sylvestris* ("Montaña Soriano-Burgalesa" provenance, ES.08) seeds were provided by Fuenteamarga forest nursery (Valladolid, Spain). *Q. ilex* ("Cuenca Central del Duero "provenance, 45/02/P/001) and *Q. pyrenaica* ("Salamanca-Sayago" provenance, 43/07/ZA/004) seeds were supplied by Junta de Castilla y León Forest Nursery (Valladolid, Spain).

2.3. In vitro antagonism study

A direct *in vitro* confrontation of pathogens and EMF was carried out with the following combinations:

- *Le. lepidum* (Ll), *A. rubescens* (Ar) and *X. ferrugineus* (Xf) against *F. oxysporum* (Fo4 and Fo5).
- *La. sanguifluus* (Ls), *T. portentosum* (Tp), *S. luteus* (Sl) and *A. sylvicola* (As) against *F. oxysporum* (Fo4 and Fo5) and *F. verticillioides* (Fv5 and Fv6).

To carry it out, the typical methodology used for the confrontation of filamentous antagonistic fungi in solid medium was followed (Poveda, 2021a). Petri dishes of 9 cm diameter with MMN culture medium were used. First, 5 mm diameter agar discs containing mycelium (from the growing edges) of each of the EMFs were deposited on the plates, 2 cm from the edge of the plate. These plates were incubated at 22°C for 2 weeks. Subsequently, the pathogenic fungus (*F. oxysporum* or *F. verticillioides*) was deposited, using the same methodology, but at the opposite end of the Petri dish. In this way, the distance between the agar discs deposited with both fungi was 5 cm. Again, the Petri dishes were incubated at 22°C and measurements of the radial growth of the pathogenic fungus on the central axis of the dish were taken before the pathogens of the negative controls completely colonized the plate surface (2 weeks after inoculation). As negative controls, Petri dishes were inoculated with an agar disc containing MMN medium without EMF. The assay was performed in duplicate, and each replicate consisted of 6 plates per condition.

The inhibition rate (IR) was calculated according to the following formula (Poveda et al., 2022):

$$IR = (R_C - R_T)/R_C \times 100$$

where R_C means the colony radius of the pathogenic fungus in the control treatment (MMN medium without EMF treatments), and R_T means the colony radius of the pathogenic fungus in EMF treatments. Once both fungi were in contact with each other, mycoparasitism was analyzed by light microscopy.

2.4. Effect of EMF cell-free filtrates on pathogen-conidial germination

To analyze the antibiosis produced by EMF on *Fusarium*, a study of the effect of EMF cell-free filtrates on the germination of pathogen conidia was carried out. To obtain the cell-free filtrates, 50 mL flasks (with 30 mL of liquid MMN) were incubated with 6 plugs of 5 mm mycelium from actively growing cultures of each of the different EMF. Liquid cultures were incubated at 20°C in the dark at 125 rpm for 30 days. The liquid culture was then filtered using 0.2 μm Minisart® syringe filters (Sartorius, Germany). Filtrates absent of EMF for use as negative control were obtained by inoculating the flasks with agar plugs without EMF.

Fusarium conidia were collected, adjusting the concentration in water to 3×10^6 sp/mL, using a hemocytometer. For this purpose, 3 mL of sterile distilled water was poured over a Petri dish completely colonized by *Fusarium*. After scraping the surface of the plate to release conidia, the water was collected and filtered with a Miracloth filter (Calbiochem, Germany), which allows only water and conidia to pass through. The final conidia concentration was obtained by quantification with a hemocytometer and subsequent dilution in sterile distilled water. Subsequently, in 1.5 mL Eppendorf tubes, 100 μL of the *Fusarium* conidia suspension was introduced together with 100 μL of each of the cell-free filtrates. The tubes were incubated at 22°C and in the dark for 24 hours. Subsequently, the percentage of conidial germination was quantified by light microscopic observation of germination tube formation or absence in 200 conidia per Eppendorf tube. Six Eppendorf tubes were used per cell-free filtrate and the assay was performed in duplicate.

2.5. In planta tests of EMF-plant-pathogen interaction

Once the *in vitro* effect of the direct interaction and the production of diffusible metabolites by EMF against *Fusarium* had been studied, *Le. lepidum* was selected to carry out the studies with *Q.ilex* and *Q.pyrenaica* seeds, and *La. sanguifluus* for studies with *P. pinea* and *P. sylvestris* seeds. In the *in planta* studies, the colonized peat methodology was used, since the effect of EMF as BCAs can be a consequence of their presence in the rhizosphere, without having to colonize the host plant.

For each EMF, a 2 L vermiculite:peat (10:1) mixture was homogenized and autoclaved (121°C, for one hour) twice. Subsequently, 1 L of liquid MMN was added to each mixture, homogenizing and leaving to settle for 24 h. The mixture was distributed in 2 L volume glass jars and autoclaved again (121°C, for 20 min). For the colonization of the mixture by the EMF, 20 plugs of mycelium growing in MMN medium, 5 mm in diameter, were introduced into each glass jar. The EMF-inoculated glass jars were incubated at 22°C in the dark for 4 months.

For the sowing of the *Quercus*- and *Pinus*-seeds, alveoli of 170 mL volume were used. To all of them, a base of 100 mL of sterile vermiculite: peat (50:50), 50 mL of the EMF-colonized substrate and 20 mL of the sterile 50:50 substrate covering the EMF-colonized substrate were applied. The treatment without EMF was carried out with the same growth substrate, without fungal and sterile inoculation.

Before sowing, all the seeds were superficially sterilized by two washings in 30 % hydrogen peroxide for 15 minutes and three subsequent washings in sterile distilled water. The sowing of the seeds was carried out on the EMF-colonized substrate. *Fusarium* infections were performed at the time of seed sowing, applying 5 mL of a 1×10^6 sp/mL conidial suspension to the surface of the EMF-colonized substrate (place where the seeds were deposited).

Subsequently, the planted pots were taken to a greenhouse. During the first 3 weeks, the pots were irrigated superficially and daily with sterile distilled water. From the 4th week, the alveoli were irrigated by spray irrigation for 10 min, twice a day. Seedling germination data were taken at 8 weeks (*Pinus*) and 14 weeks (*Quercus*) after sowing. The assay was carried out with 37 seeds per treatment and in duplicate.

2.6. Statistical analysis

Statistical analysis of the data was carried out with STATISTICA 5.5 software. The Student's t test was used for a comparison of means at $P < 0.05$; significant differences are denoted using an asterisk. One-way ANOVA using Tukey's multiple range test at $P < 0.05$ was used for pairwise comparisons; the different letters indicate significant differences.

3. Results

3.1. Effect of EMF on the growth of mycelia and germination of Fusarium conidia

Leccinum lepidium significantly inhibited the growth of *F. oxysporum* (both Fo4 and Fo5, IR 63 % and 58 %, respectively) on the Petri dishes, compared to the inhibition reported for the other EMFs. *Amanita rubescens* also inhibited *F. oxysporum* growth, but significantly less than *Le. lepidium* (IR 32 % on Fo4 and IR 27 % on Fo5). However, the effect of *X. ferrugineus* was contradictory: it was able to inhibit Fo4 growth similarly to *A. rubescens* (IR 32 %), but promoted Fo5 growth significantly (IR −15 %) (Fig. 1a and S1).

Regarding *Pinus*-EMF, only *S. luteus* inhibited the growth of *F. oxysporum* and *F. verticillioides* above 25 % (IRs 28–30 % in Fo4, Fo5, Fv5 and Fv6), being significantly higher than the inhibitions of the rest of the EMF. The next EMF that most inhibited *Fusarium* growth was *T. portentosum* (IR 4–6 % in Fo4 and Fo5; IR 9–11 % in Fv5 and Fv6). *Lactarius sanguifluus* did not differ significantly from *T. portentosum* in inhibiting the growth of Fo5 (IR 8 %) and Fv6 (IR 6 %), while it inhibited significantly less the growth of Fv5 (IR 5 %). However, it promoted the growth of Fo4 (IR −3 %). Finally, *A. sylvicola* inhibited the growth of *F. verticillioides* similarly to *La. sanguifluus* (IR 7 % in Fv5) and *T. portentosum* (IR 13 % in Fv6). In contrast, it promoted the growth of

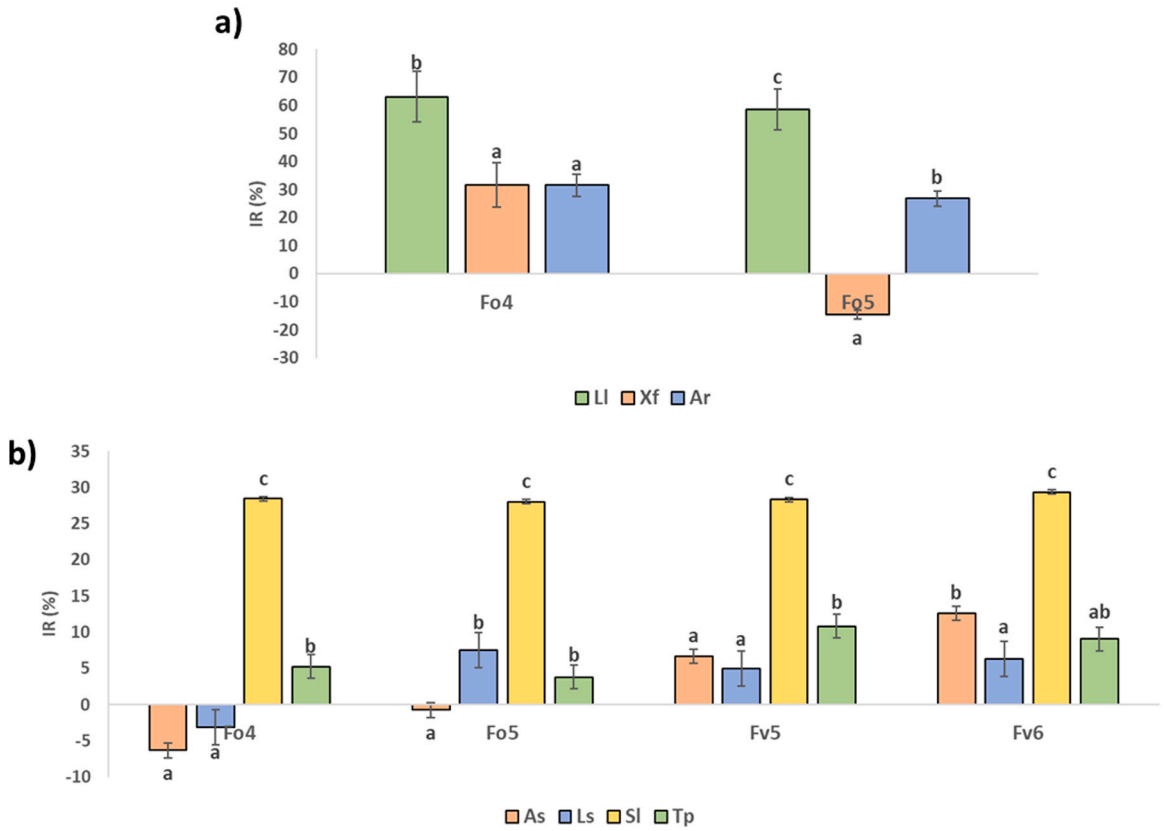

Fig. 1. : Inhibition rate (IR, %) in *Quercus*-EMF (a) (Ll: *Le. lepidum*; Xf: *X. ferrugineus*; Ar: *A. rubescens*) and *Pinus*-EMF (b) (As: *A. sylvicola*; Sl: *S. luteus*; Ls: *La. sanguifluus*; Tp: *T. portentosum*) in their antagonistic confrontation *in vitro* against *F. oxysporum* (Fo4 and Fo5) and *F. verticillioides* (Fv5 and Fv6). Data are the mean of two biological replicates for each condition with six plates in each one. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$).

F. oxysporum (IR −6 % in Fo4 and −1 % in Fo5) (Fig. 1b and S1).

With regard to the observation of the physical interaction between EMF and *Fusarium*, no mycoparasitism by any of the fungi used was observed. In addition, an antibiosis study was carried out with cell-free filtrates from EMF in the *Fusarium* conidia germination. Within the *Quercus*-AMF, only *Le. lepidum* produced cell-free filtrates capable of significantly inhibiting the *F. oxysporum* conidia germination (Fo4 and Fo5, 68 % and 87 %, respectively) compared to the control without EMF-filtrates (76 % and 97 %, respectively) (Fig. 2a).

Regarding the cell-free filtrates obtained from *Pinus*-EMF, only those from *La. sanguifluus* against Fo4 (55 %) and Fv5 (41 %), from *A. sylvicola* against Fo5 (59 %), and from *T. portentosum* against Fv5 (76 %) significantly inhibited the pathogens conidia. On the contrary, there were cell-free filtrates that promoted the *Fusarium*-conidia germination, such as those from *T. portentosum* against Fo4 (84 %), and *S. luteus* and *T. portentosum* against Fv6 (89 % and 90 %, respectively) (Fig. 2b).

3.2. Damping-off in planta control by EMF

After analyzing the BCA capacity of the different EMF, *Le. lepidum* species were chosen for the *in planta* study with *Q. ilex* and *Q. pyrenaica* seeds and the pathogen *F. oxysporum*; and *La. sanguifluus* for studies with *P. pinea* and *P. sylvestris* seeds and the pathogens *F. oxysporum* and *F. verticillioides*. In *Q. ilex* seeds, both strains of *F. oxysporum* (Fo4 and Fo5) significantly reduced germination (14 % and 17 %, respectively), compared to uninfected seeds (37 %). The presence of *Le. lepidum* in the substrate did not significantly increase germination of Fo-infected seeds (25 % and 21 %), also compared to uninfected seeds (37 %). In addition, the presence of EMF significantly reduced the germination of *Q. ilex* seeds in the absence of *Fusarium* (18 %), compared to uninfected seeds (37 %) (Fig. 3a).

In *Q. pyrenaica*, *F. oxysporum* did not significantly reduce seed germination (37 % and 40 %), compared to non-infected seeds (33 %). With respect to the presence of *Le. lepidum* in the substrate, no significant differences were reported in non-infected seeds (24 %), nor in those infected by Fo5 (40 %), compared to non-infected seeds (33 %). However, the presence of *Le. lepidum* in the substrate also infected with Fo4 significantly reduced the germination of *Q. pyrenaica* (17 %), compared to non-infected seeds (33 %) (Fig. 3b).

On the other hand, *F. oxysporum* significantly reduced *P. pinea* seeds germination (Fo4 strain only, 23 %), compared to seeds without pathogen-infection (61 %). Also, in *P. sylvestris*, the Fo4 strain significantly inhibited seed germination (4 %), followed by Fo5 (12 %), compared to seeds without pathogen-infection (40 %). The presence of *La. sanguifluus* in the substrate did not report significant differences in the germination of *P. pinea* seeds with (20 % and 54 %) and without pathogen (55 %), nor in *P. sylvestris* seeds infected with Fo5 (10 %) and uninfected (26 %), both compared to non-EMF inoculated substrates. However, *La. sanguifluus* was able to significantly increase germination of *P. sylvestris* seeds under Fo4 infection (9 %), compared to substrates without EMF application (4 %) (Fig. 4a).

Regarding the pathogen *F. verticillioides*, only strain Fv6 in *P. pinea* significantly reduced seeds germination (26 %), compared to seeds without pathogen (61 %). While in *P. sylvestris* seeds it was strain Fv5 that significantly reduced germination (21 %), compared to uninfected seeds (62 %). The presence of *La. sanguifluus* in the substrate did not significantly increase germination of *P. pinea* and *P. sylvestris* seeds, both in the presence and absence of *F. verticillioides*, both compared to non-EMF inoculated substrates. Furthermore, the presence of *La. sanguifluus* significantly reduced germination of Fv6-infected *P. sylvestris* seeds

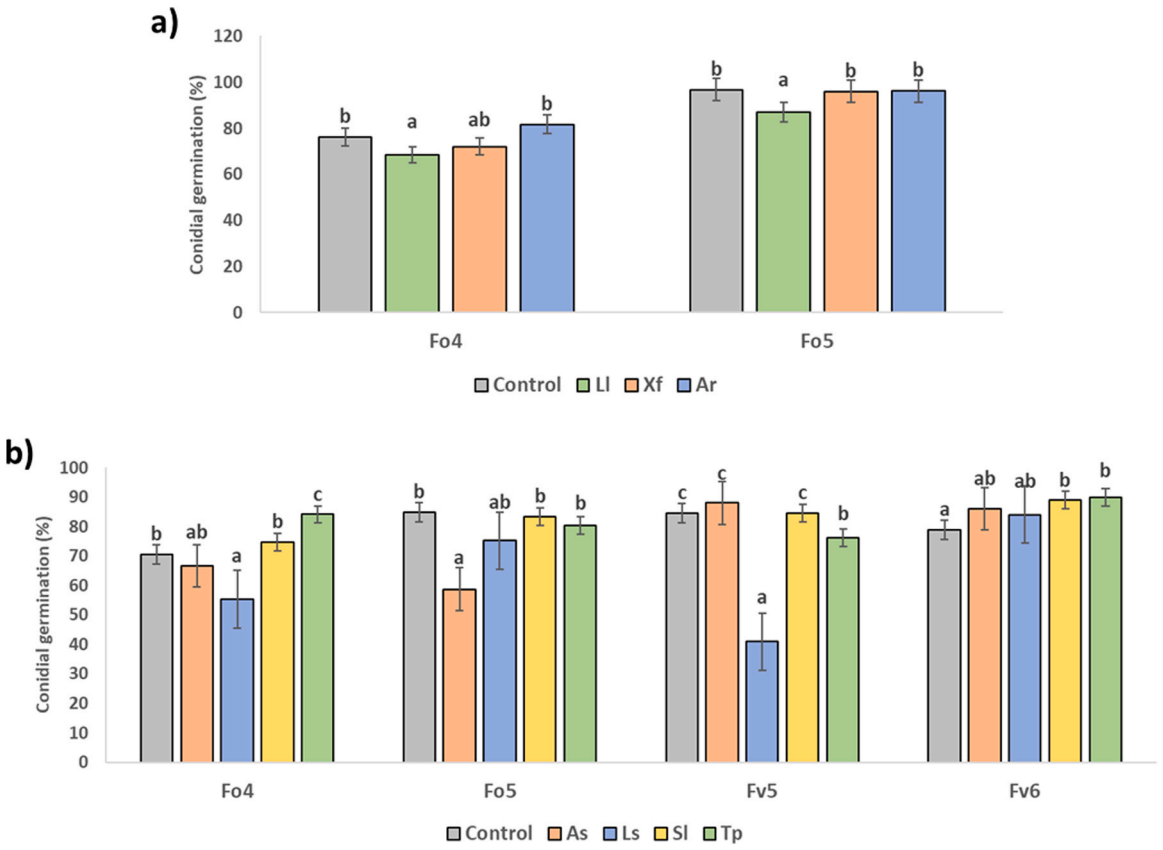

Fig. 2. : Germination percentage of *F. oxysporum* (Fo4 and Fo5) and *F. verticillioides* (Fv5 and Fv6) conidia in interaction with cell-free filtrates from *Quercus*-EMF (a) (Ll: *Le. lepidum*; Xf: *X. ferrugineus*; Ar: *A. rubescens*) and *Pinus*-EMF (b) (As: *A. sylvicola*; Sl: *S. luteus*; Ls: *La. sanguifluus*; Tp: *T. portentosum*). Data are the mean of two biological replicates for each condition with six 200 conidia in each one. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$).

(22 %), compared to the same seeds without EMF (39 %) (Fig. 4b).

4. Discussion

There is a need to search for new control strategies for forest pathogens and EMFs may prove to be an effective strategy as BCAs. In this sense, in our work we have obtained different capacities for each of the EMF species used.

Xerocomus ferrugineus is a fungus within the Boletaceae family (boletes) and within the *X. subtomentosus* complex (Šutara, 2008), a common conifer ectomycorrhizal (Karadelev et al., 2007) that in northern Europe has always been referred to as *Boletus subtomentosus* (Taylor et al., 2006). Although *X. ferrugineus* has not been previously described as BCAs against pathogenic fungi, in our study an *in vitro* reduction of the growth of the pathogen *F. oxysporum* (strain Fo4) was seen, possibly due to competition for space and nutrients or to the production of volatile compounds, since no mycoparasitism structures or antifungal effect of the diffusible compounds were observed. However, *X. ferrugineus* (referred to as *B. subtomentosus*) has recently been described as producing antiviral metabolites, such as inonotusin A against *Herpes Simplex Virus* type 2 and *Coxsackie Virus B* type 3 (Boudagga et al., 2022). In contrast, in our study *X. ferrugineus* is also able to promote the growth of strain Fo5. Possibly, the observed result is a consequence of the production of volatile metabolites, since the diffusible metabolites did not promote conidial germination of the pathogen, being a possibility reported in other BCAs (Poveda, 2021b). In this sense, we can think that there is an antagonistic pathogen-strain-specific capacity.

Amanita rubescens (European Blusher) belongs to the Amanitaceae family and is widely collected as edible mushroom with great sensory properties (Štefániková et al., 2021). In addition, it is a mushroom of great interest for soil bioremediation due to its great capacity to absorb and accumulate heavy metals (such as mercury, lead and cadmium), which makes it necessary to take precautions for its consumption (Sarı and Tuzen, 2009; Drewnowska et al., 2012). As a possible BCA, in our work we have described its ability to inhibit *in vitro* the growth of *F. oxysporum*, possibly due to competition for space and/or nutrients and/or the production of antifungal volatile metabolites, because no mycoparasitism structures or antifungal activity of the metabolites diffusible by the medium was observed. However, the antifungal ability of these metabolites to inhibit germination of *F. verticillioides* has been described for *A. rubescens* (Olaizola et al., 2018b). In this sense, the biocidal capacity of metabolites produced by *A. rubescens* has been identified, mainly against mammals, such as rubescenslysin in mice (Seeger et al., 1981) or amatoxin in humans (Vargas et al., 2011).

Agaricus silvicola is an edible fungus belonging to the family Agaricaceae (Razaq and Shahzad, 2007), of which very few studies have been carried out so far and it has never been previously described as BCA. In our work, its possible use as a BCA was also unclear, since it did not greatly inhibit the growth of *F. verticillioides* (less than 10 %) *in vitro*, and even promoted the growth of *F. oxysporum*. However, diffusible metabolites from *A. silvicola* inhibited the germination of *F. oxysporum* conidia (Fo5, not Fo4), the production of antifungal metabolites by this fungus being unknown so far.

Suillus luteus is an edible fungus of the family Suillaceae (Jaworska et al., 2014) that forms widespread ectomycorrhizal relationships with many pine species (Mateos et al., 2017). In its symbiosis with pines, *S. luteus* promotes plant growth (Bending et al., 2002), e.g., by enhancing P and K absorption (Chen et al., 2022), in addition to the accumulation in its tissues of heavy metals (such as cadmium, copper or mercury) (Adriaensen et al., 2005; Krznaric et al., 2009; Chudzyński

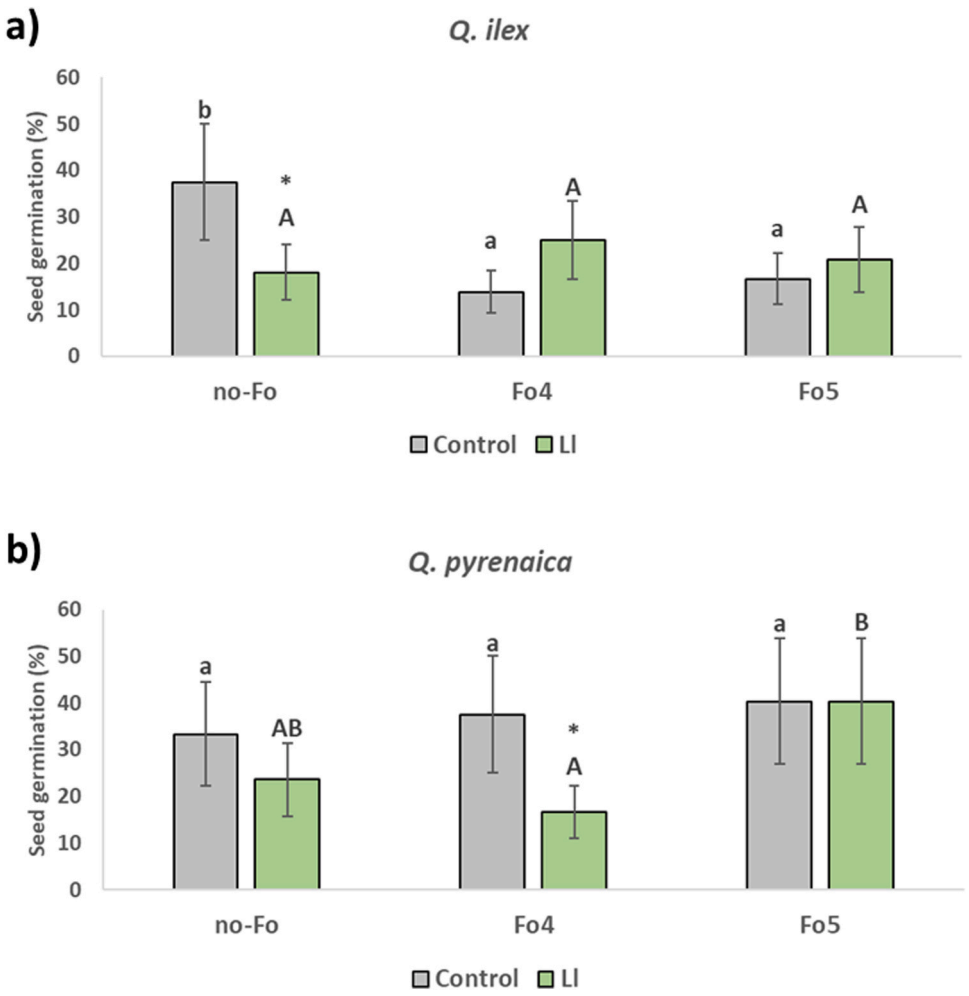

Fig. 3. : Germination percentage of *Q. ilex* (a) and *Q pyrenaica* (b) seeds infected with *F. oxysporum* (Fo4 and Fo5) or non-infected (no-Fo), and growing in a substrate EMF-colonized by *Le. lepidum* (Ll) or non-colonized (Control). Data are the mean of two biological replicates for each condition with 37 seeds in each one. One-way analysis of variance (ANOVA) was performed to compared between seeds *Fusarium*-infected and non-infected, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$), in the substrate EMF-colonized by *Le. lepidum* (uppercase letters) or non-colonized (lowercase letters). Moreover, Student's t test was performed to compared between EMF-colonized and non-colonized substrates. Asterisks denote significant differences ($P < 0.05$).

et al., 2011). As a BCA, *S. luteus* has been described as an effective antagonist of plant pathogenic fungi through the production of chitinase enzymes (Sillo et al., 2015), a possible mechanism involved in the *in vitro* antagonistic behavior also reported in our work, against *F. oxysporum* and *F. verticillioides*. In addition, *S. luteus* is able to produce several cytotoxic metabolites (Leon et al., 2008; dos Santos et al., 2013), however, these metabolites had no antifungal effect against *Fusarium* conidia, and even promoted the germination of *F. verticilloides*. Despite the absence of antifungal metabolites, direct interaction with the pathogenic fungus could be sufficient for effective disease control, as has been reported in pine seedlings against *F. oxysporum* and *F. verticillioides* (Mateos et al., 2017).

Tricholoma portentosum is an edible fungus of the family Tricholomataceae, widely distributed throughout northwestern Spain (Dıez and Alvarez., 2001), with the ability to produce antibacterial, but not antifungal metabolites (Barros et al., 2007). In our work, no antifungal activity of the metabolites produced by *T. portentosum* against *Fusarium* was described either, even these promoted the germination of *F. verticillioides* conidia. In contrast, *T. portentosum* has been described as an efficient BCA *in vitro* against *F. oxysporum* and *F. verticillioides*, by direct confrontation and production of diffusible metabolites (Olaizola et al., 2018b)

In contrast to the previous EMF, two species had a clear effect as BCAs *in vitro* against *Fusarium*, so they were also used *in planta*. In this sense, the fungus *Le. lepidum*, belonging to the Boletaceae family, has been found associated with *Quercus*-species roots as EMF (Montecchio et al., 2006). In our *in vitro* study, we describe how *Le. lepidum* inhibits the growth of *F. oxysporum*, both by direct confrontation and by inhibiting the conidial germination of the pathogen by its metabolites. Therefore, the most likely mechanism of action used by *Le. lepidum* against *F. oxysporum* would be through the release of diffusible metabolites into the medium. However, this mechanism was not identified in previous work against the same species (Olaizola et al., 2018b). *In planta*, the results obtained were not encouraging in the use of *Le. lepidum* as a BCA against *F. oxysporum*, since in no case did it increase the germination of seeds infected with the pathogen. Moreover, *Le. lepidum* inhibited the germination of *Q. ilex* seeds in the absence of the pathogen and *Q. pyrenaica* seeds in the presence of *F. oxysporum* (strain Fo4). In this regard, it is known that EMF can act negatively on the growth of their host plant, as they can act as nutrient sinks, such as N, to the detriment of the plant (Alberton et al., 2007). Furthermore, *Le. lepidum* has sometimes been described in association with decaying *Quercus* tisues (Montecchio et al., 2006).

Lactarius sanguifluus is a fungus of the Russulaceae family associated as EMF to *Pinus* species (Mattock and Kibby, 2013). In our work we describe *La. sanguifluus* as an efficient BCA against *F. oxysporum* and *F. verticillioides* both by direct confrontation and by the production of diffusible metabolites. The latter being the mechanism of action by

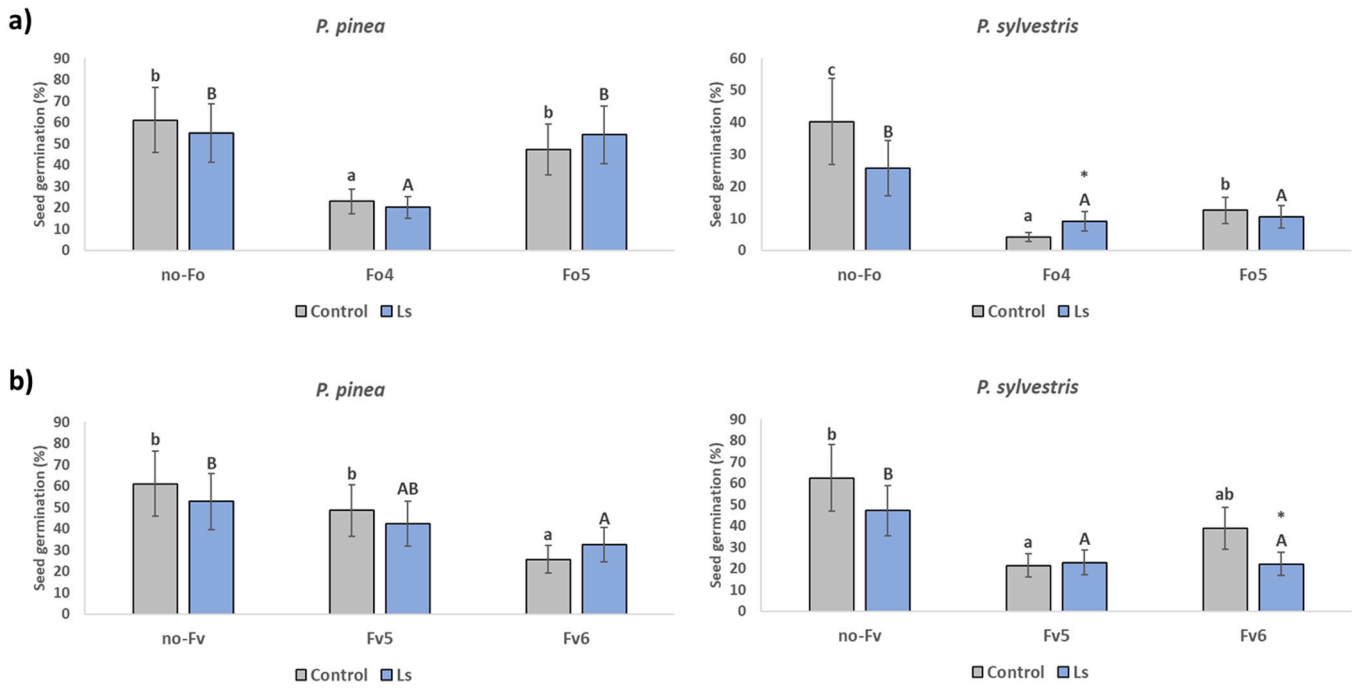

Fig. 4. : Germination percentage of *P.pinea* and *P. Pyrenaica* seeds infected with *F. oxysporum* (Fo4 and Fo5) (a) and *F. verticillioides* (Fv5 and Fv6) (b) or non-infected (no-Fo), and growing in a substrate EMF-colonized by *La. sanguifluus* (Ls) or non-colonized (Control). Data are the mean of two biological replicates for each condition with 37 seeds in each one. One-way analysis of variance (ANOVA) was performed to compared between seeds *Fusarium*-infected and non-infected, followed by the Tukey's test. Different letters represent significant differences ($P < 0.05$), in the substrate EMF-colonized by *La. sanguifluus* (uppercase letters) or non-colonized (lowercase letters). Moreover, Student's t test was performed to compared between EMF-colonized and non-colonized substrates. Asterisks denote significant differences ($P < 0.05$).

which it possibly acts, since it has been described as an efficient producer of antifungal compounds against species, such as *Candida albicans*, *Aspergillus fumigatus*, *Trichophyton rubrum*, *Epidermophyton foccosum* and *Microsporum canis* (Thakur and Sayeed, 2014; Erbiai et al., 2021). In *in planta* assays, although the presence of *La. sanguifluus* had no effect on *P. pinea* seeds, with or without pathogen, it promoted germination of *P. sylvestris* seeds infected with *F. oxysporum* (strain Fo4). Therefore, *La. sanguifluus* could be used as an efficient BCA against *F. oxysporum*, at least, in *P. sylvestris*. The disease biocontrol results reported by *La. sanguifluus* in our work were similar to those previously observed in Douglas-fir seedlings by *T. harzianum* and non-pathogenic strains of *F. oxysporum* against post-emergence damping-off caused by *F. oxysporum* and *F. commune* (Mousseaux et al., 1998; Dumroese et al., 2012).

Despite the *in vitro* antagonistic and *in planta* biocontrol results reported in our work against *Fusarium*-damping-off by different EMF, there are important drawbacks/limiters of this group of fungi for actual mass use that should be considered. The first limitation is their large-scale production, which allows their commercialization. Although bioreactors for EMF-inoculum production have been developed, both in solid and liquid form, their industrial development has not advanced sufficiently, due to EMF poor growth and easy contamination of culture media (e.g., by EMF-symbiont bacteria) (Chot and Reddy, 2023). Another drawback lies in the field application of these EMF as exogenous species, which can significantly modify the microbiota of forest soils and favor the establishment of invasive plant species (Chot and Reddy, 2023).

In conclusion, the EMF used have different *in vitro* antagonistic capacities against *F. oxysporum* and *F. verticillioides*, with *Le. lepidum* and *La. sanguifluus* being the most effective. None of the EMF species used mycoparasitism as a mechanism of action against the pathogen, being the production of diffusible metabolites the most effective possible strategy. Only *La. sanguifluus* was effective in controlling damping-off on *P. sylvestris*, while *Le. lepidum* was even detrimental to *Quercus*-species. Therefore, further research is required in the search for effective EMF species against the disease and in their actual form of application in nurseries and reforestation.

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CRediT authorship contribution statement

Julio J. Díez: Supervision, Funding acquisition, Conceptualization. **Mónica Pastor:** Methodology, Investigation. **Paula Zamora-Brauweiler:** Methodology, Investigation. **Jorge Martín-García:** Methodology, Investigation, Formal analysis, Data curation. **Jorge Poveda:** Writing – review & editing, Writing – original draft, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the

online version at doi:10.1016/j.pedobi.2024.150973.

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