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Influence of *Suillus luteus* on *Fusarium* damping-off in pine seedlings

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The role of the ectomycorrhizal (Myc) fungus *Suillus luteus* as a biological control agent against damping off caused by *Fusarium verticillioides* (Fo) and *Fusarium oxysporum* (Fm) on Scots (*Pinus sylvestris* L.) and Stone pine (*Pinus pinea* L.) was studied in a greenhouse experiment. The vegetative mycelium of *S. luteus* in a vermiculite/peat carrier was added to potting substrate before inoculation with Fo or Fm spores (macro and microconidia). Also, seedlings were inoculated only with the Myc, Fo, Fm or water for treatment comparisons. The seedling disease index (SDI) of seedlings varied significantly among pine, *Fusarium* and Myc treatments. Scots pine seedlings inoculated with *F. verticillioides* and *F. oxysporum* had a reduced SDI when co-cultured with *S. luteus*. Damage in Stone pine seedlings inoculated with *F. oxysporum* was significantly reduced in the presence of Myc fungus, but no reduction of disease symptoms was observed when inoculated with *F. verticillioides*. Mycorrhizal formation in co-cultures with *F. verticillioides* was low and absent in co-cultures with *F. oxysporum*, although *S. luteus* inoculation resulted in a greater antagonism against this latter pathogen. The protective effect of *S. luteus* against damping-off by *Fusarium* species was not related to the percent of mycorrhizal apexes in the roots of Stone and Scots pine seedlings.

Key words: Forest nurseries, ectomycorrhiza, *Suillus luteus,* damping off, *Fusarium oxysporum, Fusarium verticillioides,* biological control, *Pinus* species.

INTRODUCTION

Fusarium oxysporum Schlecht. Emend. Snyd. & Hans. and *Fusarium verticillioides* (Sacc) Niremberg (*F. moniliforme* Sheldon) are important soil borne pathogens with worldwide distribution and large host range. In forest nurseries, both species are involved in damping off disease, occurring in germinating seeds and first-year seedlings of most conifers and broadleaves around the world (Dick and Dobbie, 2002). In Spain, *F. oxysporum* and *F. verticillioides* are the main causal agents of damping off in forest nurseries (Martín-Pinto et al., 2006a,b), responsible for considerable losses, particularly in conifer species.

Several fungicides are used to control this disease, though many of them are not effective and do not protect

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the seedlings in the nurseries (Machón et al*.,* 2006). Furthermore, reducing residual toxicity from chemicals in the soil is also demanded for an environmentally acceptable nursery management (Hwang et al*.,* 1995) and therefore biological approaches to disease control are sought.

Ectomycorrhizal fungi (Myc) have a beneficial relationship with plants by improving nutrient uptake and plant growth (Smith and Read, 1997), thus enhancing the establishment of forest seedlings (Guerin-Laguette et al., 2004). Several studies have documented the protective role of ectomycorrhizae against fungal pathogens (Aleksandrowicz-Trzcinska, 2008; Manka, 2010; Zhang et al., 2011; Diez and Alves-Santos, 2012), nematodes (Diedhiou et al*.,* 2003), and insects (Halldorsson et al*.,* 2000).

Suillus luteus (L. Ex Fr.) Gray is a common agariocoid fungus in Europe (Lamaison and Polese, 2004) where it regularly forms ectomycorrhizal relationships with many pine species. In Spain, it is frequently associated with Scots pine (*Pinus sylvestris* L.) and Stone pine (*Pinus pinea* L.), two widespread tree species with quite different habitats.

Thus, the goal of this work was to study the protective effect of *S. luteus* against damping-off damage caused by *F. oxysporum* and *F. verticillioides* to Scots and Stone pine seedlings under greenhouse conditions.

MATERIALS AND METHODS

Organisms

The plant material used in the assays consisted of Scots pine ("Montaña Soriano-Burgalesa" provenance, ES.08) and Stone pine ("Meseta Castellana" provenance, ES.01) seeds provided by Fuenteamarga forest nursery (Valladolid, Spain). The damping off pathogens *F. verticillioides* (Fm-6P) and *F. oxysporum* (Fo-4P) were freshly isolated from diseased seedlings growing in commercial nurseries located at the Provinces of León (Imave nursery) and Soria (Indesfor nursery), respectively. The Myc fungi *S. luteus* (Sl-1 strain) was isolated from a fruiting body collected in a *P. sylvestris* stand located at Palencia Province. The cultures of *Fusarium* species and *S. luteus* were maintained during two months on Komada (K) medium (Komada, 1975) and modified Melin Norkrans (MMN) medium (Marx, 1969), respectively.

Greenhouse experiments

Inoculum of *Fusarium* spp. was produced by culturing the fungus in liquid Potato Dextrose Agar (PDA) medium for 7 days in the dark. Spores (macro- and microconidia) were obtained by filtration and re-suspension at a concentration of 10^6 spores/ml. Inoculum of *S. luteus* was prepared by culturing the fungus in a mixture of 1000 ml vermiculite and 100 ml peat, twice sterilised at 120°C for 60 min, and moistened with 500 ml of MMN liquid medium (pH adjusted to 5.0) in 2 L flasks. Once the culture medium was added, the flasks were autoclaved for 20 min at 121°C, and after cooling, the flasks were inoculated with the ectomycorrhizal fungus by adding 20 agar plugs (5 mm in diameter) from solid cultures on MMN medium. All the flasks were maintained at 25°C in the dark for two months. Non-inoculated flasks were also prepared for control treatments.

Twelve treatments were compared, 6 treatments for each pine species: (1) control (non-inoculated), (2) *S. luteus* (Myc), (3) *F. verticillioides* (Fm), (4) *F. oxysporum* (Fo), (5) Fm+Myc, and (6) Fo+Myc. In early February 2004, pine seeds were surface sterilized by dipping in 30% H_2O_2 for 30 min, then washed 10 times with sterile distilled water to eliminate disinfectant before sowing in multipot trays (250 cm^3 per pot) containing a mixture of 215 cm^3 of peat most and vermiculite (1:1), previously sterilized twice at 121°C for 90 min. In the treatments with Myc, 50 ml of *S. luteus* inoculum was transferred to the pots prior to seedling and laid on the surface of the sterilized peat moss (Pindstrup Mosebrug S.A.E., Burgos) mixture (165 cm^3). Immediately after sowing, a 5-ml spore suspension (10^6 spores ml^{-1}) of *F. verticillioides* or *F. oxysporum* was pipetted to each pot on the Fm and Fm+Myc, or on the Fo and Fo+Myc treatments, respectively. Control seedlings were inoculated with 5-ml of distilled water. Finally, the seeds of all the treatments were covered with 15 cm^3 of sterile peat most and irrigated daily with 20 ml of sterile distilled water during two weeks. After that, watering and other procedures were routine greenhouse practice, except that no fertilization or fungicides were applied.

Every treatment consisted of 3 replicates (trays) of 48 seeds each (one seed per pot) resulting in a total of 864 seeds (=6 treatments × 3 replicates × 48 seed per replicate) assayed for each pine species. The trays were randomly arranged on the bench and maintained in a greenhouse environment without light or temperature regulation until early July (18 weeks). Temperature during the experiment was: February (Average minimum temperature, Tmin = 1.1°C; Average maximum temperature, Tmax = 10.7°C; Average temperature, T = 5.4°C); March (Tmin = 2.5°C; Tmax = 16.1°C; T = 9.8°C); April (Tmin = 5.3°C; Tmax = 17.7°C; T = 11.5°C); May (Tmin = 8.3°C; Tmax = 22.7°C; T = 15.5°C); June (Tmin = 12.2°C; Tmax = 27.7°C; T = 20.5°C), and July (Tmin = 14.1°C; Tmax = 31.7°C; T = 23.4°C). Care was taken to minimize contamination between trays during watering and tray maintenance on the greenhouse bench.

Damping off was analysed by recording the damage to the seedlings at the end of the experiment. Three damage classes were established: (1) none to light damage (less than 10% shoot affected); (2) moderate to severe damage (over 10% of shoot affected); (3) dead seedling. Within each replication (tray), seedling damage was obtained as the mean value of the 48 seedlings and a seedling disease index (SDI) was calculated for each treatment as the mean value of seedling damage in its three replications.

Fifteen randomly selected seedlings for each treatment (dead seedlings were not included) were then taken to the laboratory and shoot height, shoot dry weight, root collar diameter, root length, and root dry weight were measured. Excised, soil-washed roots were examined for ectomycorrhizal short roots and placed in vials containing a FAA preserving solution (5 ml of formaldehyde, 5 ml of acetic acid and 90 ml of ethyl alcohol). Intensity of root colonization was expressed as the percentage of mycorrhized apexes within 250 observed plant apexes.

Statistical analysis

Untransformed data were subjected to analysis of variance (ANOVA) procedures ($p<0.05$) using Statistica 7.0 (StatSoft Inc., 1984-2005, Tulsa, Ok) software. Least significant difference (LSD) Fisher test ($p<0.05$) was applied to compare mean values when significant differences were found.

RESULTS AND DISCUSSION

The SDI of seedlings varied significantly among pine,

Table 1. ANOVA table for pine seedling disease index (SDI).

Source	d.f.	MS	F-Value	p-value
Pine	1	3.48	163.48	0.000*
Myc	1	0.34	16.20	0.000*
Fusarium	2	1.30	61.02	0.000*
Pine × Myc	1	0.08	3.93	0.058
Pine × *Fusarium*	2	0.60	28.39	0.000*
Myc × *Fusarium*	2	0.06	3.14	0.061
Pine × Myc × *Fusarium*	2	0.02	0.98	0.389

d.f.: Degrees of freedom; MS: means squares; Myc: ectomycorrhizal fungus *Suillus luteus*.

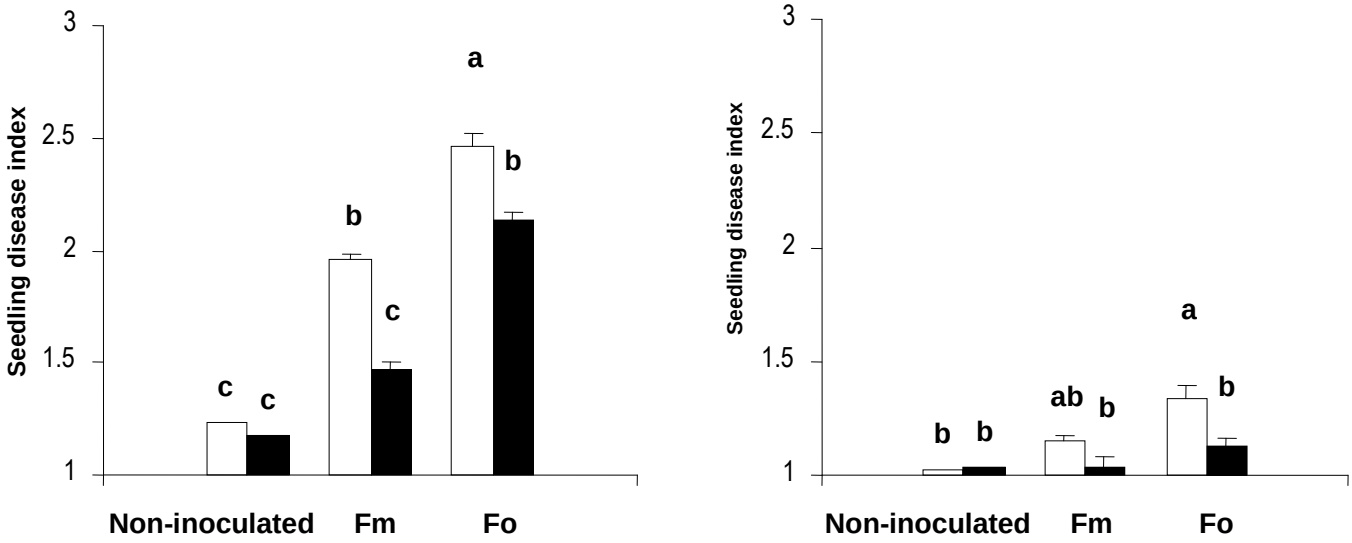

Figure 1. Effect of *Suillus luteus* inoculation (dark bars), on seedling damage Index (SDI) of Scots (left) and Stone (right) pine seedlings inoculated with *Fusarium oxysporum* (Fo) and/or *F. verticillioides* (Fm) (white bars). Vertical bars followed by different letters are significantly different (LSD Fisher test, P=0.05).

Fusarium and Myc treatments (Table 1). Moreover, SDI differences between pine species were related to the *Fusarium* isolates (Pine × *Fusarium* interaction: p<0.01). The LSD Fisher test showed *F. oxysporum* to produce a significantly higher SDI than *F. verticillioides* (Figure 1). Stone pine seedlings were less affected by *Fusarium* than those of Scots pine.

Inoculation of pine seedlings with the ectomycorrhizal fungus *S. luteus* reduced damage by *Fusarium* damping off (Figure 1). Thus, Scots pine seedlings inoculated with *F. verticillioides* and *F. oxysporum* had a reduced SDI when co-cultured with *S. luteus*. Damage in Stone pine seedlings inoculated with *F. oxysporum* was significantly reduced in the presence of Myc fungus, but no reduction of disease symptoms was observed on those inoculated with *F. verticillioides*. A similar protective effect was also obtained by other Myc fungi on Douglas-fir, Jack pine (Chakravarty and Hwang, 1991; Hwang et al., 1995), and Scots pine (Machón et al., 2006, 2009). This is the first time that *Fusarium* antagonism has been demonstrated with *S. luteus.* However, not all the Myc tested against damping-off pathogens showed a protective effect. Thus, Hwang et al. (1995) working with a close species, *Suillus tomentosus*, failed to demonstrate a positive outcome (clearly showed with the other Myc tested, *Paxillus involutus*) on Jack pine (*Pinus banksiana* Lamb.), despite the significant level of mycorrhization obtained in the plants treated with *F. verticillioides* (10%). It seems that the damping off antagonisms by Myc may be related to the mycorrhizal species selected.

Root colonization was 3.68% in the Myc treated Stone pine plants (Table 2). Mycorrhizal formation was significantly reduced when seedlings were co-inoculated with *F. verticillioides* (1.25%, p=0.049), and no mycorrhizal apexes at all were found in co-inoculations with *F. oxysporum*. Root colonization was absent in the Stone pine treatments without the Myc inoculum. Colonization in Scots pine roots was 9.34% in the Myc

Table 2. Number of mycorrhizal short roots (%) on Stone pine and Scots pine seedlings.

Treatment	Scots pine	Stone pine
Not inoculated	16.24[a]	0[b]
Fm	0[b]	0[b]
Fo	0[b]	0[b]
Myc	9.34[a,b]	3.68[a]
Fm+Myc	15.24[a]	1.25[b]
Fo+Myc	0[b]	0[b]

Fm: *Fusarium verticillioides;* Fo: *F. oxysporum;* Myc: ectomycorrhizal fungus *Suillus luteus*. Means followed by different letters within each column are significantly different. LSD Fisher test, P=0.05, n=15.

treatment, and it was apparently increased (p=0.324) when the pots were co-inoculated with *F. verticillioides* (15.24%), but no mycorrhizal apexes were found in the seedlings co-inoculated with *F. oxysporum*. As expected, no mycorrhizal formations were found in seedlings from the Fm and Fo treatments. Nevertheless, Scots pine non-inoculated treatment showed a significant number of mycorrhizal short roots, similar to those of the Myc treated seedlings (16.24%, p=0.258), despite the precaution measures used to avoid contamination. This might be one of the reasons why there were no differences between non-inoculated and Myc treated seedlings neither in their SDI nor in the plant growth variables. However, in Stone pine, where some mycorrhization was achieved, there were no differences compared to controls either.

The interaction of Myc fungi with root pathogens of pines is still not well understood. Duchesne (2000) hypothesized that root protection by the Myc may be the result of three effects: a protective barrier caused by the presence of a fungal mantle around the roots, the production of antimicrobial substances either by the mycosymbiont or by the host plant, and the competition for nutrients in the rhizosphere. Morin et al*.* (1999) found a negative correlation between the percentage of infected plants by *Cylindrocladium floridanum* and the intensity of mycorrhizae formation in the roots of black spruce seedlings. In our study, the antagonism caused by *S. luteus* was not related to the percentage of mycorrhizal apexes formed in the host roots. Thus, in Stone pine seedlings this value was only 1.25% in the Fm+Myc treatment and no mycorrhizal apexes were found in the Fo+Myc treatment, although *S. luteus* was only effective against *F. oxysporum* (Figure 1). In Scots pine seedlings, mycorrhization appeared only in the Fm+Myc (15.24%), but not in the Fo+Myc treatment, however, *S. luteus* antagonism was significant for both pathogens. It seems then that the protection exerted by *S. luteus* against Scots and Stone pine damping-off was not associated with a protective barrier by the Myc fungal mantle around the roots, as hypothesized by Duchesne (2000). Similar results were obtained by Diedhiou et al. (2003) on *Meloidogyne incognita*, where a clear relationship between mycorrhyzation and nematode control could not be established.

Several authors have reported disease suppression by Myc fungi associated to fungal-produced antimicrobial substances (Chakravarty and Hwang, 1991; Zhang et al., 2011; Diez and Alves-Santos, 2012). Toxic effects of mycorrhizal fungi have been described not only against plant pathogens, but also against insects (Halldorsson et al., 2000) and nematodes (Diedhiou et al., 2003). Antagonism of *Boletus edulis*, *Rhizopogon roseolus*, *Laccaria laccata* and *Lactarius deliciosus* against spore germination of *F. oxysporum* and *F. verticillioides* by toxic-like compounds released to the culture media has been previously confirmed in our lab (Martín-Pinto et al*.,* 2006a), besides *S. luteus* (Olaizola et al*.,* 2003). Therefore, suppression of *Fusarium* spp. damping off by *S. luteus* could be due in part to the production and release to the peat moss of antifungal compounds by this Myc.

One mycorrhizal morphotype was identified on the roots of Scots and Stone pine seedlings showing dichotomously branched mycorrhizae. This was similar to one of the three morphotypes obtained by Kieliszewska-Rokicka et al. (1998) after inoculation of Scots pine with *S. luteus in vitro*. In our study, the number of mycorrhizal short roots formed in the root of Scots (9.34%) and Stone pine (3.68%) was lower than those obtained by these authors (that reached the 90%) or by Hwang et al. (1995) with Jack pine and *S. tomentosus* (51.5%). Similar low values of mycorrhization for *S. luteus* (2.87% for Stone pine and 2.46% for Scots pine) were reported in post-emergence damping off assays using two-month old seedlings (Mateos et al., 2004).

The number of mycorrhizal apexes was higher in Scots than in Stone pine seedlings, similarly to that obtained with *Laccaria laccata* in a similar assay (Machón et al., 2004, 2009). Mycorrhization ability might be related to the host, as shown in another ectomycorrhizal fungus (Parladé et al., 2004).

Contrary to other reports (Smith and Read, 1997; Kieliszewska-Rokicka et al., 1998; Guerin-Laguette et al., 2004), a positive effect on plant growth (shoot height, shoot dry weight, root collar diameter, root length, root dry weight) in mycorrhized seedlings was not clearly shown in our study (Tables 3 and 4). This fact may be related to the poorly colonized root systems in the plants inoculated with the Myc fungi. However, Hwang et al*.* (1995) and Diedhiou et al*.* (2003) working with heavily mycorrhized seedlings also failed to demonstrate this positive effect on plant growth.

Hwang et al. (1995) observed a decrease to one-fifth of the number of mycorrhizal short roots formed in Jack pine seedlings mycorrhized with *S. luteus* after *F. verticillioides* inoculation. In our work, the presence of *F. verticillioides* lowered to one-third the percentage of

Table 3. Effect of *Suillus luteus* (Myc), *Fusarium oxysporum* (Fo) and/or *F. verticillioides* (Fm) on shoot height, shoot dry weight, root collar diameter, root length and root dry weight of Scots pine seedlings 18 weeks after sowing.

Treatment	Shoot Height (cm)	Shoot dry weight (g)	Diameter of root collar (mm)	Root length (cm)	Root dry weight (g)
Not inoculated	6.01^{a}	0.05^{a,b}	0.54^{b}	14.46^{a}	0.03^{a}
Fm	5.53^{a,b}	0.06^{a,b}	0.74^{a}	14.16^{a}	0.03^{a}
Fo	5.65^{a,b}	0.04^{b}	0.60^{b}	13.63^{a,b}	0.03^{a}
Myc	5.89^{a,b}	0.06^{a}	0.65^{a,b}	13.83^{a}	0.03^{a}
Fm+Myc	5.63^{a,b}	0.05^{a,b}	0.57^{b}	13.76^{a}	0.03^{a}
Fo+Myc	5.41^{b}	0.04^{b}	0.57^{b}	12.57^{b}	0.02^{a}

Fm: *Fusarium verticillioides;* Fo: *F. oxysporum;* Myc: ectomycorrhizal fungus *Suillus luteus*. Means followed by different letters within each column are significantly different. LSD Fisher test, P=0.05, n=15.

Table 4. Effect of *Suillus luteus* (Myc), *Fusarium oxysporum* (Fo) and/or *F. verticillioides* (Fm) on shoot height, shoot dry weight, root collar diameter, root length and root dry weight of Stone pine seedlings 18 weeks after sowing.

Treatment	Shoot Height (cm)	Shoot dry weight (g)	Diameter of root collar (mm)	Root length (cm)	Root dry weight (g)
Not Inoculated	12.39^{a,b}	0.40^{a,b}	1.48^{a}	13.46^{a,b}	0.12^{a,b}
Fm	11.38^{b,c}	0.33^{b}	1.35^{a}	13.42^{a,b}	0.09^{c}
Fo	11.61^{a,b,c}	0.34^{b}	1.49^{a}	12.67^{b}	0.09^{c}
Myc	12.97^{a}	0.45^{a}	1.52^{a}	14.27^{a}	0.13^{a}
Fm+Myc	10.47^{c}	0.32^{b}	1.44^{a}	13.40^{a,b}	0.09^{c}
Fo+Myc	12.11^{a,b}	0.37^{b}	1.34^{a}	14.43^{a}	0.10^{b,c}

Fm: *Fusarium verticillioides;* Fo: *F. oxysporum;* Myc: ectomycorrhizal fungus *Suillus luteus*. Means followed by different letters within each column are significantly different.

mycorrhizal short roots in the Myc treated stone pine plants, whereas in the seedlings inoculated with *F. oxysporum*, no mycorrhizae formation was observed, so it appears that the presence of this pathogen inhibited the mycorrhizal process. However, this effect may possibly be only linked to the pathogenic isolates of *Fusarium*, since other studies (García-Romera et al*.,* 1998; Diedhiou et al*.,* 2003) described a stimulation of mycorrhization in the presence of saprophytic *F. oxysporum* strains.

Results indicate a positive role of *S. luteus* for biological control of damping off in forest nurseries. However, the protective effect provided by *S. luteus* against damping-off by *Fusarium* spp. was not related to the percent of mycorrhizal apexes in the roots of Stone and Scots pine seedlings. More work has to be done to explore the active mechanism of disease suppression by this ectomycorrhizal fungus and also for considering its practical application in commercial nurseries.

Conflict of Interests

The authors have not declared any conflict of interests.

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