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Miren K. Duñabeitia · Susana Hormilla · Jose I. Garcia-Plazaola · Kepa Txarterina · Unai Arteche · Jose M. Becerril

Differential responses of three fungal species to environmental factors and their role in the mycorrhization of *Pinus radiata* D. Don

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Abstract Three ectomycorrhizal (ECM) isolates of *Rhizopogon luteolus*, *R. roseolus* and *Scleroderma citrinum* were found to differ markedly in their in vitro tolerance to adverse conditions limiting fungal growth, i.e. water availability, pH and heavy metal pollution. *S. citrinum* was the most sensitive, *R. luteolus* intermediate and *R. roseolus* the most tolerant species. *Pinus radiata* D. Don seedlings were inoculated in the laboratory and in a containerised seedling nursery with spore suspensions of the three ECM species. Colonisation percentage was considerably lower under nursery conditions, probably due to competition by native fungi. The effects of nursery ECM inoculation on seedling growth depended on the fungal species. Only *R. roseolus*-colonised plants showed a significantly higher shoot growth than non-mycorrhizal plants. All three fungi induced significantly higher root dry weights relative to control plants. Despite the low mycorrhizal colonisation, mycorrhization with all three species improved the physiological status of nursery-grown seedlings, e.g. enhanced root enzyme activity, shoot nutrient and pigment content, net photosynthesis rate and water use efficiency. Of the three fungal species, *R. roseolus* was the most effective; this species was also the most adaptable and showed the greatest range of tolerance to adverse environmental conditions in pure culture. It is, therefore, proposed as a promising fungal species for ECM inoculation of *P. radiata* in the nursery.

M. K. Duñabeitia (✉) · J. I. Garcia-Plazaola · U. Arteche · J. M. Becerril
Departamento Biología Vegetal y Ecología, Facultad Ciencias, Universidad del País Vasco/EHU,
Apdo. 644, 48080 Bilbao, Spain
e-mail: gvpduaum@lg.ehu.es
Fax: +34-944-4648500

S. Hormilla
NEIKER-Instituto Vasco de Investigación y Desarrollo Agrario,
Apdo. 46, 01080 Vitoria/Gasteiz, Spain

K. Txarterina
Basalan SA,
Diputacion Foral de Bizkaia,
Avda. Madariaga 1–1ª Plta, Dpto. 9, 48014 Bilbao, Spain

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Introduction

Successful reforestation depends on the early capture of site resources by tree seedlings. Early growth assures space, a continuing resource supply and vigour to resist pests, pathogens and climatic stress (Perry et al. 1987). Ectomycorrhizae (ECM) are one of the keys to optimal establishment and performance of forest tree species under natural and cultivated conditions. Much has been reported on ECM fungi and their effects on a broad range of host plants (Castellano 1996; Cairney and Chambers 1999). The ECM symbiosis can improve plant height, diameter, shoot biomass, and root system quality, increasing the survival of plants transplanted to the field (Smith and Read 1997).

Newsham et al. (1995) considered mycorrhizae to be multi-functional: different fungi produce different effects on the same host, but the same fungus can produce different effects on the same host under different environmental conditions. This natural variability makes it necessary to develop selection strategies for ECM fungi as inocula in forest nurseries. The majority of screening programmes search for isolates that increase nutrient uptake and, thus, plant growth. Recent years have seen an increased awareness of the additional benefits of a fungal partner in the root system. In this case, different criteria may be used to select inoculant mycorrhizal fungi unrelated to ability to increase growth rates or nutrient uptake (Dodd and Thomson 1994).

Inoculation with ECM fungi can increase the ability of forest plants to grow in unfavourable environmental and soil conditions (Jones and Hutchinson 1988). Water availability is one of the most limiting environmental stresses for plant production. Forests in northern Spain are generally subject to rather mild climates with high precipitation rates. Nevertheless, they suffer marked

periods of drought at irregular intervals that negatively affect growth and survival of seedlings. Moreover, in the Basque Country, acid deposition is a major environmental concern (Gonzalez-Arias et al. 1998). Acidic air pollutant deposition leads to soil acidification, altered nutrient availability and increased solubility of toxic heavy metals. Thus, acid rain creates a need for selection of fungi tolerant to pollutants to the soil.

Pinus radiata D. Don is widely distributed in northern Spain, particularly in the Basque Country, where it is of forest and economic importance. The current emphasis is on finding ECM isolates for use as inocula adapted to large-scale seedling production in the nursery.

Of the mycorrhizal fungi able to colonise radiata pine, *Rhizopogon* spp. and *Scleroderma* spp. are promising inoculants. These fungi are early ECM root colonisers producing large quantities of rhizomorphs that enhance plant water uptake (Brownlee et al. 1983; Read and Boyd 1986). Some isolates of these species promote the growth of their host and have the advantage over many other ECM fungi in that their spores can be collected en masse from mature basidiomes. Thus spore inoculum is relatively easy to prepare from field-collected material and is a cheap inoculum with low labour costs (Cairney and Chambers 1999).

The aim of this present work was to study the tolerance of pure cultures of three fungal species (*Rhizopogon luteolus, R. roseolus*, *Scleroderma citrinum*) to extremes of water availability, pH and heavy metal pollution. We also evaluated their viability as inoculum for mycorrhization of radiata pine in the nursery and determined the benefits for seedling growth.

Materials and methods

Fungal growth in pure culture

Mycelia of the three basidiomycotina *Rhizopogon luteolus* Fr. & Nordh., *R. roseolus* (Corda ex Storm) Th. Fries and *Scleroderma citrinum* Pers. were isolated from sporocarps collected in *P. radiata* plantations in northern Spain and maintained at 25°C on modified Melin-Norkrans medium (MMN) (Molina and Palmer 1982). For plant inoculation, sporocarps of each species were surface cleaned, dried at 40°C for 48 h and kept at room temperature until used.

The fungi were cultured on Petri plates as 10 replicates to evaluate the response of isolates to adverse growth conditions. After 35 days, the diameters and dry weights of fungal colonies were measured. For dry weight determination, colonies were removed from the medium by heating the plates in an autoclave at 110°C for 5 min and drying at 85°C to constant weight.

MMN growth medium was modified to achieve the desired stress conditions. Drought stress was induced using 0, 10, 25 and 30% polyethylene glycol (PEG-6000) to adjust medium water potential (Coleman et al. 1989). Final water potentials determined with a Wescor 5500 osmometer were –0.15, –0.26, –1.10 and –1.80 MPa, respectively. As PEG reduces solidification of agar, fungal isolates were grown in liquid medium. To prevent anoxic conditions, colonies were grown in Petri dishes on 7-cm-diameter Whatman filters saturated with liquid medium but placed on fine washed sea-sand to avoid submersion. The effect of pH was determined by adjusting MMN medium to pH 3, 4, 5.5, 7 and 8.

To assay heavy metal toxicity, fungal species were grown on solid MMN medium with heavy metals at 0, 3, 33 and 100 ppm. Copper and cadmium were added as sulphates ($CuSO_4$. $5H_2O$, $CdSO_4$. $8H_2O$) and lead as nitrate [$Pb(NO_3)_2$]; lead sulphate is very insoluble. Control media contained the corresponding salt of each metal. Medium pH was adjusted to 5.5, except in the case of copper (4.5, to avoid precipitation).

Seedling growth and ECM inoculation

The effects of seedling inoculation were studied under controlled laboratory conditions and after large-scale seedling inoculation in the nursery. For the laboratory study, seeds of *P. radiata* were surface sterilised for 1 h in 30% hydrogen peroxide, rinsed with sterile deionised water, and later germinated on sterilised moist sand. After 6 weeks, seedlings were transferred to PVC containers (GODET 430, Pépinière Robin, France) containing a previously autoclaved mixture of peat moss and vermiculite (1:1, v/v). Spore slurry used for inoculation was prepared according to Castellano et al. (1985), blending each sporocarp in distilled water at high speed for 2–3 min. Initial spore density was calculated using an haemocytometer and the suspension adjusted to a density of 10^5–10^6 spores ml^{-1}. Plants were inoculated with *R. luteolus*, *R. roseolus* and *S. citrinum* by watering each container with 10 ml of spore suspension. Forty-five seedlings were inoculated with each mycorrhizal fungus species. The same number of non-inoculated (control) plants were prepared. After inoculation, seedlings were maintained in an air-conditioned greenhouse at 20/25°C day/night, 14 h day length and 50/70% RH day/night. Seedlings were watered as needed with deionised water and fertilised once a month. Each plant received 10 ml of nutrient solution containing 3.65 mg N, 1.29 mg P, 3.87 mg K, 0.35 mg Fe, 0.07 mg Mg, 0.06 mg Ca, 0.06 mg B, 0.01 mg Mo and 0.01 mg Zn.

In the nursery study, seeds were sown on a mixture of peat and pine-bark (70/30, v/v) in plastic trays with 35 cavities, each 5.3×5.5×14 cm high and 200 ml in volume (Arnabat 200). Seedlings were grown in a commercial forest nursery (Garmo, Basalan, Diputacion Foral de Bizkaia). After 2 months in a greenhouse, trays were placed into a roofless greenhouse and randomly organised into four groups for controlled mycorrhization with spore suspensions of *R. luteolus*, *R. roseolus, S. citrinum*. Seedlings were inoculated by watering each container with 25 ml of the spore suspension once a month from October to January.

Seedlings received regular irrigation and fertilisation with slow release Plantacote plus (14/8/15 NPK, 2 MgO) (Aglukon, Valencia, Spain) at the recommended rate of 3 g l^{-1} at time of planting.

Analysis

Five months after inoculation, 25 seedlings from each treatment were selected randomly for determination of stem height, stem diameter and root and shoot dry weights.

To determine the degree of mycorrhizal colonisation, root systems were separated into three groups and subsamples from each group selected at random. These were examined by stereomicroscopy to determine the number of colonised short roots per total number of short roots formed.

Net photosynthesis was measured with a differential infra-red gas analyser (ADC LCA-2; The Analytical Development Co., Ltd., Hoddesdon, England, UK), as described by Lacuesta et al. (1993).

The chlorophyll and carotenoid contents of leaves were measured according to Barnes et al. (1992) using dimethyl sulphoxide for pigment extraction. Nutrient analysis (NPK) was performed on dried shoot tissue after nitric-perchloric digestion (Zazoski and Duran 1977). N and P concentrations were determined colorimetrically and K, Ca and Mg by flame photometry.

Enzyme activities of pure fungal cultures were determined as follows: 250-ml Erlenmeyer flasks containing 100 ml MMN liquid medium were inoculated with five 5-mm-diameter fungus disks taken from the edges of young colonies growing on MMN agar medium, eight replicate flasks for each fungal species. After 30 days at 25°C, the fungal colonies were filtered and washed

aseptically with deionised water. For mycorrhizal roots, short roots were excised from 10 seedlings in each treatment and cut into pieces on ice. Aliquots of 0.25 g fresh root material were used for each determination, with five replicates per treatment for each enzyme activity.

In vivo phosphatase activity was measured according to Ho (1987) by determining the amount of p-nitrophenol released after 1 h incubation in the dark at 30°C. Nitrate reductase activity was assayed according to Sarjala (1991a) as the amount of nitrite released after 2 h incubation in the dark at 30°C. Glutamine synthetase activity was measured as described by Sarjala (1991b).

Statistical analyses

Significant differences between treatments were assessed by analysis of variance (ANOVA) and treatment means were compared by least significant difference ($P<0.05$) using Student's *t* test.

Results

The effect of water stress on the growth of the three fungal species is shown in Fig. 1. All three isolates showed a general decrease in growth with increasing water stress. *R. luteolus* and *S. citrinum* appeared most sensitive, both showing colony diameter inhibition of approximately 50% and 60% at –1.1 and –1.8 MPa, respectively. Below –1.0 MPa, mycelial growth of *R. luteolus* and *S. citrinum* was reduced to a few feeble hyphae spreading throughout the surface of the culture medium. Only *R. roseolus* maintained fungal growth at the lowest water potential assayed. For all three species, growth inhibition was higher when expressed in terms of dry weight than of colony diameter.

In general, neither fungal dry weight nor colony diameter of *Rhizopogon* species were affected significantly by change in medium pH (Fig. 2). On the other hand, *S. citrinum* fungal growth was adversely affected by the lowest pH (3) and higher pH values (6.5–8) promoted growth, both as colony diameter and as dry weight. *R. roseolus* appeared to be the most pH-tolerant species.

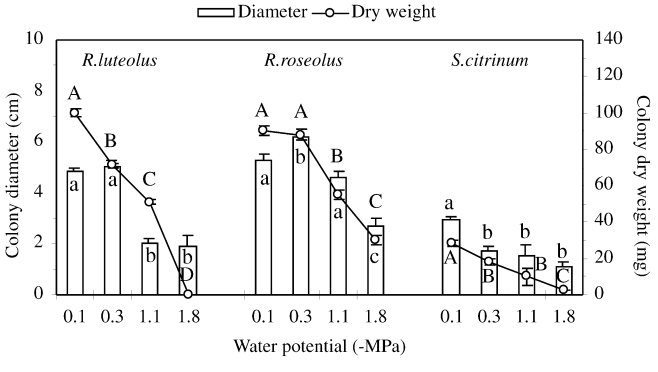

Fig. 1 Effect of water potential on the diameter and dry weight of fungal colonies grown in liquid MMN medium. For each fungus (*Rhizopogon luteolus*, *R. roseolus* and *Scleroderma citrinum*), values followed by the same letter (diameter, *lower case* and dry weight, *upper case*) are not significantly different ($P<0.05$)

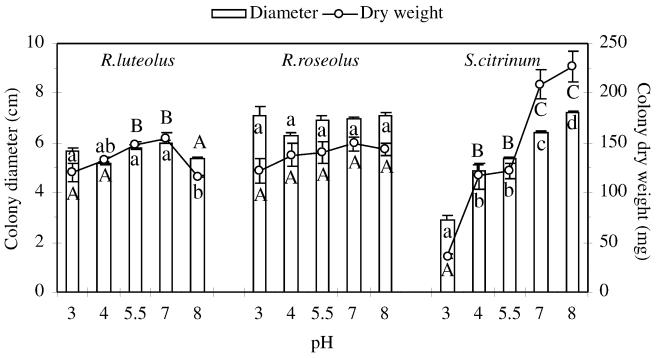

Fig. 2 Effect of medium pH on the diameter and dry weight of fungal colonies on solid MMN. For each fungus, values followed by the same letter (diameter, *lower case* and dry weight, *upper case*) are not significantly different ($P<0.05$)

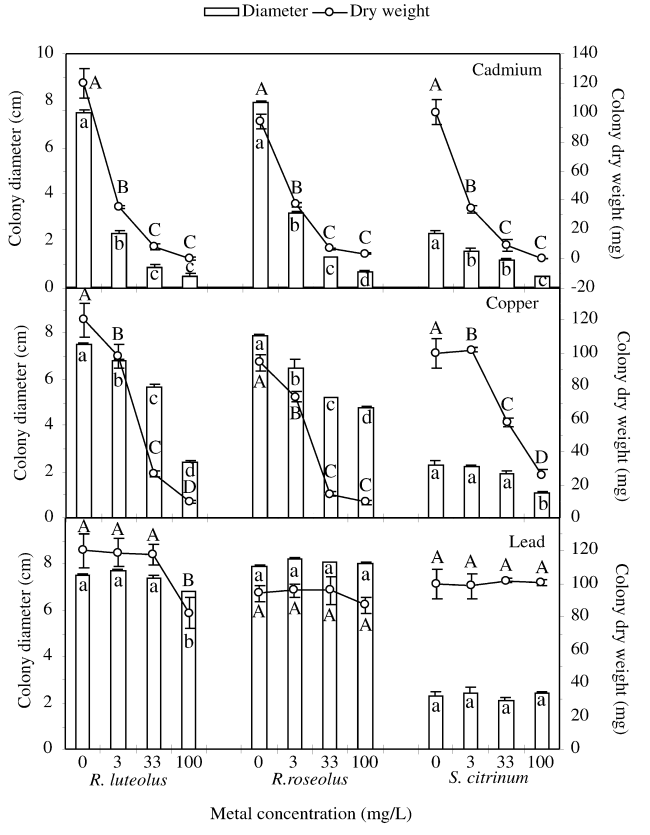

Fig. 3 Effect of cadmium, copper and lead concentrations on the diameter and dry weight of fungal colonies on solid MMN. For each fungus, values followed by the same letter (diameter, *lower case* and dry weight, *upper case*) are not significantly different ($P<0.05$)

The effects of heavy metals on the growth of the fungal species in pure culture are shown in Fig. 3. Cadmium greatly inhibited increase in biomass and colony diameter in all species, even at concentrations as low as 3 ppm. There was an almost total inhibition of growth at the highest concentrations. Copper was less inhibitory, although colony dry weight was markedly

Table 1 Mycorrhizal colonisation (%), height (cm), diameter (mm) and dry weight (g) of *Pinus radiata* seedlings inoculated with *Rhizopogon luteolus*, *R. roseolus* or *Scleroderma citrinum* under laboratory and nursery conditions. For each column, values ± SE followed by the same letter are not significantly different ($P<0.05$)

Treatment	Colonisation	Height	Diameter	Root dry weight	Shoot dry weight
Laboratory					
Control	1.8 ± 2.6 a	21.8 ± 0.6 a	3.2 ± 0.06 a	1.17 ± 0.07 a	2.22 ± 0.16 a
R. luteolus	82.9 ± 4.8 b	23.5 ± 0.7 a	3.2 ± 0.08 a	1.51 ± 0.11 b	2.64 ± 0.12 a
R. roseolus	90.4 ± 1.5 b	26.8 ± 0.5 b	3.5 ± 0.02 b	1.74 ± 0.15 b	3.38 ± 0.15 b
S. citrinum	52.4 ± 2.6 c	23.6 ± 0.9 a	3.9 ± 0.11 c	2.33 ± 0.18 c	2.75 ± 0.08 a
Nursery					
Control	15.9 ± 2.8 a	21.4 ± 1.6 a	2.4 ± 0.12 a	1.02 ± 0.06 a	1.83 ± 0.09 a
R. luteolus	48.4 ± 1.9 b	21.9 ± 1.3 a	2.6 ± 0.11 ab	1.36 ± 0.04 b	2.08 ± 0.12 a
R. roseolus	39.2 ± 1.1 c	24.7 ± 1.9 a	2.5 ± 0.14 a	1.29 ± 0.08 b	2.38 ± 0.15 b
S. citrinum	41.2 ± 3.1 c	25.5 ± 1.9 a	2.8 ± 0.19 b	1.25 ± 0.07 b	2.16 ± 0.14 a

inhibited at 33 ppm. Of the heavy metals assayed, lead was tolerated the most by all species, even at 100 ppm in the case of *R. roseolus* and *S. citrinum*. However, a clear growth inhibition was observed in diameter and dry weight of *R. luteolus* at the highest concentration.

Mycorrhizal colonisation and plant growth varied with the fungal species and seedling growth conditions (Table 1). Under controlled laboratory conditions, 85% of the plants formed ECM with the inoculated fungi. Percentage colonisation of root systems showed a large variation between *Rhizopogon* and *Scleroderma* species. *S. citrinum* colonised root systems the least (50%) but *R. luteolus* and *R. roseolus* colonised more than 80% of the short roots. Some short roots of control plants were colonised by the common greenhouse fungus *Thelephora terrestris* Ehrh.:Fr., but the percentage of mycorrhization was extremely low (0.5%). On the other hand, *Rhizopogon* and *Scleroderma* colonised container seedlings in the nursery under non-sterile conditions but to a lower extent than under laboratory conditions. In all treatments, seedlings were colonised by the naturally occurring ECM fungi in the nursery (mainly *Thelephora terrestris* Ehrh.:Fr. and *Laccaria* sp.). Colonisation by indigenous mycorrhizal fungi was higher in non-inoculated than inoculated seedlings. Responses to inoculation treatments were similar, but their extent differed significantly between the fungal species assayed.

Only radiata pine seedlings inoculated with *R. roseolus* showed a clear increase in shoot growth (height, diameter and dry weight) compared with other inoculated and control plants under laboratory conditions. Seedlings inoculated with *S. citrinum* and *R. luteolus* showed no significant differences in height or shoot dry weight relative to control plants. However, there was a significant increase in root system dry weight and stem diameter in the case of *S. citrinum*.

Under nursery conditions, although seedlings inoculated with *R. roseolus* and *S. citrinum* were rather taller and showed higher shoot dry weights than control seedlings, significant differences were found only in the case of *R. roseolus*. However, all three fungi induced significantly higher root dry weights compared with control plants.

Inoculation of radiata pine seedlings with ECM fungi had marked effects on enzyme activities (Fig. 4). In pure culture, enzyme activities differed between species depending on the enzyme assayed. *R. luteolus* showed significantly higher phosphatase activity than *R. roseolus* or *S. citrinum*. Nitrate reductase activity did not differ between fungal species, but glutamine synthetase activity was significantly higher in *R. roseolus* and *S. citrinum* than in *R. luteolus*.

Root enzyme activity showed significant differences between fungal species, correlated with that found in pure culture in the case of phosphatase. Inoculated plants showed significantly higher activities of the three enzymes compared with non-inoculated plants. The highest

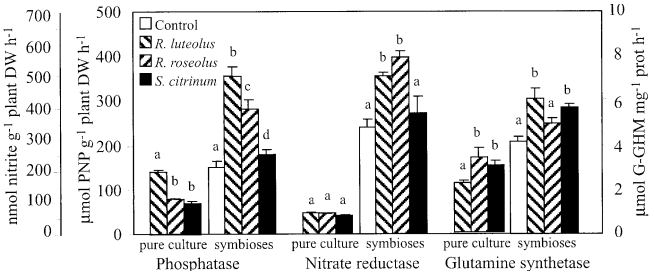

Fig. 4 Phosphatase (μmol *p*-nitrophenol g^{-1} dry weight h^{-1}), nitrate reductase (nmol nitrite g^{-1} dry weight h^{-1}) and glutamine synthetase (mol γ-GHM mg^{-1} protein h^{-1}) activities of *R. luteolus*, *R. roseolus* and *S. citrinum* isolates cultured in MMN liquid medium and of ECM formed on nursery-grown *Pinus radiata* seedlings after inoculation with spore suspensions. For each activity, values followed by the same letter are not significantly different ($P<0.05$)

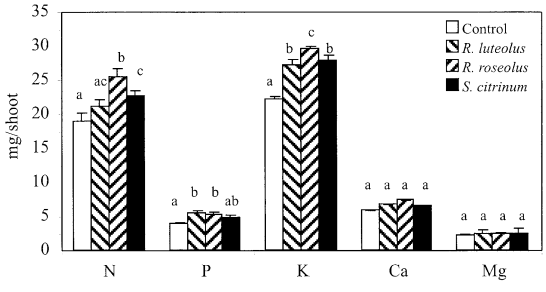

Fig. 5 Nutrient contents in shoots of 7-month-old nursery-grown *P. radiata* seedlings non-inoculated (control) and inoculated with spore suspensions of *R. luteolus*, *R. roseolus* or *S. citrinum*. For each mineral element, values followed by the same letter are not significantly different ($P<0.05$)

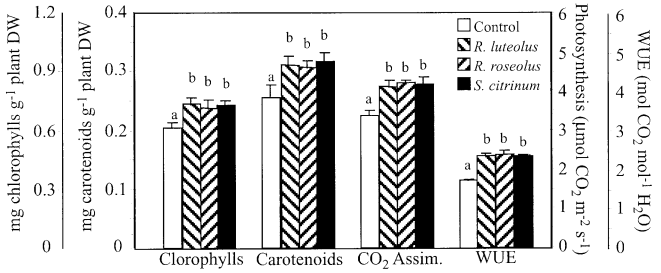

Fig. 6 Needle chlorophyll and carotenoid concentrations (mg g^{-1} dry weight), CO_2 assimilation rate (μmol CO_2 m^{-2} s^{-1}) and water use efficiency (WUE) (mol CO_2 mol^{-1} H_2O) of 7-month-old nursery-grown *P. radiata* seedlings non-inoculated (control) and inoculated with spore suspensions of *R. luteolus*, *R. roseolus* or *S. citrinum*. For each parameter, values followed by the same letter are not significantly different (P<0.05)

increase was produced by *R. luteolus*, followed by *R. roseolus* and *S. citrinum.*

Mineral nutrient content of radiata pine needles varied with treatment (Fig. 5). N, P and K significantly increased in inoculated plants. *R. roseolus*-inoculated plants showed the highest shoot nutrient contents and, in general, control seedlings showed the lowest.

Leaf pigment content, CO_2 assimilation rate and water use efficiency (WUE) were higher (P=0.01) in inoculated than in control plants (Fig. 6) with all three fungal species, but with no significant differences between inoculants.

Discussion

Fungal growth was very sensitive to low water potential in the medium. Of the three fungal species studied, only *R. roseolus* appeared drought tolerant, showing the widest range of water stress tolerance. *R. luteolus* was only tolerant of moderate water stress below –1.0 MPa. Coleman et al. (1989) reported that drought tolerance depends more on fungal species than on annual precipitation at the site of collection. In that work, *R. vinicolor* Smith was reported as drought tolerant together with *Boletus edulis* Bull.: Fr., *Cenococcum geophilum* Fr. and five *Suillus* species.

ECM associations significantly alter water relationships of host plants and this enhancement has been attributed in part to rhizomorph production and their function in water transport (Duddridge et al. 1980). It is not clear whether the drought tolerance of fungi in pure culture is transmitted to associated host plants. Parke et al. (1983) found no relationship between pure culture experiments and seedling experiments. However, we found a positive relationship in a parallel assay developed under field conditions (Ortega et al. 2003). In that study, inoculation of radiata pine seedlings in the nursery with *R. roseolus* increased dehydration tolerance.

Acid rain, which increases direct input of N, S and H ions largely as wet deposition, may have adverse effects on growth and nutrition of seedlings (Smith and Read 1997), and creates the need for fungi tolerating soil acidification and increased heavy metal availability. Soil acidity affects mycorrhiza formation and hyphal development to a degree dependent on plant species and the mycorrhizal fungus involved (Van der Heijden and Kuyper 2001). Jongbloed and Borst-Pauwels (1990) reported that low pH strongly inhibited growth in pure cultures of *Laccaria bicolor* (Maire) P. D. Orton, *L. rufus* (Scop.) Fr. and *L. hepaticus* Plowr. Ap. Boud. The same effect was observed by Hung and Trappe (1983) for *Amanita muscaria* (L.:Fr.) Per. Ex Hooker, *Cenococcum geophilum* Fr., *Hebeloma crustuliniforme* (Bull. Ex St. Amans) Quel. and *Laccaria laccata*. However, *Piloderma bicolor* (Peck.) Jülich, *Paxillus involutus* (Batsch) Fr., *Pisolithus tinctorius* (Pers.) Coker and Couch, and *Scleroderma aurantium* Pers. showed a high tolerance of acidic growth media (Willenborg et al. 1990). In the present study, *S. citrinum* was the most sensitive to acidic growth media. *R. luteolus* showed an intermediate tolerance, having only a minor growth decrease (expressed as colony dry weight) when pH was lowered to 3 or raised to 8. *R. roseolus* was again most tolerant of pH variation in the growth medium. This tolerance level leads us to consider this species as a good candidate for nursery inoculation (Hung and Trappe 1983).

Heavy metal toxicity adversely affects the duration of the lag phase, growth rate, mycelial density and biomass production (Jones and Hutchinson 1988). Different effects of each metal on each fungal species indicates different tolerance mechanisms for each metal. Heavy metals can be trapped by components of the cell wall of fungal hyphae or be captured by detoxification processes in which metalothionine peptides and proteins are involved (Gadd 1993). Fungal heavy metal tolerance is of great interest when it is maintained during the ECM symbiosis and impedes heavy metals from entering the vascular system of the plant, so conferring tolerance (Colpaert and Van Assche 1993; Blaudez et al. 2000).

According to our results, cadmium was the most toxic metal for fungi. Even at the lowest concentration tested (3 ppm), colony growth of all ECM species assayed was inhibited more than 60%. This effect was observed previously (Jongbloed and Borst-Pauwels 1990; Colpaert and Van Assche 1992). In contrast, lead had almost no effect on fungal growth. Interestingly, *R. roseolus* was most tolerant of the three metals assayed, as it was of water availability and pH. Thus, *R. roseolus* is a promising candidate for reforestation, as it may confer higher tolerance to radiata pine growing on metal-polluted soil.

As in our study, there is variability in the ECM colonisation capacity of different fungal species (Burgess et al. 1994). Colonisation was considerably lower under nursery conditions than in the laboratory for all three species tested, presumably due to the excessive humidity in the substrate and to competition by native fungi in the nursery (*Thelephora terrestris* Ehrh.:Fr. and *Lactaria* sp.).

Few studies have focussed on the lowest level of mycorrhization necessary for maintaining the effect of an introduced fungus after outplanting to the field. In the case of *Pisolithus tinctorius* (Pers.) Coker & Couch, seedlings with 50% roots colonisation have been reported to be a good indicator of inoculation effectiveness maintained in the field (Marx 1980). For other species such as *Amanita muscaria* (L.:Fr.) Hook., *Lactarius rufus* (Scopoli) Fr. or *Tricholoma albobruneum* (Pers.:Fr.) Kummer, good results have been obtained with lower percentages of mycorrhization (Stenström and Ek 1990).

With respect to the effect of mycorrhizal inoculation on seedling growth, the results obtained with different host-fungus combinations are contradictory. Some authors indicate that inoculation of containerised seedlings (grown under routine nursery conditions) rarely increases growth in the nursery phase (Castellano and Molina 1989; Colpaert et al. 1996). Castellano (1996) reported that *R. luteolus* and *R. roseolus* usually did not affect seedling growth of *Pinus* spp. seedlings. However, other authors demonstrated positive effects of both fungal species on seedling growth after nursery inoculation (Chu-Chou and Grace 1985; Parladé et al. 1996). *Scleroderma* species have also been used world-wide to increase early growth of seedlings of a range of hosts, both in the glasshouse and in the field (Castellano 1996). Nevertheless, beneficial growth responses were only reported for some combinations (Ford et al. 1985).

In the present study, despite the low level of mycorrhizal colonisation at the end of the nursery phase, mycorrhizal colonisation showed positive effects on plant growth. The benefit obtained by the inoculated plants was variable and depended on fungal species and seedling growth conditions. Seedlings grown in the laboratory in sterilised soil under environmentally controlled conditions showed better growth and mycorrhizal development than those in the nursery (in unsterilised soil). But, in both cases, inoculated seedlings tended to be taller and showed increased shoot dry weights compared with non-inoculated seedlings, although differences were only significant in the case of *R. roseolus*. Likewise, the three fungi produced a significant increase in root growth compared with controls.

ECM fungi differ in their ability to enhance enzyme activity in host plants (Colpaert et al. 1999). In our study, colonisation of root systems by *R. luteolus*, *R. roseolus* and *S. citrinum* resulted in varying increases in enzyme activities. However, in all cases, phosphatase, nitrate reductase and glutamine synthetase activities were higher in colonised roots than in non-inoculated roots. Phosphatase and nitrate reductase activities in ECM fungi in pure culture were much lower than those in their respective radiata pine mycorrhizal roots. Likewise, there was not a clear relationship between pure culture and seedlings enzyme activities for the different fungal species. As Sarjala (1991a) suggests, information obtained on enzyme activities of possible symbiotic fungus in pure culture may not correlate to natural conditions.

In the temperate forests of the northern hemisphere, availability of nitrogen and phosphorus is a major plant growth-limiting factor. After outplanting, transfer of nutrients to seedlings may be a crucial factor for young plant survival in a nutrient-limited situation (Read et al. 1985). The results obtained in this work confirm the important contribution of ECM fungi to the phosphorus and nitrogen nutrition of their hosts. In fact, growth increases in response to ECM inoculation corresponded to higher nutrient contents in plants.

The increased growth as well as the higher P and N observed in tissues of *R. roseolus*-inoculated seedlings may be partially explained by the higher enzyme activities in mycorrhizal than non-mycorrhizal roots (MacFall et al. 1991). The mycorrhization effects on plant growth may depend on the balance between carbohydrate consumption by the fungus and benefits obtained by the host plant. A change in this balance may reduce, instead of increase, plant growth. Fungal species, degree of mycorrhiza formation, as well as mycelial growth are important to this balance (Stenström and Ek 1990; Dosskey et al. 1990; Colpaert et al. 1992).

Stimulation of $C0_2$ assimilation rate in mycorrhizal plants has been observed in other tree species and with other fungi (Guehl et al. 1990; Dixon and Hiol-Hiol 1992). This modulation of the photosynthetic capacity of host plant seems to depend on the associated fungal species (Guehl et al. 1990; Guehl and Garbaye 1990). The higher net photosynthesis rate in mycorrhizal plants has been attributed to increase in photosynthate demand; colonised radical systems present a bigger sink for photosynthates than non-colonised systems (Reid et al. 1983). Colpaert et al. (1996) reported growth depression over 12 weeks in seedlings of *Pinus sylvestris* L. colonised by *S. citrinum*. Seedling root growth was more affected by mycorrhizal formation than shoot growth and, as in our case, plants showed no increase in net CO_2 assimilation rate following colonisation. These authors suggested that growth depression resulted from increased below-ground C allocation and/or high nutrient retention by the mycobiont.

In our study, we presume that the higher CO_2 assimilation rate is due to the higher N and chlorophyll contents in *R. roseolus*-inoculated seedlings, with a resultant increase in seedling growth.

Radiata pine seedlings inoculated with *S. citrinum* showed no significant differences in growth compared to non-inoculated seedlings. The absence of beneficial effects on the growth of inoculated seedlings will not necessarily continue once they are transplanted to the field. Many of the mechanisms by which fungi influence plant growth are absent during container seedling production. Nevertheless, after transplanting under adverse growth conditions, the availability of nutrients drastically decreases and the beneficial effects of a pre-established symbiosis may become more evident (Le Tacon et al. 1987; Stenström and Ek 1990).

Low survival and poor initial growth of seedlings are commonly observed in different plantation systems.

These effects can be explained by the low $C0_2$ assimilation capacity and the low and variable WUE of non-inoculated plants (Le Tacon et al. 1987). Higher WUE values were observed for colonised than non-colonised seedlings, suggesting better coupling between $C0_2$ assimilation rate and stomatic conductance. These results are in agreement with those of Guehl and Garbaye (1990) and Guehl et al. (1990). These authors suggested that improvement in WUE is related to a nutritional effect, specifically with better P uptake, which would have an influence on $C0_2$ assimilation as well as on stomatal conductance of the colonised seedlings. If we take into consideration that WUE is one of the main growth-determining factors under drought conditions (Guehl and Aussenac 1987), this result is of great importance and suggests that ECM colonisation with *R. roseolus* may help adapt host plants to drought conditions by improving water uptake and water relations.

Even though these laboratory results cannot be directly translated to the field, this study provides some evidence of the importance of forest plantations inoculated with specific ECM fungi that have proved effective in improving host physiological state. Prerequisites for the use of ECM inoculation programmes are the selection of appropriate fungal symbionts and the development of methods for large-scale production of inoculum. The results obtained in this work are of great relevance to forestry practice. Furthermore, because of the high cost involved in the use of different fungi adapted to different ecological and ambient conditions, it is of great interest to use widely tolerant species. In this sense, *R. roseolus* shows great promise for forestry practices as it tolerates various adverse environmental conditions common in nature.

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