RESEARCH NOTE

Sustained *in vitro* root development obtained in *Pinus pinea* L. inoculated with ectomycorrhizal fungi

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Summary

Stone pine (*Pinus pinea* L.) is an economically important forest tree in Mediterranean climates and has been the target for selection efforts through micropropagation. Previous attempts on microshoots, derived from mature seed cotyledons, reached incipient rooting after induction with a combination of auxin and hypertonic shock, but their development *in vitro* was not sustained. At this stage, co-culturing plantlets with some fungi isolated from ectomycorrhizas succeeded in overcoming this barrier, enabling satisfactory development in vermiculite and later in soil. About half of the fungal isolates tested helped the plants resume root growth. Although control plants (in the absence of fungi) developed roots at a later stage, i.e. during the post-transplanting acclimation in vermiculite, their growth was weaker. The root systems of some inoculated plants had ectomycorrhizas from the introduced fungi being carried over when the plants were transferred from the co-cultures to vermiculite. In conclusion, co-culturing rooted microshoots with ectomycorrhizal fungi can be an effective means to overcome the difficulties encountered in the use of micropropagation methods on this species.

Introduction

Stone pine (*Pinus pinea* L.) is one of the most important forest trees in the Mediterranean Region of Portugal, especially for seed production, but also for its ecological and ornamental aspects. The heritabilities of relevant characters such as cone weight, number of seeds per cone and seed length are 0.81, 0.81 and 0.55, respectively (M. Alpuim, personal communication, data presented at the Third Forest Congress in Portugal, 1994), thus encouraging the selection

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of seeds from 'plus' trees to serve as sources of clones with the desired characteristics by mass propagation techniques, i.e. micropropagation. Hence, an improvement in the quantity and quality of the seed production per tree is one of the main objectives of present forestry programmes in this region.

Embryo explants, preferentially from cotyledons (Aitken *et al.*, 1985), are commonly used in conifers for the induction of adventitious shoots (Paranjothy *et al.*, 1990). N^6-Benzylaminopurine (BAP), possibly the most efficient cytokinin in the induction of adventitious shoots in gymnosperms (Pierik, 1990), is normally sufficient. The adventitious shoots are produced directly on the surface of each cotyledon between 6 and 10 weeks after the beginning of the culture (Coria and Villalobos, 1990). The number of adventitious shoots produced per cotyledon varies with the species, e.g. 264 were reported for Douglas-fir (Wockhock and Abo El-Nil, 1977, cited by Paranjothy *et al.*, 1990), 180 for radiata pine (Aitken *et al.*, 1985), 20 for Chilgoza pine (*Pinus gerardiana* Wall.; Benererjee and Bjojwani, cited in Paranjothy *et al.*, 1990), or 50–215 for stone pine (*Pinus pinea* L.; A.Z. Potes, S. Figueira, C. Cavaleiro and A. Peixe, unpublished).

Still, the number of *ex vitro* established plants can be quite low, especially with gymnosperms, due to difficulties in producing adventitious roots and resulting losses during acclimation, thus limiting the applicability of micropropagation to cloning of superior genotypes at a commercial scale. For example, the rooting percentage was 25 for *Picea abies* (L.) Karst (Von Arnold and Erickson, 1985), 3 for *Pinus monticola* Dougl. (Stiff *et al.*, 1989) or 50 for *Pinus virginiana* Mill. (Saravitz *et al.*, 1991) and *Pinus canariensis* C. Smith (Pulido *et al.*, 1991). More recently, in the case of stone pine, Capuana and Giannini (1995) obtained 34.4 per cent of rooting on adventitious shoots with indole-3-butyric acid (IBA) at 10 μM.

The potential use of some ectomycorrhizal fungi for rooting of *in vitro* propagated gymnosperms is well known (other classes of root symbionts have the same potential, as reviewed by Nowak, 1998). Strains of *Hebeloma cylindrosporum* Romagnesi were shown to induce rooting in about half of the inoculated microcuttings from *Pinus pinaster* Sol. and *P. sylvestris* L. (Normand *et al.*, 1996), and spontaneous rooting of somatic embryo-derived *Larix* × *eurolepsis* Henry plantlets was much improved in the presence of at least four different ectomycorrhizal fungi, which induced elongation and branching of the initial root (Piola *et al.*, 1995). Thus it would appear likely that certain combinations of ectomycorrhizal fungi and procedures of micropropagation could be exploited for the improvement of root development of stone pine *in vitro*. The present paper reports on the effects of culturing rooted stone pine microshoots with fungi isolated from ectomycorrhizas, to sustain their root development before proceeding to later stages of acclimation for planting out, with emphasis on the methods used.

Materials and methods

Plant material

Mature seeds of stone pine were obtained on February 1999 from selected 'plus' trees (Alcácer do Sal Region, Alentejo, Portugal) and stored in a cold chamber at 4°C until used.

Fungi

Fungi were isolated from single ectomycorrhizas collected at two sites and maintained in pure culture using conventional procedures (Molina and Palmer, 1982; see also Brundrett *et al.*, 1996). Site 1, located near the Eastern border with Spain (38° 38′ N; 7° 30′ W), is a *Eucalyptus globulus* Labill. plantation at 265–270 m above sea level, with a few dispersed mature holm oaks (*Quercus ilex* L. ssp. *rotundifolia* (Lam.) Tab. Morais). At the time of collections (August and October 1995 and July 1996), the eucalypts were 9–10 years old, and the first clearcut took place in summer 1996. A total of 70 isolates were originally obtained, and for the present study a subset of 12 were used in preliminary experiments only. Site 2 is a stone pine plot from the same location as the plant material used in the study and the isolations were carried out in March 1999. The fungi from this site were from the eight most common stone pine ectomycorrhizal morphotypes observed in two soil cores located 12 m apart, totalling 40 different isolates

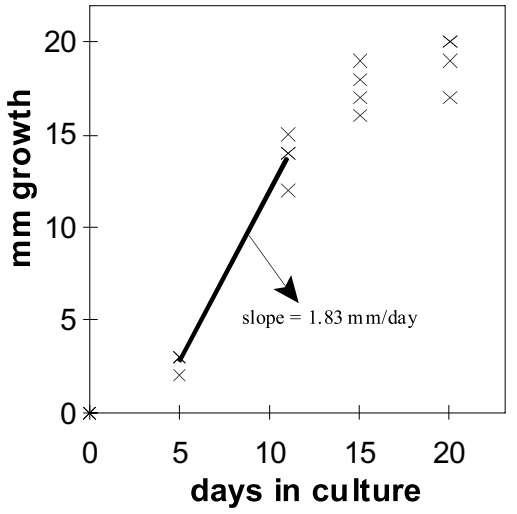

Figure 1. Example of the estimation of maximal fungal growth. For each time point there were four measurements (crosses), in this example, forming a sigmoidal growth curve having a maximal slope sometime between 5 and 11 days in culture. The regression line for that period provides an estimate for the maximal growth rate.

from which a representative sample of 12 were used in the screening experiments.

Stock cultures of each isolate were maintained in 9-cm Petri dishes containing corn meal agar (DIFCO, Detroit, MI, USA) in the dark at room temperature, with regeneration by transfer to fresh medium twice each year. Only stable cultures were used for the experiments reported.

The taxonomic identity of the isolates was not determined, but a characterization of growth in culture was done for the isolates from site 2, by replicating all stock cultures on three different agar media in 9-cm Petri dishes: MMN (Molina and Palmer, 1982), Czapek-Dox (prepared in the laboratory to reproduce the Oxoid formula, including the addition of sterile-filtered sucrose to the medium at 60°C before pouring) and corn meal (DIFCO). Most stock cultures were also replicated on potato dextrose (PDA) (DIFCO) and tryptone soya (Oxoid, Basingstoke, Hants., UK). The spread of each mycelium was measured at about 5, 10, 15 and 20 days of culture (four replicate measurements at each time point). For each growth curve, the maximum growth rate was estimated by linear regression to provide an overall standard for comparison (Figure 1). Given the unexpected diversity of stone pine ectomycorrhiza-associated fungi (the collection obtained consisted of 40 different isolates from two soil cores in site 2, P. Oliveira and J. Barriga, unpublished), a selection was made based on these maximum growth rates, in the hope of having an unbiased representation of that diversity. Thus three groups of isolates were defined: three fast-growing (>4 mm day^{-1} maximal linear growth) formed group A, four slowly growing (<1.5 mm day^{-1}) formed group C, and five moderately fast-growing formed group B. Within these groups some variations were detected, e.g. A2 grew slowly on Czapek-Dox agar, B3 and B4 grew slowly on tryptone soya agar, and C1 and C2 grew moderately fast on Czapek-Dox agar and PDA, respectively.

Shoot induction phase

The process undergone by the plant materials, from field sources to planting out micropropagated plantlets, is outlined in Figure 2. Stone pine seed shells were removed and the integuments surface-disinfected, then intact cotyledons of each seed were placed in a single Petri dish with solid woody plant medium (WPM) (Lloyd and McCown, 1980) supplemented with 5 mg l^{-1} BAP, 20 g l^{-1} sucrose, and 0.7 g l^{-1} of Bacto-Agar (DIFCO). Before autoclaving the pH was adjusted to 5.8 using NaOH (1 N). The culture chamber conditions during the shoot induction phase were 25°/19°C (day/night) temperatures with a 16-h photoperiod provided by cool-white fluorescent light at 80 μmol^{-1} m^{-2} s^{-1}. As soon as the microshoots could be easily separated from these cotyledons, they were transferred to test tubes (one per tube) with 10 ml of the same basal medium with activated charcoal (0.2 per cent) and without growth regulators. All the cuttings collected from a single seed were identified with the same clonal number.

Root induction and development

Elongated microshoots, ~2 cm long, were transferred to rooting medium based on WPM supplemented with 1 mg l^{-1} naphthalene acetic acid (NAA), 20 g l^{-1} sucrose and 0.65 per cent of

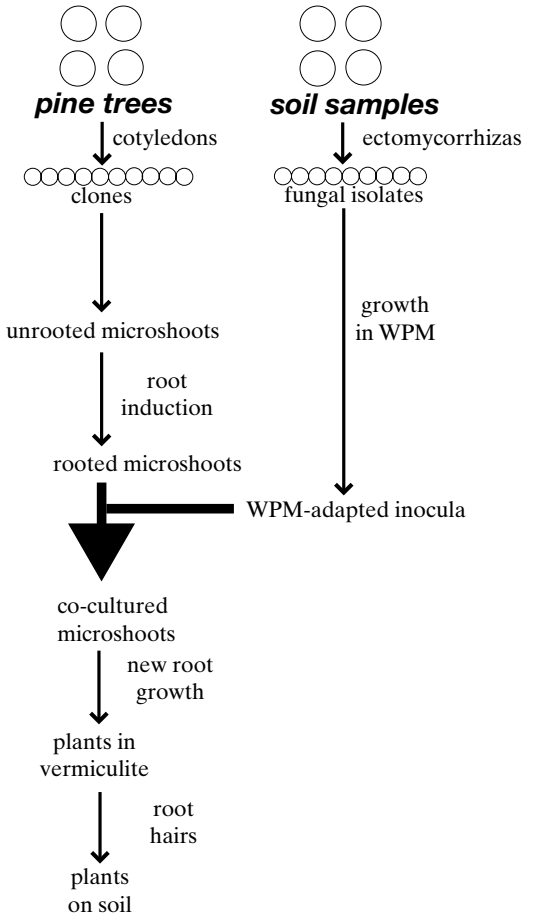

Figure 2. Scheme showing the process undergone by the plant material from field source to planting out the micropropagated plantlets.

Bacto-Agar (DIFCO). Before autoclaving the pH was adjusted to 5.8 using NaOH (1 N). This induction phase of adventitious root formation took place in the dark at 19°C for 1 week. Root expression was obtained after transferring the microshoots to the same basal medium (WPM) containing 40 g l^{-1} sucrose but no growth regulators, and incubated in a growth chamber provided with 16-h photoperiod by cool-white fluorescent light at 80 µmol^{-1} m^{-2} s^{-1} and 25°/19°C day/night temperatures. Roots appeared after 3–4 weeks under these conditions.

Co-culturing with the fungi

To obtain 'WPM-adapted' fungal mycelium (Figure 2), a small quantity from each stock culture was transferred to liquid WPM medium containing 20 g l^{-1} sucrose and antibiotics (40 µg ml^{-1} ampicillin, 30 µg ml^{-1} streptomycin and 100 µg ml^{-1} chloramphenicol) using a toothpick. Within a few days of growth at room temperature under dimmed light, with orbital rotation at 120 r.p.m., a 0.5 ml aliquot was collected from the suspension culture and spread on the surface of WPM agar, but in some cases a mycelium pellet was aseptically transferred and crushed on the agar surface using a glass rod. After a few more days of growth in the dark at room temperature, a regular 'lawn' was available as a source for the inocula (in the form of 5-mm agar plugs extracted aseptically with a cork borer) used in the co-cultures. These lawns remained usable for several weeks.

Co-cultures were carried out in 100-ml Pyrex glass bottles (4.5 cm diameter by 8.5 cm height), each containing one plant and a single fungal isolate. The plantlets were transferred to these bottles containing 30 ml co-culture agar based on the WPM formulation. The standard co-culture medium adopted for the screening experiment was a bilayer formed by 27 ml WPM agar without sucrose overlayed with 3 ml with standard WPM (containing 2 per cent sucrose). Before placing the fungal inocula, the plantlets stood for 5–7 days under the same conditions of light and temperature as described for rooting. Inocula consisted of three agar plugs placed at a distance of ~1 cm from the plantlet. At this time, the root outlines were marked on the glass surface, and the bottles returned to the growth chamber.

Acclimation

After 2–4 weeks in co-culture agar, rooted plantlets were transferred to 250-ml bottles containing 15 g of sterile vermiculite soaked with quarter strength (v/v) of WPM without sucrose, and covered with perforated aluminium foil. The plants grew in a growth chamber at 25°C and 60 per cent relative humidity with a 16-h photoperiod for 10 weeks. Plants were watered as required with alternating sterile water and the

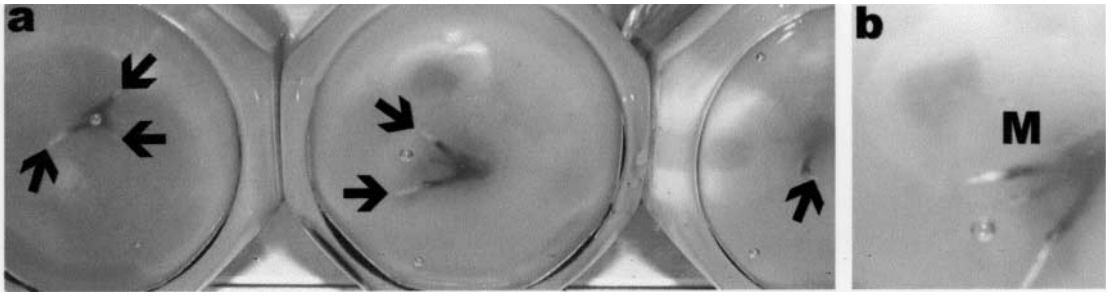

Figure 3. (a) Three examples of positive results in co-cultures as seen from below the bottles. New growth of roots is indicated by arrows. (b) A magnified image of the central bottle. M = mycelium growing at the surface of the agar (on the background).

same diluted WPM without sucrose. Aluminium foil was wrapped around the bottles to cover the substrate laterally, thus protecting roots from light. This protection was removed weekly so that root elongation and root hair development could be observed and then replaced.

Plants were then transplanted to glass bottles (250 ml) containing sterile vermiculite–peat–sand (1 : 1 : 1 by weight) as a growing substrate and maintained under the same growth conditions with regular watering. As growth continued, plants were transferred to pots containing the same substrate to prepare for planting out.

Microscopy

In one of the preliminary experiments, root materials were collected from plants during the transfer to larger pots (10 weeks after the transfer from vermiculite and 26 weeks since inoculation), fixed in formalin–ethyl alcohol–acetic acid, paraffin-embedded, sectioned for light microscopy and stained with methylene blue/azure B/basic fuchsin (Peterson, 1991) for anatomical determination of ectomycorrhizal features (presence of a fungal sheath and Hartig net in cross-section).

Results

Inoculation assays

Refinement of the culture conditions (preliminary experiments) The first experiment used 11 isolates from site 1 previously shown to be ectomycorrhizal in the growth pouch assay (Peterson and Chakravarty, 1991), and negative controls consisting of one cellulose-degrading isolate, from the same site, and an *Aspergillus niger* Tiegh. isolate. The co-cultures were on WPM agar, with at least two replicate bottles for each fungus, and growth of the inoculated fungi was observed in all cases. Negative controls without inoculation were also included. By 2–3 weeks after inoculation, newly grown roots (Figure 3) were visible in some cultures as light-coloured tips emerging at the base, laterally or apically, from preformed roots; this growth response was associated with seven of the 11 test-fungi, while no visible change in root morphology was seen after 3 weeks in the co-cultures containing any of the remainding four test-fungi or the controls. Microshoots that failed to respond to the previous root induction treatment were tested in parallel and remained unrooted, regardless of the fungus present.

A more selective set-up was introduced in a second experiment with the seven fungi that gave positive results in the first experiment: the time allowed for the observation of positive results was limited to 14 days, and the sucrose in the agar was either suppressed, lowered to 0.2 per cent, or concentrated at the surface of a bilayer. In this experiment no replication was done except for the uninoculated controls due to a temporary lack of available microshoots. Positive results (i.e. emergence of newly grown roots as in Figure 3) were obtained with five isolates in the bilayer

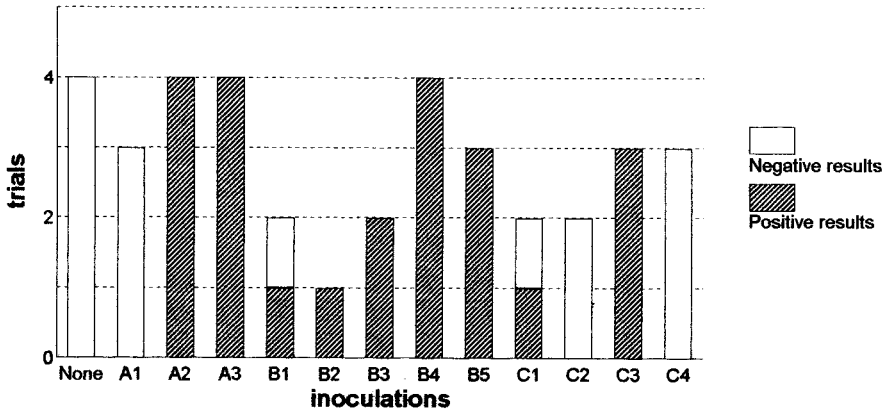

Figure 4. Results of co-culture trials using fungi from site 2 (group A, fast-growing; group B, moderately fast-growing; group C, slowly-growing) and controls without inoculation. Recovery of root growth within 3 weeks after inoculation was scored as a positive result.

setting, three with 0.2 per cent sucrose, but none without sucrose.

Screening experiment Using the bilayer setting, the selected isolates from site 2 (three fast-growing isolates, A1–A3; five moderately fast-growing isolates, B1–B5; and four slow-growing isolates, C1–C4) were tested on several rooted microshoots. Again, in all cases the inoculated fungus grew well in the co-culture, and the emergence of new root tips (the 'positive result') was observed in the presence of some of the fungi (Figure 4). While A2, A3, B3, B4, B5 and C3 produced this response repeatedly in all trials, B1 and C1 were not as consistent. Negative results were consistently obtained with A1, C2 and C4.

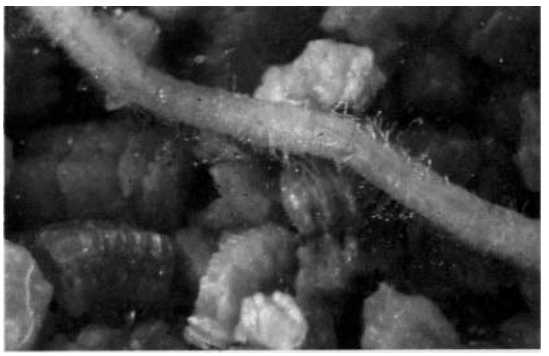

Figure 5. Further development of roots during the acclimation in vermiculite. The development of root hairs is evident in this example.

Monitoring

While in the vermiculite phase, both the shoots and root systems of all plants continued to grow, but growth was noticeably faster in plants that had positive response during the previous co-culturing step (i.e. with renewed growth in response to the inoculation). This increased growth was concurrent with the development of elongated roots with dense root hairs (Figure 5). At this stage no structures resembling ectomycorrhizas were visible. Only at the time of transferring potted plants to larger pots were the root morphologies found to be different from plant to plant (Figure 6), and the hypothesis that these specific structures were mycorrhizal was confirmed by the observation of diagnostic sheath and Hartig net profiles in cross-section (not shown).

In the second preliminary experiment, at the time of transferring potted plants to larger pots, the root morphologies were found to be different from plant to plant (Figure 6), and the hypothesis that these specific structures were mycorrhizal was confirmed by the observation of a diagnostic

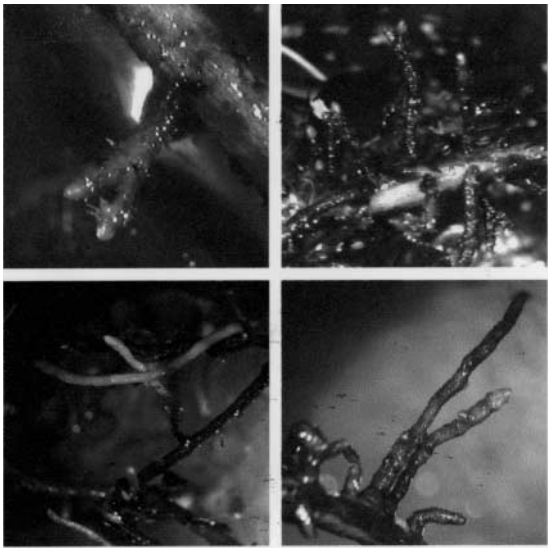

Figure 6. Examples of ectomycorrhizas on four inoculated pines from the second preliminary experiment, at the time of transfer to larger pots.

sheath and Hartig net profiles in cross-section (not shown).

The influence of renewed root growth in the co-culture step (the 'positive result') on subsequent stages of acclimation was already apparent in the vermiculite stage, with some tendency for the aerial parts to be more developed in plants with renewed root growth during co-culturing in comparison with those that did not have such growth ('negative results'), but in the pots this difference was noticed most easily (Figure 7). Plant survival during acclimation was 100 per cent in both groups, but the faster development of the plants that responded positively to inoculation shortened the schedule for planting out.

Discussion

The present study has shown that some ectomycorrhiza-derived fungal isolates can be useful in overcoming problems with achieving sustained root growth of *in vitro* propagated stone pine microshoots. By monitoring the plants after transfer to hydroponic culture and later to soil, enhanced vigour was obtained in plants that had shown positive results (renewed root growth) in the co-culture with some of the fungi tested.

Within the scope of the experiment, it was possible to ascertain that renewed root growth was restricted to rooted plantlets, and that it was still obtained with drastically reduced concentrations of sucrose, while retaining the

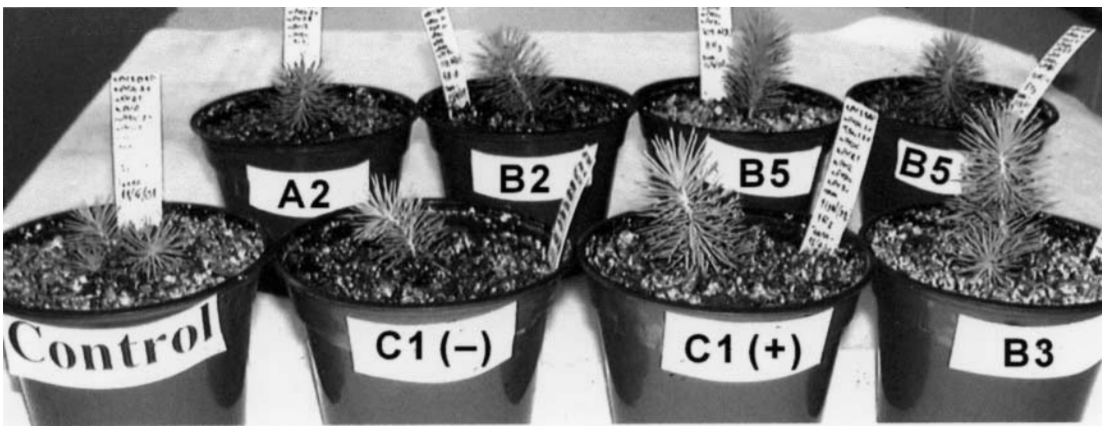

Figure 7. General appearance of potted plants from the screening experiment (fungi from site 2), at 14 weeks after transfer from vermiculite bottles. Control = uninoculated plant. The two plants inoculated with C1 are shown, the minus sign indicates the one that did not respond to the fungus and the plus sign indicates another that had new root growth in response to inoculation.

requirement for the sugar. These observations suggest that the fungi did not replicate the root initiation signalling which, in the case of *Pinus pinea* in our working conditions, was provided by a stage with auxin under cold temperature, in the presence of high sugar concentration (A.Z. Potes, S. Figueira, C. Cavaleiro and A. Peixa, unpublished).

The consistency shown by most fungi (either for positive or negative results) suggests that an isolate-specific signalling takes place between the fungus and the root system, possibly releasing a meristematic potential in cell groups located either apically, laterally or basally in the roots. The fact that isolates from oak or eucalyptus were also competent to elicit this growth in stone pine roots favours the hypothesis that such signalling might be shared among very different ectomycorrhizal plants, thus broadening the potential scope of the strategy explored here. Plausible clues for elucidating such mechanisms may come from research on ectomycorrhizal development (Barker and Tagu, 2000; Tagu *et al.*, 2000) and other *in vitro* root growth models (Stein and Fortin, 1990), and might lead to successful attempts at substituting chemically defined culture media for the fungi.

A significant proportion of the 12 fungal isolates from site 2 that were tested in the screening experiment gave positive results. Since the ectomycorrhizal community of each well-established tree is known to comprise many species (Zak, 1973; Mason *et al.*, 1983; Read, 1992; Pinho-Almeida *et al.*, 1999), and assuming that the present isolates were a relatively unbiased sample from the collection of pure cultures obtained from two soil cores, it might be expected that, for overcoming rooting limitations associated with *in vitro* culture, ectomycorrhiza-derived fungal isolates effective in restarting root growth can be picked from field samples with little effort.

Acknowledgements

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