Mycorrhiza (2002) 12:263–267
DOI 10.1007/s00572-002-0181-x

SHORT NOTE

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Pisolithus tinctorius promotes germination and forms mycorrhizal structures in Scots pine somatic embryos in vitro

Received: 23 November 2001 / Accepted: 2 May 2002 / Published online: 20 June 2002
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Abstract The results of the present study show that inoculation with the ectomycorrhizal fungus *Pisolithus tinctorius* (Pers.) Coker and Couch potentially enhances the germination of Scots pine (*Pinus sylvestris* L.) somatic embryos in vitro. Stimulation by *Pisolithus tinctorius* was only observed in the absence of direct contact between the symbionts; mature embryos were not sufficiently robust for balanced interaction with the fungus on half-strength DCR medium. Subsequently, on MMN medium with a reduced sugar concentration, direct contact between somatic embryo-derived plants and the fungus resulted in in vitro formation of mycorrhiza. Ex vitro inoculation also improved adaptation of the somatic embryo-derived plants, even though mycorrhizal structures were not observed. The reactions to *Pisolithus tinctorius* varied between different Scots pine cell lines both in vitro and ex vitro.

Keywords Ectomycorrhiza · Germination · *Pinus sylvestris* · *Pisolithus tinctorius* · Somatic embryogenesis

Introduction

Somatic embryogenesis has been suggested as a promising method for propagating coniferous species for clonal forestry purposes. Since the first report on somatic embryogenesis of *Picea abies* (L. Karst.) (Hakman et al. 1985), research has extended to a wide range of species, including pines (*Pinus* sp.). For Scots pine (*Pinus sylvestris* L.), embryogenic cultures have been established in several cell lines but very few embryo plants have been transferred to ex vitro conditions due to incomplete maturation and difficulties in germination and conversion (Keinonen-Mettälä et al. 1996; Häggman et al. 1999; Lelu et al. 1999).

Scots pine lives in a mutualistic interaction with ectomycorrhizal (ECM) fungi (Smith and Read 1997). It has been shown that the presence of compatible ECM fungi in the root system stimulates the formation of lateral roots (Karabaghli-Degron et al. 1998; Tranvan et al. 2000). The benefits of ECM fungi for adventitious root formation both in vitro (e.g. Gay 1990; Normand et al. 1996; Niemi et al. 2002) and in vivo (Linderman and Call 1977; Niemi et al. 2000) are also well documented. However, there are only two reports on attempts to promote root growth of somatic embryo plants using ECM fungi. With hybrid larch (*Larix* × *eurolepis* Henry), inoculation resulted in improved root growth and quality and subsequently in successful mycorrhiza formation in vitro (Piola et al. 1995). In contrast, mycorrhiza formation with Sitka spruce [*Picea sitchensis* (Bong.) Carr.] was observed only in ex vitro conditions (Sasa and Krogstrup 1991).

In the studies of Piola et al. (1995) and Sasa and Krogstrup (1991), inoculation was carried out after the radicle had emerged. The aim of the present study was to determine the ability of *Pisolithus tinctorius* (Pers.) Coker and Couch. to promote germination of Scots pine somatic embryos and, subsequently, to form mycorrhizas with the somatic embryo-derived plants in vitro. Effects on acclimatization of the plants to ex vitro conditions were also investigated.

Materials and methods

Biological material

Embryogenic cultures of Scots pine were derived from 1-year-old immature seed cones collected from open-pollinated elite clones 818 and 884 growing in the Punkaharju clone collection (61°48′ N,

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Table 1 Germination and subsequent root growth of Scots pine somatic embryos in the presence and absence of the ectomycorrhizal fungus *Pisolithus tinctorius*. The embryos were inoculated both in vitro on half-strength DCR medium and ex vitro in a greenhouse. Values, except for percent germination and survival, are means ± SE. Values within each cell line followed by different letters differ significantly at $P<0.05$. *I* shows percent germination after 4 weeks in culture. Non-germinated embryos were transferred to fresh medium for 4 further weeks and re-inoculated. *II* shows final percent germination after 8 weeks in culture; the fungal hyphae did not reach the germinated embryos within 4 weeks of dual culture. In the case of cell line K818/7, no statistical comparison of germination and survival percentages was performed due to the low number of embryos. Somatic embryo-derived plants from cell line K818/7 were grown for 25 weeks in a greenhouse and those from cell line K884/7 for 18 or 22 weeks, depending on the germination times

Cell line	Fungus	In vitro on half-strength DCR medium					Ex vitro in the greenhouse			
		n	% Germination		Root length (mm)	Laterals (No.)	Survival (%)	Shoot fresh wt. (mg)	Root fresh wt. (mg)	Root length (mm)
			I	II						
K818/7	Control	6	50.0	50.0	5.6±1.6a	1.0±0.5a	100	23.5±15.7b	17.5±13.5b	37.6±25.2b
	P. tinctorius	6	83.3	83.3	10.6±2.4a	2.2±0.7a	100	259.9±72.6a	297.8±85.8a	137.2±14.9a
K884/7	Control	22	22.7a	40.9a	4.9±0.5a	0.1±0.1a	66b	42.5±14.1a	47.6±17.1a	95.1±23.0a
	P. tinctorius	26	38.5a	57.7a	7.5±1.3a	0.5±0.2a	100a	40.1±14.6a	49.5±21.4a	73.5±15.4a

Table 2 Germination of Scots pine somatic embryos in the absence and presence of *P. tinctorius* on half-strength DCR medium and subsequent mycorrhiza formation on MMN2 medium. Values, except for percent germination, are means ± SE. Values within each cell line followed by the same letters did not differ significantly at $P<0.05$. *I* shows percent germination after 4 weeks in culture. Non-germinated embryos were transferred to fresh medium for 4 further weeks and re-inoculated. *II* shows final percent germination after 8 weeks in culture; the fungal hyphae did not reach the germinated embryos within 4 weeks of dual culture. In the case of cell line K884/6, control embryo-derived plants and *P. tinctorius*-treated plants were pooled and separately inoculated with *P. tinctorius* to study mycorrhiza formation in vitro. Dual culture on MMN2 medium was for 7 weeks

Cell line	Fungus	In vitro on half-strength DCR medium					In vitro on MMN2 medium					
		n	% Germination		Root length (mm)	Laterals (No.)	n	Shoot fresh wt. (mg)	Root fresh wt. (mg)	Root length (mm)	Laterals (No.)	Mycorrhizal laterals (%)
			I	II								
K884/6	Control	22	0	22.7a	11.0±5.0a	2.0±1.5a	17	28.2±9.9	28.6±12.1	29.5±10.8	30.1±14.9	72
	P. tinctorius	25	24.0	48.0a	10.8±2.1a	1.1±0.5a						
K818/9	Control	5	0	0								
	P. tinctorius	5	0	0								
K818/10	Control	4	0	0								
	P. tinctorius	5	60.0	60.0	6.0±3.2	0.6±0.6	3	42.5±4.3	43.6±11.4	22.0±2.0	10.3±3.8	81

29°17′ E). The embryogenic cultures were initiated as described by Häggman et al. (1999) and induction and proliferation of the embryogenic cultures were carried out on DCR-based media (Gupta and Durzan 1985; Becwar et al. 1990) as previously described (Sarjala et al. 1997).

Plating of embryogenic cell masses for the induction of maturation was as described by Klimaszewska and Smith (1997) with small modifications. The suspension was filtered through a moist filter paper in a Büchner funnel at low pressure. The filter paper covered with a thin layer of embryogenic mass was transferred onto DCR medium supplemented with 90 µM abscisic acid (Sigma), 7% (w/v) polyethylene glycol 4000 (PEG, Aldrich) and 176 mM sucrose for 2 weeks and then cultured for 2 weeks on the same medium lacking PEG. Finally, the maturing cultures were transferred onto DCR medium containing 88 mM sucrose and cultured as described by Häggman et al. (1999).

The ECM fungus *Pisolithus tinctorius* was originally isolated under Scots pine in Sweden (Strandberg-Arveby 1980) and was deposited in the culture collection of the Swedish University of Agricultural Sciences, Uppsala, Sweden (strain 1984a). Our previous study showed that the fungus still readily forms mycorrhizas with Scots pine seedlings (Niemi et al. 2002). For the present study, the fungus was maintained by culturing the mycelium on Melin-Norkrans (MMN1) medium (pH 5.8) (Marx 1969) modified by Heinonen-Tanski and Holopainen (1991).

Germination of somatic embryos

Culture petri dishes, 9 cm in diameter, contained 25 ml of half-strength DCR medium supplemented with 58 mM sucrose and covered by a filter paper. Preliminary experiments showed that somatic embryos thrive when close to each other and, therefore, four to six mature embryos detached from the embryogenic mass were placed on the filter paper in each petri dish. Four to five petri dishes per treatment were prepared for cell lines K884/6 and K884/7, whereas only one petri dish per treatment was used for cell lines K818/7, K818/9 and K818/10 due to the low number of mature embryos. Inoculation with *P. tinctorius* was carried out by placing a 5-mm-diameter mycelial agar plug cut from the edge of a 4-week-old culture at a distance of about 2 cm from the base of the embryos. *P. tinctorius* starts to grow relatively slowly on DCR medium containing sucrose and, thus, this distance was large enough to avoid contact between the embryos and mycelium. Direct contact between the fungus and somatic embryos was prevented because, in preliminary experiments, fungus growing close to small somatic embryos overgrew the entire embryos. In the control cultures an agar plug was substituted for the mycelial plug. The fungus and the base of the embryos were covered by a semicircular piece of moist filter paper to prevent desiccation. The fungal mycelium was also protected from light by a semicircular piece of brown paper placed on the lid of the petri dish. The petri

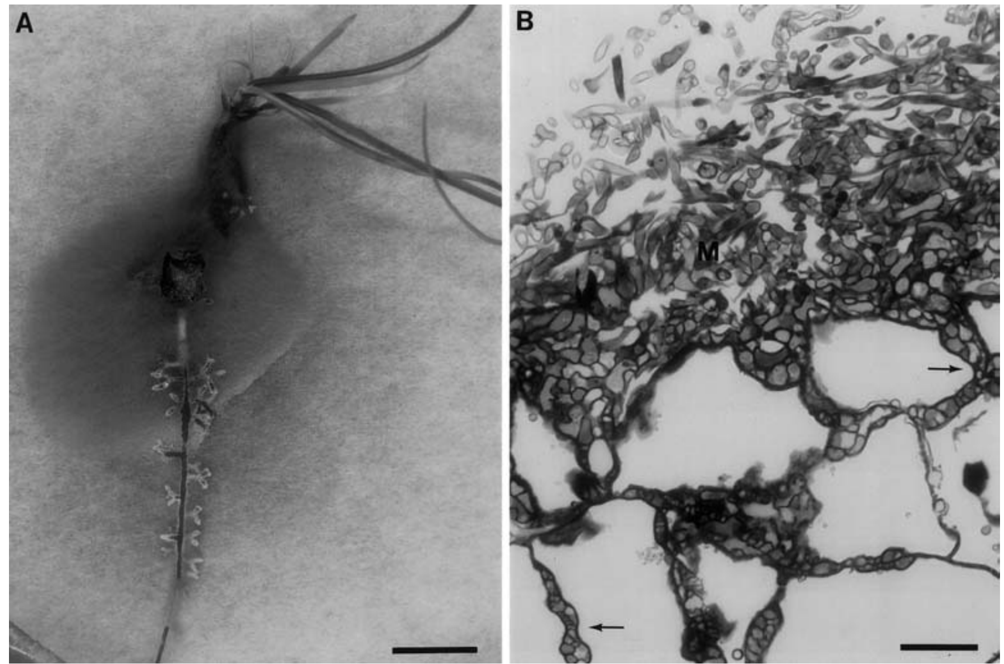

Fig. 1A, B In vitro mycorrhiza formation by *Pisolithus tinctorius* in somatic embryo-derived plants from the Scots pine cell line K884/6. The dual culture was carried out for 7 weeks on MMN2 medium supplemented with 7.3 mM sucrose. **A** Dichotomously branched lateral roots covered by a hyphal mantle; *bar* 1 cm. **B** A longitudinal section of mycorrhiza, showing the mantle (*M*) and Hartig net (*arrows*) formed by *P. tinctorius*; *bar* 25 μm

dishes were slanted at 70° and incubated in a growth chamber at 25±1°C with a 16-h photoperiod (140–150 μmol m^{-2} s^{-1}).

Germination was examined at the end of the 4-week culture period (germination I in Tables 1 and 2). At the time of harvest, root length and number of lateral roots on the germinated embryos were measured. Subsequently, the germinated embryos were either transferred to ex vitro conditions in a greenhouse or used in the study on mycorrhiza formation in vitro. The embryos that did not germinate were transferred for further 4 weeks onto fresh medium and re-inoculated with *P. tinctorius*. After harvesting, these late-germinated embryos were treated as above.

Acclimatization to ex vitro conditions

In midsummer, germinated embryos of cell lines K818/7 and K884/7 were adapted to ex vitro conditions in a greenhouse by transplanting them to a non-fertilized peat (pH 3.8, VAPO, Finland):perlite (2:1) mixture. Re-inoculation with *P. tinctorius* was carried out by placing a mycelial agar plug close to the developing root system. Control somatic embryo-derived plants were planted without any treatment. During the growth period, the plants were fertilized once with 0.2% 5-Superex fertilizer (Kekkilä, Finland). The somatic embryo-derived plants from cell line K818/7 were grown in the greenhouse for 25 weeks and those from cell line K884/7 for 22 or 18 weeks, depending on the germination time. At the time of harvest, shoot and root fresh weights and root length were determined. The number of lateral roots covered by a hyphal mantle was evaluated under a dissecting microscope.

Mycorrhiza formation in vitro

The ability of somatic embryo-derived plants of cell lines K818/10 and K884/6 to form mycorrhizas with *P. tinctorius* in vitro was studied on MMN2 medium supplemented with 7.3 mM sucrose (Marx 1969). A somatic embryo-derived plant was transferred to agar medium covered by a sterile moist filter paper and a mycelial agar plug was placed close to the root system. The root system and the fungus were covered by a semicircular piece of moist filter paper to prevent desiccation. In the case of cell line K884/6, control plants and *P. tinctorius*-treated plants were pooled and separately inoculated with the fungus. The dual cultures were incubated in a growth chamber as described above and harvested 7 weeks later. The number of lateral roots covered by a hyphal mantle was evaluated with a dissecting microscope.

Light microscopy

After analysis under a dissecting microscope, mycorrhizal root tips were chosen for further examination by light microscopy. Root tips were prefixed in 0.1 M phosphate buffer (pH 7.0) containing 2.5% glutaraldehyde for 1 day and then postfixed for 4 h in 1% osmium tetroxide and dehydrated in a graded ethanol series. The root samples were infiltrated and embedded in Ladd's LX 112 resin. The sections were cut with an LKB III Ultratome and stained with toluidine blue (Merck). Root sections were observed with a Nikon Microphot FXA light microscope.

Statistical analyses

Differences in germination frequency between control and inoculated somatic embryos from cell lines K884/6 and K884/7 were analysed by χ^2 test. This test was also used to compare ex vitro survival percentages within cell line K884/7. Differences in growth parameters between cell lines K818/7, K884/6 and K884/7 were tested using a non-parametric Mann-Whitney U-test. All statistical analyses were conducted with SPSS/PC (version 9.0).

Results

For four out of five tested cell lines, the presence of *Pisolithus tinctorius* apparently increased both the number of embryos germinating within the first 4 weeks and the final germination frequency of the somatic embryos (Tables 1 and 2). Induction of germination occurred only when the mycelium and embryo were not in direct contact (data from our preliminary experiment and the present study). This culture system did not lead to positive effects on subsequent root growth of the somatic em-

bryo-derived plants in vitro (Tables 1 and 2). Adaptation to ex vitro conditions was improved in two tested cell lines and this occurred without mycorrhiza formation by *P. tinctorius.* The fungus significantly enhanced the ex vitro growth of plants from cell line K818/7 (P<0.05) (Table 1), whereas with cell line K884/7 the percentage survival of the somatic embryo-derived plants was significantly (P<0.05) improved due to inoculation (Table 1). As was the case with *P. tinctorius*, mycorrhiza formation by fungi naturally present in the soil was poor and only a few lateral roots were covered by a thick black mycelial mantle.

Mycorrhiza formation on somatic embryo-derived plants from two cell lines was studied on MMN2 medium. After the 7-week culture period, mycorrhizas formed by *P. tinctorius* were observed on all plants from cell line K818/10 and on half of those from K884/6. The rest of the plants of cell line K884/6 did not form lateral roots. The mycorrhiza frequency, determined as the number of lateral roots covered by a hyphal mantle, was high for both cell lines (Table 2, Fig. 1A). The hyphal mantle consisted of several cell layers and Hartig net formation had started (Fig. 1B).

Discussion

Results from the present study indicate that *Pisolithus tinctorius* can induce in vitro germination of Scots pine somatic embryos. Thus, inoculation with a specific ECM fungus may be a potential tool to improve later stages of somatic embryogenesis. The degree of the responses to the fungus were dependent on the cell line, which is in agreement with our earlier observations on the specific interaction between this fungal strain and certain Scots pine genotypes during proliferation of embryogenic cell masses (Niemi et al. 1998), as well as during adventitious root formation (Niemi et al. 2000). Piola et al. (1995) inoculated somatic embryo-derived plants from one hybrid larch cell line and found enhanced root elongation and branching due to specific ECM fungi. On the other hand, in the in vitro study of Sasa and Krogstrup (1991), ECM fungi were too aggressive and overgrew Sitka spruce somatic embryo-derived plants with short radicles. This was also the case in our preliminary experiments (data not shown) in which the mycelial agar plug was placed close to the embryos on the half-strength DCR medium with a high sucrose concentration. In the present study, mycelium was not in direct contact with germinating embryos. This lack of contact suggests that improved germination is at least partly due to compounds released by *P. tinctorius*. In our earlier studies, this strain was shown to produce indoleacetic acid (Niemi et al. 2000) and diamine cadaverine (Niemi et al. 2002), both of which have been reported to be involved in rooting and root growth (Gay 1990; Normand et al. 1996; Niemi et al. 2002).

To our knowledge, this is the first time that ECM symbiosis has been established on Scots pine somatic embryo-derived plants. A low sucrose concentration in the MMN2 medium balanced the relationship between the symbiotic partners and resulted in successful mycorrhiza formation on both Scots pine cell lines tested. Piola et al. (1995) found mycorrhiza formation in the roots of hybrid larch in vitro, whereas in Sitka spruce mycorrhizas were formed only in ex vitro conditions (Sasa and Krogstrop 1991). The results of the present study are in agreement with those of Sasa and Krogstrop (1991), which suggested that the shoot and root systems must attain a certain developmental stage before physical contact with a fungus is possible.

Acclimatization of the somatic embryo-derived plants to ex vitro conditions was improved by *P. tinctorius*, although inoculation in these conditions did not result in mycorrhiza formation. Thus, the host plant may also benefit from the fungus without mycorrhiza formation. Facilitated adaptation has been found also on rooted in vitro shoots inoculated before transfer to ex vitro conditions (Supriyanto and Rohr 1994; Martins et al. 1996; Normand et al. 1996). In the study of Martins et al. (1996), the percentage of mycorrhizal root tips correlated positively with the survival of micropropagated *Castanea sativa* (Mill.) plants (Normand et al. 1996), whereas in the study of micropropagated shoots of *Pinus pinaster* (Ait.) Sol., and in the present study with Scots pine somatic embryo plants, positive responses during acclimatization were dependent on both plant and fungus genotypes.

Acknowledgements We are grateful to Ms Elina Hynynen, Ms Jaana Rissanen and Mr Jouko Lehto for their technical assistance. We also thank Dr Helvi Heinonen-Tanski for critical reading and John Derome for checking the language of the manuscript. This work was supported by the Graduate School of Forest Sciences, the Maj and Tor Nessling Foundation and the Finnish Cultural Foundation.

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