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Interaction between embryogenic cultures of Scots pine and ectomycorrhizal fungi

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Abstract Embryogenic cell masses of three Scots pine (*Pinus sylvestris*) cell lines K779, K884 and K1009 were cultivated with the ectomycorrhizal (ECM) fungi *Laccaria bicolor*, *L. proxima, Pisolithus tinctorius*, *Paxillus involutus* and two strains of *Suillus variegatus*. The average growth ratio of the slowly proliferating cell line K1009 was improved by *L. proxima* and *S. variegatus* strain H, while of the rapidly proliferating lines K779 and K884 the non-mycorrhizal controls grew best. The fungi caused two distinct reactions in embryogenic cultures. In the positive reaction, the shape and light yellow colour of the cultures resembled the controls, while in the negative reaction the embryogenic cells became brown and necrotic and the fungi grew aggressively over them. These reactions to the fungi did not correlate completely with effects on the growth ratio. All the cell lines enhanced the radial growth of *S. variegatus* H and of *P. tinctorius*, while the *Laccaria* species and *S. variegatus* strain 1 thrived better alone. This study shows that early-stage embryogenic cells of Scots pine and ECM fungi are able to interact. As some fungi produced a positive reaction or even increased proliferation, they could be used to enhance somatic embryogenesis of Scots pine. Specific fungi might be used to induce the growth of slowly proliferating cell lines, and knowledge of positive cell line-fungus interactions could be useful in work with later stages of somatic embryogenesis, such as rooting.

Key words Embryogenic culture · Ectomycorrhizal fungi · Proliferation

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Introduction

Somatic embryogenesis, which is the development of embryos from somatic cells cultured in vitro, provides the possibility both to study the process of plant differentiation and to produce genetically homogenous plant material. Although somatic embryogenesis has been reported to succeed more or less completely in many pine species (*Pinus* sp.) (e.g. Gupta and Durzan 1986; Laine and David 1990; Jones and van Staden 1995; Keinonen-Mettälä et al. 1996), only from *Pinus radiata* (D. Don) have somatic embryo plants been produced on a commercial scale (Smith et al. 1994).

Immature megagametophytes containing immature zygotic embryos have been used most often as explants for initiating somatic embryogenesis of pine species (Gupta and Durzan 1985; Laine and David 1990; Jones and van Staden 1995). This was also the case with Scots pine (*Pinus sylvestris* L.) (Keinonen-Mettälä et al. 1996; Sarjala et al. 1997). The initiation frequencies in pine species are, however, typically low: 1–5% as reviewed by Becwar and Pullman (1995) and 15% in *Pinus pinaster* (Soland *in* Ait.) (Bercetche and Paques 1995). It has been speculated that megagametophytes provide extra nutrients and / or phytohormones which may be suboptimal in the culture medium (Becwar and Pullman 1995). The growth rate of different embryogenic cell lines (i.e. genotypes) has also been shown to vary, thus indicating different nutritional requirements, e.g. in *Pinus caribea* (Morelet var. *hondurensis)* (David et al. 1995) and *Pinus pinaster* (Bercetche and Paques 1995). The main difficulties in somatic embryogenesis of pine species are, however, in the maturation and germina-

tion phases (Jones and van Staden 1995; Keinonen-Mettälä et al. 1996).

Forest trees are highly dependent on mycorrhizal symbiosis, in which an efficient mechanism for water and nutrient uptake is established. Positive effects of ECM fungi on the rooting of coniferous plantlets, nutrient uptake and survival (Gay 1990; Supriyanto and Rohr 1994; Normand et al. 1996) might also contribute to improving the somatic embryogenesis of conifers. So far, such fungi have been used only to induce root formation of somatic embryo plantlets. In both hybrid larch *Larix* × *eurolepis* Henry (Piola et al. 1995) and Sitka spruce *Picea sitchensis* (Bong.) Carr. (Sasa and Krogstrup 1991), shoot and root growth were stimulated by specific fungi, but mycorrhiza formed only in hybrid larch. To our knowledge, ECM fungi have not been used in somatic embryogenesis of Scots pine.

The aim of the present work was to study the interaction between ECM fungi and early-stage embryogenic cultures of Scots pine. This was done, firstly, to determine how ECM fungi affect embryogenic cultures lacking a differentiated root system, and, secondly, to evaluate the possibility to use ECM fungi to enhance the somatic embryogenesis of Scots pine.

Materials and methods

Plant material

At the end of June 1995, when the heat sum had reached 433 day degrees (i.e. the daily mean temperature minus the adapted +5 °C base temperature), 1-year-old immature seed cones were collected from open-pollinated elite clones K779, K1009 and K884 of Scots pine growing in the Punkaharju clone collection (61° 48'N; 29°17'E). Immature seeds were removed from the cones and the surface was sterilized for 1 min in 70% ethanol, after which the immature female gametophytes with the immature zygotic embryos were excised aseptically for culturing.

Ectomycorrhizal fungi

Pure cultures of *Laccaria bicolor* (Maire) Orton, *L. proxima* (Poud.) Pat, *Paxillus involutus* (Batsch: Fr.) Fr., *Pisolithus tinctorius* (Pers.) Coker and Couch and two *Suillus variegatus* (Sw.: Fr.) O. Kunze strains were used in dual cultures with the Scots pine cell lines. The strain *S. variegatus* 1 was isolated from central Finland and strain H from southern Finland. *L. proxima* was tested only with cell lines K779 and K1009. The collections from which the fungal species and strains were taken are listed on Table 1. The fungi were maintained in the dark at 19 °C and subcultured routinely on modified Melin Norkrans (MN) medium as described by Heinonen-Tanski and Holopainen (1991).

Table 1 Fungal species/strains ectomycorrhizal with Scots pine and used in this study, with collection details

Species/strain	Collection
Laccaria bicolor	Dept. of Forest Pathology and Mycology University of Agricultural Sciences, Sweden
Laccaria proxima	Dept. of Microbiology and Ecology University of Lund, Sweden. Strain 90003
Paxillus involutus	Dept. of Environmental Sciences University of Kuopio, Finland. Strain 7
Pisolithus tinctorius	Dept. of Environmental Sciences University of Kuopio, Finland. Strain 13
Suillus variegatus 1	Dept. of Environmental Sciences University of Kuopio, Finland. Strain 68
Suillus variegatus H	Dept. of Environmental Sciences University of Kuopio, Finland. Strain 5

Initiation and maintenance of embryogenic cultures

Immature female gametophytes, including the immature zygotic embryos with suspensor tissues, were cultivated on a slightly modified DCR medium (DCR1), which was originally used for Douglas-fir *Pseudotsuga menziesii* (Mirb.) Franco by Gupta and Durzan (1985) and later on in the initiation of embryogenic cultures of several pine species (e.g. Jones and van Staden 1995; Becwar et al. 1990; Keinonen-Mettälä et al. 1996). The basal medium (pH 5.8) included the following inorganic compounds: 5 mM NH_4NO_3, 3.3 mM KNO_3, 2.3 mM $Ca(NO_3)_2 \cdot 4H_20$, 1.5 mM $MgSO_4 \cdot 7H_20$, 1.2 mM KH_2PO_4, 0.57 mM $CaCl_2 \cdot 2H_20$, 5 μM KI, 0.1 mM H_3BO_3, 0.13 mM $MnSO_4 \cdot H_20$, 0.03 mM $ZnSO_4 \cdot 7H_20$, 1 μM $Na_2MoO_4 \cdot 2H_20$, 1 μM $CuSO_4 \cdot 5H_20$, 0.1 μM $CoCl_2 \cdot 6H_20$, 0.1 μM $NiCl_2 \cdot 6H_20$, 0.1 mM $FeSO_4 \cdot 7H_20$, and 0.1 mM Na_2EDTA. The organic compounds in the medium were 4.1 μM nicotinic acid, 2.4 μM pyridoxine-HCl, 3 μM thiamine-HCl, 26.6 μM glycine, 1.1 mM myo-inositol, 500 mg/l casein hydrolysate (Sigma), 1.7 mM L-glutamine, and 87.6 mM sucrose. The phytohormones used were 13.6 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.2 μM benzylaminopurine (BA). Aqueous stock solutions of phytohormones and L-glutamine were filter sterilized and added to the medium after autoclaving. The medium was solidified with 2.5% gelrite (Merck).

The explants were first cultured on DCR1 medium in plastic petri dishes for 4 weeks and then transferred to new positions on the same plate for a further 2 weeks. Thereafter, the embryogenic tissues were excised from responsive explants and transferred to fresh DCR1 medium (Becwar et al. 1990). Embryogenic cultures were initiated and maintained in the dark at 21 °C. To maintain the embryogenic tissues, they were subcultured every 4 weeks for several months. Embryogenic cultures of each cell line were originally derived from one explant.

Experimental designs

Three or four aliquots of embryogenic cells were weighed aseptically (70–250 mg fresh wt.) and placed equidistantly from each other onto fresh DCR1 medium in plastic petri dishes (90 mm). After 2 weeks in the dark at 21 °C, the embryogenic cell masses were inoculated with ECM fungi to give dual cultures. A mycelial plug 5 mm in diameter cut from the margin of a 1-month-old fungal colony was placed in the middle of the petri dish, about 1 cm from each embryogenic explant. The controls were embryogenic cells without fungal mycelium. Two weeks later, the embryogenic cell masses were detached from the medium, cleaned and reweighed. The growth ratio of the pieces was expressed as w2 / w1, in which w1 was the initial fresh weight and w2 the fresh weight 4 weeks later at the time of harvest. Some cultures were grown for a longer time, so that the reactions of embryogenic cells could be observed after fungal hypha had reached them. At the end of the experiment, the radial growth of the fungi in the presence or absence of embryogenic cells was also measured.

In the experiment with cell lines K779 and K1009, the medium was solidified with 1% washed agar and the medium for line K884 was solidified with 2.5% gelrite. Gelrite was chosen for K884, because this line turned brown and was not able to grow on agar.

In order to analyze the effects of the phytohormones used in proliferation medium on the radial growth of ECM fungi, the fungi were cultivated for 3 weeks on DCR media with or without 2,4-D or BA. The media used were (1) DCR without phytohormones, (2) DCR with 13.6 μM 2,4-D and 2.2 μM BA (DCR1), (3) DCR with only 13.6 μM 2,4-D, and (4) DCR with only 2.2 μM BA. All the media were solidified with 2.5% gelrite (Merck).

Scanning electron microscopy

Interactions between the fungus and the embryogenic cells of Scots pine were examined with scanning electron microscopy. Embryogenic cells in connection with a fungus were fixed in 6.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h and dehydrated in a graded series of ethanol. Some embryogenic cells of K884 were also dissolved in 5% (w/v) Ariel washing powder at 38 °C for 16–18 h to remove the cytoplasm of the embryogenic and fungal cells (Honegger 1985) and were then fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.0) for 6 h and dehydrated in a graded series of ethanol. Both glutaraldehyde and osmium tetroxide-fixed segments in pure ethanol were subjected to a critical point dryer, mounted on aluminium stubs and coated with gold and palladium (80 / 20%) in a sputter coater (Polaron E 5100). Observations were made at 15 kV in a Jeol JSM 35 scanning electron microscope.

Statistical analysis

The data on growth of embryogenic cultures and fungi were log-transformed to correct the heterogeneity of the variance. The differences between the treatments were tested by one-way ANOVA combined with the Tukey-HSD or Scheffe tests ($P<0.05$), except the data on the radial growth of fungi in the presence or absence of embryogenic tissues, which were tested by Student's *t*-test.

Results

Effects of ECM on embryogenic cultures

The mean growth ratios of the control cultures of cell lines K884 and K779 were significantly higher ($P<0.05$) than line K1009 (Fig. 1). In dual cultures, the mean growth ratios of K884, K779 and K1009 were 3.54 (SE ±0.16), 2.32 (±0.26) and 1.42 (±0.13), respectively, and differed significantly from each other. Two mycorrhizal strains, *L. proxima* and *S. variegatus* 1, significantly improved the growth of cell line K1009, while with lines K779 and K884 the control cultures grew more or the same as dual cultures (Fig. 1).

In the control cultures, immature somatic embryos with an embryogenic head cell area and elongated suspensor cells were observed. In dual cultures, the ECM fungi caused two distinct reactions. In a positive reaction, the fungus partly covered some embryogenic cells (Fig. 2a) which were translucent to light yellow in colour, like in the controls. In a negative reaction, the fungus caused browning and necrosis and grew aggressively over embryogenic cells (Fig. 2b). The changes in colour were often seen before the fungi had grown close to embryogenic cells. The cell lines K779 and K884 partly differed from K1009 in reactions to specific ECM fungi

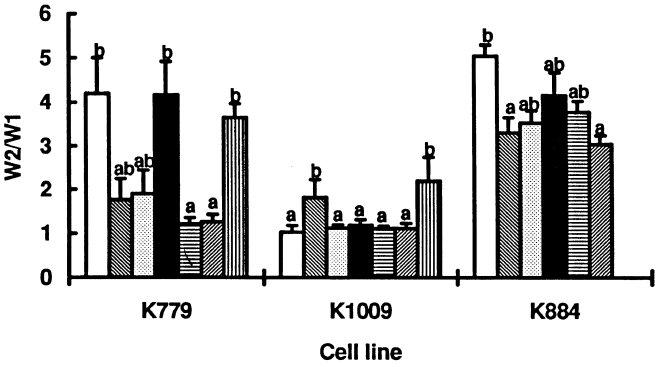

Fig. 1 Effect of ectomycorrhizal (ECM) fungi on the mean growth ratio (w2 / w1) of embryogenic cultures of Scots pine (w1 the initial fresh weight, w2 the fresh weight 4 weeks later at the time of harvest) □ control; ▨ with *Suillus variegatus* 1; ⊡ with *S. variegatus* H; ■ with *Pisolithus tinctorius*; ▤ with *Paxillus involutus*; ▨ with *Laccaria bicolor*; ▥ with *L. proxima*. The *letters a and b* indicate significant differences within the cell line at $P<0.05$ according to Tukey HSD test ($n=6$ with the cell lines K779 and K1009, $n=12$ with the line K884). The *vertical bars* represent ±SE

(Table 2). The reactions did not correlate with growth ratios, for example, *L. bicolor* caused a positive response in embryogenic cells although it decreased the growth of K779 and K884. When fungal growth inside the tissues of K884 was studied, penetration into the highly vacuolated suspensor cells, but not into head cells, was observed in all fungal treatments (Fig 2c). Penetration was, however, more regular and intensive in dual cultures with *Paxillus involutus* and *S. variegatus* 1, which caused necrotic areas in the culture.

Effects of embryogenic cultures on ECM fungi

The average radial growth of all the fungi was significantly higher ($P<0.005$) on the medium solidified with gelrite than on that solidified with agar (Table 3). All three cell lines significantly improved the radial growth of *S. variegatus* H. *Pisolithus tinctorius* thrived with

Table 2 Reactions of three different cell lines of Scots pine in the presence of an ectomycorrhizal (ECM) fungus (+ embryogenic cells looked like the controls, elongated suspensor cell with small clustures of cytoplasmic cells, light yellow, − fungus grew over embryogenic cells which were dark brown and even necrotic, *0* the fungus did not reach the embryogenic cells, *ND* not determined)

Fungus	Cell line		
	K779	K1009	K884
Laccaria bicolor	+	+	+
Laccaria proxima	+	+	ND
Paxillus involutus	−	−	−
Pisolithus tinctorius	+	−	+
Suillus variegatus 1	−	+	−
Suillus variegatus H	+	0	+

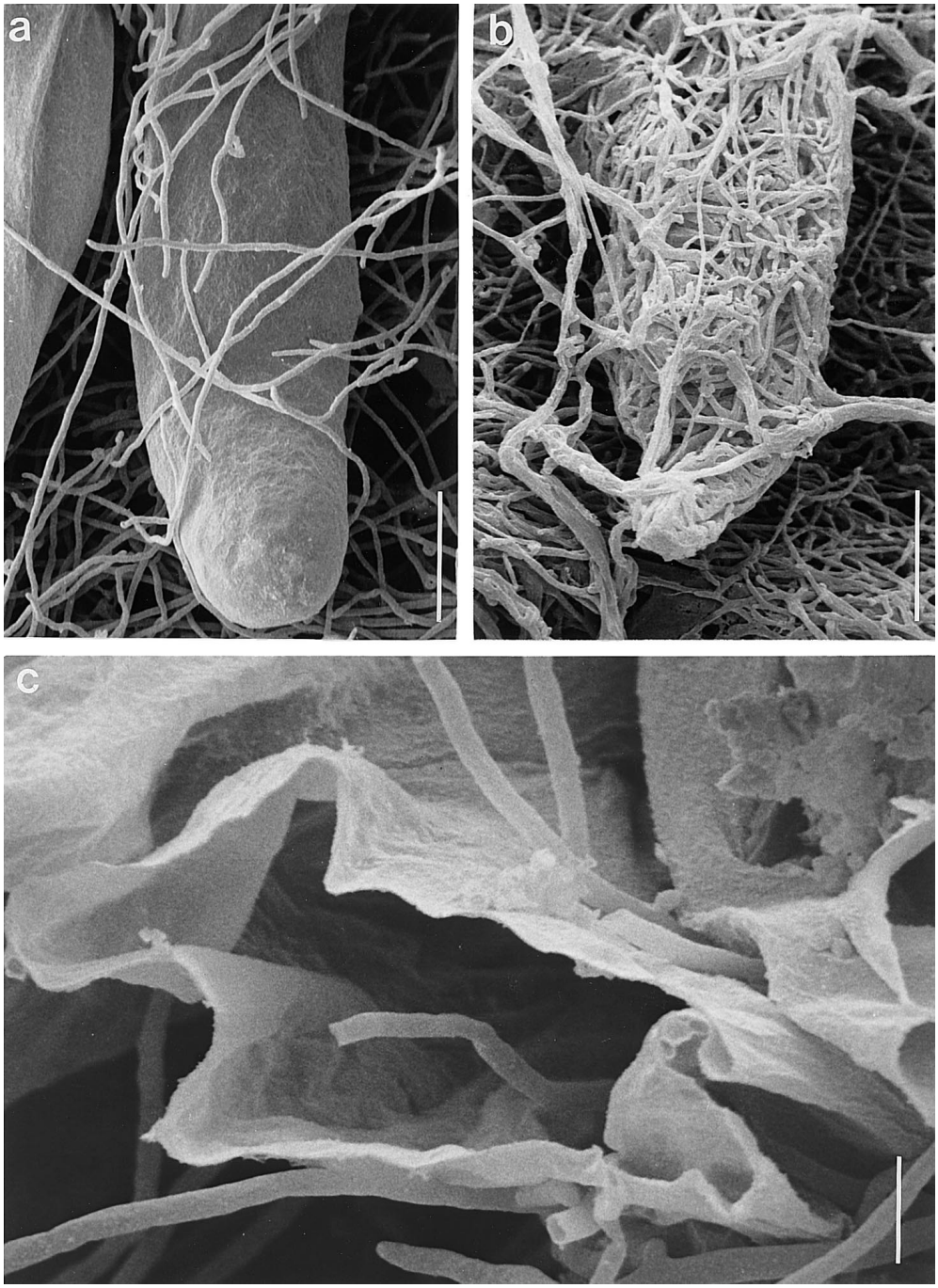

Fig. 2a–c Scanning electron micrographs of dual cultures of ECM fungi and embryogenic tissues of *Pinus sylvestris*. **a** In a positive reaction, *Laccaria bicolor* partly covers the suspensor cells of the cell line K779; *bar* 50 μm. **b** In a negative reaction, *Paxillus involutus* grows aggressively over the suspensor cells of the cell line K1009; *bar* 50 μm. **c** In a positive reaction, *Laccaria bicolor* penetrates the suspensor cell of the cell line K884; *bar* 10 μm

lines K779 and K1009, and K884 enhanced the growth *Paxillus involutus* so that the fungus grew over the whole petri plate. In contrast, both *Laccaria* species and *S. variegatus* 1 thrived as well or even better alone than in dual culture.

Both in control and dual cultures, *Paxillus involutus, Pisolithus tinctorius* and both strains of *S. variegatus* started to produce brown pigments soon after inoculation. The cell line K1009 induced the exudation of pigments by *Pisolithus tinctorius* and mycelia of *L. bicolor* were more violet in colour in the presence of all cell lines.

Growth of ECM fungi on different phytohormone media

The different phytohormone treatments had no common effect on the mean radial growth of the fungi, because only the growth of *Paxillus involutus* and *Pisolithus tinctorius* changed significantly (Table 4). The growth of *Paxillus involutus* was higher ($P<0.05$) on DCR medium with 2,4-D alone than on DCR1 supplement with both 2,4-D and BA. In the case of *Pisolithus tinctorius*, both phytohormone-free and DCR1 resulted in higher growth ($P<0.05$) than the medium with only BA.

Table 3 Radial growth of ECM fungi in the presence or absence (control) of embryogenic cultures of three Scots pine cell lines. Values are means (±SE) of three replicates. Significance of differences from the controls assessed by Student's *t*-test are indicated with asterisks * $P<0.05$, ** $P<0.01$ (*ND* not determined)

Fungal species and Scots pine cell lines	Radial growth (cm)	
	agar	gelrite
Laccaria bicolor		
Control	1.81 ± 0.10	3.06 ± 0.07
K779	1.44 ± 0.04*	
K1009	1.38 ± 0.03*	
K884		2.38 ± 0.12*
Laccaria proxima		
Control	0.80 ± 0.09	ND
K779	0.46 ± 0.03*	
K1009	0.76 ± 0.05	
K884		ND
Paxillus involutus		
Control	1.17 ± 0.10	1.83 ± 0.08
K779	1.40 ± 0.03	
K1009	1.04 ± 0.07	
K884		4.00 ± 0.01**
Pisolithus tinctorius		
Control	0.09 ± 0.02	1.46 ± 0.03
K779	0.93 ± 0.16**	
K1009	0.96 ± 0.25**	
K884		1.61 ± 0.10
Suillus variegatus 1		
Control	1.21 ± 0.04	1.63 ± 0.04
K779	1.06 ± 0.06	
K1009	1.16 ± 0.09	
K884		1.23 ± 0.08*
Suillus variegatus H		
Control	0.31 ± 0.01	0.46 ± 0.01
K779	0.56 ± 0.08*	
K1009	0.58 ± 0.01**	
K884		1.43 ± 0.07**

Discussion

Effects of ECM fungi on embryogenic cultures

Somatic embryogenesis offers an interesting way to study interactions between ECM fungi and plants at different stages of development. Until now, the rooting of somatic embryo plantlets was the only stage where the effects of ECM fungi have been reported (Sasa and Krogstrup 1991; Piola et al. 1995). In this study, early-stage embryogenic cells of Scots pine, with no differentiated roots, interacted with ECM fungi, and both positive and negative reactions of embryogenic cells were observed. These results are in agreement with those of Sirrenberg and co-workers (1995) obtained in an experiment with ECM fungi and callus cells derived from radicles of Norway spruce *Picea abies* (L.) Karst. germlings.

The effects observed here were dependent on both the cell line and the ECM symbiont. None of the fungi increased the mean growth ratio of the rapidly proliferating cell lines K779 and K884, but significant growth improvement was observed with the slowly proliferating line K1009 in dual cultures with *L. proxima* and *S. variegatus* 1. The reason for induction of proliferation is not clear, but it may be due to the presence of suitable phytohormones, like auxins, which many fungal strains have been shown to produce without exogenous precursors in aseptic cultures (Ho 1987; Rudawska and Kieliszewska-Rokicka 1997). Higher growth of K1009 in these dual cultures means that specific ECM fungi might be used to induce the growth of slowly proliferating cell lines which hamper the development of somatic embryogenesis of Scots pine.

The reason for the inhibited growth of fast-proliferating cell lines K779 and K884 in dual cultures may be changes in hormonal balance in the media caused by ECM fungi. Another possible reason for inhibition is the high concentration of sucrose (87.6 mM) needed for proliferation of embryogenic cultures. In nature, an ECM fungus is highly dependent on sugar formed by its symbiont plant. In aseptic cultures, exogenous sugar is known to increase mycelial growth and cause an imbalance in the symbiotic relationship (Duddridge 1986). In addition to higher growth, exogenous sugar induces production of fungal metabolites, like phenols, which have been suggested to reduce the growth of many tree species in closed culture systems *in vitro* (Hutchison and Piche 1995). In our study, exudation of dark brown pigments by *Paxillus involutus*, *S. variegatus* 1 and also *Pisolithus tinctorius* was very clear and was observed soon after inoculation. These compounds were proba-

Table 4 Growth of ECM fungi on DCR media supplemented with different concentrations of phytohormones 2,4-D and BA. Values are means (±SE) of three replicates. The small letters a and b indicate significant differences within the lines at $P<0.05$ according to Tukey HSD

Fungal species	Radial growth (cm)			
	No phytohormones	2,4-D + BA	2,4-D	BA
Laccaria bicolar	2.83 ± 0.10a	2.83 ± 0.11a	2.81 ± 0.03a	2.69 ± 0.06a
Laccaria proxima	1.90 ± 0.20a	1.13 ± 0.27a	1.20 ± 0.20a	1.23 ± 0.25a
Paxillus involutus	1.75 ± 0.25ab	1.55 ± 0.18a	2.43 ± 0.07b	2.33 ± 0.12ab
Pisolithus tinctorius	1.08 ± 0.13c	0.93 ± 0.05bc	0.45 ± 0.04ab	0.45 ± 0.11a
Suillus variegatus 1	1.48 ± 0.03a	1.36 ± 0.02a	1.25 ± 0.03a	1.37 ± 0.02a
Suillus variegatus H	0.40 ± 0.01a	0.38 ± 0.03a	0.42 ± 0.01a	0.46 ± 0.03a

bly the reason for the strong browning of embryogenic cells, and the cell lines that did not suffer may be tolerant of these metabolites or may have been able to degrade them.

Effects of embryogenic cultures on ECM fungi

In order to select suitable ECM fungi for development of somatic embryogenesis of Scots pine, it is important to study the reactions of fungi to embryogenic cells and also to the media used in somatic embryogenesis. In our study, all the cell lines of Scots pine induced the radial growth of slowly growing strains *S. variegatus* H and *Pisolithus tinctorius*. Previously, stimulation of fungal growth was observed in dual cultures with callus cells derived from radicles of Norway spruce germlings (Sirrenberg et al. 1995). In that experiment, normal callus cells stimulated the radial growth of all the tested ECM fungi of Norway spruce and even induced the fungi to form mycorrhizal structures, like the mantle and Hartig net. In our study with embryogenic cultures, those structures were not observed. The aggressive growth of *Paxillus involutus* (with all cell lines), *S. variegatus* 1 (with K779 and K884) and *Pisolithus tinctorius* (with K1009) over the suspensor cells was clearly harmful, because the embryogenic cells were not able to continue proliferation under a thick hyphal sheath. In addition, in both the positive and negative reactions, fungi penetrated the cells of K884, which is not typical for ECM fungi. However, it is important to note that the fungi always penetrated only the suspensor cells, which are located at the radical head of the developing embryos.

The positive effects of embryogenic cultures on the radial growth of fungi probably resulted from changes in the medium during proliferation of the embryogenic cultures. In dual culture, the invertase enzymes bound to walls of embryogenic cells might degrade sucrose to glucose and fructose after which the easily assimilated monosaccharide glucose was able to induce fungal growth. It is known that many ECM fungi are able to use glucose as an energy source much more effectively than sucrose (Laiho 1970; Salzer and Hager 1991).

Exogenous phytohormones added to media may also affect the growth of fungi. Increasing concentrations of auxins and cytokinins have been shown to inhibit the radial growth of pathogenic fungi in dual culture with Sitka spruce callus (Woodward and Pearce 1988). In the present study, the increase in the growth of *Paxillus involutus,* but not in that of *Pisolithus tinctorius* and *S. variegatus* H, was somehow dependent on the use of phytohormones by embryogenic cultures, because the first-mentioned species grew significantly better on the medium without BA than on the medium supplemented with both 2,4-D and BA.

The radial growth of *L. bicolor*, *L. proxima* and *S. variegatus* 1 was apparently inhibited by most of the embryogenic cultures used in our experiment. Woodward and Pearce (1988) found the same kind of inhibition when normal callus cells of Sitka spruce and its ECM fungi were growing together. It is known that ECM fungi activate formation of fungistatic compounds in their host plants (Krupa et al. 1973), and such compounds secreted by the embryogenic cells may have retarded the radial growth of fungi in the present study.

In conclusion, this study shows that at an early embryogenic stage Scots pine cultures are able to interact with ECM fungi. Because some fungi produced a positive reaction or even an increase in proliferation, they may offer two important possibilities for somatic embryogenesis: some fungi could be used to enhance the growth of slowly proliferating cell lines and the positive cell line-fungus interactions could be useful in later stages of somatic embryogenesis, such as rooting.

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