Mycorrhiza (2008) 18:297–307
DOI 10.1007/s00572-008-0182-5

ORIGINAL PAPER

Media formulation influences in vitro ectomycorrhizal synthesis on the European aspen *Populus tremula* L.

Ingrid Langer · Doris Krpata · Ursula Peintner · Walter W. Wenzel · Peter Schweiger

Received: 8 April 2008 / Accepted: 4 June 2008 / Published online: 2 July 2008

Abstract The effect of various media formulations on in vitro ectomycorrhizal synthesis of identified fungal strains with European aspen (*Populus tremula* L.) was tested in Petri dishes. Pre-grown seedlings were transferred to various nutrient media and inoculated with *Paxillus involutus* isolates using modified sandwich techniques. Mycorrhiza formation was evaluated macroscopically and further confirmed by microscopic examination of semi-thin sections for anatomical features of the mantle and the Hartig net. Standard media formulations did not support successful ectomycorrhiza formation because of either very poor plant survival (below 20%) or impaired fungal growth. The inclusion of micronutrients and vitamins in a Melin Norkrans (MMN)-based medium increased plant survival rate to above 60% and supported successful mycorrhizal synthesis. *P. involutus* isolates formed mycorrhizas with a characteristic Hartig net restricted to the epidermis. Mantle density and thickness varied depending on the isolate. In a follow-up experiment, the adapted medium supported successful ectomycorrhiza formation by various *Laccaria* and *Hebeloma* isolates. Our results show that an exogenous supply of vitamins and micronutrients in the medium was a prerequisite for successful mycorrhization of *P. tremula* in vitro in Petri dishes.

I. Langer (✉) · W. W. Wenzel · P. Schweiger
Institute of Soil Science, University of Natural Resources and Applied Life Sciences,
Peter Jordan-Straße 82,
1190 Vienna, Austria
e-mail: ingrid.langer@boku.ac.at

D. Krpata · U. Peintner
Institute of Microbiology, Innsbruck University,
Technikerstraße 25,
6020 Innsbruck Austria

Keywords Ectomycorrhiza · *Paxillus involutus* · *Populus tremula* (Poplar) · In vitro synthesis · Inoculation procedure · Symbiosis

Introduction

Species in the genus *Populus* have received general attention due to their use as energy crops in short rotation forestry (Dickmann 2006) and due to their great potential for carbon sequestration (Lemus and Lal-Referee 2005). *Populus* species have further attracted attention in contaminated land management based on their fast growth and considerable tolerance of increased soil heavy metal concentrations. Within, the genus, especially the European aspen *Populus tremula* L., has shown potential for use in the phytoremediation of contaminated sites (Robinson et al. 2000).

Populus tremula is one of the world's most widely distributed tree species, with its natural range extending throughout Europe to northeastern Asia and into northern Africa. It is a pioneer species that tolerates a wide range of climatic and soil conditions (Worrell 1995). *P. tremula* is also able to colonize variously disturbed habitats, including sites contaminated with heavy metals (Unterbrunner et al. 2007). Based on its ability to accumulate heavy metals in the aboveground biomass, *P. tremula* has been considered suitable for phytoextraction (DosSantos-Utmazian and Wenzel 2007).

Populus tremula generally grows in association with ectomycorrhizal (EM) fungi (Melin 1923; Krpata et al. 2008). These fungi affect heavy metal uptake by their host plants as well as within-plant heavy metal transport (Leyval et al. 1997). Only few studies examined the role of single fungal isolates on metal uptake by accumulator plants (Sell

et al. 2005; Baum et al. 2006; DosSantos-Utmazian et al. 2007). However, in most of these studies, only very low levels of EM root colonization were observed. The extent of EM root colonization is greatly dependent on the establishment of the fungus on host plant roots during mycorrhizal synthesis. Thus, experimental work focusing on *P. tremula* requires an adequate inoculation procedure.

Numerous inoculation protocols have been published that resulted in successful establishment of functional ectomycorrhizas on various hosts (Molina and Palmer 1982; Peterson and Chakravarty 1991). Some of those protocols have proven successful with many different fungal as well as host plant species. Others have been developed for specific host plant–EM fungus combinations. Inoculation techniques reported for *Populus* species predominately focus on American poplars such as *P. trichocarpa* (Baum and Makeschin 2000; Baum et al. 2002; Selle et al. 2005), its hybrids (Heslin and Douglas 1986; Tagu et al. 2001) and *Populus tremuloides* (Fortin et al. 1983; Godbout and Fortin 1985; Cripps and Miller 1995; Landhäusser et al. 2002). Further reports on mycorrhizal synthesis are available on hybrids between *P. tremuloides* and *P. tremula* (Hampp et al. 1996; Loewe et al. 2000; Selle et al. 2005). Inoculation protocols conducted with European *Populus* hybrides are scarce (Bücking and Heyser 2001; Gafur et al. 2004; Couturier et al. 2007; Langenfeld-Heyser et al. 2007). Moreover, single protocols again refer to procedures originally conducted with *P. tremula* × *tremuloides* (Gafur et al. 2004; Couturier et al. 2007). To our knowledge, *Populus tremula*, the European aspen, has not been used for studies on mycorrhizal synthesis since Melin in 1923.

In previous studies, we produced *P. tremula* plantlets by different plant propagation techniques (green cuttings vs. seedlings) and variously inoculated them with pre-grown mycelium (Perrin et al. 1996; Tagu et al. 2001; Sell et al. 2005), plugs or fungal suspensions (Landhäusser et al. 2002; Parladé et al. 2004). Plantlets were subsequently cultivated in diverse substrates such as soil, leca, sand, perlit, peat, and vermiculite. None of these inoculation

Table 1 Composition of media for cultures of EM fungi and inoculation procedures with *P. tremula* used in the present study

Compound		L-Knop [a]	MMN [b]	G-MMN [c]	L-MMN [d]
Macroelements (mg/l)	KH_2PO_4	54.440	500.000	500.000	500.000
	KNO_3	242.640			
	$(NH_4)_2SO_4$			250.000	
	$(NH_4)_2HPO_4$		250.000		250.000
	$MgSO_4$. $7H_2O$	80.720	150.000	150.000	150.000
	$CaCl_2$. $2H_2O$		50.000	50.000	50.000
	$Ca(NO_3)_2$ x $4H_2O$	240.000			
	NaCl	0.400	25.000	25.000	25.000
Microelements (mg/l)	FeNaEDTA	2.936			
	$FeCl_3$. $6H_2O$ (1%)		12.000	12.000	12.000
	H_3BO_3	0.572		15.458	15.458
	$MnSO_4$. $1H_2O$			9.295	9.295
	$MnCl_2$ x 4 H_2O	0.570			
	CuSO4 x 5 H2O	0.015		1.310	1.310
	$ZnSO_4$ x 7 H2O	0.072		5.750	5.750
	CoCl2 x 6 H2O	0.006			
	Na_2MoO_4 x 2 H2O	0.016		0.003	0.003
Vitamins (mg/l)	myo-Inositol	100.000			100.000
	Nicotinic acid	1.000			1.000
	Pyridoxine HCl	1.000			1.000
	Thiamine HCl	10.000	1.000	0.100	10.000
Carbohydrate source (g/l)	Glucose		2.5	5.0	5.0
	Sucrose	2.5			
	Malt extract		10.0	3.0	
Solidification agent (g/l)	Agar		9.0	9.0	9.0
	Gelrite	6.0			
pH		5.75	5.4	4.5	5.4

[a] New medium composition based on the Knop nutrient solution (George 1993)
[b] Modified Melin Norkrans medium (Brundrett et al. 1996)
[c] Adapted modified Melin Norkrans medium following the protocol of Gafur (Gafur et al. 2004)
[d] New medium composition based on the modified Melin Norkrans Medium

procedures, however, resulted in successful ectomycorrhizal synthesis.

The aim of the present study was therefore to develop an EM inoculation protocol specifically suitable for *P. tremula*. Synthesis experiments were conducted on *P. tremula* seedlings in vitro following several protocols in Petri dish systems. The first experiments focused on the composition of a medium, meeting the nutrient and vitamin demand of both *P. tremula* and *Paxillus involutus* isolates. Subsequently, a series of *P. tremula* inoculations was set up to confirm the suitability of the improved medium composition with *Laccaria*, *Hebeloma*, and *Paxillus* isolates.

Materials and methods

Plant material and seed germination

Populus tremula seeds were collected from a heavy metal contaminated *P. tremula* stand in southern Austria in spring 2005. *P. tremula* growing at that site has previously been shown to accumulate large amounts of both zinc and cadmium (Unterbrunner et al. 2007). In the laboratory, seeds were cleaned according to Latva-Karjanmaa et al. (2003) and stored at −18 °C (Fechner et al. 1981). For the experiments, seeds were surface sterilized with 30% H_2O_2 for 90 s and placed on a modified Knop medium (L-Knop medium; Table 1) in Petri dishes. The Knop medium (George 1993) was complemented with trace elements, vitamins (Gamborg B5 Vitamin mixture, Duchefa Biochemie B. V., The Netherlands), and sucrose (Table 1), and was solidified with 0.6% Gelrite (Duchefa Biochemie B. V., The Netherlands). Seed germination was carried out at room temperature (25 °C) with a 16/8 h day/night cycle. After 10–14 days, seedlings had developed vigorous cotyledons and a root length of 4–5 cm.

Fungal inoculum

Four *Paxillus involutus* isolates, collected in Great Britain, Austria, and Switzerland were used in the experiments (Table 2). They were cultivated on modified Melin Norkrans medium (Table 1) lacking malt extract (MMN-m) and were transferred to fresh medium every 4 weeks. For the inoculation, 6×6 mm mycelial plugs were cut and pregrown on fresh MMN-m agar until they were covered by actively growing mycelium.

Fungal cultures of three *Hebeloma* and four *Laccaria* isolates (Table 2) were cultivated on 1/2 MMN medium (MMN with half amount of carbohydrates). Mycelial plugs

Table 2 Fungal taxa and abbreviations used in this study with details on fungal hosts and original habits

Abbreviation	Fungal taxa	Isolate provenance	Details of isolation, host and origin
Pax1	*Paxillus involutus* (Batsch: Fr.) Fr.	87.017	Isolated from a fruitbody in a coal waste with *Betula pendula* in Midlothian, Scotland [a]
Pax2	*Paxillus involutus* (Batsch: Fr.) Fr.	BOKU 04. M01	Isolated from a fruitbody under *P. tremula* on a heavy metal contaminated site in Carinthia, Austria [b]
Pax3	*Paxillus involutus* (Batsch: Fr.) Fr.	WSL #37.7	Isolated from a fruitbody from a *Salix*- and *Betula* stand in Switzerland [c]
Pax4	*Paxillus involutus* (Batsch: Fr.) Fr.	WSL #37.10	Isolated from a fruitbody on a heavy metal contaminated site in Switzerland [d]
Lac1-1	*Laccaria bicolor* (Maire) P.D. Orton	S-238a	Isolated from a fruitbody under *Tsuga mertensiana* stand, Crater Lake National Park, Oregon [e]
Lac1-2	*Laccaria bicolor* (Maire) P.D. Orton	CBS 560.96	
Lac2	*Laccaria proxima* (Boud.) Pat.	CBS 592.89	
Lac3	*Laccaria laccata (*Scop.) Berk. & Broome	CBS 377.89	
Heb1	*Hebeloma cylindrosporum* Romagn.	CBS 557.96	
Heb2-1	*Hebeloma crustuliniforme* (Bull.) Quél.	85.023	Isolated from spores from a fruitbody under *Picea sitchensis* [f]
Heb2-2	*Hebeloma crustuliniforme* (Bull.) Quél.	CBS 163.46	

[a,e,f] (Finlay et al. 1992)
[b] (Krpata et al. 2008)
[c] (Sell et al. 2005)
[d] Personal communication (I. Brunner (2005) WSL, Birmensdorf, Switzerland)

of these fungi were sub-cultured every 6 weeks and prepared for the inoculation procedure as described for *P. involutus*.

Testing of media for mycorrhizal symbiosis

Populus tremula plantlets were inoculated with two strains of *P. involutus* (Pax1, Pax3) using a modified sandwich technique (Peterson and Chakravarty 1991). Mycelial plugs, one plug per Petri dish (90 mm diameter), were placed on washed and autoclaved cellophane sheets, laid over either MMN-m or L-Knop-s (modified L-Knop medium without sucrose) media. Ten-day-old plantlets from the germination plate were placed on the cellophane with their roots arranged toward the expanding mycelium. The Petri dishes contained three plants on average and each fungus-medium combination was replicated twice. Dishes were sealed with Parafilm and incubated horizontally at 25°C with a 16-h light/8-h dark regime.

Additionally, *P. tremula* was inoculated on G-MMN medium (Gafur et al. 2004). For this experimental setup, five mycelial plugs were placed in two rows on sheets of cellophane. After 4 days plantlets were arranged in one row (five plants per plate), with their roots oriented toward the mycelial plugs (Burgess et al. 1996). Sealed Petri dishes were incubated in a slanted position under controlled conditions as described above. After 5 weeks, the survival rate of the plantlets, fungal growth and fungal habit were determined. The formation of mycorrhizas was evaluated macroscopically based on morphological characteristics, such as stimulation of lateral root growth, root ramification, shape and color of root tips.

Optimization of the inoculation experiment

Populus tremula plantlets were inoculated with the four *P. involutus* isolates on a further modified nutrient medium. Based on the results obtained on the different media, this L-MMN medium contained components of the Knop as well as the MMN medium (Table 1). Three plugs per plate were placed on cellophane sheets laid over the L-MMN medium and grown for 4 days. Five-week-old *P. tremula* plantlets were transferred to the dishes with their roots arranged around the inoculum. The shoots were left to stick out of the Petri dishes through openings, cut into the sidewall with a hot needle (Wong and Fortin 1988). Petri dishes were sealed with Parafilm and autoclaved silicon. They were further wrapped in aluminum foil and positioned vertically in an incubation box, which provided high humidity to the plant shoots (16 h light; 25 °C). The experiments were performed in triplicates. After 5 weeks, the survival rate of the plantlets, fungal growth and fungal habit were observed. The roots were evaluated macroscopically for mycorrhiza formation.

Preparation of EM root tips for microscopy

Single root tips showing morphological characteristics of mycorrhiza symbiosis were fixed in 2.5% glutaraldehyde in 4-(2-Hydroxyethyl)piperazine-1-ethanesulonic acid (HEPES) buffer (0.1 M, pH 6.8). They were subsequently dehydrated using a graded ethanol series and infiltrated with Spurr resin. Semi-thin sections (1 μm) were cut, stained with toluidine blue and examined under a light microscope at ×400 magnification (Zeiss Axiovert 200M, Axiocam MRc5). Based on the presence of both mycelial sheath and Hartig net, the fungal isolates were classified as compatible or incompatible with *P. tremula*. Fungi that failed to form a characteristic Hartig net but formed a hyphal sheath were classified as intermediate. The anatomy of hyphal sheath and Hartig net formation were characterized according to Agerer (1990).

Evaluation of the general suitability of the medium composition

Plantlets were inoculated with *Hebeloma* and *Laccaria* isolates (Table 2) to evaluate the general suitability of the L-MMN medium for mycorrhizal synthesis on *P. tremula*. Based on the experiment described above, plantlets were also inoculated with *P. involutus* (Pax2) as a control treatment. The experimental setup followed the modified sandwich technique (Malajczuk et al. 1990). Mycelial plugs, one plug per Petri dish, were placed on cellophane and grown for 14 days. Thereafter, 2-week-old plantlets were arranged in the Petri dishes with their roots positioned on the expanding mycelial mats. Petri dishes, containing six plantlets each, were incubated in a horizontal position at room temperature (25°C) with a 16-h light/8-h dark regime. After 4 weeks, plant growth and fungal vitality were observed macroscopically. Single root tips showing characteristic features of mycorrhizal symbiosis were fixed and embedded in resin as described above. Semi-thin sections were cut and observed microscopically.

Results

Testing of media for mycorrhizal symbiosis

At least 50% of *P. tremula* plantlets inoculated on L-Knop-s nutrient medium survived, in contrast to only 8.5% and 20% of the plantlets treated on modified MMN media (Table 3). Plant survival was further determined by the fungal isolates, as plants inoculated with Pax1 generally had better survival rates than plants inoculated with Pax3. Plant survival was best (100%) when inoculated with Pax1 and grown on L-Knop-s medium.

Table 3 *Populus tremula* survival rate, fungal viability and mycorrhiza formation tested on three different nutrient media and with at least two *Paxillus involutus* isolates

Medium	Fungal isolate [d]	Number of plants inoculated	Plant survival rate [e]	Fungal viability [f]	Mycorrhiza formation [g]
MMN-m [a]	Pax1	6	17%	+	No
	Pax3	6	0%	+	No
			8.5% (mean value)		
L-Knop-s [b]	Pax1	6	100%	–	No
	Pax3	6	50%	–	No
			75% (mean value)		
G-MMN [c]	Pax1	10	20%	+	No
	Pax2	10	30%	+	No
	Pax3	10	10%	+	No
	Pax4	10	20%	+	No
			20% (mean value)		

[a] Modified Melin Norkrans medium (MMN, Table 1) lacking malt extract
[b] New medium composition based on the Knop nutrient solution (L-Knop, Table 1) lacking sucrose
[c] Adapted modified Melin Norkrans medium following the protocol of Gafur (Gafur et al. (2004), G-MMN, Table 1)
[d] Abbreviations of the *Paxillus involutus* isolates are listed in Table 2
[e] Plant survival rate was specified by the percentage of living plantlets
[f] Fungal viability was indicated positive (+) or negative (−) based on fungal growth and mycelial habit
[g] Mycorrhizas were determined macroscopically by morphological root characteristics indicative of mycorrhiza formation

In addition to plant survival, their appearance and growth habit were observed. Plantlets inoculated on L-Knop-s medium showed uniform growth, with just occasional discolorations and single lesions. Shoot discolorations were only observed in combination with Pax3. In contrast, plants grown on G-MMN and especially MMN-m medium showed a severe reddening of leaves and dark lesions.

Fungal viability and habit were also affected by the inoculation medium (Table 3). *P. involutus* isolates grew very well on modified MMN media, while the L-Knop-s medium markedly inhibited mycelial growth and the color of the mycelium changed from bright cream to light brown.

No mycorrhiza formation was observed, regardless of medium composition and fungal isolates (Table 3).

Optimization of the inoculation procedure and description of plant performance and EM

Use of the modified L-MMN medium resulted in a plant survival rate >50% and in vital fungal growth (Table 4). Anatomical features typical for mycorrhizas were observed with all four *Paxillus* isolates.

Populus tremula plantlets inoculated with Pax1 on L-MMN medium were delicately built but developed a vigorous root system. Macroscopic evaluation of root tips indicated mycorrhizal symbiosis. Lateral root growth was stimulated and straight and unramified or monopodial ramified mycorrhizas were formed. Cross cuttings revealed a loosely woven (plectenchymatous) mantle of at least four to five layers and single hyphae penetrating between the

Table 4 Indications of a successful inoculation procedure (plant survival rate, fungal viability and fungal compatibility) for *P. tremula* with several *P. involutus* isolates conducted on L-MMN medium

Medium	Fungal isolate [b]	Number of plants inoculated	Plant survival rate [c]	Fungal viability [d]	Fungal compatibility [e]
L-MMN [a]	Pax1	6	67%	+	+
	Pax2	6	83%	+	+
	Pax3	6	50%	+	~
	Pax4	6	50%	+	+
			62.5% (mean value)		

[a] New medium composition based on the modified Melin Norkrans Medium (L-MMN, Table 1)
[b] Abbreviations of the fungal isolates are listed in Table 2
[c] Plant survival rate was specified by the percentage of living plantlets
[d] Fungal viability was indicated positive (+) or negative (−) based on fungal growth and mycelial habit
[e] Fungi were classified as compatible (+) or incompatible (−) based on the presence of both mycelial sheath and Hartig net. Fungi which failed to form a Hartig net although enveloping the root tips with a mycelial mantle were termed intermediate (~).

epidermal cells (paraepidermal Hartig net). The cells of the epidermal layer were slightly enlarged but did not show radial elongation (Fig. 1).

Plants inoculated with Pax2 appeared robust with healthy green leaves and a vigorous root system. Mycorrhizal symbiosis was characterized by stimulated lateral root growth and the formation of simple and unramified mycorrhizas. Mycorrhizal root tips were stout and dark-colored. Cross sections revealed a dense mantle (pseudoparenchymatous mantle) and the formation of a paraepidermal Hartig net. No enlargement and radial cell elongation was detected within the epidermal cell layer.

Populus tremula plantlets in combination with Pax3 had extensive root growth and were robust in general, with few discolorations of shoots. Root systems were characterized by an enhanced production of lateral roots and dark and swollen root tips forming straight and unramified mycorrhizas. Cross sections cut near the tip proved the formation of a characteristic sheath interweaving single cells of the calyptra. Successional sections revealed hyphae of the inner mantle layer penetrating epidermal cells and growing towards the root cylinder. Thus, the symbiosis lacked a characteristic Hartig net (Table 4).

Inoculation with Pax4 resulted in poor shoot and root growth. Root systems consisted of few lateral roots with slightly clubbed and dark-colored unramified mycorrhizas. This fungus formed a very thick and dense mantle (pseudoparenchymatous) and a paraepidermal Hartig net. At tip bases, hyphae of the inner mantle were seen to penetrate cells of the epidermal layer, the cortex and the central root cylinder (Fig. 1).

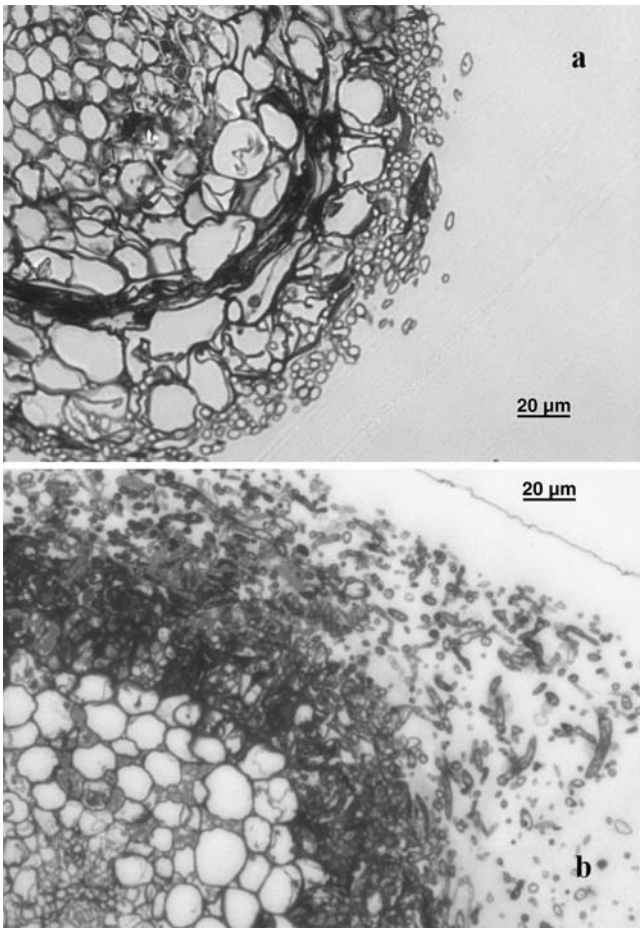

Fig. 1 *Paxillus involutus* isolates forming mycorrhizas with *P. tremula* (bars: 20µm). **a** Isolate Pax1 forming a loosely woven mantle and a paraepidermal Hartig net. **b** Cross section at the bases of isolate Pax4 mycorrhiza showing hyphal proliferation toward the root cylinder

Evaluation of the medium composition on the performance of other fungal inocula

The L-MMN medium was used for inoculation experiments with various *Laccaria*, *Hebeloma*, and *Paxillus* isolates. Plantlets, completely enclosed in the Petri dishes, achieved a survival rate of 100%. Isolates of each fungal genus were able to form mycorrhizas with *P. tremula* (Table 5). However, there was considerable variation in extent of hyphal sheath and Hartig net formation.

Populus tremula plantlets inoculated with Lac1–1 grew well and formed dark and slightly clubbed mycorrhizas. In semi-thin sections, a dense and voluminous pseudoparenchymatous mantle was visible near the root apex enclosing cells of the root calyptra. In older parts, the hyphal sheath became thin and loose. No characteristic Hartig net was observed and the epidermal cells remained small and tightly packed (Table 5 and Fig. 2)

The strain Lac1-2 severely stressed *P. tremula.* It depressed plant growth and induced distinct leaf discolorations and numerous lesions. Mycorrhiza formation could not be detected macroscopically (Table 5), whereas microscopic evaluation confirmed the formation of shortened and dark lateral roots. The screening of cross sections revealed the formation of a cohering but shallow and loosely woven sheath (plectenchymatous mantle) and the presence of a paraepidermal Hartig net (Fig. 2).

Lac2 inoculated plantlets grew vigorously, but did not form any mycorrhizas, with no hyphal sheath and no Hartig net (Table 5).

Populus plantlets inoculated with Lac3 developed poorly. Plant shoot and root growth were reduced and leaves showed severe red discolorations. No mycorrhizas were formed; however, hyphal clusters were detected within the roots growing within the vascular bundle and the endodermis (Table 5).

Heb1-inoculated *Populus* plantlets developed vigorously and produced short and distinctly clubbed lateral roots with silvery appearance. Semi-thin sections confirmed the

Table 5 Indications of a successful inoculation procedure (plant survival rate, fungal viability and fungal compatibility) for *P. tremula* with several ectomycorrhizal fungi conducted on L-MMN medium

Medium	Fungal isolate [b]	Number of plants inoculated	Plant survival rate (%) [c]	Fungal viability [d]	Fungal compatibility [e]
L-MMN [a]	Lac1-1	6	100	+	~
	Lac1-2	6	100	+	+
	Lac2	6	100	–	–
	Lac3	6	100	+	–
	Heb1	6	100	+	+
	Heb2-1	6	100	+	–
	Heb2-2	6	100	+	+
	Pax2 (control)	6	100	+	+
			100 (mean value)		

[a] New medium composition based on the modified Melin Norkrans Medium (L-MMN, Table 1)
[b] Abbreviations of the fungal isolates are listed in Table 2
[c] Plant survival rate was specified by the percentage of living plantlets
[d] Fungal viability was indicated positive (+) or negative (−) based on fungal growth and mycelial habit
[e] Fungi were classified as compatible (+) or incompatible (−) based on the presence of both mycelial sheath and Hartig net. Fungi that failed to form a Hartig net although enveloping the root tips with a mycelial mantle were termed intermediate (~).

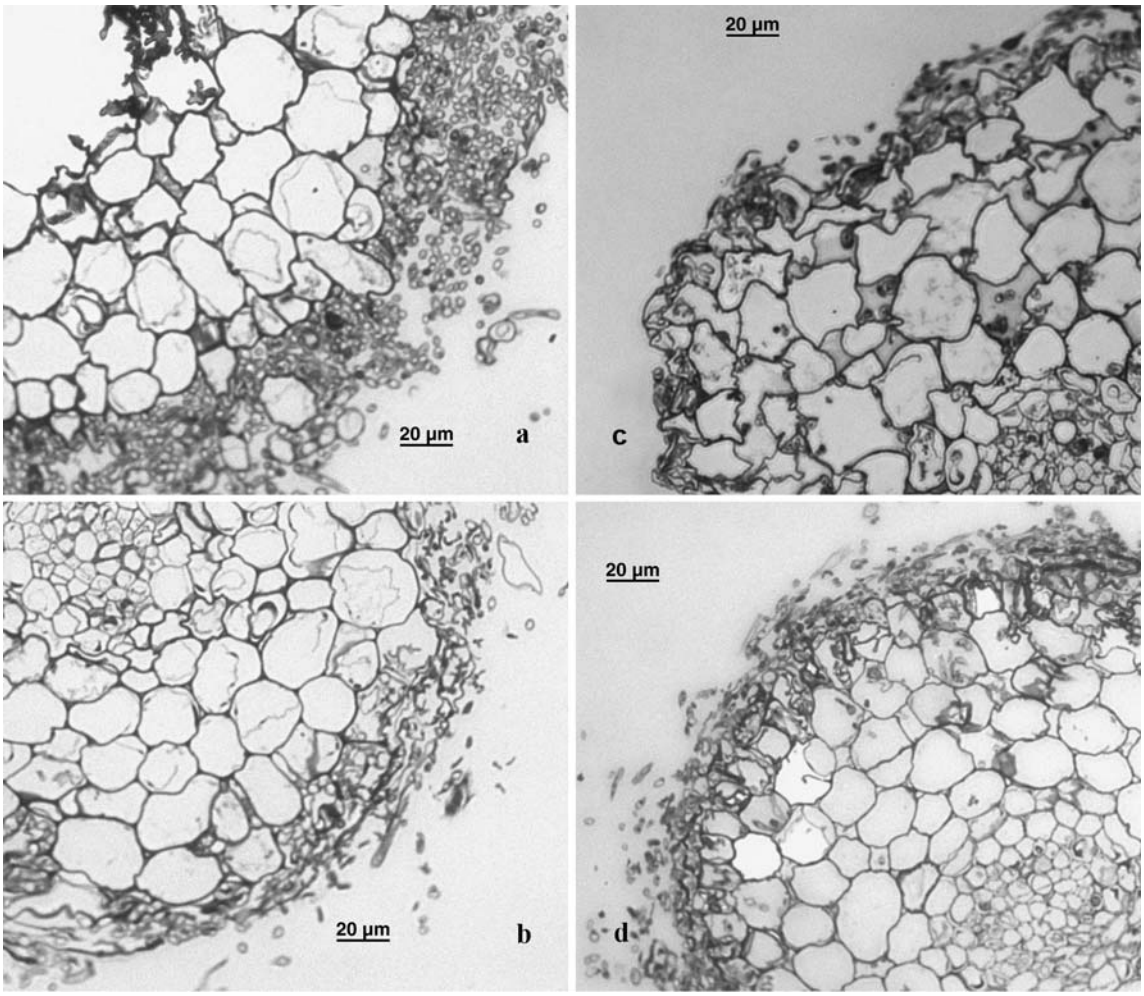

Fig. 2 *Populus tremula* mycorrhizas formed with *Laccaria bicolor* and *Hebeloma crustuliniforme* (bars: 20 µm). **a** Lac1-1 mycorrhiza showing a dens mantle but lacking a characteristic Hartig net. Epidermal cells remain small and tightly packed. **b** Lac1-2 forming a shallow and loosely woven sheath and a paraepidermal Hartig net. **c** Hyphae of Heb2-1 growing adhered to the root surface and within the root cortex. Cortex cells are loosely interconnected. **d** Heb2-2 mycorrhiza cut at the base of the root tip showing a shallow sheath and a para- to periepidermal Hartig net. Hyphae start penetrating between cortex cells toward the endodermis

presence of mycorrhizas with an extremely thick and dense hyphal sheath (pseudoparenchymatous mantle) and a para-epidermal Hartig net (Table 5).

Plantlets inoculated with the fungal strain Heb2–1 did not form mycorrhizas. In semi-thin sections, loose hyphae were seen to adhere to the epidermis and single hyphae penetrated between cells of the epidermal layer, the cortex, and the vascular cylinder. Cortex cells were interconnected loosely (Table 5 and Fig. 2).

A further strain of *Hebeloma crustuliniforme* (Heb2–2) negatively affected plant growth. Plant roots appeared very thin and reddish and showed untypical ramifications. Individual short and darkened lateral roots were observed. Their mycorrhizal status was confirmed by a plectenchymatous mantle and hyphae partly enveloping the epidermal cells (paraepidermal/ periepidermal Hartig net). In older parts of the tips, also this fungus started to penetrate between cortical cell layers toward the endodermis (Table 5 and Fig. 2).

Pax2 was confirmed as a compatible fungus for the mycorrhization of *P. tremula.* Plants inoculated with this *Paxillus* isolate appeared robust and had a well-developed root system. Microscopic evaluation confirmed mycorrhizal symbiosis (Table 5).

Discussion

Interest in inoculation of *Populus* species with ectomycorrhizal fungi has recently increased, mainly due to the use of poplars for biomass production and application in phytoremediation. Therefore, suitable information on mycorrhiza synthesis with poplar species such as *Populus tremula* is highly valuable. In the present study, *P. tremula* seedlings were variously inoculated with ectomycorrhizal fungi in Petri dishes in vitro. Synthesis experiments were conducted on several nutrient media, which clearly affected the formation of functional mycorrhizas.

The first series of experiments did not result in successful mycorrhiza synthesis mainly due to very poor plant survival on media based on the widely used MMN formulation. Only 8.5% of *P. tremula* plantlets survived on MMN-m medium whereas mycorrhiza formation on similar media was documented with *Pinus sylvestris* (Niemi et al. 2007) and *Betula pendula* (Brun et al. 1995; Blaudez et al. 1998). Modified MMN media, additionally supplemented with trace elements, facilitated plant growth and mycorrhiza formation with both gymnosperm *Larix* and *Pinus* (Wong and Fortin 1988) and broad-leafed *Eucalyptus* (Malajczuk et al. 1990) species. Moreover mycorrhiza formation was confirmed with *Populus* hybrids *P. tremula* × *tremuloides* (Hampp et al. 1996; Selle et al. 2005) and *P. tremula* × *alba* (Gafur et al. 2004).

In contrast, present experiments conducted on the G-MMN medium (Gafur et al. 2004) did not prove suitable for *P. tremula.* This may indicate the specific nutrient demand of the plantlets during mycorrhizal synthesis. Plant survival increased to 75% or more by the addition of a vitamin mixture (nicotinic acid, pyridoxine, myo-Inositol, and thiamine), usually supplemented to plant tissue culture media (George 1993). Normally, the addition of thiamine meets the vitamin requirements of plants during mycorrhizal synthesis. Malt extract, which contributes some vitamins, amino acids and growth regulants (George 1993), could not compensate for the lack of vitamins in the G-MMN medium.

It seems that *P. tremula* seedlings were not able to produce sufficient amounts of vitamins, required for normal growth and development. The demand of *P. tremula* for additional micronutrients and especially vitamins may be due to the very low seed weight of approximately 0.01 g. This is less than 10% of the average seed size of species such as *Populus alba* or *Betula pendula* (Bärtels 1989). *P. tremula* seeds lack the endosperm (Borset 1954), which markedly influences juvenile plant growth. In general, *P. tremula* mainly reproduces by root suckers and only to a much less extent by seeds (Worrell 1995). Moreover, seed quality varies considerably (Worrell 1995). Seed quality may be further impaired by environmental stress such as for example heavy metal soil contamination of the collection area (Fedorkov 1999).

Common media based on the MMN formulation did not favor survival of *P. tremula*, but promoted EM fungal growth. The L-Knop-s medium, on the other hand, promoted plant growth but failed to feed the EM fungus *P. involutus*. This fungus is in general easily cultured, and has been extensively used in studies on mycorrhizal functioning (Wallander and Söderström 1999). The failure may be due to differences between the media in carbon, nitrogen, and phosphorus supply. The L-Knop-s medium does not contain any sucrose or malt extract. The only carbon source within the inoculation procedure derives from the glucose leftover in the mycelial plug.

Contrary to our results, mycorrhiza formation has been observed on sugar-free media with several EM fungi on *Populus* (Selle et al. 2005), *Larix*, and *Pinus* species (Wong and Fortin 1988). The use of exogenous supply of glucose for mycorrhizal studies has repeatedly been disputed in the past. Duddridge (1986) cautioned against exogenous glucose supply, as mycorrhizas may be formed by partners that would be regarded incompatible in nature. High levels may also lead to the formation of an unusual host–fungus interface (Duddridge and Read 1984). However, Hutchison and Piché (1995) observed that plant infections were not necessarily increased with rising glucose concentrations, but that glucose may enhance mycelial growth. The glucose

content of the inoculum applied in our experiments obviously did not satisfy the fungal carbon demand until mycorrhizas were established.

The L-Knop-s medium contains nitrate, whereas ammonium is the predominant nitrogen source for EM fungi in soil and is supplemented to the most common media. Studies on the ability of EM fungi to utilize nitrate found generally much better growth on ammonium, although results differed considerably between fungal species and isolates (Finlay et al. 1992). None of the tested fungi, however, completely stopped growth on nitrate-supplemented media as observed in our experiment. *Paxillus involutus* isolate Pax1 was one of the best performing fungi on nitrate-supplemented medium. However, mycelial development was restricted to half of the normal growth within the first 2 months (Finlay et al. 1992). Thus, nitrate application may have contributed to depress fungal growth in our first experimental setup.

Whereas nitrogen supply of the L-Knop-s medium differs in the nitrogen compound added, phosphorus application differs in the concentration, which is reduced to one tenth normally supplied with the MMN medium. Such a reduction in phosphorus concentration was previously found to reduce the percentage of mycorrhizal root tips formed by late-stage fungi; however, it did not clearly affect linear extension of all fungi tested (Gibson and Deacon 1990).

The L-MMN-medium supported successful mycorrhizal synthesis of *P. tremula* with several EM fungi. First experiments conducted with *P. involutus* isolates revealed the compatibility of three strains, whereas one isolate failed to form a characteristic Hartig net. These differences among isolates are commonly observed (Cairney 1999) and agree with results from investigations on various *Paxillus* strains tested for the ability to form mycorrhizas with *Populus canescens* (Gafur et al. 2004).

In our study, *Paxillus involutus* isolates compatible with *P. tremula* formed a mantle of variable density and thickness. The Hartig net was consistently paraepidermal, formed by single hyphal rows within the epidermal layer. Characteristics of the mantle and the Hartig net observed here correspond to those of mycorrhizas formed by *P. tremuloides* and *P. canescens*, respectively (Godbout and Fortin 1985; Gafur et al. 2004). We did not observe radial elongation of epidermal cells, although this is frequently detected in mycorrhizas formed by angiosperms, where the Hartig net is restricted to the root epidermis.

Similar to *P. involutus*, isolates of *Laccaria bicolor* and *H. crustuliniforme* differed in their ability to form mycorrhizas with *P. tremula*. Also *L. bicolor* and *Hebeloma* spp. mycorrhizas were characterized by a paraepidermal Hartig net. This mycorrhizal characteristic is consistent with 32 EM fungi, with *P. tremuloides* as the host symbiont (Godbout and Fortin 1985).

In the last experimental setup *H. crustuliniforme*, however, seemed to enclose epidermal cells with Hartig net hyphae (periepidermal Hartig net). This agrees with investigations on *P. tremuloides* forming mycorrhizas with several fungal species (Godbout and Fortin 1985) and may be explained by the rate of fungal growth in general, the time roots are examined and the inoculation system used. However, cross sections through parts of roots with elongated epidermal cells angularly orientated toward the root apex may give the impression of several cell layers and thereby falsely be interpreted as periepidermal Hartig net.

In the present study, hyphae of the inner mantle occasionally penetrated cells of the root epidermis and grew within the cortex toward the root cylinder. Such hyphal proliferation is not typical in stable mycorrhizas but may be explained by the saprobic ability of individual EM fungi. This has particularly been found for *P. involutus* (Wallander and Söderström 1999) or single *Hebeloma* species (Marmeisse et al. 1999). Modifications of mycorrhizal anatomy generally indicate an imbalance of the symbionts based on the inoculation system utilized. Investigations by Duddridge (1986) demonstrated the impact of glucose on *Suillus grevillei* mycorrhiza ultrastructure, which underlines the relevance of a balanced nutrient supply as discussed above. A balanced mycorrhiza may further be attributed to conditions in the synthesis chamber not favoring one or the other of the symbionts (Duddridge 1986). The enclosure of only the plant roots in the last experiment may have strengthened *P. tremula* development and thereby affected the ability of the various fungi to successfully form mycorrhizas.

The inoculation systems used in our experiments mainly differed in the selective enclosure of the roots and the use of plantlets of different age. *P. tremula* inoculated in Petri dishes with their shoots sticking out had a survival rate of 62.5% on average. This relatively low value compared to the 100% observed for plantlets completely enclosed in Petri dishes may be due to the lower air humidity to which the shoots of these plants were exposed. This agrees with several observations on the high sensibility of *Populus* species to low air moisture (Hampp et al. 1996; Gafur et al. 2004). Plantlets once adapted to these environmental conditions developed vigorous shoots and a prolific root system. These benefits may be attributed to the use of more mature plantlets and to the fact that plants did not suffer from potential CO_2 deficiency or accumulation of volatile substances such as possible in a fully enclosed Petri dish system (Peterson and Chakravarty 1991).

To summarize, our results show that *P. tremula* may form mycorrhizas with EM fungi in Petri dishes in vitro comparable to American poplars and several *Populus* hybrids. However, *P. tremula* needs special nutrient support in the synthesis medium. A balanced micronutrient supply

and the addition of a number of vitamins are vital for plant growth and successful mycorrhizal synthesis.

The new medium composition used with sandwich techniques in the presented study may also be considered for other inoculation procedures. Several sterile and non-sterile inoculation techniques utilize substrates such as sand, perlite, peat, and vermiculite moistened with common nutrient solutions. These substrates may be complemented with the adapted medium composition and may thereby increase the proportion of successful EM synthesis on *P. tremula*. Apart from use in Petri dishes, it may further be employed with syntheses techniques carried out in growth pouches, jars, or containers.

Finally, ectomycorrhizal *P. tremula* plantlets may subsequently be used in studies on plant–fungus interactions such as heavy metal uptake by this accumulator plant species and within-plant heavy metal transport as affected by mycorrhizal status.

Acknowledgments We thank Ivano Brunner (WSL, Birmensdorf, Switzerland) for kindly providing the Swiss *Paxillus involutus* isolates WSL 37.7 and WSL 37.10 and Waltraud Klepal (Service Unit Cell Imaging and Ultrastructure Research; University of Vienna) for her most helpful support with sample preparation. Moreover, we thank Johann Glauninger (Institute of Plant Protection; BOKU) for kindly providing access to microscopic infrastructure and Siegrid Steinkellner (Institute of Plant Protection; BOKU) for her useful comments on the manuscript. The present study is part of the Project P170120-B04, which is financed by the Austrian Science Fund FWF.

References

Agerer R (1990) Color atlas of ectomycorrhizae. Einhorn Verlag, Munich

Bärtels A (1989) Gehölzvermehrung. Ulmer Verlag, Stuttgart

Baum C, Makeschin F (2000) Effects of nitrogen and phosphorus fertilization on mycorrhizal formation of two poplar clones (*Populus trichocarpa* and *P. tremula x tremuloides*). J Plant Nutr Soil Sci 163:491–497, doi:10.1002/1522-2624(200010)163:5<491::AID-JPLN491>3.0.CO;2-3

Baum C, Stetter U, Makeschin F (2002) Growth response of *Populus trichocarpa* to inoculation by the ectomycorrhizal fungus *Laccaria laccata* in a pot and field experiment. For Ecol Manag 163:1–8, doi:10.1016/S0378-1127(01)00534-5

Baum C, Hrynkiewicz K, Leinweber P, Meißner R (2006) Heavy-metal mobilization and uptake by mycorrhizal and nonmycorrhizal willows (*Salix* x *dasyclados*). J Plant Nutr Soil Sci 169:516–522, doi:10.1002/jpln.200521925

Blaudez D, Chalot M, Dizengremel P, Botton B (1998) Structure and function of the ectomycorrhizal association between *Paxillus involutus* and *Betula pendula.* II. Metabolic changes during mycorrhizal formation. New Phytol 138:543–552, doi:10.1046/j.1469-8137.1998.00125.x

Borset O (1954) Ospfroets spireevne. Medd Nor Skogforsoksves 44:1–44, The germination power of aspen seed

Brun A, Chalot M, Finlay RD, Söderström B (1995) Structure and function of the ectomycorrhizal association between *Paxillus involutus* (Batsch) Fr. and *Betula pendula* Roth. New Phytol 129:487–493, doi:10.1111/j.1469-8137.1995.tb04319.x

Brundrett M, Bougher N, Dell B, Grove T, Malajczuk N (1996) Working with Mycorrhizas in Forestry and Agriculture. ACIAR Monograph, Canberra, Australia

Bücking H, Heyser W (2001) Microautoradiographic localization of phosphate and carbohydrates in mycorrhizal roots of *Populus tremula* × *Populus alba* and the implications for transfer processes in ectomycorrhizal associations. Tree Physiol 21:101–107

Burgess T, Dell B, Malajczuk N (1996) In vitro synthesis of *Pisolithus-Eucalypthus* ectomycorrhizae: synchronization of lateral tip emergence and ectomycorrhizal development. Mycorrhiza 6:189–196, doi:10.1007/s005720050125

Cairney JWG (1999) Intraspecific physiological variation: implications for understanding functional diversity in ectomycorrhizal fungi. Mycorrhiza 9:125–135, doi:10.1007/s005720050297

Couturier J, Montanini B, Martin F, Brun A, Blaudez D et al (2007) The expanded family of ammonium transporters in the perennial poplar plant. New Phytol 174:137–150, doi:10.1111/j.1469-8137.2007.01992.x

Cripps CL, Miller OK (1995) Ectomycorrhizae formed in vitro by quaking aspen: including *Inocybe lacera* and *Amanita pantherina*. Mycorrhiza 5:357–370, doi:10.1007/BF00207408

Dickmann DI (2006) Silviculture and biology of short rotation woody crops in temperate regions: then and now. Biomass Bioenergy 30:696–705, doi:10.1016/j.biombioe.2005.02.008

DosSantos-Utmazian MN, Wenzel WW (2007) Cadmium and zinc accumulation in willow and poplar species grown on polluted soils. J Nutr Soil Sci 170:265–272, doi:10.1002/jpln.200622073

DosSantos-Utmazian MN, Schweiger P, Sommer P, Gorfer M, Strauss J et al (2007) Influence of *Cadophora finlandica* and other microbial treatments on cadmium and zinc uptake in willows on pollutes sites. Plant Soil Environ 53(4):158–166

Duddridge JA (1986) The development and ultrastructure of ectomycorrhizas IV. Compatible and incompatible interactions between *Suillus grevillei* (Klotzsch) sing. and a number of ectomycorrhizal hosts in vitro in the presence of exogenous carbohydrate. New Phytol 103:465–471, doi:10.1111/j.1469-8137.1986.tb02884.x

Duddridge JA, Read DJ (1984) The development and ultrastructure of ectomycorrhizas II. Ectomycorrhizal development on pine in vitro. New Phytol 96:575–582, doi:10.1111/j.1469-8137.1984.tb03592.x

Fechner GH, Burr KE, Myers JF (1981) Effects of storage, temperature, and moisture stress on seed germination and early seedling development of trembling aspen. Can J For Res 11:718–722, doi:10.1139/x81-100

Fedorkov A (1999) Influence of air pollution on seed quality of *Picea obovata*. Eur J For Pathol 29:371–375, doi:10.1046/j.1439-0329.1999.00166.x

Finlay RD, Frostegard A, Sonnerfeldt A-M (1992) Utilization of organic and inorganic nitrogen sources by ectomycorrhizal fungi in pure culture and symbiosis with *Pinus contorta* Dougl. ex Loud. New Phytol 120:105–115, doi:10.1111/j.1469-8137.1992.tb01063.x

Fortin JA, Piché Y, Godbout C (1983) Methods for synthesizing ectomycorrhizas and their effect on mycorrhizal development. Plant Soil 71:275–284, doi:10.1007/BF02182663

Gafur A, Schützendübel A, Langenfeld-Heyser R, Fritz E, Polle A (2004) Compatible and incompetent *Paxillus involutus* isolates for ectomycorrhiza formation in vitro with poplar (*Populus* × *canescens*) differ in H_2O_2 production. Plant Biol 6:91–99, doi:10.1055/s-2003-44718

George EF (1993) Plant propagation by tissue culture. Butler & Tanner, Frome, Sommerset

Gibson F, Deacon JW (1990) Establishment of ectomycorrhizas in aseptic culture: effects of glucose, nitrogen and phosphorus in relation to succession. Mycol Res 94(2):166–172

Godbout C, Fortin FA (1985) Synthesized ectomycorrhizae of aspen: fungal genus level of structural characterization. Can J Bot 63:252–262

Hampp R, Ecke M, Schaeffer C, Wallenda T, Wingler A et al (1996) Axenic mycorrhization of wild type and transgenic hybrid aspen expressing T-DNA indoleacetic acid-biosynthetic genes. Trees (Berl) 11:59–64, doi:10.1007/s004680050059

Heslin MC, Douglas GC (1986) Synthesis of poplar mycorrhizas. Trans Br Mycol Soc 86(1):117–122

Hutchison LJ, Piche Y (1995) Effects of exogenous glucose on mycorrhizal colonization in vitro by early-stage and late-stage ectomycorrhizal fungi. Can J Bot 73:898–904, doi:10.1139/b95-337

Krpata D, Peintner U, Langer I, Fitz W, Schweiger P (2008) Ectomycorrhizal communities associated with *Populus tremula* growing in a heavy metal contaminated site. Mycol Res, doi:10.1016/j.mycres.2008.02.004

Landhäusser SM, Mushin TM, Zwiazek JJ (2002) The effect of ectomycorrhizae on water relations in aspen (*Populus tremuloides*) and white spruce (*Picea clauca*) at low soil temperatures. Can J Bot 80:684–689

Langenfeld-Heyser R, Gao J, Ducic T, Tachd P, Lu CF et al (2007) *Paxillus involutus* mycorrhiza attenuate NaCl-stress responses in the salt-sensitive hybrid poplar *Populus* × *canescens*. Mycorrhiza 17:121–131, doi:10.1007/s00572-006-0084-3

Latva-Karjanmaa T, Suvanto L, Leinonen K, Hannu R (2003) Emergence and survival of *Populus tremula* seedlings under varying moisture conditions. Can J For Res 33:2081–2088, doi:10.1139/x03-129

Lemus R, Lal-Referee R (2005) Bioenergy crops and carbon sequestration. Crit Rev Plant Sci 24(1):1–21, doi:10.1080/07352680590910393

Leyval C, Turnau K, Haselwandter K (1997) Effect of heavy metal pollution on mycorrhizal colonization and function: physiology, ecological and applied aspects. Mycorrhiza 7:139–153, doi:10.1007/s005720050174

Loewe A, Einig W, Shi L, Dizengremel P, Hampp R (2000) Mycorrhizal formation and elevated CO_2 both increase the capacity for sucrose synthesis in source leaves of spruce and aspen. New Phytol 145:565–574, doi:10.1046/j.1469-8137.2000.00598.x

Malajczuk N, Lapeyrie F, Garbaye J (1990) Infectivity of pine and eucalypt isolates of *Pisolithus tinctorius* on roots of *Eucalyptus urophylla* in vitro. New Phytol 114:627–631, doi:10.1111/j.1469-8137.1990.tb00433.x

Marmeisse R, Gryta H, Jargeat P, Fraissinet-Tachet L, Gay G et al (1999) Hebeloma. In: Cairney JWG, Chambers SM (eds) Ectomycorrhizal fungi—Key genera in profile. Springer, Berlin Heidelberg New York, pp 89–127

Melin E (1923) Experimentelle untersuchungen über die birken-und espenmykorrhizen und ihre pilzsymbionten. Sven Bot Tidskr 17 (4):479–520

Molina R, Palmer JG (1982) Isolation, maintenance and pure culture manipulation of ectomycorrhizal fungi. In: Schenk NC (ed) Methods and principles of mycorrhizal research. American Phytopathological Society, St Paul, pp 115–129

Niemi K, Julkunen-Tiitto R, Haggman H, Sarjala T (2007) *Suillus variegatus* causes significant changes in the content of individual polyamines and flavonoids in Scots pine seedlings during mycorrhiza formation in vitro. J Exp Bot 58(3):391–401, doi:10.1093/jxb/erl209

Parladé J, Pera J, Luque J (2004) Evaluation of mycelial inocula of edible *Lactarius* species for the production of *Pinus pinaster* and *P. sylvestris* mycorrhizal seedlings under greenhouse conditions. Mycorrhiza 14:171–176, doi:10.1007/s00572-003-0252-7

Perrin E, Parlade X, Pera J (1996) Respectiveness of forest soils to ectomycorrhizal association: I. Concept and method as applied to the symbiosis between *Laccaria bicolor* (Maire) Orton and *Pinus pinaster* Art or *Pseudotsuga menziesii* (Mirb.) Franco. Mycorrhiza 6:469–476, doi:10.1007/s005720050149

Peterson RL, Chakravarty P (1991) Techniques in synthesizing ectomycorrhiza. In: Norris J, Read D, Varma A (eds) Methods in microbiology. Academic, U.S., pp 75–107

Robinson BH, Mills TM, Petit D, Fung LE, Green SR et al (2000) Natural and induced cadmium-accumulation in poplar and willow: implications for phytoremediation. Plant Soil 227:301–306, doi:10.1023/A:1026515007319

Sell J, Kayser A, Schulin R, Brunner I (2005) Contribution of ectomycorrhizal fungi to cadmium uptake of poplars and willows from a heavily polluted soil. Plant Soil 277:245–253, doi:10.1007/s11104-005-7084-5

Selle A, Willmann M, Grunze N, Geßler A, Weiß M et al (2005) The high-affinity poplar ammonium importer PttAMT1.2 and its role in ectomycorrhizal symbiosis. New Phytol 168:697–706, doi:10.1111/j.1469-8137.2005.01535.x

Tagu D, Rampant PF, Lapeyrie F, Frey-Klett P, Vion P et al (2001) Variation in the ability to form ectomycorrhizas in the F1 progeny of an interspecific poplar (*Populus* spp.) cross. Mycorrhiza 10:237–240, doi:10.1007/PL00009997

Unterbrunner R, Puschenreiter M, Sommer P, Wieshammer G, Tlustos P et al (2007) Heavy metal accumulation in trees growing on contaminated sites in Central Europe. Environ Pollut 148:107–114, doi:10.1016/j.envpol.2006.10.035

Wallander H, Söderström B (1999) Paxillus. In: Cairney JWG, Chambers SM (eds) Ectomycorrhizal fungi—Key genera in profile. Springer, Berlin Heidelberg New York, pp 231–252

Wong KKY, Fortin A (1988) A Petri dish technique for the aseptic synthesis of ectomycorrhizae. Can J Bot 67:1713–1716

Worrell R (1995) European aspen (*Populus tremula* L.): a review with particular reference to Scotland. I. Distribution, ecology and genetic variation. Forestry 68(2):96–105, doi:10.1093/forestry/68.2.93