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Ectomycorrhizal synthesis of *Lactarius sanguifluus* (Paulet) Fr. with *Abies pindrow* Royle Ex D. Don

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Abstract
This study was aimed to perform *in vitro* mycorrhizal synthesis between *Abies pindrow* and *Lactarius sanguifluus* was achieved. *A. pindrow* seedlings inoculated with mycelial culture of *L. sanguifluus* resulted in the formation of short, branched lateral roots which ultimately form ectotrophic mycorrhizae. Synthesized mycorrhizae were light brown to pale yellow in colour. The transverse sections of the synthesized roots showed a typical ectomycorrhizal anatomy. The anatomical structure of mycorrhiza revealed the presence of thick fungal mantle and well developed "Hartig net". Pure culture of *L. sanguifluus* was reisolated from both vermiculite peat moss mixture and synthesized ectomycorrhizae. These were compared with the original culture isolated from the fruiting bodies of *L. sanguifluus* and were found to have same cultural characteristics, thus confirming the symbiotic association.

Keywords: *Lactarius sanguifluus*, ectomycorrhiza, *in vitro*

Introduction
Lactarius sanguifluus is an ectomycorrhizal mushroom belonging in the family russulaceae grow scattered or in groups on the ground under conifers forest. Mycorrhizal diversity has been studied primarily in coniferous ecosystems (Izzo *et al*., 2005; Korkama *et al*., 2006; Kennedy *et al*., 2007; Taniguchi *et al*., 2007; Tedersoo *et al*., 2006) [14, 17, 16, 35, 37]. Mushrooms produced by mycorrhizal fungi can be observed near host trees at certain times of the year and are evidence of mycorrhizae in the soil. It should be kept in mind that not all mushroom producing fungi are mycorrhizal, and forest also have a diverse array of large, fleshy fungi that are saprophytic, parasitic or mutualistic in other ways (Pilz and Molina, 1996) [30].

These fungi play a crucial role in the growth and survival of forest trees by enhancing nutrient acquisition (Landeweert *et al*., 2001) [20], drought tolerance (Morte *et al*., 2000) [25] and pathogen resistance of their hosts (Branzanti *et al*. 1999) [2]. In return, the autotrophic hosts provide carbohydrates to their heterotrophic fungal partners. In natural forest ecosystems, the roots of single tree are almost invariably associated with several different ectomycorrhizal species (Dahlberg, 2001; Guidot *et al*., 2003) [6, 12].

It has been found that ectomycorrhizae are the dominant forms of mycorrhizae and can be found on about 90% of the trees in temperate and boreal forests (Le Tacon *et al*., 1992) [22]. During the last decades, the application of mycorrhizae in forestry has experienced a very important role. Noteworthy studies are related to the selection of those fungal species and isolates which best adapt to the environmental conditions (Mortier *et al*., 1988; Kuek *et al*., 1992) [26, 19], the most effective inoculum production techniques (Le Tacon, 1983; Mauperin *et al*., 1987; Kuek *et al*., 1992) [21, 2319], and the inoculation and production of forest tree species in the nursery (Hung and Trappe, 1987; Browing and Withney, 1991) [13, 4]. Numerous *in vitro* systems of mycorrhizal synthesis have been developed and examined the ability of fungi to form ectomycorrhizae (Chilvers *et al*., 1986; Kottke *et al*., 1987; Kasuya *et al*., 1992; Guerin-Laguette *et al*., 2000; Rincon *et al*., 2001, Eros-Honti and Jakucs, 2009; Geng *et al*., 2009) [5, 18, 15, 11, 31, 7, 9].

Abies pindrow is an important conifer which has got ecological as well as economic importance. The wood of this plant is used as fuel wood, for making slippers and light camp furniture, for planking, for preparing wood pulp and slanting roofs in hill houses. In addition this plant checks the soil erosion in higher reaches and hence playing important role in the ecology of area. Generally commercial nursery seedlings generally either lack mycorrhizal fungi or have a very limited flora associated with their root systems. Hence keeping into consideration the economic importance of *Abies pindrow* and difficulty in its natural regeneration, the present investigations have been under taken to utilize the natural mycobionts in artificial mycorrhizal synthesis.

Materials and Methods
In vitro synthesis of ectomycorrhiza was carried out following Molina (1979) [24]. The method involves using of 10ml peat, 90ml of vermiculite and 70ml of fungal medium for each 200ml synthesis tube. A disc of 5mm containing fungal culture was added to each autoclaved tube and ten replicates were used for each fungus and the uninoculated controls. After the mycelium colonise the peat-vermiculite medium for 2 weeks, sterile and aseptically germinated seedlings of *Abies pindrow* were introduced into synthesis tube and tubes were placed in growth chamber. The shoot remains outside of the synthesis tube through the slit and the root remains in aseptic condition. Tubes were sealed with aluminum foil to keep the roots and fungus in darkness. So this method provides seedlings with a natural shoot-root compartmentation by exposing the shoots to the atmosphere. Tubes were periodically checked for mycorrhization and

seedlings harvested after 5 months. For observation of early morphological changes during ectomycorrhiza formation, technique of Fortin *et al*. (1980) [8] was followed. On the conclusion of synthesis experiment, small bits of inoculum were removed aseptically from the substrate (in synthesis vessel) and also from the synthesized ectomycorrhizae and were grown in Petri plates containing nutrient medium. The isolates were compared with the original culture for growth characteristics to confirm the symbiotic association.

Results and Discussion

Lactarius sanguifluus was observed to be growing in association with *Abies pindrow* under natural conditions. This indicated that this mushroom is probably mycorrhiza former with this tree. Therefore pure culture of *Lactarius sanguifluus* was tested for their ability to form ectomycorrhiza with *Abies pindrow*. *In vitro* mycorrhizal synthesis was achieved between *A. pindrow* and *L. sanguifluus* in six months. Seedlings inoculated with mycelial culture of ectomycorrhizal mushroom resulted in the formation of short branched lateral roots which ultimately form ectotrophic mycorrhizae. Ectomycorrhizal symbiont form Light brown to pale yellow type of ectomycorrhizae (Fig. 1 and Table 1).

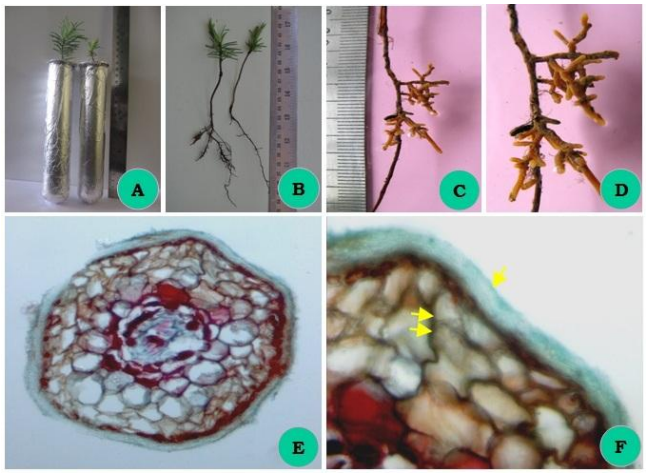

Fig 1. (A) Test tubes containing seedlings of *A. pindrow* (Seedling with large shoot inoculated with culture of *L. sanguifluus* and with smaller shoot grown in sterile medium and kept as control) for *in vitro* synthesis of ectomycorrhiza. (B) Uprooted seedlings of *A. pindrow* (after *in vitro* synthesis experiment) larger seedling showing synthesized ectomycorrhiza, smaller seedling (kept as control) without any ectomycorrhiza. (C) Root system of inoculated seedling. (D) A close view of synthesized ectomycorrhizae (E) T. S. of synthesized ectomycorrhizal root (20X). (F) Enlarged view (40X) of E showing fungal mantle (single arrow) and Hartig net (double arrow)

Similarly, Trappe (1962) [38] postulated ectomycorrhizal relationships among forest trees and homo basidiomycetes on the basis of field observations. Evidence for many of these reports is restricted to field observations of fungus plant occurrences. Alexander (1981) [1] described and compared ectomycorrhizae formed by *Lactarius rufus* and *Picea sitchensis* in the field and under aseptic conditions. Synthesized ectomycorrhizae were essentially similar to those obtained from the field except in the colour and degree of development and attributed these differences in colour and fungal sheath to ageing processes. Vaario *et al*., (2000) [39] reported the first *in vitro* aseptic synthesis of mycorrhiza between *Abies firma* and *Pisolithus tinctorius* and *Cenococcum geophilum*. Geng *et al*. (2009) [9] observed that *Tuber indicum* formed mycorrhizae after 5 months of inoculation with *Castanea mollissima* and 4 months with *Pinus armandii* respectively. Perez *et al*. (2007) [28] inoculated two species of Southern beech (*Nothofagus obliqua* and *N. glauca*) with *Tuber melanosporum*. Ectomycorrhizal development was monitored for 6 months. *T. melanosporum* readily formed ectomycorrhizae with seedlings of *N. oblique* whereas mycorrhiza formation was very sparse in *N. glauca* seedlings. Yamada *et al*. (2001) [41] tested edible ectomycorrhizal fungi in the genera *Lyophyllum*, *Tricholoma*, *Suillus*, *Rhizopogon* and *Lactarius* for *in vitro* mycorrhization with *Pinus densiflora* and observed that these genera formed ectomycorrhizae in 2-4 months after fungal inoculation. Samson and Fortin (1988) [33] gave the structural characterization of ectomycorrhizae synthesized on *larix laricina* with six species of *Fuscoboletinus* and two species of *Suillus*. Synthesized ectomycorrhizae were olivaceous yellow, white to pale yellow, white to grayish brown, white to pinkish grey.

Table 1. Morphological characteristics of *Abies pindrow* ectomycorrhiza formed during *in vitro* synthesis with *Lactarius sanguifluus*

Sr. No.	Characters	
A	**Macroscopic**	
i	Colour	Light brown to pale yellow
ii	Shape of mycorrhiza	Reticulate, coralloid and profusely branched
iii	Texture	Smooth to shiny
iv	Odour and taste	Not distinct
v	Emanating Hyphae	Infrequent
vi	Root Hairs	Absent
B	**Microscopic**	
i	Thickness of Mantle	20-25 µm
ii	Degree of development of "Hartig net"	Well developed

The transverse section of the synthesized mycorrhizal roots of *A. pindrow* showed a typical ectomycorrhizal anatomy. The anatomical structure of mycorrhiza showed the presence of thick fungal mantle and well developed "Hartig net" (Fig. 1). The roots of seedlings kept as control were non-mycorrhizal, pale in colour with prominent root hairs. No Fungal mantle and no Hartig net were present in transverse section of these roots. Pure culture of *L. sanguifluus* was reisolated from both vermiculite peatmoss mixture and synthesized ectomycorrhizae. These were compared with the original culture and were found to have same cultural characteristics, thus confirming the identity of these mycobionts. Previously, many similar studies were also carried out various workers on *in vitro* mycorrhizal synthesis. Piche and Fortin (1982) [29] synthesized *Pinus strobus* ectomycorrhizae with seven different species (*Cenococcum geophilum*, *Hebeloma cylindrosporum*, *Paxillus involutus*, *Pisolithus tinctorius*, *Suillus granulatus*, *Suillus tomentosus* and *Thelephora terrestris*) of fungi. Hartig net and mantles were observed in all cases. *P. involutus*, *P. tinctorius* and *T. terrestris* were much more rapid in forming ectomycorrhizae than the other species. Yamada and Katsuya (1995) [40] also reported mycorrhizal

synthesis between *P. densiflora* and 21 fungal species, including two species of *Russula*, which are also difficult to manipulate under *in vitro* conditions (Taylor and Alexander, 1989) [36].

Santiago-Martinez *et al.* also (2003) [34] carried out *in vitro* synthesis between *Pinus montezumae* and *Laccaria bicolor*. The synthesized mycorrhizal system ranged from simple to dichotomous and was occasionally irregular, measuring from 2.5 to 3.6 mm. The colour was light brown to dark brown. The dichotomous morphology and the penetration of the Hartig net by two to three cortical cells has already been described for *Pinus pinea* mycorrhizae with *Laccaria laccata* (Branzati *et al*., 1985) [3]. *In vitro* mycorrhizal synthesis studies provide the direct and scientifically rigorous means of determining ability of a fungal isolate to form mycorrhizae. Morphology and anatomy combined with other distinctive characters provide essential data for identifying mycobionts from field connections (Palm and Stewart, 1984; Godbout and Fortin, 1985) [27, 10]. Sagar and Lakhanpal (2005) [32] carried out pure culture synthesis of *Pinus wallichiana* ectomycorrhiza with *Suillus sibiricus* and also successfully reisolated culture of this mushroom from the synthesized ectomycorrhizae. The synthesized ectomycorrhizae were creamish yellow in colour, bifurcate to coralloid and transverse section of the mycorrhizal roots revealed a typical ectomycorrhizal anatomy.

Conclusions

The seedlings of *Abies pindrow* inoculated with mycelial culture of ectomycorrhizal mushrooms resulted in the formation of short branched lateral roots which ultimately form ectotrophic mycorrhizae. It was observed that *L. sanguifluus* formed light brown to pale yellow ectomycorrhiza. These mycorrhizal roots had no specific taste and odour. The thickness of the fungal mantle was found to be 20-25 µm and Hartig net uniformly penetrated the intercellular spaces between the cortical cells and it was well developed.

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