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Aseptic *In vitro* Synthesis of *Pinus gerardiana* Ectomycorrhizae with *Amanita ceciliae* and *Lactarius sanguifluus*

Amit Kumar Sehgal* and Anand Sagar

Department of Biosciences, Himachal Pradesh University, Summer Hill, Shimla (H.P.), india.

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The present investigations are aimed to synthesize in vitro ectomycorrhizae between Pinus gerardiana and two gilled ectomycorrhizal (ECM) mushrooms (Amanita ceciliae and Lactarius sanguifluus). To carry out in vitro synthesis, pure cultures of ECM mushrooms (A. ceciliae and L. sanguifluus) were isolated on Potato Dextrose Agar (PDA) and Modified Melin-Norkans (MMN) Medium respectively. The synthesis was achieved successfully in the surfaced sterilized seedlings of P. gerardiana geminated under aseptic conditions by using vermiculite, peat, medium for ECM fungi and inoculum of each fungus in the test tubes. Mycorrhization was checked periodically in the test tubes. P. gerardiana seedlings were lifted from test tubes after five months to observe ectomycorrhizae formation on the root system with A. ceciliae and L. sanguifluus. The synthesized ectomycorrhizae were dark brown in case of A. ceciliae whereas in case of L. sanguifluus the colour of ECM roots was yellowish brown. Anatomy of synthesized ectomycorrhizae with both ECM fungi showed fully developed fungal mantle and Hartig net. The seedlings with ECM synthesis showed a significant effect on the growth and development.

Keywords: Amanita ceciliae; Ectomycorrhiza; In vitro synthesis; Lactarius sanguifluus; Pinus gerardiana.

The ECM fungi form symbiotic interactions with roots of forest trees and play a vital role to bring about various benefits to the plant species, including enhancement in the roots absorption surface area [1], increased uptake of various nutrients [2], resistance to pathogenic microorganisms [3] and drought stress [4]. ECM symbiosis also help to increase the growth and nutrient contents of plants growing in nutrient deficient soils [5] and in return, the photosynthetic host tree species supply carbohydrates to their non-photosynthetic ECM fungal partners.

The roots of a single tree in natural forest ecosystems are almost invariably associated with many different ECM fungal species [6]. Therefore host specificity of ECM fungi is an important part of forest ecosystems, which can be utilized as a tool for managing different forest resources [7]. Natural hosts of ECM mushroom *Tricholoma matsutake* include *Pinus sylvestris* and *P. abies* [8,9], *P. densiflora* and *Quercus mongolica* in northeastern China and Korean peninsula [10,11], *P. armandii*, *P. densata*, *P. wallichiana*, *P. yunnanensis*, *Castanopsis orthacantha*, *Q. aquifolioides*, *Q. pannosa*, *Q. guyavifolia*, *Q. semecarpifolia*, *Lithocarpus* spp., and *Pasania* spp. in southwestern China and Bhutan [8,11] whereas *Amanita muscaria*, *Laccaria laccata*, *Pisolithus* sp. and *Suillus brevipes* have

*Corresponding author E-mail: aksbios@hpuniv.ac.in

been reported to produce ectomycorrhizae with *P. patula* [12].

Many systems of *in vitro* ECM synthesis have been developed by various researchers to examine the potential of different ECM fungi to form symbiosis with different forest tree species [13-18]. ECM synthesis investigations are helpful to decide fungus and host plant compatibility for morphological, anatomical and physiological investigations [19].

Pinus gerardiana is an important edible nut yielding conifer and is well known in dry fruit trade. The trade of its seeds significantly contributes to the annual income of most of the tribal families living in the area of its distribution. In district Kinnaur of Himachal Pradesh alone approximate export value of its annual produce around 18 crores rupees. It clearly reflects that the tree is not only ecologically important to the area but also has direct relevance to the economic status of the people residing over there [20]. Hence keeping into consideration the immense economic as well as ecological importance of *P. gerardiana*, the present investigation is aims to know about the ECM symbiosis between two gilled mushrooms and *P. gerardiana* also provide morphological and anatomical account of the synthesized ECM roots. These ECM fungi could be a very useful tool for the artificial inoculation in the nurseries to develop inoculated ECM seedling which further could also show better establishment in its natural habitats and helps in the reforestation and regeneration programs of *P. gerardiana* in Himachal Pradesh, India.

MATERIALS AND METHODS

Collection and isolation of pure culture of ECM fungi

The sporophore of *A. ceciliae* and *L. sanguifluus* were collected from district Kinnaur of Himachal Pradesh. The cultures were isolated from pileus region of the fresh sporophore of *A. ceciliae* and *L. sanguifluus*. Pileus was pulled gently apart and exposed interior tissue was removed with the help of sterilized scalpel and transferred into Petri plates containing PDA and MMN Medium. Then they were incubated at 25^{0C} and observed regularly for the appearance of culture. The well-grown colonies were subcultured on a PDA medium to obtain pure cultures.

Surface sterilization and seeds germination

Seeds of *P. gerardiana* were washed for 4 hours under the running tap H_2O and then treated with 10% H_2O_2 for 10 minutes. The H_2O_2 was drained and seeds were rinsed in sterilized distilled H_2O for 2 minutes. Then they were treated with 10% NaClO and Tween-20 and kept for 30 minutes. After that washed 5-6 times using sterilized distilled H_2O. Then seeds were placed in sterilized H_2O for 24 hours. After that seeds were put on sterilized wet filter paper in petri-plates and placed in low temperature for 24 hours before put in the seed germinator.

In vitro ECM synthesis

ECM synthesis was carried out by using *in vitro* synthesis experiments [21]. For this 70ml of fungal nutrient medium, 90ml of vermiculite and 10ml peat was used in each 200ml test tube. The mixture of test tubes was autoclaved and then for treatments a disc of 5mm fungal culture was placed in test tube under aseptic conditions. For both treatments and uninoculated controls 10 replicates were used and treatments were kept for mycelium colonization. After that aseptically germinated *P. gerardiana* seedling was put into each treatments and control placed in the growth chamber and observed regularly for ECM synthesis. After 5 months seedlings pulled out from test tubes and checked for mycorrhization by following the methods of Fortin et al. [22].

Confirmation of ECM synthesis

The confirmation of ECM synthesis was done following Moore et al. [23]. For this a minute inoculum of vermiculite, peat mixture was removed from test tubes and inoculated on Petri plates containing nutrient medium. The synthesized ECM roots were detached from root system of the seedlings, surface sterilized and small pieces of ECM roots were inoculated on nutrient medium. Then Petri plates were observed for the mycelial growth and fungal colonies formed from both were compared with original ECM fungal culture colonies to confirm the ECM synthesis.

Morpho-anatomical characterization of ectomycorrhiza

Morphological as well as anatomical detail of ECM roots was made by following Zak

[24]. The various growth characteristics e.g. form of ectomycorrhiza, colour of the fungal mantle, thickness of mantle etc. were recorded. The fixation ECM roots was done in F.A.A. solution and then preserved by using 70% alcohol. Anatomical details were worked out in both fresh as well as from preserved material. The sectioning of ECM roots was done following Johansen [25].

Effect of *in vitro* ECM synthesis on seedlings growth

The five *P. gerardiana* seedlings were lifted randomly from each treatment and control. The observations on various growth parameters e.g. shoot length, root length, total number of short roots, shoot fresh weight, root fresh weight, shoot dry weight and root dry weight were recorded.

Statistical analysis of the data

The data was statistically analyzed, with the help of ANOVA test and Tukey's multiple compression test was used to determine HSD (honestly significant difference) values for significance among various mean values.

RESULTS

Isolation of pure culture and sub culturing of ECM fungi

The pure cultures of *A. ceciliae* was isolated on PDA medium. The colonies form dense mycelial mat and are white in colour. The pure culture of *L. sanguifluus* has been isolated on MMN medium. The colony of mycelium form concentric

Table 1. Morpho-anatomical description of *Pinus gerardiana* ECM roots synthesized with *Amanita ceciliae* and *Lactarius sanguifluus*

No.		Ectomycorrhizal Mushrooms	
		Amanita ceciliae	*Lactarius sanguifluus*
	Macroscopic Characteristics		
1	Colour	Dark Brown	Yellowish Brown
2	Shape	Branched, Monopodial	dichotomously branched
3	Texture	Smooth to loosely wooly	Smooth to shiny
4	Odour and taste	Not distinct	Not distinct
5	Emanating Hyphae	Many long irregularly radiating outwards	Infrequent
6	Root Hairs	Absent	Absent
	Microscopic Characteristics		
7	Thickness of Mantle	25-30 µm	15-20 µm
8	Degree of development of "Hartig net"	Well developed	Well developed

Table 2. Effect of artificial inoculation on the growth of *Pinus gerardiana* seedlings

No.	Growth Characteristics	Inoculated Treatments (cm ± S.D.)		Un-inoculated (cm ± S.D.)
		A. ceciliae	*L. sanguifluus*	Control
1	Root Length (cm)	41.30±1.53**	51.24±1.22**	34.58±3.38
2	Shoot Length (cm)	15.06±1.24**	14.38±1.27**	8.88±1.21
3	Total No. of Short Roots	73.60±7.81**	62.40±5.00**	12.60±2.30
4	Root Fresh Weight (mg)	1.64±0.36**	2.59±0.34**	0.23±0.06
5	Shoot Fresh Weight (mg)	2.64±0.60**	2.36±0.55**	0.98±0.13
6	Root Dry Weight (mg)	0.27±0.10**	0.48±0.09**	0.09±0.02
7	Shoot Dry Weight (mg)	0.83±0.23**	0.78±0.25*	0.35±0.04

** $p<0.01$; * $p\leq0.05$; [NS]; Non-Significant differences as revealed through one way ANOVA and Tukey's HSD multiple comparison test.

zones during its growth. The margin of the colony was irregular. Sub culturing of ECM mushrooms was carried out on PDA medium.

***In vitro* ECM synthesis**

The pure cultures of ECM mushrooms (*A. ceciliae* and *L. sanguifluus*) were anlysed for their potential of ECM synthesis with the roots of *P. gerardiana* seedlings (Figure 1A and Figure 2A). The experiments revealed the successful *in vitro* mycorrhization between the host and two ECM mushrooms tested. The treated seedling showed the formation of many branched short lateral roots which ultimately leads to the development of ectomycorrhizae (Figure 1B and Figure 2B). The *A. ceciliae* and *L. sanguifluus* formed dark brown (Figures 1C,D) and yellowish brown types (Figures 2C,D) of ECM roots respectively. The description of synthesized ECM roots is given in Table 1.

Effect of *in vitro* ECM Synthesis on the seedlings growth

The seedlings of *P. gerardiana* were evaluated for various growth characteristics after the completion of *in vitro* experiments with both ECM fungi tested. The results on different growth characteristics are presented in Table 2. The results revealed that in both treatments (*A. ceciliae* and *L. sanguifluus*) there was a significant difference($P<0.01$ and $P<0.05$) on the overall

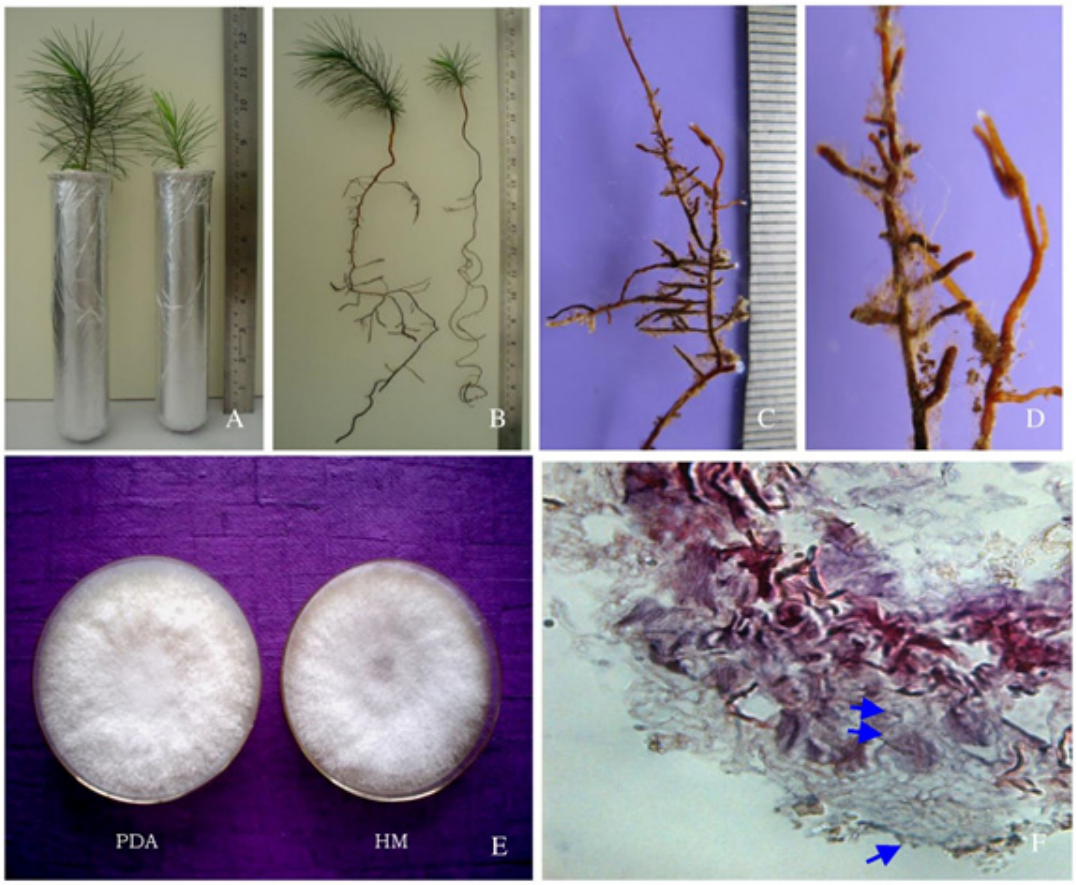

Fig. 1. *In vitro* ECM mycorrhization between *Pinus gerardiana* and *Amanita ceciliaes*: A. Test tubes showing *P. gerardiana* seedlings (Larger one with inoculation treatment with *A. ceciliae* pure culture, smaller one is control). B. Uprooted seedlings of *P. gerardiana* (larger with ECM synthesis having intensive growth in root system where as smaller one is control without any ectomycorrhization having less developed root system). C and D. Synthesized ECM roots. E. Pure cultures of *A. aeciliae* reisolated on PDA and HM nutrient medium from the synthesized ECM roots. F. T.S. of ECM root showing well developed fungal mantle (single arrow) and Hartig net (double arrow).

Fig. 2. *In vitro* ECM mycorrhization between *Pinus gerardiana* and *Lactarius sanguifluus*: A. Test tubes showing *P. gerardiana* seedlings (Larger one with inoculation treatment with *L. sanguifluus* pure culture, smaller one is control). B. Uprooted seedlings of *P. gerardiana* (larger with ECM synthesis having intensive growth in root system where as smaller one is control without any ectomycorrhization having less developed root system). C and D. Synthesized ECM roots. E. Pure cultures of *L. sanguifluus* reisolated on PDA and MMN nutrient medium from the synthesized ECM roots. F. T.S. of ECM root showing well developed fungal mantle (single arrow) and Hartig net (double arrow)

growth characteristics of the seedlings as compared to the control (Table 2). Therefore *in vitro* ECM synthesis have significant effect on the seedlings growth and development.

The anatomical details of synthesized ECM roots with both ECM mushrooms tested showed the formation fungal mantle and Hartig net (Figures 1F and Figures 2F). In case of *A. ceciliae*, the thickness of fungal mantle was 25-30 µm whereas in case of *L. sanguifluus* it was 15-20 µm (Table 1). The seedlings kept as control were non-mycorrhizal with prominent root hairs and without ECM anatomical features in the transverse section of their roots. The culture colonies of *A. ceciliae* was reisolated on PDA and Hagem's nutrient medium from both synthesized ECM roots and peat moss, vermiculite mixture (Figure 1E). Similarly the culture colonies of *L. sanguifluus* was reisolated on PDA and MMN nutrient medium from both synthesized ECM roots and peat moss, vermiculite mixture (Figure 2E)

The culture colonies of *A. ceciliae* and *L. sanguifluus* reisolated were found to have similar characteristics while compared with the culture colonies isolated from the fresh sporophores of both ECM mushrooms. Thus both (*A. ceciliae* and *L. sanguifluus*) confirming the synthesis of ECM roots with *P. gerardiana* seedlings.

DISCUSSION

During the present study two ECM mushrooms *A. ceciliae* and *L. sanguifluus* were isolated into pure culture on PDA and MMN medium respectively. The findings of the present study are supported by the results of several previous researches that reported mycelial growth of ECM fungi on various nutrient media. *Phlebopus portentosus* isolated on (Murashige and Skoog Agar) MS medium [26], *Scleroderma sinnamariense* prefer (Fungus-Host) FH medium for mycelial growth [27], *Rhizopogon roseolus* and *Tricholoma matsutake* cultures show best mycelia growth on PDA medium [28], *Pisolithus microcarpus* was isolated on (Pridham-Gottlieb Modifled by Kuek) PGK medium [29], Similarly Endo et al. [30] isolated specimens of *B. Edulis* on MA medium and subculturing was done successfully on (Malt Extract Agar) MA and (Malt Yeast Agar) MYA nutrient media. However, Agueda et al. [31] isolated *Boletus aereus*, *B. edulis*, *B. pinophilus* and *B. reticulatus* on BAF nutrient medium. The pure cultures are greatly affected by the changes made in cultivation medium and most of the ECM Boletale mushrooms favour media containing malt such as MA and MMN [29].

The genus *Pinus* is a host for numerous ECM fungi and these fungi made symbiotic association with the roots of this conifer [32]. During the present investigations *in vitro* ectomycorrhizae of *P. gerardiana* were synthesized with two ECM mushrooms (*A. ceciliae* and *L. sanguifluus*). Similarly mycorrhization between *Picea abies* and *Cenococcum geophilum* was achieved in asceptic conditions by inoculating mycelum on MMN medium [33]. Vaario et al. [34] achived ECM *in vitro* synthesis between *Abies firma* and *Pisolithus tinctorius*. *Pinus densiflora* is found to be formed ECM association in laboratory conditions with broad range of ECM fungi [35,36].

In Tibet, *Tricholoma matsutake* synthesized mycorrhizae in coniferous and fagaceous hosts tree in natural habitat and synthesized Hartig net in the ECM roots of *P. densiflora* [35]. In Canada *T. matsutake* synthesized mycorrhizae in pine and oak hosts trees [37] and also formed typical Hartig net in *P. densiflora* ECM roots.

In similar study Laiye et al. [38] utilized vermiculite, modified MMN medium and peat moss for the *in vitro* synthesis of ECM roots in the two Larch spp. seedlings with various ECM fungi. More recently, ECM *in vitro* synthesis of *Cedrus deodara* with *Rhizopogon himalayensis* [17], *Pinus gerardiana* with *Boletus edulis* and *Suillus scibricus* [18] was achived successfully.

The successes was achieved to germinate spores [39], obtaining pure culture mycelia, *in vitro* synthesis of ectomycorrhizae [40] and artificial shiro formation in potted culture system containing granite based soil substrate [41].

ECM fungi had a wide range of host specificities as reported by Kumla et al. [42] and synthesized *in vitro* ectomycorrhizae in two tree species viz. *Eucalyptus camaldulensis* and *Pinus kesiya* with single ECM fungus *Pisolithus orientalis*. Pure culture of *P. tinctorius* had potential for ECM association with *Pinus taiwanensis* in aseptic systems, but the association has not been reported in natural conditions [43]. Gomes et al. [44] reported that cultures of *P. arhizus* isolated from fruiting bodies form symbiotic association with *Quercus suber* and also had the potential to synthesize mycorrhiza with *Arbutus unedo* under laboratory and nursery conditions. The ectomycorrhizae were appears mainly on short lateral apices [45].

The results of the morpho-anatomical study were similar to the previous reports of Samson and Fortin [46] to synthesized grayish brown to pinkish grey, whitish and yellow coloured ECM roots in *Larix laricina* with two ECM fungal genus *Fuscoboletinus* and *Suillus*. *In vitro* synthesis of ECM roots between *S. sibiricus* and *P. wallichiana* was achieved successfully in vessels and formed ECM roots were bifurcate to coralloid and creamish yellow in colour [47]. Garcia-Rodriguez et al. [48] carried out synthesis to ECM roots in *Eucalyptus urophylla* and *Pinus greggii* with *Pisolithus tinctorius*, the synthesized ectomycorrhizae were simple, 1.0-2.6 mm long, yellow brown to bright yellow in colour and fungal mantle was 15.0 µm in thickness whereas ECM roots formed in *Pinus greggii* were bright yellow to yellowish brown in colour, dichotomously divided and rarely monopodial,.

The ECM roots produced during *in vitro* experiments in the present study showed similar anatonical features as that observed in

many previous studies carried out by Kumla et al. [42] and observed that *Pisolithus orientalis* has ability to synthesized fungal mantles under *in vitro* conditions with *Pinus kesiya* and *Eucalyptus camaldulensis*.The both fungal mantles were plectenchymatous and similar to the mantle synthesized by *Pisolithus tinctorius* with *Abies firma* and *Quercus ilex*, *Q. coccifera* [49], *Pisolithus microcarpus* with *Eucalyptus grandis* [50] and *Pisolithus aurantioscabrosus* with *Shorea* sp. [51], *Cedrus deodara* with *Rhizopogon himalayensis* [17], *Suillus scibiricus*, *Boletus edulis* with *Pinus gerardinan* [18]. Similar fungal mantle is synthesized by *Boletus edulis* with *Cistus* spp. [31] and *Picea abies* [52].

The culture of *A. ceciliae* and *L. sanguifluus* were reisolated from both synthesis substrate mixture as well as from synthesised ECM roots. They have similar characteristics as that of original cultures isolated from the fruiting bodies, thus validate the successful *in vitro* synthesis of ectomycorrhizae with these two ECM mushrooms. Similarly, *Scleroderma aurantium* was reisolated from both peat vermiculite mixture and synthesized ECM roots on MMN nutrient medium [53]. The fungal cultures were also reisolated from both synthesis experiment substrate mixture and from synthesized ECM roots to verify symbiotic interactions [47,16,17].

CONCLUSION

The present study has synthesized ECM roots of *P. gerardiana* under laboratory conditions by using *in vitro* experiments with the pure cultures of two ECM mushrooms (*A. ceciliae* and *L. sanguifluus*). The synthesized ectomycorrhizae with *A. ceciliae* and *L. sanguifluus* were dark brown and yellowish brown in colour respectively. The T.S. of ECM roots showed a typical ECM anatomy.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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