BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, December 2022. Vol. **19**(4), p. 1009-1018

In vitro Mycorrhization of Two Wild Edible Bolete Species with *Pinus gerardiana* - An Economically High Altitude Conifer

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http://dx.doi.org/10.13005/bbra/3050

(Received: 29 March 2022; accepted: 16 October 2022)

This paper describes for the first time in vitro mycorrhization between the two wild edible boletes (Boletus edulis and Suillus sibiricus) with Pinus gerardiana. The synthesis was carried out in a controlled growth chamber using peat, vermiculite, fungal medium and mycelial inoculum of each fungi in test tubes. The test tubes were regularly observed for mycorrhization. The seedlings of P. gerardiana were picked after five months of inoculation to examine symbiotic association between its root system with B. edulis and S. sibiricus. The B. edulis formed dark reddish brown whereas S. sibiricus synthesized light brown orange coloured mycorrhizae. The transverse sections of synthesized mycorrhizae showed a well developed fungal mantle and Hartig net for both (B. edulis and S. sibiricus) ectomycorrhizal fungi tested. The mycorrhization has significant effect on the overall growth of seedlings as compared to control.

Keywords: Boletus edulis; Ectomycorrhiza; in vitro; Suillus sibiricus.

Fungi and plants have a long history of close association. Fungi have ability to penetrate and live within plants nutritional substrate and one of the most common fungal substrates is the tissue of plants, either living or dead. The numerous ECM (Ectomycorrhizal) fungi can colonize a wide gamut of hosts whereas few show host specificity.[1,2] ECM fungi viz. *Suillus*, *Rhizopogon*, *Truncocolumella* and *Hydnangium* show specificity at the host genus level rather than the host species level.[3,4]

Melin [5,6] revealed that *in vitro* experiments could be used to synthesize ectomycorrhizae of different conifers by inoculating their seedlings with pure cultures of appropriate fungi. The *in vitro* ECM synthesis provides a method to confirm the competence of a particular fungal culture to synthesize ectomycorrhizae and many *in vitro* techniques of ECM synthesis have been standardized and investigated for the potential of different fungi to synthesize ectomycorrhizae with their host partner.[7-21]

Boletus edulis has been reported in past to have mycorrhizal associations with *Pinus massoniana* [22], *Pinus patula* [23], *Pinus sylvestris* [24], *Picea abies* [25], *Pinus mugo* [26], *Pinus virginiana* [27], *Quercus pubescens*. [28] The genus *Suillus* also exhibits a high degree of host specificity [29] and various species of this genus are found to be closely associated with trees of Pinaceae family. [30-33] Duddridge et al.[34] worked out ECM rhizomorphs between *Suillus bovines* mycelium and sterile germinated seedlings of *Pinus sylvestris*

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and also studied their role in water and mineral transport. According to Vellinga et al.[35] *Suillus lakei* and several other ECM fungi were found to have symbiotically associated with *Pseudotsuga menziesii*.

A good amount of work has been done on different aspects of mycorrhizal association in different parts of the world [36-59], but there is no report on the *in vitro* synthesis of ectomycorrhiza with *P. gerardiana*.

P. gerardiana is an economically high altitude conifer growing at elevation between 1800 to 3350 meters. This conifer has very scarce distribution and is constricted to mountainous span of Eastern India, Pakistan, Afghanistan etc. It is one of the most important cash crop and fetches good income source to the tribal people of Himachal Pradesh and Jammu and Kashmir. [60] The aim of the study is to know about the mycorrhizal synthesis between the two wild edible boletes and *P. gerardiana* as well as provide anatomical descriptions of the synthesized ectomycorrhizae.

This study is the first attempt determines the mycorrhizal synthesis of *B. edulis* and *S. sibiricus* with *P. gerardiana*. Taking into account the increasing interest of reforestation programmes of this conifer in Himachal Pradesh, India, the use of these wild edible mushrooms could be a useful tool for the artificial inoculation in the nurseries to produced artificially inoculated seedling. After assessing the inoculation success, these seedlings could also show better establishment in its natural habitats and helps in regeneration of this high altitude conifer.

MATERIALS AND METHODS

Collection and pure culture isolation of ECM fungi

Fruiting bodies of *B. edulis* and *S. sibiricus* were collected from District Kinnaur of Himachal Pradesh. For pure culture isolation the pileus of fresh sporophore of *B. edulis* and *S. sibiricus* was pulled gently apart to expose interior tissue using fingertip pressure. The exposed interior tissue was quickly removed with flame sterilized fine tip scalpel and transferred into Petri plates containing Potato Dextrose Agar (PDA) and Hagem's Agar medium. The Petri plates were incubated in BOD incubator at 25^{0C} and observed periodically for the appearance of culture. The vigorously growing colonies were subcultured on Potato Dextrose Agar (PDA) medium to get pure cultures of ECM fungi.

Surface sterilization and germination of seeds

The seeds were placed under the running water for 4 hours and then treated with 10% hydrogen peroxide for 10 minutes. The hydrogen peroxide was drained out and seeds were rinsed in sterilized distilled water for 2 minutes. After that seeds were treated with 10% Sodium Hypochlorite along with Tween-20 and kept for 30 minutes. At the end, the seeds were washed 5-6 times using sterilized distilled water. The sterilized seeds were soaked in sterilized water for 24 hours. Then seed were placed in sterile petriplates containing wet sterilized filter paper and given cold treatment for 24 hours before placing in the seed germinator.

In vitro synthesis of ectomycorrhizae

In vitro synthesis of ectomycorrhiza was carried out following protocol given by Molina. [17] This procedure involves the use of 10ml peat, 90ml of vermiculite and 70ml of fungal nutrient medium in each 200ml test tube. The test tubes containing this mixture were autoclaved and a disc of 5mm fungal culture was put in to every autoclaved test tube and the lower portion of test tube was covered with aluminum foil. Ten replicates were kept for each fungal treatment and for uninoculated controls. When mycelium colonise the peat-vermiculite mixture a sterile and aseptically germinated seedling was added into each treatments and test tubes were kept in growth chamber.

The test tubes were regularly observed for mycorrhization and seedlings picked after 5 months. The technique of Fortin et al. [61] was used to observe the morphological changes occur in the root system due to mycorrhization.

Confirmation of mycorrhization

On the completion of experiment, small piece of inoculum was removed from the peat, vermiculite mixture present in synthesis test tube. The synthesized ectomycorrhizae were removed from the seedlings and after surface sterilization small pieces were inoculated in Petri plates containing nutrient medium and observed for the growth of fungal mycelium. [62] The isolates from substrate mixture as well as from synthesized

mycorrhizae were compared with original culture of ECM fungi for their characteristics to confirm the mycorrhizal synthesis.

Morpho-anatomical Study of Ectomycorrhiza

Morpho-anatomical study of mycorrhizal roots of *P. gerardiana* was carried out by following Zak [63]. The various characteristics viz. form of mycorrhiza, colour of the fungal mantle, thickness of mantle, texture of the fungal mantle, surrounding mycelium (present or absent), odour and taste, development of Hartig net, features of the surrounding hyphae of mycorrhizal roots were recorded.

Mycorrhizal roots were fixed in F.A.A. for one day and then preserved by using 70% alcohol. Anatomical details were carried out from the fresh as well as from preserved material. Sectioning was done following Johansen [64]. The colour of the Hartig net and mantle were observed in unstained sections.

Effect of *in vitro* mycorrhization on the growth of seedlings

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

Statistical analysis of the data

The data obtained for mycelial growth under different conditions were from five replicates. All data obtained was statistically analyzed. To find out the significance of difference between the mean values, one way analysis of variance (ANOVA) test and student's t-test was applied. Tukey's multiple compression test was used to determine honestly significant difference (HSD) values for significance among means.

OBSERVATIONS AND RESULTS

Pure culture isolation of ECM fungi

The *B. edulis* pure culture was isolated by using Potato Dextrose Agar (PDA) Medium. The colour of the colonies was white. The colonies were cottony in appearance. The culture of *S. sibiricus* has been isolated on Hagem's Agar Medium. Mycelia are slow growing and colony colour changes from white to cream. Sub culturing of both mushrooms was done on PDA medium.

***In vitro* Synthesis of ectomycorrhizae**

The isolated pure cultures of two wild edible ECM mushrooms (*B. edulis*, and *S. sibiricus*) were checked for their potential to synthesize ectomycorrhiza with *P. gerardiana*. *In vitro* experiment revealed the successful mycorrhization between *P. gerardiana* and two fungi. The *P. gerardiana* inoculated with pure cultures of ECM mushrooms helped in the development of short lateral roots which were branched and leads to the formation of ectomycorrhizae. The *B. edulis* and *S. sibiricus* formed dark reddish brown (Fig. 1 c,d) and light brown orange types (Fig. 2 c,d) of ectomycorrhizae. The description of these two types of synthesized ectomycorrhizae is given in Table 1.

Table 1. Morphological and anatomical details of *Pinus gerardiana* ectomycorrhizae synthesized during *in vitro* synthesis with *Boletus edulis* and *Suillus sibiricus*

Sr. No.		Ectomycorrhizal Mushrooms	
		Boletus edulis	*Suillus sibiricus*
	Macroscopic Characters		
1	Colour	Dark Reddish Brown	Light Brown-orange
2	Shape of mycorrhiza	Monopodial, pinnate	Reticulate, coralloid and profusely branched
3	Texture	Smooth	Smooth
4	Odour and taste	Not distinct	Not distinct
5	Emanating Hyphae	Missing	Rare
6	Root Hairs	Absent	Absent
	Microscopic Characters		
7	Thickness of Mantle	15-20 µm	10-15 µm
8	Degree of development of "Hartig net"	Well developed	Fairly well developed

Effect of *in vitro* mycorrhization on the growth of seedlings

After the completion of *in vitro* mycorrhization experiments the seedlings of *P. gerardiana* were evaluated for different growth characteristics. Effects of different ECM mushrooms on the growth and development of the seedlings are presented in Table 2. The results indicate that in case of *B. edulis* there was a significant ($P<0.01$ and $P<0.05$) difference on the overall growth characteristics of the seedlings as compared to the control whereas in case of *S. sibiricus* all the growth characteristics under study were significantly ($P<0.01$ and $P<0.05$) different except for the root length of the root whose results were non-significant ($P>0.05$) as compared to control (Table 2). Thus *in vitro* mycorrhization have significant effect on the overall growth of the seedlings.

The anatomical study of the synthesized ectomycorrhiza showed thick fungal mantle and well developed "Hartig net" with the both ECM mushrooms (Fig. 1 f and Fig. 2 f). The thickness of fungal mantle was 15-20 µm in case of *B. edulis* whereas 10-15 µm in case of *S. sibiricus* (Table 1). The control seedlings were non-mycorrhizal and have prominent root hairs in their root system. Ectomycorrhizal anatomical features were absent in the transverse section. The culture of *B. edulis* was reisolated on Modified Melin Norkan's (MMN) and Potato Dextrose Agar (PDA) medium from surface sterilized ECM roots and Vermiculite peat moss mixture (Fig. 1 e). Similarly the culture of *S. sibiricus* was also reisolated on Hagem's Agar (HM) and Potato Dextrose Agar (PDA) medium (Fig. 2 e). The isolated pure cultures of *B. edulis* and *S. sibiricus* were found to have similar characteristics as that of cultures obtained from the fresh sporophores, thus confirming the *in vitro* mycorrhization of *B. edulis* and *S. sibiricus* with *P. gerardiana* seedlings.

DISCUSSION

Pinus is a host plant for many fungi which forms a mutually beneficial symbiosis when its roots are colonized by these ECM fungi. [65,66] Miller et al. [67] achieved mycorrhizal synthesis under laboratory conditions between *Amanita muscaria* and *Pinus taeda* and *Pinus virginiana*. They reisolated the fungus from the mantles and verified its identity by cultural characteristics. Theodorou & Reddell [68] selected eleven ECM fungi from the vegetation of *Pinus radiata* and *Eucalyptus* spp. and confirmed the ECM relationship by *in vitro* synthesis.

The field observations of ECM symbiosis in different plant species can be confirmed in laboratory by performing *in vitro* mycorrhizal synthesis experiments. [69] *In vitro* mycorrhization provides a way to determine the ability of a particular fungal isolate to synthesize ectomycorrhizae with the host plant. In addition it allows structural characterization of ectomycorrhizae and their extrametrical phase, which in some cases can be used as a taxonomic character to identify the genus of the fungal symbionts. [70,71]

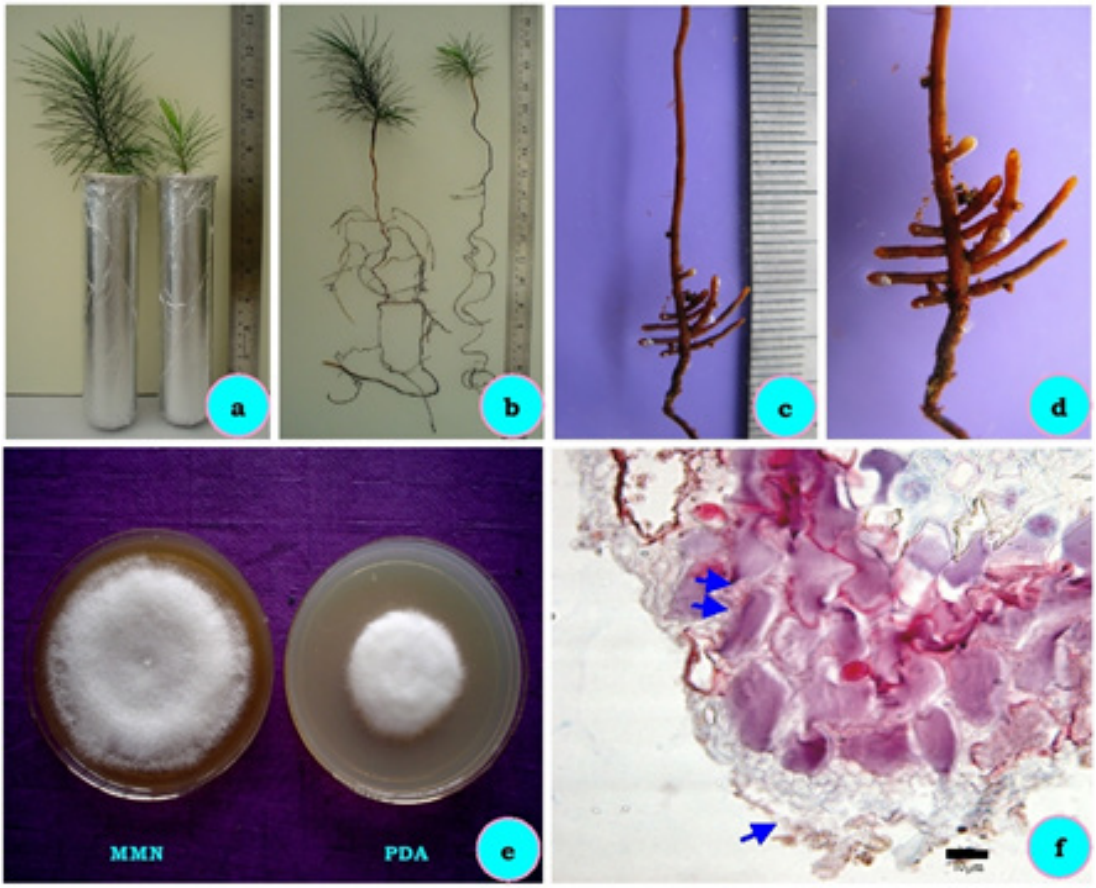
Table 2. Effect of mycorrhization on different growth characteristics of *Pinus gerardiana* seedlings

S No.	Growth Characteristics	Inoculated Treatments (cm ± S.D.)		Un-inoculated (cm ± S.D.)
		B. edulis	*S. sibiricus*	Control
1	Length of Root (cm)	38.88±2.52**	45.20±5.49NS	34.58±3.78
2	Length of Shoot (cm)	15.01±1.56**	13.72±2.04**	8.88±1.21
3	Total No. of Short Roots	56.20±7.09**	70.40±7.30**	12.60±2.30
4	Fresh Weight of Root	1.17±0.23**	2.76±0.21**	0.23±0.06
5	Fresh Weight of Shoot	2.32±0.26**	3.17±0.63**	0.98±0.13
6	Dry Weight of Root	0.27±0.06*	0.64±0.13**	0.09±0.02
7	Dry Weight of Shoot	0.68±0.15*	1.13±0.24**	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.

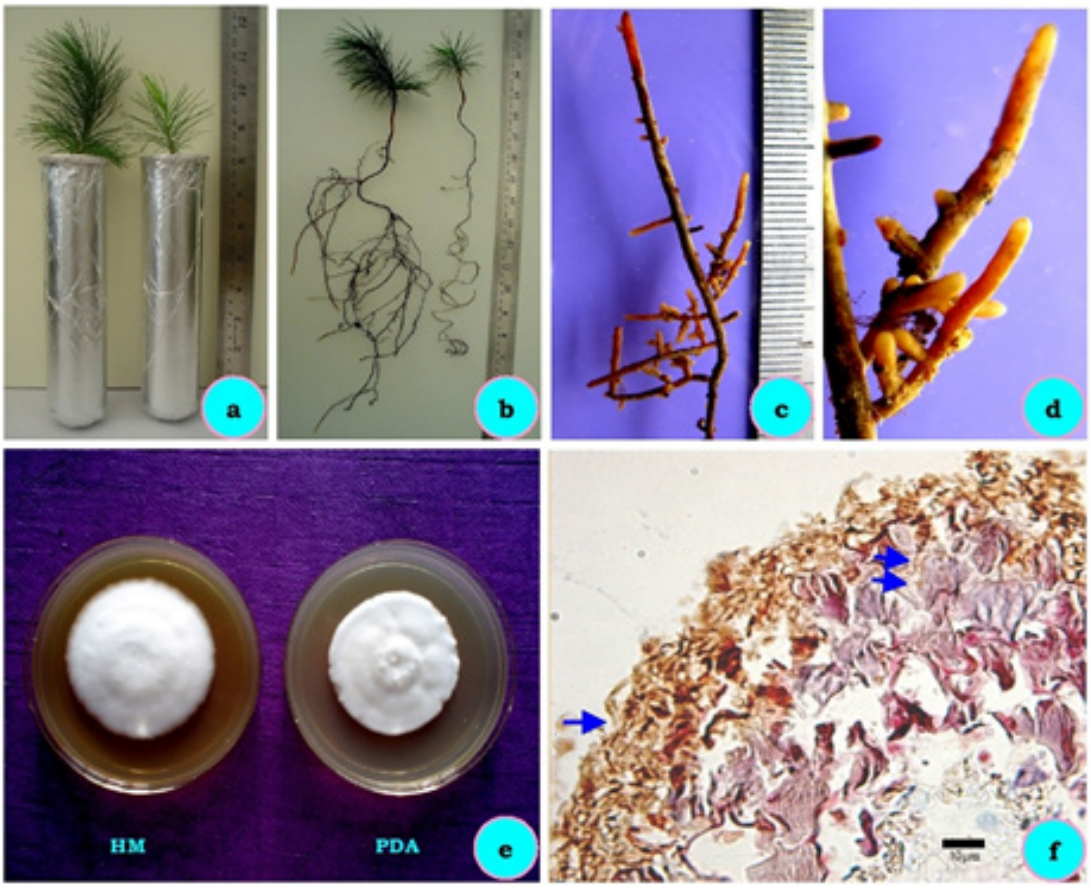
During present studies *in vitro* ectomycorrhizae were synthesized successfully between *P. gerardiana* seedlings and two wild edible ECM mushrooms i.e. *B. edulis* and *S. sibiricus*. Similar studies were also performed by many researchers in the different part of the world. The pure cultures of *B. edulis* were isolated on Malt Extract Agar medium and these cultures were used to carried out *in vitro* synthesis of ectomycorrhizae between *B. edulis* and *Pinus densiflora* successfully [72]. Richter and Bruhn [73] performed the synthesis of *P. resinosa* ectomycorrhizae with *Scleroderma aurantium* and they found that distinctive white, dichotomously-branched ectomycorrhizae were formed having fungal mantle 25-80µm in thickness. Seven different species of ECM mushrooms of genus *Cenococcum*, *Hebeloma*, *Paxillus*, *Pisolithus*, *Suillus* and *Thelephora* were found to synthesized ectomycorrhizae with *P. strobes*. [74] The many genera of edible fungi and non edible were screened out by using *in vitro* experiments for their potential to synthesized mycorrhiza with *P. densiflora* and revealed the mycorrhization in 2-4 months, [75,76] including the *Russula* spp. which are hard to handle in the process of *in vitro* mycorrhization. [77]

Samson and Fortin [78] gave the description of ectomycorrhizae synthesized with six species



a. Test tubes containing seedlings of *P. gerardiana* (Larger one inoculated with culture of *B. edulis*, smaller one kept as control). b. Uprooted *P. gerardiana* seedlings (Larger one inoculated with culture of *B. edulis* showing extensive roots system, smaller one kept as control having poorly developed root system) c and d. enlarged view of synthesized ectomycorrhizae e. Pure culture of *B. edulis* reisolated from the synthesized ectomycorrhizae on MMN Medium and PDA Medium. f. T.S. of synthesized ectomycorrhizal root (40X) showing fungal mantle ( ) and Hartig net ( )

Fig. 1. In vitro synthesis of ectomycorrhizal between *Pinus gerardiana* and *Boletus edulis*



In vitro synthesis of ectomycorrhizal between *Pinus gerardiana* and *Suillus sibiricus*: a. Test tubes containing seedlings of *P. gerardiana* (Larger one inoculated with culture of *S. sibiricus*, smaller one kept as control). b. Uprooted *P. gerardiana* seedlings (Larger one inoculated with culture of *S. sibiricus* showing extensive roots system, smaller one kept as control having poorly developed root system) c and d. enlarged view of synthesized ectomycorrhizae e. Pure culture of *S. sibiricus* reisolated from the synthesized ectomycorrhizae on MMN Medium and PDA Medium. f. T.S. of synthesized ectomycorrhizal root (40X) showing fungal mantle ( ) and Hartig net ( )

Fig. 2. In vitro synthesis of ectomycorrhizal between *Pinus gerardiana* and *Suillus sibiricus*

of *Fuscoboletinus* including two *Suillus* spp. on the roots of black larch which were white, yellow, grayish brown to pinkish grey in colour.

Santiago-Martinez et al. [79] synthesized ECM roots in *P. montezumae* with ectomycorrhizal fungus *Laccaria bicolor* successfully by performing *in vitro* experiments. Laiye et al. [80] worked out the ability of ectomycorrhizae formation in two species of larch seedlings with six species of ECM fungi (*Cenococcum geophilum*, *Lactarius hatsudake*, *Russula emitica*, *S. grevillei*, *S. laricinus* and *Tricholoma saponaceum*) by using *in vitro* synthesis technique. All six fungal isolates formed ECM association on roots of larch after ten weeks of inoculation and anatomical studies showed typical characteristics of ectomycorrhiza. *In vitro* mycorrhization of *S. sibiricus* with the seedlings of *P. wallichiana* was carried in synthesis vessels by Sagar & Lakhanpal [62]. The ectomycorrhizae formed were creamish yellow and morphologically bifurcate to coralloid. The anatomical detail confirmed the presence well developed fungal mantle and Hartig net.

Garcia-Rodriguez et al. [81] synthesized ectomycorrhizae on *Eucalyptus urophylla* and *Pinus greggii* with *Pisolithus tinctorius*. The ectomycorrhizae produced by *P. tinctorius* in roots of Eucalypt were simple, yellow brown

to bright yellow, 1.0-2.6 mm long. Mantle were around 15.0 µm, Hartig net penetrated one to two layers of cortical cells. Whereas ectomycorrhizae produced by *P. tinctorius* on pine roots were dichotomously ramified, rarely monopodial. The mycorrhizae were yellowish brown, bright yellow to opaque brown with age. Hartig net penetrated two to three layers of cortical cells. Geng et al. [13] synthesized mycorrhizae between ECM fungus *Tuber indicum* and the roots of *Pinus armandii* and *Castanea mollissima* after an inoculation period four and five months respectively and all seedlings formed well developed mycorrhizal root system. Wang et al. [82] compared the two types of inoculum for mycorrhizal synthesis between *Pinus* species native to China and ECM mushroom *Lactarius*. They revealed that mycorrhizae were formed using vegetative inoculum only.

The cultures of ECM mushrooms (*B. edulis* and *S. sibiricus*) were reisolated from *in vitro* synthesis substrate as well as from formed ectomycorrhizae and compared for their identity with the original cultures characteristics. The both have identical characteristics, thus confirming the successful mycorrhization with these mycobionts. Similarly, Richter & Bruhn [73] reisolated *Scleroderma aurantium* on MMN agar from both vermiculite-peat mixture and the excised mycorrhizae. Sagar & Lakhanpal [62], Kumar et al. [59] in similar study reisolated pure culture of ECM mycobiont from the synthesis substrate mixture and formed ectomycorrhizae to confirm symbiotic associations. Laiye et al. [83] in similar study used vermiculite and peat moss moisten with modified MMN medium for *in vitro* mycorrhiza formation on seedling of two Larch species with six different ECM fungal species.

CONCLUSON

The ectomycorrhizal synthesis between two bolete species (*B. edulis* and *S. sibiricus*) and *P. gerardiana* was successfully achieved on peat, vermiculite mixture in test tube. The seedlings of *P. gerardiana* were harvested after five months showed dark reddish brown and light brown orange coloured mycorrhizae form with *B. edulis* and *S. sibiricus* respectively. The anatomy of roots revealed the presence well developed fungal mantle and Hartig net with both wild edible mushrooms. Inoculation has significant effect on the growth and development of the seedlings as compared to control.

ACKNOWLEDGEMENT

The authors are thankful to Department of Biosciences, Himachal Pradesh University, Summer Hill, Shimla H.P. for providing the related support to compile this work.

Conflict of Interest

The authors declare that there is no conflict of interest.

Funding Sources

There is no Funding Sources.

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