Hindawi Publishing Corporation
ISRN Agronomy
Volume 2013, Article ID 317903, 12 pages
http://dx.doi.org/10.1155/2013/317903

Research Article

Effects of Media Formulation on the Growth and Morphology of Ectomycorrhizae and Their Association with Host Plant

Ferzana Islam and Shoji Ohga

Department of Agro-Environmental Sciences, Faculty of Agriculture, Kyushu University, Fukuoka 811-2415, Japan

Correspondence should be addressed to Shoji Ohga; ohga@forest.kyushu-u.ac.jp

Received 18 January 2013; Accepted 6 February 2013

Academic Editors: A. D. Arencibia, J. S. Swanston, and I. Vasilakoglou

Tricholoma matsutake and *Rhizopogon roseolus* form ectomycorrhizal (ECM) association with their host plant on natural habitats. The main objective of this study was to test mycelial growth, morphology, and host plant survival both *in vitro* and *in vivo* when treated with enriched media. Aseptically germinated seedlings of *Pinus densiflora* and *P. thunbergii* were inoculated with the strains of *T. matsutake* and *R. roseolus*, respectively. Under *in vitro* conditions mycelial growth rates performed best on pH 5 and were better on Modified-Melin-Norkrans-(MMN) based medium and Potato Dextrose Agar (PDA); addition of micronutrients and vitamins in MMN mycelial growth rates had 6–27% differences. Without ECM, plant survival rates on standard media were 30% to below 30% and by inclusion of elements they were 50% to 80%. On *in vivo*, soil containing different media with ECM allowed successful mycorrhizal association and increased seedling survival rates approximately 100%. Our findings confirm that MMN and PDA allowed higher mycelial growth but poor plant survival (<30%); however, enriched media supported 100% plant survival with successful ECM associations. The present method is advantageous in terms of giving objectivity for ECM by employing suitable media for strains and host plant, and making it possible for mass production of ECM-infected seedlings.

1. Introduction

The development and survival of many forest trees and the success of a reforestation programme depend on the symbiosis involving host tree and ectomycorrhizae—their growth and establishment. Mycorrhizal symbiosis develops capabilities of the host root system by extending the plant's ability to tolerate biological and environmental stresses such as phytopathogenic attacks, nutritional insufficiencies, pollution of heavy metal, extensive erosion, drought, and different pH [1]. These positive effects of the ectomycorrhizal symbiosis on the establishment and growth of forest plants have made the ectomycorrhizal inoculation a valuable technical tool for plant production in forestry [2]. *Pinus densiflora* and *P. thunbergii* have received extra attention due to their potential usage in pine forest reforestation programs. However, the pine forest in Japan has been under threat over the recent decades and now is facing a serious crisis to survive. The recent decline in pine forest has been aggravated by many interactive disfavourable growing conditions both for the host and the mycorrhizal fungi [2, 3]. This is ultimately reducing the production of edible mushrooms growing in pine forests which have significant economic importance and cultural value in Japan [3].

Mushrooms have become attractive as a functional food and are important as a source for the development of drugs and nutraceuticals [3, 4], especially antioxidants [5, 6] and antimicrobial compounds [7]. Alternative or substitute mushroom products are mycelia which are used as food and food-flavoring material, and also for the formulation of nutraceuticals and functional foods [8]. In the culture of Japan, *Tricholoma matsutake* and *Rhizopogon roseolus* have long been prized for its flavor, distinct taste and holds its exceptional commercial and cultural value as highly sought edible mushrooms [3, 9]. Besides, a number of bioactive compounds, antioxidants, and antifat properties have been identified in *T. matsutake* which gives this mushroom a special importance for containing medicinal properties [10, 11]. Nevertheless, *T. matsutake* has been also studied that, mycelia preparation in bulk quantity was proven to have anti-tumor activity as well as preventive activity against the formation of azoxymethane-induced precancerous lesions in case of the

colon organs [12, 13]. Besides *T. matsutake*, *R. roseolus* also holds an important position in the culture of Japan.

Some ECM mushroom fruiting bodies are difficult to grow on a large production scale. Therefore, growing mushroom mycelium on defined nutrient medium could be an alternative method for the production of ECM fungal biomass [14, 15]. For ectomycorrhizal (ECM) fungi previous research studies show that pH level plays a very important role and Modified Melin Norkrans (MMN) usually offers the best results for this group of fungi [16]. Genetic variation within species and within the strains can influence both the degree of root colonization by ECM fungi and the response to the plant to mycorrhizal symbiosis [17]. To improve forest productivity the ECM symbiosis requires fungal inoculants in a large scale level, for these reasons it is necessary to define the optimal composition of the culture medium for each fungus accounting different strains and their host plant establishment on a large variation of soil conditions.

The overall objective of this study was therefore to assess the improvement of the media formulations favorable for ECM mycelial growth, host plant survival rate *in vitro* and suitable for ectomycorrhizal association with *P. densiflora* and *P. thunbergii in vivo* conditions with best plant survival. For this, firstly the experiment had been extrapolated on *in vitro* conditions for validating the growth and development of ECM and host plant survival rates on different media adjoining the nutrients and vitamins, which sharpen the focus on the composition of a medium suitable for both ECM and their host plants without their associations. Secondly, on *in vivo* conditions inoculation of *T. matsutake* and *R. roseolus* strains were established with *P. densiflora* and *P. thunbergii* to evaluate the invariability of the improved media formulations for successful ECM association with host plant and their survival rates.

2. Materials and Methods

2.1. Under In Vitro Conditions

ECM Samples. Three different strains of *T. matsutake* and one strain of *R. roseolus* were used in this experiment. The strains of *T. matsutake* were NBRC 109050, NBRC 109051, and NBRC 109052 {NITE (National Institute of Technology and Evaluation) Biological Research Centre}. For NBRC 109050 and NBRC 109051 strains, the origin of the sources was Kyoto and strain NBRC 109052 was collected from Iwate, Japan. In case of *R. roseolus* strain RR, the origin was Fukuoka, Japan. The collected specimens were first cultured on Potato Dextrose Agar (PDA) (Wako Pure chemical Industries Ltd., Osaka, Japan) in petri dishes and to get the actively growing mycelium, mycelium plugs were cut and transferred to a fresh PDA medium every 4 weeks and pregrown there.

The Effect of Different Culture Medium and pH on Mycelial Growth. The effect of the culture media and pH on mycelial growth of *T. matsutake* and *R. roseolus* was observed on petri dishes containing 10 mL of solid medium. The following different nutritive solid mediums (Table 1) were tested: Modified-Melin-Norkrans-(MMN-) based medium, L-MMN, G-MMN {MMN, L-MMN, and G-MMN were Adapted modified media composition based on the media formulation used by Langer et al. [18]} and PDA. Each medium was adjusted to three pH levels: 4, 5, and 6 with 1 N KOH solution, and was autoclaved for sterilization. For inoculation, 4-week-old mycelial discs were cut from colonies of different strains and culture in petri dishes on different solid medium having three replicates of each and were incubated at 23 ± 2°C for 90 days in dark. Mycelial growth (colony) was measured weekly at 4 right angles during the experiment. As our research results showed that the applied pH values had no effect on the morphological characteristics on the mycelial growth so we documented these characteristics on pH 5. Meanwhile the culture media had a lot of influences on the morphological characteristic of mycelial structure. Mycelial morphological identifications were guided by the method by Barros et al. and were summarized in Table 2 [19].

Preparation of Aseptic Seedlings of Pine. Seeds of *P. densiflora* and *P. thunbergii* were collected from the University forest at Sasaguri (Kyushu University of Japan) in 2010. Growing of aseptic seedlings was guided according to the methods of Lagutte et al. and [15]. We selected 160 and 80 healthy seedlings of *P. densiflora* and *P. thunbergii*, respectively.

Testing of Improved Media for Host Plant Survival Rate. Among germinated seedlings 40 of each *Pinus* sp. seedlings were transferred to test tubes (13 × 100 mm), which contained MMN, L-MMN, G-MMN, and PDB (Potato Dextrose Broth, Wako, Japan) semisolid media (50% agar that were used in the original composition used for each media agar) with pH 5, to determine the favorable media composition for plant survival rate. For each medium composition 10 test tubes were used and each of them contained one seedling. The lower portions (root) of the seedlings were inserted into the medium and the upper portions (stem) were kept out of the test tube. The opening portions of the test tubes were sealed with parafilm tape keeping the stem out of the test tube. Seedlings were kept (incubated at 15–25°C 10–30,000 lux fluorescent light, 25 ± 2°C, and 16 hrs. photoperiod) for 7 weeks followed by the methods used by Guerin-Lagutte et al. [15] and Park et al. [20]; some of the seedlings started to turn yellowish colour with shedding their needle (pine leaf). Plant survival rates were counted by selecting the green and healthy seedlings.

Preparation of ECM Inoculum for Mixed Soil. Our finding that on *in vitro* condition pH 5 was the best condition for mycelial growth rates, so we preferred pH 5 to continue our following experimental steps. Glass flasks of 200 mL containing 100 mL of different liquid media in each of them were autoclaved. After that twenty to thirty pieces of each strain were cut from PDA medium and were transferred for culturing on different liquid media for three months at 23 ± 2°C in darkness. Before inoculation to the mixer soil containing container, the mycelial suspension from each flask was homogenized with autoclavable blender guided by Guerin-Lagutte et al. [15].

Preparation Mixed Soil with Infection Medium. Autoclave proof 160 culture containers was used. The soil used a mixture

Table 1: Composition of culture media used in this study.

Elements	Compounds	MMN[a]	L-MMN[b]	G-MMN[c]	PDA
Macroelements (mg/L)	KH_2PO_4	500.00	500.00	500.00	
	$(NH_4)_2SO_4$		250.00		
	$(NH_4)_2HPO_4$	250.00		250.00	
	$MgSO_4{\cdot}7H_2O$	150.00	150.00	150.00	
	$CaCl_2{\cdot}2H_2O$	50.00	50.00	50.00	
	NaCl	25.00	25.00	25.00	
Microelements (mg/L)	$FeCl_3{\cdot}6H_2O$	12.00	12.00	12.00	
	H_3BO_3		15.458	15.458	
	$MnSO_4{\cdot}H_2O$		9.295	9.295	
	$CuSO_4{\cdot}5H_2O$		1.310	1.310	
	$ZnSO_4{\cdot}7H_2O$		5.750	5.750	
	$Na_2MnO_4{\cdot}2H_2O$		0.003	0.003	
Vitamins (mg/L)	Thiamine HCl	1.00	10.00	0.100	
	Myo-Inositol		100.00		
	Nicotinic acid		1.00		
	Pyridoxine HCl		1.00		
Potato (g/L)					4
Carbohydrate source (g/L)	Dextrose				20
	Glucose	2.5	5.0	5.0	
	Malt extract	10.0		3.0	
Solidification agent (g/L)		9.0	9.0	9.0	15

[a,b, and c]Adapted modified media composition based on the media formulation used by Langer et al. [18].

of perlite and *Sphagnum* peatmoss at a ratio of 100 : 7–10, followed by Park et al. [20]. The bed soil was autoclaved in culture containers having 250 g of soil each for 30 min. twice per day for three days. After that, soil was mixed with 100 mL of different liquid media and was autoclaved. Each of the 40 containers contained the same medium composition. Each liquid medium, containing three month's old (cultured) mycelial suspension (of different strains) was poured into the mixed soil, wherein it was carried out on a clean bench. From total 160 container 40 of each contained same formulated medium, again 10 out of 40 contained same strain with same medium composition.

Planting of Aseptic Pine Seedlings into the Infection Soil Medium. Seedlings were aseptically immersed in the suspension of mycelia for 5 hours and their roots were carefully placed into the container with mixed soil prepared in previous step; the remaining suspension of mycelia was applied closely to the root using a sterile syringe as guided by Park et al. [20]. Each container contained 1 seedling. The open surfaces of the container were then sealed with parafilm carefully keeping the stem portion exposed to the outer environment. Following the same day they were kept in the room temperature under dark condition for hardening.

2.2. Under In Vivo Conditions. All containers were placed outside, under *in vivo* conditions, in the natural environment of Kyushu University Sasaguri forest nursery in Fukuoka, Japan. Each container was supplied with different specific liquid media containing specific strain of mycelial inoculum with the help of sterile syringe at five-day-interval. For specific medium composition, specific strain and specific *Pinus* sp. were used. Each of the containers was irrigated 50 mL of distilled water using sterile syringe every day. During the experimental period some of the seedlings started to turn yellowish with shedding their needle (pine leaf) and plant survival rates were counted by selecting the green and healthy seedlings [18]. Formation of ectomycorrhizal roots was counted in the plant roots with naked eyes which were guided according to the methods of Chung et al. [21]. Experiments were continued for seven weeks.

2.3. Statistical Analysis. Our present experiment was carried out using 3 replicates (4 radius values for each one). Data were statistically analyzed using SPSS for windows version 15 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Under In Vitro Conditions

3.1.1. The Effect of Culture Media on the Mycelial Growth. The radial growth rates of mycelia on different nutritive solid culture media (MMN, L-MMN, G-MMN, and PDA) were studied. On MMN medium the mycelial growth rates recorded were near about 3.73, 4.57, 4.48, and 4.76 cm

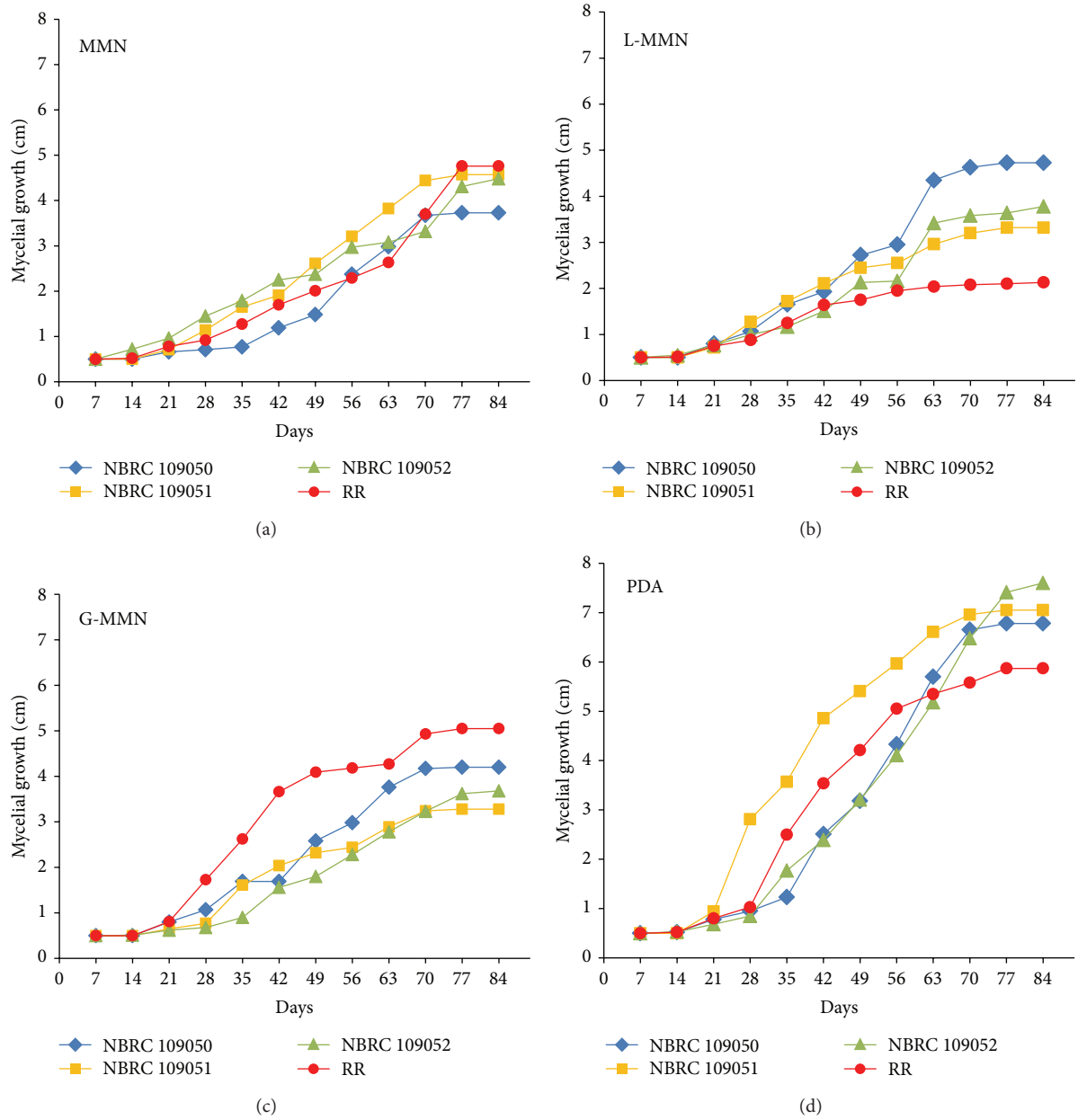

Figure 1: Effect of culture media on the mycelial growth rates of *T. matsutake* and *R. roseolus*.

for NBRC 109050, NBRC 109051, NBRC 109052, and RR, respectively (Figure 1(a)). Significant differences were found ($P < 0.01$) on MMN medium for each strain. RR strain had the highest and NBRC 109050 had the lowest mycelial growth on MMN medium. In case of L-MMN medium, NBRC 109050 had the highest mycelial growth rates and RR had the lowest growth rates. The mycelial growth rates showed highly significant ($P < 0.01$) differences on L-MMN medium and the growth rates recorded on 84th day of inoculation were around 4.73, 3.32, 3.78, and 2.13 cm for NBRC 109050, NBRC 109051, NBRC 109052, and RR, respectively (Figure 1(b)). On G-MMN medium all of the mycelial growth rates had highly significant ($P < 0.01$) differences. RR strain had the highest growth rates and NBRC 109051 had the lowest. The mycelial growth rates recorded on the last day of the experiment were more or less 4.20, 3.28, 3.68, and 5.05 cm for NBRC 109050, NBRC 109051, NBRC 109052, and RR, respectively (Figure 1(c)). For PDA medium, all of the ECM strains had highly significant ($P < 0.01$) differences. The growth rates were approximately 6.78, 7.05, 7.60, and 5.85 cm for NBRC 109050, NBRC 109051, NBRC 109052, and RR, respectively (Figure 1(d)). In case of PDA medium, the highest mycelial growth rates were recorded for NBRC 109052 strain and the lowest growth rates were recorded for RR strain. All of the ECM mycelia increased till the end of the experiment on PDA. Active mycelial growth of NBRC 109050, NBRC 109051, and NBRC 109052 strains was observed within 14 to 21 days of incubation. In case of RR strain active mycelial growth

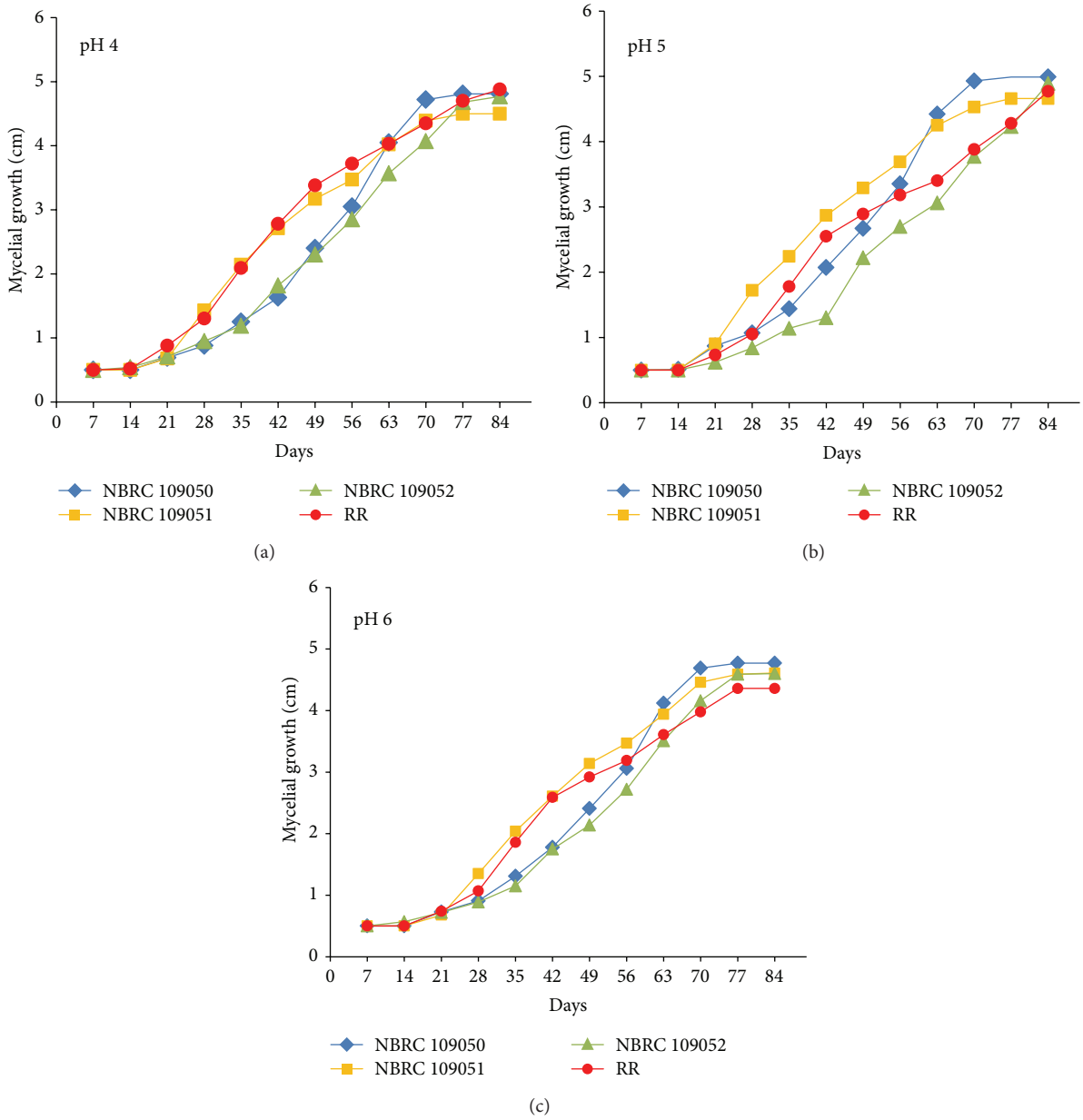

Figure 2: Effect of pH on the mycelial growth rates *T. matsutake* and *R. roseolus*.

rates were observed after 7 to 10 days of inoculation on every medium.

3.1.2. The Effect of pH on the Mycelial Growth of Different ECM Strains. On pH 4, NBRC 109050 had the highest growth rates and NBRC 109051 had the lowest growth rates; it was around 4.81, 4.50, 4.77, and 4.77 cm for NBRC 109050, NBRC 109051 NBRC 109052, and RR, respectively, (Figure 2(a)) with highly significant ($P < 0.01$) differences. With pH 5, NBRC 109050 had the highest growth rates and NBRC 109051 had the lowest, and growth rates showed highly significant ($P < 0.01$) differences; the growth rates were 4.99, 4.66, 4.89, and 4.88 cm for NBRC 109050, NBRC 109051, NBRC 109052, and RR, respectively (Figure 2(b)). On pH 6 mycelial growth rates showed highly significant ($P < 0.01$) differences; the recorded rates were 4.77, 4.61, 4.60, and 4.36 cm for NBRC 109050, NBRC 109051, NBRC 109052, and RR, respectively (Figure 2(c)), on which NBRC 109050 had the highest and RR had the lowest growth rates. In all the tested conditions, interaction with media and pH had highly significant differences ($P < 0.01$) and growth rates were highest on all media with pH 5 conditions. Mycelial growth rates on MMN with pH 5 were close to 3.81, 4.61, 4.61, and 4.93 cm for NBRC 109050, NBRC 109051, NBRC 109052, and RR, respectively. On L-MMN with pH 5 mycelial growth rates were approximately 4.89, 3.41, 4.12, and 2.30 cm for NBRC 109050, NBRC 109051, NBRC 109052, and RR, respectively. In case of G-MMN with pH 5 the growth rates were more

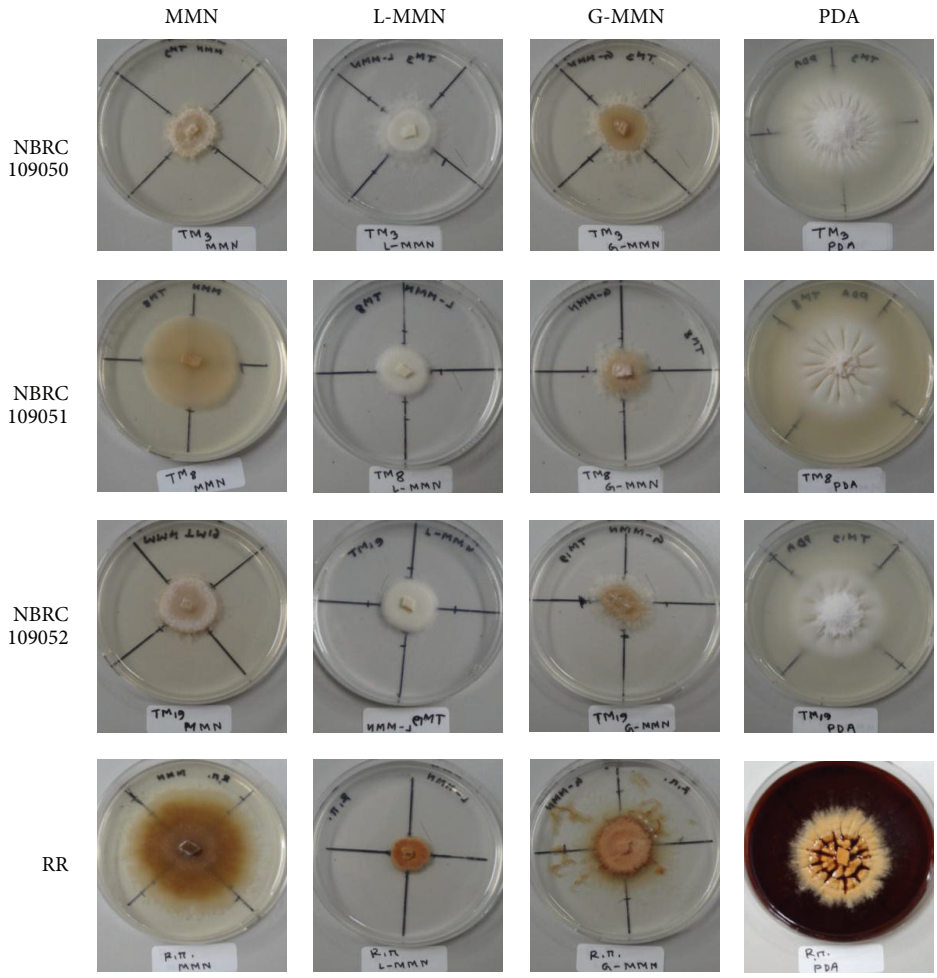

Figure 3: Appearance of *T. matsutake* and *R. roseolus* (after 80 days of inoculation) mycelial colonies, on different nutritive culture media at pH 5 level.

or less 4.32, 3.31, 3.92, and 5.31 cm for NBRC 109050, NBRC 109051, NBRC 109052, and RR, respectively. PDA with pH 5, growth rates were about 6.94, 7.31, 8.15, and 6.31 cm for NBRC 109050, NBRC 109051, NBRC 109052, and RR, respectively.

(a) *Morphological Description of NBRC 109050 Mycelial Growth*. NBRC 109050 grown under different media conditions were almost had smooth surface on MMN, L-MMN, and G-MMN, unless on PDA medium in which it became wooly (Table 2) and then after 55 to 60 days the smooth textural surface appeared around the wooly texture. Mycelial colour was bright white on PDA, light brown and light pink (after 65 days) on MMN, white on L-MMN, while it was brown and dull white (after 60 days) on G-MMN (Figure 3). The reverse colours in MMN were light brown and in G-MMN it was brown. On the other hand, the reverse colours for mycelium on PDA and L-MMN were white. Border colours were white on L-MMN, G-MMN, and PDA but light pink on MMN. Borders were clear on MMN and L-MMN but diffuse on PDA and G-MMN. Rifts and lines appeared from centre were present on PDA medium. The numbers of lines were around 26 for each petri dish, which were originated from the center of the mycelial structure. Aerial growth, media colouration, and exudates were absent.

(b) *Morphological Description of NBRC 109051 Mycelial Growth*. Mycelial textures were wooly on PDA until 50 to 55 days, then around the wooly part the mycelia formed more or less smooth structure, which appeared around wooly structure (Table 2), more or less smooth textures were found on the other medium (Figure 3). The colours of mycelial structures were white on PDA and L-MMN, but they were light brown on MMN and brown on G-MMN. The borders were diffuse on PDA and G-MMN. Clear border was observed on MMN and L-MMN. In all kinds of different media the colour of borders was found white. The reverse colour was light brown on MMN and brown on G-MMN. For PDA and L-MMN the reverse colour was found white. Aerial growth, media colouration, and exudates were absent. On the PDA, rifts and lines were found on the mycelial structure. Lines originated from the center of the mycelial structure for per petri dishes were approximately 14 in number.

Table 2: Morphological descriptions of *Tricholoma matsutake* and *Rhizopogon roseolus* on different nutritive culture media at pH 5.

Strains	Culture media	Mycelial texture	Mycelium colour	Border	Border colour	Reverse colour	Aerial growth	Medium colouration	Exudates	Rifts	Lines formed from center (no. of lines per petri dish)
NBRC 109050	MMN	Almost smooth	Light brown and light pink[b]	Clear	Light pink	Light brown	–	–	–	–	–
	L-MMN	Almost smooth	White	Clear	White	White	–	–	–	–	–
	G-MMN	Almost smooth	Brown and dull white[c]	Diffuse	White	Brown	–	–	–	–	–
	PDA	Wooly[a1] and almost smooth	Bright white	Diffuse	White	White	–	–	–	+	+ (around 26 lines)
NBRC 109051	MMN	Almost smooth	Light brown	Clear	White	Light brown	–	–	–	–	–
	L-MMN	Almost smooth	White	Clear	White	White	–	–	–	–	–
	G-MMN	Almost smooth	Brown	Diffuse	White	Brown	–	–	–	–	–
	PDA	Wooly[a2] and almost smooth	Bright white	Diffuse	White	White	–	–	–	+	+ (around 14 lines)
NBRC 109052	MMN	Almost smooth	Light brown and light pink[d]	Diffuse	Light pink	Light brown	–	–	–	–	–
	L-MMN	Almost smooth	White	Diffuse	White	White	–	–	–	–	–
	G-MMN	Almost smooth	Brown and dull white[e]	Diffuse	White	Brown	–	–	–	–	–
	PDA	Wooly[a3] and almost smooth	Bright white	Diffuse	White	White	–	–	–	+	+ (around 11 lines)
RR	MMN	Almost smooth	Dark brown	Diffuse	Dark brown	Dark brown	–	–	–	–	–
	L-MMN	Almost smooth	Dark brown	Clear	Dark brown	Dark brown	–	–	–	–	–
	G-MMN	Almost smooth	Dark brown	Diffuse	Dark brown	Dark brown	–	–	–	–	–
	PDA	Wooly[a4] and almost smooth	Brownish white and chocolate[f]	Diffuse	Chocolate	Chocolate	–	+	+	+	+ (around 16 lines)

[a]a1: Wooly until 55 to 60 days of growth, a2: Wooly until 50 to 55 days of growth, a3: Wooly until 60 to 65 days of growth, a4: Wooly until 40 to 45 days of growth.
[b]Light pink area appeared after 65 days.
[c]Dull white area appeared after 60 days.
[d]Light pink area appeared after 63 days.
[e]Dull white area appeared after 65 days.
[f]Chocolate area appeared after 45 days.

(c) *Morphological Description of NBRC 109052 Mycelial Growth*. NBRC 109052 presented almost the same morphological characteristics like NBRC 109050 on different culture media having small differences (Table 2). The mycelial textures had almost smooth surface in all nutritive media except on PDA. In PDA the textures were wooly (Figure 3) for maximum days and after that almost smooth surface appeared around the wooly structure. Smooth surface was found after 60 to 65 days of inoculation. The colour of the mycelial structure was bright white and white on PDA and L-MMN, respectively. In case of MMN it was light brown and after 63 days light pink mycelial structures were formed

around light brown area, but on G-MMN brown colour mycelial structure was developed up to 65 days. After 65 days of inoculation dull white colour mycelial structure was observed to be formed around the brown coloured area on G-MMN. The borders of the mycelial structure were diffuse on all conditions. On L-MMN, G-MMN and PDA the border colour of mycelial structure were white but on MMN it was light pink. The reversed colours were white on both L-MMN and PDA but they were light brown and brown on MMN and G-MMN, respectively. Aerial growth, medium colouration, and exudates were absent in all conditions. Rifts and lines formed from center of the mycelial structure were found only on PDA. The lines which were formed on PDA on mycelial structure were around 11 in number for per petri dish.

(d) *Morphological Description of RR Mycelial Growth*. RR mycelial structures had wooly appearances until 40 to 45 days of inoculation on PDA. After 40 to 45 days of inoculation almost smooth surface of mycelial structure was found to be formed around the wooly structures, on PDA (Table 2). The surface of mycelial growth was almost smooth on other media. Dark brown growths of mycelial structures were found on MMN, L-MMN, and G-MMN. Brownish white and chocolate (after 45 days) coloured mycelial growth structures were observed on PDA (Figure 3). Borders were clear on L-MMN but on other media they were diffuse. In all conditions both border colours and reverse colours were dark brown except on PDA, in which it was chocolate colour. Aerial growth was absent in all states of the media. Media colouration, exudates, rifts, and lines were present only on PDA. Number of lines formed from the center of the mycelial growth was near about 16 in number for each petri dish on PDA.

3.1.3. Host Plant Survival Rates on Improved Media Compositions. Seedlings on L-MMN showed uniform growth with green shoot colour on 80% planlets for both *Pinus* sp. but on PDA both species had the lowest survival rates and were about 10% and 20% for *P. densiflora* and *P. thunbergii*, respectively; they showed severe discoloration and shedding of pine needles. For MMN and G-MMN survival rates were 30% to 50% for *P. densiflora* and 30% to 60% for *P. thunbergii*, respectively. Both *Pinus* sp. have yellowish green shoot colour on MMN and they had light pale green colour on G-MMN.

3.2. Under In Vivo Conditions

3.2.1. Testing of Media Compositions for Mycorrhizal Association with Host Plant in Mixer Soil. After 2 weeks of inoculation, it was found that fungal hyphae bound soil particle together by aggregating soils (Figures 4(a) and 4(b)) during that time necessary precautions were taken not to disturb the soil portion close to the root system of the seedlings. After 7 weeks of inoculation ectomycorrhizal hyphae colonized the root surface and formed aggregated soil around the root surface; elongated lateral roots were noticed lacking root hairs (Figures 4(c) and 4(d)) which were colonized with fine discrete hyphae. Ramified and branched roots were observed in all soil conditions, for all strains the numbers (per plant) were maximum on PDB containing soil mixer maximum around 16 to 17 (Table 4) and lower in MMN containing soil mixer which were about 12. For L-MMN soil mixer they were approximately 14 to 15 and on G-MMN they were recorded 12 to 14 in number. Among *T. matsutake* strains NBRC 109052 had always the best mycorrhizal formation with the host plant, and *R. roseolus* strain formed maximum mycorrhizal root around 17 in PDA containing soil mixer and had minimum near about 12 in MMN containing soil mixer.

Table 3: Survival rates of *Pinus densiflora* and *P. thunbergii* on four different media composition at pH 5 on *in vitro* conditions.

Medium	Number of plants	Plant survival rate % on medium (without ECM)
MMN	10[a]	30
	10[b]	30
L-MMN	10[a]	80
	10[b]	80
G-MMN	10[a]	50
	10[b]	60
PDA	10[a]	10
	10[b]	20

[a] *P. densiflora.*
[b] *P. thunbergii.*
Plant survival rate was specified by the percentage of living plantlets/seedlings.

3.2.2. Host Plant Survival Rates with Ectomycorrhizal Association in Mixed Soil with Media Compositions. Seedlings in L-MMN mixed soil showed uniform growth with green shoot colour on 90% to 100% planlets for both *Pinus* sp. With MMN and PDA mixer soil both species had the lowest survival rates which were around 30% to 60%; they showed discoloration and shedding of pine needles. Both *Pinus* sp. have yellowish green shoot colour on MMN and they had light pale green colour on G-MMN. For all soil mixer conditions ECM association increased the host plant survival rates 10% to 30% (Tables 3 and 4) comparing to the survival rates without ectomycorrhizal inoculation.

4. Discussion

Fungi of ECM group have been widely studied in different parts of the world for their wide range of considerable ecological and economic importance. For this circumstance, these species are often used both for experimental research and applied purposes [22]. This research focused on physical growth rate, morphological characters of mycelia of the ECM fungi, their host plant survival rates, and ECM association with host plant.

We stimulated ECM mycelial growth by different nutritional conditions and found considerably significant differences among the applied *in vitro* conditions without host plant, which speculate that higher concentrations of nutrients may be the reason of mycelial growth rates inhibition. This

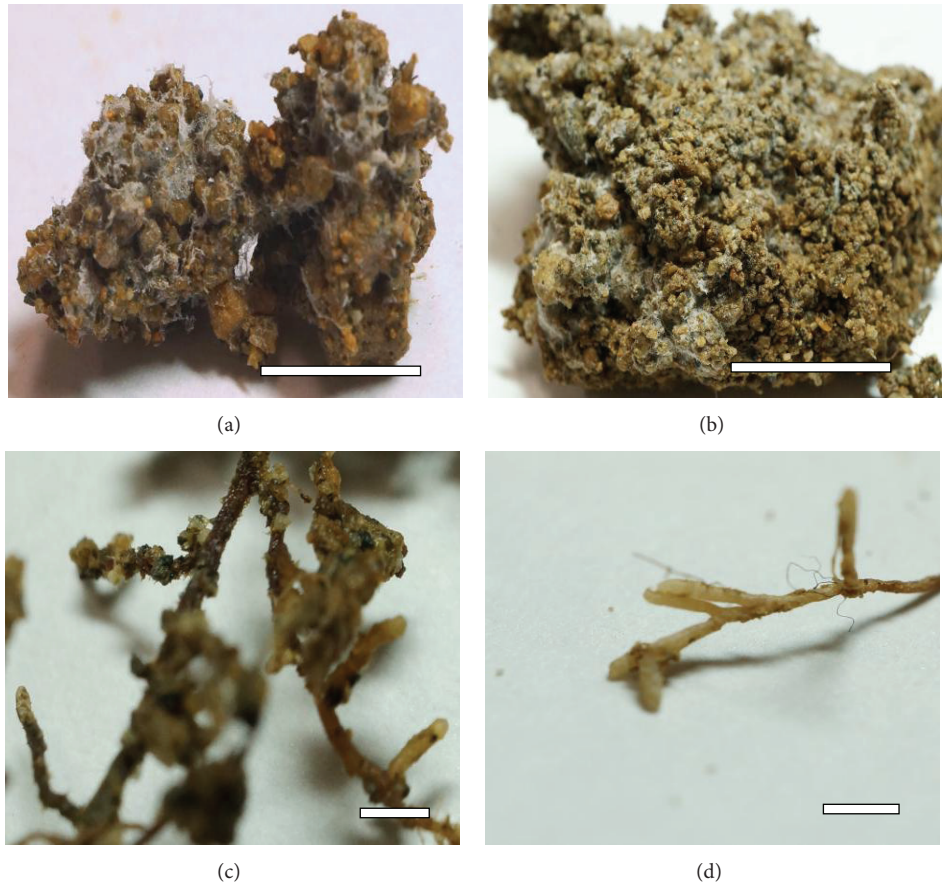

Figure 4: (a) Appearance of *T. matsutake* mycelial structure aggregates soil materials adhering to the root surface. Scale bar, 25 mm. (b) Appearance of *R. roseolus* mycelial structure aggregates soil materials adhering to the root surface. Scale bar, 30 mm. (c) Formation of ectomycorrhizal roots on *P. densiflora* (*T. matsutake*). Scale bar, 1.0 mm. (d) Formation of ectomycorrhizal roots on *P. thunbergii* (*R. roseolus*). Scale bar, 1.5 mm.

phenomenon can be explained by two ways: (i) the mycelial catabolism was suppressed by the higher concentration of nutrients because of osmotic pressure as the ectomycorrhizal fungi do not grow under high osmotic pressure and (ii) presence of high concentration of vitamin and hormones increased the activity of some enzymes which could suppress mycelial growth [23, 24]. These kinds of effect were also reported from other scientists with the application of carbohydrate components [25, 26]. It was found that higher concentration of extra glucose had an effect on catabolite suppression in the ECM fungal group. These might be an explanation of our findings for studied strains.

This study shows that media compositions with different pH levels were effective in stimulating mycelial growth rates without the host plant. It has been well known that pH had significant influences on the growth of fungi; in general they grow better between pH 5 and pH 6. Considerable growth rates were also found in some studies in between pH 3.2 and 6.5, but the optimal pH ranges from 4.5 to 5.5. Some scientists stated that optimum pH ranges are mainly related to different species, strains, enzymatic systems, important vitamin entry in the cell, mineral capture, and surface metabolic reactions [19, 27]. This supports our findings that our studied ECM strains had the highest growth on pH 5.

A relatively close study shows that there are considerable morphological differences among the studied strains of ECM on different media. We were able to distinguish them for different strains but these specific characteristics had no influences on the applied pH levels. For each strain they had similar morphological characteristics on different pH. These might be the results due to the variations among the different strains. Therefore, the results also provide evidence that the mycelial appearance not only varies with the culture media but also changes with the fungal species and strains which suggest considerable intraspecific variation among different strains [19, 23, 24, 27].

The present experiment also showed that plant survival rates were poor on widely used standard MMN formulation and on PDA which may be due to the absence of essential plant nutrients and vitamins, because with the addition of supplemented elements to G-MMN and L-MMN increased the plant survival rates. It suggests that plantlets were not able to produce satisfactory amount of nutrients and vitamins required for normal growth and development. It had been

Table 4: Survival rates of *Pinus densiflora* and *P. thunbergii*, appearance of mycelial colony in cultured soil, and formation of mycorrhizal roots (at pH 5 with mixed soil).

Medium	ECM strain	Number of plants	(ECM) Appearance of mycelial colony in cultured soil	ECM association with the plant roots	Plant survival rate % (with ECM)	Formation of ectomycorrhizal roots[c] (approximately for per plant, mean value)
MMN	NBRC 109050	10[a]	+	+	40	12
	NBRC 109051	10[a]	+	+	40	12
	NBRC 109052	10[a]	+	+	50	12
	RR	10[b]	+	+	60	12
L-MMN	NBRC 109050	10[a]	+	+	90	14
	NBRC 109051	10[a]	+	+	90	14
	NBRC 109052	10[a]	+	+	100	15
	RR	10[b]	+	+	100	15
G-MMN	NBRC 109050	10[a]	+	+	70	12
	NBRC 109051	10[a]	+	+	80	13
	NBRC 109052	10[a]	+	+	80	14
	RR	10[b]	+	+	90	14
PDA	NBRC 109050	10[a]	+	+	30	16
	NBRC 109051	10[a]	+	+	30	17
	NBRC 109052	10[a]	+	+	30	17
	RR	10[b]	+	+	40	17

[a] *P. densiflora.*
[b] *P. thunbergii.*
[c] Formations of ectomycorrhizal roots were counted in the plant roots with naked eyes and were determined by morphological root characteristics indicative of mycorrhizal formation.

reported that nutrients especially micronutrients and vitamins play an important role on plant survival rates, growth, development establishment, and resistance capacity to a wide range of variations [28, 29]. This phenomenon supports our findings, which confirms that *Pinus* sp. need sufficient amount of nutrients and vitamins for survival with normal growth and development.

We found that the numbers of ramified and branched roots were higher in case of all strains on PDA mixed soil but plant survival rates were poor. Kusuda et al. [26] suggested that the supply of carbohydrate plays an obligate role for some of mycorrhizal formation; this might be a reason that the strains in our study formed maximum mycorrhizal roots in soil mixer containing PDA. They also stated that exogenous carbohydrates sometimes also reduce the fungal requirement for root carbohydrates which may be a reason for the variations of mycorrhizal colonization of the studied strains. Results showed that the mycorrhizal associations of studied strains increased plant survival rates 100% with L-MMN mixed soil which contained minimum amount of carbohydrate among the used medium compositions. This may prove the definite nutrient demand of the plantlets during mycorrhizal synthesis. Plant survival rates reached 90–100% with ectomycorrhizal inoculation by the addition of vitamin mixture (thiamine, myo-inositol, nicotinic acid and pyridoxine, usually supplemented to plant tissue culture) which also indicated that pine seedlings required symbiotic helper such as the ectomycorrhizal fungi for their highest survival rates. Normally, the addition of thiamine meets the vitamin demands of plants during mycorrhizal association. Fungal colonization on roots can benefit the tree by forming hyphal network that effectively increases plant nutrient absorptive surface area and benefited each other by symbiotic associations [30]. These phenomena support our results, that culturing pine seedlings with ectomycorrhizal inoculation increased plant survival rates.

The results have important attribution to future research with these strains and might have some important implication for ECM inoculation along with their mycelial production. Observations indicate that NBRC 109050, NBRC 109051, NBRC 109052, and RR are suitable for inoculation to pine seedlings due to their adaptability under a wide range of *in vitro* and *in vivo* conditions, but among them strain NBRC 109052 and RR were the best for *in vitro* and *in vivo* conditions, which indicates the growth variation between and within the species [23, 27]. Trappe [31], Parladé et al. [32] and Marix et al. [33] suggested that intraspecific variability of fungi plays an important role on controlling inoculation and exhibits the physiological capacity to form abundant ECM on the desired host. Habitat differences might be reflected on the *in vitro* growth conditions; also in the colonization patterns they indicate some degree of specialization or host preference among the strains [32, 33].

To summarize, our findings show that formulations of media on *in vitro* and *in vivo* conditions varied the mycelial growth of the studied ECM strains whereas the additional

nutrients are essential for plant establishment for its own potentiality. It also confirms that *Pinus* sp. may form successful ECM association *in vivo* with number of special nutrient and vitamin support. A balanced nutrient and the inclusion of different vitamins are vital for plant establishment and successful ectomycorrhizal association. Several new techniques may be complemented with adapted medium composition and may thereby increase the proportion of successful ECM association on *Pinus* sp. The procedure of enriching media can successfully increase the host plant survival rates without ECM and satisfactory ECM associations with host plant. With ECM association the plant survival rate achieved the highest survival rates. The mycelial growth of *T. matsutake* and *R. roseolus* performed best on MMN and PDA media without host plant *in vitro* condition, whereas host plant survival rates were poor without ECM association *in vitro* condition on MMN and PDA. Methods based on enriching media formulations for ECM with new strains can have further scope for future research work.

5. Conclusion

To conclude, formulation of media on *in vitro* and *in vivo* conditions varies the mycelial growth and development of ECM strains, whereas additional nutrients are essential for plant survival. Our findings confirm that *Pinus* sp. forms successful associations with ECM on *in vivo* when supplied with a number of special nutrients and vitamins. A balanced nutrient composition and inclusion of vitamins are essential for successful ectomycorrhizal associations and highest host plant survival. Several new techniques may be complemented with adapted media formulation and may thereby increase the proportion of successful ECM association with *Pinus* sp. The findings of our research may further be employed with different synthesis techniques carried out in plantation and reforestation areas with different ECM fungi. This opens new prospective in enriching plantation forest research where ectomycorrhizal association can flourish to protect the decline of ECM mushroom and pine forest in Japan.

References

[1] S. Smith and D. J. Read, *Mycorrhizal Symbiosis*, vol. 640, Academic Press, London, UK, 1996.

[2] S. E. Smith and D. J. Read, *Mycorrhizal Symbiosis*, Academic Press, London, UK, 2008.

[3] F. Islam and S. Ohga, "The response of fruit body formation on in situ condition *Tricholoma matsutake* by applying electric pulse stimulator," *ISRN Agronomy*, vol. 2012, Article ID 462724, 6 pages, 2012.

[4] S. T. Chang, "Global impact of edible and medicinal mushrooms of human welfare in the 21 century: non-green revolution," *International Journal of Medicinal Mushrooms*, vol. 1, pp. 1–7, 1999.

[5] G. C. Yen and C. Y. Hung, "Effects of alkaline and heat treatment on antioxidative activity and total phenolics of extracts from Hsian-tsao (*Mesona procumbens* Hemsl.)," *Food Research International*, vol. 33, no. 6, pp. 487–492, 2000.

[6] I. C. F. R. Ferreira, P. Baptista, M. Vilas-Boas, and L. Barros, "Free-radical scavenging capacity and reducing power of wild edible mushrooms from northeast Portugal: individual cap and stipe activity," *Food Chemistry*, vol. 100, no. 4, pp. 1511–1516, 2007.

[7] L. Barros, R. C. Calhelha, J. A. Vaz, I. C. F. R. Ferreira, P. Baptista, and L. M. Estevinho, "Antimicrobial activity and bioactive compounds of Portuguese wild edible mushrooms methanolic extracts," *European Food Research and Technology*, vol. 225, no. 2, pp. 151–156, 2007.

[8] C. C. Weng, *Taste quality of Grifola frondosa, Morcbella esculenta and Termitomyces albuminosus mycelia and their application in food application in food processing [M.S. thesis]*, National Chung-Hsing University, Taichung, Taiwan, 2003.

[9] W. Yun, I. R. Hall, and L. A. Evans, "Ectomycorrhizal fungi with edible fruiting bodies 1. *Tricholoma matsutake* and related fungi," *Economic Botany*, vol. 51, no. 3, pp. 311–327, 1997.

[10] N. Ohnuma, K. Amemiya, R. Kakuda, Y. Yaoita, K. Machida, and M. Kikuchi, "Sterol constituents from two edible mushrooms, *Lentinula edodes* and *Tricholoma matsutake*," *Chemical and Pharmaceutical Bulletin*, vol. 48, no. 5, pp. 749–751, 2000.

[11] H. W. Lim, J. H. Yoon, Y. S. Kim, M. W. Lee, S. Y. Park, and H. K. Choi, "Free radical-scavenging and inhibition of nitric oxide production by four grades of pine mushroom (*Tricholoma matsutake* Sing.)," *Food Chemistry*, vol. 103, no. 4, pp. 1337–1342, 2007.

[12] T. Ebina, T. Kubota, N. Ogama, and K. I. Matsunaga, "Antitumor effect of a peptide-glucan preparation extracted from a mycelium of *Tricholoma matsutake* (S. Ito and Imai) Sing," *Biotherapy*, vol. 16, no. 3, pp. 255–259, 2002.

[13] K. Matsunaga, T. Chiba, and E. Takahashi, "Mass production of Matsutake (*Tricholoma matsutake*) mycelia and its application to functional foods," *Bioindustry*, vol. 20, pp. 37–46, 2003.

[14] V. P. Cirillo, W. A. Hardwick, and R. D. Seeley, "Fermentation process for producing edible mushroom mycelium," USA Patent no. 2, 928, 210; 1960.

[15] A. Guerin-Laguette, L. M. Vaario, W. M. Gill, F. Lapeyrie, N. Matsushita, and K. Suzuki, "Rapid in vitro ectomycorrhizal infection on *Pinus densiflora* roots by *Tricholoma matsutake*," *Mycoscience*, vol. 41, no. 4, pp. 389–393, 2000.

[16] R. Molina and J. G. Palmer, "Isolation, maintenance, and pure culture manipulation of ectomycorrhizal fungi," in *Methods and Pricciples of Mycorrhizal Research*, N. C. Schenck, Ed., pp. 115–129, American Phytopathological Socity, St Paul, Minn, USA, 1982.

[17] R. L. Peterson and S. M. Bradbury, "Use of plant mutants, interspecific variants and non-hosts in studying mycorrhiza formation and function," in *Mycorrhiza: Structure, Function, Molecular Biology and Biotechnology*, A. K. Varma and B. Hock, Eds., Springer, Berlin, Germany, 1995.

[18] I. Langer, D. Krpata, U. Peintner, W. W. Wenzel, and P. Schweiger, "Media formulation influences in vitro ectomycorrhizal synthesis on the European aspen *Populus tremula L.*," *Mycorrhiza*, vol. 18, no. 6-7, pp. 297–307, 2008.

[19] L. Barros, P. Baptista, and I. C. F. R. Ferreira, "Influence of the culture medium and pH on the growth of saprobic and ectomycorrhizal mushroom mycelia," *Minerva Biotecnologica*, vol. 18, no. 4, pp. 165–170, 2006.

[20] M. C. Park, S. G. Sim, and W. J. Cheon, "Methods of preparing *Tricholoma matsutake*-infected young pine by coculturing aseptic pine seedlings and *T. matsutake*," US Patent no. 7,269,923 B2; 2007.

[21] H. C. Chung, D. H. Kim, and S. S. Lee, "Mycorrhizal formation and seedling growth of *Pinus densiflora* by *in vitro* synthesis with

the inoculation of ectomycorrhizal fungi," *Mycobiology*, vol. 30, no. 2, pp. 70–75, 2002, Copyright by The Korean Society of Mycology.

[22] I. R. Hall and Y. Wang, "Methods for cultivating edible mycorrhizal mushroom," in *Mycorrhiza Manual*, A. Varma, Ed., pp. 99–114, Springer, Berlin, Germany, 1998.

[23] T. Hatakeyama and M. Ohmasa, "Mycelial growth characteristics in a split-plate culture of four strains of the genus *Suillus*," *Mycoscience*, vol. 45, no. 3, pp. 188–199, 2004.

[24] T. Lubbehusen, V. González Polo, S. Rossi et al., "Protein kinase A is involved in the control of morphology and branching during aerobic growth of *Mucor circinelloides*," *Microbiology*, vol. 150, no. 1, pp. 143–150, 2004.

[25] T. Hatakeyama and M. Ohmasa, "Mycelial growth of strains of the genera *Suillus* and *Boletinus* in media with a wide range of concentrations of carbon and nitrogen sources," *Mycoscience*, vol. 45, no. 3, pp. 169–176, 2004.

[26] M. Kusuda, M. Ueda, Y. Konishi, K. Yamanaka, T. Terashita, and K. Miyatake, "Effects of carbohydrate substrate on the vegetative mycelial growth of an ectomycorrhizal mushroom, *Tricholoma matsutake*, isolated from Quercus," *Mycoscience*, vol. 48, no. 6, pp. 358–364, 2007.

[27] L. L. Hung and J. M. Trappe, "Growth variation between and within species of ectomycorrhizal fungi in response to pH *in vitro*," *Mycologia*, vol. 75, pp. 234–241, 1983.

[28] E. J. Jokela, W. W. McFee, and E. L. Stone, "Micronutrient deficiency in slash pine: response and persistence of added manganese," *Soil Science Society of America Journal*, vol. 55, no. 2, pp. 492–496, 1991.

[29] A. Shalata and P. M. Neumann, "Exogenous ascorbic acid (vitamin C) increases resistance to salt stress and reduces lipid peroxidation," *Journal of Experimental Botany*, vol. 52, no. 364, pp. 2207–2211, 2001.

[30] J. V. D. Rousseau, D. M. Sylvia, and A. J. Fox, "Contribution of ectomycorrhiza to the potential nutrient-absorbing surface of pine," *New Phytologist*, vol. 128, no. 4, pp. 639–644, 1994.

[31] J. M. Trappe, "Selection of fungi for ectomycorrhizal inoculation in nurseries,"" *Annual Review of Phytopathology*, vol. 15, pp. 203–222, 1977.

[32] J. Parladé, J. Pera, and J. Luque, "Evaluation of mycelial inocula of edible *Lactarius species* for the production of *Pinus pinaster* and *P. sylvestris* mycorrhizal seedlings under greenhouse conditions," *Mycorrhiza*, vol. 14, no. 3, pp. 171–176, 2004.

[33] D. H. Marix, S. B. Maul, and C. E. Cordell, "Application of specific ectomycorrhizal fungi in world forestry,"" in *Frontiers in Industrial Mycology*, G. F. Leatham, Ed., Chapman & Hall, New York, NY, USA; Kluwer Academic Publishers, Dordrecht, The Netherlands, 1992.