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Growth responses of ectomycorrhizal isolates on two synthetic culture media

Muzna Zahur, A.N. Khalid and A.R. Niazi

Department of Botany, University of the Punjab Lahore 54590, Pakistan

Abstract

Thirty-three different ectomycorrhizae isolated from rhizosphere of 12 tree species have been tested for in vitro isolation on MMN and MEA synthetic media. Out of these, twenty-seven mycorrhizae have successfully been cultured on MMN and MEA media. Six ectomycorrhizae did not show any response on MEA medium. Successful isolation of most of the ectomycorrhizal species on both media show their non specificity for nutrients . Six species, that show no response on any of the media, due to the nonavailability of particular nutrients in the medium. These ectomycorrhizal isolates have been tested for their growth, culture and hyphal characteristics so as to select the most suitable species having rapid growth rate in synthetic media.

Key words: Ectomycorrhizae, MMN, MEA, isolates, cultures.

Introduction

Among the mycorrhizal fungi, ectomycorrhizal(ECM) fungi are unique in the sense that these can be cultured on synthetic medium without their host species. In vitro culture studies provide insight into the basic biology and processes of the fungal symbionts. comparative study of diverse fungal species and different isolates of a species provide the basis for selecting suitable isolate for the inoculation of nursery stock (Trappe, 1977). Comparatively fast growing cultures are preferred because they are considered to be physiologically more active and, therefore, better source of inoculum (Molina and Palmer, 1982).

A mere 3% of phanerogams are ECM, all of which are woody species, trees and shrubs (Werner, 1992; Carlile and Watkinson, 1994; Smith and Read, 1997). Despite this small percentage, the global importance of the ECM association is greatly increased by the large area covered by these plants globally and their economic value as the main source of timber (Smith and Read, 1997). In total, 140 genera, in 43 plant families have been identified as forming ECM (Carlile and Watkinson, 1994). New plantations benefit considerably from ECM inoculation, especially on ground previously uncolonized by ECM trees. Similarly, exotic trees nearly always fail to grow in other parts of the world unless suitable fungi are introduced with them (Jackson and Mason, 1984). Many media types, which are optimized for particular fungus, have been developed (Molina and Palmer, 1982, Fries, 1983, Ohta, 1990). Not all ectomycorrhizal fungi require same inoculation and nutritional supplements (Marx and Daniel, 1976).Culturability was also different among the isolates of different or same genus. Schenk (1982) found that ectomycorrhizal fungi can differ strongly in ease of isolation and growth in culture. The preliminary studies, which concern optimizing culture condition for particular fungi and comparing inoculum forms are a pre-requisite for large-scale inoculum production for use in forestry.

To distinguish particular isolates from the growth media, morphological, characters such as colour and texture of mycelial colonies can often be used. A wide range of information like growth rates at different temperatures, tolerance to different chemicals, culture morphology, microscopic morphology (clamp connections, shape, thickness, color, growth pattern of hyphae) etc are obtained by observation or by experimentation can be used to help, identify or confirm the identity of ECM fungi growing in culture (Brundrett *et al*., 1996, Hutchinson, 1991, 1992).

Several ectomycorrhizal types had been isolated from different regions of Pakistan (Afshan *et al*., 2003; Kazmi, 2003) and their growth is tested on different culture media.

Materials and Methods

Description of sampling sites

Sampling was conducted from the areas of Murree and Sakesar hills. In these hills, trees were

selected from Khanspur, Nathia Gali, Dunga Gali, Ayubia Church, Ayubia National Park and Mukshpuri Peak and PAF base Sakesar. These areas fall under moist temperate forests of Himalayan range where *Pinus wallichiana* A.B.Jackson, *Pinus roxburghii* Sargent *Abies pindrow* Roxb. *Cedrus deodara* (Roxb.) Loud, *Picea smithiana* (Wall.) Boiss, *Quercus dilatata* Lindl.ex ex. Royle, *Populus ciliata* Boenm, *Prunus padus* H.Kf. non-L., and *Alnus rubra* Bongn, grow. The area is at elevation of 1,373 – 3,050 m above sea level approximately, spreading over 32 km (Hussain, 1995). Root samples were collected during June, August and September 2003.

Root Sampling

Blocks of soil along with the root system of selected trees were dug out from the upper horizon, 10 cm of the forest floor, with the help of a digger. These were wrapped in polythene bags to avoid evaporation.. The soil samples were labeled and brought to the lab.

Washing

The soil blocks were soaked in tap water to loosen the adhering soil particles from ectomycorrhizal roots. The attached soil particles were removed with the help of camel hairbrush under a stereomicroscope. These washed roots were kept in McCartney bottles with a few drops of washing liquid to wash the roots more cleaner. This washing was repeated three to four times.

Surface Sterilization

The washed roots were surface sterilized with 15% H_2O_2 for 20-30 seconds, depending upon the thickness of the roots, following by then made several washings with distilled, sterilized water. The roots were kept in streptomycin and chloromycetin solution for one minute.

Media Used

Two types of growth media were used for ectomycorrhizal roots 'Modified Melin Norkans Medium' (MMN) and 'Malt Extract Agar (MEA) medium' (Marx and Daniel, 1976).

Culturing

Surface sterilized roots were cutup into fine sections from their tips with a sharp sterilized razor. The pieces were inoculated to solid media in McCartney bottles and Petri dishes with a sterilized forceps in laminar flow hood. The cultures were incubated at 20ºC.

Culture Studies

Cultures were investigated periodically (colony shape, color, growth etc). Mycelium started growing within three to four weeks depending on type of mycorrhizae.

Sub culturing

Subcultures were prepared on MMN medium, as soon as sufficient mycelium developed from the primary inoculum. A small piece of mycelial colony from its edge was taken with the help of sterilized spatula and transferred to fresh media in Petri dishes under sterilized conditions.

Results

The results are described in tables and graphical form.

Table-1: Culture Characteristics of Ectomycorrhizae isolated From Murree and Sakesar Hills

Isolate	Host	Growth on media	Habit of the colony	Appearance
Abirhiza dichotoma	*Abies pindrow*	Fast	Surface to submerged	Brownish yellow, circular
Abirhiza claynica	*Abies pindrow*	Difficult	Surface to aerial	White cottony colony,oval
Abirhiza irregulata	*Abies pindrow*	Fast	Surface to aerial	White, brown circularly spread colony
Abirhiza bentata	*Abies pindrow*	Fast	Surface to aerial	White circular colony
Alnirhiza plectina	*Alnus rubra*	Difficult	Surface	Hyaline to white, semi-circular
Cedrirhiza cystidica	*Cedrus deodara*	Difficult	Surface to aerial	White and cottony colony, small circle
Piceirhiza mukshpuriana	*Picea smithiana*	Difficult	Aerial	White, pink circular colony
Pinirhiza cubiculata	*Pinus roxburgii*	Moderate	Surface to aerial	White to brown circular colony
Pinirhiza smoothiana	*Pinus roxburgii*	Moderate	Surface to aerial	Light brown to off-white, irregular colony
Pinirhiza elliptica	*Pinus roxburgii*	Fast	Surface to aerial	Grayish black, circular

Pinirhiza spinulata	*Pinus roxburgii*	Fast	Surface to aerial	Black and off-white, circular rings
Pinirhiza citrina	*Pinus wallichiana*	Fast	Surface to aerial	Off-white to brown, circular
Pinirhiza beadulata	*Pinus wallichiana*	Moderate	Surface to aerial	White with reddish tinge, circular
Pinirhiza coralloidia	*Pinus wallichiana*	Moderate	Surface to aerial	Off-white to dark brown, circular
Pinirhiza variegate	*Pinus wallichiana*	Moderate	Surface to aerial	Greenish yellow with irregular margins
Pinirhiza nigra	*Pinus wallichiana*	Moderate	Surface to submerged	White, cottony, circular colony
Pinirhiza irregulata	*Pinus wallichiana*	Fast	Surface to aerial	White, spread colony
Populinirhiza lustrata	*Populus ciliata*	Fast	Surface	White, spread colony
Populinirhiza pinnata	*Populus ciliata*	Moderate	Aerial	White with brown margins, spread
Populinirhiza copperina	*Populus ciliata*	Moderate	Surface to aerial	Yellowish brown, irregular colony
P-Sk 122	*Prunus persica*	Difficult	Surface to aerial	Yellowish shiny white to brown, circular
P-Sk 123	*Prunus persica*	Fast	Surface to aerial	Off-white to brown, irregular colony
Prunirhiza grainata	*Prunus padus*	Difficult	Surface	Grayish black, circular
Pyrus II	*Pyrus aucuparia*	Fast	Surface to aerial	White, circular, spread colony
Pyrus I	*Pyrus aucuparia*	Fast	Surface to aerial	White to brown, circular
Quercinirhiza nigra	*Quercus dilatata*	Moderate	Surface	Off-white and brown, circular patches
Quercus I	*Quercus dilatata*	Difficult	Surface	Small, white colony,a small circle

Table-II: Growth Characteristics of Isolated Ectomycorrhizae from Murree and Sakesar Hills.

Isolate	Host	Days of observat-ions	Growth rate (cm/day)		Colony diameter (cm)		Growth rate in sub-cultures (cm)
			MMN	MEA	MMN	MEA	
Abirhiza dichotoma	*Abies pindrow*	20	0.06	0.03	1.2	0.6	0.2
Abirhiza claynica	*Abies pindrow*	22	0.01	0.009	0.3	0.2	0.03
Abirhiza irregulata	*Abies pindrow*	20	0.05	0.04	1.0	0.8	0.3
Abirhiza bentata	*Abies pindrow*	30	0.1	0.01	3.0	0.3	0.17
Alnirhiza plectina	*Alnus rubra*	25	0.004	0	1.0	0	0.05
Cedrirhiza cystidica	*Cedrus deodara*	15	0.02	0	0.3	0	0.06

Piceirhiza mukshpuriana	*Picea smithiana*	22	0.09	0.03	2.0	0.8	0.07
Pinirhiza cubiculata	*Pinus roxburgii*	20	0.05	0.02	1.0	0.4	0.1
Pinirhiza smoothiana	*Pinus roxburgii*	10	0.15	0.03	1.5	0.3	0.11
Pinirhiza elliptica	*Pinus roxburgii*	12	0.1	0.05	1.2	0.6	0.3
Pinirhiza spinulata	*Pinus roxburgii*	12	0.04	0.04	0.5	0.5	0.15
Pinirhiza citrina	*Pinus wallichiana*	15	0.03	0.03	0.5	0.5	0.15
Pinirhiza beadulata	*Pinus wallichiana*	15	0.05	0.05	0.75	0.75	0.14
Pinirhiza coralloidia	*Pinus wallichiana*	15	0.04	0.04	0.6	0.6	0.1
Pinirhiza variegata	*Pinus wallichiana*	12	0.04	0	0.5	0	0.1
Pinirhiza nigra	*Pinus wallichiana*	12	0.07	0.05	0.9	0.6	0.2
Pinirhiza irregulata	*Pinus wallichiana*	20	0.08	0.01	1.8	0.2	0.28
Populinirhiza lustrata	*Populus ciliata*	20	0.03	0.03	0.6	0.6	0.16
Populinirhiza pinnata	*Populus ciliata*	25	0.05	0.07	0.1	1.7	0.12
Populinirhiza copperina	*Populus ciliata*	20	0.03	0.03	0.6	0.6	0.1
P-Sk 122	*Prunus persica*	12	0.08	0	1.0	0	0.06
P-Sk 123	*Prunus persica*	12	0.03	0.03	0.4	0.4	0.15
Prunirhiza grainata	*Prunus padus*	20	0.03	0.03	0.6	0.6	0.06
Pyrus II	*Pyrus aucuparia*	40	0.1	0.01	4.0	0.5	0.44
Pyrus I	*Pyrus aucuparia*	10	0.1	0	1.0	0	0.46
Quercinirhiza nigra	*Quercus dilatata*	25	0.01	0.01	0.4	0.4	0.1
Quercus I	*Quercus dilatata*	15	0.05	0.04	0.8	0.6	0.03

Table-III: Hyphal characteristics of ectomycorrhizae isolated from Murree and Sakesar Hills

Isolate	Host	Branching	Clamp connections	Hyphal surface	Wall thickness
Abirhiza dichotoma	*Abies pindrow*	Absent	+	Smooth	Thick
Abirhiza claynica	*Abies pindrow*	Dichotomous, Y. and T-shaped	+	Smooth	Thick
Abirhiza irregulata	*Abies pindrow*	Dichotomous	+	Smooth	Thin
Abirhiza bentata	*Abies pindrow*	Y-shaped	+	Smooth	Thin
Alnirhiza plectina	*Alnus rubra*	Dichotomous	+	Rough	Thin
Cedrirhiza cystidica	*Cedrus deodara*	Dichotomous	+	Rough	Thick
Piceirhiza mukshpuriana	*Picea smithiana*	Y-shaped	—	Rough	Thin

Pinirhiza cubiculata	*Pinus roxburgii*	Dichotomous	+	Slightly rough	Thick
Pinirhiza smoothiana	*Pinus roxburgii*	Dichotomous	—	Rough	Thin
Pinirhiza elliptica	*Pinus roxburgii*	Dichotomous	+	Rough	Slightly thick
Pinirhiza spinulata	*Pinus roxburgii*	Y-shaped and T-Shaped	—	Smooth	Both thin and thick walled
Pinirhiza citrina	*Pinus wallichiana*	Y-shaped	+	Smooth	Thin
Pinirhiza beadulata	*Pinus wallichiana*	Y-shaped	+	Smooth	Thin
Pinirhiza coralloidia	*Pinus wallichiana*	Rarely branched	+	Smooth	Thick
Pinirhiza variegata	*Pinus wallichiana*	Highly branched	+	Slightly rough	Thin
Pinirhiza nigra	*Pinus wallichiana*	Dichotomous	—	Rough	Thin
Pinirhiza irregulata	*Pinus wallichiana*	Y-shaped	+	Smooth	Thick
Populinirhiza lustrata	*Populus ciliata*	Y-shaped	±	Smooth	Slightly Thin
Populinirhiza pinnata	*Populus ciliata*	Rarely branched	+	Smooth	Thick
Populinirhiza copperina	*Populus ciliata*	Dichotomous	+	Smooth	Thick
P-Sk 122	*Prunus persica*	Dichotomous	+	Smooth	Thin
P-Sk 123	*Prunus persica*	Rarely branched	+	Smooth	Thick
Prunirhiza grainata	*Prunus padus*	Dichotomous	—	Smooth	Thick
Pyrus II	*Pyrus aucuparia*	Dichotomous	+	Rough	Slightly thin
Pyrus I	*Pyrus aucuparia*	Unbranched	±	Smooth	Thick
Quercinirhiza nigra	*Quercus dilatata*	Unbranched	+	Smooth	Thin
Quercus I	*Quercus dilatata*	Y, and T-shaped	+	Smooth	Thin

Hyphae of all the mycorrhizae were hyaline and 5.4-6.6 (6µm) in width. Positive (+) showing the presence and negative (—) showing the absence of clamp connections.

Discussion

Ectomycorrhizal species isolated from *Abies pindrow* differ in their growth and culture characteristics. Among these, three had fast growth; only *Abirhiza claynica* showed restricted growth. *Abirhiza irregulata* was fast growing(Table1)in culture, because its media requirements were simple, and could be easily grown on MEA. All these had circular colonies but appearance and color of the colonies differ strongly. Colony habit was also similar, from surface to aerial except *Abirhiza dichotoma* in which colony grew from surface to submerged habit (Table I). It could be concluded that the above-mentioned mycorrhizae from *Abies pindrow* can easily be cultured and used for testing the efficiency in colonization.

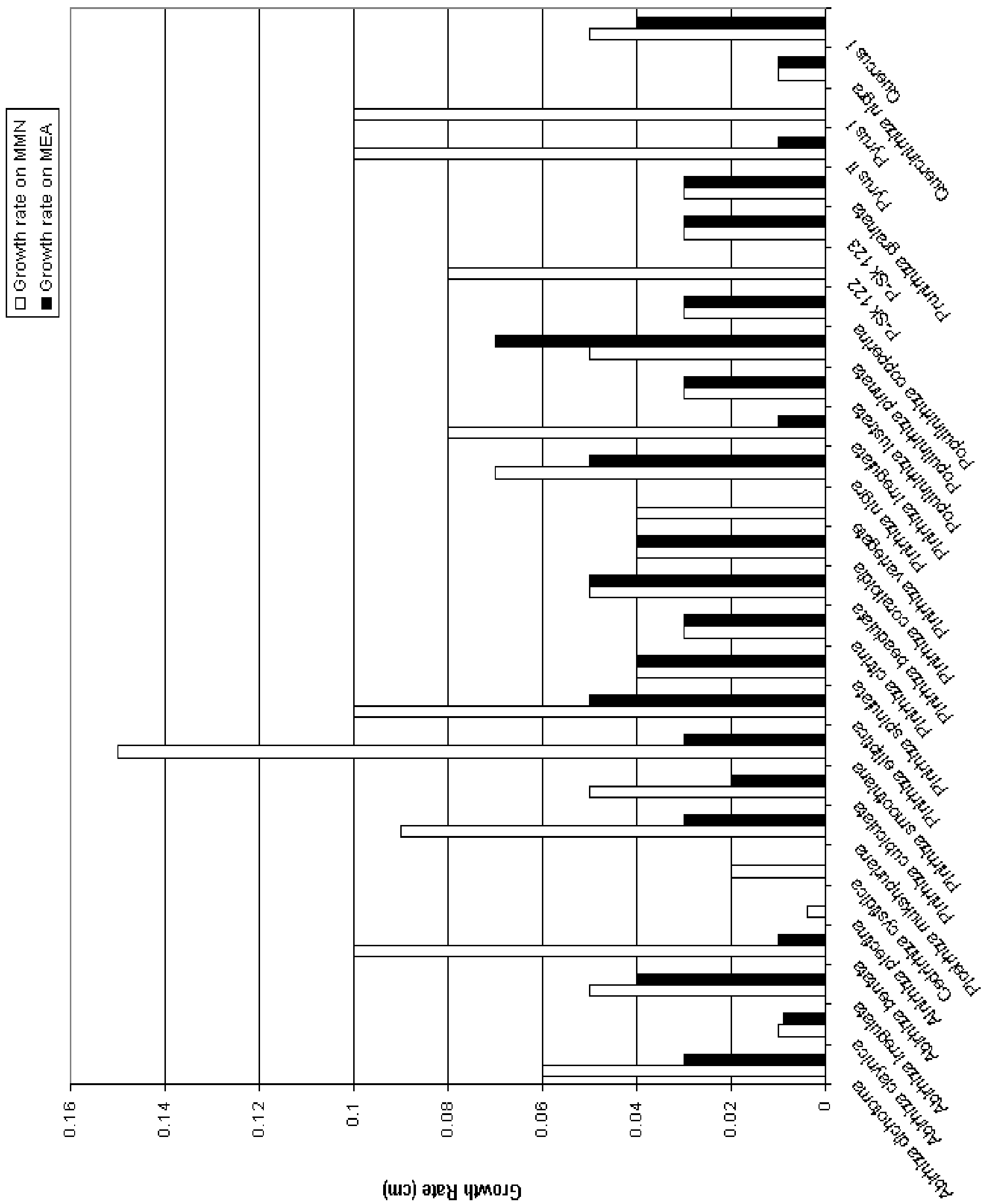

Fig.1: Ectomycorrhizal isolates showing growth rate at MMN and MEA media.

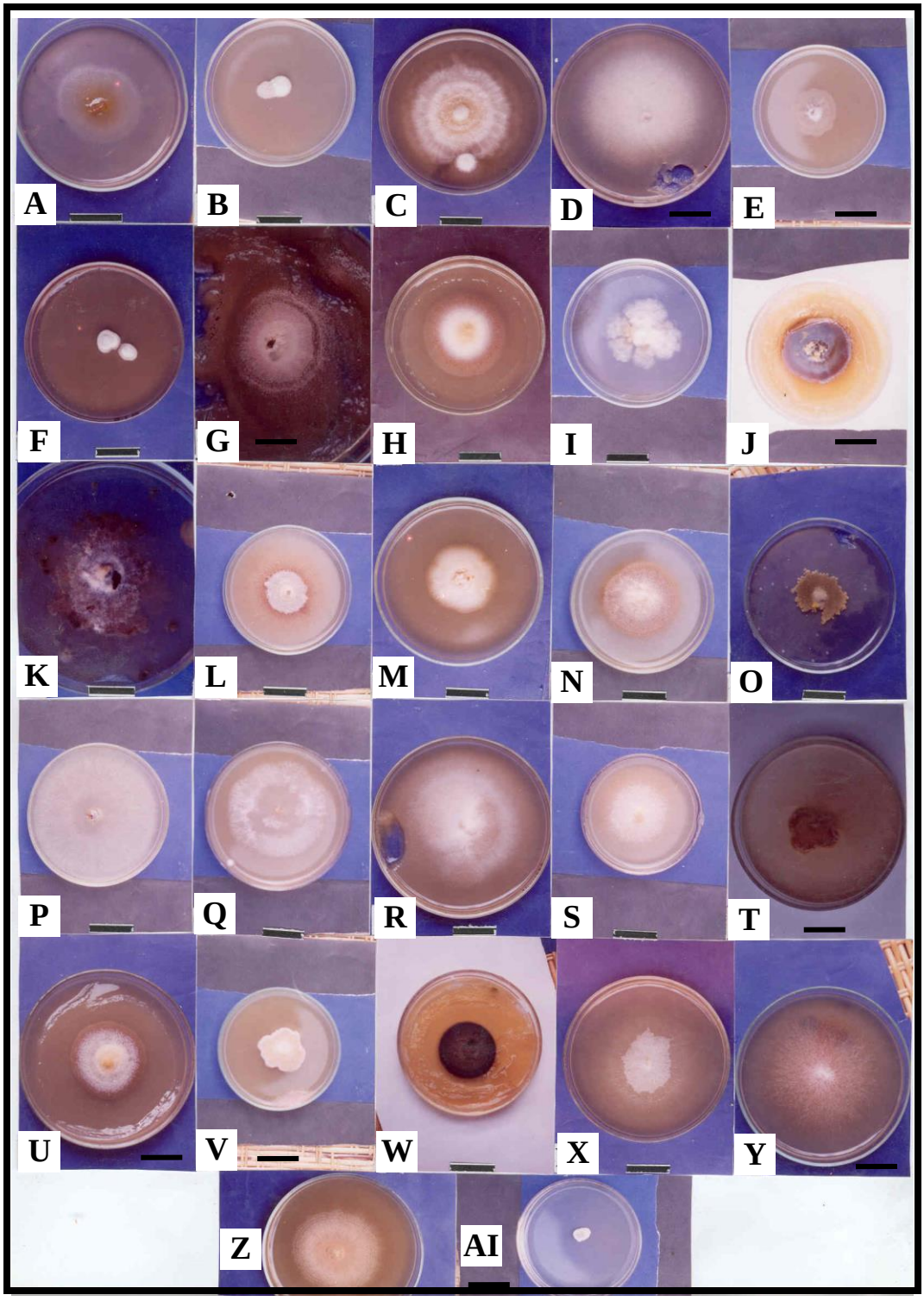

Plate 1: Cultures isolated on different media.
A: *Abirhiza dichotoma*, **B**: *A. claynica*, **C**: *A. irregulata*, **D**: *A. bentata*, **E**: *Alnirhiza plectina*, **F**: *Cedrirhiza cystidica*, **G**: *Picierhiza mukshpuriana*, **H**: *Pinirhiza cubiculata*, **I**: *P. smoothiana*, **J**: *P. elliptica*, **K**: *P. spinulata*, **L**: *P. citrina*, **M**: *P. beadulata*, **N**: *P. coralloidia*, **O**: *P. variegata*, **P**: *P. nigra*, **Q**: *P. irregulata*, **R**: *Populinirhiza lustrata*, **S**: *P. pinnata*, **T**: *P. copperina*, **U**: *P. sk 122*, **V**: *P. Sk 123*, **W**: *Prunirhiza grainata*, **X**: *Pyrus II*, **Y**: *Pyrus I*, **Z**: *Quercinirhiza nigra*, **AI**: *Quercus I*.
Scale bar = 2.5 cm (A, C, F, H, M, T, U, X, Z); 3 cm (B, E, I, J, L, N, O, P, Q, S, V, W, AI); 2.25 cm (G, K, R, Y).

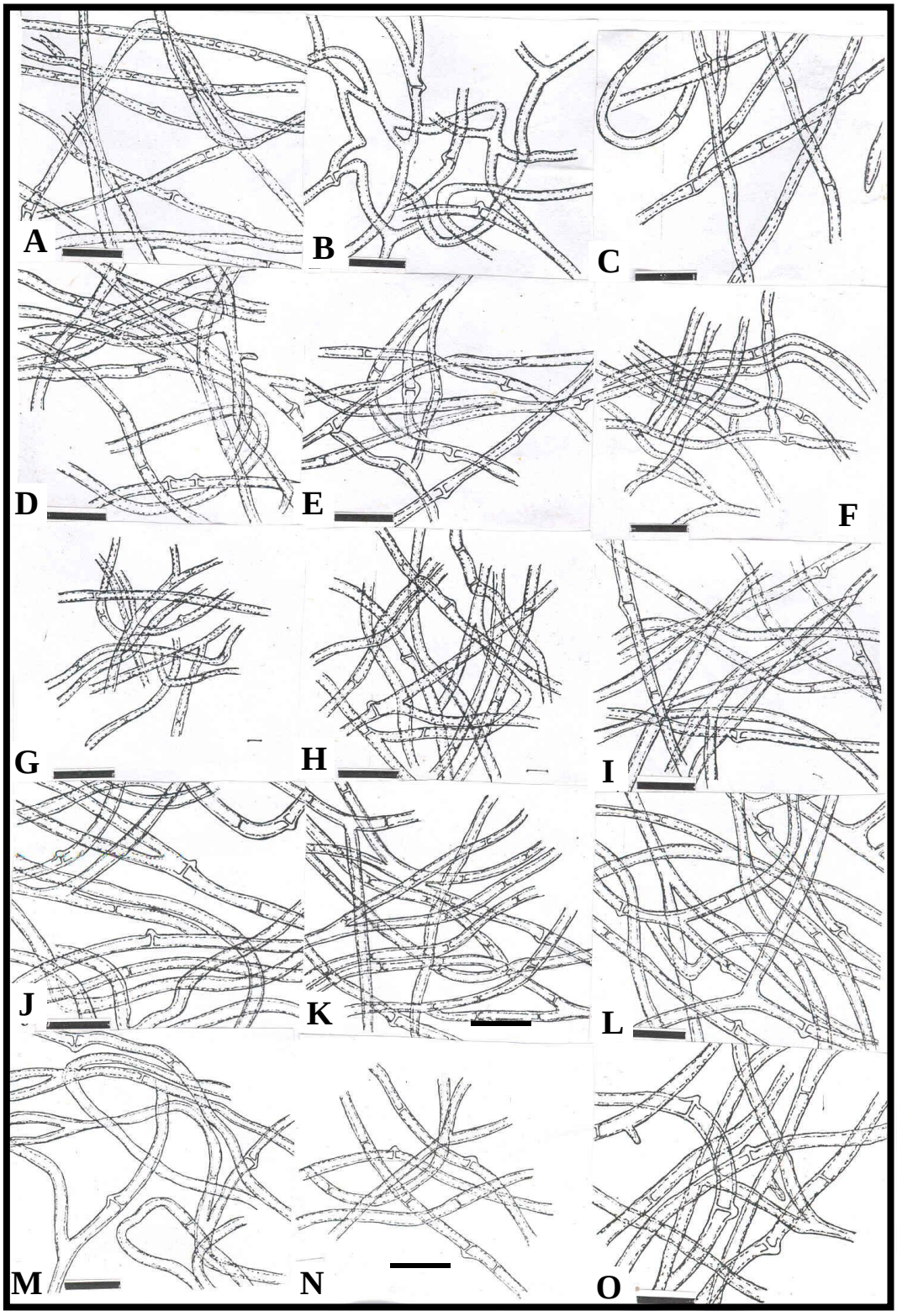

Plate 2: Line drawings of hyphae (scale bar = 6 µm).
A: *Abirhiza dichotoma*, **B**: *A. claynica*, **C**: *A. irregulata*, **D**: *A. bentata*, **E**: *Alnirhiza plectina*, **F**: *Cedrirhiza cystidica*, **G**: *Picierhiza mukshpuriana*, **H**: *Pinirhiza cubiculata*, **I**: *P. smoothiana*, **J**: *P. elliptica*, **K**: *P. spinulata*, **L**: *P. citrina*, **M**: *P. beadulata*, **N**: *P. coralloidia*, **O**: *P. variegata*.

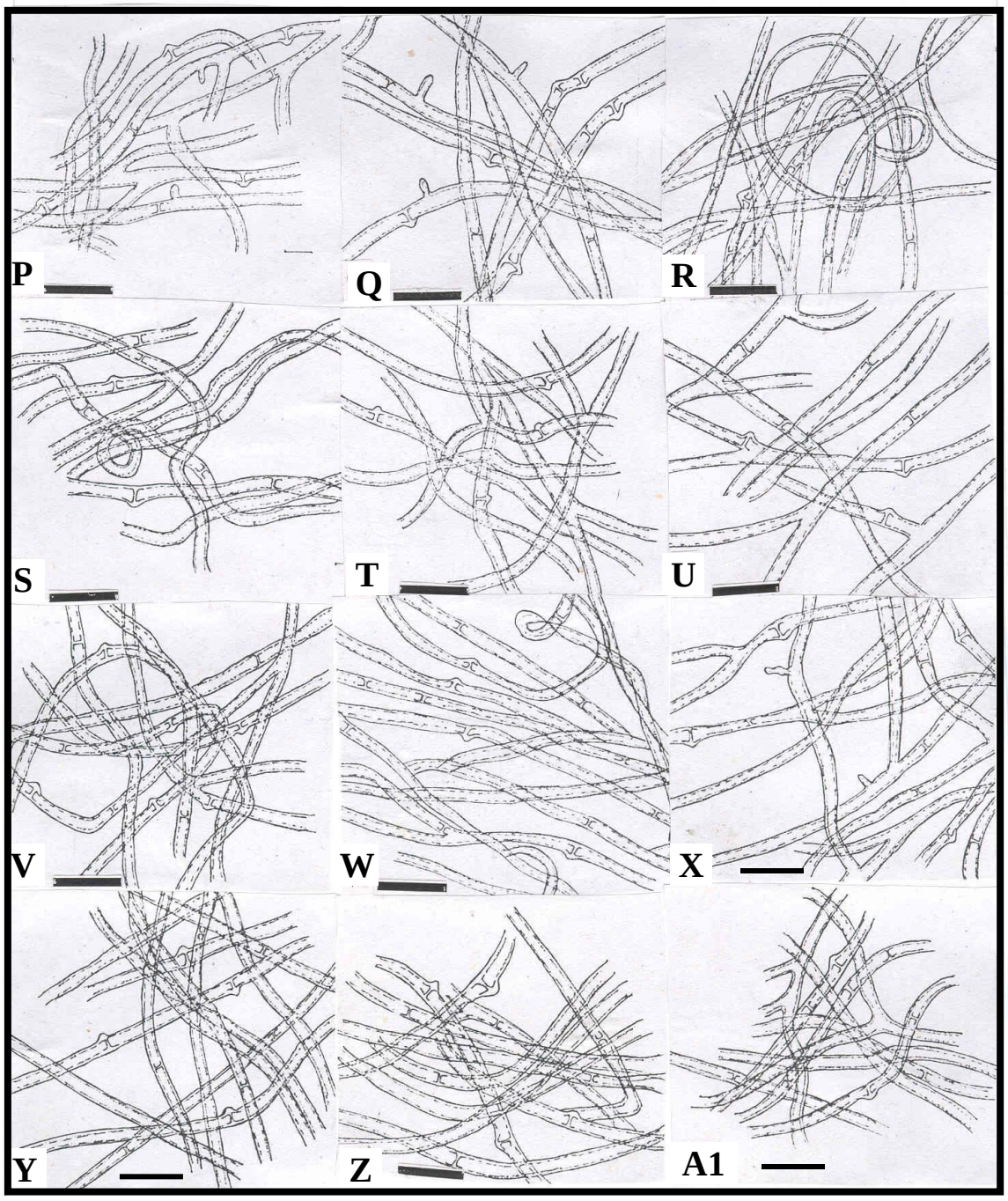

Plate 3: P: *P. nigra*, **Q**: *P. irregulata*, **R**: *Populinirhiza lustrata*, **S**: *P. pinnata*, **T**: *P. copperina*, **U**: *P. sk 122*, **V**: *P. sk 123*, **W**: *Prunirhiza grainata*, **X**: *Pyrus II*, **Y**: *Pyrus I*, **Z**: *Quercinirhiza nsigra*, **AI**: *Quercus I*. (Scale bar = 6 µm).

Alnirhiza plectina isolated from *Alnus rubra* was difficult to culture. Growth rate is 0.004 cm/day on MMN while on MEA, colonies did not appear at all. Colony growth increased slightly in sub culture and grew to 2.5 cm within 45-50 days.

Cedrirhiza cystidica, isolated from *Cedrus deodara* grew well only on MMN medium where moderate growth rate of 0.02 cm/day was observed. No growth was seen on MEA. When transferred to sub culture, growth rate increased to 0.06 cm/day and 1.0 cm wide colony appeared within 15 days of incubation. Hyphae in culture were similar to natural hyphae in branching having thick walls and clamp connections. Protuberances present in natural hyphae are not found in cultural ones.

Piceirhiza mukshpuriana isolated from the *Picea smithiana* seemed slow growing on MEA i.e.0.03 cm/day but on MMN, it is 0.09 cm/day. This rate does not change much on sub culturing.. Hyphae resemble mostly with natural ones but are thinner (6.1 µm in diameter) than the natural hyphae (19.9um diameter).

All ectomycorrhizal species isolated from *Pinus roxburgii* were moderate to fast growing (0.02-1.0) in culture (Table II). Their colonies had surface to aerial habit. At young stage of the growth these had white to light brown mycelium however at maturity they totally differ in their colony morphology (Table I). All showed better growth on MMN than MEA except *Pinirhiza spinulata*, which had same growth rate on both of the media (Table II).

The mycorrhizal species isolated from *Pinus wallichiana* were easy to culture and had moderate to fast growth rate (0.03-0.8 cm / day) in culture. Colonies had same habit but differ in their appearance widely (Table I). All these were culturable on MMN and MEA except *Pinirhiza variegata*, which did not grow on MEA and *Pinirhiza irregulata*, which showed very slow growth on MEA.

Three ectomycorrhizae isolated from *Populus ciliata i.e. Populinirhiza pinnata* and *Populinirhiza copperina*, *Populinirhiza lustrata* showed a moderate growth rate (0.03-0.05 cm / day) and almost same colony habit. In contrast to the other two, it grew on MMN medium and did not response when cultured on MEA.

Two ectomycorrhizae isolated from *Prunus persica*, differ widely in their culture characters and colony appearance. These were not given names due to lack of sufficient data so these were designated by their numbers. *P-Sk 122* and p-sk 123. Both had same colony habit (Table I). Their media requirements were also totally different. P-Sk 122 grew only on MMN with growth rate 0.08 cm/day but did not give any response on MEA. When sub cultured on MMN, growth was enhanced and colony attained 3.0 cm width within 48 days. P-Sk 123 had same growth pattern on both media showing growth rate of 0.3 cm/day in both (Table II). Hyphal characteristics of both are the same.

Prunirhiza grainata isolated from *Prunus padus* had similar growth rate on MMN and MEA media like P-Sk 123 however its cultural characters resemble with P-Sk 122. In sub culture growth increased with the passage of time and within 45 days colony growth was 3.5 cm. Hyphae in culture were different in diameter i.e. 5.9 um and hyaline, from naturally occurring emanating hyphae with diameter 19 um and dark brown in color. Other hyphal characters were similar and also resemble to those isolated from *Prunus persica.*

One of the two ectomycorrhizae isolated from *Pyrus aucuparia*, *Pyrus II* grew luxuriously on MMN medium with 0.1 cm/day growth rate but on MEA, colonies grew slowly (0.01 cm/day). Sub culture when grown on MMN medium grew fast and within 15 days a colony of 6.6 cm in diameter was formed.

Other mycorrhizal strain from the same isolate *Pyrus I*, which grew at the same rate on MMN as the former; but did not show growth on MEA. Increase in growth rate in sub culture was also the same but it had different pattern of colony so these could be distinguished only by their colony character (Table I). So their cultures could easily be obtained on MMN.

Two ECM species from the *Quercus* trees did not show significant change in growth rate on MMN and MEA media (Table II). Thus MEA can be easily used for them due to simple handling and less use of nutrients. Thus mycorrhizae from *Quercus sp* .can easily be cultured on MEA to prepare inoculum.

Among the above-described mycorrhizae, *Pinirhiz smoothiana* found to be the most efficient according to their fastest growth in culture media and easy to isolate on MEA and MMN media. Some other species which were easily culturable on both media are: *Abirhiza dichotoma, A. irregulata, A. bentata, pinirhiza elliptica, P. spinulata, P.citrina, P. irregulata, Populinirhiza lustrata*, and *P-Sk 123*.

The mycorrhizal species with least efficiency were: *Abirhiza claynica, Quercus I, Alnirhiza plectina, Cedrirhiza cystidica, Picirhiza mukshpuriana, P-Sk 122 and Prunirhiza grainata*. These were not only slow in their culture growth

but also these couldn't be grown on MEA medium. The remaining ectomycorrhizae showed intermediate growth rate in culture.

Isolation of fast growing Pines mycorrhizae and from other tree species on cheap and simple media is an achievement in the sense that these can be utilized in pre-inoculation to the nursery stock for forest establishments specially for pine forests. Easy isolation of mycorrhizae on MEA to get inoculum for afforestation and reforestation programmes is economical.

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