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About TERI

A dynamic and flexible organization with a global vision and a local focus, TERI (The Energy and Resources Institute, formerly known as the Tata Energy Research Institute) was established in 1974. TERI's focus is on research in the fields of energy, environment, and sustainable development, and on documentation and information dissemination. The genesis of these activities lie in TERI's firm belief that the efficient utilization of energy, sustainable use of natural resources, large-scale adoption of renewable energy technologies, and reduction of all forms of waste would move the process of development towards the goal of sustainability.

Biotechnology and Management of Bioresources Division

Focusing on ecological, environmental, and food security issues, the activities of the Biotechnology and Management of Bioresources Division include working with a wide variety of living organisms, sophisticated genetic engineering techniques, and, at the grass-roots level, with village communities.

Mycorrhiza Network

The Biotechnology and Management of Bioresources Division's Mycorrhiza Network works through its three wings—the MIC (Mycorrhiza Information Centre), the CMCC (Centre for Mycorrhizal Culture Collection), and *Mycorrhiza News*.

The MIC is primarily responsible for establishing databases on Asian mycorrhizologists and on mycorrhizal literature (RIZA), which allows information retrieval and supplies documents on request. The CMCC has the main objectives of procuring strains of both ecto and vesicular arbuscular mycorrhizal fungi from India and abroad; multiplying and maintaining these fungi in pure cultures; screening, isolating, identifying, multiplying, and maintaining native mycorrhizal fungi; developing a database on cultures maintained; and providing starter cultures on request. Cultures are available on an exchange basis or on specific requests at nominal costs for spore extraction or handling.

Mycorrhiza News

Mycorrhiza News – a quarterly newsletter publishing articles compiled from RIZA – invites short papers from budding and senior mycorrhizologists, giving methodologies being used in mycorrhizal research, providing information on forthcoming events on mycorrhiza and related subjects, listing important research references published during the quarter, and highlighting the activities of the CMCC.

Contents

Amino acid utilization, protease activity, and protein patterns in mycorrhiza

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Organic nitrogen in the form of proteins of dead green plants, dead animals, and faeces is first broken down into amino acids, then to ammonium salts and nitrites, and ultimately to nitrates by bacteria and fungi to enable the plant roots to absorb nitrates for their metabolic activities. Mycorrhizal fungi are able to break up organic matter into nitrates with the help of enzymes for their utilization.

Amino acid utilization by mycorrhizal fungi

Studies conducted at the Department of Biological Science, King Abdul Aziz University, Jeddah, Saudi Arabia, showed that three ecologically important ectomycorrhizal fungi, *Suillus bovinus*, *Amanita muscaria*, and *Hebeloma crustuliniforme*, were able to utilize aspartic and glutamic acids, and amides, together with arginine, alanine, and serine. Growth in each fungus was comparable with that on ammonium as a mineral nitrogen source. Cysteine, methionine, proline, threonine, tryptophane, and tyrosine were, however, not utilized. There were interspecific differences in the abilities to utilize the remaining amino compounds (Abuzinadah and Read 1988).

In studies conducted at the Department of Microbial Ecology, University of Lund, Ecology Building, Helgonavagen 5, Lund, Sweden, mycelial discs, 25 mm in diameter, were cut from 15-day-old colonies of *Paxillus involutus*, and grown on cellophane-covered agar medium containing MMN (modified Melin–Norkrans) medium. These mycelial discs were then incubated between 1 and 24 hours in small dishes containing 1 ml nitrogen-free MMN supplemented with either ^{14}C-glutamate or ^{14}C-glutamine. Effects of pH and ^{14}C source (glutamate or glutamine) on total ^{14}C absorption were examined by scintillation counting of total tissue digests. Incorporation of label was fastest for glutamine. Distribution of radioactivity into individual amino acids separated by HPLC (high performance liquid chromatography) was further investigated in time-course experiments using the enzyme inhibitors MSX (methionine sulphoximine), AZA (azaserine), and AOA (aminooxyacetate). Turnover of labelling between glutamate and glutamine was rapid and followed by rapid incorporation of label into y-aminobutyric acid, alanine, aspartate, citrulline, and asparagines (Chalot, Brun, Finlay, *et al.* 1993).

Further studies conducted at the above-mentioned University showed that *P. involutus* efficiently took up exogenously supplied (^{14}C) alanine and rapidly converted it to pyruvate, citrate succinate, fumarate, and CO_2 (carbon dioxide), thus providing direct evidence for the utilization of alanine as a respiratory substrate. (^{14}C) alanine was further actively metabolized to glutamate, glutamine, and aspartate. Exposure to aminooxyacetate completely suppressed $^{14}CO_2$ evolution and greatly reduced the flow of carbon from ^{14}C alanine to tricarboxylic acid cycle intermediates and amino acids. It was thus concluded that alanine amino transferase plays a pivotal role in alanine metabolism in *P. involutus* (Chalot, Brun, Finlay, *et al.* 1994).

Further studies conducted at the above-mentioned University on factors affecting amino acid uptake by *P. involutus* using tracer kinetic experiments showed that amino acid uptake by *P. involutus* was dependent on the pH of the incubation medium. Uptake of L-glutamate and L-glutamine had a distinct pH optimum of about 4.0 and declined sharply between pH 4.5 and 6.4. Uptake of alanine and aspartate was also highest at low pH values but declined more slowly with increasing pH. Total uptake of labelled amino acids was unaffected by 0.05 mM or 0.5 mM ammonium while 5 mM ammonium decreased amino acid uptake. Nitrate or glucose did not significantly affect amino acid uptake but glucose greatly increased the proportion of carbon that was respired. Uptake of different amino acids decreased by a factor of 4 to 10 with increasing age (1–5 weeks), and the proportion of absorbed ^{14}C that was respired increased steadily with age (Chalot, Kytoviita, Brun, *et al.* 1995).

Studies conducted at the Plant Science Department, Scottish Agricultural College, UK, on effects of DFMA (difluoromethylarginine) and DFMO (difluoromethylornithine) on polyamine concentration and metabolism, and on the activities of enzymes of polyamine biosynthesis and breakdown in *Laccaria proxima*, showed that *L. proxima* possessed both ADC (agrinine decarboxylase) and ODC (ornithine decarboxylase) activities. DFMA depleted putrescine and did not inhibit ODC activity. Incubation of mycelium with a (U–^{14}C) arginine substrate led to ^{14}C putrescine formation. DFMA completely inhibited the formation of polyamines from (U–^{14}C) arginine. DFMO inhibited bound biosynthetic enzyme activity and the formation of putrescine from (U–^{14}C) ornithine. *L. proxima* thus may not be susceptible to DFMO (Zarb and Walters 1994a).

In further studies at the above-mentioned College, radiolabelled products that co-chromatographed with authentic standards of cadaverine, APC (aminopropylcadaverine), and N, N bis (3-aminopropyl) cadaverine (3 APC) were isolated following the decarboxylation of a (U–^{14}C) lysine substrate by fungal lysine decarboxylase extracts. The identity of 3 APC was confirmed by NMR (nuclear magnetic resonance) spectroscopy. The inhibition of the enzymes AdoMetDC (S-adenosylmethionine decarboxylase) and spermidine synthase led to significant reductions in the recovery of radiolabelled 3 APC. It was concluded that a range of ectomycorrhizal and plant pathogenic fungi could convert lysine into the higher homologues of cadaverine. It was suggested that these cadaverine homologues were formed via the action of AdoMetDC and spermidine and spermine synthases, although the operation of an additional route for the biosynthesis of these compounds from L-aspartic-beta-semialdehyde was thought to be a possibility (Zarb and Walters 1994b).

Production of extracellular proteinases by hyphae of ectomycorrhizal fungi

Studies conducted at the Department of Forest Science, University of Alberta, Edmonton, Canada, showed that *H. crustuliniforme* produced an extracellular acid proteinase in a liquid medium containing BSA (bovine serum albumin) as the sole nitrogen source. The proteinase was purified 26-fold with 20% activity recovery and was shown to have a molecular weight of 37 800, as indicated by SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis), and an isoelectric point of 4.8 ± 0.2. The enzyme was most active at 50 °C and pH 2.5 against serum albumin and was stable in the absence of substrates at temperature up to 45 °C and pH between 2.0 and 5.0. Pepstatin A, diazoacetyl-DL-norleucine, metallic ions Fe^{2+} and Fe^{3+}, and phenolic acids severely inhibited the enzyme activity, while antipain, leupeptin, N-alpha-p-tosyl-L-lysine chloromethyl ketone and trypsin inhibitor inhibited the activity moderately. The proteinase hydrolysed BSA and cytochrome c rapidly compared with casein and azocasein but failed to hydrolyse any of the low-molecular-weight peptide derivatives tested (Zhu and Dancik 1990).

In studies conducted at the Laboratory of Food Microbiology, Department of Food and Nutrition, Kinki University, Higashiosaka, Japan, carboxyl proteinases were found in all cultures of the ectomycorrhizal fungus, *Tricholoma matsutake,* tested in a liquid potato glucose medium of pH 5.1. The isoelectric points were between pH 3.85 and 5.76, commonly ca. 4.5. For most stocks of *T. matsutake*, the optimum temperature for enzyme activity was ca. 70 °C, and for most of the related species (except *Tricholoma ponderosum*) it was ca. 40 °C (Terashita and Kono 1989).

Studies were conducted at the above-mentioned Laboratory on proteinases of *T. matsutake* in still culture. One proteinase having optimum pH at about 3.0 was detected in the culture filtrate of this fungus. This enzyme activity was completely inhibited by carboxyl proteinase inhibitors such as S-PI (streptomyces–pepsin inhibitor, pepstatin A), DAN (diazoacetyl-DL-norleucine methyl ester), and EPNP (1, 2 epoxy-3- (p-nitrophenoxy) propane). In addition, two kinds of proteinases, having optimum pHs at about 3.0 and 6.5, were detected in the vegetative mycelium and the fruit-body extract, respectively. The enzymes having optimum pH in the acid range

were inhibited by S-PI, DAN, and EPNP. The other was inhibited by metal proteinase inhibitors such as talopeptin (MK-I) and EDTA (ethylene diamine tetra acetic acid). The three carboxyl proteinases were purified and characterized. They showed single bands on polyacrylamide gel electrophoresis, with the exception of the vegetative mycelium. The carboxyl proteinases in the culture filtrate and in the fruit-body extract were 1469-fold (18% recovery) and 3281-fold (7% recovery) purified over the original crude materials, respectively. These carboxyl proteinases differed from other carboxyl proteinases of basidiomycetes in their enzymatic properties such as S-PI-sensitivity (high-affinity) and optimum temperature (about 70 °C) (Terashita and Kono 1987).

In studies conducted at the University of Guelph, Department of Microbiology, Guelph, Ontario, Canada, *H. crustuliniforme* was grown in defined liquid media containing different combinations of ammonium, glucose, and BSA as sources of nitrogen and carbon for proteinase induction or repression. The production of extracellular proteinase was highest in the medium containing BSA as sole nitrogen source and it was not affected by the addition of ammonium at concentrations below 3.2 mM, but higher ammonium concentrations repressed the proteinase production. Proteinase synthesis required simple carbon as an energy source and was not repressed by glucose at concentrations from 0.5% to 2%. Optimal culture pH for proteinase production was between 4 and 5. At pH 3, the fungus yielded biomass that was only 10% of that at pH 5, though proteinase production per mg dry weight of mycelium was not affected. The fungus was unable to grow and produce proteinase at pH 7 or above. The proteinase was most active at pH 2.5–3 and was stable at pH 2.5–5.5. These results suggested that the regulation of extracellular acid proteinase in *H. crustuliniforme* involved protein induction and partial nitrogen repression but not carbon catabolite repression (Zhu, Dancik, and Higginbotham 1994).

Production of extracellular proteinases by hyphae of ericoid mycorrhizal fungi

In studies conducted at the Department of Animal and Plant Science, University of Sheffield, Sheffield, UK, the proteinase activity of *Hymenoscyphus ericae* was initially detected using a crude but rapid assay of protein utilization based upon coummassie blue. A more sensitive assay based upon a fleximetric principle was then developed. Protease activity was measured in buffered cell-free culture filtration over the pH range of 1.0–9.0. Significant activity was restricted to the pH range of 1.0–5.0 with optimum at 2.2. In further studies, proteinase of *H. ericae*, isolated from roots of *Calluna vulgaris* growing in soil of pH 3.5, was compared with a similar enzyme from an endophyte of the calcicolous alpine shrub *Rhodothamnus chamaecistus,* growing in soil of pH 6.5. The fungi were grown in liquid culture at pH values ranging from 3.0 to 8.0 with pure protein, BSA, as sole source of nitrogen. Both fungi yielded an extracellular acid proteinase with pH optimum for activity between 2.0 and 3.0. The production and activity of these enzymes were strongly affected by pH of the culture medium. Maximum enzyme production during exponential growth occurred in both fungi at a culture pH of 4.0–5.0, whereas higher pH treatments severely inhibited enzyme production. The acid proteinase of *H. ericae* was tolerant to extreme acidity and retained near-optimal activity in solutions of pH 2.0. In contrast, the activity of the enzyme from the *Rhodothamnus* endophyte retained activity at much higher pH values than did the proteinase from *H. ericae*. Unlike *H. ericae*, the isolated endophyte of *Rhodothamnus* was able to grow and use protein as sole source of nitrogen at pH 7.0 and 8.0 (Leake and Read 1987, 1990).

In further studies conducted at the above-mentioned University, *H. ericae* was grown in liquid culture with either pure protein or with protein hydrolysate as sole source of nitrogen and carbon, and the effect of supplementation with ammonium and glucose was investigated. Proteinase production was induced as effectively by protein hydrolysate as by pure protein, but in each case was enhanced in the presence of low concentration (10 mg/ml) of ammonium. Maximum enzyme production was found in those cultures that were supplied with protein or hydrolysate as sole carbon sources, a feature associated with the release of additional ammonium. This ammonification coincided with an increase of pH to a final value of 6.45, at which point the enzyme appeared to be inactivated. Glucose strongly repressed proteinase production when the endophyte was grown with protein, but not when grown with hydrolysate. It was suggested from these contrasting responses that conditions leading to induction exert a stronger influence over enzyme production compared to conditions leading to repression. In addition, protein hydrolysis products must be present before full enzyme production is induced (Leake and Read 1991).

Further studies conducted at the above-mentioned University on extracellular proteinases of three ericoid and nine ectomycorrhizal fungi showed that despite the wide taxonomic range of the species examined, which included both Ascomycetes and Basidiomycetes, their proteolytic enzymes showed remarkable similarities. Many of the features of the

fungi and their enzymes are known to he appropriate to the particular edaphic environments associated with heathland and boreal forests in which the ability of mycorrhizas to access polymeric organic nitrogen is likely to be of greater benefit to host plants (Leake 1990).

Production of proteases by mycorrhizal roots/mycorrhizal fungi

In studies conducted at the Department of Botany, University of Wyoming, Wyoming, USA, enzymatic activity in ectomycorrhizal and saprotrophic Basidiomycetes was visualized by placing glass fibre filter paper soaked with agar containing one of several reaction mixtures in contact with ectomycorrhizal root systems and mycelia of saprotrophic fungi. Protease, along with cellulase and phosphatase enzyme systems, was visualized by staining residual substrate following exposure. Protease activity was also visualized by using a protein substrate linked to a fluorescein isothiocyanate chromophore, and activity could be directly observed after exposure or could be visualized with blue or UV (ultraviolet) light. In both ectomycorrhizal and saprotrophic fungi, protease activity, although greatest near the hyphal front, was also evident in older hyphae (Miller 1993).

In further studies conducted at the above-mentioned University, both saprotrophic and ectomycorrhizal fungi were grown on microscope slides coated with agar containing FITC (fluorescein isothiocyanate chromophore)–BSA as a protein substrate. The FITC is capable of fluorescing only upon proteolysis; so fluorescence should be limited to sites of proteolytic activity. Direct examination of the growing mycelia with epifluorescent microscopy revealed that proteolytic activity was localized on the wall or plasma membrane and was not freely diffusible into the agar. In a second experiment, fungal mycelia grown in nutrient solutions with BSA were homogenized and fractionated in an isotonic sucrose medium. Each fraction was subjected to the FITC–BSA assay and fluorescence quantified. The greatest proteolytic activity was again found in the cell wall/plasma membrane fraction with a lower amount of activity in the cytoplasmic fraction. No proteolytic activity was detected in the culture filtrate. However, electrophoretic separation of proteins extracted from culture filtrate following cell lysis indicated that there was substantial release of proteases into the filtrate. Bound proteases have now been detected in both ectomycorrhizal and saprotrophic Basidiomycetes. In addition, there also appear to be highly active intracellular proteases released upon lysis. These findings suggest that there may be several strategies for protein utilization by ectomycorrhizal fungi under different environmental conditions (Miller and Ghosh 1993).

In studies conducted at the Department of Animal and Plant Sciences, University of Sheffield, Sheffield, UK, FHOM (fermentation horizon organic matter) was collected from a birch forest and placed in transparent observation chambers with (M) or without (NM) plants of *Betula pendula*, the roots of which were infected with *Paxillus*. The fungus colonized the FHOM of the M chambers. The activities of protease and polyphenol oxidase increased in organic matter that had been occupied for 28–50 days and remained elevated in FHOM that had been occupied for 50–98 days (Gray and Read 1995).

Protein synthesis by mycorrhizae or mycorrhizal fungi

In studies conducted at the Department of Botany, Erindale Campus, University of Toronto, Mississauga, Canada, *Pinus resinosa* seedlings in test tubes were inoculated with *P. involutus* or with discus of sterile MMN medium. Control tubes were also inoculated with *P. involutus* in the absence of *P. resinosa* seedlings. In vivo labelling of proteins in *P. resinosa* roots and in *P. involutus* mycelium was carried out using ^{35}S-L-methionine 1, 2, 3, 4, 5, and 7 days after inoculation. SDS–PAGE of the protein extracts from the four treatments and autoradiography demonstrated that the presence of root exudates altered protein synthesis in *P. involutus* as three major bands disappeared when the fungus was exposed to root exudates. Protein synthesis in *P. resinosa* roots was also altered when *P. involutus* was introduced into the tubes, since at least two bands were more intense when seedlings were inoculated with fungus in comparison with control roots. No difference was observed in the growth and the label incorporation of *P. involutus* growing with or without root exudates. Ectomycorrhizal roots were not formed during this experiment. Gene regulation in this ectomycorrhizal association occurs, therefore, prior to the formation of ectomycorrhizal roots (Duchesne 1989).

In studies conducted at the Rothamsted Experimental Station, Harpenden, Hertfordshire, UK, inhibitors known to be capable of blocking the synthesis of DNA, RNA, and protein were used to investigate the metabolic processes taking place during spore germination and growth of *Glomus caledonium*. The results obtained could be compared with the known effects of these compounds on other fungi. In general, the response of *G. caledonium* was similar to that observed with saprophytic fungi rather than other obligate biotrophs, and there seemed no obvious limitation to the synthesis of nucleic acid or protein,

which would explain the dependence of this fungus on a living host (Hepper 1979).

In studies conducted at the Lund University, Department of Microbial Ecology, Ecology Building, Lund, Sweden, five types of pine ectomycorrhizae (*Piloderma*, tuberculate, *Lactarius*-like, *Russula*-like, and unidentified pink) collected from bedrock and moraine sites in mature *Pinus sylvestris* forest on four occasions over a period of one year were used for further biochemical assays. Total protein extractions were performed in sequence for each sample of distinct mycorrhizal morphotype. Differences between sites (bedrock moss vs moraine) from which the morphotypes originated were small, except for an increase in protein in *Piloderma* and tuberculate mycorrhizae on moraine sites in July and January. However, there were significant variations in protein concentrations, both between seasons and mycorrhizal types. Protein concentrations were significantly higher in the winter samples for *Piloderma*, tuberculate, and pink mycorrhizae, and were minimum in the early summer (Wallander, Massicotte, and Nylund 1997).

Protein patterns in vesicular arbuscular mycorrhizal plants and vesicular arbuscular mycorrhizal fungi

In studies conducted at the YE de Fisiologia Vegetal, Instituto de Investigaciones Agrobiologicas de Galicia, Apdo, Sandiago de Compostela, Spain, a comparison of protein contents and patterns in root extracts from non-mycorrhizal and VAM (vesicular arbuscular mycorrhizal) (with *Glomus mosseae*) red clover (*Trifolium pratense* L.) was made. Soluble protein content was higher in mycorrhizal than in non-mycorrhizal roots. After separation by DEAE (diethylaminoethyl)–cellulose chromatography and electrophoretic analysis, several additional polypeptides appeared in the mycorrhizal extracts. In addition, it seemed that one of the additional proteins was a plant-induced superoxide dismutase isozyme (Arines, Palma, and Vilarino 1993).

In studies conducted at the University of Pisa, Ctr Studio Microbil Suolo, Via Del Borghetto, Pisa, Italy, spore proteins of different species and isolates of VAM fungi were compared by PAGE. Reproducibility of protein patterns was assessed by using cultures of the same species either grown on different host plants, or produced during successive propagation cycles and stored up to five years. The results consistently showed that host species – different generations and storage – did not affect protein profiles, thus validating the accuracy of the method. Comparison among different geographical isolates of the same species revealed consistent protein patterns. The stability and diversity of spore protein profiles suggested that PAGE could be used to differentiate and identify AM fungal species and isolates. By contrast, the physiological state of spores affected the quality and quantity of bands, with germinating spores showing marked profile changes, as compared to quiescent spores, both in denaturating and native analytical conditions. The disappearance of some polypeptides in germinated spores might be related to the occurrence of storage proteins in AM fungi (Avio and Giovannetti 1998).

Studies conducted at the Thuringer Landesanstalt Landwirtschaft, Naumburger, Jena, Germany, on the protein content and protein patterns of VAM *Panax ginseng* roots of one-year-old plants in pot showed that the shoots and roots of plants inoculated with *Glomus intraradices* had lower protein content than non-inoculated plants. This may give rise to a lower growth of mycorrhizal plants than non-mycorrhizal ones. About 80% of all soluble proteins of ginseng roots separated by SDS-PAGE (coumassie blue staining method) were concentrated on two dominant bands with molecular masses corresponding to 25–29 kDa (kilodatton). These two bands appeared to be stronger in mycorrhizal roots in spite of lower protein concentrations. Other quantitative or qualitative differences between non-mycorrhizal and mycorrhizal roots could hardly be detected on the coumassie blue stained gels. Using the high sensitive silver staining method, three additional peaks (polypeptides with molecular weights of approximately 82 kDa, 31 kDa, and 20 kDa) were detected in the extract derived from the mycorrhizal treatment. Therefore, it was assumed that these polypeptides were induced in ginseng roots by mycorrhization (Domey, Leinhos, Dautz, *et al.* 1998).

Amino acid production by ectomycorrhizal fungi/plants

Studies conducted at the United States Department of Agriculture, Forest Service, Northeastern Forest Experiment Station, Forest Physiology Laboratory, Beltsville, Md, USA, on analysis of amino acids produced in vitro by the ectomycorrhizal fungus, *Pisolithus tinctorius* by gas–liquid chromatography showed that glutamic acid was the major component (21%) of the amino acids in the total pool (bound + free) and bound fraction (total – free) during the exponential phase of growth. Alanine, however, was the major component in the free pool during the acceleration phase (35%) and in the early days of the exponential phase (33%), but subsequently decreased rapidly. Arginine, an ornithine cycle intermediate, was present in substantial amounts (7%) in the total pool and in the bound fraction during the exponential

phase. The pattern of ammonia production was very similar to that of arginine in the total and bound fractions. Citrulline and ornithine, along with ornithine cycle intermediates, were present in all fractions. Differences in the proportions of other amino acids at different stages of growth indicated diversity in the production of proteins. A specific pattern of amino acid synthesis was exhibited by *P. tinctorius*. This pattern suggested that alanine as well as glutamic acid may have been a route by which amino compounds were formed from inorganic nitrogen. The presence of arginine, citrulline, ornithine, and ammonia in the free pool was an indication that the ornithine cycle is operative in this fungus. It was also suggested that this pattern of amino acid synthesis may be similar to that of the other ectomycorrhizal fungi that have demonstrated their ability to alter and enhance nitrogen metabolism in a host plant (Booker 1979).

Amino acid production by vesicular arbuscular mycorrhizal fungi

In studies conducted at the Faculty of Agriculture, Yamagata University Tsuruoka, Yamagata Prefecture, Japan, six non-mycorrhizal (Cruciferae and Chenopodiaceae) and four mycorrhizal (Graminae and Leguminosae) plants were grown in unsterilized soil that contained indigenous mycorrhizal fungi. White clover was grown in soil of three P (phosphorus) levels. Rhizosphere soil solution was collected with porous ceramic cup buried inside a pot. Six plant species of different low P tolerance were grown in water culture of four P levels, and root extract and root exudate were collected. Amino acid and reducing sugar contents in rhizosphere soil solution were higher in P-deficient solution. Proportion of glutamate in amino acids of the non-mycorrhizal root was higher than the mycorrhizal root. Some relationships were observed between mycorrhizal infection and each amino acid content. It was concluded that amino acids were leaked from root cells, thus, controlling the first step of mycorrhizal infection when root membrane permeability was increased at abnormal composition of phospholipid of root membrane under P-deficient conditions. Optimal amino acid level, especially of glutamate, is necessary for growth of VAM fungi within root cells (Tawaraya, Wagatsuma, and Sasai 1990).

Studies conducted at the Institute of Soil Science, Academia Sinica, Nanjing, China, on cotton (*Gossypium hirsutum*) plants grown on an autoclaved calcareous soil and found seriously depressed by excess Mn_2^+ in the steamed soil (regardless of phosphate addition) showed that inoculation of the plants with *Glomus* spp. alleviated the toxicity and kept the plants surviving and growing much better than uninoculated plants. Total amount of free amino acids in non-mycorrhizal roots was found to be three times higher than that in mycorrhizal plants, but they mainly existed in Asp (aspartic acid), Thr (threonine), Glu (glutamic acid), and Gly (glycine). These four accounted for 93% of the total amount in non-mycorrhizal roots compared with 59% in mycorrhizal roots. However, the situation for basic or partially basic amino acids was evidently reversed. Both the amount and percentage of Arg (arginine), Lys (lysine), Leu (leueine), Ile (isolucine), and Val (valine) were higher in mycorrhizal roots. A hypothesis that basic amino acids might stimulate polyphosphate synthesis by the fungi and thereby complex excess metal in polyphosphate or in a form of metal–polyphosphate–arginine (or other basic amino acids) within mycorrhizae was discussed (Xiong and Shi 1990).

In studies conducted at the Department of Botany, Jawahar Lal Nehru Vishwa University, Jodhpur – 342001, Rajasthan, India, the efficacy of VAM fungus, *G. fasciculatum* was evaluated towards amino acid levels in *Prosopis cineraria* under glass house conditions. A total of 12 amino acids were quantified in mycorrhizal and non-mycorrhizal plants. VAM inoculation resulted in increased level of all the amino acids as compared with control. The investigation can be important for the production of highly nutritive leafy fodder of *P. cineraria* (Mathur and Vyas 1996).

Superoxide dismutase activity in vesicular arbuscular mycorrhizal fungi

Studies were conducted at the YE de Fisiologia Vegetal, Instituto de Investigaciones Agrobiologicas de Galicia, CSIC, Apdo 122, 15080-Sandiago de Compostela, Spain, comparing protein patterns in Rhizobium-nodulated non-mycorrhizal and VAM (with *G. mosseae*) red clover (*T. pratense* L.). Red clover plants were grown in a sterilized sand–vermiculite (1:1) mixture, uninoculated or inoculated with soil inoculum of *G. mosseae* in growth chamber, under controlled humidity and light conditions. Uninoculated plants received more phosphate than inoculated ones. Ten weeks later, roots with the rhizosphere-attached material were powdered under liquid nitrogen and the powder was extracted with phosphate buffer at pH 7.2 with several additives. Soluble protein content was higher in mycorrhizal than in non-mycorrhizal roots. After separation by DEAE-cellulose chromatography and electrophoretic analysis, several additional polypeptides appeared in mycorrhizal extracts and one of the additional proteins was a plant-induced SOD (superoxide dismutase) isozyme. After IEF (isoelectric focusing) analysis,

some proteins in the mycorrhizal root extract at acidic pH showed SOD activity not inhibited by CN-. One Cu, Zn-SOD was found in spore from *G. mosseae*, while one Mn-SOD (Mn-SOD I) and two Cu, Zn-SOD (II and III) were present in mycorrhizal and non-mycorrhizal root extracts. Mycorrhizal roots showed further two new isozymes, Mn-SOD II and Cu, Zn-SOD I), having 37 800 and 33 300 Da MW (molecular weight), respectively. Cu, Zn-SOD I was found to be exclusive of mycorrhizal roots, whereas Mn-SOD II was also coincident with a Mn-SOD expressed in Rhizobium nodules. The results obtained suggest that both isozymes Mn-SOD II and Cu, Zn-SOD I were induced in the plant in response to colonization by Rhizobium or *G. mosseae*, Cu, Zn-SOD I being exclusively expressed after colonization by the mycorrhizal fungus (Arines, Palma, and Vilarino 1993; Palma and Arines 1992).

Further studies conducted at the above-mentioned Institute showed that soluble extracts of spores of *G. mosseae* contained a cuprozinc-containing SOD (Cu, Zn-SOD). And this was the first report of the presence of SOD isozymes in VAM fungi. Extracts of roots and leaves of non-mycorrhizal *T. pratense* plants contained one Mn-SOD and two different Cu, Zn-SOD isozymes. In addition, roots of *T. pratense* soil inoculated with *G. mosseae* contained a Cu, Zn-SOD exclusive to mycorrhizal roots and a different Mn-SOD. It was thus concluded that these two isozymes were induced in *T. pratense* roots in response to infection with mycorrhizal fungi, possibly as a result of an increase in the generation of $O_2(-)$ radicals in plant roots (Palma, Longa, Rio, *et al.* 1993).

Further studies conducted at the above-mentioned Institute on protein pattern and SOD activity in mycorrhizal and non-mycorrhizal roots of peas (*Pisum sativum*) cv. Petit Provencal grown in soil inoculated with *G. mosseae* showed that a polypeptide with a molecular weight of 32.0 kDa and a protein with an isoelectric point of pI 4.9 were strongly expressed in mycorrhizal roots. There was higher SOD activity in mycorrhizal compared to non-mycorrhizal roots, although both root types showed the same isoenzymatic pattern for SODs. Two Mn-SODs (I and II) and two Cu, Zn-SODs (I and II) were detected. Cu, Zn-SOD was the most abundant isozyme in both types of roots. A similar pattern of SOD isozymes was also found in nodules of mycorrhizal and non-mycorrhizal roots. In nodules, Mn-SOD II was the main isozyme. This enzyme is postulated to be of bacterial nature (Arines, Quintela, Vilarino, *et al.* 1994).

Studies conducted at the CSIC, Estacion Expt Zaidin, Department Microbiol Suelo Sistemas Simbiot, Apdo 419, Granada, Spain, on total activity and the isoenzymatic pattern of SOD (EC 1.15.1.1) at different growth times in the symbioses of *T. pratense–G. mosseae*, *T. pretense–G. intraradices*, *Allium cepa–G. mosseae*, and *A. cepa–G. intraradices* showed that in non-AM red clover, total SOD activity increased two-fold in 50- and 80-day-old plants, compared to 15- and 30-day-old plants. However, in plants inoculated with AM fungi, the activity only rose to the same level after 80 days of growth. In contrast, no changes in SOD activity were detected in similar experiments carried out with onion. Analysis of SOD isozymes in the symbioses studied showed the presence of one Mn-SOD and two Cu, Zn-SODs (Cu, Zn-SOD I and Cu, Zn-SOD II) in each plant species at 15 days of growth. In both red clover and onion, two new Cu, Zn-SODs (named Cu, Zn-SOD 1 and Cu, Zn-SOD 2) were detected in tissues older than 30 days, besides the constitutive isozymes indicated above. Both *T. pratense*, *G. mosseae*, and *A. cepa*, *G. mosseae* symbioses also expressed a specific isozyme (mycCuZn-SOD), which was absent in the symbioses with *G. intraradices*. The expression of mycCuZn-SOD could only be detected in material older than 30 days (Martin, GarciaRomera, Ocampo, *et al.* 1998).

Superoxide dismutase activity in ectomycorrhizal fungi

Studies conducted at the Department of Plant Physiology, Polish Academy of Sciences, Slawkowska 17, Poland, showed that sulphite at a concentration of 1 mM did not strongly affect the growth of mycelium of the ectomycorrhizal fungus, *Rhizopogon roseolus*. Higher concentrations of 5–20 mM almost completely inhibited the growth of mycelium and SOD (EC.1.15.1.1) activity. The activity of this enzyme was not detectable on polyacrylamide gels. The lack of induction of SOD and the resulting oxidative stress may in part be responsible for the growth inhibition caused by high concentrations of sulphite (Miszalski and Botton 1996).

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Research finding papers

Ectomycorrhizal mass and root tip morphology in oak and pine seedlings in relation to light and nutrients: a case study from Indian Central Himalaya

Manoj Tiwari[*1] *and S. P. Singh*[2]

Introduction

Almost all higher plants in the terrestrial ecosystem are known to be associated with mycorrhizal fungi (Smith and Read 1997). In many ecosystems, these symbiotic fungi play an important role in sustaining plant productivity by increasing its nutrient and water uptake from the soil (Tiwari, Pandey, and Singh 2004; Tiwari, Singh, Tiwari, *et al*. 2003) and, in return, consume considerable part of assimilated carbon for establishing and maintaining this association (Vogt, Publicover, and Vogt 1991). Information on ectomycorrhizal status (number and mass of mycorrhizal tips) of forest trees should provide an index of change in forest ecosystem under anthropogenic and/or natural stresses.

Ectomycorrhizal development is influenced by a variety of direct and indirect factors that influence root growth, including availability of light and nutrients (Andersen and Rygiewicz 1991; Kieliszewska-Rokicka, Rudawska, Leski 1997). While a number of reports are available on the beneficial effects of ectomycorrhiza on the growth of host, surprisingly, there are no reports providing evidence, in the form of resource response curve, of the benefits to the plant from, and the morphological behaviour of ectomycorrhiza, when plant growth is limited by reduced resource availability. Photosynthates being the predominant carbon source for ectomycorrhizal fungi, a strong relationship exists between the capacity of plants to fix carbon and the amount of mycorrhizal mass that it produces and supports (Vogt, Publicover, and Vogt 1991).

It is essential to increase our understanding on how ecological factors, like light and nutrients, influence effectiveness of ectomycorrhizae, and appreciate their role in natural ecosystems. The host growth is negatively affected under reduced availability of soil-derived nutrients and light; the latter limits photosynthesis. Further, reduced photosynthesis may reduce the below-ground carbon availability, which, in turn, results in poor mycorrhizal status (Jean-Francois and Jean-Baptiste 1997; Quoreshi and Timmer 2000). The reduced mycorrhizal status of host roots could be due to reduced size of individual mycorrhiza or reduction in the number of mycorrhizal tips without any effect on the size of individual mycorrhiza.

Quercus leucotrichophora A. Camus (oak) and *Pinus roxburghii* Sarg. (pine) are two dominant forest tree species of Indian Central Himalaya and occur at a height between 1200 m and 2200 m. Both are evergreen with about one year of leaf lifespan (Singh and Singh 1987a, b). While the occurrence of late successional oak is associated with more mesic and nutrient-rich soils (Champion and Seth 1968), early successional pine is known for its ability to colonize bare sites, including extremely dry and sterile soils developed on sandstone and quartzite rocks (Singh and Singh 1992). The present investigation was undertaken to record changes in the mass and morphology of ectomycorrhizal roots of pine and oak under reduced light and soil nutrient conditions. The major objectives of the study were (i) to analyse the effect of reduced light and nutrient conditions on ectomycorrhizal roots of both species, and (ii) to characterize the morphological changes in ectomycorrhizal roots under reduced light and soil nutrient conditions.

Materials and methods

Cultural conditions, ectomycorrhiza, and seedling establishment

Seeds were collected from single mother trees growing in a natural forest stand, at Kailakhan near Nainital in the Indian Central Himalaya (1900–2000 m altitude).

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Soils of the various forests were used for seed germination of each species. Soil in the oak forest is residual brown earth derived from limestone, quartzite and shales, and sandy loam (sand 75%, silt 14%, and clay 11%) with 4.5% organic carbon and 0.55% total nitrogen. In the pine forest, it is derived from chlorite-sericite schists and micaceous quartzites, weakly podzolic, sandy loam (sand 66.5%, silt 18.3%, and clay 15.2%) with 2.3% organic carbon and 0.20% total nitrogen.

Seedlings, in excess (150 no.), of the two species were raised in separate plastic trays (60 × 90 × 30 cm) containing autoclaved (non-mycorrhizal) or unautoclaved (mycorrhizal) soil. The seedlings raised in trays containing unautoclaved soil were associated with the ectomycorrhizal fungi as in case of natural stands. Eight weeks after germination, one seedling of both the species from these trays was transferred to plastic bags containing approximately 8 kg of seived, natural soil or natural soil diluted with washed sand (described below) and autoclaved (twice at 15 lb for 1 h for two days). Previous work with these species had shown that neither the root growth nor ectomycorrhizal infection was restricted in bags of this volume. The bags were kept in a greenhouse and were reared at ambient air temperature averaging 18–25 °C with relative humidity ranging between 65% and 85% (within the range of temperature and relative humidity experienced by the species in nature). Bags were watered to field capacity with deionized water and precautions were taken to prevent possible nutrient loss by leaching.

Experiment 1: Light condition

The bags with seedlings were placed in the open (full or high light), or under one (intermediate light) or two (low light) layers of shade cloth such that the irradiance at leaf level was 250, 105 or 40 μmole m^{-2} s^{-1}, respectively.

Experiment 2: Soil nutrient concentration

Nutrient levels were achieved by diluting the soils collected from the natural stands by washed commercial white sand taken from a nearby river bank to high nutrient (no dilution), medium nutrient (1:1 v/v, soil:sand) or low nutrient (1:3 v/v, soil:sand). These mixtures had pH of 6.1±0.3 (in a 1:1 soil:water extract), which is within the range reported from the oak and pine forest soils (Singh and Singh 1992).

Experimental design, observations, and statistical analysis

Levels of light and nutrients were arranged in a complete randomized block design. Bags were kept at appropriate distances from one another to avoid self-shading and edge effect and moved frequently. Five seedlings of each species were harvested from each nutrient and light levels at 4-, 8-, and 12-month interval from the date of seed germination. At each harvest, seedlings were carefully washed from the soil and separated into root and shoot components. After cleaning roots, mycorrhizal fine roots were separated and were divided according to their branching pattern as monopodial branching (morphotype I), dichotomous branching (morphotype II), and densely branching (morphotype III). Number of tips in each morphotype and non-mycorrhizal tips were also counted. Stem, coarse root, non-mycorrhizal fine root, and mycorrhizal fine root mass of each morphotype were recorded separately, after drying till constant weight at 70 °C was reached. Data from final harvest was subjected to three-way analysis of variance, Tukey's significant honest t-test, and Pearson correlation analysis (SAS Institute Inc., Cary, NC). The whole data sets for final harvest were used in correlation analysis. Logarithmic transformations were used in fungal mass data to normalize distributions. Proportional mycorrhizal data was arc-sine transformed prior to conducting ANOVA.

Results

Effect of light levels

The reduction in light intensity generally reduced the growth of both oak and pine seedlings (Figure 1, A1–A4). Between the two species, the effect of light reduction was greater on pine than on oak seedlings, and in pine the root mass was more severely affected than the above-ground mass (Figure 1, A2). Three types of mycorrhizal morphotypes observed during the studies are described in Table 1. Pearson's correlation coefficient between mycorrhizal and growth parameters of seedlings of both species along the light gradient is given in Table 2.

The number of mycorrhizal tips as well as mycorrhizal mass was more in oak than in pine (both $p < 0.01$, Figure 2A), and in both the species they were suppressed with the deepening of shade (Figure 2A). The two species also differed in the branching of the mycorrhizal tips. In pine, root tips were evenly divided between monopodialy branched (morphotype I) and dichotomously branched (morphotype II) root tips ($p > 0.05$; Figures 2A and 3). In oak, an additional morphotype, densely branched (morphotype III), also occurred (Figure 3). The light intensity also affected the proportion of morphotypes in oak. At low and intermediate light levels, morphotype I and morphotype II were equally represented and were more than the morphotype III ($p < 0.05$). However, at the highest light intensity, morphotype III was most common, and was followed by morphotype II ($p < 0.01$, Figure 2A). Among the three morphotypes recorded in oak, morphotype III

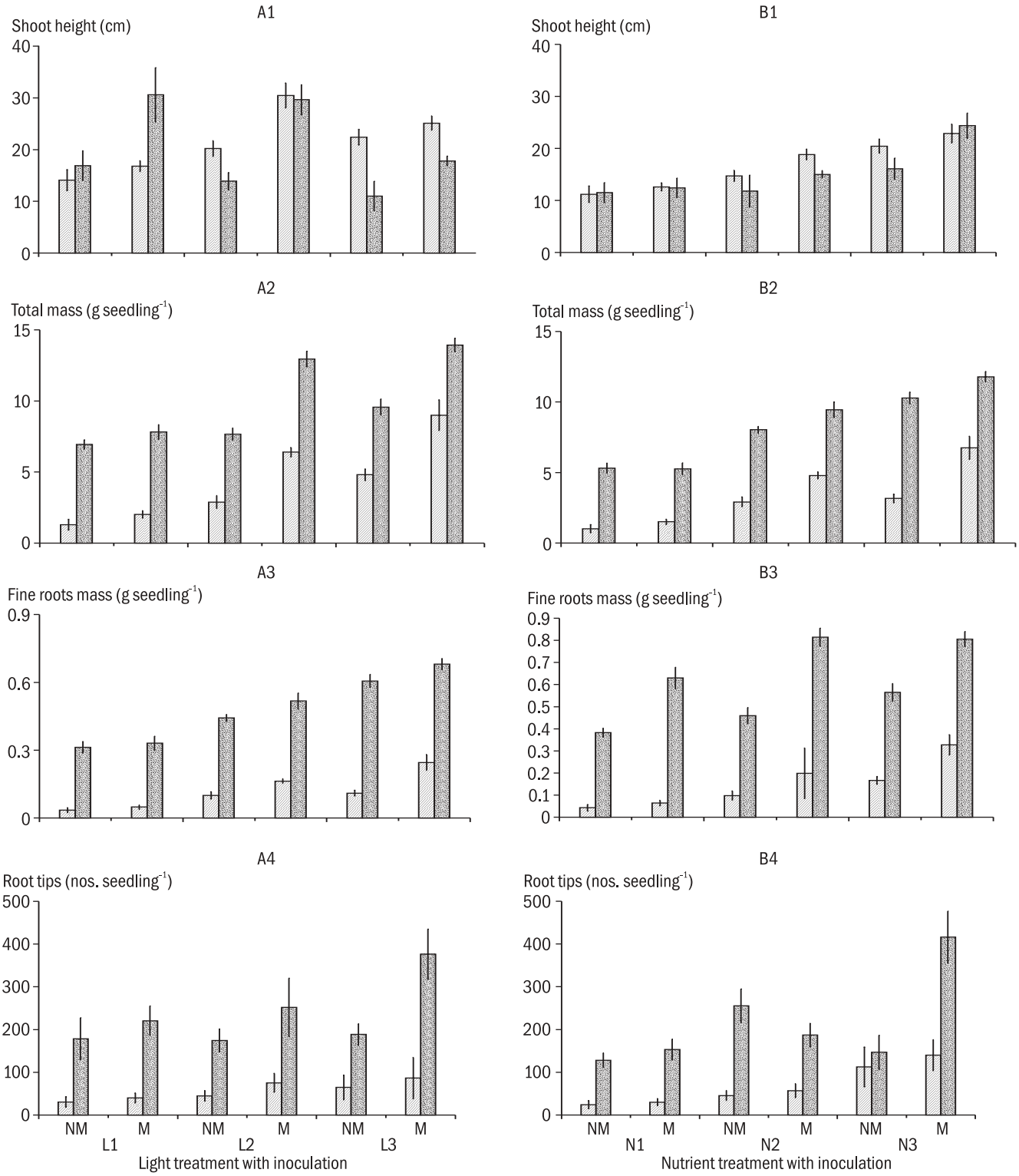

Figure 1 Above-ground height, seedling mass, fine root mass, and number of root tips of NM (non-mycorrhizal) and M (mycorrhizal) pine (▭) and oak (▭) seedlings along the light (A, L1 low, L2 medium, L3 high) and nutrient (B, N1 low, N2 medium, N3 high) gradients. Vertical bars represent ±1SE (n = 5).

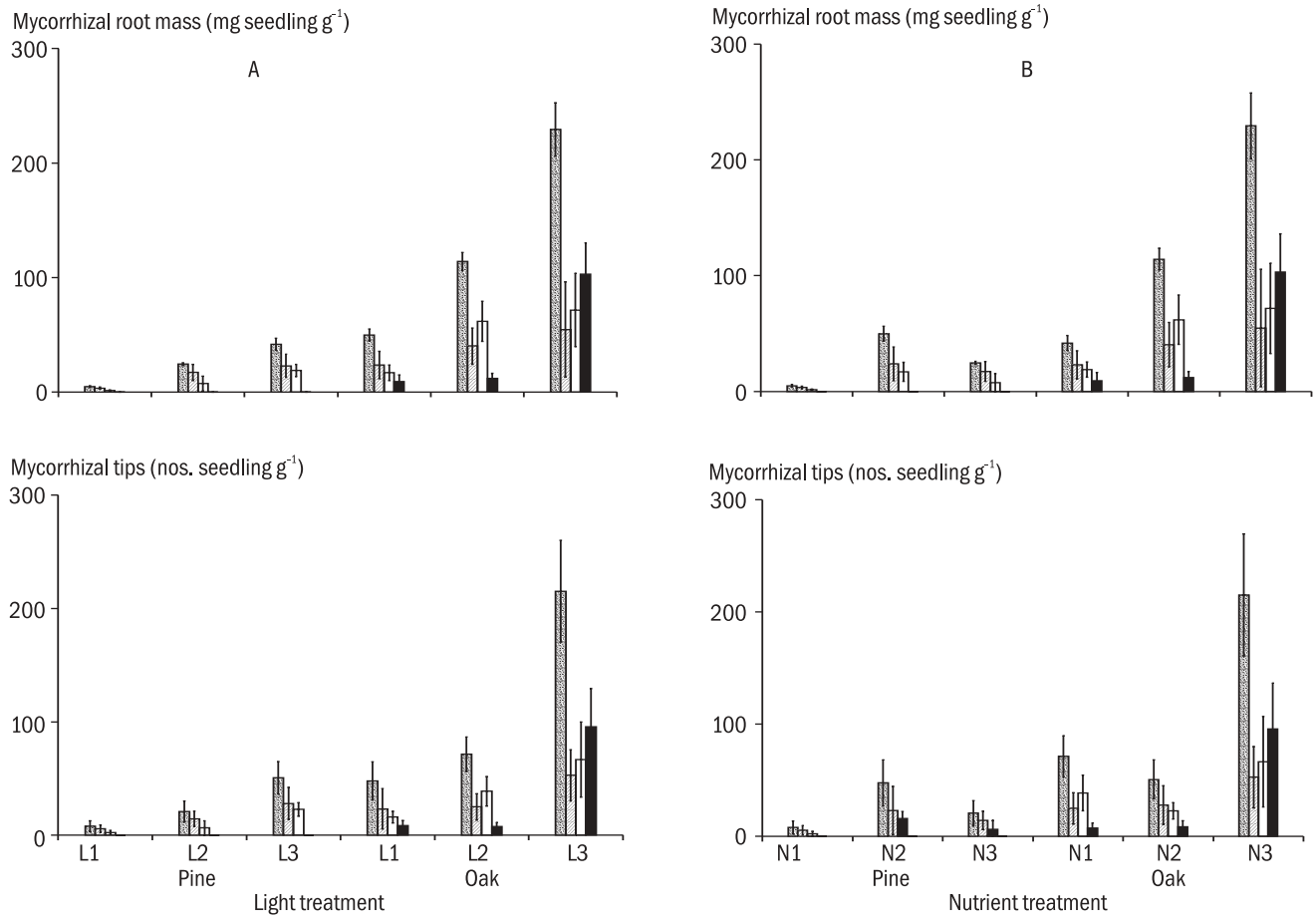

Figure 2 Mass and number of mycorrhizal tips; total ( ) morphotype I ( ), II ( ), and III ( ) of pine and oak seedlings along the light (A) and nutrient gradient (B). Vertical bars represent ±1SE (n = 5).

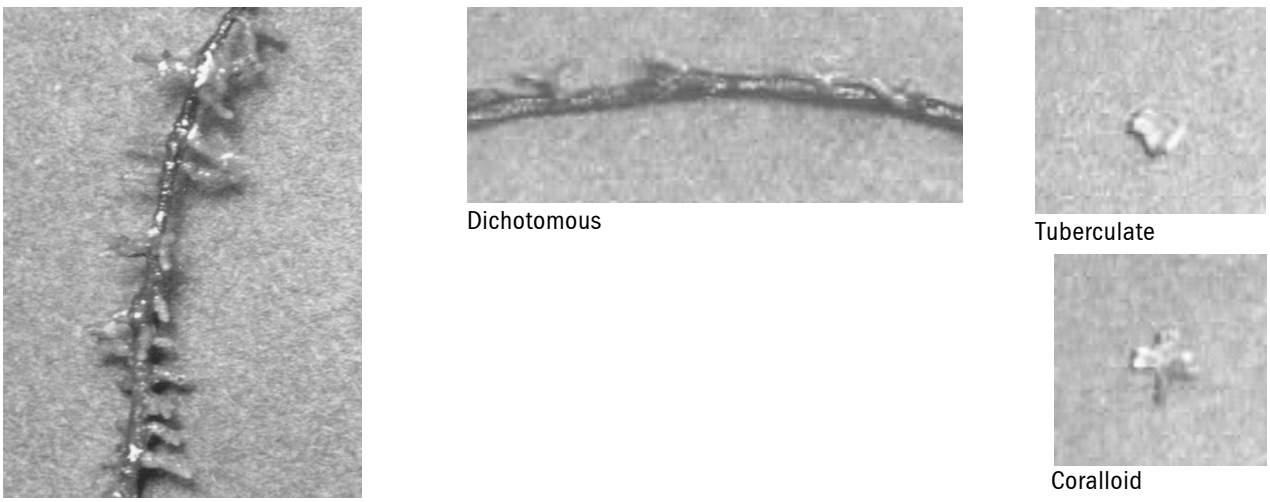

Figure 3 Ectomycorrhizal morphotypes as observed on seedlings of the study species (not to scale).

Table 1 Description of ectomycorrhizal morphotypes encountered in the experiments

Morphotype	*Description*
Type 1	Monopodialy branched, pinnate or pyramidal; infrequently branched; all types of tips shape with variable diameter and length; light brown or silvery in colour, rarely white
Type 2	Dichotomously branched; brown to dark brown; thicker than type 1; sometimes repeatedly and irregularly branched; tips straight, beaded or tortuous
Type 3	Densely branched, tuberculate or coralloid; light to dark brown; mantle surface shiny and smooth

Table 2 Pearson's correlation coefficient (r) between mycorrhizal and growth parameters of seedlings of both species along the light gradient

	Seedling height	*Seedling mass*	*Fine root mass*	*Number of root tips*
Mycorrhizal fine root mass	0.3661 (0.047)	0.8583 (0 .000)	0.9194 (0.000)	0.7993 (0.000)
Type I mass	0.3661 (0.047)	0.8115 (0.000)	0.9195 (0.000)	0.7944 (0.000)
Type II mass	0.1452 (0.444)	0.8930 (0.000)	0.9245 (0.000)	0.8565 (0.000)
Type III mass	0.1362 (0.473)	0.7275 (0.000)	0.7745 (0.000)	0.6411 (0.000)
Mycorrhizal root:plant root ratio (%)	0.4568 (0.011)	0.6326 (0.000)	0.6933 (0.000)	0.4481 (0.013)
Number of mycorrhizal tips	0.1929 (0.307)	0.8074 (0.000)	0.8618 (0.000)	0.7158 (0.000)
Type I number	0.0676 (0.723)	0.8583 (0.000)	0.8484 (0.000)	0.7678 (0.000)
Type II number	0.2073 (0.272)	0.8100 (0.000)	0.8801 (0.000)	0.7522 (0.000)
Type III number	0.2239 (0.234)	0.6646 (0.000)	0.7355 (0.000)	0.5619 (0.001)
Number of mycorrhizal tips per gram root dry weight	0.4525 (0.012)	0.5289 (0.003)	0.7144 (0.000)	0.5120 (0.004)

p values are given in parentheses

was most sensitive to reduction in light ($p < 0.01$, Figure 2A). Monopodial root type (morphotype I) did not show any significant reduction in number and mass from high to intermediate light level ($p > 0.05$); morphotype II appeared to be equally sensitive to reduction in light condition from high to intermediate and intermediate to low light level, and thus falls between morphotype I and morphotype III. Similar to mycorrhizal tips and mycorrhizal mass, mycorrhizal tips per gram of root dry weight and mycorrhizal mass as percentage of total root mass declined with reduced light conditions (Figures 4 and 5).

Effect of nutrient levels

The reduction in soil nutrients adversely affected all growth parameters in both the species, but the response was found to differ (Figure 1, B1–4). For each parameter, the effect of low nutrient was more severe on pine seedlings than on oak seedlings. The nutrient reduction from high to low levels (average of inoculated and non-inoculated seedlings) was about 45% and 40% in height (Figure 1, B1), 76% and 60% in seedling mass (Figure 1, B2), 74% and 50% in fine root mass (Figure 1, B3), 79% and 54% in the number of root tips (Figure 1, B4) in pine and oak seedlings, respectively ($p < 0.01$). Pearson's correlation coefficient between mycorrhizal and growth parameters of seedlings of both species along the nutrients gradient is given in Table 3.

In pine seedlings, the maximum number of mycorrhizal tips and their mass were recorded at intermediate and minimum at low nutrients level ($p < 0.01$, Figures 2B and 3). As in case of light treatments, in nutrient treatments also, only morphotypes I and II were recorded in pine seedlings, sharing equal numbers and mass values ($p > 0.05$). In oak seedlings, number of mycorrhizal tips and their mass continuously decreased with decreasing levels of soil nutrients. Three mycorrhizal morphotypes, observed on oak seedlings, followed a pattern similar to that seen in light treatments. The decrease in soil nutrients from high to intermediate levels suppressed both the number and mass (more so in case of number than the mass). Reduction from intermediate to low nutrient level suppressed only the mass (Figures 4 and 5), not the number of root tips.

Discussion

Several factors are known to affect the formation and development of ectomycorrhizal roots (Sharpe and Marx 1986), light and nutrients being two of them. At critical levels, light and nutrients are decisive factors,

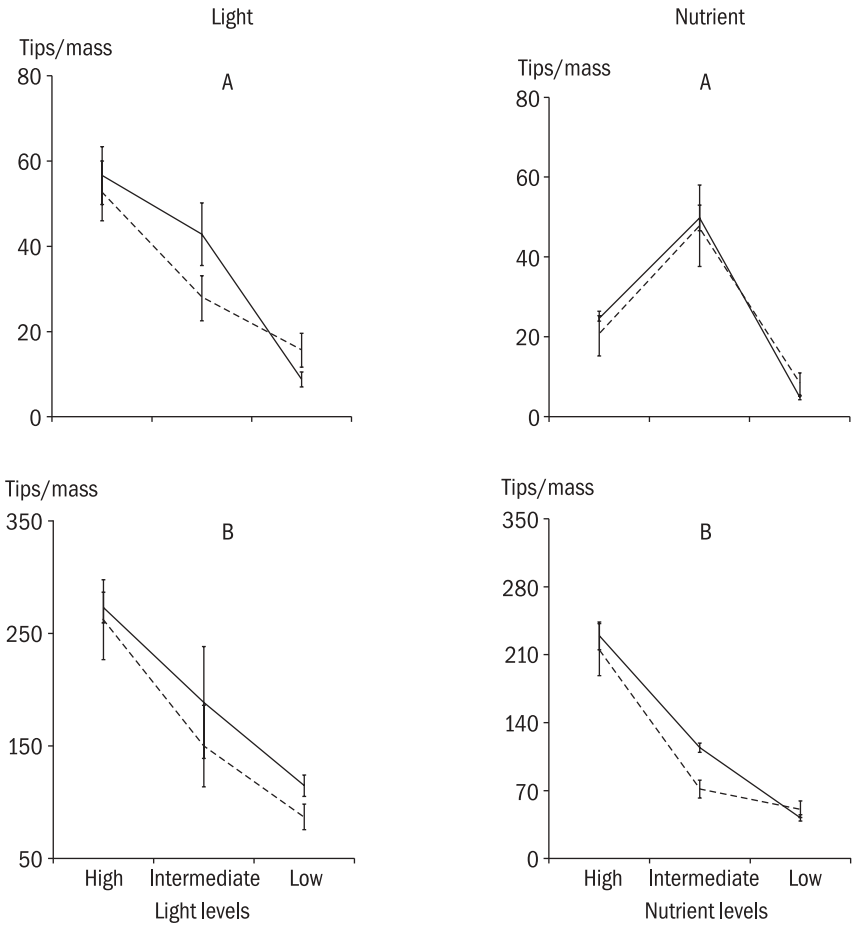

Figure 4 Effect of variables light and soil nutrient on number of mycorrhizal tips (broken lines) and mycorrhizal mass (unbroken lines) of pine (A) and oak (B) seedlings
ANOVA: All means for high, intermediate, and low levels of light and nutrient in both species were significantly different (p = 0.05, Tukey's significant honest t-test) except means for number of mycorrhizal tips at medium and low nutrient levels.

Table 3 Pearson's correlation coefficient (r) between mycorrhizal and growth parameters of seedlings of both species along the nutrient gradient

	Seedling height	*Seedling mass*	*Fine root mass*	*Number of root tips*
Mycorrhizal fine root mass	0.8087 (0.000)	0.7758 (0.000)	0.7399 (0.000)	0.8363 (0.000)
Type I mass	0.7408 (0.000)	0.7425 (0.000)	0.6767 (0.000)	0.7294 (0.000)
Type II mass	0.8404 (0.000)	0.6899 (0.000)	0.5927 (0.001)	0.6951 (0.000)
Type III mass	0.6736 (0.000)	0.7465 (0.000)	0.7628 (0.000)	0.8519 (0.000)
Mycorrhizal root: plant root ratio (%)	0.4578 (0.011)	0.8925 (0.000)	0.9509 (0.000)	0.9456 (0.000)
Number of mycorrhiza tips	0.6263 (0.000)	0.8528 (0.000)	0.8821 (0.000)	0.9300 (0.000)
Type I number	0.6531 (0.000)	0.8347 (0.000)	0.8246 (0.000)	0.8473 (0.000)
Type II number	0.5825 (0.001)	0.9011 (0.000)	0.9097 (0.000)	0.9377 (0.000)
Type III number	0.6510 (0.000)	0.7361 (0.000)	0.7573 (0.000)	0.8354 (0.000)
Number of mycorrhizal tips per gram root dry weight	0.5646 (0.001)	0.7138 (0.000)	0.7612 (0.000)	0.8407 (0.000)

p values are given in parentheses

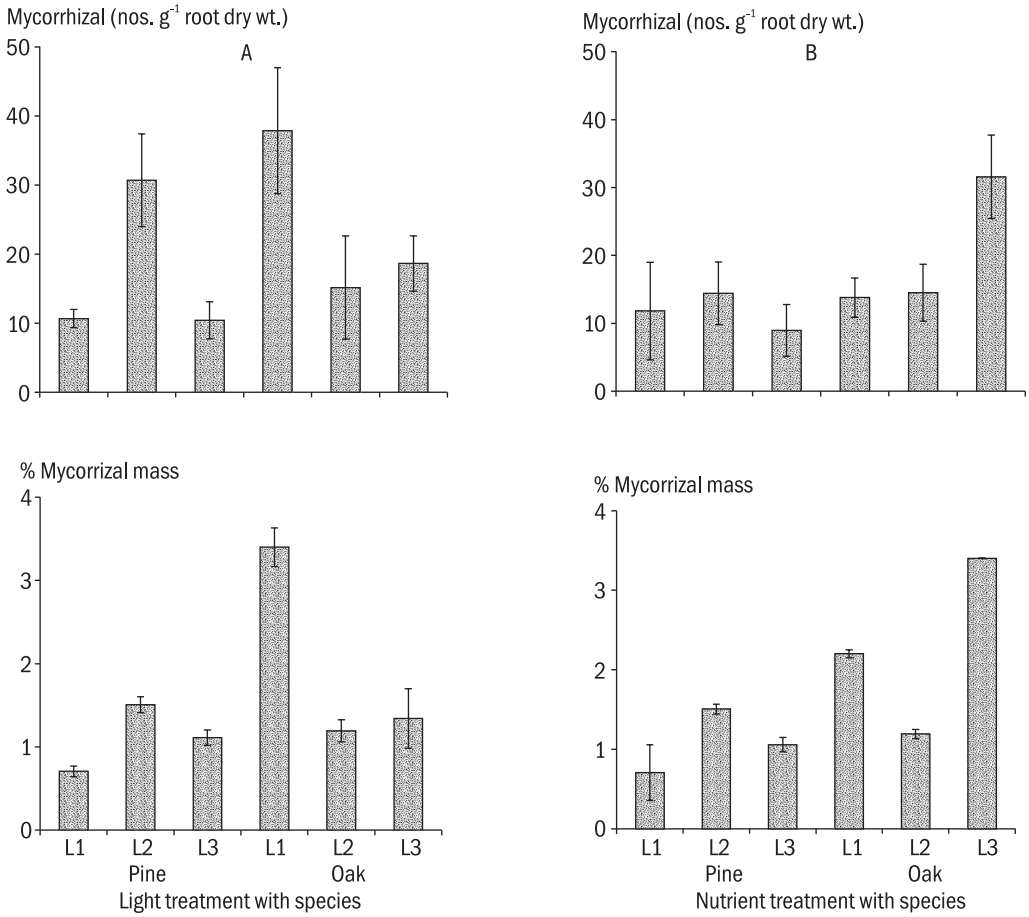

Figure 5 Number of mycorrhizal tips per gram root dry weight and mycorrhizal mass as per cent of total root mass of pine and oak seedlings along the light (A) and nutrient gradient (B). Vertical bars represent ±1SE (n = 5).

influencing the frequency of mycorrhizal development in forest soils (Marx, Hatch, and Mendicino 1977; Vare 1990). The present study on oak and pine ectomycorrhizae clearly shows that changes in nutrient or/and light conditions markedly influence the mycorrhizal status of these seedlings, morphological characters of root tips, and seedling growth. For example, intermediate nutrient and high light levels are best suited for pine ectomycorrhizal status.

Nutrient status is the prime factor in determining the intensity of mycorrhizal infection (Dubey, Pandey, and Tripathi 1998; Wallander 1995). A moderate deficiency of nitrogen, phosphorus, potassium, and other nutrients promotes mycorrhizal formation, but a severe deficiency might be detrimental (Bjorkman 1970; Gagnon, Langlois, and Fortin 1988; Wallander 1995). Pine in the present study, with its low tissue nutrient concentration, is well adapted to low fertility (Ralhan and Singh 1987; Singh and Bisht 1992; Singh and Singh 1987a, 1992). Because of the low tissue nutrient requirements, the dependence of pine on mycorrhizae is expected to decline with increase in soil nutrient supply. In pine seedlings, the maximum number of mycorrhizal tips and their mass were recorded at intermediate nutrient level, not at low or high nutrient level. It seems that at low nutrient level, pine was unable to accumulate enough photosynthate to support a large mycorrhizal biomass. On the other hand, oak comes on sites with a relatively rich nutrient supply (Singh and Bisht 1992; Singh and Singh 1987a, b) and has a higher tissue nutrient concentration (Ralhan and Singh 1987; Singh and Singh 1986; Singh and Singh 1992). Even the highest nutrient concentration in the present investigation seems to be inadequate for the optimal growth of oak, therefore it still had a large mycorrhizal support resulting in a continuous increase in mycorrhizal mass from low to high level. Evidently, the host responses to ectomycorrhizal associations along the gradient of soil nutrient differ from species to species, and may have implications on the overall nutrient cycling and retention of nutrients in the soil component of forest ecosystem.

In general, decrease in light intensity results in reduction in mycorrhizal formation (Harley and Waid 1955; Yasman 1995). In the present experiment, light intensity did not show a linear relationship with the amount of ectomycorrhizal infection both in pine and oak seedlings. There is a critical level of light intensity required for the growth of ectomycorrhizae, and a further reduction in light intensity adversely affects ectomycorrhizae. Increasing the rate of photosynthesis by increasing light intensity is directly related to available carbohydrates. Its absence or presence in very small quantities in root could be directly related to factors that limit mycorrhizal formation. Mycorrhizal fine root mass and number in both the study species decreased with decreasing light availability, showing that ectomycorrhizal mass was reduced due to reduction in its number and not due to reduction in the size of individual mycorrhiza.

Because seedlings of both species were raised in soil collected from their natural habitats (exposing them to mycorrhizal inoculum present in their natural stand), they are likely to be associated with several ectomycorrhizal fungi. Their presence may result in different branching patterns and deformations. It is likely that a given form of branching is associated with a specific group of ectomycorrhizal species. No attempts were made to identify these associations in the present study. Overall, morphotypes I and II shared equal mass and number, and were equally sensitive to adverse nutrient or light conditions. In oak, the morphotype III is ecologically most important as it shared the largest proportion of ectomycorrhizal mass and number. It is most sensitive to adverse light or nutrient condition. Morphotype I on oak was least affected by these adverse conditions but its contribution is least to ectomycorrhizal number and mass in case of oak.

Contrast in the species strategies

The relationship between plant and mycorrhizae is broadly symbiotic, but the cost and benefit ratio to the partners may vary particularly in relation to environmental conditions. The fine roots become increasingly deformed as ectomycorrhizal mass increases. In oak, as an example, in coralloid form, in which root deformation is most pronounced, ectomycorrhizal mass is the highest. These mycorrhizal roots are expected to have a longer lifespan compared to non-mycorrhizal once (Majde, Damm, and Nylund 2001), resulting in lower carbon cost of root production. They are also efficient soil nutrient absorber, particularly nutrients that do not readily move in soil (Read 1991). These benefits need to be weighed against the costs of maintaining ectomycorrhizal association (Smith and Read 1997). Nutrients in mycorrhizal plants come from soil in two ways: directly through root uptake and via mycorrhizal fungi to roots. The dependence of plants on soil nutrients is substantially reduced because of resorption from senescing leaves. Processes involved in all the three forms of nutrient acquirement, namely, nutrient uptake by the root's surface, nutrient uptake via mycorrhizae, and reuse of nutrients withdrawn from senescing parts, have costs. The relative costs of these determine what balance of nutrients from the three sources end up in leaves and other similar tissues.

The two important forest species occupying a similar altitudinal zone in the Indian Central Himalaya greatly differ in their nutrient use strategy (Singh and Singh 1992; Singh, Rawat, and Chaturvedi 1984). The overall tissue nutrient concentration in the oak is nearly 70% greater than in pine. The proportional absorption of N from the senescing leaves, as an example, is 58% in pine compared to only 25% in oak. A major consequence of this is a massive nutrient (128 kg N ha^{-1} $year^{-1}$ compared to only 69 kg N ha^{-1} $year^{-1}$ in pine) return to soil through litter fall in oak forest (Singh and Singh 1992). Our present data shows that oak continues to increase ectomycorrhizal support with the increasing soil fertility, which is in contrast to pine and several other species (Singh and Singh 1992). This strategy of maximizing ectomycorrhizal support with increasing soil fertility enables oaks to have a strong control over soil nutrients generated through the massive nutrient return via litter fall year after year. The access to such a nutrient pool, along with round-the-year active photosynthesis, results in generation of enough photosynthate on which ectomycorrhizae thrive. The pine, in contrast, is a species of low nutrient status, with an efficient nutrient use efficiency (Singh and Singh 1992). It saves the carbon cost of supporting a large ectomycorrhizal mass, particularly when the soil is fertile. Thus, both pine and oak forests conserve nutrients but deploy different mechanisms. While pine has a strong within-the-tree nutrient control, oak deploys a mechanism that enables it to have an efficient extra-plant ecosystem-level control. Interestingly, this response of oak to resource enrichment is also seen in case of sunlight. The species showed both the highest root deformation and ectomycorrhizal mass in the highest light condition.

The details of control mechanism in a given situation may be very complex. We do not even know whether it is ectomycorrhizae that exhibit a given condition characterized by carbohydrate status of host, its need to acquire nutrient, soil fertility, light condition, and other environmental factors, or the host has capacity to keep a check on ectomycorrhizal development. For example, whether the host can restrict ectomycorrhizal growth when it does not find it cost-effective.

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Inter-species variation in mycorrhizal efficiency of arbuscular mycorrhizal fungus species isolates with respect to three different hosts in three different soils

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AM (arbuscular mycorrhizal) fungal species vary in their mycorrhizal efficiency towards plant hosts (Smith and Read 1997). Selection of an efficient AM species-isolate for inoculum production is an important step towards adopting mycorrhiza inoculation technology in crop production. We studied the variation in plant growth and phosphorus nutrition promotion efficiency of three common AM fungus species-isolates with respect to three AM-responsive hosts – *Sesbania grandiflora* (sesban), *Citrus reticulata* (mandarin orange), and *Citrus sinensis* (sweet orange) – in three different soils.

Maize-root-based cultures of three AM species – *Glomus mosseae*, *Glomus fasciculatum*, and *Gigaspora margarita* – were developed with single spores of the species isolated from rhizosphere soils of grass hosts in fallow alluvial soil of the University Farm. The mother cultures were maintained in steam-sterilized sand–soil mixture in PVC (polyvinyl chloride) pots in isolation under transparent plastic sheet cover in a polynet house. Infected mother culture roots were used to inoculate formalin-sterilzed sand–soil mixture (pH 6.8, available phosphorus 7 PPM [parts per million]) for the production of maize-root-based inoculum in bulk by repeat crop growth cycles. Fifty grams of air-dried inoculum root pieces of each species, shredded and sieved through a 1-mm sieve, and appropriately diluted with uninfected root pieces, were added to 1 kg solarized experimental soil to raise infective inoculum density of the experimental soil to 75 per gram for each species. Heat-killed roots of maize in appropriate quantity were in the control set. Standard methods of soil and mycorrhizal analyses were followed for making the preparations and determining the physical, chemical, and mycorrhizal properties of soil (Dewis and Freitas 1984; Schenck 1984).

Sesban in costal zone saline soil, mandarin orange in hill zone brown forest soil, and sweet orange in red-laterite zone laterite soil of the state were grown

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Table 1 Properties of the experimental soils under study for the determination of host response to three different arbuscular mycorrhizal fungal species inocula

Soil	*Mechanical analysis (%)*				*EC_e (m. mhos/ cm)*	*Organic carbon (%)*	*Total nitrogen (%)*	*Available phosphorus (PPM)*	*Available potassium (PPM)*
	Sand	*Silt*	*Clay*	*pH*					
Coastal zone saline soil	34	35	31	7.4	3.00	0.75	0.17	8.3	216.5
Hill zone brown forest soil	67	15	18	5.2	0.01	1.29	0.12	12.5	80.2
Red-laterite zone laterite soil	54	20	26	5.5	0.03	0.18	0.03	5.4	110.6

PPM – parts per million; EC_e – electrical conductivity

Table 2 Plant growth and phosphorus uptake of three hosts inoculated with three different arbuscular mycorrhizal fungal species-isolates in three different soils.

Inoculation with	*Sesban in saline soil*			*Mandarin orange in brown forest soil*			*Sweet orange in laterite soil*		
	Plant height (cm)	*Plant weight* (g)	*Plant P-content* (mg/plant)	*Plant height* (cm)	*Plant weight* (g)	*Plant phosphorus content* (mg/plant)	*Plant height* (cm)	*Plant weight* (g)	*Plant phosphorus content* (mg/plant)
Glomus fasciculatum	29.4b	3.47c	1.87c	21.2c	2.06c	4.00c	17.9a	1.76a	3.56a
Glomus mosseae	34.2a	4.13a	2.44a	27.5a	2.60a	5.16a	14.7b	1.24b	2.41b
Gigaspora margarita	28.1b	3.62b	2.03b	25.6b	2.32b	4.30b	13.0c	1.02c	1.92c
Heat-killed inoculum (control)	25.1c	2.30d	1.06d	15.9d	1.75d	3.18d	10.0d	0.83d	1.55d
F (one-way ANOVA)	29.2*	121.8*	265.9*	93.1*	58.2*	221.8*	47.8*	140.7*	769.1*
CV (%)	5.2	4.7	4.3	5.3	4.9	3.0	7.7	6.2	3.0
SE_m	0.7	0.07	0.04	0.5	0.05	0.05	0.5	0.03	0.03
$LSD_{0.05}$	2.7	0.21	0.11	1.6	0.14	0.16	1.5	0.10	0.10

ANOVA – analysis of variance; CV – coefficient of variance; SE_m – standard error mean; LSD – least significant difference
Values followed by same letters in the columns were not significantly different at $P < 0.05$
*$P < 0.01$

against AM inoculation with the above-mentioned three species isolates during spring–summer season under partially controlled conditions in a polynet house. Basic characters of the experimental soils are given in Table 1. Plants raised from seeds in sterile sand–soil–FYM (farm yard manure) mixture, for one month in case of sesban, and two months in case of sweet and mandarin orange, were transplanted to 2 kg inoculated experimental soil in PVC pots in 5 × 5 replicates. Plants were maintained with irrigation alone as and when needed. Plant height, dry matter yield, and phosphorus content were measured from whole plant harvest at 120 days.

Results of the experiment (Table 2) showed significant variation in the promotion of growth and phosphorus acquisition by the host plants through the exotic AMF (AM fungal) species-isolates. Performance of *G. mosseae* as inoculant was significantly superior to the other two AMF species with two hosts, sesban in saline soil, and mandarin orange in brown forest soil. Performance of *G. fasciculatum* surpassed others in case of other host, sweet orange in laterite soil. Results of the experiment showed that mycorrhizal efficiency of an AMF species-isolate would vary in interaction with a host plant and also the soil. Results pointed out the importance of location and plant-specific selection of AMF species isolates for inoculum production to promote adoption of AM technology in plant culture.

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Arbuscular mycorrhizal fungi in early land plants

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Introduction

The AM (arbuscular mycorrhizal) fungi probably originated more than 450 million years ago (Redecker 2000) and arbuscule-like structures have been found in the fossil of *Algalophyton,* the earlier known vascular plant (Remy, Tayler, Hass, *et al*. 1994). The co-evolution of plants and AM fungi may have been the key factors in the evolution of the first rootless plants to colonize the land (Pirozynski and Mulloch 1975; Schubler 2002; Simon, Bousquet, Levesque, *et al.* 1993). According to Edwards, Duckett, Richardson (1995); Qui and Lee (2000); and Wellman, Osterloff, and Mohiuddin (2003) extant liverworts and hornworts had features of earlier plants that colonized land. Both groups often form symbiosis with fungi (Read, Duckett, Francis, *et al*. 2000). AM fungi have been colonizing many species of simple thalloid liverworts in metgerniales (*Pellia* and *Fossombrionia*) and calobryales (*Hoplomitrium*) as well as complex thalloid species in the marchantials (Boullard 1988; Carafa, Duckett, and Ligrone 2003; Read, Duckett, Francis, *et al*. 2000; Russell and Bulman 2005).

Materials and methods

Species of *Riccia, Notothyllus,* and *Astrella* were collected, along with rhizospheric soil, from the Botanical garden of Dr H S Gour University, Sagar, situated on Patharia Hills, and *Hypnum* species was found growing on the rocks and walls of the buildings of the Department of Botany and was collected from there. Sagar is situated near the tropic of cancer, occupying almost central position in the country. The climate of Sagar is seasonal with three well-marked seasons, that is, rainy, winter, and summer. At Sagar, average temperature remains between 13 °C and 42 °C, average rainfall is 60–150 cm, and average relative humidity 60%. Sagar is situated at an average height of 2030 feet from the MSL (mean sea level).

Samples were collected during the rainy season, from July to September, in 2003 and 2004. The identification of the samples was done with the help of Prof. N K Soni. Extraction of VAM (vesicular arbuscular mycorrhizal) fungal spores was done as per the method of Gerdemann and Nicolson (1963). Spore population was calculated from 100 g of soil. Rhizoidal parts of the thallus were stained using the method of Phillips and Hayman (1970) with slight modification: instead of 10% KOH (potassium hydroxide), 5% NaOH (sodium hydroxide) solution was used. Per cent colonization was calculated as per the method of Giovannetti and Mosse (1980). Soil analysis was done as per the method of Mishra (1968). Identification of AM spores was done using the key of Schenck and Perez (1987).

Results

The data obtained from the experiment is depicted in Table 1. It is clearly evident from the observation that all the six genera of bryophytes showed VAM association. However, all the three species of *Ricci*a showed greater colonization in comparison to other three bryophytes—*Notothyllus, Astrella,* and *Hypnum*. Comparatively, *Hypnum* had lesser colonization. Maximum number of VAM spores were found in *Riccia discolor* and minimum was recorded in *Hypnum*. Intra-radical mycelium was found in all the six genera but arbuscules were found in *R. discolor* and *Riccia* sp, and vesicles were observed in *Riccia fertile, Riccia* sp., *Astrella* sp., and *Notothyllus* sp. Eight VAM fungal species were recorded with *R. discolor,* followed by five VAM fungi in *R. fertile* and *Astrella* sp. Four species of VAM fungi were recorded in *Riccia* sp. *Notothyllus* sp., and *Hypnum* sp. Though, the numbers of spp. were same in *Riccia* sp., *Notothyllus* sp., and *Hypnum* sp., spp. of VAM fungi varied from plant to plant.

Among the VAM species, *Glomus* sp. was found to be dominating and eight species, that is *Glomus aggregatum (*LAGR), *G. mossae* (LMSS), *G. fasciculatum* (LFSC), *Glomus maculosum* (LMCL), *Glomus margricaule* (LMGL), *Glomus pustulotum* (LPST), *Glomus occultum* (LOCT), and *Glomus hoi* (LHOI), were recorded. Three spp. of *Acualospora*, that is, *Acaulospora scrobiculata* (ASCB), *Acaulospora nicolsonii* (ASCN), and *Acaulospora spinosa* (ASPN), were recorded. Single species of *Gigaspora, Gigaspora candida* (GCDD), was recorded. Among the *Glomus* species, *G. hoi* was found associated with all the six genera, *G. mosseae* was not found with *Notothyllus* sp., *G. aggregatum* was found to be associated with *R. discolor*, *Astrella* sp., and *Hypnum* sp., *G. margricaule* was found to be associated with *R. fertile*, *Riccia* sp., and *Notothyllus* sp. *G. fasiculatum* was found to be associated with *R. discolor* and *Notothyllus* sp.*,* while *G. occultum* and *G. maculosum* were seen only with *R. discolor* and *G. pustulotum* was found only with *R. fertile*. *A. scrobiculata* and *A. nicolsonii* were found to be associated with *R. discolor* and *Astrella* sp. *A. spinosa*

Table 1 Arbuscular mycorrhizal fungi colonization and distribution in bryophytes isolated from Sagar (Madhya Pradesh)

Name of bryophytes	*Per cent colonization*	*Spore population /100 g soil*	*Mycorrhizal status* M	A	V	*Occurrence of arbuscular mycorrhizal species*[a]
Riccia discolor	63	1244	+	+	–	ASCB, ASCN, LHOI, LAGR, LMSS, LFSS, LOCT, LMCL
Riccia fertile	60	1127	+	–	+	LMSS, LHOI, LMGL, LPSC, ASPN
Riccia sp.	54	1054	+	+	+	LHOI, LMSS, LMGL, ASPN
Astrella sp.	48	947	+	–	+	LHOI, LMSS, LAGR, ASCB, ASCN
Notothyllus sp.	50	1050	+	–	+	LHOI, LFSC, LMGL, ASPN
Hypnum sp.	39	813	+	–	–	LHOI, LMSS, LAGR, GCDD

M – mycelium; A – arbuscules; V – vesicles
[a]Species codes are as per Perez and Schenck (1990)

was found to be associated with *R. fertile*, *Riccia* sp., and *Notothyllus* sp. No *Acaulospora* sp. was found to be associated with *Hypnum* sp. However, single species *of Gigaspora* was recorded with *Hypnum* sp.

Discussion

The liverworts have generally been assigned an important place in the evolution of plants. Morphologically and biologically, they show a wide range of variation in structure of gametophyte and sporophyte (Bhargava and Thampi 1960). The results obtained from the present study suggest that although bryophytic flora is poorly distributed on Patheria Hills, Sagar, it shows association with VAM fungi. Earlier, Bhargava, and Thampi (1960) reported poor occurrence of bryophytes and at that time concluded that the climatic conditions were unsuitable for luxuriant and rich growth of bryophytes in Sagar. However, occurrences of all the bryophytes mentioned in this study were also described by Pillai (1960); and Bhargava and Thampi (1960).

Almost 150 years ago, Schach (1854) reported occurrence of symbiosis between hepatics and fungi. Later, Janse (1897) provided illustrations of swollen rhizoids in apices occupied by fungi in Zoopsis, a leafy jungermannialean hepatic. Bernard (1909) used the widespread occurrence of fungus–hepatic association on the basis of his theory that vascular cryptogams descended from macrophytic bryophytes. Ridler (1922) and Gavandan (1930) confirmed that associations of fungus and bryophytes were normal features of hepatic biology.

In recent years, the combination of histochemistry, LM (light microscopy), and EM (electron microscopy) has provided further refinement to our understanding. These have confirmed that zygomycetous infection of the AM fungi occur in *Phaseoceros* (Ligrone 1988), *Astrella* (Ligrone and Duckett 1994), and *Pellia* (Peacock and Duckett 1985). Occurrence of AM fungi in bryophytes has also been reported by Carafa, Duckett, and Ligrone (2003); Russell and Bulman (2005); Selosse (2005); and Smith and Read (1997).

In this study, we have found that among glomealian fungi, *Glomus* sp. dominated. Few species of *Acaulospora* were also observed. Other AM fungi were not found to be associated with bryophytic flora of Sagar. Among *Glomus* sp., *G. hoi* and *G. mosseae* were dominating species. This might be due to the local environments that provide conducive conditions for the occurrence of *Glomus* sp. in abundance. Douds and Millner (1999) suggested that a fungus may be a significant member of the vegetation community, but, because of date of sampling, local environment or host plant regulation of carbon expenditure may produce spores and may well be able to persist to the following year as infective hyphae in root or soil.

Dominance of *Glomus* in the soils of Sagar has earlier been reported by the authors (Vyas and Soni 2004; Vyas, Mishra, Singh, *et al.* 2006).

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Response of five medicinal plants to vesicular arbuscular mycorrhizal inoculations in unsterile soil

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Introduction

Plants generally owe their virtues as medical agents to the secondary metabolites that they synthesize, and have contributed more than 7000 different compounds that are in use today as heart drugs, laxatives, anti-cancer agents, hormones, contraceptives, diuretics, antibiotics, decongestants, analgesics, anaesthetics, ulcer treatments, and antiparasitic compounds. Extensive screening of plants is being carried out to identify chemical compounds that may be useful in treating human diseases. Efforts are being made to discover still more potent plant drugs that can come to our rescue.

Improvement in drug quality implies an improvement of in situ growth of medicinal plants. Growing them in situ is a better option rather than growing them in vitro, as the compounds or active ingredients in the drug should be in the right proportion and should also be of right configuration. Besides, in situ conservation would be the ideal approach because it not only conserves a given species in its natural habitat but also carries all its associated elements (for example, mycorrhiza, pollinators, and predators), ensuring intrinsic value because of the belief that drugs prepared from plants collected in the wild are somehow more efficacious compared to those prepared from cultivated plants (Fuller 1991). The improvement phase of medicinal plants starts from the time the domestication of plants is undertaken. It is certainly necessary to follow certain models like collection and evaluation, clonal selection, hybridization, mutation, and other methods. One such model perhaps may be the use of VAM (vesicular arbuscular mycorrhizal) fungi for growth improvement.

Kumar and Mahadevan (1984) have reported the absence of VAM association in medicinal plants and attributed this to the presence of various secondary metabolites in the host plants. However, there are many reports of medicinal plants with secondary substances harbouring VAM associations in their root systems (Abbott and Robson 1982; Basu and Srivastava 1998; Rao, Suresh, Suresh, *et al.* 1989; Lakshman and Raghavendra 1992; Manjunath and Reddy 2003; Prasad and Reddy 1998; Selvaraja and Subramanian 1992; Sullia and Sampath 1992; Taber and Trappe 1982).

Kumar and Murugesh (2002) have tested the efficacy of three VAM fungal species *(Glomus mosseae, Glomus fasciculatum*, and *Glomus monosporum)* on 10 medicinal plants and showed clearly that mycorrhizal inoculation is beneficial in improving their growth. Basu and Srivastava (1998) have tested 13 species of VAM fungi on five medicinal plants and observed that VAM fungal association had not only enhanced the growth of medicinal plants but also improved the productivity of medicinal compounds. Therefore, there is a need for research in improving the quality and quantity of drugs produced from native medicinal plants in relatively shorter period and at lower expense by using selective VAM fungi.

Material and methods

In the present study, five medicinal plants (*Mucuna pruriens, Clitoria ternatea, Hyptis suaveolens, Ocimum sanctum*, and *Ocimum gratissimum*) were selected for

assessing their response to a VAM fungus, *Glomus aggregatum*. This fungus is the most prevalent, and compared to other species has proved to be highly effective in its performance with respect to local crops besides its ability to colonize roots and survive in the soil (Reddy and Bias 1990). Pot culture experiments were conducted using natural unsterile soil. Five sets of six pots each were taken and each pot was filled with 5 kg soil. Three pots in each set were treated as inoculated by mixing 500 g/pot of pure culture of *G. aggregatum* inoculum having 50 ± 5 spores/1 g of soil, and the other three were treated as control as there was no addition of inoculum. Seeds of medicinal plants were sown at the rate of 15–20 seeds per pot in both treated and control pots. On germination, the plants were thinned keeping five to six plants in each pot. The plants were maintained in greenhouse beds by regular watering once in two days. The plants at the flowering stage (120 days) were harvested along with their root systems, taking care not to damage the roots system, and the roots were cleared by dipping them in water several times till the adhering soil particles were removed.

Few representative root samples from both treated and untreated plants were cut into 1-cm bits and fixed in standard FAA (formalin acetoalcohol) and processed further for the assessment of VAM colonization (percentage mycorrhizal association) by staining (Phillips and Hayman 1970). Then the plants were oven-dried for 72 h at 70 °C. Root and shoot systems were then weighed.

MD (mycorrhizal dependency) of all the five medicinal plants was determined using the formula given by Gerdemann (1975).

$$\text{MD} = \frac{\text{Dry weight of inoculated plants}}{\text{Dry weight of uninoculated plants}} \times 100$$

Mycorrhizal efficiency of *G. aggregatum* in enhancing the growth was also calculated by taking the average dry weights of the plants grown in unsterile control soil and VAM-fungus-inoculum-treated soil, using the modified formula originally given by Singh and Tilak (1990).[1]

Results

It is clearly evident from the data that the root systems of all the five plant species grown in both control and VAM-fungus-infested unsterile soils were invariably found to harbour VAM association (Table 1). However, in plant roots grown in unsterile soil without the addition of VAM inoculum, the per cent VAM association (22% to 46%) and the intensity of formation of intrametrical VAM fungal structures, vesicles (10% to 30%) and arbuscules (12% to 24%), were found to be very less and insignificant (Table 1) as compared to plant roots grown in soils infested with *G. aggregatum* inoculum. In the inoculated plants, the per cent VAM association (96% to 100%) and the intensity of formation of vesicles (74% to 90%) and arbuscules (62% to 70%) were very high and significant (Table 1).

Response of Mucuna pruriens

It is evident from the present data (Table 1) that the VAM fungus (*G. aggregatum*) inoculation had a significant effect on the growth of *M. pruriens* when grown in unsterile soil. The per cent intensity of endophytic colonization by *G. aggregatum* in its roots increased to 100% as compared to 28% in plants grown in unsterile control soils. Similarly, the intensity of formation of vesicles and arbuscules also increased, respectively, from 22% to 90% and 16% to 70% in inoculated plants. The increase in dry weight of both shoot and root systems due to inoculation of VAM fungus was also found to be considerably high. The dry weight of shoot was found to have increased by 19.22% and that of the root by 16.16%. The efficiency (MEI) of *G. aggregatum* in enhancing the plant's growth in unsterile local soil was found to be 15.24%.

Response of Clitoria ternatea

The plants of *C. ternatea* also readily responded to the endophytic colonization by VAM fungi (Table 1); however, *G. aggregatum* inoculation had increased the per cent colonization significantly. The per cent VAM colonization in control plants (grown in unsterile soil) was 46% while in inoculated plants it was 100% (Table 1), and similarly, the intensity of formation of VAM fungal structures, both vesicles and arbuscules, in the root cortex region increased by 30% to 84% and by 20% to 72%, respectively (Table 1). Total dry matter production was also found to have increased significantly (t-test significant at 5% level) due to *G. aggregatum* inoculation. The dry weight of shoot was found to have increased by 43.60% and the dry weight of root increased by 81.13%. The efficiency (MEI) of inoculation in the plant's growth enhancement was 35.75%.

Response of Hyptis suaveolens

It is apparent from Table 1 that both the intensity of endophyte colonization under the influence of

[1] MEI (mycorrhizal efficiency index) = $100 \times 1 \frac{\text{Dry weight of plant grown in uninocu1ated sc}}{\text{Dry weight of plant grown in VAM fungus inocu1a}}$

Table 1 Intensity of VAM formation and its effect on the growth of five selected medicinal plants grown in unsterile soil infested with *Glomus aggregatum* (at the age of 120 days)

	VAM status						*Growth response*					
	Control plants			*VAM plants*			*Control plants*		*VAM plants*			
	%VAM association (g)*	*Intensity (%) of formation*		*%VAM association*	*Intensity (%) of formation*		*Dry weight* (g)*		*Dry weight* (g)*			
Name of the plant		*V*	*A*		*V*	*A*	*Shoot*	*Root*	*Shoot*	Root	MD	MEI
Mucuna pruriens	28	22	16	100	90	70	17.17	11.64	20.47	13.52	117.98	15.24
Clitoria ternatea	46	30	20	100	84	72	8.99	4.24	12.91**	7.68**	156.63	35.75
Hyptis suaveolens	36	26	24	96	74	62	9.00	4.54	17.00**	7.18	*178.58*	44.00
Ocimum sanctum	22	10	12	100	80	68	3.44	1.88	4.00	2.36	*119.55*	16.36
Ocimum gratissimum	40	30	21	96	74	70	21.33	13.33	41.33**	21.33**	*180.78*	44.68

* Average value/plant

** 't' test significant at 5% level.

VAM – vesicular arbuscular mycorrhizal; V – vesicles; A – arbuscules; MD – mycorrhizal dependency; MEI – mycorrhizal efficiency index

G. aggregatum inoculum in unsterile soil and the intensity of formation of endophyte structures were found to increase considerably in the roots of *H. suaveolens* as compared to the roots of uninoculated control plant (Table 1). The intensity of VAM colonization increased from 36% to 96%, intensity of formation of vesicles increased from 26% to 74%, and that of arbuscules increased from 24% to 62% due to *G. aggregatum* inoculation.

VAM inoculation has also increased the total dry matter production, particularly the dry weight of shoot (17.00 g/plant), to a significant proportion (t-test significant at 5% level) (88.88%) compared to control plants (9 g/plant). The increase in the dry weight (7.18 g) of root was 58.15% as compared to that weight of root of control plant (4.54 g/plant). The efficacy (MEI) of *G. aggregatum* in enhancing the growth of *H. sumeolens* was found to be 44.00%.

Response of Ocimum sanctum

The data (Table 1) indicates that the mycorrhization of plant roots of *O. sanctum* due to VAM fungus (*G. aggregatum*) inoculation in unsterile soil has increased to 100% as compared to 22% in control plants grown in uninoculated unsterile soils. Also, the intensity of formation of vesicles and arbuscules increased from 10% and 12% to 80% and 68%, respectively, indicating that the plants of *O. sanctum* had readily responded to VAM fungal colonization. Similarly, as in other plants, VAM inoculation resulted in increased dry matter production over control plants. The dry weight of shoot was found to increase by 16.28% and that of the root by 48.00%. The mycorrhizal efficacy (MEI) of *G. aggregatum* in unsterile local soil for this plant was found to be 16.36%.

Response of Ocimum gratissimum

The data presented in Table 1 clearly indicates that, as in other plants, not only the intensity of mycorrhization (per cent colonization) and the formation of endophytic VAM fungal structures (both vesicles and arbuscules) but also dry matter production (both shoot and root weight) significantly (significant at 5% level) increased in *O. gratissimum* due to the addition of *G. aggregatum* inoculum in unsterile soil as compared to control plants. The dry weight of the shoot was found to increase by 93.77% while that of the root increased by 60.00%. The effectiveness (MEI) of *G. aggregatum* in enhancing the plant growth was found to be 44.68%.

Discussion

It is clearly evident from the present investigation on the effect of VAM inoculation on mycorrhization and growth response (Table 1) in five select medicinal plants (*M. pruriens, C. ternatea, H. suaveolens, O. sanctum,* and *O. gratissimum*) that these plants have readily responded to VAM colonization when grown in unsterile soil infested with *G. aggregatum* inoculum. These results confirm the earlier reports by Rao, Suresh, Suresh, *et al.* (1989), who have shown that medicinal plants known to contain several secondary metabolites do respond to VAM colonization and are not averse to VAM colonization when inoculated artificially. A pronounced growth response following inoculation with *G. aggregatum* was also observed in all the five medicinal plants (Table 1) when grown in unsterile soil. All the plants inoculated with VAM fungus inoculum have shown improved growth and development as compared to control plants. Though, the control plants also have had VAM association varying from 22% to 46% (Table 1)

in their root systems when grown in unsterile soil because of the presence of low level of natural inoculum, a significant increase not only in per cent VAM associations varying from 96% to 100% but also in biomass production (dry weight of root and shoot) was observed due to inoculation with *G. aggregatum* in all the five plant species (Table 1). Increased growth due to increased mycorrhization upon inoculation with *G. aggregatum*, as seen in the present situation (Table 1), has largely been attributed to improved acquisition of nutrients, especially that of less mobile phosphorus by host (Bagyaraj and Manjunath 1980). Increased uptake of phosphorus by mycorrhizal plants is well documented (Gilmore 1971), and earlier studies have indicated that phosphorus deficiency is one of the most important factor limiting plant growth in calcareous soils (Grime and Curtis 1976). Grime (1965) reported that phosphorus deficiency increases dramatically in calcareous soils above soil pH 7.0. The Gulbarga soils being calcareous with high pH (Naik, Seetharam, Ravindranath, *et al.* 1994) might not support good growth unless plant develops symbiotic union with VAM fungi for phosphorus forage. Examples of plants infected with VAM fungi that takes more phosphorus from phosphorus-eficient soil, are well documented (Tinker 1975).

Host plants growing in soils with relatively low nutrient level, as in Gulbarga soils (Reddy and Goud 1989), are predisposed to effective endophytic fungal invasion (Russel 1975). In the present study, similar situation was observed in all the five plants as there were 96% to 100% VAM associations in them (Table 1).

The overall root and shoot biomass (dry weight) of the inoculated plants was found to be significantly higher than that of corresponding controls in all the five medicinal plants except *O. sanctum* (Table 1). Basu and Srivastava (1998) have earlier reported such enhanced growth in medicinal plants due to VAM fungal association. Similar improved growth response was also observed in 10 medicinal plants when inoculated with three VAM fungal species *(G. mosseae, G. fasciculatum*, and *G. monosporum)* for their efficacy by Kumar and Murugesh (2002). They suggested that mycorrhizal inoculation is more advantageous in obtaining healthy vigorous transplantable seedlings and results in higher biomass of medicinal plants that were found to grow better in the field.

MD is used as an index to compare receptivity of different plant species to VAM fungus at a given set of conditions (Gerdemann 1975). The MD values presently calculated are 117.98 for *M. pruriens,* 156.63 for *C. ternatea,* 178.58 for *H. suaveolens,* 119.55 for *O. sanctum*, and 180.78 for *O. gratissimum* (Table 1). These figures suggest that *G. aggregatum* has greater influence on the growth of *C. ternatea, H. suaveolens*, and *O. gratissimum* than on that of *M. pruriens* and *O. sanctum.* The MD of a host plant can be altered by factors such as soil type, soil phosphorus content, and mycorrhizal species (Azcon and Ocampo 1981; Menge, Johnson, and Platt 1978).

Several reasons were proposed for differences in MD in different plant species or species of the same genus. Baylis (1967) reported that root hair length and root thickness can determine the MD level. The variation in the extent of increase in growth of plants inoculated with VAM fungus over that of control plants could be due to observed variation in the growth-promoting ability of *G. aggregatum* MEI (Table 1).

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New approaches

Rapid and reliable DNA extraction techniques

Two improved DNA extraction techniques involving trypan-blue-stained root fragments were developed by Ishii S and Loynachan T E (*Mycorrhiza* 14: 271–275, 2004) and compared for rapid and reliable analysis. In method A, 1 cm trypan-blue-stained mycorrhizal root fragments were individually isolated, crushed by bead beating, and purified with Chelix-100 (Bio-Rad). In method B, DNA extraction was carried out using an ultra-clean microbial DNA isolation kit (Mo Bio Laboratories). DNA was extracted from the mycorrhizal roots of plant species, quantified by UV (ultraviolet) absorbance and PCR (polymerase chain reaction)-amplified with primers specific to arbuscular mycorrhizal fungi. Although PCR inhibitors might still exist when using method A, appropriate dilution and employment of nested PCR overcame this problem. Method B removed PCR inhibitors but sometimes, depending on the mycorrhizal colonization within the root fragments, it also required nested PCR. In conclusion, both methods enabled to handle many samples in a short time. Method B provided greater reliability and method A provided better cost performance. Both techniques can be used for PCR-based application to identify species and estimate species composition after measuring mycorrhizal colonization rate with trypan blue staining.

Centre for Mycorrhizal Culture Collection

Characterization of CMCC germplasm, TERI

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AM (arbuscular mycorrhizal) fungi are ubiquitous in soils around the globe and have been associated with improved plant growth for over 100 years. They are generally known to increase the absorption and translocation of mineral nutrients from soil to the host plant, improve the tolerance of host plants towards biotic and abiotic stresses, and build up macro-porous structure of soil, which allows penetration of water and air and prevents erosion.

Wide diversity exists among the AM fungi. There are over 150 species within the order Glomales, the diversity of which shows up in morphology, physiology, and functions. To conserve and exploit this diversity, the CMCC (Centre for Mycorrhizal Culture Collection) was established in 1993 at TERI (The Energy and Resources Institute), with the aid of the Department of Biotechnology, Government of India. This mycorrhizal culture depository houses and maintains cultures from 12 different agro-ecological zones out of 20 identified for the country, and has an impressive collection of over 450 isolates of mycorrhizal fungi. Apart from these cultures, the centre also has a culture collection of AM fungal isolates from the agricultural fields of wheat. These cultures were collected from 11 agro-climatic regions of India, resulting in additional 132 trap isolates and 300 polysporal/monosporal cultures.

This germplasm collection forms an invaluable reservoir of genetic diversity of agriculturally and industrially important AM fungi, many of which are being lost worldwide to habitat destruction and intensive agriculture. Maintaining distinct strains of these beneficial micro-organisms is important because these strains may contain genes that improve plant productivity and aid in the continuing fight against pests and diseases; even those strains, overall characteristics of which may not be attractive economically, are used. Since the beginning of agriculture era, huge variability has occurred in all micro-organisms by the processes of mutation, hybridization, artificial selection, and adaptation to a wide range of conditions. The problem lies in finding, preserving, and using the genetic variability of these fungi.

The cultures in this collection have already been morphologically characterized. However, morphological characterization, where there are morphologically similar species, is prone to ambiguity. Therefore, molecular and biochemical characters are now employed as an important supplement to morphological characters used to characterize the order Glomales. Describing the diversity within a natural community or habitat is important for exploiting it.

Currently, classification and identification of glomalean fungi are based mostly on morphological characters of spores. Comparative taxonomic analyses have been hindered by inadequately defined characters and ambiguous distinctions between morphologically similar species. Thus, molecular and biochemical characters are important supplements to morphological characters used to define and relate taxa in the order Glomales. The abundance of lipids in spores and vesicles of colonized roots is a potentially useful biochemical characteristic for taxonomic purposes and quantification of glomalean fungi in host root tissue. Advances in gas–liquid chromatography of lipids have generated widening interest in the use of FAME (fatty acid methyl ester) profiles for the purpose of identification and elucidation of relationships among organisms. FAME is based on the extraction of 'signature' lipid biomarkers from the cell membranes and walls of micro-organisms. These phospholipids are an essential component of the membranes of all cells. Once extracted from cells with organic solvents, the lipids are concentrated and fractionated. Individual FAME is then analysed by GC/MS (gas chromatography/mass spectrometry) to know its composition. A profile of the fatty acids and other lipids is then used to determine the characteristics of the microbial community.

Arbuscule-forming fungi in the order Glomales form obligate endomycorrhizal associations with plants, which makes it difficult to quantify them; taxonomy of the group is only beginning to be objectively understood. FAME profile is a potential tool that assesses the diversity of these fungi by analysing the diversity and quantity of fatty acids. Majority of AM fungi gave a characteristic fatty acid profile, sharply distinct from other organisms.

The lipid composition of glomalean fungi in mycorrhizal roots was first determined by Cooper and Losel (1978). Substantial information has been reported on the composition of fatty acids in endomycorrhizal citrus and soybean roots (Nagy, Nordby, and Nemec 1980; Nordby, Nemec, and Nagy 1981, Pacovsky and Fuller 1988), fungal spores (Beilby 1980), and vesicles isolated from roots (Jabaji-Hare, Deschene, and Kendrick 1984). The potential of lipids and fatty acids as taxonomic characters for glomalean fungi was first pointed out by Jabaji-Hare (1988) who examined FAMEs in three *Glomus* species and one *Gigaspora* species. Sancholle and Dalpe (1993) characterized FAMEs in seven *Glomus* species, one *Acaulospora* species, and one *Scutellospora* species. Bentivenga and Morton (1994) assessed the potential of FAME profiles as taxonomic tool by comparing profiles of up to four successive 'generations' of pot cultures of eight isolates, representing all families in Glomales, and found FAME profiles to be stable and heritable. Several fatty acids have been reported as common in lipids from AM structure. Beilby and Kidby (1980), for example, found that the fatty acids 16:1, 16:0, and 18:1 dominated in lipids from spores of *Glomus caledonium*. The fatty acids 16:1ω5, 18:1ω7c, 18:3, 20:3, 20:4, and 20:5 have been detected in several studies either exclusively, or in higher amounts, in plants colonized by AM fungi (including several *Glomus* species) compared to uncolonized roots (Nordby, Nemec, and Nagy 1981). These fatty acids have, therefore, been suggested as indicators of the biomass of AM fungi. Graham, Hodge, and Morton (1995) analysed the FAME profile of 53 isolates of 24 glomalean species. Spores yielded reproducible FAME profiles from replicate spore collections extracted from soil pot cultures despite being grown in association with a host plant, in the presence of contaminating micro-organisms. Unweighted pair group analysis revealed relatively tight clusters of groups at the intraspecific, specific, and generic levels; however, lipid profiles at the family level were convergent. Thus, FAME profile comparisons provide a robust measure of similarity below the family level.

The most advanced and accurate method of identification is molecular characterization. The use of molecular-based techniques such as PCR (polymerase chain reaction)–DGGE (denaturing gradient gel electrophoresis) offers great possibilities to detect, identify, and characterize these symbiotrophic fungi. Souza, de Leeflang, Smit, *et al.* (2001) developed and tested different PCR–DGGE approaches for identification and characterization of AM fungi species and to study AM fungi community structure in field conditions. Applying this technique, differentiation among closely related *Gigaspora* species and other AM fungi was possible. This novel approach provides a robust means to characterize rDNA heterogeneity in Glomales.

At the CMCC, the AM fungi are already characterized on the basis of morphology. Fact sheets having detailed morphology of 50 AM fungal species have already prepared. A total of 140 trap cultures and 300 monosporal cultures from varied agro-climatic regions are being multiplied and are a good source of germplasm material. As these materials are pure and

in good amount, they could be directly used for analysing FAME profiling and molecular characterization of different AM fungal species and isolates. This would further strengthen the taxonomic characterization.

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Recent references

The latest additions to the network's database on mycorrhiza are published here for the members' information.

The Mycorrhiza Network will be pleased to supply any of the available documents to bonafide researchers at a nominal charge. This list consists of papers from the following journals.

- *Agriculture Ecosystems & Environment*
- *Annals of Microbiology*
- *Applied Soil Ecology*
- *Biological reviews*
- *Environmental Pollution*
- *European Journal of Agronomy*
- *Journal of Plant Research*
- *Journal of Tropical Ecology*
- *Journal of the American Society for Horticultural Science*
- *Mycorrhiza*
- *Mycological Research*
- *Periodicum Biologorum*
- *Plant Biology*
- *Plant and Soil*
- *Planta*
- *Symbiosis.*
- *Tree Physiology*
- *New Phytologist*

Copies of papers published by mycorrhizologists during this quarter may please be sent to the Network for inclusion in the next issue.

Name of the author(s) and year of publication	Title of the article, name of the journal, volume no., issue no., page nos (address of the first author or of the corresponding author, marked with an asterisk)
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