Vol. 18 No. 2
July 2006

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.

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Phosphorus mobilization by mycorrhizal fungi—Part 1. Production and detection of phosphatases

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Production of phosphatases by mycorrhizal fungi/roots

Many isolates of ectomycorrhizal fungi excrete enzymes, which are capable of digesting organic matter. Phosphates become available following the degradation of litter components by fungal phosphatases. Phytates and various other forms of organic phosphorus are degraded and phosphates are absorbed in the soil.

Acid phosphatases contribute to the mineralization of organic phosphorus compounds in soil, thus enhancing the biological availability of released inorganic phosphorus. Both the roots and mycorrhizal symbionts produce acid phosphatases.

Production of phosphatases by ectomycorrhizae

Studies conducted at the Institute of Terrestrial Ecology, Merlewood Research Station, Grange-over-Sands, Cumbria, UK, on acid phosphatase production (determined by phenol release from di-sodium phosphate) by non-mycorrhizal and ectomycorrhizal roots of pine (*Pinus contorta*) and birch (*Betula pubescens*) seedlings in liquid cultures of six mycorrhizal and two saprophytic fungi (Agaricales), showed that phosphatase production by mycorrhizal roots was lower than in non-mycorrhizal roots of birch. But in roots of pine, phosphatase production of mycorrhizal and non-mycorrhizal roots was not significantly different, except for mycorrhiza of *Hebeloma crustuliniforme* where it was as in birch (Dighton 1983).

Studies conducted at the Universitat Hohenheim, Institute fur Planzenernahrung, Stuttgart, Germany, on inorganic and organic phosphate, measured in soil solutions of bulk soil, rhizosphere soil, and mycorrhizal rhizoplane soil in 80-year-old Norway spruce (*Picea abies*) stands showed that about 50% (range of 35%–65%) of total phosphate was present as organic phosphate. Compared with bulk soil, the concentration of readily hydrolysable organic phosphorus was lower in rhizosphere and rhizoplane soils, and this difference was particularly marked in the humus layer. In contrast, the concentration of inorganic phosphate was the same or greater. Acid phosphatase activity present was 2–2.5 times greater in the rhizoplane soil than in the bulk soil. Also, there was a positive correlation between phosphatase activity and the length of mycelial hyphae. The studies stress the role of organic phosphate and acid phosphatase in the rhizosphere in phosphate uptake by mycorrhizal roots of Norway spruce trees on acid soils (Haussling and Marschner 1989).

Studies conducted at the Laboratoire de Biologie des Ligneux, Universite de Nancy, Vandoeuvra-les-Nancy, Cedex, France, showed that the interface of *Pisolithus tinctorius* mycelium (strain H 445) with the *Pinus sylvestris* root system was characterized by the development of polysaccharide fibrillae, which join together fungal and root surfaces. The development of such fibrillae is the result of mutual recognition and corresponds to the phase of fungal attachment to the host plant, leading to mycorrhizal formation. Parietal, plasmalemma, vacuolar, and cytoplasmic phosphatases, localized using immunocytochemistry, were evident only in the fungus. Extracellular

* This paper has been compiled from TERI records in RIZA.

phosphatases secreted in the medium were detected around hyphae; however, they were absent in the space separating hyphae from the root surface (Gourp and Pargney 1991).

Studies conducted at the Department of Biology, Clarkson University, Potsdam, USA; Department of Science, Blubbton College, Blafftion, Ohio, USA; and Institute of Terrestrial Ecology, Grange-over-Sands, Cumbria, UK, on American beech (*Fagus grandifolia)*, occurring in a climax stand and gray birch (*Betula populifolia*), growing in mid-successional stands, showed that all the root types collected early or late in the growing season on a common soil type demonstrated similar oxygen consumption rates on both a root length basis and dry weight basis. All the root types demonstrated measurable phosphatase activities at pH 5.0 with P-nitrophenyl phosphate, bis-p-nitrophenyl phosphate, and phytic acid as substrates, and thus, the potential to mineralize monoester and diester forms of organic phosphorus. The activity varied seasonally and among ectomycorrhizal morphotypes. Comparison of uptake of 32P from inositol phosphate by gray birch roots suggested that phosphatase activity was linked to phosphorus uptake from this source (Antibus and Dighton 1993; Antibus, Bower, and Dighton 1997).

In studies conducted at the Department of Botany, University College Dublin, Belfield, Dublin, Ireland, ectomycorrhizal fungi (*Paxillus involutus*, *Suillus grevillei*, and two unidentified basidiomycetes from excised Sitka spruce mycorrhizae) were isolated from stands of Sitka spruce either in monoculture or in mixture with Japanese larch in an Irish conifer plantation. They were grown for 35 days on modified Melin-Norkran liquid medium containing ferric phytate as the phosphorus source. The cultures were then separated into wall and membrane-bound, cytoplasmic and extracellular fractions and assayed for phosphatase. Wall- and membrane-bound fractions contained the most active acid phosphatase. The unidentified basidiomycetes showed a lower substrate affinity and higher velocity of reaction than *P. involutus* and *S. grevillei*. Wall- and membrane-bound and cytoplasmic phosphatase activities were optimum over a broad pH range (4.0–6.0). Various methods were used to release wall-bound phosphatase and a high proportion (49%–88%) appeared to be tightly held within the wall. Phosphatase released from the wall by sonication had a similar Km to wall-bound phosphatase, but Vmax was lower. The use of a number of substrates demonstrated a high affinity for inorganic pyrophosphate and sodium beta-glycerophosphate, but a low phytase activity (McElhinney and Mitchell 1993).

In studies conducted at the Department of Botany, University of Helsinki, Unioninkatu, Helsinki, Finland, on morphologically similar ectomycorrhizae between Scotspine (*Pinus sylvestis*) and different geographical isolates of *Suillus variegatus* and *Suillus bovinus* were synthesized under semi aseptic conditions in sand-peat growth mixture containing urea formaldehyde as the organic nitrogen source. Acid phosphatase isozyme activities were detected in the ectomycorrhizae but were restricted to a single isozyme known to be common to both fungal species (Sen 1990).

Production of phosphatases by VA-mycorrhizal fungi

Studies conducted at the ICRISAT (International Crops Research Institute for the Semi-Arid Tropics), Patancheru, Andhra Pradesh, India, showed that in sterilized soil, acid and alkaline phosphatase activities in the rhizospheres of mycorrhizal and non-mycorrhizal plants were higher than in non-rhizosphere soil. No significant differences were noted between the rhizospheres of mycorrhizal and non-mycorrhizal plants. In unsterile soil, differences in alkaline phosphatase activity among the three treatments were not significant. With acid phosphatase, the activity was highest in the rhizosphere of mycorrhizal plants, followed by rhizosphere of non-mycorrhizal plants and non-rhizosphere soil (Krishna and Bagyaraj 1985).

Studies conducted at the Department of Biology, Clarkson University, New York, USA and Institute of Terrestrial Ecology, Cumbria, UK, on red maple (*Acer rubrum*), growing in a mid-successional stand, demonstrated oxygen consumption rates similar to American beech and gray birch root material collected early and late in the growing season on a common soil type. All the root types examined demonstrated measurable phosphatase activity at pH 5 with p-nitrophenyl phosphate, bis-p-nitrophenyl phosphate, and phytic acid as substrates, with rates of hydrolysis greatest with p-nitrophenyl phosphate. VAM (vesicular arbuscular mycorrhiza) infected roots of red maple consistently demonstrated lowest phosphatase activity on both length and dry weight basis. Comparison of 32p uptake from inositol phosphate by red maple and gray birch roots suggested that the phosphatase activity was linked to phosphorus uptake from this source (Antibus and Dighton 1993).

Comparative effect of different mycorrhizal fungi on phosphatase activity in soil and/or plant roots

In studies conducted at the Biological Laboratory, University of Kent, Canterbury, Kent, UK, the plants of rape, wheat, and onion were inoculated singly with *Glomus geosporum*, *G. mosseae*, and *G. monosporum* or were left uninoculated and were grown in sand with little available phosphorus. Root and rhizosphere levels of acid phosphatase activity were higher for plants inoculated with *G. geosporum* and *G. mosseae* than for control plants. Infection by *G. monosporum* did not result in a similar increase in phosphatase activity, but plant growth was improved.

In further experiments on wheat, increases in phosphatase induced by *G. geosporum* or *G. mosseae* became apparent 25–51 days after sowing (Dodd, Burton, Burns, *et al.* 1987).

Studies conducted at the Indian Institute of Horticultural Research, Hessaraghatta, Bangalore, India, on phosphatase activity in papaya (*Carica papaya*) roots showed that inoculation with AMF (arbuscular mycorrhizal fungi) enhanced the activity of both acid and alkaline phosphatases (25%–114%) on the root surface as determined in enzyme extracts from root and soil surrounding the root region. Acid phosphatase activity was much greater than alkaline phosphatase activity. Plants inoculated with *Glomus mosseae* showed better activity than those with *G. fasciculatum* (Mohandas 1990).

In studies conducted at the Faculty of Horticulture, Chiba University, Matsudom, Chiba, Japan, marigold (*Tagetes patula*) and leek (*Allium porrum*) were inoculated with each of the three AMF – namely, *Glomus mosseae, G. etunicatum,* and *Gigaspora rosea* – to compare the phosphatase localization in the intraradical hyphae. The mycorrhizal roots were harvested at 3, 4, 5, and 6 weeks after sowing, treated with a digestion solution containing cellulase and pectinase, and then stained for phosphatase activities at pH 5.0 and pH 8.5. *G. mosseae* formed fine-branched (mature) arbuscules only at the early phase of infection (3–4 weeks after sowing). Mature arbuscules of *G. etunicatum* and *G. rosea* were observed from the early phase (4 weeks after sowing) up to the end of experiment. At pH 5.0, the localization of the phosphatase activities of the three fungi was similar, irrespective of the host plant species. The activity appeared in the mature arbuscules and intercellular hyphae, whereas the collapsed arbuscules were inactive. Ten millimolar NaF, an acid phosphatase inhibitor, inhibited the phosphatase activities of *G. mosseae* and *G. etunicatum*, but did not inhibit the activities of *G. rosea*. At pH 8.5, a difference among the fungal species was found in the localization of phosphatase activity while there was no such difference between host species. The mature arbuscules were also the active sites in all three species. Only *G. rosea* showed the activity in the intercellular hyphae while the two *Glomus* spp. did not. Five millimolar EDTA (ethylene diamine tetraacetic acid), asynthetic chelate, inhibited the activity of *G. rosea* at pH 8.5 while the activities of *G. mosseae* and *G. etunicatum* were not affected by either 5 mM EDTA or 10 mM KCN (both are alkaline phosphatase inhibitors) (Ezawa, Saito, and Yoshida 1995).

In studies conducted at the Laboratory of Developmental Biology, Institute of Botany, KatholieKe Universiteit Leuven, K Mercierlaan, Leuven, Belgium, activities of extracellular enzymes were determined in organic matter of beech (*Fagus sylvatica*) leaf litter, colonized by the litter decomposer *Lepista nuda* or by vegetative mycelium from the mycorrhizal fungi, *Thelephora terrestris* or *Suillus bovinus,* and buried for up to six months in plant containers in which mycorrhizal or non-mycorrhizal *P. sylvestris* seedlings were cultivated at a low rate of nutrient addition. After different periods of colonization, the activities of phosphomonoesterase and protease, the two enzymes involved in nitrogen and phosphorus mobilization, were determined in colonized and uncolonized litter. The activities of cellulase, beta-xylosidase, beta-glucosidase, and polyphenol oxidase (catechol oxidase) were investigated as indicators of the decomposing capacity of fungi in the litter. Low activities of all enzymes tested occurred in uncolonized beech leaves. Phosphomono-esterase activity was high in litter colonized with *L. nuda* or *S. bovinus*, and was intermediate in the *T. terrestris* treatment. For all other enzymes, the activities in the organic matter inoculated with the white-rot litter decomposer were considerably larger than those detected in litter colonized by ectomycorrhizal basidiomycetes. Cellulase activity was low in the control, as well as in the mycorrhizal treatments. Beta-xylosidase and beta-glucosidase were detected in the litter with mycorrhizal mycelium, whereas polyphenol oxidase activity was only clearly increased in the *S. bovinus* treatment. These results demonstrated the low lignocellulase activity of both mycorrhizal fungi. This reduced the capacity of the mycorrhizal fungi to exploit fresh beech leaf litter, whose endogenous nitrogen was associated with or shielded by refractory compounds (Colpaert and Laere 1996).

Studies conducted at the CNRS (Centre national de la recherche scientifique), Centre de Pedologie Biologique, Vandoeurize, Nancy, France, on extraradical hyphae of *Glomus intraradices* or *G. claroideum*, extracted from root-free sand of two-compartment pot cultures and used to determine fungal phosphatase activity (p-nitrophenyl phosphate hydrolysis) showed that *G. intraradices* had the highest external phosphatase activity in two experiments and *G. claroideum* had the same activity in one experiment. The activity reached 184 µ mol p-nitrophenyl phosphates hydrolysed per mg dry weight H^{-1} (Joner and Johansen 2000).

Detection methods for phosphatase production by mycorrhizal fungi

Ectomycorrhizal fungi

In studies conducted at the Laboratoire de Recherches dur les Symbiotes des Racines, INRA-ENSA (Institut National de la Recherche Agronomique–Centre national de la recherche scientifique), Place Vala, Montpellier, France, acid phosphatase activities were measured in a phosphate-depleted medium of *Hebeloma cylindrosporum* mycelia. These activities showed a great variability depending on the substrate (sodium para nitrophenyl phosphate or sodium polyphosphate) and the strain

of *H. cylindrosporum* (wild dikaryotic strain or homokaryotic strains issued from this dikaryon). The purification tests were carried out on the phosphatase secreted by the wild dikaryon. After filtration, concentration, and desaltation of the culture solution, the phosphatases were separated by a cation exchange chromatography (carboxy methyl trisacryl). Two fractions of phosphatase activities were collected, one of which was eluted using a gradient of sodium chloride. The electrophoretograms of the fractions showed a high purity and phosphatase were directly injected to a rabbit. The appearance of antibodies in the serum was detected by the ELISA (Enzyme-Linked Immunosorbent Assay) test. It was concluded that the antibodies were useful to study the regulation of the fungal phosphatase activities in the presence of orthophosphate or the phanerogam host (Deransart, Chaumat, Cleyet-Marel, *et al.* 1990).

In further studies conducted at the above institute, an acid phosphatase of *Pisolithus tinctorius* was purified and used to prepare polyclonal antibodies. These antibodies were applied in homologous tests on the mycelium and mycorrhizae of several fungi. This immunochemical approach seemed to be a way to characterize and detect fungi when a particular protein is used as a marker (Clayet-Marel, Bousquent, and Mousain 1990).

In the studies conducted at the Department of Biochemistry and Microbiology, G B Pant University of Agriculture and Technology, Pant Nagar, Uttar Pradesh, India, alkaline phosphatase purified from mycorrhizal and non-mycorrhizal French bean (*Phaseolus vulgaris*) gave a single band on 5%–6% gradient PAGE (polyacrylamide gel electrophoresis). A single protein peak was observed by UV spectrophotometry with an absorption maximum at 275 nm. The Vmax and Km differed slightly for the two preparations but they had similar pH (8.5) and temperature 40 °C optima. The alkaline phosphatase from non-mycorrhizal beans had wider substrate specificities than that from mycorrhizal beans (Kumari, Mishra, and Johri 1990).

The pNPPase (p-nitrophenol phosphomonoesterase assay), commonly used to measure cell-wall-associated and extracellular phosphatase activity of soil fungi, is usually done in the context of fungal nutrition, where inorganic phosphorus supply may be enhanced by mineralization of organic phosphorus in the soil. Series of experiments were conducted at the Bournemouth University School, Conservation Science, Talhot Campus, Dorset, UK, with the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum*, that highlight components of accepted methodology that might impinge on the reliability of the assay. These include the loss of pNPPase after filtration, inaccuracies in measuring wall-associated enzyme, and the ample pool of intracellular pNPPase can be mistakenly measured as external pNPPase if cells are accidentally damaged (Tibbett, Sanders, Grantham, *et al.* 2000).

VAM fungi

In studies conducted at the Institute fur Pflanzenbau and Tierhygience in den Tropen and Subtropen, University of Gottingen, Grisebachstrabe, Gottingen, Germany, methods are reported for separation and identification of organic acids, including phenolic acids, and on the determination of phosphatases from VAM roots. For gas-chromatographic preparation of the samples containing the organic acids, silylation and methylation were tested, as well as various solvents and silylating reagents. Columns of different polarity were examined for their separation properties. Gas-chromatographic separation of the acids was incomplete on the packed column OV 7. With the capillary column PVMS 54, separation was much better, but improvements are still needed. MSP (mycorrhiza specific phosphatases) appearing as three bands were found by electrophoresis with porosity gradient gels in mycorrhizal roots of Allium, Sorghum, and Eupatorium plants. In these three species, each MSP was located at the same position. Inoculated onion roots enzymatically digested with cellulase and pectinase showed no root specific phosphatases, but still revealed MSP (Fabig, Vielhauer, Moawad, *et al.* 1989).

In further studies conducted at the above university, *Eupatorium odoratum*, Sorghum, and onion plants were inoculated with *Acaulospora longula*. The plants were harvested after 8 and 16 weeks, respectively, and cell-free root extracts were prepared for electrophoresis. The improved separation of mycorrhizal proteins through a 3%–30% polyacrylamide gradient gel revealed three MSP instead of only one diffuse MSP band, which is to be seen in a 10% homogeneous gel. It is suggested that fluctuations in total MSP activity during the vegetation period are the result of fluctuations in single phosphatases sharply distinguished from one another (Vielhauer 1990).

In the studies conducted at the Institute Coltivazioni Arboree dell' Universita, Via Giuria 15, Turin, Italy, activities of acid and alkaline phosphatase, esterase, and succinate dehydrogenase were assessed in extraradical mycelium of *Glomus clarum* by histochemical methods. Staining was observed for all enzymes with the exception of acid phosphatase. The amount of mycelium showing activity decreased after initial infection. In older hyphae, stained deposits were irregularly distributed inside the hyphae. It is suggested that in some hyphal tracts, vacuolization takes place and enzymatic activity is reduced (Schubert and Mazitelli 1990).

In studies conducted at the Central Arid Zone Research Institute, Jodhpur, India, filter papers treated with a mixture of 1-naphthyl phosphate as substrate and the diazonium salt Fast Red TR as an indicator were used for the nondestructive visual demonstration of the release of acid phosphatase by VAM fungi. Wheat was grown for six weeks in

sterilized soils in specially designed pots with five compartments with or without hyphal and root barriers. The plants were inoculated with *G. mosseae* and then treated filter paper was placed at the outer surface of the hyphal compartment. Acid phosphatase activity was visualized as a red-coloured hyphal print on the filter paper (Tarafdar 1995).

Studies conducted at the National Grassland Research Institute, Nishinasuno, Tochigi, Japan, showed that an alkaline phosphatase in the intraradical hyphae of VAM fungi was closely related to an improvement of plant growth. To detect the phosphatase activity in a crude extract of mycorrhizal roots, phosphatase isozymes in mycorrhizal and non-mycorrhizal onion roots were compared with those in *Gigaspora margarita* by electrophoresis. A mycorrhiza-specific band was found when the phosphatase was stained under alkaline conditions. To clarify the origin of this phosphatase, the phosphatase extracted from intraradical hyphae was also compared with the phosphatase from mycorrhizal roots by electrophoresis. The intraradical hyphae were isolated from mycorrhizal roots by enzyme digestion followed by Percoll gradient centrifugation. The soluble protein was extracted from the hyphae by ultrasonication after treatment with chitinase. A phosphatase in the hyphal soluble protein showed a similar, but slightly higher, relative mobility on the gel, compared with the mycorrhiza-specific phosphatase from roots. By adding the hyphal extract to the root extract, the relative mobility of the mycorrhiza-specific phosphatase was slightly changed and became identical to that of the phosphatase in the hyphae. This indicates that the specific band of phosphatase found in the crude extract from mycorrhizal roots was of intraradical hyphal origin (Kojima, Hayatsu, and Saito 1998).

Inter- and intra-strain variations of phosphatase activity in ectomycorrhizal fungi

In studies conducted at the Centre de Recherche en Biologie Forestiere, Universite Laval, Sainte-Foy, Quebec, Canada, the variation in acid phosphatase activity among the monokaryotic F1 progeny from two different synthesized dikaryotic cultures of the ectomycorrhizal basidiomycete, *Laccaria bicolor*, was examined. The progeny of one of the dikaryons showed variation in acid phosphatase activity up to 10 times that of the lowest value. The progeny of the other dikaryon were much less variable, showing differences of up to five times the lowest value. Both sets of monokaryotic progeny showed distributions indicative of polygenic inheritance for acid phosphatase activity in this fungus (Kropp 1990a).

Further studies conducted at the above university on acid phosphatase activity of monokaryotic progeny from controlled crosses in *L. bicolor* indicated that acid phosphatase activity in this fungus was polygenic. A biometric analysis was done to partition genetic and environmental components of the variations among dikaryons created by pairing selected monkaryotic cultures. The results showed that a relatively small proportion of the phenotypic variability observed in this study was due to environmental factors. Further partitioning showed that the genetic variation could be attributed primarily to additive rather than non-additive components. The heretability of acid phosphatase activity in *L. bicolor* was thus quite high, indicating that strains having an elevated acid phosphatase activity could be created by breeding (Kropp 1990b).

Studies conducted at the Universite Claude Bernard Lyon 1, Unite d'Ecologie Microbienne, Associee au Centre National de la Recherche Scientifique, Cedex, France, on interstrain variation with 11 wild strains and intra-strain variability with 20 sib-monokaryons and 50 reconstituted dikaryons progeny of *Hebeloma cylindrosporum* strain showed that the range of variation of acid phosphatase activity among wild dikaryotic mycelia was the same as that among sib-monokaryons or dikaryons belonging to the progeny of a single strain. The total phosphatase activity of the wild strains ranged from 5.7 to 96.0 TmU (total milliunits). It ranged from 11.1 to 120.5 TmU within sib-monokaryons and from 34.2 to 178.1 TmU for reconstituted dikaryons. Specific phosphatase activity of wild dikaryons ranged from 48.5 ImU (international milliunits) to 675.6 ImU, whereas the ranges of variation among sib-monokaryons and reconstituted dikaryons were, respectively, 85.3 to 71.0 and 270.7 to 816.1 ImU. On average, sib-monokaryons and reconstituted dikaryons had lower activity than their parental dikaryon. However, four reconstituted dikaryons had a higher specific activity than the original dikaryon, *H. cylindrosporum*. The growth of the studied mycelia also varied, but in a narrower range (from 97.1 to 151.6 microg protein per culture for wild dikaryons, from 130.1 to 199.1 microg for sib-monokaryons, and from 160.6 to 275.9 microg for reconstituted dikaryons). No correlation could be detected between specific acid phosphatase activity and growth rate in pure culture within the different monokaryotic or dikaryotic populations studied. The results demonstrate the possibility of obtaining, by intra-strain crossing, mycelia having higher phosphatase activity than the parental wild strains (Meysselle, Gay, and Deband 1991).

Studies conducted at the Department of Forest Science, University of Alberta, Edmonton, Canada, on mycelial extracts of 43 isolates of *Suillus tomentosus* from forest regions subjected to starch gel electrophoresis showed that a total of 21 bands were resolved from eight different enzymes presumably representing 13 loci. Six loci were polymorphic among these isolates. Cluster and principal components analysis demonstrated that the intra-specific genetic variation existed among and within

the forest regions. Polymorphic loci of acid phosphatase and alkaline phosphatase exhibited the greatest genetic similarity among the isolates within forest regions. Habitat isolation and host selection could be the sources of the isozyme variation among forest regions in this fungal species (Zhu, Higginbotham, and Dancik 1987).

In studies conducted at the United States Department of Agriculture, Pacific Northwest Research Station, Forestry Sciences Laboratory, Corvallis, Oregon, USA, six isolates of *Laccaria laccata* (S-167, from a forest nursery and S-238, S-283, S-236, S-444, and S-472 isolates from natural forests) were analysed for acid phosphatase, alkaline phosphatase activity, acid phosphatase isozyme patterns, production of other enzymes and hormones. Differences in enzyme activity and phytohormone production were prominent among the isolates. The patterns of acid phosphatase isozyme could be clearly divided into three host-related groups. Two polymorphic gene loci could be identified as coding for enzymes of acid phosphatase. Two of these gene loci, Acp-b and Acp-c, were characterized by mostly constant, host dependent frequencies. The other, Acp-d, exhibits allele frequencies related to different habitats. Five isolates share the same Acp-a habitat, four isolates share the Acp-b habitat, two the Acp-c habitat, and only one isolate, S-238, was from a high elevation at the Acp-d habitat. The isolate from a forest nursery differed strikingly in several characteristics from other isolates, all of which were from a natural forest. This suggested that nursery soil management practices may select isolates for particular edaphic ecotypes of mycorrhizal fungi (Iwan 1987).

Relation of phosphatases with mycorrhizal infection in plants

In studies conducted at the Laboratoire de Phytoparasitologie, INRA-CNRS, Station de Genetique et d, Ameliozation des Plants, INRA, Dijon, Cedex, France, a histochemical procedure was developed to visualize and estimate the proportion of arbuscular mycorrhizal infections showing alkaline phosphatase activity and was compared with the total amount of fungal tissue (indicated by trypan blue staining) and of living mycelium (indicated by succinate dephyclrogenase activity). In roots of leeks and *Platanus acerifolia*, only a small proportion of living intraradical mycelium showed alkaline phosphatase activity during early infection by *Glomus* spp. but this increased greatly just before the mycorrhizal growth response of the host plant. The infection revealed by all three strains reached a maximum at six weeks after inoculation, after which the level of trypan blue-stained infection remained constant, while the proportion showing succinate dehydrogenase and alkaline phosphatase activity declined as the infection aged. Alkaline phosphatase activity was absent from virtually all abortive entry point hyphae formed on roots of a resistant myc (-) nod (-) mutant of pea, although succinate dehydrogenase activity was detected. These observations suggest that the alkaline phosphatase activity is induced by colonization of host roots and that this fungal enzyme could find a useful marker for analysing the symbiotic efficiency of arbuscular mycorrhizal infection (Tisserant, Gianinazzi-Pearson, Gianinazzi, *et al.* 1993).

In studies conducted at the Western Regional Research Centre, USDA-ARS (United States Department of Agriculture–Agriculture Research Service), Albany, California, USA, two cultivars of *Phaseolus vulgaris* (Mexico 309 responsive to VAM, Rio Tibagi less responsive to VAM) were grown in a greenhouse in Leonard jars containing sand/vermiculite supplied with nutrient solution containing low concentrations of nitrogen and phosphorus, and were inoculated or not with soil that contained spores and hyphae of *Glomus etunicatum*. The plants were harvested three and four weeks post emergence. Mexico 309 beans grew faster supported proportionately more VAM fungi and assimilated more phosphorus than did Rio Tibagi plants. SPUR (specific phosphorus uptake rate) was 35% greater in non-inoculated Rio Tibagi compared with Mexico 309 roots. Colonization by *G. etunicatum* more than doubled the SPUR of each cultivar compared to controls. Acid and alkaline phosphatase activities were significantly higher in Mexico 309 mycorrhizae than in controls but in VAM and control Rio Tibagi roots, there were no significant differences. New acid phosphatase isoenzymes that were not present in controls appeared in VAM colonized roots in both cultivars and high molecular weight acid phosphatases decreased concomitantly in the host. Polyphosphate hydrolase activity increased in mycorrhizae of both cultivars compared to uninfected roots. Malate dehydrogenase, peroxidase, and esterase activities were also higher in VAM colonized roots than in controls and usually new isozymes appeared in mycorrhizae that were either undetectable in control roots or were expressed to only a very small extent. Total peroxidase activity was higher although two host bands disappeared in mycorrhizae and one new band having more than 50% of total activity appeared. These results indicated that the dependence of a host on VAM fungi increased when nutrients that mycorrhizae can provide were limited. The greater the increase in absorption or utilization capacity following VAM colonization, the greater would be the dependence of the host. More importantly, by identifying enzyme activities that affect these plant microbe associations, specific genes can be targeted that code for these enzymes for future manipulation (Pacovsky, Silvada, Carvalho, *et al.* 1990).

In studies conducted at the Faculty of Horticulture, Chiba University, Matsudo 271, Japan, *Tagetes patula* cv. Bonanza spray, *Tagetes patula* ev

Bonanza spray, *T. patula* cv. Disco and *T. erecta* cv. Discovery were inoculated with *Glomus etunicatum* and in additional experiments, Bonanza spray was inoculated with *G. mosseae* or *Gigaspora* spp. also in order to investigate the origin and properties of the phosphatase specific to VAM infection. Soluble phosphatases were extracted from six-week-old plants and analysed using electrophoresis. The infection specific phosphatases were commonly detected in all these associations and the Rf values were almost the same (0.11–0.12). Weak phosphatase activity was observed at the same Rf value as that of infection specific phosphatase in non-mycorrhizal plants. No major phosphatase band was observed at Rf 0.11–0.12 in the extracts of germinating and resting spores of *G. etunicatum*. It was thus suggested that infection specific phosphatase was of the host origin. The infection specific phosphatase was partly purified from the roots of Bonanza spray infected with *G. etunicatum* about 170-fold and characterized. The optimum pH of 5.0, the hydrolysis of various phosphate esters, and the inhibition of fluoride, molybdate, phosphate, and vandate were indicative of the typical characteristics of non-specific acid phosphates (E.C. 3.1–3.2). In addition, since the enzyme hydrolysed the pyrophosphate bond effectively, it was suggested that the enzyme was an acid phosphatase originating from the plant (Ezawa and Yoshida 1994).

Studies conducted at the University of West Indies, Centre for Biotechnology, Kingston 7, Jamaica, on the effect of VAM fungus, *Glomus pallidum*, on the phosphatase activity in cowpea (*Vigna unguiculata*) roots at successive stages of plant growth showed that both acid and alkaline phosphatase activities were significantly higher (P=0.05) in mycorrhizal than in non-mycorrhizal roots 30 days after inoculation (Thiagarajan and Ahmad 1994).

Studies conducted at the Laboratoire de Phytoparasitologie, INRA-CNRS, Station de Genetique *et al*. Amelioration des plantes, Dijon, Cedex, France, on soyabean and pineapple (having different growth rates and response to phosphate) to see the effects of phosphate fertilization on physiological activities of VAM fungi showed that total mycorrhizal infection estimated by trypan blue staining was reduced by phosphate fertilization in phosphorus sufficient soils. In phosphorus deficient soil, fungal infection in pineapple roots was not modified by phosphate application. The level of total mycorrhizal infection was not related to plant growth. Fungal alkaline phosphatase staining, and to a lesser extent, succinate dehydrogenase activity showed a relationship with plant growth. There is thus a potential of this procedure for estimating endomycorrhizal infection as a marker of efficiency of the symbiosis (Guillemin, Orozco, Gianinazzi-Pearson, *et al*. 1995).

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Research findings

Impact of arbuscular mycorrhizal fungus (*Glomus fasciculatum*) and phosphate solubilizing bacterium (*Pseudomonas striata*) on growth and nutrient status of *Azadirachta indica* L.

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AMF (arbuscular mycorrhizal fungi) are obligate symbionts that colonize the roots of more than 90% of plants in all terrestrial environments. The beneficial effect of AMF on plant health improvement is now well established (Prasad 2000, 2002). Recent investigations have brought to light instances where biological activities are markedly enhanced in two or three member associations of organisms. The phosphate solubilizing micro-organisms interact well with AMF in phosphorus-deficient soils. So far, no systematic investigations have been made on the growth response of *Azadirachta indica* to AMF individually and/or in combination with PSB (phosphate solubilizing bacteria). The present research work deals with the effects of single and dual inoculation of *Glomus fasciculatum* and phosphate solubilizing bacterium *(Pseudomonas striata*) on *Azadirachta indica* plant.

Materials and methods

Preparation of AM fungal inoculum: *Azadirachta indica* (neem) trees were surveyed and AMF spores were isolated from the rhizospheric soil by the wet sieving and decanting method (Gerdmann and Nicolson 1963). Quantification of AM fungal spore density from the air-dried soil was carried out using the method given by Gaur and Adholeya (1994). Single AM fungal spores were purified and maintained as pure cultures by the standard technique using maize *(Zea mays)* as the host plant. Diagnostic slides of spores were prepared using PVLG (polyvinyl alcohol lactoglycerol) as a mountant. AMF spores were identified using relevant literature (Mortan and Benny 1990; Schenck and Perez 1990; Prasad and Rajak 1999). Soil based *G. fasciculatum* AMF inocula was prepared by growing sterilized seeds of *Paspalum notatum* in pots containing sterilized sandy loam soil (sand:soil, 1:1, w/v). The substrate containing spores, sporocarps, and root pieces served as a stock culture of AMF inoculum.

Procurement of PSB: The culture of PSB was obtained from the IARI (Indian Agricultural Research Institute), New Delhi, India.

Earthen pot preparation and inoculation: A pot culture experiment was conducted to study the effect of *Glomus fasciculatum* and PSB (*P. striata*) on *A. indica* seedlings under nursery conditions. The soil was ground, passed through a 4 mm sieve, and was autoclaved for 2 consecutive days. The soil was deficient in phosphorus. Eight kilograms of such processed soil was used to fill in the earthern pots (30 cm diameter).

The topsoil was mixed with 200 ml soil containing finely chopped, heavily infected root pieces of *G. fasciculatum* inoculum comprised of 150–200 spores/plant. Healthy seeds of *A. indica* were surface sterilized and treated with soil and charcoal mixture (1:3) based carrier inoculant of *P. striata*, wherever required. Autoclaved soil without any microbial inoculum served as control. The treatment was replicated ten times and arranged in a randomized block design. The plants in pots were maintained to grow for 90 days in a mist house with temperature of 27–35 °C, 70%–80% humidity, and light intensity of 15000Lux–19000Lux. Plant samples were taken at intervals of 30 days. Growth parameters were observed by well-known methods.

Estimation of AM fungal infection: Freshly collected roots were washed in water, cleaned with 10% KOH (potassium hydroxide), acidified with 1N HCL (hydrochloric acid) and stained in 0.05% trypan blue (Phillips and Hayman 1970). Quantification of AMF root infection was carried out using the slide method (Giovannetti and Mosse 1980).

Estimation of shoot NPK (nitrogen, phosphorus, and potassium): Nitrogen was estimated by the Kjeldhal method, phosphorus was determined by the Ammonium molybdate vandate method, and potassium was determined using the Flame photometer as described by Jackson (1973).

Results and discussion

The inoculation with AMF in general resulted in higher root infection than the uninoculated plants (Table 1). Dual inoculation of *G. fasciculatum* and *P. striata* has significantly increased root infection, spore population, stem height, number of leaves, total dry weight compared to single inoculations, and control. The stem height, number of leaves, and dry weight were directly proportional to percentage colonization in roots, and AMF spore population in soil during the plant growth period and data were statistically significant at P=0.05. Effect of AMF and

Table 1 Influence of *Glomus fasciculatum* and *Pseudomonas striata* on root colonization, spore density, stem height, number of leaves, and dry weight of Azadirachta *Indica* under nursery conditions.

Treatment	*No. of days*	*% root colonization*	*No. of AMF spores/ 50g of soil*	*Stem height* (cm)	*No. of leaves*	*Total dry weight* (mg/plant)
Control	30	–	–	5.18[c] ± 0.106	4.3 [c] ± 0.260	74.3 [c] ± 0.263
	60	–	–	6.46[c] ± 0.040	6.4 [c] ± 0.371	155.5 [b] ± 0.342
	90	–	–	7.80[b] ± 0.157	8.8 [c] ± 0.221	201.9 [c] ± 0.251
	30	25 ± 0.472	56 ± 1.248	8.41[b] ± 0.072 (62.36)	8.3 [a] ± 0.260 (93.02)	98.6 [b] ± 0.234 (32.71)
Glomus fasciculatum	60	35 ± 0.944	68 ± 2.162	11.81 [a] ± 0.136 (82.95)	10.7 [b] ± 0.260 (67.19)	285.5 [c] ± 0.265 (83.60)
	90	45 ± 0.944	89 ± 1.416	15.05 [b] ± 0.151 (92.98)	13.65 [b] ± 0.334 (55.11)	342.4 [a] ± 0.214 (69.59)
P. striata	30	–	–	7.72 [b] ± 0.181 (49.03)	6.8 [b] ± 0.243 (58.14)	94.88 [c] ± 0.223 (27.69)
	60	–	–	10.84 [b] ± 0.144 (67.80)	8.7 c ± 0.266 (35.94)	281.4 [a] ± 0.211 (80.96)
	90	–	–	14.01[b] ± 0.154 (79.62)	11.8 [b] ± 0.243 (34.09)	331.4 [b] ± 0.172 (64.14)
Glomus fasciculatum + P. striata	30	45 ± 0.144	65 ± 1.248	10.42 [a] ± 0.145 (101.16)	10.4 [a] ± 0.344 (141.86)	105.4 [b] ± 0.145 (41.86)
	60	55 ± 0.148	85 ± 2.162	13.37 [a] ± 0.247 (106.97)	13.2 [a] ± 0.139 (106.25)	308.3 [a] ± 0.185 (98.26)
	90	85 ± 0.188	105 ± 1.416	18.45 [a] ± 0.133 (136.54)	16.4 [a] ± 0.134 (86.36)	415.4 [a] ± 0.194 (105.75)

AMF - Arbuscular mycorrhizal fungi

Each figure represents the mean of ten replicates; ±SE values are mean of ten observations;

Figures in the parantheses denote per cent increase over the control; values without common letters differ significantly at LSD=0.05

P. striata inoculation statistically increased the stem height of *A. indica* plants when compare with control. Maximum (136.54%), (92.98%), and (79.62%) increase in stem height was registered in the plants raised after dual inoculation with *G. fasciculatum + P. striata* and single inoculation of *G. fasciculatum* and *P. striata* in 90 days of plant growth, respectively. A higher number of leaves (16.4) were recorded in the plants raised after dual inoculation of *G. fasciculatum + P. striata* after 90 days of plant growth followed by *G. fasciculatum* (13.65) and *P. striata* (11.8) in individual inoculation. Similarly, variable enhancement compared to the control was also noticed in total dry weight ranging from 27.69% to 41.86%, 80.96% to 98.26%, and 64.14% to 105.75% in 30 days, 60 days, and 90 days of plant growth, respectively, after inoculation of AMF and *P. striata* singly and/or dually. As evident from Table 1, the highest dry weight (415.4 mg/plant) was recorded in *G. fasciculatum* and *P. striata* inoculated plants. A statistically significant difference in dry weight compared to control was found in the *A. indica* plants raised after singly and/or dually treated plants.

Seed inoculation with *P. striata* together with soil inoculation with AMF increased the NPK uptake in *A. indica*. Inoculation of soil with *G. fasciculatum* resulted in an increase in the percentage of NPK over control. However, the results were not significant further. The seed inoculation with *P. striata* in conjunction with soil inoculation with *G. fasciculatum* produced significantly higher NPK uptake than the soil inoculation with AMF alone. The interaction of both the organisms brought about a significant increase in nutrient uptake by the plant (Table 2).

Overall assessment of the data indicates that inoculation of AMF and/or in dual combination with *P. striata* caused an increase in stem height, number of leaves, biomass, shoot NPK, AMF infection in roots, and population in soil of *A. indica*. Dual inoculation of *G. fasciculatum* and *P. striata* brought maximum improvement in the studied parameter compared to the other single inoculations.

On the basis of the aforementioned results, it can be concluded that single inoculation of AMF or bacterium did not significantly enhance plant growth, but dual inoculation of AMF and bacteria

Table 2 Influence of *Glomus fasciculatum* and *Pseudomonas striata* on NPK status of Azadirachta indica under nursery conditions

Parameter/ treatment	*No. of days*	*Nitrogen* (mg/plant)	*Phosphorus* (mg/plant)	*Potassium* (mg/plant)
Control	30	0.85[c] ± 0.043	0.181[c] ± 0.094	0.910[c] ± 0.120
	60	3.95[c] ± 0.243	0.385[c] ± 0.043	3.620[c] ± 0.095
	90	5.04[c] ± 0.970	0.641[c] ± 0.067	4.650[b] ± 0.093
Glomus fasciculatum	30	2.19[b] ± 0.029 (157.65)	0.458[a] ± 0.085 (153.04)	2.12[a] ± 0.058 (132.97)
	60	6.15[b] ± 0.141 (55.70)	0.656[b] ± 0.093 (70.39)	6.041[b] ± 0.690 (66.88)
	90	7.66[b] ± 0.041 (51.98)	0.914[c] ± 0.131 (42.59)	7.455[b] ± 0.071 (60.32)
P. striata	30	7.98[b] ± 0.145 (132.94)	0.425[a] ± 0.094 (134.81)	2.10[a] ± 0.456 (130.77)
	60	5.91[b] ± 0.149 (49.62)	0.645[b] ± 0.0.67 (67.53)	6.019[ba] ± 0.098 (66.27)
	90	6.34[b] ± 0.089 (25.80)	0.814[c] ± 0.139 (26.99)	7.310[b] ± 0.075 (57.20)
Glomus fasciculatum	30	2.43[b] ± 0.133 (185.88)	0.554[a] ± 0.143 (206.08)	2.25[a] ± 0.195 (147.25)
+ P. striata	60	7.45[a] ± 0.199 (88.61)	0.759[a] ± 0.083 (97.14)	7.14[a] ± 0.119 (97.24)
	90	8.96[a] ± 0.143 (77.78)	1.41[a] ± 0.591 (119.97)	8.744[a] ± 0.079 (88.04)

NPK – nitrogen, phosphorus, and potassium
Each figure represents the mean of three replicates; ±SE values are mean of three observations;
Figures in the parantheses denote per cent increase over the control; Values without common letters differ significantly at LSD=0.05

significantly enhanced plant growth characteristics. The present study also confirmed that significant combinations (AMF and PSB) can be used as biofertilizer for improved production of *A. indica*. Hence, this dual inoculation technology will be useful in successful afforestation and waste land management programmes where neem is extensively included.

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Arbuscular mycorrhizal fungi in the reclamation and restoration of soil fertility

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Introduction

Wastelands remain unexploited due to scarce marginal soil, severe drought conditions, and various anthropogenic activities. Such sites usually support xerophytic vegetation, which can adapt to harsh soil conditions. Present studies were conducted to evaluate the role of VA (vesicular-arbuscular) mycorrhizal fungi in adaptation of xerophytic vegetation in wastelands, which may ultimately help in the reclamation of wastelands by restoration of soil fertility.

Materials and methods

The research work was carried out at a site in Chunkankadai Hills supporting thin, xerophytic vegetation. It is located in Kanyakumari district, the southern most part of the state of Tamil Nadu. The wasteland under study has *Cymbopogon flexuous s* Nees ex Steud, *Jatropha glandulifera* Roxb and *Calotropis procera*, R. Br., as dominant vegetation. AM (arbuscular mycorrhizal) fungal association and spore content of the rhizosphere soil of the pioneer plants and two tuber yielding plants, *Dioscorea alata* and *Manihot esculenta*, were examined during this study.

Soil samples from the root zone of each plant species grown in different locations of the site were randomly collected; physical and chemical properties of the soil such as texture, pH, electrical conductivity, available nitrogen, phosphorus, and potassium were determined by standard analytical methods (Jackson 1973).

Rhizosphere soil samples with tender roots of plants were collected randomly. The first sampling was done in August 2003, and the second in February 2004. Roots were separated, washed, and cut into small segments, cleared in KOH (potassium hydroxide), and stained with trypan blue (Phillips and Hayman 1970). VA mycorrhization of each plant species was determined by estimating the percent root colonization (Giovannetti and Mosse 1980).

For AM fungal spore enumeration, 100 g of the substratum was dispersed in 1 litre water. After 15 minutes, the suspension was decanted through 710 and 48 µm sieves, and the remains on the sieves were washed into beakers. The supernatant was filtered through grided filter papers. Each filter paper was spread onto a micro-slide and observed under a dissecting microscope at x40 magnification. The intact AMF spores were counted. The AM fungal spores were picked up with a wet needle and mounted in poly-vinyl alcohol lactophenol on a micro-slide for identification.

Table 1 Soil characteristics of the study site

Parameter	*Unit*	*Mean value*
Mechanical composition	Percentage	
Coarse sand	”	52
Fine sand	”	20
Silt	”	18
Clay	”	10
Texture		Yellowish red sandy clay
pH		5.9
Organic carbon	Percentage	0.32
Available nitrogen	Kg/ha^{-}	152.8
Available phosphorus	”	38.5
Available potassium	”	192.5

The intact and the crushed spores were examined under a compound microscope and identified according to Schenck and Perez (1987).

Results and discussion

Jatropha glandulifera Roxb is a small evergreen plant of 4–8 feet, with a stout trunk and 3–5 lobed glandular leaves. *Cymbopogon flexuosus* Nees ex Steud is a perennial grass, while *Calotropis procera*, R. Br., is an erect shrub with a normal root system. VA mycorrhizal association was observed in all the plants examined. Vesicles, arbuscules, and extramatrical spores characteristic of AMF were observed in the roots. The highest amount of AM colonization was found in roots of Cassava (80%) and the lowest in *Calotropis* (47%) (Table 2). The roots of introduced plants showed appressoria, well developed arbuscules, and vesicles of various shapes. Both thick walled and thin walled hyphae were observed. In *Dioscorea* species, arbuscules showed deeply stained bead like aggregations. Degenerate arbuscules were seen with yellow pigmentation. Cassava showed small vesicles of varied shapes.

The pioneer plant *Cympopogon flexuosus* produced finely branched roots. Root squash showed extensive AMF hyphae, uniformly thin, running parallel to the long axis. Extramatrical hyphae were more abundant. Penetration points more then 10/mm were observed. These plants thrived well under extreme conditions, and survival capacity was directly proportional to the extent of AMF mycorrhization. Examination of rhizosphere soils of all plants

revealed the presence of a large population of extramatrical spores of AM fungi (Table 2).

The largest population of AM spores was observed in the rhizosphere soil of native *Cymbopogon* (450 spores/100 g soil). The average spore count in the rhizosphere soils of all the five plants was 280 spores/100 g soil. Spores of seven AMF – namely, *Acaulospora scrobiculata* Trappe, *Glomus aggregatum* Schenck and Smith emend Koske, *G. mosseae* (Nicol. and Gerd) Gerd and Trappe, *G. geosporum* (Nicol. and Gerd) Walker, *Gigaspora margarita* Becker and Hall, *Sclerocystis* sp., and *Scutellospora pellucida* (Nicol. and Schenck) Walker and Sanders – were observed.

The study site was a unique habitat where the vegetation was adapted to stress arising from marginal soil and anthropogenic activities. The results of the present investigation indicate that the dominant vegetation of the area had significant AMF association. The most important beneficial effect of mycorrhizal association is its role in the increased uptake of certain nutrients, especially phosphorus by plants. Soil pH plays an important role in phosphorus availability in soil and uptake by plants (Wang, Stribley, Tinker, *et al.* 1985). Soil pH, thus, has significant importance in VA mycorrhizal symbiosis and distribution of AMF (Janardhanan, Abdul-Khaliq, Naushin *et al.* 1994). The study site became unexploited due to scarce marginal soil and drought. Among the AMF associated with the plants of the area, *Glomus* constitute the dominant genus. *G. geosporum* and *G. aggregatum* could thrive well under stressful conditions.

Plants belonging to the *Poaceae* family were found to be extensively associated with AMF under stressful and ecologically unfavourable conditions (Stahl, Williams, and Christian 1988). Present observations support this findings. According to Dodd (2000), AMF are primarily responsible for nutrient transfer from soil to plant, soil aggregation, and protection of plants against drought stress. This study also confirmed the possible role of VAM fungi in the restoration of soil fertility of unexploited marginal soil. Mycorrhizal networks might link many of the plants within this habitat because of the general lack of host specificity of these fungi.

In *Cymbopogon*, extensive and finely branched roots with abundant AMF greatly influenced the survival capacity of the grass during stressful conditions. AM propagules and its inoculation in drought tolerant root crops help in the efficient utilization of marginal soils and, subsequently, its restoration.

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Table 2 Native AMF found associated with plants under study

Name of the plant	Family	AMF Colonization HC	AC	VC	Spore No 100/g soil	AMF species
Jatropha glandulifera	*Euphorbiaceae*	51.32 ± 2.3	13.92 ± 1.31	11.38 ± 3.1	210.00	*Acaulospora scrobiculate* Glomus aggregatum
Cymbopogon flexuosus	*Poaceae*	62.36 ± 5.2	15.18 ± 1.36	20.86 ± 3.05	450.50	G. mosseae G. geosporum
Calotropis procera	*Asclepiadaceae*	47 ± 5.1	27.38 ± 5.18	5.17 ± 3.95	250.4	Gi. margarita *Sclerocystis* Sp
Manihot esculenta	*Euphorbiaceae*	80.0 ± 7.5	36.27 ± 5.63	22.15 ± 2.13	365.75	Scutellosproa pellucida
Dioscorea alata	*Dioscoreaceae*	71.98 ± 5.01	18.96 ± 3.21	32.55 ± 2.50	143.75	

AMF – arbuscular mycorrhizal fungi; HC – hyphal colonization; AC – arbuscules; VC – vesicles

Effect of bio-inoculant organisms on growth and yield of *Coleus forskohlii* Briq.—an endangered medicinal plant

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Material and methods

The investigation was carried out at the Department of Medicinal and Aromatic Plants, Kittur Rani Channamma College of Horticulture, Arabavi during the year 2003/04. Seven AM (arbuscular mycorrhiza) fungal species, *viz*., *Glomus intraradices*, *G. fasciculatum*, *G. monosporum*, *G. mosseae*, *Sclerocystis dussii*, *Gigaspora margarita*, and consortia-I formed the eight treatments along with control. Consortia-I consisted of *Azotobactor chroococcum*, *Azospirillum brassilence*, *Pseudomonas striata*, and *Trichoderma harzianum*.

Completely randomized block design was followed with three replications. A spacing of 60 × 20 cm was followed. The crop was harvested after 150 days and various morphological and yield characters were recorded (Tables 1 and 2). Chlorophyll content in leaves was estimated as per the procedure given by Shoaf and Lium (1976).

Forskolin content (%) was estimated in dry tubers of different treatments using HPLC (high performance liquid chromatography).

Results and discussion

The plants inoculated with different bioinoculants showed more vigorous growth than the uninoculated control plants (Table 1). Among different treatments, establishment of cutting was better in consortia-I treated plants (91.66%) and minimum was in control (80%). As *Trichoderma harzianum* has

Table 1 Effect of AM fungi on growth characters in *Coleus forskohlii* Briq.

Treatment	*Percent establishment of cuttings*	*Plant height* (cm)	*Plant spread* (cm)		*No. of branches* per plant	*Leaf length* (cm)	*Leaf breadth* (cm)	*Inter-nodal length* (cm)	*Stem diameter* (cm)	*Petiole length* (cm)	*Total chlorophyll* (mg/g)
			East-West	*North-South*							
Glomus intraradices	85.00	52.66	52.80	55.20	58.40	5.54	2.58	1.23	2.57	1.16	0.39
Glomus fasciculatum	86.66	53.40	50.63	56.96	57.66	5.82	2.45	1.24	2.26	1.17	0.37
Glomus monosporum	83.33	50.73	51.86	51.20	52.50	5.37	2.27	1.26	2.40	1.22	0.43
Glomus mosseae	91.66	56.76	56.00	52.50	52.50	6.12	2.76	1.58	2.35	1.32	0.61
Gigaspora margarita	91.66	61.23	55.16	52.00	55.83	6.24	2.70	1.57	2.34	1.33	0.24
Sclerocystis dussii	90.00	57.76	53.50	53.46	53.50	5.76	2.60	1.41	2.33	1.32	0.30
Consortia-I	93.33	58.36	56.16	52.36	53.46	5.59	2.56	1.54	2.35	1.29	0.21
Control	80.00	49.23	54.30	54.16	48.13	5.39	2.41	1.26	2.24	1.17	0.41
Mean		55.02	54.17	53.48	54.00	5.73	2.54	1.38	2.35	1.25	
S.Em±	2.530	1.406	1.190	1.122	0.769	0.113	0.080	0.047	0.020	0.031	0.003
C.D. at 5%	7.673	4.265	3.609	3.402	2.334	0.343	0.242	0.144	0.062	0.094	1.24
CV (%)	5.00	4.43	3.80	3.63	2.47	5.91	5.43	5.91	1.50	4.30	–

pathogen controlling activities (Sukhada 1999) and plant growth promoting substances (Crafts and Miller 1974), the consortia-I inoculated plants showed better rooting.

Plants inoculated with *Gigaspora margarita* had maximum plant height (61.23 cm) and leaf length (6.24 cm); the influence on leaf breadth and internodal length was also good, followed by consortia-I, *Glomus mosseae,* and *Sclerocystis dussii*. The plants inoculated with *Glomus mosseae* recorded the highest plant chlorophyll (0.61 mg/g), leaf breadth (2.76 cm), internodal length (1.58 cm), and also had a good influence on plant spread, leaf length, and petiole length, which was on par with *Gigaspora margarita* and consortia-I over uninoculated plants. Increased plant height with application of *Glomus mosseae* has been reported by Boby and Bagyaraj (2003) in *Coleus forskohlii*.

Among different treatments, plants inoculated with consortia-I, *Gigaspora margarita*, and *Sclerocystis dussii* exhibited significant influence on yield attributes (Table 2). The plants inoculated with consortia-I recorded maximum length of tuber (13.52 cm), number of tubers (16.8), and also had a good influence on fresh tuber weight, dry tuber weight, and tuber diameter over uninoculated ones.

In the present experiment, the fresh weight of tubers per plant (g) and dry weight of tubers per plant (g) were found to be higher in the plants inoculated with *Gigaspora margarita* and consortia-I, which also had good influence of tuber diameter and tuber length, compared to the uninoculated plants. An increase in dry tuber yield of more than 25% was recorded due to the application of these organisms. This is mainly due to higher expression of morphological characters in inoculated plants, which might have, in turn, helped in higher photosynthesis and better accumulation of dry matter. Increased economic yields were reported in different crops due to the application of bio-inoculant organisms by other workers in chilli (Bagyaraj and Sreeramulu 1992) and vetiver (Neelima, Gautam, and Verma 2002).

Bio-inoculant organisms are known to produce growth-promoting hormones (Allen, Moove, and Christensen 1980; Azcon, Azcon-Aguilar, and Barea 1978) and to increase the availability of micronutrients like copper, manganese, iron, magnesium, and zinc in addition to higher phosphorus-uptake (Sreenivasa 1992; Gurumurthy and Sreenivasa 1996; Adivappar, Patil, Patil, *et al.* 2004). A similar beneficial effect of consortia-I could be attributed in the present study also for the increased dry weight of tuber per plant in the treated plants. Similar results were reported by Earanna, Malikarjuniah, Bagyaraj, *et al.* (2001) in *Coleus aromaticus*, Priyarani, Aggarwal, and Mehrotra (1999) in *Acacia nilotica,* and Abdul–Khaliq, Gupta, and Anskumar (2001) in peppermint.

Among the different treatments where forskolin estimation was done (Table 2), the forskolin content and yield was the maximum in consortia-I (0.403%

Table 2 Effect of AM fungi on tuber yield and forskolin content in *Coleus forskohlii*

Treatment	*Number of tubers per plant*	*Length of tubers* (cm)	*Diameter of tubers* (cm)	*Fresh tuber yield*		*Dry tuber yield*		*Forskolin content* (%)	*Forskolin yield* (mg/plant)
				(g/plant)	(q/ha)	(g/plant)	(q/ha)		
Glomus intraradices	9.26	11.33	1.25	107.78	89.82	14.08	11.85	–	–
Glomus fasciculatum	11.60	11.03	1.27	120.74	100.62	15.78	13.15	0.329	19.30
Glomus monosporum	10.73	11.24	1.25	126.26	105.21	16.43	13.69	–	–
Glomus mosseae	9.53	11.54	1.32	125.40	104.48	16.39	13.65	–	–
Gigaspora margarita	10.53	13.28	1.36	160.09	133.40	20.92	17.36	0.307	17.28
Sclerocystis dussii	13.53	13.42	1.37	133.06	110.89	17.39	14.62	0.274	15.56
Consortia-I	16.80	13.52	1.34	159.46	132.89	20.85	17.35	0.403	26.07
Control	9.80	10.38	1.30	127.60	106.33	16.69	13.91	0.330	20.75
S.Em±	1.119	0.576	0.020	7.01	5.84	0.94	0.80	–	–
C.D. at 5%	3.393	1.746	0.061	21.25	17.70	2.86	2.44	–	–
CV (%)	4.709	2.424	0.085	9.16	9.15	9.43	9.65	–	–

and 26.07 mg/plant), followed by *Gigaspora margarita* (0.307% and 17.28 mg/plant), and *Sclerocystis dussii* (0.274% and 15.56 mg/plant), as against control (0.33% and 20.75 mg/plant). The application of consortia-I recorded an increase of more than 22% of forskolin over control.

The present investigation thus confirmed the role of microbial consortia-I and AM fungi in the improvement of both tuber yield and forskolin content in *Coleus forskohlii*.

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New approaches

Sustained in-vitro pine root development

Oliveira P, Barriga J, Cavaleiro C, Peixe A, and Potez A Z (2003) succeeded in sustained in-vitro root development of stone pine (*Pinus pinea*) by the use of ectomycorrhizal fungi (Forestry Proceedings 76 (5): 579–587, 2003). Stone pine is an economically important forest tree that grows in Mediterranean climate and has been the target for selection efforts through micro propagation. Previous attempts on micro shoots, derived from mature seed cotyledons, reached incipient rooting after induction with a combination of auxin and hypertonic shock, but their development in-vitro was not sustained. At this stage, the authors succeeded in overcoming this barrier by co-culturing plantlets with some fungi isolated from the ectomycorrhizae, enabling satisfactory development in vermiculite and later in soil. About half the fungal isolates tested helped the plants resume root growth. Although control plants (in the absence of the fungi) developed roots at a later stage–that is, during the post-transplanting acclimation in vermiculite–their growth was weaker. The root systems of some inoculated plants had ectomycorrhizae from the introduced fungi being carried over when the plants were transferred from the co-cultures to vermiculite.

In conclusion, co-cultured rooted micro shoots with ectomycorrhizal fungi can be an effective means to overcome the difficulties encountered in the use of micro propagation methods on this species.

Centre for Mycorrhizal Culture Collection

Functioning and relevance of heavy metal uptake and translocation by mycorrhizal fungi

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Introduction

Heavy metal, by definition, refers to the metallic elements with a specific mass higher than 5 g/cm^3, and the ability to form sulphides (Adriano 1986). Trace element would, therefore, be a more correct form since the latter is based only on concentration (less than 0.1% in the soil or 100 mg/kg in dry matter of biological samples). However, the term 'heavy metal' is generally used and accepted in environmental studies. The toxicity of metals in the soil is dependant on their bioavailability, defined as their ability to be transferred from the soil compartment to a living organism (Juste 1988). According to Berthelin, Munier-Lamy, and Leyval (1995), metal bioavailability is a function not only of their total concentration, but also their physio-chemical (pH, Eh, organic matter clay content) and biological (for example, biosorption, bioaccumulation, and solublization) factors. Soil microorganisms, including mycorrhizal fungi, are affected by the presence of high metal concentration in the soil (Giller, Witter, and Macgrath 1998). But the organisms, in turn, influence the availability of the metals in soil either directly, through alterations of pH, Eh, biosorption or uptake, or indirectly in the rhizosphere through their effect on plant growth, root exudation, and resulting rhizosphere chemistry. Because plants function as the principle entry point of heavy metals into the food chain leading to animal and man (Rauser 1990), it is very important to analyse the distribution of various heavy metals in plants. A unique trait of mycorrhizal fungi is that they form a direct link between the soil and the root, providing a path for movement of elements in the rhizosphere. These aspects of interaction between mycorrhizae and heavy metals in soil are the topic of this article.

Heavy metal uptake by arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi are the root symbionts found in most plant species and habitats (Harley and Smith 1983). While the enhanced absorption of trace metals (Zn, Cu, Co) from deficient or non-enriched soils by AM (arbuscular mycorrhizal) plants has been documented (Faber, Zasoski, Burau, *et al*. 1990), relatively little attention has been paid to the role of AM fungi in metal contaminated soils.

Arbuscular mycorrhiza has been shown to improve plant tolerance to heavy metal stress in polluted soils (Leyval, Haselwandter, and Tarnau 1997). Glomelean hyphae may contribute directly to the uptake and translocation of metals to the host roots, including micronutrients such as Cu and Zn, as well as the toxic element Cd (Burkert and Robson 1994; Li, Marchner, and George 1991b; Leyval, Haselwandter, and Tarnau 1997). AM fungi may sequester toxic elements, thereby reducing their availability to the plants (Kaldorf, Khun, Schroder, *et al*. 1999; Turnau, Kottke, and Oberwinkler 1993). Such metal sequestration may alter metal translocation patterns in plants, leading to metal accumulation in the mycorrhizal roots and reduced metal transfer to the above ground biomass (Dehn and Schuepp 1989; Shetty, Hetrick, Figge, *et al*. 1994).

Uptake into hyphae may be influenced by adsorption on hyphal walls as chitin has a metal-binding capacity. More indirect effects of AM fungi on rhizospheric soil include changes in pH (Li, Marchner, and George 1991b), microbial communities (Olsson, Francis, Read, *et al*. 1998), and root exudation patterns (Laheurte, Leyval, and Berthelin 1990)—factors that are known to influence metal mobility or availability. Evidence for heavy metal-binding has also been shown in AM fungi (Turnau, Kottke, and Oberwinkler 1993).

Since AM fungi cannot be cultivated without a host plant, metal tolerance cannot be evaluated through fungal growth on axenic media. Hence, the mechanism of heavy metal tolerance in AM fungi has not yet been elucidated. Co-tolerance to Cd and Zn was suggested from one isolate from a soil polluted with long-term application of Zn through sewage sludge. Increased metal tolerance has also been observed for AM fungi isolated from a soil naturally high in heavy metals (Weissenhorn, Glashoff, Leyval, *et al*. 1994).

Heavy metal uptake by ectomycorrhizal fungi

Since many ectomycorrhizal fungi can be grown in axenic culture, metal tolerance has been tested as mycelial growth on axenic media containing increasing concentrations of heavy metal (Ray, Tiwari, Reddy, *et al*. 2005). In ectomycorrhizae, there are numerous examples of reduced metal uptake relative to non-mycorrhizal controls (Colpaert and Vanassche 1993; Denny and Wilkins 1987; Marchner, Goldbold, and Jentschke 1996), although there are cases where no amelioration occurs (Jentschke, Fritz, and Goldbold 1997; Jones and Hutchinson 1988a,b). Again, fungal sorption could be a major mechanism, but complexion by elements (P and S) enriched in or on mycorrhizal structures (Marchner, Jentschke, and Goldbold 1998) or specific proteins (Howe, Evans, and Ketteridge 1997) and a dilution effect due to enhanced nutrition and plant growth in mycorrhizal plants may also explain some of the differences.

Heavy metal uptake by ectomycorrhizal fungi grown on fly ash amended media clearly indicated that high uptake ability for one metal does not imply high uptake ability for other metals by ectomycorrhizal fungi. Therefore, it can be said that a strong interspecfic and intraspecfic variation exists in metal tolerance by ectomycorrhizal fungi (Ray, Reddy, Lapeyrie, *et al*. 2005).

Heavy metal translocation by mycorrhizal fungi

There has been considerable interest in the potential use of arbuscular mycorrhiza in agricultural systems. Experiments have shown that mycorrhizal effects can be explained by mechanisms such as enlargement of the absorbing area and volume of accessible soil, decreased soil pH near the fungal hyphae due to exudation of organic acids and other ions, a high affinity to P on the membranes of arbuscular mycorrhizal fungi and efficient hyphal transport in the form of polyphosphate, and utilization by mycorrhiza of P sources that are unavailable to plant roots (Smith and Read 1997). In the similar line, transport of mineral elements into plants through the hyphae of AM fungi has been demonstrated for P (Li, Marchner, and George 1991a,b), N (Ames, Reid, Porter, *et al*. 1983), Zn (Kothari, Marchner, and Romheld 1991; Burkert and Robson 1994), Cu (Li, Marchner, and George 1991b), Cd (Joner and Leyval 1997), and Fe (Caris, Hordt, Hawkins, *et al*. 1998). For other elements, although the uptake of Sr, Co, and Cs were found to be increased by AM colonization (Jackson, Miller, and Franklin 1973; Rogers and Williams 1986), the contribution of the hyphae of the AM fungus to the uptake of these elements has not been elucidated to a great extent. Suzuki, Kumagari, Oohashi, *et al*. (2001) suggested that hyphae of the AM fungal could absorb Zn, Na, Rb, Se, Sr, and Y from the soil and transport these elements to the plants. They further suggested that the absorption and transport ability of Be, Sc, Cr, Mn, Fe, Co, Zr, and Tc by AM fungus hyphae may be low.

Since AM fungi cannot be cultivated without a host plant, it is more difficult to demonstrate the intrinsic metal uptake by their hyphae. Using culture systems with separate extra radical hyphae from roots, it has been shown that extra radical hyphae can accumulate and translocate 65Zn that may differ between species (Cooper and Tinker 1978). Adding 109Cd to a hyphal compartment, Joner and Leyval (1997) showed that extraradical hypahe may transport Cd from soil to roots.

Functioning and relevance

Heavy metals cannot be chemically degraded. Therefore, remediation of metal polluted soils is mainly limited to immobilization or extraction/ concentration techniques. Among remediation options for metal contaminated sites,

phytoremediation methods have recently attracted much attention (Anderson and Coats 1994). The principle of phytoremediation methods is to promote plant growth, reduce or eliminate bioavailability of metals, minimize wind and water erosion, improve soil quality, and reduce leaching of metals. Treatments include appropriate fertilization, either reduction in metal availability using different amendment and/or using metal tolerant plant species.

Ectomycorrhiza has been proposed as a bioindicator of air pollution (Fellner 1989). Fungal fruit bodies are potentially useful as bioindicators of radioactive contaminants of the environment (Berreck, Ohenoja, and Haselwandter 1992). The suitability of wild growing mushrooms, including ectomycorrhizal species as bioindicators of heavy metal pollution, has been investigated (Gast, Jansen, Bierling, *et al*. 1988). A study carried out in the Chernobyl area of Ukraine revealed a close correlation between the radiocesium content in the area where the fruit bodies are collected (Grodzinskaya, Berreck, Wasser, *et al*. 1995). This underlines the potential use of fungal fruit bodies as bioindicators for the heavy metal pollution of the biosphere as long as the number of samples per species and the species collected are representative.

Like ectomycorrhizal fungi, AM species have potential which can be employed in biomonitoring programmes. The decline of AM fungal occurrence and infectivity in metal polluted soils can be used as bioindicators of soil contamination (Leyval, Sing, and Joner 1995). Mycorrhizal colonization of plant roots after soil remediation can be a sign that the metal concentration or bioavailability has decreased. Since metal tolerance evolves in some fungi from metal contaminated soils, a sensitive AM fungi can be used and tested for its ability to colonize roots in the metal polluted soils, providing useful information about their metal toxicity. In various investigations on the long-term effect of metals on AM fungi in arable fields where metal contamination sludge has been applied for ten years, preliminary results have shown that the diversity of AM fungi changes even at low metal concentrations.

The use of mycorrhizal fungi as bioremediation agents has been reviewed by Donnelly and Fletcher (1994). Mycorrhizal fungi appear to partially protect plants against the toxicity of heavy metals. On the other hand, the host plant may give the fungus a selective survival advantage at a contaminated site. This mutual benefit would make this mycorrhizal association superior to the application of single organisms, either non-mycorrhizal plants or free living microorganisms, for bioremediation purposes.

Conclusion

To improve the understanding of the interactions between the heavy metals, mycorrhizal fungi, and roots in contaminated soils, parameters like the tolerance of the mycorrhizal fungi and host plant, the nutritional status of both organisms, soil properties of the metals and their specific behaviour should be considered. The extent to which mycorrhizal fungi can alleviate metal toxicity to plants under field conditions remains to be established.

The protective effect of ectomycorrhizal and ericoid mycorrhizal fungi against metal toxicity in plants is quite clear and various mechanisms have been demonstrated, the main one being fungal structures acting like barriers to metal uptake in plants. More work remains to be done on AM fungi to understand the mechanism involved in metal immobilization in the hyphae inside and/or outside the roots. The compartment system mentioned already is a promising approach for such studies. Relatively large amounts of extraradical hyphae can be produced and separated from the roots, which should allow the study of metal adsorption and uptake by AM hyphae, transfer to the roots, and translocation to the shoots. The functioning of different fungi could be compared in such systems. Other experimental devices including normal or transformed roots might also be useful to study metal tolerance of AM fungi. Results with ectomycorrhizal fungi show metal specific tolerance mechanisms and competition between metals for uptake. This should also be investigated for all kind of mycorrhizae, and especially for the possible use of mycorrhizal fungi in phytoremediation.

There is no doubt that genetic engineering aimed at producing plants which are more tolerant and resistant to heavy metals may play an important role in the future. But such attempts must not overlook the possible changes in the susceptibility of the plants to mycorrhizal infection. A metal-resistance plant breed should still be susceptible to mycorrhizal symbiosis, and when colonized by a metal-resistant mycorrhizal fungus, genetically modified or derived from the natural resources, should be of great value for the rehabilitation of metal contaminated soils. The management of soil microorganisms including mycorrhizal fungi is a prerequisite for the success of future restoration programmes.

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Recent references

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*
- *American Naturalist*
- *Annals of Forest Science*
- *Applied Soil Ecology*
- *Aquatic Botany*
- *Basic and Applied Ecology*
- *Chemosphere*
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- *Environmental Microbiology*
- *Fems Microbiology Reviews*
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- *Microbiological Research*
- *Molecular Ecology Notes*
- *Molecular Phylogenetics and Evolution*
- *Mycological Research*
- *Mycorrhiza*
- *New Phytologist*
- *Plant Molecular Biology*

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

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Printed and published by Dr R K Pachauri on behalf of the The Energy and Resources Institute, Darbari Seth Block, IHC Complex, Lodhi Road, New Delhi – 110 003, and *printed at* Multiplexus (India), C-440, DSIDC, Narela Industrial Park, Narela, Delhi – 110 040.