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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, was established in 1974 as the Tata Energy Research Institute. While initially, TERI's focus was mainly on documentation and information dissemination, research in the fields of energy, environment, and sustainable development was initiated towards the end of 1982. The genesis of these activities lay in TERI's firm belief that 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.

The Biotechnology and Management of Bioresources Division

Focusing on ecological, environmental, and food security issues, the division's activities include working with a wide variety of living organisms, sophisticated genetic engineering techniques, and, at the grass-roots level, with village communities. The division functions through two areas—the Centre for Mycorrhizal Research, and Plant Tissue Culture and Molecular Biology. It is actively engaged in mycorrhizal research. The Mycorrhiza Network has specifically been created to help scientists across the globe in carrying out research on mycorrhiza.

The Mycorrhiza Network and the Centre for Mycorrhizal Culture Collection

Established in April 1988 at TERI, New Delhi, the Mycorrhiza Network first set up the MIC (Mycorrhiza Information Centre) in the same year and the CMCC (Centre for Mycorrhizal Culture Collection) – a national germplasm bank of mycorrhizal fungi – in 1993. The general objectives of the Mycorrhiza Network are to strengthen research, encourage participation, promote information exchange, and publish the quarterly newsletter *Mycorrhiza News*.

The MIC has been primarily responsible for establishing an information network, which facilitates sharing of information among the network members and makes the growing literature on mycorrhiza available to researchers. Comprehensive database on Asian mycorrhizologists and mycorrhizal literature (RIZA) allow information retrieval and supply documents on request.

The main objectives of the CMCC are to procure strains of both ecto and VA mycorrhizal fungi from India and abroad; multiply and maintain these fungi in pure culture; screen, isolate, identify, multiply, and maintain native mycorrhizal fungi; develop a database on cultures maintained; and provide starter cultures on request. Cultures are available on an exchange basis or on specific requests at nominal costs for spore extraction or handling.

Contents

Chitinases, chitin synthases, and utilization of chitin by mycorrhizal fungi

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Chitinases are commonly induced in plants upon attack of pathogens and by various sources of stress, which lead to association of them with the plant defence. Host defences are, however, less induced when plants are infected by mycorrhizal fungi than when plants are attacked by pathogenic fungi. This is because plant chitinases are able to inactivate elicitors from mycorrhizal fungi.

Main enzymes that synthesize fungal walls are chitin synthase and glucans synthetase. Chitin synthase is activated by protease and glucans synthetase is activated by guanosine triphosphate.

Chitinase activity in plants induced by ectomycorrhizal fungal infection

In studies conducted at the INRA (Nancy), Laboratoire de Microbiologie Forestiere, 54 280, Champenoux, France, chitinase activity patterns of *Eucalyptus globulus* ssp *bicostata* roots and shoots were investigated, following infection by nine *Pisolithus tinctorius* strains isolated from carpophores collected from under the *eucalyptus* trees in Australia. Strains have previously been classified into three groups (low, moderate, and high aggressiveness) based on the rate of mycorrhizal development in vitro. Non-aggressive isolates did not form a Hartig net. Aggressive isolates rapidly developed typical mycorrhizae with a thick mantle, a Hartig net, and radially elongated epidermal cells. Roots were contacted with the living fungal colonies as well as crude fungal extracts. Stimulation of root and shoot chitinase activity above that of uninoculated control was detected 24 hours after contact with all strains tested. In addition, 2, 4, and 7 days after inoculation, a definite relationship between stimulation of root chitinase activity and mycorrhizal infection could be established. Aggressive strains induced a significantly greater stimulation of chitinase activity than the poorly aggressive strains. In shoots, no close relationship could be established between aggressiveness of strains and intensity of chitinase activity. Stimulation of chitinase activity of roots following contact with living mycelium or fungal extracts indicates that fungal penetration between cortical cells cannot be in itself, responsible for chitinase induction (Albrecht, Burgess, Dell, *et al.* 1993).

In further studies conducted on the same subject at the above institute, chitinase and peroxidase activities were measured in roots of *E. globulus* ssp *bicostata* during colonization by *Pisolithus* sp isolated from under the *eucalyptus* trees. Ten fungal isolates from poor to good root colonizers were selected to represent a range of ectomycorrhizal aggressivity. Induction of chitinase and peroxidase activities was strongly related to aggressiveness of fungal strains. Only good colonizers, strains which rapidly formed differentiated ectomycorrhizae, induced a strong response in plant. It can, therefore, be concluded that enzymes are not responsible for poor root colonization by the less-aggressive strains. This suggests that a major component of differential induction observed in-vivo is the consequence of root colonization, tissue penetration, and ability to deliver elicitors to the plant prior to and during root colonization (Albrecht, Burgess, Dell, *et al.* 1994).

In further studies at the above laboratory, early changes in *E. globulus* ssp *bicostata* root and shoot chitinase activity patterns were investigated,

following contact with fungal colonies or fungal extracts of a symbiotic strain of *P. tinctorius* and pathogens as the previous observations strongly suggested that early events leading to recognition between symbionts could be closely related to those implicated in plant–pathogen interactions. Chitinases are well-known enzymes induced in many plants in response to abiotic and biotic stress, such as infection by pathogens. Chitinase activity was fully induced in roots and shoots 24 hours after contact between symbiotic hyphae and roots. Chitinase activity remained constant over seven days of symbiosis establishment. Chitinase electrophoretic patterns of seedlings in contact with pathogens or the symbiotic fungus differ only on 2-D gels. The patterns of activity were similar whether roots were in contact with fungal colony or treated with crude fungal extract. After 24 hours of contact on 1-D gels, the first band chitinase activity was stimulated by a factor of 2.5 following treatments with *P. tinctorius* extracts. The second bond responded much more intensely and its activity was increased 20-fold (Albert and Lapeyrie 1993).

In further investigations conducted at the above institute, a comparative study was made on activities induced by the ectomycorrhizal basidiomycete *P. tinctorius* and the pathogenic fungus *Phytophthora cinnamomi* on two chitinases constitutively present in *E. globulus* ssp *bicostata* roots. When seven-day-old seedlings were challenged with the ectomycorrhizal fungus, root chitinase activity was stimulated within six hours during the very early stages of ectomycorrhizal colonization. Comparing chitinase electrophoretic patterns induced by symbiotic strains more or less compatible with *eucalyptus*, a strong stimulation of the chitinase activity indicated successful interaction, which evolved quickly towards the root infection and mature mycorrhiza formation. Root chitinase activity remained constant over seven days during the establishment of symbiosis. No new chitinase band was induced by the pathogen when compared with the symbiotic fungus. Chitin activity was only stimulated quantitatively after pathogenic infection. Root chitinase activities were also stimulated by fungal cell extracts of both the species applied in vitro. Such stimulation mimicked precisely the stimulation by living fungi. The intensity of plant response to fungal extracts was related to the aggressiveness of the fungal strain (Albrecht, Asselin, Piche, *et al.* 1994).

In studies conducted at the Department of Plant and Soil Sciences and the Department of Molecular and Cell Biology, University of Aberdeen, Meston Building, Aberdeen, UK, four ectomycorrhizal basidiomycetes, namely *P. tinctorius, Paxillus involutus, Suillus variegatus, Boletinus cavipes*; two pathogenic root-infecting basidiomycetes, namely *Haterobasidium annosum, Armillaria ostoyae,* and one oomycete, namely *Phytophthora cinnamomi* were screened for chitinolitic activity in liquid culture. N-acetyl glucosaminidase, exo- and endo-chitinase activities were distinguished using fluorogenic 4-methylumbelliferone substrates. N-acetyl glucosaminidase was the prominent enzyme activity and endo-chitinase was the predominant chitinase activity. Haterobasidium and Armillaria had higher specific activities than the ectomycorrhizal fungi. The ectomycorrhizal fungi showed a variable enzyme activity and only one species *B. cavipes* showed activity against all substrates. Phytophthora showed no chitinolitic activity but only N-glucosaminidase activity. In general, chitinolitic activities were highest when chitin was present in growth medium and declined in presence of glucose. Diammonium phosphate did not repress the chitinolitic activities (Hodge, Alexander, Gooday 1993; 1995a).

In further studies conducted at the above University, chitinolitic activities of *Pinus sylvestris* and *Eucalyptus pilularis* roots were measured after an exposure for 24 hours to either ectomycorrhizal fungus *P. tinctorius* or root-infecting pathogenic fungi *Heterobasidium annosum* and *P. cinnamomi*. *P. sylvestris* roots not exposed to the fungal hyphae had low levels of N-acetyl glucosaminidase activity. This activity greatly increased after exposure to *H. annosum* but not after exposure to *P. cinnamomi* or *P. tinctorius*. N-acetyl glucosaminidase activity in *E. pilularis* roots was unchanged after exposure to *P. cinnamomi* or *P. tinctorius*. Exo- and endo-chitinase activities increased after *P. sylvestris* roots were challenged with *H. annosum* but not by other fungi. The results suggest that N-acetyl glucosaminidase might be an early response to chitinous pathogens (Hodge, Alexander, Gooday 1995b).

Studies at the Institute of Botany, University of Basel, Hebelstr 1, Basel, Switzerland, showed that rapid reactions comprising efflux of K^+ and Cl^-, phosphorylation of a 63 kDa protein (pp63), extracellular alkalinization, and synthesis of H_2O_2 were equally induced in cells of *Picea abies* (spruce) by chitotetraose, colloidal chitin, and cell wall elicitors from the ectomycorrhizal fungus *H. crustuliniforme*, an ectomycorrhizal partner of spruce. Cleavage of fungal cell wall elicitors and of artificial chitin elicitors to monomeric and dimeric fragments by apoplasmic spruce chitinases (36 – kDa class 1 chitinase pi 8.0 and 28 kDa chitinase pi 8.7, EC 3.2.1.14) equally prevented induction of these rapid reactions. Also, N-acetyl glucosamine oligomers and elicitors from fungal cell walls showed a similar dependence of activity upon the degree of polymerization. From these results, it was suggested that during ectomycorrhiza formation, only some of the chitin-derived elicitors reached their receptors at the plant plasma membrane, initiating reactions of hypersensitive response in host cells. The remaining fungal elicitors would be degraded to varying extents by wall-localized chitinases of host roots, reducing the defence reactions of the

plant and allowing symbiotic interactions of both organisms (Salzer, Hebe, and Hager 1997).

In further studies at the above institute, two chitinases (EC 3.2.1.14) and two beta-1, 3 glucanases (3.2.1.39) were purified from the culture medium of spruce (*P. abies*) cells to study their role in modifying elicitors, cell walls, growth, and hyphal morphology of ectomycorrhizal fungi. The 36-kD class I chitinase (isoelectric point pi 8.0) and the 28 kD chitinase (pi 8.7) decreased the activity of elicitor preparations from *H. crustuliniforme, Amanita muscaria,* and *S. variegatus* as demonstrated by using the elicitor-induced extra-cellular alkalinization in spruce cells as a test system. In addition, chitinases released monomeric products from walls of these ectomycorrhizal fungi. The beta 1-3 glucanases (35 kD, pi 3.7 and 3.9), in contrast, had little influence on the activity of fungal elicitors and released some polymeric products only from the walls of *A. muscaria.* Furthermore, chitinases alone and in combination with beta 1–3 glucanases had no effect on growth and morphology of hyphae. Thus, it is suggested that apoplasmic chitinases in the root cortex destroy elicitors from the ectomycorrhizal fungi without damaging fungus. By this mechanism, the host plant could attenuate the elicitor signal and adjust its own defence reactions to a level allowing symbiotic interaction (Salzer, Hubner, Sirrenberg, *et al.* 1997).

In studies conducted at the Department of Plant and Soil Science, University of Aberdeen, Aberdeen, UK, localization of chitinolytic activities in *Fagus sylvatica* (beech) mycorrhizae was examined using a range of fluorogenic 4-methyl umbelliferyl (4-MU-[GlcNAc] 1-4) substrates in order to distinguish between exochitinase, endochitinase, and beta-N-acetyl glucosaminidase activities. Validity of the technique was confirmed using onion epidermal cells. In beech mycorrhiza, endochitinase activity was not detectable above the background fluorescence. Exochitinase activity was detected in fungal sheath and Hartig net. Beta-N-acetyl glucosaminidase activity was also mainly associated with the fungal sheath and Hartig net. Individual fungal hyphae extending from these structures also showed substantial beta-N-acetyl glucosaminidase activity. Cortical cell walls of the host in the Hartig net region also fluoresced brightly. Localization of beta-N-acetyl glucosaminidase was confirmed using a choromogenic histochemical reagent S-bromo-4-chloro-3-indolyl-N-acetyl –beta – d – glucosaminide (Hodge, Alexander, Gooday, *et al.* 1996).

Chitinase activity in plants induced by VAM fungal infection

In studies conducted at the Abeteilung Pflanzenphysiologie, Botanisches Institute der Universite, Basel, Switzerland, chitinase (EC 3.2.14) activity was measured in roots of *Alliun porrum* during development of the VA-mycorrhizal symbiosis with *Glomus versiforme*. During the early stages of infection, between 10–20 days after inoculation, specific activity of chitinase was higher in mycorrhizal roots than in uninfected controls. However, 60–90 days after inoculation when symbiosis was fully established, mycorrhizal roots contained much less chitinase than control roots. Chitinase was purified from *A. porrum* roots. An antiserum against bean-leaf chitinase was found to cross react specifically with chitinase in extracts from the non-mycorrhizal and mycorrhizal *A. porrum* roots. This antiserum was used for immuno-cytochemical localization of the enzyme with fluorescent and gold-labelled probes. Chitinase was localized in vacuoles and in intracellular spaces of non-mycorrhizal and mycorrhizal roots. There was no immuno-labelling on fungal cell walls in intercellular and intracellular phases. It was thus concluded that chitin in the fungal walls was inaccessible to the plant chitinase. This cast doubts on the possible involvement of this hydrolase in the development of mycorrhizal fungus. However, fungal penetration did appear to cause a typical defence response in the first stages that was depressed later (Spanu, Boller, Alexander, *et al.* 1989; Spanu, Bonfante-Fasolo, and Boller 1990).

In further studies at the above university, transgenic *Nicotiana sylvestris* plants, expressing different forms of tobacco chitinase under control of the expression signals of the 35S transcript of the cauliflower mosaic caulimovirus were used to compare colonization of the root system by *Rhizoctonia solani* and *Glomus mosseae*. Plants of *N. sylvestris* expressing the vacuolar tobacco chitinase A or the same chitinase without the N-terminal chitin-binding domain were less colonized by *R. solani* than control plants and showed less reduction of the root fresh weight when exposed to an attack of this fungus. Plants of *N. sylvestris* expressing chitinase A without the C-terminal signal for vacuolar targeting showed no enhanced resistance as they were colonized by *R. solani* to the same extent as control plants. All transgenic *N. sylvestris* plants investigated were equally colonized by *G. mosseae*, indicating that enhanced resistance conferred by the constitutive expression of chitinase in transgenic plants did not interfere with VA-mycorrhizal symbiosis (Vierheilig, Alt, Neuhaus, *et al.* 1993).

In another study conducted at the above university, activities of chitinase and beta-1, 3 glucanase and ethylene production, two parameters often induced in plants in response to pathogenic fungi, were measured in roots of various plants after inoculation with *G. mosseae* and after mock inoculation in absence of the VA-mycorrhizal fungi. Tomato, a host plant for VAM fungi, was

compared in this respect with three species of non-host plants, namely rape, spinach, and lupin. In case of rape, two cultivars, Jet Nauf (rape 0) and Arabella (rape 00) were examined. In roots of tomato, spinach, and lupin, chitinase activity was not affected during the first days after inoculation but was depressed afterwards in comparison to the mock-inoculated controls. In roots of rape 0 and rape 00, chitinase was weakly induced upon inoculation. Levels of beta 1, 3-glucanase were not altered in tomato roots during the VA-mycorrhiza's initial stages after inoculation. In a later stage, most inoculated non-host plants showed less activity than mock-inoculated plants. It is thus concluded that both, host and non-host plants perceive VA-mycorrhizal fungus and respond to its presence. As different non-host plants showed different response patterns, it was suggested that none of the reactions studied could be taken as a general indicator for the inability of plants to entertain VA-mycorrhizal symbiosis (Verheilig, Alt, Mohr, *et al*. 1994).

Further studies were conducted at the above university to determine the response of bean (*Phaseolus vulgaris*) roots to infection by the VA-mycorrhizal fungus *G. mosseae* and the pathogen, *Fusarium solani* f. sp. *phaseoli* as the plant roots enter symbiotic as well as pathogenic interactions with fungi in rhizosphere. In a time course study of symbiotic interaction between bean roots and *G. mosseae,* covering all stages of mycorrhiza development, little change was detected in expression of defence-related genes, chitinase, beta 1, 3-glucanase, and phenylalanine ammonia lyase compared with non-mycorrhizal control roots. The only difference observed was a transient increase in chalcone synthase transcripts at a later stage of mycorrhizal root colonization. In interactions with pathogens, a marked induction of chitinase and phenyl alanine ammonia lyase expression was observed at the level of both, transcripts and enzyme activities. Class 1 beta-1, 3-glucanase levels strongly increased at the transcript level but there was little change in the overall beta-1, 3-glucanase enzyme activity. In non-host interactions between common bean and *F. solani* f.sp.*pisi*, defence responses increased only slightly and transiently, if at all (Mohr, Lange, Boller, *et al*. 1998).

In another study conducted at the above university, isoforms of endo chitinase in soybean were studied in relation to root symbiosis. Five selected cultivars differing in their nodulation potential were inoculated with two strains of *Bradyrhizobium japonicum*, the broad host range *Rhizobium* sp, NGR 234, and with the mycorrhizal fungus *G. mosseae*. Total chitinase activity in nodules was up to seven-fold higher than in uninoculated roots and in mycorrhizal roots. Chitinase activity in nodules varied depending upon the strain–cultivar combination. On semi-native polyacrylamide gels, four acidic isoforms were identified. Two isoforms (CH2 and On. 4) were constitutively present in all analysed tissues. The other two isoforms (CH 1 and CH 3) were strongly induced in nodules and were stimulated in mycorrhizal roots as compared to uninoculated roots. Induction of CH 1 varied in nodules depending upon the soybean cultivar. This isoform was also stimulated in uninfected roots when they were treated with tri-iodobenzoic acid, rhizobial lipochitooligosaccharides, and chitotetraose. On. 3 was not affected by these stimuli, indicating that this isoform could represent a marker for enzymes induced in later stages of the symbiotic interactions (Xie, Staehelin, Wiemken, *et al*. 1999).

Studies conducted at the Laboratoire de Phytoparasitologie INRA–CNRS, Station de Genetique et d'Amelioration des Plantes, Dijon, Cedex, France, showed that by using a two-dimensional PAGE system, one major additional bond with chitinase activity could be detected in leek (*Allium porrum*) root extracts after colonization by *G. versiforme* or *G. intraradices* when compared with root extracts of control plants. After separation under native conditions in the first dimension, the band was observed both in the Davis system (designed to separate native acidic or neutral proteins) and in the Reisfeld system (designed to separate basic proteins). After the second dimension in SDS (sodium dodecyl sulphate)–PAGE (polyacrylamide denaturing gels) under non-reducing conditions, its apparent MW was determined to be c. 30 kDa. In *G. fasciculatum*-colonized root extracts of onion (*Allium cepa*), four additional bonds with chitinase activity were found. The same additional bonds were obtained in onion roots colonized by other *Glomus* species. Among the four bonds, one of them was separated in the first dimension in both, the Davis system and the Reisfeld system. Two were only detected as acidic and neutral isoforms while the last one was present as a basic isoform. Their apparent MWs were estimated at 33, 35, and 50 kDa in VAM (vesicular arbuscular mycorrhiza)-colonized pea root extracts. One acidic chitinase isoform was strongly stimulated while the other acidic isoform was less highly induced in various mycorrhizal interactions. Their apparent MWs were 30 and 47 kDa. Chitosanase activities were detected in leek and onion roots colonized by different VAM fungi while no chitosanase activity was detected in either non-mycorrhizal or VAM-colonized pea root extracts. In VAM-colonized leek root, the main chitosanase activity had an estimated apparent MW of 20 kDa (Dumas-Gaudot, Grenier J, Furlann, *et al*. 1992).

In further studies conducted at the above laboratory, chitinase activities were compared in tobacco roots (*Nicotiana tabacum* cv. Ocanthi) infected by pathogenic fungus *Chalara elegans* or three species of VA-mycorrhizal fungi, namely *G. versiforme, G. intraradices,* and *G. fasciculatum*, using native PAGE (polyacrylamide gel electrophoresis).

All previously known acidic chitinase isoforms were stimulated in roots by pathogenic fungus and by VAM fungi while two new acidic chitinase isoforms were specifically induced in response to the VA-mycorrhizal association. After separation in SDS–PAGE under non-reducing conditions, the estimated apparent molecular mass for these additional acidic chitinase isoforms from VAM-colonized samples was 33 kDa compared to 30 kDa for the main activity stimulated in *C. elegans*-infected root extracts (Dumas-Gaudot, Grenier, Furlan, *et al*. 1992.

In another study conducted at the above laboratory, chitinases were studied in an endomycorrhizal-resistant mutant and a wild-type pea (*Pisum sativum*) in order to characterize plant hydrolases specific to pathogenic (*Aphanomyces euteiches* and *C. elegans*) or mycorrhizal (*G. mosseae*) root interactions. Stimulation of constitutive and induction of new chitinase activities were detected by native PAGE for acidic proteins in both pea genotypes inoculated with pathogenic fungi. In contrast, a different additional chitinase isoform was induced in *G. mosseae*-colonized roots. This isoform was also not elicited in chemically stressed roots, confirming its mycorrhizal specificity. Investigations of basic chitinase and beta 1, 3 – glucanase activities provided further evidence for differential pea responses during pathogenic and symbiotic interactions (Dassi, Dumas–Gaudot, Asselin, *et al*. 1996).

In studies conducted at the Faculty of Agriculture, Hebrew University of Jerusalem, Rehovot, Israel, chitinase along with CHI (chalcone isomerase) and PAL (phenyl alanine ammonia lyase) activities and flavonoid accumulation was followed during the early colonization of lucerne cv. Gilboa roots by *G. intraradices*. Chitinase and CHI activities increased in inoculated roots prior to colonization by VAM fungus whereas increase in PAL activity coincided with colonization. Formononetin, the early flavanoid, showed a consistent increase in inoculated roots. The increase depended only on the presence of fungus in plant rhizosphere and no colonization of root tissues was required. After reaching a maximum, activities of all enzymes declined to below those of uninoculated roots. PAL inactivation was not caused by a double inhibitor. It was concluded that VAM fungi initiated a host defence response in the lucerne roots, which was subsequently suppressed (Volpin, Elkind, Okon, *et al*. 1994).

Chitin synthases in VA-mycorrhizal fungi

In studies conducted at the University of Laval, Faculty of Foresterie and Geomat, Ctr Rech Biol Forestiere Pavillon Ce Marchand, St Foy, Canada, on activities of chitin synthase in VAM fungi, different concentrations of nikhomycin Z, a competitive inhibitor of chitin synthase, were applied to the AM fungi *G. margarita* and *G. intraradices* under in-vitro conditions. These two fungi were known to differ in structure and composition of their cell walls. The two AM fungi were able to grow in presence of higher concentrations of this antibiotic than so far reported for other fungi. Fluorescence, electron microscopic, and cytochemical studies showed that blocking of the fungal chitin synthase activity induced alteration in hyphal morphology, a reduction in fungal wall thickness, and several other changes in the hyphal wall structure and organization (Bago, Chamberland, Goulet, *et al*. 1996).

In studies conducted at the CNR, Centro de Studio Sulla, Mycologia del Terreno del CNR, University of Turin, Turin, Italy, chitin synthase genes of AM fungus *G. versiforme* were sought in an investigation of molecular basis of fungal growth. Three DNA fragments (Gvchs1, Gvchs2, and Gvchs3) corresponding to the conserved regions of distinct chitin synthase (chs) genes were amplified by means of PCR (polymerase chain reaction) with two sets of degenerate polymers. Gvchs1 and Gvchs3 encode two class I chitin synthase whereas Gvchs3 encodes a class IV chitin synthase. A genomic library was used to obtain the Gvchs3 complete gene (1193 amino acids), which shows a close similarity to the class IV chitin synthase of *Neurospora crassa* (Lanfranco, Garnero, and Bonfante 1999).

In further studies conducted at the Dipartimento Biol Vegetale of the above university, genomic DNA from spores of endomycorrhizal fungus *G. margarita* were amplified with two sets of degenerate primers designed on the conserved regions of chitin synthase polypeptides. Three chitin synthase (chs) gene portions: Gimchs 1, Gimchs 2, and Gimchs 3 were identified, encoding one class II and two class IV chitin synthases, respectively. Gimchs 1 and Gimchs 3 transcripts were detected by reverse transcription polymerase chain reaction (RT-PCR) during root colonization whereas none of the three mRNAs were revealed during spore germination (Lanfranco, Vallino, and Bonfante 1999).

In studies conducted at the Plant Science Department, McGill University, Lakeshore, Ste-Anne-de-Bellevue, Qc, Canada, AM fungus *G. intraradices* was grown in-vitro with Ri T–DNA-transformed *Daucus carota* roots on plates divided in two compartments. One side of the plates contained only fungal hyphae and spores while the other side contained mycorrhizal roots. Using this technique, limitations of insufficient biological material available for molecular analysis of this obligate symbiont were overcome. Fungal material from the first compartment was used as a pure source of *G. intraradices* for genomic DNA extraction. PCR amplification as well as southern hybridization were conducted using this DNA.

Sufficient pure genomic DNA and mRNA were obtained to carry out the southern analysis, achieve optimum PCR results with 25–35 cycles, and conduct RT–PCR. A differential expression of chitin synthase class I and class II was detected in *G. intraradices*. Using degenerate primers specific to the fungal chitin synthase sequences, a single amplification product obtained after 25 cycles of PCR was cloned. Sequencing fragments revealed a similarity to other fungal chitin genes. PCR with these primers and additional primers allowed amplification of chitin synthase fragments from spores of different isolates of *G. intraradices* as well as *G. mosseae, G. margarita, Acaulospora scrobiculata,* and *Entrophospoa colombiana*. A total of 21 chitin synthase sequences from different species and isolates of various AM fungi were successfully amplified. Sequencing of these fragments permitted their classification into class I and class II of the chitin synthase groups (Ubalijore, Hamel, Clung, *et al.* 2001).

Chitin synthases in ectomycorrhizal fungi

In studies conducted at the Departmento di Biologia Vegetale, del' Universitat and Centro de Studio Sulla Mycologia del Terreno del CNR, Turin, Italy, on chitin synthase homologs in ectomycorrhizal truffles, degenerate PCR primers were used to amplify a conserved gene protein coding chitin synthase from genomic DNA of six species of ectomycorrhizal truffles. DNA was extracted from both, hypogeous fruit bodies and in-vitro growing mycelium of *Tuber borchii*. A single fragment of about 600 bp was amplified for each species. The amplification products from *Tuber magnatum, T. borchii,* and *T. ferrugineum* were cloned and sequenced, revealing a high degree of identity (91.5%) at the nucleotide level. On the basis of the deduced amino acid sequences, these clones were assigned to class I chitin synthase. Southern blot experiments performed on the genomic DNA showed that amplification products derived from a single copy gene. Phylogenetic analysis of nucleotide sequences of class I chitin synthase genes confirmed the current taxonomic position of the genus Tuber and suggested a close relationship between *T. magnatum* and *T. uncinatum* (Lanfranco, Garnero, Delpero, *et al.* 1995).

In further studies at the above university in the Dipartmento Biology Vegetale, Mattioli 25, Turin, Italy, chitin synthase genes of ectomycorrhizal fungus *Tuber magnatum* were sought in order to investigate the molecular basis of its growth. Primers designed from highly conserved chs domains were used to identify an rhs gene portion. A genomic library was used to obtain the full gene sequence (TM chs 4) with an open reading frame, which encodes a predicted protein of 1230 amino acids. The sequence is similar (62%) to the class IV chitin synthase from *N. crassa*. The putative protein showed a hydrophobicity pattern that suggested the presence of several intra membranous domains and other peculiar features common to other rhs of class IV. Northern experiments demonstrated that the gene was expressed in ascomata sampled at different maturation steps. These data suggest that ascomata growth requires new chitin deposition based on a chs gene activation and not only on an enzymatic activity (Garnero, Lazzari, Mainieri, *et al.* 2000).

In studies conducted at the Institute of Microbiology, Swiss Federal Institute of Technology, Zurich, Switzerland, DNA sequences of a single copy gene coding for chitin synthase (UDP – N – acetyl – D – glucosamine: chitin 4 – B – N acetyl glucosaminyl transferase, EC: 2.4.1.16) was used to characterize ectomycorrhizal fungi. Degenerate primers deduced from short, completely conserved amino acid stretches flanking a region of about 200 amino acids of zymogenic chitin synthases allowed amplification of DNA fragments of several members of this gene family. Different DNA band patterns were obtained from basidiomycetes because of a variation in the number and length of amplified fragments. Cloning and sequencing of most prominent DNA fragments revealed that differences were due to various introns at the conserved positions. The presence of introns in basidiomycetous fungi, therefore, has a potential use in identification of the genera by analysing PCR-generated DNA fragment patterns. Analysis of the nucleotide sequences of cloned fragments revealed variations in nucleotide sequences from 4%–45%. By comparison of the deduced amino acid sequences, a majority of the DNA fragments were identified as members of the genes for chitin synthase class II. The deduced amino acid sequences from the species of the same genus differed only in one amino acid residue whereas identity between the amino acid sequences of ascomycetous and basidiomycetous fungi within the same taxonomic class was found to be approximately, 43%–66%. Phylogenetic analysis of the amino acid sequence of class II chitin synthase encoding gene fragments by using parsimony confirmed the current taxonomic groupings. In addition, data revealed a fourth class of putative zymogenic chitin synthase (Brigitta, Ivano, and Gerhard 1994).

Utilization of chitin as nitrogen source by mycorrhizal fungi

In experiments conducted at the Department of Animal and Plant Biology, University of Sheffield, Sheffield, UK, two ectomycorrhizal fungi *P. involutus* and *Rhizopogon roseolus,* and three isolates of ericoid fungi (two isolates of *Hymenoscyphus ericae* and one isolate of *Oidiodendron griseum*) were tested

for their ability to utilize chitin as a sole source of nitrogen. All ericoid fungi readily degraded chitin. The ectomycorrhizal fungi, in contrast, used it only sparingly. The results extend the range of polymeric nitrogen sources known to be utilized by mycorrhizal fungi and imply that some mycorrhizal fungi may be involved in recycling of nitrogen from the structural component of hyphal walls in soil (Leake and Read 1990).

In studies at the Institut fur Forstbotanic der Universitat, Göttingen, Germany, seedlings of *P. sylvestris* were grown in defined nutrient solutions of carbon filters, either sterile or infected with ectomycorrhizal fungus *S. variegatus*. After the mycorrhizae were established, shoot of the seedling was subjected to ^{14}C photosynthesis. ^{14}C–labelled photo assimilates were translocated to both mycorrhizae and non-infected root tips. Microautoradiographs of mycorrhizae indicated that omission of external sugars did not affect formation of mycorrhizae: 14C-labelled photo assimilates were supplied to cortex, Hartig net, and the mantle of hyphae surrounding rootlets. When soluble nitrogen was omitted from nutrient solution and replaced by chitin precipitated on the filter-bearing mycorrhizae, fungus appeared strongly labelled in the mantle where fungal chitinase provided soluble nitrogen compounds necessary for growth of the seedlings (Bauer, Blechschmidt-Schneider, and Eschrich 1991).

Studies were conducted at the IACR Rothamsted, Harpenden, Herts, England, to test the hypothesis that the chitin present in mycelial walls of soil fungi could be rendered accessible to ericaceous plants by their fungal endophytes. Measurements of chitin contents of the rooting horizons of a tropical moor-humus heath land soil indicated that chitin can contain in excess of 20% of the total nitrogen in the litter (L) horizon and 30% in the fermentation (F) horizon. Much of the chitin nitrogen was thought to be contained in mycelial walls of soil fungi. Mycelium of ectomycorrhizal fungus *Suillus bovinus* and the ericoid endophyte *H. ericae* were grown in liquid cultures before being killed and added either in the intact condition or after fractionation as a sole source of nitrogen to sterile media, upon which were grown either *H. ericae* in pure culture or mycorrhizal and non-mycorrhizal plants of *Vaccinium macrocarpum* and *Colluna vulgaris*. *H. ericae* was able to produce a significantly higher yield when grown on intact fungal necromass than when provided with equivalent concentrations of nitrogen in the form of ammonium. Its yields on mycelial fractions were lower but significantly greater than those obtained in controls lacking nitrogen. Significantly greater yields and nitrogen content were also found in ericaceous plants grown with these nitrogenous substrates in the mycorrhizal condition. Without mycorrhizal fungus, they had no access to the substrates (Kenley and Read 1997).

Chitin decomposing organisms associated with VAM spores

In studies conducted at the United States Department of Agriculture, Agricultural Research Service, Western Regional Research Centre, Albany, California, spores of *Glomus macrocarpum* were sieved from a calcareous silty clay loam soil, washed in sterile water and plated on chitin agar for isolation of actinomycetes. Of the 190 spores examined, 100 were colonized by one or more chitin-decomposing organisms: 82 were colonized by actinomycetes, 17 by bacteria, and one by fungi. Fifty-one isolates of actinomycetes were classified on the basis of morphological, cultural, and biochemical criteria. Tentative identifications to the genera and number of different isolates were Streptomyces (29), Nocardia (3), Streptosporangium (1), Streptoverticillium (1), Intrasporangium (1), Nocardiodes (1), and unidentified (15) (Ames, Mihara, and Bayne 1989).

In further studies conducted at the above centre, 12 actinomycetes originally isolated from the spores of *G. macrocarpum* were inoculated into unsterilized soil containing *G. macrocarpum* and *G. mosseae*. Chitin-decomposing actinomycetes were strongly antagonistic to the fungi, bacteria, or actinomycetes or combinations of these, based on prior studies. Eight species of Streptomyces, two of Nocardia, and two unidentified species were tested for their effect on mycorrhiza development, plant growth, and nutrient content in onion (*Allium cepa*), soil hyphal density, and soil population of bacteria and actinomycetes. None of the actinomycetes adversely affected plant growth or nutrient uptake. With a few exceptions, soil microbial populations and saprophytic hyphal fungal densities were not significantly different from controls. Seven of the twelve actinomycetes significantly increased the per-cent mycorrhizal root colonization and four significantly increased the density of hyphae more than 5 μ in diameter, predominantly those of VAM fungi (Ames 1989).

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Research findings

Incidence of arbuscular mycorrhizal colonization of *Pinus wallichiana* A B Jackson in different forest stands of Kashmir Himalaya

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Introduction

Pinus wallichiana (blue pine) is a socio-economically and ecologically important plant, constituting the major forest species in Kashmir Himalaya. Members in pinaceae characteristically harbour ectomycorrhizal symbionts (Harley and Smith 1983) but there are occasional reports of endomycorrhizal associations as well (Golubinskaya 1967; Cazares and Trappe 1993; Cazares and Smith 1996). These reports, however, could not logically explain the reason for dual association of some pinaceae members and hence, called for further studies to ascertain the reasons. Though Horton, Cazares, and Bruns (1998) reported ectomycorrhizal, vesicular-arbuscular, and dark septate fungal colonization of *Pinus muricata* seedlings after fire and attributed the phenomenon to rich VAM inoculum in soils, in respect of *P. wallichiana,* no earlier report exists for the AM incidence. The present communication highlights the significant correlation between differential colonizations and rhizospheric soil characteristics.

Materials and methods

Study area

P. wallichiana seedlings were collected from two ecologically different habitats of Kashmir Himalaya, including Dachigam National Park (protected) and Kangan forest (partially protected). Dachigam National Park (34°, 4' to 34°, 14' N Lat and 74°, 48' to 75°, 85' E long), covering an altitude of 1600–4250 m amsl (above the mean sea level) is part of the Great Zanskar range of the Western Himalaya, with rectangular shape and an approximate length and breadth of 22.5 and 8 km, respectively, enjoying a the submediterranean climate. Kangan forest (34°–16' N Lat and 74°, 50' E long, elevation; 1833 m amsl) in the Sind Valley lies about 4 km towards north-west of Ganderbal, sustaining a sizeable population of *P. wallichiana* and *Cedrus deodara*.

P. wallichiana seedlings were collected from time to time from each of the different habitats and 100 root pieces from each seedling were cleared in 15% KoH solution and stained with trypan blue, followed by de-staining in 50% lactic acid. The AM colonization was evaluated by compound microscopy using '+' and '–' for presence and absence of association, respectively. The marked presence of vesicles reflected GLOMUS-type association, which was confirmed by J M Trappe (personal communication). Rhizospheric soil was regularly collected twice a month during the study period (October 2003–September 2004) and analysed for different physico-chemical characteristics, such as pH, moisture content, field capacity, organic carbon, calcium, magnesium, and phosphorus content. pH was measured by a digital pH meter. Soil moisture content was obtained by the burning alcohol method following Singh, Hukker, and Rajput (1970) and field capacity by field method (Veihmoyer and Hendricksen 1949). Organic carbon was determined by the wet-oxidation method (Walkley 1947) and organic matter content by the rapid-titration method (Black 1965). Calcium and magnesium were estimated by the routine EDTA-titration method following Barrows and Simpson (1962). The available phosphorus was obtained by extraction with sodium bicarbonate (Olsen, Cole, Watanabe, *et al.* 1954) whilst the total phosphorus was measured after Jackson (1958).

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Results and discussion

Responding to the call of Cazares and Trappe (1993) to investigate the overlooked phenomenon of the AM colonization of pinaceae members, seedlings of *P. wallichiana* from two different ecological habitats of Kashmir Himalaya for AM association were evaluated. The per cent AM colonization of *P. wallichiana* seedlings from different habitats is shown in Table 1. Data reveals relatively high (54%) AM colonization in seedlings from Dachigam National Park, in contrast to

Table 1 Per cent VAM colonization of *P. wallichiana* seedlings from different ecological habitats of Kashmir Himalaya

Sampling site	*Per cent colonization*		
	Vesicles only	*Arbuscles only*	*Hyphae only*
Dachigam National Park	32	14	08
Kangan Forest Stand	4.3	2.1	4.3

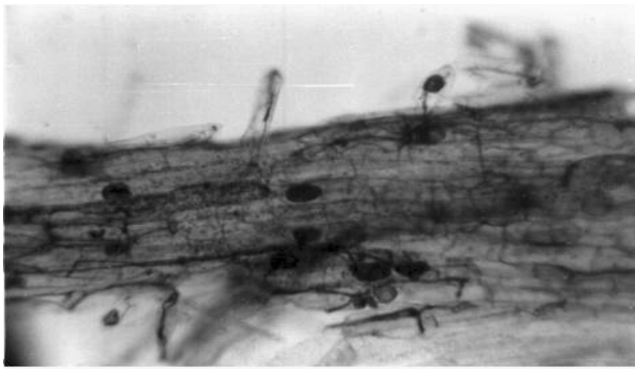

Figure 1 Glomus-type vesicles in root of *P. wallichiana*

seedlings from Kangan forest stand. Seedlings from Kangan forest stand showed just 10.7% AM colonization. The percentage of vesicles, arbuscles, and hyphae was estimated at 32%, 14%, and 8%, respectively. The *Glomus* type association was characterized by the predominance of vesicles (Figure 1) and relatively less frequency of arbuscles and hyphae. Differences in the physico-chemical characteristics of rhizospheric soil of Kangan forest stand and Dachigam National Park are presented in Table 2. The two sites show evident differences in pH, moisture content, total and available phosphorus, and organic carbon. Furthermore, a slight variation between the two sites was observed with regard to the field capacity and magnesium content.

The phenomenon of dual colonization in some ectomycorrhizal hosts like *Pinus* has been variously interpreted. Francis and Read (1995) suggested that presence of vesicles in the otherwise EM hosts indicates a pathogenic interaction whilst Lapeyerie and Chilvers (1985) hypothesized that VAM play an important role in seedling establishment in the nutrient-poor sites, which subsequently are succeeded by the EM fungi. Smith, Johnson, and Cazares (1998) reported that *Pseudotsuga menziesii* seedlings inoculated with *Glomus intraradices* had higher foliar phosphorus levels than the uninoculated controls.

Table 2 Average physico-chemical characteristics of rhizospheric soil of Dachigam National Park and Kangan Forest Stand (October 2003–September 2004)

Parameter	*Dachigam National Park*		*Kangan Forest*	
	Range	*Mean*	*Range*	*Mean*
pH	6.29–7.52	6.39	4.91–5.51	5.34
Moisture content (%)	20.58–29.2	22.78	4.23–6.21	5.46
Field capacity (%)	32.21–38.21	36.41	30.21–35.12	32.3
Magnesium (meq/100g)	0.3–0.9	0.5	0.1–0.4	0.3
Organic carbon (%)	3.58–5.21	4.17	1.92–3.45	2.88
Available phosphorus (kg/ha)	15.0–30.5	27.8	32.2–48.5	40.2
Total phosphorus (kg/ha)	212–275	248	448–562	463
Calcium (meq/100g)	4.5–6.5	5	2.8–4.2	3.7
Magnesium (meq/100g)	0.3–0.9 2.88	0.5	0.1–0.4	0.3

We attribute the differential AM colonization of *P. wallichiana* of the studied sites to the presence of rich VAM inoculum as Dachigam National Park characteristically harbours a dense population of *Themeda anathera*, an ideal AM host (Shah and Zafar, in press). Nevertheless, Kangan forest stands do not support such a dense population of the AM host plants and as such *Pinus* seedlings from this site showed relatively low AM colonization of just 10.7%.

Given the gap in understanding the reasons for AM colonization in the otherwise ectomycorrhizal hosts, an attempt was made to correlate it with the physico-chemical characteristics of their rhizospheric soils (Table 2). Rhizospheric soil pH may be a factor responsible for the differential AM colonization of pine seedlings in the three study sites. Dachigam soils, because of a higher soil pH (6.39) show higher levels of AM propagules. In contrast, Kangan soils, because of acidic pH (5.34), presumably do not support the AM propagules as acidic soils are known to have a fungistatic effect on spores of *Glomus* species (Siquiere, Hubbell, and Mahmud 1984). Recently, Ingrid, Aarle, Olsson, *et al.* (2002) evaluated the AM fungal response to substrate pH whilst Abott and Robson (1985) showed that the spread of extraradical mycelium by a *Glomus* isolate was strongly inhibited at a low pH.

Difference in the average soil moisture content of the two sites (Dachigam = 22.78%, Kangan = 5.46%) is possibly another factor responsible for differential AM incidence in *P. wallichiana* seedlings. Infact, Auge, Stodola, Tims, *et al.* (2001) demonstrated a change in both, the soil structure and its moisture characteristic curve, relative to non-mycorrhizal soils having similar root densities. The soil moisture content is improved by VAM fungi through its influence on the soil structure, aggregate stability, and efficient exploitation of bound water even accessing waters below the wilting point as reflected by a comparatively higher field capacity (36.41%) of Dachigam soils than of Kangan soils (32.39%).

Relatively high levels of exchangeable calcium and magnesium in soils of Dachigam than Kangan may be yet another factor for the higher AM incidence in the former than the latter. This is in agreement with the findings of Hepper and O'Shea (1984); Gryndler, Vejsdova, Vancura (1992);

Habte and Soedarjo (1995); and Jarstfer, Koppanol, and Sylvia (1998), showing the critical influence of calcium and magnesium on the AM colonization and sporulation.

As the AM hyphae and associated products decompose easily as compared to other below-ground sinks, such as the EM hyphae and root exudates (Johnson, Leake, Ostte, *et al*. 2002), the higher organic carbon level (4.17%) of Dachigam soils than kangan soils (2.88%) is on expected lines. Studies have shown that the EM fungi suppress while the AM fungi support the bacterial numbers and activity in *Pinus contorta* rhizosphere.

As higher phosphorus is known to suppress the mycorrhizal association, the higher per cent colonization of Pinus seedlings of Dachigam forest stand than of Kangan stand is supported by comparatively lower phosphorus content of the former than the latter (Table 2).

For further unraveling of the mechanism and significance of the AM association in the otherwise EM hosts, the present study stresses the need for evaluation of the effects of different AM isolates on members of Pinaceae. An important next step in this direction will be to determine the functional differences among the different Pinaceae members, viz-a-viz individual and combined endo- and ecto-mycorrhizal inoculation treatments. Such studies may help in elucidating the contribution of VAM association in the seedling establishment of *P. wallichiana*, particularly in nutrient-poor sites followed subsequently by ectomycorrhizal succession, and would help in the establishment of pine seedling nurseries for eco-restoration of the degraded pine forests.

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Distribution of arbuscular mycorrhizal fungi in soybean (*Glycine max* [L.] Merrill) rhizosphere

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Introduction

Majority of the vascular flora have mycorrhizal association, dominated primarily by the AM (arbuscular mycorrhizal) fungi (Brundrett 1991). The presence of AM fungi has been reported from different habitats and from a wide range of plants (Bhat and Kaveriappa 1997). Occurrence of the AM fungi, associated with different host plants grown in various types of soils is well documented (Gupta, Basak, and Das 2002; Alloush and Clark 2001). Importance of the AM fungi has received considerable attention in the recent years owing to their beneficial response in improving crop productivity (Abdel-Fattah 1997; Raverkar and Tilak 2002; Delfin, Paterno, Ocampo, *et al.* 2003; Hemalatha and Selvaraj 2003; Klironomos, Mc Cune, and Moutoglis 2004; Goswami, Jamaluddin, and Dadwal 2005).

Soybean is an important crop of a very high economic and commercial value and is reported to have a mycorrhizal association (Tilak, Rathi, and Saxena 1995; Raverkar and Tilak 2002). The effect of mycorrhization on vegetative growth, nutrient uptake, and yield of soybean has been reported by Lukiwati and Simanungkalit (2002). However, studies on the association of AM fungi with different soybean cultivars in the cropping field of Jabalpur (Madhya Pradesh) are scanty. Hence, in the present investigation, an extensive survey on distribution of various AM fungi in rhizosphere of different soybean cultivars being grown in the Jabalpur region of Madhya Pradesh has been made so as to study the AM fungi diversity in the soybean cropping fields.

Materials and methods

Root samples along with rhizospheric soil of five soybean cultivars, namely JS-335, JS71-05, NRC 2, NRC 7, and NRC 12, of the Jabalpur (Madhya Pradesh, India) region were collected during the kharif season 2002/03. Roots and rhizospheric soil samples were brought to the laboratory in polythene bags and stored at 4 °C and at room temperature, respectively.

Roots of soybean were washed in water, cleared with 10% KOH, acidified with 1 N HCl, and stained in 0.05% trypan blue in lactoglycerol (Phillips and Hayman 1970). Quantification of root colonization of the AM fungi was carried out using the slide method (Giovannetti and Mosse 1980).

Spores of the AM fungi were extracted from rhizospheric soil using wet-sieving and decanting method (Gerdemann and Nicolson 1963). Quantification of AM fungal spore density was carried out using the method given by Gaur and Adholeya (1994). Diagnostic slides with spores/ sporocarps were prepared using PVLG (polyvinyl alcohol lactoglycerol) as mountant. Identification of the AM fungi was done using relevant literature (Morton and Benny 1990; Schenck and Perez 1990; Prasad and Rajak 1999).

Results and conclusion

In the present study, intense AM colonization was restricted in the epidermal and cortical parenchymatous cells near the root bases. Roots exhibited a fairly good colonization of the AM fungi, which was characterized by the presence of hyphae, arbuscules, and vesicles (Plate 1).

Variable mycorrhizal infection in five different soybean cultivars (62.6% in JS 335, 69.2% in JS 71-05, 69% in NRC 2, 66.6% in NRC 7, and 66.0% in NRC 12) was recorded (Table 1).

In the RS (rhizospheric soil) of five different soybean cultivars collected from Jabalpur region, maximum number of AMF spores (per 50 g of soil) were recorded (Table 1) in the RS of cultivar NRC 2 (100.2) followed by cultivar NRC 7 (95.0), cultivar JS 71-05 (89.9), cultivar NRC 12 (85.8), and cultivar JS 335 (74.4). Twenty-two types of AMF spores belonging to six genera, namely *Acaulospora*, *Glomus*, *Gigaspora*, *Scutellospora*, *Sclerocystis, and Entrophosphora* (Plates 1 and 2), have been tentatively identified. Of these, *Glomus*, *Acaulospora*, and *Gigaspora* were commonly found in RS of all five soybean cultivars. Amongst the identified genera, *Glomus* was the most dominant genus and amongst *Glomus* species, *Glomus intraradices* was the most prevalent. In the present study, four species of *Acaulospora*, three of *Gigaspora*, ten of *Glomus*, two of *Sclerocystis*, one of *Entrophosphora*, and two of *Scutellospora* were tentatively identified (Table 2).

Table 1 Per cent of AM fungal infection in roots and infestation in rhizospheric soil of five soybean cultivars

Name of cultivar	*Per cent infection in roots*	*Spore density in soil*/ 50 g soil*
JS 335	62.6**	74.4
JS 71 05	69.2	89.4
NRC 2	69.0	100.2
NRC 7	66.6	95.0
NRC 12	66.0	85.8

* Data are mean of three replicates

** Data are mean of five replicates with 100 root segments for each replicate

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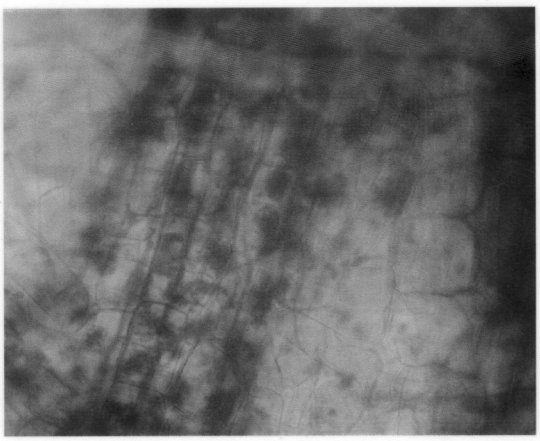
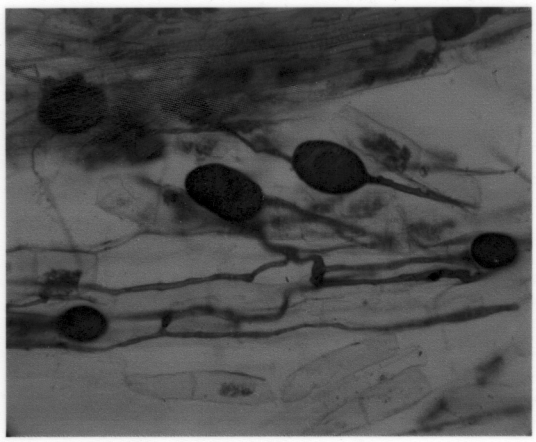


Root showing abundant arbuscles (X100)



Root showing hyphae and vesicles (X100)

Table 2 Occurrence of arbuscular mycorrhizal fungal spores in rhizospheric soil of five soybean cultivars

AM fungal species	*Cv. JS 335*	*Cv. JS 71-05*	*Cv. NRC 2*	*Cv. NRC 7*	*Cv. NRC 12*
Acaulospora laevis	+	–	+	+	–
Acaulospora tuberculata	+	+	+	+	+
Acaulospora lacunosa	–	+	+	–	+
Acaulospora foveata	+	+	–	–	–
Gigaspora gigantea	+	+	+	+	+
Gigaspora candida	–	+	–	+	+
Gigaspora sp.	+	–	–	+	–
Glomus aggregatum	+	–	+	–	+
Glomus constrictum	–	+	–	+	+
Glomus fasciculatum	+	+	+	+	+
Glomus macrocarpum	+	+	–	–	+
Glomus geosporum	–	–	+	–	+
Glomus leptotichum	+	–	+	+	–
Glomus intraradices	+	+	+	+	+
Glomus epigacum	+	+	–	–	–
Glomus sp.	+	–	–	+	–
Glomus sp.	–	+	+	–	+
Sclerocystis rubiformis	+	+	–	–	–
Sclerocystis sp.	–	–	+	+	–
Entrophosphora sp.	+	–	–	+	+
Scutellospora nigra	–	+	+	–	+
Scutellospora sp.	+	–	–	+	+

+ present; – absent

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Multiplication and trapping of vesicular arbuscular mycorrhiza fungi in soil of dumps of limestone quarries

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Introduction

Mine overburden dumps are physically, chemically, and nutritionally disturbed ecosystems. Due to the altered soil profile, physio-chemical properties of soil are changed to a great extent. The vegetative spectrum in such areas is found to be almost nil. The status of vegetational spectrum, microorganisms, and nutrients is altered by mining (Paone, Struthers, and Johnson 1978. Such infertile soils do not suit the establishment of vegetation. It takes a long time to restore the ecosystem in such stress sites.

VAM (vesicular arbuscular mycorrhiza) fungi play a pivotal role in the establishment and growth of plants under natural as well as stress conditions, particularly in nutrient-deficient soils. The role of VAM fungi in plant growth and productivity has been well-documented (Menge, Davis, Johnson, *et al*. 1978; Bolan 1991; Suryaprakash 1995). As the naturally occurring VAM fungi are well-adopted to conditions prevailing in a given place, their application as biofertilizers may be helpful in the establishment of plants and in further afforestation of degraded lands.

The present study deals with the development of effective techniques for multiplication of VAM. It investigates the occurrence and status of VAM fungi occurring in limestone-mined areas.

Material and methods

In order to carryout the study, soil samples were collected from limestone-mined soils of different age groups, including five-year and ten-year-old, and fresh dumps. Soil samples were collected up to a depth of 10 cm. Samples were collected in fresh polyethylene bags and their openings were tied with rubber bands. The experiment was conducted in the nursery of the forest pathology division, Tropical Forest Research Institute, Jabalpur.

Plastic buckets were filled with sterilized nursery soil and limestone-mined overburden dumps soil in the 1:1 ratio. Separate buckets were managed for soil dumps of each age group in three replications.

Zea mays (maize) was selected as the host plant for VAM association. The healthy seeds of Zea mays (20 seeds) were sown in each plastic bucket during June 2004. All buckets were kept in iron angle beds in order to avoid contamination in nursery. Watering in experimental buckets was done daily with the help of fresh and clean water, using fine rose cane. Maize plants were removed after maturation and the soil along with roots was collected for the study of VAM fungi.

Wet-sieving and decanting technique of Gerdemann and Nicolson (1963) was employed for isolation of VAM spores. Viable spores were extracted by using the sucrose-centrifugation technique (Daniel and Skipper 1982). Counting of viable VAM spores was done in a nematode-counting dish under a dissecting binocular microscope. The method of Phillip and Hayman (1970) was applied for staining of roots. The percent root colonization was assessed by slide method of Giovannetti and Mosse (1980). Genera of VAM fungi were identified on the basis of morphology of

their resting spores. In the present investigation, different genera of VAM fungi were identified with the help of the manual of Schenck and Perez (1990).

Results and discussion

It is evident from Table 1 that the population of VAM spores is increasing with the aging of dumps. Un-sterilized nursery soil contains the highest number of spores, that is, 850 per 100 g of soil with 70% root colonization.

In soils of limestone-mined overburden dumps, there was a significant difference in the spore population and further root colonization.

Soil from ten-year-old overburden dumps has the highest spore population, that is, 760 per 100 g soil with 70% root colonization. While soils from five-year-old and fresh overburden dumps was found to be less populated with VAM spores, it contains 540 and 240 spores per 100 g of soil and 50% and 40% root colonization, respectively.

The number of spores in sterilized dump soil and nursery soil was negligible and root colonization was also negligible. Jamaluddin and Chandra (1999) observed an increase in the growth of bamboo species in the nursery soil as well in coal mine overburden soil. It is well-established that many plants cannot grow adequately without the VAM fungi, especially in disturbed mining dumps (Jasper, Abott, and Robson 1989). Arbuscular mycorrhizal fungi have been found of immense application in the agroforestry, horticulture, plant disease control, and reclamation of wastelands (Vander-heijden, Klironomos, Vrsic, *et al*. 1999; Mukherjee, Singh, and Chamola 2000).

It was observed that there was a considerable difference in the population of VAM spores in the soil of limestone mine overburden dumps of different age groups, with a highest number in 10-year-old dumps. The procedure followed in this study indicated the status of VAM fungi and its multiplication in mined overburden dump soils. This is the easiest technique to study the VAM fungi and its effectiveness towards developing infection in trap plant.

Table 1 Multiplication of VAM fungi in limestone mine dumps

S. No.	Soil of limestone quarries amended with sterilized soil	*Number of spores/100 gm soil*	*Per-cent root colonization*	*Genera of VAM fungi identified*
1.	Sterilized soil plus ten-year-old limestone mine overburden dumps soil	760	70	Glomus, Acaulospora, Gigaspora, and Scutellospora
2.	Sterilized soil plus five-year-old limestone mine overburden dumps soil	540	50	Glomus, Acaulospora, and Gigaspora
3.	Sterilized soil plus fresh limestone mine overburden dump soil	240	40	Glomus and Acaulospora
4.	Un-sterilized nursery soil	850	70	Glomus, Acaulospora, Gigaspora, and Scutellospora
5.	Sterilized limestone mine overburden dump soil	Negligible	Negligible	—
6.	Sterilized nursery soil	Negligible	Negligible	—

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New approaches

Protocol for *Agrobacterium rhizogen*-transformed roots of *Medicago truncatula* for study of nitrogen fixing and VAM associations

Medicago truncatula, a diploid autogamous legume, is currently being developed as a model plant for study of root endosymbiotic associations, including nodulation and mycorrhizal colonization. An important requirement for such a plant is the possibility of rapidly introducing and analysing chimeric gene constructs in root tissues. For this reason, Boisson D A, Chabaud M, Garcea F, Becard G, Rosenberg C, Banker D G (2001) developed and optimized a convenient protocol for *Agrobacterium rhizogen*-mediated transformation of *M. truncatula* (*Molecular Plant–Microbe Interactions* [*Proceedings*] **14**(6): 695–700. This unusual protocol, which involves inoculation of sectioned seedling radicals, results in rapid and efficient hairy root organogenesis and subsequent development of vigorous composite plants. In addition, authors found that kanamycin can be used to select for co-transformation of hairy roots directly with gene constructs of interest. *M. truncatula*-composite plant hairy roots have a similar morphology to the normal roots and can be nodulated successfully by their nitrogen-fixing symbiotic partner *Sinorhizobium meliloti*. *M. truncatula* hairy root explants can be prepared in-vitro and as demonstrated by authors, these clonal lines can be colonized by VAM fungi, such as *G. intraradices*, with the formation of arbuscules within cortical cells. The results suggest that *M. truncatula* hairy roots represent a particularly attractive system with which endosymbiotic associations can be studied in transgenically modified roots.

An efficient, fast-track method for reclamation of industrially contaminated lands and management of industrial waste waters

In recent years, effluent discharged from industries such as distilleries, and pulp and paper mills into land and water bodies has become a problem of immense importance. Both, distilleries and pulp and paper mills have been categorized among the seventeen most-polluting industries in the country by the CPCB (Central Pollution Control Board), New Delhi. An increasing public concern about the disposal of potential toxicants and anticipated future stringent regulatory measures to control the discharge of pollutants from this industry have led to the development of several abatement processes. Unfortunately, none of the existing waste water treatment processes offer a perfect solution.

Distillery industry

In India, more than 30 billion litres of spent wash is generated annually by 285 cane-molasses-based distilleries. After alcohol distillation, large amounts of highly coloured spent wash remain in the still to be disposed of. For every litre of ethanol produced, 10–15 litres of spent wash is generated. Therefore, a typical distillery generates over half-a-million litres of spent wash effluent daily. In addition to being highly coloured, spent wash is difficult to treat by normal biological processes, such as activated sludge or anaerobic lagooning. It also has an extremely high COD load. In India, the primary spent wash is generally utilized in an anaerobic digestion step to utilize its high COD load for fuel production (methane) while reducing its organic load. The secondary spent wash produced by the anaerobically DMSW (digested primary molasses spent wash) effluent is darker in colour, needing huge volumes of water to dilute it and is currently used in irrigation water causing gradual soil darkening. Usually, this biomethanated effluent is stored in lagoons or disposed of on land for solar drying.

Pulp and paper mills

The Indian paper industry is more than 100 years old. Apart from a few exceptions of new modernized mills, most mills are based on obsolete process technologies for effluent handling. Pulp and paper industry generates about 90–175 m^3 depending upon the facilities made available in mills, of waste water per tonne of paper produced. Of the different waste waters generated by the pulp and paper industry, bleach plant effluents are considered to be the most polluting. If untreated or partially treated effluents from such paper and pulp mills are discharged, it results in persistence of colour in the receiving body of water over a long distance. In India, small paper mills, whose two main raw materials are lignocellulosics, such as straw and bagasse, account for about half of the total installed capacity. The combined pollution load of these mills, in terms of lignin, is about four times higher than that of larger paper mills. Waste water discharged by this industry is highly heterogeneous as it contains compounds from wood or other raw materials, processed chemicals, as well as compounds formed during processing.

In India, there are 515 paper and board mills with an aggregate capacity of approximately, 5.0 million tonnes. Of of the 515 mills, approximately, 180 are in medium and small agro-based sector, ranging 10 to 80 TPD (tonnes per day) capacities and with exception of 8–9 number of mills. The others are without any chemical recovery plants and discharge their black liquor after secondary biological treatment system (http://www.ippta.org/dlpia1.htm). The small units using agro-residues are a more serious threat to the environment as these units discharge black liquor along with unrecovered chemicals, which are difficult to treat. The presence of caustic in black liquor results in an increase in dissolved solids in waste streams. Sodium concentration renders sodium hazard if the effluent is discharged on soil for irrigation. Discharge of untreated black liquor also results in a loss of valuable chemicals. Another problem is low concentration of black liquor from the agricultural residues.

For small-scale paper mills using agricultural residues as their raw material, the installation of CRP (chemical recovery plant) for soda recovery has been suggested. However, the high cost of

establishing a conventional CRP is a major deterrent for most small paper mills. There is another option of soda ash recovery, which is slightly cheaper to establish and is presently being exercised by some paper mills like Shreyans papers, Ahmedgarh, Punjab. There is an apprehension among paper mills that if all of them go for soda ash recovery, it would not be economically feasible to get a good market for it.

Taking into consideration the above facts, there is a real need for development of technology related to low cost materials that could be applied to the industrial effluent in order to reduce their pollution load on land and in water bodies. Both distilleries as well as pulp and paper industries are likely to face more stringent regulations on the quality of effluent entering receiving waters. Problems faced by these industries relate to high organic load, residual COD, toxicity, and colour.

Phytomicrobial remediation

Phytomicrobial remediation is a low-cost alternative to the conventional waste water treatment systems for industries, which use large areas of cultivable land for effluent disposal. This is a promising technology where selected plant species are used for reclamation as well as for providing an option of loading huge volumes of waste waters in a small area of land with no obvious side effects. The technology envisages the use of dynamic multi-component soil systems as a live filtration device to renovate pretreated waste water through absorption, ion exchange, precipitation, and stabilization of pollutants through microbial degradation. Also, earthworms present in the system provide aerobic conditions for stabilization of organic contents in waste water through a network of furrows.

Plant-based remediation (phytoremediation) is defined, according to Cunningham and Berti (1993) as the use of green plants to remove, contain, or render harmless environmental contaminants. To be effective, phytoremediation projects must successfully integrate both the plant-based and engineering aspects of a system: this requires an interdisciplinary approach.

It is important to note that this includes the use of vegetation for in-situ treatment of water, sediments, soils, and air. In this process, specially selected or engineered plants can be used for extraction of toxic metals from soil or water, including the removal of radioactive elements, removal of toxic organic compounds, and, if possible, their mineralization.

Benefits of phytomicrobial remediation

- Works on natural treatment processes and is thus, eco-friendly
- Facilitates development of forest and green belt around factory premises
- Generates sink potential for air pollutants
- Low-energy and cost requirements compared to conventional waste water treatment systems
- Ease of installation and simplicity of operations
- Biomass generation and revenue returns

Role of mycorrhiza

The central role of symbiotic AMF in plant population dynamics is through their control of soil-nutrient uptake, protection against root pathogens, and intra- and interplant species linkages. Plants in most plant communities are able to form symbiotic associations with one or more AMF species. Populations of AMF are essential for soil development and successful plant establishment. The presence of AMF as obligate plant symbionts in the majority of plant species may reduce the negative effects of stress caused by a lack of nutrients or organic matter resulting from adverse soil structure or extreme pH. Regeneration succession on highly degraded sites mostly results in a low diversity of plant communities and associated microbial populations. In order to promote and accelerate succession in restoration processes and to develop sustainable revegetation practices, it is necessary to study the implementation possibilities of phytomicrobial complexes (plants–symbiotic fungi–beneficial rhizosphere bacteria) that are tolerant to various stresses. To accomplish this objective, it is essential to restore or reintroduce functional populations of beneficial AMF in soil. Mycorrhizal symbiosis develops when sufficient numbers of AMF propagules are in soil. However, the number of spores and root colonization are often reduced by soil disturbance. Therefore, if the AMF have deteriorated or have been eliminated, they should be reintroduced by inoculation of soils. Efforts are constantly being made to reclaim natural or anthropogenically eroded lands by distributing seeds and fertilizers. However, results of these efforts are quite variable as it usually takes years of fertilization, followed by reseeding, to obtain permanent vegetation cover. The use of mycorrhiza can substantially enhance this process.

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Recent references

Latest additions to the network's database on mycorrhiza are published here for 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*
- *Applied and Environmental Microbiology*
- *Austral Ecology*
- *Cellular Microbiology*
- *Ecological Applications*
- *Ecological Entomology*
- *Ecology Letters*
- *Fems Microbiology Letters*
- *Forest Ecology and Management*
- *Genetica*
- *Geoderma*
- *Journal of Environmental Biology*
- *Journal of Environmental Quality*
- *Journal of Microbiological Methods*
- *Journal of Plant Nutrition*
- *Molecular Plant-Microbe Interactions*
- *Mycologia*
- *New Phytologist*
- *Nutrient Cycling in Agroecosystems*
- *Plant Molecular Biology*
- *Plant Physiology*
- *Plant Physiology and Biochemistry*
- *Planta*
- *Proceedings of the National Academy of Sciences of The United States of America*
- *Renewable Agriculture and Food Systems*

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 asterick]
Dickie I A,* Schnitzer S A, Reich P B, Hobbie S E. 2005	**Spatially disjunct effects of co-occurring competition and facilitation** *Ecology Letters* **8**(11): 1191–1200 [Landcare Res, POB 69, Lincoln, Nebraska, US]
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Forthcoming events

Conferences, congresses, seminars, symposia, and workshops

Ca' Tron (Treviso), **Italy**
6–10 March 2006

Workshop on Introduction to biosafety and risk assessment for the environmental release of genetically modified organisms (GMOs): Theoretical approach and scientific background
The International Centre for Genetic Engineering and Biotechnology, Padriciano 99, 34012 Trieste, Italy
Fax +39 040 2265 55 • *E-mail* courses@icgeb.org
Tel. +39 040 3757333 • *Website* www.icgeb.org/MEETINGS/CRS06/6_10marzo.pdf

Peradeniya, **Sri Lanka**
22–24 March 2006

Regional Practical Training Programme on Detection of genetically modified organisms (GMO) and genetically modified food (GMF)
Prof. Athula Perera, Department of Agricultural Biology, Faculty of Agriculture, University of Peradeniya, Sri Lanka
Fax +94 81 2388239 • *E-mail* profapererasltnet.lk
Tel. +94 81 2395247, 2387250 • *Website* www.icgeb.org/~bsafesrv/bsfn0510.htm#srilanka, http://www.icgeb.org/MEETINGS/CRS06/Meetings2006.htm

Concepción, **Chile**
27 March–7 April 2006

ICGEB - UNU/BIOLAC Theoretical and Practical Course 'Forest genetics and biotechnology
Dr. Sofia Valenzuela Aguila, Facultad de Ciencias Forestales, Universidad de Concepción, Casilla 160-C, Concepción, Chile
Fax +56 41 227455 • *E-mail* sofvalenudec.cl
Tel. +56 41 204848, 204906 • *Website* www.icgeb.org/MEETINGS/CRS06/Meetings2006.htm

Lima, **Peru**
4–7 April 2005

IX Plant Virus Epidemiology Symposium
International Potato Center, P O Box 1558, Lima 12, Peru
Fax +51 1 317 5326 • *E-mail* plant—virus-epidemiology-symp@cgiar.org
Tel. +51 1 349 6017 • *Website* www.cipotato.org/training/PlantVirusEpidemSymp05/index.htm

Trieste, **Italy**
10–13 April 2006

Theoretical Course RNA structure and function
ICGEB - Conferences and Meetings, Padriciano 99, I-34012 Trieste, Italy
Fax +39 040 226555 • *E-mail* coursesicgeb.org
Tel. +39 040 3757333 • *Website* www.icgeb.org/MEETINGS/CRS06/Meetings2006.htm

Nashville, **Tennessee**
30 April–3 May 2006

28th Symposium on biotechnology for fuels and chemicals
Jonathan Mielenz, Conference Chairman, Oak Ridge National Laboratory, Post Office Box 2008, Oak Ridge, Tennessee 37831, USA
E–mail mielenzjr@ornl.gov
Tel. +865 576 8522 • *Website* www.simhq.org/html/meetings.html

Florence, **Italy**
15–19 May 2006

Practical Course Biosafety II: Practical course in evaluation of field release of genetically modified plants
ICGEB (International Centre for Genetic Engineering and Biotechnology) Conferences and Meetings, Padriciano 99, I-34012 Trieste, Italy
Fax +39 040 226 555 • *E-mail* coursesicgeb.org
Tel. +39 040 3757 333 • *Website* www.icgeb.trieste.it/MEETINGS/CRS06/15_19maggio.pdf

Belgrade, Serbia, and Montenegro
15–19 May 2006

Workshop on 'Evolutionary approaches to the study of nuclear mitochondrial genome interactions in metazoa'
Prof. Cecilia Saccone, CNR - Institute of Biomedical Technologies, Via G Amendola, 122/D, 70126 Bari, Italy
Fax +39 080 5929 690 • *E-mail* cecilia.sacconeba.itb.cnr.it
Tel. +39 080 5929 680 • *Website* www.icgeb.org/MEETINGS/CRS06/Meetings2006.htm

Boston, MA, **USA**
18–21 June 2006

2006 AAPS National biotechnology conference
American Association of Pharmaceutical Scientists
2107 Wilson Blvd, Suite 700, Arlington, VA 22201-3042, USA
Fax + 703 243 9650
Tel. + 703 243 2800 • *Website* www.aapspharmaceutica.com/nationalbiotech/

Granada, **Spain**
23–27 July 2006

Profesor Albareda 1, E-18008 Granada, Spain
Fax + 34 958 129600 • *E-mail* francisca.gonzalez@eez.csic.es
Tel. + 34 958 181600 • *Website* http://www.eez.csic.es/icom5/Index.htm

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