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सत्यमेव जयते

SOIL MICROBIAL DIVERSITY FROM THE HIMALAYA : NEED FOR DOCUMENTATION AND CONSERVATION

Anita Pandey, Pankaj Trivedi, Bhavesh Kumar, Bhaskar Gharasia and L.M.S. Palni

Environmental Physiology and Biotechnology,
G.B. Pant Institute of Himalayan Environment and Development,
Kosi-Katarmal, Almora, 263 643.

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For further information, please contact

The Chairman
National Biodiversity Authority
475, 9th south cross street,
Kapaleeswarar Nagar,
Neelangarai,
Chennai – 600 041.

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1. INTRODUCTION

Microorganisms are ubiquitous in nature and form vital components of all known ecosystems on earth. Their ubiquity is attributed mainly to the small size, easy dispersal, ability to survive and multiply in diverse habitats, including anaerobic and other extreme conditions, their metabolic versatility and flexibility to utilize wide substrates as nutrient source. One of the fascinating aspects of microorganisms is that some have evolved to thrive under conditions that are too harsh for the animals as well as plants. Extremes of temperature, pH, oxidation-reduction potentials, salinity and humidity, and various combinations of these, found in diverse terrestrial and aquatic habitats are colonized only by the microorganisms. During the major climatic and geological events, the least affected life forms are microorganisms. It has been widely recognized, particularly in the last two decades, that majority of such environments are inhabited by surprisingly diverse microbial communities. The microorganisms that thrive under extreme environments, from polar deserts to geothermal springs, are referred as extremophiles. The number of extremophiles known to exist, and even thrive, in biotopes with environmental extremes has rapidly grown in recent years. This increase in the known microbial biodiversity is based both on laboratory cultures of isolated microorganisms and on the basis of characterization of 16S rDNA sequences recovered directly from the environment (Stolp, 1988; Seckbach, 2000; Budhiraja et al., 2002; Satyanarayana et al., 2005). With advances in our understanding in this area, the importance of documentation of biodiversity of world's resources and conservation of the biological gene pool has been receiving increasing attention (Sly et al., 1990).

2. BACTERIAL, ACTINOMYCETES AND FUNGAL DIVERSITY IN RHIZOSPHERE AND NON RHIZOSPHERE SOILS

Bacteria, actinomycetes and fungi are three major groups of soil inhabiting microorganisms. Diverse vegetation, including trees, harbour a variety of specific rhizospheres allowing colonization by selected groups of soil microorganisms. While edaphic as well as climatic conditions affect the number and nature of microbial diversity in general, factors like root exudates and age of the host plants affect the microflora associated with a given rhizosphere, in addition. Soil samples collected from different altitudes in Sikkim and Uttarnachal Himalaya have been investigated for general as well as rhizospheric soil microbial diversity. The rhizosphere effect exerted by ecologically important tree species of Himalaya has also been worked out.

a. Microbial populations along an altitude:

In a case study, microbial analyses of soil samples collected from three altitudes, viz. Kamrang, 1200 m; Chamgaon, 1600 m and Jaubari, 1900 m amsl in Mamlay watershed, Sikkim Himalaya were conducted (Pandey and Palni, 1998a). The watershed is situated in the southern part of the state of Sikkim, extending from 27^0 13′ to 27^0 16′ 15′′ N and 88^0 19′ 2′′ to 88^0 23′ 30′′E. The watershed has a great altitudinal variation, from 300 m to 2650 m amsl. The climate of the watershed is mainly subtropical up to an elevation of about 1100 m and temperate conditions prevail above this altitude. Kamrang experiences climate more representative of the subtropical region whereas conditions at Chamgaon and Jaubari are typically temperate. The mean monthly temperature was recorded 15^0 (max) and 12^0 (min) at subtropical and 10^0 (max) and 4^0C (min) at temperate sites. The annual rainfall (mm) was 1826 and 2227 at subtropical and temperate sites, respectively. The soil pH was 6.27 at Kamrang, 6.41 at Chamgaon and 6.46 at Jaubari (Sharma et al., 1992)

Reysenbach AL, Wickham GS, Pace NR. 1994. Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus spring, Yellow Stone National Park. *Applied and Environmental Microbiology* 60 (6), 2113–2119.

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Analyses of three groups of microorganisms, viz. actinomycetes, fungi and bacteria (including *Bacillus*, *Pseudomonas* and pigmented bacteria as a separate group) were taken into consideration. In general, microbial population of the three groups, bacteria, actinomycetes and fungi decreased along the increasing altitude. In case of bacteria and actinomycetes, a sharp and statistically significant decline ($P < 0.05$) above 1600 m was recorded. Fungal population appeared to be more sensitive to the altitudinal changes. The fungal counts decreased significantly ($P<0.05$) at Chamgaon (1600 m). On the other hand, interesting results were obtained in case of three specific groups of bacteria. The population of pigmented bacteria at 1600 and 1900 m altitudes were almost two times higher than their respective population at 1200 m. However, another group of one of the dominant bacterial species belonging to the genus *Bacillus* did not show much variation in terms of counts, probably on account of its endospore forming ability. The bacteria under this category were aerobic to facultative anaerobic, making slimy to irregular to rhizoidal colonies on nutrient agar. Microscopically, these were gram positive rods, occurring singly, in clusters or in chains and biochemically positive and capable of hydrolyzing starch and Tween 80. In the third group of bacteria, slimy and mucoid colonies (initially cream turning brown on prolonged incubation at lower temperature) were obtained on a nitrogen free media. This group of bacteria, like the pigmented ones, also showed an increasing trend in the population with increasing altitude. These bacteria were aerobic in nature, gram negative oval rods, catalase and oxidase positive, and unable to hydrolyze starch and ferment lactose. A number of bacterial isolates from this group were able to grow at 4^{0}C. These bacteria were placed under the genus *Pseudomonas*. The populations of various microbial communities recorded at three altitudes are presented in Fig. 1.

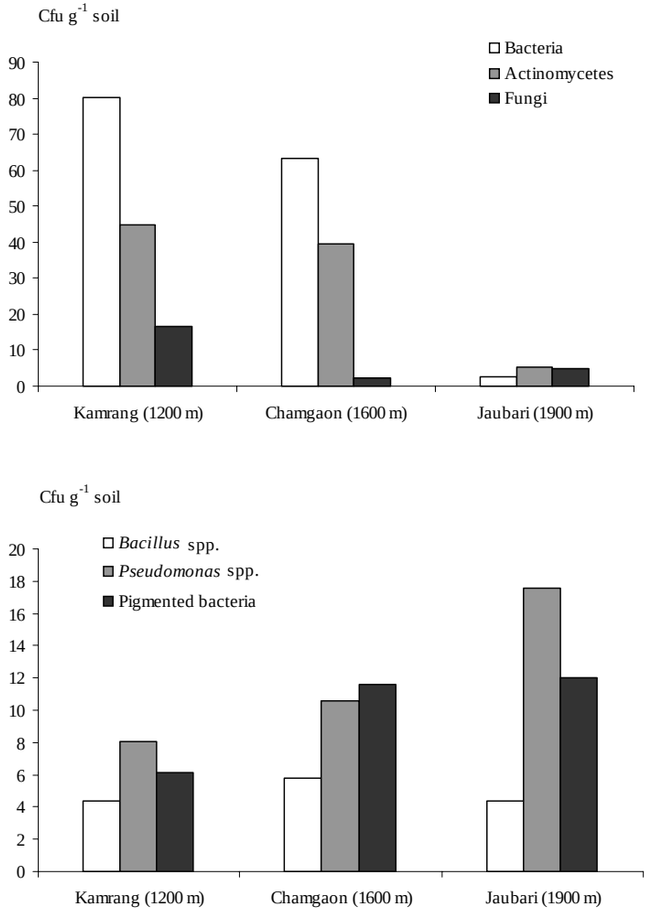

Fig. 1. Microbial populations along an altitudinal gradient in Mamlay Watershed, Sikkim

Amongst fungi, in the soil samples collected from both Sikkim and Uttaranchal Himalaya, species of *Penicillium*, namely, *P. aurantio-griseum*, *P. chrysogenum*, *P. citrinum*, *P. janthinellum*, *P. javanicum*, *P. oxalicum*, *P. pinophillum*, *P. purpurogenum* and *P. raistrickii* were most frequently occurring species in soil at higher altitudes. Species of *Aspergillus*, *Cladosporium*, *Epicoccum*, *Fusarium*,

Pandey A, Palni LMS, Mulkalwar P, Nadeem M. 2002. Effect of temperature on solubilization of tricalcium phosphate by *Pseudomonas corrugata*. *Journal of Scientific and Industrial Research* 61, 457–460.

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Gangronella, *Myrothecium*, *Paecilomyces* and *Trichoderma* also contributed to the fungal flora. Dominance of *Penicillium* spp. has been reported from various environmental niches. Baath (1981) reported the dominance of *Penicillium* spp. in pine forest soil in Central Sweeden, and Widden (1987) reported the dominance of *Penicillium* spp. along an elevational gradient ranged from 350 m to 880 m in Northern England. Various actinomycetes, mainly those that produce diffusible pigmented metabolites, were also isolated from these soils (Pandey and Palni, 1998a). Representative examples of selected and frequently occurring species of bacteria, actinomycetes and fungi are shown in Fig. 2 (A-F).

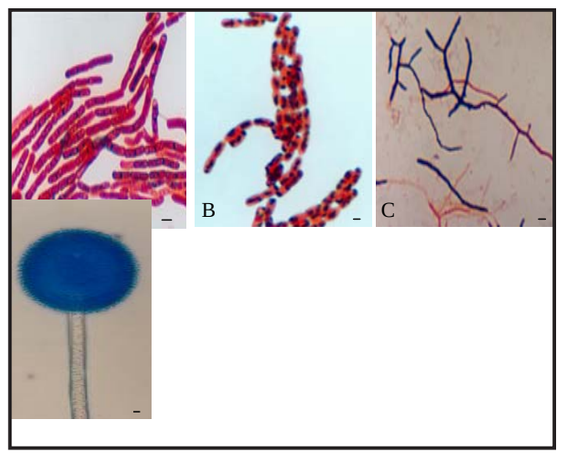

Fig.2. Microbial diversity-*Bacillus* spp. (A&B), *Streptomyces* spp. (C&D), *Penicillium* & *Aspergillus* spp. (E&F)

b. Rhizosphere effect exerted by Himalayan trees:

The influence of a particular plant species on the rhizospheric microbial population is referred as rhizosphere effect and conveniently illustrated by comparing the values for rhizosphere and non rhizosphere (R:S ratio) populations. The rhizosphere effect exerted on the microbial communities by ten representative and important tree species of the

Indian Himalayan region which covered a wide altitudinal range (1200 to 3610 m above mean sea level) representing sub tropical to sub alpine climatic conditions was studied. Based on the altitude, the regions fall within the subtropical (1200 to 1800 m amsl); temperate (1800 to 2800 m amsl); and sub alpine (2800 to 3800 m amsl) zones. Barring a few exceptions, the trees of sub tropical and temperate regions exerted a slight stimulatory effect on the rhizospheric microorganisms. Conifers of the sub tropical and temperate locations, namely *Cedrus*, *Pinus* and *Taxus* supported relatively higher microbial population in the rhizosphere in comparison to non-coniferous species. *Abies pindrow* (a conifer of sub alpine region) exerted a distinct negative rhizosphere effect. Similarly two other species of the sub alpine region, *Betula* and *Rhododendron,* also exerted suppressive effect on the rhizospheric communities. In case of *Betula utilis* and *Rhododendron campanulatum* (3040 m amsl), the soil samples were collected from the mixed forest sites where the two species were growing in close proximity, with their roots intermingled (unpublished observations). The classical concept of rhizosphere effect is based on the stimulation of microbial populations, at times fairly intense in the region adjacent to the roots, as against the bulk soil. This has been based on the results of research conducted largely on short duration plants and only a few tree species (Hale and Moore, 1979; Curl and Truelove, 1985; Lynch, 1990; Rovira, 1991; Waisel et al., 1991; Whipps, 2001; Pinton et al., 2001; Walker et al., 2003). In our investigations, it was considered that the rhizoflora of long lived plant species experiencing hard climatic conditions, such as low temperatures, heavy rainfalls and snow falls (e.g., temperate and sub-alpine climates), going through various successions due to various biotic and abiotic pressures, may result in dominance of selected microbial communities or populations upto an extent of exerting a negative rhizosphere effect.

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3. THERMOPHILIC MICROORGANISMS FROM HOT SPRINGS

Temperature is considered as one of the most important environmental factors controlling the activities and evolution of organisms (Pace, 1991). Microorganisms which grow between temperature range of 45-113^{0}C are defined as thermophilic microorganisms (Seckbach, 2000). The high temperature environments are associated with volcanic activity, such as hot springs, since these natural habitats have probably existed throughout the time in which organisms have been evolving on earth (Reysenbach et al., 2002). Hot springs are manifestations of geological activity that represent extreme environments that support a variety of microorganisms, filamentous organisms in particular. Occurrence of a pink filamentous community in the Octopus Spring and a black filamentous community in association with the thermal springs at Calcite in Yellow Stone National Park, USA, has been reported (Reysenbach et al., 1994; 2000). Recently, soil samples collected from two hot springs, Soldhar (latitude 39^{0}29' 25'', longitude 79^{0}39' 29'', altitude 1900 m amsl), and Ringigad (latitude 30^0 33' 14'', longitude 79^{0}40' 0.06'', altitude 1850 m amsl) both located in the Chamoli district of the Garhwal region of Uttaranchal Himalaya, were analysed in our laboratory for their physical, chemical and microbial components (Kumar et al., 2004). The approximate area of both these sites was about 45 m^2, while the hot water outlet (90^{0}C) was present in the middle of the mound at Soldhar, the mouth of the hot spring was located along slope towards a cliff at Ringigad creating a temperature gradient along the slope. At this site the soil temperature around the water outlet was 78^{0}C. The soil from both sites was slightly alkaline, light brown in colour with the same particle size of >60 µm. The water holding capacity of the soil from Soldhar

was almost three times that of Ringigad; carbon, nitrogen and potassium were not detected in these soil samples. The soil from Soldhar had higher amounts of Cu, Fe and Mn; Cu was absent from the Ringigad sample. Soil samples from the Ringigad site were found to possess higher phosphorus content.

Microbial analyses of soil samples exhibited dominance of bacteria, in addition to filamentous forms. While the microorganisms were isolated from the soil samples on various media across a broad temperature range (21^0 to 80^{0}C), the optimum temperature for isolation of microbial population was 50^{0}C. Moderate numbers were isolated on diluted tryptone yeast extract agar plates, which also helped to enumerate a few colonies that were morphologically different from those that developed on full strength medium. A total of 59 morphologically distinct isolates (58 aerobic and one anaerobic) were isolated and developed as pure cultures. The isolates were grouped in various categories, such as thermotolerants, thermophiles and hyperthermophiles (Table 1, Fig. 2 G–H, Kumar et al., 2004). High temperature, alkaline nature and poor nutrient status of the soil prevailing at the hot spring sites seem to provide a unique environment for the development of specialized microbial communities.

Thermophillic filamentous organisms (Fig. 2 G&H)

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Table 1. Grouping of the total aerobic organisms isolated from hot spring sites based on physiological parameters

GROUP	RANGE	NUMBER OF STRAINS
Thermotolerants	30–55 ºC	46
Thermophiles	45–65 ºC	9
Hyperthermophiles	55–85 ºC	3
Halophiles	0.5–2.0%	3
	2.0–5.0%	16
	10%	38
Alkaliphiles	8-9	31
	10	12
	11	15
Acdiophiles	6	29
	5	17
	4	12

(Kumar et al., 2004)

The extremophiles, thermophiles in particular, have been a subject of extensive investigation, with a view to the production of industrially useful enzymes, such as amylase, protease, cellulase, and cellulose free xylanase, and for their industrial applications in the detergent, leather, pulp and paper and other industries, etc., (Pennisi, 1997; Srinivasan and Ingale, 1999; Budhiraja et al., 2002). For example, *Thermus aquaticus* was the first such organism to be used for the production of Taq polymerase, followed by *Pyrococcus furiosis*, for the production of Pfu (Mani and Sakker, 2001). Kinetics of amylase activity of *Sacharomycopsis fibuligera* isolated from the hot spring site in Garhwal Himalaya has recently been reported from this laboratory (Kumar et al., 2005). *S. fibuligera* was the only eukaryote that was obtained from the soil samples of the hot spring sites. The yeast was

found to produce extracellular amylase on soluble starch, having 73 units ml^{-1} activity and temperature and pH tolerance from 4 to 60^0C (optimum 40^0C) and 4–11 (optimum 6 pH), respectively. *S. fibuligera* has been reported to be a weak fermentative organism with known ability to improve the quality of fermentation products when used as a blend (Verachtert and Mot, 1990). Knowledge of the natural habitat of a given microbe does indicate the potential uses that the organisms can be put to, as it defines the growth parameters likely to be encountered. Occurrence of another species of yeast, *Debaryomyces vanriji*, in association with thermophilic bacteria has been reported from a hot spring in Russia (Rikhvanov et al., 1999). The amylase family of enzymes produced by the microorganisms is of great significance due to wide applications (Pandey et al., 2000).

4. MYCORRHIZAL ASSOCIATIONS IN HIMALAYAN TREES

Mycorrhizae are beneficial associates of plants that influence plant growth in terms of increased nutrient and water absorption from the soil, protection from pathogens, increased tolerance to soil toxins, and root elongation (Trofymow and van den Driessche, 1991; Podila and Varma, 2005). Seven types of mycorrhizal associations namely- ectomycorrhizae, arbuscular mycorrhizae, ecto-endo mycorrhiza, arbutoid mycorrhiza, monotropoid, ericoid and orchidoid mycorrhizae are known (Smith and Read, 1997; Mukerji et al., 2002). The status of ectomycorrhizae and their importance in plant growth in the context of Himalayan trees has been described by Lakhanpal and Sagar (1994).

The belowground diversity of vesicular arbuscular mycorrhizal fungi is a major factor in the maintenance of plant diversity and ecosystem functioning (van der Heijden et al., 1998). Detailed

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investigations conducted on the arbuscular mycorrhizal (AM) fungi associated with *Taxus baccata* and five species of rhododendrons found in Uttaranchal Himalaya were recently reported from our laboratory (Chaurasia et al., 2005a &b). In case of *Taxus baccata*, root and rhizosphere soil samples were collected from three sites in the Himalayan region, representing sub temperate to temperate, as well as the transition zone between the temperate and alpine conditions. At site A (Jageswar Forest- 29^0 35′-29^0 39′ N and 79^0 53′-79^0 59′ E, 1800 m amsl, Dist. Almora) *T. baccata* trees were found under the canopy of deodar (*Cedrus deodara*) growing along a water channel. *Quercus floribunda*, *Q. leucotrichophora*, *Aesculus indica*, and *Rhododendron arboreum* were other tree associates. At site B (Vinayak forest- 29^0 27′-29^0 29′ N and 79^0 23′-79^0 25′ E, 2200 m amsl, Dist. Almora) *T. baccata* trees were found under the thick canopy of *Abies pindrow* and *Pinus wallichiana*. Site C (Khaljuni Forest- 30^0 6′-30^0 8′ N and 79^0 57′-79^0 59′ E, 2450 m amsl, Dist. Bageswar) represented a transition zone between the temperate and alpine conditions. The upper forest canopy was dominated by *T. baccata*, *A. pindrow* and broad-leaved *Q. semecarpifolia*. The second story mainly consisted of *Q. leucotrichophora*, *R. arboreum*, *Alnus nepalensis* and *A. indica*. At this site *T. baccata* forms open canopy with other tree species.

The colonization by the AM fungi was characterized by the presence of arbuscules, inter/intra cellular hyphae, vesicles and extraradical chlamydospores. The degree of colonization observed at the three sites was in the order: site C> site A> site B, and the average number of intraradical vesicles were >20 at site C and < at site B, these vesicles were not found in the root samples collected from site A.

Formation of ellipsoidal and spherical vesicles indicated the presence of genus *Glomus* and irregular or rectangular shaped vesicles were indicative of the presence of genus *Acaulospora* in the roots of *T. baccata*. The occurrence of different shaped vesicles in the same root segment indicated AM colonization by more than one species. The absence of vesicles at site A was characteristic of root colonization by *Gigaspora* and *Scutellispora*. The extraradical chlamydospores (genus-*Glomus*) were also associated with fine feeder roots of *T. baccata*; such roots were covered with a sheath or mantle of fungal mycelium, confirming the presence of ectomycorrhizae in *T. baccata* roots. Such observations have been reported in other studies as well (Tommerup, 1988; Morton, 1990; Onguene and Kuyper, 2001).

Five genera of the order-Glomales, namely *Acaulospora*, *Entrophospora*, *Glomus*, *Gigaspora* and *Scutellispora* were isolated from the rhizosphere soil of *T. baccata*. Genus *Sclerocystis* was not observed in any of the soil samples. Species richness of AM fungi was highest at site C (9 species), followed by site A (6 species) and B (5 species). The major component of AMF composition at site C was on account of genus *Glomus* (44 %), while the same at site A was equally shared by genus *Glomus* and *Gigaspora* (33.33 % each). *Glomus fasciculatum* and *G. aggregatum* were dominant in the rhizosphere soil samples from sites B and C, respectively. Several workers (Ragupathy and Mahadevan, 1993; Dalpe and Aiken, 1998) have reported predominance of *G. fasciculatum*, under various climatic conditions, ranging from tropical to high arctic. The distributional pattern is known to vary with the prevailed climatic conditions in a particular region (Vestburg, 1995).

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AM associations in rhododendrons were investigated in all the five species of rhododendrons (*Rhododendron anthopogon*, *R. arboreum*, *R. campanulatum*, *R. barbatum* and *R. lepidotum*) of temperate, sub-alpine to alpine zones of Pindari Glacier region (33^0 5′–30^0 10′ N to 79^0 48′–79^0 52′ E) of Uttaranchal Himalaya (Chaurasia et al., 2005b). A total of five species of rhododendrons (family Ericaceae), distributed in the altitudinal range from 1500 to 4500 m amsl (up to timber line), are found in this region. *R. arboreum* is most widely distributed and starts appearing around 1500 m elevation; it increases in importance with elevation especially in oak dominated forests. Beyond 2800 m it is replaced by *R. barbatum* in the birch forest. Eventually, vegetation is represented by scrubs or heaths (above 3400 m amsl), where *R. campanulatum* and *R. anthopogon* are the sole representatives of woody vegetation, and *R. lepidotum* forms sparse bushy appearance in the alpine meadows. The pH in these soils ranged from 4.60 to 5.32. In the cited study, the distribution of AM fungi along with details of externally associated functional structures (extra radical mycelium, vesicles and chlamydospores) as well as internal associations (intraradical hyphal coiling, intraradical vesicles and chlamydospores) is described. Extraradical spores and vesicles as well as intraradical vesicles and spores were observed. Extraradical as well as intraradical spores and vesicles varied from spherical to ellipsoidal in shape. Colonization of AM fungi was almost similar in all the rhododendron species. Maximum arbuscular mycorrhizal percent colonization was observed in *R. arboreum* (42 %) followed by *R. barbatum* (40 %), *R. anthopogon* (37 %), *R. campanulatum* (33 %) and *R. lepidotum* (28 %). Intraradical vesicles were not observed in

R. lepidotum samples. Arbuscules were not detected in the root segments of all the species, however, the hyphal coiling and constriction in hyphae were observed. The results indicated that there was no correlation between the arbuscular mycorrhizal colonization and spore population. The number of species of arbuscular mycorrhizal fungi associated with the rhizosphere soil samples of all the rhododendrons showed a limited correlation with arbuscular mycorrhizal colonization.

Spore populations were found to belong to five genera, namely- *Acaulospora*, *Glomus*, *Gigaspora*, *Sclerocystis* and *Scutellispora*. A total of 16 spore forming arbuscular mycorrhizal fungi were observed. Among these seven species belonged to the genus *Glomus*, three each to *Gigaspora* and *Scutellispora*, two to *Sclerocystis* and one to *Acaulospora*. The rhizosphere soil of all the five species of rhododendrons appeared to be dominated by the species of *Glomus*. *G. fasciculatum* was found to be the most abundant and common species except in *R. barbatum* where it was not observed. Genus *Gigaspora* was found to occur frequently associated with *R. campanulatum* along with the genus *Glomus*. *Acaulospora foveata* was absent from the soil samples of *R. anthopogon* and *R. lepidotum*. *Acaulospora* sp., *Gigaspora* sp., and *Sclerocystis* sp. were not detected in the rhizosphere soil samples of *R. lepidotum*. The study also reflected a trend indicating decrease in the richness and diversity of arbuscular mycorrhizal fungi with the increasing altitude. It coincided with decreased richness and diversity in the vegetational flora along an increase in the altitude.

Smith and Smith (1997) reviewed structural diversity of arbuscular mycorrhizal and recognized two types of colonizing pattern, viz., *Arum*-type and *Paris*-type. In the *Arum*-type, extensive intercellular

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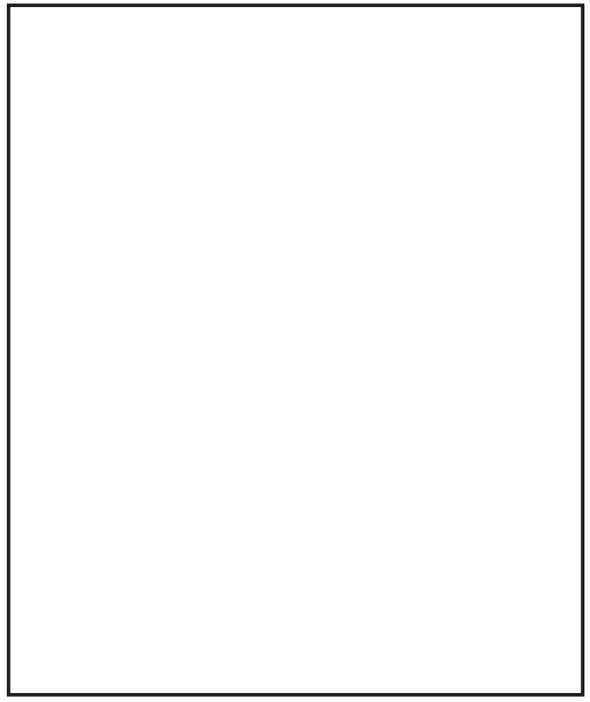
hyphae and arbuscules develop, while in the *Paris*-type these structures are absent and hyphal coils occur commonly. The *Paris*-type colonization of arbuscular mycorrhizal fungi was found to be prominent in all the five species of rhododendrons. These structures may have a role in bidirectional transfer of nutrients in the absence of arbuscules. In two of the rhododendron species, namely *R. campanulatum* and *R. anthopogon*, presence of dark septate (DS) fungi was also observed. Functional role of DS fungi in rhododendrons is a matter of investigation. The occurrence of DS fungi in high stress environments such as alpine and arctic habitats suggests a mutualistic role of these fungi (Jumponen, 2001). DS fungal structures have also been reported from antarctic and sub-arctic sites (Christie and Nicolson, 1983; Treu et al., 1996).

Arbuscular mycorrhizal fungi are widely distributed common soil fungi being abundant in phosphorus and other mineral deficient soils and also obligate symbionts which affect host plant positively. Around 70–80 % of plants ranging from bryophytes to flowering plants including aquatic plants have the obligate symbiotic association of AM fungi. AM fungi are unique as they are present partly inside the host and partly outside the root. The vesicles, arbuscules and hyphae are formed inside the root and do not encounter competition and antagonism from other soil communities. Arbuscules are the key sites for nutrient exchange. There is a great potential for AM fungi as biofertilizer subjected to their multiplication, mass production of inoculum and commercialization (Manoharachary, 2000, 2004). Some of the frequently occurring spores of AM fungi isolated from rhizosphere of temperate trees are shown in Fig. 2 I–L.

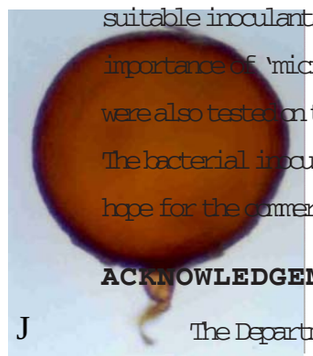


Spores of arbuscular mycorrhizae (I-L). Bar=2µm (A-H); Bar=20µm (Fig. 2 I-L).

5. BIOTECHNOLOGICAL APPLICATIONS

The extraordinary activity of microorganisms is based on their remarkable metabolic diversity and genetic adaptability. This resulted in the development of technologies for the production of antibiotics and other therapeutic agents, management of pests and pathogens, bioleaching of metals, increasing soil fertility, generating biofuels, monitoring air pollution, destroying persistent pollutants, waste water treatment, bioremediation, and serving tools in biomedical research (Atlas and Bartha, 1998). While major efforts have focused on generating the basic information on the occurrence, isolation and

The use of biological fertilizers, in recent times, is gaining attention mainly due to (1) the environment friendly nature of bioinoculants, (2) the long term hazards associated with the continued use of chemical fertilizers, and (3) increased acceptability of natural "organic" products globally. In practice, the use of biological fertilizers is limited due to the unavailability of suitable climate based inoculants. In view of the knowledge of ecological specificity associated with naturally occurring microorganisms, consistent efforts from research laboratories are required for selecting and developing microbial inoculants suited to a specific set of climatic conditions. Significant initiatives have been taken in our laboratory for developing bacterial inoculants for colder regions in the mountains. Naturally occurring native species of *Bacillus* and *Pseudomonas* due to their positive effects on a variety of agricultural and forest species have been rated as most suitable inoculants for use under temperate conditions. In view of the importance of 'micropropagation technology' the bacterial inoculants were also tested on tissue culture raised plants with encouraging results. The bacterial inoculants in the form of appropriate formulations provide hope for the commercialization of this technology.



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of colder regions in the mountains is essential from the point of view of both basic and applied nature.

Identification of areas with extreme environments and investigations on associated microbial wealth for biotechnological applications are of great importance. Investigations on thermophiles have recently got much attention due to the biotechnological potential associated with these microorganisms and their cellular products (Rakshit, 2005). Thermostable proteins from thermophiles have a wide range of applications as they are more robust than other proteins and are active in extreme conditions. The study sites for isolation for thermophilic organisms are often fragile due to various reasons including natural disturbances. The hot springs of Garhwal Himalaya got disturbed due to the blasting process applied for road construction work. Use of these hot springs for domestic activities, e.g., boiling of food materials such as rice, potatoes, etc., also disturb the microbial diversity. Drill holes and wells, constructed for providing hot water to nearby villages have also reduced the geothermal activity and discharge of hot water. Isolation, characterization and preservation of the microbial diversity obtained from these two hot springs is an effort towards the conservation of the microbial diversity associated with the two hot springs, which seem to be in grave danger. These investigations are important for understanding (1) the microbial diversity associated with the hot springs, (2) the biotechnological applications of the microbial isolates, and (3) the strategies adapted by the microbial community for the survival under extreme environment (Trivedi et al., 2006).

characterization of various microbial communities in different niche areas spread across the Himalayan region, selected pure cultures have been investigated for some of the important biotechnological applications. Screening for the desirable traits and selection of efficient strains has been carried out continuously in the laboratory. For example, development of microbial inoculants for improving the plant productivity, and investigations related to the production of secondary metabolites and important enzymes have resulted in useful findings of applied value.

a. Microbial inoculants for colder regions:

The use of biological fertilizers, in recent times, is receiving attention mainly on account of increased global preference for natural "organic" products. Isolation of microorganisms, screening for desirable characters, selection of efficient strains, production of inoculum, and preparation of carrier based formulations are important steps in the use of this microbe-based environment friendly and sustainable technology. With a view of developing microbial inoculants suitable for field applications in the colder mountainous regions, a systematic long term investigation was conducted. At the very outset, field inoculation trials were carried out at higher elevations using available bacterial inoculants originally isolated from the warmer regions. Inoculation trials using three strains of *Azotobacter chroococcum* and two of *Azospirillum brasilense* were carried out on farmers fields at two elevations, representing subtropical (1200 m altitude) and temperate (1900 m altitude) climates, in a watershed in Sikkim Himalaya. Maize, a major cereal crop in Sikkim, which is cultivated at altitudes varying from the foothills to 2000 m amsl under rainfed conditions, was used as a test crop. One of the objectives of this study was to find out if the above

mentioned and well known growth promoting bacterial inoculants were effective at higher locations, characterized by low temperature and high rainfall. Statistically significant increment in grain yield, growth promotion, and yield attributing characters was observed, at the subtropical site, in this study. Almost 1.5 fold yield enhancement over control was recorded with one of the strains of *Azotobacter chroococcum*. Contrary to this, bacterial inoculations were found to be ineffective at the temperate site. This probably resulted from the inability of introduced bacteria to establish and/or survive at lower temperatures. The bacterial inoculants used in this study were originally soil isolates from tropical areas, and were obtained from the Division of Microbiology, Indian Agricultural Research Institute, New Delhi.

This field based study clearly indicated the need for fresh isolations of native beneficial rhizobacteria, for selection and further development as inoculants for use at the higher elevations (Pandey et al., 1998). The study confirmed effectiveness of bacterial inoculants at the subtropical site, no effect was, however, observed at the temperate site. Soil samples were, therefore, collected from various temperate and alpine locations spread across the Indian Himalayan region in order to isolate as well as screen potential plant growth promoting native bacteria. A culture collection of native "high altitude bacteria" was developed, and the selected isolates were characterized for plant growth promotion and biocontrol with special reference to their adaptability to low temperatures. Systematic screening experiments based on petridish assays, bioassays, greenhouse and field trials have resulted in the selection of four bacterial species, namely *Bacillus megaterium*, *B. subtilis*, *Pseudomonas corrugata* and *P. putida*. Efficient bacterial species of

terms of particular conditions for incubation, modified growth media and high incubation will be required. For maintenance of thermophiles agar flakes (containing the organism) were developed by prolonged incubation at the optimum temperature in agar plates (agar concentration 2 %). The cultures could be revived easily by introducing small piece of dried agar on fresh medium (Kumar et al., 2004). The pure isolates of mesophiles and psychrophiles are being maintained by regular subculturing and deep-freezing in 10% glycerol at -20^{0}C.

7. CONCLUSION

The documentation and conservation of biodiversity including microbial diversity is important as it provides the potential source of biological resources. Microbial culture collections are well recognized as germplasm banks which contribute resource pools for biotechnological research and development (Srinivasan, 1992). The techniques used to preserve the microbial strains are of critical importance. It is essential that the full potential of the strains are retained (Kurtboke and Swings, 2004). Through application of molecular methods, such as direct extraction of nucleic acids from the environment, application of PCR (Polymerase chain reaction) technologies and sequence analysis, the assessment of the biodiversity of prokaryotes including culturable and non-culturable as well is being taken up. The identity of a microbial species can be assessed by analysis of its genetic material, preferably by 16S ribosomal DNA (Stackebrandt and Liesack, 1993). Initiatives on exploration, documentation and preservation of microbial diversity of Himalayan region have been taken up by this laboratory with special reference to their biotechnological applications. It can be concluded that, the exploration of below ground biodiversity

acid sequences, and gene banks, thereby ensuring the maintenance of all species as well as the endangered ones. It must be understood that large amounts of biological materials need not be stored physically, in future. It may be adequate only to store tissues, small samples of soil, etc., as frozen "blueprints" or "micro-ecosystems" (Sly et al., 1990).

The cultures held in the World's microbial culture collections represent the known culturable microbial diversity. Almost one million cultures are known to be preserved and maintained in the major collections, and considerably more are likely to be held by research scientists and the industry in private or personal collections. These cultures represent a valuable genetic resource (Sly, 1994). Microorganisms often require special preservation methods in order to ensure optimal viability, storage, purity and stability of individual strains. A culture collection of microbial isolates isolated from various locations in the Indian Himalayan region has been developed in this laboratory. Following preliminary characterization, 'the microbial isolates' are regularly being accessioned in the National or International repositories. Bacterial, actinomycetes and yeast cultures are being accessioned by the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, and fungal cultures by the Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi. Two of the plant growth promoting rhizobacteria, *Bacillus subtilis* and *Pseudomonas corrugata*, have been accessioned at the Agricultural Research Culture Collection, International Depository Authority, Illinois, USA. With continued explorations on extreme environments, e.g., for the isolation and maintenance of thermophilic microorganisms under laboratory conditions, special requirements in

Bacillus and *Pseudomonas* have also been evaluated in form of carrier based formulations, for application in relatively colder regions.

b. *Bacillus* and *Pseudomonas* spp.: Novel inoculants for colder regions:

Since Hiltner's (1904) pioneering work, there have been continuous efforts to gain an in-depth understanding of the "rhizosphere microbiology" focused towards the isolation and selection of rhizosphere microorganisms for use in plant growth promotion and biocontrol (Fravel, 1988; Weller, 1988; Kloepper et al., 1989; Pandey and Kumar, 1989; Pandey and Palni, 1998b; Whipps, 2001). As every plant provides a somewhat species specific site for the stimulated microbial activity in form of a "rhizosphere", every rhizosphere, in turn, also provides an opportunity for the isolation of microbial isolates that can be used for biotechnological applications. The rhizosphere microorganisms which are closely associated with plant roots have been termed as plant growth promoting rhizobacteria (PGPR) (Glick, 1995). The rhizosphere and rhizoplane often provide better potential sites for the isolation of beneficial organisms than the bulk soil (Weller, 1988; Pandey et al., 1998). Natural rhizospheres are often inimical to pathogens since they harbour antagonists as part of the rhizosphere community (Lynch, 1990). Four major groups of plant-microbe associations on record are: (1) *Rhizobium*-legumes, (2) free living microorganisms-plant species, (3) *Frankia*-actinorhizal plant species, and (4) mycorrhizae-host plants. The best known examples of the plant growth promoting or biocontrol agents are species of *Agrobacterium*, *Azotobacter*, *Azospirillum*, *Bacillus*, and *Pseudomonas* (bacteria), *Frankia* and *Streptomyces* (actinomycetes), and *Trichoderma*, *Gliocladium* and *Glomus* (fungi), (Kerr and Tate, 1984; Papavizas, 1985;

Fravel, 1988; Weller, 1988; Pandey and Kumar, 1989; Whipps, 2001).

While conducting the investigations on soil microbial diversity of Himalayan region, a culture collection was established consisting of microorganisms isolated from the soil samples collected from various temperate / alpine locations (upto 3600 m amsl) including higher altitudes of Himachal Pradesh, Sikkim, Uttaranchal and West Bengal. The microbial analyses established the dominance of *Bacillus* and *Pseudomonas* species in these soils. In view of the importance of these bacteria in plant growth promotion and disease control, the isolates were subjected to biochemical and physiological characterization, including "desirable traits" such as phosphate solubilization, nitrogen fixation, antagonism against disease causing fungi and survival at low temperatures. For detailed investigations, two species of *Bacillus* (*B. subtilis* and *B. megaterium*) and two of *Pseudomonas* (*P. corrugata* and *P. putida*) were selected.

The selected strains of *Bacillus* species isolated from rhizosphere soil samples exhibited strong antifungal activity against a range of saprophytic as well as pathogenic fungi. *Bacillus subtilis* gave the best results (Pandey and Palni, 1997, Pandey et al., 1997). *Bacillus* species including *B. subtilis* are known for their antifungal properties, hence their importance in the biological control of a number of plant and animal diseases (Broadbent et al., 1977; Fravel, 1988; Weller, 1988; Milner et al., 1996; Pandey et al., 1997; Ryder et al., 1999; Whipps, 2001). Three mechanisms of biocontrol involve competition, parasitism / predation, and antibiosis (Baker, 1968). An efficient antagonistic strain of *Bacillus subtilis*, originally isolated from the rhizosphere of established tea, and selected through screening was found to cause structural

dry alginate microbeads and gum-arabic preparations of bacterial inoculants like *Azospirillum brasilense* Cd, *Pseudomonas fluorescens*, and *Rhizobium* sp. have been evaluated (Bashan, 1998; Forestier et al., 2001; Bashan et al., 2002). For commercialization, viability of the bioinoculant(s) in a prescribed formulation for a defined period, with preservation of strain characteristics is of utmost importance (Fages, 1992; Smith, 1992). Alginate beads have also been reported to preserve the beneficial properties of PGPRs during storage (Russo et al., 2001). Lewis and Papavizas (1985) suggested storage of alginate pellets carrying the strains of *Trichoderma viride* and *Gliocladium virens* at 5^0C. Lower temperatures ($4 - 10^0$C) are known to slow down the process of division and retard metabolic activities of bacterial cells, resulting in concomitant reduction in the consumption of nutrients and reduced moisture loss from the carriers, thus improving the keeping quality of inoculants (van Shrevan, 1970).

6. CULTURE COLLECTIONS AND THE CONSERVATION OF MICROBIAL DIVERSITY

The idea of establishing culture collections was conceived during the period 1880–1890, at a time when solid culture media such as those based on potato, gelatin, and agar were being devised. This led to the isolation of pure cultures of microorganisms, and at this stage, need for the "preservation" of such pure cultures for future work was also felt. This is important from the point of view of the ready availability of basic microbial resource for carrying out fundamental studies, as well as for providing the raw material for developing novel products. Culture collections have responded to this challenge by providing repositories for the safe "live" storage of germplasm, including nucleic

tumefaciens 60 to 97 % root formation (1–3 roots per explant) within 8 to 10 weeks of infection was observed against only 13 % in control seedlings (Mihaljevic et al., 1996). In *Leucaena leucocephala*, over 80 % survival of in vitro raised plants could be obtained when co-cultured for two weeks with *Rhizobium* (NGR 8) in screw cap bottles on quartz with inorganic nutrient salts (Dhawan and Bhojwani, 1987). Similarly ectomycorrhizae as well as vesicular-arbuscular mycorrhizae have also been tried for the hardening of micropropagated plants (Wang et al., 1993; Varma and Schuepp, 1996; Reddy and Satyanarayan, 1997; Hernandez-Sebastia et al., 1999; Sahay and Varma, 2000).

d. Storage, viability and carrier based formulations:

Appropriate formulations of identified inoculants in easy to use form are required for the field application. Maintaining viability of the inoculum during storage and transport is an important consideration towards commercialization of this microbe-based technology (Whipps, 1997; Bashan, 1998). Two of the selected bacterial isolates (*Bacillus subtilis* and *Pseudomonas corrugata*) were tested using five formulations (three alginate based, one coal based and one broth based) for inoculant delivery in a maize based bioassay. The viability of bacterial formulations was evaluated following storage either at 4^{0}C or at room temperature, upto six months. Rhizosphere competence of the inoculated bacteria was highest after 6 weeks of growth in the plants treated with alginate based formulations. Maximum viability of bacterial inoculants was recorded in alginate bead based formulations, even after 180 days of storage at 4^{0}C (Trivedi et al., 2005). Due to the limitations of direct inoculation and in the use of various solid-phase bacterial inoculants, several polymer based formulations, such as alginate beads, wet and

deformities in phytopathogenic fungi, under *in vitro* culture conditions; this was attributed to the production of diffusible and volatile antifungal compounds (Table 2).

(Table 2) Inhibition in the radial growth of pathogenic fungi caused by diffusible or volatile compound(s) produced by *Bacillus subtilis*

Pathogen	Per cent inhibition in radial growth					
	24h		72h		120h	
	Diffusible	Volatile	Diffusible	Volatile	Diffusible	Volatile
Alternaria alternate	39.10	20.00	49.50	60.00	71.70	65.20
Cladosporium oxysporum	27.70	15.90	46.00	41.00	53.30	52.10
Fusarium oxysporum	17.50	34.40	45.30	40.90	66.10	60.00
Paecilomyces lilacinus	35.00	20.00	50.00	42.10	67.00	52.10
P. variotii	26.50	33.30	48.10	46.80	59.20	62.80
Pythium afertile	29.20	30.00	35.10	84.00	67.90	84.00

(Chaurasia et al., 2005)

Out of six test fungi, four (*Alternaria alternata*, *Cladosporium oxysporum*, *Fusarium oxysporum* and *Pythium afertile*) were phytopathogenic, while the remaining two (*Paecilomyces lilacinus* and *P. variotii*) were of clinical importance. The pathogens were isolated from the soil samples collected from temperate and alpine forests of Himalayan region and have been reported to cause diseases (*Fusarium oxysporum*- *Fusarium* wilt and rots, *Pythium afertile*- damping off of seedlings and *Pythium* blight, *Alternaria alternata*-leaf spot and leaf blight, *Cladosporium oxysporum*- fruit and crop rots, *Paecilomyces lilacinus* and *P. variotii*- causal agents of various human diseases (Bilgrami et al., 1991; Dhindsa et al., 1995; Fletcher et al., 1998).

The bacterial strain successfully restricted the growth of all the test fungi in dual cultures, and induced several morphological abnormalities such as mycelial and conidial deformities. For example, deformation in mycelial, hyphal or conidial structures was common in all tested fungi. The transverse as well as longitudinal septa completely disappeared in *A. alternata* and the conidia became thick walled and spherical or irregular in shape. In various instances conidia formation was arrested, and only the vegetative mycelium was observed. In *C. oxysporum* the conidiophores became vegetative and stunted; formation of normal conidia was also hampered. Prominent lysis of fungal hyphae, and vacuolation as well as granulation in mycelial structures was observed in *F. oxysporum*. In this case, the conidia became swollen and thick walled. Similarly in *P. afertile* also lysis of fungal hyphae, and vacuolation and granulation of mycelial structures were observed. Formation of normal sporangium and oogonium was found to be greatly suppressed in *P. afertile*. Morphological abnormalities were also recorded in both the species of *Paecilomyces*; swollen and broad conidiophores due to vacuolation of mycelium were common to both, *P. lilacinus* and *P. variotii*. The inhibitory effect was much greater on account of volatile than that caused by diffusible compounds (Chaurasia et al., 2005). Antimicrobial compounds may act on the phytopathogenic fungi by inducing fungistasis, inhibition of spore germination, lysis of fungal mycelia, or by exerting fungicidal effects (Gloud, 1990). Antagonism is known to be mediated by a variety of compounds of microbial origin, e.g., bacteriocins, enzymes, toxic substances, volatile compounds, etc. The effect of volatile compounds has received only limited attention in comparison to the antagonism affected by diffusibles.

'tissue culture' or 'micropropagation' is performed under 'aseptic' and 'controlled' environmental conditions. Therefore the tissue cultured plants lack the required resistance towards major and minor pathogens in the absence of any previous exposure to various microbial communities present in the open environment. When such aseptic plants are transferred to soil they encounter soil microbial communities for the first time. Bacterial inoculations (*Bacillus subtilis* and *Pseudomonas corrugata*) were used for the hardening of tissue culture raised plants of tea (*Camellia sinensis*) and *Picrorhiza kurrooa* (an alpine medicinal herb). The analyses of rhizosphere and rhizoplane soil samples suggested fungal attack (mainly *Fusarium* sp.) to be the major cause of mortality during lab to land transfer. Inoculation with selected bacteria at the time of transplantation provided the first line of defense to tissue culture raised plants, and resulted in near 100 % survival against 45–55 % survival of control plants in tea. In case of micropropagated plants of *P. kurrooa*, inoculations with *B. subtilis* or *P. corrugata*, at the time of lab to land transfer, resulted in 92.5 % or 85.0 % survival of treated plants, respectively, against 37.5 % survival in untreated controls. The overall growth of inoculated plants was also superior in both the cases (Pandey et al., 2000; 2002).

Biological hardening refers to the use of 'biological agents' at the time of field transfer of tissue culture raised plants for better survival and establishment. Application of selected microorganisms for this purpose is gaining attention (Bhojwani and Dhawan, 1989; Kozai, 1991; Ziv, 1995; Pandey et al., 2002). For example, when in vitro raised *Pinus nigra* seedlings, cut from the hypocotyl end, were co-cultivated with the wild strains of *Agrobacterium rhizogenes* and *A.*

selective antibiotics (Josey et al., 1979; Kluepfel, 1993). The individual populations of introduced bacteria were found to differ significantly during the complete period of growth. *P. corrugata* maintained higher population during winter in comparison to *B. subtilis*, and exhibited greater tolerance to low temperature. The bacterial inoculants were found to be efficient rhizosphere colonizers and positively influenced various growth parameters, e.g., shoot length and stem girth in all four clones of tea (Trivedi et al., 2005). Similar pattern of root colonization in the rhizosphere of wheat by *Bacillus* sp. L324-92R$_{12}$ and *Pseudomonas* sp. 2-79RN$_{10}$ has been reported (Kim et al., 1997). In several experiments conducted at the subtropical locations in the mountains, *Azotobacter chroococcum* was used for comparison, and in most cases it gave positive results (Pandey et al., 1998; 1999; Bisht et al., 2003). The significance of nitrogen fixation combined with root colonization in diazotrophic pseudomonads has been recognized while developing bacterial inoculants for temperate regions in the Canadian High Arctic (Lifshitz et al., 1986).

Plant tissue culture technology has gained importance not only for its use in 'micropropagation' per se but also as an effective tool for the 'conservation' of rare and endangered species. The technology has so far received limited acceptance and success at the commercial scale. Major efforts have been on in-depth research for developing protocols under aseptic and controlled environment. The plants raised in the 'controlled' environment are 'delicate' with a 'fragile' root system and experience heavy mortality during lab to land transfer. Besides many 'physiological' and 'anatomical' deficiencies that are often associated with tissue culture raised plants, one major cause of high mortality that needs serious consideration is 'biological' in nature. The

The available literature suggests that only a few bacteria, the aerobic bacilli and anaerobic clostridia, in particular, are capable of forming spores that are resistant to environmental stresses (Stolp, 1988). Species of *Bacillus* can survive under adverse conditions due to their spore forming nature that can be activated by a variety of treatments, notably exposure to heat (Sneath, 1984; Slepecky and Hemphill, 1992). The present study confirmed the importance of established natural rhizospheres for the isolation, screening and selection of efficient biocontrol agents. Such agents exhibiting antagonistic effect on a range of pathogenic fungi should be preferred in the disease management programmes.

In a detailed investigation conducted on young to established (4 to 123 years old) tea bushes of *chinery* as well as *assamica* types growing in well maintained to abandoned gardens, and representing monsoonal, subtropical to temperate locations in the Indian Himalayan region, several characteristic features have been identified. Occurrence of a negative rhizosphere effect, exerted by the established tea bushes in contrast to the normal stimulatory effect exhibited by the young tea bushes was the first and foremost feature associated with the tea plants. Preponderance of greater antagonistic populations consisting of species of *Bacillus*, *Streptomyces*, *Trichoderma* and *Penicillum*, and lowering of the soil pH were other important characteristic features of the tea rhizosphere (Pandey and Palni, 2004). Amongst the species of *Bacillus*, *B. subtilis* and *B. mycoides* were the most dominant ones. The two species comprised a major part of the bacterial population, even during unfavourable periods. The bacterial species were isolated during extreme winters when the soil temperature was recorded sub zero. However,

under laboratory conditions, the pure isolates did not grow below 14^{0}C. These species of *Bacillus* also exhibited their tolerance for a wide range of pH (4.0–12.5). Survival of these bacterial species under unfavourable climatic conditions was considered on account of the spore forming property of the genus *Bacillus* (Pandey and Palni, 1997).

Species of *Pseudomonas*, both fluorescent as well as non fluorescent were frequently isolated from the soil samples collected from colder regions. Non fluorescent strains of *Pseudomonas corrugata* were initially isolated from the maize fields of Sikkim Himalaya and subsequently from other sites, including the rhizosphere of trees growing at the higher elevations. Two strains of *Pseudomonas corrugata*, *P. corrugata* 1, a rhizosphere associate isolated from subtropical environment, and *P. corrugata* 7, a rhizoplane associate isolated from temperate environment were investigated for nitrogenase, phosphate solubilization and antifungal properties (Pandey and Palni, 1998b). Both the strains of *P. corrugata* were found to possess nitrogenase activity ranging from 0.344 to 0.743 nmol of C_2H_2. Production of diffusible antifungal metabolites against a range of saprophytic and pathogenic fungi, namely *Alternaria alternata*, *Aspergillus niger*, *Cladosporium oxysporum*, *Fusarium moniliforme*, *Macrophomina phaseolina*, *Paecilomyces variotii*, *Penicillium funiculosum*, *P. janthinellum*, *P. javanicum*, *P. purpurogenum*, *P. raistrickii*, *Rhizoctonia solani* and *Trichoderma viride* was observed in vitro. In a plant based bioassay suppression of three major phytopathogens, namely *Pythium ultimum*, *P. arrhenomanes* and *Fusarium graminearum* was investigated. While the two strains were sensitive for gentamycin and rifampicin, they exhibited resistance against ampicillin, carbenicillin

and suppress the fungal flora; colonization of roots by the myconrhizal fungi improved in all treatments. Bacterial treatments also resulted in higher phosphorus values in shoots and grains in the inoculated rice plants (Trivedi et al., 2006).

Using a number of microbial inoculants (*Laccaria laccata*, *Trichoderma viride*, *Bacillus subtilis*, *Pseudomonas corrugata* and *Azotobacter chroococcum*) trials were also conducted to study their influence on seed germination, seedling survival, nutrient uptake and plant growth in *Cedrus deodara* (Roxb.) Loud, an economically important species of the Indian Himalayan region (Bisht et al., 2003). The inoculations positively affected the above mentioned parameters in treated plants over control, in the following order: *A. chroococcum* > *P. corrugata* > *B. subtilis* > *T. viride* > *L. laccata*. In view of the high demand of this species for plantation programmes, raising healthy seedling nurseries is an important prerequisite. The major problems associated with this species are poor seed germination and fungal attack at the seedling stage, resulting in high mortality. One of the bacterial inoculants, *B. subtilis*, resulted in 76 % seed germination compared to 54 % in control. Increased biomass and growth promotion due to bacterial inoculations have been reported in many other conifers (Chanway et al., 1991; Holl and Chanway, 1992).

Net house trials were conducted using four clones of seedling as well as cutting raised tea. The presence of "introduced" bacteria in the rhizosphere was confirmed using a set of antibiotic markers. The use of genetic markers such as intrinsic levels of resistance to various antibiotics is one of the simple and rapid methods of strain identification, and enumeration of the introduced bacteria that exhibit resistance to

introduced bacteria to colonize roots, rhizosphere or rhizoplane, or both, is an important and desirable attribute of the inoculant(s). *Pseudomonas corrugata* inoculations were found to positively influence growth and yield of two crops (*Amaranthus paniculatus* and *Eleusine coracana*) grown in the mountains. The inoculations resulted in greater colonization of the rhizosphere in *Amaranthus*, and moderate colonization of the rhizosphere and the rhizoplane in *Eleusine* (Pandey et al., 1999). In this pot based and in earlier field based experiments (Pandey et al., 1998), it was observed that the inoculants, which are usually taken as higher titres, seem to act as stimulants resulting in the promotion of native rhizoflora, subsequently contributing towards improved plant growth.

A number of landraces of rice are cultivated in the rainfed upland farming systems of Uttaranchal Himalaya (Agnihotri et al., 2000). The use of blue green algae, generally recommended for the rice fields in lowland areas, is not feasible in the rainfed mountain regions. *B. megaterium*, *B. subtilis* and *P. corrugata*, the promising bacterial inoculants, were tested for their influence on a local landrace of rice (*Oryza sativa* L.; landrace: *dudil*) through pot and field based assays. Observations were recorded in respect of the rhizosphere microflora, mycorrhizal infection, phosphorus content (in plant and soil), and growth and yield. The bacterial treatments (broth based in pots and charcoal based in field experiments) resulted in improved plant performance. Out of the three treatments, *B. subtilis* gave the best performance resulting in 1.66 and 1.55 fold increase in the grain yield in pot and field trials, respectively. Inoculations were also found to stimulate the rhizosphere associated native bacterial and actinomycetes populations,

and penicillin (Pandey et al., 2001). The species was also found to produce siderophores and volatile metabolite(s) causing inhibition of fungal growth. Various chemicals and enzymes of biocontrol importance produced by *B. megaterium*, *B. subtilis* and *P. corrugata* are presented in Table 3 and various activities are shown in Fig. 3.

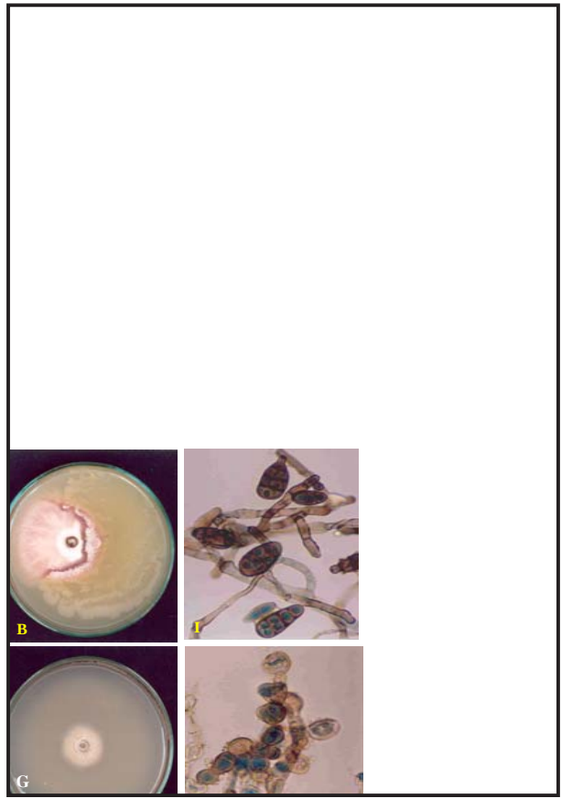

Fig.3. Antagonistic activities of bacteria: Antifungal activity of (A) *B. subtilis* against *A. alternata*; (B) *B. megaterium* against *F. oxysporum*. Antagonism through volatile compounds (C and E) Fungal growth of *A. alternata* and *F. oxysporum*; (D and F) Inhibition in growth of respective fungus; (G) Siderophore production by *P. corrugata*; (H) Protease production by *B. subtilis*. Morphological abnormalities in phytopathogenic fungi (I) Conidia of *A. alternata* (normal); (J) Deformed conidia of *A. alternata*; (K) Hyphae with macroconidia of *F. oxysporum* (normal); (L) swelling of hyphal tip and conidia deformation in *F. oxysporum*.

Table 3. Production of antifungal substances and enzymatic activity of antagonistic bacteria

Bacteria	HCN	Ammonia	Siderophore	Pectinase	Cellulase	Lipase	Amylase	Protease
B. megaterium	–	+	–	–	–	+	+	+
B. subtilis	–	+	+	–	–	+	+	+
P. corrugata	–	+	+	–	–	+	–	+
P. putida	+	+	+	–	–	+	–	+

Another antagonistic and phosphate solubilizing bacterial species of *Pseudomonas*, *P. putida* strain BO, was isolated from a sub-alpine Himalayan forest site (Pandey et al., 2006). The bacterial strain exhibited several properties associated with biocontrol and plant growth promotion. In petridish assays, the bacterium tested positive for inhibition of the growth of two phytopathogenic fungi, *Alternaria alternata* and *Fusarium oxysporum*. In quantitative estimations, the bacterium was found to produce chitinase, ß-1,3-glucanase, salicylic acid, siderophore and hydrogen cyanide. The production of these compounds by 14 strains of *Pseudomonas fluorescence* has recently been reported. Chitinase and siderophore production by *P. putida* BO is at par with the most effective strain, i.e., *P. fluorescence* PfMDU2, while salicylic acid and HCN production was higher than 12 strains in the study reported by Nagarajkumar and his coworkers (2004). The effect of *P. putida* in the control of major pathogens, such as, *Phytophthora parasitica* and *Fusarium* sp. has been reported by other workers as well (Scher and Baker, 1982; Yang et al., 1994). Several species of *Pseudomonas*, including *P. putida*, have been known for the production of secondary metabolites, diffusible as well as volatile antimicrobial compounds (Buyer et al., 1990; Ellis et al., 2000; Meyer, 2000; Molina et al., 2000; Bano and Musarrat, 2003). Upadhyay and

2006). Both the species appeared to be psychrotrophic in view of their in vitro growth. While the range of temperature tolerance was 4^0C to 35^0C for *P. corrugata*, *P. putida* could grow from 0^0C to 35^0C; optimum temperature for growth for both the species was 25^0C.

The beneficial effects of plant growth promoting rhizobacteria, particularly those belonging to the genus *Bacillus* or *Pseudomonas*, in enhancing growth and overall health of the plants, as well as controlling a range of plant diseases have been reported (Weller 1988; Glick 1995; Pandey et al., 1998; Whipps 2001; Sharma and Johri 2003; Tilak et al. 2005). The beneficial soil microbes influence plant growth in the following ways: growth promotion by providing fixed nitrogen to the host plant; production of phytohormones; phosphate solubilization; production of metabolites, including antibiotics that protect the host against one or more pathogens; and siderophores (Pandey and Kumar, 1989; 1990; O'Sullivan and O'Gara 1992; Budzikiewicz 1997; Reddy et al., 1997; Duffy and Defago, 1999; Ellis et al., 2000; Meyer, 2000). In our investigations, besides plant-microbe interactions, ecological specificity was given considerable importance in the selection of *Bacillus subtilis*, *B. megaterium* and *Pseudomonas corrugata* as promising inoculants for the mountains (Pandey et al., 1999; 2004).

c. Bioassays for evaluation of efficient microbial inoculants:

The bacterial inoculants were evaluated for their root colonization, growth promotion and biocontrol properties through several greenhouse, net house and field based assays. Initial experiments indicated that the inoculants were effective in seed, cutting as well as tissue culture raised plants, in terms of improved field establishment, augmentation of growth and overall plant performance. The ability of

Utilization of Carbon sources	Positive for Arabinose, Dextrose, Fructose, Galactose, Mannitol, Mannose, Trehalose, Xylose Negative for Adonitol, Cellobiose, Dulcitol, Inositol, Inulin, Lactose, Maltose, Raffinose, Rhamnose, Salicin, Sorbitol
Temperature, pH and salt tolerance	0–35^{0}C, optimum 25^{0}C 3–12, optimum 8.0 upto 4% (w/v)
Antibiotic sensitivity ($\mu g ml^{-1}$)	Ampicillin 1500, Carbenicillin 1500, Chloroamphanicol 50, Gentamycin 5, Kanamycin 5, Nalidixic acid 200, Penicillin 1500, Rifampicin 200, Streptomycin sulphate 10, Tetracycline 200

Phosphorus, an essential element for plant nutrition can only be assimilated as soluble phosphate. A variety of bacteria and fungi have been known to solubilize rock phosphate. Selected microorganisms have been developed as microbial inoculants and recommended as an ecofriendly alternative to chemical fertilizers (Gaur, 1990; Tilak, 1993). Various mechanisms have been reported for phosphate solubilization, the most recognized one is through the production of organic acids (Nahas, 1996). Production of organic acids, like citric, gluconic and oxalic acid, has been recognized for phosphate solubilization by several microorganisms (Asea et al., 1988; Kucey et al., 1989; Cunningham and Kuiack, 1992; Illmer et al., 1995; Bagyaraj et al., 2000; Rashid et al., 2004). Occurrence of phosphate solubilizing microorganisms has been reported from different environmental niches (Banik and Dey, 1982; Illmer and Schinner, 1992; Nautiyal et al., 2000; Vanquez et al., 2000; Chen et al., 2002). Microbial phosphate solubilization is often considered as one of the parameters related to plant growth promotion.

While occurrence of *P. corrugata* was reported from subtropical and temperate locations (Pandey and Palni, 1998b), *P. putida* was isolated from still higher altitudes, i.e., sub-alpine site (Pandey et al.,

Jayaswal (1992) had suggested the induction of morphological abnormalities and inhibition of conidiation in phytopathogenic fungi by an antagonistic bacterium (*Pseudomonas cepacia*) as a possible mechanism of biological control.

Both the species of *Pseudomonas*, *P. corrugata* and *P. putida* have been examined for phosphate solubilizing activity along a temperature range (psychrophilic to mesophilic). The quantitative estimations in broth following spectrophotometric methods were carried out upto day 30 of incubation (3 days interval). The maximum activity in case of *P. corrugata* was recorded (155 $\mu g\ ml^{-1}$) on day 18 at 21^0 C that declined to 70 $\mu g\ ml^{-1}$ after day 30 of incubation. While, in case of *P. putida*, maximum solubilization (247 $\mu g\ ml^{-1}$ of P) was seen on day 15 after which it continued to decline and reached 139 $\mu g\ ml^{-1}$ of P on day 30 of incubation. The bacteria solubilized TCP at 28^{0}C but generally with a lower efficiency than recorded at 21^{0}C. The pH of the broth was found to decline in each case due to bacterial activity; lowering of pH coincided with an increase in efficiency of phosphate solubilizing activity (Fig. 4).

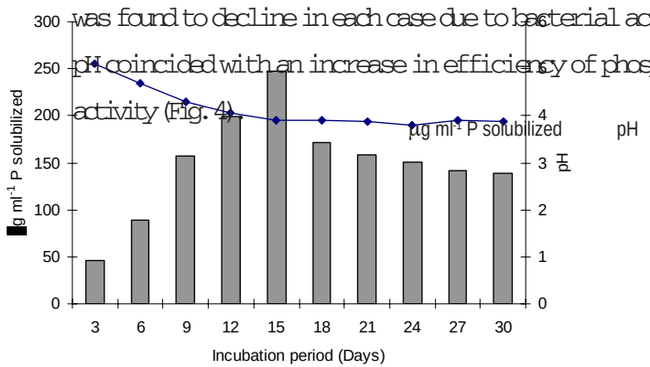

Fig. 4. P solubilized and corresponding lowering in pH of the broth at 21 ºC due to phosphate solubilizing activity of *P. putida*.

Table 4. General characteristics of plant growth promoting species of *Bacillus* and *Pseudomonas*

Bacillus megaterium	
Colony morphology	Undulate, spreading, circular, rough, whitish, 2.0–3.0 mm (dia.) colonies on Tryptone yeast extract agar at 25°C after 48 h incubation
Microscopic features	Gram positive thick rods, 1.6 x 2.4 µm with central oval endospore
Extra and Intracellular enzyme activity	Positive for Catalase, Citrate utilization, Casein hydrolysis, Gelatin liquefaction, Lysine decarboxylase, Nitrate reduction, Ornithine decarboxylase, Oxidase, Starch hydrolysis, Urea hydrolysis Negative for Arginine dehydrolase, H_2S production, Indole production, Methyl red
Utilization of Carbon sources	Positive for Allobiose, Cellobiose, Dextrose, Fructose, Glucose, Inulin, Lactose, Maltose, Mannitol, Mannose, Raffinose, Salicin, Sorbitol, Sucrose, Trehalose, Xylose Negative for Arabinose, Adonitol, Dulcitol, Galactose, Melibiose, Rhamnose
Temperature, pH and salt tolerance	14–45°C, optimum 25°C 4–11, optimum 6.0 Upto 5 % (w/v)
Antibiotic sensitivity	Ampicillin 150, Carbenicillin 10, Chloroamphanicol 5, Gentamycin 10, Nalidixic acid 5, Penicillin 150, Rifampicin 5, Streptomycin sulphate 10, Tetracycline 10
Bacilus subtilis	
Colony morphology	Irregular, matted with dull to rough surface, spreading, circular, 2.0–3.0 mm (dia.) on Tryptone yeast extract agar at 25°C after 48 h incubation
Microscopic features	Gram positive rods, single or short chains, 0.8x1.8 µm with ellipsoidal subterminal spores
Extra and Intracellular enzyme activity	Positive for Arginine dehydrolase, Catalase, Citrate utilization, Casein hydrolysis, Gelatin liquefaction, Methyl red, Nitrate reduction, Oxidase, Starch hydrolysis Negative for H_2S production, Indole production, Lysine decarboxylase, Ornithine decarboxylase, Urea hydrolysis
Utilization of carbon sources	Positive for Arabinose, Cellobiose, Dextrose, Dulcitol, Fructose, Galactose, Glucose, Maltose, Mannitol, Mannose, Raffinose, Rhamnose, Salicin, Sorbitol, Sucrose, Trehalose, Xylose Negative for Adonitol, Allobiose, Inulin, Melibiose

Temperature, pH and salt tolerance	14–50°C, optimum 25°C 4–11, optimum 6.0 upto 10 % (w/v)
Antibiotic sensitivity	Ampicillin 100, carbenicillin 10, chloroamphanicol 5, gentamycin 5, penicillin 100, rifampicin 5, streptomycin sulphate 10
Pseudomonas corrugata	
Colony morphology	Non fluorescent, entire, circular, yellowish colonies (2–3 mm dia) on Pseudomonas isolation agar at 25°C after 48 h incubation
Microscopic features	Gram negative single oval rods, 0.4 x 1.1 µm
Extra and Intracellular enzyme activity	Positive for Catalase, Citrate utilization, Gelatin hydrolysis, Indole production, Lysine decarboxylase, Cytochrome oxidase, Urea hydrolysis, Voges Proskauer Negative for Casein hydrolysis, Methyl red, Nitrate reduction, Ornithine decarboxylase, Starch hydrolysis
Utilization of Carbon sources	Positive for Allobiose, Cellobiose, Dextrose, Fructose, Glucose, Maltose, Mannitol, Mannose, Melibiose, Raffinose, Salicin, Sorbitol, Sucrose, Trehalose, Xylose Negative for Arabinose, Adonitol, Allobiose, Dulcitol, Galactose, Inulin, Lactose, Melibiose, Rhamnose
Temperature, pH and salt tolerance	4–35°C, optimum 25°C 4–9, optimum 7 upto 4% (w/v)
Antibiotic sensitivity ($\mu g ml^{-1}$)	Ampicillin 1500, Carbenicillin 2000, Chloroamphanicol 100, Gentamycin 10, Kanamycin 100, Nalidixic acid 250, Penicillin 2500, Rifampicin 10, Streptomycin sulphate 100, Tetracycline 100
Pseudomanas putida	
Colony morphology	Fluorescent, yellowish colonies (2–3 mm dia) on Pseudomonas isolation agar at 25 °C after 48 h incubation
Microscopic features	Gram negative, single, motile rods, 0.6–0.8 X 1.2–1.8 µm
Extra and Intracellular enzyme activity	Positive for Catalase, Citrate utilization, Gelatin hydrolysis, Cytochrome oxidase Negative for Casein, Starch and Urea hydrolysis, Nitrate reduction