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Cover photo: **Top middle**: Vegetation on the banks of river Teesta; **Top left**: Antagonism of fluorescent *Pseudomonas*; **Immediately below**: *Aspergillus clavatus*;**Top right**: Flower of *Tacca integrifolia*; **Immediately below**: Conidia of *Curvularia lunata*; **Bottom left**: DNA band on agarose gel; **Immediately right**: *Paenibacillus alvei*; **Bottom right**: Wild mushroom from Teesta basin; **Immediately left**: a protein folding model.

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REVIEW ARTICLE

Plant Defense Proteins

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Plants are compelled to withstand stresses of all kinds, be it biotic, abiotic or anthropogenic as a consequence of their immobility. The initial infection process involving adhesion/recognition events between plants and fungal pathogens is essential for the establishment of pathogenesis (Chakraborty,1988). The extracellular matrix (ECM) is a biologically active part of the cell surface composed of a complex mixture of macromolecules that, in addition to serving a structural function, profoundly affect the cellular physiology of the organism (Roberts, 1989; 1990). During the past two decades it has become evident that the cell wall is a dynamic organization that is essential for cell division, enlargement and differentiation as well as responding to biotic and abiotic stress. ECM is also the source of signals for cell recognition within the same or between different organisms (Brownlee, 2002). Cell walls are natural composite structures, mostly made up of high molecular weight polysaccharides, proteins and lignins (Fry, 2004). Lignins are only found in specific cell types. *Arabidopsis thaliana* cell wall proteins (CWP) that can be involved in modifications of cell wall components, wall structure and signaling as well as interactions with plasma membrane proteins at the cell surface has been reviewed (Jamet *et.al*, 2006). All available *Arabidopsis* cell wall proteome data were screened using bioinformatic tools to select only those proteins containing a signal peptide but devoid of known retention signals for the endomembrane system. These 281 proteins from the database of CWP were assembled in functional classes and subclasses according to the presence of functional domains in the protein.

The defense strategy of plants against stress factors involves a multitude of tools, including various types of stress proteins with putative protective functions, A group of plant-coded proteins induced by different stress stimuli, named "pathogenesis-related (PR) proteins" is assigned an important role in plant defense against pathogenic constraints and in general adaptation to stressful environment (Datta and Muthukrishnan, 1999; Chakraborty, 2005). Such protective plant proteins specifically induced in pathological or related situations have been intensively studied from the agricultural interest. On the other hand, many of the reserve proteins accumulated in seeds and fruits take the constitutive defense function against microbial pathogens and invertebrate pests in addition to their storage function. These inducible or constitutive defense mechanisms of higher plants are relatively conserved in the course of evolution. Accordingly, most plants produce or accumulate similar proteins under certain situations irrespective of their morphological differences.

Terminology

The introduction of polyacrylamide electrophoresis by which proteins could be separated on the basis of their combination and charge was a chief innovation in unearthing PR-proteins - the new protein first observed in tobacco, (*Nicotiana tabacum*) cultivars reacting hypersensitively to tobacco mosaic virus (TMV). Since the discovery of pathogenesis related proteins (initially named as "b" poteins) by two independently investigating groups (Van Loon and Van Kammen, 1970; Gianinazzi *et.al,* 1970) have focused an increasing research interest in view of their possible involvement in plant resistance to pathogens. Since the proteins were induced under specific pathological conditions, they were named pathogenesis-related proteins abbreviated as PRps. By definition, PRps were described as "proteins coded for by the host plant but induced specifically only in pathological or related situations". Later, however, it turned out that this proteins are induced not only in resistant, but also in susceptible plant-pathogen interaction, as well as in plants, subjected to abiotic stress factors. To be included among the PRps, a protein has to be newly expressed upon infection but not necessarily in all pathological conditions. Pathological situations refer to all types of infected states, not just to resistant, hypersensitive responses in which PRps are most common; they also include parasitic attack by nematodes, insects and herbivores. Induction only by abiotic stress conditions is not a sufficient criterion for inclusion as a PRps. These considerations imply that the characteristics of the induction of PRps take priority over other identifying features, such as chemical properties or cellular localization.

Occurrence and properties of PR-proteins

The occurrence of these proteins was not pathogen specific but determined by the type of reaction of the host plant, indicating that these proteins were of host origin. PRps are most often of low molecular weight, selectively extractable in low *p*H, highly resistant to proteolytic degradation/or endogenous proteases and localized predominantly in the intercellular space. PRps were found in small amounts in senescing leaves of flowering plants and in relatively larger quantities when necrosis was more severe. This led to the assumption that these polypeptides were stable proteolytic breakdown products of larger leaf proteins. PRps that have been found in many plant species (Table-1) can be classified into seventeen families.

Table 1. Plant species in which PR-proteins have been identified

Plant species	Family
Arabidopsis thaliana	Cruciferae
Brassica nigra	Cruciferae
Brassica napus	Cruciferae
Camellia sinensis	Theaceae
Chenopodium amaranticolor	Chenopodiaceae
Chenopodium quinoa	Chenopodiaceae
Citrus sinensis	Rutaceae
Glycine max	Papilionaceae
Lablab purpureus	Papilionaceae
Gomphrena globosa	Amaranthaceae
Picea abies	Pinaceae

On the basis of similarities in molecular weights, amino acid composition, and serological properties, and confirmed by nucleotide sequencing of corresponding cDNAs, the 10 major acidic PRps of tobacco were grouped into five families, designated PR-1 to PR-5. This classification has set a standard for other plant species, in which PRps with properties homologous to the tobacco PRps are now similarly designated by these family numbers.

To accommodate further classes of PRps with different properties, additional families were adopted. The criteria for inclusion of new families of PRps were (i) protein(s) must be induced by a pathogen in tissues that do not normally express the protein(s), and (ii) induced expression must have shown to occur in at least two different plant-pathogen combinations, or expression in a single plant-pathogen combination must have been confirmed independently in different laboratories. Each PR-family is numbered and the individual family members are assigned lower case letters in the order in which they are described. In accordance with the recommendations of the Commission for Plant Gene Nomenclature, PR-genes are designated as *ypr*, followed by the same suffix as of the family. Later on three more peptides, which were capable of inducing defense responses of plants, were identified. Based on the grouping of PRps into plant-wide families sharing amino acid sequences, serological relationship, enzymatic or biological activity seventeen families of PRps have been recognized (Table 2).

Table 2. Recognized and proposed families of pathogenesis-related proteins

Family	Type member	Properties	Gene symbols
PR-1	Tobacco PR-1a	Antifungal?14-17kD	*ypr1*
PR-2	Tobacco PR-2	Class 1,II and III endo-β 1,3-glucanase, 25-35kD	*ypr2*
PR-3	Tobacco P,Q	Class I, II, IV, V, VI and VII endochitinases about 30kD	*ypr3*
PR-4	Tobacco R	Antifungal, *win*-like proteins endochitinase activity, 13-19kD	*ypr4*
PR-5	Tobacco S	Antifungal, thaumatin-like Proteins, osmoins, zeamatins	*ypr5*
PR-6	Tomato inhibitor I	Proteinase inhibitor, 6-13kD	*ypr6*
PR-7	Tomato P	Endoproteases	*ypr7*
PR-8	Cucumber chitinase	Class III chitinase	*ypr8*
PR-9	Tobacco"lignin-forming peroxidase"	Peroxidase	*ypr9*
PR-10	Parsley PR-1	Ribonucleases	*ypr10*
PR-11	Tobacco classV Chitinase	Endochitinase Class 1	*ypr11*
PR-12	Radish Ps-AFP3	Plant defensins	*ypr12*
PR-13	*Arabidopsis* THI2.1	Thionins	*ypr13*
PR-14	Barley LTP4	Nonspecific lipid transfer Proteins (ns-LTPs)	*ypr14*
PR-15	Barley OxOa	Oxalate oxidase	*ypr15*
PR-16	Barley OxOLP	Oxalate-oxidase-like proteins	*ypr16*
PR-17	Tobacco PRp27	Unknown	*ypr17*

Besides proteins newly defined mRNAs (cDNAs) are often considered as additional members of the existing families where shown to be induced by pathogens or specific elicitors. Thionins and defensins both families of small basic, cysteine-rich polypeptides, qualify for inclusion as new families of PRps. However, because PRps are generally defined by their occurrence as protein bands on gels, and classified within each family once the protein has been characterized, cDNA or genomic sequences without information on the corresponding protein cannot be fitted in to the adopted nomenclature. Thus for naming it is necessary to gather information at both the nucleic acid and the protein level when dealing with a stress-related sequence falling within the definition of PRps. Conversely, homologies at the cDNA or genomic level may be encountered without information on the expression or characteristics of the encoded protein. Such sequences obviously belong to the PR-type families, but yet cannot, be considered to correspond to pathogen-induced PRps and named accordingly. In more than a few situations, it is difficult to distinguish PRps from related proteins/ mRNAs that are present in some organs or appear during specific developmental stages. Homologous proteins/ mRNAs in healthy tissues in which no induction by pathogen infection has yet been demonstrated, are to be termed PR-like proteins (PRLs) and their genes *yprl*.

Induction

Besides the known PRps inducers of biotic origin (pathogen, insects, nematodes, herbivores), a new type of biotic inducers, *Orobanche* weeds, has been reported in tobacco. Pathogen-derived elicitors are potent PRps inducers. Well characterized are glucan and chitin fragments derived from fungal cell walls, fungus-secreted glycoproteins, peptides and proteins of elicitin family (Edreva *et.al,* 2002). Protein products of avirulence genes in fungi and bacteria are capable of PRps induction. Chemicals, such as salicylic, polyacrylic and fatty acids, inorganic salts as well as physical stimuli (wounding, UV-B radiation, osmotic shock, low temperature, water deficit and excess), are involved in PRps induction. A special class of PRps inducers are hormones (ethylene, jasmonates, abscisic acid, kinetin, auxins). Reactive oxygen species (ROS)-mediated PRps formation has largely been recognized (Schultheiss *et.al*, 2003).

PRps synthesis can also be triggered by internal plant developmental stimuli. The presence of PRps in different flower parts (Fraser, 1981), their appearance in abscission zones as well as their relation to seed germination and somatic embryogenesis point that they are developmentally controlled. It is noteworthy that developmentally-induced PRps are accumulated in an organ and tissue specific manner (Kombrink *et.al,* 2001).

Functional Properties of PR-proteins

Elucidation of the biochemical properties of the major, pathogen-inducible PRps of tobacco and subsequent cloning of their cDNAs and/ or genes revealed proteins with substantial similarity to the classical PRps, which are mostly acidic and extracellular proteins, the homologous counterparts are mostly basic and localized intracellularly in the vacuole. As far as it has been possible to deduce, they possess the same type of enzymatic activities, but their substrate specificity and specific activity may be rather different (Kauffmann *et.al*, 1987; Legrand *et.al,* 1987).

PRps are, as such, a collective set of novel proteins which a plant produces in reaction to a pathogen mainly in incompatible interactions and thus impedes further pathogen progress. The "related situations" in which PRps were found to be induced, seem to prove the point: application of chemicals that mimic the effect of pathogen infection or induced some aspects of the host response, as well as wound responses that give rise to proteins that are also induced during infections, can induce both PRps and acquired systemic resistance (SAR). Few of the inducible acidic PRps associated with SAR have been shown to possess significant anti-pathogenic activity (VanLoon, 1997).

The occurrence of homologous PRps as small multigene families in various plant species belonging to different families, their tissue-specificity during development and consistent localization in the apoplast as well as in the vacuolar compartment and their differential induction by endogenous and exogenous signaling compounds suggest that PRs may have important functions extending beyond their apparently limited role in plant defense. During the hypersensitive reaction cellular damage and death is a major stress to the plant, as exemplified by high increases in abscisic acid and ethylene. It is possible, therefore, that PRps are stress proteins directed to alleviate harmful effects of cellular degradation products on thus far untouched neighboring cells.

Both acidic and basic PRps may be induced by high concentrations of ethylene (Lotan *et.al,* 1989) or physiological necrosis (Edreva, 1990), plasmolysis or wounding. Such induction in the absence of pathogenic attack might be taken to indicate protection of cellular structures, either physically to stabilize sensitive membranes or macromolecules, or chemically to keep potentially harmful saprophytic microorganisms on tissue surfaces or in intercellular spaces in check. In virtually any natural stress condition e.g., heat, cold, drought, osmotic stress, water logging, anaerobiosis, metal toxicity, etc., plants are known to react by the synthesis of novel, and sometimes partly overlapping, sets of proteins (Wasternack and Parthier 1997). The various conditions under which PRps occur are reminiscent of those under which heat-shock proteins (HSP) are induced. These proteins are ubiquitous in living organisms and associated with the acquisition of thermotolerance to otherwise lethal temperatures, but a causal connection is not evident.

Interestingly, the promoters of all three tobacco PR-1 genes that are expressed, as well as of a different type of PR in parsley, contain a heat shock regulatory element (Somssich *et.al,* 1988) but the proteins are not induced to detectable levels by heat shock. Nevertheless, PRps might have an analogous function, though quite different, chaperonin-like function: unlike PRps, HSP are intercellular proteins that do not accumulate during heat shock. However, the specific occurrence of individual PRps in some floral organs, but not in others, points to other, more specific roles.

The relative ineffectiveness of PRps in determining resistance to pathogens does not preclude an involvement in defense. As first proposed (Mauch and Staehelin,1998) acidic, extracellular PRps might be involved in recognition processes, releasing defense-activating signal molecules from the walls of invading pathogens. This would hold particularly for chitinases and glucanases that could liberate elicitor-type carbohydrate molecules from fungal and bacterial cell walls. β-1,3-glucanase induced in soybean seedlings by infection or chemical stress releases

elicitor-active fragments from cell wall preparations of the fungus *Phytophthora megasperma* f.sp. *glycinea* (Ham *et.al,* 1991). Such elicitors could help stimulate defense responses in adjacent cells and thus accelerate and enhance these reactions, as well as induce acquired resistance to further infection.

Demonstration that the PR-2 family are β-1,3-endoglucanases and the PR-3,-4,-8, and -11 families consist of chitinases with or without lysozyme activity, immediately suggested that these PRps are directed against cell walls of fungi and bacteria Homology of the thaumatin-like proteins of the PR-5 family with a bifunctional l-amylase/trypsin inhibitor from maize seeds seemed consistent with a role in protection against phytophagous insects. However, no proteinase inhibitor activity has been demonstrated for PR-5 proteins.Resistance to insect attack is taken to be conferred primarily by wound-inducible proteinase inhibitors, which have now been grouped into the family PR-6.

A role of PRps as specific internal signal generating enzymes would be consistent both with their occurrence in specific organs and with their induction during the development and in response to stressful pathogen infections. The major chitinase of bean leaves first described (Boller *et.al,* 1983) to be induced by ethylene and located in the vacuole, appears to be also induced in abscission zones at the stem petiole-junction (Del and Lewis, 1992) together with a PR-1-like protein, two isoforms of β-1,3-glucanase, other chitinases, and a thaumatin-like protein. However, the natural substrates for chitinases in higher plant cell walls remain to be determined. The tobacco PR-2 glucanases vary 250-fold in specific activity on laminarin (Kauffmann *et.al,* 1987) and their relative activities on different substrates vary greatly, suggesting that their normal actions may be diverse. Expression studies of PR-2d in transgenic tobacco suggest that this protein functions developmentally in seed germination by weakening the endosperm, thus allowing the radicle to protrude (Vögeli-Lange *et.al,*1994).

The occurrence of almost all types of PRps in various floral tissues also suggests specific physiological functions during flower development rather than a role in general defense against pathogen invasion. This notion is supported by the presence in floral organs of additional PR-like proteins, glucanases (Coté *et.al,* 2002) and thaumatin-like proteins. In petunia flowers, chitinase activity is localized in the petals (about 15%) and stigma (about 85%). In the stigma it increases about five-fold following anther dehiscence, strongly suggesting that the chitinase has a specific function in reproduction.

Major interest has been devoted to plant hydrolases, β-1,3-glucanases (E.C. 3.2.1.39) (PR-2) and chitinases (E.C. 3.2.1.14) (PR-3), as they are capable of cleaving fungal cell walls resulting in pathogen growth inhibition, and moreover, the products of the hydrolysis can act as elicitors of further defence responses (Boller, 1995). Both β-1,3-glucanases and chitinases are highly abundant proteins in plants involved in diverse physiological and developmental processes. They can act either alone or in combination strengthening their antifungal activity (Mauch *et.al,* 1988). Their accumulation is not restricted only to resistant plants but is often observed in compatible plant-pathogen interactions or even non-pathogenic combination.

Activation of natural weapons before infection, called systemic acquired resistance (SAR) is initiated by pathogens, pathogen- or pathogen-derived elicitors, as well as a number

of chemical compounds. Among the main defence genes, which are switched on in response to pathogen infection, belong those encoding pathogenesis related (PR) proteins. The association of PRps with SAR, but not with ISR, has led to the hypothesis that accumulation of PRps is not a pre-requisite for the induction of resistance, but that PRps contribute to the protective state. SAR is dependent on the accumulation of SA, but not JA or ethylene. It appears that only when increases in the levels of any of these signals occur, PRps become detectable in the infected plants. The observations indicate that individual PRps are induced to various extents by these different signals. Consequently, the mixture of signals released or produced upon microbial stimulation appears to determine the magnitude of the plant's response and its effectiveness to inhibit further infection.

In *Arabidopsis*, SA-dependent expression of PR-1, PR-2 and PR-5 is required for increased protection against the biotrophic fungus *Perenospora parasitica*, whereas SA-independent but JA-dependent induction of the plant defensin gene *pdf1.2*, as well as of PR-3 and PR-4, is associated with the induced resistance against the necrotrophic fungi *A. brassicicola* (Penninckx *et.al,* 1996), *Botrytis cinerea* (Thomma *et.al,* 1998) and *Fusarium oxysporium* f.sp. *matthiolae* (Epple *et.al,* 1998). These results suggest that the SA- and JA-dependent defense pathways in *Arabidopsis* contribute to resistance against distinct microbial pathogens. As a result, PRps and similarly induced antimicrobial proteins appear to contribute differentially to the induced resistance against different pathogens.

Biosynthesis

Biosynthesis of PRps in sugar beet has been intensively investigated both on plants infected by pathogens and treated with synthetic inducers of SAR: salicylic acid and its functional derivates 2,6-dichloroisonicotinic acid (INA) and benzo-1,2,3-thiadiazole-7-carbothioic acid S-methyl ester (BTH). BTH was shown to induce resistance to a number of fungal and viral pathogens, e.g. *Arabidopsis*, wheat, tobacco. Analysis of extracellular fluid isolated from BTH-treated sugar beet leaves revealed the accumulation of acidic and basic proteins displaying both chitinase and b-1,3-glucanase activities indicating the ability of BTH to activate defence reactions in sugar beet. However, in contrast to there was no increase in accumulation of transcripts encoding three chitinase isozymes (including Ch4) and β-1,3-glucanase Glu2 in sugar beet leaves following the treatment with INA, compound similar to BTH, even though the INA pretreatment completely inhibited the development of *Cercospora beticola*. Recently, the BTH capability of inducing SAR to root pathogens was shown on cucumber plants against *Pythium* damping-off as well as to *Phytophthora* root rot and even to root-parasitic weed *Orobanche cumana*, suggesting that BTH-induced or potentiated defence mechanism might be of more general character. Similarly, the cross-activity of defence responses against diverse pathogens has been demonstrated on rhizomania-diseased sugar beet and *Heterodera schachtii* root nematodes.

Cellular and tissue localization

Localization of the major, acidic PRps in the intercellular space of the leaf seems to guarantee contact with invading fungi or bacteria before these are able to penetrate. In localization studies *in planta*, labelling for β-1,3-glucanases and chitinases was especially pronounced over fungal cell walls confirming their role in plant defence. With most of the investigations devoted to leaf tissues. In roots, expression of defence genes was studied on

infection by pathogens, arbuscular mycorrhizal fungi, antagonistic fungus *Trichoderma harzianum* or non-pathogenic bacterium *Pseudomonas fluorescens* and differences in the expression of distinct classes of chitinases and β-1,3-glucanases were reported in dependence on the particular microbial inducer. Antimicrobial proteins extracted from *Exobasidium vexans* inoculated resistant tea varieties were electrophoretically resolved on SDS-gels, analysed by EDAS and characterized immunologically after probing with PAb-chitinase (Chakraborty *et.al*, 2004, 2005; Sharma and Chakraborty, 2004). Induction of resistance in tea varieties against blister blight pathogen was attempted with a few abiotic inducers (Chakraborty *et.al*, 2005a) Salicylic acid and hexaconazole treated leaves of *Camellia sinensis* (L.) O. Kuntze were reacted with polyclonal antibodies of chitinase (PR-3) and labeled with FITC, bright apple green fluorescence was observed in the epidermal and mesophyll tissues (Sharma and Chakraborty, 2005). Similar results of localization of PR-3 proteins have been reported in potato leaves infected by *Phytophthora infestans*.

Subcellular localization of PR-1 proteins was studied in roots of resistant *Nicotiana tabacum* cv. *Xanthi* uninfected or infected *in vitro* by the black root rot fungus *Chalara elegans*, using polyclonal or monoclonal antibodies raised against PR-1 protein. In healthy tobacco roots, the PR-1 proteins were found to be present in low amounts in intercellular space material, over cell walls and over secondary thickening of xylem vessels. All these cell compartments were significantly enriched in the PR-1 proteins in infected tobacco root tissues. Their accumulation over the cell walls of inter- and intracellular hyphae of *C. elegans* colonizing tobacco roots may reflect an eventual role of these proteins, in association with other PR-proteins like β–1,3-glucanases and chitinases in directly hindering hyphal growth of the pathogen. Transmission electron microscopic observations of tea leaf tissues treated with salicylic acid and labeled with PAb-chitinase and PAb-β-1,3-glucanase revealed intense labeling corresponding systemic accumulation of both the PRps (PR-2 and PR-3) in treated plants. Accumulation of the PRps in treated tea plants was observed in cell walls and extracellular spaces.

Ribosome inactivating proteins (RIPS)

Ribosome-inactivating proteins or RIPs are divided into two groups. Type 1 RIPs are single A-chain molecules (30 kDa) exhibiting the ribosome-inactivating activity. Type 2 RIPs additionally contain a lectin B-chain (30 kDa) connected to the active A-chain via a single disulfide bond. The lectins of most type 2 RIPs specifically recognize galactose or N-acetylglucosamine. The antifungal activity of this protein was synergistically enhanced in combination with either a basic class IV glucanase or basic class II chitinase from barley seeds. The basis of the antifungal activity of this RIP may rely on its inhibitory effect on fungal ribosomes, although it has not been demonstrated that this occurs *in vivo*. Better documented is the antiviral activity of several type 1 RIPs. When purified RIPs are applied on plants together with viruses, they drastically suppress virus multiplication and symptom development. It is supposed that RIPs enter cell together with the viruses and exert their adenosine glycosidase activity in the cytosol to affect either host ribosomes or possibly viral RNA.

Applications

Plant protection is a major challenge to agriculture worldwide. One of the effective strategies for disease resistance in plants has been the incorporation of disease resistant genes into commercially acceptable cultivars. Experimental evidences substantiated the utility of

PRps genes to develop disease resistance in transgenic plants. This practical aspect of PRps gene research resulted in the release of agronomically important crops resistant to various diseases of economical interest. The most attractive initial candidates for manipulation of the single gene defense mechanism approach are genes encoding chitinases or β-1,3-glucanases because these two enzymes hydrolyze chitin and β-1,3-glucans which are structural components of the cell walls of several fungi. Chitinase gene from *Rhizopus oligosporus* has been shown to operate as an antifungal system in transgenic tobacco. Transgenic cucumber harboring the rice chitinase genes exhibited enhanced resistance against gray mold, *Botrytis cinerea.* While it is clear that it is possible in several cases to alter the expression of chitinase transgenes to generate plants with increased resistance to the pathogen, it is not clear whether constitutively expressed chitinase alone is responsible for the reduction of disease symptoms as observed in the case of tobacco and canola. Introduction of bacterial chitinase gene from *Serratia marcescens* in transgenic tobacco cells showed up to an eightfold increase in amount of chitinase protein in the plants and conferred resistance to *Rhizoctonia solani.* Expression of β-1,3-glucanase in transgenic tobacco plants was shown to result in enhanced resistance to *Alternaria alternata.* The constitutive over expression of tobacco class 1 PR-2 and PR-3 transgenes I potato plants enhanced their resistance to Phytophthora infestans, the causal agent of late blight. *Brassica napus* transgenic plants, constitutively expressing a chimeric chitinase gene, display field tolerance to fungal pathogens. Combined expression of PR-2 (β-1,3-glucanase) and PR-3 (chitinase) gives effective protection against fungal infection as they have been shown to act synergistically.

Conclusion

Increasing amount of data enlarged the knowledge on the relevance of PRs to important plant performances, such as development, disease resistance and general adaptation to stressful environment. As defense related proteins usually provide a plant with resistance to stresses, varieties that are apt to intensively induce such proteins are agriculturally important and are drawing much attention of plant breeders. The knowledge gained by such studies also provides a base for the development of novel agrochemicals for disease control and also for the development of disease-resistant crops by regulating the system in plants through genetic manipulation which encouraged the application of PR genes in gene-engineering technologies for crop improvement. However, fundamental aspects of PRs gene studies remain little understood, particularly the exact mechanisms of gene regulation; thus, the receptors, signal transducing cascades and moleclular targets involved in PRs induction are a challenge for both fundamental and applied studies.

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REVIEW ARTICLE

Anthrax and Bioterrorism

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Abstract

Bioterrorism is the deliberate discharge or dissemination of viruses, bacteria, or other germs used to cause sickness or death in people, animals, or plants. Though these agents are found in nature, they are usually modified to increase their ability to cause disease, make them resistant to current antibiotics, or to increase their ability to be spread into the environment. Biological agents can be spread through the air, through water, or in food. Terrorists may use biological agents because they can be extremely difficult to detect and do not cause illness for several hours to several days. Among these biological agents, Anthrax is considered to be the Category-A agent because of its high potency for adverse public health impact and serious effect on large-scale dissemination. Though there is a big hue and cry in the recent past about bioterrorism particularly after 9/11 attack, the history of bioterrorism is fairly old. In the present paper, we have sketched the history and the evolution of bioterrorism with reference to anthrax, particularly microbiology and pathogenesis of the disease causing agent and its clinical symptoms and treatments.

Keywords: Anthrax, Bio-terrorism, *Bacillus anthracis*

Introduction

In recent times, the increased threat of terrorism has attracted the attention of society to the risk posed by various microorganisms as biological weapons. Biological warfare agents are more potent than conventional and chemical weapons. During recent past, immense progress in biochemistry, genetic engineering and, biotechnology has simplified the development and production of biological warfare agents. Wide availability and ease of production has increased the demand for biological agents by various developed and developing nations. Among the numerous bioterrorism agents, anthrax may be an effective biological weapon since it is easy to culture, can be aerosolized, and readily forms spores which remain viable for years, infecting soil and other materials long after initial attack (Anonymous, 2004a). Besides this, it can cause wide spread illness and death that can cripple a city or region (Ridel, 2004).

The Center for Disease Control and Prevention (CDC), Atlanta, Georgia has categorized bioterrorism agent into three categories. Organisms in category A (e.g. *Bacillus anthracis*) are high priority because they pose a risk to national security. Category A organisms can be easily disseminated or transmitted from person to person, resulting in high mortality rate. The potential for major public health impact leading to public and social disruption creates the need for public health preparedness. Transmission of *Bacillus anthracis* is well known, and following an intentional release, results in inhalation of anthrax spores (Anonymous, 2004b). Mortality rates in inhalation anthrax patients are very high despite appropriate antibiotic treatment. In 1972, a convention on the "Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction" also

known as BWC, was held. It prohibited offensive bio-weapons research and development and was signed by most countries (Ridel, 2004). However, the former Soviet Union and Iraq, both signatories of the convention, have subsequently acknowledged bio-weapons research and production (Ridel, 2005). Following the World Trade Center attack in September 11, 2001, the "anthrax letter" created widespread psychosocial breakdown and uncertainty. The use of anthrax as a bio-terrorist weapon is likely in coming era. This threat requires the medical community to educate and prepare itself for a response to this possible terrorism.

History

Anthrax is primarily a disease of herbivorous animals, although all mammals (including humans) and some avian species can contract it. The disease has world-wide distribution and is a zoonosis. From the earliest historical records until the development of effective veterinary vaccine and antibiotics, the disease was one of the foremost causes of uncontrolled mortality in herbivores i.e. cattle, sheep, goats, horses and pigs. The major sources of human anthrax infection in its various forms (inhalation, cutaneous and gastro- intestinal) are through direct or indirect contact with infected animals, or occupational exposure to infected or contaminated animal products. Other possible sources are rare and epidemiologically trivial. The bacillus responsible for causing anthrax has a long history of interaction with humans. Described as far back as 1500 BC, it is thought to be the etiologic agent of the fifth Egyptian plague (Anonymous, 2002). It has been suggested in texts of antiquity that the famous plague of Athens (430-427 BC) was an epidemic of inhalation anthrax (Mcsherry and Kilpatrick,1992).The species name is derived from Greek word "anthracites", means coal like, indicative to the typical black eschar seen in the cutaneous form of the disease. There are accurate records of infections in ancient Rome depicted by the Roman Poet Virgil (70-19 BC) in his third "Georgics" which is devoted to animal husbandry and contains a section of veterinary medicine. He detailed an epizootic that occurred in the Noricum district of Rome (ancient name of the Danube River delta and the eastern Alps), where disease affected cattle, horses, sheep as well as dogs and other domestic and wild animals (Virgil, 1956). Over the centuries, it was known as the "Black Bane" and the "malignant pustule" due to its cutaneous manifestations, although it is neither pustulant nor necessarily deadly. Other names include "Rag pickers' disease" Charbon, Milzbrand, "Tanners' disease", Siberian (splenic) fever etc. It was also known as " Wool sorters' disease" because of occupational hazards frequently observed among mill workers exposed to animal fibers contaminated with *B. anthracis* spores (Riedel, 2005; Anonymous, 2002).

In 19th century, anthrax was a major point of interest in biological research. Pierre Rayer and his assistant Casimir – Joseph Davaine in 1850 were the first to observe non motile, filiform bodies in the blood of sheep having died of anthrax. In 1855 Pollender, 1857-58 Brauell, 1859 Fuchs and 1860 Delafond also identified that same agents in anthrax affected animals. Among them Brauell, in 1857-58 first reported the transmission of anthrax from man to sheep. In a series of studies between the years 1863 and 1868, Davaine definitely established the presence of filiform bodies in the blood of animals, which had died of anthrax. He gave the name "bacterides" to these bodies (Merchant and Parker, 1983).

In 1871, Tiegel and in 1877 Louis Pasteur and Joubert, showed that anthrax was caused by the small "bacterides" of Davaine because filtrate from which the organisms had

been removed did not produce disease (Merchant and Parker, 1983). In 1877, Koch postulated that the anthrax bacilli could be transmitted from one host to another and he grew the organism in vitro and induced the disease in healthy animals by inoculating them with materials from these bacterial cultures (Riedel, 2005). He was able to trace the complete life cycle of this bacilli for first time (1876) and also explored that it remain viable for long periods in unfavourable environment (Carter, 1988). Koch proved his postulates by isolating the organism from the infected animal (Merchant and Parker, 1983).

Contributions to the immunity of anthrax were made in 1880 by Chauveau and in the same year by Toussaint. Final and undoubted proof of the value of vaccination, however, resulted from the famous experiments of Pasteur at Pouilly-le-Fort; a small village out-side of Paris, in 1881. He inoculated 25 cattle with his anthrax vaccine, which contained live attenuated organisms. Subsequently, he inoculated the vaccinated as well as other cattle with a virulent strain of anthrax bacilli. All of the vaccinated animals survived, but others died (Merchant and Parker, 1983; Carter, 1988). At this time, both Koch and Pasteur pave the way for further work in medical microbiology.

Anthrax is world wide in its distribution. It is particularly prevalent in countries where no organized control of animal disease exists. India, China, Siberia, Russia, northern Africa, some parts of South America and Mexico has anthrax as a major livestock problem. Germany, France, Italy, Great Britain and the United States keep the disease well under control. Anthrax is enzootic in southern India but is less frequent to absent in the Northern Indian states where the soil is more acid, while in Nepal it is endemic. Hansen believes that anthrax was introduced into the Ohio River Valley during the early days of westward migration, and he cites that Kercheval was the first person to describe an outbreak of the disease in cattle and infection in four farmers in the United States in 1824. An extensive epizootic of anthrax occurred in northeastern Oklahoma and southeastern Kansas in 1957, with a loss of 1627 animals on 741 premises as reported by Van Ness and associates (Merchant and Parker, 1983). As countries become free of anthrax or the annual incidence of outbreaks approaches unity, the numbers of animals, affected in an outbreak increase. This seems to be due to the decreasing veterinary experience in recognizing cases and in dealing appropriately with outbreaks. The mere absence of reported livestock anthrax does not mean that a country is free of the disease. Reporting deficiencies and insufficient examination of unexpected livestock deaths are common throughout the world (Anonymous, 1997 a,b).

Circumstantial evidence indicates that man is moderately resistant to anthrax. Before vaccines and antibiotics became available, and at a time when understanding of industrial hygiene was relatively basic, workers in industrial occupations processing animal products were exposed to significant numbers of anthrax spores on a daily basis. In Britain, 354 cases of Anthrax in such industries were notified during 13-year period 1899-1912 (Anon, 1918). Although the numbers of persons exposed is not known, it must have been many thousands, and the number of cases represented only a very small proportion of the number exposed. With improvements in industrial hygiene practices and restrictions on imported animal products, the number of cases fell in considerable level in latter parts of the 20th Century. However, death rates remained high (>85%) when inhalation anthrax occurred (Riedel, 2005).

Historical analysis of epidemiological data globally reveals the following approximate ratios: a) One human cutaneous anthrax case to ten anthrax livestock carcasses; b) One incident of enteric human anthrax to 30-60 anthrax-infected animals eaten; c) in humans, 100-200 cutaneous cases for each enteric case that occurs. Industrial anthrax incidence data can be inferred from the volume and weight of potentially affected materials handled or imported, taking into account the quality of prevention, such as vaccination of personnel and forced ventilation of the workplace. These relationships are essentially all that can be used for many countries where human anthrax is infrequently, erratically or incompletely reported. In addition, certain countries suppress anthrax reporting at the local or national levels.

Human case rates for anthrax are highest in Africa, the Middle East and central and southern Asia. Where the disease is infrequent or rare in livestock it is rarely seen in humans (Turnbull *et al.*, 2002). In the 1950s, US Army Chemical Corps was developed human anthrax vaccine which was replaced by new and improved vaccine in 1970 with its license. In 1997, the US armed forces mandated vaccination for all reserve and active troops (Riedel, 2005; Morris, 1999). Cutaneous anthrax is said to account for 95% or more of human cases globally. However, serological and epidemiological evidence suggest that undiagnosed low-grade gastrointestinal tract or pulmonary anthrax with recovery can also occur, and may not be infrequent among exposed groups (Brachman *et al.*, 1960; Norman *et al.*, 1960).

An outbreak in a mill in New Hampshire, USA, in 1957 was not associated with any unusual change in occupational exposure, but seems to have been an isolated event within a prolonged period of exposure. The US Department of Defense bases its strategies on an estimate that the LD50 for humans is 8000 to 10,000spores. However, in this relation it was found that workers were found to be inhaling 600 to 1300 anthrax spores over an 8-hour shift without ill effect. *B. anthracis* was recovered from the nose and pharynx of 14 of 101 healthy persons. Furthermore, it is well established that, spores above 5 mm have increasing difficulty in reaching the alveoli of the lung. The likelihood of inhaled spores penetrating far enough to induce inhalation anthrax therefore depends greatly on the size of the particles to which they are attached (Turnbull *et al.*, 2002; Meselson *et al.*, 1994; Dahlgren *et al.*, 1960).

Outbreaks and epidemics do occur in humans, sometimes these are sizeable, such as the epidemic in Zimbabwe which began in 1979, was still smouldering in 1984-85 and had by that time affected many thousands of persons, albeit with a low case fatality rate (Turner, 1980; Davies, 1982).Typically, gastrointestinal anthrax follows the consumption of insufficiently cooked contaminated meat. In 1987, 14 cases of gastrointestinal and oropharyngeal anthrax were reported from northern Thailand (Kunanusont *et al.*, 1989).

Anthrax related biological warfare

If we go through the history of attempts of using diseases in biological warfare, it illustrates the difficulty of differentiating between a naturally occurring epidemic and an alleged or attempted biological warfare attack. This problem has continued into present times. The conception of Koch's postulates and the development of modern microbiology during the 19th century made the isolation and production of stocks of specific pathogen possible. Many countries have worked to develop these agents for biological warfare purpose (Ridel, 2004; Riedel, 2005). So, the use of biological warfare became sophisticated during the nineteenth century.

World War I

Substantial evidence suggests the existence of an ambitious biological warfare program in Germany, England and France during World War-I. This program allegedly featured covert operations. During World War I, reports circulated of attempts by German to ship horses and cattle inoculated with disease producing bacteria, such as *Bacillus anthracis* (anthrax) and *Pseudomonas pseudomalli* (glanders), to the USA and other countries (Riedel, 2005; Hugh-Jones, 1992). Though no hard evidence of using such arms was found, an international diplomatic meeting was held on 17th June, 1925. The "Protocol for the Use in War of Asphyxiating, Poisonous or Other Gases and of Bacteriological Methods of Warfare" (aka Geneva Protocol) was signed by 108 nations. Ultimately, it was meaningless since several countries (including Canada, Belgium, France, Great Britain, Italy, the Netherlands, Japan, Poland and the Soviet Union) began to develop biological weapons soon after its official implementation. The USA remained apart from the Geneva Convention until 1975 (Ridel, 2004).

World War II

During World War II Germany, Canada, United Kingdom, Japan, the Soviet Union, and the USA began an ambitious biological warfare research program. Various allegations and counter charges clouded the events during and after World War II. Japanese biological warfare program was known as "Unit 731" and was conducted in occupied Manchuria near the town of Pingfan from 1932 until the end of World War II. The program was under the direction of Shiro Ishii (1932-1942) and Kitano Misagi (1942-1945) and consisted of more than 150 buildings, 5 satellite camps, and a staff of more than 3000 scientist (Eitzen and Takafuzi, 1997; Christopher *et al.*, 1997). *Bacillus anthracis* was one of the organisms of interest which had been extensively researched and used. The Japanese government accused the Soviet Union of experimentation of biological warfare agents *like B. anthracis*, *Shigella, V. cholerae* based on recovery of such agents from Russian spies (Ridel, 2004). German medical researchers infected prisoners with disease producing organisms like *Rickettsia prowazeki*, hepatitis A virus, and malaria. Despite this effort, a German offensive biological warfare program was not completely materialized (Hugh-Jones, 1992).

On the other hand, German officials accused the allies of using biological warfare agents as weapons. It was believed by many that the British were experimenting with at least one organism as biological warfare agent (i.e. *Bacillus anthracis)* under the control of Dr. Paul Fildes in 1940s. Bomb experiments of weaponized spores of *B. anthracis* were conducted on Gruinard Island near the northwest coast of Scotland. This led to a heavy contamination of the island with persistence viable spores. In 1986, the island was finally decontaminated by using formaldehyde and seawater (Christopher et al., 1997; Manchee and Stewart, 1988). Offensive Biological warfare program was begun in USA on 1942 at Camp Detrick (recent name Fort Detrick), Maryland where the organism of interest was *B. anthracis*. Canada also started biological warfare program with small number of workers where *B. anthracis* was also under consideration during World War II.

After World War II

During the Korean War, the Soviet Union, China, and North Korea accused the USA of using anthrax spores against the Chinese and North Koreans, although this has not been proved. The credibility of the USA in this respect remained questionable due to its failure to

ratify Geneva Protocol of 1925, and because of suspicions of collaboration with former Unit 731 scientist for its own offensive biological warfare program (Ridel, 2004; Anonymous, 2002).

Biological Weapon Convention 1972

It was obvious that Geneva Protocol was a toothless measure for control & proliferation of biological weapons. Risks of an unpredictable nature as well as lack of epidemiological control measures for biological weapons became a serious concern internationally during late sixties. In 1969, Great Britain and the Warsaw Pact Nations submitted a proposal for the prohibition of biological weapons. This proposal was strengthen by a report issued by World Health Organization on November, 1969 regarding the possible consequences of the use of biological weapons. The report revealed that anthrax is a dangerous threat because it showed that release of 50Kg aerosolized anthrax agents by aircraft over a population center of 500,000 causes 95,000 deaths and 1, 25,000 severe incapacitations (Ridel, 2004; Riedel, 2005; Anonymous ,1971; Anonymous, 1970). In the way of on-going process, in 1972 "Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction" known as the BWC, was developed. The BWC was signed by 103 nations and implemented officially in April 1972. This measure helped to prohibit development, production, stockpiling, and technological transfer of biological warfare agents.

Post BWC Scenario

Despite BWC agreement, some signatories continued their offensive bio-warfare program. In April, 1972 a large epidemic of inhalation anthrax occurred among citizens of Sverdlovsk (now Ekaterinburg), Russia. The epidemic was suffered by those who lived and worked near a soviet military microbiology facility, known as Compound 19. Many livestock also died of anthrax in same area within 50 Km from that Compound in Sverdlovsk. European and US intelligence suspected that this epidemic might be attributed to an accidental release of anthrax spore from Compound 19 where biological warfare research was conducted. In February 1980, the well circulated German daily "Bild Zeitung" revealed a story about the accident in Soviet military settlement in Sverdlovsk in which an anthrax cloud had resulted. Soviet officials attributed the human cases of anthrax from the ingestion of contaminated meat.

The epidemic of Sverdlovsk, which claimed thousands of lives, required independent scientific investigation. In 1986, Matthew Meselson (Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts) conducted several unsuccessful trials, which ultimately led to an invitation to come to Moscow for discussion with 4 Soviet physicians engaged in Sverdlovsk outbreak. The outcome of this meeting was that epidemiological and pathoanatomical data was needed for further investigation. The Soviet Union maintained that this outbreak was due to consumption of anthrax contaminated meat from the black market.

After collapse of the Soviet Union in 1992, the Russian President Boris Yeltsin admitted that the compound 19 was a part of offensive Biological warfare program and the epidemic was caused by an accidental release of anthrax spores. Returning to Russia, Meselson and his team joined in the investigation and reviewed demographic, ecologic, atmospheric as well as private pathologist's data. This led them to the conclusion that the 42 cases of fatal anthrax

bacteremia and toxemia were typical of inhalation anthrax as seen in experimentally infected nonhuman primates. The data indicated that the outbreak resulted from an aerosol that originated from Compound 19. After this incident, the research was continued at a remote military facility in the isolated city of Stepnogorsk in Kazakhstan, for producing more virulent anthrax strain (Ridel, 2004; Riedel, 2005; Messelson *et al.*, 1994; Caidle, 1997).

In August 1991, during the process of UN inspection, representatives from the Iraqi government announced to the UN Special Commission Team that Iraq had conducted research on offensive biological weapons including *B. anthracis*. Later, a bioterrorism attack in Japan using anthrax and botulinum toxin was also conducted unsuccessfully by Aum Shinrikyo before March 1995 (Ridel, 2004; Messelson *et al.*, 1994).

2001 Scenario

Demonstration of "anthrax letters" associated with intentional release of the anthrax organism following World Trade Center attack in September 2001, was first confirmed in a journalist in Florida on October 2001. Later some cases of cutaneous and inhalation anthrax was seen in postal workers who handled those types of mail. Analysis of these cases suggested that this bioterrorism was conducted by using Ames strain of *B. anthracis* (Ridel, 2004; Riedel, 2005).

In November 2, 2001 a sample from an envelope containing suspicious white powder received by the office of Deputy Chief Minister Chhagan Bhujbal has tested positive for the presence of anthrax spores, making this the first confirmed case of "anthrax mail" in India. This sample was confirmed by Molecular Diagnostics Pvt. Ltd. in Thane (Anonymous, 2001a).

Microbiology

The genus *Bacillus* is comprised of about 70 species with diverse characteristics, including: *B. anthracis, B. cereus, B. licheniformis, B. mycoides, and B. thuringiensis*. Members of the *Bacillus cereus* group (B. *anthracis, B. cereus and B. thuringeinsis)* are really pathovars of a single species (Riedel, 2005). Most species are saprophytes with no pathogenic potential. However, they often contaminate clinical specimens and laboratory media. *B. anthracis* is the most important pathogen in the group.

Most *Bacillus* species are large, Gram-positive, endospore forming rods up to 4 to 10 µm by 1-1.5 µm in size. They are aerobic or facultative anaerobic. With the exception of *B. anthracis* and *B. mycoides* they are motile. In blood smears of tissue or lesion fluid from diagnostic specimens, these chains are two to a few cells in length. In suspension made from agar plate cultures, they can appear as endless strings of cells which are responsible for the tackiness of the colonies. Also characteristic is the square-ended i.e. "box car" shaped appearance traditionally associated with *B. anthracis* vegetative cells, although this is not always very clear. Ellipsoidal central spores, which do not swell the sporangium, are formed at the end of the exponential growth phase in presence of oxygen. Under anaerobic condition the bacilli in infected tissue secretes a polypeptide [poly- (D-glutamic acid)] capsule, but this character is lost when the bacterium is grown aerobically in vitro. The capsule can be induced by incubating in defibrinated horse blood for at least 5 hrs, or by culturing the isolate on

nutrient agar containing 0.7% sodium bicarbonate with incubation at 37°C in presence of 5% to 10% carbon dioxide. This capsule can be identified under microscope by the staining with polychrome methylene blue (McFadyean's reaction), where capsule stains pink and bacillus cells stains dark blue or may be highlighted with negative staining (Riedel, 2005).

Bacillus anthracis grows readily on different types of media at 37°C. A slightly alkaline medium, pH 7.5 to 7.8 is most conclusive to good growth. *B. anthracis* colonies are up to 2 mm to 5 mm in diameter, flat, dry, grayish and with a "ground glass" appearance after incubation for 48 hours. At low magnification, curled outgrowths from the edge of the colony impart a characteristic, "medusa head" appearance. In gelatin stab culture, filaments of growth radiate from the line of puncture and give the appearance of an inverted fir tree. It is generally non-haemolytic on sheep blood agar media. It also shows weak and slow lecithinase activity on egg yolk agar. Biochemically it is non-lactose fermenting, indole and H_2S are not produced, nitrates are reduced to nitrites, and ammonia is produced. The organism is Methyl Red positive and Voges-Proskauer variable. Its diagnostic feature is that it shows Mc Fadyean reactions, susceptibility to diagnostic gamma phage and penicillin and its colony characteristics (Merchant and Parker, 1983;Anonymous, 2001b).

Bacilli will form spores in the environment outer from host body. Under favorable conditions, anthrax spores germinate and rapidly multiply into vegetative form. Little information exists regarding lifestyle of this pathogen outside of the host. Recently it was found that spores of *B.anthracis* have the capacity to germinate in the rhizosphere of grass plants and to establish populations of vegetative cells that could support horizontal gene transfer in the soil and helps in the evolution of this species under *Bacillus cereus* group as a saprophytic organism outside the host (Saite and Kochler, 2006).

Multiple locus enzyme electrophoresis (MEE) and multiple locus sequence typing (MLST) have shown the lack of genetic diversity of *B.anthracis*. It is one of the most molecularly monomorphic bacteria known. Amplified fragment length polymorphism (AFLP) analysis helps to detect difference between *B. anthracis* isolates and to examine phylogenetic relationship between *B. anthracis* and its close relatives. Multiple locus variable number tandem repeat analysis (MLVA) which, unlike AFLP designed to subtype *B. anthracis* specifically and can not be used to address phylogenetic relationship between *Bacillus* species. MLVA determines the copy numbers of variable number tandem repeat (VNTR) in the region of VrrA genes as well as on two plasmids to differentiate into 89 distinct genotypes of these bacilli. Besides this, single nucleotide polymorphism (SNP) also helps in genome-based analysis of bacilli.

Besides this, full confirmation of virulence can be carried out using the polymerase chain reaction (PCR). Template DNA for PCR can be prepared from a fresh colony of *B. anthracis* on nutrient agar by resuspension of a loop-full of growth in 25 ml sterile deionized (or distilled) water and heating to 95^0 C for 10 minutes. Following cooling to approximately 4^0 C, and brief centrifugation, the supernatant can be used for the PCR reaction (Table-1 for primers information). PCR can be carried out in 50 ml volumes using the above primers, 200mM each of dATP, dCTP, dTTP and dGTP, 1.5mM MgCl and 2.5 units of Amplitaq polymerase, all in NH_4 buffer, followed by addition of 5 ml of template DNA.

Alternatively,"Ready-To-Go™" beads are available from Pharmacia Biotech (Uppsala, Sweden, product number 27-9555-01). These are premixed, predispensed, dried beads, stable at room temperature, containing all the necessary reagents, except primer and template, for performing 25 ml PCR reactions. The template can be added in a 2.5 ml volume. For strains of *B. anthracis* lacking both pX01 and pX02, the primers specific to the S-layer can be included to confirm the presence of *B. anthracis* chromosomal DNA (Table-1). It is probably advisable to run the S-layer primers alone rather than in multiplexed with PA and Capsule primers. The following PCR cycle can be used: 1 x 95° C for 5 minutes; 30 x (95° C for 0.5 minutes followed by 55° C for 0.5 minute followed by 72° C for 0.5 minute); 1 x 72° C for 5 minutes; cool to 4° C (Turnbull *et al.*, 2002).

Table 1. Suitable primers for confirming the presence of pX01 and pX02 plasmids

Target	Primer ID	Sequence 5´-3´	Product size	Conc.
Protective Antigen (PA)	PA5 3048-3029 PA8 2452-2471	TCCTAACACTAACGAAGTCG GAGGTAGAAGGATATACGGT	596bp	1mM
Capsule	1234 1411-1430 1301 2257-2238	CTGAGCCATTAATCGATATG TCCCACTTACGTAATCTGAG	846bp	0.2mM
S-layer	Upper 391-413 Lower 1029-1008	CGCGTTTCTATGGCATCTCTTCT TTCTGAAGCTGGCGTTACAAAT	639bp	0.2mM

Pathogenesis

The virulence of *B. anthracis* derives from the presence of a capsule and the ability to produce a complex toxin. Both virulence factors are encoded by plasmids and are required for disease production. The expression of virulence factors is regulated by host temperature and carbon dioxide concentration. The capsule is composed of high molecular weight poly peptide (poly-D-glutamic acid) and is encoded by the pXO2 plasmid. This small plasmid is 95.3 kilo base pairs and encodes the three genes *capB*, *capC*, *capA*. The capsule inhibits phagocytosis of the vegetative form of *B. anthracis*.

Another plasmid pXO1 is of 184.5 kb pairs and encodes toxin complex, which consists of three synergistically acting proteins i.e., Protective Antigen (PA,83 KDa), Lethal Factor (LF, 87 KDa) and Edema Factor (EF, 89 KDa) is produced during the log phase of growth of *B. anthracis* (Riedel, 2005; Turnbull *et al.*, 2002). Recent study provides evidence that pXO2 is necessary for the maximal expression of pXO1(Lamonica *et al.*, 2005). Individually each factor lacks toxic activity in experimental animals, although protective antigen induces antibodies which confer partial immunity. LF in combination with PA forms lethal toxin and EF in combination with PA for oedematoxin. Both these toxins are now regarded as responsible for characteristic signs and symptoms of anthrax.

According to the currently accepted model, Protective Antigen binds to receptors on the host cells and is activated by a host protease which cleaves off a 20 KDa piece, thereby exposing a secondary receptor site for which LF and EF compete to bind. The PA+LF or PA+EF are then internalized and the LF and EF are released into the host-cell cytosol. Edema

Factor or EF is a Calmodulin-dependent adenylate cyclase which by catalyzing the abnormal production of cyclic-AMP (cAMP) produces them altered water and ion movements that lead to the characteristic oedema of anthrax. High intracellular cAMP concentrations are cytostatic but not lethal to host cells. EF is known to impair neutrophil function and oxidative process and its role in anthrax infection may be to prevent activation of the inflammatory process.

Lethal Factor or LF appears to a Calcium and Zinc- dependent metalloenzyme endopeptidase. It has recently been shown that it cleaves the amino terminus of two mitogen-activated protein kinases and thereby disrupts a pathway in the eukaryotic cell concerned with regulating the activity of other molecules by attaching phosphate groups to them. This signaling pathway is known to be involved in cell growth and maturation, but the manner in which its disruption leads to the known effects of LF has yet to be elucidated. On the basis of mouse and tissue culture models, macrophages are major target of lethal toxin which is cytolytic in these. The initial response of sensitive macrophages to lethal toxin which is the synthesis of high level of Tumor Necrosis Factor i.e., TNF alpha and Interleukin-1 beta cytokines and it seems probable that death in anthrax infections results from a septic shock type mechanism resulting from the release of these cytokines.

The endothelial cell linings of the capillary network may also be susceptible to lethal toxin and the resulting histologically visible necrosis of lymphatic elements and blood vessel walls is presumably responsible for systemic release of the bacilli and for the characteristic terminal hemorrhage from the nose, mouth and anus of the victim (Turnbull *et al.*, 2002).

Infection with anthrax occurs after introduction of spores through a break in skin in case of cutaneous anthrax or entry through the mucosa in case of gastrointestinal anthrax. After spores are ingested by macrophages at the port of entry, germination of the vegetative form occurs and the bacilli rapidly multiply. Rapid extra cellular multiplication is accompanied by the production of the lethal toxin and oedema factor.

In inhalation anthrax, spores (1-2 μm in diameter) are inhaled and deposited in the alveolar spaces. From there, they are transported to local lymphatic and the mediastinal lymph nodes, where they germinate and cause hemorrhagic lymphadenitis. Vegetative bacilli then further spread via the blood stream and lymphatics, causing septicemia. The large amount of toxin produced by the bacilli, together with the host response i.e., release of TNF-alpha and Interleukin-1 are responsible for the rapid decline and the overt symptoms of the host organism. Recently, several newly identified putative virulence factors were observed, these include enolase, high affinity zinc uptake transporter, the peroxide stress related alkyl hydogen peroxide reductase, isocitrate lyase, and the cell surface protein A (Riedel, 2005; Lamonica *et al.*, 2005).

Clinical symptoms

Anthrax in human is classically divided in two ways. The first type of classification reflects how the occupation of the individual led to their exposure to anthrax. The non-industrial type occurrs in farmers, butchers, knackers, veterinarians and so on. The other type is industrial anthrax, occurring in those employed in bones, hides, wool and other animal products. Industrial anthrax distinguishes between cutaneous anthrax acquired through a skin lesion, or insect bite,

gastrointestinal tract anthrax contracted from ingestion of contaminated food, primarily meat from an animal that died of disease, or conceivably from ingestion of contaminated water and pulmonary (inhalation) anthrax from breathing in air borne anthrax spores.

Cutaneous anthrax is said to account for 95% or more of human cases globally. In cutaneous cases, incubation period ranges as little as 9 hours to 2 weeks, mostly 2 to 7 days. *B. anthracis* (usually as spores) entered through skin lesion like cut, abrasion, insect bite etc. A small pimple or papule appears; gradually a ring of vesicles develops around it. Marked oedema starts to develop. Painful lymphadenitis may occur in the regional lymph nodes. The original papule ulcerates to form the characteristic eschar. Oedema extends some distance from the lesion. Clinical symptoms may be more serve if the lesion is located in the face, neck or chest. In these more serve forms, clinical findings are high fever, toxemia, regional painful adenomegaly and extensive oedema, shock and death may ensue. Generally, eschar resolves within six weeks. In untreated cutaneous anthrax, about 20% of patients develop septicemia and die. However, with the use of appropriate antibiotics, the mortality rate is < 1% (Lew, 1995). In this context, seven confirmed and four suspected cases of cutaneous anthrax were identified during the 2001 out break. Skin trauma was not associated with these cases of cutaneous anthrax. Exposure to contaminated mail was the apparent source of infection in all patients.

There are two clinical forms of gastrointestinal anthrax which may present following ingestion of *B. anthracis* in contaminated food or drink. Intestinal forms include symptoms like nausea, vomiting, fever, abdominal pain, haematemesis, bloody diarrhea and massive ascites. Unless treatment commences early enough, toxemia and shock develop, followed by death. There is evidence that mild, undiagnosed cases with recovery can occur (Brachman *et al.*, 1960). In this form ulcers and necrosis usually form in the wall of the terminal ileum; sometimes caecum, colon, stomach and duodenum can also be involved (Riedel, 2005). In oropharyngeal form, the main clinical features are sore throat, dysphasia, fever, regional lymphadenopathy in the neck and toxemia. Even with treatment, the mortality is about 50% (Doganay *et al.*, 1986). The suspicion of anthrax depends largely on awareness and alertness on the part of the physician as to the patient's history and to the likelihood that he/she had consumed contaminated food or drink.

In case of pulmonary form of anthrax symptoms prior to the onset of the final hyperacute phase are non-specific and suspicion of anthrax depends on the knowledge of the patient's history. Early symptoms are non-specific and "flu-like" begin insidiously with mild fever, fatigue lasting one to several days (Plotkin *et al.*, 1960). Headache, muscle aches, chills, fever, drenching sweats, minimally productive cough, nausea or vomiting, mild chest pain were symptoms recorded in diseased patient's chest radiography at initial examination showed mediastinal widening, paratracheal fullness, hilar fullness, pleural effusions or infiltrates or both; chest computed tomography scan is helpful in detecting hemorrhagic mediastinal lymph nodes and oedema, peribronchial thickening, hyper dense mediastinal and hilar adenopathy findings seen in inhalation anthrax. This mild initial phase was followed by the sudden development of dyspnoea, cyanosis, disorientation with coma and death. Death occurred within 24 hours onset of the hyper acute phase. In 2001 outbreak of bioterrorism related anthrax 11

patients were identified for inhalation anthrax are believed to have been exposed to mail containing or contaminated with *B. anthracis* spores.

Meningitis due to anthrax is a serious clinical development which may follow any of the other three forms of anthrax. The case fatality rate is almost 100%, the clinical signs of meningitis with intense inflammation of meninges, markedly elevated CSF pressure and the appearance of blood in the CSF (the meningitis of anthrax is a hemorrhagic meningitis) are followed rapidly by loss of consciousness and death (Turnbull *et al.*, 2002).

Sepsis develops after the lymphohaematogenous spread of *B. anthracis* from a primary lesion (cutaneous, gastrointestinal or pulmonary). Clinical features are high fever, toxemia and shock, with death following in a short time (Turnbull *et al.*, 2002). Where anthrax has not been suspected prior to post mortem, characteristic signs are dark haemolysed unclotting blood, enlarged hemorrhagic spleen, petechial hemorrhages throughout the organs and a dark edematous intestinal treat, ulcerated or with areas of necrosis (Turnbull *et al.*, 2002). Histopathological findings from patients with disseminated anthrax showed hemorrhage, oedema, necrosis, fibrin deposition and a variable degree of inflammatory cell infiltrate, predominantly consisting of neutrophils (Abramova *et al.*, 1993).

Treatment and Prophylaxis

Prompt and timely antibiotic therapy usually results in dramatic recovery of the individual or animal infected with anthrax. Almost all isolates of *B. anthracis* can be expected to be highly sensitive to penicillin and being cheap and readily available in most parts of the world, this remains the basis of treatment schedules, particularly in animals and in humans in developing countries. In mild uncomplicated cases of cutaneous anthrax, Penicillin V, 500 mg orally every 6 hours for 5 to 7 days is adequate, but the treatment usually recommended is 3 to 7 days of intramuscular procaine penicillin, 600 mg (1 million units), every 12-24 hours or intramuscular benzyl penicillin (penicillin G), 250,000 units at 6 hours intervals. Cutaneous lesions usually become sterile within the first 24 hours of such regimens, and early treatment will limit the size of lesion.

In severely affected patients or when pulmonary or gastrointestinal anthrax is suspected, the initial treatment is penicillin G, 1200 mg (2 million units per day, by infusion or by slow intravenous injection (< 300 mg / min) every 4-6 hours until the patient's temperature returns to normal. At this point treatment should continue in the form of intramuscular procaine penicillin. Streptomycin, 1-2 gm per day intramuscularly, may act synergistically with penicillin. In the event of allergy to penicillin, several antibiotics are effective alternatively such as tetracycline, chloramphenicol, gentamicin and erythromycin. Trimethoprim is not effective.

In case of anthrax meningoencephalitis suggest that penicillin (2 million units of crystalline penicillin intravenously every 2 hours initially) remains the antibiotic of choice because it diffuses in the cerebrospinal fluid through highly inflamed meninges. Chloramphenicol (1 gm intravenously every 4 hours) is a suitable alternative for hypersensitive patients. Essential supportive therapy includes the early institution of oedema measures, such as 100 ml of 20% manitol intravenously every 8 hours and hydrocortisone, 100 mg every 6 hours (Turnbull *et al.*, 2002).

All the *B. anthracis* isolates associated with 2001 bioterrorism related anthrax outbreak were sensitive to the quinolones, rifampin, tetracycline, vancomycin, imipenem, meropenem, chloramphenicol, clindamycin and the aminoglycosides. The isolates have intermediate-range susceptibility to the macrolides but are resistant to extended-spectrum cephalosporins, including third generation agents like ceftriaxone, and to trimethoprim- sulfamethoxazole 9 (Anonymous, 2001c).

The decision regarding the use of penicillins or amoxicillin, the drugs historically used for treatment and prophylaxis of anthrax, is complicated against the Ames strains causing these recent infection (Riedel, 2005; David *et al.*, 2002). Genomic sequence data show two beta-lactamases: a potential penicillinase (class A) and a cephalosporinase (class B). Concern about the use of penicillin arises because an inducible penicillinase could be activated in the face of treatment with beta-lactams, particularly if the number of organisms present is high, as appears typical with inhalation disease. Concerns have also been raised about the poor penetration of beta-lactams in macrophages, the site where *B. anthracis* spores germinates (David *et al.*, 2002).

Ciprofloxacin and doxycycline may be recommended as a good first line agent. Thus recommendation for initial treatment of inhalation anthrax is a multidrug regimen of ciprofloxacin or doxycycline along with one or more agents to which the organisms is typically sensitive, this treatment also allows empiric coverage for other pathogens. Clindamycin has been suggested to have antitoxin properties. Steroids have been used to control oedema of cutaneous disease and have been suggested for the treatment of meningitis or substantial mediastinal oedema.

During the recent bioterrorist attacks, interim Center for Disease Control and Prevention (CDC), Atlanta recommendation for anthrax prophylaxis include ciprofloxacin or doxicyclin, this are also approved by the Food and Drug Administration (FDA), for prophylaxis of inhalation anthrax infection. The optimal duration of prophylaxis is uncertain, however, 60 days was recommended, primarily on the basis of animal studies of anthrax deaths and spore clearance after exposure. In one human case during the Sverdlovsk outbreak in former Soviet Union on 1979, anthrax developed 43 days after spores were release into the atmosphere (Friendlander *et al.*, 1993). It has been postulated that germination of dormant spores may occur as late as 60 days after infection (Riedel, 2005). There options are now offered for prophylaxis: i) 60 days of antibiotic prophylaxis; ii) 100 days of antibiotic prophylaxis and iii) 100 days of antibiotic prophylaxis, plus anthrax vaccine as investigational post-exposure treatment [3 doses over a 4 week period] (Anonymous, 2001d).

Normally, vaccination seems the most effective form of mass protection. Pasteur produced the first anthrax animal vaccine in 1881. Four decades of research have shown that protection against anthrax in the susceptible host is dependent almost entirely on that host is immune response to a single antigen – the protective antigen (PA) component of the anthrax toxin, a well defined protein of molecular weight 83 KDa. The effectiveness of both animal and human vaccines is dependent on the induction of anti- PA antibodies. However, the immune response is complex and involves cellular immunity in some as yet unidentified manner (Turnbull *et al.*, 2002).

Most anthrax vaccines in use today utilize the toxigenic, non-capsulating (pXO1+ / pXO2-) *B. anthracis* strain 34F2 isolated in 1937 by Sterne. Earlier Pasteur – type vaccines are still in Italy. In Central and Eastern Europe, an equivalent pXO2 – derivative, "Strain 55", is the active ingredient of the current livestock vaccine. Another Strain "1190R- Stamatin" is used for vaccine production in Romania (Turnbull *et al.*, 2002). A vaccine for use in humans was licensed in 1970 by the Food and Drug Administration. This vaccine was based on cultures in a synthetic medium, which was initially termed "528 medium" (Riedel, 2005). In China and in the countries of the former USSR, live spore vaccines are prepared for human use from Strain A16R and Strain STI (Sanitary Technical Institute) respectively (Turnbull *et al.*, 2002). In most other countries, live spore vaccine are not licensed for use in humans. In the United Kingdom and USA, non-living human vaccines developed in the 1950s and 1960s are available (Turnbull *et al.*, 2002). The UK vaccine is an alum-precipitated cell- free culture filtrate of Strain 34F2 while the US vaccine is an aluminum hydroxide – adsorbed cell free culture filtrate of a non-capsulating, non-proteolytic derivative of bovine isolate V770.

Considerable effort has been made in recent years to develop newer and safer anthrax vaccine free from any dangerous side effects in any species. Future vaccines are likely to take two forms: i) vaccines that contain recombinant protective antigen, ii) genetically manipulated vaccines that either contain deletions of genes for PA which in fact helps in deletion of undesired toxin factors or recombinant constructs of PA gene. In this context a new type of plant based vaccine was prepared very recently where PA gene was inserted into chloroplast of the cell of tobacco plants and each plant produce 150 milligrams of PA antigen, which adds up to 360 million doses worth of PA from one acre of tobacco plant and this vaccine is much cleaner than recently used vaccines (44). The aims for prospective new vaccine are that they should be cheap, environmentally acceptable, orally administrable, safe and protective after a single dose (Turnbull *et al.*, 2002).

Conclusion

Though anthrax is a common and naturally occurring disease in domestic animals, but it is gradually coming up on the surface as a devastating biological weapon. Research and development of anthrax as a biological weapon during the first half of the 20th century focused on easy dissemination of the bio-weapon and the development of multidrug resistant strains (Riedel, 2005). But this biological weapon would not only cause sickness and death but also aim to create fear, panic and paralyzing uncertainty. Its goal is disruption of social and economic activity, the breakdown of government authority and the impairment of military responses. Besides this, anthrax causes residual contamination of the ground for a long period along with great devastation in the civilized population as estimated by WHO in 1970 (Ridel, 2004).

Recently demonstrated by the "anthrax letter" in the aftermath of the World Trade Centre attack in September 2001, the occurrence of only a small number of infections can cause an enormous psychosocial breakdown. More importantly, these attacks fueled fears that future attacks might be more extensive.

Despite improvements in treatment and prophylaxis, anthrax is considered a fatal infection. Biological warfare attacks with anthrax agents are now a serious possibility. Primary prevention depends on creating a strong global norm that stops development of such weapons.

Secondary prevention depends on early detection and its prompt treatment. As BWC is preparing to assist nations that have been targets of biological weapons, the medical community must be ready to face the challenge of biological warfare because nobody knows what will happen next.

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Searching for phosphate solubilizing fungal isolates from soil

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Abstract

A total of 354 fungal isolates were obtained from soil samples collected from forests, river basins and agricultural fields of North Bengal using serial dilution, direct soil plating, serial root washing and root maceration techniques. Cultural characteristics of the isolated fungi were studied and microscopic observations were made for identification of these isolates. All the isolates were screened for their phosphate solubilizing activities in vitro. A total of 70 fungal isolates showed phosphate solubilizing activities as detected in Pikovskaya's agar medium. Quantitative evaluation of phosphate solubilization in liquid medium supplemented with two phosphate sources (tricalcium phosphate and rock phosphate) was carried out for all the isolates showing phosphate solubilizing activity. Maximum phosphate solubilizing capacity was shown by three isolates of A. niger while A. clavatus showed minimum activity. Genomic DNA was extracted from sixteen isolates showing high activity and PCR amplification of DNA from nine isolates was done.

Introduction

Microorganisms form a vital component of all known ecosystems of earth. Soil bacteria and fungi play pivotal roles in various biochemical cycles (BGC) (Molin and Molin, 1997) and are responsible for the cycling of organic compounds. Soil microorganisms also influence above-ground ecosystems by contributing to plant nutrition (George *et al.*, 1995), plant health (Smith and Goodman, 1999), soil structure (Wright and Upadhyaya, 1998) and soil fertility (Yao *et al.*,2000). An estimated 1,500,000 species of fungi exist in the world (Giller *et al.*, 1997). In a study conducted on the dicersity of filamentous fungi on decomposing leaf and woody litter of mangrove forest of south west coast of India, it was found that the frequency of occurrence of *Aspergillus, Cirrenalia, Penicillium* and *Trichocladium* was more than 10% and that woody litter collected during summer season showed highest fungal diversity than in the monsoon (Ananda and Sridhar, 2004). The role of rhizospheric organisms in mineral phosphate solubilization was known as early as 1903. Since then, there have been extensive studies on mineral phosphate solubilization by naturally abundant rhizospheric microorganisms. Important genera of mineral phosphate solubilizers include *Bacillus* and *Pseudomonas* (Illmer and Schinner, 1992), while *Aspergillus* and *Penicillium* form the important fungal genera. The high proportion of PSM is concentrated in the rhizospheres and is known to be more metabolically active than those isolated from sources other than the rhizosphere. Conversely, the salt-, pH- and temperature-tolerant phosphate-solubilizing bacteria have been reported to be maximum in the rhizoplane followed by the rhizosphere and root-free soil in alkaline soils (Johri *et al.*, 1999). Among PSMs, fungi perform better in acidic soil conditions (Ahmad and Jha, 1968). Species of *Aspergillus, Penicillium* and yeast have been widely reported solubilizing various forms of inorganic phosphates (Asea *et al.*, 1988; Whitelaw, 2000). Fungi have been reported to possess greater ability to solubilize insoluble phosphate than bacteria.

In this context, the present work has been envisaged for studying the microbial diversity of the north Bengal region which encompasses diverse habitats including high altitude regions, forests at different altitudes, rivers, cultivated lands as well as plantations. Since these fall under the Biodiversity Hotspots, microbial diversity is also expected to be high. Most of these regions have not yet been previously worked out.

Material and Methods

Isolation and Identification of Microorganisms

Soil samples were collected from three districts (Darjeeling, Jalpaiguri and Cooch Behar) of North Bengal. Source of soil samples were forests (Sukhna, Lohagarh, Cinchona, Mongpong, Gorumara) riverine soil from river basin (Balasan, Mahananda, Dhorola, Torsa, Raidak) agricultural land (paddy, bamboo) rhizosphere of tea, rubber, mandarin (plantation crops) and *Cryptomeria.* Fungi from these soil samples were isolated following Warcup's soil plate method (1950) for isolating fungi from the rhizosphere with a few modifications.

Screening for phosphate solubilizing activity

Screening for primary phosphate solublizing activity of the isolates was carried out by allowing the fungi to grow in selective media, i.e., Pikovskaya's agar (Pikovskaya, 1948) for 7 to 10 days at 25 °C. The appearance of a transparent halo zone around the fungal colony indicated the phosphate solublizing activity of the fungus.

Estimation of phosphorus

Phosphorus solubilizing ability of fungal strains was tested in two different types of Pikovskaya (PVK) liquid medium (Pikovskaya, 1948) amended with with 0.5% tricalcium phosphate ('P' = 997 μg/ml) and 0.25% (w/v) Rock phosphate (RP-140) having P_2O_5 ("P" 18.8 %) pH of the medium was adjusted to 7 before autoclaving. Dissolved phosphate concentration in the culture filtrate was determined by ammonium molybdate-ascorbic acid method (Kundsen and Beegle, 1988). Difference between the amount of tricalcium phosphate and Rock phosphate added and their remainder after incubation gave the exact phosphate solubilizing potential of the isolates.

Isolation of fungal genomic DNA

Isolation of fungal genomic DNA was done as outlined by Boreman (1996) with modifications.

PCR amplification

PCR amplification of fungal isolates with ITS specific primer HCHITSF-1 5' GCGGAAGGATCATTACTGAG 3' and HCHITSF-2 5' GGGTATCCCTACCTGATCCG 3' was carried out in a PCR-Thermocycler Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, 2.5 mM dNTP mix, primers and Taq polymerase (1U). Polymerase Chain Reaction was performed in a total volume of 100 μl,.containing. PCR was programmed with an initial denaturing at 94°C for 5 min. followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 40 s and extension at 70 °C for 90 s and the final extension at 72 °C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 μl) was mixed with loading buffer (8 μl), and then loaded in 0.8% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis.

Results and Discussion

A total of 354 fungal isolates were obtained from soil samples collected from Darjeeling, Jalpaiguri and Cooch Behar districts of North Bengal. Source of soil samples from forest, riverine and agricultural land (rhizosphere of plantation and agricultural crops) yielded 117, 52 and 185 fungal isolates respectively (Tables 1, 2 & 3). Cultural characteristics of the isolated fungi were studied and microscopic observations were made for identification of these isolates (Fig 1). A total of 70 fungal isolates obtained from different sources showed phosphate solubilizing activities as detected in Pikovskaya's agar medium (Table 4) showing halo zones after 4-5 days of incubation (Fig 2) In a study carried out by Pradhan and Sukla (2005). The phosphate solubilization potential of *Aspergillus* isolates was determined in three different liquid medium where PVK medium showed maximum phosphate solubilization. In the present study isolates were further taken up for evaluation of phosphate solubilization potential in liquid medium, isolate RHS/P 51 and FS/L04 showed maximum solubilization when medium was amended with tricalcium phosphate, whereas isolate RHS/R 12, FS/L 13 and FS/L 17 showed maximum amount of phosphate solubilization when the medium was amended with rock phosphate, with an average drop in the pH from 7 to 3.5. Acid production and drop in the pH of the medium have been reported in the earlier studies (Abd Alla, 1994; Whitelaw, 2000), however no significant relationship could be established in terms of phosphate solubilization and drop in the pH of the liquid medium. Some researchers have suggested that of the medium increasing P concentration in the phosphate solubilizing fungus containing medium was releated to the organic acid- types metabolities, which should correlate with the pH (Illmer and Schinner, 1992). Many studies have showed the ineffectiveness of rock phosphate use due to low solubility of its P content. Phosphate solubilizing fungus (PSF) have demonstrated the utilization of these poorly soluble phosphate source and PSFs were used as bioactivators of poorly soluble Rock phosphate (Didiek *et al.*2000).

Total genomic DNA of selected isolates were obtained efficiently following the method as outlined (Fig 2 A). ITS-PCR finger prints obtained with the primer HCHITSF-1 and HCHITSF-2 of *Aspergillus niger*, *A. melleus* and *A.clavatus* yielded single molecular weight product of 168 bp (Fig 2 B). Ribosomal DNA (rDNA) is suited for phylogenetic studies and developing molecular markers because the degree of conservation differs from one component of rDNA to another (Hibbett 1992).Internal transcribed spacer regions (ITS I and ITS II) are found to be more variable than the three ribosomal gene subunits in an organism (Molina *et al.* 1994). ITS I region targeted in the present study to utilize ITS region to develop molecular markers for *Aspergillus* group of phosphate solubilizing fungus gave a clue that ITS specific universal primers can be used for further investigation and analysis. It has been reported that universal primer do not work consistently for some fungi (Li *et al.* 1992), the PCR products could be obtained in case of all the DNA samples taken for amplification.

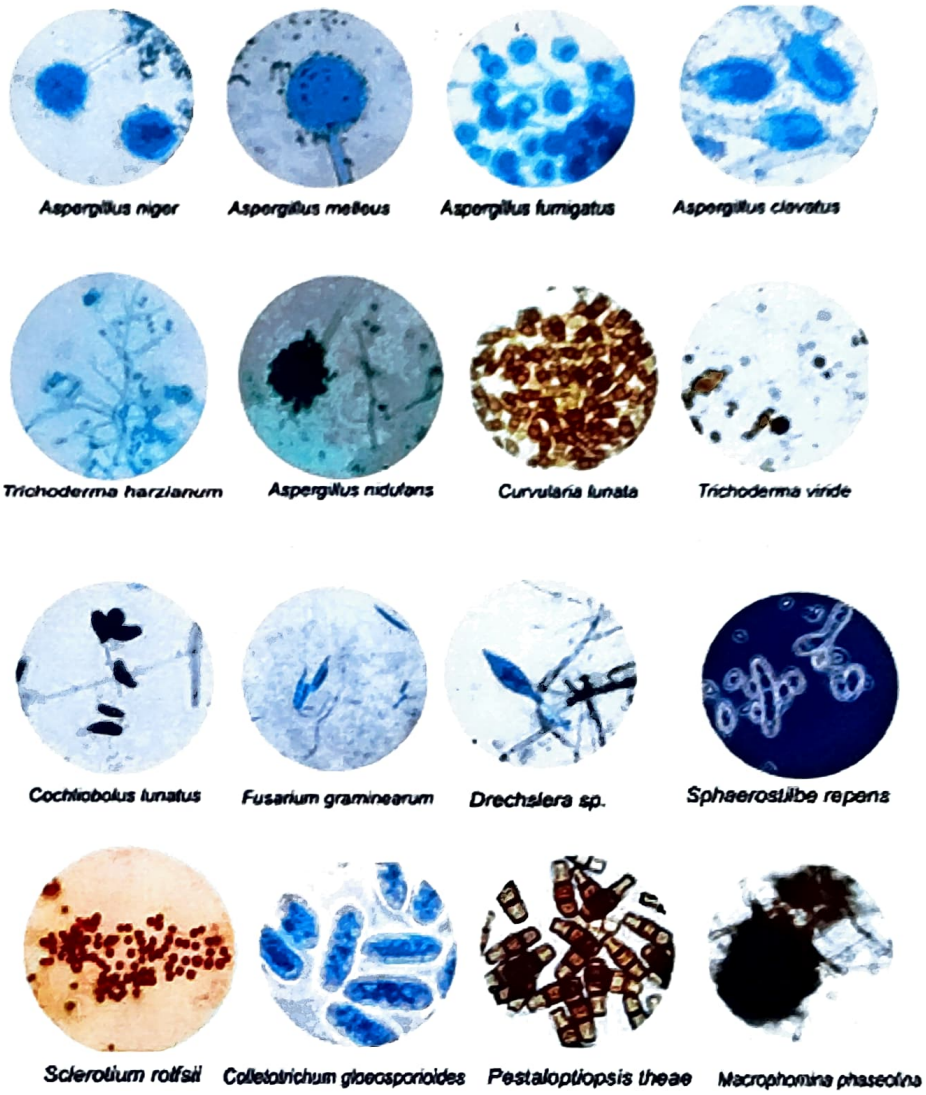

Fig 1. Microscopic view of selected fungal isolates

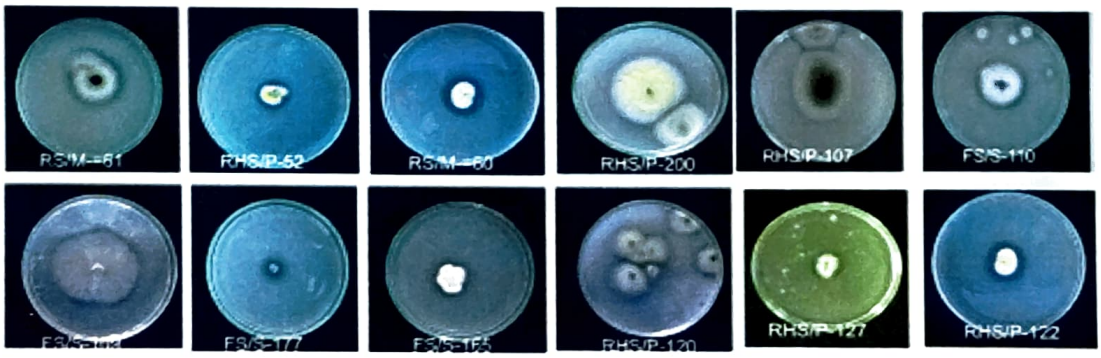

Fig 2. Screening of fungal isolates on Pikovskaya's agar medium showing of halo zones after 4-5 days of incubation indicating the phosphate solubilization activity of the isolates

Table 1. Fungal isolates obtained from forest soil

Name of the forest	Geographical location	Isolated fungi	Total
Sukna Forest	Darjeeling	FS/S-63, FS/S-64, FS/S-108, FS/S-109, FS/S-110, FS/S-185, FS/S-186, FS/S-187, FS/S-188, FS/S-189, FS/S-278, FS/S-279, FS/S-280, FS/S-281, FS/S-282, FS/S-311, FS/S-312, FS/S-313, FS/S-314, FS/S-315, FS/S-316, FS/S-352, FS/S-353, FS/S-354, FS/S-355, FS/S-356, FS/S-357, FS/S-358, FS/S-359, FS/S -165, FS/S -166, FS/S -167, FS/S -168, FS/S -169, FS/S -170, FS/S -171, FS/S -172, FS/S -173, FS/S -174, FS/S -175, FS/S -176, FS/S -177, FS/S -178, FS/S -179	44
Lohagarh Forest	Darjeeling	FS/L-4, FS/L-11, FS/L-13, FS/L-16, FS/L-17, FS/L-18, FS/L-19, FS/L-20, FS/L-24, FS/L-39, FS/L-40, FS/L-41, FS/L-42, FS/L-55, FS/L-56	15
Cinchona Forest	Darjeeling	FS/C-140, FS/C-141, FS/C-142, FS/C-143, FS/C-144, FS/C-145, FS/C-146, FS/C-147, FS/C-148, FS/C-149, FS/C-150, FS/C-151, FS/C-152, FS/C-153, FS/C-154, FS/C-155, FS/C-156, FS/C-157 FS/C-160	19
Mongpong Forest	Darjeeling	FS/M-111, FS/M-112, FS/M-113, FS/M-255, FS/M-256, FS/M-257, FS/M-258, FS/M-259, FS/M-260, FS/M-261, FS/M-262, FS/M-263, FS/M-264, FS/M-265, FS/M-266.	15
Gorumara Forest	Jalpaiguri	FS/G-226, FS/G-228, FS/G-317, FS/G-318, FS/G-319, FS/G-320, FS/G-321, FS/G-322, FS/G-323, FS/G-324, FS/G-325, FS/G-326, FS/G-327, FS/G-328, FS/G-329, FS/G-330, FS/G-360, FS/G-361, FS/G-362, FS/G-363, FS/G-364, FS/G-365, FS/G-366, FS/G-367.	24

Table 2. Fungal isolates obtained from riverine soil

Name of the river	Geographical location	Isolated fungi	Total
Panighata	Darjeeling	RS/P-01, RS/P-02, RS/P-03, RS/P-05, RS/P-14, RS/P-15	6
Mahananda	Darjeeling	RS/M-60, RS/M-61, RS/M-62, RS/M-161, RS/M-162, RS/M-163, RS/M-164	7
Dhorola	Jalpaiguri	RS/D-274, RS/D-275, RS/D-276, RS/D-277, RS/D-283, RS/D-284, RS/D-285, RS/D-286, RS/D-287, RS/D-289, RS/D-374, RS/D-375, RS/D-376, RS/D-377, RS/D-378, RS/D-379, RS/D-380	17
Torsha	Cooch Behar	RS/T-30, RS/T-31, RS/T-32, RS/T-33, RS/T-34, RS/T-57, RS/T-58, RS/T-59, RS/T-74, RS/T-75, RS/T-76, RS/T-85, RS/T-86, RS/T-182, RS/T-183, RS/T-184	16
Raidak	Cooch Behar	RS/R-88, RS/T-89, RS/T-115, RS/T-116, RS/T-118, RS/T-119	6

Table 3. Fungal isolates obtained from agricultural soil

Name of the Plant	Geographical location	Isolated fungi	Total
Tea	Darjeeling	RHS/T-267, RHS/T -268, RHS/T -269, RHS/T -270, RHS/T -272, RHS/T-273	06
Tea	Jalpaiguri	RHS/T-70, RHS/T-71, RHS/T-72, RHS/T-73, RHS/T-90, RHS/T-90, RHS/T-91, RHS/T-99, RHS/T-100, RHS/T-225, RHS/T-227	11
Rubber	Darjeeling	RHS/R-06, RHS/R-07, RHS/R-08, RHS/R-09, RHS/R-10, RHS/R-10, RHS/R-12	07
Mandarin	Darjeeling	RHS/M-01, RHS/M-02, RHS/M-03, RHS/M-04, RHS/M-05, RHS/M-06, RHS/M-07, RHS/M-08, RHS/M-09, RHS/M-10, RHS/M-11, RHS/M-12, RHS/M-13, RHS/M-14, RHS/M-15, RHS/M-16,	16
Large cardamom	Darjeeling	RHS/LC-21, RHS/LC-22, RHS/LC-23, RHS/LC-25, RHS/LC-26, RHS/LC-27, RHS/LC-28	07
Paddy	Darjeeling	RHS/P-43, RHS/P-44, RHS/P-45, RHS/P-46, RHS/P-47, RHS/P-48, RHS/P-49, RHS/P-50, RHS/P-51, RHS/P-52, RHS/P-53, RHS/P-54, RHS/P-65, RHS/P-66, RHS/P-67, RHS/P-68, RHS/P-69, RHS/P-120, RHS/P-121, RHS/P-122, RHS/P-123, RHS/P-124, RHS/P-125, RHS/P-12, RHS/P-127, RHS/P-128, RHS/P-129, RHS/P-130, RHS/P-131, RHS/P-132, RHS/P-133, RHS/P-134, RHS/P-135, RHS/P-136, RHS/P-137, RHS/P-138, RHS/P-139, RHS/P-221, RHS/P-222, RHS/P-223	42
Paddy	Cooch Behar	RHS/P-35, RHS/P-36, RHS/P-37, RHS/P-38, RHS/P-105, RHS/P-106, RHS/P-107, RHS/P-114, RHS/P-117, RHS/P-180, RHS/P-181, RHS/P-196, RHS/P-197, RHS/P-198, RHS/P-199, RHS/P-200, RHS/P-201, RHS/P-202, RHS/P-203, RHS/P-204, RHS/P-205, RHS/P-206, RHS/P-207, RHS/P-208, RHS/P-209, RHS/P-210, RHS/P-211	31
Bamboo	Cooch Behar	RHS/B-218, RHS/B-219, RHS/B-220	03
Bamboo	Darjeeling	RHS/B-241, RHS/B-242, RHS/B-243, RHS/B-244, RHS/B-245, RHS/B-246, RHS/B-247, RHS/B-290, RHS/B-291, RHS/B-292, RHS/B-293, RHS/B-294, RHS/B-295, RHS/B-296, RHS/B-297, RHS/B-298, RHS/B-299, RHS/B-300, RHS/B-301, RHS/B-302, RHS/B-303, RHS/B-304, RHS/B-305, RHS/B-306, RHS/B-307, RHS/B-331, RHS/B-332, RHS/B-343, RHS/B-344, RHS/B-345, RHS/B-346, RHS/B-347, RHS/B-348, RHS/B-350, RHS/B-351	35
Bamboo	Jalpaiguri	RHS/B-247, RHS/B-248, RHS/B-249, RHS/B-250, RHS/B-251, RHS/B-252, RHS/B-253, RHS/B-254, RHS/B-368, RHS/B-369, RHS/B-370, RHS/B-371, RHS/B-372, RHS/B-373	14
Cryptomeria	Darjeeling	RHS/C-308, RHS/C-309, RHS/C-310, RHS/C-333, RHS/C-334, RHS/C-335, RHS/C-336, RHS/C-337, RHS/C-338, RHS/C-339, RHS/C-340, RHS/C-341, RHS/C-342	13

Table 4.Fungal isolates showing phosphate solubilizing activity

Soil type	Isolate code	Total no. of isolates
Forest soil	FS/L04, FS/L-13, , FS/L-17,FS/L-18,FS/L-24, FS/L-40, FS/L-41, FS/L-42, FS/S-63, FS/S-64, FS/S-108, FS/S-109, FS/S110, FS/S-112, FS/S-113, FS/C-140, FS/C143, FS/C-160, FS/S-165, FS/S-173, FS/S-177, FS/S-262, FS/S-278, FS/G-226,	23
Rhizosphere soil	RHS/R-12, RHS/P-43, RHS/P-45, RHS/P-46, RHS/P-47, RHS/P-48, RHS/P-49, RHS/P-50, RHS/P-50, RHS/P-51, RHS/P-52, RHS/P-54, RHS/P-65, RHS/P-82, RHS/P-120, RHS/P-112, RHS/P-125, RHS/P-127, RHS/P-130,RHS/T-99, RHS/T-190, RHS/T-191, RHS/P-37, RHS/P-38, RHS/P-105, RHS/P-106, RHS/P-107, RHS/P-114, RHS/P-117, RHS/P-198, RHS/P-200, RHS/P-201, RHS/P-202, RHS/P-205, RHS/P-209, RHS/B-220	37
Riverine soil	RS/P05, RS/P/14 RS/M-60, RS/M-61, RS/D-288, RS/T-57, RS/T-58, RS/T-59, RS/R-115, RS/T-182, RS/T-183,	10

Table 5.Evaluation of fungal isolates for phosphate solubilization potential in liquid medium amended with 0.5% tricalcium phosphate (TCP) and 0.25% (w/v) Rock phosphate (RP-140)

Isolates	TCP	RP	Isolates	TCP	RP	Isolates	TCP	RP
RHS/R-12	810	385	RHS/P-200	838	345	FS/S110	842	367
RHS/P-37	807	345	RHS/P-201	836	342	FS/S-112	842	354
RHS/P-38	799	288	RHS/P-202	829	350	FS/S-113	848	360
RHS/P-43	812	350	RHS/P-205	842	340	FS/C-140	824	344
RHS/P-45	842	287	RHS/P-209	827	331	FS/C143	821	345
RHS/P-48	841	342	RHS/P-114	838	335	FS/C-160	824	346
RHS/P-46	829	360	RHS/B-220	837	344	FS/S-165	830	352
RHS/P-47	811	348	RHS/P-105	807	349	FS/S-173	802	343
RHS/P-49	849	350	RHS/P-106	813	344	FS/S-177	843	341
RHS/P -50	830	342	RHS/P-107	807	355	FS/G-226	847	352
RHS/P-51	849	374	RHS/P-117	837	360	FS/S-262	795	360
RHS/P -52	830	351	FS/L04	856	366	FS/S-278	829	339
RHS/P -54	839	350	FS/L-13	817	381	RS/P05	854	370
RHS/P-65	851	340	FS/L-17	820	379	RS/P/14	852	360
RHS/P-82	838	350	FS/L-18	821	376	RS/T-57	809	352
RHS/T-99	832	341	FS/S-24	810	338	RS/T-58	802	354
RHS/P-112	797	355	FS/L-40	847	370	RS/T-59	830	350
RHS/P-120	808	355	FS/L-41	843	214	RS/P -60	840	340
RHS/P-125	819	342	FS/L-42	830	360	RS/P -61	847	343
RHS/P-127	820	345	FS/S -63	839	332	RS/R-115	836	338
RHS/P-130	839	332	FS/S-64	842	211	RS/T-182	810	309
RHS/T-190	825	350	FS/S-108	808	350	RS/T-183	850	317
RHS/T-191	827	351	FS/S-109	802	355	RS/D-288	830	350
RHS/P-198	841	346						

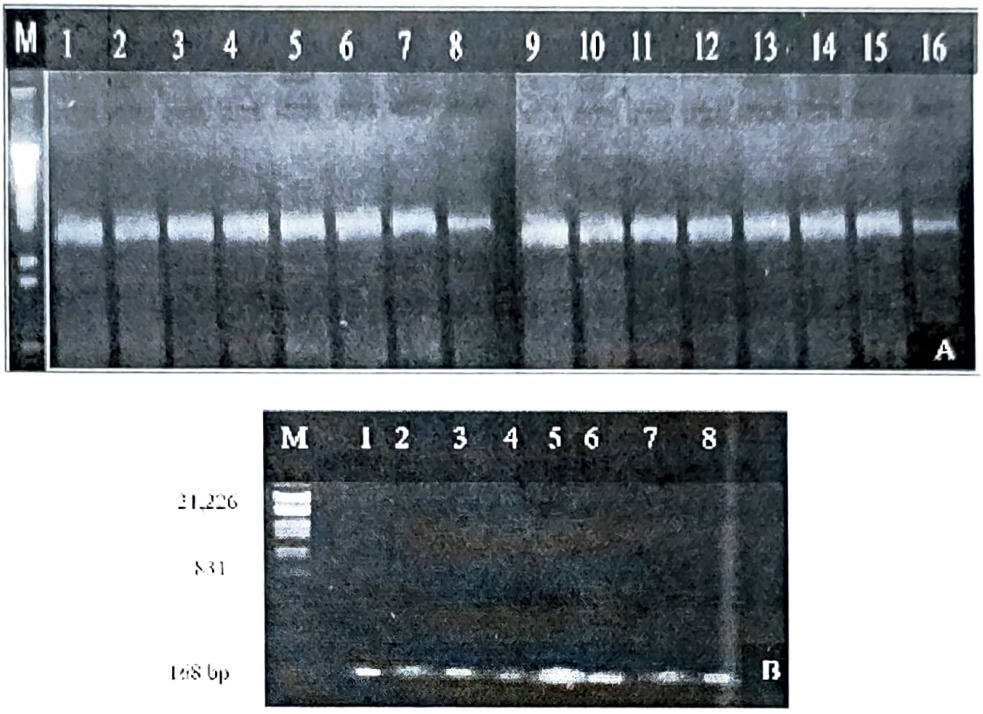

Fig 3. (A) Agarose gel electrophoresis of genomic DNA of 1-RHS/P-82, 2-FS/S-108, 3-FS/S-109, 4-FS/S110, 5-FS/S-112, 6-FS/S-113, 7-RHS/P-120, 8-RHS/P-112,9-RHS/P-125, 10-RHS/P-127, 11-RHS/P-130, 12-FS/C-140, 13-FS/C143, 14-FS/C-160, 16-FS/S-165. (B) ITS-PCR finger prints obtained with the primer HCHITSF-1 and HCHITSF-2 of *A. niger* (lanes 1-3), *A. melleus* (lanes 4-7) and *A.clavatus* (lane 8). M - DNA Ladder Eco R1 HindII double digest

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Diversity of Trees in the Darjeeling Foothill Region of Eastern Himalaya

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Abstract

Phytosociological investigation using 20 x 20 m quadrats has been made on seven major forest types of Darjiling foothill region within an altitudinal range of 135 m to 1200 m leading to the record of 215 species of trees, 11 species of liana and three species of palms. Analysis of data revealed that the East Himalayan Sal forest showed highest species diversity but have moderate species richness and concentration of dominance. High species diversity and species richness were recorded from Semi Evergreen Mixed forest, Wet Mixed Deciduous Forest & Lower Bhabar Sal Forest with correspondingly low concentration of dominance. Bamboo brake had the lowest species diversity with high concentration of dominance. Computation of similarity index showed a poor relation between different forest types and reflected the developing nature of vegetation. Also, these forests have a high regeneration status with a good species composition. It has also been predicted that the anthropogenic interferences may cause the abrupt change in the structure of these vegetation types.

Keywords: Concentration of dominance, Darjiling forests, Phytosociology, Regeneration potential, Species diversity, Species richness, Tree diversity.

Introduction

Eastern Himalaya is well known for its extremely rich biodiversity and has attracted the attention of many plant and animal scientists from different corners of the world at least during the last three centuries (Das 1995). The Himalayan region has favoured the development of extremely rich vegetation and it ranges from tropical to subalpine and evergreen forests to coniferous stands (Champion & Seth 1968, Bhujel 1996). Various climatic factors, soil characteristics, diversified landforms, aspects and altitudinal variation in the region played important roles in determining the species composition and consequently the forest structure. Classification of Indian forests by Champion & Seth (1968) has undergone tremendous change with time and much is needed to understand the dynamics of forest and their community composition (Rai & Das 2003). Tropical and Subtropical regions are more diverse with respect to the species composition with great number of tree species occurring in this belt (Grierson & Long 1983; Spur & Burnes 1980). At the same time, future composition of forest is very much dependent on the regenerating power of the existing population which is determined by the differences in micro-climatic condition, inter-specific competition and available space (Pandey *et al* 2002). However, survivability of seedlings and saplings depends on the cumulative interaction of different components of the ecosystems and the age of the community.

The present investigation was carried out to find out the vegetation composition of different forest types as recognized by Champion & Seth (1968), similarity between different forests, their regeneration power, possible future compositional change, to assess the pattern of forest

growth and overall biological richness.

Study Area

Study was carried out in different reserve forests, national parks and wildlife sanctuaries along the low altitude hills (foothills) of Darjiling, Kurseong and Kalimpong sub-divisions in the Darjiling District of West Bengal, India. Entire horizontal stretch of Darjiling hills extending from foothills up to 1200 m was selected for the study. However, the rolling plains of Terai and Duars of the district have been excluded in the present study. The region is well represented by seven forest types as recognized by Champion and Seth (1968), under tropical and subtropical vegetation and is categorized under *Group 2B* and *Group 3C*. The overall climate of the area is subtropical with relatively warm days except a brief cold spell during December to February. The average day temperature ranges from 5.6 – 23° C. The area receives heavy rainfall due to monsoon for four months, June – September with an average of 325.5 cm per annum of which only 0.3 cm is received during January and a maximum of 149.4 cm during July. The mean Relative Humidity of the area is 71.4%.

Methods

The present study was carried during January 2001 to March 2003. Seven forest types were identified from the foothill region of the Darjiling part of Eastern Himalaya based on the phenological conditions and association of dominant species. These forest types are:

Type I: **Eastern Himalayan Sal Forest:** dominated by sal, distributed along the foothills, from Panighata to Teesta Valley.

Type II: **Bamboo Brakes:** dominated by *Dendrocalamus hamiltonii* which are sporadic in occurrence and invading natural forests as it is found at places in Balasun Valley.

Type III: **Riverine Forests:** along the low lying river valleys of Balasun, Nandi and Teesta.

Type IV: **Semi-evergreen Mixed Forests:** occurring along the steep slopes in the Mahananda Wildlife Sanctuary and Najoek of Kalimpong sub-division.

Type V: **Wet Mixed Deciduous Forests:** occurring in Rung Dung valley of Darjiling sub-division and Shivkhola of Kurseong sub-division.

Type VI: **Lower Bhabar Sal Forests:** occurring along the lower valley of river Teesta.

Type VII: **Upper Bhabar Sal Forests:** distributed along the upper reaches of river Teesta, Relli and Najoek in Kalimpong sub-division.

Random samplings were made taking at least 6 quadrates for each forest type to get a representative picture. Modification of methods as worked out by Mishra 1966; Malhotra 1973; Das & Lahiri, 1997 was adopted with 20 x 20 m as the size of the quadrat so that variability / homogeneity of species against small areas are taken care of. The girth at breast height (GBH) above 15 cm are treated as trees; 5 – 15 cm as saplings and below 5 cm as seedlings. Lianas with girth exceeding 10 cm are also recognized in the present analysis as trees.

Data compilation and analysis was carried out as per the standard procedures (Mishra, 1966) to determine the Relative Density (RD), Relative Frequency (RF), Relative Dominance (RDm) and Importance Value Index (IVI) for different species of trees. Species Diversity using Shanon-Weaver Index (Shannon & Weaver 1963), Concentration of Dominance (Simpson 1949) and Species Richness by Menhinick's Index (Menhinick 1964) for these forest types were determined. Similarity Index (community coefficient) among different types was calculated as per Sorenson (1948) and Kadir (2001). To asses the uniformity of vegetation, frequency distribution pattern of Raunkiaer (1934) was followed.

The entire tree stand was grouped according to GBH with an interval of 25 cm to understand the dynamics of the forest growth. Number of seedlings and saplings calculated for five dominant species in each forest type to predict the future compositional change of vegetation.

Results and Discussion

Arboreal Diversity

A total of 215 species including 11 lianas and 3 palms having over 15 cm GBH have been recorded from the study area. Highest number of 96 species has been recorded from Type-IV (*Semi-evergreen Mixed Forests*) and with minimum number of 28 species from Type-VII (*Upper Bhabar Sal Forests*). Other forest types also showed high diversity for matured trees. Type–I (*Eastern Himalayan Sal Forest*) 66 species; Type – II (*Bamboo Breaks*) 54 species; Type – III (*Riverine Forests*) 42 species; Type – V (*Wet Mixed Deciduous Forests*) 42 species and Type – VI (*Lower Bhabar Sal Forests*) 63 species [Tables 1 – 7].

Girth Class Distribution Pattern

Girth class distribution pattern of different forest types shows relatively higher number of population in lower girth class for all the forest types (Figure 1). The middle canopy species are relatively lesser in number and shall not affect the interpretative quality. Thus, suggesting that either these forests are on the seral stage or the secondary forests coming up after the elimination of the primary structure. The proportion of different age classes across a landscape and over time is one of the fundamental characteristics of the vegetation mosaic (Spies & Turner 1999). There is a relatively higher number of species in seedling and sapling class, which is indicative of the disturbed ecosystem and is in confirmation with the observation by Pandey and Singh (1985). Moreover, higher number of seedlings and saplings along with the good number of species in these forests are also indicative of its rapidly developing vegetation rather than climax. Distribution pattern among different species in the same interval of the GBH shows a gradual decreasing pattern with increase in the girth class for all the forest types. Sites I, II & VI are well marked with tree-species of lower girth class, which shows an excellent adaptation of these species in this area. However, Sites I, III, IV & VI showed more or less stable population in higher girth class and hence is less disturbed as is expected for a protected area. Site II shows much skewed pattern with a much less number of population in the middle girth class indicating a heavy interference of forest by different factors. With lower population in higher girth class and low number of seedling and sapling with lesser number of species occurrence in Site II & V is indicative of matured forest and also appears to be disturbed.

Table 1. Phytosociological attributes in Eastern Himalayan Sal Forest (Type I)

Sl No.	Names of Plants	Density $400m^{-2}$	TBA $400m^{-2}$ (cm^2)	RF	RD	RDm	IVI
1	*Shorea robusta*	106.67	33.99	13.043	46.57	66.37	125.983
2	*Pinus roxburghii*	10.48	8.413	3.106	4.574	10.49	18.17
3	*Schima wallichii*	5.71	123.26	3.727	2.495	5.243	11.465
4	*Terminalia bellirica*	7.14	701.826	4.348	3.119	1.688	9.155
5	*Phoenix loureiri*	10	1842.87	2.484	4.366	0.666	7.516
6	*Lagerstroemia parviflora*	5.24	9.722	4.969	2.287	0.181	7.437
7	*Syzygium cumini*	4.76	157.05	4.969	2.079	0.209	7.257
8	*Sterculia villosa*	7.14	24.754	3.106	3.119	1.017	7.242
9	*Bauhinia purpurea*	6.19	33.654	1.863	2.703	2.02	6.586
10	*Grewia optiva*	5.71	287.445	3.106	2.495	0.425	6.026
11	*Mallotus philippensis*	4.76	21.781	3.106	2.079	0.392	5.577
12	*Terminalia alata*	3.33	20.485	2.484	1.455	1.194	5.133
13	*Aporosa octandra*	4.76	45.81	1.863	2.079	0.849	4.791
14	*Bridelia retusa*	2.86	62.86	2.484	1.247	0.066	3.797
15	*Michelia champaca*	0.48	1421.86	0.621	0.208	2.406	3.235
16	*Terminalia chebula*	2.38	45.806	1.863	1.04	0.041	2.944
17	*Stereospermum colais*	1.43	113.113	1.863	0.624	0.413	2.9
18	*Actinodaphne sikkimensis*	1.43	154.152	1.863	0.624	0.2	2.687
19	*Phyllanthus emblica*	1.43	51.191	1.863	0.624	0.072	2.559
20	*Heynea trijuga*	2.38	8.413	1.242	1.04	0.219	2.501
21	*Mucuna macrocarpa*	1.9	65.737	1.242	0.832	0.06	2.134
22	*Dillenia pentagyna*	1.43	26.717	1.242	0.624	0.189	2.055
23	*Lannea coromendalica*	1.43	116.572	1.242	0.624	0.173	2.039
24	*Tectona grandis*	1.9	8.413	0.621	0.832	0.571	2.024
25	*Anthocephalus cadamba*	0.48	143.738	0.621	0.208	0.996	1.825
26	*Castanopsis hystrix*	0.95	6.319	1.242	0.416	0.167	1.825
27	*Litsea monopetala*	0.95	130.164	1.242	0.416	0.121	1.779
28	*Wrightia arborea*	0.95	46.909	1.242	0.416	0.104	1.762
29	*Grewia disperma*	0.95	65.568	1.242	0.416	0.051	1.709
30	*Acacia pennata*	0.95	80.993	1.242	0.416	0.037	1.695
31	*Bauhinia vahlii*	0.95	33.654	1.242	0.416	0.024	1.682
32	*Careya arborea*	0.48	51.191	0.621	0.208	0.769	1.598
33	*Dalbergia stipulacea*	1.43	117.264	0.621	0.624	0.107	1.352
34	*Archidendron clypearia*	1.43	30.363	0.621	0.624	0.04	1.285
35	*Oroxylum indicum*	0.95	106.832	0.621	0.416	0.214	1.251
36	*Aglaia chittagonga*	0.48	112.066	0.621	0.208	0.379	1.208
37	*Syzygium balsameum*	0.48	23.371	0.621	0.208	0.291	1.12
38	*Myrsine semiserrata*	0.95	72.43	0.621	0.416	0.069	1.106
39	*Dillenia indica*	0.95	4450.685	0.621	0.416	0.029	1.066
40	*Quercus sp*	0.48	45.806	0.621	0.208	0.236	1.065
41	*Sarcosperma arboreum*	0.48	27.951	0.621	0.208	0.179	1.008
42	*Syzygium sp*	0.48	63.568	0.621	0.208	0.171	1
43	*Ficus oligodon*	0.48	198.182	0.621	0.208	0.078	0.907
44	*Gmelina arborea*	0.48	48.461	0.621	0.208	0.07	0.899
45	*Ixora undulata*	0.48	58.696	0.621	0.208	0.063	0.892
46	*Casearia vareca*	0.48	44.547	0.621	0.208	0.061	0.89
47	*Pterospermum acerifolium*	0.48	881.890	0.621	0.208	0.043	0.872
48	*Buchanania lanazan*	0.48	79.123	0.621	0.208	0.034	0.863
49	*Cordia obliqua*	0.48	436.15	0.621	0.208	0.028	0.857
50	*Holmskoildia sanguinea*	0.48	330.403	0.621	0.208	0.028	0.857

Table 1 (contd.). Phytosociological attributes in Eastern Himalayan Sal Forest (Type I)

Sl No	Names of Plants	Density $400m^{-2}$	TBA $400m^{-2}$ (cm^2)	RF	RD	RDm	IVI
51	*Persea sp*	0.48	808.034	0.621	0.208	0.026	0.855
52	*Terminalia arjuna*	0.48	547.987	0.621	0.208	0.026	0.855
53	*Bridelia sikkimensis*	0.48	125.363	0.621	0.208	0.025	0.854
54	*Casaeria sp*	0.48	254.571	0.621	0.208	0.025	0.854
55	*Millettia extensa*	0.48	538.457	0.621	0.208	0.025	0.854
56	*Bassia butyracea*	0.48	38.727	0.621	0.208	0.018	0.847
57	*Holarrhena pubescens*	0.48	33.654	0.621	0.208	0.018	0.847
58	*Syzygium formosum*	0.48	316.493	0.621	0.208	0.018	0.847
59	*Litsea glutinosa*	0.48	263.816	0.621	0.208	0.013	0.842
60	*Wrightia tinctoria*	0.48	315.564	0.621	0.208	0.009	0.838
61	*Acacia sp*	0.48	48.461	0.621	0.208	0.005	0.834
62	*Antidesma acidum*	0.48	208.101	0.621	0.208	0.005	0.834
63	*Dalbergia sp*	0.48	15.114	0.621	0.208	0.005	0.834
64	*Dysoxylum thyroideum*	0.48	95.819	0.621	0.208	0.005	0.834
65	*Zizyphus xylocarpa*	0.48	16.49	0.621	0.208	0.005	0.834
66	*Glochidion gamblei*	0.48	9.573	0.621	0.208	0.003	0.832
	Total	229.05	17014.67				

D: Density $400m^{-2}$; RF: Relative Frequency; RD: Relative Density; RDm: Relative Dominance; IVI: Importance Value Index

Table 2. Phytosociological attributes in Bamboo Brake forest (Type II)

Sl No.	Names of Plants	Density $400m^{-2}$	TBA $400m^{-2}$ (cm^2)	RF	RD	RDm	IVI
1	*Dendrocalamus hamiltonii*	814.21	18.226	14.286	89.994	32.881	137.161
2	*Schima wallichii*	6.84	125.771	4.511	0.756	19.644	24.911
3	*Bombax ceiba*	8.95	285.991	6.767	0.989	5.102	12.858
4	*Albizia odoratissima*	3.68	1092.283	3.008	0.407	7.909	11.324
5	*Stereospermum colais*	2.11	532.069	3.008	0.233	4.838	8.079
6	*Heteropanax fragrans*	8.42	129.608	5.263	0.931	1.498	7.692
7	*Terminalia alata*	6.84	78.616	4.511	0.756	2.188	7.455
8	*Sterculia villosa*	5.26	42.321	5.263	0.582	1.315	7.16
9	*Albizia chinensis*	4.21	546.782	3.008	0.465	2.367	5.84
10	*Castanopsis indica*	0.53	290.154	0.752	0.058	4.799	5.609
11	*Bauhinia purpurea*	2.63	42.321	3.759	0.291	0.407	4.457
12	*Terminalia bellirica*	1.05	45.007	1.504	0.116	1.95	3.57
13	*Lagerstroemia parviflora*	2.11	109.053	3.008	0.233	0.103	3.344
14	*Bischofia javanica*	2.11	114.633	0.752	0.233	2.262	3.247
15	*Alstonia scholaris*	1.05	10.58	1.504	0.116	1.101	2.721
16	*Gynocardia odorata*	1.05	284.715	1.504	0.116	1.085	2.705
17	*Mallotus philippensis*	2.11	4639.174	1.504	0.233	0.656	2.393
18	*Macaranga pustulata*	1.05	62.572	1.504	0.116	0.761	2.381
19	*Engelhardtia spicata*	0.53	123.76	0.752	0.058	1.447	2.257
20	*Castanopsis tribuloides*	1.05	73.07	1.504	0.116	0.589	2.209
21	*Trema orientale*	0.53	20.547	0.752	0.058	1.37	2.18
22	*Callicarpa vestita*	1.58	14.92	1.504	0.175	0.338	2.017
23	*Albizia procera*	3.16	455.652	0.752	0.349	0.781	1.882
24	*Firmiana colorata*	1.58	1399.233	1.504	0.175	0.198	1.877
25	*Crateva religiosa*	1.05	753.22	1.504	0.116	0.256	1.876
26	*Callicarpa arborea*	1.05	63.757	1.504	0.116	0.237	1.857
27	*Dalbergia stipulacea*	1.05	10.58	1.504	0.116	0.151	1.771
28	*Cordia obliqua*	1.05	10.58	1.504	0.116	0.129	1.749
29	*Grewia eriocarpa*	1.05	56.311	1.504	0.116	0.116	1.736
30	*Drymicarpus racemosus*	1.05	524.32	1.504	0.116	0.031	1.651

Table 2 (contd.). Phytosociological attributes in Bamboo Brake forest (Type II)

Sl No.	Names of Plants	Density $400m^{-2}$	TBA $400m^{-2}$ (cm^2)	RF	RD	RDm	IVI
31	*Erythrina stricta*	0.53	90.534	0.752	0.058	0.779	1.589
32	*Pandanus nepalensis*	2.11	158.868	0.752	0.233	0.349	1.334
33	*Duabanga grandiflora*	0.53	24.777	0.752	0.058	0.471	1.281
34	*Pentapanax fragrans*	0.53	139.031	0.752	0.058	0.346	1.156
35	*Tetrameles nudiflora*	0.53	367.828	0.752	0.058	0.222	1.032
36	*Pterospermum acerifolium*	0.53	158.631	0.752	0.058	0.216	1.026
37	*Rhus semialata*	1.05	83.691	0.752	0.116	0.135	1.003
38	*Kydia calycina*	0.53	53.562	0.752	0.058	0.164	0.974
39	*Wrightia arborea*	1.05	84.239	0.752	0.116	0.091	0.959
40	*Macaranga indica*	0.53	334.764	0.752	0.058	0.144	0.954
41	*Bassia butyracea*	0.53	69.474	0.752	0.058	0.134	0.944
42	*Nayariophyton zizyphifolium*	0.53	208.339	0.752	0.058	0.087	0.897
43	*Phyllanthus emblica*	0.53	65.134	0.752	0.058	0.072	0.882
44	*Ostodes paniculatus*	0.53	9.299	0.752	0.058	0.055	0.865
45	*Bridelia sikkimensis*	0.53	1460.748	0.752	0.058	0.047	0.857
46	*Bauhinia vahlii*	0.53	127.107	0.752	0.058	0.044	0.854
47	*Bridelia retusa*	0.53	1169.265	0.752	0.058	0.044	0.854
48	*Talauma hodgsonii*	0.53	20.003	0.752	0.058	0.021	0.831
49	*Acrocarpus fraxinifolius*	0.53	162.696	0.752	0.058	0.019	0.829
50	*Careya arborea*	0.53	942.734	0.752	0.058	0.011	0.821
51	*Ficus semicordata*	0.53	8.266	0.752	0.058	0.011	0.821
52	*Glochidion gamblei*	0.53	214.249	0.752	0.058	0.011	0.821
53	*Salacia brunoniana*	0.53	1324.221	0.752	0.058	0.01	0.82
54	*Terminalia chebula*	0.53	44.16	0.752	0.058	0.009	0.819
	Total	904.74	19277.444				

D: Density $400m^{-2}$; RF: Relative Frequency; RD: Relative Density; RDm: Relative Dominance; IVI: Importance Value Index

Table 3. Phytosociological attributes in Riverine Forest (Type III)

Sl No.	Names of Plants	Density $400m^{-2}$	TBA $400m^{-2}$ (cm^2)	RF	RD	RDm	IVI
1	*Sterculia villosa*	14.44	628.06	5.825	7.182	23.486	36.493
2	*Terminalia bellirica*	16.67	155.31	6.796	8.287	10.47	25.553
3	*Terminalia alata*	8.89	1133.04	2.913	4.42	12.2	19.533
4	*Syzygium cumini*	12.22	68.78	4.854	6.077	6.421	17.352
5	*Holarrhena pubescens*	18.89	469.26	4.854	9.392	1.541	15.787
6	*Dillenia pentagyna*	8.89	738.48	3.883	4.42	6.565	14.868
7	*Aglaia spectabilis*	7.78	1392.7	3.883	3.867	3.83	11.58
8	*Careya arborea*	6.67	41.71	3.883	3.315	4.035	11.233
9	*Lagerstroemia parviflora*	5.56	2464.81	4.854	2.762	3.197	10.813
10	*Syzygium claviflorum*	11.11	31.5	1.942	5.525	1.931	9.398
11	*Aphanamixis polystachya*	7.78	176.68	2.913	3.867	1.586	8.366
12	*Acacia catechu*	7.78	1699.6	1.942	3.867	2.123	7.932
13	*Pterygota alata*	3.33	2452.82	2.913	1.657	2.726	7.296
14	*Lannea coromendalica*	2.22	68.4	1.942	1.105	2.922	5.969
15	*Strebulus asper*	4.44	86.99	1.942	2.21	1.516	5.668
16	*Choerospondias axillaris*	2.22	660.4	1.942	1.105	2.38	5.427
17	*Firmiana colorata*	2.22	100.86	1.942	1.105	2.369	5.416
18	*Nayariophyton zizyphifolium*	2.22	187.8	1.942	1.105	2.04	5.087
19	*Wrightia arborea*	4.44	1324.3	1.942	2.21	0.652	4.804
20	*Casearia vareca*	3.33	3025.57	2.913	1.657	0.06	4.63
21	*Wrightia tinctoria*	3.33	3116.66	1.942	1.657	0.518	4.117
22	*Stereospermum colais*	2.22	125.99	1.942	1.105	0.877	3.924
23	*Albizia lucidor*	3.33	28.27	1.942	1.657	0.1	3.699
24	*Acacia pennata*	2.22	28.27	1.942	1.105	0.15	3.197
25	*Grewia optiva*	2.22	2112.15	1.942	1.105	0.084	3.131

Table 3 (contd.). Phytosociological attributes in Riverine Forest (Type III)

Sl No	Names of Plants	Density $400m^{-2}$	TBA $400m^{-2}$ (cm^2)	RF	RD	RDm	IVI
26	*Trewia nodiflora*	2.22	179.08	0.971	1.105	1.018	3.094
27	*Litsea cubeba*	1.11	34.9	0.971	0.552	1.505	3.028
28	*Pterospermum acerifolium*	1.11	2521.53	0.971	0.552	1.217	2.74
29	*Persea sp*	2.22	1882.1	0.971	1.105	0.173	2.249
30	*Sapindus rarak*	2.22	143.31	0.971	1.105	0.138	2.214
31	*Ventillago denticulata*	2.22	42.23	0.971	1.105	0.042	2.118
32	*Bauhinia variegata*	1.11	3741.68	0.971	0.552	0.357	1.88
33	*Heynea trijuga*	1.11	907.97	0.971	0.552	0.319	1.842
34	*Crateva religiosa*	1.11	784.97	0.971	0.552	0.085	1.608
35	*Litsea glutinosa*	1.11	400	0.971	0.552	0.061	1.584
36	*Hiptage bengalensis*	1.11	1208.94	0.971	0.552	0.049	1.572
37	Unidentified Liana	1.11	3158.4	0.971	0.552	0.033	1.556
38	*Shorea robusta*	1.11	1445.56	0.971	0.552	0.02	1.543
39	*Premna mucronata*	1.11	1053.72	0.971	0.552	0.017	1.54
40	*Combretum decandrum*	1.11	43.01	0.971	0.552	0.015	1.538
41	*Mallotus philippensis*	1.11	337.57	0.971	0.552	0.014	1.537
42	*Millettia pachycarpa*	1.11	357.61	0.971	0.552	0.014	1.537
	Total	201.11	40560.94				

D: Density $400m^{-2}$; RF: Relative Frequency; RD: Relative Density; RDm: Relative Dominance; IVI: Importance Value Index

Table 4. Phytosociological attributes in Semi – evergreen Mixed Forest (Type IV)

Sl No	Names of Plants	Density $400m^{-2}$	TBA $400m^{-2}$ (cm^2)	RF	RD	RDm	IVI
1	*Aglaia chittagonga*	20.67	23.278	5.143	8.611	6.196	19.95
2	*Shorea robusta*	11.33	214.426	2.286	4.722	11.903	18.911
3	*Pterospermum acerifolium*	9.33	152.705	5.143	3.889	7.333	16.365
4	*Schima wallichii*	14.67	437.589	2.857	6.111	5.348	14.316
5	*Michelia champaca*	13.33	725.602	2.857	5.556	5.637	14.05
6	*Terminalia bellirica*	5.33	899.889	2.857	2.222	8.276	13.355
7	*Duabanga grandiflora*	6	536.195	2.857	2.5	4.938	10.295
8	*Tetrameles nudiflora*	0.67	952.796	0.571	0.278	6.22	7.069
9	*Aglaia spectabilis*	6	1177.875	1.143	2.5	2.983	6.626
10	*Albizia lebbek*	4.67	17.433	1.714	1.944	2.877	6.535
11	*Sapindus rarak*	2.67	121.976	1.143	1.111	4.15	6.404
12	*Mallotus philippensis*	6.67	88.011	3.429	2.778	0.132	6.339
13	*Pterygota alata*	3.33	229.424	1.714	1.389	2.883	5.986
14	*Terminalia myriocarpa*	3.33	378.229	1.143	1.389	2.859	5.391
15	*Chukrasia tabularis*	2.67	3457.665	1.143	1.111	2.705	4.959
16	*Dillenia pentagyna*	5.33	141.07	2.286	2.222	0.428	4.936
17	*Combretum decandrum*	5.33	52.018	2.286	2.222	0.316	4.824
18	*Persea odoratissima*	4	349.803	2.286	1.667	0.775	4.728
19	*Dalbergia stipulacea*	6	15.129	1.714	2.5	0.114	4.328
20	*Crateva unilocularis*	4.67	15.129	1.714	1.944	0.487	4.145
21	*Nayariophyton zizyphifolium*	4.67	1340.16	1.143	1.944	0.9	3.987
22	*Rhus insignis*	3.33	22.685	1.143	1.389	1.112	3.644
23	*Lagerstroemia parviflora*	4	1889.835	1.143	1.667	0.618	3.428
24	*Toona ciliata*	1.33	234.999	1.143	0.556	1.55	3.249
25	*Ficus elastica*	2	1480.34	0.571	0.833	1.814	3.218
26	*Syzygium cumini*	3.33	1297.547	1.714	1.389	0.09	3.153
27	*Cinnamomum bejolghota*	1.33	1340.16	1.143	0.556	1.185	2.884
28	*Knema erratica*	2.67	48.581	1.143	1.111	0.576	2.83
29	*Bauhinia purpurea*	3.33	86.476	1.143	1.389	0.204	2.733
30	*Grewia disperma*	4	152.376	0.571	1.667	0.368	2.606
31	*Bombax ceiba*	0.67	234.999	0.571	0.278	1.579	2.428
32	*Castanopsis hystrix*	1.33	41.042	0.571	0.556	1.224	2.351
33	*Bridelia sikkimensis*	2.67	27.722	1.143	1.111	0.096	2.349
34	*Ficus hookeriana*	2.67	117.257	1.143	1.111	0.085	2.339
35	*Litsea monopetala*	0.67	19.919	0.571	0.278	1.412	2.261
36	*Grewia sp*	1.33	1201.258	0.571	0.556	1.048	2.175
37	*Gynocardia odorata*	0.67	544.649	0.571	0.278	1.265	2.114
38	*Callicarpa vestita*	1.33	544.649	1.143	0.556	0.32	2.019
39	*Acacia pennata*	0	141.554	1.143	0.833	0.032	2.008
40	*Alseodaphne owdenii*	1.33	1323.722	0.571	0.556	0.87	1.997
41	*Ficus hispida*	2.67	116.086	0.571	1.111	0.212	1.894
42	*Bridelia retusa*	1.33	46.434	1.143	0.556	0.129	1.828
43	*Holarrhena pubescens*	2.67	286.59	0.571	1.111	0.094	1.776

Table 4 (contd.). Phytosociological attributes in Semi – evergreen Mixed Forest (Type IV)

Sl No.	Names of Plants	Density $400m^{-2}$	TBA $400m^{-2}$ (cm^2)	RF	RD	RDm	IVI
44	*Machilus villosa*	1.33	21.084	1.143	0.556	0.071	1.77
45	*Cedrela toona*	0.67	278.973	0.571	0.278	0.863	1.712
46	*Albizia procera*	1.33	158.359	0.571	0.556	0.49	1.617
47	*Stereospermum colais*	2	182.099	0.571	0.833	0.104	1.508
48	*Pandanus nepalensis*	2	134.164	0.571	0.833	0.073	1.477
49	*Cinnamomum glaucescens*	0.67	1147.617	0.571	0.278	0.612	1.461
50	*Rhus semialata*	2	2769.315	0.571	0.833	0.041	1.445
51	*Castanopsis indica*	2	18.898	0.571	0.833	0.031	1.435
52	*Ficus semicordata*	2	48.999	0.571	0.833	0.029	1.433
53	*Ficus neriifolia*	1.33	51.211	0.571	0.556	0.262	1.389
54	*Alstonia scholaris*	0.67	130.875	0.571	0.278	0.538	1.387
55	*Bauhinia vahlii*	1.33	302.374	0.571	0.556	0.21	1.337
56	*Gordonia excelsa*	1.33	315.442	0.571	0.556	0.166	1.293
57	*Adina cordifolia*	1.33	188.46	0.571	0.556	0.14	1.267
58	*Bassia butyracea*	1.33	225.367	0.571	0.556	0.111	1.238
59	*Premna bracteata*	1.33	32.719	0.571	0.556	0.108	1.235
60	*Persea minutiflora*	1.33	123.572	0.571	0.556	0.082	1.209
61	*Magnolia pterocarpa*	0.67	3091.215	0.571	0.278	0.356	1.205
62	*Oroxylum indicum*	1.33	77.53	0.571	0.556	0.065	1.192
63	*Heynea trijuga*	1.33	15.129	0.571	0.556	0.045	1.172
64	*Colebrookea oppositifolia*	1.33	779.177	0.571	0.556	0.044	1.171
65	*Drypetes lancifolia*	1.33	28.845	0.571	0.556	0.018	1.145
66	*Engelhardtia spicata*	0.67	617.052	0.571	0.278	0.249	1.098
67	*Erythrina stricta*	0.67	20.94	0.571	0.278	0.249	1.098
68	*Salix tetrasperma*	0.67	15.129	0.571	0.278	0.185	1.034
69	*Beilschmiedia dalzellii*	0.67	249.238	0.571	0.278	0.173	1.022
70	*Juglans regia*	0.67	281.508	0.571	0.278	0.138	0.987
71	*Garcinia stipulata*	0.67	70.777	0.571	0.278	0.127	0.976
72	*Morus macroura*	0.67	53.1	0.571	0.278	0.114	0.963
73	*Celtis timorensis*	0.67	57.009	0.571	0.278	0.107	0.956
74	*Cryptocarya amygdalina*	0.67	89.885	0.571	0.278	0.107	0.956
75	*Actinodaphne angustifolia*	0.67	282.81	0.571	0.278	0.098	0.947
76	*Tectona grandis*	0.67	117.683	0.571	0.278	0.098	0.947
77	*Knema tenuinervia*	0.67	1146.674	0.571	0.278	0.086	0.935
78	*Gmelina arborea*	0.67	1262.326	0.571	0.278	0.072	0.921
79	*Ficus cunia*	0.67	486.719	0.571	0.278	0.065	0.914
80	*Horsfieldia kingii*	0.67	29.682	0.571	0.278	0.06	0.909
81	*Saurauja roxburghii*	0.67	405.398	0.571	0.278	0.057	0.906
82	*Litsea hookeri*	0.67	2271.257	0.571	0.278	0.056	0.905
83	*Terminalia alata*	0.67	125.692	0.571	0.278	0.043	0.892
84	*Persea glaucescens*	0.67	532.145	0.571	0.278	0.026	0.875
85	*Wrightia tomentosa*	0.67	1532.885	0.571	0.278	0.02	0.869
86	*Dalbergia sp*	0.67	8.847	0.571	0.278	0.019	0.868
87	*Talauma hodgsonii*	0.67	76.099	0.571	0.278	0.019	0.868
88	*Litsea citrata*	0.67	21.966	0.571	0.278	0.015	0.864
89	*Miliusa roxburghiana*	0.67	41.042	0.571	0.278	0.01	0.859
90	*Helicia nilagirica*	0.67	214.426	0.571	0.278	0.009	0.858
91	*Aporosa roxburghii*	0.67	95.096	0.571	0.278	0.008	0.857
92	*Caryota urens*	0.67	2264.87	0.571	0.278	0.007	0.856
93	*Castanopsis armata*	0.67	1251.944	0.571	0.278	0.007	0.856
94	*Maesa chisia*	0.67	13616.235	0.571	0.278	0.007	0.856
95	*Millettia pachycarpa*	0.67	1696.14	0.571	0.278	0.007	0.856
96	*Sorindeia madagascarensis*	0.67	44.026	0.571	0.278	0.004	0.853
	Total	238	62026.627				

D: Density $400m^{-2}$; RF: Relative Frequency; RD: Relative Density; RDm: Relative Dominance; IVI: Importance Value Index

Table 5. Phytosociological attributes in Wet Mixed Deciduous Forest (Type V)

Sl No.	Names of Plants	Density $400m^{-2}$	TBA $400m^2$ (cm^2)	RF	RD	RDm	IVI
1	*Schima wallichii*	80	108.757	4.348	26.087	17.898	48.333
2	*Bombax ceiba*	6.67	126.687	2.174	2.174	37.924	42.272
3	*Bassia butyracea*	6.67	104.7	4.348	2.174	10.926	17.448
4	*Castanopsis lanceifolia*	6.67	13951.275	2.174	2.174	10.304	14.652
5	*Gynocardia odorata*	6.67	4842.375	2.174	2.174	4.784	9.132
6	*Acacia pennata*	13.33	530.044	4.348	4.348	0.17	8.866
7	*Duabanga grandiflora*	3.33	171.446	2.174	1.087	4.307	7.568
8	*Litsea cubeba*	3.33	157.181	2.174	1.087	3.897	7.158
9	*Litsea glutinosa*	13.33	104.7	2.174	4.348	0.489	7.011
10	*Callicarpa arborea*	6.67	13156.209	4.348	2.174	0.123	6.645
11	*Morus macroura*	10	251.542	2.174	3.261	0.85	6.285
12	*Ostodes paniculata*	3.33	67.008	2.174	1.087	2.624	5.885
13	*Dendrocnide sinuata*	10	218.43	2.174	3.261	0.383	5.818
14	*Morus laevigata*	10	1006.167	2.174	3.261	0.354	5.789
15	*Bridelia retusa*	10	325.704	2.174	3.261	0.201	5.636
16	*Pandanus nepalensis*	6.67	11000.044	2.174	2.174	0.346	4.694
17	*Stereospermum colais*	6.67	817.97	2.174	2.174	0.32	4.668
18	*Grewia disperma*	6.67	126.687	2.174	2.174	0.212	456
19	*Macaranga indica*	6.67	163.593	2.174	2.174	0.205	4.553
20	*Sterculia villosa*	3.33	176.943	2.174	1.087	1.286	4.547
21	*Crateva unilocularis*	6.67	270.78	2.174	2.174	0.171	4.519
22	*Ficus hookeri*	6.67	6108.46	2.174	2.174	0.128	4.476
23	*Talauma hodgsonii*	6.67	94.492	2.174	2.174	0.118	4.466
24	*Engelhardtia spicata*	6.67	973.972	2.174	2.174	0.064	4.412
25	*Dalbergia sericea*	3.33	9953.044	2.174	1.087	0.394	3.655
26	*Lagerstroemia parviflora*	3.33	312.137	2.174	1.087	0.381	3.642
27	*Brassaiopsis hainla*	3.33	261.357	2.174	1.087	0.208	3.469
28	*Trema orientalis*	3.33	138.466	2.174	1.087	0.181	3.442
29	*Pyrularia edulis*	3.33	115.432	2.174	1.087	0.126	3.387
30	*Cinnamomum bejolghota*	3.33	301.361	2.174	1.087	0.098	3.359
31	*Ficus semicordata*	3.33	723.216	2.174	1.087	0.069	333
32	*Mallotus philippensis*	3.33	104.7	2.174	1.087	0.054	3.315
33	*Ailanthus integrifolia*	3.33	58.894	2.174	1.087	0.05	3.311
34	*Ficus elastica*	3.33	6700.8	2.174	1.087	0.05	3.311
35	*Phoebe pallida*	3.33	442.358	2.174	1.087	0.05	3.311
36	*Michelia champaca*	3.33	126.687	2.174	1.087	0.045	3.306
37	*Alnus nepalensis*	3.33	320.644	2.174	1.087	0.041	3.302
38	*Casearia glomerata*	3.33	1904.449	2.174	1.087	0.041	3.302
39	*Myrsine semiserrata*	3.33	3283.392	2.174	1.087	0.041	3.302
40	*Helicia nilagrica*	3.33	408.983	2.174	1.087	0.037	3.298
41	*Cleidion javanicum*	3.33	151.292	2.174	1.087	0.026	3.287
42	*Oroxylum indicum*	3.33	461.727	2.174	1.087	0.023	3.284
	Total	306.67	123469.3				

D: Density $400m^{-2}$; RF: Relative Frequency; RD: Relative Density; RDm: Relative Dominance; IVI: Importance Value Index

Table 6. Phytosociological attributes in Lower Bhabar Sal Forest (Type VI)

Sl No.	Names of Plants	Density $400m^{-2}$	TBA $400m^{-2}$ (cm^2)	RF	RD	RDm	IVI
1	*Shorea robusta*	45.71	82.984	6.742	17.112	34.16	58.014
2	*Lagerstroemia parviflora*	20	258.459	5.618	7.487	2.364	15.469
3	*Aglaia spectabilis*	15.71	137.419	3.371	5.882	5.99	15.243
4	*Tectona grandis*	10	926.595	2.247	3.743	8.803	14.793
5	*Syzygium cumini*	17.14	6733.519	5.618	6.417	0.988	13.023
6	*Dillenia pentagyna*	7.14	161.986	1.124	2.674	6.502	10.3
7	*Mallotus philippensis*	11.43	28.718	3.371	4.278	0.558	8.207
8	*Cedrela toona*	2.86	754.289	2.247	1.07	4.347	7.664
9	*Holarrhena pubescens*	7.14	2424.067	3.371	2.674	1.125	7.17
10	*Spondias axillaris*	1.43	44.871	1.124	0.535	4.985	6.644
11	*Casaeria glomerata*	7.14	97.203	1.124	2.674	2.062	5.86
12	*Ailanthus interigifolia*	1.43	1470.437	1.124	0.535	3.957	5.616
13	*Duabanga grandiflora*	1.43	35.168	1.124	0.535	3.797	5.456
14	*Firmiana colorata*	5.71	701.924	1.124	2.139	1.544	4.807
15	*Schima wallichii*	7.14	64.615	1.124	2.674	0.493	4.291
16	*Dalbergia stipulacea*	4.29	36.346	2.247	1.604	0.29	4.141
17	*Callicarpa vestita*	2.86	75.833	1.124	1.07	1.728	3.922
18	*Oroxylum indicum*	2.86	164.524	2.247	1.07	0.368	3.685
19	*Polyalthia simiarum*	2.86	70.112	1.124	1.07	1.304	3.498
20	*Acacia gageana*	5.71	2212.924	1.124	2.139	0.195	3.458
21	*Eugenia bracteata*	5.71	6461.486	1.124	2.139	0.17	3.433
22	*Bridelia retusa*	2.86	137.419	2.247	1.07	0.114	3.431
23	*Careya arborea*	5.71	1308.451	1.124	2.139	0.083	3.346
24	*Terminalia alata*	1.43	72.411	1.124	0.535	1.625	3.284
25	*Bombax ceiba*	1.43	195.471	1.124	0.535	1.424	3.083
26	*Pterospermum acerifolium*	1.43	656.665	1.124	0.535	1.386	3.045
27	*Syzygium ramosissimum*	4.29	25.24	1.124	1.604	0.168	2.896
28	*Michelia champaca*	4.29	473.954	1.124	1.604	0.117	2.845
29	*Terminalia bellirica*	1.43	217.178	1.124	0.535	1.08	2.739
30	*Mangifera sylvaticum*	1.43	214.541	1.124	0.535	1.063	2.722
31	*Lannea coromendalica*	2.86	431.214	1.124	1.07	0.36	2.554
32	*Grewia optiva*	2.86	382.933	1.124	1.07	0.252	2.446
33	*Engelhardtia spicata*	1.43	287.385	1.124	0.535	0.769	2.428
34	*Ficus altissima*	2.86	306.529	1.124	1.07	0.23	2.424
35	*Maesa chisia*	1.43	25.24	1.124	0.535	0.672	2.331
36	*Litsea monopetala*	1.43	1077.363	1.124	0.535	0.633	2.292
37	*Sapium baccatum*	1.43	54.294	1.124	0.535	0.595	2.254
38	*Nayariophyton zizyphifolium*	1.43	1144.334	1.124	0.535	0.558	2.217
39	*Alstonia Scholaris*	1.43	118.755	1.124	0.535	0.443	2.102
40	*Phoebe attenuate*	1.43	1809.328	1.124	0.535	0.419	2.078
41	*Persea minutiflora*	1.43	66.186	1.124	0.535	0.411	2.07
42	*Gmelina arborea*	1.43	949.479	1.124	0.535	0.279	1.938
43	*Gynocardia odorata*	1.43	312.754	1.124	0.535	0.253	1.912
44	*Adina cordifolia*	1.43	100.961	1.124	0.535	0.152	1.811
45	*Grewia disperma*	1.43	700.106	1.124	0.535	0.128	1.787
46	*Wrightia arborea*	1.43	356.336	1.124	0.535	0.116	1.775
47	*Alangium chinensis*	1.43	1109.782	1.124	0.535	0.095	1.754
48	*Aegle marmelos*	1.43	2358.554	1.124	0.535	0.081	1.74
49	*Elaeocarpus aristatus*	1.43	1012.412	1.124	0.535	0.081	1.74
50	*Semecarpus anacardium*	1.43	167.752	1.124	0.535	0.072	1.731

Table 6 (contd.). Phytosociological attributes in Lower Bhabar Sal Forest (Type VI)

Sl No.	Names of Plants	Density $400m^{-2}$	TBA $400m^{-2}$ (cm^2)	RF	RD	RDm	IVI
51	*Pavetta polyantha*	1.43	122.162	1.124	0.535	0.059	1.718
52	*Dalbergia sissoo*	1.43	1816.539	1.124	0.535	0.045	1.704
53	*Dalbergia volubilis*	1.43	32.42	1.124	0.535	0.041	1.7
54	*Celtis timorensis*	1.43	8483.504	1.124	0.535	0.038	1.697
55	*Litsea glutinosa*	1.43	140.12	1.124	0.535	0.032	1.691
56	*Brassaiopsis hispida*	1.43	95.314	1.124	0.535	0.026	1.685
57	*Zanthoxylum nitidum*	1.43	2139.902	1.124	0.535	0.026	1.685
58	*Trema orientalis*	1.43	1837.934	1.124	0.535	0.024	1.683
59	*Cyathea spinulosa*	1.43	2765.09	1.124	0.535	0.021	1.68
60	*Sorindeia madagascarensis*	1.43	3698.303	1.124	0.535	0.019	1.678
61	*Alstonia neriifolia*	1.43	40.496	1.124	0.535	0.017	1.676
62	*Garuga pinnata*	1.43	197.883	1.124	0.535	0.015	1.674
63	*Lasiococca symphyllifolia*	1.43	44.871	1.124	0.535	0.015	1.674
	Total	26571	60431.045				

D: Density $400m^{-2}$; RF: Relative Frequency; RD: Relative Density; RDm: Relative Dominance; IVI: Importance Value Index

Table 7. Phytosociological attributes in Upper Bhabar Sal Forest (Type VII)

Sl No.	Names of Plants	Density $400m^{-2}$	TBA $400m^{-2}$ (cm^2)	RF	RD	RDm	IVI
1	*Shorea robusta*	94	114.489	10.638	34.815	31.186	76.639
2	*Schima wallichii*	16	391.4	6.383	5.926	30.266	42.575
3	*Terminalia alata*	24	8673.086	6.383	8.889	7.513	22.785
4	*Lagerstroemia parviflora*	26	70.673	6.383	9.63	2.746	18.759
5	*Engelhardtia spicata*	2	883.406	2.128	0.741	10.783	13.652
6	*Mallotus philippensis*	10	1477.683	8.511	3.704	0.803	13.018
7	*Wrightia arborea*	12	704.997	4.255	4.444	1.249	9.948
8	*Terminalia bellirica*	6	221.702	6.383	2.222	0.359	8.964
9	*Anthocephalus cadamba*	2	377.077	2.128	0.741	5.403	8.272
10	*Glochidion gamblei*	14	17310.68	2.128	5.185	0.682	7.995
11	*Dillenia pentagyna*	6	48.267	4.255	2.222	0.414	6.891
12	*Bombax ceiba*	4	160.819	4.255	1.481	1.034	6.77
13	*Grewia disperma*	8	122.185	2.128	2.963	1.477	6.568
14	*Tectona grandis*	8	35.336	2.128	2.963	1.472	6.563
15	*Pandanus nepalensis*	4	156.399	4.255	1.481	0.254	5.99
16	*Entada rheedii*	6	592.942	2.128	2.222	0.09	4.44
17	*Terminalia myriocarpa*	2	339.047	2.128	0.741	1.57	4.439
18	*Albizia procera*	4	257.745	2.128	1.481	0.488	4.097
19	*Ficus altissima*	4	277.036	2.128	1.481	0.152	3.761
20	*Bauhinia vahlii*	2	203.537	2.128	0.741	0.55	3.419
21	*Bridelia sp*	2	60733	2.128	0.741	0.439	3.308
22	*Sterculia villosa*	2	1088.322	2.128	0.741	0.426	3.295
23	*Emblica officinale*	2	684.11	2.128	0.741	0.235	3.104
24	*Mangifera indica*	2	590.587	2.128	0.741	0.173	3.042
25	*Erythrina stricta*	2	191.915	2.128	0.741	0.1	2.969
26	*Albizia julibrissin*	2	2520.652	2.128	0.741	0.071	2.94
27	*Aralia foliolosa*	2	1005.12	2.128	0.741	0.044	2.913
28	*Ficus hookeriana*	2	334.124	2.128	0.741	0.022	2.891
	Total	270	44906.64				

D: Density $400m^{-2}$; RF: Relative Frequency; RD: Relative Density; RDm: Relative Dominance; IVI: Importance Value Index

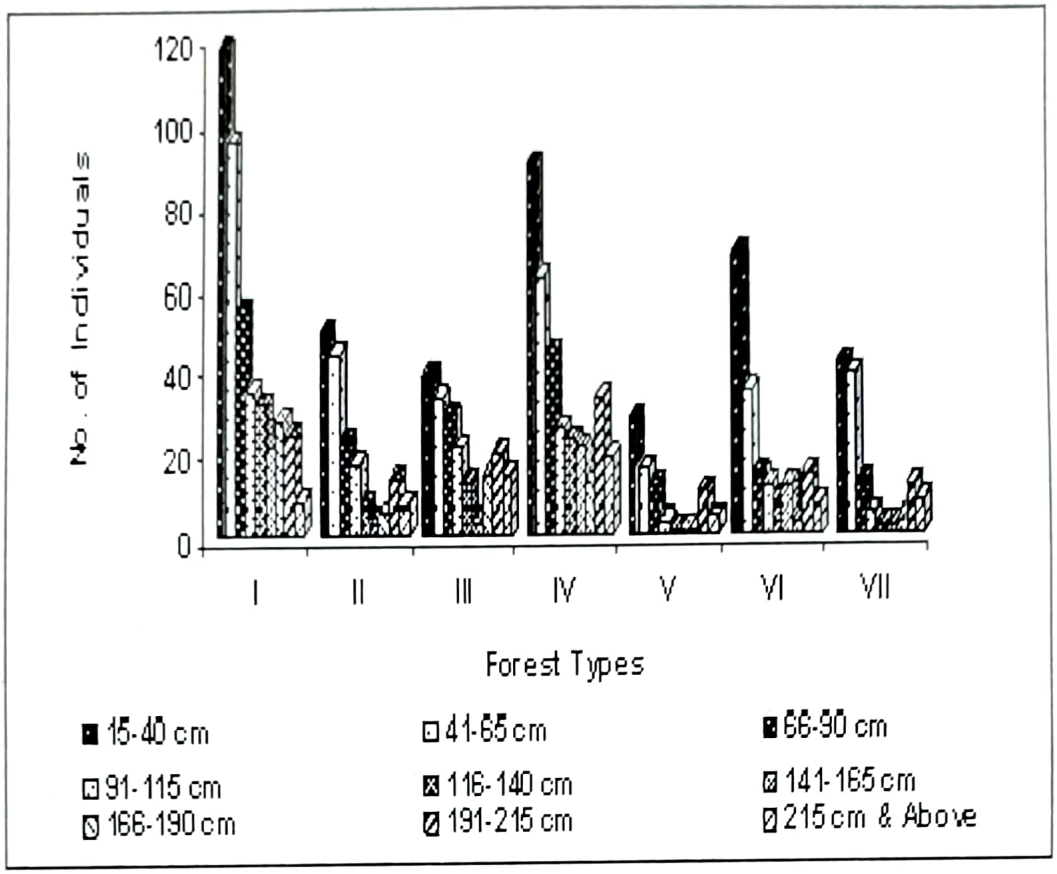

Fig 1. Graph showing girth class distribution in different forest types

Phytosociological Structure

Quantitative analysis of vegetation in 7 different forest types are shown in Tables 1 – 7, with their Density 400m^{-2}, total Basal Area (TBA) 400 m^{-2}, RD, RF, RDm and IVI. *Shorea robusta* is the most predominant species in Types I, VI & VII, whereas Type-II is dominated by *Dendrocalamus hamiltonii*. However, Type-III is quite heterogeneous with *Sterculia villosa*, *Terminalia bellirica*, *Terminalia alata* and *Syzygium cumini*, among the important species, co-habiting the same niche and in Type IV *Shorea robusta* is nearly equally predominant with *Aglaia chittagonga*. This Type is quite heterogeneous as the species like *Pterospermum acerifolium*, *Schima wallichii*, *Michelia champaca* and *Terminalia bellirica* are also highly predominating. On the other hand, Type-V is more pronounced with the presence of *Schima wallichii*, *Bombax ceiba*, *Bassia butyracea* and *Castanopsis lanceifolia*. Considering the total density 400 m^{-2} of forests, Type-II is with most dense (904.74) vegetation, which is followed by Types-V with 306.67 and the Type-III showed the least density of 201.11 only. Remaining Types (VII, VI, IV & I) had a moderate density with 270, 265.71, 238 & 229.05 respectively.

If the RF of different species are considered, except for the Types I, II & VII where only one species is most commonly found, in other Types there are no such plants, instead, the predominant species are nearly equally dispersed in the vegetation. This has also been reflected in the RD values of high IVI plants in these Types. RDm is calculated over the basal area of different species. It is expected that the trees with very high RD values will also score high RDm values. However, this can be interfered by the species with excessively high GBH values.

In Types I, II, VI & VII the species with highest RD and RDm values are same. But, this has been shifted in other Types. In Type III *Holarrhena pubescens* is with highest RD value. But, the highest RDm value has been recorded by *Sterculia villosa* with its much thick trunk in comparison to the dominating species. So, the thick trunk and comparatively better RD and RF values all together made this species most important in this Type.

Sal is not a frequent species in Type IV and is fourth in its RD value. But, this has scored highest RDm value. Also, the species is not at all well distributed in the vegetation. So, the high GBH of its individuals made it to score highest RDm.

The most interesting situation developed in Type- V, where *Schima wallichii* is recorded with highest RD, RF & IVI scores but for RDm *Bombax ceiba* emerged highest with a much better score. This is certainly due to the very large girth of this very fast growing tree species.

But, the situations in these forests are no doubt more congenial for the very aggressive and tolerant species *Shorea robusta*. However, in newly formed habitat plants like *Bombax ceiba*, *Litsea glutinosa* etc. try to dominate. *Tectona grandis* is not a naturally growing plant in the region but perform nicely if planted. In forest enrichment program, sometimes teak is planted in comparatively open areas inside natural forests.

So, all the seven types of vegetation showed a profound degree of heterogeneity in their species composition but some are with one clearly dominating species. It is expected that, in matured vegetation one species will be dominating as in the cases of I, VI & VII. However, in the complete dominance of *Dendrocalamus hamiltonii* in Type II is not a similar situation. Bamboos generally spread in open areas in the forest. Creation of such areas is generally the result of anthropogenic or other biotic activities or due to some sudden natural disaster. *D. hamiltonii* is now spreading nicely in this Type and reintroduced the vegetation of the Type into the developmental phases. However, the co-dominance of a few species in vegetation indicates that the forest is still in its seral stages (Odum 1971). The low number of individuals for the high girth class and much more for small girth and the thin distribution of mature trees in the vegetations of this region are indicative of excessive anthropogenic, biotic and natural interferences to the natural forest ecosystem.

Similarity Index (SI)

Similarity indices among different forest types are given in Table 8. It is interesting to note that no two forest types are same with over 50% as their SI value. The highest value has been recorded between the Types-IV & VI (SI = 40.51%) and the lowest for Types III & V (16.67%). Forest types IV & VI are situated almost in the similar altitude but the nature of dominants created the difference. The clear dominance of *Shorea robusta* in Type VI caused the difference in the selection of co-habiting species of trees in the forest. On the other hand, Type III is a riverine vegetation, which should be extremely different from that of a mixed deciduous vegetation (Type V) situated in higher altitude hill-slope locality. However, this low level of SI values between different forest types of the region is probably mainly due to (i) extremely high biodiversity in area and (ii) numerous locality factors including edaphic, precipitation, aspect etc. In addition, anthropogenic factors might have some role.

Table 8. Similarity Index within different Forest Types in Darjiling foothill region

Forest types	No. of spp A+B	Total species	Common	Similarity Index
Type IV / Type VI	158	126	32	40.51
Type I / Type III	108	87	21	38.89
Type II / Type IV	149	121	28	37.58
Type II / Type V	96	78	18	37.5
Type II / Type VII	82	67	15	36.59
Type I / Type VI	128	105	23	35.94
Type IV / Type V	137	113	24	35.04
Type I / Type VII	94	78	16	34.04
Type I / Type IV	161	134	27	33.54
Type I / Type II	120	100	20	33.33
Type II / Type VI	117	98	19	32.48
Type III / Type VI	105	88	17	32.38
Type V / Type VI	105	90	15	28.57
Type VI / Type VII	91	78	13	28.57
Type IV / Type VII	123	106	17	27.64
Type III / Type IV	138	119	19	27.54
Type V / Type VII	70	61	9	25.71
Type II / Type III	96	84	12	25
Type I / Type V	108	95	13	24.07
Type III / Type VII	70	62	8	22.86
Type III / Type V	84	77	7	16.67

Type I: Eastern Himalayan Sal Forest; Type II : Bamboo Brake; Type III : Riverine Forest; Type IV : Semi-evergreen Mixed Forest; Type V : Wet Mixed Deciduous Forest; Type VI : Lower Bhabar Sal Mixed Forest; Type VII : Upper Bhabar Sal Mixed Forest

This appears to be very interesting or, in other words, this is the expression of the extremely high phytodiversity in the region. With little change in habitat conditions almost a new set of species is selected for a particular Type of vegetation. But, there are at least two species [*Lagerstroemia parviflora* and *Mallotus philippensis*] which are present in all the seven Types. Similarly three species [*Schima wallichii, Terminalia alata* and *Terminalia bellirica*] are present in six Types. Similarly, ten species are present in five Types, 15 species in four Types, 19 species in three Types, 37 species in two Types and 118 species in one Type of vegetation only. This is one very interesting situation and is possible only due to the reasons expressed in the earlier paragraph.

Diversity Indices

Diversity can be expressed in different ways. Following types of tests have been made to understand the species diversity in the selected types of forests (Table 9):

Table 9. Diversity Indices of trees in different types of forests in Darjiling foothill region

Indices / Forest Types	Simpson's Index (CD)	Shanon-Weaver Index (SD)	Menhinick's Index (SR)
Type I	0.23	7.23	3.15
Type II	0.81	0.98	1.37
Type III	0.04	5.02	3.64
Type IV	0.03	5.86	5.17
Type V	0.08	4.66	4.38
Type VI	0.05	5.13	4.75
Type VII	0.15	3.66	2.41

I. *Species Richness* (SR): Menhinick Index of SR value for different forest types ranges from 1.37 (Type- II) to 5.17 (Type- IV). Forests in Types VI, V, III & I are also with high SR values of 4.75, 4.38, 3.64 & 3.15 respectively. And, for Type VII, where only 28 species of trees occur is also with a very low SR value (2.41). However, this forest type has moderate species richness, and concentration of dominance. Out of these, Types III, IV & VI showed high species diversity (SD) and SR with correspondingly low concentration of dominance (CD). Type II showed poor species diversity and species richness with correspondingly high CD value. May be the anthropogenic interference and aggressive invasion by bamboo population together caused this substantial change in different diversity indices.

II. *Species Diversity* (SD): Shanon – Weaver Index of SD for these forest types ranged between 0.98 (Type-II) to 7.23 (Type-I). However, it is over 5.00 in Types III, IV & VI (5.02, 5.86 & 5.13) and very close to this middle value i.e. 4.66 in Type-V.

III. *Concentration of Dominance* (CD): This is the measurement of the diverseness among the dominants in the vegetation. Lower the value, more diverse is the vegetation and *vice versa* with highest value being 1.00. The clear dominance of one particular species can not be good for the associated species. But, a situation where a number of species are co-dominating that must be congenial for the occurrence of a larger number of species. The determined value ranges between 0.03 and 0.81. In Type-II, where *Dendrocalamus hamiltonii* is with extremely high IVI and highest CD values is not favouring other species of trees to flourish. This is followed by Type-I with 0.23 and Type-VII with 0.15. The CD values for Types-III, IV, V & VI are quite low thereby indicating a good assemblage of different species in these forest types.

Frequency Class Distribution

To understand the nature of the forest community Raunkiaer's *frequency class* distribution was preformed with frequencies categorized into five classes with an interval of 20 and compared with the Raunkiaer's formulation A>B>C=D<E. Frequency class distribution in different forests (Fig. 2) show varying degree of deviation from the normal model in all the forest types, which again is indicative of biotically disturbed community without uniform distribution pattern. Although forest Types I & II appear to be uniform, but the absence of tree species in the frequency percentage class C and D clearly indicate some form of interference in the forest. Similarly, absence of higher frequency percentage class E in forest Types III, IV & V also points to severe biotic stress in these forests.

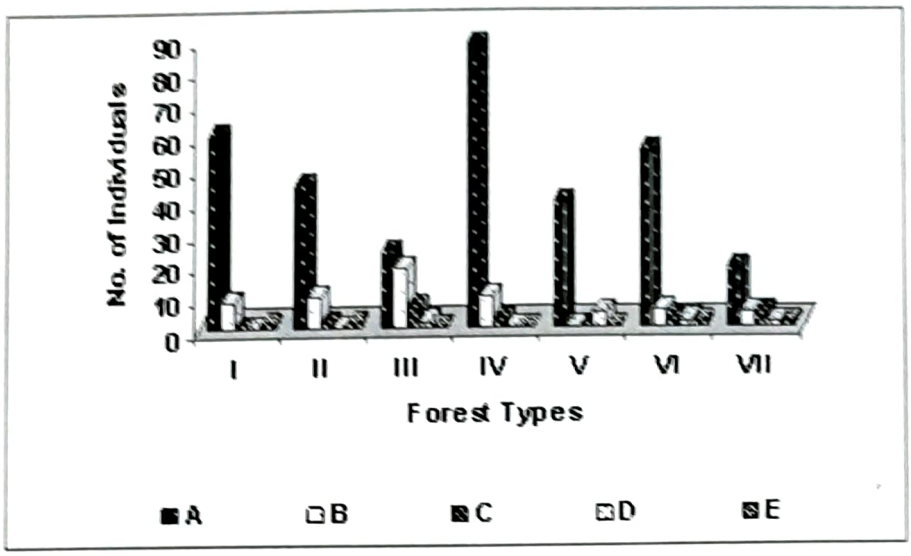

Fig 2. Showing the frequency class distribution in different forest types

Regeneration Potential of Seedlings and Saplings (RPS)

This is the measurement of the capacity of regeneration of different species in a particular forest. The level of RPS in a forest determines its future composition. RPS values in these forests are presented in Table 10. Type-I is found to be vibrant with highest number of 135.428 seedlings and saplings $400m^{-2}$ followed by Types-VII & VI with 99.4 & 87.572 $400m^{-2}$ respectively. Type-V had the lowest number of only 54 seedlings and saplings $400m^{-2}$. Types-II, III & IV also showed low level of regeneration. But with respect to number of regenerating species Type-II is richer with 75 species followed by Type-I with 63 species. The intermediate disturbance hypothesis (Connell 1978; Gurevitch *et al* 2002) states that the species diversity will be highest at intermediate level of disturbance and this appear to be true for Type-II. Type V is poor with only 18 regenerating species. Other Types had also a fairly good level of recruitment.

Table 10. Regeneration potential of seedlings and saplings in different forest types

Forest Type	No. of Species	Seedlings $400m^{-2}$	Saplings $400m^{-2}$
Type I	63	117.332	18.096
Type II	75	40.844	14.896
Type III	45	44.112	9.92
Type IV	47	51.132	15.332
Type V	18	44.332	9.668
Type VI	39	73	14.572
Type VII	31	86.8	12.6

Possible Compositional Changes

To predict the possible compositional change in these forests RPS of five dominant species were computed on the basis of the number of seedlings and saplings (Table 11).

Table 11. Distribution pattern of five different highly regenerating species (seedling & saplings) 400m^{-2} in different forests

Name	Type I	Type II	Type III	Type IV	Type V	Type VI	Type VII
Actinodaphne ovata	-	4.16	-	-	-	-	-
Aglaia spectabilis	-	-	3.112	-	-	-	-
Bauhinia purpurea	-	-	-	-	-	-	4.4
Bauhinia vahlii	-	-	-	-	-	6.144	-
Crataeva religiosa	-	-	2.776	-	-	-	-
Cryptocarya amagydina	-	-	-	-	-	-	6
Dalbergia sericea	4.904	-	-	-	-	-	-
Dalbergia tamarindifolia	-	-	-	-	2.668	-	-
Heynea trijuga	7.144	-	-	-	-	-	-
Lagerstroemia parviflora	-	-	-	-	-	-	6.4
Litsea monopetala	4.192	5.684	-	-	-	-	-
Litsea salicifolia	-	-	4	-	-	-	-
Macaranga peltata	-	-	-	4.332	-	-	-
Mallotus philippensis	11.144	6	-	9.136	11.668	14.716	7.6
Morus macroura	-	-	-	-	2.668	-	-
Ostodes paniculata	-	-	-	-	4.332	-	-
Premna mucronata	-	-	-	-	-	3.428	-
Pterospermum acerifolium	-	-	6.888	4	-	-	-
Pterygota alata	-	-	8.112	-	-	-	-
Schima wallichii	-	4.08	-	6.336	15.332	-	-
Shorea robusta	65.24	-	-	5.332	-	18	32.6
Syzygium cumini	-	-	-	-	-	4.716	-
Terminalia bellirica	-	1.792	-	-	-	-	-

1. *Shorea robusta* has an excellent regeneration status of seedlings and sapling in Types I, IV, VI & VII and is expected to maintain the major skeleton of vegetation there. This species showed an excellent power of generation in Types-I & VII and contributing highest number of recruitments. *Schima wallichii, Heynea trijuga Litsea monopetala, Pterospermum acerifolium, Cryptocarya amagydina & Bauhinia purpurea* may be its close associates in these forests.

2. *Shorea robusta* is making rapid ingress also into the semi-evergreen mixed forest and may form a major constituent if not dominating in years to come.

3. In the middle canopy *Mallotus philippensis* will dominate in all the forest Types except for Type III with *Dalbergia sericea*,

4. Liana like *Bauhinia vahlii* will be an important component of Type VI forest.

5. But, the most important factor is the anthropogenic interference which is difficult to predict. Human population in these areas is increasing at an alarming rate which will certainly interfere at least with the population of timber yielding species like *Shorea robusta, Lagerstroemia parviflora, Schima wallichii, Syzygium cumini* etc.

6. Under such disturbed situation, it is expected that plants like *Mallotus philippensis, Litsea monopetala, Actinodaphne ovata, Ostodes paniculata, Crataeva religiosa, Premna mucronata* and liana like *Bauhinia vahlii, Dalbergia sericea, D. tamarindifolia* etc. will flourish.

So, under natural process of regeneration or self recruitment it is expected that *Shorea robusta* will continue to remain as the most dominating tree in sal dominating forest types and in future it will also dominate in some other forest types which are now in different seral stages. However, in Type II forest *Dendrocalamus hamiltonii* will increase its dominance and will certainly interfere with the survival of not only the tree species but with the inhabitants in all other strata in the forest.

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Variation of antioxidant properties and phytochemical constituents of tea cultivated under various agronomic conditions at Terai region of North Bengal

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Abstract

The studies were conducted during 2007-2008 in three cultivars of tea (*Camellia sinensis*) namely, TV26, TV29 and Dangri Manipuri Jat, at tea estates of Terai, North Bengal. Commercial standard plucked tea leaves were assayed to determine antioxidants activity and related phytochemical constituents. DPPH was used to determine the antioxidant properties of bud+leaf (B+L), bud+two leaves (B+2L), bud+three leaves (B+3L) and matured leaves (L). Subsequently, total phenolics, flavonols and hydrolysable tannins were estimated using standard methods. The result showed that free-radicals scavenging potentiality and chemical composition varies significantly at different growing conditions and leaf maturation. Statistical analysis showed significant relation between antioxidant and some phytochemical composition of dry tea leaves, among which phenolics ($R^2 = 0.904$, $P < 0.001$), tannins ($R^2 = 0.567$, $P < 0.05$) and flavonols ($R^2 = 0.314$, $P < 0.05$) bear significant correlation. Soil physicochemical parameters like *p*H ($R^2 = 0.537$, $P < 0.05$), nitrogen ($R^2 = 0.618$, $P < 0.01$), K_2O ($R^2 = 0.106$, insignificant), P_2O_5 ($R^2 = 0.730$, $P < 0.01$), S ($R^2 = 0.157$, insignificant) and soil moisture ($R^2 = 0.745$, $P < 0.01$) influence free-radical scavenging activity in tea leaves. Antioxidant quality of tea varies with maturation stages of leaves and the pattern is specific for a selected varieties. From our observation it may be concluded that antioxidant quality is dependent on some fertility parameters of soil and maturation of leaf. These results suggests that appropriate agronomic practices and proper choice of plucking may help to preserve the tea antioxidant quality.

Keywords: Tea cultivars, free-radical scavenging activity, phytochemical composition, agro-climatic conditions.

Introduction

In the wake of rising global production of tea and stiff competition, the quality has gained considerable importance. The tea should not only pass the minimum quality standards prescribed by the various agencies but also meet the expected levels in antioxidants quality. The quality of tea begins in the field where the leaves accumulate the necessary chemical substances. The chemical composition of tea shoots varies with genotype, season and cultural practices. During the course of processing of plucked leaves, various biochemical changes take place in stored phytoconstituents of leaves, resulting in the degradation and formation of compounds; responsible for liquor, briskness, colour and aroma of cuppa.

Processed tea that is widely consumed now as a popular health drinks/bevarage is commercially manufactured from the young tender leaves of the tea plant (Cabrera *et al.*, 2003). Tea elixir has continued to be considered as medicine since the ancient time because of its richness in phytochemical constituents. Research on the effects of tea on human health has been fueled by the growing need to provide natural healthy diets that includes plant derived polyphenols. Research is going on to elucidate how functional components in tea cultivar could expand the role of diet in oxidative disease prevention and treatment (Misra *et al*, 2003,

Hitchon *et al.*, 2004, Marian *et al.*, 2004, Pajohk *et al.*, 2006). There are evidences that tea constituents play therapeutic role in more than sixty different health conditions (Pandey *et al.*, 2005, Vanessa *et al.*, 2004, Yamamoto *et al.*, 2004, Paola *et al.*, 2005, Hang *et al.*, 2003 and Hakim *et al.*, 2004). Therefore tea appears to be an effective chemopreventive agent for toxic chemicals which are produced in the body during normal metabolic pathways or introduced from the environment. Many plant phenolics have been reported to have antioxidant properties that are even much stronger than vitamin-C and E (Karori *et al.*, 2007). In addition, currently available synthetic antioxidant like BHA, BHT and Gallic acid esters have been suspected to initiate negative side effects (Amie *et al.*, 2003, Aquil *et al.*, 2006) and hence the need to substitute them with natural antioxidants like that from tea with broad-spectrum action. Despite the upsurge of interest in the therapeutic potential of tea plants as sources of natural antioxidants, limited studies have been carried out in various growing conditions using different cultivars. Information on the tea antioxidant properties varies in tea cultivar and degree of leaf maturation, which are rare and grossly lacking. In this research, a set of 36 tea samples; from three cultivar; grown in different agro climatic conditions of Terai were analyzed for antioxidant quality.The objective of this study was to compare the antioxidants quality with some chemical constituents of three tea cultivars and correlate them with soil agronomic parameters for determining better cultivation practices to restore the antioxidant quality of tea.

Material and methods

Plant materials

Description of cultivar

Fairly drought resistance, Indochina type, Aphids susceptible tea cultivar TV26 variety is very common cultivated plant in Terai region. Leaves are semi-erect, light, medium, lanceolate to ovate shaped leaflets and petiole bearing brown pubescence hair especially at axils of leaflets. Shoot size is medium.

Indochina, triploid TV29 is also popular cultivar of Terai region due to its drought tolerant capacity and high yield index. Dark semi-erect, medium leaves are lanceolate to ovate shaped. Low pubescence hair is found in axils of leaflet (Singh *et al.*, 1994). Indochina, Jat Dangri Manipuri is a hardy plant, difficult to descript morphological character due to seed jat, wide variation found in plant to plant and very common throughout Terai region.

Collection of shoots and extraction process

Experimental three variety of tea leaves are collected from the three commercial garden of Terai regions, Darjeeling, West Bengal in November and December month, 2007. The collected sample shoots comprisede of [Bud =B, Leave = L)] B + L, B + 2L, B + 3L and mature leaves (L) were dried in hot air oven at temperature between 50 to 60° C for 12 h after which 10 g of respective plant components were chopped and ground. The ground plant materials (400 mg) was soaked in 50 ml. of methanol:water:: 4:1 and boiled for 30 min on a hot plate with glass beads. The flask containing methanol extracts were kept overnight for cold percolations. The extracts were filtered through Whatman-42 filter paper and volume was reduced by evaporation under reduced pressure in rotary evaporator. Residue was washed three times and extracts were concentrated (20 mg/ml). Methanolic extract were used for evaluation of DPPH and determination of total phenolics, flavanols and tannin contents.

Antioxidants assay

DPPH based free radical scavenging activity

The free radical scavenging activities of each fraction were assayed using a stable DPPH, as per Blois (1958). Percentage of free radical scavenging activity was expressed as percentage inhibition as per the formula given below and 50% inhibition concentration was estimated.

$$\% \text{ inhibition} = \frac{\text{Abs. of control - Abs. of sample}}{\text{Abs. of control}} \times 100$$

Soil sampling and determination of physicochemical properties of soil

Soil samples were collected from four spots surrounding a plant, 0-45 cm depth at 1 ft apart from the collar region. Composite soil samples were prepared by mixing of four samples from each plant. Moisture content, *p*H, organic carbon (available organic nitrogen), available potassium as K_2O, available phosphorus as P_2O_5 and available sulfur were determined as per the procedure prescribed by the International Association of Soil Chemist (Baruah *et al.*, 1997).

Determination of phytochemical constituents

Estimation of total phenolics

Total phenols estimation was carried out in methanolic extracts of tea (dry mass) with Folin-Ciocalteu reagent as per Malick *et al*, 1980. The amount of total phenols was expressed as percent of extract catechol equivalent.

Estimation of flavonols

Flavonol content was determined spectrophotometrically in tea samples according to Mahadevan *et al.* (1986). The amount of flavonols was expressed as percent of extract, equivalent to phloroglucinol.

Estimation of tannins

Folin-Denis method was used for the determination of tannins as per Sadasivam *et al.* (1992) which is based on the non-stoichiometric oxidation of the molecules containing phenolics hydroxyl group, equivalent to tannic acid.

Statistical analysis

All the experiments were repeated three times and the data were represented as means and were analyzed by SPSS (ver. 11.0) for determining significance (P value < 0.001, 0.01, and 0.05 were represented as "***", "**" and "*" respectively).

Results and discussion

From the preliminary phytochemical evaluation of methanol extract of dried tea leaves (20 mg/ml concentration) in average standard plucking, it was confirmed that major groups of phytochemicals were phenols, flavonols and tannins. Fig. 1 and 3 represents the correlation between antioxidant activity with phenols and tannins and flavonols content did not execute significant level of the antioxidant activity Fig. 2.

The free-radical scavenging activity was demonstrated by DPPH, which is a stable free-radical and accept electron or hydrogen radical to become a stable diamagnetic molecules (Blois, 1958). Certain plants show antioxidant activity with DPPH S because of their phenolics constituents. Hydrolysable tannins and flavonols are a broad class of low molecular weight secondary metabolites widely distributed in plants. The beneficial effect of total phenolics and hydrolysable tannins are attributed to their antioxidant and chelating ability (Heim *et al.*, 2002). Phytochemical analysis confirm the presence of phenolics especially tannins and flavonols at varying degree in the three different cultivar viz. diploid, triploid and seed jat which represent all types of clonal varieties irrespective of genetic make up and cultural practices. Results have indicated that the antioxidant effect is related to development of reductones. Reductones are reported to be terminators of free-radical chain reaction (Dorman *et at.*, 2003).

Irrespective of cultivar and leaf maturation the antioxidant capacity of tea plant was significantly varied with soil physicochemical parameters. Fig.4 shows that DPPH (IC_{50}) based free-radical scavenging activity was significantly correlated with soil *p*H, because soil *p*H significant affects on the availability plant nutrients and microbial activity surrounding the rhizosphere. In very low (< 4.00) and high (6.00) *p*H, the availability of major as well as micro nutrients drastically affected and immediately plant suffers from nutritional stress. Therefore soil amendment will be performed in such a way that *p*H should be in between $4.00 < pH < 6.00$. From the Fig. 4, it may predicted that antioxidant activity restoration in tea leaves is optimized when the pH range in between 4.00 -5.00. There is a correlation between available from of organic nitrogen and antioxidant quality of tea leaves (Fig. 5). Nitrogen play significant role on preservation of antioxidant potentiality up to certain limit of 0.13 % after which antioxidant value decreases. Nitrogen is a basic constituent of protein and nucleic acid, integral part of chlorophyll, imparts vigorous vegetative growth, delay in maturity of plants, related to carbohydrate utilization and enhance sink strength. Excess nitrogen is a possible cause of Zn deficiency in citrus plant (Das, 1996). Potassium show the insignificant role (Fig. 6) on preservation of antioxidant activity in tea plant, our observation clearly indicates that 80-100 ppm soil potash maintain this important property, above or below this limit may be detrimental to radical scavenging activity of tea due to catalytic nature, over activate the enzyme and disturb the osmotic pull. The effect of phosphorus on free-radical scavenging activity is presented in Fig. 7, Soil available phosphorus show the varying degree of radical scavenging action of tea leaves but significant activity was observed within 15- 45 ppm level. Harbone (1980) observed that the phosphorus deficiency increases antioxidant constituent levels; same finding was also reflected in our experiments. Sulphur is a vital part of ferredoxins which is important for light and dark reaction of photosynthesis process and important constituents of some protein, so maintenance up to certain range very much essential. In Fig. 8, insignificant action of sulphur on antioxidant was observed, above 13 and below 7.00 reduces the tea antioxidant quality. Moisture level at 15% decreases the nutrients availability of tea

plants. Oxidative stress generated within the cell as a result of low water availability increases its antioxidant property so, Fig. 9 shows the significant results within 15-20 % moisture level.

For normal growth and development when nutrients are not limiting the important weather variables are solar radiation, temperature, saturation deficit of the air and rainfall (Carr *et al.*, 1992). The growth processes are manifested with the expansion of leaves, shoots, production and storage of dry matter and partitioning of secondary metabolites between the various plant organs. Maturation of tea leaf affects the accumulation pattern of antioxidant capacity as executed in Fig. 10. This may be due to higher allocation of carbon in secondary metabolites path way with up regulation of maturation. Minimum temperature 12-13^0 C maximum 30^0 C and 700 mm/ annum precipitation is ideal for tea cultivation. Below minimum temperature and above maximum temperature, storage and development of antioxidant compounds in tea leaves may be hampered. Yao *et al.* (2005) was observed the seasonal variation of phenolics compounds in Australia-grown tea. This is also another agreement of our result of agro-climatic variability of antioxidant quality of tea.

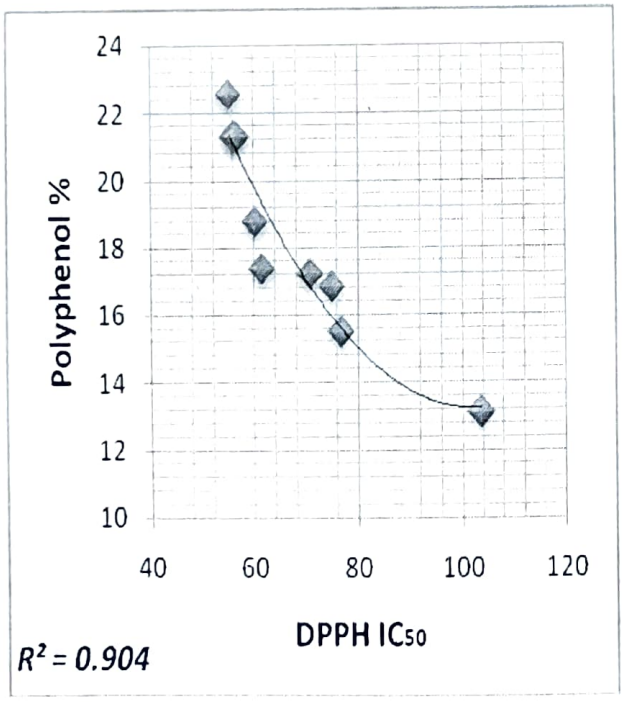

Fig 1. Relation between polyphenols and antioxidant activity (DPPH IC50)

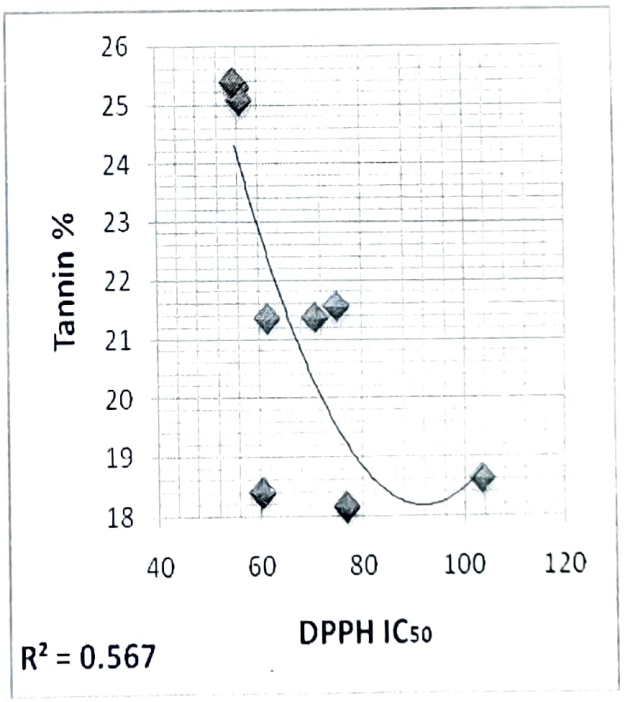

Fig 2. Relation between Tannins and antioxidant activity (DPPH IC50)

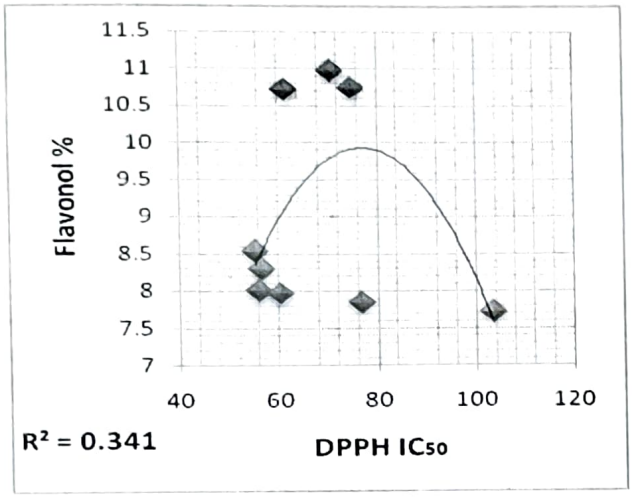

Fig 3. Relation between Flavonols and antioxidant activity (DPPH IC50)

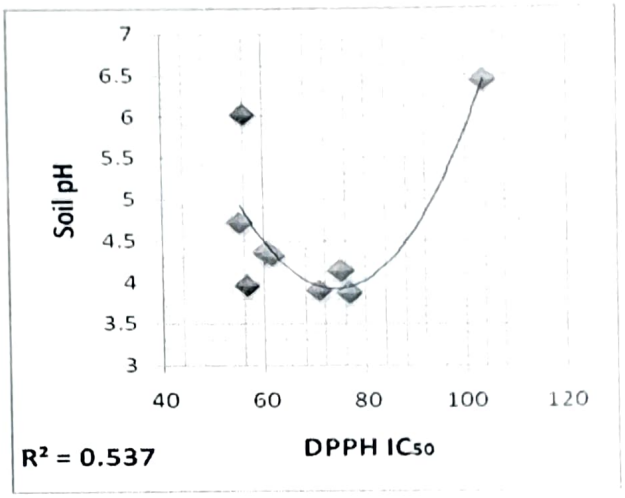

Fig 4. Relation between Soil *pH* and antioxidant activity (DPPH IC50)

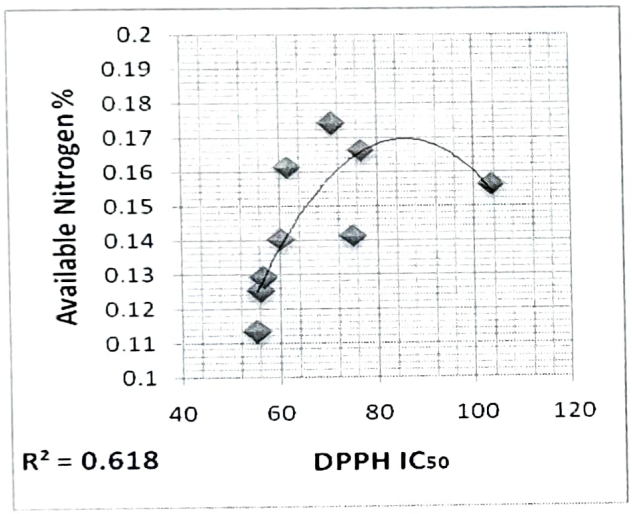

Fig 5. Relation between available Nitrogen and antioxidant activity (DPPH IC50)

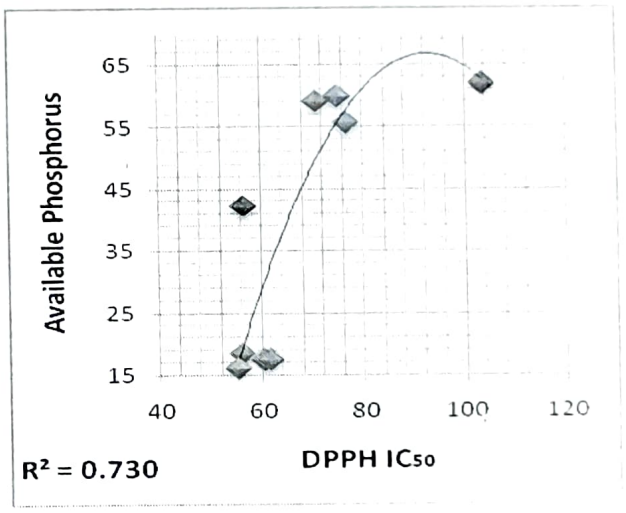

Fig 6. Relation between available Phosphorus and antioxidant activity (DPPH IC50)

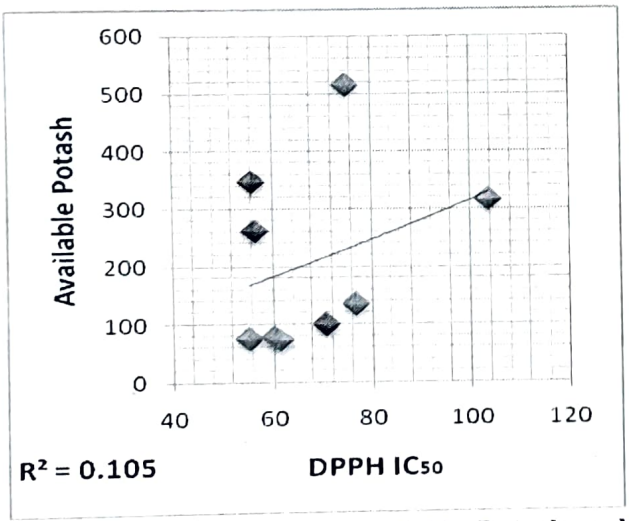

Fig 7. Relation between available Potash and antioxidant activity (DPPH IC50)

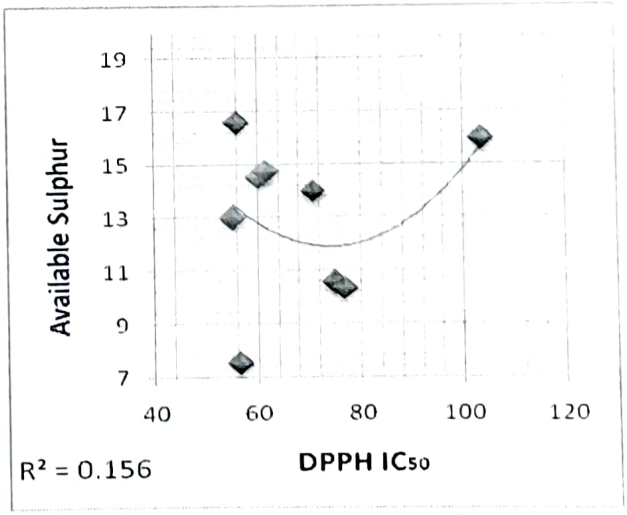

Fig 8. Relation between available Sulphur and antioxidant activity (DPPH IC50)

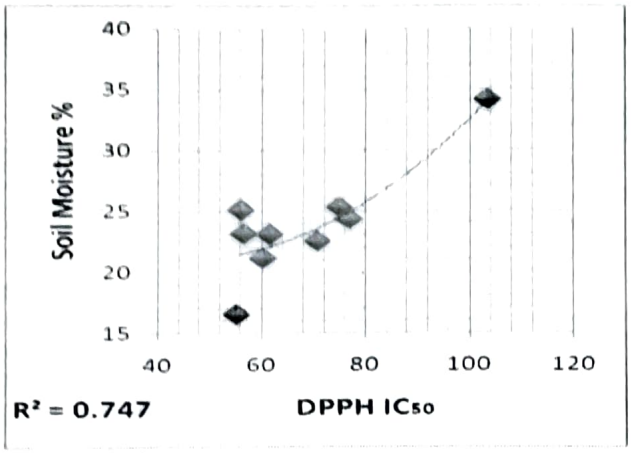

Fig 9. Relation between Soil moisture and antioxidant activity (DPPH IC50)

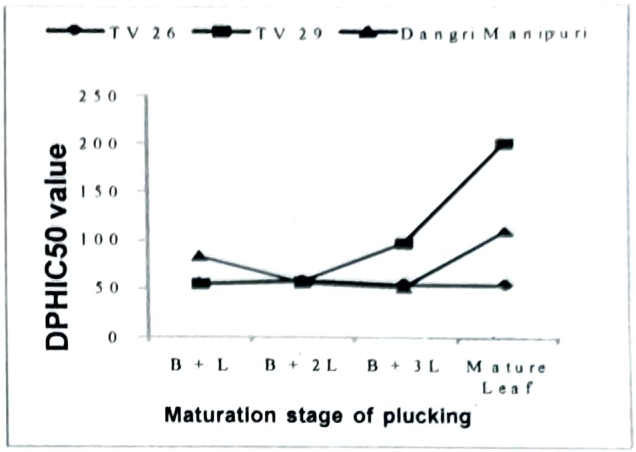

Fig 10. Pattern of antioxidant activity with leaf maturation

Conclusions

To conclude, the results have undoubtedly shown that the antioxidant quality of tea is best, if the soil *p*H is in between 4.50-5.00, nitrogen status 0.13%, available phosphorous as P_2O_5 is in between 15-45 ppm and moisture at 15-20% level. This is a preliminary report on antioxidant activity of tea under different soil agronomic practices and leaf maturation. Further studies are undertaken for the characterization of individual components of bioactive fractions of tea extracts to elucidate the mechanisms for restoring high antioxidant activity.

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Bacterial strain isolated from the Mango field and identified on the basis of 16S rDNA sequence information and optimization of the alkaline cellulase production

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Abstract

A bacterial strain was isolated from the soil of mango (*Mangifera indica*) orchards. The isolate was identified as *Paenibacillus alvei* [MTCC 7809] through phylogenetic analysis based on the 16S rDNA gene sequencing and also by conventional biochemical processes. The scanning electron microscopic analysis revealed the morphological details of the strain. The isolate is Gram positive, motile, rod shaped, spore forming bacterium with extreme salt tolerance. It can hydrolyze starch and gelatin, and catalase and oxidase were produced. The cellulase activity of the culture broth was determined by measuring the reducing sugar released from carboxymethyl cellulose (CMC). Different cultural conditions were tested to reach the optimum growth conditions in order to obtain large-scale production of alkaline cellulase for commercial importance. Crude cellulase from *Paenibacillus alvei* (MTCC 7809) showed activity and stability at moderately high temperature and pH and the activity remained stable at 40°C and pH 8 after 2h of incubation making the strain a good choice for industrial applications. The rDNA sequence of 1509bp is submitted into the NCBI GenBank database (Accession no. EF429201).

Keywords: Cellulase, *Paenibacillus alvei*, 16S rDNA gene sequencing.

Introduction

Cellulose is the largest renewable carbon source that is frequently found in close association with other compounds such as hemicellulose, lignin and other polysaccharides (Person *et al.*, 1990). In nature microorganisms synthesize different enzymes to degrade cellulosic plant cell wall. Cellulase is one of the most demanded industrial enzymes and also used in plant protoplast isolation, plant virus studies, metabolic investigations and genetic modification experiments (Ray *et al.*, 2007). However, among all cellulases the industrial applications of alkaline cellulases as laundry detergent additive (Horikoshi, 1999) led many microbiologists to carry extensive research on alkaline cellulase producers. Fungi were considered as the chief cellulase producers but recently there is an increasing interest in cellulase production by bacteria (Li and Gao, 1996) because of the high growth rate of bacteria compared to fungi (Ariffin *et al.*, 2006).

We isolated many bacteria from the soil of different mango (*Mangifera indica*) orchards of Malda district, W.B., India (longitude 24°40′20″ N to 25°32′08″ N and latitude 87°45′50″ E to 88°28′10″ E) and some of these isolates are really important from industrial perspectives (Roy and Chattopadhyay, 2008). Among these microbes *Paenibacillus alvei* (MTCC 7809) was the potent cellulase producer. The isolate was identified on the basis of 16s rRNA gene sequencing as this process has 90% accuracy for speciation (Moor *et al.*, 2006) and also by other conventional biochemical processes. The size and morphology of the strain were studied by using scanning electron microscope (SEM).

The present investigation is aimed at the isolation of the cellulolytic bacteria *Paenibacillus alvei* (MTCC 7809) from the soil of mango orchards of the Malda district of West Bengal, India and determining the optimal conditions for alkaline cellulase enzyme production and identification on the basis of 16S rDNA gene sequencing.

Materials and Methods

Isolation and Screening of Cellulase Producer

Soil samples from different mango orchards were collected for the isolation of bacterial colonies. The soil dilutions were prepared according to the methods described in Benson's Microbiological Applications (Brown, 2005) and 1ml of the soil suspension was transferred to nutrient agar medium. The preliminary qualitative analysis for cellulolytic activity of the isolates was conducted by using Congo red dye. The isolates were screened for cellulase production on a medium containing (g/L) KH_2PO_4 - 1.0, $MgSO_4.7H_2O$ - 0.5, NaCl - 0.5, $FeSO_4.7H_2O$ - 0.01, $MnSO_4.H_2O$ - 0.01, NH_4NO_3 - 0.3, CMC - 10.0, Agar - 15.0, (pH8.0) (Ariffin *et al.*, 2006), for 3 days at 37°C. After the end of incubation period the CMC agar plates were flooded with aqueous Congo red (1%w/v) solution for 15 min followed by destaining with 1M NaCl for 15 min after pouring off previous stain. The colonies that produce clear zones of hydrolysis around it indicate cellulose degradation (Ariffin *et al.*, 2006) and were picked and maintained in nutrient agar. The biochemical, physiological and morphological tests were performed to determine the strain identity.

Enzyme Assay

For quantitative assay of cellulase production by the strain liquid media was used. Cellulase activity was determined by the release of reducing sugar from carboxymethyl cellulose (CMC) using 0.05M sodium citrate buffer (Ekperigin, 2007 and Ariffin, 2006). 0.5 ml of culture supernatant fluid was incubated with 0.5 ml 1% CMC in 0.05M sodium acetate buffer, pH7.5 at 40°C for 1 h. The reducing sugar released was assayed by dinitrosalisylic acid (DNS) method (Miller, 1959). One unit (U) of cellulase was defined as the amount of enzyme liberating 1mg of glucose equivalent to per min under the assay conditions.

Optimization of Fermentation Conditions

A variety of factors like inoculums size (carbon source and cellulose quality), pH value, temperature, growth time etc. appears to control the cellulase yields (Immanuel *et al.*, 2006). To determine the optimum pH and temperature for the fermentation process media was adjusted at different levels of pH ranging from 6 to 8.5 and for temperature fermentation was carried out at an intervals of 5°C ranging from 25°C to 45°C. Another important parameter for enzyme production is fermentation period and for it the process was carried out up to 120h (Ray *et al.*, 2007). The fermentation medium was seeded with 1.0%, 2.0%, 3.0%, 4.0%, and 5.0% seed culture (tryptone soya broth) for optimizing the inoculum volume and incubated at 37°C (Ray *et al.*, 2007).

Scanning Electron Microscopy of the Strain

The morphological details of the strain *Paenibacillus alvei* (MTCC 7809) was analyzed by scanning electron microscopy (SEM).The specimen was prepared according to the methods of Felgenhauer (1987); fixed in 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.0) at room

temperature for 3h and then dehydration was done with ethanol followed by post fixation in 1.0% osmium tetroxide (OsO_4) in buffer for 2h. The sample was then coated with gold and the micrograph was taken with a JEOL 840 scanning electron microscope (Figure 2).

DNA Extraction for 16s rRNA Gene Sequencing

The strain was grown on nutrient agar medium for 48 h at 37°C for extraction of DNA. DNA was extractted using 250 ml lysis buffer (10 mM/l Tris HCl, 1 mM/l EDTA pH 8.0 with 3 mg/ml lysozyme) and incubated at 37°C for 15 min. 36 ml of 10% (w/v) sarkosyl were added and was further incubated at 37°C for another 30 min. NaCl was added (46.5 ml of a 5 M/l stock) to give a final concentration of 0.7 M/l; 332 ml of a 10% CTAB (prewarmed) solution in 0.7 M/l NaCl were added and the mixture was incubated for 20 min at 60°C. Solvent extraction by 664 ml chloroform was followed by centrifugation at 13,000 ´g for 5 min. DNA was precipitated after the addition of equal volume of isopropanol and chilling to -20°Ç and was pelleted by centrifugation at 13,000 ´g for 5 min. The DNA pelletes were then dissolved in 50 ml TE buffer (pH 8.0) (Bell *et al.*, 1999).

PCR Amplification

PCR amplification was carried out in a reaction mixture containing 50 ng template DNA, PCR buffer (10 mM Tris HCl, pH 8.3; 50 mM KCl; 2.5 mM $MgCl_2$, 0.001% gelatin), two universal primers each with 0.2 mM concentration, 2.5 U *Taq* DNA polymerase (Genei, Bangalore), and four deoxynucleoside triphosphate each in 0.2 mM concentration. The primer sequence selection was done from the regions of 16s rDNA and the 16s rDNA was amplified by using universal forward primer 5′-TGGAGAGTTTGATCCTGGCTCAG-3′ and universal reverse primer 5′-TACCGCGGCTGCTGGCAC-3′ (Hamasaki *et al.*, 2005; Hall *et al.*, 2003). After an initial denaturation for 2 min at 95°C, 25 cycles were completed each consisting of 1min at 94°C, 50 sec at 60°C annealing temperature, and 1.5min at 72°C. A final extension of 7 min at 72°C was applied. 10 ml of PCR product was loaded onto 2% agarose gel with ethidium bromide staining to determine the size (Lopez *et al.*, 2003). The PCR product was cloned in pGEMT Easy Vector (Roy and Chattopadhyay, 2008) and vector DNA was bi-directionally sequenced using the forward, reverse and an internal primer. Sequence data was aligned and analyzed for finding the closest homologs for the microbe.

Results

Soil dilutions on nutrient agar plates after 7 days of incubation at 37°C were found to contain 36 different bacterial colonies indicating the bacterial flora richness of soil of mango orchards. After the initial screening for cellulase producing bacteria by Congo red method 3 isolates shown positive results but it was the *Paenibacillus alvei* (MTCC 7809) that produces the largest diameter of clear zone of CMC hydrolysis [Fig. 1] and was the strain of interest. The biochemical, morphological and physiological test analysis for the identification of the strain was performed and the results are summarized in Table 1. The rod shaped of the bacterium was confirmed based on the analysis of scanning electron microscopy (SEM) [Fig. 2] and the size ranging from 1.5 to 2 μm.

Cellulase yield was gradually increased with the increase of pH up to 8.0 and then declined beyond pH 8 [Fig. 3]. The temperature profile for fermentation condition is depicted in Figure 4. The maximum enzyme yield was observed at 40°C. Cellulase production was

found increase with the gradual increase in incubation period and was at the peak after 96 h incubation but decreased thereafter [Fig. 5]. The result of the effect of inoculum size reflects that 3% inoculum size was optimum for highest enzyme production though not significantly different from that in 2% inoculum size [Fig. 6]. PCR product of 16S rDNA was fractionated on 1% agarose gel electrophoresis [Fig. 8].

The sequence analysis of 1509 bp of the 16s rRNA gene of the strain was determined and compared using Ribosomal Database project and GenBank database. Based on nucleotides homology and phylogenetic analysis the microbe was identified to be *Paenibacillus alvei* and its nearest homologue species found to be *Paenibacillus apiarius* (ABO73201). The phylogenetic position of the strain was determined by constructing the phylogenetic tree based on comparison of the 16s rRNA gene sequences of the reference *Paenibacillus* sp. [Fig. 7]. Information about other close homologues for the microbe can be found from the alignment view in Table 2.

Table 1. Biochemical, morphological and physiological profiles of the cellulase producing strain *Paenibacillus alvei* (MTCC 7809)

Tests	Results	Tests	Results
Gram's reaction	+ *ve*	H_2S production	–
Endospore	+	Cytochrome Oxidase	+
Motility	+	Catalase Test	+
Fluorescence (UV)	–	Gelatin hydrolysis	+
Growth under anaerobic condition	+	Arginine dihydrolase	+
		Lysine decarboxylase	–
Indole Test	–	Acid production from carbohydrates	
Methyl Red Test	–	Arabinose	+
VP Test	–	Dextrose	+
Starch hydrolysis	+	Dulcitol	–
Citrate utilization	–	Galactose	+
Casein hydrolysis	+	Lactose	–
Urea hydrolysis	–	Mannitol	+
Nitrate reduction	+	Maltose	+
Growth on MacConkey Agar	–	Sucrose	–

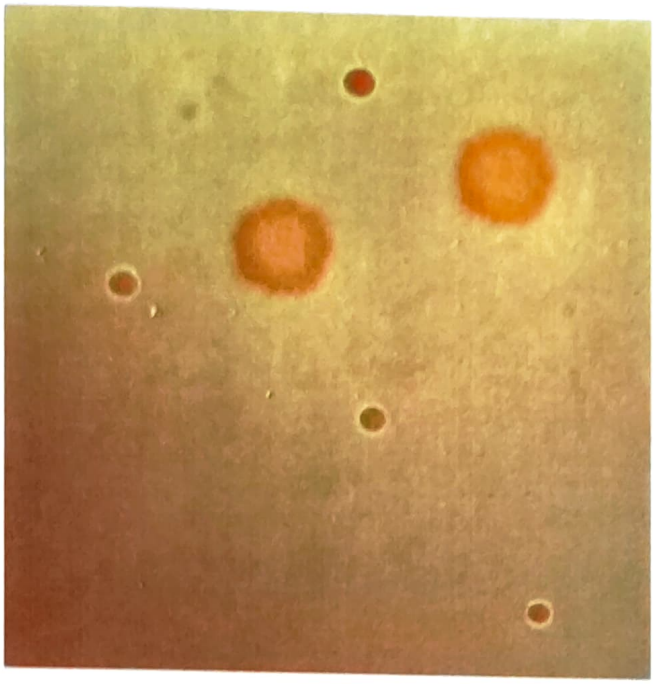

Fig 1. Production of the halo zones around the colonies of *Paenibacillus alvei* (MTCC 7809) after staining with Congo red that indicates the cellulase hydrolysis

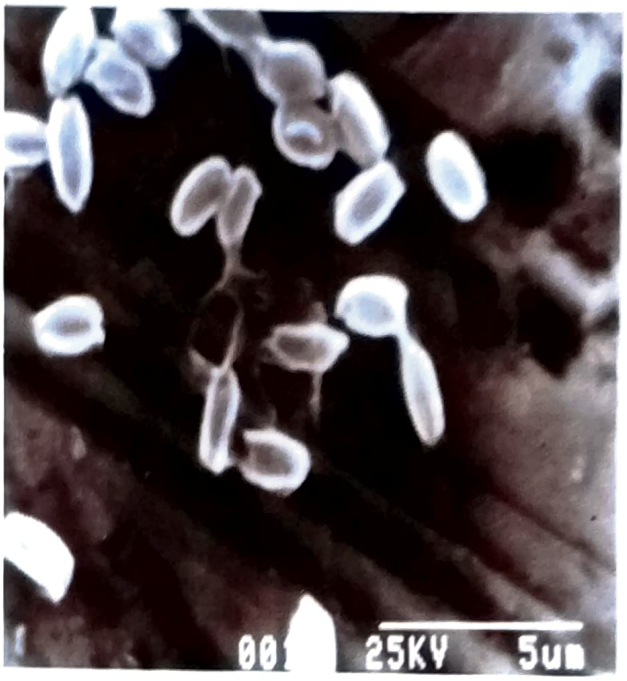

Fig 2. Scanning electron micrograph of *Paenibacillus alvei* (MTCC 7809) after dehydration and fixation indicating the morphological details

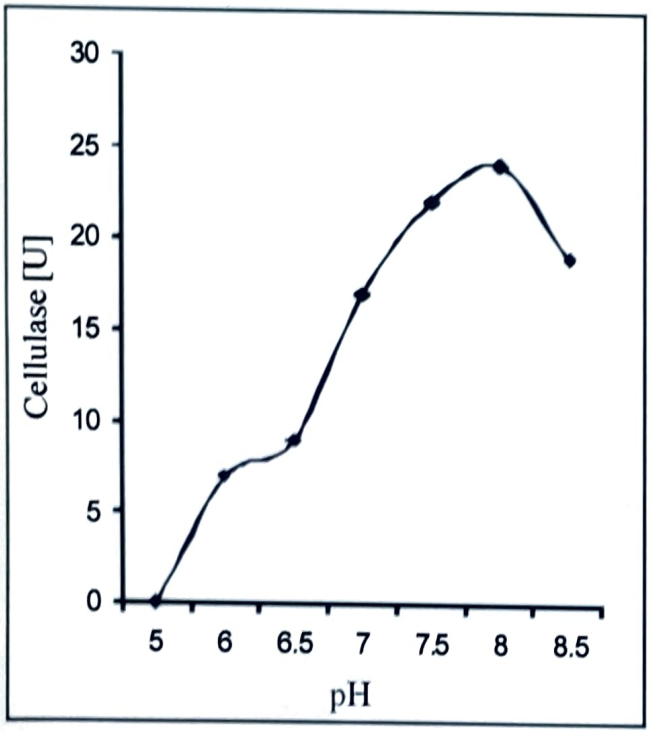

Fig 3. Effect of pH on cellulase production by *Paenibacillus alvei*

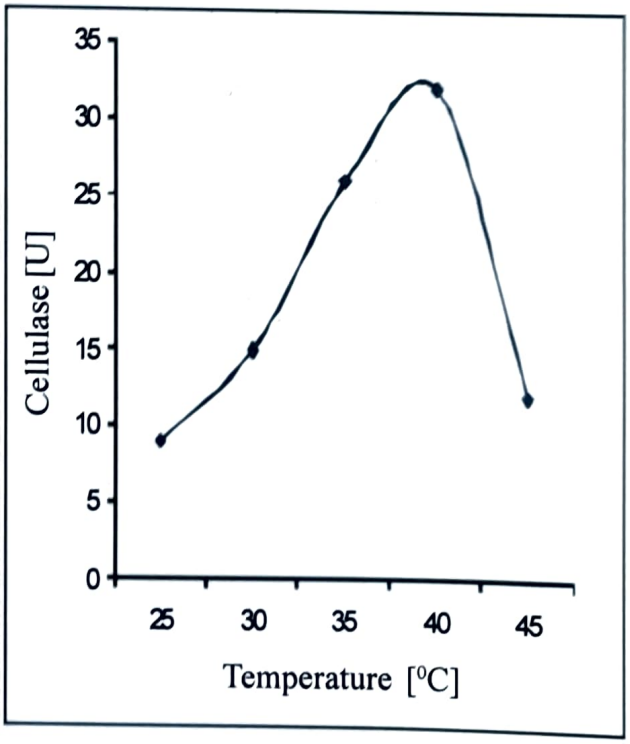

Fig 4. Effect of temperature on cellulase production by *Paenibacillus alvei*

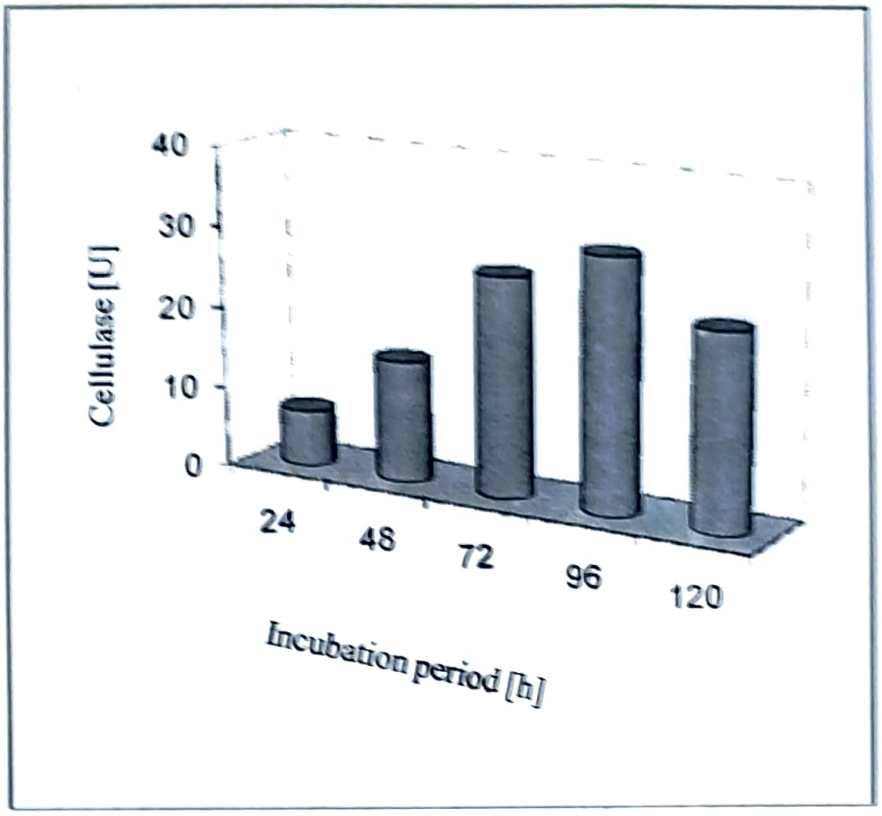

Fig. 5. Effect of incubation period on cellulase production by *Paenibacillus alvei*

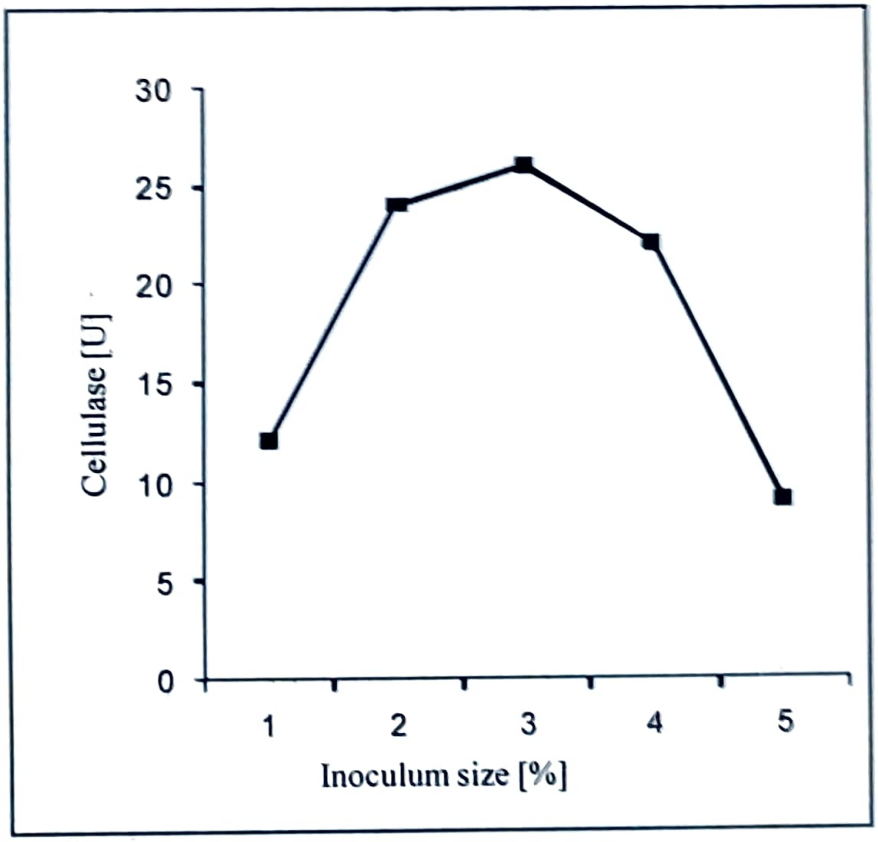

Fig. 6. Effect of percentage of inoculum size on cellulase production by *Paenibacillus alvei*

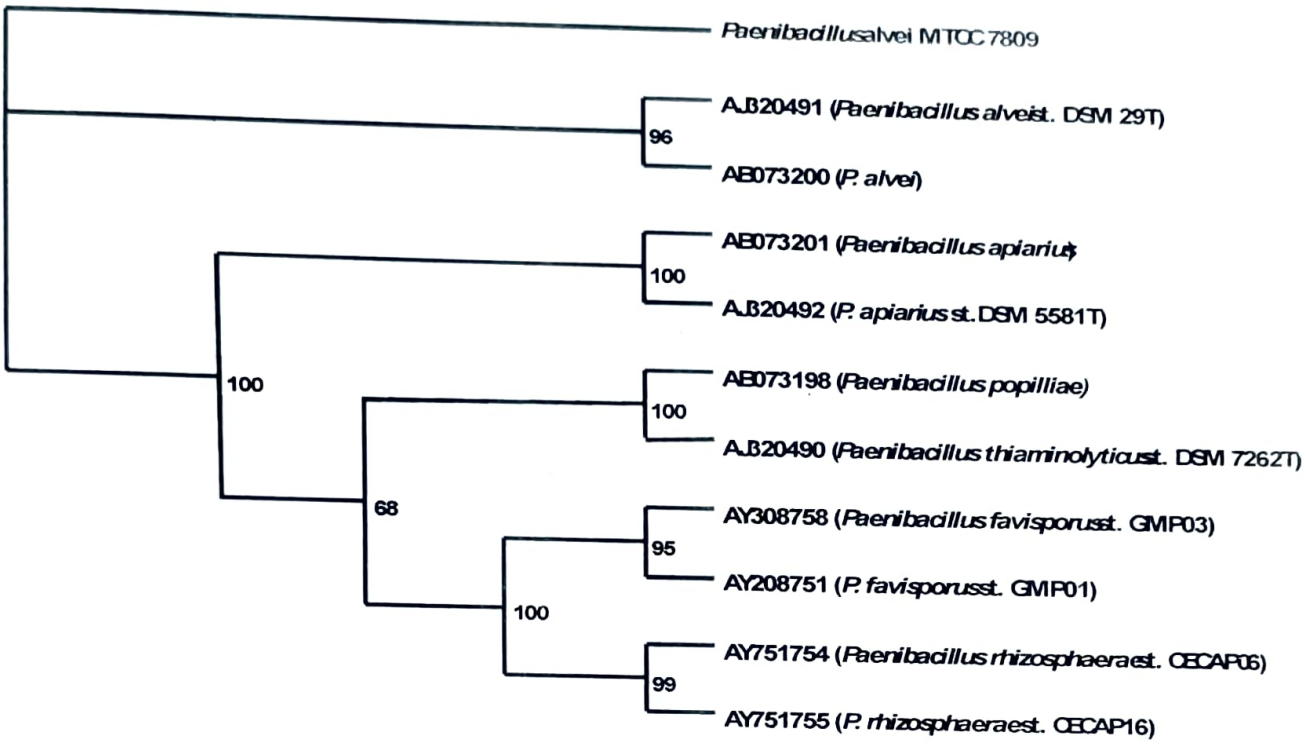

Fig 7. Phylogenetic dendrogram based on 16s rDNA sequence data indicating position of the isolate among the other representatives of the genus *Paenibacillus*. The sequences used in the analysis were obtained from Ribosomal Database project and GenBank. The scale bar indicates evolutionary distance

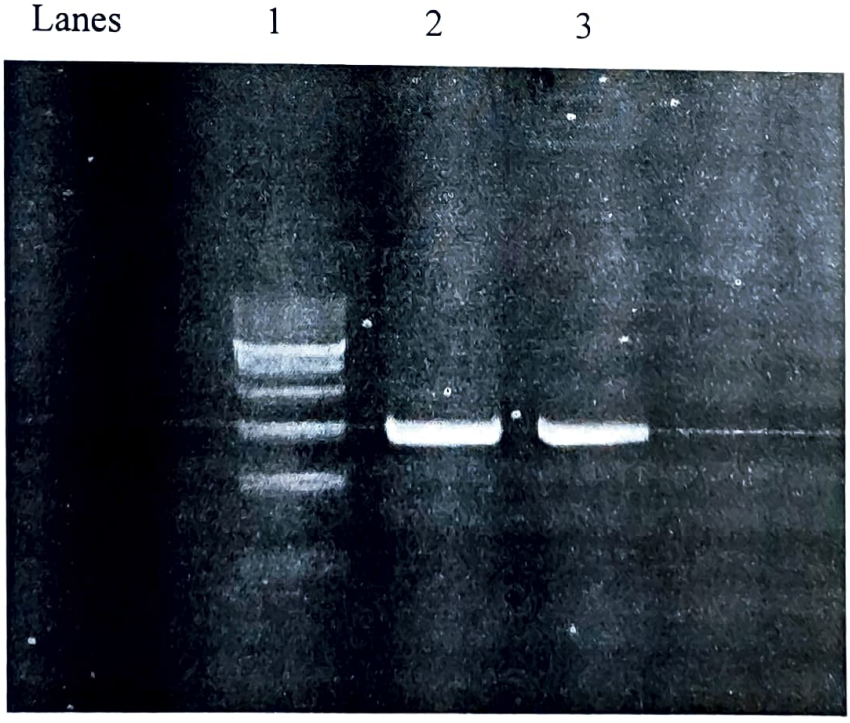

Fig 8. PCR product of 16rDNA amplification was fractionated on 1% agarose gel electrophoresis after staining with ethidium bromide Lane1, 500 bp ladder DNA marker and lanes 2 &3 showing the 1.5 kb band of 16S rDNA of *Panibacillus alvei* MTCC 7809 in duplicate

Table 2. Percentage homology based on nucleotide sequence to determine the close homologues of the strain

Sl. No.	Isolates	Percent Homology										
		1	2	3	4	5	6	7	8	9	10	11
1	*Paenibacillus alvei* MTCC 7809	*	98	98	95	93	94	93	93	93	92	92
2	AJ320491		*	99	96	94	95	94	94	94	93	93
3	AB073200			*	96	94	95	94	94	94	94	94
4	AB073201				*	97	95	95	94	94	94	94
5	AJ320492					*	93	93	92	92	92	92
6	AB073198						*	99	93	93	93	93
7	AJ320490							*	93	93	93	92
8	AY308758								*	99	99	99
9	AY208751									*	99	99
10	AY751754										*	99
11	AY751755											*

Discussions

Cellulase productions depend on a complex relationship involving a variety of factors, which are illustrated. Established fermentation techniques allow microbial enzymes to be produced in large quantities. The enzyme production is controlled in microorganisms and therefore, to improve the productivity the parameters can be exploited and modified (Ray *et al.*, 2007). Commercially available cellulases have optimum activity at pH ranges from 4.0 to 6.0 (Horikoshi, 1999). However, the use of alkaliphiles, which produces alkaline cellulases for laundry purpose in industrial scale has an advantage for their high pH range. Alkaline cellulase from *Bacillus* sp. KSM635 was found to have detergent effects on cotton cloth with reduced washing time and allow washing at lower temperature under European washing conditions (Hoshino *et al.*, 2000). Optimum cellulase activity of *Penicillium* sp. CR-316 (Picart *et al.*, 2007) was recorded at 65°C, that is higher than *Paenibacillus alvei* (MTCC 7809) and far lower pH at 4.5. The thermostable alkaline cellulase strictly alkaliphilic strain of *Bacillus* sp. KSM-S237 (Hakamada *et al.*, 1997) was reported to have optimum pH and temperature of 8.6 to 9.0 and 45°C respectively which is nearly similar to that of our strain. Dasilva *et al.*, (1993) reported *Bacillus* sp. strain B38-2 and *Streptomyces* sp. strain S36-2 with an optimum pH and temperature for the crude enzyme activity ranging from 7.0 to 8.0 at 60°C and 6.0 to 7.0 at 55°C respectively.

The above discussion indicates that the thermostability with high pH tolerance of the enzyme from *Paenibacillus alvei* (MTCC 7809) supports its potential use in detergent industry. However, more research is suggested before its commercial exploitation.

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Influence of culture media and environmental factors on mycelial growth, sporulation and spore germination behaviour of *Curvularia eragrostidis* (P. Hennings) Mayer

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Abstract

Curvularia eragrostidis, is a foliar fungal pathogen of young tea plants. It causes leaf spot disease of tea. Mycelial growth, sporulation and spore germination behavior of the pathogen were studied. Six different media were tested for mycelial growth. Among these, potato carrot agar (PCA) was found best for the mycelial growth and sporulation. Maximum mycelial growth was attained after 15 days of incubation. Mycelial growth was also studied in different temperatures and pH. Optimum temperature of growth was 25 °C and best growth was obtained at pH 6.0. Glucose and peptone were best carbon and nitrogen sources respectively for growth and sporulation of the fungus. The optimum conditions of spore germination were found to be at pH 7.25 and at incubation temperature of 25 °C.

Introduction

Tea is grown as a major plantation crop in the sub-Himalayan agro climatic zone of north-east India and forms the basis of economy of this region. *Curvularia eragrostidis* (P. Hennings) Mayer was discovered as a foliar fungal pathogen of young clonal cuttings of tea (Saha *et al.*, 2001). Mycelial growth, sporulation and spore germination behavior of a pathogen are essentially required for routine handling of a pathogen in the laboratory or in field studies. Hence, the present investigation was undertaken to observe the growth of *C. eragrostidis* in different media supplemented with different carbon and nitrogen sources. Different environmental factors were evaluated for optimization of spore germination conditions of *C. eragrostidis*.

Materials and methods

Fungal culture

Curvularia eragrostidis (P. Hennings) Mayer was originally isolated from naturally infected tea leaves of young clonal cuttings raised in the nursery of Muhurgong and Gulma Tea Estate of Darjeeling district. It was identified (Identification no. 4150.2 k) using resources of Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi. The fungal culture was maintained on freshly prepared sterile slants of potato dextrose agar (PDA) medium throughout the duration of the work.

Assessment of mycelia growth for determination of optimum growth period

To determine the optimum growth period *C. eragrostidis* was grown in potato dextrose broth (PDB) medium. Mycelial discs of 4mm diameter were inoculated in 250 ml Ehrlenmeyer

flask containing 40 ml PDB. Flasks were harvested at 5 days intervals and the dry weights of the mycelia were recorded until 25 days.

Assessment of mycelial growth in different media

To assess the mycelial growth in different media, *C. eragrostidis* was grown in petriplates containing 20 ml sterile test medium. Six different media viz. potato dextrose agar (PDA), malt extract agar (MEA), czapek dox agar (CDA), oat meal agar (OMA), potato carrot agar (PCA) and Richards' agar (RA) were used for the study. Each plate was inoculated by mycelial disc of 4 mm, cut from the advancing zone of 7 day old culture of *C. eragrostidis*. Diameter of the mycelia grown on the plates was recorded at 2 day intervals until 8 days.

Effect of different carbon sources on growth and sporulation

To study the effect of different carbon sources on mycelia growth and sporulation of *C. eragrostidis*, a basal medium (glucose 1%; asparagines 0.2%; KH_2PO_4 0.1%; $MgSO_4$, $7H_2O$ 0.005%; Zn^{++}, Mn^{++} and Fe^{++} 2 ml/ml) was used in which the fungus was cultured and glucose was replaced individually by the different carbon sources tested in equivalent quantities of carbon as present in 1% glucose. Each flask (250 ml) containing 40 ml basal media substituted with different carbon sources was inoculated in triplicate with 4 mm mycelia discs and incubated at 25 ±1°C. Control flasks did not contain any carbon compound. Sporulation and mycelial dry weight were recorded at 5 days intervals until 25 days.

Effect of different nitrogen sources on growth and sporulation

Modified Asthana and Hawker's basal medium 'A' (glucose 1 g; KNO_3 3.5 g; KH_2PO_4 1.75 g; $MgSO_4$, $7H_2O$ 0.75 g; distilled water 1 L) was used for studying the effect of different nitrogen sources on the mycelia growth and sporulation of the fungus. The quantity of various nitrogen sources was adjusted by replacing KNO_3 so as to give the same amount of nitrogen as furnished by 3.5 g KNO_3 in the basal medium. The basal medium only served as control. Sterilized media were inoculated with 4 mm mycelia discs and incubated at 25 ±1°C. Extent of sporulation and mycelia dry weight were recorded at 5 days intervals until 25 days.

Influence of different pH on mycelial growth

Potato dextrose broth (PDB) medium (40 ml taken in 250 ml Ehrlenmeyer flask) was adjusted to different pH (pH 5, 5.5, 6 and 6.5) by adding 1N HCl or 1N NaOH. Following inoculation with 4 mm mycelia discs, the flasks were incubated at 25 ±1°C. Each treatment was replicated thrice. Mycelia dry weight was recorded until 25 days at 5 days interval.

Influence of temperature on mycelial growth

Potato dextrose broth (PDB) medium (40 ml taken in 250 ml Ehrlenmeyer flask) were inoculated with 4mm mycelia disc and incubated at different temperatures (0°C – 45°C at intervals of 5°C) taking three flasks for each temperature conditions. Mycelial dry weight was recorded after 15 days of incubation.

Assessment of spore germination

Spore suspension was prepared from 7 day old sporulated culture of *C. eragrostidis* following the method as described by Saha and Chakraborty (1990). The concentration of the spores in the suspension was adjusted to 10^5 spores ml^{-1} following hemocytometer count. Thirty microlitre of spore suspension drops were then placed on clean, grease free glass

slides. Slides were incubated for 24 hours at 25 ± 1 °C in a sterile humid chamber, stained with cotton blue-lactophenol and observed under microscope. Percent germination and germ tube elongation were recorded at 2 hour intervals. In another set, slides containing spore suspension drops were incubated at different temperatures (0 °C - 45 °C) at intervals of 5 °C in a sterile humid chamber. After 24 hours, the slides were stained with cotton blue-lactophenol and were observed under microscope. Percent germination and germ tube elongation were also recorded.

Assessment of germination of spores in different pH

Six different phosphate buffer of 0.1 M were prepared by mixing sodium dihydrogen phosphate and disodium hydrogen phosphate solutions and the final solutions were adjusted to different pH (pH 4, 6, 6.5, 6.75, 7.25 and 9). Thirty microlitre buffer of each pH were mixed with 30 ml of spore suspension separately and finally mounted on clean, grease free glass slides. After 24 hours of incubation percent germination and germ tube elongation were determined.

Results and Discussions

From the results (Fig.1) it was evident that the maximum growth (mycelial dry weight of 193 mg) of the fungi was after 15 days of incubation. After that period lysis started and mycelial dry weight was found to be declined due to autolysis and depletion of media.

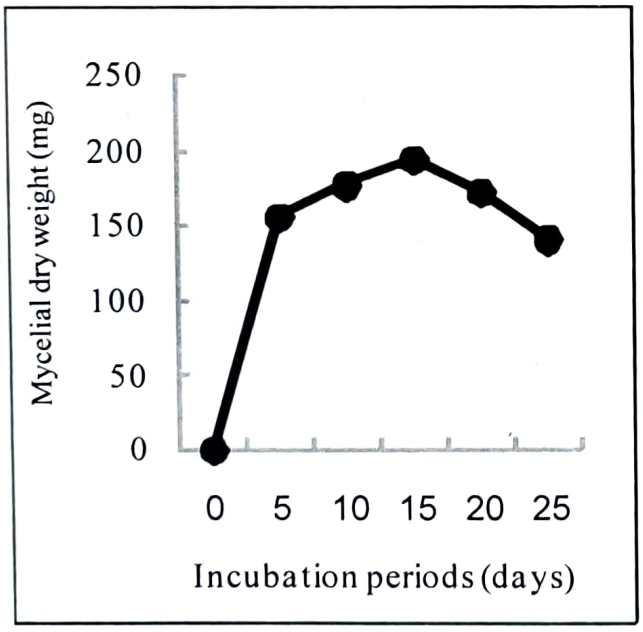

Fig 1. Mycelial growth of *C.eragrostidis* in diferent days of incubation

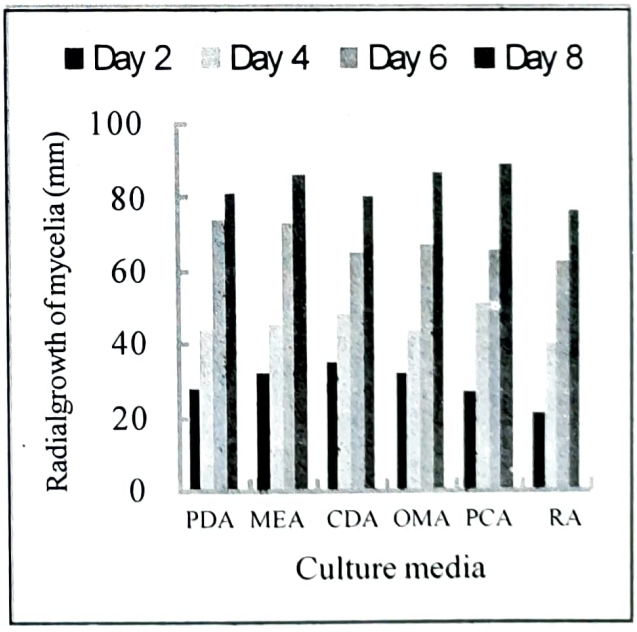

Fig 2. Mycelial growth of *C. eragrostidis* in different solid media

[Abbreviations : PDA = Potato Dextrose Agar, MEA = Malt Extract Agar, CDA = Czapek Dox Agar, PCA = Potato Carrot Agar, OMA = Oat Meal Agar, RA = Richards' Agar]

Among the six solid media tested, PCA showed maximum growth (89 mm in diameter after eight days of incubation) and was found to be the most suitable solid media for mycelia growth. Growth was observed in all the other media tested (Fig.2).Richard's agar and potato dextrose agar supported good growth and sporulation of *C. gloeosporioides* isolated from cashew anthracnose as shown by Sandhyarani and Murthy (2004). Several other workers also stated that PDA was best media for growth and sporulation of some other fungi (Xu *et al* 1984; Maheswari *et al* 1999). Saha *et al* (2008) stated that tea root extract supplemented potato dextrose broth was best for the growth of *Lasiodiplodia theobromae*.

Mycelial growth was observed to be much higher in presence of all the carbon sources tested compared to control, which did not contain any carbon compound (Table 1). Among the various carbon sources tested, glucose and sucrose containing media showed highest growth with mycelia dry weight of 310 mg and 289 mg after 15 days of incubation respectively. Media having galactose as carbon source recorded minimum mycelia growth (115 mg) after 5 days of incubation. Sporulation, which was started after 5 days in all the cases, was found excellent in glucose after 15 days of incubation. In control, *C. eragrostidis* showed insignificant growth which increased up to 25 days and no sporulation was observed. Our result was similar to that reported by Saha *et al.* (2008) who showed that glucose and sucrose was the best carbon sources for the mycelia growth of *Lasiodiplodia theobromae*. Our observation was also partly similar to that of Ray (2004) who reported that lactose and glucose had similar effect on growth of *Botryodiplodia theobromae*.

Table 1. Mycelial growth and sporulation of *C. eragrostidis* in different carbon sources

Carbon sources	Incubation periods									
	5 days		10 days		15 days		20 days		25 days	
	Mwt (mg)*	Spn**	Mwt (mg)	Spn	Mwt (mg)	Spn	Mwt (mg)	Spn	Mwt (mg)	Spn
Glucose	254.0 ±0.50	+	292.5 ±0.81	++	310.0 ±0.61	++++	296.0 ±1.15	++++	272.0 ±1.00	++++
Sucrose	215.5 ±0.76	++	266.7 ±0.87	++	289.0 ±1.00	+++	270.0 ±0.81	+++	246.0 ±0.58	+++
Mannitol	124.0 ±0.58	+	176.3 ±0.65	++	206.0 ±0.72	+++	188.0 ±0.58	+++	156.0 ±0.23	+++
Galactose	115.0 ±0.53	+	150.0 ±0.92	+	169.0 ±0.81	++	145.0 ±0.87	++	121.5 ±0.76	++
Control	9.4 ±0.92	-	12.0 ±1.15	-	15.8 ±0.42	-	18.0 ±0.55	-	23.5 ±0.87	-
CD at 5%	5.69		4.37		6.66		5.07		6.76	

*Mwt =Mycelial dry weight; Spn = sporulation

** Mean of three replicates; Data after ± represents standard error.

Among the seven nitrogen sources tested (Table 2), maximum mycelial growth (384.5 mg) of *C. eragrostidis* was found in the basal media supplemented with organic nitrogen source peptone. Other organic nitrogen sources also showed better growth than the inorganic nitrogen sources tested. Sporulation was poor in all the nitrogen sources tested and no sporulation was recorded in the basal media. *Alternaria protenta*, a pathogen of sunflower showed abundant sporulation on glucose peptone agar and leonien agar but not on dextrose nitrate agar (Wu and Wu, 2003). Holb and Chauhan (2005) observed that peptone was the best nitrogen source that produced quickest growth of *Monilia polystoma*. Saha *et al.* (2008) suggested the suitability of peptone and potassium nitrate as organic and inorganic nitrogen sources respectively for the growth of *L. theobromae*.

Table 2. Mycelial growth and sporulation of *C. eragrostidis* in different nitrogen sources

Nitrogen source	Incubation periods									
	5 days		10 days		15 days		20days		25 days	
	Mwt (mg)*	Spn**	Mwt (mg)	Spn	Mwt (mg)	Spn	Mwt (mg)	Spn	Mwt (mg)	Spn
Inorganic										
Potassium nitrate	144.0 ±1.00	+	172.0 ±0.64	+	215.0 ±0.53	++	199.0 ±0.72	++	187.0 ±0.53	++
Sodium nitrate	120.0 ±0.58	-	155.0 ±0.58	+	189.0 ±0.72	++	199.0 ±0.71	+++	185.0 ±1.08	+++
Ammonium nitrate	105.0 ±0.71	+	133.0 ±0.92	++	175.0 ±0.90	+++	169.0 ±0.53	+++	150.0 ±0.90	+++
Ammonium sulphate	135.0 ±1.02	-	172.0 ±0.58	+	201.0 ±0.50	+++	188.0 ±1.15	+++	175.0 ±0.78	+++
Organic										
Peptone	315.0 ±0.49	+	358.0 ±1.15	++	384.5 ±0.87	+++	336.0 ±0.58	+++	269.0 ±0.60	+++
Yeast extract	155.0 ±0.75	+	224.0 ±0.58	+	265.0 ±0.50	++	207.5 ±0.76	++	144.0 ±0.82	++
Beef extract	210.0 ±0.95	+	290.2 ±0.42	+	355.0 ±0.69	++	329.0 ±0.81	++	272.0 ±0.79	++
Control	4.4 ±0.81	-	8.1 ±0.66	-	11.1 ±1.21	-	14.4 ±0.70	-	20.6 ±0.87	-
CD at 5%	5.99		8.06		5.92		5.78		5.812	

*Mwt =Mycelial dry weight; Spn = sporulation

** Mean of three replicates; Data after ± represents standard error.

The growth of *C. eragrostidis* was evaluated in potato dextrose broth adjusted to four different pH. Mycelial dry weight was maximum (235.0 mg) after 15 d when the fungi was grown in the media at pH 6.0 (Table 3). These results correlate with the work of Mendoza *et al.* (2005) where the influence of the composition of the culture media and the pH for the growth, sporulation and morphology of the conidia of *S. schenckii* was emphasized. Kang *et al.* (2003) observed that optimum growth of the phytopathogenic fungus *C. gloeosporioides* was around the pH 6.0. Thakare and Patil (1995) suggested that the optimum pH for growth of *C. gloeosporioides* was 4.1-6.8.

Table 3. Mycelial growth of *C. eragrostidis* in different pH

Medium of growth	pH	Mycelial dry weight (mg)*				
		5 days	10 days	15 days	20 days	25 days
PDB	5.0	156±0.53	160±0.85	195±0.32	172±0.26	140±0.71
	5.5	150±0.64	180±0.80	205±0.60	190±0.58	175±0.72
	6.0	154±0.20	210±0.40	235±0.30	220±0.26	199±0.58
	6.5	160±1.11	183±0.11	213±0.56	198±0.26	185±0.60
CD at 5%		6.44	5.44	4.18	3.20	5.69

*Mean of three replicates; Data after ± represents standard error.

C. eragrostidis was grown in different temperatures to find the optimum temperature of growth. From the results (Table 4) the optimum temperature of growth of the fungi was recorded to be 25°C.

Table 4. Mycelial growth of *C. eragrostidis* in different temperature

Medium of growth	Temperature (°C)	Mycelial dry weight (mg)*
PDB	0°C	40.60±0.83
	5°C	127.50±1.04
	10°C	188.40±0.78
	15°C	210.00±0.72
	20°C	253.80±1.13
	25°C	290.50±0.78
	30°C	225.00±0.53
	35°C	130.50±0.55
	40°C	77.60±0.50
	45°C	22.50±0.29
CD at 5%		5.34

*Mean of three replicates; Data after ± represents standard error.

Since spore germination is a determining factor at the onset of host colonization by a fungal pathogen, several studies were undertaken to evaluate the influence of environmental factors like pH, temperature, incubation periods etc. on the germination of spores *in vitro*. *C. eragrostidis* showed maximum spore germination (88.80%) and germ tube elongation (46.40 mm) after 24 hours of incubation (Fig.3).Germination started within 2 hours in case of *C. eragrostidis*. *In vitro* studies with the spores of *B. carbonum* showed that germination started between 2-4 hours (Saha and Chakraborty 1990). The maximum spore germination (76.23%) and germ tube elongation (53.00 mm) were found at 25 °C. At 45 °C spore germination of *C. eragrostidis* was completely inhibited (Fig. 4). It was observed by Saha and Chakraborty (1990) that spore germination of *B. carbonum* reduced to 27% when pretreated at 50 °C but pretreatment at 0 °C for even 12 hours had no effect on germination and germ tube elongation. The high temperature also reduced the viability of spores (Flett and Wehner, 1989). Achar (2000) reported that conidia from *Stenocarpella maydis* exposed at temperatures below 22 °C germinated only after 17 hours of incubation and rate of germination increased from 22-27 °C, after which the germination rate declined.

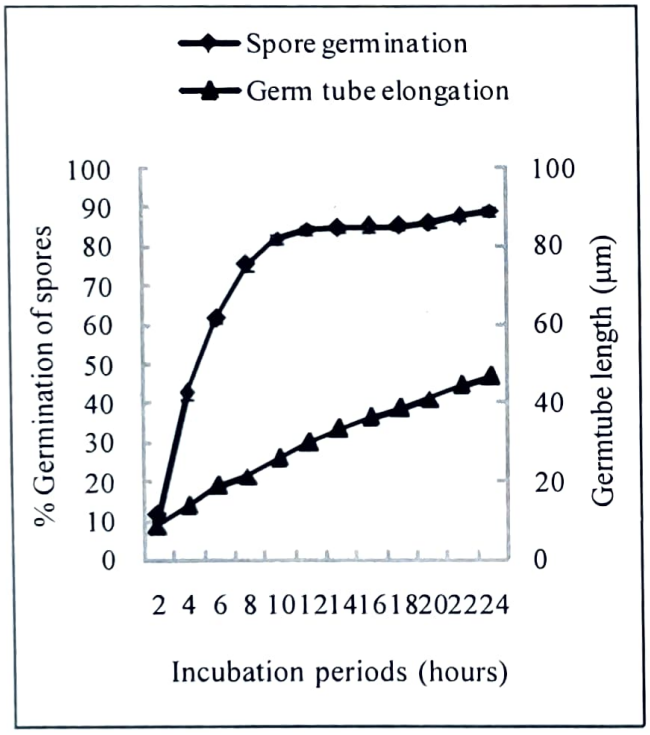

Fig 3. Percent germination of spores and germtube elongation of *C. eragrostidis* in different incubation periods (hours)

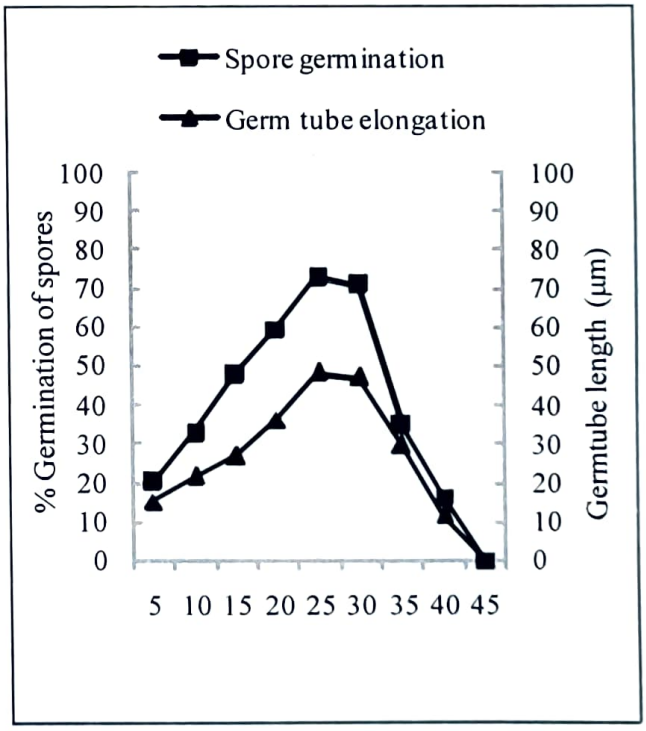

Fig 4. Percent germination of spores and germtube elongation of *C. eragrostidis* in different temperatures

Table 5. Spore germination and germ tube elongation of *C. eragrostidis* at different pH after 24 hours

pH	Percent germination when control raised to 100	Germ tube length (μm)
4.00	40.90±0.97	30.00±0.50
6.00	44.23±0.62	46.40±0.70
6.50	47.16±0.58	47.50±0.36
6.75	55.55±0.29	49.20±0.40
7.25	83.15±0.44	50.40±0.30
9.0	26.60±0.83	29.20±0.42
CD at 5%	4.83	2.23

*Mean of three replicates; Data after ± represents standard error.

It has been established that pH has some role on spore germination behavior of *C. eragrostidis*. Highest Spore germination (83.15 %) and germ tube elongation (50.40 mm) were recorded at pH 7.25 (Table 5). Saha and Chakraborty (1990) reported that pH 6.75 was best for germination of spores of *B. carbonum* while pH 7.2 was best for germ tube elongation. More than 97% of conidia of *Basidiobolus ranarum* germinated at pH range of 7-9 (Callaghan, 1974).

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Ethnic uses of some pteridophytic weeds of tea gardens in Darjeeling and Terai

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Abstract

Pteridophytes occupy a recognisable position in the flora of Terai and the hills of Darjiling. They are also well represented as weed in the Tea Gardens of this area. Recent survey recorded the occurrence of 86 species of pteridophytes from nine such gardens covering both Terai and Darjiling Hills. Of these, 25 species (30%), covering 21 genera and 20 families, are used by the Tea Garden workers in different manner like (i) 10 species as food, (ii) 02 species for fermenting traditional liquor, (iii) 01 species as fodder and (iv) 22 species as medicine for human diseases. In addition, some of these plants are used for basketry, playing carom, ceremonial decoration, ornamentals etc.

Keywords: Ethnobotany, Terai, Darjiling Hills, Pteridophytes.

Introduction

The documentation of ethnic uses of plants and other natural resources is extremely important (Al Corn 1995). Man, during his survival within the natural surroundings learnt the uses of innumerable types of natural resources to meet up his wide range of needs like food, medicine, house building materials, religious articles, fodder for his pets, ornamental and decorative articles, etc. But, in the present developing society the existence of traditional knowledge is becoming endangered. It is also now realized that in this 21st century the traditional knowledge will play a big role in health care and that includes mainly food, medicine and cosmetics (Pushpangadan 2002).

In addition, even today there are thousands of human settlements in different corners of this planet where facilities of modern civilization are yet to arrive or difficult to utilize. And, in those areas people are surviving with the help of their traditional knowledge on the uses of local resources. But, in most of the places, around the world, the inroad of industry and market dependent life style is forcing the local people to forget or to give up their traditional practices (Cotton 1996).

Three T's, "*Tea, Timber & Tourism*" formed the economic backbone for the people of Darjiling district of West Bengal, Terai and Duars. With the steady decline of forest cover timber economy is minimized and the tourism depends on numerous factors. But, the number of Tea Gardens in this area is increasing even today. A very large proportion of area of Darjiling, Terai and Duars are covered with Tea Gardens though most of these areas were basically forest covered. That means, Tea Gardens have replaced basically biodiversity-rich vegetation (Das 2004).

After the replacement of natural vegetation with tea plantations many local species tried to survive within the gardens as weeds. Recent survey on Tea Garden weeds in Darjiling Hills recorded the existence of large number plants. And, the ethnobotanical part of the survey revealed

the usefulness of such weeds (Ghosh *et al* 2004). The distribution and harm/usefulness of tea weeds has attracted the attention of many other workers like Dutta (1983), Haridas & Sharma (1972), Haridas & Venkataramani (1972), Harikrishnan (1978), Mustafee (1972, 1981, 1988, 1998), and Ramachandran (1978).

Tea Gardens need a big workforce to maintain it's all the activities and numerous people belonging to different tribal communities like Santhals, Oraons, Mundas from outside and different local groups like Rajbansies, Lepchas and Nepali communities work in these gardens round the year. Most of these TGs are situated in remote areas where the basic amenities of modern civilization are unavailable and in addition, they are very poorly paid. And, that is why they are quite dependent on local natural resources for their survival. As part of their daily needs they use numerous local plants, majority of which are weedy and, in general, are not directly useful in the civilised society (Ghosh 2006). The present study was undertaken to record the ethnic knowledge of some pteridophytic weeds of Darjeeling Tea Gardens and the understanding of these people with the local species of plants.

Study Area

The present study was undertaken in nine tea gardens of Darjiling District, namely:
(1) *Gungaram Tea Estate* [±122 m amsl; 26° 37' 409" N Latitude & 88° 18' 167" E Longitude],
(2) *Hansqua Tea Estate* [±125 m amsl; 26° 37.784' N Latitude & 88° 19.068' E Longitude],
(3) *Matigara Tea Estate* [±130 m amsl; 26° 42' 500" N Latitude & 88° 22' 142" E Longitude]
(4) *Atal Tea Estate* [±152 m amsl; 26° 40' 576" N Latitude & 88° 13' 082" E Longitude],
(5) *Kamalpur Tea Estate* [±154 m amsl; 26° 42.341' N Latitude & 88° 18.428' E Longitude],
(6) *Mohorgong & Gulma Tea Estate* [±154 m amsl; 26° 47.203' N Latitude & 88° 22.866' E Longitude], (7) *Makaibari Tea Estate* [± 1100 m amsl; 26° 62' 59" N Latitude & 88° 16' 43" E Longitude], (8) *Soom Tea Estate* [± 1200 m amsl; 27° 04 590/ N Latitude & 88° 13 723/ E Longitude] and (9)*Tamsong Tea Estate* [±1300 m amsl; 27°02.318/ N Latitude & 088°09.992/ E Longitude]. A GPS (GARMIN 12CX) was used to determine the location of these gardens. The work was done during 2002 – 2004.

Materials and methods

During the present survey, wide range of ethnobotanical information was recorded from the resourceful persons with the help of local field guides, local herbal practitioners, vendors, priests, spiritual healers known as *Jhankri* (in general) and experienced senior rural folks of different ethnic communities in workers colonies and also from nearby villages. Also the local people of different age groups, sex and profession were interviewed following a prepared questionnaire. Most of the common people provided information on various types of uses of local plants like edible, fodder-producing, dye yielding, thatching, etc. herbal practices, on the other hand shared their knowledge on herbal drugs.

All the voucher specimens were spotted and collected by these people and were recorded in the field note book along with many other information like (i) vernacular name, (ii) useful part, (iii) purpose of use, (iv) other ingredients etc. All the collected specimens were processed and identified in the Taxonomy & Environmental Biology Laboratory of the Department of Botany, University of North Bengal and are preserved in the NBU-Herbarium. However, Jain (1981,

1987, 1991); Rai *et al* (1998); Rai & Bhujel (1999), Rai (2002) among others, were followed for overall methodology.

Results

As much as 86 species of pteridophytes has been recorded from the Tea Gardens under study (Ghosh 2006). Of these 25 species has been recorded to use by these people. The plants and their uses have been enumerated below:

Adiantum capillus-veneris L. [ADIANTACEAE]

Exiccatus: Makaibari TE, **AP Das & Chandrâ 1966**, dated 30.06. 2003

Used as medicine in pectoral demulcent and as expectorant and tonic. It is boiled in wine that is given in cases of hard tumours of the spleen, liver and other viscera.

Adiantum philippense L.; *Adiantum lunulatum* N. Burman [ADIANTACEAE]

Exiccatus: Tamsong TE, **AP Das & Chandrâ 2398**, dated 05.11. 2003.

Used in blood diseases, in epileptic fits and in rabies; rhizomes prescribed for strangery and in fever due to elephantiasis. Fronds are burnt in oil and used to pacify itching.

Angiopteris crassipes Wallich *ex* C. Presl; *Angiopteris evecta sensu auct. multi.* Beddome [MARATTIACEAE]

Exiccatus: Makaibari TE, **AP Das & Chandrâ 2821**, dated 23.02. 2004.

Massive short stem, full of starch, is edible and form the basis of an intoxicating drink. Leaves used as head dresses and temporary beds. Stem is reported to be very effective in curing white patches on skin.

Asplenium filix-femina var. ***parasnathensis*** Bernham [ASPLENIACEAE]

Exiccatus: Tamsong TE, **AP Das & Chandrâ 2431**, dated 05.11. 2003.

Anthelmintic, Diuretic, Poultice, female diseases; relieving labour pains; internal ailments such as cancer of the womb; general body pains; breast pains caused by childbirth and to induce milk flow in caked breasts; externally to heal sores; liquid root extract is an effective anthelmintic.

Blechnum orientale L.; *Asplenium orientale* (L.) Bernhardi [BLECHNACEAE]

Exiccatus: Kamalpur TE, **AP Das & Chandrâ 1183**, dated 18.10.2002.

Rhizome edible and also used in urinary disorders and as an anthelmintic; poultice applied to boils.

Ceratopteris thalictroides (L.) Brongniart; *Acrostichum thalictroides* L. [PARKERIACEAE]

Exiccatus: Hansqua TE, **AP Das & Chandrâ 1664**, dated 13.11. 2002.

Vegetative fronds are edible both as green salad and after cooking as potherb.

Dicranopteris linearis (N. Burman) Underw.; *Polypodium lineare* N. Burman [GLEICHENIACEAE]

Exiccatus: Tamsong TE, AP **Das & Chandrâ 2299**, dated 05.09. 2003.

Rhizomes anthelmintic; fronds used in asthma; rachis used for making mats, chairs, seats, pouches,

caps, fishing traps, baskets, belts etc. Fronds are ingredients for making local beverages.

Diplazium esculentum (Koenig *ex* Retzius) Swartz; *Hemionitis esculenta* Koenig ex Retzius [ATHYRIACEAE]

Exiccatus: Mohurgong & Gulma TE, **AP Das & Chandrâ 0009**, dated 27.01.2002; Makaibari TE, **AP Das & Chandrâ 1861**, dated 10.06. 2003.

Young unopened fronds eaten in salad or as cooked vegetable.

Dryopteris filix-mas (L.) Schott; *Polypodium filix-mas* L. [DRYOPTERIDACEAE]

Exiccatus: Hansqua TE, **AP Das & Chandrâ 1153**, dated 14.06.2002.

Young fronds cooked and eaten. Roots anodyne, antibacterial, anti-inflammatory, antiviral, astringent, febrifuge, vermifuge, worm-expellant; checking internal haemorrhage, uterine bleeding, mumps and feverish illnesses. Pregnant women and people with heart complaints should not consume this plant.

Equisetum diffusum D. Don [EQUISETACEAE]

Exiccatus: Tamsong TE, **AP Das & Chandrâ 2229**, dated 05.09. 2003; Soom TE, **AP Das & Chandrâ 3294**, dated 26.06. 2004.

Used as clotting agent in nose bleeding, wounds and coughing up of blood. Also used in bleeding urinary tract, cystitis and prostrate diseases due to its astringent property. It has cooling effect in gonorrhea.

Equisetum ramosissimum Desf. Subsp. ***debile*** (Roxburgh *ex* DC.) Hauke; *Equisetum debile* Roxburgh *ex* Vaucher [EQUISETACEAE]

Exiccatus: Mohurgong & Gulma TE, **AP Das & Chandrâ 0033**, dated 27.01.2002; Tamsong TE, **AP Das & Chandrâ 1944**, dated 30.04. 2003.

Used as refrigerant and given in gonorrhoea.

Hypolepis punctata (Thunberg) Mettenius *ex* Kuhn; *Polypodium punctatum* Thunberg [HYPOLEPIDACEAE]

Exiccatus: Makaibari TE, **AP Das & Chandrâ 2850**, dated 25.03. 2004.

Fronds used as poultice on boils.

Lycopodiella cernua (L.) Pichi-Sermolli; *Lycopodium cernuum* L. [LYCOPODIACEAE]

Exiccatus: Tamsong TE, **AP Das & Chandrâ 3014**, dated 10.04. 2004; Soom TE, **AP Das & Chandrâ 3459**, dated 12. 10. 2004; Mohurgong & Gulma TE, **AP Das & Chandrâ 1525**, dated 22.10. 2002.

Decoctions used in lotions in beriberi, cough and uneasiness in the chest; embrocation of ashes in vinegar recommended for skin eruption. Plants used for stuffing pillows after drying.

Lycopodium pseudoclavatum Ching; *Lycopodium clavatum auct non* L. [LYCOPODIACEAE]

Exiccatus: Soom TE, **AP Das & Chandrâ 3392**, dated 12. 10. 2004; Tamsong TE, **AP Das & Chandrâ 1956**, dated 30.04. 2003.

Spores are used as dusting powder for playing carom; used against wounds, cracks and fissures. The plants are formed a part of the ceremonial and religious decoration. It is also to stop haemorrhage after childbirth.

Lygodium japonicum (Thunberg) Swartz; *Ophioglossum japonicum* Thunberg [LYGODIACEAE]

Exiccatus: Tamsong TE, **AP Das & Chandrâ 2885**, dated 10.04. 2004; Hansqua TE, **AP Das & Chandrâ 1381**, dated 20.10.2002.

Used as expectorant, decoction of fertile fronds as diuretic and cathartic.

Lygodium microphyllum (Cavan) R. Brown; *Ugena microphyllum* Cavan [LYGODIACEAE]

Exiccatus: Mohurgong & Gulma TE, **AP Das & Chandrâ 0975**, dated 04.05. 2002; Hansqua TE, **AP Das & Chandrâ 0254**, dated 09.02.2002.

Young leaves eaten; used as poultice in skin diseases, swellings; decoction used in dysentery; old rachis for basket making and plaiting.

Lygodium salicifolium C. Presl; *Lygodium flexuosum sensu* Beddome [LYGODIACEAE]

Exiccatus: Mohurgong & Gulma TE, **AP Das & Chandrâ 0014**, dated 27.01.2002; Hansqua TE, **AP Das & Chandrâ 0172**, dated 03.02.2002; Tamsong TE, **AP Das & Chandrâ 2134**, dated 30.04. 2003.

Used as expectorant, fresh roots applied externally against rheumatism, sprain, scabies, eczema, wounds and particularly for carbuncles.

Marsilea quadrifolia L. *Marsilea crenata* Presl. [MARSILEACEAE]

Exiccatus: Kamalpur TE, **AP Das & Chandrâ 0457**, dated 17.04.2002.

Leaves eaten as vegetable. Also, used against insomnia.

Nephrolepis auriculata (L.) Trimen; *Polypodium auriculatum* L., *Nephrolepis cordifolia sensu auct. pl., non* (L.) Presl [NEPHROLEPIDACEAE]

Exiccatus: Makaibari TE, **AP Das & Chandrâ 2473**, dated 11.11. 2003.

Root-tubers are edible and taken as remedy of jaundice; decoction of the freshly collected fronds given in cough; fronds used in decoration.

Odontosoria chinensis (L.) J. Smith; *Trichomanes chinense* L. [LINDSAEACEAE]

Exiccatus: Soom TE, **AP Das & Chandrâ 3596**, dated 12. 10. 2004.

Prescribed in chronic enteritis.

Onychium siliculosum (Desvaux) C. Christensen; *Pteris siliculosa* Desvaux [TAENITIDACEAE]

Exiccatus: *Golden Fern*

Decoction of the fronds used in dysentery.

Pityrogramma calomelanos (L.) Link; *Acrostichum calomelanos* L. [HEMIONITIDACEAE]

Common Name: *Silver Fern*

Exiccatus: Soom TE, **AP Das & Chandrâ 3100**, dated 03.05. 2004

Constituent of a decoction used in kidney troubles. Rhizomes used as an anthelmintic and leaf smoked for colds in head and chest.

Pteridium aquilinum (L.) Kuhn subsp. ***aquilinum*** var. ***wightianum*** (J. Agardh) Tryon [PTERIDIACEAE]

Exiccatus: Mohurgong & Gulma TE, **AP Das & Chandrâ 0317**, dated 16.02. 2002; Tamsong TE, **AP Das & Chandrâ 2255**, dated 05.09. 2003.

Boiled rhizomes eaten during scarcity, or grounded into flour for making bread. Rhizomes used for brewing local beer. Young fronds used as vegetables, as soup, fodder and dried fronds as packing material. Rhizome astringent and anthelmintic; proves fatal when consumed more and solely.

Selaginella bisulcata Spring [SELAGINELLACEAE]

Common Name: *Spike Moss*

Exiccatus: Soom TE, **AP Das & Chandrâ 2640**, dated 27.12. 2003.

Used for decoration. Uprooted plants are sold in Indian markets as resurrection plant and as ornamental. It is reputed as '*Sanjeevani*' and is sold during summer as cooling agent.

Tectaria coadunata (J. Smith) C. Christensen [TECTARIACEAE]

Exiccatus: Soom TE, **AP Das & Chandrâ 3204**, dated 26.06. 2004; Tamsong TE, **AP Das & Chandrâ 2167**, dated 30.06. 2003.

Used against acute cases of diarrhoea in children and other stomach troubles and eaten as salad.

Discussion

Recorded 25 species of pteridophytes with ethnic use are coming under 21 genera and 20 families. Of these only three families (Equisitaceae, Lycopodiaceae and Selaginellaceae) are not ferns. These three families are represented by four genera and five species. Except Lycopodiaceae (*Lycopodiella* and *Lycopodium*) all other families are represented by one genus only. Among the genera *Lygodium* is represented with three species and two other genera, *Adiantum* and *Equisetum* are represented with two species. All the remaining 22 genera are represented with one species only.

At least ten of these 25 pteridophytes are edible in different form.Young fronds of *Diplazium esculentum* and *Tectaria coadunata* are marketed in huge quantity. In addition, two plants are used for the production or fermenting of local traditional liquor. On the other hand only one plant is used as fodder. However, a much larger number of 22 plants are recorded to have medicinal uses. An wide range of diseases and/or symptoms are treated with these plants and covers diseases like rabbis, elephantiasis, many female diseases, cancer in womb, gonorrhoea, mumps, beriberi, dysentery, carbuncle, insomnia, kidney trouble etc. In addition some plants are also used as anthelmintic, antibiotic, antiviral, expectorant, vermifuge, analgesic etc. However, tribal tea garden workers suggested that *Pteridium aquilinium* should not be consumed for a long period and should not be eaten by pregnant women as it may produce lethal effects.

While *Lycopodiella cernua* is used for stuffing pillow, *Lycopodium pseudoclavatum* spores are used for playing carom.Two species, *Dicranopteris linearis* and *Lygodium microphyllum* are used for basketry and similar other works. *Dicranopteris linearis* is also used for making fishing-traps.

Nephrolepis auriculata and *Selaginella bisulcata* are used as ornamental and plants like *Lycopodium pseudoclavatum* used as ceremonial and religious decoration. And, *Selaginella bisulcata* is used as a coolant.

Now it can be conclude that over 30% pteridophytic tea garden weeds are useful for the society as a considerably large population is using those plants for over a hundred years of their settlement in the tea garden areas of Darjiling and Terai.

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Antagonism of fluorescent *Pseudomonas* BRL-1 against *Curvularia lunata*

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Abstract

A potato rhizospheric isolate, fluorescent *Pseudomonas* BRL-1, showed *in vitro* antagonistic activity against *Curvularia lunata*. Microscopic studies of the mycelium after antagonism illustrated hyphal shriveling, swelling, vaculation, short branching and granulation of cytoplasm resulting in lysis of hyphae of *C. lunata*. Association of antagonistic property of the isolate has been found to be coupled with chitinolytic and proteolytic activity as well as by the production of indole acetic acid and siderophore.

Keywords: *fluorescent Pseudomonas BRL-1, Curvularia lunata*

Introduction

Curvularia lunata is an important seed and soil-borne plant pathogen distributed throughout the world. The pathogen mediate leaf spot of *Citrus* sp., *Cymbopogon citrates*, *Musa paradisica*, *Sorghum vulgare* (Chaudhury, 1936; Agarwal and Beliram, 1960; Subramonium, 1953) fruit spot of *Mangifera indica*, *Psidium guajava* (Srivastava *et al.*, 1964; Tilak and Rao, 1968), Karnels spot of *Oryza sativa* (Padmanavan, 1949), cotyledon spot of soybean (Muchovej *et al.*, 1988) blight of Zoysiagrass (Roberts and Tredway, 2008). The species of *Curvularia* along with *Fusarium* pathogen causes viability loss up to 100% in sorghum (Christopher and Clint, 2003). Infection by *C. lunata* in human beings results in allergic fungal sinusis, broncho pulmonary fungal diseases and fatal cerebral phaeohyphomycosis (Rohwedder, *et al.*, 1979; Berry *et al.*, 1981; Carter and Boudreaux, 2004).

Florescent pseudomonads have drawn attention worldwide due to the ability of production of secondary metabolites viz. siderophore (Neilands, 1981), antibiotics (O'Sullivan and O'Gara, 1992; Keel *et al.*, 1992) volatile compound, HCN (Wei *et al.*, 1992) enzymes, phytohormones (O'Sullivan and O'Gara, 1991; Keel *et al.*, 1992) and rhamnolipids (Stanghellini and Miller, 1996). These have been implicated in reduction of plant pathogenic fungi and harmful rhizobacteria with simultaneous induction of growth of crop plants. Biological control of plant diseases with bacterial antagonists is a potential alternative to chemical control, because chemical control is expensive and hazardous to ecosystem. Taking it as an objective, an attempt has been made to isolate a potential antagonistic organism having lethal effect on this pathogenic fungi *C. lunata*.

Materials and Methods

Organisms

The fungal pathogen was obtained from Molecular and Applied Mycology and Plant Pathology Laboratory.

The antagonist was isolated from the rhizosphere of potato and its biocontrol competence was proved against several phytopahogens including *Fusarium oxysporum* (Sen *et al.*, 2005) *Sclerotium rolfsii* (Sen *et al.*, 2006a), *Aspergillus niger* (Sen *et al.*, 2006b), *Mucor hiemalis* (Sen *et al.*, 2006c), *Erwinia carotovora* (Sen *et al.*, 2006d) *Alternaria alternata, Myrithecium rodidum, Colletrotrichum capsisi* (Sen *et al.*, 2006e) etc. The antagonist was subcultured and maintained on TSA medium for subsequent use.

Interaction of fluorescent Pseudomonas BRL-1 against C. lunata in dual solid and liquid culture

During *in vitro* antagonistic study, dual culture technique was performed on peptone glucose agar (PGA) solid (Skidmore and Dikinson. 1976) and liquid (Basha and Ulaganathan, 2002) media. In the former experiment bacterial isolate was streaked on PGA plate in a circular / O shaped and semicircular / U shaped pattern. In the later case 50 ml of PG broth was inoculated with 1ml the bacterial suspension (10^7 cfu ml^{-1}). Then spore suspension of *C. lunata* was subsequently point inoculated at the center of O or U shaped region on the PGA plate and in dual liquid culture technique PG broth was reinoculated with mycelial mat (5mm diameter) of 3 days old culture of *C. lunata*. Sole inoculation with pathogen in both solid and liquid media was served as control. Plates were incubated for 5 days at 30°C and inhibition of colony growth was measured and compared with the control, where as broths were kept at 30°C for 72 hours in a rotary shaker and the mycelial dry weight were estimated. The liquid culture was filtered through pre weighted Watman No. 1 filter paper and was dried for 24 h at 70°C and compared with the control set. Microscopic studies were also performed to detect physical and / or morphological changes of mycelia.

Mode of action of fluorescent Pseudomonas BRL-1

To investigate the inhibitory effect of fluorescent *Pseudomonas* BRL-1 a range of experiments were performed. For the production of volatile compound 'inverted plate technique' was followed (Dennis and Webster, 1971). Spores of fungal pathogens were point inoculated on the centre of the petriplate containing 20 ml of PGA. A loopful of 2 days old fluorescent *Pseudomonas* BRL-1 culture was strike on petriplate having 20 ml of PGA then inverted over the plates inoculated with the fungal pathogen. Two plates were sealed together (mouth to mouth) with parafilm, control plates consist of fungal pathogens inverted over uninoculated PGA plate. The plates were incubated at 30°C and three replicates were maintained for each treatment. After 5 days of incubation colony diameter of the pathogen was measured and compared with the control.

Production of hydrogen cyanide was tested qualitatively according the method of Wei et al., (1991). The antagonist was inoculated in TSA medium supplemented with amino acid glycin (4.4 g l^{-1} of medium). A strip of sterilized filter paper saturated with a solution containing picric acid 0.5% (yellow) and sodium carbonate (2%) was placed in the upper lid of the petridish. The petridishes were then sealed with parafilm and incubated at 30°C for 4 days. A change of colour of the filter paper strip from yellow to light brown, brown or reddish brown was recorded as weak, moderate or strong cyanogenic potential, respectively.

To test the chitinolytic property of the isolate it was inoculated on LB medium (Gunasekaran, 1995) supplemented with 0.5% colloidal chitin as principal source of carbon. Plates were incubated at 30°C for three days. Formation of a clear halo region around the colonies indicates chitinase activity of the strain (Basha and Ulagnathan, 2002).

IAA production was quantified spectrophotomertically, growing the screened fluorescent *Pseudomonas* in 10 ml of minimal salt media supplemented with 100 mg ml^{-1} of tryptophan, and incubated at 30°C under shaking for 48 h. Broth culture was centrifuged at 7500 rpm for 10 min. To 1 ml of culture supernatant, 2 ml of Salkovsky reagent was added and incubated at 30°C for 25 min. Absorption was read at 530 nm and levels quantified from standard curve of IAA (Gaur et al., 2004).

Chrome Azurol S (CAS) agar medium was prepared as described by Schwyn and Neilands (1987) to detect the siderophore production. CAS agar (blue agar) was inoculated at the center of the plate with 24 h old fluorescent fluorescent *Pseudomonas* BRL-1 and kept for incubation at 30°C for 72 h. The change of the blue colour of the medium to orange or presence of yellow to light orange halo surrounding the bacterial colony indicates the production of siderophore.

Chemical assay to detect the nature of siderophore

Tetrazolium test were performed to detect the hydroxamate nature. Triphenyltrtrazolium chloride (about 1 mg) was dissolved in a drop of culture supernatant of fluorescent *Pseudomonas* BRL-1, on a tile and a drop of 2 *N* of NaOH added. Immediate development of deep red colour was taken as a positive reaction (Snow, 1954).

Arnow's test was performed to detect catecholate siderophore. This nature of siderophore on reaction, in succession with nitrous acid, molybdate and alkali, yield a pink chromogen that absorbs maximally at 515 nm (Arnow, 1937).

Shenker's spectophotometric test for the detection of carboxylate siderophore. The copper complex formed was observed for absorption maxima between 190-280 nm. There is no specific wavelength at which the copper complex is absorbed. The entire wavelength 190-280 nm was scanned to observe the peak of absorption of siderophore (Shenker, 1992).

Production of hydrolytic enzyme was qualitatively assayed in minimal medium containing gelatin, starch, pectin and carboxymethyl cellulose (CMC) for protease, amylase, pectinase and cellulase respectively. Plates were incubated for 48 h at 30°C and formation of clear zone around bacterial colonies was read as positive (Gaur *et al.*, 2004).

Results and Discussion

During dual culture, considerable growth inhibition of *C. lunata* by fluorescent *Pseudomonas* BRL-1 was observed both in solid and liquid culture. In solid culture, mycelial growth was restricted near bacterial streak and continued away from it, as evident from figure 1A-1C. Increase in incubation period was proportionate to growth inhibition of *C. lunata* upto 5 days (Fig. 1F). Microscopic study of the mycelia from the interacting zone showed hyphal shriveling, mycelia deformities, swelling, fragmentation, short branching and finally resulting into lysis (Fig. 1E). Co-culture of *C. lunata* and fluorescent *Pseudomonas* BRL-1 in liquid media resulted in 69 percent reduction in the mycelial dry weight when compared

Table1. Different secondary metabolites production and enzymatic activity of fluorescent *Pseudomonas* BRL-1

Different Metabolites	Rate of Production
Siderophore production	+++
Antibiotic Substances	-
HCN production	-
Volatile substances production	-
Chitinase activity	+
Protease activity	++
IAA production	+++

'+++' Stronger production, '++' Moderate production, '+' Low production, '-' No production

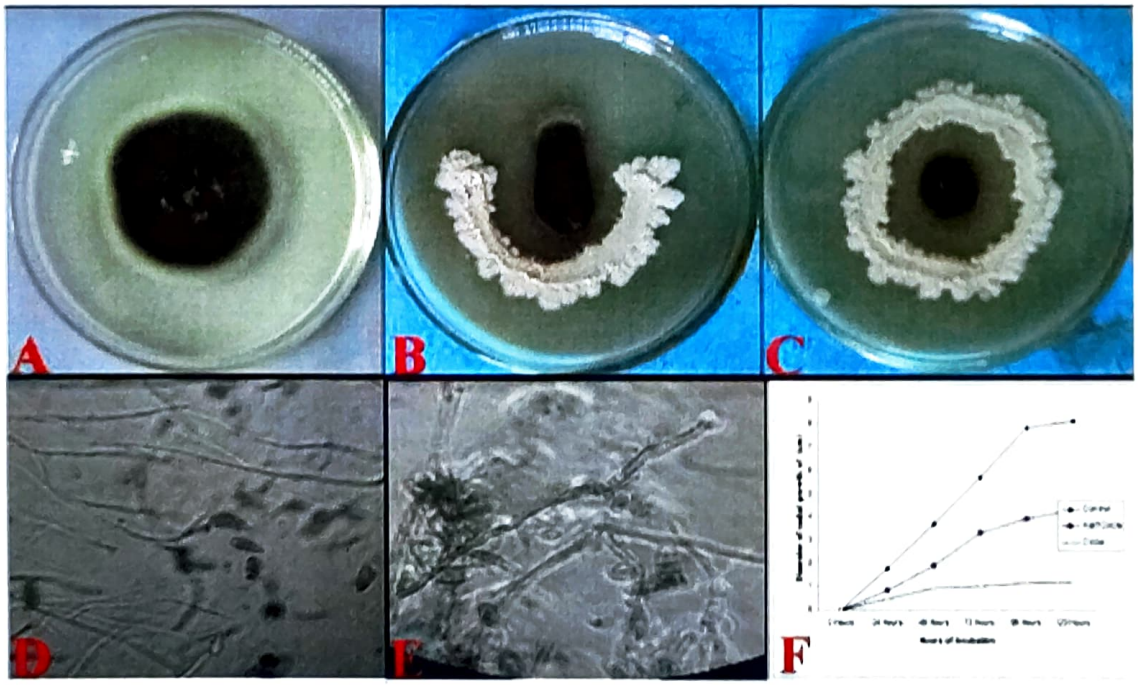

Fig 1. Inhibitory effect of fluorescent *Pseudomonas* BRL-1 on *Curvularia lunata*. (A) Control; *C. lunata* colony in pure culture showing more rapid growth at 30°C; (B) Colony of *C. lunata* showing restricted growth, but growing freely towards the top end away from the U shaped streak culture of the antagonist and (C) Colony *C. lunata* showing restricted growth due to the bacterium inoculated as a circular streak. (D) Microscopic observations of mycelium from set 'A' (control) (E) Microscopic observations of mycelium from set 'C' (treated) (F) Incubation period verses radial growth of the fungi in control and treated sets. Data represents the mean of triplicate sets of experiments.

to the control. Among different biochemical tests performed for the detection of inhibitory effect of fluorescent *Pseudomonas* BRL-1, the isolate showed significant protease, chitinase, IAA and siderophore producing activity (Table 1). The antagonist has been found to produce chitinase and protease, when it grown in chitin and gelatin media respectively, as a sole carbon source. Moreover, Lorito *et al.*, (1994) and Dunne *et al.*, (1997) reported earlier that the exposure of selected phytopathogenic fungi to lytic enzymes such as chitinase, protease could result in the degradation of the structural matrix of the fungal cell wall. So the reduction of mycelial dry weight might be coincided by the chitinase and protease activity. In general Indole acetic acid is universally accepted as a plant growth promoter. The level of IAA was quantified spectrophotomatrically. It was found that the isolate produce 24 $\mu g\ ml^{-1}$ IAA at 48 h of incubation, increase in incubation period was proportionate to gradual decline in the production level of IAA upto 6 days. Infact, Chirst and Mosinger (1989) reported that this phytohormone could also induce resistance through PR protein production; Sharaf and Farrag (2004) reported that IAA reduce spore germination, mycelial dry weight and protein content of the pathogenic fungi and thus prevent significantly any chance for disease induction by pathogens. Our study complements their findings by demonstrating significant production of IAA by fluorescent *Pseudomonas* BRL-1. The isolate did not produce hydrocyanic acid (HCN). Infact, it was reported that production of HCN proved to be deleterious to the plant (Alstrom and Burns, 1989) CAS agar (blue agar) plate assay indicated the hydroxamate nature of siderophore production by fluorescent *Pseudomonas* BRL-1 as indicated by orange halo around the colony. This colour change is based on the principle that the blue colour of the CAS medium is due to the Fe-dye complex and when siderophore is produced by the fluorescent *Pseudomonas* BRL-1 the iron is released from the Fe-dye complex resulting in the change in colour to orange which indicate the siderophore production. The isolate produced hydroxamate siderophore as evidenced by positive tetrazolium test and negative in case of Arnow and Shenker's tests. Siderophore have been shown to play a role in increased growth response of certain plants to treat the planting material with fluorescent pseudomonads (Ahl *et al.*, 1986; Kloepper and Schroth, 1981; Scher and Bakker, 1982). The response is thought to involve suppression of deleterious rhizospheric microorganism (Ahl *et al.*, 1986; Kloepper and Schroth, 1981; Schippers *et al.*, 1987).

Thus the present study concluded that the fluorescent *Pseudomonas* BRL-1 showed considerable antagonistic property through combined and / or individual effect of siderophore, proteolytic enzyme, IAA and chitinolytic activity. Considerable attention has been paid to plant growth promoting rhizobacteria (PGPR), as the best alternative to chemicals to facilitate eco-friendly biological control of soil and seed borne pathogen. These observations and further study might help in developing this PGPR (fluorescent *Pseudomonas* BRL-1) as a potential biocontrol agent against *C. lunata.*

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