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Application of molecular markers in plant genome study

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Abstract
The development of molecular techniques for genetic analysis has led to a great increase in our knowledge of plant genetics and our understanding of the structure and behaviour of plant genome. During last three decades, several powerful DNA based marker technologies have been developed for the assessment of genetic diversities and molecular marker assisted breeding technology. In plant systems, the prospects of DNA profiling and fingerprinting is becoming indispensable in the context of establishment of molecular phylogeny, assessment of somaclonal variants, characterization of plant genomics, marker-based gene tags, map-based cloning of agronomically important genes, variability studies, synteny mapping, marker-assisted selection of desirable genotypes etc. In this review article, various molecular markers are reviewed with emphasis on specific areas of their application in higher plants.

Keywords: Molecular markers, Plant genome, DNA fingerprinting

Identification of different genotypes of crop species and varieties is important particularly when new crop varieties are to be released, different accession of wild species are to be characterized and purity of germplasm is to be determined. For a long time such identification has been based on the phenotypic differentiation and morphological features which are indicative of the genotype like maturity of grain in wheat (Hu *et al.*, 1996), morphology of leaves, flowers and grain quality in rice (Villareal *et al.*, 1999; Lee *et al.*, 1999; Mandal *et al.*, 2000). Though it can provide unique description of cultivated varieties, however, morphological characters are represented only by a few loci because there are not a large enough number of characters available. Moreover, these characters are also affected by the environment and growth practices. To have an accurate and reliable estimate of genetic relationship and diversity, a large number of polymorphic markers are essentially required. Similarly, introgression lines, which are sometimes different from the parent by only few base pair sequences, are difficult to differentiate from each other on morphological and phenotypic basis.

To circumvent such difficulties biochemical markers such as isozymes were developed and have been used successfully for the last few decades (Arus *et al.*, 1982; Sangwan *et al.*, 2003). Seed storage protein (Gupta and Robelen, 1986) and High Performance Liquid Chromatography (Buchler *et al.*, 1989) were also used for cultivar identification. Since the level of polymorphism detected through these techniques is low and tissue specific, therefore, their utilization has become limited.

With the advent of molecular markers, a new generation of markers has been introduced over the last two-three decades, which has revolutionized the entire scenario of biological sciences. DNA-based molecular markers have acted as versatile tools and have found their own position in various fields like taxonomy, physiology, embryology, genetic engineering, etc. In this article, DNA markers developed during the last three decades of molecular biology research and utilized for various applications in the area of plant genome analysis are reviewed.

1. Various types of markers

Polymorphism among the different genotypes of a species is the raw material for genome analysis. To be a genetic marker, the marker locus has to show experimentally detectable variations among the individuals in the test population. The variation can be observed at different biological levels, from the simple heritable phenotype to detection of variation of a single nucleotide. Once the variation is identified and the genotypes of all the individuals in the test population are known, the frequency of recombination events between loci is used to estimate the linkage distances between markers for the construction of a linkage map. Target traits or target genes in a segregating population can be identified with the assistance of linked genetic markers to accelerate traditional breeding programs. This process is known as marker assisted selection (Zheng *et al.*, 1995).

An ideal genetic marker should be highly polymorphic in nature, show co-dominant inheritance (which can be determined both the homozygous and heterozygous

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states of diploid organisms), occupy frequency in the genome, show selective neutral behavior (the DNA in the genome of any organism is neutral to environmental conditions or management practice), should be easily available, have an easy and fast assay with high reproducibility, show no effect of alternate alleles on the plant morphology and should have low or no interaction among markers allowing the use of many markers at the same time in a segregating population.

In genome analysis three types of markers have been used: Morphological markers, protein markers and DNA - based markers. High quality markers, based on the detection of polymorphism in proteins and DNA, have been developed during last three decades (Reviewed by Winter and Kahl, 1995; Mohan *et al.*, 1997; Joshi *et al.*, 1999). They have been termed as molecular markers (Tanksley, 1993). A molecular marker is confined to a discrete difference in the genetic information possessed by two individuals within a species that can be detected by molecular methods. Such markers can be detected by inferring the genotype on the basis of observed marker phenotype. The marker phenotype can be observed by assaying a primary to secondary product of a gene or by analyzing a specific DNA sequence.

The molecular markers have most or all the requisite properties of good genetic markers. For these reasons, their potential as tools for the plant breeding is much higher than that of morphological markers.

1.1 Morphological Markers

Early mapping studies concentrated on discrete traits with simple Mendelian inheritance, which often had one to one correspondence with the genes controlling the traits and phenotypic differences (polymorphisms) between parents were co-inherited. In such cases, morphological characters (phenotypes) can be used as reliable indicators for specific genes. Morphological characters have been the subject of numerous studies in population genetics and agriculture and used for the identification of species, families and genera. However, morphological markers are strongly influenced by environment and identification of morphological markers with each genotype is difficult. To distinguish true genotypic variation from phenotypic variation, special breeding programs and experimental designs are required. Besides these difficulties involved, a large number of morphological markers have been studied and mapped for maize, tomato, rice and many other plants. In the last two decades molecular markers have developed to circumvent some or all the problems associated with the morphologic markers.

1.2 Protein Markers

Protein polymorphisms are detected by separation through gel electrophoresis followed by specific staining of a discrete protein sub-class. Much of the detectable protein variations identify allelic sequence variations in the structural genes encoding the proteins. Alternately, some protein variation is due to post-translational modification. Two classes of proteins, isozymes and allozymes are used as markers. Isozymes are allelic variants of the some enzyme, generally encoded by different loci (Tanksley and Orton, 1983; Weeden *et al.*, 1988), while allozymes are different proteins encoded by different genes performing the same enzyme function.

Isozymes are the most commonly used of protein markers. Markert and Moller in 1959 introduced the term isozyme to define each one of the possibly many multiple forms of an enzyme existing in the same population of an organism. In isozyme analysis, a tissue extract is separated according to their net charge and size by electrophoresis using a starch or polyacrylamide gel. The gel is stained for a particular enzyme by a substrate and a dye under the appropriate reactions, resulting in bands (s) at the position to where the enzyme has migrated. Depending on number of loci, their stable of homo or heterozygosity in an individual, and the enzyme molecular configuration one to several bands are visualized. The positions of the bands are polymorphic and thus informative.

Isozyme linkage maps have also been established for several plant species, including important crops such as tomato (Tanksley and Rick, 1980), rye (Wehling *et al.*, 1985), rice (Wu *et al.*, 1988; Ranjhan *et al.*, 1988). In *Cymbopogon* isozyme marker was used to study genetic diversity (Sangwan *et al.*, 2003).Although the isozyme markers are limited and their expression is often restricted to specific developmental states or tissues, due to the ease of detection through electrophoresis and specific staining, they are used in conjugation with DNA markers.

1.3 DNA Markers

A DNA marker is a small segment of DNA showing sequence polymorphism in difference in individuals within a species. A wide variety of techniques have been developed in the past few years for visualizing the degree of polymorphism. Two basic marker systems have emerged for polymorphism detection in the desired segment, hybridization based markers and PCR based markers.

1.3.1 Hybridization based DNA markers

The realization, that length polymorphism in restriction fragments between individuals could be detected on DNA blots using radioactively labeled probes that hybridize to target sequence in the genome led to revolution in plant genetics. Such hybridization-based markers were applied to a wide range of plant species.

RFLP: In Restriction Fragment Length Polymorphism (RFLP), DNA is digested with restriction endonucleases, which cut genomic DNA at specific palindromic recognition sequences; the resulting fragments are separated by gel electrophoresis, blotted onto a filter and probes are hybridized to the separated

fragments. The polymorphism of fragment size, which depends on the presence and distances between the recognition sequences is detected by autoradiography. Variations in fragment length between individuals or species arise either when mutations alter restriction sites or as a result of insertions or detections in the recognition sites. RFLP markers are co-dominant, allowing detection and characterization of multiple alleles at a given RFLP locus among individuals in a population. They are reliable markers in linkage analysis and breeding and can easily determine if a linked trait is present in a homozygous or heterozygous state in individual, information that is highly desirable for recessive traits (Winter and Kahl, 1995). Bostein *et al.*, (1980) used for the first time RFLP marker to construct a genetic map. Later on, this hybridization-based marker was used widely for mapping of the genomes of many plants such as rice (Mc Couch *et al.*, 1988), tomato (Tanskley *et al.*, 1989; Saliba *et al.*, 2000), *Arabidopsis* (Reiter *et al.*, 1992), potato (Gebhardt *et al.*, 1989), wheat (Anderson *et al.*, 1992). RFLP markers have proved their importance in gene tagging and are very useful in locating and manipulating quantitative trait loci (QTL) in a number of crops. Besides their usefulness, generation and application of RFLP markers are time consuming and expensive. Firstly, one out of several markers provides a polymorphism. This Problem is serious, especially in crosses between closely related cultivated breeding lines. Secondly, for every polymorphic locus tested is cross, a single experiment has to be performed and this is a formidable task with saturated maps such as those of rice, tomato and maize, with hundred or more markers. In addition a large amount of DNA is required for restriction digestion and southern blotting. The requirement of radioactive isotope makes the analysis relatively expensive and hazardous. Finally, the inability to detect single base change restricts the use of RFLPs in detecting point mutations occurring within the regions at which they are detecting polymorphism. Information based on RFLP markers has been used to generate PCR based markers in crops.

Sequence Tagged sites (STS) Sequence tagged sites (STS) are short unique fragments of DNA (approx. 300bp). RFLP markers linked to desire traits are converted into PCR based STS markers from the nucleotide sequence of the probe polymorphic band pattern, or amplicons specific to different genotypes. Using this technique tedious hybridization procedures involved in RFLP analysis are avoided. This approach is extremely useful for studying the relationship between various species at a specific locus (Bustos *et al.*, 1999). STS marker was used in genome analysis of plants (Mazur and Tingy, 1995; Bustos *et al.*, 1999) and integrated into plant breeding programs for marker-assisted selection of the trait of interest.

Expressed Sequence Tags (EST) Theses are introduced by Adams *et al* (1991) and are obtained by partial sequencing or random c-DNA clones. In this approach, the sequence of 300-500bp or one or both ends of each of a large number of randomly chosen c-DNA clones are determined, which in most cases unambiguously identify the corresponding genes. Once generated, they are useful in cloning specific genes of interest and synteny mapping of function genes in related organisms. ESTs are popularly used in full genome mapping of functional genes in related organisms. Moreover, an EST that appears to be unique helps to isolate new genes. EST markers are identified to a large extent for rice, *Arabidopsis*, etc. wherein thousands of functional c-DNA clones are being converted into EST markers (Sasaki, 1994: Cook *et al.*, 1996).

Allele-Specific Associated Primers (ASAPs) To obtain an allele specific marker, a specific allele (either in homozygous or heterozygous state) is sequenced and primers are designed for amplification of a single DNA fragment from the specific allele at stringent annealing temperatures. These markers tag specific alleles in the genome and are more or less similar to SCARs (Mohler and Jahoor, 1996). ASAPs have exhibited their utility in genotyping of allelic variants of loci that results from both size differences and point mutations, e.g. waxy gene locus in maize (Stattuck-Eidens *et al.*, 1991)

Single Strand Conformational Polymorphism (SSCP) SSCP can detect DNA sequence alterations as small as a single nucleotide change (Orita *et al.*, 1989). This method exploits the tendency of single stranded DNA to form intramolecular base pairs, resulting in a sequence dependent conformation with a specific mobility in acrylamide gels. Changes in DNA sequences, even in a single base pair, can cause alterations in the conformation, which result in the changes in electrophoretic mobility. In practice, SSCPs are detected by digesting the DNA with restriction enzymes, denaturing the DNA, separating the DNA by conformation through polyacrylamide gel electrophoresis, followed by Southern blotting using a specific fragment as probe. In another method, PCR is used
to amplify a specific fragment separated on a high-resolution polyacrylamide gel. It was found useful in the detection of heritable human diseases. In plants, however, it is not well developed although its application in discriminating progenies can be exploited, once suitable primers are designed for agronomically important traits (Fukuoka *et al.*, 1994)

Restriction Land Mark Genomic Scanning (RLGS) Introduced by Hatada *et al* (1991) for genomic DNA analysis of higher organisms, this method is based on the principle that restriction enzyme sites can be used as landmarks. It employs direct labeling of genomic DNA at the restriction sites, and 2-dimensional (2D) electrophoresis to resolve and identify these landmarks. The technique has proven its utility in genome analysis of closely related cultivars and for obtaining polymorphic markers that can be cloned by the spot target method (Hirotsune *et al.*, 1993). It has been used to isolate DNA spots specific for a mutable slender-glume gene in rice (Teraishi *et al.*, 1995).

1.3.2 PCR Based DNA Markers

The development of the Polymerase Chain Reaction (PCR) for amplifying DNA sequences led to a revolution in the applicability of molecular methods and a range of new technologies were developed which would overcome the technical limitations of hybridization based markers. In PCR, arbitrary or known sequence primers are used to amplify one or several discrete DNA segments that can be separated in agarose or polyacrylamide gels. Each product is derived from a region of the genome containing two DNA sites with sequences complementary to the primer(s) on opposite strands and sufficiently closer for amplification to work.

Randomly Amplified Polymorphic DNA (RAPD) RAPD uses single short oligonucleotide primers of arbitrary sequence for the amplification of randomly distributed segments of genomic DNA (Williams *et al.*, 1990; Welsh and Mc Clelland, 1990). If the binding sites of primers are on the opposite strands of the DNA in inverted orientation, the segment of DNA flanked by the primer is amplified. Polymorphism in RAPD results from different types of changes in the genomic DNA: base pair substitution, insertion and deletions, which modify or eliminate the primer annealing sites; insertions in the genomic sequence that changes the intervening length of the DNA between the primer sites; and insertions which separate the primer sites to a distance that will not permit amplifications (Williams *et al.*, 1990). This procedure usually amplifies 1-15 DNA fragments from a single primer PCR reaction (Reiter *et al.*, 1992). The amplified DNA band is highly polymorphic between individuals in a population. The primers are usually 10bp length with GC content of at least 50% and have a low annealing temperature (36-40^{0}C). RAPD is an inexpensive and easy technology for fingerprinting, mapping and related research (Rafalski *et al.*, 1991; Waugh and Powell, 1992; Hadrys *et al.*, 1992; Tingey and Del-Tufo, 1993; Williams *et al.*, 1993). In *Cymbopogon*, RAPDs have been used to study genetic diversity among elite varieties (Sangwan *et al.*, 2001, 2003; Shasany *et al.*, 2000), identification of somaclonal variants (Nayak *et al.*, 2003), to establish species relationships and to study the genetic polymorphism of somatic embryo derived plantlets (Bhattacharya *et al.*, 2008).

The RAPD approach has had several modifications, the use of shorter primers (less than 10 nucleotides) in combination with PAGE and highly sensitive silver staining results in DNA amplification fingerprinting (DAF; Caetano-Anolles *et al.*, 1993) and AP-PCR that uses arbitrary primers (Welsh and Mc Clelland, 1990).

Paran and Michelmore (1993) introduced the technique of SCAR (Sequence characterized amplified regions for amplification of specific bands), in which the RAPD marker termini are sequenced. Longer primers, extending into the genomic DNA 3' to the initial RAPD primer, with higher and stringent annealing temperatures are designed for specific amplification of a particular locus; the presence or absence of the band indicating polymorphism. SCARs are dominant markers but may be converted to codominant markers by digesting them with restriction enzymes followed by separation in a denaturing gel. RAPD markers have successfully used for cultivar analysis of various plant species including rice (Ko *et al.*, 1994; Rana *et al.*, 1999), cauliflower (Hu and Quiros, 1991), banana (Kaemmer *et al.*, 1992), *Brassica* (Demeke *et al.*, 1992), cotton (Khan *et al.*,2000),wheat(Farooq *et al.*, 1994). Similarly, in *Cymbopogon* RAPD markers were also used to study genetic diversity, and to establish species relationship (Sangwan *et al.*, 2001, 2003; Khanuja *et al.*, 2005). RAPD markers are reliable and produce reproducible results for phylogenetic relationship. Various molecular markers are also widely used to detect and characterize somaclonal variation at the DNA level (Ford-Llod *et al.*, 1992). Of the available techniques, RAPD is mostly used to identify variant somaclones (Rani *et al.*, 1995; Rout *et al.*, 1998). In a number of plant species RAPD markers were applied to detect gross genetic changes of somaclones (Dey *et al.*, 1997; Hossain *et al.*, 2003; Patzack *et al.*, 2003; Godwin *et al.*, 1997).

In contrast to RFLP markers, RAPD markers not only require extremely small amounts of genomic DNA but also eliminate the need for blotting and use of radioactive probes. RAPD have considerable appeal for surveys of genomic variation and marker assisted selection since they are relatively inexpensive, randomly sample a potentially a large number of loci and prior sequence information is not needed for primer designing. However, the reproducibility of the process needs to be optimized.

Microsatellites and Minisallites Microsattelites and minisattelites are short tandem repetitive DNA sequences dispersed through out the eukaryotic genome. The term microsatellite was coined by Litt and Lutty (1989), while the term minisatellites was introduced by Jeffrey (1985). They were reported first in humans (Bell *et al.*, 1982) and now available in genomes of nearly all higher eukaryotes including plants (Tautz and Renz, 1984; Langercrantz *et al.*, 1993). These DNA elements frequently change their length by slipped-strand mispairing and other less understood process (Levinson and Gutman, 1987; Jeffreys *et al.*, 1988; Zischler *et al.*, 1992), which generate varying number of repeats at a given locus. However, the flanking single copy sequences are normally conserved, which provide a valuable source of polymorphisms for linkage analysis (Lathrop *et al.*, 1985; Jeffreys *et al.*, 1986; Nakamura *et al.*, 1987; Wells *et al.*, 1989), identification of species and cultivars (Weising *et al.*, 1992), and marker assisted selection (Beckman and Soller, 1990).

Minisatellites are tandem repeats of sequences ranging from 9-100bp in the genome; the number of repeats varies, but usually less than 1000. For minisattelites, the loci that vary in the number of repeat units between genotypes are referred to as variable number of tandem repeats (VNTRs; Nakamura *et al.*, 1987), or hypervariable regions (HVRs). These loci, containing tandem repeats within a genome, generate high level of

polymorphism between individuals in a population (Jeffreys *et al.*, 1985).

Microsatellites are stretches of DNA, consisting of tandemly repeating mono-, di-, tri-, tetra- or penta nucleotide units, which are hypervariable and ubiquitously distributed through out the genomes of most eukaryotic species. Microsatellites are short tandem repeats (STRs) or simple sequence repeats (SSRs). The SSRs are favoured over minisattelites as the former are evenly distributed in the genome (while the later are usually confined to the telomeric region), found in abundance, and are easier to discover and employ using the PCR.

There are two commonly used ways to identify micro satellite loci suitable for use as genetic markers. For some plant species, such as *Arabidopsis*, rice, where a large amount of DNA sequence data has already been accumulated, microsatellites may be identified by searching through the DNA sequence data bases for sequences containing simple repeats. Primers are commonly designed directly from sequence data. However, for most plant and animal species, a large effort using hybridization and sequencing is needed to identify microsatellites suitable for use as genetic markers. Hybridization using simple repeats as probe to screen genomic clones can be used to identify clones containing microsatellite loci. The conserved DNA sequences flanking SSRs have been used for designing suitable primers for amplification of SSR loci. Any such pair of primers, when used to amplify a SSR locus of a number of genotypes, will reveal SSR polymorphism in the form of differences in the length of the amplified product; each length representing an allele at that locus. The length differences are attributed to the variation in the number of repeating units of a particular SSR locus. As polymorphism is based on differences in the number of repeats of simple sequences, polymorphism revealed by SSRs is also termed as simple sequence length polymorphism (SSLP) and sequence tagged microsatellite sites (STMS).

The uniqueness and value of the microsatellite arise from their multi-allelic nature, co-dominant transmission, ease of detection by PCR, relative abundance, extensive genomic coverage and requirement for only a small amount of genomic DNA. In addition, primer sequence information of the markers may be distributed between laboratories, thus providing a common language for collaborative research and acting as universal genetic mapping tools.

Saturated microsatellite maps have been developed for several plant species including rice (Temnykh *et al.*, 2000; Chen *et al.*, 1997; Akagi *et al.*, 1996), maize (Senior and Heun, 1993; Chin *et al.*, 1996; Taramino and Tingey, 1996), barley (Becker and Heun, 1995), wheat (Roder *et al.*, 1998), Arabidopsis (Bell and Ecker, 1994) and soybean (Akkaya *et al.*, 1995).

Inter Simple Sequence Repeat markers (ISSR) This technique uses primers based on microsatellites to amplify regions between microsatellite loci (Zietkiewicz *et al.*, 1994). The resulting bands are electrophoresed in agarose gels, stained with ethidium bromide and viewed under UV light. This technique is more reproducible and generates 3 to 5 times the variation of RAPD bands/ marker (Nagaoka and Ogihara, 1997). ISSR markers have been shown to be more reliable and conform closely to dominant Mendelian inheritance, which makes them useful for genotype analysis and genome mapping elucidate genetic relationships among blueberry cultivars (Levi and Rowland, 1997), peas (Lu *et al.*, 1996), wheat (Nagaoka and Ogihara, 1997), and used as a probe linked to particular characters in rice (Akagi *et al.*, 1996).

Randomly Amplified Microsatellite Polymorphism (RAMP) In this PCR- based strategy, genomic DNA is first amplified using arbitrary (RAPD) primers, the amplified products electrophoretically separated, and the dried gel is hybridized with microsatellite oligonucleotide probes. This technique combines the advantages of oligonucleotide fingerprinting (Epplen *et al.*, 1991), RAPD (Williams *et al.*, 1990), and microsatellite-primed PCR (Weising *et al.*, 1995). The advantages are the speed of the assay, the high sensitivity and the high level of variability detected and the non-requirement of prior DNA sequence information (Richardson *et al.*, 1995). RAMP has been successfully employed in the genetic fingerprinting of tomato, kiwi fruit and closely related genotypes of *Dioscorea bulbifera* (Richardson *et al.*, 1995).

Amplified Fragment Length Polymorphism (AFLP): AFLP combines the reliability of the RFLP markers with the ease of PCR (Vos *et al.*, 1995). The relatively high polymorphism inherent in the random placement of restriction sites between different genomes as detected by RFLP and the ease of PCR combined with the potential nucleotide sequence variability within a short stretch of DNA directly flanking these restriction sites makes AFLP a highly informative assay for both plant and animal genomes.

The AFLP procedure involves digestion of genomic DNA with two restriction enzymes, a rare cutter and a frequent cutter, ligation of double stranded adapters (corresponding to the restriction sites) to both ends of the digested fragments, pre-amplification of the ligated DNA fragments with primers complementary to the adapters, and selective amplification of a subset of the pre-amplified DNA fragments using primers with one to three arbitrary selective nucleotides at the 3' ends. By changing the selective nucleotides of the primers, different sub-sets of the genome can be amplified. The amplified fragments are detected through radioactive or non-radioactive labeling followed by electrophoresis on a polyacrylamide sequencing gel. AFLP assays are reproducible, require no sequence information and use a small number of generic primers.

AFLP analysis can be applied regardless of the origin and complexity of the genome. Most AFLP fragments correspond to unique positions on the genome and hence can be exploited as landmarks in genetic and physical mapping (Vos *et al.*, 1995). AFLPs are useful as tools for DNA fingerprinting (Hongtrakul *et al.*, 1997) and for cloning and mapping of variety-specific genomic DNA

sequence (Yong *et al.*, 1996). AFLPs are abundant in rice (Cho *et al.*, 1996; Mackill *et al.*, 1996) and a map consisting of 208 AFLP markers is available for rice (Maheswaran *et al.*, 1997). Similar to RAPDs, the bands of interest obtained by AFLP can be converted into STSs (Paglia *et al.*, 1998). Thus, AFLP provides a newly developed, important tool for a variety of applications.

Conclusion: The above review reveals that in recent times DNA fingerprinting technologies in plant systems have been a matter of immense interest among the plant scientists throughout the world. In the context of utilization and protection of plant genetic resources, molecular marker assisted breeding strategies and assessment of somaclonal variants has become the important issues. An ever-increasing number of reports are being published in each year directing towards new dimension of research for this promising technique. The different methods on DNA based markers of presently reviewed article revealed their utility in establishing genetic identity and estimating the extent of genetic relationships. Moreover, genome mapping with various molecular markers has tremendous potentialities to bring new dimensions in the field of plant molecular genetics and for understanding molecular evolution.

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Major plant viruses: an overview

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Abstract

Plant viruses cause severe diseases leading to enormous crop loss. The present day viral researches of economic plants are centered on identification of virus, molecular characterization and management of viral diseases. Till date more than thousand viruses have been classified into several families. It is desirable to know about the different virus families along with their type genus and/or important genus. But due to an enormous volume of literature published on this aspect, it becomes difficult to study all of them. Hence the present review has highlighted the salient features of the major plant viruses which have been classified at the family level. Most of the virus families have been discussed with important/type genus of each family. Some viruses which could not be placed in any family have been grouped as 'no family'. Importance of molecular data, immunological data and data on protein configuration of coat proteins along with bioinformatics and its predictive power have been highlighted.

Keywords: Plant virus, virus classification, coat protein

Virus is an obligate parasite. It does not have inherent replication machinery which can replicate outside a living organism. Thus virus is unique. Plant viruses are those viruses which use higher plants as their obligate hosts and use the plants for their replication. Enormous losses of our crops have been reported due to viral attack (Pappu, 1997; Palukaitis *et al*., 1992; Hema and Prasad, 2004; Bateson and Dale, 1995; Espion *et al*., 1990; Robert and Lemaire, 1999; Lokhande *et al*., 1992). Presently, more than one thousand viruses are known (Jiskani, 2007). The nomenclature of virus and creation of new species is very important. The International Committee on Taxonomy of Viruses (ICTV) has suggested for creation of new genus if the molecular similarity is found less than 89% with the nearest species in case of geminivirus (ICTV, 2006). The present day viral researches of economic plants are centered on identification of virus, molecular characterization and management of viral diseases. Although it seems, the molecular characterization and nomenclature has taken central attention but to a beginner the knowledge of conventional techniques and classification rules are still important. To understand the whole scenario of role of virus in plant diseases the present review has been done with a view to proper understanding of a virus nomenclature.

More than thousand viruses have been classified into several families. All the families have been characterized by a type species. The virus families along with the type genus and/or important genus have been presented in the following lines. Among the different families mentioned some families are very important for their destructive role in economic crops. The other viruses although less important but they may provide some important information regarding viruses and their possible infecting capacity in continuously changing genotypes of our crops. Important families with salient molecular characters along with important plant diseases producing genus have been presented in the following lines. Most of the molecular informations given in the following lines have been presented from the ICTV reports. ICTV reports has summarized by Matthews, 1983a, 1985a,b. ICTV has published seven reports up to 2000 in Archives of Virology (Hull, 2002). The most recent report of ICTV has been edited by Fauquet *et al*., 2005.

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Important families of plant virus:

Caulimoviridae: Members of Caulimoviridae are replicated by reverse transcription. All the viruses have circular DNA. The genome is discontinuous at specific sites. The members are divided into two groups: isometric particles containing *Caulimoviruses* and bacilliform particles containing *Badnaviruses*. *Cauliflower mosaic virus* (CaMV), the type member of the genus *Caulimovirus*, is one of the most common and important viruses of brassica crops in the world. CaMV has double-stranded DNA as their genetic material. This virus is wide spread in temperate regions wherever species of *Brassica* are grown (Sutic *et al*. 1999). CaMV has isometric particles about 50nm in diameter, composed of 420 capsid protein subunit and a circular double-stranded DNA genome which contains seven major ORFs (I to VII). Of these ORFs only the capsid protein (and/or its polyprotein precursor GAG), the polymerase polyprotein and the transactivator/ viroplasmin are essential for virus replication (Hohn and

Futterer, 1997 and Kobayashi and Hohn, 2003) whereas the movement protein (MP) and the virion associated protein (VAP) are additionally required for induction of plant disease (Kobayashi *et al.*, 2002). Virus is replicated by the reverse transcription of an RNA intermediate (Haas *et al.*, 2002). Caulimoviruses have a restricted host range, usually one or two families. CaMV mainly infects members of Cruciferae and Solanaceae (Chenault *et al.* 1992). This virus induces a variety of systemic symptoms (Chlorosis, mosaic, vein clearing and stunting). On many cruciferous plants, particularly on various *Brassica campestris* and *B. oleracea* cultivars, often in mixed infection with *Turnip mosaic virus* (TuMV) (Shepherd, 1981). The virus is not transmissible by seed or pollen (Blanc *et al.*, 2001). The three viruses of genus '*Soybean chlorotic mottle virus-like*', '*Cassava vein mosaic virus-like*' and '*petunia vein clearing virus-like*' resemble genus *Caulimovirus* but their genome organizations are different. Two other genus '*Bodnavirus*' and '*Rice tungro bacilliform virus-like*' are in the group which contain bacilliform particles. Both the viruses are important in tropical agriculture.

Geminiviridae: The name of the family comes from geminate virus particles which contain the genome of the virus. Two incomplete icosahedra form the geminate particles (Kirthi *et al.*, 2002). The genome of the family is of circular ssDNA type (Rojas *et al.*, 2005). Geminiviridae and Circoviridae are the two families of plant viruses that have circular ssDNA. *Geminiviruses* constitute the largest, most diverse and economically important family of plant DNA viruses (Rojas *et. al*, 2005). They infect a broad range of plants and cause devastating crop diseases, particularly in tropical and subtropical regions of world (Mansoor *et al.*, 2006; Moffat, 1999 and Morales and Anderson, 2001). single-stranded DNA (*ss*DNA) genomes display high levels of genetic variability (Seal *et al.*, 2006). The family *Geminiviridae* is classified into four genera, *Begomovirus*, *Curtovirus*, *Topocuvirus* and *Mastrevirus*, based on their genome organization, host range and insect vectors (Faquet *et al.*, 2003). The largest genus of Geminivirus family is *Begomovirus*. *Begomovirus* have one or two genome components (designated as DNA-A and DNA-B) which infect dicots and are transmitted by *Bamisia tabaci*. A significant increase in the frequency and severity of *Begomovirus* diseases over the past 20 years have been well documented by Rashed, 2006. The DNA-A component of *Begomovirus* contains four genes (AC1, AC2, AC3 and AC4) on their c-sense strand and two genes (AV1 and AV2) on their v-sense strand. AC1 encodes Rep, replication initiator protein (Elmer *et.al*, 1988, Etessami *et al.*, 1991), AC2 gene encodes TrAP, transcription activator protein (Sunter and Bisaro, 1991, Hartiz *et al.*, 1999), AC3 encodes REn, Replication enhancer (Elmer *et al.*, 1988) and AC4 gene encodes C4 protein. AV1 gene encodes coat protein and AV2 encodes pre-coat protein. Similarly, the DNA-B component contains two ORFs which encode for proteins that assist in intra-cellular and inter-cellular viral movement- BC1/NSP (nuclear shuttle protein) and BV1/MP (movement protein) (Lazorawitz, 1999). The invariant TAATATT↓AC sequence, located in the intergenic regions, contains the initiation site (↓) of rolling circle DNA replication. Among the geminiviridae tomato leaf curl virus is one of the important virus which cause substantial loss of crops (Magar *et al.*, 2008). *Tomato leaf curl virus* (ToLCV) is a whitefly (*Bamisia tabici*) transmitted *Geminivirus* (family *Geminiviridae*, genus *Begomovirus*) causing destructive disease of tomato in many parts of India (Vasudeva and Sam Raj, 1984; Sastry and Singh, 1973; Muniyappa and Saikia, 1983; Saikia and Muniyappa, 1989; Harrison *et al.*, 1991; Reddy *et al.*, 2005; Kirthi *et al.*, 2002; Paximadis *et al.*, 2001; Ramappa *et al.*, 1998). Besides tomato *Begomovirus* infects variety of other plants including Kenaf (Paul *et al.*, 2006), *Dimorphotheca sinuta* (Raj *et al.*, 2007), Bimili jute (Raj *et al.*, 2007a), Bun ochre (*Urena lobata*)(Chatterjee *et al.*, 2007), *Mimosa invasa* (Koravich *et al.*, 2008), Bitter gourd (Rajinimala and Rabindran, 2007), *Datura stramonium* (Ding *et al.*, 2007), Papaya (Wu and Zhou, 2006), *Duranta repens* (Tahir *et al.*, 2006), Chilli (Shih *et al*, 2006), Methi (Raj *et al.*, 2001), common bean (Papayiannis *et al.*, 2007), *Ageratum conyzoides* (Wong *et al.*, 1993), potato (Garg *et al.*, 2001), pepper (*Capsicum annum*) (Strenger *et al.*, 1990) of India and other parts of the world. *Begomovirus* infected diseases are characterized by severe leaf curling, shrinking of leaves, stunted growth of the plants, yellowing of the veins (Chakraborty *et al.*, 2003). Jatropha (*Jatropha curcas* L.) an important biofuel producing plant is infected by JMIV. The disease cause stunted growth and several other symptoms. The disease cause low production of fruits which is used for extraction of biofuel (Rangaswamy *et al.*, 2005; Aswatha Narayana *et al.*, 2006).

Circoviridae: Like the geminiviridae this family also possesses genome of ss DNA but they possess much smaller circular DNA. The virus particle is also very small (approximately 20nm in diameter). Out of the two genus of the family only one genus (*Nanovirus*) infects plants. One of the species *Banana bunchy top virus* (BBTV) of *Nanovirus* is economically important. The icosahedral virus particle contains six or more circular ssDNA molecules of approximately 1 kb in size. Banana bunchy top disease is caused by BBTV, a multi-component, circular single-stranded DNA virus of the family Nanoviridae that is transmitted plant-to-plant by the aphid vector *Pentalonia nigronervosa*. The family Nanoviridae consists of aphid transmitted viruses with isometric virions, approximately 18–20 nm in size, and is divided into two genera; (BBTV is the only member of the genus), and Nanovirus. Nanovirus contain *Subterraneanclover stunt virus* (SCSV) (Boevink *et al.*, 1995), *Faba bean necrotic yellows virus* (FBNYV) (Katul, 1997 and 1998) and *Milk vetch dwarf virus* (MDV) (Sano *et al.*, 1998). Placement of *Coconut foliar decay virus* remains uncertain as only a single component of the virus has been described (Rohde *et al.*, 1990). The component described as satellitelike molecule associated with a geminivirus (Briddon *et al.*, 2006). The genome of BBTV has been reported to have at least six components of approximately 1100 nucleotides. The six components are DNA-R, DNA-S,

DNA-C, DNA-M, DNA-N and DNA-U3 which encode a rolling-circle replication initiator protein [Rep], coat protein [CP], cell-cycle link protein [Clink]), movement protein [MP], nuclear shuttle protein [NSP]) and undetermined respectively (Vetten *et al.*, 2005).

Reoviridae: The viruses of the family contain ds RNA. The most important part of the family is that some members of the family infect plants and those viruses also infect their vectors (invertebrate). All the three genera (*Fijivirus*, *Oryzavirus* and *Phytoreovirus*) which attack plants can be separated by their dsRNA segment number and particle size diameter. *Fijivirus* mostly attack mocotyledonous plants of poaceae and liliaceae. The third genus Phytoreovirus can attack both monocotyledonous and dicotyledonous plants. *Rice dwarf virus* (RDV) is a member of the genus *Phytoreovirus* of the family *Reoviridae*, which also includes animal *Reovirus*, *Orbivirus*, and *Rotavirus*. RDV replicates both in insects and in graminaceous plant cells, but it can be transmitted only by insects such as the leafhopper (*Nephotettix cincticeps* or *Resilia dorsalis*) (Suzuki *et al.*, 1994). RDV does not induce neoplasia but induce stunted growth, develop characteristic chlorotic flecks, and fail to bear seeds. This virus is widespread among rice plants in southern China and other Asian countries. The RDV genome is composed of 12 double-stranded RNA segments, designated S1 to S12 in ascending order of their mobility on a polyacrylamide gel (Fujii-Kawata and Fuke, 1970). The complete sequences of all of these segments have been determined and 90% sequence similariry have been reported between a Japanese isolate (Uyeda *et al.*, 1994) and a Chinese isolate (Zhang *et al.*, 1997).

Partitiviridae: The plant infecting viruses of this family are commonly known as 'cryptic virus'. These viruses produce no symptoms or negligible symptoms. The viruses are characterized by non-enveloped bi-segmented dsRNA genome. The two plant infecting genera are *Alphacryptivirus* and *Betacryptovirus*. Plant cryptoviruses are associated with latent infections of their hosts. Pollen/seed transmission is the only means of virus dispersal. No cell-to-cell movement of the virus has been reported. The virus can only move when cell division takes place (Boccardo *et al.*, 1987). One genus *Partitivirus* have been reported from fungi (Ghabrial *et al.*, 2005).

No family: Varicosavirus genus could not be ascertained to any defined families and kept as no family. The type species is '*Lettuce big vein virus*'. Two ds RNA molecules are there in the genus and the shape of the virions are rods.

Rhabdoviridae: Rhabdoviruses are characterized by bacilliform to rounded structure enveloped by a membrane. The outer surface is characterized by glycoprotein spikes. The viruses are (-) strand RNA viruses. There are two well defined viral genera in the family although several plant rhabdoviruses could not be assigned to any genus due to inadequate data and information required for assigning a genus. All those viruses are kept in a group as 'unassigned Rhabdoviridae'. *Potato yellow dwarf virus* (PYDV) is a species of plant virus under the genus *Nucleorhabdovirus*. The virus was first identified in the USA by Barrus and Chupp (1922). The virus has several effects including stunted growth, dwarfing, apical yellowing, tuber cracking and malformation. PYDV has been transmitted artificially to species of the families Apocynaceae, Asteraceae, Brassicaceae, Fabaceae, Lamiaceae, Polygonaceae and Scrophulariaceae (Black, 1970; OEPP/EPPO, 1980). It occurred naturally during 1986-88 in Minnesota (USA) on the ornamental herbaceous plants *Mirabilis jalapa*, *Nicotiana alata*, *Tagetes erecta* and *Zinnia elegans*, causing severe stunting, chlorosis, vein yellowing and systemic vein and leaf necrosis (Lockhart, 1989). PYDV isolates can be distinguished by their serological reactions and their vector specificity. There are two serotypes: one transmitted by the leafhopper *Aceratagallia sanguinolenta* and another *Aceratagallia* sp. ("*Sanguinolenta yellow dwarf virus*"), the other by the leafhopper *Agallia constricta* ("*Constricta yellow dwarf virus*"). Both forms are transmitted by *Agalliota quadripunctata*. The California isolate is closely related to the sanguinolenta serotype (Falk & Weathers, 1983). Both serotypes have a long incubation period in their respective vector leafhoppers (at least 6 days), during which they multiply (Chiu *et al.*, 1970). Nymphs, adult male and female insects transmit PYDV. *Leucanthemum vulgare* serves as the principal virus source for infecting potato crops. *Catharanthus roseus* is a natural host in California. PYDV is carried through tubers derived from infected plants. The virus is mechanically transmissible to seven species of tobacco (Falk *et al.*, 1981).

Bunyaviridae: Bunyaviridae are characterized by spherical virions with glycoprotein spikes. The viruses are either of (-) strand RNA or in an ambisense arrangement [where both (-) and (+) strands of RNA are present]. Altogether three RNA segments are found. This is one of the large families. Most of the genus attack vertebrates and invertebrates. Only one genus (*Tospovirus*) attack plants and invertebrate vectors. Out of the three RNA segments of Tospovirus the largest one is of (-) strand and the other two are of ambisense type. Tomato spotted wilt (caused by *Tomato spotted wilt virus*) was first described in Australia in 1919. It is an important disease of several crops grown in temperate and subtropical regions of the world. TSWV infect approximately in 174 plant species (Zitter *et al.*, 1989). The common hosts are tomatoes, peppers, celery, lettuce, eggplant, peanuts, pineapple, many legumes, many ornamentals, and many weeds such as field bindweed and curly dock. Symptoms of TSWV are numerous and varied. TSWV is transmitted from infected plants to healthy plants by at least nine species of thrips. Thrips transmit the virus in a persistent manner, which means that once the insect has picked up the virus, it is able to transmit the virus for the remainder of its life (Goldberg, 2000). There are significant differences in tospoviruses and their relationship with specific thrips species, so what is presented herein on TSWV is not applicable to all tospovirus-thrips interactions (Sherwood, 2003). The current understanding of the TSWV gene functions

indicates that the NSm serves as a movement gene, NSs is a silencing suppressor and the glycoproteins contain determinants for thrips transmission. Management of TSWV has proven to be challenging due to the wide host range of both the virus and its vector, and emergence of resistance breaking strains. However, increased understanding of the biology, genetics, epidemiology and molecular biology of TSWV resulted in development of practical and effective integrated disease management programs for reducing the impact of TSWV in some crops (Pappu, 1997).

No family: Two genera *Tenuivirus* and *Ophiovirus* could not be placed in any family due to inadequate informations. These two genera have some similarities with the members of Bunyaviridae. Tenuivirus is spreaded by grass hoppers but the vector for *Ophiovirus* is unknown. *Tenuivirus* attack plants of the family Poaceae whereas the other genus can attack both monocotyledons and dicotyledons.

Bromoviridae: Members of this family contain tripartite (+) strand RNA. The viruses of the family are isometric with generally bacilliform in shape. Size varies in different genera. Five different genera are *Bromovirus, Cucumovirus, Ilarvirus, Alfamovirus* and *Oleavirus*. Among the genera *Cucumovirus* are most important as it has a wide host range. Largest number of plant species (approximately 1000 plant species) is infected by the virus (Kim *et al.*, 2010). The genome of the virus consists of plus-sense single stranded three RNAs (RNA 1, RNA 2, and RNA 3) and a subgenomic RNA (RNA 4) which is encoded by the 3'-half of RNA 3 (Palukaitis *et al.*, 1992) and which is involved in encapsidation (Suzuki *et al.*, 1991). *Cucumber mosaic virus* (CMV) isolates are classified into two sub-groups, I and II, according to various converging criteria which include symptomatology, serology and nucleic acid homology etc. (Palukaitis *et al.*, 1992). RNA 3 contains both the viral coat protein (CP) gene and movement protein gene involved in the cell-to-cell and long-distance movements (Davies and Symons, 1988). After mapping for the determination of CMV pathogenicity (Mossop and Francki, 1977), pseudorecombinants created by exchanging gel-eluted genomic RNAs revealed that each of the three genomic RNAs plays its determinant role by various host-strain interactions (Palukaitis *et al.*, 1992). Asian strains of CMV have been placed in subgroup IB (Palukaitis and Zaitlin 1997). Several workers are working in elucidating the role of different proteins in different functions of the viral activities. *Alfalfa mosaic virus (AMV)* has a tripartite single-stranded genome. RNAs 1,2,3 and subgenomic RNA 4 are separately encapsidated into baciliform particles which are 18nm wide and have lengths characteristic of the RNA encapsided (about 56,43,35 and 35 nm respectively) (Thole *et al.* 1998). The genomic RNAs are not infective. Infection can start only in the presence of RNA 4 or its translation protein product (CP). AMV mostly infects herbaceous plants, but several woody species are included in the natural host range (Edwardson and Christic, 1997).

Comoviridae: *Comoviridae* are made up of two ssRNA of positive strand. Capsid proteins are very important. The capsid proteins are named for their functions. Encapsidation is only one feature of an extremely diverse array of structural, functional, and ecological roles played during viral infection and spread (Callaway *et al.*, 2001). The capsid protein is multifunctional; in addition to having a role in encapsidation; it affects virus movement in plants (Kaplan *et al*, 1998), transmission, symptom expression, and host range (Shintaku and Palukaitis, 1990). *Comovirus*, *Fabavirus*, and *Nepovirus* are the three genera of the family. The capsid of Comovirus is made up of two proteins of different size. The capsid of *Fabavirus* are made up of two proteins of similar size but the capsid of *Nepovirus* are made up of a single protein. Cow pea mosaic virus of genus *Comovirus* is one of the important virus which infect *Vigna radiata*. Foliage turns yellowish green with areas of light and dark green tissue. Infected leaves are often stunted and frequently have a puckered appearance. Susceptible host species are *Datura stramonium, Glycine max, Gomphrena globosa, Nicotiana tabacum, Phaseolus vulgaris, Pisum sativum, Spinacia oleracea, Vicia faba, Vigna angularis, Vigna radiata, Vigna unguiculata* (Gomase and Kale, 2008; Gopinath *et al.*, 2003).

Tombusviridae: The virus particles are in the size range of 30 to 35 nm in diameter. The viruses contain single species of RNA of positive ss type. Members have narrow host range. The eight genera are *Tombuvirus*, *Aureus virus*, *Avenavirus*, *Carmovirus*, *Machlomovirus*, *Necrovirus*, *Panicovirus* and *Dianthovirus*. Members either infect monocotyledons or infect dicotyledons but no virus attack both types of plants. *Tomato bushy stunt virus* (TBSV) RNA genome of approximately 4.8 kb, encodes five major open reading frames (ORFs) (Brunt *et al.*, 1996). Viral replication require an ORF1and an ORF2 (Scholthof *et al.*, 1995a). Coat protein and viral movement protein are encoded by ORF3 and ORF4 respectively. Cell to cell movement and symptom determination on certain host plants are controlled by ORF4 (Scholthof *et al.*, 1995b). Product of ORF5 has a role in the induction of necrotic symptoms and in the long-distance spread of the virus, depending on the host. TBSV has been reported from tomato of Ireland, North and South America, Europe, Africa, and Japan (Ohki *et al.*, 2005). TBSV has been divided on the basis of serological reactivity into three major strains. Because of serological reactivity and of the high sequence homology in their genomes, it was also proposed that *Petunia asteroid mosaic virus* and *Artichoke mottled crinkle virus* (AMCV) are also strains of TBSV (Luis-Arteaga *et al.*, 1996).

Sequiviridae: The virus particle of 30nm size is made up of three different proteins of different sizes. Single positive ssRNA encodes poly proteins which after cleavage produce functional proteins. Two genera are *Sequivirus* and *Waikavirus* infect plants of limited importance.

Closteroviridae: The particles of different members of the family are flexuous and filamentous. The nucleic acid is linear (+) strand ss RNA. Depending on genus

the nucleic acid may be monopartite or bipartite. The transmissions of the viruses are of different types. The viruses are generally restricting themselves in the phloem. The genera may be classified on the basis of transmission vectors like aphid-transmitted, mealy bug-transmitted and whitefly transmitted. *Closteroviridae* is represented by *Closterovirus* and *Crinivirus*. *Citrus tristeza virus* (CTV), an aphid-transmitted *Closterovirus*, has been the most important viral pathogen of citrus for the last ninety years. CTV has a huge destructive role and have been reported from almost all over the world (Harper *et al.*, 2009). *Citrus tristeza virus* (CTV) has been reported from various Indian states also (Ahlawat and Raychaudhuri, 1988; Capoor, 1963; Narani *et al.* 1965; Vasudeva and Capoor, 1958). Vector specificity and reaction in differential hosts are the two criteria which have been followed to determine mild or severe strains in India (Capoor and Rao, 1967; Balaraman and Ramakrishnan, 1977; Capoor and Chakraborty, 1980). Among the seven aphid species reported as vectors of CTV, *Toxoptera citricidus* has been found to be the most efficient vector (Capoor and Rao, 1967).

Luteoviridae: 'Luteus' mean yellow in Latin. Most of the genus produce yellows type disease. Hence the name of the family is *Luteoviridae*. The members of the family contain (+) sense ss RNA. Three important genera are *Luteovirus*, *Poleovirus* and *Enamovirus*. Eleven viruses of family have not been assigned to any genus due to insufficient data availability for characterization. *Potato leafroll virus* (PLRV) of the genus *Polerovirus* infects potato crops worldwide (Robert & Lemaire, 1999). It is transmitted in a persistent manner by a limited number of aphid species like *Myzus persicae;* the most efficient and important vector (Harrison, 1984). Its natural host range is mainly restricted to a few solanaceous plants (*Solanum tuberosum*, *Physalis oridana*, *Datura stramonium* and *Lycopersicon esculentum* (Thomas, 1993). Moreover, a few plants of other families can be infected by PLRV, either experimentally (Harrison, 1984) or naturally (Lizarraga *et al.*, 1996). Six main open reading frames (ORFs) numbered from 0 to 5 are there in the genome of the virus (Miller *et al.*, 1997). Six different proteins (P0 to P5) are formed from the ORFs. P0 is essential for replication of the PLRV genome (Sadowy *et al.*, 2001) and that P1 has a hydrophobic domain, thought to be involved in membrane attachment (Mayo & Ziegler-Graff, 1996). P2 is translated by a rarely occurring ribosomal frameshift from ORF1. P3, P4 and P5 being translated from a subgenomic RNA correspond to the major capsid protein (CP), the putative movement protein (MP) and a protein (of readthrough domain) which is translated by suppression of the ORF3 stop codon. Ashoub *et al.* (1998) identified two further putative ORFs encoding two proteins, P6 and P7, the roles of which remain unknown.

Potyviridae: Members of *Potyviridae* have flexuous particles 650-900 nm long and 11-15 nm in diameter. The genome is (+) sense ss RNA with a VPg (a genome-linked protein covalently bound to the 5'end) and with a 3' poly A portion. Inclusion bodies are found in all the hosts infected by the members of the family. Out of the six genera (*Potyvirus*, *Ipomovirus*, *Macluravirus*, *Rymovirus*, *Tritimovirus* and *Bymovirus*) *Potyvirus* is the largest and also the largest of plant viruses. Some economically important species are included in this family. Bean common mosaic virus was first reported in Russia in 1894 and has since been distributed worldwide (Agrios, 1988; Boss 1971, Hampton *et al.*, 1983; Sherf and Macnab, 1986). *Bean common mosaic virus* (BCMV) on bean cause systemic mosaic, malformation of leaves and pods and rugosity of lower leaves, sometimes even necrosis. Viruses cause substantial loss (Hall, 1994). The virus is transmitted in non-persistent manner by insect vectors (e.g. *Acyrthosiphon pisum*, *Aphis craccivora*, *A. fabae* and *Myzus persicae*). It affects primarily the French bean (*Phaseolus vulgaris*) but also *Phaseolus* sp and faba bean (Bos, 1971; Sherf and Machnab, 1986; Smith, 1972). Mechanical inoculation and seed-transmission (in *P. vulgaris* up to 83%) have been reported by Brunt *et al.*, 1996. *Papaya ring spot virus*, first described by Lindner *et al.* (1945), induces irregular mosaic patterns on leaves, ring on petioles and yellow ring on fruit (Shinde *et al.*, 2005) of papaya where it is grown (Purcifull *et al.*, 1984). The PRSV strains that infect papaya, designated as PRSV-P are distinct from the PRSV-W strain of cucurbits. Papaya trees infected with PRSV-P are stunted, produce deformed fruits with some ringspot, vein clearing, mottling, malformed leaves, streaks on fruit, stems and petioles (Kuan *et al.* 1999). Bateson *et al.* 1994 reported that PRSV-P evolved from PRSV-W, presumably by mutation. This was first indicated by the very close sequence similarity of the coat protein (CP)-coading region of P and W isolates. Another member of the genus *Potyvirus* is *Potato virus Y* (PVY). The isolates of PVY have been classified in three main strains: PVY^N, PVY^O and PVY^C (De Bokx and Huttinga, 1981). The virus can infect potato, tobacco, tomato and pepper as well as wild species, especially those in the *Solanaceae* family (Ellis *et al.*, 1997). The serological classification of PVY isolates is a matter of discussion. Coat protein-directed polyclonal antibodies do not discriminate between PVY strains so monoclonal antibodies specific to O and N strains have been used to characterize selected PVY isolates (Gugerli and Fries, 1983; Hataya *et al.*, 1998)

Conclusion

Although molecular data, immunological data and protein configuration of coat proteins are most important, bioinformatics is being increasingly used in virus classification due to its strongest predictive power. Bioinformatics is particularly important when information from several techniques is combined, including experimental confirmation of protein antigenicity predictions etc. (Gomase and Changbhale, 2007; Gomase *et al*, 2007). Sequence data of genome of different viruses are being available in the gene bank and all such sequences may be used to find out the closeness of the different isolates, species and genus of the viruses. Most importantly in several cases the sequence data have solved the problems of classification. Still several sequence data demand

considerable reorganization of the classification.

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In vitro multiplication of *Curcuma longa* Linn.–an important medicinal zingiber

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Abstract
Curcuma longa Linn. is a herbaceous perennial plant belonging to the family Zingiberaceae. *In vitro* protocol for the regeneration of plantlets from the sprouted rhizomes of turmeric was optimized. Murashige and Skoog media supplemented with different concentrations and combinations of cytokinins and varied percentage of sucrose were experimented. Murashige and Skoog media supplemented with benzyl amino benzene (BAP) at the concentration of 2mg/l and sucrose 3% showed the best regeneration in comparison to Kinetin when used singly. Combination of 2mg/l BAP and 3mg/l Kinetin resulted in highest number of shoots. The same media showed spontaneous rooting. Healthy regenerated plantlets were selected for hardening in sterile mixture of garden soil and sand in the ratio of 1:1. Ninety three percent of the micropropagated plantlets survived to maturity when transferred to soil.

Keywords: Benzyl amino purine, Cytokinin, *Curcuma longa*, Kinetin, micropropagated plantlets.

Turmeric (*Curcuma longa* Linn.) belongs to the family Zingiberaceae. It is a perennial rhizomatous herb supposed to have Indian origin (Ghani, 1998) and is used both as food and medicine. Also known as the golden spice as well as the spice of life, it is found growing well in the hilly areas of both the Western and Eastern Ghats. Currently it is cultivated in both the tropical and subtropical parts of the world (Ghani, 1998).

Turmeric is traditionally used mainly as spices, pigments and medicine. Turmeric has efficacy in both pharmacological as well as clinical studies used to cure different diseases like dyspepsia (Thamlikitkul *et al.* 1989), peptic ulcers (Prucksunand *et al.* 2001), and gastric ulcers (Muderji *et al.* 1981; Sakai *et al.* 1989; Rafatullah *et al.* 1990; Kositchaiwat *et al.* 1993; Masuda *et al.* 1993). The essential oil obtained from the plant can be used as antacid, carminative, stomachic and tonic (Ghani, 1998; Kirtikar *et al.*, 1996). The pharmacology of curcumin contained in turmeric was investigated and it proved that curcumin has anti-inflammatory activity, which returns the levels of serum glutamic oxaloacetic transaminase and serum glutamic transaminase to normal after its administration to inflamed rats (Srimal and Dhawan, 1973).

In recent years, tissue culture technique has been used for the large scale propagation of many plants. Micropropagation of turmeric is done conventionally by using recurrent rhizomes. Flowering of turmeric is very rare. Even when it flowers, hardly any seed is produced. So, at least one healthy bud containing rhizome pieces are used by turmeric cultivors as seed. Because of this reasons it is necessary to retain 20-30% of annual production for raising the following season crop. Moreover, its rhizome multiplication is very low. Besides these, they are prone to damages due to different factors such as adverse environment, insect and pathogen attack etc. Due to the low yield, vulnerability and higher price of seed, there are major constraints faced by the growers for the production of rhizomes. Now a days, micropropagation techniques have been used advantageously to conquer such problems in many vegetatively propagated crops as well as decorative and horticultural plants.

Because of high demand and greater economic importance along with its medicinal importance, it is essential to develop a suitable protocol for mass production of disease resistant plants through tissue culture technique. The suitable protocols for in vitro regeneration of plantlets of turmeric have also been reported (Balachandran *et al.*, 1990; Nadagauda *et al.*, 1978).

In the present paper an attempt was made to set up a protocol for the *in vitro* propagation of *Curcuma longa* to get disease free plantlets.

Materials and Methods

Sprouted rhizomes were collected from the field grown plants of turmeric (*Curcuma longa* Linn.). They were then brought to the laboratory and processed for aseptic culture. They were washed carefully under running tap water to remove the soil. Buds which emerged on rhizomes were excised with sharp blade and were surface sterilized by immersing in 1% extran for 10 min and then washed 5 times with double distilled water for 5 min each. After that, these were dipped in 0.1% mercuric chloride for 10 min, then again washed 5 times with sterile double distilled water to remove the traces of mercuric chloride under a laminar flow cabinet. Next, they were treated with 70% ethanol for 1 min and

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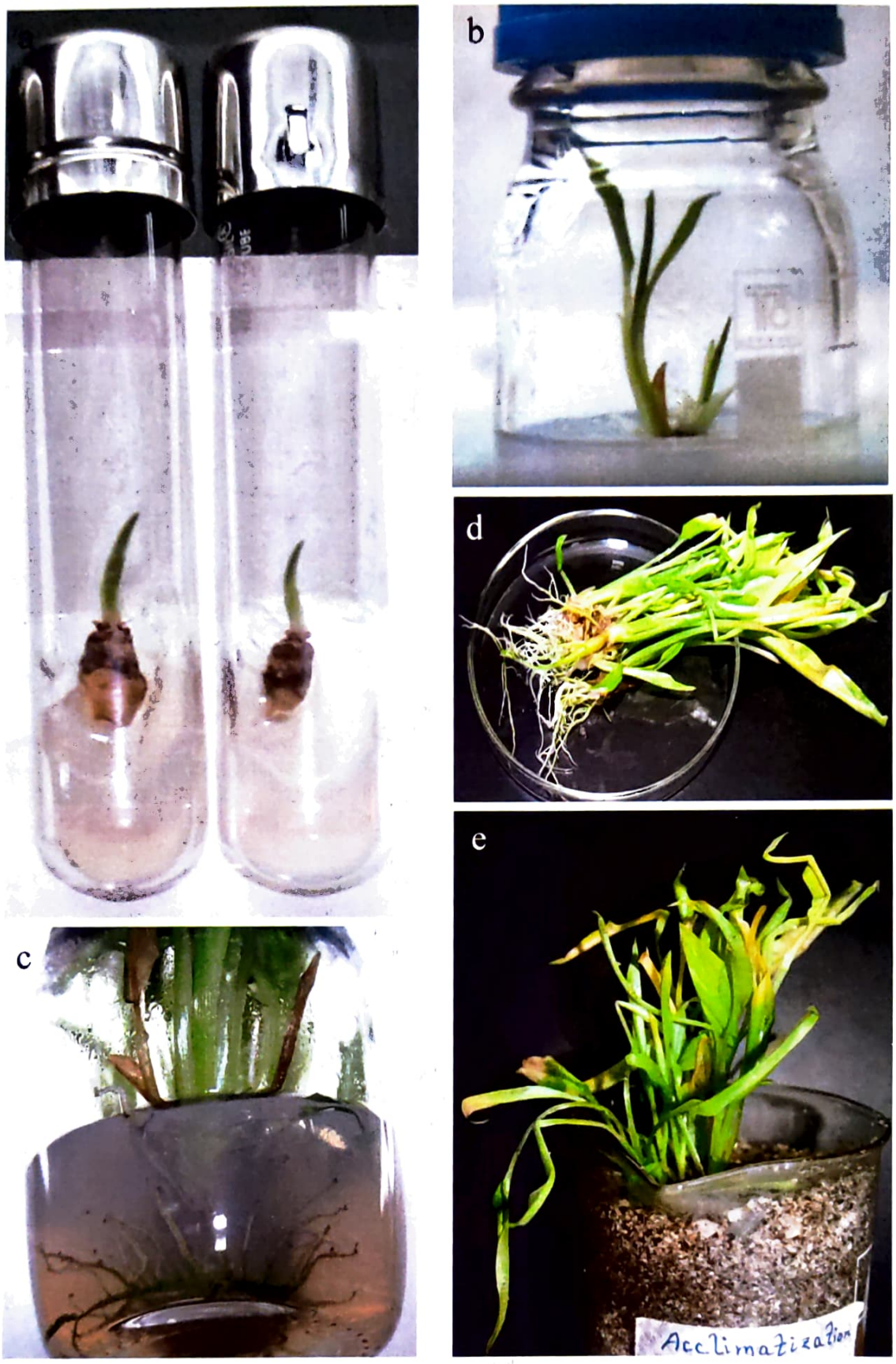

Fig1: Stages of micropropagation of *Curcuma longa* Linn. (a) Rhizomatous bud of *Curcuma* inoculated on MS media. (b) Formation of plantlets. (c) and (d) rooting from *in vitro* regenerated plantlets. (e) Transfer and hardening of *in vitro* grown plantlets in beaker containing soil and sand.

Table1: Effect of BAP and Kinetin on the regeneration of *Curcuma longa* Linn. in MS media with 3% sucrose when used alone.

Hormones (mg/l)	M S Medium with 3% sucrose	
	No. of Shoots	Height of Shoots (cm)
BAP		
1	1.93±0.34	1.10 ± 0.10
2	8.41 ± 0.33	5.82 ± 0.13
3	8.12 ± 0.41	6.10 ± 0.60
4	6.34 ± 0.49	6.63 ± 0.21
5	4.80 ± 0.10	4.93 ± 0.20
Kinetin		
1	1.93 ± 0.34	1.12 ± 0.11
2	2.97 ± 0.23	3.30 ± 0.20
3	3.87 ± 0.49	4.38 ± 0.50
4	5.47 ± 0.29	6.27 ± 0.42
5	4.67 ± 0.06	4.89 ± 0.14

finally, washed several times with sterile double distilled water for 10 min each.

After surface sterilization, rhizome buds were trimmed in 2-3 mm height and blotted dried on sterile blotting paper and inoculated on the Murashige and Skoog medium (Murashige and Skoog, 1962) for shoot and root development. Different concentrations and combinations of cytokinins like BAP (benzyl amino purine) and Kinetin were experimented in this study. To access the effect of different phytohormones sucrose was used as a carbon source at the rate of 30mg/l in the present experiment. Agar at the rate of 8mg/l was used as a solidifying agent in the culture media. The pH of the media was adjusted to 5.7 ± 0.1 with 0.1N NaOH or 1N HCl prior to the addition of agar, followed by autoclaving at 121 ° c for 20 min at 15 psi. The growth regulators were filtered sterilized and poured to the culture media. Then slants were prepared in a laminar flow cabinet. After 20 min the media became solidified in the test-tubes and inoculations of explants were done carefully. Cultures were incubated at 25 ° c with a photoperiod of 16 hrs at 2000-3000 lux light intensity of cool white fluorescent light.

From the *in vitro* regenerated plantlets, the healthy plantlets having good numbers of root were selected for hardening. The plantlets were removed from the media and were washed with double distilled water properly to get rid of the traces of agar sticking to the roots. The plantlets were then transplanted into beaker containing autoclaved mixture of soil and sand (1:1). Later they were transferred to the garden and planted in the field after a month.

Data related to height of the plants, number of shoots produced per plant were recorded.

Results and Discussions

The explants proliferated within 20-25 days after inoculation. But after few days of inoculation it was found that some of the explants were contaminated with fungi and bacteria. Such contaminated cultures were rejected and kept out from the culture room. Surface sterilization with 0.1% $HgCl_2$ solution for about 5 min resulted in fungal contamination of the cultured explants after few days of incubation. But when the explants were treated with 0.1% $HgCl_2$ for about 10 min more or less contamination free cultures were obtained. These explants remained green and healthy growth and proliferation of axillary shoots can be observed.

Explants grown on the basal medium did not produce shoots, roots or callus. To find out the optimum culture condition for maximum shoot and root proliferation from the cultured explants, different experiments were conducted with different hormonal concentrations.

Different stages of micropropagation of *Curcuma long* Linn (Fig. 1). MS media supplemented with 2mg/l BAP showed the maximum rate of shooting as well as rooting (Table 1).

Explants grown on MS media supplemented with different concentration of BAP showed some good results. It was found that sucrose concentration has a profound effect on the proliferation and growth of explants (Table 2). Maximum regeneration was noticed when explants were cultured on MS media supplemented with 2mg/l BAP and 3% sucrose. Extremely low regeneration was observed when the media was supplemented with 1% sucrose supplemented with 2mg/l BAP(2.1 shoot/ explant) and 3mg/l BAP (1.47 shoot/explant), where as MS media supplemented with 3% sucrose along with 2mg/l BAP(8.41 shoot/ explant) and 3mg/l(8.12 shoot/explant) had high regeneration rates. Thus it can be inferred from this that the concentration of sucrose in the media plays a vital role in regeneration of plantlets along with the phytohormones.

To observe the effects on regeneration different types of cytokinins like BAP and Kinetin were added to the media (Table 3). Media supplemented with BAP at a concentration of 2mg/l produced maximum number of plantlets (8.41), while maximum number of plantlets in case of Kinetin was observed at a concentration of 4mg/l (5.47). All the cultures resulted in moderate to spontaneous rooting. Rahman *et al.* 2004 also observed high effectiveness of BAP in turmeric tissue culture. Lower concentration of cytokinin in media led to less number of plantlets while it declined above a critical level. This may be inferred as high concentration of cytokinin in the media may produce some inhibitory effect and thus the rate of shooting is declined.

Different combinations and concentration of BAP and Kinetin were also tried to see the effect on regeneration. Media containing 2mg/l BAP and 3mg/l Kinetin showed the maximum number of plantlets/ explant (9.60) followed by 3mg/l BAP and 4mg/l Kinetin (8.53), where maximum shoot height (8.30) was obtained in media with 2mg/l BAP and 4mg/l Kinetin followed by 2mg/l BAP and 3mg/l Kinetin (7.60). Moderate to profuse rooting was observed in the most of the combinations. The number of plantlets regenerated and the rate of rooting was found to be proportional. Thus it can be concluded that combinations of BAP and Kinetin is more effective than when used singly.

In plant tissue culture, the ultimate success of *in vitro* propagated plantlets lies in its growth in the external

Table2: Effect of different concentration of sucrose (%) and BAP (mg/l) on the regeneration of *Curcuma longa* Linn. in MS media.

Concentration of Sucrose (%)	BAP (mg/l)	No. of Shoots
1	2	2.10
1	3	1.47
2	2	3.47
2	3	2.95
3	2	8.41
3	3	8.12

environmental conditions in pots. The in vitro propagated plantlets with well developed root and shoot system were successfully transferred to the beaker containing autoclaved mixture of soil and sand. After hardening the regenerated plantlets were transferred to the field where the plantlets showed 93% survival to maturity.

Thus based on these observations, we can conclude that this paper may prove useful to get huge number of desired cultivar within a very short span of time.

Table3: Effect of different concentrations of BAP and Kinetin on the regeneration of *Curcuma longa* Linn. in MS media with 3% sucrose.

BAP+Kinetin (mg/l)	MS Medium with 3% sucrose	
	No. of Shoots	Shoot Height (cm)
1+1	1.99±0.34	1.70±0.61
1+2	3.32±0.41	2.30±0.20
1+3	5.40±0.25	3.34±0.32
1+4	5.61±0.32	6.17±0.27
2+1	5.73±0.59	2.60±0.80
2+2	7.42±0.18	2.99±0.22
2+3	9.60±0.59	7.60±0.29
2+4	7.40±0.47	8.30±0.62
3+1	5.84±0.13	2.71±0.12
3+2	7.08±0.20	2.93±0.03
3+3	7.23±0.29	4.59±0.50
3+4	8.53±0.30	7.59±0.19

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Time-course accumulation of metabolites and expression of anti-oxidative enzymes in *Glycine max* under temperature stress

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Abstract
One month old seedlings of four cultivars of *Glycine max* (L.) Merrill were subjected to a cold stress of 5°C for a period of 0-24 h and analyses were done every 2 h. Results revealed that among the antioxidative enzymes, catalase and ascorbate peroxidase showed an initial decline upto 4 h after which there was an increase. The other 3 enzymes- superoxide dismutase, glutathione reductase and peroxidase exhibited increased activities during the early periods of stress followed by a decline. In all cases, 2 peaks were obtained- one during the early periods of stress, and a second during the late period. Decrease in catalase and ascorbate peroxidase activities were concomitant with increase in accumulation of H_2O_2 during initial stress. Accumulation of small antioxidants – ascorbate and carotenoids showed differences among the 4 cultivars. In JS 71-05 and JS 335 both these antioxidants decreased initially, whereas in the other 2 cultivars- Rossio and NRC 37, an initial increase was evident. In these 2 cultivars, besides the 3 antioxidative enzymes, ascorbate and carotenoids also may play a role in conferring tolerance. Total phenols increased initially in all cultivars. Protein accumulation during the different periods of stress also varied with the cultivars. It is quite clear from the results that the time of over expression of enzymes or accumulation of antioxidants varies with different cultivars and this differential time related response may be involved in tolerance.

Keywords: cold stress, soybean, antioxidants, antioxidative enzymes

Among the abiotic stresses temperature is one of the most important environmental stress that a plant encounters and it is also a major factor limiting the growth of plants. Temperature stress as heat, cold or freezing is a principal cause for yield reduction in crops (Boyer,1982) and ROS (reactive oxygen species) generated by these stresses have been shown to injure cell membranes and proteins which lead to oxidative stress (Larkindale and Knight, 2002). The effects of chilling on cell membranes have been looked at as an oxidative stress that results in the production of highly reactive substances, such as hydrogen peroxide and oxygen free radicals (Elstner and Oswald 1994, Prasad *et al.* 1994 a, b). Not only temperature but also the rate of temperature changes (Steffen *et al.* 1989) and duration of exposure determine the degree of injury (Rajashekar *et al.* 1983). Tropical and subtropical plant species are prone to injury at chilling temperatures of 0-15°C (Zhang *et al.* 1995)

Leguminous oil seed crop like *Glycine max* (L.) Merr. are hot season annuals and the plants are more sensitive to cold season. It is assumed that at low temperatures, the antioxidant systems of heat-loving plants fail to overcome the increasing level of ROS and peroxides arising there from; such failure is an initial stage of injury (Hariadi and Parkin, 1993).

The present study was undertaken to investigate how the responses of 4 cultivars of soybean to cold temperature, with special emphasis on antioxidative responses, proceed with increasing duration of stress at a specific temperature.

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Materials and Methods

Plant material and induction of cold stress

The seeds of 4 different cultivars of soybean (Rossio, NRC 37, JS 335, JS 71-05) were obtained from the National Centre for Soybean Research, Indore, M.P. and one (Rossio) from ICAR Gangtok. Viability was checked in laboratory and seedlings of the different cultivars were then raised from this stock of seeds. Seeds were soaked overnight in distilled water after surface sterilization with 0.1% $HgCl_2$ and grown in petriplates. For experimental purposes small seedlings were transferred to pots containing sandy loam soil mixed with farmyard manure. Plants were watered regularly and maintained properly. One month old seedlings were exposed to low temperature of 5°C for 0-24 hours, during cold temperature treatment, plants were kept in plant growth chamber, with controlled humidity and light.

Antioxidant enzyme extraction and assays

For extraction of enzymes, leaf samples were initially ground to powder in liquid nitrogen and then extracted with 0.05 M sodium phosphate buffer, pH 6.8-7.2 (peroxidase, ascorbate peroxidase and catalase) and 0.1 M potassium phosphate buffer, pH 7.6 (glutathione reductase and superoxide dismutase) using polyvinylpyrrolidone under ice cold conditions. The homogenates were then centrifuged at 10,000 rpm for 15 min. Supernatants were used as crude enzyme extracts.

Table 1: Accumulation of H_2O_2 in different cultivars of soybean at different time periods of cold treatment

Time*	H_2O_2 content (mM/g tissue)			
	Rossio	JS-335	JS-71-05	NRC37
0	1.384	1.049	1.222	1.084
2	2.108	1.161	1.411	1.268
4	2.125	1.678	1.661	1.691
6	3.108	2.488	1.947	1.887
8	2.106	2.381	3.214	1.643
10	2.089	2.328	2.25	1.286
12	2.053	2.238	1.822	1.268
14	1.628	2.22	1.643	1.233
16	1.138	1.5	1.553	1.22
18	2.048	1.358	1.483	2.125
20	2.822	1.358	2.286	2.214
22	1.411	2.75	2.322	3.105
24	1.286	3.697	2.911	3.161

*Treatment time in hours; treatment at 5°C; average of 3 replicates

Peroxidase (POX: EC. 1.11.17)

Peroxidise activity was assayed spectrophotometrically in UV VIS spectrophotometer (Model 118 SYSTRONICS) at 460 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2 (Chakrabotry *et.al.*, 1993). Specific activity was expressed as ΔA_{460mg} protein $^{-1}$ min $^{-1}$

Ascorbate peroxidase (APOX : EC.1.11.1.11)

Activity was assayed as decrease in absorbance by monitoring the oxidation of ascorbate at 290 nm according to the method of Asada *et.al* (1987) with some modification. Enzyme activity was expressed as ΔA_{290}mg protein $^{-1}$ min $^{-1}$

Catalase (CAT: EC.1.11.1.6)

Catalase activity was assayed as described by Chance and Machly (1955) by estimating the breakdown of H_2O_2 which was measured at 240 nm in a spectrophotometer. The enzyme activity was expressed as ΔA_{245}mg protein $^{-1}$ min $^{-1}$.

Superoxide dismutase (SOD: EC 1.15.1.1)

Activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Dhindsa *et al.* (1981) with some modification. The absorbance of samples were measured at 560nm and 1 unit and activity was defined as the amount of enzyme required to inhibit 50% of the NBT reduction rate in the controls containing no enzymes.

Glutathione reductase (GR: EC 1.6.4.2)

Glutathione reductase activity was determined by the oxidation of NADPH at 340 nm as described by Lee and Lee (2000). Enzyme activity was finally expressed as μM NADPH oxidized mg protein^{-1} min^{-1}.

Isozyme analysis

Isozyme analysis was carried out by PAGE following standard techniques. Following electrophoresis, gels were treated

Quantification of hydrogen peroxide

The hydrogen peroxide was extracted and estimated following the method of Jena and Choudhuri (1981) using titanium sulphate. Concentration of H_2O_2 was calculated using the extinction coefficient (0.28μmol^{-1}cm^{-1})

Non- enzymatic antioxidants

Ascorbate

Ascorbate was extracted and estimated by following the method of Mukherjee and Choudhuri (1983). The concentration of ascorbate was calculated from a standard curve plotted with known concentration of ascorbic acid.

Carotenoids

Carotenoids were extracted and estimated following the method described by Lichtenthaler (1987). Extraction was done in methanol and the extract was filtered. Absorbance of the filtrate was noted at 480nm, 663nm and 645nm in a VIS spectrophotometer and the carotenoid content was calculated using the following standard formula.

Phenols

Phenol was extracted by following the method of Mahadeven and Sridhar (1982). Total phenol was estimated by following the method of Bray and Thorpe (1954).

Protein quantification

Total soluble protein extracted in 0.05M sodium phosphate buffer were used as crude protein extract for quantification and analysis analysis of protein pattern. Proteins were estimated following the method of Lowry (1951).

Results

Effect of low temperature treatments on antioxidative enzymes

The seedlings were exposed to 5^{0}C for a period of 24 h and sampling was done every 2 h as described in Materials and Methods. Catalase activities showed an

Table 2: Accumulation of carotenoids in different cultivars of soybean at different time periods of cold temperature treatment

Time*	Carotenoid content (mg/g fresh wt.)			
	Rossio	JS-335	JS-71-05	NRC37
2	0.02	0.044	0.012	0.017
4	0.02	0.019	0.003	0.018
6	0.02	0.019	0.004	0.019
8	0.04	0.021	0.026	0.02
10	0.05	0.031	0.033	0.02
12	0.07	0.042	0.045	0.021
14	0.08	0.061	0.049	0.022
16	0.09	0.029	0.057	0.023
18	0.04	0.027	0.084	0.024
20	0.01	0.027	0.029	0.028
22	0.01	0.025	0.027	0.019
24	0.01	0.025	0.027	0.019

*Treatment time in hours; treatment at 5°C; average of 3 replicates

interesting trend. Initially, activity declined in all cultivars upto 4 h, with the decline being very steep in Rossio and NRC 37. After 4 h, there was an increase in activity till 22 h in Rossio, and 16 h in NRC 37. In JS 335 and JS 71-05, a sort of plateau was observed between 10-14 h and then there was a further decline (Fig.1).

Ascorbate peroxidase activities also showed an initial decline till 4-6 h in all cutivars. In all cultivars activities further increased till 14- 18 h of treatment and declined again after that (Fig.2). In case of peroxidase activity, Rossio showed enhanced activity till 12 h, a decline thereafter and a further increase after 20 h. In all the other 3 cultivars, activities initially increased till 6 h and then declined (Fig.3). Glutathione reductase and superoxide dismutase activities increased significantly in Rossio till 8-10 h and then declined. In the other 3 cultivars, there was an initial increase in activities till 4-6 h following which there was a decline (Figs. 4 and 5).

Accumulation of H_2O_2

This was also monitored for 24 h and results taken every 2 h. It was observed that in all cultivars, two peaks were evident in the accumulation pattern- one during the early hours (6-8 h) and the other towards the end , ie., 20 h in Rossio and 24 h in all other 3 cultivars (Table 1).

Effect of low temperature on small antioxidants

Total phenols increased initially in all 4 tested cultivars, followed by a decline and another increase following longer duration of stress. However, while in JS 335, JS 71-05 and in NRC 37, decline started after 4-6 h, in Rossio it was maintained till 10 h (Table 2). In case of ascorbate, in 2 of the cultivars, JS 335 and JS 71-05, a decline in accumulation was obtained even during the early periods, and only after 20 h of stress an increase was obtained. However, in Rossio and NRC 37, there was an initial increase till 4-6 h, followed by a decline and another increase later on (Table 3). Similar trend was also obtained in case of accumulation of carotenoids- i.e., in JS 335 and JS 71-05, a decline in accumulation was obtained till 8 h after which there was an increase.

Table 3: Accumulation of ascorbate in different cultivars of soybean at different time periods of cold temperature treatment

Time*	Ascorbate content (mM/g fresh wt.)			
	Rossio	JS-335	JS-71-05	NRC37
2	6.13	1.60	1.48	1.70
4	8.58	1.25	1.48	2.26
6	5.83	1.60	1.20	2.80
8	4.17	1.00	1.20	2.30
10	2.77	0.73	1.03	1.33
12	1.35	0.4	0.60	5.02
14	0.85	0.33	0.90	5.14
16	5.14	0.14	0.90	5.70
18	8.16	0.63	1.50	4.70
20	9.48	0.30	0.73	4.40
22	8.95	0.23	0.65	3.50
24	8.13	0.23	0.59	3.30

*Treatment time in hours; treatment at 5°C; Average of 3 replicates

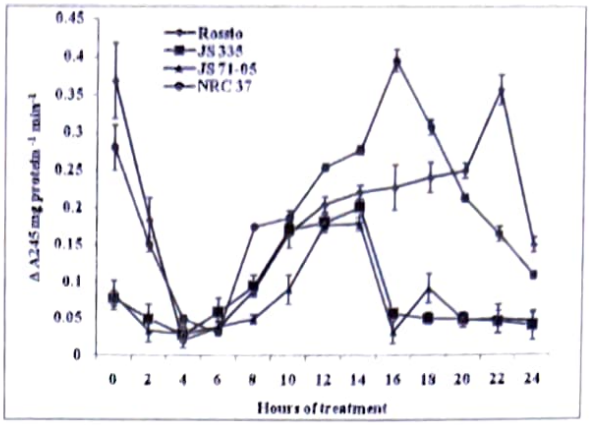

Fig. 1: Catalase activities in different cultivars of soybean subjected to a temperature of 5°C for different durations

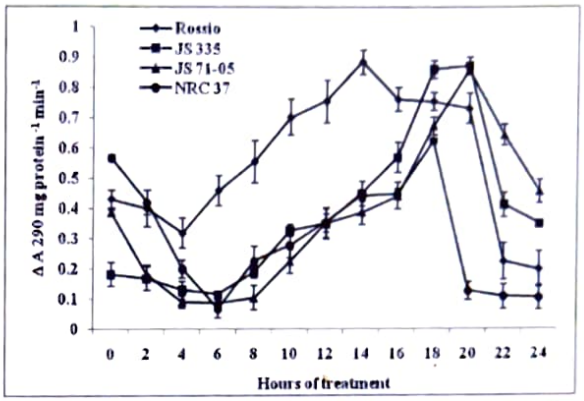

Fig. 2: Ascorbate peroxidase activities in different cultivars of soybean subjected to a temperature of 5°C for different durations

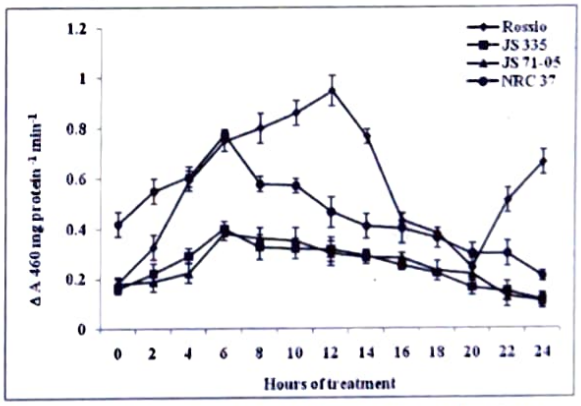

Fig. 3: Peroxidase activities in different cultivars of soybean subjected to a temperature of 5°C for different durations

However, in Rossio and NRC 37, there was an initial increase till 4-6 h, followed by a decline and another increase later on (Table 4).

Effect of cold stress on protein accumulation

Pattern of protein accumulation in seedlings of soybean over the period of stress varied among the four cultivars. In 3 of the cultivars, a decrease in protein accumulation was evident till 14-16 h after which there a slight increase followed by further decline. However, in Rossio, an increase in accumulation was evident initially till 6 h followed by a decline and another peak was obtained at 20 h of cold treatment (Table 5). SDS-PAGE

Table 4: Accumulation of phenols in different cultivars of soybean at different time periods of cold temperature treatment

Time*	Phenol content (mg/g tissue)			
	Rossio	JS-335	JS-71-05	NRC37
2	2.4	2.3	3.8	2.8
4	2.5	2.9	3.8	2.9
6	2.6	2.9	2.85	2.2
8	3.6	2.7	2	1.6
10	4.4	1.7	1.85	0.8
12	3.0	1.6	1.95	1.0
14	2.4	1.4	3.9	1.4
16	2.0	2.8	4.05	1.8
18	1.9	3.5	5.3	2.8
20	1.8	3.9	5.3	3.4
22	2.7	3.3	4.4	4.7
24	2.6	2.9	3.4	3.6

*Treatment time in hours; treatment at 5°C; Average of 3 replicates

analysis of proteins revealed the accumulation of few new proteins during cold stress.

Discussion

The time-course dependent biochemical responses of soybean seedlings, specially the antioxidant responses were determined in 4 cultivars exposed to a cold temperature of 5°C for a period of 24 h, with analyses being done every 2 h. Among five antioxidative enzymes tested, results revealed that both catalase and ascorbate peroxidase showed an initial decline in activity for 4-6 h before being enhanced. This decline in activity could be correlated with an increase in accumulation of H_2O_2 detected during the early hours. It is quite clear that in the early period of oxidative stress, there is an accumulation of H_2O_2 which, besides being an ROS, is also involved in signaling (Chakraborty, 2005). It is now clear that ROS, besides being toxic molecules causing damage to proteins and DNA, are also involved in signaling substances for guard cell functioning, photoprotection, pathogenesis and development (Desikan *et al.* 2004; Einset *et al.* 2007).

With increase in the duration of cold stress, catalase and ascorbate peroxidase activities increase resulting in breakdown of H_2O_2. Thus, during the early period of

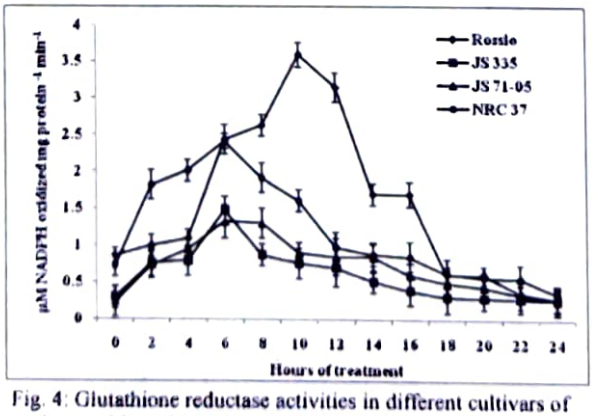

Fig. 4: Glutathione reductase activities in different cultivars of soybean subjected to a temperature of 5°C for different durations

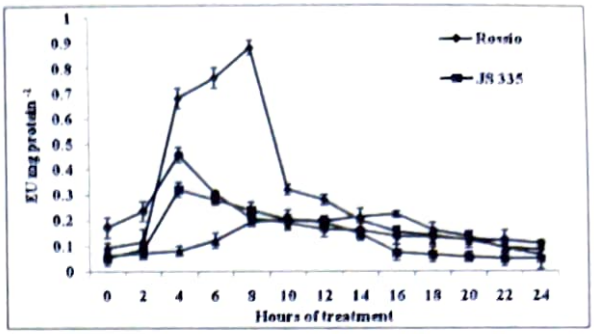

Fig. 5: Superoxide dismutase activities in different cultivars of soybean subjected to a temperature of 5°C for different durations

stress, protection against cold-stress is provided by activities of peroxidase, superoxide dismutase and glutathione reductase which are enhanced initially and lead to a certain degree of protection against oxidative stress. Prolonged period of stress or increasing the stress intensity, however, leads to a decline in activity indicating that the plants succumb to oxidative stress after an initial resistance. It was reported by Queiroz *et al.* (1998) that in coffee seedlings subjected to chilling stress of 15-10°C, ascorbate peroxidase and catalase activities did not change, in contrast with the activities of guaicol peroxidase, which increased. Cellular damage caused by superoxide and lipid peroxidation might be reduced or prevented by protective mechanisms like free radical processing by enzymes such as SOD, CAT, POX and APOX (Asada and Takahashi, 1987). Ascorbate peroxidase gene expression and activity has been reported to be rapidly induced by various stress conditions including chilling (Prasad *et al.* 1994, Keshavant and Naithani, 2001). Lukatkin (2002) compared SOD activity in various plant species differing in their cold-resistance during chilling. According to him, in resistant cultivars, chilling sharply activated SOD production. It has been reported that under stress conditions different plants and tissues respond to SOD induction differently with regard to SOD induction suggesting that different mechanisms may be involved

Table 5: Accumulation of proteins in different cultivars of soybean at different time periods of cold temperature treatment

Time*	Protein content (mg/g tissue)			
	Rossio	JS-335	JS-71-05	NRC37
2	151	144	200	224
4	157	104	192	210
6	290	102	188	204
8	129	84	182	200
10	113	84	176	184
12	103	176	192	136
14	100	261	192	152
16	130	200	224	184
18	130	200	240	160
20	150	168	256	144
22	120	148	250	116
24	110	140	240	108

*Treatment time in hours; treatment at 5°C; Average of 3 replicates

in protection against oxidative stress (Blokhina *et al.* 2003). Huang and Guo (2005) reported that, under chilling conditions, SOD activity of tolerant rice cultivar remained similar to control, while that of susceptible cultivar decreased after chilling and remained low throughout the chilling period. In the present study also, the nature of responses varied among the cultivars. Rossio could maintain antioxidant responses for more prolonged period than the other cultivars. NRC 37 also showed responses more or less similar to Rossio, while the other 2 cultivars varied.

Besides, antioxidative enzymes, small antioxidants such as phenols, ascorbate and carotenoids are also involved in stress tolerance. In the present study, accumulation of these was also determined at specific time intervals. It was observed that while total phenols initially increased in all cultivars, increased accumulation was maintained in Rossio for longer periods than in the other 3. The other 2 antioxidants- ascorbate and carotenoids decreased in JS 335 and JS 71-05 with increase in the duration of cold stress up to 8h after which they increased. In Rossio and NRC 37, the responses were different, with an initial increase, followed by a decline. It seems probable that in Rossio, and NRC 37, to some extent, which are more tolerant than the other two, small antioxidants also play a role initially, whereas, in the more susceptible cultivars, they are not involved in protection.

In conclusion, it may be stated that soybean plants respond to cold-stress by an initial enhancement of antioxidant activity which however, declines with prolonged stress. Among the cultivars, Rossio, which is grown in the hilly regions exhibited more tolerance towards cold-stress as evidenced by maintenance of antioxidant activities for longer periods in comparison to the other cultivars. Higher activities of antioxidative enzymes during chilling, along with accumulation of other antioxidants, could be associated with tolerance.

Acknowledgement

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Bacillus megaterium mediated growth promotion and biological control of crown rot disease of *Oncidium* incited by *Sclerotium rolfsii* Sacc.

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Abstract

Crown rot disease of orchid caused by *Sclerotium rolfsii* is a major constraint in orchid cultivation especially during prolonged hot, humid weather. The disease causes the death of the infected plants, spreading infection to the nearby plants and destroying the whole plantation. Despite its aggressive nature, a number of microorganisms have been found to limit the growth. *Bacillus megaterium* de Bary TRS-3 isolated from rhizosphere of tea plants has been used as a biological control agent in the present study in controlling the growth of *S. rolfsii* in one of the most widely cultivated orchid genus-*Oncidium* sp. and to determine the role of defense enzymes in induction of systemic resistance and disease suppression. The bacterium suppressed the mycelial growth of fungal pathogen as well as sclerotial germination. *In vitro*, it produced volatiles which could inhibit the growth of the pathogen. *B. megaterium* induced resistance to fungal pathogen by eliciting the increase in activity of both chitinase and β-1,3- glucanase as well as peroxidase and ascorbate peroxidase and reduction in the activity of catalase of *B. megaterium* TRS-3 treated orchids plants. Bacterium treated plants also showed increase in content of total chlorophyll as well as both chlorophyll a and b. The present study proved the potentiality of *B. megaterium* TRS-3 as a biological control. .

Keywords: *Sclerotium rolfsii, Bacillus megaterium, orchid, stem rot*

Orchids are the top selling herbaceous perennials worldwide and cultivated in a large scale. However, the cultivation of orchid is often plagued by a disease called crown rot or stem rot that damages many plants in various stages of growth. This fungal disease, caused by *Sclerotium rolfsii*, begins its life cycle with germination of sclerotia and can severely damage established host within a week, and is difficult to eradicate. Symptoms begin to appear on host after prolonged hot, humid weather. The lower leaves begin to turn yellow, and then brown, and wilt from the margins back toward the base. The base of these damaged petioles shows a brown discoloration and mushy texture. Fluffy white threads (mycelium) of the crown rot fungus are often found to be present on the rotted tissue and surrounding soil (Fig. 1). The leaves begin to collapse and soon the plant dies rapidly spreading infection to the nearby plants and destroying the whole plantation.

Microorganisms that grow in the rhizosphere are ideal for use as biological control agents against soil borne pathogens since the rhizosphere provides the front line defense for roots against attack by pathogens. A number of microorganisms have been found to limit the growth, including *Bacillus* sp. *Pseudomonas* sp. *Gliocladium virens*, *Penicillium*, *Trichoderma harzianum* and *Trichoderma viride* (Kokalis-Burelle *et al.* 2006, Murphy *et al.* 2000, Zhang *et al.* 2002 and van Peer *et al.* 1991). *Bacillus megaterium* isolated from rhizosphere of tea plants has been used as a biological control agent in the present study because of its innate ability to form endospore which makes it easier to formulate the commercial biocontrol agent.

The present investigation was undertaken to test the ability of *B. megaterium* as potent biocontrol agent in controlling the growth of *S. rolfsii* in one of the most widely cultivated orchid genus like *Oncidium* sp. and to determine the role of defense enzymes in providing resistance and disease suppression to the plant.

Materials and methods

Antagonistic tests

Bacillus megaterium isolated from the rhizosphere of healthy tea plants growing at Darjeeling hills were used in the present study. The antagonism was determined *in vitro* by dual culture test against *S. rolfsii*. Observations were recorded from 72 h of growth onwards in petri plates and diameter of growth of the pathogen (fungal growth) was measured and compared to control growth without the bacterium. Each experiment was run in triplicate. Results were expressed as mean percent inhibition of the growth of *S. rolfsii* in presence of *B. megaterium* de Bary TRS-3.

Percent inhibition was calculated using the formula:
% inhibition=(1-fungal growth/control growth)X100

Volatile production

Inhibition of pathogen by volatile compound released by *B. megaterium* was also tested. For this purpose , *B. megaterium* TRS-3 was streaked on the one half of the petri plate containing PDA and 7mm disk of the freshly growing *S. rolfsii* was placed at the center of another petri plate. Both the half plates were sealed to prevent

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Table 1: Effect of *B. megaterium* on crown rot disease in *Oncidium* plants caused by *Sclerotium rolfsii*

Treatment	Percent disease incidence* Days after inoculation		
	15	30	45
S rolfsii	36	87	92
S rolfsii+*B megaterium*	12	23	42

* PDI was calculated as the number of diseased plants out of 25 inoculated plants in each treatment

the loss of volatiles formed. The plates were incubated at 28°C. The growth of the pathogen was measured and compared to control developed in the absence of the bioantagonist. Each experiment was performed in triplicate.

In vivo application

For application of bacteria in nursery grown plants, fresh bacterial culture was centrifuged at 10,000 rpm and pellets were collected and suspended in sterile distilled water at a concentration of $3x10^6$ cfu. The bacterial suspension thus obtained was sprayed on the plants. For assessment of disease control the plants were initially treated with *B. megaterium* followed by challenge inoculation with *S. rolfsii*. The percentage of disease incidence was calculated after 15, 30 and 45 days of inoculation as the number of diseased plants over the total number of inoculated plants.

Active principle extraction and testing

The active principle was extracted from bacterial lawn using acetone and partially purified. Extracts were then tested in vitro against growth of S.rolfsii.

Extraction and assay of enzyme activity

The enzyme activity of the plant was studied after 72 hrs of bacterial application and compared to that of the untreated control plants. For this the plant tissues were weighed and ground to powder in liquid nitrogen and extracted with 0.05 M Na_2HPO_4/ NaH_2PO_4 buffer (pH 6.9) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulphonylfluoride (PMSF) and 20mg of polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 4°C for 20 minutes at 15,000 g. The supernatant was used for the activity assay of peroxidase, ascorbate peroxidase and catalase.

Peroxidase activity was assayed spectrophotometrically as increase in absorbance at 460 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2 (Chakraborty *et al.*1993).

Ascorbate Peroxidase activity was assayed as decrease in absorbance by monitoring the oxidation of ascorbate at 290 nm (Asada, 1994).One unit of activity was defined as 1 µM of ascorbate oxidized by the enzyme in 1 minute.

Catalase activity was measured according to Chance and Machly (1955) by monitoring the disappearance of H_2O_2 at 240 nm in a UV-VIS Spectrophotometer and enzyme activity was expressed as enzyme units/mg protein.

Chitinase was extracted activity was assayed using 0.1 M sodium citrate buffer (pH 5) following the method described by Boller and Mauch (1998).

Extraction and assay of activity of β-1, 3- glucanase was done following the method described by Pan *et al.* (1991).

Results and discussion

In vitro antagonism of B.megaterium

Antagonism of *B. megaterium* TRS-3 against *S. rolfsii* was investigated by dual culture assay in solid medium. *B. megaterium* TRS-3inhibited the hyphal growth by 55.5% compared to control. The zone of inhibition was found to be 2.3 cm after 7 days of incubation. Several strains of *Pseudomonas* sp. and *Bacillus* sp. have been reported to suppress soil borne diseases caused by fungal pathogens (Powell,1992). *B. megaterium* TRS-3 released some volatile compound which inhibited the mycelial growth of fungal pathogen *in vitro*. The compound extracted from bacterial lawn of *B. megaterium* TRS-3 was found to be highly active and inhibited the growth of fungal pathogen completely when added to the media in 1:5 ratio. It also inhibited sclerotial germination. None of the treated sclerotia germinated whereas the water treated sclerotia showed full germination.

In the present study it is evident that *B. megaterium is* antagonistic to *S. rolfsii* and also produced some volatile component which significantly inhibited the growth of pathogen

In vivo tests

When applied in field *B. megaterium* TRS-3 was effective in reducing crown rot disease incidence. The percentage of disease incidence after 45 days of inoculation was 92% in untreated *Oncidium* plants which reduced to 42% on pretreatment of plants with *B. megaterium* TRS-3 (Table 1). In several cases, disease suppression has been attributed to the ability of the bacteria to produce antibiotic compounds (Homma and Suzui, 1989; Howell and Stipanovic, 1979; Dahiya *et al.* 1988).

Table 2: Changes in enzyme activities in *Oncidium* induced by *B megaterium* and *S.rolfsii*

Treatment	Enzyme activities				
	Peroxidase[1]	Ascorbate[2] peroxidase	Catalase[3]	Chitinase[4]	β-1,3 glucanase[5]
Control	0.18	0.76	2.08	3.93	11.20
B megaterium	0.58	2.07	1.81	6.16	13.00
S. rolfsii	0.28	0.91	2.32	4.58	12.50
B megaterium + *S rolfsii*	0.39	1.38	1.93	4.67	12.25

1=ΔA_{460} g^{-1} fresh wt. tissue; 2=ΔA_{290} nm mg^{-1} fresh weight min^{-1}; 3=Enzyme unit/mg protein/min; 4=µg GlcNAc g^{-1} tissue; 5=mg glucose g^{-1} tissue min^{-1}

Fig 1: Healthy plant of Oncidium (A&B); Sclerotium rolfsii Sacc. Infected plants showing the leaf yellowing typical of stem rot (C&D); Sclerotium rolfsii Sacc. Mycelia showing mustard like sclerotia.

Effect of B.megaterium on enzyme activities

Changes in the activities of different enzymes were tested in the plants treated with *B. megaterium* TRS-3 alone, *B. megaterium* TRS-3 treated plants challenged with *S. rolfsii* and *S. rolfsii* inoculated plants and compared their activity with that of control plants.

Assay of chitinase and β-1,3-glucanase was used to evaluate whether *B. megaterium* could induce resistance to fungal pathogen. *B. megaterium* elicited increase in activity of both chitinase and β-1,3- glucanase in *B. megaterium* treated *Oncidium* plants compared to untreated control and pathogen treated plants. *B. megaterium* TRS-3 also elicited significantly greater peroxidase and ascorbate peroxidase activities in leaves of treated *Oncidium* plants compared to untreated and pathogen inoculated plants. Catalase activity on the other hand was found to be significantly reduced in *B. megaterium* TRS-3 treated plants with respect to untreated control plants (Table 2).

Results of the present finding therefore, indicate that *B. megaterium* TRS-3 is a potential biological control and can be utilized for the control of *S. rolfsii* in orchid plantations successfully after field trial.

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Evaluation of genetic variation among fourteen rice (*Oryza sativa* L.) varieties (Landraces) of North Bengal using morphological traits

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Abstract
The Northern region (North Bengal) of West Bengal possesses a rich genetic diversity of rice (*Oryza sativa* L.). This region is known for growing one important rice variety Tulaipanji with GI number in the district of North Dinajpur and many other varieties of local landraces with tolerance to biotic and abiotic stresses. A considerable range of diversity and variation exists among the genetic resources of rice landraces of North Bengal. Prominent fourteen (14) landraces such as Sadanunia, Kalonunia, Dhepi, Banni, Dudkalam, Malsira, Lalpanati, Bhadoi, Ashami, and Gobindabhog, Enda, Chenga, Katharibhog, Tulaipanji are known for their special quality attributes were collected from the different districts of North Bengal. Genetic variation among these landraces was characterized by means of morphological traits such as seed sizes, shape, volume, colour, awn character, surface texture with husk (Microscopic observation) and pericurp colour. The Karl Pearson's simple matching coefficient was used to calculate the genetic diversity among the varieties. Similarity coefficient was ranged from 0.94 to 0.64 with an average of 0.79 among the 14 varieties. Cluster analysis based on genetic similarity of these varieties gave rise to three distinct groups. The results suggested that the level of genetic diversity within this group of rice varieties of North Bengal was sufficient for breeding programs and can be used to establish genetic relationships among them on the basis of morphological traits.

Keywords: Rice, genetic variation, cluster analysis, morphology

Rice (*Oryza sativa* L.) is the principal food crop under the family Poaceae (Graminae) globally providing food and livelihood security to a large section of society at least 2/3rd of the world population. Over the last three decades in response to the declining availability of water and land, rice production has been declined at an alarming level (Childs, 2004). As a consequence, there will be serious food shortage in near future (Sakamoto and Matsuoka, 2008). Attempts are to be made to improve the existing crop production to feed the 9.0 billion world population by 2050. Due to increase in the consumption of rice along with a tremendously growing demand, there is a need to conserve the germplasm of the landraces which is expected to serve as the major way to develop the new high-yielding varieties and to raise the maximum yield potential. Collection, conservation, evaluation of diverse rice germplasm is important for the purpose. It is estimated that about 120000 varieties of rice exist in the world (Khush, 1997), consequent to selection by farmers to suit different habitats and growing conditions across the region. However, the aggressive introduction of modern verities in this region resulted on the loss of many valuable landraces from the farmer's field. Only few varieties/accessions have been employed in breeding programs, hence there is a large amount of rich diversity of rice landraces left untapped. This creates a narrow genetic base rendering the high-yielding varieties vulnerable to unpredicted biotic and abiotic stresses. Extensive screening of germplasm accessions and local landraces of rice for desirable traits can help in identifying landraces suitable for specific breeding programs (Kasem *et al*., 2009). The genetic architecture of local landraces of rice is shaped and stabilized by natural (biotic and abiotic) and artificial (human) selection, hence they harbour variability for adoptive as well as to some extent productive characteristics. Thus, the local landraces or the wild relatives are more adaptive but less productive. And on contrary, the high-yielding varieties are more productive and less adaptive. The morphological architecture of local landraces of rice seeds is governed by and reflected in forms of their wide range of phenotypic parameters, like weight of the seed, length of the seed, volume of the seed, presence or absence of awn, colour of the pericarp, colour of the seed itself and many more. These landraces can be short, medium or long in terms of seed length. Some local varieties are found to be endowed with a pleasing aroma. Rice seeds also come in many different colours like yellow, deep brown, reddish brown, blackish brown, *etc*. Similarly, the pericarp of these varieties may exhibit colours of different types like yellowish white, light brown, deep brown, *etc*. There is no report on characterization of rice landraces of North Bengal so far carried out still to date.

In the present study, we report on the evaluation of genetic variation existing among the fourteen (14) landraces of rice varieties of North Bengal on the basis

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Table 1: Morphological characteristics of 14 rice (landraces) seeds are summarized in this table

Varieties (Landraces)	Weight (mg)	Volume (mm^3)	Length (mm)	Presence of awn	Awn length (mm)	Color of pericarp	Aroma	Color of husk
Sadanunia	15.8	0.159	7.7	Awned	28.6	Yellowish white	Present	Yellow
Kalonunia	12.9	0.127	6.4	Awned	9.7	Yellowish white	Present	Deep brown
Dhepi	23	0.287	7.8	Awned	12.2	Light brown	Absent	Reddish brown
Banni	26	0.273	8.9	Awned	17	Yellowish white	Absent	Blackish brown
Dudkalam	22	0.206	8.1	Awnless	-	White	Absent	Yellow
Malsira	21	0.214	8.1	Awned	12.8	Yellowish white	Absent	Reddish brown
Lalpanati	24.16	0.238	8.5	Awnless	-	Light brown	Absent	Reddish brown
Bhadoi	19.8	0.210	7.6	Awnless	-	Deep brown	Absent	Blackish yellow
Gobindabhog	10.57	0.115	5.6	Awnless	-	Yellowish white	Present	Blackish yellow
Ashami	21.63	0.217	7.7	Awnless	-	Crème color	Absent	Reddish yellow with black spots
Tulaipanji	16.28	0.138	7.9	Awned	31.9	Yellowish white	Present	Reddish yellow
Katharibhog	9.8	0.115	5.3	Awnless	-	Yellowish white	Present	Reddish yellow
Enda	24.5	0.263	7.3	Awned	13.4	Light brown	Absent	Yellowish brown
Chenga	23.7	0.233	7.5	Awnless	-	Light brown	Absent	Blackish brown

of morphological traits such as seed shape, size, colour and texture of the seeds with husk and clustering them using simple matching coefficient for their conservation purposes.

Materials and Methods

Plant materials

Fourteen varieties of rice (landraces) seeds were collected from different districts of North Bengal and maintained in the Plant Genetics & Tissue Culture Laboratory, DRS Department of Botany, University of North Bengal. These are Sadanunia, Kalonunia, Ashami, Dudkalam, Lalpanati, Banni, Malsira, Bhadoi, Dhepi, Gobindabhog, Tulaipanji, Enda, chenga and Katharibhog.

Morphological traits

Weight of single seed: Average single seed weight was measured by measuring total 5 gm of seed of each of the 14 varieties and then divided by the total numbers of rice seeds were taken. Weight of one seed was calculated in the following way- Seeds of 5gm weight/ Total No. of seeds = weight of per seed.

Volume measurement per seed: The volume of each of the seed was measured using a measuring cylinder containing 20 ml of water. The seeds were added to it one by one until the volume of the water reached 25 ml, and then the seeds were taken out and counted. The volume of each of the seeds was enumerated as follows -

Final vol. - Initial vol./Total No. of seeds = Volume of single seeds ≈ mm^3

Measurement of length: Fourteen rice seeds from each of the varieties were taken and then the length was measured using a millimeter paper, and finally the average length was calculated for each varieties of rice.

Study of other phenotypic traits (characteristics): Other phenotypic characteristics like presence of awn, and its length, presence of aroma, color of pericarp, and color of husk were carefully observed in each variety and summarized in table 1.

Morphological data analysis: The work was based on general similarity as judged by the comparison of many characters, each given equal weight. Karl Pearson's coefficient of correlation (expressed as r), was used to determine the correlation between each pair of traits (summarized in table 2). The following formula was used to compute correlation coefficient (Sneath and Sokal, 1973) between two taxonomic units x and y:

$$r = \frac{\sum xy}{N \times SD_x \times SD_y} = \frac{\sum xy}{N \times \frac{\sqrt{\sum x^2}}{N} \times \frac{\sqrt{\sum y^2}}{N}}$$

Here r stands for coefficient of correlation, x stands for (x- arithmetic mean of X), y stands for (y- arithmetic mean of Y), S.D. stands for standard deviation of x/y series.

RESULTS AND DISCUSSION

Morphological traits such as seed texture were studied under Stereomicroscope (E330-ADUI.2x, Olympus) in all the fourteen rice varieties. Surface texture of the fourteen rice land races of North Bengal were depicted in figure 1. Each variety has distinct type of seed husk texture and specifying a unique genetic trait. Seed colour varies from one variety to other (given in table 1) ranging from yellow, brown, blackish to blackish brown. Several characters in relation to morphology of rice seeds of 14 landraces (North Bengal) were studied (given in table 1) and analyzed in detail following which an association co-efficient so called correlation coefficient was numerically calculated for every pair, thereby comparing every variety with the every other variety (Table 2). It was revealed that while the weight of each seed in mg varied from 9.80 recorded in case of Katharibhog to 24.50 recorded in Enda. Volume in mm^3 per seed was the parameter with certain variation among the landraces. Single seed weight was lowest in Katharibhog (9.8 mg) and highest in Banni (26 mg). Similarly lowest volume recorded in Katharibhog (0.115 mm^3) and highest in Dhepi (0.287 mm^3). Length was varies from 5.3 mm (in Katharibhog) to 8.9 mm (in Banni). Awn was present in the following rice varieties *viz.* Sadanunia, Kalonunia, Dhepi, Banni, Malsira, Tulaipanji, and Enda. Awn length was varies from 9.7 mm to 31.9 mm. Specific aroma was recorded in five rice varieties out of fourteen varieties, these are sadanunia, Kalonunia, Gobindobhog, Tulaipanji and Katharibhog. Seed colour varies from yellow to brown to blackish brown (Table 1). The pericarp colour was also different in different varieties. Seed size was defined by its length and the seeds were categorized as 'very long (>7.5 mm), long (6.5-7.5 mm), medium (5.5-6.5 mm), and short (<5.5 mm). Most of the rice seeds were very long, the remaining are being medium sized. Sadanunia (7.7 mm), Dhepi (7.8 mm), Banni (8.5 mm), Dudkalam (8.1 mm), Malsira (8.1 mm), Lalpanati (8.5 mm), Bhadoi (7.8 mm), Ashami (7.7 mm), Tulaipanji (7.9 mm) had length greater than 7.5 mm, Enda (7.3 mm), Chenga (7.5 mm) belonged to the category of long seeds. Gobindabhog (5.6 mm) and Kalonunia (6.4 mm) were the two medium sized varieties while Katharibhog (5.3 mm) was the only exclusively short variety. Awn was reported to be present in a very few varieties like Sadanunia, Kalonunia, Dhepi, Enda, Lalpanati, with a variation in length from 9.7 mm in Kalonunia to 31.9 mm in Tulaipanji. Only five of the fourteen rice landraces were gifted with a pleasing form of aroma. The red, black, brown, purple colour in rice pericarp due to the presence of anthocyanin pigment. Anthocyanins have antioxidant properties, which have positive human health benefits including suppression of tumor cell growth. The rice landraces having high percentage of similarity, as worked out in terms of the association co-efficient so called correlation co-efficient, were grouped together while constructing the dendrogram (Figure 2). Correlation co-efficient had values lying between 0.0 (no matches) to 1.0 (100% matches). Highest percentage of similarity worked out in terms of correlation co-efficient was recorded as 0.94 between Banni and Lalpanati and lowest in between Katharibhog and Tulaipanji (0.78) (Table 2). Dendrogram has shown three distinct clusters comprising the 14 rice varieties of North Bengal. First cluster consisting of five varieties namely Gobindabhog, Sadanunia, Kalonunia, Katharibhog and Tulaipanji. All the varieties are with unique aroma of their own and with yellow seed colour except Kalonunia. Second cluster comprises of Dudkalam, Dhepi, Lalpanati and Banni. Third cluster

Table 2: Similarity matrix was calculated based on correlation-coefficient among the varieties using morphological traits

	Enda	Banni	Malsira	Dhepi	Kalo nunia	Tulai panji	Sada nunia	Kathari bhog	Gobinda bhog	Dud kalam	Bhadoi	Ashami	Lal panati	Chenga
Enda	1													
Banni	0.765	1												
Malsira	0.941	0.706	1											
Dhepi	0.765	0.882	0.706	1										
Kalonunia	0.882	0.765	0.824	0.765	1									
Tulaipanji	0.824	0.824	0.765	0.824	0.941	1								
Sadanunia	0.706	0.824	0.647	0.824	0.824	0.882	1							
Katharibhog	0.824	0.706	0.765	0.706	0.941	0.882	0.765	1						
Gobindabhog	0.706	0.824	0.647	0.824	0.824	0.882	0.94	0.765	1					
Dudkalam	0.882	0.882	0.824	0.882	0.882	0.941	0.824	0.824	0.824	1				
Bhadoi	0.882	0.765	0.824	0.765	0.882	0.824	0.824	0.824	0.824	0.765	1			
Ashami	0.941	0.706	0.882	0.824	0.824	0.765	0.647	0.765	0.647	0.824	0.824	1		
Lalpanati	0.824	0.824	0.765	0.941	0.824	0.765	0.765	0.765	0.765	0.824	0.824	0.882	1	
Chenga	0.94	0.765	0.941	0.765	0.882	0.824	0.706	0.824	0.706	0.882	0.882	0.941	0.824	1
	Enda	Banni	Malsira	Dhepi	Kalo nunia	Tulai panji	Sada nunia	Kathari bhog	Gobinda bhog	Dud kalam	Bhadoi	Ashami	Lal panati	Chenga

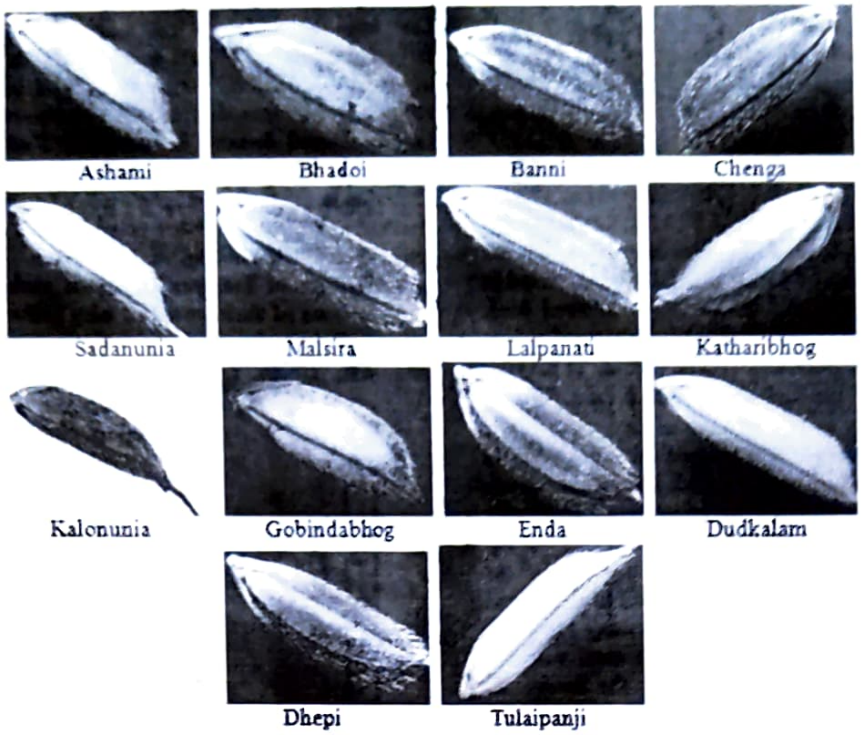

Fig1: Stereomicroscopic images of fourteen rice seeds (Landraces of North Bengal) showing different shapes, sizes and colour (Photographs were taken using Olympus Stereomicroscope E330-ADU1.2x/7c24087). Variety name was mentioned below.

consisting of the following varieties Chenga, Ashami, Bhadoi, Malsira and Enda. There are certain genetic variations existing among the 14 rice varieties under study and can be used in breeding program for crop improvement in future. Remote areas of North Bengal are yet to be assessed for their landraces. A complete picture on rice diversity will only be available when the unexplored areas of North Bengal and North-eastern regions of our country are fully explored. To safeguard the gene pool, we should let the farmers be allowed to conserve and multiply the traditional rice germplasm and hence, the introduction of high yielding varieties may be discouraged specially in the rural areas of the country where large number of rice diversities are available (Hore, 2005). All these rice landraces that are adapted under such acclimatized eco-system must be protected in *in situ* or on-farm conditions, so that their evolutionary process may be continued naturally (Rana *et al.*, 2009). It is predicted that the world population will need an additional 50 million tones of rice annually by 2015. Under these circumstances, rice gene-pool stored in landraces and other relatives is going to play a crucial role to ensure future food security (Kumar et al., 2010). It is therefore nessecary to manage the continuing genetic erosion and address the issues of germplasm conservation of rice landraces and optimum utilization of what remains on-farm and conserved in the gene banks.

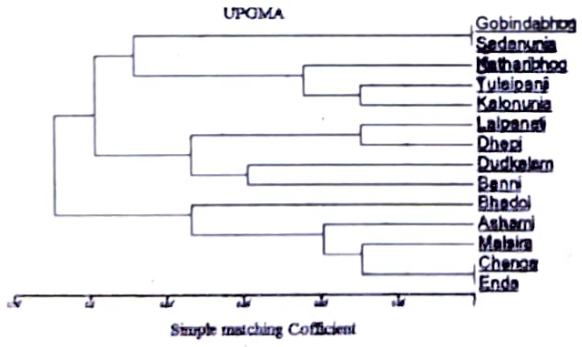

Fig 2: Dendrogram showing the three distinct clusters of 14 rice varieties (landraces) based on the morphological traits

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Regulation of trehalose metabolism by protein methylation in a mutant strain of *Saccharomyces cerevisiae*

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Abstract

Trehalose is a non reducing disaccharide occurring in a wide range of organisms, from bacteria, yeast, to lower and higher plants and insects. It is economically important as a potent stress protectant, protein and biological membrane stabilizer; hence biosynthesis of trehalose is an extremely important event. Regulation of trehalose metabolism by protein methylation has been reported from previous works of this laboratory. Trehalose metabolism was monitored during different stages of growth of *Saccharomyces cerevisiae*. HPLC and enzymatic determination of trehalose, glucose, trehalose 6 phosphate phosphatase (TPP) and trehalose 6 phosphate synthase (TPS) were carried out. It was noticed that trehalose, glucose, TPP and TPS peaked at A_{660} ~20 during growth but during this period the hydrolyzing enzymes, acid trehalase (AT) and neutral trehalase (NT) are found to be low. Effect of a potent universal methyl group donor S-adenosyl-L-methionine (AdoMet), and a methylation inhibitor, oxidized adenosine (Adox) on trehalose metabolism has been studied. Trehalose metabolism is altered when YPD grown cells were incubated for 1 hr in presence of either 1 mM Adox or AdoMet at 30°C and pH 6.0 TPS showed a slight increase in specific activity in cells incubated with AdoMet over Adox. Trehalose level of Adox treated cells were seen to be lower than control throughout the period of incubation. Trehalose level initially decreased upto 4 hours in all the sets by utilizing pre-synthesized trehalose, thereafter the AdoMet incubated cells showed sharp increase in trehalose content, in 8 hours 3 fold, in 24 hours nearly 5 fold with respect to others. At the point of 48 hours, cells reached stationary phase in all sets and trehalose level was found to increase.

Keywords: S-adenosyl-L-methionine (AdoMet), Acid Trehalase (AT), Oxidized adenosine (Adox), Neutral Trehalase (NT), Trehalose-6-phosphate synthase (TPS), Trehalose-6-phosphate phosphatase (TPP), Trehalose.

Trehalose, commonly known as "insect sugar", is a major storage carbohydrate and is found to be present in a wide variety of microorganisms, plants, and invertebrates except mammals. Accumulation of trehalose in organisms plays a role in enhancing the stress tolerance. This sugar was first isolated from the ergot of rye, in 1832 by Wiggers. Chemically this sugar is α-D-glucopyranosyl- α-D-glucopyranoside. Two glucose molecules are linked together in a α, α 1, 1-glycosidic linkage; hence, trehalose has no reducing power. Due to the absence of reducing ends in the chemical structure, trehalose is highly resistant to heat, pH and Millard's reaction. It accumulates intracellularly during periods of starvation, desiccation and after exposure to mild heat stress, where it serves as a potent membrane stabilizer. Besides stabilizing different cellular structures from adverse effects of freezing and drying induced dehydration and stabilization of proteins against denaturation, trehalose is also important for the control of glucose influx during the cellular response to adverse condition. Trehalose is a multifunctional molecule (Elbein et al, 2003) and the importance of this sugar lies in both economic and biotechnological fields. It is 45 % as sweet as sugar, highly soluble in both water and aqueous ethanol and comparatively less hygroscopic. Industrially it is used for the preservation of color and taste of food products and for protecting vaccines in hot climates. Human red blood cells can be freeze-dried while maintaining a high degree of viability by loading trehalose into them.

Trehalose biosynthesis is controlled by the activity of two synthesizing enzymes, TPS and TPP and two hydrolyzing enzymes AT and NT (Basu et al, 2006; Londesborough and Varimo 1984). Our aim was to find out the regulation of trehalose biosynthesis by post-translational modification of trehalose biosynthesizing enzymes. Protein methylation is one of the modes of protein modification regulating protein function (Aletta et al, 1998). Using AdoMet as the methyl group donor and Adox as the methylation inhibitor, the effect of protein methylation and methylation inhibition on overall trehalose metabolism is being studied.

Materials and Methods

Materials

Trehalose-6-phosphate, Uridine 5'-diphosphoglucose (UDPG), glucose-6-phosphate (G-6-P), Periodate

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Oxidized adenosine (AdOx), S-adenosyl-L-methionine (AdoMet), phenylmethylsulphonyl fluoride (PMSF), benzamidine hydrochloride, 2-mercaptoethanol, Bradford reagent were purchased from Sigma, USA. All the other chemicals and medium components used were of analytical grade and were purchased locally.

Methods

Organism and culture condition

The Kanamycin resistant mutant diploid strain of yeast, *Saccharomyces cerevisiae* MAT aKnα was used. Yeast cells were allowed to grow in YPD medium at 30 ± 1°C shaker incubator till desired growth phase is obtained, which is measured by taking absorbance at 660nm. Cells were harvested by centrifugation at 10 000×g, at 4–5°C for 10min.

Cell lysis and preparation of enzyme solution

Harvested cells were suspended in lysis buffer and cells were disrupted by passing twice through a FRENCH® Pressure Cell Press (SLM Instruments, USA) at 18,000 lb psi. The homogenate was then centrifuged at 10,000 × *g*, 4 -5 °C for 10 min and the supernatant was dialyzed against 1 l of dialysis buffer with 4- 5 changes following the method of Chaudhuri et al., 2007. Precipitate which appeared after dialysis was removed by centrifugation at 10,000 × g for 10 min. The resulting supernatant was used as crude enzyme solution for different enzyme assays. It was also used to measure protein for determining specific activity of the enzymes.

Assay of different enzymes of trehalose biosynthesis pathway

Trehalose-6-phosphate phosphatase (TPP) was measured according to assay protocol of Matula M., Mitchell M., and Elbein A.D., 1971. Trehalose-6-phosphate synthase (TPS), Acid Trehalase (AT) and Neutral Trehalase (NT) activities were measured according to published protocol of Chaudhuri et al., 2008; Biswas and Ghosh, 1996 respectively.

Trehalose-6-phosphate (1mM) was used as substrate for TPP assay, incubated with $MgCl_2$ at 37°C for 30 min. Phosphate group liberated was quantified spectrophotometrically at A_{630} nm. TPS activity was assayed at 37 °C for 15 min using 5 mM UDPG and 5 mM G-6-P as substrates. Trehalose-6-phosphate (T-6-P) formed was determined by anthrone color reagent after neutralizing all other sugars. Final solution was spectrophotometrically analyzed at A_{625} nm(Chaudhuri et al., 2008). Trehalose (13.2mM) was used as substrate for AT and NT activities. Incubations were for 15 min at 30 °C. Glucose liberated enzymatically was determined by glucose oxidase- peroxidase (GOD-POD) method (Bergmeyer H.U, 1974).

Unit of enzyme activity (U) was expressed in micromole (μmole) of Phosphate liberated (for TPP); μmole of T-6-P synthesized (for TPS) and μmole of reducing sugar liberated (for AT and NT), per min under assay conditions.

Protein estimations

The protein content of enzyme solutions was measured by Bradford Reagent as per technical bulletin provided by the manufacturer, Sigma, USA. Protein content of whole cell homogenate was determined by the modified method of Lowry (Bergmeyer and Bernt, 1974). Standard protein solution used was bovine serum albumin (BSA) from Sigma, USA.

Intracellular metabolite extraction and their estimation

Intracellular metabolites from known mass of cells were extracted with 0.5 N cold perchloric acid according to the protocol of Sutherland and Wilkinson, 1971. AdoMet was estimated by HPLC using a cation-exchange column Partisil 10 SCX (4.6 × 250 mm, Whatman, England) (Biswas and Ghosh, 1997). Known volume of metabolite extract was injected, and amount of AdoMet present was determined from standard curve generated by injecting standard AdoMet solutions. AdoHcy was also determined from the same chromatograms by injecting AdoHcy standards, and comparing peak areas of AdoHcy of injected samples with standard curve. Trehalose was measured following the protocol of Parrou and Francois, 1997 using purified AT by DEAE Sephadex A50 column chromatography. Intracellular trehalose was also quantified enzymatically by measuring amount of glucose produced due to hydrolysis of trehalose by purified acid trehalase (Chaudhuri et al., 2008). Intracellular metabolite content was measured as μmole (g wet wt)$^{-1}$.

Study of Trehalose Metabolism during different stages of Growth

Yeast cells were grown in YPD medium, aliquots were collected at different hours of growth to get A_{660} ~10, A_{660} ~15, A_{660} ~20, A_{660} ~25, A_{660} ~30, and A_{660} ~35. The cells of different absorbance values were then separately harvested by centrifugation. In each case, total cell mass was divided into two parts. One part was kept for enzyme preparation and another part was used for metabolite extraction by perchloric acid. The enzymes TPS, TPP, AT, NT and the intracellular metabolites trehalose, glucose, glucose 6 phosphate, AdoMet and AdoHcy were extracted and measured.

Biosynthesis of Trehalose during incubation of yeast cells with 1 mM AdOx and AdoMet [short term incubation]

YPD grown cells (A_{660}~20) were harvested by centrifugation and re-suspended under shaking at 30°C in 25mM MES-KOH buffer,[pH6.0] containing either 1 mM AdOx or AdoMet. The cells were incubated for 1 hour in presence of the chemicals. Aliquots were collected at different intervals in ice cold water to terminate the reactions and centrifuged at 5000 x g for 5 min to collect the cell mass. Cells were washed thoroughly to remove all traces of adhering chemicals and stored at 0-4°C. A portion was used for crude enzyme preparation while another portion was used for intracellular metabolite extraction. (Bhattacharyya et al, 2005)

Biosynthesis of Trehalose during incubation of yeast cells with 0.1 mM Adox and AdoMet [long term incubation]

YPD grown cells from diauxic phase (A_{660} ~20) were harvested and the total cell mass was divided into three parts. Of the three parts, one part was re-suspended in fresh YPD medium in absence of Adox and AdoMet (control) and the other two parts were resuspended in medium containing either 0.1 mM AdOx or AdoMet. Those cells were harvested at different intervals of 4, 8, 24, 48 hr. Aliquots collected were washed and stored at 0 - 4 °C for further studies. Trehalose metabolic profile, including intracellular trehalose level and activities of trehalose metabolic enzymes was monitored. Intracellular AdoMet and AdoHcy content were also measured by HPLC method. (Bhattacharyya et al, 2005).

Results

Study of Trehalose Metabolism during different stages of Growth

The results indicate that the intracellular trehalose content was found maximum at O.D 20 and at that particular growth stage the activity of the trehalose synthesizing enzymes, TPP and TPS were also highest. The activity of the hydrolyzing enzyme AT was found to be very low nearly 350 times lower than TPS activity and NT was found to show the lowest activity. Concurrent with this, the intracellular Glucose level was low showing that rate of anabolism was much greater than the catabolic activity. [Fig: 1]

Biosynthesis of Trehalose during incubation of yeast cells with 1 mM AdOx and AdoMet [short term incubation]

Trehalose content increased during AdoMet treatment

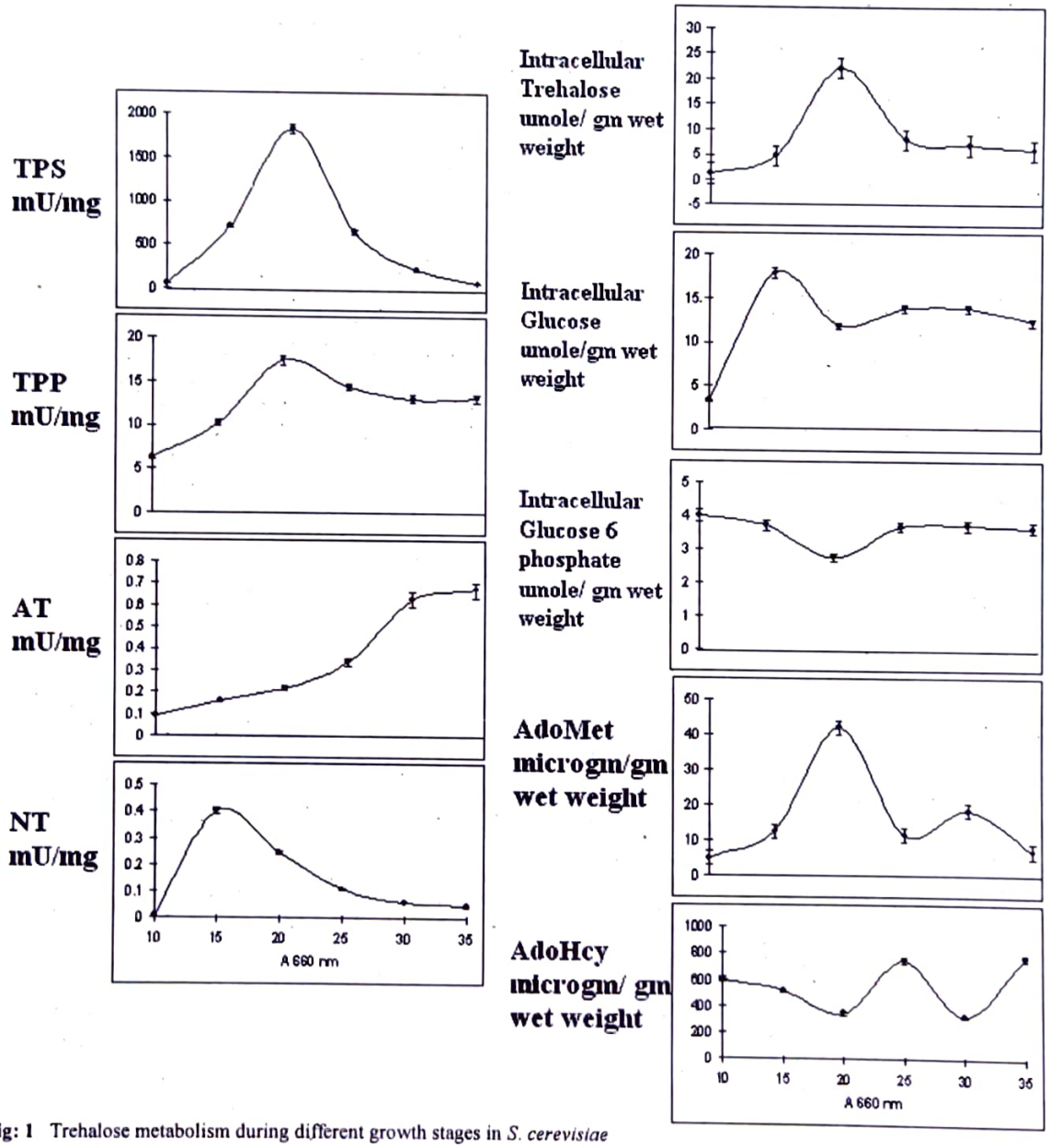

Fig: 1 Trehalose metabolism during different growth stages in *S. cerevisiae*

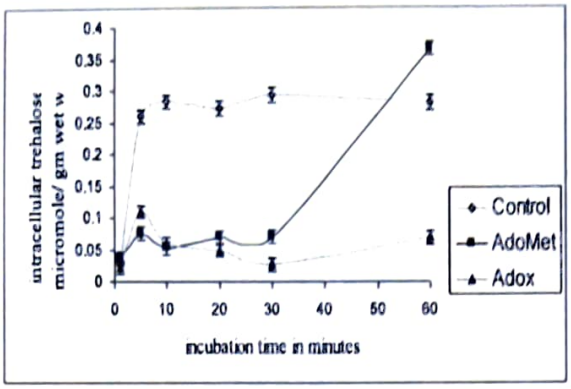

Fig: 2. Effect of short term incubation on trehalose content of yeast cells in 1 mM AdOx and AdoMet treatment. Yeast cells at A_{660} ~ 20 were suspended in MES-KOH buffer containing 1mM AdOx, AdoMet or water and incubated for 1 h. Level of trehalose of aliquots collected at 1, 5, 10, 20, 30 and 60 min of incubation was monitored. Data given are the mean of at least three sets of experiments.

from 20 mins onwards. [Fig: 2]

G6P content increased upto 10 mins and then decreased in AdoMet treated cells. Intracellular Glucose level was found to remain lower throughout the period of incubation in AdoMet treatment than both Adox treated and Control sets. Intracellular AdoMet and AdoHcy level was opposite to each other during the different treatments, while AdoMet content increased in AdoMet treated sets over Adox treatment and Control cells. [Table: 1]

TPS content increased during AdoMet treatment over Adox treated cells. But both were lower than Control sets. TPP activity peaked during AdoMet treated sets over Adox treated cells, but both showed lesser specific activity than control sets. [Table: 2]

Biosynthesis of Trehalose during incubation of yeast cells with 0.1 mM Adox and AdoMet [long term incubation]

Intracellular trehalose level of Adox treated cells were seen to be lower than Control throughout the period of incubation. Trehalose level initially decreased upto 4 hours in all the sets. Thereafter the cells with AdoMet treatment showed sharp increase in trehalose content, i.e., in 8 hours 3 fold, in 24 hours nearly 5 fold increases with respect to Adox treated and Control sets. But at the point of 48 hours, cells reached stationary phase and trehalose level increased irrespective of treatment. From 4 hour incubation onwards in both the synthesizing enzymes TPS and TPP, specific activity was found to increase in AdoMet treated cells over Adox treated sets and Control sets. AT and NT was found to show lower activity in AdoMet treatment over Adox treatment. Intracellular AdoMet content increased in AdoMet treated sets over Adox treatment and Control cells [Fig: 3] while intracellular AdoHcy level was found to be just opposite to AdoMet content. [Table 3]

Intracellular Glucose and Glucose 6 phosphate levels were higher in Adox treatment over AdoMet treatment throughout the period of incubation. [Table: 3]

Discussion

During the growth of yeast, trehalose accumulation begins at the diauxic shift, continues till entering stationary phase and degradation of trehalose starts once cells have entered stationary phase (Werner- Washburne et al. 1993). In *S. cerevisiae*, trehalose is synthesized by a two step pathway that requires trehalose synthase

Table 1: Effect of short term incubation on intracellular glucose, G-6-P, AdoMet and AdoHcy contents of yeast cells in 1 mM AdOx and AdoMet treatment. Yeast cells at A_{660} ~ 20 were suspended in MES-KOH buffer containing 1mM AdOx, AdoMet or water and incubated for 1 h. Level of trehalose of aliquots collected at 1, 5, 10, 20, 30 and 60 min of incubation was monitored. Data given are the mean of at least three sets of experiments AX=Adox, AM=AdoMet, C=Control

Incubation Time in mins	Intracellular Glucose*			Intracellular G-6-P*			Intracellular AdoMet*			Intracellular AdoHcy*		
	AX	AM	C	AX	AM	C	AX	AM	C	AX	AM	C
1	0.0508	0.0479	0.0574	2.1534	2.1171	2.4034	415.22	368.97	257.81	649.32	442.21	558.67
5	0.0428	0.0366	0.099	2.1145	2.1562	2.3339	249.52	445.83	234.35	1033.04	201.32	944.88
10	0.044	0.0379	0.0576	2.1617	2.2145	2.07	144.18	627.52	154.29	743.92	543.71	933.12
20	0.0471	0.0371	0.0469	2.2062	2.0839	2.1284	189.13	1417.04	114.65	717.11	443.42	2351.17
30	0.0442	0.037	0.0527	2.2006	2.2228	2.2312	88.58	1467.31	59.85	645.84	433.5	549.59
60	0.047	0.0394	0.0489	2.2228	2.195	2.3034	48.15	319.21	67.04	1237.67	744.29	1214.53

*µmole per gram wet weight

Table 2: Effect of short term incubation on trehalose biosynthesizing enzymes TPP, TPS, AT and NT contents of yeast cells in 1 mM AdOx and AdoMet treatment. Yeast cells at A_{660} ~ 20 were suspended in MES-KOH buffer containing 1mM AdOx, AdoMet or water and incubated for 1 h. Level of trehalose of aliquots collected at 1, 5, 10, 20, 30 and 60 min of incubation was monitored. Data given are the mean of at least three sets of experiments. Ax=Adox, AM=AdoMet, C=Control

Incn.§	TPP*			TPS*			AT#			NT#		
	AX	AM	C	AX	AM	C	AX	AM	C	AX	AM	C
1	1.09	20.06	26.02	762.87	165.83	203.18	8.62	1.46	5.62	6.11	1.007	18.11
5	1.19	17.78	17.65	934.58	439.18	3812.41	1.54	0.97	9.49	2.94	1.07	14.41
10	15.74	17.08	41.38	954.3	1460.44	1943.76	3.75	0.86	4.4	3.35	1.66	4.66
20	18.94	22.99	25.66	264.4	727.21	2590.85	3.63	0.73	10.74	3.42	1.9	3.66
30	15.84	25.93	27.33	118.55	244.67	618.43	2.86	0.77	6.63	2.58	1.66	3.78
60	21.98	17.17	17.88	304.71	1508.35	11109.29	3.34	0.9	7.4	5.97	1.36	18.62

*U/mg, # mU/mg ; §Incubation time in minute

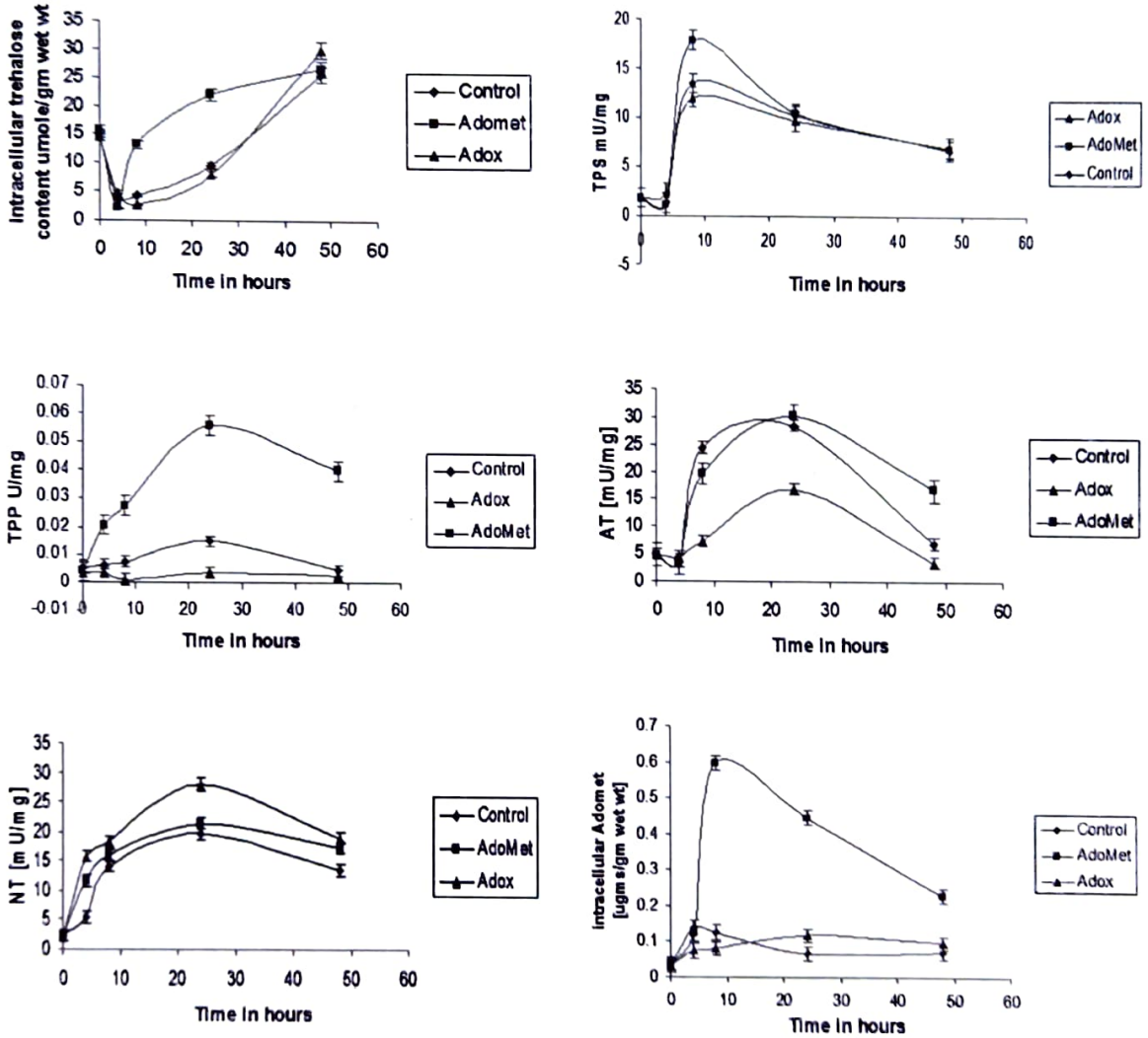

Fig 3:. Effect of AdoMet and Adox on Trehalose metabolism during 48 hours incubation. YPD grown cells from A_{660} ~20 were harvested by centrifugation and re-suspended under shaking in fresh YPD medium containing 0.1 mM AdOx or AdoMet for 48 h incubation. In either case a positive control (minus added chemicals) was maintained. After different time intervals, aliquots were collected in ice-cold water to terminate the reactions and centrifuged in cold at 10,000 × g to obtain cell pellets. Changes in stored trehalose level and corresponding enzyme activities of TPS, TPP, AT and NT; and intracellular AdoMet level of aliquots collected at 4, 8, 24 and 48 h were studied. Data given are the mean of at least three sets of experiments.

enzyme complex which consists of (i) trehalose-6-phosphate synthase (TPS) subunit AND (ii) trehalose-6-phosphate phosphatase (TPP) subunit. Trehalose-6-phosphate is formed from Glucose-6-phosphate (G6P)

Table 3: Effect of AdoMet and Adox on Trehalose metabolism during 48 hours incubation. YPD grown cells from A_{660} ~20 were harvested by centrifugation and re-suspended under shaking in fresh YPD medium containing 0.1 mM AdOx or AdoMet for 48 h incubation. In either case a positive control (minus added chemicals) was maintained. After different time intervals, aliquots were collected in ice-cold water to terminate the reactions and centrifuged in cold at 10,000 × g to obtain cell pellets. Changes in intracellular glucose, G-6-P and AdoHcy levels of aliquots collected at 4, 8, 24 and 48 h were studied. Data given are the mean of at least three sets of experiments. Ax=Adox, AM= doMet, C=Control

Incubation time (h)	Intracellular Glucose*			Intracellular G-6-P*			Intracellular AdoHcy#		
	AX	AM	C	AX	AM	C	AX	AM	C
0	0.00134	0.00166	0.00199	3.002	3.258	3.3	0.1452	0.16729	0.1672
4	0.00134	0.00134	0.00067	3.9856	3.574	3.15	0.40081	0.19017	0.38566
8	0.00199	0.0025	0.000335	3.952	3.692	3.119	0.1862	0.38555	0.256
24	0.00299	0.0025	0.0032	4.675	3.149	3.169	0.1318	0.2263	0.2052
48	0.00366	0.00299	0.0035	3.0653	3.01	3.778	0.1388	0.0862	0.26965

*µmole/gm wet weight, #µgm/gm wet weight

and Uridine 5' diphosphoglucose (UDPG) by TPS which is then dephosphorylated to trehalose by TPP. The enzyme responsible for trehalose hydrolysis is trehalase existing in two different forms; one is cytosolic neutral trehalase (NT) and the other one is vacuolar acid trehalase (AT) (Cabib and Leloir, 1958). Since the synthesis of trehalose is catalyzed by the enzyme TPS and TPP, level of trehalose in the cells is dependent on the activity profile of the enzymes. Observation from our present study indicates an increase in TPS and TPP activity from exponential phase of growth (A_{660} ~ 10) till early diauxic phase (A_{660} ~20), and decrease in TPS and TPP activity with entry into stationary phase, corresponding to the increase in intracellular trehalose level throughout the growth of the yeast cells.

The intracellular trehalose level increased with time in the yeast cells incubated with AdoMet and decreased when treated with Adox with respect to control throughout the period of incubation. Initially, the trehalose level decreased upto 4 hours in both the treatments (AdoMet and Adox) and this may be due to the fact of complete utilization of pre-synthesized trehalose already present in the cell. Thereafter, the cells incubated with AdoMet showed sharp increase in trehalose content. As the cells had reached the stationary phase at 48 hours, trehalose level showed an increase irrespective of the treatment.

Concurrent with this observation the two trehalose synthesizing enzymes, TPS and TPP activity were higher during AdoMet treatment with respect to Adox indicating the possible effect of Methylation on trehalose biosynthesis. In case of the hydrolyzing enzymes, AT and NT, the effect of AdoMet was found lower than Adox which suggests no prominent effect of methylation on the activity of the hydrolyzing enzymes. Intracellular Glucose level was found very low due to this reduced hydrolysis of trehalose. This could be attributed to the fact of glucose getting converted to G6P in the first step of Glycolysis. Moreover, the variation of TPS and TPP activity and intracellular trehalose content in different treatments has been supported by the intracellular AdoMet and AdoHcy contents which have been determined by HPLC. This may be justified with the mechanism of a methylation reaction of proteins in which a protein is transformed into their methyl esters where methyl group donor is AdoMet. During the reaction AdoMet gets converted to AdoHcy (Adenosine Homocysteine), which by further hydrolysis splits up into Adenosine and Homocysteine residues by the enzyme AdoHcy Hydrolase. Adox plays a role as an indirect trans-methylation inhibitor. It blocks the conversion of AdoHcy to its components and thus in cells treated with Adox, the level of AdoHcy increases. This AdoHcy is a competitive inhibitor of all Methyl Transferases. Thus the transfer of Methyl groups from AdoMet to Protein stops. Adox thus causes indirect inhibition of methylation. The gene encoding PIMT is missing in *S. cerevisiae*, but our findings clearly indicate an elevation in trehalose biosynthesis by methylation. This leads us to conclude that there is possibility of presence of a new enzyme responsible for methylation of either TPS or TPP or both in *S. cerevisiae*.

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In vitro antioxidant activity of two edible Timbur fruits of Darjeeling Himalaya

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Abstract
Free radicals are implicated for many chronic, painful and near-fatal diseases including Diabetes mellitus, arthritis, cancer, apoptosis, neurodegenerative disorders etc. In treatment of these diseases, antioxidant therapy has gained an utmost importance and current research is now directed towards finding naturally occurring antioxidants of plant origin. Edible Timburs namely *Zanthoxylum acanthopodium* DC. and *Litsea cubeba* (Loureiro) Persoon are traditionally used as spices in different Nepali recipes. In the present study, the fruits of these plants were extracted with aqueous methanol (1:4) to examine the *in vitro* antioxidant property, phenol content and phytochemical constituents. The scavenging activities on DPPH free radicals, superoxide anions and per-oxidized lipid molecules were determined as well as the flavonoid and phenolic constituents of the extracts. The extracts exhibited significant scavenging activity towards DPPH free radicals and high anti-lipid peroxidation values due to the presence of relatively high total phenol contents. Also, these spices contain a vast array of different phytochemicals in their dry form. These results suggest that both *Zanthoxylum acanthopodium* and *Litsea cubeba* fruits are endowed with antioxidant phytochemicals and could serve as basal ingredients for nutraceutical formulations.

Keywords: Antioxidant, DPPH, *Zanthoxylum acanthopodium*, *Litsea cubeba*

An extensive diversity of medicinal plants and edible fruits is observed in Darjeeling hills. Two plants of these hills viz. *Zanthoxylum acanthopodium* DC. and *Litsea cubeba* (Loureiro) Persoon are locally known as 'Boke Timbur' and 'Sil Timbur' respectively. These plants are wild, tree like in habit under the families of Rutaceae and Lauraceae and are widely spread in the forest of Darjeeling hills. Fruits of these plants ('Timburs') have been used traditionally for healing diarrhea, vomiting, and gastric ulcer and as warm killer. These fruits are also used as spices in different Nepali recipes. Several studies have been conducted to determine the antioxidant properties of many plants, especially those used in traditional medicine (Jang *et al.* 2007, Surveswaran *et al.* 2007). Currently, there is a great interest in the field of antioxidant substances mainly due to the findings concerned with the effects of free radicals in the organism. Free radicals have significant role in creation of several metabolic, mutagenic and age-related disorders like diabetes, cirrhosis, cancer and cardiovascular diseases (Hertong and Feskns 1993). Reactive oxygen species (ROS), which include free radicals such as superoxide anion (SO^{2-}), hydroxyl radicals (OH) and non-free-radical species like H_2O_2 and singlet oxygen ($^1O_2$) are various forms of activated oxygen (Gulcin *et al.* 2002, Halliwell and Gutteridge 1999, Yildirim *et al.* 2000). It is commonly recognized that antioxidants can neutralize potentially harmful reactive free radicals in body cells before they cause lipid and protein oxidation and may reduce potential mutation risks and therefore, help to prevent cancer or heart diseases. Recently, there is a growing attention on the discovery of natural antioxidants because epidemiological and clinical evidences suggest that consumption of vegetables and fruits reduce the risk of developing chronic diseases like cancer and in this respect phytochemicals are generally safer than synthetic chemicals (Dastmalchi *et al.*, 2007). Therefore, the search for natural antioxidants as alternatives to synthetic ones is of great interest among researchers. Plants contain a wide variety of free radical scavenging molecules like phenols, flavonoids, vitamins, terpenoids etc. that are rich in antioxidant activity (Cai *et al.*, 2003). However, the use of natural antioxidants are limited due to lack of knowledge about their molecular composition and dynamics, amount of active ingredients in the source material and the availability of relevant toxicity data (Shahidi *et al.* 1994). Natural antioxidants tend to be safer and they also posses antiviral, anti-inflammatory, anti-tumour and hepatoprotective properties (Lim and Murtijaya, 2007). Information related to antioxidant activity and phenolic compounds on traditional Darjeeling medicinal and underexplored edible plants is scarce. Literature survey revealed no relevant phyto-pharmacological records on *Zanthoxylum acanthopodium* and *Litsea cubeba*, and the fruits of these plants have not yet been screened for their antioxidant activity. This present study, therefore investigated the phytochemical compositions and polyphenol content, the *in vitro* antioxidant, lipid peroxidation and superoxide scavenging potential of this plant.

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MATERIALS AND METHODS

Plant materials

Two fruit samples of the selected species *viz.*, *Zanthoxylum acanthopodium* (Figure 1) and *Litsea cubeba* (Figure 2) were collected from the Chwak Bazar market of Darjeeling Town, Darjeeling , West Bengal and species authentication were done by the Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal.

Animal material

Goat liver, used for anti-lipid peroxidation assay, were collected from slaughter house immediately after slay and the experiment was conducted within one hour after collection.

Chemicals

Methanol; 2,2-diphenyl-1-picryl hydrazyl (DPPH); nitro blue tetrazolium (NBT); reduced nicotinamide adenine dinucleotide phosphate sodium salt, monohydrate (NADPH); phenazine methosulphate (PMS); trichloroacetic acid (TCA); thiobarbituric acid (TBA); $FeSO_4$, $7H_2O$; acetic acid; Folin-Ciocalteu reagent; NaOH; Arnow's reagent; quercetin; $NaNO_2$; $AlCl_3$; sodium carbonate (Na_2CO_3); ferric chloride solution; Fehling's solution, cupper acetate solution; petroleum ether; ninhydrin reagent; ammonia; lead acetate; acetic anhydride; olive oil; ferric chloride; hydrochloric acid; Dragendroff's reagent; pyridine; sodium nitroprusside; chloroform; Conc. H_2SO_4 were either purchased from Sigma Chemicals (USA) or Merck (Germany). All the chemicals and reagents, used for experimental purposes were of analytical grade.

Extraction procedure

Under Soxhlet extractor powdered drug of fruits were separately extracted with methanol water in ratio 4:1 for eight hours. The refluxed samples were separated from the residues by filtering through Whatman No. 1 filter paper. The filtrates were dried under reduced pressure and their total extractive values were calculated on dry weight basis by the formula:

$$\text{\% extractive value (yield \%)} = \frac{\text{Weight of dry extract}}{\text{Weight taken for extraction}} \text{X100}$$

The samples were then kept in freeze for further use.

DPPH based free radical scavenging activity

The scavenging reaction between (DPPH•) and an antioxidant (H-A) can be written as:

$$(\text{DPPH}\bullet) + (\text{H-A}) \rightarrow \text{DPPH-H} + (\text{A}\bullet)$$

Purple Yellow

Antioxidants react with DPPH•, which is a stable free radical and is reduced to the DPPH-H and as consequence, the absorbance is decreased from the DPPH• radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability (Benabadji *et al.* 2004).The free radical scavenging activities of each fraction were assayed using a stable DPPH, following standard method of Blois (1958). The reaction mixture contained 1.8 ml of 0.1mM DPPH and 0.2 ml of each serial dilution (200 mg/ml, 40 mg/ml, 20 mg/ml, 8 mg/ml FW) of methanolic fruit extracts. Simultaneously, a control was prepared by replacing extracts with methanol. The reaction mixture was shaken and allowed to incubate for 30 min at room temperature (25° C) in the dark and OD values were measured at 517 nm with a spectrophotometer. Radical scavenging activity was expressed as percent inhibition from the given formula:

$$\text{Percent inhibition of DPPH radical} = \frac{\text{Abs. of control} - \text{Abs. of sample}}{\text{Abs. of control}} \text{X100}$$

Superoxide radical scavenging assay

Measurement of superoxide radical scavenging activity of *Zanthoxylum acanthopodium* and *Litsea cubeba* were done by using standard method followed by Nishikimi *et al.*, 1972 with minor modifications. The reaction mixture contained 1 ml of NBT solution (312 µM prepared in phosphate buffer, pH-7.4), 1ml of NADH solution (936 µM prepared in phosphate buffer, pH-7.4) and differentially diluted sample extracts. Finally, reaction were accelerated by adding 100 µL PMS solution (120 µM prepared in phosphate buffer, pH -7.4) to the mixture. The reaction mixtures were incubated at 25° C for 5 min and absorbance was measured at 560 nm against methanol as control. Percentage inhibition was calculated using the same formula mentioned above.

Anti-lipid peroxidation (ALP) assay

The anti-lipid peroxidation activity of the extracts of two timbur fruits was determined by the standard method (Bauchet *et al.*, 1998) followed by slight modification with the goat liver homogenate. 2.8 ml of 10% goat liver homogenate, 0.1ml of 50mM $FeSO_4$ and 0.1 ml extract was mixed. The reaction mixture was incubated for 30 minutes at 37°C. 1 ml of reaction mixture was taken with 2ml 10%TCA-0.67%TBA in acetic acid (50%) for blocking the reaction. Then the mixture was boiled for 1hour at 100°C and centrifuged at 10,000 rpm for 5 minutes. Supernatant was taken for absorbance at 535nm. Vitamin E was used for standard. ALP % was calculated using the following formula:

$$ALP\ percent = \frac{Abs\ of\ Fe^{2+}\ induced\ peroxidation - abs\ of\ sample}{Abs\ of\ Fe^{2+}\ induced\ peroxidation - abs\ of\ control} \text{x100}$$

Total phenol Estimation

Total phenolic compounds of fruit extracts were determined by Folin-Ciocalteu method (Folin and Ciocalteu, 1927). For the preparation of the calibration curve, 1 ml aliquot of 0.025, 0.05, 0.075, 0.1, 0.2 and 0.3 mg/ml methanolic gallic acid solution was mixed with 5 ml of Folin-Ciocaltue reagent (10 times diluted) and 4 ml sodium carbonate (75 g/L). The absorbance at 765 nm was measured after 1 hr. at 20° C and the calibration curve was drawn. To the same reagent, 1 ml methanolic fruit extracts (10 mg/ml) was mixed as described above and after 1 hr. the absorbance was measured. Total phenolic content in fruit methanolic extracts in Gallic Acid Equivalents (GAE) was

measured by the formula:

$C = c.V/m$

Where, C - total content of phenolic compounds, mg/g of plant extract, in GAE; c - the concentration of gallic acid deduced from the calibration curve (mg/ml); V - the volume of extracts (ml); m - the dry weight of the plant material.

Total flavonoids determination

Aluminum chloride spectrophotometric method was used for flavonoids determination (Sultana *et al.*, 2009). . Each fruit methanol extracts (0.5 ml of 200mg/ml FW) were separately diluted with 4 ml double distilled water. Then the diluted extracts of fruits were mixed with 5% (0.3 ml) $NaNO_2$ 10% aluminum chloride was then added with reaction mixture. After 6 minute 2ml (1.0 M) NaOH and 2.4 ml D.D. water was added and mixed well. Thereafter, absorbance was measured at 510 nm in spectrophotometer. Standard solutions quercetin (0-500 mg L^{-1}) was used as calibration curve.

Phytochemicals evaluation of the crude extracts

The methanolic crude extract (200 mg/ml) of the fruits of the plant was subjected to various chemical tests in order to determine the secondary metabolites present by employing the use of various methods as follows:

Test for Reducing Sugars

To 0.5ml of the extract, 2ml of a mixture (1:1) of Fehling's solution I (A) and Fehling's solution II (B) was added and the mixture was boiled in a water bath for five minutes. A brick-red precipitate indicated the presence of free reducing sugars (Brain and Turner, 1975).

Test for Flavonoids

To 1ml of methanolic extract, a few drops of 10 % ferric chloride solution were added. A green or blue colour indicated the presence of phenolic nucleus (Brain and Turner, 1975).

Test for resins

0.5ml of extracts were evaporated and dissolved in 2ml of petroleum ether, 2ml of 2% cupper acetate solution was then added and the mixture was shaken vigorously and allowed to separate, a green colour indicated the presence of resin (Trease and Evans, 1983).

Test for amino acid

0.5 ml methanolic fruit extracts were treated with few drops of ninhydrin reagent, heated in water bath, a purple colour indicated the presence of amino acids (Kumar *et al.*, 2009).

Test for anthraquinones

1ml methanolic fruit extracts were evaporated and dissolved in 2ml chloroform. 2ml of ammonia was added. Occurrence of Red/orange colour suggested the presence of anthraquinones (Kumar *et al.*, 2009).

Test for tannin

0.5 ml methanolic extract of each fruit was added with 0.5 ml 1% lead acetate; a yellow colour precipitation indicated the presence of tannin (Kumar *et al.*, 2009).

Test for triterpenoids

0.5ml of methanolic fruit extracts were evaporated and dissolved in 1ml chloroform.1ml acetic anhydride was then added and chilled. After cooling, conc.H_2SO_4 was added. If reddish violate colour appeared, the existence of triterpenoids was confirmed (Kumar *et al.*, 2009).

Test for alkaloids

0.5 ml of each fruit extract was added with 0.2ml of 36.5% hydrochloric acid and 0.2 ml Dragendroff's reagent. Production of orange or red precipitation denoted the presence of alkaloids (Kumar *et al.*, 2009).

Test for glycosides

0.5 ml methanolic extract of fruits were added with 2ml of 50% hydrochloric acid. The mixtures were hydrolyzed for 2 hrs on a water bath. After that 1ml pyridine, few drops of 1% sodium nitroprusside solution, and 5% sodium hydroxide solution were added. Pink to red colour designated the presence of glycosides (Kumar *et al.*, 2009).

Test for steroid

0.5ml methanolic fruit extracts were evaporated and dissolved in 2ml chloroform. 2ml of conc. H_2SO_4 was introduced carefully by the side wall of the test tube. Formation of red colour ring confirmed the presence of steroid (Kumar *et al.*, 2009).

Test for Saponins

2ml of double distilled water was added with 1ml of each methanolic extract. Few drops of olive oil were added and agitated. Formation of soluble emulsion indicated the presence of saponin (Ngbede *et al.*, 2008).

Test for cardiac glycoside

0.5ml of methanolic fruit extracts were evaporated and dissolved in 1ml glacial acetic acid. One drop of 10% ferric chloride was then added. 1ml of conc.H_2SO_4 was added by the side of the test tube. Appearance of brown colour ring at the interface indicated of presence of cardiac glycosides (Ngbede *et al.*, 2008).

RESULTS AND DISCUSSION

Free radicals are involved in many disorders like neurodegenerative diseases, cancer and AIDS. Antioxidants through their scavenging power are useful for the management of those diseases. DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts (Koleva *et al.*, 2002). Unlike other free radicals like hydroxyl radicals and superoxide anions, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelating and enzyme inhibition (Jayasri *et al.*, 2009). Figure 3 shows that the methanol extracts of *Zanthoxylum acanthopodium* and *Litsea cubeba* have antiradical activity by inhibiting DPPH radical with the maximum inhibition value of about 98.32% and 85.12% in their plateau phase at a concentration range of 200 mg FW/ml. The inhibition percentage of DPPH radical at variable concentration of fruit extracts obey logarithmic equation at correlation co -efficient (R^2) of about 0.984 and 0.987 for *Zanthoxylum*

Table 1: Phytochemical profile of two different timbur fruits (semi-quantitative screening)

Plant samples	1	2	3	4	5	6	7	8	9	10	11	12
Sil Timbur	+++	+	++	+++	++	+++	+++	+++	+++	+++	++++	+++
Boke Timbur	++	++	+	++	+++	+++	++	++	++	++	++	++

1. Alkaloid 2. amino acid 3. anthraquinones 4. steroids 5. glycosides 6. flavonoids 7. saponins 8. tannins 9. reducing sugar 10. triterpenes 11. cardiac glycosides 12. resin

Fig 1: Fruits of Boke Timbur [*Zanthoxylum acanthopodium* DC.]

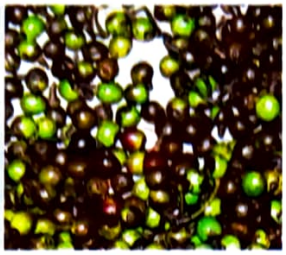

Fig 2: Fruits of Sil Timbur [*Litsea cubeba* (Loureiro) Persoon]

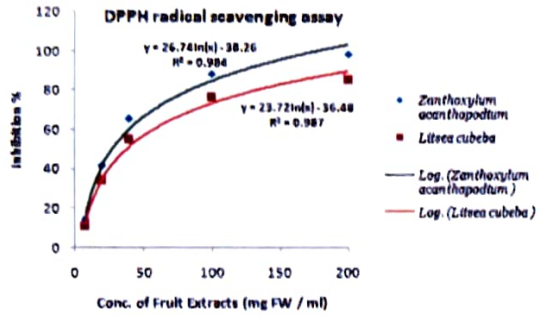

Fig.1: DPPH radical scavenging assay

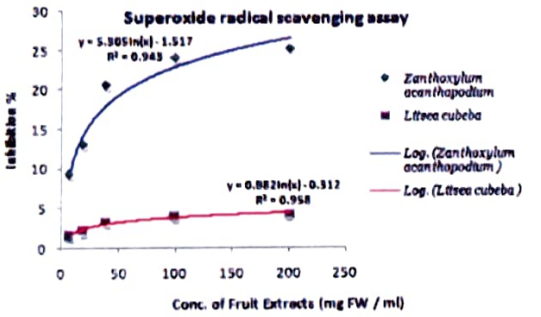

Fig.2: Superoxide radical scavenging assay

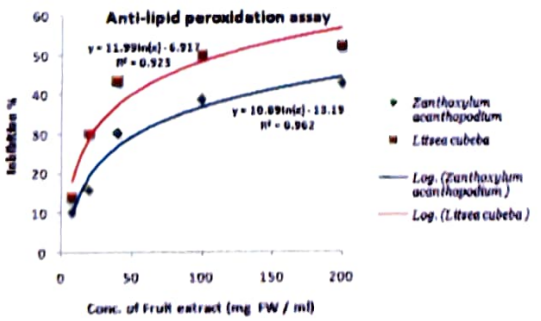

Fig. 3: Anti-Lipid Peroxidation Assay

and *Litsea* respectively. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become stable diamagnetic molecule. Antioxidant molecules can quench DPPH free radicals (i.e., by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them to a colourless molecule (i.e., hydrazine or a substituted analogous of hydrazine), resulting in a decrease in absorbance at 517 nm. It appears that extracts of two timbur fruits possess hydrogen donating abilities to act as an antioxidant.

Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that could be generated; it also has the ability to change to other harmful reactive oxygen species like hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA (Yen and Chen, 1995; Pietta, 2000). In the PMS / NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS / NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. Addition of the extracts of two fruits in above coupling reaction showed minimum decrease in absorbance (Figure 4). The extracts of *Zanthoxylum acanthopodium* demonstrated a logarithmic (R^2=0.943) dose-response inhibition of superoxide anion radicals with maximum value of 25.1%; whereas in case of *Litsea cubeba*, inhibition was almost insignificant (Figure 4).

Free radicals react with serum lipoprotein (LDL) and cause the formation of atheromatous plagues or react with the cell membranes' lipid and cause the peroxidation of polyunsaturated fatty acids. TBA method was used for evaluating the extent of lipid peroxidation. The extracts with liver homogenate undergo rapid peroxidation when incubated with $FeSO_4$ and produce peroxide (Aruma, 1996), which attack the biological material. This leads to the formation of MDA

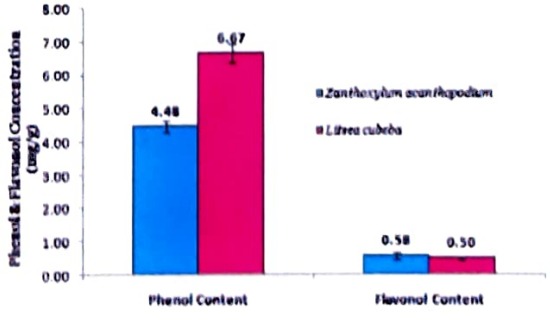

Fig.4. Phenol and flavonol content

(malonaldehyde) and other aldehydes, which form a pink chromogen with TBA, absorbing at 535nm (Kosugi *et al.*, 1987). It was observed (Figure 5) that methanol extract of two timbur fruits have high anti-lipid peroxidation effect against goat liver. Addition of Fe^{2+} to the liver, cause increased rate of lipid peroxidation. The peroxides of lipids react with ferrous chloride to form a reddish ferric chloride pigment. In this method the concentration of peroxide decreases as the antioxidant activity increases. The dose dependent logarithmic inhibition curve of lipid peroxidation was observed in Figure 5 and both *Zanthoxylum* and *Litsea* showed potential bioactivity with optimized inhibition of 42.64 and 52.1% at their saturated point.

Phenolic compounds such as flavonoids, phenolic acids and tannins are widely researched, naturally occurring antioxidant components of plants, and their effects on human nutrition and healthcare are considerable. These phenolic compounds, found in medicinal plants as well as fruits and vegetables, play important roles in preventing degenerative diseases, including inflammation, cancer and arteriosclerosis, when they are consumed as a part of daily diet (Sato *et al.*, 1996, Li *et al*, 2008). The mechanism of action of flavonoids is through scavenging or chelating the harmful radicals (Cook and Samman, 1996, Kessler *et al.*, 2003). Basically phenolics are a class of antioxidant agents which act as free radical terminators (Shahidi and Wanasundara, 1992). The flavonoid contents of the *Zanthoxylum* and *Litsea* fruit extracts in terms of quercetin equivalent (the standard curve equation: $y = 0.0067x + 0.0132$, $r^2 = 0.999$) were 0.58 and 0.50 mg/g respectively (Figure 6). Figure 6 also demonstrated the contents of total phenols that were measured by Folin Ciocalteu reagent in terms of gallic acid equivalent (by standard curve equation: $y = 0.05x + 0.0545$, $r^2 = 0.9873$). The total phenol content of *Zanthoxylum* and *Litsea* were about 4.48 and 6.67 mg/g respectively on dry weight basis.

It is reported that the phenolic compounds constitute a major group of compounds that acts as primary antioxidants (Hatano *et al.*, 1989). Though *Zanthoxylum* contains lower amount of total phenolics, as compared with *Litsea*; both Methanol Extractive Yield and radical scavenging activity is higher in *Zanthoxylum* (Methanol extractive value=105 [mg /g FW], % Yield in methanol=10.35) than in *Litsea* (Methanol extractive value=85 [mg /g FW], % Yield in methanol=8.40). So the positive correlation between antioxidant activity and phenolic compounds, as stated by several authors (Surveswaran *et al.*, 2007), is not the universal rule for all plant extracts. It also indicates that the metabolites other than the phenolics are equally responsible for antioxidant activity in case of *Zanthoxylum*. The result of phytochemical screening of methanol extract of two timbur fruits showed the presence of alkaloid, amino acid, anthraquinones, steroids, glycosides, flavonoids, saponins, tannins, reducing sugar, triterpenes, cardiac glycosides and resin (Table 1), most of which are higher in *Litsea cubeba* (Sil Timbur).

CONCLUSION

The present study confirmed that the methanolic extracts of fruits of *Zanthoxylum acanthopodium* and *Litsea cubeba* contain high levels of total phenolic and flavonoid compounds and have high DPPH radical scavenging ability along with potential anti-lipid peroxidation properties. The study also indicated that these fruits contain versatile group of secondary metabolites, even when their moisture levels were sufficiently reduced. So these underexplored spices are promising for more detailed investigation of their antioxidant properties, development of therapeutic products to protect against certain diseases and show potential for use in food supplements, subject to evaluation of toxicity and immunogenicity. Additional studies are required to analyze in detail the individual compounds related to antioxidant activity of these fruits, and further scientific investigation must proceed to ensure that the medicinal properties of these spices *in vivo* correlate with its antioxidant activity.

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Phytochemical screening and *in vitro* evaluation of crude extracts of moss *Funaria hygrometrica* (Funariaceae) for potential antibacterial activity

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Abstract
Traditionally bryophytes are known to possess some bioactive components and, therefore, used throughout the world as drugs and remedies to cure various diseases. In the present study, bioassay for antibacterial activities was carried out using whole plant of *Funaria hygrometrica* Hedw. Different solvent fractions and aqueous extracts of this moss were obtained and dried in vacuum. Antibacterial effect of these fractions was determined by disc diffusion technique on different human pathogenic gram positive bacteria i.e. *Bacillus subtilis* and *Staphylococcus aureus*, gram negative bacteria i.e. *Escherichia coli* and *Pseudomonas aeruginosa*. The result was then compared with the standard antibiotic streptomycin. Both the aqueous and organic crude extracts showed considerable activity against all the bacteria but maximum antibacterial activity was observed in ethanol extract against *Staphylococcus aureus*. The phytochemical analysis of the extract indicated the presence of steroid, flavonoids, alkaloids starch and oil.
KEYWORDS. .

Keywords: *Funaria hygrometrica*, moss, crude extracts, agar well diffusion, microorganism, antibacterial activity, phytochemical constituents

Bryophytes are the second largest group of land plants after the flowering plants with about 20,000 to 28,000 species. Three main groups of bryophytes are hornworts (Anthocerotopsida), liverworts (Marchantiopsida) and mosses (Bryopsida).They live in all zonobioms from the desert to the polar, but not in the seas (Sabovljevic 2006). Bryophytes are closely linked with civilization, culture, beliefs, and ethics of humankind (Pant 1990). Bryophytes are used by different cultural groups for cuts, wounds and skin diseases suggesting that they protect the skin and open wounds from microbial pathogen (Subramoniam 2005). Extracts of many bryophytes have been shown to possess varying levels of antibacterial and anticancer activities in vitro (Ando 1984; Adio 2004 ; Subramoniam 2003) and many chemical constituents were isolated from bryophytes (Asakawa 1982)

Chemical components of these plants can be used as biologically active agents since many compounds isolated from bryophytes have shown interesting biological activity with particular reference to their application in medicine and agriculture for all round benefit of living beings (Pant 1998). In Asia, already 500 bryophytes have been studied with respect to their chemistry, pharmacology and application as cosmetics and medicinal drugs. (Asakawa, 2001 a). Compounds like polygodial from *Porella*, Norpiguisone from *Conocephalum conicum* and Lunularian from *Lunularia cruciata*, 4-hydro-3-methoxybibenzylel 1 and a-and b-pinine-alloromadendrine from *Plagiochila stevensoniana* are useful as antimicrobial compounds (Kamory 1995; Lorimeres 1993). *Plagiochila fasciculate* shows inhibitory effect on P388 cells (Leukemia), Herpes simplex type 1, Polio type1, *Bacillus subtilis*, *Escherichia coli*, *Candida albicans*, *Trichophyton mentagrophytes* and *Cladosporium resinae* (Lorimeres 1994). The antifungal activity of *Herberta aduncus* against *Botrytis cinerea, Rhizoctonia solani, Pythium debaryanum* is also well illustrated (Matsuo 1983). Members of *Fissidens* and *Polytrichum* were used as diuretic and hair growth stimulating drugs (Basile 1998).

Funaria hygrometrica Hedw. belong to family Funariaceae and the plants grow in loose to compact tufts, in large patches, green to yellowish green, simple or branched. Stem slender, erect, 11 to 12.5 mm height. Lower leaves small sparse; Costa poorly developed, upper leaves large crowded at apex; leaves (dry) curved and folded, (moist) spreading up to 3mm long and 1mm broad, concave, obovate to oblong lanceolate, acute,entire; costa strong, ending below the apex. Plants grow on moist soil or on rocks in large patches; or on slopes near water resources.

In this context, antibacterial potential of *Funaria hygrometrica* against some pathogenic bacteria was studied. The study includes effect of some organic and aqueous extracts of *Funaria hygrometrica* against four

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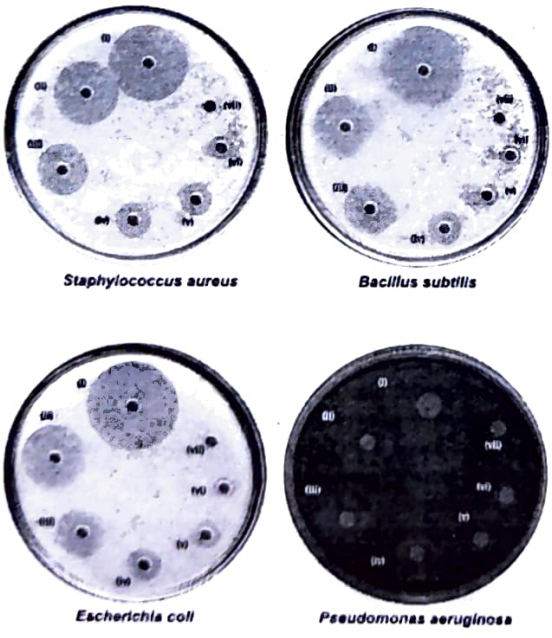
bacterial strains. Preliminary phytochemical screenings were also conducted.

Materials and Methods

Collection of plant material. *Funaria hygrometrica* was collected from Nakki Lake (Alt.1160) and Sunset point (Alt.1195) of Mount Abu district of Sirohi, Rajasthan, in the month of September 2006. The plants were identified and voucher specimens have been deposited in Bryology Laboratory, Dept. of Botany, Univ. College of science, Udaipur for future reference.

Extraction procedure and phytochemical screening: The plant material was carefully cleaned from attached litter and dead material under running tap water and finally with sterile distilled water. Air-dried and powdered approximately 20 g plant material of *Funaria hygrometrica* was extracted by cold percolation in either petroleum ether, benzene, acetone, methanol, ethanol or about 200 ml autoclaved water. The extracts were decanted, filtered with whatman No.1 filter paper and concentrated at reduced pressure below 40°c through rota vapour and lyophilized (Buchi, Labconco, US) to obtain dry extract. 0.1mg crude extracts were taken up for biological screening and also to observe the presence and absence of different phytochemical constituents.viz. alkaloids (Dragendorff's test), saponins (foam formation), flavonoids (using magnesium (Mn) and dil.HCl), terpenes (Liebermann-Burchard's test) according to standard methods (Sofowora, 1982, Trease and Evans, 1987).

Test microorganisms. Four test microorganisms were used in antibacterial sensitivity test were procured from Microbial Type Culture Collection And Gene Bank (IMTECH, Chandigarh,India), ie., the Gram positive bacteria *Bacillus subtilis* (MTCC-441) and *Staphylococcus aureus* (MTCC-740) and Gram negative bacteria *Escherichia coli* (MTCC-41) and *Pseudomonas aeruginosa* (MTCC-424). All the bacterial strains were maintained at 4°C on nutrient agar slants and sub cultured as required.



(i) 1000µg/ml (ii) 800µg/ml (iii) 500µg/ml (iv) 250µg/ml (v) 125µg/ml (vi) 65µg/ml (vii) DMSO

Fig1: Control plates of various bacteria against antibiotic Streptomycin as +ve and DMSO as –ve control.

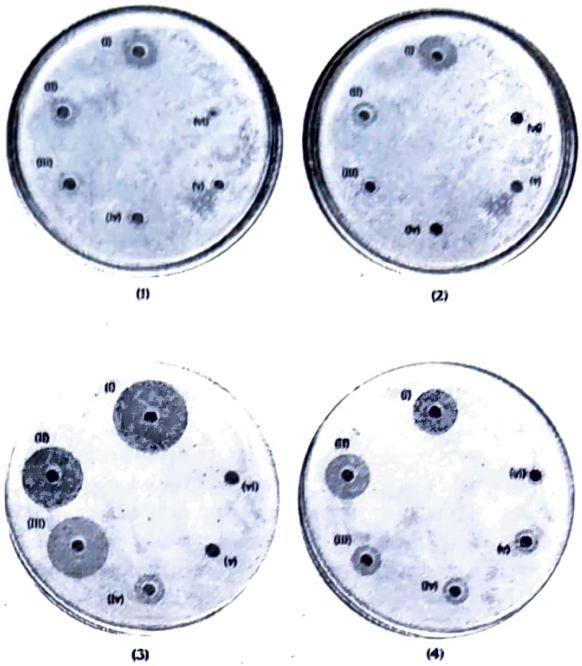


Fig 2. Antibacterial screening of benzene and acetone extract of *F. hydgrometrica* against *B. subtilis* (1,2) activity of ethanol and methanol extract against *Staphylococcus aureus* (3,4)

Antibacterial activity. The agar well diffusion method (Murray et al., 1995) evaluates the antibacterial activity. Bacteria were cultured overnight at 37°C in nutrient broth (Hi-media, Bombay) and were used as inoculum. 20 ml nutrient agar medium was poured in sterilized petri plates and allowed to solidify at room temperature. 24 h broth culture of test bacteria was used as inoculum under sterile condition. The freshly activated 100µl of organisms was set to 0.5 optical density and spread with a sterile L shaped bent glass rod. Using cork borer several wells of 6mm diameter were punched. To each well 100 µl crude extracts of various concentration (1000, 800, 500, 250, 125, 65µg/ml) were added. DMSO (Dimethyl sulfoxide) were used in making of extracts concentration and neutralized with 0.1N NaOH and 0.1N HCl. The plates were incubated at 37°C. Streptomycin and DMSO were used as positive and negative control respectively. The experiment was performed in triplicate and average results were recorded. Finally the diameter of zone of inhibition including laterally around the well was measured with antibiotic zone scale in mm

Results and Discussion

Preliminary phytochemical screening of the plant showed the presence of steroids, alkaloids and flavonoids in *Funaria hygrometrica* while saponins were absent (Table-1). The results of testing the

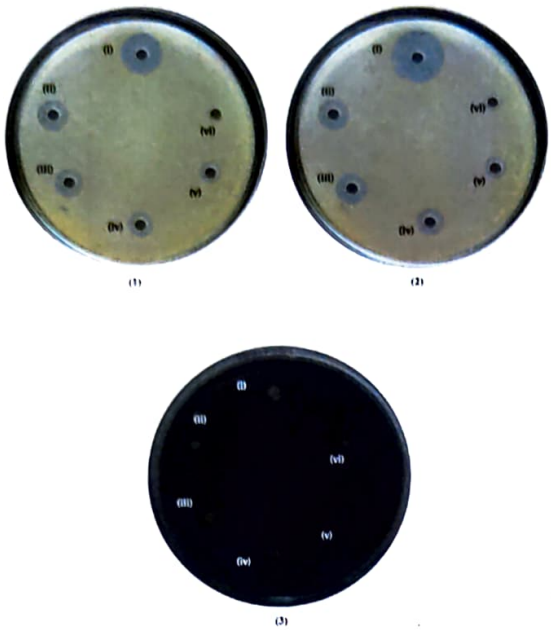


Fig 3. Antibacterial screening of benzene and acetone extract of *F. hydgrometrica* against *E. coli* (1,2) activity of ethanol and methanol extract against *Pseudomonas aeruginosa* (3)

antibacterial activity of crude extracts of moss *F.hygrometrica* are presented. These were obtained by the disc diffusion method against various bacterial strains. Streptomycin and DMSO were used as positive and negative control respectively. Plant was extracted using both aqueous and organic solvents. The maximum antibacterial activity (approximately 24mm zone of inhibition) was observed in ethanol extract of the plant against the *Staphylococcus aureus* and minimum zone of inhibition observed in petroleum ether extract against *P.aeruginosa* (Figs.1-3)

Data obtained demonstrates that the antibacterial activity of plant depends largely upon the types of solvent used for extraction. Data indicate that almost all organic extracts of the plant showed antibacterial activity against the tested bacterial isolates (Table-2).

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DNA and its bending: a glimpse of mechanism and implication in bacteriology

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Abstract

The double helical DNA structure varies from one species to other. Bend DNA is also available in the cell. Bending is expressed by the supercoiling of DNA structure. The mechanism of DNA bending is based on two basic models: the A-Tract model where the phasing of 6/A residues are important and in-junction bending model, where the heteronymous poly (dA), poly (dT) and B-DNA junction are responsible for causing bend in the helix axis. DNA bending has several implications, like transcription, replication, regulation etc., that induce the protein- protein and protein-DNA interaction. This DNA bending mechanism with multidimensional attributes has important applications such as drug preparation, cancer therapy and so on.

The biochemical investigation on DNA began with Friedrich Miescher (1868). Miescher isolated a phosphorus – containing substance which he called as nuclein from the nucleus of leukocytes and he also found that, 'nuclein' consists of an acidic portion and a basic portion, i.e. protein. The acidic portion, today is known as DNA. The first direct evidence that DNA is the bearer of genetic information came in 1944 through a discovary made by Oswal T. Avery, Colin Macleod and Maclyn McCarty. These investigators found that, the DNA which is extracted from a virulent strain of *Streptococcus pneumoniae* genetically transformed a non-virulent strain of this organism to a virulent form.

Avery and his colleagues extracted the DNA from heat killed virulent pneumococci, removing the protein as completely as possible and added this DNA to non-virulent strain, which were permanently transformed to virulent strain and when injected to mouse, it died. They concluded that, the DNA which is extracted from the virulent strain carried the inheritable genetic message for virulence. But this concept was not accepted to all, as protein impurities present in the DNA could be the carrier of genetic information.

But this idea was soon eliminated after the second important experiment in 1952 by Hershey and Chase. They provided the evidence that DNA carries genetic information. They used radioactive phosphorus (32 P) and radioactive sulfer (35 S) and when infected by bacteriophage (T2 infects the host cell i.e. *Escherichia coli*) it was found that the phosphorus containing DNA of viral particle enters into the host cell instead of the sulfur containing protein particle of the viral coat. The DNA material entered into the host cell for different functions like viral replication, transcription and translation. Thus, they proved that DNA is the genetic material rather then a protein.

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DNA Structure

Rosalind Franklin and Maurice Wilkins used the powerful method of X-ray diffraction to analyze DNA fibres. The beams are diffracted or broken down by the atoms in a pattern that is characteristic of the atomic weight and the spatial arrangement of molecules. Later, Franklin concluded that, DNA is a helical structure with two distinctive regularities of 0.34 nm and 3.4 nm along the axis of the molecule.

A most important clue to the structure of DNA came from the work of Erwin Chargaff and his colleagues in late 1940s.

They found that the four nucleotide bases of DNA occur in different ratios in the DNA of any organism and the amount of certain bases are closely related. They concluded that-

The base composition of DNA in a given species does not change with organism's age, nutritional state or changing environment.

In all cellular DNA the number of adenosine resideues is equal to the number of thymidine residues i.e. A = T and the number of guanosin residues is equal to the no of cystocin residue, i.e. G = C

Thus it can be said, that $A + G = T + C$

This quantitative relationship is sometime called as "Chargaff's rules"

It is very difficult to understand the DNA structure in two dimensional plane. The best way to conceptualise DNA is in three dimensional plane. In 1953, Watson and Crick postulated a three dimensional model of DNA structure that accounted for all the available data, i.e. x-ray diffraction data of Franklin and base equivalence

Table 1: DNA Characteristics

DNA:	Polymer of Deoxyribonucleotide
Linked by :	5' – 3' phosphodiester bonds
Nucleotide :	Sugar + base + phosphate
Sugar :	Deoxyribose sugar having 5' – OH group
Base :	Purine and Pyrimidine
Purines :	A and G
Pyrimidines :	T & C
Base Pairing :	A with T G with C
H bonding :	A = T G ° C
Rationale of base pairing :	A – G large enough to fit with in 2.0 nm dia of DNA. T – C small enough to fit with in 2.0 nm dia of DNA.

observed by Chargaff (Table 1).

For the sake of simplicity it is assumed that the base pair step is coplanar but it is not exactly so (Mohan and Yathinadra, 1992). There is an element of propeller twist as a result of which the 2H bonded bases may be twisted some what like a propeller. The H-bonding may result not only between two bases in a base pair but also between the units of the adjacent base pairs also. Propeller twist is partly responsible for this.

Steric hindrance between two adjacent purines is again partly influenced by propeller twist. The DNA stability is due to the double helical structure. This stability is not only maintained by H-bond of complementary base pairs but also by electronic interactions between the stacked bases as well as hydrophobic interactions. The two anti-parallel strands are not identical- rather they are complementary to each other.

WHY HELIX

If the DNA is considered as an untwisted ladder, then a considerable space would be present in between which would be accessible to water. But the bases are oily or hydrophobic in nature, they would try to expel those water molecules from this space. This phenomena will occur only when the bases slide over each other or by twisting. Sometime sliding of bases occur through twisting. Thus twisting conformation is more stable.

The bases are not exactly coplanar somewhat like propeller twist occurs. Thus, the H-bonding is not only present in between two bases in a base pair but between the units of adjacent base pair also. This propeller twist also cause the steric hindrance between the adjacent purines by which the helical conformation of DNA is maintained (Mohan and Yathinadra, 1992). If the base pairs are forced to open by EtBr and chloroquine like elements, then it is shown that DNA can get back to its untwisted conformation. Thus, it can be said that the way of base stacking plays an important role in the structure of the DNA which favours the twisted form for stability. The bases are stacked in such a manner that the bases are particularly accessible from the major groove side than the minor groove side. If the base stacking manner are tried to unstack it will require energy and a tension will build up in DNA structure unable to rotate freely.

STRUCTURE VARIATION

The basic of DNA structure is the dinucleotides. The dinucleotides also undergo deformation in various ways. The deformation occurs due to the movement of bases along three different axes (Tolstorukov et al., 2007) like, Twist axis, Roll slide axis, Front back axis. So, they are deformed in major 3 ways: Roll, Twist, Slide. Along the twist axis the dinucleotide twists, along the roll or slide axis, dinucleotides roll or slide.

There may be positive roll and negative roll, also positive slide and negative slide. When two adjacent dinucleotides move away from each other then it is known as +ve slide, where as when they move closer to each other then it is –ve slide. 'Steric hindrance' is caused when two adjacent bases come closer, there is a repulsive interaction i.e. steric hindrance, where the bases twist to each other. In another case, when two purines are located on the opposite strand like, G-G, A-T that repulsion occurs due to the propeller twist. This phenomenon is known as purine clash. To avoid the purine clash, the purines must slide away from each other (+ve slide) or slide over one another (-ve slide) and pyrimidines will have to rotate away from one another.

Deformation of dinucleotide also occur in case of charge -charge repulsion. G-C base pair possess dipole as G contain '- ve' charge and C contain '+ve' charge. Thus when one G-C base pair stacked over another there is repulsion due to same charge and thus it causes sliding and/or rolling deformation of dinucleotide. But in case of A-T it has no dipole, thus it need not to deform due to the charge difference it only deform by the steric hindrance phenomena and propeller twist phenomena.

Aternative double helical structure

Variations in the conformation of the dinucleotide of DNA associated with conformation of its varieties. The double helical structure of DNA are present in 6 different forms i.e. A, B, C, D, E & Z. Among these the A, B and Z forms are important (Table 2).

Certain DNA sequences adopt unusul structures. When in DNA identical DNA sequences are present in the reverse orientation they are called inverted repeat. The sequences of inverted repeats together is called palindrome. The inverted repeat may cause the formation of secondary structure in SSDA or in dsDNA. In a longer palindromic sequence the complementary sequences pair to form a hairpin. In dsDNA the formation of two opposite hair pin is produced forming cruciform structure, which is so called because it represents the junction of four duplex regions.

Other types of DNA structure

Besides double stranded helical structure, DNA also exists in certain unusual structure and believes as important for molecular recognition of DNA by protein

Table 2: Comparison of structural features of different conformations of double helical DNA.

Types of DNA	B[1]	A[2]	Z[3]
Helix type	Right handed	Right handed	Left handed
Helical diameter (nm)	2.3	2.5	1.8
Distance per each complete turn (nm)	3.4	3.2	4.5
Rise per base pair (nm)	0.34	0.29	0.37
No. of bp per complete turn (nm)	10.5	11	12
Base till normal to the helical axis.	6°	20°	7°

[1]B-form: Most predominant form
[2]A form: Occurs in DNA-RNA hybrid, conformation similar with the dsRNA
[3]Z-form; Available *in vitro* under high salt condition.

and enzymes.

A. Triple stranded DNA

Triple stranded DNA formation may occur due to additional H-bonds between the bases. T can selectively form 2 Hoogsteen

hydrogen bond to A, of A-T pair forming T-A-T where as a protonated C can also form two Hoogsteen H-bond with G of G-C pair forming C^+ - G-C. The N-7, O^6 of guanine and N^6 of Adenine atoms are involved in Hoogsteen H-bonding; thus these are referred to as Hoogsteen positions in non Watson-Crick base pairing, first described by Karst Hoogsteen (1963).

By this Hoogsteen pairing the triplex DNA is formed (Aishima *et al*, 2002). The triplex DNA strands are less stable than double helix, because of the 3 negatively charged back bone. Strands in triple helix produce increased electrostatic repulsion. The triple DNA is most stable in low pH because. C ≡ G C+ require a protonated C. Some triplex DNA contain two pyrimidine strand and one purine strand or vice versa, called H-DNA having alternating pyrimidine and purine tract.

B. Four stranded DNA

Polynuceotide with very high content of guanine can form a novel tetrameric structure called G-quartates. These structure are planar and are connected by Hoogsteen H-bonds. (Aishima *et al*, 2002) Antiparallel four stranded DNA structures are referred to as G-tetraplex.

The end of the eukaryotic chromosomes- namely telomeres are rich in guanine and therefore form G-tetraplexes. Telomeres have become the targets of anticancer chemotherapy. G-tetraplexes have been implicated in the recombination of immunoglobulin genes and in dimerization the dsRNA of HIV. The G-tetraplex is quite stable over a wide range of conditions.

C. Bent DNA

In general a base containing DNA tract is rigid and straight but bent conformation also occurs. Bending in DNA structure happens due to photochemical damage or mispairing of bases. Certain antitumor drugs e.g. cisplatin induce bent structure in DNA such changed structure can take up protein that damage DNA.

DNA supercoling

The DNA double helix represents DNA as a linear molecule. But DNA *in vitro* generally shows a closed structure. It lacks free end. Cellular DNA is extremely compacted, implying a high degree of structural organization. The folding mechanism must not only pack the DNA but also permit access to the information in the DNA.

Before considering its accomplishment in processes like replication, transcription, it is important to understand the property of DNA supercoiling.

Supercoils, the coiling of a coil, occur in DNA when a duplex is twisted in space around its own axis. The twisting introduced by supercoiling places a DNA molecule under torsion. Supercoil occurs only in closed structure because an open molecule can release the torsion simply by untwisting. A closed molecule must not have any breakage on either strand of DNA; any break even in one strand of a circular molecule allows untwisting.

A molecule that lacks supercoiling whether is open or closed is said to be relaxed. Supercoiling is of two types: Negative supercoiling and Positive supercoiling.

Negative supercoils twist the DNA around its axis in the opposite direction from the clockwise turn of the right handed double helix. This allows in principle to relieve the torsional pressure of DNA by adjusting the structure of the double helix. The relief takes the form of loosening the winding of the two strands about each other- DNA with negative supercoiling is said to be underwound.

The opposite type of effect is caused if DNA is supercoiled in the same direction as the intrinsic winding of the double helix. The positive supercoils tighten the structure applying torsional pressure to wind the double helix even more tightly. DNA with positive supercoil is known as overwound. It has been shown that many circular DNA molecules remain highly supercoiled even after they are extracted and purified, freed from protein and other cellular components. This indicates that, supercoiling is an intrinsic property of DNA tertiary structure. It occurs in all cellular DNA and are highly regulated.

Supercoiling is the combination of Twisting number (Tw) and Writhing number (Wr)

Twisting number (Tw):

It is property of the double helical structure itself, representing the rotation of one strand about the other. It represents the total number of turns of a duplex and determined by number of bp per turn. The twist angle 34^0 in B-DNA results in a helical repeat of about 10.5 bp/turn.

Writhing number (Wr):

It is the turning of the axis of the duplex in space. The global wrapping of the axis of the double helix around itself.

Linking number (Lk)

It is the number of times one DNA strand wraps the other when the molecule lie in a plane. Thus, Lk is the number of revolution that one strand make around the other when the DNA is considered to lie flat on a plane surface. Lk is equal to the sum of Wr and Tw, be written as: L=Tw+Wr

The DNA supercoil can be of two types: Toroidal and Plectonemic. In a protein free state, the DNA will remain plectonemicly supercoiled but in the presence of DNA binding protein it may be present as toroidal supercoil.

In toroidal supercoil DNA is stable. But isolated in laboratory condition, it may be in plectonemic form, as all proteins have been removed from it. Supercoiling is intricately linked to biological activity of the DNA. DNA supercoiling has an important role to bring the distal segment of DNA in close proximity as it is required for recombination event. Thus, DNA compaction requires for the DNA supercoiling.

DNA Bending

DNA does not exist as linear form in a cell rather, in twist, turn and supercoil. The supercoiling is required for compaction and also for several biological functions. For the twist, turn or supercoiling DNA requires to be flexible enough, initiating the bending process.

Nucleosomal bending

Nucleosome model is very useful model for DNA bending. In nucleosome model 2 nm DNA is wrapped around the central core i.e. the histone octamer. DNA (160 bp) wrapped into core molecule via roll and slide with the bp twisting. The tight bending of DNA in the nucleosome suggests its ability to bend in a particular direction, that's a crucial factor. Experiments performed to understand if there was any intrinsic sequence dependence and revealed a periodic modulation of dinucleotide AA/TT and a dinucleotide GC but this happens in opposite direction. (Goodsell *et al*, 1993) The phasing of AA/TT and GC/CG in the nucleosomal model consider that AA step is rigid and they present at the low roll position, where as GC is localized in the high roll position (Tolstorukov *et al*, 2007).

The sequence of the bound DNA affects the binding of DNA to histones in nucleosome cores. The histone core does not bind randomly to the DNA, rather they tend to place themselves at certain locations. This positioning is not fully understood. In some cases, it appears to depend on abundance of AT base pairs in the DNA helix where it is in contact with histones. The tight wrapping of the DNA around the nucleosome- histone core require compression of the minor groove of the helix and a cluster of 2 or 3 A=T bp makes this compression more likely. (Goodsell *et al*, 1993)

Mechanisms for DNA bending

The relationship between DNA sequence, structure and function has been studied and discussed extensively in last few decades. To reveal the structural basis of DNA bending/curvature effort has been directed towards the structure of a short run of 4-6 A-T residues known as A-tract. The DNA curvature is induced when the 'A-tract' that is inserted in phase, with the helical periodicity.

The structural basis of 'A-tract' that induce 'DNA bending' has remained enigmatic. Because no single structure could explain the whole phenomenon, it is necessary to rely on several models which have been confirmed by 'gel migration data' (Kerppola, 1997) This data suggests that the center of curvature is towards the minor groove of the 'A-tract' and towards the major groove of intervening general sequence.

DNA bending at A-T tracts

It is quite common to use the variation in gel mobility for mapping a bending locus contained in a sau 3 - a restriction fragment isolated from *Leishmania tarentolae* kinetoplast DNA and the sequence identified as–

GAATTCCCAAAAAGTCAAAAAATAGGCAAAAA ATGCSAAAAATCCCAAAC– (Wu and Crothers, 1986). This is a striking feature that a regular repeat of the sequence element $CA_{5\text{-}6}T$ with 10bp periodicity is present around the center of the bend.

Presumably, each A-tract produces a small bend in the DNA helix axis repetition of these elements in phase with the helix screw results in their coherent addition to form a large overall bend. Thus, for DNA bending, it requires a continuous run of 'A-tract' residues and the potential role of the junctions with other bases flanking the A-tract. For DNA bending phasing is also very essential (Kerppola, 1997). It is shown that bending elements must be repeated in phase with the helix screw in order. The importance of continuous run of A-residues for the bending phenomenon was investigated by interrupting the A-tract with another nucleotide at the central base it is shown that the single bp change causes the gel mobility to revert nearly to normal.

It appears that the continuous run in 'A' residues is the basis for bending phenomenon. In this case the purine 'A', and 'G' are not equivalent. In addition, it is also found that the sequence of 'G' has normal gel electrophoretic mobility which conform the special role of A-tracts. It is found that 'A' tract of length 3 produces only a minor electrophoretic anomali, the effect is substantial with 4 in a row and increases to maximum for a tract of length 6, that becomes another important factor for DNA bending (Crothers *et al*, 1990). It is the junctions of A-tracts on which the extent of bending would depend on the flanking bases. It is probable that the greatest degree of bending is seen when the 5'-flanking base is C and the 3'-base is T as it is found at natural bending locus in *L. tarentolae*. When C is present in both 3' and 5' end, it is less bent and when G is present in both 3' and 5 end the bending property reduces further (Crothers *et al*, 1990)

There are two general classes of models for the origin of sequence – directed DNA bending (Goodsell and Dickerson, 1994). Class I includes the sequence –

periodicities. Sequeunces should favour bending by compressing the minor groove, where as pyrimidine – purine sequence should bent into the major groove. All these observations support the 'A-tract' DNA bending.

Class-2 model shows that poly (dA) poly (dT) has an anomalous structure as revealed by fibre diffraction, its 10.1 bp helical screw, its Raman spectrum and its inability to be reconstituted into nucleosomes. It is possible that oligo dA-dT tracts in DNA can adopt one or more alternative structures in addition to the B-configuration. This model appeals to a longer range structural polymorphism at the A-tract to focus the properties of junctions between it and adjacent B-DNA. According to this, the heteronymous poly (dA) poly (dT), then a bend in the helix axis is expected at the junction with B-DNA. The junction bending model serves as an effective working hypothesis (Nadeau and Crothers, 1989). This model holds that the 'A-tract' adopts a conformation similar to the poly (dA) poly (dT) structure deduced from fibre diffraction studies the key feature of which is the substantial tilt of the bp relative to the helix axis. When a segment of B-DNA is adjoined to the filled bases of the A-tract the helix axis is deflected at the junction between the two dissimilar structure for maintaining favourable base stacking. Concerted phasing of these local junction bends then yields to a global curvature of the DNA.

The distinguishing assumption of the original junction model is that 'A-tract' residue will remain in a B-conformation unless they are long enough to overcome the free energy barriers to nucleation of altered structure responsible for bending (Nadeau and Crothers, 1989). The structures of several similar 'A-tract' containing duplexes have been determined by x-ray crystallography, each of which is bent and displays a minor groove narrowed by strong propeller twisting of the A-T pairs without significant bp tilt. The bends are at the roll of the junction and the responsible factors are groove compression, whether results from propeller twisting bp tilt, some other structural features or combination of several.

In order to inhibit gene expression proteins need to bind to specific DNA target site such that recognition is often accompanied by 'DNA bending'. Scanning force microscopy (SFM) studies revealed crucial differences in DNA bending induced by protein of non specific and specific sequence. However, the significance of DNA bending is not clearly understood.

From the studies on 'Cro' protein i.e. the protein that regulates the genes in bacteriophage, it has been shown that 'Cro' first binds loosely to DNA at non specific sites and produces 'binding wave' and travel along the chain until it recognize a specific target site where it binds very tightly. The SFM studies revealed an increase in DNA bending angle where the protein is bound to a specific site. This molecular recognition is not only important in gene regulation but in many other biological processes. Thus it can be said that DNA bends to bind (Kanhere and Bansali, 2004)

DNA Bending and transcription

The $\alpha_2B\beta'\sigma$ *E. coli* RNA polymerase holoenzyme is capable of promoter binding, accurate initiation and response to diverse transcriptional regulators some of which alter the conformation of DNA in the upstream promoter region. Architectural changes in DNA may be important to initiate the formation of a DNA loop around RNA polymerase. Wrapping of DNA around *E. coli* RNA polymerase in the preinitiation complex has been observed.

The largest dimension of *E.coli* RNA polymerase holoenzyme is 160 A^0, but footprints of holoenzyme on promoter DNA in both closed and open complexes extend to 310 A^0 or more (Coulombe and Burton, 1999). This footprinting is explained by wrapping the DNA round RNA polymerase. The prominent common features of the RNA polymerase is the presence of finger like projections in RNA polymerase that is close to form a channel large enough to accommodate DNA. For *E. coli* RNA polymerase the channel is open in the initiating holoenzyme and remains closed to the elongating core enzyme. This suggests that DNA penetrates the channel which closes around template during elongation to prevent transcription.

In both the closed and open complex the topology of promoter is altered to introduce negative supercoiling. Upstream promoter DNA may be partially wrapped around RNA polymerase in closed complex, with a significant DNA bend near the – '35' region of the promoter (Perez – Martin and Lorenzo, 1997). During transcription there is a conformational change in the holoenzyme that closes the 'hand' of RNA polymerase around the DNA and introduces another major DNA bend near the transcriptional start site. This conformational change has the effect of developing significant strain on the DNA helix around + 1.

It is proved that two DNA bends are spaced in such a manner that as the DNA wrap is tightened, the short stretch of helix between the 2 bends are forced to unwind.

DNA upstream and downstream of the two DNA bends to act as two levers and to force the helix into the unwound conformation that preceeds open complex formation. Transcriptional regulators can affect this mechanism by pulling the upstream DNA lever the downstream lever or both.

DNA Bending and transcriptional control

The α-subunit of RNA polymerase are important for interaction with upstream promoter DNA and regulators. The α-structure comprises an amino-terminal domain (α -NTD) and a CTD (α-CTD) domain separated by a flexible central region.

A DNA wrapping model describes catabolite gene activator protein (CAP) cyclic AMP (CAMP) medicated activation of 'lac' promoter. CAP binding sites can be located at variable positions relative to the transcriptional start site but the face of the helix on which CAP lies relative to RNA polymerase for

activation (Perez – Martin and Lorenzo, 1997) Maintaining the orientation of CAP relative to RNA polymerase is to serve two purposes- (i) to allow specific CAP-RNA polymerase interactions, and (ii) to maintain the directionality of the CAP-induced bend in DNA towards RNA polymerase.

CAP-CAMP bends DNA by 90^0, bending promoter DNA back to RNA polymerase. CAP-CAMP on the lac promoter make specific contact with activation region on the α-CTD. DNA bending and α-binding by CAP-CAMP might contribute to promoter strength in two ways: i) by enhancing RNA polymerase binding to promoter and ii) by promoting isomerisation (Coulombe and Burton, 1999). Initial binding of holoenzyme induce DNA bending in the upstream promoter region, DNA bending by CAP-CAMP could stimulate binding. RNA polymerase binding is also expected to be stimulated by interaction bent CAP-CAMP and α-subunit of RNA polymerase.

According to DNA wrapping model, however appropriately phased bends in upstream DNA initiate DNA wrapping round RNA polymerase so that DNA bending in the upstream promoter region can also contribute to isomerisation (Spronk *et al*, 1999). Formation of appropriate DNA loop around holoenzyme requires bending and wrapping of the DNA, facilitated by a multitude of protein-protein and protein-DNA contacts.

Thus DNA bending by CAP-CAMP in upstream promoter region, appear to cause DNA to wrap around RNA polymerase.

DNA bending and regulation of gene expression

The most common example regarding gene expression is the 'lac operon'. The lac repressor protein is one of the key enzyme in the lactose digestion chain of *E.coli* bacteria. This protein turns off the genes that are responsible for lactose digestion when lactose is absent from the bacterial environment.

The lac repressor functions through clamping two out of the three DNA sites i.e. O_1, O_2, O_3 called operator site. The DNA bend - site is forced to form a loop which interferes with the reading the genes by another protein i.e. RNA polymerase (Mahadevan and Schulten, 1999). A single repressor molecule binds to two operators that are between 93 and 401 bp apart. One suggested mechanism is that the repressor tetramer binds to separate operator sequences forcing the DNA to conform to a loop structure.

For DNA interaction to occur there must be a sufficient length between the operator sequence in addition to certain other properties of DNA. The formation of the loop structure makes RNA polymerase binding even more unfavourably than only one repressor tetramer bound to the DNA. DNA loop induced by the lac repressor between the operator sites O_1 and O_3.

Conclusion

It is experimentally proved that DNA bending depends on the binding competition between monovalent and multivalent components. DNA bound p53 core domain causes local DNA conformational change, that results in DNA bending. Local distortion mechanism, induced by polyamine treatment, could be related to DNA condensation. Further implication for transcription activation lies with p53 induced DNA binding. Since p53 is an important factor for cancer suppression thus DNA bending can be used in cancer therapy. Based on a few examples it is speculated that updating of knowledge suggests that there may be a relation between drug molecule and DNA bending where structural and conformational change in the DNA and drug molecule occurs. Thus to understand the mechanism of drug action, DNA-drug interaction during DNA bending is very essential.

Thus DNA curving is also very important in biotechnological field which helps to regulate the synthetic plasmid replication and transcription. When polyamine ion is immobilized in the middle of major groove, the DNA chain could bent upto the largest angle which is sterically allowed.

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Bioinformatics of codon usage pattern in pathogenic proteobacteria *Burkholderia mallei*

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Abstract
Burkholderia mallei are pathogenic *gram* negative β Proteobacteria those are available in Africa, Asia, Middle East, Central and South America and abundantly known as the causal organism of glanders.In this study, the synonymous patterns of four *Burkholderia mallei* (*Burkholderia mallei* ATCC 23344, *Burkholderia mallei* NCTC 10229, *Burkholderia mallei* NCTC 10247, *Burkholderia malle* SAVP1) genome were compared and analyzed to each other. It was observed that *Burkholderia mallei* have high G+C content and moderately biased. Using cogon adaptation index (CAI) as a numerical estimator of gene expression level where ribosomal protein coding genes were considered as a reference of highly expressed genes. Here, we also studied the functional analysis of the PHX genes, gene expression level, correspondence analysis and horizontally transferred pathogenicity related genes activity. COGs are also associated with metabolism especially those linked to carbohydrate metabolism and amino acid transport.

Keywords: *Burkholderia mallei*, Codon bias,Correspondence analysis, PHX, COG.

Abbreviation: **BMAL-44**-*Burkholderia mallei* ATCC 23344, **BMAL-29**- *Burkholderia mallei* NCTC 10229, **BMAL-47**-*Burkholderia mallei* NCTC 10247, **BMAL-VP1**-*Burkholderia malle* SAVP1, **PCG**-Protein coding gene, **RPG**-Ribosomal protein genes, **PRG**-Pathogenecity related genes, **HTG**- Horizontally transferred genes, **PHX**-Predicted highly expressed genes, **COG**-Clusters of orthologous groups of protein, **CAI**-Codon adaptation index.

Horizontal gene transfer refers to the transfer of genes or genetic material directly from one individual to another by processes similar to infection. It is distinct from the normal process of *vertical* gene transfer - from parents to offspring - that occurs in reproduction. Genetic engineering bypasses reproduction altogether by exploiting horizontal gene transfer, so genes can be transferred between distant species that would never interbreed in nature. For example, human genes are transferred into pig, sheep, fish and bacteria. Toad genes are transferred into potatoes. Completely new, exotic genes can therefore be introduced into food crops.

Horizontal gene transfer is the lateral movement of genes between organisms. HGT may be designated as infectious transfer. This is a very common mechanism among bacteria even they are distantly related. By this way one bacterium become drug resistance. Transformation, transduction and conjugation are three classical mechanisms of HGT. Most of the organisms are affected by HGT.

The purpose behind this study was to implement a comparative analysis of the synonymous codon usage patterns, predicted expression levels for the protein coding genes in these pathogenic bacterial strains with special reference to pathogenicity related genes, study of horizontally transferred pathogenicity related genes to detect their presence in the strains and scrutinize the nature of highly expressed genes upon their lifestyles. We consider that the result of this study would be helpful in further work on these bacteria.

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Materials & Methods:

The complete genome sequences for four strains of *Burkholderia mallei* (*Burkholderia mallei* ATCC 23344, *Burkholderia mallei* NCTC 10229, *Burkholderia mallei* NCTC 10247, *Burkholderia malle* SAVP1, henceforth referred to as BMAL-44, BMAL-29, BMAL-47and BMAL-VP1 respectively) bearing Gene Bank accession numbers NC_006348, NC_006349,NC_008836, NC_008835,NC_009080, NC_009079, NC_008785 and NC_008784 were obtained from the IMG website (www.img.jgi.doe.gov). The gene sequences are retrieved in the FASTA format. All the protein coding genes, horizontally transferred genes, pathogenicity related genes and ribosomal protein genes were examined using Codon W software (http://bioweb2.pasteur.fr) (Peden, 1999) and CAI Calculator2 (http://www.evolvingcode.net/codon/CalculateCAIs.php) (Wu *et al.*, 2005) Codon W (Peden, 1999) was used to determine the GC3 content, effective number of codons (Nc) (Wright,1990) and the frequency of optimal codons (Fop) (Peden, 1999). The effective number of codons (Nc) is a straightforward measure of codon bias (Wu *et al.*, 2005). It generally ranges between 20 and 61. Fop (Peden, 1999) calculates the section of synonymous codons that are optimal. Its value varies from 0 to 1.0. All negative Fop values were adjusted to zero. The 'codon adaptation index' (CAI)

(Peden, 1999) values were computed using 'The CAI Calculator 2 (http://www.evolvingcode.net/codon/CalculateCAIs.php) (Wu *et al.*, 2005) taking the ribosomal protein genes as a reference. The CAI value varies from 0 to 1.0 with higher CAI values suggestive of the fact that the gene of study has a codon usage pattern like that of the reference genes (Sharp and Li, 1987).

An analysis of the horizontally transferred pathogenicity related genes among the *Burkholderia* strains were carried out to recognize whether they were located in all the strains or indigenous to a specific strain.

Among different types of genes, the pathogenicity related horizontal gene transfer mechanisms in the studied strains were sorted out acquires genes. Using the Integrated Microbial Genomes database (www.img.jgi.doe.gov) the sorted pathogenicity related genes for each strain were subjected to IMG Genome BLAST against the studied strains to find out the sequence homologs. The minimum percent identity was set at 90% and the maximum E value 1e-2.

Correspondence analysis (COA) is a sensitive method identifying non-random usage of synonymous codons in many organisms. Correspondence analysis of codon count was performed with Codon W (http://bioweb2.pasteur.fr) (Peden, 1999). This method investigates the key trends in codon and amino acid disparity among the genes.

Results and discussion

Codon usage patterns in four strains of *Burkholderia mallei* genome:

It was proved that codon usage pattern is more advanced for the prokaryotes than for the eukaryotes; much of this information is based on the relatively few species that have been subjected to a concerted molecular genetic analysis. It is truth that codon usage among the Gram-negative proteobacteria is much more advanced than in any other group of species (Grantham *et al.*, 1980; Gouy and Gautier, 1982). Generally most bacterial genome with a balanced AT/GC genome content had high degree of heterogeneity in codon usage pattern. Codon heterogeneity was closely associated with gene expression level. Thus highly expressed genes contain a high frequency of codons i.e. translationally optimal (Ikemura, 1981; Lafay *et al.*, 2000; Ikemura, 1985). The studied strains of *Burkholderia mallei* genomes have moderately high G+C content, GC3 and Nc values that help to determine the existence of codon heterogeneity among them. The results from Nc versus GC3 plots, suggested the codon usage variations among genes of the same genome showed that the Nc values of the genes ranging from 21-61 for the four strains of *Burkholderia mallei* genomes suggesting the considerable heterogeneity is present in these GC-rich genomes. The RPGs, which were expected to be expressed at high levels during rapid cell growth are recognized and represented in Nc vs GC3 plot. The clustering of most RPGs in *Streptomyces* (Wu *et al.*, 2005) *Xanthomonas Frankia, Azotobacter* (Sen *et al.*, 2007; 2008) genome remain at low end of the Nc vs GC3 curve that signify stronger codon bias in the RPGs. The RPGs and PRGs, in all the studied bacteria formed similar clusters at the low end of the Nc versus GC3 plot that indicate stronger codon bias in RPGs and PRGs of *Burkholderia mallei*.

Codon usage is similar in closely related species but difference depends on phylogenetic distance (Peden, 1999). Genes having < 40 Nc value tend to have stronger codon bias manipulated by mutational pressure (Cameron and Aguade, 1998). Table 1 show that the mean Nc values of protein coding genes is 34 for all the studied strains with mean standard deviation value is around 6 where as Nc value of HTGs are high ranging from 38-40. The GC3 values for the four strains of *Burkholderia mallei* were quite high ranging from 84-85 in PCGs but the GC3 values of PRGs are higher than that. PCGs, PRGs and RPGs have higher Fop values in compared to HTGs. GC value of the studied genomes is very similar in the respective indices which are also shown in the Table 1.

Table-1: Mean Values of Nc, GC%, GC3%, Fop, CAI of the studied genes of *Burkholderia mallei* (Mean ± Standard deviation

Strain	Gene	Mean Nc	MeanGC (%)	MeanGC3 (%)	Mean Fop	Mean CAI
BMAL-44	PCG	34.661±6.22	67.918±0.048	84.5±0.09	0.576±0.05	0.600±0.14
	RPG	33.471±4.83	61.747±0.029	80.0±0.04	0.607±0.05	0.700±0.07
	PRG	34.713±5.25	68.702±0.046	86.2±0.07	0.584±0.04	0.612±0.09
	HTG	40.31±7.65	63.609±0.064	76.0±0.10	0.539±0.073	0.512±0.151
BMAL-29	PCG	34.104±5.64	68.368±0.049	85.3±0.08	0.579±0.057	0.612±0.12
	RPG	33.384±4.607	61.716±0.032	80.0±0.051	0.609±0.053	0.697±0.076
	PRG	33.106±4.351	68.789±0.035	87.8±0.054	0.587±0.043	0.645±0.091
	HTG	38.248±6.800	64.268±0.066	78.3±0.100	0.550±0.187	0.540±0.137
BMAL-47	PCG	34.419±5.96	68.352±0.049	84.6±0.090	0.576±0.059	0.600±0.136
	RPG	33.219±4.65	61.908±0.034	80.0±0.052	0.607±0.053	0.700±0.083
	PRG	33.249±4.14	68.969±0.036	87.7±0.057	0.587±0.044	0.639±0.089
	HTG	39.013±7.03	63.939±0.063	76.7±0.103	0.545±0.068	0.519±0.145
BMAL-VP1	PCG	34.297±5.822	68.238±0.050	84.7±0.089	0.577±0.058	0.617±0.129
	RPG	33.636±4.891	61.964±0.033	79.9±0.052	0.606±0.049	0.703±0.089
	PRG	33.164±3.916	68.677±0.033	87.4±0.055	0.586±0.039	0.652±0.068
	HTG	38.443±6.732	63.916±0.062	77.1±0.100	0.546±0.071	0.543±0.137

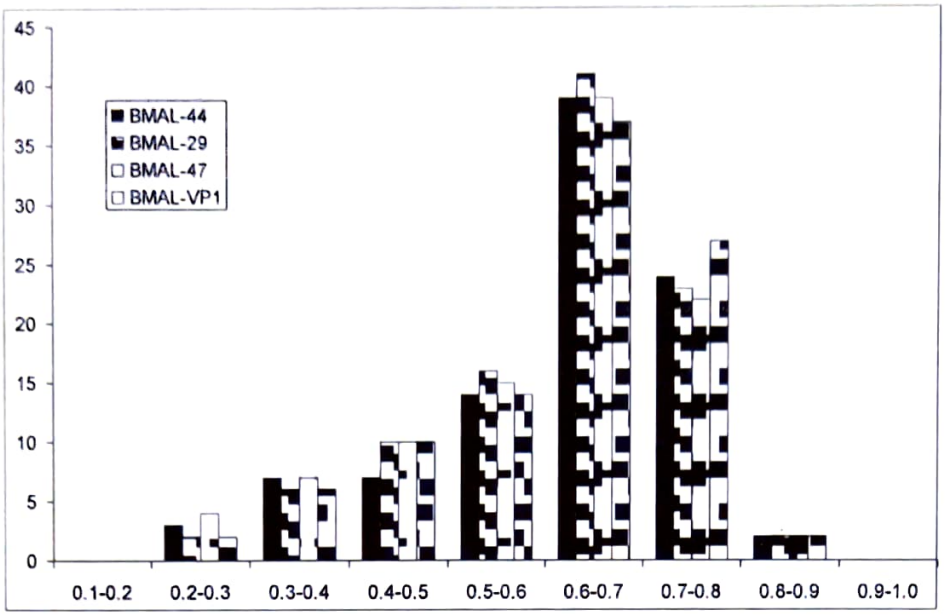

Figure 1: Frequency distribution of CAI values for all coding genes in the four *Burkholderia mallei* genome.

Analysis of horizontally transferred pathogenicity related genes:

Gene transfer is generally associated with transposon-like elements or insertion sequences (Groisman *et al.*, 1992; Groisman *et al.*, 1993; Simon, *et al.*, 1980). Horizontally gene transferred mechanism is not easy process and there are some transfer barriers that prevent the delivery of genetic information from a donor cell that block inheritance of newly acquired genes (Matic *et al*,. 1995). But genes acquired by horizontal transfer often have atypical G+C content, codon bias and repetitive elements (Medigue *et al.*, 1991) and only approach the characteristic codon usage and G+C content of their host after millions of years (Groisman *et al.*, 1993). The studied strains of *Burkholderia mallei* contained 391, 428, 513 and 468 HTGs for BMAL-44, BMAL-29, BMAL-47and BMAL-VP1 respectively. Amongst them, the number of PRGs was 47,6,12 and 10 for BMAL-44, BMAL-29, BMAL-47and BMAL-VP1 respectively.

IMG BLAST results revealed homologs having sequence identity with a number of similar horizontally transferred pathogenicity related gene coding proteins in the four studied strains of *Burkholderia mallei*. Among them, BMAL-44 was the most pathogenic strain containing virulence factors essential for pathogenicity. These are extra cellular capsule, *Salmonella typhimurium*-like type III secretion system and type VI secretion system (Schell *et al.*, 2008). It was observed that out of 47 horizontally transferred pathogenicity related genes in BMAL-44, 33,36 and 32 horizontally transferred homologs are identical (percent identity ranging from 90-100) in BMAL-29, BMAL-47and BMAL-VP1 respectively.

Correspondence Analysis:

Correspondence analysis (CA) is a multivariate method and, as such, its aim is to summarize data structures in high-dimension space by projection onto low-dimension subspaces, while loosing as little information as possible. The principal factors are therefore along the directions of maximum variability in the dataset. Correspondence Analysis (COA) of synonymous codon usage of all protein coding genes in *Burkholderia mallei* strains were performed on simple codon count. Correspondence analysis of PCGs, RPGs and PRGs revealed the position of first and second major axis showed the position of above-mentioned genes. In BMAL-44, the Protein Coding Genes scattered as two indistinguishable clouds where as the PRGs and PCGs clustered from -0.5 to +0.5 on X-axis and Y-axis respectively. In BMAL-29, the PCGs distributed on the both axes but PRGs were not overlapping on RPGs that means the RPGs are not highly expressed. In BMAL-47, the scattering of studied genes were almost like BMAL-44and in BMAL-VP1, all these genes formed a clumped horizontal line where PRGs and RPGs were overlapped with each other. However, correlation with first major axis and GC3, GC, Nc, CAI values revealed some interesting results. The position of the genes on the first major axis of variation showed strong positive correlation with GC3 ($r = 0.941$,$r = 0.922$; $p<0.001$) for BMAL-44 and BMAL-29 and a moderate positive correlation with GC3 ($r = 0.602$,$r =0.444$; $p<0.001$) for BMAL-47 and BMAL-VP1. The position of the genes on the first major axis of variation showed positive correlation with Nc ($r =0.795$,$r =0.705$, $r =0.447$; $p<0.001$) for BMAL-44, BMAL-29, BMAL-47 and negative correlation with Nc ($r = -0.335$; $p<0.001$) for BMAL-VP1.On the other hand, the genes of the first major axis showed strong negative correlation with CAI ($r = -0.951$,$r = -0.945$, $r = -0.617$; $p<0.001$) for BMAL-44, BMAL-29, BMAL-47 and positive correlation with CAI ($r = 0.413$; $p<0.001$) for BMAL-VP1. From these results, we can predict that the genes virulent strain has significant negative correlation with first major axis and CAI whereas avirulent has simple positive correlation.

Identification of predicted highly expressed genes:

The effective measures of synonymous codon usage bias is the codon adaptation index (CAI).Gene expression was measured by calculating CAI value in the four strains of *Burkholderia mallei*. Here, RPGs were taken as reference set of highly expressed genes (Sharp and Wen-Hsiung, 1987). The distribution of CAI

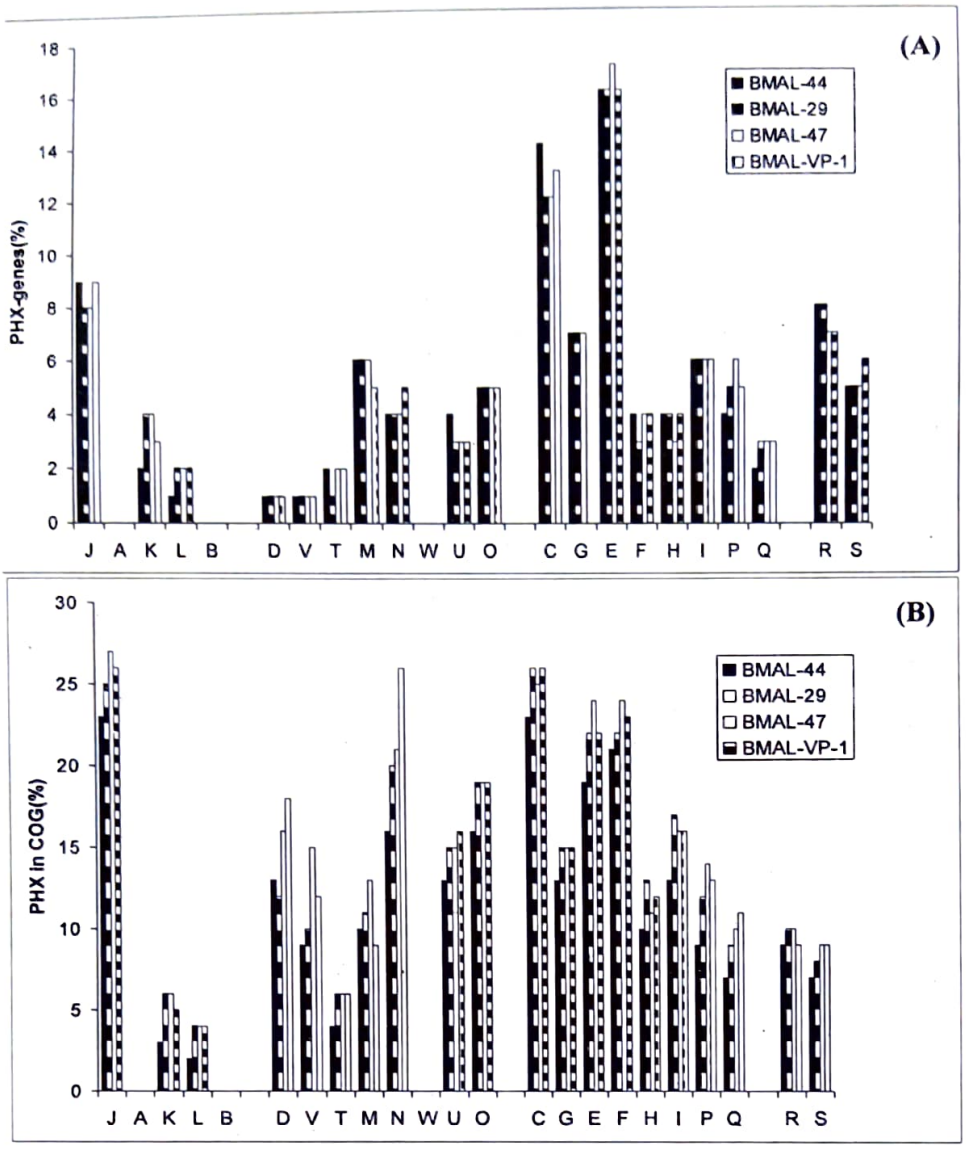

Figure 4 (A-B): Distribution of *Burkholderia mallei* predicted highly expressed genes within functional COG groups (as in text)

value of different genomes are shown in the Figure 1 .The CAI value ranges from 0.09-0.86, 0.12-0.86,0.12-0.86 and 0.11-0.87 for BMAL-44, BMAL-29, BMAL-47 and BMAL-VP1 respectively. The mean CAI value of PCG, PRG and HTG were from 60-61, 61-65 and 51-54 where the mean CAI value of highly expressed RPG was quiet high (Table1).

According to descending order of CAI value, the top 10% of total CAI value were classified as the predicted highly expressed genes (PHX), and corresponded to CAI cutoff values are 0.751, 0.74, 0.739 and 0.748 for BMAL -44, BMAL-29, BMAL-47and BMAL-VP1 respectively. BMAL-44 had 449 PHX genes, including 15 RPG, 22 PRG and 11 HTG. BMAL-29 had 554 PHX genes, with 16 RPG, 16 PRG and 19 HTG. BMAL-47 had 589 PHX genes, including 18 RPG, 24 PRG and 23 HTG. BMAL-VP1 had 521 PHX genes, including 17 RPG, 23 PRG and 20 HTG.

Functional analysis of the PHX genes:

Orthologs are evolutionary significant as they are evolved from common ancestor and opposed to paralog. Basically, orthologous proteins have the same structural domain and the same function. In order to realize the functional distribution of the PHX genes among the four *Burkholderia mallei* genomes the Cluster of Orthologous Groups (COG) of Proteins were studied. For these *Burkholderia mallei,* genomes, 23 COG categories were analyzed.Fig.-2 (A and B) showed the portion of the PHX into each COG category based on the total PHX genes and the title genes within the COG groups and expressed as percentage. To support the analysis, each of the COG categories were classified into 4 COG groups: *Information and storage processing* comprising to COG connected to J-Translation; A-RNA processing; K-Transcription; DNA replication ,recombination and repair; B-Chromatin structure and dynamics,(COG-1);*Cellular process and signaling* comprising of COGs related to D-Cell divisions-Defense mechanism-Signal transduction-Cell envelope biogenesis; N-Cell motility and secretion; W-Extra cellular structure; U-Intracellular trafficking; O-

Table-2: Pathogenecity related PHX genes of BMAL-44, BMAL-29, BMAL-47, BMALVP1 and their CAI value

Locus tag of PRG	Description	CAI value
	BMAL-44	
BMAA0440	hypothetical protein	0.793
BMA0504	unknown function	0.786
BMAA0455	hypothetical protein	0.786
BMAA0755	putative outer membrane nitrite reductase	0.785
BMA_IS407A-37	endoribonuclease, L-PSP family	0.776
BMAA1542	type III secretion system protein BsaQ	0.771
BMAA1633	HrpB2-like protein	0.769
BMAA1107	alcohol dehydrogenase, iron-containing	0.767
BMAA1457	putative lipoprotein	0.766
BMAA1534	type III secretion system protein BsaY	0.764
BMA0994	copper ABC transporter, periplasmic copper-binding protein, putative	0.763
BMA0995	nitrous-oxide reductase precurser	0.762
BMAA1786	alkyl hydroperoxide reductase, subunit c	0.761
BMAA1449	putative syringomycin synthesis regulator SyrP	0.761
BMAA1628	type III secretion inner membrane protein SctR	0.759
BMAA1812	putative sugar ABC transporter, periplasmic sugar-binding protein	0.758
BMA3346	unknown function	0.757
BMAA1531	BipB protein	0.755
BMA0991	protein disulfide isomerase NosL, putative	0.755
BMAA1610	putative type IV pilus biogenesis protein PilN	0.754
BMAA0405	hypothetical protein	0.754
BMAA1536	type III secretion system protein BsaW	0.751
	BMAL-29	
BMA10299_A3397	putative lipoprotein	0.773
BMA10299_A3203	Enoyl-[acyl-carrier-protein] reductase [NADH] (EC 1.3.1.9) (IMGterm)	0.744
BMA10299_A3014	putative outer membrane porin	0.766
BMA10299_A2984	ompA family protein	0.78
BMA10299_A2858	phospholipase C	0.77
BMA10299_A2852	radical SAM domain protein	0.785
BMA10299_A2805	acyl carrier protein	0.835
BMA10299_A2742	chaperonin, 60 kDa	0.821
BMA10299_A2542	endoribonuclease, L-PSP family	0.774
BMA10299_A2476	putative transcriptional regulator	0.748
BMA10299_A2336	thioesterase domain protein	0.758
BMA10299_A2129	putative threonine efflux protein	0.776
BMA10299_A2029	sodium/bile acid symporter family protein	0.763
BMA10299_A1467	putative ABC transporter, permease protein	0.765
BMA10299_A1465	putative syringomycin synthesis regulator SyrP	0.768
BMA10299_A0751	ketol-acid reductoisomerase (EC 1.1.1.86) (IMGterm)	0.844
	BMAL-47	
BMA10247_0398	ketol-acid reductoisomerase (EC 1.1.1.86) (IMGterm)	0.845
BMA10247_2943	ABC transporter, ATP-binding protein	0.802
BMA10247_2950	LysE family protein	0.8
BMA10247_3221	putative outer membrane porin	0.789
BMA10247_1750	radical SAM domain protein	0.786
BMA10247_A1658	putative outer membrane nitrite reductase	0.782
BMA10247_3517	putative lipoprotein	0.781
BMA10247_3387	putative threonine efflux protein	0.774
BMA10247_A0750	type III secretion system protein BsaY	0.772
BMA10247_A0742	type III secretion system protein BsaQ	0.769
BMA10247_2893	putative syringomycin synthesis regulator SyrP	0.768
BMA10247_A1276	alcohol dehydrogenase, iron-containing	0.767
BMA10247_2255	sodium/bile acid symporter family protein	0.764
BMA10247_2860	SCO1/SenC family protein	0.762
BMA10247_A2046	alkyl hydroperoxide reductase, subunit C	0.76
BMA10247_A0637	type III secretion inner membrane protein SctR	0.76
BMA10247_3050	aldehyde dehydrogenase family protein	0.759
BMA10247_A0753	BipB protein	0.754

Table-2: *Continued*

Locus tag of PRG	Description	CAI value
BMA10247_A0658	type IVB pilus formation outer membrane protein, R64 PilN family	0.754
BMA10247_A0748	type III secretion system protein BsaW	0.752
BMA10247_0916	arginine/ornithine antiporter	0.748
BMA10247_1384	Enoyl-[acyl-carrier-protein] reductase [NADH] (EC 1.3.1.9) (IMGterm)	0.746
BMA10247_A0371	GatB/Yqey family protein	0.743
BMA10247_A1232	putative sugar ABC transporter, periplasmic sugar-binding protein	0.741
	BMAL-VP1	
BMASAVP1_A1115	ketol-acid reductoisomerase (EC 1.1.1.86) (IMGterm)	0.849
BMASAVP1_A0789	thioredoxin-disulfide reductase	0.83
BMASAVP1_A0165	cytochrome c oxidase, subunit III	0.801
BMASAVP1_A3005	flagellar hook-associated protein 3	0.799
BMASAVP1_A3526	lipoprotein, putative	0.792
BMASAVP1_0593	putative outer membrane nitrite reductase	0.791
BMASAVP1_A1655	FimA	0.788
BMASAVP1_A2959	putative threonine efflux protein	0.782
BMASAVP1_A1654	fimbrial biogenesis outer membrane usher protein	0.778
BMASAVP1_A0708	endoribonuclease, L-PSP family	0.775
BMASAVP1_A0123	putative syringomycin synthesis regulator SyrP	0.775
BMASAVP1_0107	alcohol dehydrogenase, iron-containing	0.775
BMASAVP1_A3064	sodium/bile acid symporter family protein	0.772
BMASAVP1_A3320	aldehyde.dehydrogenase family protein	0.77
BMASAVP1_A2581	OmpA family protein	0.767
BMASAVP1_0779	alkyl hydroperoxide reductase, subunit C	0.765
BMASAVP1_A3080	aminotransferase (EC 2.6.1.-) (IMGterm)	0.763
BMASAVP1_A1549	butyryl-CoA:acetate CoA transferase (EC 2.8.3.8) (IMGterm)	0.762
BMASAVP1_A1622	TonB-dependent siderophore receptor	0.761
BMASAVP1_1518	GatB/Yqey family protein	0.758
BMASAVP1_0065	putative sugar ABC transporter, periplasmic sugar-binding protein	0.757
BMASAVP1_A2111	Enoyl-[acyl-carrier-protein] reductase [NADH] (EC 1.3.1.9) (IMGterm)	0.754
BMASAVP1_A1282	putative homoserine/threonine efflux protein	0.752

Post transnational modification, protein turnover and chaperons,(COG-2); *Metabolism* consisting of COGs related to C-Energy production and conversion-Carbohydrate transport and metabolism-Amino acid transport and metabolism's-Nucleotide transport and metabolism-Coenzyme transport and metabolism; I-Lipid transport and metabolism-Inorganic ion transport and metabolism-Secondary metabolites biosynthesis and transport, (COG-3);*General function prediction and unknown function* comprising of COGs related to R-General function prediction; S-Unknown function, (COG -3). Some pathogenicity related genes found in PHX category which are shown in the Table-2. There were 22, 16, 24 and 23 PR-PHX genes in BMAL-44, BMAL-29, BMAL-47and BMAL-VP1 respectively. Another study revealed that the most pathogenic strain of *Burkholderia mallei* i.e. BMAL-44 has 17 known PR-PHX genes, those are iron-containing alcohol dehydrogenase, alkyl hydroperoxide reductase, BipB protein, copper ABC transporter- periplasmic copper-binding protein, endoribonuclease, L-PSP family, HrpB2-like protein, nitrous-oxide reductase precursor, putative outer membrane nitrite reductase, putative lipoprotein, putative sugar ABC transporter, periplasmic sugar-binding protein, putative syringomycin synthesis regulator SyrP, protein disulfide isomerase NosL, putative, putative type IV pilus biogenesis protein PilN, type III secretion system protein BsaY,type III secretion system protein BsaQ,type III secretion inner membrane protein SctR and type III secretion system protein BsaW with mean CAI-value is 0.76±0.008 that is quiet high.but the CAI-value of rest three strains are 0.70±0.108, 0.70±0.103 and 0.68±0.122 for BMAL-29, BMAL-47and BMAL-VP1 respectively. It indicates that in BMAL-44, the virulence gene expression level was comparatively high.

Conclusion:

Moderate codon bias has been observed in the *Burkholderia* strains. Codon heterogeneity is associated with the gene expression levels. A number of pathogenesis related genes in the studied *Burkholderia* strains were horizontally transferred and had somewhat different pattern of codon usage compared to other genes. Correspondence analysis revealed variability among different sets of genes. A number of pathogenicity related genes directly associated with the virulence and toxicity in *Burkholderia* belonged to the PHX category. COGs associated with metabolism especially those linked to carbohydrate metabolism and amino acid transport and metabolism had the lion's share of PHX genes implying their significance in influencing the lifestyle of the *Burkholderia* strains as pathogens.

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Solubilization of rock phosphate by *Azotobacter spp*

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Abstract

To find out a potent phosphate solubilizing strain of diazotrophs, fifteen *Azotobacter* strains were isolated from local sources. They have been tested for the ability to solubilize rock phosphate *in vitro* and production of acid in the medium. Almost all the strains solubilized rock phosphate which ranged up to 45.5%. *Azotobacter* sp. R12 was found to be a potent one, which solubilized 30 to 46% phosphate from different rock phosphates. Almost maximum solubilization of P and acid production was achieved at four days ofgrowth of the organism. The strain produced ascorbic acid in the culture medium, the primary cause of rock phosphate solubilization. Addition of $(NH_4)_2SO_4$ in the culture medium reduced the solubilization of phosphate with increasing concentration beyond 0.25mg /ml for Jordon rock phosphate. Increasing concentration of $CaCl_2$ and $CaCO_3$ also reduced the P solubilization from Jordon rock phosphate by the strain. Kinetics of solubilization and acid production showed a linear relationship till the 5th day of incubation, then P level becomes static. Adding EDTA at 5mg/ml as chelating agent of calcium ions to 5 days old culture increased the P solubilization to 81%. Increasing EDTA concentration in 3 days old culture showed linear relationship between concentration of EDTA and P solubilization.

Keywords: *Azotobacter*, Rock Phosphate, Chelation, calcium activity

Phosphorus is one of the most essential plant nutrients. It plays a vital role in energy transfer and regulation. It is also an important constituent of macromolecules such as phosphor-lipids and nucleic acids. Most of the essential plant nutrients, including phosphorus, remain insoluble in soil. Crop plants get these nutrients from nature and from applied chemical fertilizer. A large portion of inorganic phosphate applied to soil as fertilizer is rapidly immobilized soon after application and become unavailable to the plants (Dey 1988). Applying phosphate solubilizing soil microbes to soil as biofertilizer may alleviate this problem by solubilizing these immobilized products. Several soil bacteria (Darmwal *et al.* 1989, Gaur *et al.* 1987, Loganathan *et al.* 2004, Rashid *et al.* 2004, Pandey *et al.* 2006), Cyanobacteria (Roychaudhury *et al.* 1989) and fungi (Agnihotri 1970, Chhonkar *et al.* 1967) have the property of solubilizing different inorganic and rock phosphates. So phosphatic rock may be used as a cheap source of phosphate fertilizer for crop production. In different states of India, there are about 40 million tons of phosphatic rocks deposited (Roychaudhury *et al.* 1989). We have screened 15 strains of *Azotobacter*, from natural sources of Burdwan district, which is an important part of cereal producing area in India. These bacteria fix atmospheric nitrogen and may be used as biofertilizers (Vincent 1970). Our primary object of this investigation was to assess the ability of isolated strains of *Azotobacter* to solubilize rock phosphate. We also studied the effects of several other factors on the extent of rock phosphate solubilization by these strains.

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Materials and Methods

Fifteen strains (Table 1) were isolated from roots of fully matured rice plants of alluvial soil from Burdwan district and screened to select potent phosphate solubilizers.The strains were maintained by routine transfer on Burk's N-free medium supplemented with 0.025% yeast extract.

The composition of the Burk's medium used to study rock phosphate solubilization by the strains of *Azotobacter* sp was as follows: D-glucose 20g;mgSO_4 $7H_2O$ 0.2g; $CaCl_2$ 0.09g; Fe-Mo Solution ($FeSO_4$ $7H_2O$ 50mg; $NaMoO_4$ $2H_2O$, 2.52mg); water to 1000ml; powdered phosphate rock (~240 mesh Size) at 2g per litre was added as a source of insoluble phosphate. The pH of the media was adjusted to 7.00 before autoclaving. The flasks were inoculated with cells of a stationary phase culture at 4% and incubated for 3 days on a rotary shaker at 28°C.

Contents of the culture flasks at the end of incubation period were centrifuged at 15000 rpm for 15 minutes to remove biomass and insoluble matter. The supernatant was taken as a test material. Quantitative estimation of soluble phosphate was done following the method of Chen et al.(1956). The reagent for phosphate estimation was prepared fresh before each experiment. One volume of 6(N) H_2SO_4, two volume of double distilled water and one volume of ammonium molybdate (1%) were taken together to which one volume of 10% ascorbic acid was added and mixed thoroughly 4ml of the reagent was added into each tube containing an aliquot of test material and the final volume of the reaction mixture was adjusted to 8ml and (incubated at 37° C for 1 hour). Absorbance was noted at 829 nm against a

Table 1: Strains of *Azotobacter* spp. isolated and used

Strains	Host	Locality*	Marker character
R3	*O sativa*	Kalna2,	Tet^r
R4	*O sativa*	Memari1	Amp^r, $Chlo^r$
R6	*O sativa*	Kalna 2	Amp^r
R7	*O sativa*	Kalna 1	Amp^r
R10	*O sativa*	Kalna 2	$Chlo^r$
R11	*O sativa*	Kalna 2	Amp^s,$Chlo^s$,Tet^s
R12	*O sativa*	Memari2	Tet^r
T1	*S indicum*	Kalna 2	Tet^r
T2	*S indicum*	Kalna 1	Tet^r
R20	*O sativa*	Memari 2	Amp^r
R21	*O sativa*	Monteshwar	Amp^r
R23	*O sativa*	Memari2	$Chlo^r$
R27	*O sativa*	Memari1	Amp^s,$Chlo^s$,Tet^s
R33	*O sativa*	Memari2	Amp^s,$Chlo^s$,Tet^s
R34	*O sativa*	Monteshwar	Amp^r,$Chlo^r$,Tet^r

*Locations are in Bardwan District, West Bengal India

corresponding blank in a spectrophotometer and at 619 nm in a visible spectrophotometer. The amount of solubilized phosphate was determined from a standard curve prepared by using K_2HPO_4 as standard and expressed as equivalent P.

The phosphate rocks are used as source of high grade and low grade phosphatic materials. Two phosphate rocks were collected from Phosphate Company, Rishra, Hooghly, P1 (off white Jordon Phosphate rock) and P2 (pale brown Udaypur Phosphate rock). P3 (yellowish gray Purulia Phosphate rock) was obtained from Sriniketan Agricuture farm, Sriniketan, Visva Bharati and P4 (black Mussoric phosphate rock) was collected from Das enterprise, Prof Lakshmi Narayan Das, Sure Kalna, Burdwan. The composition of those four phosphate rocks, as determined by the method of Shapiro and Brannock (1962), are given as Jordon phosphate rocks (JPR) P1 which contained 34.00% P_2O_5, 41.64% CaO, Udaypur phosphate rocks (UPR) P2 containing 31.00% P_2O_5, 46.82% CaO, Purulia phosphate rocks (PPR) P3 containing 32.7% P_2O_5, 51.20% CaO, were source of high grade phosphate. On the other hand Mussorie phosphate rocks (MPR) P4 contained 17.00% P_2O_5, 48.90% CaO and were source of low grade phosphate . In the media containing either P1, P2, P3 or P4 phosphate rock at 0.2%, the total phosphorus equivalents added were 362.5, 301, 310 or 189mg/ml respectively, if solubilized completely.

The pH of the spent culture was measured by Systronic Digital pH meter 802.The organic acids were extracted after passing the spent culture broth through Dowex 50WX8-400 column (H^+ form). The eluent was concentrated by lyophilization. The acids were identified by Thin Layer Chromatography on silica gel plates and in comparison with authentic samples. The chromatograms were developed in a solvent system of butanol: acetic acid: water (2:1:1). Spots were identified by spraying with glucose-aniline and heating at 125°C for 5 min or by spraying with aniline phthalate.

Results and Discussion

The ability to solubilize rock phosphate by a number of

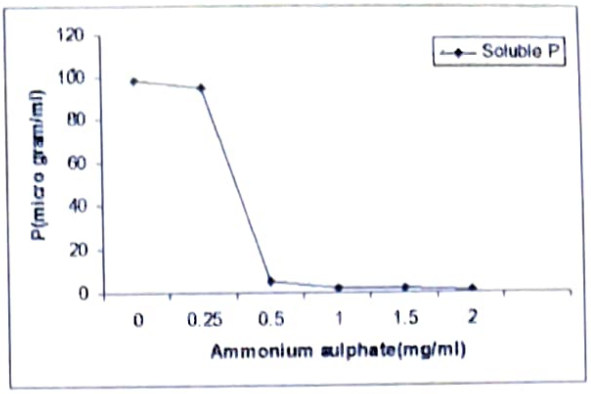

Fig.1: Effect of ammonium salt on solubilization of Jordon rock phosphate by the culture of *Azotobacter* sp. R 12. Ammonium sulfate in increasing level was added to Burk's medium. Release of phosphate, expressed as equivalent phosphorus, was measured after 4 days

Azotobacter strains (Table 1) isolated from local sources was evaluated. The strains were found to be Gram negative, small rods having cysts in their cells when stained with acrydine orange, produce smooth colonies, were indole and catalase positive and starch non utilizers.

After 6 days incubation, the end pH of the culture and the amount of phosphate solubilization from phosphatic rock by the strains in N_2 free Burks's medium are presented (Table 2). The pH of the culture media after autoclaving was near the pH 7.00 and level of soluble phosphate was 0.03mg/ml. After incubation the end pH was measured to find out whether solubilization of P was accompanied by the production of acid.

In Table 2 the data show that the solubilization of rock phosphate by strains of *Azotobacter spp.* ranged from 4.88% (R 21) to 29.89% (R 12) from P1, 6% (R 34) to 31.10% (R 12) from P 2, 3.75%(R 6) to 32.3% (R 12) from P 3 & 4.67%(R 21) to 45.5% (R 12) from P 4. The strain R 12 solubilized more than 32, 31, 32 and 45% of P from P 1, P 2, P 3 and P 4 respectively. The Strain (R 12) was found to be highest P solubilizer in all rock phosphates among the tested strains of *Azotobacter*. The pH of the media lowered by the production of acid, after incubation pH of the medium ranged from 6.00 (R 10) to 4.01 (R 12) in P1 , 5.88(R 10) to 3.92 (R 11) in P2 , 5.30 (R 4) to 4.32 (R 34) in P 3 , 6.1 (R 4) to 4.1 (R 12) in P4.

It is evident from the results (Table 2) that the strain R 12 of *Azotobacter sp. was* the highest P solubilizer of all the strains studied. Jordon phosphate rock (P1) proved to be a good source of P, so further investigation was carried out to characterize the process of P solubilization form only P1 with this strain. Bacteria belonging to the genera *Rhizobium*, *Bradyrihizoum*, *Bacillus*, *Enterobacter*, and *Pseudomonas* are known to bring about dissolution of insoluble phosphatic compound (Rashid *et al.* 2004, Roychaudhury *et al.* 1989, Halder *et al.* 1990, Rojas *et al.* 2001) along with production of organic acids. The degree of solubilization of P from the rock phosphates by the strains of *Azotobacter is* very comparable to that by other bacteria reported. All the tested organisms produced acid and lowered the medium

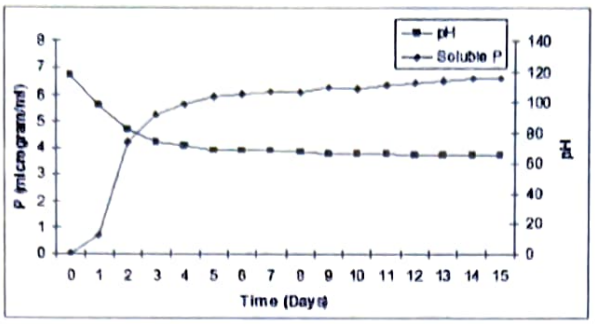

Fig.2: Kinetics of solubilization of phosphate and acid production by *Azotobacter* sp. R 12. The strain was cultured in Burk's medium containing 0.2% Jordon phosphate rock. Release of phosphate expressed as equivalent phosphorus, was measured at different time intervals.

pH and it showed that the acids produced by organisms were responsible for the P solubilization.

It has been considered that effective P solubilizing microorganisms decreased the pH during their growth (Agnihotri 1970, Halder *et al.* 1990, Seshadri *et al.* 2000, Arora *et al.* 1979). Characteristic of P solubilization is not dependent on the taxonomic grouping.

To study the effect of ammonium salt on solubilization of rock phosphate by the strain of *Azotobacter* sp., ammonium sulphate as ammonium salt was added in increasing concentrations to the composition of Burk's medium with powdered P1 phosphate rock at 2gm / litre. The results of phosphate solubilization from the phosphatic rock and end pH of the culture after 4 days incubation are presented (Figure 1). The strain released comparable amount P from P 1 in the culture adding 0.25mg /ml with that of zero $(NH_4)_2SO_4$. The results show that (Fig. 1) in *Azotobacter* the presence or absence of NH_4SO_4 in medium has no effect on P solubilization in lower dose. But higher dose of $(NH_4)_2SO_4$ beyond 0.25mg/ml in culture inhibits the phosphate solubilization.

Kinetics of solubilization of phosphate and acid production by the strain R 12, are presented (Figure 2). The figure shows the kinetics of solubilization of P and corresponding values of culture pH in their respective medium at different time intervals for 15 days. It appeared from the results that as the mean pH decreased, the level of solubilized P increased linearly till the 4th day of incubation and the pH was near 4, after reaching in highest level of P solubilization , it became static and further incubation did not improved the extent of solubilization drastically. Complete solubilization of the rock phosphate was never achieved. In term of percentage, about 32% of solubilization of P was achieved after 15 days of incubation. Further lowering of pH beyond 4th day was probably due to the accumulation of soluble P in the form of phosphoric acid (H_3PO_4).The organic acid produced by R12 turned out to be ascorbic acid as compared with the authentic sample.

Adding increasing amounts of calcium as $CaCO_3$ to the culture of strain R 12, in medium change of phosphate was recorded. The data (Figure 3) indicated that after 4 days incubation the pH of the cultures became higher as compared to control. The extent of P solubilization was reduced gradually with an increasing concentration of $CaCO_3$. The dissolution of P was reduced to zero at the concentration of 2mg/ml of $CaCO_3$. It also indicated that increasing amounts of calcium as $CaCl_2$ to the culture of strain R 12 in medium made little difference in the culture pH after 4 days of growth from that of the control culture with no $CaCl_2$ added, the extent of P solubilization reduced significantly with addition of

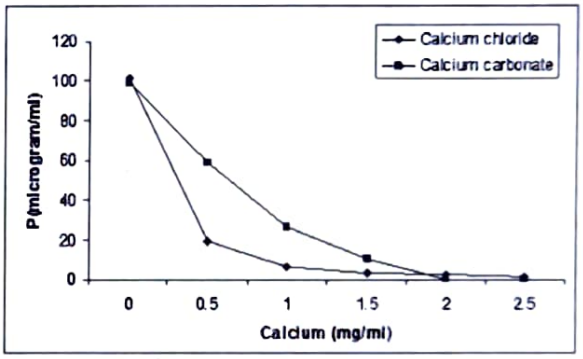

Fig.3: Effect of calcium supplement on phosphate solubilization from Jordon phosphate rock by culture of *Azotobacter* sp. R 12. Calcium in increasing levels was added to the Burks medium. Release of phosphate expressed as equivalent phosphorus, was measured after 4 days

Table 2: Solubilization of rock phosphate by the strains of *Azotobacter spp* on N-Free Burks medium after 6 days and end pH of cultures

Strains	P1		P2		P3		P4	
	%P*	pH	%P	pH	%P	pH	%P	pH
R3	27.7	4.8	26.66	4.82	20.47	5.12	23.2	5.7
R4	28.04	4.6	19.62	5.77	17.81	5.3	21.05	6.1
R6	5	5.7	8.88	5.6	3.75	4.62	7.1	5.8
R7	23.31	5	28.51	5.07	16.32	4.96	25	5.1
R10	7.43	6	17.66	5.88	11.89	5.07	16.42	6.24
R11	20.6	4.2	18.51	3.92	17.44	4.1	19.07	5.7
R12	29.89	4.0	31.1	4.8	32.3	4.77	45.5	4.1
T1	20.71	4.8	28.51	5	23.05	4.85	23.68	5.36
T2	28.37	4.68	27.77	4.72	20.44	4.75	32.89	5.77
R21	4.88	5.1	7.32	4.77	9.32	5.22	4.67	5.46
R23	14.74	4.8	12.8	4.91	10.52	4.76	15.21	4.86
R27	12.26	4.8	8.31	5.02	7.28	5.16	11.53	4.91
R33	6.5	5.0	9.11	4.98	8.02	5.06	6.32	5.12
R34	5.86	5.0	6.45	4.79	9.9	4.32	7.02	5.04
R20	13.12	4.9	10.66	4.84	5.36	4.86	9.72	5

*%age of soluble P

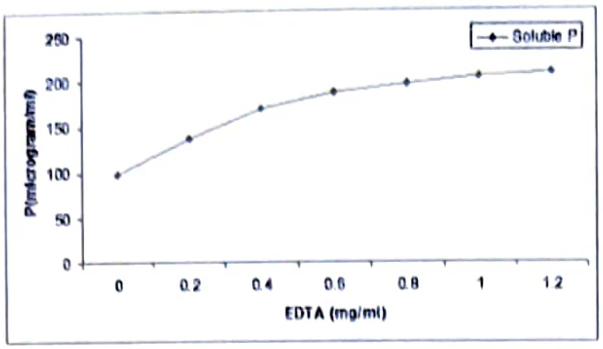

Fig.4:Effect of adding EDTA on phosphate solubilization from Jordon phosphate rock in culture of *Azotobacter* sp. R12. The strain was allowed to grow in the presence of . rock phosphate for 4 days in Burk's medium when neutralized EDTA was added to the culture in increasing concentration. Soluble phosphate, expressed as equivalent phosphorus, was measured in the culture filtrate after incubation of 24 h

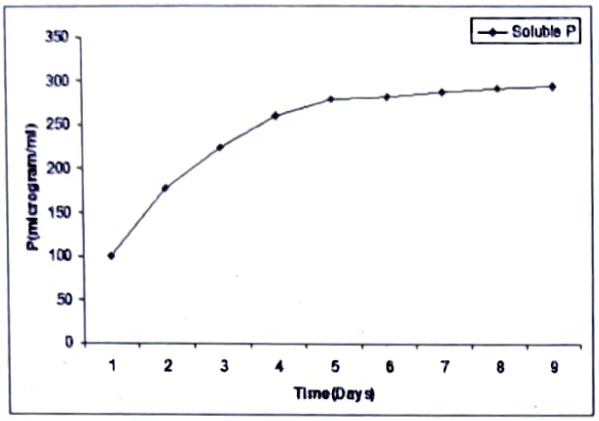

Fig.5: Kinetics of solubilization of phosphate by *Azotobacter* sp. R12 upon addition of EDTA to a 5 days old culture. The strain was allowed to grow in the Burk's medium containing 0.2% Jordon phosphate rock for 5 days when nutralised EDTA was added to the cultures to a final concentration of 5mg perml. Release of soluble phosphate, expressed as equivalent phosphorus, was then measured at time intervals

$CaCl_2$ to their medium. The role of calcium activity in the dissolution of P from phosphate rocks is important. Studies of Wilson & Ellis (1984) involving soil solution suggested the calcium activity as an important factor controlling the rate and extent of dissolution of rock phosphate.

Adding di-sodium salt of ethylenediamine tetra acetic acid (EDTA) neutralized to pH 7.0 to 4 days old cultures of R 12 and further incubation of the culture for another 24 h showed that increasing concentration of EDTA increased the solubilization of P from rock phosphate (Figure 4). The extent of P solubilization increased up to 55%. It is apparent that probably calcium which inhibits P solubilization as observed in Figure 3 are chelated by EDTA when applied to the medium[13]. Adding neutralized EDTA to a final concentration of 5mg/ml to a 5 day old culture gradually increased the solubilized P upon incubation for the next 5 days. The P reached a plateau when more than 81% of the P from P I was released (Figure 5).

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Genetic relatedness between some saprophytic and parasitic macrofungi of Darjeeling Hills

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Abstract
Eight dominant saprophytic and parasitic macro fungi collected from Darjeeling hills [N 26°31'27.13' - E 87-59'-88.53'] of North Bengal region were studied using internal transcribe spacer (ITS) and RAPD PCR. rDNA region of saprophytic and parasitic macro fungi with ITS1 and ITS4 primers produced range between 400-800bp products. The genetic relatedness among these macro fungi were analyzed with four random primers. RAPD profiles showed genetic diversity among the isolates with the formation of two clusters. Analysis of dendrogram revealed that similarity coefficient ranged from 0.34 – 0.86.

Keywords: Saprophytic , parasitic macro fungi , rDNA, RAPD

The fungi are an immensely diverse group of organisms, encompassing a huge range of forms from microscopic single celled yeasts to large macrofungi, as exemplified by the well-known mushrooms and toadstools and the largest of fruitbodies, the giant puffball. An estimates for the number of fungal species as reported by Hawksworth et al., 1995 revealed that 72,065 species spread across 11 phyla in 7745 genera . New fungal species continue to be described, and a year 2009 estimate based on the description rate in The Index of Fungi would be of the order of 80–1,00, 000 species of fungi. The great majority of the 80 000+ fungal species so far named and described are likely to occur in the soil environment at some stage in their life-cycle. Fungi therefore have many different functions in soils, which include both active roles, such as the degradation of dead plant material and inactive roles where propagules are present in the soil as resting states. The development of molecular techniques has provided a new range of tools that can provide clear insights into specific interactions and activities in soil environments. The combination of broad spectrum polymerase chain reaction (PCR) detection, coupled with RAPD and single strand conformation polymorphisms (SSCP) or denaturing gradient gel electrophoresis (DGGE), can give more accurate answers to fundamental questions on ecosystem diversity. Macrofungi are defined here to include ascomycetes and basidiomycetes with large, easily observed spore-bearing structures that form above or below ground. Although macrofungi have perhaps the longest history of diversity studies of any group of fungi, they are nevertheless understudied over most of the world. More data are available from North America and Europe than from any other region, but knowledge of macrofungal diversity is incomplete even for these regions. Macrofungi are distinguished by having spore-bearing structures visible to the naked eye (mushrooms, brackets, puffballs, false-truffles, cup fungi, etc.). Most macrofungi are Ascomycota or Basidiomycota, but a few are Zygomycota.

The peak mushrooms and macrofungi season for each region is differ from each ecological climate. Each year is a little different; the season may be early or late depending on rainfall and temperature (Arora, 1991). Most terrestrial macrofungi are saprobes or mycorrhizal symbionts, but some are pathogens of plants or fungi. Fungi fruiting on woody substrata are usually either saprobes or plant pathogens.

In the present investigation two types of macrofungi, (a) saprophyte and (b) parasitic on insects were considered for their molecular characterization through RAPD. There is no organic matter which is not attacked and destroyed by fungi and bacteria. Everything that goes to form organic substances comes from nature and reenters the natural "economic" cycle because of the action of microorganisms. The breakdown of organic substances is achieved by fungi in the mycelial stage, i.e. as mold, even though, in many cases, this mold will produce a fruit-body. .

In natural conditions the breakdown of given types of matter is carried out exclusively by specific species of saprophytic fungi. (Pacioni, 1981). There are numerous associations between insects and fungi. Of particular importance among them, because of their possi-ble practical implications, are those which are established with the *entomopathogenic* fungi, i.e. fungi which are parasitic on insects: these fungi could well offer mankind an alternative weapon in the war against insects. In temperate climates members of the genus *Cordyceps* are relatively common; these usually have thread- or club-shaped carpophores, often brightly col-ored. The commonest species, *Cordyceps militaris* forms orange-red clubs on members of Lepidoptera and Co-leoptera. *Cordyceps sinensis*, parasitic on Lepidoptera, has been used in China for medicinal purposes since earliest times. (Pacioni, 1981). Four dominant saprophytic fungi and four macrofungi parasitic on insects collected form Darjeeling hills were selected for analysis of their genetic relatedness.

Materials and methods :

Fungal strain and culture

Macrofungi were collected from the forest area of Darjeeling. The specimens were collected,

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Fig 1: A. *Clavariadelphus pistillaris* (DG 26 MF 4), B. *Russula ochroleuca (22-6-006MF 5)* C. *Inocybe fastigiata* (22-06-007 MF 6) D. *R. cyanoxantha* (GL-201MF 3) E. *Cordyceps sp.* (IS-001 MF1) F. *Cordyceps sp.* (IS-004 MF 2) G. *Cordyceps sp.* (IS-007 MF 10) H. *Cordyceps sp.* (IS-009 MF 11)

photograpghs were taken and kept in plastic bags. The locations of collection area of macrofungi were recorded by GPS tools (Garmin). These fungi grow on top of dead and decomposed leaves and logs. Specimens at different stages of development were collected by digging (not pulling) them up so as not to damage their bases. For those attached on dead logs and woods, efforts were made to scrape a piece of the wood or bark on which the specimen was attached. A paper slip indicating the place where it is collected and placed in each plastic bag. Upon returning from the collecting trip, the materials were examined and morphological characteristics were recorded. The specimens were measured, noted down for the shape, size, color and other characteristics. The information on characters affected by drying was also noted down. Each specimen was identified following comparison with the literatures. Fungal tissue cultures from each of eight selected fungi from the somatic tissue of saprophytes (IS-001MF1, IS-004MF2, IS-007 MF10, IS-009MF11) and macrofungi parsitic on insects GL-201MF3, DG 26 MF 4, 22-6-006MF 5, 22-06-007 MF6) were prepared aseptically and culture stored in 4°C for future work.

Genomic DNA Extraction

Isolation of genomic DNA from each of macrofungi was done by growing the fungi for 3-4 days in malt dextrose agar. The mycelia were incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH8.0), 100 mM NaCl and 2% SDS, for 1 hr at 60°C followed by centrifugation at 12,000 rpm for 15 min. The supernatant was then extracted with equal volume of water saturated phenol and further centrifuged at 12,000 rpm for 10 min; the aqueous phase was further extracted with equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) and centrifuge at 12,000 rpm for 15 min; the aqueous phase was then transferred in a fresh tube and the DNA was precipitated with chilled ehanol (100%). DNA was pelleted by centrifuging at 12000 rpm for 15 min and washed in 70% ethanol by centrifugation. The pellets were air dried and suspended in TE buffer (pH 8.0).

Qualitative and quantitative estimation of DNA

The extraction of total genomic DNA from the isolates as per the above procedure was followed by RNAase treatment. Genomic DNA was re suspended in 100 µl 1 X TE buffer and incubated at 37°C for 30 min with RNAse (60µg). After incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol as described earlier. The quality and quantity of DNA was analyzed both spectrophotometrically and in 0.8% agarose gel. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

PCR amplification of ITS region

All isolates of Macro fungi were taken up for ITS-PCR amplification. Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94°C for 5 min. followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 59 °C for 30 sec and extension at 70 °C for 2 min 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 ml) containing 0.25% bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis.

RAPD of Macro fungi isolates

For RAPD, random primers i.e. BAS 359, OPA-1; OPD-6; OPA-4; were selected . PCR was programmed with an initial denaturing at 94°C for 4 min. followed by 35cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min 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 ml) containing 0.25 % bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination by horizontal electrophoresis.

Scoring and data analysis

The image of the gel electrophoresis was documented through Kodac gel documentation system and NTSYSPc analysis software. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed *In Silico* into similarity matrix using NTSYSpc (Numerical Taxonomy System

Table 1: The nucleotide sequence used for ITS and RAPD PCR

Primer	Primer Seq 5'-3'	Mer	TM	% GC
	ITS-Primers pairs			
ITS 1	TCTGTAGGTGAACCTGCGG	19	63.9	57%
ITS4	TCCTCCGCTTATTGATATGC	20	61.5	45%
	RAPD primers			
BAS 359	AGGCAGACCT	10	31.0	60%
OPA-4	AATCGGGCTG	10	39.3	60%
OPD6	GGGGTCTTGA	10	32.8	83%
OPA1	CAGGCCCTTC	10	38.2	70%

Table 2: Analysis of the polymorphism obtained with RAPD markers in macro fungi isolates

Seq Name	Total no RAPD bands	Band size (bp). (approx.)		Poly-morphic bands	Poly-morphic (%)
		Min	Max.		
BAS359	07	100	2000	07	100
OPA-4	06	100	1000	06	100
OPD-6	05	100	1000	05	100
OPA1	04	100	1000	04	100
Total	22			04	100

Biostastiscs, version 2.11W) The SIMQUAL program was used to calculate the Jaccard's coefficients. The RAPD patterns of each isolate was evaluated, assigning character state "1" to indicate the presence of band in the gel and "0" for its absence in the gel. Thus a data matrix was created which was used to calculate the Jaccard similarity coefficient for each pair wise comparison. Jaccard coefficients were clustered to generate dendograms using the SHAN clustering programme, selecting the unweighted pair-group methods with arithmetic average (UPGMA) algorithm in NTSYSpc (Rohlf, 1993.).

Result and Discussions:

Morphological characteristic of macrofungi.

The detail descriptions of four sprophytes are as follows:

Clavariadelphus pistillaris (DG 26 MF 4)

GIS Location : N26°50'.181''E88°13'.876'']

Source : Forest floor

Description : Carpophore 8-25 cm high with a maximum d ameter of 2.5 cm, club-shaped, slender or stout, surface smooth then sulcate lengthwise, light yellow tending to grayish brown. Flesh white, bruising brownish (Fig:1 A)

Russula ochroleuca (22-6-006MF 5)

GIS Location : N26°50'.323''E88°13'.431''

Source : Forest floor and trees of *Castronopsis* of eastern himalaya

Description : Carpophore 4-11 cm, yellow but variously tinged, pale lemon-yellow, yellowish ochre spotted with orange or brownish at center, late in season often light olive-yellow, gray at center, convex-umbilicate then flat, slightly depressed, cuticle half detachable, moist and shiny, margin thick, curved, sometimes lobate. Gills pale cream-colored or faintly pale yellow with a few small brownish markings with age, averagely crowded, unequal, intervenose, ventricose, slightly obtuse frontwards. Stipe 3-7 x 1.5-2.5 em, white, slightly grayish, spotted with brownish yellow from base upward, cylin-drical, sometimes club-shaped, flared beneath gills, full, soft, slightly pithy at top, Flesh white, grayish at top of stipe, thick, soft then tough. Odor pleasant, flavor varying from piquant to sweet. Spores white, ovoid, aculeate, 7-9 X 6.3-9 microns, amyloid. (Fig :1 B)

Inocybe fastigiata (22-06-007 MF 6)

GIS Location : N26°50'.276''E88°13'.765''

Source : Forest floor of *Picea* trees of eastern himalaya

Carpophore 2-7 cm, pales straw, conical then raised at the edge; tinged with olive, then brownish, crowded, narrow, with lighter edges. Stipe 3-8X0.4-1 cm whitish, cylindrical at the base, tapering, fibrillose. Flesh whitish, fibrous in the stipe. Spermatic odor, no flavor. Spores brownish, elliptical, smooth, 7-10x4-5 microns. (Fig:1 C)

Russula cyanoxantha (GL-201MF 3)

GIS Location : N26°50'.921''E88°13'.434''

Source : Forest floor

Description : Carpophore 5-15 cm, blackish-violet, pale purple at edge and conspicuous green at disc, varying to slate-gray with lighter areas, or bluish violet or even a uniform green when mature, rounded then convex, flat, fairly depressed, cuticle two-thirds detachable, thin, viscous in damp weather, shiny, with radial fibrils and grooves, margin curved inward then ob-tuse, sometimes striate when mature. Gills white tinged bluish green, fairly crowded, unequal, forked, intervenose, ventri-cose. Stipe 5-10 X 1.5-4 cm, white, sometimes tinged lilac or reddish, with brownish markings, sturdy, even, narrowing and rooting at base, fleshy, soft then spongy, pruinous, slightly ru-gose. Flesh white, sometimes grayish when mature, thick, soft, moist. Odor pleasant, flavor first sweet then unpleasant. Spores white, elliptical, with small isolated warts, 7-10 X 6-7.5 microns, amyloid. (Fig :1 D)

The detail descriptions of four macrofungi parasitic on insects are as follows:-

***Cordyceps sp.* (IS-001 MF1)**

GIS Location : N26°50'.621''E88°13'.575''

Source : Insect larva from forest floor

Description : concentrically zoned with contrasting shades of yellow, red, brown, zones alternately velvety and smooth. Tubes: white to pale yellow; pores 3-5 per mm, angular. Stalk: absent. (Fig:1 E)

***Cordyceps sp.* (IS-004 MF 2)**

GIS Location : N26°50'.633''E88°13'.555''

Source : Insect larva from forest floor

Description : Whitish, , smooth; base white-hairy., edges fringed. Stalk: very short, compressed, hairy. elliptical, smooth, odourless. (Fig:1 F)

***Cordyceps sp.* (IS-007 MF 10)**

GIS Location : N26°50'.311''E88°13'.785''

Source : Wasp from forest floor

Description : Concentric bands of color ; tough and leathery, becomimg brittle and hard when dry; velvety with different tomentum from one color zone to another, reflecting different growth conditions. Tubes up to 3 mm deep; cinnamon to rusty brown. (Fig:1 G)

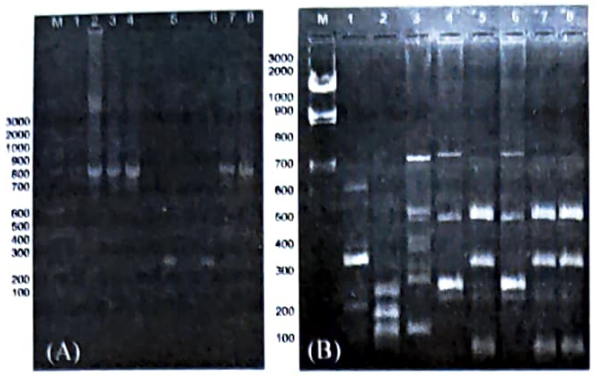

Fig 2 (A) 1% Gel electrophoresis of PCR of ITS region and (B) RAPD PCR with primer BAS359. Lane M : Low range DNA marker, Lane 1: *Russula cyanoxantha* (GL-201MF 3), 2: *Clavaridelphus pistillaris* (DG 26 MF 4), 3: *Russula ochroleuca* 22-6-006MF5, 4: *Inocybe fastigiata* (22-06-007 MF 6), 5:*Cordyceps sp.* (IS-001MF1), 6: *Cordyceps sp.* (IS-004MF2), 7: *Cordyceps sp.* (IS-007 MF 10), 8:*Cordyceps sp.* (IS-009MF 11) in both the photograph

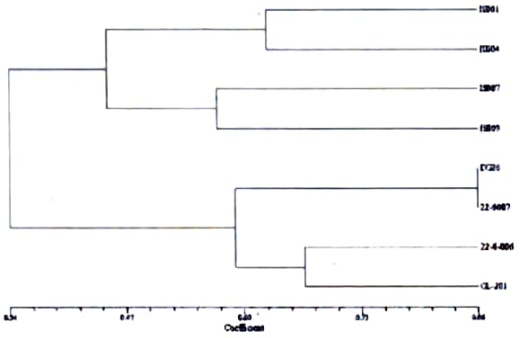

Fig 3: Dendrogram showing the genetic relationships among eight microfungi based on RAPD analysis.

***Cordyceps sp.* (IS-009 MF 11)**

GIS Location : N26°50'.201'' E88°13'.445''

Source : Insect larva from forest floor

Description : Concentric bands of color whitish ; tough and leathery, becomimg brittle and hard when dry; velvety with different tomentum from one color zone to another, reflecting different growth conditions. (Fig:1 H)

For the study of molecular characterization the ribosomal RNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome [Kindermann et. al. 1998). They also occur in multiple copies with up to 200 copies per haploid genome [Lieckfeldt, E. and K.A. Seifert 2000, Taylor et. al. 1999] arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S, and the 28S large subunit (LSU) genes. Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species [Druzhinina et. al. 2004]. In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies, In the present study, we focused on the ITS regions of ribosomal genes for the construction of primers that can be used to identify macrofungi. ITS region of rDNA was amplified using genus specific ITS-1 and ITS4 primers. Amplified products of size in the range of 400-800bp was produced by the primers (Fig 2A). The ITS PCR has helped to detect polymorphism at ITS region of rDNA among the macrofungi isolates.

The genetic relatedness among eight isolates of macrofungi were analyzed by four random primers BAS359, OPA-1, OPD-6 and OPA-4 to generate reproducible polymorphisms. All amplified products with the primers had shown polymorphic and distinguishable banding patterns which indicate the genetic diversity of macrofungi isolates. A total of 22 reproducible and scorable polymorphic bands ranging from approximately 100bp to 2000bp were generated with four primers among the eight macrofungi isolates (Table 2).

RAPD profiles showed that primer BAS359 scored highest bands which ranged between 100bp to 2000bp (Fig 2B). Relationships among the isolates was evaluated by cluster analysis of the data based on the similarity matrix.

The Dendogram was generated by unweighted pair-group methods with arithmetic mean (UPGMA) using NTSYSpc software (Fig 3). Based on the results obtained all the nineteen isolates can be grouped into two main clusters. One cluster represents the isolates saprophyte macro fungi (GL-201MF3, DG 26 MF 4, 22-6-006MF 5 and 22-06-007 MF6) and other parasitic macro fungi (IS-001MF1, IS-004MF2, IS-007 MF10, and IS-009MF11) . The similarity coefficient ranges from 0.34 – 0.86. There is formation of two distinct group at 0.34 similarity.

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