BIOLOGY OF USEFUL PLANTS AND MICROBES

Alpha Science

Editor
Arnab Sen

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Editor
Arnab Sen
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Dedicated to the memory of
my father
Sri Paresh Chandra Sen
(1940-1994)

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Foreword

The importance of research in the field of economically important plants especially medicinal plants and plants for herbal products and cosmetics and whole lot of bacterial community used for biotechnological purposes for the production of medicines and vaccines etc. are much more relevant today than the last century. Bioinformatics, on the other hand is fast emerging as an important discipline for academic research and industrial applications. Research and development in bioinformatics and computational biology requires the co-operation of specialized personnel from the field of general biology, Computer science, Statistics etc. The overall goal of the subject is to elucidate cell function and physiology from a comprehensive set of measurements. Various computational methods are being widely used to address a broad spectrum of problems including the research development and management of economically important plants and microbes

Keeping this in view, in the last winter, Dr. Arnab Sen organized a conference on "Biology and Bioinformatics of Economically Important Plants and Microbes" at University of North Bengal, Siliguri. In this conference, hundreds of eminent scientists gathered and expressed their views on useful plants and microbes of the planet. The idea of the present volume was put forward in a brainstorming season in that conference.

The edited volume "Biology of useful plants and microbes" (edited by Dr. Arnab Sen) consists of 28 articles and virtually has two parts. In one part, modern biological research and development of plants and microbes were discussed and in the other part various techniques of computational biology and bioinformatics were covered. The chapters were written by renowned scientists and science writers in the field of biology and bioinformatics.

However, the best part of the book is perhaps its editing. I must congratulate this young editor for this compilation and for bringing out this book within short time. Dr. Arnab Sen with all his editorial skill and experience including the experience of editing Indian Journal of Microbiology (a Springer publication) has brilliantly abridged the chapters in such a way that the present volume has become a treasure in the field of economic biology.

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Preface

There is perhaps an everlasting quest in biology and that is to learn about ourselves, human being in particular and the living community as a whole. However, through this learning procedure man has also learnt to exploit the living world for its well being. Since the dawn of the civilization, he started to realize about the importance of plants, animals and microbes.

This has become more apparent as we entered in to the 21st Century. More than ever before, human communities are now dependent on plants and microbes. Therefore, research in the field of medicinal, cosmetics and other products from native plants and microbes has once again taken the centre stage of biological research.

Another important aspect is the coordinated interaction between microbes and plants. This interaction is of paramount importance for their healthy association in almost all agricultural settings.

Realizing the importance of plants, microbes and their everlasting association, modern techniques are widely used these days which has led to the development of new disciplines in biology. One such discipline is Bioinformatics.

Bioinformatics is the field of science in which biology, computer science and information-technology merge to form a single discipline. Its ultimate goal is to enable the discovery of new biological insights as well as to create a global perspective from which unifying principles in biology can be discerned. These days, techniques and principles of bioinformatics have been widely used in analyzing the genomes of various beneficial and harmful microbes as well as economically important plants. This way bioinformatics has come up as a major tool for studying the modern day biology. It is in this context Department of Botany, University of North Bengal in association with NBU Bioinformatics Facility organized a three day National Conference on "Biology and Bioinformatics of Economically Important Plants and Microbes". In that conference it was decided that a proceeding volume will be published on the papers presented. The present volume is a culmination of that effort. There are all together 28 articles in this book which may be broadly divided into two parts. One part is biology of economically important plants and microbes and the other part is bioinformatics study of various plants and microbes along with description of various bioinformatics tools.

This book couldn't have been materialized without the active support of University of North Bengal, financial support from Department of Biotechnology, Government of India and Narosa Publication House, New Delhi for agreeing to publish this volume. I am also thankful to Dr. T. Madhanmohan, Adviser, DBT, Government of India for providing help as and when required and also for writing a foreword for this book. I am also thankful to the contributors for conferring

considerable confidence on me and contributing in this volume. I would like to thank my colleagues in the Department of Botany, NBU as well as in the laboratory, especially Mr. Arvind K. Goyal for giving me great editorial support. Thanks are also due to my mother, wife and children for allowing me to spend most of the time working on this book which I should have spent with them. Above all I would like to thank the Almighty God, without whose blessings no work can be done, I believe.

Arnab Sen

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Biology of useful plants and microbes
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Chapter 1

Molecular characterization of the *Citrus tristeza* virus (CTV) genome

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Abstract

Citrus tristeza virus (CTV) is a viral species of the *Closterovirus* genus that causes the most economically damaging disease to *Citrus* plants. CTV is with a long flexuous virion of 2000 X 11 nm size containing positive sense, mono-partite, single stranded RNA about 19.2-19.3kb long enclosed by two types of capsid proteins. The size of its genome makes CTV one of the largest RNA viruses known. The CTV genome contains 12 open reading frames, which could encode at least 17 proteins. CTV exhibits a wide range of diversity in the natural environment. Analyses of complete genomes sequence of many CTV isolates available in GenBank database have shown that the 3' terminal half of the genome is well conserved, whereas the 5' terminal half exhibits high sequence diversity. Genetic diversity studies involving Indian isolates have confirmed that Indian isolates form distinguishable phylogenetic groups, and majoritiy of them are in phylogenetic association with VT genotype.

Keywords

Citrus tristeza virus (CTV), virion, genome, genetic diversity

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Introduction

Citrus tristeza virus (CTV) is the longest known, phloem-limited, plant virus under genus *Closterovirus* in the family Closteroviridae. CTV is with a long flexuous virion of 2000 X 11 nm size containing positive sense, mono-partite, single stranded RNA of 19.2-19.3 kb with 12 open reading frames (ORFs)[1]. Virions contains about 6% RNA content that are helically constructed with a basic pitch of about 3.7 nm^2, about 8-9 capsids per helix turn and a central hole of 3-4 nm. CTV was first *Closterovirus* to be biophysically characterized. Unlike the virions of other elongated plant viruses they posses cylindrical nucleocapsids and like the other members of *Closteroirida*[3], CTV virions are bipolar and contain two coat proteins of 25 kDa (major CP) and 27kDa (minor CP, CPm) that encapsulate about 97 and 3% of virion length respectively[4]. Virions consist of long helical body and short tail. The tail corresponds to 5' end region of the viral genome and the particle tail of other Closteroviruses has been associated with small amount of p61 and p65.

Molecular characterization of the CTV genome

The 19.3 kb single stranded and positive sense gnomic RNA (gRNA) of CTV is divided almost equally into two parts, the 5' part consisting of ORF lab and lb harboring the viral replication machinery and the 3' half harboring ten ORFs encoding a range of structural proteins and other gene products involved in virion assembly and host and vector interactions[1]. Complete nucleotide sequences of atleast 18 distinct CTV isolates have been determined[5]. Interestingly while the sequences of the 3' half of all the sequenced CTV isolates shared 97% and 89% identity when comparing the A' non translated regions (NTRs) and rest of the 3' half respectively, the 5' half sequences often differ considerably with 60-70% identities in 5' NTR and coding region[1,5].

A remarkable feature of CTV isolates are the close identities (97%) of their 3'NTR and considerable divergence of their 5' NTR sequences. Sequences of 5' NTR are of three types, but surprisingly even dissimilar sequences of the 5' NTRs from different strains are predicted to contain two stem loops (SLR and SL2) separated by a short spacer region[6]. Mutations disrupting secondary structure abolished replication whereas compensatory mutations allowed replication to resume, suggesting the essentiality of the predicted secondary structure for replication than primary structure. However, some sequences of the 5'-UTR were necessary for both replication and virion assembly[4]. The 3' UTR, a highly conserved region[6] is critical for recognition by the replicase complex. The secondary structure of this region comprises a series of ten stem-loops with variable ability to support mutations affecting the primary or the secondary structure. Core of the 3' replication signal might be located in three of the central stem-loops (SL) mapped to be 230 nt within 3'

NTR, mutations introduced in this region completely prevented replication suggesting critical recognition signal for replicase complex in this particular region[7]. A terminal 3' triplet CCA seems essential for efficient replication[7].

Genome organization and Function

The single stranded positive sense genomic RNA of CTV is organized into twelve open reading frames (ORF's), potentially encoding at least 17 protein products, and two untranslated regions (UTR's) of about 107 and 273 nt at the 5' and 3' termini, respectively[8]. These 12 ORFs of CTV are expressed through a variety of mechanisms including proteolytic processing of the poly protein, translational frameshifting, and production of ten 3'- terminal genomic RNAs[9]. The first two mechanisms are used to express proteins encoded by the 5' half the genome while the third mechanism is use to express ORF's 2-11[10]. ORFs 1a and 1b encode proteins of replicase complex, whereas ORF 2-11, spaning the 3' terminal half of JRNA, encode proteins p33, p6, p65, p61, p27, p25, p18, p13, p20 and p23. The coding region of the gRNA is divided into, replication module (ORFs la and lb), a five-gene module (p6, p65, p61, p27 and p25) encoding proteins involved in virion assembly and transport, which is unique signature block, conserved in all members of the family *Closteroviridae,* the p20 gene, a homologue of the p21 gene of *Beet yellow virus* (BY), and four genes encoding proteins with no homologue in other *Closteroviruses* (p33, p18, p13 and p23)[11]. The ORF la encodes a 349 kDa poly protein containing two papain-like protease and methyl transferase like and helicase like domains. Translation of the poly protein could also continue through the 54 kDa, RNA dependent RNA polymerase (RdRp) like domain (ORF lb) by +1 frameshift[12]. The function of p33 (ORF2) is unknown, but is required for infection of a subset of the viral host range. The next protein products encoded by the 3' five -gene module include a transmembrane protein (p6), a homologue of the HSP70 plant heat-shock proteins (p65), two diverged copies of the capsid protein (p25 and p27)[13], and an additional protein (p61) also regarded as a diverged CP copy[11]. The p6 homologue in BYV has been shown to be a movement protein and required for systemic invasion of host plants[14]. The coordinate action of p65 (ORF4, cellular heat –shock protein homologue, HSP70) and p61 (ORF5), in addition to the CP and CPm coat proteins, is required for proper virion assembly[15].

These two proteins, p65 and p61 probably bind to the transition zone between CP and CPm and restrict CPS to the virion tail[4]. The p20 protein is a major component of CTV-induced amorphous inclusion bodies[16], and p23, an RNA binding protein with a Zn finger domain, regulates asymmetrical accumulation of the positive and negative strands during RNA replication[7]. Both p20 and p23, in addition to the CP, act as RNA silencing suppressors in *N.*

benthamiana and *N. tabacum* plants, with p23 inhibiting intercellular silencing, CP intracellular silencing, and p20 both inter- and intracellular silencing[17]. Deletion mutants lacking genes p33, p18 and p13 were capable of replication and assembly[15,18], indicating that they are not required for these functions thus their role in CTV biology remains unknown. Protein p23 ORF 11 is unique in CTV as no homologue found in other *Closteroviruses*[19], it is a multifunctional protein which binds cooperatively both ss RNA and ds RNA, contains a zinc-finger domain that regulate the synthesis of minus strand molecules, controls the level of genomic and subgenomic negative stranded RNAs and is an inducer of CTV like symptoms in transgenic *Citrus aurantifolia* plants[1].

Replication of the CTV gRNA involves synthesis of negative strands that serve as template for the generation of new positive strands, although the latter accumulate 10-20 times more than the negative strands[7]. During replication large number of less than full length RNAs are produced. These include ten 3' co-terminal sg RNAs[20], and ten negative stranded sg RNAs corresponding to the ten 3' sg mRNAs, plus ten 5'-coterminal sgRNAs that apparently are produced by termination just 5' of each of the ten ORFs[21,22].

Infected cells also contain huge amount of two other small 5-coterminal positive stranded sg RNAs of about 600 and 800 nt designated as low molecular weight tristeza (LMT1 and LMT2) produced by premature termination of the gRNA synthesis, thus totaling more than thirty sg RNAs in infected cells [9,22,23].

Frequently, CTV-infected tissues also accumulate large amounts of positive- and negative-stranded defective RNAs (D-RNAs) that contain the 3' and 5' termini of the gRNA but lack variable portions of the central region with extensive internal deletions. These viral RNAs, which are easily observed by electrophoretic analysis of plant extracts enriched in double-stranded RNA (dsRNA), are very common in field isolates[23-26]. CTV D-RNAs accumulate abundantly even when their genome contains less than 10% of the viral genome. Most D-RNAs are 2.0-5.0 kb in size, but large D-RNAs comprising ORFs la and lb in their 5' proximal moiety, or ORFs 2-11 in their 3' terminal moiety, have been reported[27]. Sequence analysis of the junction site and flanking regions suggest that most D-RNAs must be generated by a template- switching mechanism induced by different factors[28]. The minimal replication signals required for D-RNA replication in trans are located in the 5' proximal 1 kb and at the 3'-UTR of the D-RNA sequence[29,30].

The biological role of CTV D-RNAs is presently unknown, as no specific associations have been established for any of the D-RNAs. Their wide occurrence in CTV isolates suggests that they may provide some advantage. At least in one case, the presence of a D-RNA was reported to modulate CTV symptom expression[31]. The interaction of CTV with host factors

is largely unknown, but examination of D-RNA from Alemow plants infected with seedling yellow (SY) and non-SY inducing isolates revealed mostly a major single D-RNA of 4.5 or 5.1 kb in non-SY plants and two different D-RNAs of 2.4 or 2.7 kb in SY plants. These results suggested the possibility that D-RNAs might play a role in suppression of SY symptoms[31]. Most CTV isolates occur in mixed population of different variants[32] and defective RNAs. However, some CTV isolate consists principally of one genotype with minor concentrations of other variants.

Manjunath *et al.*[33] cloned and sequenced coat protein (CP) gene of four selectively Indian CTV isolates. Eight ORFs of the total 12 ORFs in CTV genome of Florida severe isolate T36 have been cloned and sequenced for the first time (Figure 1)[34]. After comparison in the annotated amino acid sequences of CTV ORFs with other annotated amino acid of viral ORFs available in database it was reported that genome of CTV have been arisen as a result of multiple recombination events[34]. Complete genome of 18 CTV isolates from different geographical areas inducing different phenotypes has been sequenced and deposited in GenBank. They are T36 (U16304), and T30 (AF260651) from Florida, VT (AF001623) from Israel, T385 (Y18420 and T318A (DQ151548)) from Spain, SY568 (AF001623) from California, NuagA (AB046398) from Japan, Qaha (AY340974) from Egypt, B165 (EU076703) from India, one isolate Mexico (DQ272579) from Mexico, five isolates, NZ-B18 (FJ525436), NZRB-G90 (FJ525432), NZRB-M12 (FJ525431), NZRB-M17 (FJ525435), NZRB-TH28 (FJ525433) and NZRB-TH30 (FJ525434) from New Zealand,

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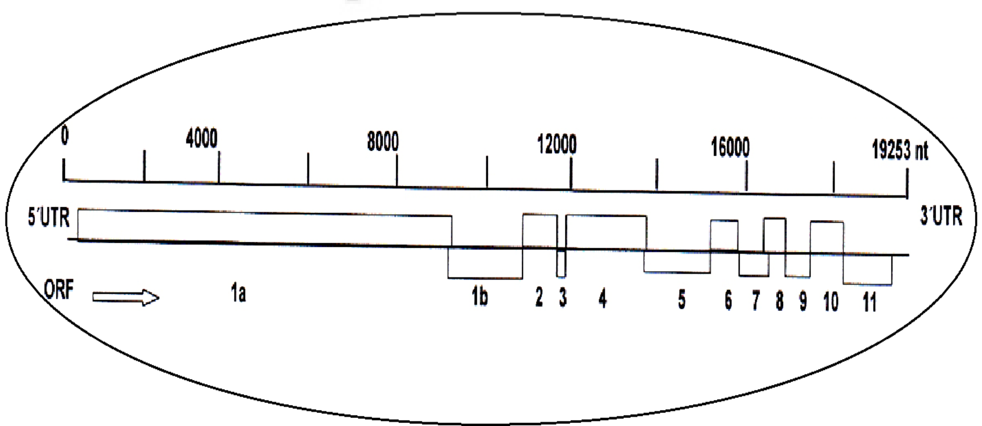

Figure1: Diagram of the organization of CTV complete genome (20- kb) with 12 open reading frames

and two isolates, HA16-5 (GQ454870) and HA18-9 (GQ454869) from Hawaii.

Genetic diversity

CTV has been shown to exhibits a wide range of diversity in the natural environment[35,36] and existence of several divergent CTV isolates have been reported from many citrus growing countries[37]. Nucleotide sequence analysis has been proved to be an accurate procedure for estimation of genetic diversity of CTV[38] and using this method several divergent isolates have been reported from citrus growing countries[39,40]. Various procedures has detected variations in viral RNA, including: (1) differences in dsRNA pattern between isolates[25,41], later shown to be due to the presence of D-RNAs in CTV isolates; (2) hybridization pattern with cDNA or cRNA probes of several gRNA regions[42], (3) restriction fragment length polymorphism (RFLP) analysis of the CP gene[43], (4) RT-PCR amplification patterns with primer sets specific for several CTV genotypes[37]), for 5-UTR sequence types I. II and III[44,45] or for three. groups of isolates differing by their p23 sequence[46], and (5) single-strand conformation polymorphism (SSCP) analysis of different gRNA regions[47,48]. This SSCP technique has been used to characterize the population structure of CTV isolates and select specific variants for sequencing, thus allowing estimates of the genetic diversity within and between isolates[49]. The values obtained were in the range of those calculated for other plant viruses[50], but higher than those estimated for other members of the family Closteroviridae[38].

Genetic variation is unevenly distributed along the CTV ERNA, the most conserved region being the 5'UTR, with over 95% identity between various isolates, and most variable being the 5UTR with identity values as low as 44-55 % between sequence types of different groups[6]. Analysis of genomes of two CTV isolates VT and T36, more similarity (89 %) at 3' half than that at the 5' half of the CTV genomes were noticed which suggests that diversity of CTV isolates was not resulted from a recent recombination event[51]. Later on studies on the genetic diversities of CTV isolates began in citrus growing countries of the world. Sequence diversity in some CTV isolates is shown to be uniform throughout the whole genomes, while in other isolates it is asymmetrical[18]. By genome comparison of various CTV isolates it was found that CTV exhibits about 30% sequence diversity in the 5' half, while 10% in 3' half of the genomes[52]. Analyses of complete genomes sequence of many CTV isolates available in GenBank database have shown the pattern of sequence variation among the isolates with > 90% in 3' proximal 8 kb, and much greater divergence in the 5' proximal 11 kb of the genome[37], concluding the 3' terminal half of the genome is well conserved, whereas the 5' terminal half exhibits high sequence diversity[53].

Overall, sequence comparisons between CTV isolates of different geographical origin and

pathogenicity characteristics showed a high degree of conservation between CTV genomes separated in time and space, with a limited repertoire of genotype[45], and a population structure variable between isolates, with some consisting of a predominant sequence and some closely related variants, and others having a complex structure with highly divergent sequence variants[49]. CTV genome contains three RNA silencing suppressors viz., P20, P23, and CP[54].

CTV isolates based on sequence diversity in various gnomic regions were divided in to different groups viz., three groups[6]. Sequence variation is comparatively uniform in genome of CTV-VT, whereas it is very divergent with other CTV isolates like T36 and primers based on diverged sequences detected only a few CTV isolates[55]. Complete nucleotide sequences of Florida mild isolate (T30) which are identical to a Spain mild isolate (T385), suggesting parents of these isolates might have common origin probably in Asia, followed by dispersion throughout the world by movement of citrus[42].

Classification scheme based on sequence analysis of 5' and 3'end of CTV genome has been proposed, and two major groups VT and T36 have been identified by Hilf *et al.*[37]. The VT group members had sequence divergences which were distributed in constant proportion throughout the genome, while the T36 group members had highly divergent and conserved sequences at 5' and 3' ends respectively.

Rubio *et al.*[38] analyzed sequences of four ORFs of 30 CTV isolates from Spain and California and concluded that sequence variants originated due to effect of recombination between diverged sequences variants. Roy *et al.*[43] in USA sequenced CP genes four Indian CTV isolates and compared the nucleotide, and amino acid sequence, with other international isolates and considerable genetic variability among them was not found. Roy and Brlansky[56] based on the ORFla, overlapping region of RdRp and P33 sequence analysis of 21 Indian CTV isolates, divided them in to five distinct groups. Most of the analyzed isolates were found of VT genotype and first time reported T36, T30 (occurring in mixed infections) like isolates under Indian conditions. Population structure of CTV isolate SY568 revealed that it composed of sequence types occurring with varying nucleotide frequency[57].

Conclusion

Genetic diversity of Indian CTV isolates has been studied earlier using limited number of isolates[40]. Analyzing 15 Indian isolates with specific primers, it is proposed Indian isolates are of VT genotype[37]. Using 21 Indian CTV isolates based on sequences from 5'ORFla (position 697-1484 nt) it is reported that Indian isolates form distinguishable phylogenetic groups, and majorities of them are in phylogenetic association with VT genotype[58].

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Chapter 2

Molecular detection of arbuscular mycorrhizal fungi and their application for plant health improvement

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Abstract

Arbuscular mycorrhizal fungi (AMF) the most widespread symbionts on earth receiving attention because of the increasing range of their application in sustainable agriculture and ecosystem management. Root colonization with AMF is a dynamic process which is influenced by several edaphic factors. Mycorrhiza form critical link between the plant and soil structure and make a direct contribution to soil fertility and quality through increased uptake of immobile nutrients especially phosphorus that are mobilised by the fungus. However there is increasing evidence that AMF have a range of other effects for example protection against plant parasite water stress tolerance alleviation of salt stress and in sustainable maintenance of plant health and soil fertility. Molecular analysis has provided the first opportunity to consistently identify the AMF taxa present in the plant roots Molecular methods allow the identification of AMF taxa independently of morphological criteria and potentially at low levels of colonization. During colonization AMF can also prevent root infections by reducing the access sites and stimulating host defense. In order to efficiently use mycorrhizal fungi in agriculture it is necessary to understand their ecophysiology and metabolic functioning Concerted efforts should be made to develop bioformulations which can be used as biofertizers and bioprotectors for improvement of plant health status. However stringent quality control measures must be adopted so that farmers get quality products.

Keywords

Arbuscular mycorrhiza, plant growth, symbiosis, DGGE, ITS

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Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate biotrophs which live symbiotically in the roots of about 80% of plant species. Most AMF form spores in the soil which are able to germinate and grow from a quiescent-like state in response to different edaphic and environmental conditions but are unable to produce extensive mycelia and to complete their life cycle without establishing a functional symbiosis with a host plant[1]. The key developmental switches occurring in the fungal organism from the germination of an individual spore to the formation of an extensive hyphal network in the soil involve a sequence of morphogenetic events represented by spore germination and pre-symbiotic mycelial growth differential hyphal branching pattern in the presence of host roots appressorium formation root colonization arbuscule development extraradical mycelial growth and spore production[2].

Root exudates compounds and growth of AMF

The lack of host-regulated spore germination contrary to what happens with many pathogenic biotrophic fungi could have represented a strong selective disadvantage. Nevertheless AMF are considered evolutionary successful "living fossils" having survived and evolved for 460 millions years their ancestral nature having been shown by diverse fossil records and DNA sequence data[3]. Their persistence indicates that they must have evolved efficient strategies to overcome the lack of spore germination regulation and to allow the survival of individuals and populations. The phenomenon of spore dormancy has concerned researchers since Godfrey's early studies on spore germination[4]. As early as Mosse[5] suggested the storage of collected spores on damp filter paper at 5°C for 6 weeks in order to obtain the regular germination of resting spores of an *Endogone* sp. (presumably *Glomus mosseae*). Eighty percent of spores treated in this way germinated within 3–4 days. The problem of erratic spore germination has been mentioned in many reports and Tommerup[6] gave a clear-cut definition of spore dormancy making a distinction between dormancy and quiescence. A dormant spore was defined as one failing to germinate when exposed to physical and chemical conditions which support germination of apparently identical spores defined as quiescent spores. Differences in cytoplasmic organization between young and old resting spores were described in *Acaulospora laevis* and *Glomus* species wherein dormant spores the oil globules enlarged at the expense of the cytoplasm which was restricted to small interstitial spaces[7]. A fine network of cytoplasmic material was also described by Sward[8] in dormant spores of *Gigaspora margarita*. The relief of dormancy by storage was reported by many authors Hepper and Smith[1] found that spores of *G. mosseae* from freshly harvested sporocarps germinated slowly compared to spores

detached from sporocarps and stored at 6°C for 5 weeks. The same results were obtained with a North American isolate of *G. mosseae* which showed a marked difference in germinability between freshly isolated and 10°C stored spores[9]. Diverse species of the genus *Glomus* exhibited spore dormancy such as *Glomus intraradices, G. clarum, G. caledonium, G. monosporum*[10]. Other species such as *G. coronatum* showed erratic germination even after cold treatments lasting one year[11]. A marked dormancy was shown by spores of *A. laevis* which germinated after 6 months storage in two different experimental conditions. Other species within the genus *Acaulospora* exhibited the same behaviour and in a laboratory experiment only a small proportion of spores stored for two months germinated while most spores germinated well after storage for 4–6 months[12]. Similarly *Acaulospora longula* showed complete relief of dormancy after 8 weeks storage at 23°C in soil[13]. Not all the species and genera of AMF show spore dormancy. Spores of *Gigaspora gigantea* collected throughout the year from sand dunes did not show any dormancy and were able to germinate as early as one day after incubation either when they had been surface sterilized or not while newly formed spores showed a period of endogenous dormancy[14]. Germ tubes of *G. margarita* emerged after 72 h incubation on water agar or within 3–5 days on agar media without any storage treatment[15]. Similarly spores of *Scutellospora fulgida* and *S. persica* did not possess any dormancy showing mycelial growth and the formation of auxiliary cells after two weeks in the dark at 24°C[16]. Propagule dormancy may contribute to the survival of AMF in adverse environments but despite many different experimental reports on spore dormancy of many species of AMF a complete understanding of the phenomenon has not been obtained. We still do not know whether dormancy in more species or genus than isolate correlated because experiments have often been carried out on different isolates. Moreover no studies have been performed on the molecular bases of dormancy. We ignore whether it may be affected by the presence of compounds in young spores which inhibit germination or by the occurrence of compounds in mature old spores which enhance germination. The molecular signals which relieve spore dormancy and activate the cell cycle still remain unknown though different environmental conditions triggering the initiation of germination in genera and species of AMF have been investigated. In fact resting spores of many AM fungal species germinate both in soil and in agar under adequate physical chemical and microbiological conditions. Many germination factors have been identified which play important roles in growth activation of quiescent spores. Although complex interactions among different factors probably play the most important role in spore germination in nature. Many investigators have studied germination factors such as pH temperature moisture mineral and organic nutrients host plants and microorganisms as if they were independent triggers.

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Root colonization with AMF and benefits to plants

The inability of the plants to support mycorrhizal colonisation may be due to the accumulation of chemicals like alkaloids cyanogenic glucosinolates and antifungal compounds in the roots which fail to elicit differential hyphal branching[17,18]. There are different types of mycorrhizal interactions which have been classified into ectomycorrhiza and endomycorrhiza based on the presence of various extraradical or intraradical hyphal structures[19]. Seven mycorrhizal types have been identified but the most common endomycorrhizas are the Arbuscular mycorrhizas [20]. Other types such as Ectendomycorrhiza Arbutoid and Monotropoid mycorrhizas are grouped under ectomycorrhizas. These are characterised by the formation of a hartig net and a mantle or sheath around the plant roots. Orchid and Ericoid mycorrhizas are other forms of endomycorrhizas that are known for their ability to penetrate the outer root cells to form intracellular hyphal coils swellings or branching. They differ from the arbuscular mycorrhizal fungi by having septate hyphae that are restricted to the epidermal cells of plant roots. All these mycorrhizal types differ from each other by the characteristic host plant that they associate with fungal species involved and morphology within roots[21].

Specificity infectivity and effectivity are the three major parameters in determining root colonisation. Specificity refers to the ability of the fungus to colonise root cells of particular plant species infectivity the amount of colonisation and effectivity the plant's response to colonisation. The presence of AM colonisation is usually undetected by the naked eye because there are no morphological root changes mycelial mantle or large fungal fruit bodies but when cleared stained and examined microscopically visible root colonisation is observed. Detection of AM fungal colonisation in roots is essential for mycorrhizal research. Hence ranges of methods have been employed to achieve this such as light microscope based techniques biochemical methods and molecular techniques. However the standard technique for visualisation and quantification of root colonisation by AM fungi remains the non-vital staining technique with various stains such as Trypan and Cotton Blue Chlorazol Black E or an appropriate ink.

The major benefits of AM fungi to symbionts includes enhanced nutrient uptake increased tolerance to root pathogens drought resistance tolerance to toxic heavy metals and improved soil aggregation and structure. AM fungi are known to enhance mainly the uptake of the macronutrient phosphorus P from the soil which is then translocated to the host plant through hyphal networks in the soil. Their ability to also take up other micronutrients such as Cu, Zn, Ni, Pb and Fe etc; has been demonstrated by researchers using different host plants and soil type management Phosphorus is the second essential nutrient after nitrogen

(N) required for plant growth and is found in many soils in organic and complex inorganic forms (phytic acid). Due to its low solubility and mobility plants cannot readily utilise P in an organic or complex inorganic form Inorganic phosphate present in soluble forms in the soil can be readily utilised by plants but usually in limited amounts. Thus AM fungi intervene to enhance nutrient uptake through the spread of extraradical hyphae (ERH) into the surrounding soil and hydrolysing any unavailable sources of P with the aid of secreted enzymes such as phosphatise. The enzyme phosphatase produced by AM fungal extraradical hyphae hydrolyses and releases P from organic P complexes and facilitates the absorption of P and other nutrients thereby creating a depletion zone around the roots. These depletion zones limits the rate of phosphorus uptake by non-mycorrhizal plants but gives mycorrhizal plants a greater advantage. Sylvia *et al.*[22] reported that under nutrient deficient conditions the effectiveness of AM fungi is exercised by the ability of the ERH to bridge the nutrient depletion zones of host plant roots. But when nutrients are available to the plant root length growth is increased and the mycorrhizal dependency of the plant to take up nutrient is reduced.

Although P is the main nutrient transported by AM fungi to plants N is of great importance for plant growth and should not be over-looked. Nitrogen is obtained by the ERH of AMF in different forms ranging from amino acids peptides ions to recalcitrant organic nitrogen forms. It has been recorded that the extraradical hyphae of different *Glomus* sp. can assimilate and metabolise both organic and inorganic sources of nitrogen perhaps by glutamate synthetase activity. It can be stated therefore that the concentration of P and N in the soil can determine the rate of other micro (Fe Cu Mn Zn) and macronutrient (K Ca) uptake by mycorrhizal plants. Due to the potential of mycorrhizal fungi to enhance nutrient uptake this benefit has however brought about the suggested use of AM inoculum instead of some chemical fertilisers for plant productivity growth and restoration of polluted soils or in revegetation.

Along with accessing soil nutrients the hyphae of AM fungi allows greater access to water through mechanisms such as stomatal regulations increased root hydraulic conductivity osmotic adjustments and maintenance of cellular water pressure and cell wall elasticity changes. Mycorrhizal infection of maize with *G. mosseae* and *G. intraradices* helped the plant to maintain higher leaf water potential compared to non-mycorrhizal plants. The ability of AM fungi to effectively alleviate drought stress has been studied in terms of nutrient uptake of N and P photosynthesis and cytokinins. However due to the possible interference of drought in the mobility of NO_3^- to the root surface the role of nitrogen uptake by *G. fasciculatum* under such conditions was tested using a radio labeled nitrogen (^{15}N).

Results showed that under optimal water supply the amount of ^{15}N was the same in both mycorrhizal inoculated and non-mycorrhizal inoculated plants but four times higher in the mycorrhizal inoculated plants under water stressed conditions. Other recent studies have shown the capability of AM fungi to influence plant growth crop quality and adaptability to stress conditions.

Soil structure is improved by AMF through the secretion of a glue-like proteinaceous water-soluble and heat stable substance from their hyphae called glomalin. This compound aids in soil aggregation by binding soil particles together thereby influencing soil porosity which promotes aeration and water movement essential for good root growth root development and microbial activity. Glomalin a recalcitrant iron-containing glycoprotein is indeed responsive to ecosystem fluctuations such as elevated atmospheric CO_2 concentrations global warming and agricultural practices. Due to the positive correlation observed between glomalin land-use and soil carbon-nitrogen ratio this glycoprotein can be used to assess changes in soil C in various land-use types. Hence glomalin can be regarded as an indicator for soil aggregation and stability. Glomalin is easily assayed and cannot be produced from uncolonised plant roots as it is AM fungal specific. Therefore it can be used to determine AM hyphal growth and activity in the soil.

The toxicity of metals lies in the concentrations in which they are present in the soil. These metals can arise from a variety of sources in the form of acid rain dust containing these metals wash waters from polluted soils or from atmospheric factors produced as a result of mining smelting burning of fossil fuels industrial or agricultural activities. Heavy metals have been said to affect some developmental stages of AM fungi or eliminate their establishments. However it is reported that the level at which heavy metals such as Zn, Cd, Al, Cu and Pb affects plants and mycorrhizal fungi varies and is dependent on their actual concentration oxidation state in the soil soil pH organic matter content cation exchange capacity and redox potential. *In vitro* effect of Cd, Pb and Zn on critical life stages of two AMF (*Glomus etunicatum* and *G. intraradices*) showed that these two species differ in their metal sensitivity but generally were able to survive under metal stress. Thus isolation of AMF from metal contaminated soils has proven their potential ability to thrive on such soils. Additionally AMF alleviate plant stunting caused by toxic metals by binding to these metals in the root zone with the aid of the extraradical mycelium and altering the plant cells ability to capture the metals. The polyphosphates produced by AMF are proposed to be the reason behind this sequestration though this has not been confirmed[23].

Molecular detection of AMF

Molecular analysis of AMF has provided the first opportunity to consistently identify the

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taxa present in the roots of plants[24,25]. Molecular methods allow the identification of AMF taxa independently of morphological criteria and potentially at low levels of colonisation. Analysis of AMF from field samples relies on the Polymerase Chain Reaction (PCR) that allows the DNA from small quantities of starting material to be bulked up into sufficient quantities for analysis. AMF can be targeted either within DNA of a plant root or from small amounts of template such as a single spore. The data from PCR depend upon the design of the primers used to initiate the reaction and the specificity of the primer determines the range of sequences that may be designed to amplify sequences from a single isolate up to those that recognise all AMF.

The majority of nucleic acid information derived from the Glomales is from the ribosomal RNA genes. In most organisms the ribosomal RNA genes are present in multiple copies arranged in tandem arrays with each repeat unit consisting of a small subunit (SSU or 18S) and a large subunit (LSU or 28S) ribosomal RNA gene separated by an internal transcribed spacer (ITS) that includes the 585 rRNA gene. The multiple copies are normally very similar in sequence due to the genetic processes of unequal crossing over and gene conversion. It is expected that the ribosomal genes of the Glomales are similarly arranged but an unusual feature that has come to light is the frequent occurrence of multiple rRNA gene sequences sometimes quite divergent in a single spore or culture.

Primers developed

First molecular analyses of DNA isolated from AMF spores (*Glomus intraradices* and *Gigaspora margarita*) were done[26] and the SSU rRNA gene sequenced using PCR fragments generated with universal eukaryotic primers. It was possible to amplify sequences from several AMF directly from plant roots by using VANS1 a taxon-specific primer. A phylogenetic analysis of sequences from spores using VANS1 showed that the molecular phylogeny was congruent with the family classification and that the sequence divergence was consistent with the fossil record Group specific primers VAGLO (*Glomus*) VAACAU (*Acaulospora*) VALETC (*Getunicatum*) and VAGIGA (*Gigaspora* and *Scutellospora*) were subsequently designed (Table 1) However using VANS1 it was found difficult to amplify products directly from roots[27-29].

Based on a large amount of reference sequence information that was available to earlier studies another primer AM1 was designed to amplify. AMF sequences by excluding plant sequences AM1 amplifies a section of the SSU gene in combination with the general primer NS31 that has a high degree of information sequence variation. However drawback of this primer combination is that it also amplifies sequences from some other fungi of Pyrenomycetes. While the AMF that fall within the three long - accepted families can be

Table 1: Primers used to amplify SSU rDNA and LSU rDNA sequences from Mycorrhizal roots

SSU rDNA	
VANS1	5'GTCTAGTATAATCGTTATACAGG
NS21	5'AATATACGOCTATTGGAGCTGG
VALETC	5'ATCACCAAGGTTTAGTTGGTTGC
VAGLO	5'CAAGGGAATCGGTTGCCCGAT
VAACAU	5'TGATTCACCAATGGGAAACCCC
VAGIGA	5'TCACCAAGGGAAACCCGAAGG
VANS22	5'TAAACACTCTAATTTTTTCAA
VANS32	5'AAGCTCGTAGTTGAATTTCGG
SS1492'	5'GCGGOCCGCTACGGMWACCTTGTTACGACTT
LSU rDNA	
LSU 0061[1,2]	5'AGCATATCAATAAGCGGAGGA
LSU 3f[1]	5'AGTTGTTTGGGATTGCAGC
LSU 4f[3]	5'GGGAGGTAAATTTCTCCTAAGGC
LSU 6f[1]	5'AAATTGTTGAAAGGGAAACG
LSU 9f[1]	5'ATTCGTTAAGGATGTTGACG
LSU 5r[1]	5'CCCTTTCAACAATTTCACG
LSU 7r[3]	5'ATCGAAGCTACATTCCTCC
LSU 8r[1]	5'GGGTATCCGTTGCAATCCTC
LSU 0599[2]	5'TGGTCCGTGTTTCAAGACG
LSU 0805[1,2]	5'CATAGTTCACCATCTTTCGG

[1] Primers used for sequencing; [2] Primers used for primary PCR amplification; [3] Primers used for the second nested-PCR amplification

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detected successfully using this primer it does not match the SSU sequences of the more divergent clades which are now designated as the new families. Archaeosporaceae and Paraglomaceae SSU primers that are specific for each of the five Glomalean families have been described[3].

Other studies have focused on the ITS region of the rRNA family Universal sequences ITS1 and ITS4 have been used[30] to obtain PCR- restriction fragment length polymorphism (RFLP) and sequence information from spores collected from the field[31] and to study the relationships among AMF species[32]. As high level of diversity is observed within species and even within spores utility of ITS variation for field identification is limited. Other taxon -specific primers have been designed to amplify fragments (ca 45 bp) of the large subunit (LSU) rRNA gene. The design of PCR primers for community analysis necessarily evolves as more information becomes available. The history of molecular analysis of AMF is one of the evolutions of more precise targeting[29]. No single system will satisfy all purposes and the regular evaluation of a system is necessary as more reference sequence information becomes

available and a better understanding of the genetic diversity of AMF is required.

Species specific Primers

The use of family or species-specific primer sets has demonstrated the power of molecular approaches for positive identification of fungi (Table 2). Targets have been the SSU of the ribosomal gene complex the LSU and the ITS Specific primers have also been generated from random amplified polymorphic DNA (RAPD) analysis of spores[33,34] or characterization of highly repeated DNA sequences[35] Species- specific primers were used to investigate the influence of two host plants on the community structure of *Glomus mosseae*[36].

Gigasporaceae- specific primer - VANS1 was found to perform poorly for investigation of the structure of natural AMF communities specially. *Scutellospora* colonising the roots of bluebell using the primer combinations SS38/VAGIGA and VANS1/VAGIGA. These primer sets were used to amplify sequences from bluebell roots and field spores and surprisingly high level of SSU sequence variation with single spores has been noticed.

Additionally it was noted that several of the AMF sequences obtained from spores would

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Table 2 : Primers for amplification of fungal nuclear ribosomal RNA genes

rRNA	Gene primer[a]	Product size (bp)[b]
Nuclear small		
NS1	5' GTAGTCATATGCTTGTCTC	555
NS2	5' GGCTGCTGGCACCAGACTTGC	
NS3	5' GCAAGTCTGGTGCCAGCAGCC	597
NS4	5’ GTTCCGTCAATTCCTTTAAG	
NS5	5’ AACTTAAAGGAATTGAGGGAAG	310
NS6	5’ GCATCACAGACCTGTTATTGCCCTC	
NS7	5’ GAGGCAATAACAGGTCTGTGATGC	377
NS8	5’ TCCGCAGGTTCACCTACGGA	
Nuclear ITS		
ITS1	5’ TCCGTAGGTGAACCTGCGG	290
ITS5	5’ GGAAGTAAAAGTCGTAACAAGG	315
ITS2	5’ GCTGCGTTCTTCATCGATGC	290
ITS3	5’ GCATCGATGAAGAACGCAGC	330
ITS4	5’ TCCTCCGCTTATTGATATGC	580
ITS1F	5’ CTTGGTCATTTAGAGGAAGTAA	700
ITS4B	5’ CAGGAGACTTGTACACGGTCCAG	
TW13	5’ GGTCCGTGTTTCAAGACG	1200

[a] All odd-numbered primers are 5’ primers; even numbers indicate 3’ primers Sequence are written 5’–3’;
[b] Product sizes are approximate based on rRNA genes of *Saccharomyces cerevisiae*

not have been amplified using VANS1. This illustrates the limited value of using specific primers to study community diversity in any situation in which the starting material is not well characterised with regard to its inherent sequence variability. Specific primers are ideally suited for the experiments where the aim is to rapidly test for the presence /absence of fungi added from well characterised pot cultures but they are not appropriate for field collected samples unless the aim is to focus on just one component of the community.

Primers Intended to Target all Glomalean Fungi

An allied approach is to use primers designed to amplify Glomalean templates but exclude other sequences likely to contaminate DNA extracts. Studies of this kind have all used cloning and PCR-RFLP to separate classes and sequencing to give insight into the identity of the clone. Interest has again concentrated on the ribosomal gene complex in particular the SSU and ITS regions.

PCR-DGGE for AMF

Denaturing Gradient Gel Electrophoresis (DGGE) is a DNA-based technique which generates a genetic profile or “fingerprint” of the microbial community. Individual DNA sequences or “bands” from this profile can be excised and sequenced to identify the dominant members of the microbial population. Molecular techniques provide a vigorous means of characterizing AMF to enhance our understanding of the phylogeny[37] ecology[38] and evolution[39] of this group of fungi. Molecular characterization of AMF was initiated by Simon *et al.*[26] and for the first time applying DGGE Kowalchuk *et al.*[40] assessed AMF diversity in sand dune soil and root samples. A polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) procedure for the detection of fungal 18S ribosomal RNA gene was developed by Ma *et al.* [41] with reference cultures of seven isolates (representing five AMF species). These reference cultures were chosen because isolates of their species were putatively identified in a previous survey of farm field soils in the province of Saskatchewan Canada. A reference PCR-DGGE profile was generated using DNA extracted and amplified from the spores of these cultures. The effectiveness of the procedure was tested by its application to soil samples from 38 farms. Prominent bands from the PCR-DGGE profiles of these samples were excised for sequence analysis. The total number of species recovered was low in comparison to other AMF community surveys of temperate climate locations. The majority of the sequences recovered were *Glomus* species *Scutellospora calospora* a previously undetected AM fungus in Saskatchewan was also found PCR-DGGE analysis using a new primer was shown successfully to discriminate five species of AM fungi from grasslands which includes *Glomus claroideum, G. etunicatum, G. clarum, Gigaspora margarita* and *Archaeospora leptoticha*[42]. Though not

without its drawbacks this approach to community composition analysis of AMF was faster than conventional trap cultivation methods.

Mycorrhiza helper bacteria

Mycorrhiza helper bacteria (MHB) are organisms that specifically promote mycorrhiza formation especially ectomycorrhizal fungi by producing growth metabolites that encourages easy proliferation of the fungal hyphae thereby increasing the chances of the fungal hyphae to colonise plant roots with a large surface area. When plant growth promoting rhizobateria (PGPR) are found to stimulate mycorrhizal formation they can be regarded as MHB[43] this interchangeable characteristic brings about the overlap that exists between the two groups. Generally the microbes in the mycorrhizosphere affect mycorrhizal functioning and thus some bacteria may interact with the mycorrhizal fungi on more than one metabolic level. It has been reported that some organisms especially those belonging to the genera *Bacillus* can be multifunctional. This means that they are able to perform functional roles such as being N_2 fixers P solubilizers or grouped as PGPR or MHB. Few studies have also been carried out on the interaction between AMF and Actinomycetes. Research conducted using both organisms to determine their effect on plant growth showed their individual enhancement whereas dual inoculation of organisms adversely affected plant growth and exhibited antagonistic interaction towards each other. Actinomycetes was said to be responsible for the suppression of AM fungi due to its antagonism and inhibitory effect in the rhizosphere. The production of inhibitory compounds by actinomycetes could be seen as the organism's way of competing with other organisms for nutrients. *Frankia* were able to form a synergistic relationship with AM fungi when inoculated together in actinorrhizal plants such as tibetan seabuckthorn (*Hippophae tibetana*) and *Discaria trinervis*[44]. *Streptomyces* is a common soil organism belonging to the actinomycetes. Their effect on AM fungi varies according to species. For example the colonisation of finger millet roots by *Glomus fasciculatum* was shown to be inhibited by *Streptomyces cinnamomeous* while *Streptomyces orientalis* produced volatile compounds that stimulated germination of the resting spores of *G. mosseae Gigaspora margarita* and *Scutellospora heterogama* when cultured auxenically[45]. Hence the interactions of AM fungi with soil bacteria can either stimulate or inhibit each other's processes in the rhizosphere.

Mycorrhizal fungi are the best-known examples of fungal and bacterial interactions as the hyphae offer good ecological niches for other microbes. AM fungal spores harbour Bacteria Like Organisms (BLOs) also referred to as endosymbionts in their cytoplasm and these organisms complete their life cycle within the eukaryotic cells giving rise to a further level of symbiosis[46]. To demonstrate this symbiosis a combination of morphological and

molecular techniques were conducted and it was concluded that the AM fungal spores of *Gigaspora margarita* and *Glomus versiforme* spores harboured these BLOs in their cytoplasm. Analysis of the bacterial 16S rRNA gene sequence obtained from the extraction of spore DNA of *G. margarita* inferred that these bacteria are related to the genus *Burkholderia*. Investigation of two geographically separated isolates of *Gigaspora margarita* and four other isolates *G. gigantea, G. rosea* and *Scutellospora persica* showed that four out of the five species had endosymbionts the exception being *Grosea*. This demonstrates that BLOs are common features in the *Gigaspora* and can possibly be used as a genetic marker for members of this genus. Following further analysis of the morphological and molecular similarities between the endosymbionts found in *G. margarita, S. persica and S. castenea* through the amplification and sequence of partially complete 16S rRNA revealed that all endosymbionts obtained from the three AM fungal species were over 98% similar to each other.

Plant protection by AMF

In the development of sustainable crop production practices the use of microbal inoculants as replacement for chemical fertilizers and pesticides is receiving attention. From the plant's perspective biological control can be considered a net positive result arising from a variety of specific and non-specific interactions. Mutualism is an association between two or more species where both species derive benefit. Sometimes it is an obligatory lifelong interaction involving close physical and biochemical contact such as those between plants and mycorrhizal fungi.

Different hypotheses have been proposed to explain bioprotection by AM fungi. These include (a) improvement of plant nutrition and root biomass in Mycorrhizal plants which could contribute to an increased plant tolerance and compensate for root damage caused by a pathogen (b) changes in root system morphology (c) modification of antagonistic microbial populations in the mycorrhizosphere and (d) competition between AM fungi and pathogenic fungi to colonize root tissues with the possible induction of resistance mechanisms.

Many of the microbes isolated and classified as biocontrol agents (BCAs) can be considered facultative mutualists involved in protocooperation because survival rarely depends on any specific host and disease suppression will vary depending on the prevailing environmental conditions. Further down the spectrum commensalism is a symbiotic interaction between two living organisms where one organism benefits and the other is neither harmed nor benefited. Most plant-associated microbes are assumed to be commensals with regards to the host plant because their presence individually or in total rarely results in overtly positive

or negative consequences to the plant. And while their presence may present a variety of challenges to an infecting pathogen an absence of measurable decrease in pathogen infection or disease severity is indicative of commensal interactions. Neutralism describes the biological interactions when the population density of one species has absolutely no effect whatsoever on the other. Related to biological control an inability to associate the population dynamics of pathogen with that of another organism would indicate neutralism. In contrast antagonism between organisms results in a negative outcome for one or both. Competition within and between species results in decreased growth activity and/or fecundity of the interacting organisms. Biocontrol can occur when non-pathogens compete with pathogens for nutrients in and around the host plant. Direct interactions that benefit one population at the expense of another also affect our understanding of biological control. Parasitism is a symbiosis in which two phylogenetically unrelated organisms coexist over a prolonged period of time In this type of association one organism usually the physically smaller of the two (called the parasite) benefits and the other (called the host) is harmed to some measurable extent. The activities of various hyperparasites i.e. those agents that parasitize plant pathogens can result in biocontrol. And interestingly host infection and parasitism by relatively avirulent pathogens may lead to biocontrol of more virulent pathogens through the stimulation of host defense systems. Lastly predation refers to the hunting and killing of one organism by another for consumption and sustenance. While the term predator typically refer to animals that feed at higher trophic levels in the macroscopic world it has also been applied to the actions of microbes e.g. protists and mesofauna e.g. fungal feeding nematodes and microarthropods that consume pathogen biomass for sustenance. Biological control can result in varying degrees from all of these types of interactions depending on the environmental context within which they occur. Significant biological control as defined above most generally arises from manipulating mutualisms between microbes and their plant hosts or from manipulating antagonisms between microbes and pathogens.

The role of Mycorrhizal fungi in control of various soil borne plant diseases has been reviewed by many workers and recently Adholeya[47] has discussed positive impact of AMF in sustainable agriculture. A few cases have been tabulated below (Table 3). Besides the association of AM fungi with plant nematodes and the beneficial effect of mycorrhizal symbiosis on plant growth had also led to investigations into the potential of AM fungi to limit yield losses due to nematodes[48].

During colonization AMF can prevent root infections by reducing the access sites and stimulating host defense. Various mechanisms also allow AMF to increase a plant's stress

Table 3 Crop disease management using AMF

Crop	Disease	Pathogen/Organism	Management by AMF
Zea mays	Black bundle	*Cephalosporium acremonium*	*Glomus fasciculatum G. mosseae* and *Acaulospora laevis*
Phaseolus vulgaris	Root rot	*Fusarium solani*	*G. mosseae G. intraradices G. clarum Gigaspora gigantea* and *Gi margarita*
Solanum torvum	Wilt	*Verticillium dahliae*	*G. mosseae*
Vigna unguiculata	Root rot	*Rhizoctonia solani*	*G. clarum*
Triticum aestivum	Take-all	*Gaeumannomyces graminis*	*G. mosseae*
Asparagus officinalis	Root rot	*Fusarium oxysporum* f sp. *asparagi* (Foa)	*Gi margarita G. fasciculatum* and *Glomus* sp. R10(GR)
	Root rot	*Phytophthora parasitica*	*G. mosseae* and *G. intraradices*
	Root rot	*Fusarium oxysporum* f sp. *lycopersici*	*G. mosseae*
Lycopersicon esculentum	Root rot	*Fusarium oxysporum* f sp. *radicislycopersici*	*G. intraradices*
	Root rot	*F oxysporum* f sp. *lycopersici*	*G. etunicatum*
	Wilt	*F oxysporum*	*G. mosseae*
Piper nigrum	Wilt	*F oxysporum*	*G. mosseae*
Medicago sativa	Wilt	*Verticillium albo-atrum F oxysporum* f sp. *medicaginis*	*Glomus* sp. *G. fasciculatum* and *G. mosseae*
Vigna unguiculata	Wilt	*F oxysporum*	*G. fasciculatum*
	Root rot	*Macrophomina phaseolina*	*G. fasciculatum*
Gossypium sp.	Root rot	*Verticillium dahliae*	*G. mosseae G. vesiformae* and *Scutellospora sinuosa*
Cicer arietinum	Wilt	*Fusarium oxysporum*	*G. fasciculatum*
	Root rot	*Fusarium udum*	*G. fasciculatum*
Cajanus cajan	Wilt	*Fusarium udum*	*G. margarita*
	Wilt	*Fusarium udum*	*G. mosseae*
Allium cepa	White rot	*Sclerotium cepivorum*	*Glomus* sp.
	Root rot	*Aphanomyces euteiches*	*G. intraradices*
Pisum sativum	Root rot	*Aphanomyces euteiches*	*G. fasciculatum*
Arachis hypogaea	Root rot	*F solani R solani*	*G. mosseae*
Citrus sp.	Root rot	*Phytophthora nicotianae*	*Acaulospora tuberculata*
Citrus reticulata	Root rot	*Fusarium solani*	*G. mosseae G. fasciculatum*
Pyrus sp.	Pear decline	Pear decline (PD) Phytoplasma	*G. intraradices*
Camellia sinensis	Charcoal stump rot	*Ustulina zonata*	*G. mosseae*
	Sclerotial blight	*Sclerotium rolfsii*	*G. mosseae G. fasciculatum*

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tolerance. This includes the intricate network of fungal hyphae around the roots which block pathogen infections AMF protect the host plant against root-infecting pathogenic bacteria. The damage due to *Pseudomonas syringae* on tomato may be significantly reduced when the plants are well colonized by mycorrhizae[49]. The mechanisms involved in these interactions include physical protection chemical interactions and indirect effects. The other mechanisms employed by AMF to indirectly suppress plant pathogens include enhanced nutrition to plants; morphological changes in the root by increased lignification; changes in the chemical composition of the plant tissues like antifungal chitinase isoflavonoids etc alleviation of abiotic stress and changes in the microbial composition in the mycorrhizosphere.

Mycorrhizal colonization significantly reduced the percentage of disease severity and incidence in infected bean plants. Among the potential mechanisms involved in the resistance of mycorrhizal systems the induction of plant defenses is the most controversial where a number of biochemical and physiological changes have been associated with mycorrhizal colonization. Alteration in isoenzymatic patterns and biochemical properties of some defense-related enzymes such as chitinases chitosanases and β-13-glucanases have previously been shown during mycorrhizal colonization[50]. These hydrolytic enzymes are believed to have a role in defense against invading fungal pathogens because of their potential to hydrolyze fungal cell wall. Stimulating the host roots to produce and accumulate sufficient concentrations of metabolites which impart resistance to the host tissue against pathogen invasion have also been reported[51]. AM colonization can reduce root diseases of horticultural crop[52] and plantation crop[53] caused by fungal pathogens.

Direct (via interference competition including chemical interactions) and indirect (via exploitation competition) interactions have been suggested as mechanisms by which AM fungi can reduce the abundance of pathogenic fungi in roots. These have generally been proposed in response to observations of negative correlations in the abundance of AM fungal structures and pathogenic microorganisms in roots. Presumably pathogenic and AM fungi exploit common resources within the root including infection sites space and photosynthates within the root. Interference competition may also arise if carbon availability within intercellular spaces and the rhizosphere or the number of infection loci within the root system is reduced as a result of AM fungal colonization. Moreover increasing the richness of AM fungal taxa colonizing the root system may result in more intense competition with a pathogenic fungus.

TERI (The Energy and Resources Institute) has developed an *in vitro* based mass production technology which successfully produces pure inoculum in large quantities and overcomes

the limitations of the soil based system. This inoculums has a long shelf life and is very concentrated with a small quantity itself carrying a very large number of propagules. This facilitates the application of desired amounts as it can be easily transported and mixed with a substrate of choice and applied as required[47]. They have been able to develop modern biotechnologies concerning mycorrhization with proven impact.

This technology coupled with bacterization would be more meaningful to 'biotize' *in vitro* propagated plantlets for transient transplant shock during acclimatization. The modern 'hairy-root technology' has played a significant role in establishment of AMF with transgenic host plants. The use of dual technology is promising as micropropagated plantlets are suitable platforms for understanding the mystery of host-endophyte interaction excessive production of secondary metabolites heavy metal tolerance bioprotection bioremediation and growth promoting activity. This will also help to unravel impediments for the growth and development of AMF in culture. Besides this technology offers an opportunity to apply molecular approaches to understand host-symbiont interactions secretion of flavonoids and signal transduction pathways. This may result in a more efficient and at the same time agriculturally and environmentally sustainable use of soil microorganisms for crop production.

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Chapter 3

Economically important *Bacillus* and related genera: a mini review

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Abstract

Members of the aerobic spore-forming *Bacillus* and related genera can be recovered from almost every niche in the environment. They are frequently isolated from both the natural habitat (soil and growing plants) and foods. In recent decades, there has been a growing interest in their biotechnological and economic importance. Members of *Bacillus* and related genera are known for the synthesis of wide range of medicinal, agricultural, pharmaceutical and industrial products. The present review is aimed to provide an overview of the various potentials that the organisms belonging to *Bacillus* and related genera have in biotechnological applications.

Keywords

Bacillus, aerobic bacteria, PGP, biocontrol

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Introduction

Microbial diversity is a major resource for biotechnological products and processes. Exploration of microbial diversity holds great promise because of the role of microbes in nutrient cycling, environmental detoxification and novel metabolic abilities in pharmaceuticals and industrial processes and act as a major resource for agricultural, industrial, and medicinal applications[1-3]. Bacteria are the most dominant group of this diversity which exists in diverse ecological niches, including extreme environments present in both lithosphere and hydrosphere, where their metabolic abilities play a critical role in geochemical nutrient cycling[2] and producing a wide range of products of industrial significance.

The gram-positive bacteria form an important part of the microbiota in many soils. In particular, the low G+C content gram-positive bacteria, which are divided into three classes — clostridia, mollicutes, and bacilli[4], play major roles in the mineralization of plant-derived materials, humus, pesticides and hydrocarbons in soil[5]. The traditional genus *Bacillus* represents one of the most diverse genera in the class bacilli. The members of this genus exhibit a wide range of DNA base compositions and major amino acid compositions of the cell walls[6,7]. It includes aerobic and facultatively anaerobic, rod-shaped, gram-positive spore -forming bacteria[5]. Recently, 16S rRNA gene sequence analysis has revealed a high level of phylogenetic heterogeneity in this genus, on the basis of which a division into different genera was proposed: *Bacillus, Alicyclobacillus, Paenibacillus, Brevibacillus, Aneurinibacillus, Virgibacillus, Salibacillus, and Gracilibacillus*[8]. Here the term "*Bacillus* and related genera" is used as an operational term to indicate these organisms.

Common physiological traits important to their survival include production of a multilayered cell wall structure, formation of stress-resistant endospores and secretion of peptide antibiotics, peptide signal molecules, and extracellular enzymes[9].

Members of *Bacillus* and related genera are used for the synthesis of a very wide range of important medical, agricultural, pharmaceutical and other industrial products. These include a variety of antibiotics, enzymes, amino acids and sugars[8]. They are major elements of the agronomic environment of plants, inhabiting the soil. Several reports have described the biodiversity, antibiotic production and plant growth-promoting effects of *Bacillus* and related genera (Table 1).

Bacillus and related genera as Plant growth promoting (PGP) agents

Microbial production of secondary metabolites that can promote or constrain plant growth has often been found to be finely tuned, controlled by environmental conditions, medium

Table 1: Economically important *Bacillus* and related genera

***Bacillus* and related genera**	**Functions/activities reported**
Bacillus spp.[12,13]	Plant growth promoting (PGP)
Bacillus spp.[15]	PGP (increasing nutrient availability of the plants)
Bacillus licheniformis[16]	Biofertiliser
Bacillus spp.[17]	Increase plant health and survival rates of micropropagated bananas
Bacillus spp[18]	Increase the yield, growth and nutrition of raspberry plant
Bacillus megaterium[19]	Improve different root parameters (rooting performance, root length and dry matter content of root) in mint
Bacillus megaterium var. phosphaticum[20]	Phosphate solubilising
Bacillus mucilaginosus[21]	Potassium soubilising
Bacillus pumilus 8N-4[22]	Bio-inoculant for biofertilizer production
Brevibacillus brevis[23]	Improve *in vitro* spore germination and growth of *Glomus mosseae*
Paenibacillus spp[11]	Plant growth promoting (PGP)
Paenibacillus polymyxa[25]	Nitrogen fixing ability
Paenibacillus polymyxa[26]	Produce plant growth promoting compounds similar in activity to indole-3-acetic acid
Paenibacillus polymyxa[27-29]	Releases iso-pentenyladenine and unknown cytokinin-like compound which promotes seed germination, *de novo* bud formation, release of buds from apical dominance, stimulation of leaf expansion and reproductive development and retardation of senescence
Paenibacillus polymyxa[30]	Affects growth parameters of wheat and spinach plants
Paenibacillus elgii[31]	Chitinolytic, antifungal, and mineral phosphate solubilization abilities and enhanced the growth of groundnut and tobacco plant
Lysinibacillus sp. NF-4[32]	A potential N_2 fixing bacteria, harbouring *nifH* gene
Lysinibacillus fusiformis[33]	Plant growth promotion of ginseng
Bacillus subtilis[34]	Biocontrol agent, produce different biologically active compounds with a broad spectrum of activity
Bacillus megaterium[35]	Produce antifungal metabolite
B. thuringiensis[36]	Potent biopesticides
Bacillus sphaericus[36]	Potent biopesticides
B. thuringiensis	Infects protozoa, nematodes, flatworms, mites and insects that are either plant pests or human and animal health hazards
Bacillus thuringiensis (kurstaki)[37]	Control lepidopteron pests

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***Bacillus* and related genera**	**Functions/activities reported**
Bacillus sphaericus[38]	Control lepidopteron pests
Paenibacillus lentimorbus NRRL B-30488[24]	Biocontrol activity against a phytopathogenic *Fusarium oxysporum*
Paenibacillus polymyxa strain P13[39]	Produce and secrete a compound, named Polyxin, effective against a wide range of gram-positive and gram negative bacterial species including food-borne pathogens
Brevibacillus laterosporus strain BPM3[40]	Strongly inhibited growth of phytopathogenic fungi and gram -positive bacterium
Lysinibacillus fusiformis[41]	Biological activity against *Phytophthora cinnamomi*
Lysinibacillus sphaericus C3-41[42]	Used as an insecticide
Bacillus subtilis MA9[48]	Produce thermostable α-amylase enzyme
Bacillus cereus MK8[48]	Produce thermostable α-amylase enzyme
Bacillus subtilis (natto)[49]	Mediated fermentation of soybean food, *natto*
B. subtilis (natto)[50]	Produces amylases, cellulases and important proteases
B. subtilis [51]	Produce significant amounts of D-ribose
B. pumilus[51]	Produce significant amounts of D-ribose
Bacillus spp.[52]	Produce antibiotics (low-molecular-weight peptides) which possess different biological activities, including antimicrobial, antiviral, and antitumor activities
Paenibacillus polymyxa strains[53-55]	Produce cell wall degrading enzymes such as β-1,3-glucanases, cellulases, chitinases, proteases, xylanase
Paenibacillus polymyxa[56]	Cell wall β-glucans that act as immunostimulants or as adjuvant of some animal vaccines
Brevibacillus thermoruber 438[57]	Produce exopolysaccharides which plays an important role in medicine, dairy industry, biofilms and corrosion, and their applications in the field of biotechnology

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components, and influenced by root activities. Plant-stimulatory effects exerted by plant growth-promoting bacteria (PGPB) might also be due to an enhanced availability of limited plant nutrients such as nitrogen, phosphorus, vitamins and amino acids in the rhizosphere, caused by phosphate-solubilizing and diazotrophic bacteria[10,11].

Bacillus is the most abundant genus in the rhizosphere, and the PGP activity of some of these strains has been known for many years, resulting in a broad knowledge of the mechanisms involved[12,13]. There are a number of metabolites that are released by these strains[14], which strongly affect the environment by increasing nutrient availability of the plants[15]. Being present naturally in the immediate vicinity of plant roots, *Bacillus subtilis* is able to maintain stable contact with higher plants and promote their growth. *Bacillus licheniformis* when inoculated on tomato and pepper shows considerable colonisation and can be used as a biofertiliser without altering normal management in greenhouses[16]. Jaizme-

Vega *et al*.[17] evaluated the effect of a rhizobacteria consortium of *Bacillus* spp. on the first developmental stages of two micropropagated bananas and concluded that this bacterial consortium can be described as a prospective way to increase plant health and survival rates in commercial nurseries. *Bacillus* is also found to have potential to increase the yield, growth and nutrition of raspberry plant under organic growing conditions[18]. *Bacillus megaterium* is very consistent in improving different root parameters (rooting performance, root length and dry matter content of root) in mint[19]. The phosphate solubilizing *Bacillus megaterium* var. *phosphaticum* and potassium solubilising *Bacillus mucilaginosus* when inoculated in nutrient limited soil consistently increased mineral availability, uptake and plant growth of pepper and cucumber, suggesting its potential use as fertilizer[20,21]. The *Bacillus pumilus* 8N-4 has been proposed to be used as a bio-inoculant for biofertilizer production to increase the crop yield of wheat variety Orkhon in Mongolia[22].

Brevibacillus brevis was found to improve *in vitro* spore germination and growth of *Glomus mosseae* by increasing the presymbiotic growth (germination rate growth and mycelial development)[23]. *Paenibacillus* spp. isolated from milk was found to possess PGP traits[24]. Nitrogen fixing ability by *Paenibacillus polymyxa* was demonstrated by Guemori-Athmani *et al*.[25]. The production of plant growth promoting compounds by *P. polymyxa* similar in activity to indole-3-acetic acid has been suggested to stimulate growth in crested wheatgrass[26]. It also releases iso-pentenyladenine and one unknown cytokinin-like compound during its stationary phase of growth which promotes seed germination, *de novo* bud formation, release of buds from apical dominance, stimulation of leaf expansion and reproductive development and retardation of senescence[27] in wheat[28,29]. The effect of inoculation with *P. polymyxa* on growth parameters of wheat and spinach plants was observed[30]. *Paenibacillus elgii* which was positive for chitinolytic, antifungal, and mineral phosphate solubilization abilities enhanced the growth of groundnut in terms of shoot height, root length, total chlorophyll, and fresh and dry weight when applied alone or in combination with chitosan. The plant growth-promoting activity of *P. elgii* was seen in tobacco in a specially designed gnotobiotic setup indicating its capability to promote growth of at least groundnut and tobacco[31].

A potential N_2 fixing bacteria, *Lysinibacillus* sp. NF-4 harbouring *nif*H gene, was isolated from raw coir pith[32]. *Lysinibacillus fusiformis* was reported to be positive for most of the plant growth promoting traits, indicating their role in growth promotion of ginseng[33].

Bacillus and related genera as biocontrol agents

Production of extracellular enzymes by biologic control bacteria is a well-documented phenomenon that has long been thought to be involved in the lysis of the phytopathogenic

fungal cell wall. *Bacillus subtilis* is widely recognized as a powerful biocontrol agent. In addition, due to its broad host range, its ability to form endospores and produce different biologically active compounds with a broad spectrum of activity, *B. subtilis* as well as other *Bacilli* are potentially useful as biocontrol agents[34]. *Bacillus megaterium* from tea rhizosphere is able to produce antifungal metabolite and thus it helps in the plant growth promotion and reduction of disease intensity[35]. Research of almost 85 years reveals that *Bacillus* spp., especially *B. thuringiensis* and *Bacillus sphaericus* are the most potent biopesticides[36]. Available information depict that *B. thuringiensis* is a versatile pathogen capable of infecting protozoa, nematodes, flatworms, mites and insects that are either plant pests or human and animal health hazards[37].Two strains [*Bacillus thuringiensis* (kurstaki) and *Bacillus sphaericus*] have the ability to help in the control of the lepidopteron pests[38].

Paenibacillus lentimorbus NRRL B-30488 isolated from milk showed biocontrol activity against a phytopathogenic *Fusarium oxysporum* by alteration and distortion of the hyphal cell wall through the action of its chitinase and β-1,3-glucanase enzymes[24]. *P. polymyxa* strain P13, isolated from Argentinean regional fermented sausages, was found to produce and secrete a compound, named polyxin, that inhibited the growth of *Lactobacillus* strains. This antimicrobial compound is effective against a wide range of gram-positive and gram negative bacterial species including food-borne pathogens[39].

Brevibacillus laterosporus strain BPM3 isolated from mud of a natural hot water spring of Nambar Wild Life Sanctuary, Assam, India, strongly inhibited growth of phytopathogenic fungi (*Fusarium oxysporum f. sp. ciceri, F. semitectum, Magnaporthe grisea* and *Rhizoctonia oryzae*) and gram-positive bacterium (*Staphylococcus aureus*)[40].

A strain of *Lysinibacillus fusiformis* isolated from the roots of *Persea americana* (avocado) trees was identified as a potential biocontrol agent as it showed biological activity against *Phytophthora cinnamomi*[41]. *Lysinibacillus sphaericus* C3-41(previously, *Bacillus sphaericus* C3-41) is used as an insecticide because its spores release endotoxins that kill mosquito larvae. Because the bacteria is short-lived and are not harmful to humans and other animals, *Lysinibacillus sphaericus* C3-41 is an ideal insecticide[42].

Bacillus and related genera as biotechnological agents in industrial processes and production

Each single strain of organism produces a large number of enzymes which include the function such as hydrolyzing, oxidizing or reducing, and metabolic in nature. But the absolute and relative amounts of the various individual enzymes produced vary markedly between species and even between strains of the same species. Amylase, lipase, protease

and cellulase constitute a very important part of microbial enzymes that are used in food, pharmaceutical, textile, paper, leather, and other industries. The world market for industrial enzymes is estimated to be US$1.6 billion, split between food enzymes (29%), feed enzymes (15%), and general technical enzymes (56%)[43].

It is estimated that *Bacillus* spp. enzymes make up about 50% of the total enzyme market[44]. Bacterial amylase preparations at elevated temperatures give rapid liquefaction of starch. A significant application of the bacterial enzyme is in the continuous process for desizing of textile fabrics[45,46]. Another is in preparing modified starch sizing for textiles[45] and starch coatings for paper[47]. Two *Bacillus* strains [*Bacillus subtilis* MA9 and *Bacillus cereus* MK8] isolated from eastern Himalayan region has been reported to produce thermostable α-amylase enzyme[48].

Food fermentations mediated by *Bacillus*, can provide insight into some of the potential industrial properties of the genera or species involved. *Bacillus subtilis* (natto) is used in Japan for producing the fermented soybean food, natto[49]. While *B. subtilis* (natto) produces many enzymes, including amylases and cellulases, the most important enzymes in the production of natto are proteases; two proteases having a pH optima of 8.5 and 10.3–10.8 have been characterized[50]. The proteases are responsible for the main flavor, through hydrolysis of soybean protein.

D-Ribose is frequently used as a flavor enhancer in food, health food and animal feed. Several strains of *B. subtilis* and *B. pumilus* and their mutants are reported to produce significant amounts of D-ribose[51]. Recent developments in genetic engineering and fermentation technology have contributed to improvements in D-ribose productivity by *Bacillus* fermentations.

Most members of the genus *Bacillus* are able to produce antibiotics. Interestingly, the majority of these antibiotics are low-molecular-weight peptides, which possess different biological activities, including antimicrobial, antiviral, and antitumor activities[52].

Different strains of *Paenibacillus polymyxa* were reported to produce cell wall degrading enzymes such as β-1,3-glucanases, cellulases, chitinases, proteases[53,54] and xylanase[55]. β-glucans, or glucose polymers found in the cell walls of *Paenibacillus polymyxa*, have been found to have beneficial effects on the immune system of experimental animals. This investigation supported the idea that β-glucans isolated from *Paenibacillus polymyxa* can be used as immunostimulants or as adjuvant of some animal vaccines[56].

In their study, Radchenkova *et al.*[57] isolated a thermophilic bacterial strain, *Brevibacillus thermoruber* 438, which was a high producer of exopolysaccharides which plays an

important role in medicine, dairy industry, biofilms and corrosion, and their applications in the field of biotechnology.

Conclusion

The reports reviewed in this work demonstrate just how effective *Bacillus* and its related genera are in playing important roles as dominant bacteria in biotechnological applications as well as industrial processes and products. And, with recent advances in biotechnology, the economic contributions that these organisms can make in biotechnological applications and industrial processes can be exploited further for large scale benefit of mankind.

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Chapter 4

Plant derived anti-diabetics and antioxidants: potential health benefits in free radical associated diseases

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Abstract

In today's modern world the risk of diseases due to oxidative stress is compounded by unhealthy lifestyle, exposure of chemicals, pollution, cigarette smoking, drugs, illness, and different kinds of stress. With increasing percentage of world population suffering from complex metabolic disorders such as diabetes mellitus and other oxidative stress related diseases, there is an urgent need for safe health care solutions. In this context, the use of plant-derived compounds with almost no side effects for cure of such complex disorders provides a tempting solution. A good amount of research is now going on in this field and exploration of yet unexplored regions to identify plants with potential curative compounds which can be commercially used is in progress. For this purpose, many ethno-botanical surveys on medicinal plants used by the local population of different regions have been performed in different parts of the world and a number of plants have been identified which contain high amount of antioxidants and/or anti-diabetic compounds. However, further studies are in progress to isolate and characterize such compounds which may be developed into clinically useful medicines. Besides, for a better understanding of how these compounds can be fully utilized, a thorough knowledge of their mechanisms of action and their targets in the human body are needed and efforts are on throughout the world to unravel the mechanisms. In the present review, efforts have been made to collate the available data on plants with hypoglycaemic and antioxidative effects.

Keywords

Diabetes, Oxidative stress, Anti-diabetic compounds, Antioxidants

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Introduction

Diabetes mellitus is metabolic disorder characterized by hyperglycemia and alterations in carbohydrate, fat and protein metabolism, associated with absolute or relative deficiencies in insulin secretion and/or insulin action[1]. Such a deficiency results in increased concentrations of glucose in the blood, which in turn damage many of the body's systems, in particular the blood vessels and nerves. In particular, type 2 diabetes mellitus (T2DM) is the most encountered form of diabetes, accounting for more than 80% of the total cases of diabetes[2,3]. Glucose metabolism disturbances are major factors leading to diabetes. The insulin released by the pancreatic β-cells is the hormone responsible for glucose homeostasis[4,5]. Insulin stimulates hepatocytes, myocytes, and adipocytes to uptake glucose from the circulatory system. Depending on need, glucose can either be used as an energetic source by glycolysis, or alternatively, stored as glycogen inside muscle or liver cells. The inappropriate utilization of insulin leads to insulin resistance, which is characterized by the inability of cells to respond to normal levels of circulating insulin[2], thus leading to the occurrence of the disease. As the number of people with diabetes multiplies worldwide, the disease takes an ever-increasing proportion of national and international health care budgets. It is projected to become one of the world's main disablers and killers within the next 25 years. Regions with greatest potential are Asia and Africa, where DM rates could rise to two to three-folds than the present rates[6]. It is the most prevalent disease in the world affecting 25% of population and afflicts 150 million people and is set to rise to 300 million by 2025[7]. It causes number of complications like retinopathy, neuropathy, and peripheral vascular insufficiencies[8]. Diabetes is still not completely curable by the present anti diabetic agents. Insulin therapy is the only satisfactory approach in diabetic mellitus, even though it has several drawbacks like insulin resistance[9], anorexia, brain atrophy and fatty liver in chronic treatment[10]. Herbal drugs are gaining popularity in the treatment of diabetes mellitus[11].

Another area of concern in today's world is the fast life style of people, along with their changing food habits leading to stress and diseases associated with free radicals. Free radicals induced oxidative stress is now believed to be a fundamental mechanism underlying a number of human cardiovascular, neurologic and other disorders. Treatment of such diseases depends on the ability of compounds, i.e., antioxidants to scavenge the harmful free radicals and help to decrease the incidence of oxidative stress induced damage. Plants play a major role as sources of antioxidants and in traditional herbal medicines, dietary foods were the main source of antioxidant for ancient people that protected them from the damage caused by free radicals.

Medicinal plants, since time immemorial, have been used in virtually all countries as a

source of medicine. It has been estimated that about 80-85% of population both in developed and developing countries rely on traditional medicine for their primarily health care needs and it is assumed that a major part of traditional therapy involves the use of plant extracts or their active principles[12-14]. Globally, about 85% of the traditional medicines used for primary healthcare are derived from plants. Traditional medicine and ethnobotanical information play an important role in scientific research, particularly when the literature and field work data have been properly evaluated[15].

Thus, there is no doubt that plants are the sources of various compounds useful for the treatment of a variety of disease. The present article will focus on two very important properties of plants which has been traditionally exploited- antidiabetics and antioxidative.

Antidiabetic activities

India is a varietal emporium of medicinal plants and is one of the richest countries in the world in regard to genetic resources of medicinal plants. It exhibits a wide range in topography and climate, which has a bearing on its vegetation and floristic composition.

There are more than 1000 plant species being used for the treatment of T2DM worldwide[16]. In parts of the world where the population has restricted access to the healthcare system, the use of medicinal plants for the treatment of T2DM is widespread. In many cases, very little is known about the mechanism of action of traditionally used antidiabetic plants, thus preventing them from being used in standard diabetes care. Recently, more research is being focused on elucidating the action of these plants and their active constituents. Herbal medicine has played an important role in treating diabetes in Asia, India and Africa for centuries. Jung *et al.*[17] reviewed the hypoglycemic effects of many plants that are used as anti-diabetic remedies, as well as anti-diabetic natural products discovered during 2001–2005. With the rapid advancement of novel technologies and the increased research on anti-diabetic natural products, many new plants, their extracts, and their active principles have been found to exhibit anti-diabetic effects, which may provide us with valuable leads to develop as novel anti-diabetic agents to supplement the current chemotherapies[18]. Table 1 presents a number of plants that are currently used for their antidiabetic properties, together with their active biomolecules.

Indian context

Grover *et al.*[61] has reviewed 45 such plants and their products (active, natural principles and crude extracts) that have been mentioned/used in the Indian traditional system of medicine and have shown experimental or clinical anti-diabetic activity. Indian plants which are most effective and the most commonly studied in relation to diabetes and their complications are:

Table 1: List of reported anti-diabetic compounds from various plants

Plants	Antidiabetic compound	Reference
Allium sativum	Allicin and *S*-allyl cysteine sulfoxide	19, 20
Catharanthus roseus	Vindoline, vindolinine and vleurosine	21
Trigonella foenum-graecum	Saponins, amino acid 4-hydroxyisoleucine, trigonelline	22,23
Gymnema sylvestre	Gymnemic acids I-VII, gymnemosides a-f, protein-bound polysaccharide components and glycosaminoglycans	24,25
Ephedra sinica and E. distachya.	Glycans and ephedrans A-E	26
Gallega officinalis	Galegine	27,28
Cornus officinalis	Ursolic acid and oleanolic acid	29
Curcuma longa	curcumin, demethoxycurcumin, bisdemethoxycurcumin, ar-turmerone	30
Artemisia dracunculus	Davidigenin, sakuranetin, 2',4'-dihydroxy-4-methoxydihydrochalcone, 4,5-di-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, 6 demethoxycapillarisin	31,32,33, 34
Aegle marmelos	Aegeline 2, an alkaloidal-amide	35
Cinnamonum zeylanicum	Cinnamaldehyde	36
Angelica keiskei	4-hydroxyderricin and xanthoangelol	37
Aronia melanocarpa	Anthocyanins	38
Pongamia pinnata	Pongamol and karanjin	39
Salacia reticulata	Salacinol, kotalanol, de-O-sulfated salacinol, de-O-sulfated kotalanol, ponkolanol, salaprinol	40
Syzygium cumini	Mycaminose	41,16
Swietenia macrophylla	Swietenine(tetra-nortriterpenoid)	42
Bidens pilosa L.	Polyacetylenic glucosides	43,16
Bauhinia forficate	Kaempferol-3-neohesperidoside (insulin mimetic)	44,16
Capparis moon	Gallotannins (chebulinic acid derivatives)	45
Swertia punicea	Methylswertianin, bellidifolin	46
Panax japonicus	Polyacetylenes, phenolic compounds, one sesquiterpenoid, one sterol glucoside	47
Stevia rebaudiana	Alkaloids, flavonoids	48
Phaseolus vulgaris L.	Alkaloids, flavonoids, fiber, proteins, tannins, terpenoids, saponins, quercetin, anthocyanin, catechin	49
Rhododendron tomentosum	Quercetin	50
Ruta graveolens	Rutin	51
Pinus pinaster	Polyphenols: proanthocyanidins, catechin, epicatechin	52,53

Table 1: continued to next page

Table 1: *continued from previous page*

Plants	Antidiabetic compounds	Reference
Eleutherine americana	Eleutherinoside A	54
Aquilaria sinensis	Mangiferin, iriflophenone 2-*O*-α-L-rhamnopyranoside, iriflophenone 3-*C*-β-D-glucoside, iriflophenone 3,5-*C*-β-D-diglucopyranoside	55
Carissa carandas	Gallic acid, flavonoids	56
Piper retrofractum	Piperidine alkaloids: piperine, pipernonaline, dehydropipernonaline	57
Marrubium vulgare	Flavonoids	58
Morus alba	(2S)-euchrenone, chalcomoracin, moracin C, moracin D, moracin N, (2R)/(2S)-euchrenone, moracin N, quercetin, norartocarpetin, several flavanes	59
Ocimum sanctum	Polyphenols, caffeic acid, p-coumaric acid	60
Solanum xanthocarpum	Polyphenols, caffeic acid	60

Allium cepa, Allium sativum, Aloe vera, Cajanus cajan, Coccinia indica, Caesalpinia bonducella, Ficus bengalenesis, Gymnema sylvestre, Momordica charantia, Ocimum sanctum, Pterocarpus marsupium, Swertia chirayita, Syzigium cumini, Tinospora cordifolia and Trigonella foenum graecum. Among these they have *evaluated M. charantia, Eugenia jambolana, Mucuna pruriens, T. cordifolia, T. foenum graecum, O. sanctum, P. marsupium, Murraya koeingii and Brassica juncea*. All plants have shown varying degree of hypoglycemic and anti-hyperglycemic activity. Chakraborty and Das[62] reported on the anti diabetic activities of *Cinnamomum tamala* and it was also reported by them that *Scoparia dulcis* had antihyperglycemic effect in mice where diabetes had been induced by streptozotocin[63]. Many plant-based products have been suggested as potential antidiabetic agents, but few have been shown to be effective in treating the symptoms of Type 2 diabetes mellitus (T2DM) in human studies, and little is known of their mechanisms of action. Extracts of *Gymnema sylvestre* (GS) have been used for the treatment of T2DM in India for centuries. In a study conducted by Al-Romaiyan *et al.*[64], authors reported the effects of a novel high molecular weight GS extract, Om Santal Adivasi, (OSA(R)) on plasma insulin, C -peptide and glucose in a small cohort of patients with T2DM. Oral administration of OSA (R) (1 g/day, 60 days) induced significant increases in circulating insulin and C-peptide, which were associated with significant reductions in fasting and post-prandial blood glucose. *In vitro* measurements using isolated human islets of Langerhans demonstrated direct stimulatory effects of OSA(R) on insulin secretion from human ß-cells, consistent

with an *in vivo* mode of action through enhancing insulin secretion. These *in vivo* and *in vitro* observations suggest that OSA(R) may provide a potential alternative therapy for the hyperglycemia associated with T2DM.

Targets for anti-diabetic medicinal plants and isolated natural products

There is no doubt that a large number of plants possess antidiabetic properties and in most cases the chemical nature of the active principles have also been worked out. However, the mechanism of action of the compounds- and their targets in the human body are still being worked out.

In an analysis of 728 patent applications claiming diabetes as an indication during 2008–2010, the highest patent counts were associated with eight anti-diabetic targets: 11b-HSD1, DGAT1, DPP-4, glucokinase (GK), GPR119, PPAR-a, -d, -g, SGLT1/2, and stearoyl-CoA desaturase1 (SCD1)[65]. 11b-Hydroxysteroid dehydrogenase 1 (11b-HSD1) localizes to the ER and mediates the inactivation of glucocorticoids (mentioned above), as well as catalyzes the inter-conversion of cortisone and cortisol. The possibility that blockage of 11b-HSD1 can be utilized in the treatment of type-2 diabetes is quite high considering the role of glucocorticoids in the development of whole-body insulin resistance and the over expression of 11b-HSD1 in visceral adipose[65]. The initial step in glycolysis is catalyzed by Glucokinase (GK) which is a key determinant of carbon flux through the glycolytic, glycogen synthesis, pentose phosphate shunt, and gluconeogenic and lipogenic pathways. Carpino and Goodwin[65] suggested that activation of GK in the liver and pancreas will be an effective strategy for lowering blood glucose by up-regulating hepatic glucose utilization, down-regulating hepatic glucose output, and normalizing glucose-stimulated insulin secretion. Renal re-absorption of glucose is critical in the maintenance of plasma glucose levels, and this re-absorption is mediated by two sodium-dependent glucose co-transporters, SGLT1 and 2[66]. According to these authors, the most compelling evidence in support of targeting renal glucose re-absorption for the management of type-2 diabetes comes from human genetics studies, indicating that individuals with renal glycosuria (mutations in SGLT gene) rarely exhibit hypoglycemia or hypovolemia. Phlorizin, a non-selective inhibitor of SGLT1 and 2, lowered blood glucose levels, however, with unwanted side effects[67,68] while sergliflozin and remogliflozin, which are selective SGLT2 inhibitors, stimulated urinary glucose excretion without any increase in insulin secretion or any discernable effects on normoglycemia or electrolyte balance[69,70]. SCD1 has been implicated in non-alcoholic fatty liver disease, which can often lead to insulin resistance. Global SCD1 inhibition or antisense -mediated SCD1 inhibition in adipose and liver has been shown to decrease lipogenesis and increase fatty acid oxidation in rodents maintained on high-fat diets. However, there are side

effects associated with the systemic SCD1 inhibitors, such as closed eye fissure and skin barrier dysfunction, which have limited the safety profiles[71].

Role of Antioxidants in disease reduction

Cellular damage or oxidative injury arising from free radicals or reactive oxygen species (ROS) now appears the fundamental mechanism underlying a number of human neurodegenerative disorders, diabetes, inflammation, viral infections, autoimmune pathologies and digestive system disorders. Free radicals are generated through normal metabolism of drugs, environmental chemicals and other xenobiotics as well as endogenous chemicals, especially stress hormones (adrenalin and noradrenalin). Accumulated evidence suggests that ROS can be scavenged through chemoprevention utilizing natural antioxidant compounds present in foods and medicinal plants. Antioxidants are our crucial defense against free radical induced damage, and are critical for maintaining optimum health and wellbeing. It has been estimated that ~ 5% of inhaled oxygen is converted into several damaging ROS species like superoxide, hydroxyl and hydrogen peroxide by equivalent reduction of oxygen[72]. In today's modern world the risk of diseases due to oxidative stress is compounded by unhealthy lifestyle, exposure of chemicals, pollution, cigarette smoking, drugs, illness, and stress etc. Exogenous consumption of antioxidants from plant, animal, and mineral sources have proved beneficial to human health and effective to reduce the incidence of free radical induced diseases.

Most of the information available regarding the use of traditional medicines which have been prevalent in Indian, Chinese, Egyptian, Greek, Roman and Syrian traditional medicine reveal that all of them are based on different plants/ plant products. From ancient times, knowingly or unknowingly people have been taking different fruits, vegetable, food or medicine from plant, animal and mineral sources which contain antioxidant constituent and are responsible for several health benefits. In recent years, on a global scale there is substantial and justifiable recognition of the many benefits of orthodox medicine. Many compounds from natural sources have been used as drugs, either in their original or semi-synthetic form. Plant derived constituents can also serve as drug precursors, drug prototypes, and pharmacological probes[73,74,75]. With the advancement in science, one of the foremost areas of research involves the isolation, characterization, purification and testing of plant products to be used in medicine.

Phytochemicals as antioxidants

Plant constituents are the major source of antioxidants. The majority of these phytochemicals are redox active molecules; hence they are active to maintain redox balance

and are therefore defined as antioxidants. In the body, the balance between pro oxidants and anti-oxidants are generally well maintained, but if the system favors pro-oxidants at any time, this leads to oxidative stress followed by various diseases. The chemicals from plants are generally classified as primary or secondary constituents, depending on their role in plant metabolism. Antioxidant phyto-constituents are the secondary constituents or metabolites found naturally in plants such as fruits and vegetables. Plants produce an extremely impressive array of antioxidant compounds such as carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols and tocotrienols to prevent oxidation of the susceptible substrate. These plant-based antioxidants are believed to have a better biological effect than the synthetic ones, because phytoconstituents are a part of the physiological functions of living flora and hence they are believed to have better compatibility with human body. Antioxidants of plant origin with free-radical scavenging properties could have great importance as prophylactic and therapeutic agents in several diseases caused due to oxidative stress[76,77]. A list of some of the antioxidant compounds

Table 2: List of antioxidants from plants

Plants	Antioxidant compound	Reference
Withania somnifera	Steroidal lactone, withanolides, glycine, withanine	78,79
Solanum nigrum	Polyphenolic compounds, Flavonoids, steroids	80
Daucus carota	Carotenes, carotenoids, glycosides, Flavonoids, sugars, quaternary bases	81
Cuscuta reflexa	Flavonoids, dulcitol,bergenin, coumarins, glycosides, lac-tone	82
Asplenium bulbiferum	Antioxidant flavonoids:Kaempferol glucosides	83
Dryopteris crassirhi-zoma	Flaspidic acid PB, flavaspidic acid AB	84
Helminthostachys zey-lanica	Flavonoids, ugonins	71
Glycyrrhiza glabra	Glycyrrhizin, Flavonoids, liquiritin, isoliquiritin, rhamno-liquiritin, 2-methylisoflavones	85
Huperzia selago	Huperzine A	86
Equisetum telmateia	Flavan-3-ol, kaempferol, A-type proanthocyanidins, afzelechin	87
Capsicum annuum	Trans-p-feruloyl-*β*-D-glucopyranoside, trans-p-sinapoyl- *β* --D-glucopyranoside, quercetin 3-O-R-L-rhamnopyranoside-7-O-*α*-D glucopyranoside, arabino-pyranoside, lutoeolin	88
Abacopteris penangiana	Flavan-4-ol, glycosides, abacopterins	89
Psidium guajava	quercetin, quercetin-3-*O*-glucopyranoside and morin	90
Equisetum arvense	Isoquercetin, di-E-caffeoyl-mesotartaric acid	91
Acer tegmentosum	6’-*O*-galloylsalidroside, Quercetin	92

from different plant species has been provided in Table 2.

The relation between free radicals, antioxidants and functioning of various organs and organ systems is highly complex and the discovery of redox signaling is a landmark in this regard. In recent years, antioxidants have gained a lot of importance and are emerging as potential prophylactic and therapeutic agents in many diseases. The discovery of the role of free radicals in pathogenesis of different disease has led to a medical revolution that is assuring a new paradigm of healthcare.

Mechanisms

Antioxidants act against free radicals by an array of different mechanisms including (a) affecting the catalytic systems to neutralize or divert ROS, (b) binding or inactivation of metal ions preventing generation of ROS by Haber-Weiss reaction, (c) suicidal and chain breaking antioxidants scavenge and destroy ROS, (d) absorbing energy, electron & quenching of ROS. Antioxidants may also function as immune modulators and can be used for prophylaxis or therapy of certain diseases along with the mainstream therapy. Supplements of exogenous antioxidants can act directly to quench the free radical or free radical reactions, prevent lipid peroxidation and also boost the endogenous antioxidant system and hence deliver the prophylactic or therapeutic activity.

Conclusion

Many novel approaches and significant findings have come to light in the last few years. The natural food, spices and medicinal plants are rich sources of antioxidants and can be prime resources to treat oxidative stress induced diseases. Several antioxidants epigallocatechin-3-*O*-gallate, lycopene, ellagic acid, coenzyme Q10, indole-3-carbinol, genistein, quercetin, vitamin C and vitamin E have been found to be pharmacologically active as prophylactic and therapeutic agents. Therefore targeting oxidative stress or boosting the endogenous levels of antioxidants by the use of antioxidants is likely to have beneficial outcome in the management of several disorders[93,94].

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Chapter 5

Trends in biochemical and molecular characterization of rhizobia and their nitrogen fixation mechanism: a review

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Abstract

Bacteria of the genus *Rhizobium* are a genetically diverse and physiologically heterogeneous group of microorganisms that are nevertheless classified together by virtue of their ability to nodulate groups of plants of the family Leguminosae, where atmospheric nitrogen is being fixed. These rhizobial isolates were traditionally characterized using biochemical and molecular tools, however, physiological characters such as growth rates as well as morphological characters such as colony shape, size, cell shape etc., were also invariably utilized for this purpose. In recent research, these traditional characterizations have been replaced largely by molecular methods. The traditional methods together with serological techniques are of special interest during modern day's research. Molecular characterization was initially done by mere comparison of plasmids followed by DNA: DNA homology followed by simple PCR amplification using rhizobial oligonucleotide primers for intergenic spacer (IGS) regions of 16S-23S ribosomal genes (rDNA). Additionally, diversity studies of different strains of rhizobia are generally done using random amplified polymorphic DNA (RAPD), Amplified fragment length polymorphism (AFLP), Restriction fragment length polymorphism (RFLP) etc. however, 16S rDNA of conserved regions have been invariably used for such studies. Modern research, however, focus on the expression of different cloned *Rhizobium nif* D, *nif*H, or *nif*DH. However, nitrogen fixation is of prime importance and it is brought about by the rhizobial-legume symbiosis. The regulation of the *nod* genes is strictly maintained for symbiosis and

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strong expression has been shown to inhibit nodulation. Synthesis of Nod factors are a major determinant of host range and its production is mediated by proteins encoded by other *nod* genes. Nod factors also participate in signal transduction especially during host rhizobial symbiosis interaction. The induction of the *fix* genes and the switch to microaerobic respiration is one of the most critical developments in bacteroid differentiation and must occur before nitrogenase biosynthesis, due to the enzymes oxygen labile nature. The *nod*, *nif* and *fix* genes of *R .leguminosarum* all lie within a 36 kb region of the sym plasmid pRL10 with a characteristic low G+C ratio. The *nifA*, *nifD*, *nifK* and *nifH* are the structural and regulator genes for dinitrogenase and dinitrogenase reductase activities. The hierarchical regulatory cascade of rhizobia displays transcription of *nifA* and *fixK* at an oxygen concentration where NifA activity is prevented. This enables expression of the *fix* genes required for microaerobic growth at the relatively higher oxygen concentration found in certain areas of the nodule without expression of *nif* genes, at a concentration where nitrogenase would be inactivated.

Keywords

Biochemical tests, polymerase chain reaction, molecular characterization, nitrogen fixation, *nif,* dversity

Introduction

The genus *Rhizobium* can be described as a heterogeneous group of Gram-negative, hetero-trophic, aerobic, non-sporeforming rods which have the ability to invade and form nodules on the roots of leguminous plants[1] of agricultural and environmental importance, in a process of biological nitrogen fixation[2-3]. Rhizobia are among the best known beneficial plant-associated bacteria because of the importance of the nitrogen fixation that occurs during the *Rhizobium*-legume symbiosis[4]. This genus have been divided into two groups[1,5] based on their growth habits. One is fast grower, which produce acid on yeast-mannitol-agar medium. This type includes *R. phaseoli, R. trifolii, R. leguminosarum* and *R. meliloti* and the other is slow growing species which produce alkali on yeast-mannitol-agar medium. This slow growing species comprise species like *R. lupine, Bradyrhizobium japonicum* and rhizobia of cowpea, miscellany[6].

Although the process of nitrogen fixation is not solely restricted to members of the genus *Rhizobium*, it is the root-nodule-symbiosis with plants of the family Leguminosae, which is almost exclusively a property of this group of microorganisms[5]. Here a symbiotic state exists between the rhizobial partner (microsymbiont) and the plant partner (macrosymbiont) within these root-nodules. One result of this symbiosis is the fixation of atmospheric dinitrogen into ammonia by the rhizobial partner, in exchange for protection and a source of

photosynthetically fixed carbon, provided by the host plant.

Discovery of *Rhizobium*

The present classification of *Rhizobium* mainly depends on the work of Frank[7]. Based on its ability to form nodules on the roots of legumes, the genus *Rhizobium* was established by Breed *et al.*[8]. The genus *Rhizobium* along with the genera *Agrobacterium* and *Chromobacterium* comprise the family Rhizobiaceae[9]. The six species of *Rhizobium* (i.e. *R. leguminosarum* (type species), *R. japonicum*, *R. lupini*, *R. meliloti*, *R. phaseoli* and *R. trifolii*) are recognized mainly based on differences in effectiveness associated with certain biochemical tests. Apart from the non-effective rhizobia, host legume reaction still remains to be valid test in the identification of rhizobia and its differentiation from agrobacteria.

Present classification of the family *Rhizobiaceae*

Based on data derived from the use of traditional tests such as biochemical and physiological tests the rhizobia are broadly classified into two groups, specially, on the basis of growth rate. These two groups are (i) Fast growing and (ii) Slow growing[1]. These rhizobia are physiologically heterogeneous and genetically diverse group of bacteria that are otherwise classified together on the basis of their ability to form nodules on various members of legumes. At the present moment the taxonomy of the rhizobia is in a flux state, where the proposals of new name have been flooding. This has been stimulated by the hectic process like isolation of symbionts from new sources and recently by the advances in various molecular methods, which have greatly increased the confidence which can assign strains to species and describe the relationship among species[10]. For assessing phylogenetic relationships among bacteria, sequence comparisons of the small subunit from the ribosomal RNA (SSU rRNA), have become the standard method. Such studies also show the diversity of rhizobia very clearly. All rhizobia, so far discovered, are members of the alpha subdivision of the proteobacteria, but the branch also includes other bacteria that are not root nodule symbionts[11]. However, the reliability of using the SSU rRNA gene alone as a sole approach to estimate phylogenic relationships of bacteria has limitation, particularly for closely related species. Sequences of the small subunit SSU rRNA can vary within a single isolate and the species *R. leguminosarum* has a more dramatic polymorphism[12]. A correlation between SSU rRNA and DNA-DNA reassociation data from the member of *Rhizobiaceae*[13] is not linear. Nevertheless, for a parametric species definition the SSU rRNA nucleotide sequence data are still most reliable to estimate relatedness when these genes share more than 97% similarity[14].

In the classification of rhizobia, the ability of certain rhizobial species to infect and nodulate

particular group of legume is important. Thus, rhizobia are broadly classified according to host-based system. The cross inoculation system has provided a useful classification system for rhizobial strains[10]. However, some major problem has been existed with its application.

It is very difficult to draw a direct line between closely related species by conventional method. A reasonably stable philosophical basis for the taxonomic scheme for grouping of rhizobial strains have provided by cross inoculation group. Similarly, varieties of methods, including evaluation of DNA hybridization, numerical taxonomic procedures, DNA base ratio, serology, intrinsic antibiotic resistant, as well as genetic marker have been applied to taxonomy in transition. Combination of these techniques have created new horizon in the concepts of species designation derived by more classical method as well as to detect occurrence of specific strains in soil and nodule[15]. Workers are of the opinion that the comparison of nucleotide sequence is capable of revealing the phylogenetic relation among bacteria which may also be helpful in the classification of the bacteria probable at genus or lower level[16]. A new *Rhizobium* species was reported for the first time on the basis of phenotypic characterization, 16S rRNA gene sequence and DNA-DNA hybridization[17].

This newly formed species belongs to the phylogenetic tree branch, which includes *R. leguminosarum.* The possibilities of utilizing Polymerase Chain Reaction and Restriction Fragment Length Polymorphism techniques (PCR-RFLP) analysis of full-length 16S rRNA gene, 16S rRNA gene analysis of the first 300 bp sequence, bacteriophage typing and amplication of the genomic region by random primer while establishing the phylogenetic relationship[18]. They recommended that *R. huakuii* bv. Rengei should be classified into sub-species of the new genus *Mesorhizobium* and renamed *M. huakuii* sub sp. *rengei*.

Identification and Characterization of *Rhizobium*

In order to classify bacteria, several phenotypic and genotypic methodologies have been utilized. Though phenotypic methods play a key role in identification, in recent days genotypic techniques are thought to be more authentic, reliable and useful for identification and also to study the diversity of bacterial isolates. Phenotypic methods rely mainly on biochemical tests, morphological tests, carbohydrate metabolism and enzyme production tests[19], fatty acid analysis[20], intrinsic antibiotic resistance[21], fluorescent antibody technique and polyacrylamide gel electrophoresis of total proteins[22,23]. Genotypic methods include PCR, PCR-RAPD (Polymerase Chain Reaction-Randomly Amplified Polymorphic DNA) Rep-PCR, (DNA-RNA, DNA-DNA) hybridization, TP-PCR (Two primer-Polymerase chain reaction)[24], LMW (Low molecular weight) RNA profiles[25] and RFLP (restriction fragment length polymorphism).

A large numbers of molecular methods based on PCR reaction have been proposed to characterize the *R. leguminosarum* strains and to provide a high degree of differentiation of these strains[26].

Fast and slow growing rhizobia

The genus *Rhizobium* can be broadly divided into two major groups depending upon their growth rates and effects of growth on the pH of yeast extract mannitol (YEM) culture medium[1,13,26-32]. Mean generation time for the "fast-growing" rhi-zobia are between two and four hours while that of the slow growers fall between 6 hours and longer ,the former decreasing the pH of the culture medium while the latter do not lower the pH of the media[5]. Generally speaking, *R. leguminosarum*, R. *meliloti, R. phaseoli, R. trifolii*, and *Rhizobium* spp. capable of nodulating *Leucaena* and *Sesbania* are characterized as fast-growing and acid--producing, while *R. japonicum, R. lupini*, and the rhizobia belonging to "cowpea group" are characterized as slow-growing and alkali -producing. In addition to the slow-growers, there are also fast-growing rhizobia which are able to form nodule soybeans[33]. Graham[34] proposed, after Adansonian analysis of 83 strains of *Rhizobium* (but including only 6 strains of *R. japonicum*), that the differences between the 'fast' and 'slow' growing strains were so great as to warrant a separate genus for the slow growing rhizobia. He further suggests classifying some agrobacteria with the fast-growing rhizobia. A later study supports the findings of Graham[35].

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In contrast to the previous workers,'t Mannetje[36], using different clustering techniques and the same six *R. japonieum* strains used by Graham[34] found high affinity between slow and fast growing strains of *R. japonicum.* Further,'t Mannetje[36] recommended against dividing the genus *Rhizobium* into different genera and further suggested that the genus *Agrobacterium* not be consolidated with *Rhizobium*. However, the conclusions of 't Moffett[37] and Graham[38] was supported by the results of DeLey *et al.*[38] who compared deoxyribonucleic acid base ratios and the degree of DNA homology of various strains and species of rhizobia and also *Agrobacterium* spp. These workers, however, concluded that there is a close relationship between the fast growing rhizobia and *Agrobacterium.* Interestingly, they found that the slow-growing species are not closely related to the fast growers. Again, they included very less number of *R. japonicum* strains in their studies, and also the lack of homology was thought to be enough to suggest separation of the fast and slow rhizobia into separate genera, at later stage, it was, however, recommended that more strains should be examined[39]. Elkan[40] further described the close relationship between the fast growing strains of rhizobia and agrobacteria by a number of transformation and

hybridization studies. Numerous morphological, biochemical, physiological, serological, and ecological techniques are available for the study of *Rhizobium* taxonomy, but comparatively little biochemical information is available for the 'slow' growing species[40].

Biochemical attributes of rhizobia

Consistent with the division of rhizobial strains into fast and slow-growing groups based on their growth rate and effect on the pH of YEM culture medium the fast-growing rhizobia may be differentiated from the slow-growing ones by the presence of other characteristics like methylene blue-staining, cytoplasmic granules and by colony size on YEM agar[28]. The fast growing rhizobia have such granules and produce much larger colonies than the latter. As was pointed out by Frank[7], Graham and Parker[28], and Vincent[4], fast-growing rhizobia tend to utilize a wider variety of carbohydrates than do the slow-growers. Although the utilization of a specific sugar is not a useful tool for differentiating rhizobia, but there exists a clear difference in the pattern of utilization of a large number of carbohydrates by fast and slow-growing strains. Glenn and Dilworth[41] indicated that the slow-growers apparently lack both uptake systems and catabolic enzymes for disaccharide utilization. For instance, fast-growing rhizobia have B-galactosidase activity, while the slow-growers lack this enzyme. Thus, rhizobia can also be separated into fast and slow-growing groups based on the presence or absence of enzymes of the pentose phosphate pathway as it was found that both fast and slow-growing rhizobia have NAD-linked 6-phosphogluconate activity (6-PGA), only the fast-growers have NADP-linked 6PGA activity[42-43].

Separation of fast and slow growing rhizobia can also be done based on their relative tolerance to pH and NaCl. Previous studies of rhizobia[28,31,44-45] showed that fast-growers were relatively more alkali-tolerant and acid--sensitive, than the slow-growers. Further, Graham and Parker[41] showed that among rhizobia, tolerance to 2% NaCl was restricted to the fast-growing *R. meliloti.* The responses of rhizobia in litmus milk[6,28] and their relative resist-ance to antibiotics[46] have also been used to separate rhizobia into these fast and slow growing groups. Unlike the slow growers, the fast-growers tend to produce acid in medium and peptonization reactions in litmus milk can be invariably noticed and they are in most cases found to be sensitive to antibiotics.

Thus, biochemical tests are still one of the main criterion utilized to characterize the different isolates of rhizobia including different species of *Rhizobium.* The various biochemical tests which were carried out for the phenotypic characterization of *Rhizobium*

Table 1. Biochemical characterization of *Rhizobium* spp

Species	Tests	Results	References
Rhizbium isolates	95 carbon sources	Out of 95 only 8 carbon sources were assimilated by 43 strains.	Hernandez[47]
15 rhizobial strains of chick-pea	Colony characters, osmotic potential, symbiotic effectiveness, phosphate solubilization, whole cell protein profile	All isolates showed constant colony characters, cell size and diversity in their osmotic potential. Out of 15 only 4 isolates showed phosphate solubilizing activity. The protein profile indicates that the patterns are isolate specific and can be used as fingerprints.	Shahida *et al.*[48]
Bradyrhizobium elkanii	Acid-alkaline production and indole-3-acetic acid production.	Out of 44 isolates from various tree legumes most of the isolates produced alkaline and only 4 could produce IAA	Manassila *et al.*[49]
Rhizobium japonicum	pH reaction in litmus milk, growth rates, antibiotic test and serological test.	Slow, scant growth and little or no acid formation were noted. Alkaline reaction, with no serum zone formation in litmus milk was noted. Only 5 of 50 strains have an acid reaction in xylose and rhamnose broth. They were found to be resistance to erythromycin, chloramphenicol and polymyxin but sensitive to novobiocin.	Elkan[40]
Rhizobium strains	Colony morphology, phenotypic features, NaCl, temperature and pH tolerance, C and N assimilation, antibiotic and heavy metal tolerance.	85% of cultures produced mucus, all strains grew well in pH 5 and 8 but variations were noted in pH 3 and 9. All strains could grew in 0.1 to 0.3 % NaCl and very few grew in 0.5 % NaCl. All the isolates grew well at 20, 25, 30 and 37°C, however some strains grew well up to 40°C. All the isolates could not utilize starch, casein and L-Lysine. However, glycine was found to be better N source. Over 85% of isolates were tolerant of Zn, Cr and Ni.	Kucuk and Kivanc[50]
Rhizobium strains	Colony morphology, mucus production, change in pH, growth rate,.	Colony were having sticky appearance with mucous. Colonies were round and whitish colouration. The pH were changed from 7 to 6. Single cells was rod and gram negative.	Singh *et al.*[51]
Fast growing and slow growing *Rhizobium*	Cell and colony morphology, Tolerance to pH and NaCl, litmus milk test, 3-ketolactose and hydrogen sulfide production tests, urease activity, citrate utilization test, penicillinase and oxisadase activities,	Only 1-5% fast growing and 80-90% slow growing-cells were motile. Fast growing isolates were sensitive to the lower pH as well as 2% NaCl and tolerant of high pH, however reverse were found with the slow growing isolates. All the isolates were catalase, urease and oxidase positive. Almost all isolates produced penicillinase and all isolates reduced nitrate. Non of the isolates produced 3-ketolactate from lactose, produced hydrogen sulphideor utilize citrate as sole carbon source. Fast growing showed clear zone in triptone agar medium where as slow growing were negative in this test. The litmus test showed varied results.	Sadowsky *et al.*[52]

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by different workers are given (Table1).

Diversity of *Rhizobium leguminosarum*

The diversity of different species of *Rhizobium* is large and has been well documented in several studies[54-56]. The populations of those strains were characterized using different strain typing methods which depict a considerable genetic heterogeneity between strains. Among these methods, serotyping has been widely used to identify isolates, detect nodule occupancy and track the distribution of antigenically distinct strains[56]. However, the involvement of animals and technical concerns in antiserum production has deterred some researchers from using serological methods[57]. Studies carried out with *Rhizobium* and *Bradyrhizobium* species have shown that individual serotypes may be comprised of more than one strain[58].

Methods used to study diversity

Molecular techniques have been one of the most widely used methods for assessing strain diversity within species. Characterization of the *Rhizobium* genome at the molecular level is the most discriminating method for assesses the variability among strains and isolates of the bacteria[54,59]. Molecular tools for the identification of bacteria are now available and are used routinely in laboratories.

Intraspecific variation and intragenomic heterogeneity also have limitations for the study of diversity and phylogeny of rhizobia[12]. Sequences of the small subunit ribosomal RNA (SSU rRNA) can vary within a single isolate and the species *R. leguminosarum* has a more dramatic polymorphism[12]. However, several authors have constructed phylogenies based on *nod* gene sequences and have concluded that they are not congruent with those based on SSU rRNA. For instance, the *nodABC* and *D* genes of *R. trifolli* are closer to *R. viciae* and *S. meliloti.* Therefore, some authors have considered that the study of the genes involved in nitrogen fixation must be worked out separately from that of the bacteria that now carry them[12].

The Pulsed-field gel electrophoresis (PFGE) fingerprinting of large DNA fragments obtained by cutting with restriction endonuclease enzymes is among the other methods that rely on the analysis of features distributed over the whole genome of *Rhizobium,* furthermore, one of the disadvantages of this technique is that standard protocols involve time-consuming steps such as the DNA preparation, lengthy restriction enzyme digestion and extended electrophoresis times, which for *Rhizobium* can take up to 6 days[60].

Intrinsic antibiotic resistance

An intrinsic antibiotic resistance (IAR) study is other phenotypic approaches for the study of

rhizobial diversity. This method uses the natural resistance of strains to a given level of various antibiotics for identification[57] and the detection of antibiotic markers is considered more practical than other methods because the methodology is simple, reliable and non-expensive[61-63]. In separate experiment different antibiotics were used to characterize the rhizobial diversity from a dry loamy sand of Thal[64]. However, the intrinsic multiple antibiotic markers for strain identification were found to be stable after passing through soil and host conditions and could be used for ecological studies. It was further revealed that the overall efficiency of a strain is the combined effect of characters like compatibility, competitiveness and inherent capacity to fix nitrogen[65]. Intrinsic antibiotic resistant can also be used to select the rhizobial strains which nodulate pea plant for nitrogen fixation potential[66].

Carbon utilization pattern

Carbon utilization patterns have widely been used to distinguish isolates and strains among the *Rhizobiaceae* family. The ability of *Rhizobium* to metabolize a broad range of sugars, organic acids and aromatic compounds is well documented[67]. The studies in carbon nutrition and metabolism in free-living cells has provided a baseline for comparison among strains. For *R. leguminosarum* bv. *trifolii*, the carbon utilization patterns have been used for phenotypic comparison between plasmid-cured strains[68]. The BIOLOG substrate utilization patterns have been used to confirm phenotypic similarities between electrophoretic types given by multilocus enzyme electrophoresis (MLEE)[58].

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Molecular techniques:

Plasmid

Casse *et al.*[69] utilized a method for the isolation of covalently closed circular DNA of high molecular weight, followed by agarose gel electrophoresis of the crude extracts, for the detection of plasmids with molecular weights of more than 250 × 10 from *Rhizobium, Agrobacterium tumefaciens* and *Pseudomonas putida* species. This technique was subsequently used for a survey of plasmids in 25 strains of *Rhizobium meliloti* from various geographical origins. Of these, 22 strains were found to carry at least one large plasmid. By electron microscopy and measurement of electrophoretic mobility in gels, the molecular weights of most of the plasmids were estimated to range from 90 × 10 to 200 × 10.

The electrophoretic screening of large plasmids, although limiting the analysis to the extrachromosomal elements, has a particular importance in the screening of *Rhizobium.* These bacteria have large plasmids (> 50 kb in size) that account for a substantial portion of their genome and contain several genes coding for nodulation and nitrogen fixation[70]. These

plasmids are often present in variable numbers and sizes; traits that provide the basis for an accurate strain characterization when the extracted plasmids are separated by gel electrophoresis[60]. However, the use of results from plasmid profile analysis to explain diversity in the population of *R. leguminosarium* bv. *trifolii* must be taken carefully, since plasmids maybe transfer from one cell to another and might not be related to chromosomal variation[71]. Strains may loose or regain those plasmids, and rearrangements or deletions may occur during laboratory cultivation and in the natural environment[72,73].

Random amplification polymorphic DNA (RAPD) analysis

Since its discovery, Polymerase Chain Reaction (PCR) has been utilizing for the analysis of natural microbial diversity[74] particularly for identification of specific microbial strain[75]. A modification of PCR referred to as Randomly Amplified Polymorphioc DNA (RAPD) analysis has been developed[76]. The polymorphic fragments of DNA named RAPD can be used as genetic makers[77-78]. This method is based on amplification of genomic DNA sequence by using a single oligonucleotide as primer. However, this oligonucleotide may not be complimentary to specific DNA sequence in the genome but under low stringency conditions a number of different sites of annealing are present on genome depending on the length, the nucleotide sequence and the G+C content of primer which allow amplification. The RAPD technique is a potential tool for the identification of the genetics and systematic of different populations. This technique utilizes arbitrary primers to detect changes in the DNA sequence at sites in the genome which anneal by the primer. Phylogenetic relationships are constructed from RAPD fingerprints by statistical analysis. The preliminary findings indicated that RAPD could provide us with a rapid and efficient method for screening the differences between genomes. The RAPD markers are useful in the construction of genetic maps, referred to as RAPD mapping. Therefore, the RAPD technique is a potentially useful tool for the study of genetics and systematic[79]. The development of RAPD markers provided a new tool for investigating genetic polymorphisms in many different organisms, including bacteria[80-82], and this method has been used for *Rhizobium* identification and *Bradyrhizobium* genetic analyses[83].

Genomic DNA fingerprinting using random amplification of polymorphic DNA (RAPD) has been found to be useful in differentiating very closely related rhizobial strains[84]. However, reproducibility is poor which may result in incorrect genotype interpretations[85].

Restriction fragment length polymorphism (RFLP)

To establish genetic relationships as well as to characterize *Rhizobium* strains at the species and higher levels, Restriction fragment length polymorphism (RFLP) analysis has been

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widely used[86]. RFLP analysis can detect mutations or rearrangements in the genome which alter the distribution of specific restriction endonuclease recognition sites within defined regions of the genome. RFLP analysis has also demonstrated the diversity of Sym (symbiotic) plasmid types within naturally occurring populations of *R. leguminosarum* including bv. *Trifolii*[54]. Romdhane *et al.*[87] utilized RFLP technique to study the genetic diversity of rhizobia which nodulate *Acacia tortolis*, where they found huge heterogeneity among the studied strains. Similarly, Hung *et al.*[88] studied different rhizobia from different host plants where they characterized those rhizobia using RFLP and after sequencing the specific bands common to all the cultures, they found that most of the isolates belong to *Rhizobium, Bradyrhizobium* and *Agrobacterium.* Nkot *et al.*[89] studied forty-two strains from *Arachis hypogaea* were examined by restriction fragment length polymorphism (RFLP) analysis of 16S–23S rDNA genes amplified by polymerase chain reaction (PCR). A considerable level of genetic diversity was determined among those peanut isolates. Eight composite genotypes were obtained from the combined data of the RFLP analysis with four endonucleases. A correlation between land use system and the diversity of peanut isolates was observed in the current study.

Enterobacterial repetitive intrergenic consensus sequence and extragenic palindromic

Oligonucleotides derived from enterobacterial repetitive intergenic consensus (ERIC) sequences and extragenic palindromic (REP) elements have been found to be very useful for fingerprinting a variety of Gram-negative and Gram-positive bacteria using the polymerase chain reaction (PCR)[55,90-91]. However, strain diversity within species of rhizobia may be high, both genetic and phenotypic[12]. Therefore, it is necessary to define the level of diversity which is appropriate to characterize particular genera and strains.

Numerical cluster analysis

Numerical cluster analysis of an adequate number of strains and comparison with rhizobial type strains can permit the characterization of large populations. Numerical taxonomy basically combines the classic data on physiology and morphology in a rigorous and less subjective manner that gives quantitative measures of similarity[12]. In this regards, minimum standards for the description of new genera and species of *Rhizobium* were given by Graham *et al.*[92].

Deoxyribonucleic acid base ratios and homology

Rhizobial strains and also their species can be separated into several groups based on nucleic acid hybridizations and DNA base ratios. While DNA base ratios, mole percent G+C, for *Rhizobium* are relatively broad ranged [values ranging from 59-

66%[38]], indicate relationships between strains. Further, it was pointed out that the peritrichously flagellated fast-growing rhizobia, *R. leguminosarum, R. phaseoli,* and *R. trifolii*, tend to have low percentage of G+C values, ranging from 59-63%, while the polar to sub-polarly flagellated slow-growers such as *R. japonicum, R. lupini*, and some of the cowpea miscellany, have relatively high percentage of G+C values, 63-65%[38]. Heberlein *et al.*[39] reported that with the exception of *Agrobacterium pseudotsugae* and *R. japonicum*, the agrobacteria and rhizobia, which they examined, had about the same percentage of G+C content, about 59-63%. *Rhizobium japonicum* on the other hand, had G+C values of about 64-65%. However, on examination of 25 strains of *R. japonicum* it was found that their mole percentage of G+C were relatively homogeneous[94]. It was further indicated that although percentage of G+C values were significantly different between some strains of *R. japonicum*, these values fall within the range of other fast and slow-growing rhizobia and thus, these characters were of limited taxonomic value.

DNA-DNA homology

The DNA-DNA homologies have been utilized by several workers[93,95] to study the degree of genetic relatedness between different strains and isolates of *Rhizobium*. The homology studies of Gibbins and Gregory[95] and Heberlein *et al.*[39] have sharply indicated that there is a distinct line of demarc-ation between the fast-growing species (e.g., *R. leguminosarum, R. trifolii, R. phaseoli,* and *R. meliloti*) and the slow -growing ones (e.g., *R. japonicum and R. lupini*). Hollis *et al.*[93] have shown that the slow-growing strains of *R. japonicum* could be separated into at least three distinct DNA homology groups. They further indicated that there was little homology between DNA from several *R. japonicum* strains and *R. leguminosarum, R. trifolii, R. meliloti, R. phaseoli,* and *Agrobacterium tumefaciens*. Similarly, in other experiment it has been shown that the 113 fast-growing and 9 slow-growing strains of rhizobia could be placed into 4 DNA homology groups, and that the fast-growers were clearly separated from the slow-growers[96]. Interestingly, these authors also indicated that there was little homology between fast and slow-growing lotus rhizobia. The two groups were more closely related to each other than to the other fast and slow-growing rhizobia which were examined. Thus, fast and slow-growing rhizobia can also be separated into two groups on the basis of DNA-DNA homologies.

DNA hybridization techniques may be used to estimate the genetic relationships of strains labelled as *R. japonicum* to clarify their taxonomic status[93]. They carried out reassociation

using DNA from various *Rhizobium* and *Agrobacterium* species and the relationships of DNA sequences were also estimated by comparing the thermal stability of heterologous DNA duplexes with that of the homologous DNA duplex. The results indicated that strains labeled as *R. japonicum* can be separated into at least three DNA homology groups. Reference strains of other *Rhizobium* species, with the exception of *R. zupini,* were not closely related to these homology groups.

16S rRNA sequence analysis

Sequence comparisons of the small subunit of the ribosomal RNA (SSU rRNA) have become the standard method for assessing phylogenetic relationships among bacteria[97] and particularly of rhizobial strains[98]. Comparison of 16S rDNA nucleotide sequences can be applied for classification of isolates at species and higher levels[99]. The 16S rRNA or rDNA contain highly conserved regions because of their crucial structural and functional constraints, however, they also contain highly variable signatures. Conserved regions proximal to the 5' and 3' termini are found in all prokaryotic 16S rRNA sequences and are, therefore, used as primer sequences in PCR reactions to amplify almost the entire gene[100]. Several studies have further shown that 16S rRNA sequences of non-symbiotic isolates were closely related to those of the symbiotic rhizobial isolates[98,101]. However, it is clear that the degree of 16S rRNA sequence similarity between strains of rhizobia does not always accurately reflect overall genomic similarity, as has been found for other bacteria with more than 97% 16S sequence similarity[102].

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Serological relationships

Serological techniques have been widely used in the study of rhizobia: (1) to obtain information about their antigenic composition[103-104], (2) for strain identification[105-108], (3) to investigate the serological relatedness of strains and species of *Rhizobium*[109-113] and (4), for ecological studies[105,107-108,114-118]. Three techniques in particular have found wide acceptance in serological investigations of the Rhizobiaceae (i) agglutination, (ii) immunodiffusion (Ouchterlony Gel Diffusion), and (iii) immunofluorescence.

Twenty-nine cultures of *Rhizobium* spp. were studied for chickpea (*Cicer arietinum* L.) and were characterized by serological techniques by Kingsley and Bohlool[119] further they performed analysis of intrinsic antibiotic resistance (IAR). Fluorescent antibodies (FAs) against the somatic antigens of eight of the inoculum-quality strains grouped all cultures into at least four distinct somatic serogroups. Serogroup I (Nitragin 27A8) contained the majority of the strains. Serogroups II (Nigragin 27A3) and III (TAL-480) each crossreacted

with one strain, while serogroup IV (USDA 3HOa9) consisted only of the homologous strain. Nine of the chickpea strains and fifty other cultures from among strains of *R. leguminosarum*, *R. lupini*, *R. japonicum*, and *Rhizobium* spp. (*Vigna*, *Sesbania*, and *Stylosanthes*) did not react with any of the four specific FAs. Immunofluorescence cross adsorption and immunodiffusion analyses of cultures within serogroup I revealed that four of the strains, most commonly, recommended for inoculum used were serologically identical. The intrinsic antibiotic resistance patterns of these four cultures were also identical. Serogroup II was shown to contain two distinct serotypes (Nitragen 27A3 and 27A16); while the two strains in serogroup III (TAL 480 and 622) were found to be the same. Their studies on antigenic uniqueness of the chickpea rhizobia and their lack of serological crossreaction with any other *Rhizobium* species (especially with those in *R. leguminosarum*, where they had previously been classified), as well as the results of other workers on their nodulation specificity for *Cicer*, led support to the proposal that *C. arietinum* and its root nodule bacteria constitute a separate cross-inoculation group.

Agglutination techniques

The agglutination techniques were the first of the sero-logical methods applied to the study of rhizobia[120]. The early serological studies were concerned mainly with the relationships between serological groupings and host-specificity. Results of most studies indicated that rhizobia are a serologically heterogeneous group of organisms. Stevens[121] and Wright[122] found that different strains isolated from the same host-plant species could be serologically unrelated. Hughes and Vincent[123] found that even strains isolated from different nodules on the same plant could be unrelated serologically. However, in their study, Bushnell and Sarles[124] found that some strains from different cross-inoculation groups were sero-logically related. The ability of a rhizobial strain to nodulate a particular host-plant is not necessarily related to it serological characteristics as detected by agglutinations[125]. Bushnell and Sarles[124] studied the rhizobial strains from the soybean, cowpea, and lupin cross-inoculation groups and found no correlation between the ability of certain strains in one group to cross-inoculate another, and their ability to cross-agglutinate. In addition, Stevens[121] and Bushnell and Sarles[124] indicated that due to their serologic hetero-geneity, all strains within the same *Rhizobium* species cannot be identified by agglutination reactions with a limited number of antisera.

Despite the inability to show correlations between host-specificity and serological groupings, agglutination reactions have been used to assess the serological relatedness of strains and species of *Rhizobium*. Kleczkowski and Thornton[125], using whole

-cell antisera against 4 strains of *R. trifolii* and 2 strains of *R. leguminosarum*, examined the agglutination cross-reactions of antigens from 161 strains of *R. trifolii*, 29 *R. leguminosarum*, 5 strains of *R. meliloti* and *R. lupini,* and 13 non-*Rhizobium* soil isolates. Results of their study indicated that while no cross-react-ions occurred outside of the clover and pea groups and with the 13 soil isolates, some cross-reactions occurred between the two groups. And, while some antisera were quite spe-cific, others were relatively non-specific.

In their studies on the serological relationships of 25 strains of the slow-growing *R. japonicum*, Koontz and Faber[126] identified 6 somatic serogroups using agglutination adsorption reactions. Wright *et al.*[122] similarly found 6 serogroups among the *R. japonicum* they examined; however, these authors did not differentiate between somatic and flagellar antigens. Date and Decker[127] analyzed 28 strains of *R. japonicum* and found 17 somatic serogroups on the basis of cross-reactions and agglutination cross-adsorptions.

Graham[128] tested 113 strains of *Rhizobium* for agglutination by whole cell antisera produced against 58 strains of *Rhizobium* and 16 *Agrobacterium* strains. The results of his study of whole- and somatic-cell antigens indicated that the rhizobia could be separated into three serologically distinct groups: (1) *R. trifolii, R. leguminosarum,* and *R. phaseoli;* (2) *R. japonicum, R. lupini,* and *Rhizobium* spp. of the cowpea miscellany; and (3), *R. meliloti*. While there were no cross-reactions between the groups, there were cross--reactions within the groups. Graham[128] also indicated that strains of *R. meliloti* showed some serological relatedness to *Agrobacterium tumefaciens* and *A. radiobacter* and that agglutination cross-reactions were greater with whole-cell antigens than with somatic-cell antigens.

Immunodiffusion techniques

This technique has been used extensively to invest-igate the serological relationships between strains and species of *Rhizobium*[111,112,129-132]. The technique relies on the separation of soluble, diffusible antigens through an agar-gel matrix. Relationships between various antigens and antisera are determined by examining the nature of the interaction at the junction of precipitin bands from various wells. Gel diffusion methods have been used in the study of rhizobia because they permit the rapid enumeration of soluble antigens, the techniques are relatively simple, and they can be used to study serological relationships of strains at the single antigen level. Dudman[120] has indicated that while agglutination reactions can be used to separate rhizobia into serological groups, agglutination techniques lack the resolving power

of immunodiffusion in distinguishing between antigenically identical and closely related, but not identical strains.

Dudman[111] was the first investigator to use immuno-diffusion techniques to study the serological relatedness of strains and species of *Rhizobium*. In his study of the extra-cellular soluble antigens of 2 strains of *R. meliloti*, Dudman[111] found that the two strains examined shared all antigens accept several fast-moving ones. He proposed that since the strains did not cross-agglutinate, that these strain-specific antigens could be used for identification purposes.

Using gel immunodiffusion, Skrdleta[132] divided the 11 slow-growing *R. japonicum* which he examined into two basic somatic serogroups. While he detected the same sero-groups using agglutination reactions, he found that immuno-diffusion allowed him to show serological relationships between strains that were not agglutinated by the same antisera. Skrdleta[132] also indicated that the somatic antigens were more specific than flagellar ones in differ-entiating individual strains. Dudman[112] in his study of seven strains of *R. japonicum* found that pretreatment of antigens (by boiling or ultrasonic disruption) was required for the proper immuno-diffusion analysis of these slow-growing strains. Gibbins[113] found that while ultrasonic disruption prevented the formation of precipitin bands in immunodiffusion reactions, band formation could be restored by heating the sonicated antigen preparations.

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While the use of somatic antigens (heat-stable anti-gens) have been more specific than flagellar ones (heat-labile) in differentiating individual strains of *Rhizobium*, internal antigens have also been reported[133] to provide some insight into the serological relatedness of fast- and slow-growing rhizobia. Using whole-cell antisera against three strains of *R. japonicum*, Vincent *et al.*[110] studied the internal antigens of sixty-nine strains of *Rhizobium* and 5 *Agrobacterium* strains.

Immunodiffusion reactions indicated that at least one common antigen was present in 13 strains of *R. japonicum*, 4 strains of *R. lupini*, and 4 strains of the slow-growing cowpea and lotus rhizobia. Their results also indicated that the forty-six fast-growing rhizobia examined were readily distinguished from the slow-growing strains and that the 5 strains of agrobacteria grouped with the fast-growing rhizobia. More recently, Pankhurst[134] studied the immunodiffusion cross-reactions of somatic and interal antigens from 62 fast and slow-growing strains of lotus rhizobia. Results of his study indicated that while the fast- and slow-growers shared no common somatic anti-gens, internal antigens were shared by all of the fast-growing strains,

and with seven exceptions, by all of the slow-growing strains.

The fluorescent antibody technique

It is the method of choice for the direct examination and identification of strains of rhizobia in culture and nodules[108] and for the enumeration of specific strains_directly in soi1[118]. The major advantages of immunofluorescence over other techniques is that only small amounts of antigen and antibody are needed[117], the procedures are relatively rapid, and its the only technique readily applicable to the study of rhizobia *in situ*[116].

Vincent[133] in his recent review of the literature has pointed out that when serological[110] and other taxo-nomic evidence[13,29] are taken together, clear relationships among rhizobia can be recognized i.e. (1) there is a closer relationship between the fast-growing species of *Rhizobium* and *Agrobacterium* than there is between the fast and slow-growing groups of rhizobia; (2) *R. trifolii* and *R. phaseoli* should be made separate biovars of the species *R. leguminosarum*; (3) *R. meliloti* is so different from other species of *Rhizobium*, that it requires its own species status; (4) among the slow-growers, the slow-growing soybean rhizobia should remain as a separate species group and be included in the new genus *Bradyrhzobium*; and (5) the fast and slow-growing lotus rhizobia fall within the genera *Rhizobium* and *Bradyrhizobium* respectively.

Molecular studies on *Rhizobium*

Walton and Moseley[135] studied efficiency of a variety of common mutagens in producing mutation in *Rhizobium trifolii P3*. Ethyl methanesulphonate, methyl methanesulphonate, decarbamoyl mitomycin C, nitrous acid and gamma radiation did not mutate *R. trifolii P3. N* -Methyl, N'-nitro, N-nitrosoguanidine (MNNG) and ultraviolet radiation were both mutagenic, the former being the more effective. Transposon mutagenesis with Tn5 yielded the same frequency and range of auxotrophs as did MNNG. Zimmerman *et.al.*[136] investigated the expression of specific symbiotic genes during the development of nitrogen-fixing root nodules and conducted a systematic analysis of nodule specific proteins and RNAs produced after the inoculation of alfalfa roots with a series of *Rhizobium meliloti* mutants generated by site-directed transposon Tn5 mutagenesis. The mutagenized region of the *Rhizobium* genome covered approximately 10 kilobases and included the region encoding the nitrogenase polypeptides. All mutant strains that were analyzed produced nodules, but with several strains the nodules failed to fix nitrogen (Nod^+ Fix^- phenotype). All Fix nodules accumulated reduced levels of the host plant protein leghemoglobin. In

addition, Tn5 insertions in the nitrogenase operon (nifHDK genes) eliminated some or all of the nitrogenase polypeptides and nifHDK RNA transcripts, depending on the site of insertion. Finally, mutation of a region approximately 5 kilobases upstream from the nitrogenase operon resulted in the absence of all three nitrogenase polypeptides and their corresponding RNAs, suggesting that this region may serve a regulatory function during nitrogen fixation. The studies indicated that site-directed mutagenesis coupled with biochemical analysis of nodule proteins and RNAs allows the identification of products of specific gene regions as well as the assignment of specific functions to previously unidentified regions of the *R. meliloti* genome.

Schetgens *et al.*[137] performed extensive studies on the structural organization and regulation of the expression of the nitrogenase gene cluster in *Rhizobium leguminosarum*. Site-directed Tn5 mutagenesis was applied to a nif DH-specific clone and subsequently the transposon transferred back into the wild-type rhizobial genome by homologous recombination. Phenotypic effects of Tn5 region of the structural *nif*-genes were determined by measuring acetylene reduction in nodulated analysis of bacteroid-specific proteins. Expression of different cloned *Rhizobium nif* D, *nif* H, or *nif* DH, was achieved in *Escherichia coli* minicells dependent upon the presence of a strong promoter sequence. Gene products were identified by immunoprecipitation with specific antisera. Endogenous transcriptional start signals in one case (*nif* H) seemed to be recognized at a low rate by the *E. coli Rhizobium* ribosome binding sites for all three structural nif-genes functioned normally in minicells. Arp[138] purified *Rhizobium japonicum* hydrogenase from soyabean root nodules by four column chromatography steps after solubilization from membranes by treatment with a nonionic detergent. The specific activity was from 40 to 65 μmol H^2 oxidized min^{-1} mg $protein^{-1}$ and was increased 450-fold relative to that in bacteroids. The yield of activity was from 7 to 12%. The molecular weight of the native enzyme was 104,000 as determined by sucrose density gradient centrifugation. Electrophoresis in the presence of sodium dodecyl sulfate revealed two subunits with molecular weights of 64,000 and 35,000, indicating subunit structure. The amino acid content of the protein indicated 20 cysteine residues. Analysis of the metal content indicated 0.59 ± 0.06 mol Ni/mol hydrogenase and 6.5 ± 1.2 mol Fe/mol hydrogenase. Antisera prepared to the hydrogenase cross-reacted with the enzyme in bacteroid extracts at all stages of the purification but did not cross-react with extracts of *Alcaligenes eutrophus* grown under chemolithotrophic conditions. A collection of symbiotically defective mutants of *Rhizobium meliloti* JJ1c10 was derived by Tn5 mutagenesis using the suicide vector pGS9[139]. The mutants were found to be heterogenous in acetylene reduction activity and in the ultrastructure of the nodules which they induced.

Over 90% were found to contain bona fide Tn5 insertions in a variety of DNA restriction fragments. When Tn5-carrying DNA segments cloned from 24 of the mutants were introduced into the equivalent location in the genome of the wild strain by recombination-mediated replacement, only eight produced a symbiotically defective phenotype similar to that of the original mutant. DNA segments apparently containing mutated *fix* genes but not containing Tn5 were found in eight mutants by identifying cosmids carrying type DNA which complemented their symbiosis defects. Probing of the DNA of these mutants with their complementing cosmids revealed no detectable physical alteration of the homologous DNA. A segment of DNA including the *hsn* and *nifHDK* genes was favoured for these non-Tn5 mutations. Three regions of the genome were identified. One of the known megaplasmid *nod-nif* region. The other two regions, designated *fix-e5* and *fix* found to be chromosomal. Surin *et al.*[140] performed DNA sequencing of the *nodlJ* region from *Rhizobium leguminosarum* biovar *trifolii* and revealed the *nodT* gene immediately downstream of *nodJ*. DNA hybridizations using nodT-specific probe showed that *nodT* is present in several *R. leguminosarum* strains. Interestingly, a flavonoid-inducible *nodT* gene homologue in *R. leguminosarum* bv. *viciae* was found not to be located in the *nodABCIJ* operon but is located downstream of *nodMN*. The sequence of the *nodT* gene from bv. *viciae* was determined and a comparison of the predicted aminoacid sequences of the two *nodT* genes showed them to be conserved; the predicted protein sequences appeared to have a potential transit sequence typical of outer-membrane proteins. Mutations affecting *nodT* in either biovar had no observed effect on nodulation of the legumes tested. Roche *et al.*[141] made extensive studies on the molecular basis of host specificity in *Rhizobium meliloti*. They reported that *R. meliloti* nodulation (*nod*) genes determined the production of acylated and sulfated glucosamine ollgosaccharide signals. They showed that the biochemical function of the major host-range genes, *nodH* and *nodPQ*, is to specify the 6-O-sulfation of the reducing terminal glucosamine. Purified Nod factors (sulfated or not) from *nodH*+ or *nodH* strains exhibited the same plant specificity in a variety of bioassays (root hair deformations, nodulation, changes in root morphology) as the bacterial cells from which they were purified. The results provided strong evidence that the molecular mechanism by which the *nodH* and *nodPQ* genes mediated host specificity.

Cubo *et al.*[142] performed molecular characterization of the rhizosphere expressed genes rhiABCR that can influence nodulation by *Rhizobium leguminosarum* biovar *viciae*. Although mutation of the rhi genes does not normally affect nodulation, in the absence of the closely linked nodulation genes nodFEL, mutations in the rhi genes influenced the nodulation of the vetch *Vicia hirsuta*. The DNA sequence of the rhi gene region revealed

four large open reading frames, three of them constituting an operon (rhiABC) transcribed convergently toward the fourth gene, rhiR. rhiABC were under the positive control of RhiR, the expression of which was repressed by flavonoids that normally induce nod gene expression. This repression, which required the nodD gene product (the transcriptional activator of nod gene expression), was due to a *cis* effect caused by a high level of NodD-dependent expression from the adjacent nodO promoter that was transcribed divergently from rhiR. This rhiR shows significant similarities to a subfamily of transcriptional regulators that included the LuxR and UvrC-28K proteins. The rhiA showed limited homology to a short domain of the lactose permease, LacY, close to a region thought to be involved in substrate binding The biochemical role of the rhi genes was not been established, but it appeared that they may play a role in the plant-microbe interaction, possibly by allowing the bacteria to metabolize a plant-made metabolite.

Maxwell *et al.*[143] performed molecular characterization and expression of the alfalfa isoliquiritigenin 2-*O*-methyltransferase, an enzyme specifically involved in the biosynthesis of an inducer of *Rhizobium meliloti* nodulation genes. The cDNA clone was identified on the basis of N-terminal sequence identity to purified *S*adenosyl- L-methionine: isoliquiritigenin 2-*O*-methyltransferase (chalcone OMT) and expression of enzymatically active chalcone OMT protein in *Escherichia coli*. The deduced amino acid sequence showed significant similarities to other OMTs. Chalcone OMT was encoded by a small gene family in alfalfa and related sequences are present in other legumes. The chalcone OMT gene was expressed primarily in alfalfa roots; transcript levels were highest during the first 2 weeks of development. The OMT transcript was also detected, to a much lesser extent, in root nodules. Chalcone OMT transcripts were elicitor-inducible in alfalfa cell suspension cultures, although only low levels of methoxychalcone accumulated. The implications of these results for plant-microorganism interactions were far reaching. Soto *et al.*[144] reported nucleotide sequence and characterization of a 3345 base-pair DNA section of the *nfe* region. Sequence analysis revealed four open reading frames (ORFs), two of them with rightward polarity, termed *nfe1* and *nfe2*, are preceded by functional *nif* consensus sequences and NifA -binding motifs. An additional, NifA independent, transcriptional start site for *nfe1* was also found. Two other ORFs with leftward polarity, designated ORFA and ORFB, were identified upstream from *nfe1* and *nfe2* but no *nif* consensus sequences were found. The gene products of *nfe1* and *nfe2* were identified using *in vitro* transcription/translation and bacteriophage T7 RNA polymerase/promoter system, respectively. In addition, *nfe1* shows homology with the upstream non-coding DNA region of the *fixABCX* operon. Putative ORFB encoded protein contains a helix-turn-helix motif that resembles the DNA binding

consensus sequence proposed for many prokaryotic regulatory proteins.

Many researchers[145-147] studied the exchange of signals in *Rhizobium* legume symbiosis. They used molecular and biochemical techniques to study the mechanism of perception of Nod factors and to characterize nodule organogenesis. The fact that specific lipochitooligosaccharidic structures are active atnpicomolar concentrations on the legume plant suggested that Nod factors are perceived by specific high affinity receptors. They characterised such receptors by i) studying binding sites for Nod factors in extracts of roots and cell cultures of *Medicago* and ii) isolating clones encoding binding proteins. In order to define particular organogenesis we have isolated clones for a large number of genes induced during nodulation and are using these to characterize the different steps. This work includeed studies on the regulation of expression of these genes and the biochemical functions of the encoded proteins. Following the establishment of the nodule the rhizobia fix dinitrogen into ammonium which is then assimilated by the plant. Krol *et al.*[148] identified three genes pssCDE in *Rhizobium leguminosarum bv. trifolii TA1*. Gene products of pssCDE genes shared significant homology to prokaryotic glycosyl transferases involved in exopolysaccharide synthesis. The Tn5 insertion in pssD created the nonmucoid mutant that induced non-nitrogen-fixing nodules.

Extensive studies concerning the use of marker genes for competitive studies were extensive performed[149]. Reporter genes such as the ß-glucuronidase gene (gusA) or a thermostable ß-glucosidase gene (celB) allowed detection of rhizobial strains in nodules when they are still attached to the root system. The detection technique was highly suitable for the study of rhizobial competition and studies using gusA-marked strains of *Rhizobium*. By making use of gusA and celB, differentially marked strains can be produced and distinguished easily on roots. The availability of two marker genes permitted competition studies of two or more than two strains and analysis of dual nodule occupancy. Jaiswal *et al.*[150] analysed indigenous rhizobia isolated from root nodules of lentil plants collected from various agro-climatic regions of India. These isolates together with four standard lentil *Rhizobium strains* were screened for sensitivity against eight phages. Four strains, USDA 2431, BHULR 104, BHULR 113 and BHULR 115 having restricted sensitivity to lytic phages LRP-1, LRP-4, LRP-13 and LRP-15 respectively, were characterized for both physiological and molecular characters. Genomic and phylogenetic relationships among the strains were examined by randomly amplified polymorphic DNA and 16S rRNA sequence analysis. Genetic distance varied from 0.09 to 0.23 among the strains, and the primer OPL-11 was found to be suitable for the discrimination of these strains. The 16S rRNA sequence analysis revealed 99–100% similarity with *Rhizobium leguminosarum bv. viciae*. The results indicated that phage

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sensitivity is a useful marker for discriminating locally soil-adapted rhizobial strains forming effective nodules in lentil. Zhang *et al.*[73] isolated 360 *Rhizobium leguminosarum bv. viciae* strains from three brown-coal mining restoration fields of different age and plant cover (without and in the first and second year of alfalfa, *Medicago sativa*, cultivation) using two host species (*Vicia hirsuta* and *Pisum sativum*) as capture plants. The strains were genetically typed by restriction fragment length polymorphism analysis of polymerase chain reaction (PCR)-generated 16S-23S ribosomal DNA intergenic spacer regions (IGS-RFLP) and characterized by plasmid profiles and RFLP analysis of amplified nodABC genes. The *R. leguminosarum bv. viciae* population was dominated by the same group of strains. According to type richness, the genetic diversity of indigenous *R. leguminosarum* in the second year of restoration was lower than in the first year and it resembled that of the fallow field, except for plasmid types, in which it was higher than that of the fallow field. Some of the less frequent nodABC genotypes were associated with distinct chromosomal IGS genotypes and symbiotic plasmids (pSyms) of different sizes, indicating that horizontal transfer and rearrangements of pSym can occur in natural environments. However it was found that the dominant pSym and chromosomal genotypes were strictly correlated suggesting a genetically stable persistence of the prevailing *R. leguminosarum bv. viciae* genotypes in the absence of its host plant. The biodiversity of rhizobial strains nodulating *Cicer arietinum L.* in representative soils from various areas of Morocco were determined[151]. Restriction fragment length polymorphism (RFLP) of PCR-amplified 16S rDNAs were compared with those of reference strains. Numerical analysis of the phenotypic characteristics showed that the 48 strains studied fell into three distinct groups. RFLP analysis of 16S rRNA genes revealed an additional heterogeneity and four ribotypes were identified.

The occurrence of thermophilic reversible 2-resorcylate decarboxylase (2 -RDC) in the cell extract of a bacterium isolated from natural water, *Rhizobium* sp. strain MTP-10005 were found, and purified the enzyme to homogeneity[152]. The molecular mass of the enzyme was determined to be about 151 kDa by gel filtration, and that of the subunit was 37.5 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme was induced specifically by the addition of 2-resorcylate to the medium. The enzyme required no coenzyme and did not act on 2,4 dihydroxybenzoate, 2,5-dihydroxybenzoate, 3,4-dihydroxybenzoate, 3,5-dihydroxybenzoate, 2-hydroxybenzoate, or 3-hydroxybenzoate. It was relatively thermostable to heat treatment, and its half-life at 50°C was estimated to be 122 min; furthermore, it catalyzed the reverse carboxylation of resorcinol. The values of kcat/Km (mA−1 Cs−1) for 2-resorcylate and resorcinol at 30°C and pH 7 were 13.4 and

0.098, respectively. The enzyme contained 327 amino acid residues, and sequence identities were found with many others.The genes (graA [1,230 bp], graB [888 bp], and graC [1,056 bp]) that are homologous to those in the resorcinol pathway also exist upstream and downstream of the 2-RDC gene. Judging from the results, the resorcinol pathway also existed in *Rhizobium* sp. strain MTP-10005, and 2-RDC probably catalyzed a reaction just before the hydroxylase in it does. Lafay and Burdon[153] analysed molecular diversity of Legume Root-Nodule Bacteria in Kakadu National Park of Australia. Their study concentrated upon symbiotic relationships between leguminous plants (family Fabaceae) and nodule-forming bacteria in Australia. They also characterized an entirely novel nodule-forming lineage, phylogenetically distant from any previously described rhizobial and non-rhizobial legume-nodulating lineage within the Rhizobiales. Overall, their results supported the hypothesis of tropical areas being centres of biodiversity and diversification for legume root-nodule bacteria.

Twenty-eight *Rhizobium* strains were isolated from the root nodules of faba bean (*Vicia faba* L.) collected from 11 governorates in Egypt[154]. A majority of these strains (57%) were identified as *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) based on analysis of a *nodC* gene fragment amplified using specific primers for these faba bean symbionts. The strains were characterized using a polyphasic approach, including nodulation pattern, tolerance to environmental stresses, and genetic diversity based on amplified ribosomal DNA-restriction analysis (ARDRA) of both 16S and 23S rDNA. ARDRA indicated that the strains could be divided into six 16S rDNA genotypes and five 23S rDNA genotypes. Sequence analysis of 16S rDNA indicated that 57% were *Rlv*, two strains were *Rhizobium etli*, one strain was taxonomically related to *Rhizobium rubi*, and a group of strains were most closely related to *Sinorhizobium meliloti*. Results of these studies indicate that genetically diverse rhizobial strains are capable of forming N2-fixing symbiotic associations with faba bean and PCR done using *nodC* primers allows for the rapid identification of *V. faba* symbionts. Naz *et al.*[155] studied *Rhizobium* isolates from arid soils. They studied genetic diversity among the isolates through RAPD-DNA finger printing and PCR was done for the presence of 16S-rRNA gene. RAPD tests categorized isolates into two clusters. The RAPD results were further analyzed by MVSP software; similarity matrix was measured and converted into dendrogram through UPGMA clustering method. Elboutahiri *et al.*[156] performed extensive analysis of rhizobia, *Sinorhizobium meliloti* and *Rhizobium sullae*, from root nodules and soils from Morocco. They used three PCR-based techniques namely rep-PCR, RAPD and ARDRA techniques for genotypic characterization of 10 isolates of *S. meliloti* and *R. sullae*, in order to identify rapid and reliable techniques for applications in population genetic

analysis of these species. The analysis revealed characteristic banding patterns for *S. meliloti* and *R. sullae* isolates by all the three techniques, even though the isolates are from a narrow geographic region in Morocco. Furthermore, the results showed that the rep- PCR with REP and ERIC primers was more efficient than RAPD and ARDRA technique for genotyping *S. meliloti* isolates; and rep-PCR with REP primers and the ARDRA technique with restriction enzyme HinfI, were more efficient than the other rep-PCR and RAPD-PCR techniques for genotyping *R. sullae* isolates.

The NifA-RpoN complex is a master regulator of the nitrogen fixation genes in alphaproteobacteria[157]. Based on the complete *Rhizobium etli* genome sequence, they constructed an *R. etli CFN42* oligonucleotide (70-mer) microarray and utilized this tool, reverse transcription (RT)-PCR analysis (transcriptomics), proteomics, and bioinformatics to decipher the NifA-RpoN regulon under microaerobic conditions (free life) and in symbiosis with bean plants. The *R. etli* NifA-RpoN regulon was determined to contain 78 genes, including the genes involved in nitrogen fixation, and the analyses revealed 42 new NifA-RpoN-dependent genes. More importantly, the study demonstrated that the NifA-RpoN regulon is composed of genes and proteins that have very diverse functions, that play fundamental and previously less appreciated roles in regulating the normal physiology of the cell, and that have important functions in providing adequate conditions for efficient nitrogen fixation in symbiosis. The *R. etli* NifA-RpoN regulon had some components in common with other NifA-RpoN regulons described previously, but the vast majority of the components have been found only in the *R. etli* regulon, suggesting that they have a specific role in this bacterium and particular requirements during nitrogen fixation compared with other symbiotic bacterial models. Recently Lopez *et al.*[158] performed extensive analysis of the bacterial endophytic community present in different *Phaseolus vulgaris* (bean) cultivars using 16S ribosomal RNA gene sequences of cultured isolates derived from surface disinfected roots and immature seeds. A novel rhizobial species *Rhizobium endophyticum* was recognized on the basis of DNA– DNA hybridization, sequence of 16S rRNA, *recA*, *rpoB*, *atpD*, *dnaK* genes, plasmid profiles, and phenotypic characteristics. *R. endophyticum* is capable of solubilizing phytate, the type strain is CCGE2052 (ATCC BAA-2116; HAMBI 3153) that became fully symbiotic by acquiring the *R. tropici* CFN299 symbiotic plasmid.

Jida and Assefa[159] studied symbiotically efficient native lentil nodulating rhizobia endowed with different plant growth-promoting (PGP) characteristics. About, 30 lentil nodulating rhizobial isolates were isolated from soils collected from different farmer fields found in Central and Northern parts of the country. All isolates were characterized based on morphological, physiological, symbiotic and PGP characteristics. The result of the study

showed that these isolates have exhibited interesting features such as wide range of carbon-sources and nitrogen sources utilization, tolerance to acidic and alkaline pH, metal toxicity and antibiotics. Symbiotic characterization indicated that all tested isolates have showed great diversity in their capacity to nodulate their host plant and produce shoot dry matter yield under glasshouse conditions. In addition, they showed PGP characteristics such as IAA production and inorganic phosphate solubilization. Out of all tested isolates 36.7% of them were IAA producer while only 16.7% were insoluble inorganic phosphate solubilizer. Wadhwa *et al.*[160] looked into the molecular diversity of native rhizobia trapped field pea genotypes in Indian soils. Selected 54 rhizobia, from all cultivars, were authenticated as rhizobia by plant infectivity test. Along with nodulation, symbiotic effectiveness in terms of symbiotic ratios showed wide range of effectiveness of pea rhizobia from 1.11 to 5.0. DNA of all the 54 rhizobia were extracted and amplified by PCR, using ERIC and 16S rDNA primers. Dendrogram based on ERIC profiles for 54 rhizobia showed the formation of 13 subclusters at 80% level of similarity. Dendrogram based on RFLP of 16S rDNA by three restriction endonucleases; *Msp* I, *Csp* 6I and *Rsa* I; also formed 13 subclusters at 80% level of similarity. However, positioning of subclusters was different from that of ERIC based dendrogram. Majority of the isolates i.e. 64.8% by ERIC profiles and 44.4% by RFLP of 16S rDNA formed one cluster. Isolates from same nodule were not 100% similar. Considering each cluster representing a rhizobial genotype, both techniques used to assess molecular diversity indicated the presence of 13 genotypes of field pea rhizobia. Mazur *et al.*[161] analyzed the distribution and sequence variability of markers located on chromosomes and extrachromosomal replicons of *Rhizobium leguminosarum bv. trifolii* strains originating from nodules of clover grown in the same site in cultivated soil. On the basis of sequence similarity of repA and repC replication genes to the respective counterparts of chromids reported *in R. leguminosarum bv. viciae 3841* and *R. etli CFN42*, chromid-like replicons were distinguished from the pool of plasmids of the nodule isolates studied. Variability of the gene content was analyzed in the different genome compartments, i.e., the chromosome, chromid-like and 'other plasmids'. The stable and unstable chromosomal and plasmid genes were detected on the basis of hybridization data. Displacement of a few unstable genes between the chromosome, chromid-like and 'other plasmids', as well as loss of some markers were observed in the sampled strains. Analyses of chosen gene sequences allowed estimation of the degree of their adaptation to the three genome compartments as well as to the host. The results showed that differences in distribution and sequence divergence of plasmid and chromosomal genes can be detected even within a small group of clover nodule isolates recovered from clovers grown at the same site. Substantial divergence of genome organization could be detected especially taking into account the content of

extrachromosomal DNA. Despite the high variability concerning the number and size of plasmids among the studied strains, conservation of the location as well as dynamic distribution of the individual genes (especially replication genes) of a particular genome compartment were demonstrated. The sequence divergence of particular genes may be affected by their location in the given genome compartment. The 'other plasmid' genes were less adapted to the host genome than the chromosome and chromid-like genes. Acosta *et al.*[162] studied genomic lineages of *Rhizobium etli* revealed by the extent of nucleotide polymorphisms and low recombination. They identified high levels of DNA polymorphism in *R. etli*, and found that there was an average divergence of 4% to 6% among the tested strain pairs. DNA recombination events were estimated to affect 3% to 10% of the genomic sample analyzed. In most instances, the nucleotide diversity (π) was greater in DNA segments with recombinant events than in non-recombinant segments. However, the degree of recombination was not sufficiently large to disrupt the congruence of the phylogenetic trees, and further evaluation of recombination in strains quartets indicated that the recombination levels in this species are proportionally low. Their data suggested that *R. etli* is a species composed of separated lineages with low homologous recombination among the strains. Horizontal gene transfer, particularly via the symbiotic plasmid characteristic of this species, seems to play an important role in diversity but the lineages maintain their evolutionary cohesiveness. Weilbo *et al.*[163] examined the link between physiological traits and bacterial competitive ability in eighteen *Rhizobium leguminosarum bv. viciae* (Rlv) isolates during root nodule colonization using laboratory and field experiments. The competitive ability of R/v strains was measured as the percentage of root nodules colonized by gusA-tagged rhizobia in two types of host plants, peas and vetch. The competitiveness of Rlv strains was significantly affected by soil type and the identity of the host plant. The amount of acylated homoserine lactones (AHL) produced by the strains was less important in influencing competitiveness. Finally, the preactivation of strains with flavonoids or the addition of AHL to gus-tagged Rlv strains did not significantly enhance competitiveness: of the gus-tagged inoculants in comparison to indigenous soil populations of vetch microsymbionts.

The rhizobia and regulation of symbiotic nitrogen fixation

Nitrogen is one of the essential nutrients for plant growth and as such fertilizers are frequently used in commercial crops for proper growth and development to enhance the nitrogen supply available to the plant. As concerns mount to the growing input of reactive nitrogen into our environment, as part of a “nitrogen cascade”, an increased need to understanding biological nitrogen fixation has become of paramount importance. Thus, bio-

fertilizers, microbial inoculants capable of nitrogen fixation to supplement the nitrogen requirement of plants, are frequently applied to the crop field to replace chemical fertilizers in sustainable agriculture.

Nitrogen fixation [i.e. the reduction of gaseous nitrogen (N_2) to ammonia (NH_3) *via* the action of the enzyme nitrogenase] is confined to the prokaryotes (diazotrophs) and is widespread amongst both the eubacteria and proteobacteria[164]. Nitrogenase is an oxygen-labile enzyme and rhizobia are obligate microaerophiles. However, the ability for free living prokaryotes to fix nitrogen is reasonably widespread only a few bacteria have evolved the ability to form nitrogen-fixing symbioses with higher plants. The rhizobia comprise several genera of gram-negative bacteria that are members of the family *Rhizobiaceae*, an α-subdivision of proteobacteria that are common in soil and has an ability to form root nodule with certain leguminous plants to facilitate nitrogen fixation. It has been estimated that the fixation of nitrogen by *Rhizobium*-legume symbiosis contributes over 50% of the available nitrogen in the biosphere and is the largest single input into the nitrogen cycle[15]. The occurrence site of this mutualistic association is root nodules, found on the members of the *Leguminosae*, where nitrogenase-forming strains of rhizobia differentiate to form membrane enclosed pleomorphic cells, referred to as bacteroids. This symbiotic association works on the principal that the bacteroids fix nitrogen in an energy expensive process and supply it to the plant in the form of ammonia on the other hand the plant reduces carbon dioxide and provide the bacteroid with a carbon and energy source.

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Regulation of the *nod* genes

The root systems of leguminous plants secrete compounds such as flavonoids and betaines, produced via the isopropanoid pathway, into the rhziosphere that trigger in rhizobia the induction of *nod* genes essential to initiate symbiosis[165]. The *nod* genes are very essential for determining host range and except for *nodABC* vary between species to species[166]. The genetic organization of the *nod* genes is such that they are found either as part of a sym island on the chromosome or on a separate transmissible sym plasmid. In *R. leguminosarum* the 13 *nod* genes are grouped into five distinct operons all found on the Sym plasmid pRL10;*nodABCIJ, nodD, nodFEL,nodMNT and nodO*[167-169].

In *R. leguminosarum* NodD protein is localized in the cytoplasmic membrane and act as both sensor and transcriptional regulator for the other *nod* operons and production of Nod factor[170-172]. NodD is a member of the LysR transcriptional regulator family and in response to flavones positively regulates transcription of approximately 25 genes required for bacterial synthesis and export of Nod factor[173-174]. The N-terminus of NodD is highly conserved and believed to be involved in DNA-binding, whereas the C-terminus is

comparatively variable and thought to be the regulatory domain that senses flavonoids or other inducers[175]. NodD is a *trans*-acting regulator that binds to a *cis*-regulatory element, the "*nod* box", preceding the transcriptional start site of genes associated in the *nod* response[176-177]. The typical *nod* box is located 25 to 75 of the relative transcriptional start site, and contains two half-sites containing the imperfect repeat ATC-N9-GAT-N16-ATC-N9-AAT that is critical for NodD binding[177-179]. NodD is a homo-tetramer that folds by action of the chaperone GroEL to form a dimer of dimers. This arrangement confers a V-shape where interactions between the four DNA-binding motifs of the subunits and the two target repeats of the *nod* box bend the DNA to form a V-shape also[180-182]. Whilst the DNA-binding of most NodDs *in vitro* occurs independently of flavonoids, however, it has also been shown that compatible inducer molecules must interact with the DNA bound NodD to initiate transcription of *nod* genes[183-185]. In *R. leguminosarum* the response of NodD to the binding of inducer molecules is to activate transcription by facilitating a sharpening of the bend in the *nod* box target DNA that forms the V-shape on interaction with NodD.

The regulation of the *nod* genes is strictly maintained for symbiosis and strong expression has been shown to inhibit nodulation[186-188,]. Differing strategies for regulation have evolved, however, all centre on the regulation of transcription by NodD. Species such as *R. leguminosarum bv. viciae* and *bv. trifolii* have only one copy of *nodD* whereas species of other rhizobia, such as *B. japonicum* and *S. meliloti*, encode two and three copies respectively[189-190]. In *R. leguminosarum nodD* expression is constitutive but autoregulates its expression. Transcription of *nodD* occurs divergently from *nodA* and binding of NodD to the *nod* box upstream of *nodA* competes with RNA polymerase binding at its own promoter[191-193]. In *S. meliloti* NodD1, NodD2 and NodD3 all act as positive regulators of the *nod* genes and mutation of all three is required to abolish nodulation[194-196]. Transcription of *nodD*3 is positively regulated by another LysR regulator *syrM* in response to NodD2, so repression of *nodD*2 by NolR also silences the expression of *nodD*3[197]. In *B. japonicum* NodD1 and NodD2 have very distinct functions. Unlike in other species transcription of *nodD*1 is not constitutive but is induced in response to flavonoids[198]. Transcription of *nodD*1 and *nodD*2 appears constitutive but regulation occurs through repression by NolR binding to the *nodD*1, *nodD*2 and *nodA* promoter regions[170,199-200]. NodD1 functions as a positive regulator for transcription of the *nod* genes, whereas NodD2 has a negative effect on *nodABC* transcription and acts as a repressor of *nodD*1 transcription[201]. Expression of *nodD*2 occurs in response to the MerR like positive regulator NolA whose transcription in turn is regulated in response to an as yet unknown plant derived inducer[202-203]. However, NodD1 mutants of *B. japonicum* still show ability to nodulate and this led to the

characterisation of the twocomponent sensor regulator NodVW that is also capable of initiating transcription of *nodA* in response to flavonoids[204]. Mutation of *nodVW* allowed only nodulation to occur only with soybean demonstrating that *nodVW* provides *B. japonicum* with the flexibility to nodulate a number of plants such as cowpea, mungbean and sirato[201].

Nod factors

Nod factors are lipo-chitooligosaccharides with a conserved β-1,4-linked acetylglucosamine backbone of usually 4 or 5 residues with an n-acyl chain attached to the C-2 position of the non-reducing terminal glucosamine[205]. Depending on the *Rhizobium* species Nod factors vary through differences in the structure of this acyl chain as well as substitutions to the reducing and non-reducing terminal glucosamine residues, which include addition of sulphuryl, methyl, carbamoyl, acetyl, fucosyl and arabinosyl groups to various positions on the Nod factor backbone[206-207]. Synthesis of Nod factors, as well as modifications and additions, are a major determinant of host range and production is mediated by proteins encoded by other *nod* genes. The *nodABC* genes are those conserved among rhizobia and encode an acyltransferase (NodA), a deacetylase (NodB) and a *N*-acetylglucosaminyltransferase (NodC), which function together to catalyze the synthesis of the core Nod factor structure required in nodule formation[208-209]. Also conserved among species are *nodIJ* which are co-trancribed with *nodABC*. These encode a putative ABC-transport system that facilitates secretion of Nod factor[210-212].

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Whilst *nodABCIJ* are the conserved *nod* genes, other accessory *nod* genes differ in both combination as well as action between species to achieve variation in Nod factor and so host range. *R. leguminosarum* lacks genes *nodH* and *nodPQ*, which when mutated in *S.meliloti* lead to a loss of the ability to activate early host responses. NodH, NodP and NodQ bring about the 6-O-sulfation of the reducing terminal glucosamine that is essential for host plant perception, as *R. leguminosarum* lacks *nodH* and *nodPQ* its Nod factor is non-sulphated[141,213-214]. The homology of *nodFE* between species is greater than that of *nodABC*, so it would appear that NodF and NodE of both *R. leguminosarum* and *S. meliloti* would have a similar function in the conversion of Nod factor. However, whilst a change to the acyl chain of Nod factor is facilitated by *nodEF* and *nodL* in both species, the nature of the change differs between species. Mutation of *nodF* and *nodL* of *S. meliloti* produces mutants able to initiate an early plant response but are not capable of forming infection threads[215]. *S. meliloti* double mutants in *nodF* and *nodH* produce Nod factor with a C18:1 *N*-acyl attachment rather than the C16:2 group usually secreted[216]. Similarly, in *R. leguminosarum* mutation of *nodE* produces Nod factor with the C18:1 *N*-acyl attachment. Modification of

the acyl chain is mediated by NodE, NodF and NodL to produce the mixture of C18:1 and C18:4 groups produced by wild-type, which changes the *biovar* specificity in *R. leguminosarum* from clover to pea[217]. However, mutation of *nodE* alone in *R. leguminosarum* is not sufficient to bring about a reversion in plant nodulation phenotype. Mutation of both *nodE* and *nodO* is required in *R. leguminosarum* to abolish nodulation as single mutations of both have no effect[218].

Finally, NodO forms ion-selective channels regulated by Ca^{2+} concentrations that allow the movement of monovalent cations (K^+ and Na^+) across membranes[219]. The exact reasons as to why only single mutation in either *nodE* or *nodO* does not abolish infection thread formation are unknown. It has been suggested that NodO has some role in the plant uptake of Nod Factor or induces lipooligosaccharide depolarisation of the plasma membrane of leguminous plants.

Nod factor perception and signal transduction

Attachment of the rhizobia to the root hair occurs by adhesins and cellulose fibrils[220]. In response to molecular recognition of Nod factor root hairs undergo a radical developmental change with root hairs swelling and becoming deformed as a result of altered growth. Root hairs begin to curl shortly after and this is accompanied by activation of plant nodulation genes. The root hair tip curls by gradual and constant reorientation of the growth direction of the root hair to trap the rhizobia within the pocket of the curl[221]. Whilst purified Nod factor has been shown to elicit root hair curling they are not sufficient to bring about the tightly curled "shepherds crooks" that are the usual sites of entry for rhizobia[222]. It has been suggested that prolonged, localised Nod factor production and sensing is required to redirect the off axis tip growth to bring about tight curling[174,223-224].

The rapid and specific response of root hair and epidermal cells to Nod factors indicate that these cells directly perceive them through very specific receptors, as concentrations as low as 10^{-12} M can induce responses[70]. Nod factor perception has been proposed to occur as a two-event lock and key mechanism. Initial Nod factor recognition is less stringent and brings about root hair curling whilst the other is more stringent and allows initiation of infection threads and activation of plant nodulation genes[215,225]. Advances in microarray analysis of early plant nodulation events as well as characterized mutant phenotypes has led to the unravelling of 46 genes that are differentially regulated or proposed to play a role in the plants early Nod factor response[226]. The early less stringent recognition event in *L. japonicus* is facilitated by a heterodimer consisting of two LysM serine/threonine receptor-like kinases NFR1 and NFR5 specific to perception of *M. loti* Nod factor. These LysM receptor-like kinases are membrane spanning with each containing three extracellular

receiver domains as well as an intracellular kinase domain[227]. The extracellular domains show high similarity to the LysM binding domains of chitin and peptidoglycan binding proteins suggesting involvement in recognition of the Nod factor backbone[228]. *L. japonicus* mutated in either *NFR1* or *NFR5* were shown to be non-nodulating and also unresponsive to purified Nod factor[228]. *NFR5* was shown to have orthologs in both *P. sativum* and *M. truncatula*, *SYM10* and *NFP* respectively whilst orthologs of *NFR1* were identified first in *P.sativum* as *SYM2* and later in *M.truncatula* as *LYK3* and *LYK*[229]. This suggests that the early stage less stringent response of binding to the Nod factor backbone is also conserved between species and would explain how species can have multiple hosts. However, although it is established that NFR1 and NFR5 and their orthologues are the initial receptors for Nod factor perception they may also function in some way at a later stage in conjunction with other more stringent receptors for Nod factor binding and recognition. An *NFR1* deletion mutant of *L. japonicus* abolished early nodulation responses whereas gene silencing of *LYK3*/*LYK4* lead to root hair curling and entrapment of rhizobia. This root hair initiation was aborted soon after at a still early stage, indicating that it functions both in initial recognition and the late stage more stringent response[225]. The gene silencing method used to abolish *LYK3*/*LYK4* expression is not as clean as deletion mutant and subsequently it appeared that enough residual *LYK3*/*LYK4* expression remained to allow the early stage recognition events.

Nod factor perception at the later more stringent stage of infection brings about root hair curling, oscillations in calcium concentration and activation of plant nodulation genes. The initial nodulation events are induced by the depolarization of cells at the root hair tip by Ca^{2+} ion influx, which is thought to influence changes in the actin bundles of root hair cells. This act to bring about curling, deformation and also inhibit and the reinitiation of root hair tip growth[230-231]. It has also been implicated as a way of transducing the Nod factor perception signal and brings about activation of plant nodulation genes[232]. Calcium flux has been observed in a variety of legumes and is followed by the efflux of Cland K^+ ions causing the cytoplasm to fluctuate in pH and bring about alkinisation[233-234].

Calcium flux acts to accentuate the calcium gradient that occurs down from the root hair tip, where there is a high Ca^{2+} concentration, and is important in the later calcium spiking. After the early characteristic nodulation events observed in a variety of legumes, such as root hair curling and calcium flux, there is a lag time of approximately 10 minutes before oscillations in the calcium gradient occur. This is known as calcium spiking[231]. Addition of EGTA, a Ca^{2+} chelator, blocks calcium spiking by preventing root hair membrane depolarisation and this leads to a subsequent loss of expression for the plants nodule specific genes, indicating

that the modulation of intracellular calcium concentrations is required for signal transduction[235-236]. The genes required to initiate and respond to calcium spiking have been characterised in *M. truncatula* to structure a model of signal transduction that we can apply to other legumes where orthologs have been identified. *M. truncatula* mutants of *dmi1*, *dmi2* still show the initial nodulation events observed in response to Nod factors, such as root hair swelling, but not the subsequent calcium flux and early plant nodulation gene expression[237].

DMI1 and its orthologues, SYM4 and SYM8 in *L. japonicus* and *P. sativium* respectively, are predicted to be membrane spanning proteins with weak homology to cation channels. DMI2 and its orthologs, SYM2 and SYM19 in *L. japonicus* and *P. sativium* respectively, are predicted to function as receptor kinases that contain a leucine-rich-repeat (LRR) domain[238]. The LRR domains are proposed to function in protein-protein interactions and after binding of a ligand could induce a phosphorylation cascade which leads to DMI1 modulating calcium spiking[229]. DMI1 and DMI2 appear to act in concert to induce calcium spiking as mutation in either abolishes induction of plant nodulation genes. Therefore it is proposed that DMI2 acts as a sensor for Nod factor perception and DMI1 acts as a cation transporter that brings about calcium flux and spiking. In addition to DMI1 and DMI2 a calcium calmodulin protein kinase, DMI3, has also been identified in *M. truncatula,* which translates the calcium spiking signal and activates the cascade of genes required for nodulation[226,239-240,]. Modification of this Ca^{2+}/calmodulin-dependent protein kinase leads to autoactivation of the plants nodulation signalling pathway demonstrating its role in the key regulatory role upstream of later gene responses[241-242]. It has been proposed that increased bacterial numbers leads to an accumulation of Nod factor to sufficient levels that activate calcium flux and drive infection thread growth[243].

Nodule formation and structure

Bacterial cells that become trapped by the root hair curl initiate infection thread formation. Infection thread formation occurs either by local degradation of the plant cell wall or by new cell wall growth around the encompassed bacteria. This invagination grows down the body of the root hair into the body of dividing epidermal cells, known as the nodule primordium, within the root cortex. The rhizobia within the infection thread continue to grow and divide down the infection thread and until they reach the base of the root hair where cells are released and engulfed by endocytosis to form the symbiosome[244]. Growing cells of *S. meliloti* that formed infection threads with alfalfa formed two or three columns of sister cells within each infection thread and growth was restricted to a region approximately 60 μM from the growing tip[245]. The growth of various mutant strains in infection threads indicates that the plant provides the necessary carbon and nitrogen requirements for bacterial growth.

As bacteria grow down the infection thread they accumulate large PHB reserves that are lost in *phaC* mutants indicating they receive a plentiful carbon supply from the host plant[246]. Amino acid auxotrophs are also able to invade developing nodules suggesting that the plant provides the necessary amino acids to complement for growth down the infection thread[247]. Developing infection threads may fuse with membrane vesicles, similar to that occurring in pollen tube growth, and provide nutrients that would explain why growth occurs only at the tip[247-249]. More recently this restriction of growth to the tip of the infection thread was proposed to be a result of hydrogen peroxide mediated solidification of the luminal matrix[250]. Control of oxidative stress may allow the plant to control the abortion of infection thread progression and hence control the nodule number[250-251].

The release of bacterial cells from the infection thread, to form symbiosomes, occurs by formation of infection droplets that occur at positions where the cell wall gets disrupted. Rhizobial cells come into direct contact with the plasma membrane and are endocytosed[250]. The symbiosome consists of differentiated bacterial cells facilitating nitrogen fixation that are surrounded by a continuous plant membrane[251-253]. Formation is either accompanied by division of the plant membrane, as occurs in pea nodules that results in bacteria becoming singly enclosed, or it does not divide as occurs in bean that gives multiple cells enclosed by the same membrane. Differentiation of bacterial cells in indeterminate nodules occurs after several periods of division and the symbiosome membrane is relatively impermeable to metabolites. Later in the infection process a burst of carbon metabolism by rhizobia is proposed that is required to convert free-living cells into much larger differentiated bacteroids[246].

The plant-derived membrane around the bacterial cells is termed the peri-bacteroid membrane (PBM) and segregates the bacteroids from plant cytosol, with the intervening peri-bacteroid space (PBS)[248,252-253]. The peri-bacteroid membrane is poorly permeable to metabolites leaving the bacteroids reliant on the plant host for nutrients. Aquaporins, an ammonium channel, a dicarboxylate transporter and an aspartate/H+ symporter have all been demonstrated are suggested to facilitate transport and control of metabolites, required by the bacteroid for nitrogen fixation, across the PBS[254,255]. In addition H^+ ATPases, both P-type and Vtype, have been identified at the PBM that function to pump H^+ into the peri-bacteroid space, which in conjunction with H^+ liberated by the bacterial electron transport chain into the PBS creates an electrochemical and pH gradient between the plant cytosol, PBS and the baceteroid[255-256]. This acidic environment may be important for the movement of ammonia out of the bacteroid as it promotes formation of $NH4^+$ from NH_3 maintaining a diffusion gradient[257-259]. The transport of ammonia and amino acid across the peri-bacteroid

membrane will be addressed further in depth later.

Rhizobia are capable of forming two distinct types of nodule, determinate and indeterminate, depending on the host plant. Legumes that form determinate nodules are typically *Glycine max*, *Phaseolus vulgaris* and *L. japonicus* whereas those that form indeterminate nodules are *M. sativa*, *M. truncatula, Vicia faba* and *P. sativum*. The main distinguishing characteristic is that determinate nodules lack a persistent apical meristem and have no obvious development gradient. The infection thread does not continue to extend and instead infection occurs via division of pre-infected cells to give evenly distributed bacteria. Nodule growth occurs by cell enlargement to give nodules a characteristic round shape. In comparison indeterminate nodules have a persistent apical meristem caused by elongation and branching of the infection thread that continues to divide giving rise to a meristematic zone where new cells are subsequently infected to form new nodule tissue. This causes indeterminate nodules to become elongated and have clear zones at different stages of development. The bacteria-free meristematic cells and the subsequent infection of them can be seen as zones I and II. The area where the bacteria begin to differentiate into bacteroids occurs between zones II and III and is characterised by layers of host cells of high starch content and sees expression of the *nif* genes required for nitrogenase. Zone III is the where mature differentiated bacteroids carry out nitrogen fixation and zone IV is where they begin to show signs of senescence[260].

Bacterial cell surface polysaccharides of rhizobia consist of capsular polysaccharides (CPS), exopolysaccharides (EPS), lipid polysaccharides (LPS) and cyclic β-1,2 glucans and are essential for invasion and nodule development[261]. The precise role of these cell surface sugars is still unclear but mutations in EPS of both *S. meliloti* and *R. leguminosarum* has demonstrated that they are essential for nodule formation[262]. EPS biosynthesis and modification in *R. leguminosarum* is carried out by the *pss* genes and mutation in a number of these genes has shown to produce strains that are nonmucoid and produce non-nitrogen fixing nodules. Whilst the strains are capable of eliciting root hair curling the initiation of an infection thread is abolished suggesting that cell surface recognition is also required for nodule formation[263-264]. To further illustrate this mutations of the genes required for synthesis of the core element of LPS in *R. leguminosarum* have been shown to reduce colonization efficiency in a similar manner[265]. Plant derived glycoproteins bind to the cell surface of free-living *R. leguminosarum* and are also detectable in bacteroids following isolation from mature nodules. Mutation of the genes required for core LPS production produces a strain incapable of binding to the plant derived glycoproteins *in vitro*[267]. Further evidence for a required interaction is that more subtle mutations that affect the correct length

of the O-antigen side chain produce strains that are capable of infection thread formation and release but form abnormal symbiosomes. These abnormal symbiosomes show defects in the synchronized division of bacteroids and peribacteroid membrane suggesting that interaction between plant glycoproteins and LPS is required[266-267].

Nitrogen fixation by mature bacteroids is carried out in a microaerobic environment and regulation of the oxygen tension in the nodule is essential to balance the oxygen labile nitrogenase with the need for respiration. The plant cell cytoplasm contains leghaemoglobin that acts to regulate the supply of oxygen to nodule tissue and gives nodules the characteristic pink red colour[268].

Leghaemoglobin binds free oxygen and releases it when the concentration drops below a certain level to provide the bacteria with enough for respiration but maintains a low free oxygen environment[269].

The oxygen status of the nodule is a key factor in activation of the *nif* and *fix* genes, required for microaerobic respiration and nitrogenase biosynthesis, with both being transcriptionaly co-ordinated in response to the microaerobic environment[270].

The *fix* genes and bacteroid differentiation

Genes identified as essential for nitrogen fixation were typically characterised either through their shared homology to those genes required for nitrogen fixation amongst freeliving bacteria, *nif* genes, or their identification as genes required for nitrogen fixation that are specific to bacteria that form symbiotic relationships, *fix* genes[271-274]. The induction of the *fix* genes and the switch to microaerobic respiration is one of the most critical developments in bacteroid differentiation and must occur before nitrogenase biosynthesis, due to the enzymes oxygen labile nature. The *nod*, *nif* and *fix* genes of *R .leguminosarum* all lie within a 36 kb region of the sym plasmid pRL10 with a characteristic low G+C ratio. The *fixABCX* genes are transcriptionaly coupled to *nifAB* and this operon is divergently transcribed from *fixNOQP* and *fixGHIS*. A second copy of *fixNOQP* and *fixGHIS* are found on pRL9 and would appear to be in an operon with the distal regulatory gene *fixK2*. Also divergently transcribed from this are the other more orthodox other regulatory genes *fixKL*[169,275]. The reason for the presence of two copies of *fixNOQP* and *fixGHIS* is unknown as one intact copy of either is sufficient for effective nitrogen fixation[275]. The *fixNOPQ* operon is predicted to encode an alternative terminal oxidase with high oxygen affinity that would be required for bacteroid respiration in a microaerobic environment, whilst the *fixGHIS* genes encode a cation pump and accessory genes required for correct FixNOPQ synthesis[275-277].

The two principal regulatory proteins that bring about transcription of the *nif* and *fix* genes

are the transcriptional regulators NifA and FixK. Regulation of *nifA* and *fixK* in response to the oxygen status of the nodule was first demonstrated in *S. meliloti*. The *fixL* and *fixJ* genes encode a two-component regulator in which FixL acts in response to the oxygen status to phosphorylate the response regulator FixJ[278-280]. The phosphorylated FixJ then activates the expression of the transcriptional regulator FixK, a member of the FNR/CRP family, to bring about activation of the *fix* genes and *nifA*[281-283]. However, whereas in *S. meliloti* the FixLJ cascade regulates expression of both *nifA* and *fixK* it was noted that in *B. japonicum* it acts only to initiate expression of *fixK2*, which positively regulates the genes required for microaerobic growth. In *B. japonicum* expression of *nifA* in aerobic conditions is significant but is increased in microaerobic conditions by action of the two-component regulator RegS/ RegR, which senses the redox state of the cell[284]. The genetic structure and regulation of the fix genes of *R. leguminosarum* and *R. etli* is remarkably similar and again the regulation differs from that previously discussed for *B. japonicum* and *S. meliloti*. *R. leguminosarum bv. viciae* and *R. elti* have no FixJ homologue and instead encode a FixL like protein that is a hybrid of both FixL and FixJ[285-286]. FixL performs the function of both proteins to activate expression of *fixK* and *fnrN*, encoding two FNR/CRP like transciptional regulators that are important for expression of the *fixNOQP* and other[285,287]. *R. leguminosarum bv. viciae* and *R. etli* have two copies of *fixNOQP* and it is FnrN rather than FixK that is primarily responsible for expression of both operons, however only a double mutation in *fixK* and *fnrN* results in a Fix- plant[275,285,287]. Whilst strains that had a single mutation in *fnrN* where Fix+ there was a significant reduction in the rate of nitrogen fixation, indicating its role as the dominant transcriptional regulator for *nifNOQP* expression.

The *nif* genes and nitrogenase biosynthesis

The reductive breakage of the strong triple bond between N_2 molecules is an energetically expensive reaction that is catalysed by the nitrogenase enzyme to yield NH3.

$$N_2 + 8\ H^+ + 8\ e^- + 16\ ATP \rightarrow 2\ NH_4^+ + H_2 + 16\ ADP + 16\ P_i$$

Nitrogenase is composed of two structurally and mechanistically conserved metalloenzymes, dinitrogenase and dinitrogenase reductase, which are encoded by the *nif* genes in rhizobia[288]. Dinitrogenase is a molybdenum-iron, MoFe, containing protein that is 220 – 240 kDa and forms an $\alpha_2\beta_2$ tetramer of two pairs of two metaloclusters[289-290]. Each αβ pair contains one MoFe cofactor, MoFe7S9 homocitrate, which contains the site of substrate reduction, and one Pcluster, Fe - 7S[291]. The α-subunit is encoded by *nifD* and contains the MoFe cofactor whilst the β-subunit is encoded by *nifK* and associates with the α-subunit, with a P-cluster present at the αβ interface[292]. Dinitrogenase reductase is a smaller dimeric iron containing protein of 60 kDa encoded by *nifH*. Dinitrogenase reductase acts as the

obligate electron donor to dinitrogenase and contains a single 4Fe-4S cluster at the subunit interface as well as two Mg-ATP binding sites, one on each subunit[292-293]. The oxygen labile nature of nitrogenase is due to this surface exposed 4Fe-4S cluster on the dinitrogenase reductase as the enzyme mechanism requires electron transfer from here to the P-cluster of the dinitrogenase and subsequently to the FeMo cofactor, the site of substrate reduction. Nitrogenase is a relatively large and slow enzyme with a turnover time of approximately 5 s^{-1} and can account for up to 30% of the bacteroid's protein content[294-295].

The role of *nifA*, *nifD*, *nifK* and *nifH* as the regulatory and structural genes for dinitrogenase and dinitrogenase reductase has already been discussed above. However, a number of other *nif* genes are required for the biosynthesis of the metalloclusters and the correct polypeptide folding associated with nitrogenase maturation. The genetic organisation of the *nif* genes in *R. leguminosarum bv. viviae* is such that *nifAB* and *nifHDKEN* are found closely localised to the *fix* genes on the sym plasmid pRL10, *nifU* is found on the chromosome and *nifS* on pRL8[169,296]. NifB, NifN, NifE, NifV and NifH have all been assigned roles in the FeMo cofactor biosynthesis whilst NifU and NifS are proposed to have a role in Fe-S cluster biosynthesis[297-298]. The products of other non-*nif* genes under the regulation of NifA, such as GroEL, have also been identified that have a role in maturation of nitrogenase. GroEL is a general chaperone that forms transient interactions with NifH, NifD and NifK and has been shown to be vital for correct interaction of the FeMo cofactor and form the active site[299]. However, there is still much to be determined as to how and by what the Fe-S and Pclusters are formed and then associate with the polypeptides.

Transcriptional activation of the *nif* genes is mediated via the transcriptional regulator NifA and the RNA polymerase sigma factor σ^{54}[300]. NifA was first identified as the regulator required for transcription of *nifH* and other genes associated with rhizobia nitrogenase biosynthesis through addition of purified NifA from *K. pneumoniae*, then later through cloning and mutation[301-302]. The symbiotic regulation of *nifA* in *R. leguminosarum bv. viviae* UPM791 was recently shown to be autoregulated as promoter mapping indicated a NifA binding site upstream of the *fixABCX nifAB* operon[303]. A second weak symbiotically expressed promoter also exists between the intergenic region of *fixX* and *nifA* that is most likely required to initiate the transcription of *nifA*, as it has no discernable NifA binding site upstream, so that transcription from the primary promoter can be initiated. NifA is a member of the enhancer-binding protein family (EBP) and shares the conserved architecture of an amino terminal regulatory domain, a carboxyl terminal DNA binding domain and a central conserved AAA+ ATPase domain. The C-terminal DNA binding domain contains a helix-turn-helix motif that is required for recognition of an upstream activating element

(UAS) -24 / -12 of the promoter, in *S. meliloti* the σ54 polymerase core binds to the consensus sequence 5'-CTTTGTCGATATCCGACAAAG-3'[202,304]. The N-terminus of typical NifA proteins from *K. pneumoniae*, *A. brasilense* and *A. vinelandii* all contain cGMP -specific phosphodiesterase stimulated *Anabaena* adenylate cyclases and an *E. coli* FhlA (GAF) sensory input domain, both required for regulation of NifA activity in non-symbionts. Interaction at these sites appears to be involved with sensing of the nitrogen status of the cell either by interaction of GlnB or 2-oxoglutarate[305]. Whilst NifA mediated expression of *nifH* in rhizobia has not been demonstrated in response to the nitrogen status of the cell the N-terminal region has been implicated in post-translational regulation. Partial deletion of the N-terminal domain of NifA leads to an increase of transcription of the *nif* genes indicating its regulatory role[306]. Finally the central AAA^+ ATPase domain is required for interaction with the σ54 RNA polymerase haloenzyme via DNA looping to form a closed complex. ATP hydrolysis via the AAA^+ domain then brings about the change in the σ54 binding surface to form an open complex and initiate transcription of the *nif* genes. This central region would also appear to confer NifA with its oxygen sensitive nature, due to a region of conserved, indispensable and invariant cysteine residues that lie between the AAA^+ domain and the DNA binding domain that are believed to be involved in the binding of metal ions[295,307].

NifA is itself reactive to oxygen and so displays both transcription and posttranslational control that could allow for disparate oxygen sensing, bringing about the differential timing of expression between the *fix* and *nif* genes. The hierarchical regulatory cascade of *B. japonicum* displays transcription of *nifA* and *fixK* at an oxygen concentration where NifA activity is prevented[308-309]. This enables expression of the *fix* genes required for microaerobic growth at the relatively higher oxygen concentration found in certain areas of the nodule without expression of *nif* genes, at a concentration where nitrogenase would be inactivated. When a lower relative oxygen concentration is reached in the areas of nodule tissue that nitrogen fixation occurs NifA is no longer inactivated and so transcription of the genes required for nitrogenase biosynthesis occurs. The oxygen reactive nature of NifA was certainly attributed to be the reason as for why microarray data comparing *S. meliloti* microaerobic growth with symbiotic conditions showed only 31 genes induced in both conditions[310]. Of these 31 genes the transcriptional regulators *nifA* and *fixK* were both shown to be transcribed and whilst the genes subject to FixK regulation were also detected those under the control of NifA were not. They attribute this to the fact that their microaerobic environment was kept at a concentration of just under 1 μM dissolved oxygen whereas the free oxygen reported in soybean nodules is 18 nM[311]. Microarray analysis of *S.*

meliloti nifH and *nifA* mutants, relative to wild-type, have further demonstrated the regulatory function of NifA on symbiotic genes compared to that of a structural gene involved in nitrogenease biosynthesis. Mutation in *nifA* caused a decrease in the transcription of 310 genes, compared to a decrease of 150 genes in response to mutation in *nifH*, indicating possible transcriptional control by NifA over 160 genes during symbiosis[304].

Conclusion

Nitrogen is a life sustaining element for all living organisms. Nature has several pathways by which this element is biologically recycled. Many leguminous plants trap the abundant pool of nitrogen in the atmosphere through symbiotic association with nitrogen-fixing microorganisms. Growing interest in the unexplored biogeographical regions has provided evidence of enormous diversity among the nitrogen-fixing and nodulating bacteria[88]. Rhizobia are such nitrogen fixing bacteria which are genetically diverse and heterogeneous in nature. The rhizobia can be characterized either by using traditional methods or molecular techniques may also be utilized. Therefore, there is wide scope to explore the possibility to select better strains which can fix nitrogen efficiently and can adopt with the local environment. The various molecular mechanisms involved in the process of nitrogen fixation have been widely studied globally and these tools can be utilized successfully.

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Chapter 6

The science of tea (*Camellia sinensis*): a review

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Abstract

Tea (*Camellia sinensis*) is the oldest caffeine containing beverage, and has been used for over two to three thousand years. In this paper an attempt has been made to provide comprehensive review on various aspect of Tea research carried out in India and worldwide. This review provides deep insight into the taxonomic background, genome diversity, economic importance and health benefits of tea. Histological studies, conventional and *in vitro* methods of propagation, DNA fingerprinting studies, genetic transformation including gene construction and vectors are also discussed.

Keywords

Camellia, tea, cash crop, taxonomy, propagation, molecular diversity

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History of tea (*Camellia sinensis*)

History of tea world wide

Tea is the oldest caffeine containing beverage, and has been used for two to three thousand years in south-east China[1]. It is also the most widely consumed hot beverage as being the cheapest. To date, the habit of tea drinking has become well established for more than half of the world's population. India is also the largest consumer of tea and accounts for 22% of global tea consumption. Based on FAO records[2], 2.7 million hectares of land are under tea cultivation and 2.2 million tons are produced every year in the world. Over 80% of the world's tea exports come from India and Srilanka. Exports from China rank next to these two countries. The United Kingdom is the largest importer of the world, importing over 20% of the production. The largest importer of the world is the United States, but there only about half a pound per person is drunk[3] India with 5.11 lakh hectares under tea with an average annual production of 850 million Kg is the largest producer of tea in the world. It accounts for a fifth of the global tea area and nearly a third of the total world production. At present tea is grown in 16 states in India. Major tea growing states are Assam (50.7%), West -Bengal (22.1%), Tamil-Nadu (15.9%) and Kerala (8.3%)[4].

The centre of origin of the tea plant is considered to be near the source of the Irrawady River and further North. It is variously stated that tea is found wild in Assam and Upper Burma and South Yunnan in upper Indo-China[1,5], but its real origin is still the subject of various theories. In China, cultivation of tea has long been established, while in Japan tea was introduced during the 12th century from China.

From the main centers of its primary origin in South-East Asia, tea has spread far and wide into tropical and subtropical areas and adopted broad characteristics corresponding to regions of tropical rain forest, tropical Savannah and summer rain areas[6-9].

In India, tea seeds from China were brought and sown at Botanical Garden, Calcutta in 1780[10]. Tea (China type) was introduced in North East India in 1836, although in 1823, Major Robert Bruce discovered tea plants growing wild in some hills near Ragpur (now Sibsagar), the then capital of Assam[11]. In South India, one Dr. Christy has experiments on growing tea in Nilgiris in 1832[12]. But the tea did not get under way until 1893 when the planting of tea increased rapidly[13].

In the early part of the eighth century, tea was introduced into Japan from China and in Srilanka; seeds were imported from Assam in 1839 and planted in Royal botanic Gardens at Peradeniya near Kandy[14]. The commercial planting in Srilanka was taken up from 1867[15]. In Taiwan, tea growing on a considerable scale started at the conclusion of the Sino-Japanese war of 1895[16]. In Indonesia, tea seeds were imported from Japan in 1826 and planted in the

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Bogor (Buitenzorg) Botanical Gardens though commercial planting started only in 1878 using Assam seeds collected from Srilanka[17]. Tea was introduced in Bangladesh in 1839[14], and in Vietnam it took commercial shape in 1918[18].

However, in other countries tea cultivation was introduced as a commercial production since the discovery of the black tea variety *assamica* by R. Bruce in 1823.

In Africa, tea was first grown near south of Limpopo river in 1850[19]. The commercial production in Natal, South Africa, dates from 1878[20]. Tea seeds obtained from Botanic Garden in Edenburgh were introduced into Malawi in 1878 but there were no survivors. Another batch of seeds was brought in 1886 from Edinburgh and Kew Gardens in 1888[21]. In Kenya, tea was introduced at Limuru in 1903 but commercial tea growing, however, did not start until 1920s. In Uganda and Tanzania, experimental planting of tea was made in 1900 and 1904 and commercial production did not begin until 1931 and 1926 respectively[14,22].

In Turkey, tea was first planted in 1888 but it was unsuccessful and later cultivation started in 1939-1940 dominated by China hybrid seeds obtained from Georgia in the former Soviet Union[23]. Cultivation of tea started in Russia in 1885 in Chakva village near Batumi city with the seeds transported from China[24].

In addition to the above countries to the above countries, tea is also grown in Iran, Malaysia, Mayanmar, Nepal, Thailand, Burundi, Mauritius, Mozambique, Etiopia, Cameroon, Republic of Congo, Rwanda, Zimbabwe, Argentina, Brazil, Ecuador, Peru, Papua New-Guinea and Australia. The latitude of these countries ranged from 41°N to 33°S.

History of Tea in India

The credit for creating India's vast tea empire goes to the British, who discovered tea in India and cultivated and consumed it in enormous quantities between the early 1800's and India's independence from Great Britain in 1947. The Scottish adventurer, Robert Bruce, discovered tea plants growing in Assam in the 1820's. At this time, no one thought that tea existed in India; however Major Bruce discovered the plants growing wild in the jungles controlled by the tribal chiefs. The British East India Company's monopoly in China ended in 1832 and it became necessary to find other sources to supply the English consumers of tea. In 1834, a tea committee was appointed to investigate the possibility of cultivating tea in India. After a thorough investigation and study of the crop, the first commercial batch of tea ever produced outside of China came from Assam in 1839. Two of India's major teas are the Darjeeling tea and the Assam tea. India’s famed Darjeeling tea is named after the summer capital of the Government of Bengal, where tea is cultivated at altitudes of 4,000-10,000 feet in the Darjeeling hills. India's other major tea, Assam tea is named for the district in which it is grown, which lies in northeast India along the border between India

and Burma. This region produced more black tea than any other area in the world, with the exception of some parts of China.

Tea has been addressed under various botanical names viz. *Camellia thea* Dyer, *Camellia sinensis* (L.) O. kuntze[25], *Thea sinensis*, *T. bohea* or *T. viridis*[6]. It belongs to Thea section of the *Camellia* genus under Theaceae family. However, today tea is botanically referred to as *Camellia sinensis*, irrespective of species specific differences. But it has been found that tea has various distinct species, or forms and subspecies. Different authors have recognized various intra-specific categories of *Camellia sinensis*[25-27] but the only ones which have met general acceptance were described by Kitamura, quoted by as *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica* (Masters). For simplicity, these two species are generally referred to as *C. sinensis* and *C. assamica*[6,15,27,28].

A third form which resembles Planchon's Thea *lasiocalyx*[27],was proposed by Wight[35] as subspecies of *C. assamica* and named *C. assamica* subsp. *lasiocalyx* (Planchon ex Watt). A fourth group called hybrid variety with mixed taxonomic characters of cultivated China, Assam and Cambod types and to a lesser extent some species hybrids from other species of *Camellia*[15,27,28], occupies the largest tea area under cultivation through seed propagation[29].

Genome diversity

The genus *Camellia* had 82 species in 1958[30] and accounts for more than 325 species in 2000[31] that indicates genetical instability and high out-breeding nature of the genus. Presently, world-wide over 600 cultivated varieties are available, of which many have unique traits such as high caffeine content, blister blight disease tolerant etc. Owing to extensive internal hybridization between different *Camellia* taxa, several intergrades, introgressants and putative hybrids have been formed. These can be arranged in a gradient based on morphological characters that extend from China types through intermediates to those of Assam types. Indeed, because of the extreme homogenization, existence of the pure archetypes of tea is doubtful[32]. Till date, numerous hybrids currently available are still referred to as China, Assam or Cambod tea depending on morphological proximity to the main taxon[33]. Tea breeds well with wild relatives and thus taxonomists have always been interested to identify such hybrids due to suspected involvement in tea genetic pool. Two particularly interesting taxa are *C. irrawadiensis* and *C. taliensis* whose morphological distributions overlap with that of tea [33]. It has also been postulated that some desirable traits such as anthocyanin pigmentation or special quality characters of Darjeeling tea might have introduced from wild species[34].

Other *Camellia* species, which are suspected to have contributed to the tea genetic pool by hybridization, include *C. flava* (Pifard) Sealy, *C. petelotii* (Merrill) Sealy[35] and possibly *C.*

lutescens Dyer[28]. The role of *C. taliensis* is however, not clear because the species itself is considered to be a hybrid between *C. sinensis* and *C. irrawadiensis*[34,32]. Therefore, it is generally agreed that at least three taxa i.e. *C. assamica*; *C. sinensis*; *C. assamica* sub sp. *lasiocalyx* and to an extent *C. irrawadiensis* have mainly contributed to the genetic pool of tea. The term 'tea' should therefore, cover progenies of these taxa and the hybrids thereof or between them.

Economic importance and health benefits

The economic importance of the genus *Camellia* is primarily due to the tea. Tea was initially used as a medicine and subsequently as beverage and now has proven well to be a future potential as an important raw material for the pharmaceutical industry. Tea is mainly consumed in the form of 'fermented tea' or 'black tea'. However, 'non-fermented' or 'green tea' and semi-fermented or 'oolong tea' are also popular in some countries e.g. Japan and China. Apart from being used as beverage, green leaves are also used as vegetables such as 'leppet tea' in Burma and 'meing tea' in Thailand. Though the tea seed oil is used as lubricant, yet extraction from seed is not economical[36]. Additionally tea seed cakes contain saponins but has got poor value as fertilizer as well as unfit for animal feed due to low nitrogen, phosphorus and potassium content but can be used successfully in the manufacture of nematocide[36]. Tea leaves have more than 700 chemical constituents, among which flavonoides, amino acids, vitamins (C, E, K), caffeine and polysaccharides are important to human health. Importantly, the vitamin C content in leaves is comparable to that of lemon. Tea drinking' is now being associated with cell-mediated immune responses of the human body and reported to improve the growth of beneficial micro flora in the intestinal. Tea also imparts immunity against intestinal disorders, protects the cell membranes from oxidative damages, prevents dental caries due to presence of fluorine, normalize blood pressure, prevents coronary heart diseases due to lipid depressing activity, reduces the blood-glucose activity and normalizes diabetes[37]. Tea also possesses germicidal and germistatic activities against various gram-positive and gram negative human pathogenic bacteria such as *Vibrio cholera*, *Salmonella*, *Clostridium* etc[37]. Both green and black tea infusions contain a number of antioxidants like catechins and have anti-carcinogenic, anti-mutagenic and anti-tumorous properties. Among the different catechins, epigallo catechin-gallate is the most active component. Several epidemiological studies have also proved that tea consumption plays a protective role against human cancer.

Conventional propagation and breeding

Tea is propagated either through seeds or cuttings. Usually seeds are collected from orchard, stratified in sand and then sown in polythene sleeves in the nursery where it takes 12–18

months before transferring to the field. Nevertheless, seed-grown plants show a high degree of variability. Therefore, the alternative choice is through vegetative propagation of the elite variety wherein single leaf internode cuttings, with an axillary bud are planted in polythene sleeves under shade for 12–18 months followed by the transfer of these rooted plants to the field. Recently, grafting as an alternative propagation technique has gained considerable popularity. In this technique, fresh single leaf internode cutting of both root-stock and scion are generally taken. Scion, commonly a quality cultivar, is grafted on root-stock, which is either drought tolerant or high yielding cultivar. Upon grafting, the scion and stock influence each other for the characters and thus composite plants combine both yield and quality characters resulting 100% increase of yield with better quality than either of the non-grafted cultivar. Further, a modified improved 'second generation' grafting has been developed where tender shoots were grafted on the young seedlings of tea which have an additional advantage over conventional grafting due to presence of tap root system[38]. Tea breeding consists of hybridization as well as selection. Hybridization can be either natural or hand pollination. In natural hybridization, based on better performance of yield, quality or diseases resistance capability, two parents are planted side by side in an isolated place and allow them to bear fruits. Subsequently seeds (F1) are harvested, raised and planted. If average performance of these plants is found to be better than either parent, then seeds (F1) are released as hybrid seed or Bi-clonal seed. However, some of the outstanding performers among the progenies are marked and verified for multi-locational trial and still if found suitable released as clone. These clones are geographically specific and most of the tea research institutes in the world have generated clones for their own region. Sometimes more than two parents are used and known as poly-clonal seeds. The idea is to introduce more variability among the F1 seeds. Since it is difficult to know about the pedigree of the cultivars (as pollen may come from any male), hence the chance of reproducibility is low and least preferred presently. Alternatively, pollination or control cross, despite being an important approach has made a limited success in tea breeding. However, recently, few clones have been released in Kenya and Malawi using this technique. Selection is the most popular, age-old practice in tea breeding. Since commercial tea gardens earlier were established with seeds, hence lot of variability exit among them. Many instances the elite plant has been identified the existing bushes and released as clones. Majority of the tea clones have been developed through selection. However, pedigrees of the clone remain unknown. Though breeding work is limited up to F1 progenies presently yet F2 population holds greater promise for varietal improvement of tea. The advantage of this approach is better segregation of characters and with the help of molecular biology this can be exploited for marker assisted selections for a particular trait and construction of linkage map which is

till not available for tea. Although, conventional tea breeding is well established and contributed much for tea improvement over the past several decades, but the process is slow due to some bottlenecks. Specifically in tea, they are:

- perennial nature,
- long gestation periods,
- high inbreeding depression,
- self-incompatibility,
- unavailability of distinct mutant of different biotic and abiotic stress,
- lack of distinct selection criteria,
- low success rate of hand pollination,
- short flowering time (2–3 months),
- long duration for seed maturation (12–18 months),
- clonal difference of flowering time and fruit bearing capability of some clones.
- Similarly, vegetative propagation is an effective method of tea propagation, yet it is limited by several factors such as:
- slower rates of propagation,
- unavailability of suitable planting material due to winter dormancy, drought in some tea growing area etc.,
- poor survival rate at nursery due to poor root formation of some clones and
- seasonal dependent rooting ability of the cuttings.

Therefore, micropropagation technique appears to be an ideal choice for circumvention of the problems related to conventional propagation. Additionally, transgenic technology has the potential for varietal improvement of tea through means other than conventional breeding. However, central to any successful transgenic technology is an efficient *in vitro* regeneration protocol. While an efficient regeneration protocol is essential for introduction of the foreign gene into plant tissues, micropropagation is important for the transfer of large number of genetically modified plants to the field within a short span of time.

Camellia sinensis being a polymorphic species offers a lot of practical difficulties in the field of breeding and evolution of pure line races from them. Thus, there occurs a large variation in several important and desirable characters from bush to bush in the existing tea populations. These variations offers a ready means to exploit such plants and develop

improved planting material in this perennial crop by careful selection for high yield, excellence of cup characters of made tea, fair resistance to drought and some important pests and diseases and adaptations for different environment. Since commercialization of tea, improved planting materials have been developed primarily by selection of the desired plant type. Conventional tea breeding is well established, though time-consuming and labor intensive due to its perennial nature and long gestation period (4—5 years). Vegetative propagation is standard, yet limited by slow multiplication rate, poor survivability of some clones, and need for copious initial planting material. Seed-borne plants are heterogeneous due to their highly allogamous nature; consequently, it is difficult to maintain their superior character. Additionally, tea breeding has been slowed by lack of reliable selection criteria. Although few morpho-chemical markers are available for identification of superior cultivars, these markers are greatly influenced by environmental factors and show a continuous variation with a high degree of plasticity. To overcome these problems, a limited number of isozyme markers have been used, resulting in less polymorphism. With the advancements of molecular biology, however, efforts have shifted to using various DNA markers. Understanding genetic diversity at the molecular level of tea germplasm will help to: (1) preserve the intellectual property rights of the tea breeder; (2) identify individual tea cultivars through use of a molecular passport; (3) prevent duplicate entry of different genotypes into the tea gene pool; (4) increase efficient selection of varieties for hybridization, composite plant production, etc.; (5) classify tea genotypes taxonomically using molecular markers; and (6) improve tea varieties for agronomically important characteristics through marker assisted selection. Consequently, biotechnological tools appear to be the ideal choice to circumvent problems of conventional tea breeding.

DNA finger printing study of tea

Morphological markers such as leaf pose, dry matter production, partitioning, flesh evenness, etc. and biochemical markers such as total catechin/polyphenol content, caffeine, etc. are used to identify the superior tea plant. However, tea breeders are often unable to use markers effectively because they are greatly influenced by environmental factors and show a continuous variation with a high degree of plasticity. Hence, to overcome these problems, research has shifted to using more sensitive DNA marker technology.

Morphological markers

Tea has been classified into different taxa by morphological characters. Barua[27] provided morphoanatomical descriptions, which later was elaborated by Bezbaruah[39]. Morphological parameters such as leaf architect, growth habits and floral biology are important criteria used by tea taxonomists[33]. While bush vigour, pruning weight, period of recovery from

pruning time, plant height, root mass, root-shoot ratio, plucking point density, dry matter production and partitioning are considered as yield indicator of tea[33], caffeine, volatile compounds[40], green leaf pigmentation[33], leaf pubescence[41], total catechine content and total tannin content etc.[42] have been used as potential determinants for tea quality. Despite the several disadvantages, these are the most adopted markers used by tea breeder globally.

Cytological markers

Cytological markers of the genus *Camellia* were elaborately studied in the early 1970s with many interesting features. Chromosome number has been established for the most available taxa of *Camellia* including tea[39], which was reviewed by Kondo[43]. Generally tea chromosomes are small in size and tend to clump together due to ‘stickness’. Tea is diploid (2n=30; basic chromosome number, X=15) and karyotype ranges from 1.28μ to 3.44μ[39]. The *r* value (ratio of long arm to short arm) for all the 15 pairs of chromosomes range from 1.00 to 1.91. This consistency in diploid chromosomes number suggests a monophyletic origin of all *Camellia* species. However few higher ploidy level such as triploids e.g. TV-29, HS-10 A, UPASI-3, UPASI-20 (2n=45), tetraploids (2n=60), pentaploids (2n = 75) and aneuploids (2n +1 to 29) have also been identified[44,45]. Karyotypic data had also been accumulated in past for the other species of this genus[46-51]. In karyotype analysis, unfortunately, grouping by chromosome size was difficult in the *Camellia* taxa, since the chromosome grade imperceptibly from the largest to the smallest. Furthermore, even in the best preparation, homologous chromosome pairs could not be appeared identical in *Camellia*[48-50]. Relatively little intraspecific karyotypic variation had been observed in the cultivated species of *Camellia* studied[48,51]. Sat-chromosomes in karyotypes within mass accessions of certain *Camellia* species are morphologically and quantitatively variable. Thus karyotypes including characteristics of sat-chromosomes are not of taxonomic significance for *Camellia* taxa. Among the diploid species of *Camellia* studied, *C. japonica* L. *sensu lato* showed the greatest karyotypic variation, many of the accessions studied indicated similar karyotypic patterns to each other[48]. For instance, *C. japonica* L. var. *Spontanea* (Makino), *C. japonica* L. var. *macrocarpa* Masamune, *C. japonica* L. subsp. *rusticana* (Honda) Kitamura and four cultivars namely ’Aka-Wabisuke’, ’Fukurin- Wabisuke’, Kuro-Wabisuke’, and ’Wabisuke’ carried same, most common standered acetoorcein-stained karyotype if presence of satellites is not considered;16 metacentric, 8 submetacentric and 6 subtelocentric chromosomes. Actually, *C. japonica* L. var. *Macrocarpa* Masamune had satellites on four submetacentric chromosomes and the other accessions had satellites on two submetacentric chromosomes[52]. Later, it was shown by Kondo and Parks[53] that the C-banding method can be applied to the somatic mid-metaphase chromosomes in *Camellia* taxa. These differentially stained bands in somatic midmetaphase chromosomes permit the

identification of individual chromosomes and make it possible to match the homologous pairs of chromosomes more precisely and possibly even measure chromosome divergence between different clones within the same species with same or similar karyotypes. Karyotypic variability and divergence among the seven accessions of *C. japonica* L. *sensu lato* with same aceto-orcein stained karyotype were revealed by C-banding method[54]. These way cytological markers were used to sort and classify the vast number of cultivars. However, due to the development of more sensitive biochemical techniques, attention was shifted towards the search of biochemical markers.

Biochemical markers

Biochemical markers were widely used for characterization of different plant germplasm[55]. Presence of calcium oxalate crystals and it's quantity in paranchymatous tissue of leaf petioles nomenclatured as phloem index, have been suggested to be a suitable criterion for classifying tea hybrids[29]. The variations in quantity and morphology of the sclereids in the leaf lamina were also utilized for differentiating tea taxa[56,57,]. Takeo[58] suggested a chemo-taxonomic method of classifying tea clones based on a ratio referred to as the Terpene Index (TI), which expresses the ratio between linalools and linalools plus geraniols. However, with the advancement of high performance liquid chromatogarphy, considerable success has been achieved in the identification of tea quality indicators[59,60]. These indicators have also found wider use in distinguishing the two main species of tea, namely *C. sinensis* and *C. assamica* and their respective clones[61]. Although not fully exploited, the polyphenol oxidase activity, individual polyphenols, amino acids and chlorophyll content are considered to be potential parameters in tea taxonomy[62]. The presence or absence of certain phenolic substances in tea shoots has also been used in establishing relationships among various taxa[63]. Quantitative changes in chlorophyll-*a*, chlorophyll-*b* and four carotenoids (β-carotene, lutein, violaxanthine and neoxanthine) were used for characterization of Assam, China, and Cambod[64]. Total catechin concentration and the ratio of dihydroxylated to trihydroxylated catechins of green leaf were used to establish genetic relationship among the 102 Kenyan tea accession[65]. Upon multivariate analysis, accumulation of the various catechins separated the tea clones into 3 major and 5 minor groups, according to their phylogenetic origin. They found that Cambod tea had the highest ratio (7:10) followed by China tea (3:5) while Assam tea had the lowest ratio (1:4). This biochemical differentiation indicates that there is potential for broadening the genetic base of mainly Assam tea in Kenya with the putative China and Cambod tea. Though detection accuracy is higher, yet accumulation of such chemicals is subjected to post-transcriptional modification, which restricts the utility of biochemical markers[66].

Isozymes markers

Genetic analysis of isozyme variation was used for cultivar identification in a wide range of plants[67,68]. Similarly in tea also, isozymes have been analysed by several workers[69-71] for studying the genetic tendencies, cultivar identifications and implications in hybrid breeding. Among the isozymes, peroxidase and esterase are extensively studied in different tea cultivars[72-77]. However, other isozymes such as tetrazolium oxidase, aspartate aminotransferase and alpha-amylase were also studied among 7 different tea cultivars along with 3 different species[78]. The electrophoretic analysis revealed both the qualitative and quantitative variation in the isozyme banding pattern among different species of tea and their clones. The tetrazolium oxidase enzyme system showed the highest variability among all the enzymes. Cluster analysis using isozyme banding pattern produced a dendrogram which clearly differentiated characteristics of both the clones and species studied. In general, isozyme studies in tea were limited to few enzymes with inadequate polymorphism[79]. However, isozymes were extensively studied in *C. japonica*. Wendel and Parks[80] reported the analysis of 17 isozymes in different cultivars of *C. japonica*. They found that 15 isozymes produced 2-9 polymorphic loci while 2 produced 1–3 monomorphic bands. Based on the segregation of 12 loci by 8 enzymes, they suggested that genes of aspartateamino-transferase with phospho-glucomutase and 6-phosphogluconate dehydrogenase with phosphoglucomutase are linked. Further, the same group[81] resolved isozyme variation at 15 loci from 12 enzymes in 205 cultivated genotypes of *C. japonica*. All loci were polymorphic and a total of 64 alleles were detected. The sensitivity of electrophoresis in distinguishing clones was high so much so that 95.4% of the clones uniquely characterized by their 15-locus genotypes. Isozyme analysis was also used to study the genetic linkage and segregation pattern of alcohol dehydrogenease genes[82]. They found that alcohol dehydrogenease isozymes in *C. japonica* are encoded by two genes Adh-1 and Adh-2. Both loci are expressed in seeds and their products are randomly associated with intragenic and intergenic dimmers. Electrophoresis of leaf extracts produces only the products of Adh-2. Formal genetic analysis indicated that the two Adh loci are tightly linked. Most segregations fit expected Mendelian ratios but in some families distorted segregation was also observed at Adh-1, Adh-2 or both. Starch gel electrophoresis was used to score allelic variation at 20 loci resolved from 13 enzyme systems in seeds of *C. japonica* collected from 60 populations distributed over throughout the Japan. The genetic diversity within the population was higher i.e. 66. 2% of loci were polymorphic per population on an average within a mean number of 2. 16 allele per locus; the mean observed and heterozygosities were 0.230 and 0.265, respectively. They also reported genotypic proportions at most of the loci in majority of the population fit the Hardy-Weinberg

expectations[83]. The dispute of identification of two oldest *C. japonica* plants at New Zealand as cv. Middle Mist Red was concluded using 10 different isozymes[84]. Among them 3 isozyme could not detected polymorphism. The analysis based on rest of the 7 enzymes confirmed that those plants were not actually belonging to the Middle Mist Red cultivars. However, limited number of loci of isozyme showed the lesser polymorphism but with the advancement of molecular biology such efforts were shifted towards various DNA based markers.

Plant genetics and by association plant breeding, is based on the analysis of inheritance of characteristic and traits and elucidation of gene expression as related to the genotype and environmental interaction. Biochemistry and molecular biology have impacted these activities through the application of molecular markers. Initially molecular markers were based on isozymes (i.e. polymorphic mobility variants of enzymes detected usually in starch gels using activity staining), which are valuable but not numerous enough to cover the extensive regions of genome found in most plants. Recently, a large number of DNA markers are in use as tools for large scale genetic studies providing the best estimate of genetic diversity and are not influenced by the environmental factors. Various classes of the molecular markers are available but those based on PCR are considered to meet many applications in genetic studies[85]. By using the polymerase chain reaction, smaller amounts of DNA are needed, facilitating analysis of badly preserved samples (e.g. field material, museum specimen, forensic samples, plant parts). Most PCR based marker technologies use arbitrary primers. One interesting alternative has been the use of telomere-associated sequence derived PCR primers[86].

DNA based markers

In addition to the problems described earlier in the 'Conventional propagation and breeding' section, progress of tea breeding has also been slowed down due to the lack of reliable selection criteria[9]. Though a number of morpho-biochemical markers has been reviewed in past[14,87,88], yet they have only marginally improved the efficacy of selection for desired agronomic traits. This is mainly due to the fact that most of the morphological markers defined so far, are influenced greatly by the environmental factors and hence show a continuous variation with a high degree of plasticity. Therefore, these markers cannot be separated into discrete groups for identification[89]. Recently, development of the molecular biology has resulted in alternative DNA-based markers for crop improvement of tea. These markers can assist the process of traditional breeding with several efficacies. The greatest advantages of molecular markers are:

- free from the environmental influence and

- detection of polymorphism at an early stage.

The different markers, which have been employed for varietal improvement of tea, are reviewed below.

Various types of molecular markers studied are as follows:

Restriction Fragment Length Polymorphism (RFLP)

The first DNA-based markers were RFLPs (restriction fragment length polymorphisms), which were detected using either random genomic or cDNA clones. RFLPs are still of great value today and effective co-mapping requires near complete map saturation as is achieved in only a few plant species such as Arabidopsis, maize and rice.

By determining the segregation of molecular polymorphism in segregating families, it is possible to determine their degree of linkage and recombination. This generates genetic maps in which distances are measured in units of recombination (called centimorgans) or physical distances (in Kilo or megabases)[90]; In plant genetics maps are usually based on F2 families or thereof derived recombinant inbred line populations[91,]. The latter have the advantage of immortality, inexhaustibility of DNA, ability to be shared globally, as well as homozygosity at each locus, facilitating the application of molecular markers which detect dominant loci only[93-95] (e.g. DAF, RAPD and AFLP markers). Potential applications are frequently thwarted by the requirement for significant quantities of DNA in the case of RFLP analysis or by lack of relevant sequence information in the case of PCR based techniques. Recent criticisms are that DNA fingerprinting requires special molecular training, is labour-intensive and is relatively expensive.

Random Amplified Polymorphic DNA (RAPD)

Williams *et al*[94], described a novel type of genetic marker based on DNA amplification, which does not require prior information of target DNA sequences. These markers called RAPD (Random Amplified Polymorphic DNA) markers are generated by the amplification of random DNA segments with single primers of arbitrary chosen primers. However not much work has been done on the studies of genetic diversity of tea worldwide, though Wachira *et al*[79] and Chen & Yamaguchi[96] have done some studies in a very limited way. Use of random amplified polymorphic DNA (RAPD) markers, detected by PCR amplification of small inverted repeats scattered throughout the genome, adds a new technology of DNA fingerprinting to the molecular analysis of relatedness between genotypes. The PCR based RAPD technique[94] is an attractive complement to conventional DNA fingerprinting. RAPD analysis is conceptually simple. Nanogram amounts of total genomic DNA are subjected to PCR using short synthetic oligonucleotides of random

sequence. The amplification protocol differs from the standard PCR conditions[97] in that only a single random oligonucleotide primer is employed and no prior knowledge of the genome subjected to analysis is required. When the primer is short (e.g. 10 mer), there is a high probability that the genome contains several priming sites close to one another that are in an inverted orientation. The technique essentially scans a genome for these small inverted repeats and amplifies intervening DNA segments of variable length. The profile of amplification products depends on the template-primer combination and is reproducible for any given combination. The amplification products are resolved on agarose gels and polymorphisms serve as dominant genetic markers, which are inherited in a Mendelian fashion[94,98,99]. Amplification of non-nuclear RAPD markers is negligible because of the relatively small non-nuclear genome sizes. Since discovered, random amplified polymorphic DNA (RAPD) assay[94] is being used for a number of areas in plant taxonomy. At the present it is the most preferred DNA markers due to greater speed, easy-to-perform and non-requirement of radioactive materials etc. In tea and other species of *Camellia*, a considerable amount of work has been carried out which are summarized below.

Germplasm characterization

Present day tea plantation is developed largely from the selected genotypes based on the performance of yield, quality, biotic and abiotic stress resistance amongst the previously existing planting materials. As a consequence, widespread cultivation of clonal tea can diminish the genetic diversity if care is not taken to use clones of disparate origin. Therefore germplasm characterization at molecular level of tea will help:

- varietal improvement of tea for agronomically important character.
- to preserve the intellectual property right of tea breeder.
- identification of individual tea cultivar by making a molecular passport.
- prevention of duplicate entry of different genotypes in tea gene pool.
- efficient selection of the varieties for hybridization program, graft compatibility in composite plant production etc and
- taxonomic classification of tea genotypes on the basis of molecular markers.

Wachira *et al*.[79] were the first to characterize 38 different cultivars of Kenyan tea. A total of 23 primers which were used could generate 157 polymorphic bands. The maximum polymorphism of 20 bands was detected by primer SC10-56. The amplified fragments and similarity matrix ranged from 0.3 to 3 kb and 43% to 96%, respectively, among the clones. Based on average linkage cluster analysis, a dendrogram was constructed which clearly discriminate different varieties of Assam, Cambod and China tea. Further, to examine the

evolutionary relationship the principal co-ordinate analysis (PCA) was undertaken which was able to classify into three varietal types of tea in a manner consistent with both the present taxonomy of tea and with the known pedigrees of some clones. In a preliminary study, Tanaka *et al*.[100] used several 10-mer and 12-mer primers to detect variation among Korean, Japanese, Chinese, Indian and Vietnamese tea. Among all primers, OPF- 2 was found to be the most polymorphic. Their study concluded that after introduction from China, Korean tea undergone little genetic diversification. On the contrary, Japanese tea showed a closer relationship with their Chinese and Indian counter part, which reveal the fact that tea in Japan might have brought from China as well as India. Twenty-five Indian tea cultivars and 2 ornamental species were characterized using RAPD markers[101]. In a separate study, the diversity of 27 accessions comprising Korean, Japanese and Taiwanese tea was examined with RAPD markers. The RAPD system has been used in linkage map construction, insect resistance gene localization[103], hybrid origin identification[104], and breeding utilization[105,106]. RAPDs may also be useful for the design of collection strategies to maximize the sampling of genetic variation within the available gene pool[107-110]. Moreover RAPD markers are capable of detecting variation in non-coding regions of the genome.

Detection of genetic fidelity among *in vitro* raised plants by RAPD analysis

The most important part of any *in vitro* propagation system is mass multiplication of plantlets which are phenotypically uniform and genetically akin to the mother plant, otherwise the advantage of desirable characters of elite supreme clones will not be achieved. Several approaches have been applied for identifying variants among micropropagated plants. These are phenotypic variation[111], karyotypic analysis of metaphase chromosomes[112] and biochemical analysis[113]. Importantly, a major disadvantage of these techniques is the limited number of informative markers and the influence of environmental conditions or developmental process[114]. Besides these limitations, the above approaches are not fully suitable for detecting DNA sequence polymorphisms of *in vitro* raised plants. On the other hand, RAPD has been used very advantageously for number of crop species to detect genetic diversity among micropropagated plants[113-115].

Cultivar identification

RAPD markers were also employed for identification of the true-crossing progenies in tea breeding programme and to determine relationship between parents and their hybrids. For example, two Japanese tea cultivars, Yutakamidori and Meiryoku for which parentage identification for registration documents were identified using this marker[116]. Wright *et al*. used the same technique to characterize 5 different South African tea cultivars namely SFS

150, SFS 204, PC1, PC81 and MFS87. Of the 20-arbitary primers tested, only one (ABI-17) yielded a unique set of fingerprints for each cultivar, which allowed cultivar discrimination. Singh *et al*.[117] isolated DNA from 10 different processed dried commercial black and green tea samples. The isolated DNA was subjected for PCR amplification by random primers. Thus they demonstrated that this method has tremendous potential for testing the originality of commercial tea and for the identification of cultivars used by a tea manufacturer for a particular brand. Mondal *et al*.[118] described a simple method of DNA isolation from eight different polyphenol rich genus as well as from 20 commercially important tea cultivars. The method does not require liquid nitrogen or phenol purification step. The DNA was successfully used as a template in PCR amplification, which indicated the wide applicability of the marker. Liang *et al*.[119] investigated the possibility of classification and identification of tea as well as closely related species. The results showed that the RAPD markers could specifically discriminate between species and varieties. While both Assam and China tea had a specific band, Japanese tea was closer to Chinese tea than others. Some of the tea varieties from Vietnam were the hybrids of Assam and China. Tanaka *et al*.[120] used RAPDs to identify the pollen parent of popular Japanese green tea cultivar 'Sayamakaori'. They have screened the female parent 'Yabukita' along with 78 putative male tea plants, most of which were introduced from China and concluded that pollen parent of 'Sayamakaori' was not present amongst the putative population. However, due to dominant in nature and limited degree of polymorphism, attention was given for alternative advance markers.

DNA Amplification fingerprinting (DAF)

Alpha Science

Two modifications of detecting RAPD markers have been described as DNA Amplification Fingerprinting (DAF) and Arbitrarily Primed Polymerase Chain Reaction (AP-PCR). An alternative technology, although not as widely used as RAPD is DNA Amplification Fingerprinting commonly called DAF[93,121]. This procedure uses single arbitrary primers to amplify multiple target regions using low template concentrations (20-100pg/µl) and high primer concentrations (usually about 3µM, but 30µM for mini-hairpin primers. With these major differences to RAPD, DAF is more optimized, can produce from 30-50 amplification products if separated on MiniProtean II gel rigs (BioRad Inc., uses primers of shorter length (5-mers can work, although 8-mers or mini-hairpin primers are commonly used), is possible with annealing temperatures of 55°C, and is resolved using thin 5 to 10% denaturing polyacrylamide gels commonly stained with silver[93,122]. DAF markers are usually dominant, are mainly inherited as nuclear markers, have been mapped on the soybean map[123] can detect maternal inheritance and can be cloned from gels[124]. DAF markers were shown to have the same resolution as RFLP markers, but at high efficiency caused by the larger number of assayed loci, and to confirm the genetic pedigree in a number of soybean lines.

DAF was used to determine genetic distinctiveness in bacteria[125,126], plants, humans, viruses and animals[93]. The ability to amplify populations of DNA molecules by arbitrary primers laid the basis for molecular expression studies using mRNA derived cDNA populations[127,93] (such as Differential Display, Liang and Pardee 1992; cf. Caetano-Anolles *et al* 1991 refer to this possibility by coining the term cDAF for cDNA amplification fingerprinting). DAF uses short random primers of 5-8 bp and visualizes the relatively greater number of amplification products by polyacrylamide gel electrophoresis and silver staining[93] (Caetano-Anolles, Bassam & Gresshof 1991). AP-PCR uses slightly longer primers (such as universal M13) and amplification products are radioactively labelled and also resolved by polyacrylamide gel electrophoresis[128,129]. Standard RAPD analysis is performed according to the original methods[94] using short oligonucleotide primers of random sequence which are commercially available (Operon Technologies, Inc., Alameda, Calif.). Only high molecular weight i.e. non degraded DNA should be subjected to RAPD analyses. Amplification products can be resolved by gel electrophoresis on 1.4% agarose gel.

Amplification Fragment Length Polymorphism (AFLP)

A combination of RFLP, PCR and DAF is found in the recently developed AFLP procedure[95]. The term AFLP was originally used by Caetano-Anolles *et al*[93] to describe DAF polymorphisms. Independently KeyGene Inc. in Wageningen (The Netherlands) developed a related technique and called it AFLP. To avoid confusion, the term AFLP should be used in context of the KeyGene procedure. The technique is rather laborious and expensive. It combines elements of PCR, RFLP and DAF. Large amounts of genomic DNA are isolated and restricted with two restriction nucleases (usually a 6 base cutter (*EcoRI*) and a four base cutter (*MseI*). Restricted fragments are annealed to adapter molecules specific for the cohesive restriction site. PCR primer, specific for each adapter molecule, but extended by 2 or 3 nucleotides on the 3' end provide selectivity to amplify the interstitial DNA fragment (assuming its size does not exceed 2 to 3 kb). This produces about 100 bands per amplification, which are resolved on a polyacrylamide sequencing gel and visualized by autoradiography. Alternatively the separation can occur in a modern DNA sequencer allowing automatic data acquisition. Most AFLP markers are dominant, cluster in chromosomal regions and represent predominantly repeated DNA. They thus are of great value for identity determination (DNA fingerprinting) but not for marker-assisted selection (MAS) or map-based cloning (positional cloning). Additionally AFLP's require large amounts of template DNA as well as high input costs (US$600-700 per kit)[130].

Microsatellites

Microsatellites arise in genomes by the repetition of single sequences[131]. The tandem repeats

like microsatellites or simple sequence repeats (SSR) are densely interspersed in eukaryotic genomes[132-134]. These are usually 2-5bp long, short DNA sequence motif that occur at multiple sites[135,136] and reveal a high degree of allelic diversity which can be typed via polymerase chain reaction[137]. As PCR technology finds increased use in genetic analysis, additional novel variations of this technique are emerging. PCR analysis using anchored simple sequence repeats primers have gained attention recently as an alternative means of characterizing complex genomes. This approach employs oligonucleotides based on an SSR anchored at either the 5' or 3' end with two to four purine or pyrimidine residues, to initiate PCR amplification of genomic segments flanked by inversely oriented, closely spaced microsatellite repeats[138]. The PCR products thus generated reveal multiple polymorphic products which can be resolved on agarose gel electrophoresis. The Inter SSR-PCR (ISSR-PCR) strategy is especially attractive because it avoids the need to carry out costly cloning and sequencing inherent in the original microsatellite-based approach. Moreover, locus specific probes and microsatellite containing sequences of interest can be developed by isolating and cloning or reamplifying individual bands[138,139]. ISSR-PCR has been profitably used for genetic characterization of various plant species[140-143]. Because of greater length of ISSR primers, they may show greater repeatability and stability of map position in the genome when comparing genotype of closely related individual[138]. Simple sequence repeats (SSR) were derived from *C. japonica*, a closely related species of tea in Japan. Using these primer pairs, 53 *C. japonica* ecotypes were genotyped and population genetic parameters calculated. Later, the same group investigated the spatial genetic structure of *C. japonica* using four of these microsatellite primers. Spatial distribution of individuals was also assessed to obtain an insight into spatial relationships between individuals and allele. Use of molecular markers in the characterization and management of plant genetic resources including tea is well documented[79,144].

Thus, SSR's are PCR-based, which is powerful because of ease of detection, large numbers of alleles per locus, and universal abundance. However, they require prior investment for their discovery and thus are of value only for elite crops such as corn, soybean, and tomato.

Organelle DNA analysis

Because of the relative resistance to evolutionary changes of organelle DNA than nuclear DNA, chloroplast (cp DNA) and mitochondrial DNA (mt DNA) sequences have been widely used to investigate interspecific relationships[145-147]. Chloroplasts DNA encodes for many agronomically important genes including large subunit of Rubp carboxylase oxygenase[148], the 32 kd thylakoid membrane protein[149] and some other coupling factors[150] etc. Non-coding regions display higher rates of evolution than coding regions; hence former is desirable target for phylogenic studies. The resolutions of many such non-coding regions

have been amplified by the universal PCR primers[151,152]. However, the relatively high frequency of insertion/deletions may even, in some cases, make it possible to use the size of PCR product as a genetic marker. The choice of cp and mt DNA sequences that maximize phylogenic information however, depend upon the evolutionary time scale of the plant system. To date, systematic organelle DNA analysis for tea is not reported, though, we made a first attempt to study the cp DNA of tea. Good quality of cp DNA using a discontinuous sucrose gradient was isolated from 10 different tea cultivars. To overcome the phenolic problem, 0.1% polyvinylpyrolidone along with 0.1% BSA was used in the homogenization buffer. The clear greenish yellow bands of intact chloroplasts were collected from the interface of 20–45% sucrose gradient from where cp DNA was isolated. Wachira *et al.*[153] analysed species introgression into cultivated gene pool of tea using 5 different organelle-specific primers in 19 taxa as well as 9 tea cultivars. Out of the 5, 3 non-coding chloroplast regions as well as one mitochondrial region that amplified with universal primers did not reveal any polymorphism. Remaining one cp DNA specific PCR product revealed a single-strand conformation polymorphism (SSCP). This SSCP in the inter-genic spacer between the *trnL* (UAA) 3_ exon and *trn* F (GAA) indicated that 4 species namely *C. furfuracea, C. assimilis, C. nokoensis* and *C. tsaii* shared a common haplotype. This may indicate a possible hybridization between species of the sections involved. Thakor[154] re-examined taxonomic relationship among the genus *Camellia* with the help of 5 cp DNA sequences from 25 *Camellia* species covering 4 subgenera of tea family. Out of which, 4 showed a low degree of variability (less than 2%). Remaining one revealed a much higher degree of variability (3.8–20%). Interestingly, the phylogenic analysis using parsimony (PAUP) analysis of these sequence contradicted the sectional or subgeneric grouping of either Sealy's or Chang's monograph. Prince and Parks[155] analyzed the evolutionary relationship in tea subfamily Theoideae based upon two cp DNA regions, namely *rbc*L and *mat*K sequence data, for 4 species of subfamily Ternstroemioideae and 24 species from Theoideae. Later on, the same workers also examined the same cp DNA region (*rbc*L and *mat* K) to confirm the family Theacea, a natural group as well as to evaluate the validity of circumscription of tribes and genus of its subfamily Theoideae[156]. The nucleotide sequences of *rbcL* gene in chloroplast DNA are determined on the native tea varieties of Japan, Korea, China, South East Asia, Sri Lanka and India. Direct sequencing of the amplified cp DNA products were carried out. The nucleotide sequences of the *rbcL* gene in cpDNA of China and Assam type were presented. Alignments were obtained by assuming two substitutions, at nucleotide position 40 (adenine in China tea) and 948 (guanine in China tea). The nucleotide sequences of the *rbcL* gene in China and Assam were 99.8% similar. On the other hand, the 1370 nucleotide sequences of *rbcL* gene among *C. irrawadiensis C. taliensis*

and Assam tea were the same except a different base at position 627. At this position in *C. irrawadiensis* and *C. taliensis*, thymine and adenine were observed, respectively, as specific bases[157].

Various Molecular marker techniques applied to *Camellia* -Milestones

The genetic diversity of tea has been studied by restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD)[79,158], amplified fragment length polymorphism (AFLP)[153].

Randomly amplified polymorphic DNA (RAPD)[94] has proven quite useful in plant discrimination studies[159-165]. Recently RAPDs have also been used for the investigation of genetic relationship[79,153], identification of parentage[120], genetic diversity[166], genetic mapping[167] of tea (*C. sinensis*), as well as for the molecular systematics of the section Thea[168].

In Japan, a wide range of markers has been used with various applications. The markers used for genetic characterization of different green tea cultivars are RAPD, AFLP, SSR, CAPS, and RFLP. Importantly, the RFLP technique was also applied in Japan to prevent adulteration of higher grade with lower grade tea. Several other minor tea-producing countries have used different molecular markers to characterize the tea gene pool of introduced tea cultivars available to that country. Such efforts were made using RAPD in Portugal, ISSR in Taiwan, and RAPD in South Africa. All work focused on the genetic characterization and molecular taxonomy of the introduced variety available in the respective countries. Similarly, South Korea and China tea cultivars were characterized through RAPD or AFLP, and RAPD, respectively.

Japanese researchers have isolated the cDNA chalcone synthase (CHS) gene as well as ß-tubulin gene from the Japanese green tea cultivar 'Yabukita'. More recently, a few important genes such as phenyl ammonia lyase (PAL), caffeine synthetase, and primeverosidase have been isolated. The genetic diversity of tea *Camellia sinensis* (L.) O. Kuntze, including the two main cultivated *sinensis* and *assamica* varieties, was investigated based on PCR-RFLP analysis of PAL, CHS2 and DFR, three key genes involved in catechin and tannin synthesis and directly responsible for tea taste and quality. Polymorphisms were of two types: amplicon length polymorphism (ALP) due to the presence of indels in two introns of PAL and DFR, and point mutations detected after restriction of amplified fragments with appropriate enzymes. A progeny test showed that all markers segregated in a Mendelian fashion and that polymorphisms were exclusively co-dominant. CHS2, which belongs to a multi gene family, allowed for greater variation than the single copy PAL gene. Based on Nei's gene diversity index. Var. *sinensis* was revealed to be more variable than

var. *assamica*, and that a higher proportion of overall diversity resided within varieties as compared to between varieties. Even though no specific DNA profile was found for either tea varieties following any single PCR-RFLP analysis, a factorial correspondence analysis carried out on all genotypes and markers separated the tea samples into two distinct groups according to their varietal status. This reflects the large difference between var. *sinensis* and var. *assamica* in their polyphenolic profiles.

Researchers in different countries have made fingerprints of tea cultivars in their countries of origin. In India also RFLP technique was used to detect adulteration with cashew husk in 10 different tea samples. Some work has also been done on analysis of tea using the simple sequence repeat anchored polymerase chain reaction (SSR-anchored PCR) or Inter SSR-PCR (ISSR)[169].

Work is ongoing to develop a complete tea database with chemical as well as molecular data, which will assist with easy identification of the different cultivars. Simple sequence repeats (SSR) were derived from *C. japonica*, a closely related species of tea in Japan. Using these primer pairs, 53 *C. japonica* ecotypes were genotyped and population genetic parameters calculated[170]. Later, the same group investigated the spatial genetic structure of *C. japonica* using four of these microsatellite primers. Spatial distribution of individuals was also assessed to obtain an insight into spatial relationships between individuals and alleles.

Although techniques like RFLP and AFLP have been applied successfully and have provided considerable genetic information in a number of plant species[95,171,172] these techniques are comparatively slow and expensive requiring the nucleotide information and are not amenable for assessment of genetic studies. SSR markers require prior information of target DNA sequences and sophisticated electrophoretic systems with computer software for accurate band separation and scoring[173,174]. RAPD techniques overcome these limitations; a number of polymorphic markers can be obtained with ease from small amount of genomic DNA without the knowledge of target sequences. There is only one drawback of this technique i.e., lack of reproducibility that may arise if the experimental conditions applied are not standardized properly[175]. In spite of this fact RAPD methodology provides informative data consistent with other markers, especially at the intraspecific level[176,177]. Moreover it is cost effective for large scale population genetic analysis. In preliminary studies the importance of molecular markers for the characterisation of tea genetic resources and estimation of genetic diversity were reported by various workers[79,178]. In the north east region of India some work was done on five varieties of tea by Bera and Saikia[179] using sixteen RAPD markers. But the work mainly covered only the molecular characterization and genetic diversity of five tea varieties. Amplified fragment length polymorphism (AFLP) markers were also studied in depth to detect diversity and genetic differentiation of several

important tea clones, including the famous 'Darjeeling tea', mainly to protect cultivars for intellectual property rights purposes.

***In vitro* culture studies in tea**

The potential of tissue culture in various aspects of plant improvement has already been recognized and attracted the attention of scientists. Rapid multiplication of propagation materials through tissue culture, particularly in the initial selection, assumes importance in plant improvement programmes. Tissue culture offers opportunity to utilize genetic, physiological and biochemical procedures in developing ideotypes[180]. It also holds promise in the development of pure lines through haploid technology, production of triploids through endosperm culture, culture of embryos of incompatible crosses and isolation of somaclonal variants. Banerjee[33] suggested further possibilities of genetic manipulation for production of pure line of tea for inbreeding, breeding and propagation of interspecific hybrids derived from the various combination of crosses by cotyledon and embryo culture. Somatic cell hybridization using protoplast fusion has been tried to transfer the Darjeeling tea flavour of China clones to the Assam cultivars possessing strong and brisk liquors[180]. In Japan, the tissue culture is being used to produce new tea varieties. A tissue culture study in tea is also being carried out at a number of centres in Taiwan, Srilanka, Thailand and the former USSR[181-183].

Since the chemical composition of calli in the tea plant was analyzed[184,185], callus induction and organogenesis have been reported in several papers from the year 1980 to 2001. Callus induction has been obtained from numerous organs in high frequency[186,187]. Wu[188] have successfully obtained new clones derived from cotyledon callus. They reported from Taiwan that the callus-derived plantlets grew strongly and these plantlets were used as a female to cross with one of the highest-quality variety which appeared weak in growth. F_1 hybrids were expected to improve the yield and quality of oolong tea. Doi[189] obtained roots from anther callus but not buds. The callus from the stem showed different capacities of differentiation, depending on the origin of the explant[181]. Although plantlets were regenerated from stem callus, in the callus of shoot tip, leaf and root bud formation has not been observed. The embryoids were formed directly from slices of cotyledon[187,190]. Haploid plants derived from anther culture were established in China[191]. Chen and Liao[191] cultured anthers which were stored at 5°C for 2 days. Shiny calli were formed on the N6 medium supplemented with 0. 5mg/l 2, 4-D, 2mg/l Kin, 100mg/l serine and 800mg/l glutamine. Transfer of shiny calli to N6 medium containing 2mg/l Zeatin, 20mg/l adenine and 10mg/l Casein permitted shoot growth or continued proliferation of new shiny calli. Rooting was obtained on the 0. 1mg/l IAA medium. The chromosome number of plantlets was observed with squashed root tips. Embryoids were obtained from anthers cultured on N6 medium

supplemented with 0. 05mg/l 2, 4-D and 0. 2 mg/l Kin[192]. Haploid plants provide production of homozygous plants; they are of great importance in tea breeding, tea being heterogeneous.

Cell suspension culture has been investigated[193], but it gained small aggregates with a few free cells. Since Creze[194] reported that axillary buds of *C. japonica* were cultured and elongated, the propagation of tea and other plants in *Camellia* genus has been reported by different workers. Numerous buds, as well as shoots, were induced from lateral buds of 3 month old tea seedlings[195]. Cultured shoot tip explants from 4 to 5 month old seedlings of *C. japonica* showed shoot proliferation followed by root initiation and later adaptation to soil[196,197]. Also in *C. japonica* and *C. chrysantha*, embryoids formed directly or via callus of cotyledons[198,187,190,199], but the regeneration from callus of organs other than the cotyledon scarcely has been reported to date in these plants[3].

In tea research the previous era held major emphasis on standardizing parameters of the *in vitro* protocol, such as using a suitable explant, overcoming microbial contamination, and optimizing media composition combined with growth regulation for better proliferation[3]. Following this era the efforts turned towards hardening micro-shoots to achieve a higher survival percentage. Accordingly, several non- conventional approaches, such as a CO_2-enriched hardening chamber, biological hardening, and micrografting, were developed till 2001[200]. Presently, attention is increasingly focused on evaluating field performance of the transformed *in-vitro* grown whole plantlets. Although there is no stable technique developed so far to produce transformed *in-vitro* grown whole plantlets of tea except the one made by Mondal *et al.*[200] in 2001 by micrografting seedling grown roots on the *in-vitro* grown shoots of tea.

The development of micropropagation, a rapid *in vitro* multiplication method, of tea has passed through three phases. Until the 1980s, emphasis was on standardizing parameters of the in vitro protocol, such as using a suitable explant, overcoming microbial contamination, and optimizing media composition combined with growth regulation for better proliferation. It is now accepted that nodal segments (0. 5-1 cm) cultured on MS medium with BAP (1-6 mg/l) are best for multiplication of shoots, along with either a high dose (500mg/l) pulse treatment or a low dose (1-2mg/l) long duration treatment of auxin such as IBA for in vitro rooting. Until the1990s, efforts turned toward hardening micro-shoots to achieve a higher survival percentage. Accordingly, several non-conventional approaches, such as a CO_2-enriched hardening chamber, biological hardening, and micro grafting, were developed. Presently, attention is increasingly focused on evaluating field performance of the micro propagated plant. One prerequisite for genetic transformation of tea is an efficient system of regenerating the complete plant from a single cell. Until today, somatic embryogenesis in

tea was considered the most efficient regeneration system. Unlike micro propagation, tea somatic embryogenesis started in the late 1980s. Thus, emphasis was focused on standardizing parameters, such as genotypes, seed maturity, media formulation, growth regulator, physical condition, etc. A bioreactor system for repetitive embryogenesis in tea has also been developed in Australia in which uniform sizes of globular somatic embryos were obtained for a bioreactor technology called the temporary immersion system (TIS). By controlling immersion cycles, synchronized multiplication (24 fold) and embryo development were achieved with greater consistency and with a high rate of plant recovery. Plantlets recovered through this method were hardy, with a well-formed taproot. Therefore, this technique was the first significant step for commercial application of bioreactor technology to produce large-scale tea somatic embryos.

The ultimate success of any *in vitro* protocol depends upon performance of plants in the field compared to vegetative counterparts. For the last several years, researchers at the Research and Development Department of Tata Tea Ltd, India, have transferred more than 45,000 plants of eight tea cultivars to the field, from which leaves are harvested regularly to manufacture black tea. A systematic study at 1. 7, 4, and 8 year-old field-grown micro propagated and vegetatively propagated tea plants demonstrated that overall yields and quality were comparable. Although different physiological parameters such as photosynthetic rate, chlorophyll content, etc. remained the same, two morphological variations were noticed. First, the number of lateral shoots produced after `centering' were significantly greater in micropropagated-raised plants compared to vegetatively propagated plants. This is perhaps due to effects of various growth regulator treatments applied under *in vitro* conditions. Second, root volumes of tissue culture plants were also greater than in vegetatively-propagated plants. Micropropagated shoots were treated with IBA to induce rooting, which may be responsible for better root development in the field. Therefore, the conclusion was drawn that the micropropagation protocol should be used only when required to produce a large number of plantings from a limited source. Other techniques have been applied in tea with specific objectives, efforts to improve these techniques are ongoing at laboratories worldwide[201].

Histological study

Somatic embryogenesis involves control of 3 consecutive steps:

- Induction of embryogenic lines from sporophytic cells
- Maintenance and multiplication of embryogenic lines
- Maturation of somatic embryos and conversion into viable plantlets[94]

Induction of embryogenic lines and their subsequent conversion into plantlets have been much studied by many workers[202,203,204] but the multiplication step has been comparatively less studied although it directly contributes to the ability of the resulting embryos to germinate and develop into growing plantlets[205].

Two main problems have been reported concerning the multiplication step. The first one is the difficulty in obtaining stable and subculture-suitable lines that will produce embryos for long periods of time[203,204]. The second is the lack of synchrony in embryo development and the risk of morphological abnormalities such as pluricotyledony, multiple apex formation, fused cotyledons and/ or fasciation.

In angiosperm species, multiplication of embryogenic lines can be achieved either by regular subculturing of explants taken from compact or friable embryogenic calli[203], or by the formation of new embryos from the previously developed somatic embryos themselves[206,204,94]. This second case is reffered to as secondary embryogenesis.

In Quercus, initiation of somatic embryogenesis has been described from a variety of sporophytic explants, namely stem segments, leaves and zygotic embryos. The multiplication of the embrogenic lines was first achieved from calli ageing on the same culture medium[207,208] or via successive transfers onto fresh culture media with different growth regulator supplements[207,209]. Embryogenic response from anthers and ovary tissues was also obtained using similar procedures[210].

Researchers have noted that

- Within one embryogenic line the somatic embryos could occur from different histological origin, as observed for example in *Theobromo cacao*[211].

- The growth regulator composition of the culture medium influenced the histological origin of the somatic embryos[212,213], *Elaeis guineesis*[214].

- Depending on their origin somatic embryos exhibited different potentials for germination and further growth[212].

In tea, initiation of somatic embryogenesis has been described from explants, like stem segments and cotyledons[3,200]. In all the reports of embryogenesis, emphasis has been given to manipulate the nutrient composition, growth regulators in culture medium, physical conditions of incubation and other stress treatments to induce somatic embryos. However, there is no report on the histological study of *in vitro* grown embryos of tea. None of these studies investigated the histological origin and structural organization of the somatic embryos.

Genetic transformation of tea

Alpha Science

Production of desired ecotypes of human choice through conventional breeding is an age-old practice. Transgenic technology offers several advantages such as:

- transferability of genes of any origin to plant,
- time effectiveness and
- self-expression of transgene i.e. free from any epistatic and dominant/recessive interaction etc.

Recent advances in tissue culture and recombinant DNA technology have opened new avenues in transformation of higher plants, which consequently produced many transgenic plants with new genetic properties. Transgenic plants broadly speaking refers to those plants in which functional foreign genes have been inserted in their genomes[215]. For the improvement of crop species these developments in molecular biology and gene transfer technology have equipped scientists with powerful tools of biotechnology. Previously impossible techniques like gene identification, cloning and transfer have now enabled the workers to introduce specified alterations in plants. Today it is possible to transfer useful genes from sexually incompatible species or unrelated organisms to crop species of agricultural importance for developing transgenic crop plants. A number of methods are available for transfer of gene to plants and many transgenics have been generated with a number of useful traits in an array of crop species. The methods include a modified Ti plasmid system in *Agrobacterium tumefaciens* and the direct gene transfer including PEG-induced DNA uptake, microinjection of DNA into cultured cells and plant organs, electroporation, microprojectile bombardment and some other innovative means. The refinement in plant regeneration from cultured cells, efficient vector constructs and availability of defined selectable marker genes has resulted in the production of transgenic plants. Though several techniques are available[216-220], yet few have been employed to produce the transgenic tea, detail accounts of which are elucided below.

Agrobacterium mediated genetic transformation

Plant transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium, has become the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants. *A. tumefaciens* naturally infects the wound sites in dicotyledonous plant causing the formation of the crown gall tumors. The first evidences indicating this bacterium as the causative agent of the crown gall goes back to more than ninety years[221]. Since that moment, for different reasons a large number of researches have focused on the study of this neoplastic disease and its causative pathogen. During the first and extensive period, scientific effort was devoted to disclose the

mechanisms of crown gall tumor induction hoping to understand the mechanisms of oncogenesis in general, and to eventually apply this knowledge to develop drug treatments for cancer disease in animals and humans. When this hypothesis was discarded, the interest on crown gall disease largely decreased until it was evident that this tumor formation may be a result of the gene transfer from *A. tumefaciens* to infected plant cells. *A. tumefaciens* has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease[222,223]. T-DNA contains two types of genes: the oncogenic genes, encoding for enzymes involved in the synthesis of auxins and cytokinins and responsible for tumor formation; and the genes encoding for the synthesis of opines. These compounds, produced by condensation between amino acids and sugars, are synthesized and excreted by the crown gall cells and consumed by *A. tumefaciens* as carbon and nitrogen sources. Outside the T-DNA, are located the genes for the opine catabolism, the genes involved in the process of T-DNA transfer from the bacterium to the plant cell and the genes involved in bacterium-bacterium plasmid conjugative transfer[219,224]. Virulent strains of *A. tumefaciens* and *A. rhizogenes*, when interacting with susceptible dicotyledonous plant cells, induce diseases known as crow gall and hairy roots, respectively. These strains contain a large megaplasmid (more than 200 kb) which plays a key role in tumor induction and for this reason it was named Ti plasmid, or Ri in the case of *A. rhizogenes*. Ti plasmids are classified according to the opines, which are produced and excreted by the tumors they induce. During infection the T-DNA, a mobile segment of Ti or Ri plasmid, is transferred to the plant cell nucleus and integrated into the plant chromosome. The T-DNA fragment is flanked by 25-bp direct repeats, which act as a *cis* element signal for the transfer apparatus. The process of T-DNA transfer is mediated by the cooperative action of proteins encoded by genes determined in the Ti plasmid virulence region (*vir* genes) and in the bacterial chromosome. The Ti plasmid also contains the genes for opine catabolism produced by the crown gall cells, and regions for conjugative transfer and for its own integrity and stability. The 30 kb virulence (*vir*) region is a regulon organized in six operons that are essential for the T-DNA transfer (*virA, virB, virD,* and *virG*) or for the increasing of transfer efficiency (*virC* and *virE*)[219,224,225]. Different chromosomal-determined genetic elements have shown their functional role in the attachment of *A. tumefaciens* to the plant cell and bacterial colonization: the loci *chvA* and *chvB*, involved in the synthesis and excretion of the b -1,2 glucan[226]; the *chvE* required for the sugar enhancement of *vir* genes induction and bacterial chemotaxis[227,228,229] the *cel* locus, responsible for the synthesis of cellulose fibrils[230]; the *pscA* (*exoC*) locus, playing its role in the synthesis of both cyclic glucan and acid succinoglycan[231,229]; and the *att* locus, which is

involved in the cell surface proteins[232]. The initial results of the studies on T-DNA transfer process to plant cells demonstrate three important facts for the practical use of this process in plants transformation. Firstly, the tumor formation is a transformation process of plant cells resulted from transfer and integration of T-DNA and the subsequent expression of T-DNA genes. Secondly, the T-DNA genes are transcribed only in plant cells and do not play any role during the transfer process. Thirdly, any foreign DNA placed between the T-DNA borders can be transferred to plant cells, no matter where it comes from. These well-established facts, allowed the construction of the first vector and bacterial strain systems for plant transformation[219,233-235].

Agrobacterium tumefaciences-mediated transformation has been the most widely used technique in woody plants. Despite the fact that several transgenic plants have been produced in a wide range of genera, production of the transgenic tea plant remains difficult till recently mainly due to low transformation efficiency as well as difficult regeneration system. This may be perhaps due to the presence of high levels of polyphenols in tea explants, which has germicidal property[236]. Nevertheless, genetic transformation of tea through *Agrobacterium tumefaciens* has been attempted by several workers using different explants such as *in vitro* leaves, somatic embryos etc[236-241]. While only stable transformed callus could be achieved from leaf explants[236,238,239,242], the first healthy transgenic plants were produced by Mondal *et al.*[240], using somatic embryos as explants. The later group found that while pre-culturing of explants and wounding of somatic embryos had no effect on the transformation efficiency, bacterial growth phase (OD value=0. 60), cell density (109/ml), co-cultivation periods (5 days) and pH of co-cultivation medium (5.6) enhanced the transformation efficiency significantly. These parameters were optimized on the basis of *β-glucuronidase* (*GUS*) activity evidenced by the blue spots that appeared on the somatic embryos after 48 h of co-cultivation. Eventually blue spots increased in size and became uniformly dispersed all over the embryo surface. Very strong GUS positive signals could also be detected in the leaf tissue from 1 year old tea plants recovered through germination of kanamycin resistant somatic embryos. However, no endogenous *GUS* expression was detected in the un-infected somatic embryos or tissues. Interestingly, though acetosyringone, had no effect to increase the transformation efficiency of tea as experienced by Mondal *et al.*[240], yet, concentration at500 μM improved the transformation efficiency in tea as reported by Matsumoto and Fukui[238]. Additionally, two more important factors affect the efficiency of transformation. They are:

- efficiency of selection of transgenic tissue on antibiotics such as kanamycin, hygromycin etc. and

- an effective use of bactericidal antibiotics.

In some *Camellia* hybrids, Tosca *et al*.[243] detected a lethal dose of 75 mg l−1 kanamycin for selection of internode explants. A similar response was also observed by Mondal *et al*.[240] who reported that 50 mg l−1 kanamycin followed by an elevation of 75 mg l−1 kanamycin were optimum for the effective selection of transformants for tea somatic embryos. However, Matsumoto and Fukui[238] found that 200 mg l−1 kanamycin was effective when leaves were used as explants. Scant systematic effort has been made on the effect of bactericidal antibiotics for tea transformation. However, Mondal *et al*.[244] tested the bactericidal effects of three antibiotics and found that sporidex at 400 mg l−1 not only ensured a total restriction of the *Agrobacterium tumefaciens* overgrowth but also confirmed negligible effect on the growth retardation of the somatic embryos. The next best antibiotics were found to be carbenicillin followed by cefotaxime. Therefore, sporidex in combination with carbenicillin was preferred as it was cheaper and locally available. Siswanto and Chaidamsari[242] reported that the development of the transformed *in vitro* leaves to calli produced a mucilage-like compound covering the calli that hinder the further development. Though the authors did not mention any further detail, yet it seems that *Agrobacterium* overgrowth, which produces a mucilage-like compound on the explants, if not controlled properly after co-cultivation stage. Further, the putative transformants were also confirmed through molecular technique. While Matsumoto and Fukui[237,238] have reported stable transformations in callus after molecular characterization through PCR and southern hybridization, it was Mondal *et al*.[240] who confirmed the stable integration of transgene after molecular characterization through southern hybridization. This indicated that the presence of the marker gene *npt-II* was linked with that of *gus* as a single 'T-DNA strand' in the genomic DNA of the transformed plant. Luo and Liang[241] contructed a vector containing *Bt* gene which was subsequently transformed to tea. The pGA471 plasmid containing *Bt* gene cry I A (c) was digested with *Hind III* and *Bgl II* and inserted into the vector pCAMBIA-2301. The constructed plasmid containing *Bt* gene, *GUS* intron and *npt-II* was transformed into *E. Coli* and introduced into *Agrobacterium* strains such as LBA 4404, EHA 105 and pRi15834 through triparental mating. They detected the transient expression of *GUS* gene in transgenic calli and leaves of putative transgenic tea plants. They found the optimum concentration for hygromycin and kanamycin as 20 g l−1 and 50 gml−1 for screening the tea leaves. However, no transgenic plants were produced.

Agrobacterium rhizogenes

Agrobacterium rhizogenes, causal agent of the 'hairy root' disease is characterized by its ability to cause root proliferation at the sites of infection of the susceptible hosts. The

phenomenon of profuse root growth on hormone free media under *in vitro* conditions has been extensively exploited in a large number of plant species specially for secondary metabolite production. For the first time, in tea, Zehra *et al*.[245], infected 35-day-old *in vitro* leaves with *Agrobacterium rhizogenes* strain A4. The explants were co-cultivated with bacterial cell (108/ml) in dark for 2 days. The excessive bacterial solution was blotted dry on sterile filter paper and leaves were then cultured on MS for 35 days for inducing hairy root. Mannopine from these roots were analysed through paper electrophoresis to confirm the stable integration of this gene. Konwar *et al*.[246] also transformed *in vitro* tea shoots of 4–6 months old at the basal end followed by co-cultivation in liquid basal MS medium supplemented with IBA (5 mg l−1) and rifampicin (100 mg l−1). Roots were initiated after 32–45 days culture among the 66% explants from the basal end which enabled hardening of the microshoots in nursery beds. However, the technique has not been exploited commercially to produce the secondary metabolites, which will be immense useful for a crop like tea.

Biolistic

Biolistic mediated genetic transformation is an alternative method that has been successfully used in production of transgenics in a wide variety of plant species. Though no transgenic tea plants could be produced by this technique, yet a preliminary study on transient expression was reported by Akula and Akula[247]. Tea somatic embryos were bombarded with gold particles (1.5–3m diameter), coated with plasmid p2k7, a vector originally derived from pBI 221. The marker and reporter gene was *npt-II* and *β-glucuronidase* respectively under the control of 35S cauliflower mosaic virus (CaMV) promotor. Various factors such as the distance between the site of delivery of the microprojectile and the target tissue, helium pressure and the state of target tissue to obtain transient expression was optimized on the basis of *β-glucuronidase* (*GUS*) assay after 30–40 h of bombardment. Following which they achieved the highest transient expression levels up to 1085 blue spots/shot. However, further details of regeneration of somatic embryo were not mentioned. Though beginning, yet the different protocols that have been standardized using both *Agrobacterium* and biolistic-mediated methods hold a tremendous potential for producing transgenic tea with useful genes.

Gene constructs and vectors

A number of gene constructs (either genomic or chimeric) have been investigated both at callus and plant level. Particular significance to achieve the reproducible transformation would depend on the avilability of selectable genes conferring resistance to antibiotics; bleomycin, chloramphenicol, hygromycin, kanamycin and streptomycin. Furthermore,

several reporter genes including *CAT*, *ß-glucuronidase* (*GUS*), *luciferase* and *neomycin phosphotransferase* (*npt-II*) have been employed for the analysis of precise nature of promoter activities. Some demonstrated instances of foreign gene expression are given below.

Herbicide tolerance genes

Transfer of aroA bacterial gene from *Salmonella typhimurium* into tobacco and a hybrid clone of *Populus*[248]. The transformed tobacco plants exhibited a high degree of tolerance to glyphosate. De Block *et al*[249] inserted the bar gene from *Streptomyces hygroscopicus* into tobacco, tomato and potato. These transgenic plants showed an increased level of resistance to the herbicide phosphinothricin which is a potent inhibitor of glutamine synthetase in plants.

Insect tolerance genes

Bacillus thuringiensis is a bacterium that forms insecticidal proteinaceous crystals during sporulation. The crystal proteins are specifically toxic to lepidopteran insects. The commercial *B. thuringiensis* gene has been introduced into tomato and tobacco and the transgenic plants thus produced show an increased level of resistance to lepidopteran insects[250]. The introduction of toxin genes into plants seems to a practical method for providing protection against certain insect pests.

Seed storage protein genes

In legumes and most other dicots, the major storage proteins are the salt-soluble globulins. In monocots, prolamins (alcohol-soluble) and glutelins (acid- or base- soluble) are the predominant seed proteins. The tissue-specific expression and abundance of mRNA of these proteins have made it possible to clone and transfer these genes into various plant species. Some examples of transgenic plants carrying seed storage protein genes include: a 17kbp soybean DNA fragment with the *lectin* gene in tobacco[251]; the *legA* gene of pea into *N. plumbaginifolia* petunia[252]. Transgenic tobacco plants have been obtained by introducing a 17kbp soybean DNA fragment containing the *lectin* gene and atleast four nonseed protein genes are expressed in correct tobacco developmental stages but at different quantitative levels.

Coat protein genes for virus protection

The practical control of plant viruses depends on methods that prevent or restrict virus infection. Recently it has been shown that the coat protein of the virus has an important role in systemic cross-protection. Insertion of a cloned coat protein (CP) gene delayed the virus disease development and subsequent systemic spread of the virus in transgenic plants. The

expression of the CP gene of tobacco mosaic virus (TMV), of alfalfa mosaic virus (AIMV), of potato virus X (PVX) and of cucumber mosaic virus disease development and subsequent systemic spread of the virus in transgenic plants resulted in transgenic plants resulted in protection of these plants from infectious viruses. Abel *et al*[253] produced transgenic tobacco plants expressed TMV mRNA and CP as a nuclear trait. Inoculation with TMV showed that seedlings expressing the CP gene delayed in symptom development. Plants accumulating AIMV-CP were highly resistant to infection with AIMV nucleoproteins.

Light regulated gene

Many light regulated genes are tissue specific in expression. Transgenic plants offer a unique opportunity for the analysis of such genes as the small subunit of ribulose-1, 5-bisphosphate carboxylase (rbcS) and chlorophyll a/b-boinding protein (Cab). The cis elements responsible for tissue-specific expression of light regulated genes have been investigated extensively. It is not known how the pathways leading to tissue specific and light-induced transcription are related. The expression of the pea rbcSW-3A gene has been studied in transgenic tobacco plants[254]. This gene is expressed at low levels in dark adapted plants but after exposure of the plants to light, transcript levels increase 20-50 folds. The upstream region containing an enhancer like element and a 280bp region (-330 to-50 relative to the transcription start site) is sufficient for regulated expression. The transcription of a chimeric gene with a 1.2 kbp 5'-upstream promoter was shown to be light inducible in tobacco plants.

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Conclusion

From the above discussion it is apparent that the importance of tea in the economy of Eastern Countries is paramount. India has a long history of tea cultivation and research way back in 1800. The British discovered tea and started cultivation in India. Since than tea remains one of the major cash crops and contributed significantly in the development of Indian economy. Besides these tea remains as one of the most popular health drinks. Tea is mostly cultivated through colonal propagation, however like other crops tea has some cultivation and disease problems which needs to be sorted out with the help of modern biotechnological techniques like micropropagation and production of genetically modified crops. India is rich in tea germplasm varieties, these varieties must be documented with the help of molecular documentation techniques. DNA fingerprinting techniques may play a significant role in this regard.

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Chapter 7

In vitro regeneration of some lesser known medicinal Zingibers: a review

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Abstract:

Medicinal plants have a glorious history. Many medicinal plant species are disappearing at an alarming rate due to rapid agricultural and urban development, uncontrolled deforestation and indiscriminate collection. *In vitro* cell and tissue culture is mean for germplasm conservation, rapid mass propagation for large-scale regeneration and for genetic manipulation studies.

The Zingibers have numerous references in medical literature. *In vitro* clonal propagation strategies have been developed for a number of economically important plant species. In Zingiberaceae plant breeding is seriously handicapped by poor flowering and seed set. *In vitro* culture offers a method for producing and exploring variations for crop improvement and an alternative means of plant propagation. The most common explant used for regeneration of Zingibers is the rhizome sprout or the emerging shoot buds. Murashige and Skoog's medium supplements with sucrose and plant growth regulators were extensively used in the regeneration of the Zingibers. In the regeneration of Zingibers plant growth regulators like TDZ, BA, Kn, NAA, IAA, TRIA, 2, 4-D, Dicamba etc were mostly used. Direct *in vitro* shooting was preferred in regeneration of Zingibers over somatic embryogenesis through callus culture. In cultures of the members of Zingiberaceae additional plant growth regulator may or may not be required for *in vitro* rooting. In hardening of regenerated tissue cultured plantlets of the family Zingiberaceae showed a high rate of survival of regenerated plantlets.

Keywords

Zingiberaceae, medicinal, *in vitro*, plant growth regulators, regeneration, hardening

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Introduction

Medicinal plants have been the subject of man's curiosity since time immemorial[1]. Almost every civilization has a history of medicinal plant use[2]. Approximately 80% of the people in the world's developing countries rely on traditional medicine for their primary health care needs, and about 85% of traditional medicine involves the use of plant extracts[3]. Interest in phytomedicine has exploded in the last few years, and different plant species are used as key ingredients and many are still being collected from the wild. The resurgence of public interest in plant-based medicine coupled with rapid expansion of pharmaceutical industries has necessitated an increased demand for medicinal plants, leading to over-exploitation that threatens the survival of many rare species. Also, many medicinal plant species are disappearing at an alarming rate due to rapid agricultural and urban development, uncontrolled deforestation and indiscriminate collection. Combinations of *in vitro* propagation techniques[4] and cryopreservation may help in conservation of biodiversity of locally used medicinal plants. Cryopreservation is a suitable method for long-term preservation of the germplasm of endangered species. Several medicinal plant species have been successfully cryopreserved[5,6]. *In vitro* cell and tissue culture methodology is envisaged as a mean for germplasm conservation to ensure the survival of endangered plant species, rapid mass propagation for large-scale regeneration, and for genetic manipulation studies.

Systematic position of the family Zingiberaceae

The family Zingiberaceae has been placed differently by different taxonomists. According to Bentham and Hooker Zingiberaceae is a suborder under the order Scitaminae belonging to the series Epigynae. Engler placed the family in the order Scitaminae. According to Hutchinson, this family belongs to the order Zingiberales, under the division Calycifereae.

Occurrence of the genera

Zingiberaceae are distributed mostly in tropical and subtropical areas. The center of distribution is in South East Asia. The greatest concentration of genera and species are in the Malaysian region (Indonesia, Malaysia, Singapore, Brunei, the Philippines and Papua New Guinea).

The genera under study are mainly found concentrated in the Asian countries. *Alpinia galanga*, a perennial herb, is found mainly in the Eastern Himalayas and South-West India[7]. *Aframomum corrorima* (Korarima or the Ethiopian cardamom) is a renowned spice and medicinal crop native to Ethiopia[8]. *Alpinia officinarum* is distributed throughout the tropical and subtropical Asian region[9]. *Mantisia wengeri* is a highly fascinating Zingiber endemic to a single population in Lunglei, Mizoram, North-East India[10]. *Etlingera elatior* (Torch

ginger), which is locally known in Malaysia as "kantan" is a species native to Sumatra, Indonesia and widely cultivated throughout the tropics[11]. *Curcuma aeruginosa* is found abundantly in Thailand[12]. *Costus speciosus* commonly known as 'spiral ginger' is a rhizomatous perennial herb distributed below 1500m altitude in tropical forests throughout India. About 150 species of *Costus speciosus* have been reported from the tropical regions of the world[13]. *Boesenbergia rotunda* is believed to have originated and found from India and South-East Asia region[14]. *Alpinia purpurata* is found in the southern part of India[15]. *Curcuma amada* (Mango ginger), is an important medicinal plant cultivated in India. *Curcuma kwangsiensis* is cultivated exclusively in South China since the ancient times. *Zingiber petiolatum* is a rare perennial herb found in the evergreen forest of Pattani, a southern province of Thailand[16]. *Curcuma zedoaria* is found in Brazil[17]. *Curcuma zedoaria* and *Zingiber zerumbet* are found abundant in Malayasia[18]. *Amomum krervanh* (Krawan), also known as Siam cardamom or best cardamom, is a common spice and medicinal plant species native to Thailand and Cambodia[8]. The genus *Cornukaempferia* is a new genus in Zingiberaceae family from Thailand[19]. *Zingiber montanum* Koenig syn. *Zingiber cassumunar* is native to India[52].

Economic importance and health benefits

The Zingibers have a very old and glorious history. There are numerous references to ginger and turmeric in Sanskrit literature and in Chinese medical treatise. The Sanskrit name *Singabera* gave rise to the Greek Zingiberi and to the Latin Zingiber. Ginger has been used in India from Vedic period and is called maha-aushadi meaning the great medicine. *Alpinia galanga* is used in traditional medicine in certain countries and also as condiment and spice. Phytochemical constituents of *A. galanga* rhizome include antitumour, antiulcer and anticalculi activity[20,21]. Seeds of *Alpinia galanga* also have cytotoxic and antifungal diterpenes[22]. The rhizomes of *Alpinia officinarum* is used for respiratory troubles, arthiritis, cancer, microbial and gastrointestinal disorders[9]. *Alpinia purpurata* rhizome has a sharp odour, improves appetite, taste and voice. It is also used for headache, rheumatism, sore throat and renal disorders. The flowers used as decoction are a relief against cough[23,25]. Dried fruits of *Aframomum corrorima* are part and parcel of the daily dishes of the Ethiopians. They are also used as a carminative, purgative and tonic in the traditional medicine[24]. Korarima oil has similar chemical composition with that of its famous relative, the Indian cardamom (*Elettaria cardamomum*), except for its reduced content of terpinyl acetate, which is the major component in the latter[25]. *Mantisia wengeri* rhizome has been used as a remedy for bone fracture and gastrointestinal ailments in the past by local people[10]. The decoction of *Etlingera elatior* fruits has been used to treat earache and the

leaves are used to clean wounds[26,27]. In Australia and Costa Rica, it is cultivated for its inflorescences as cut flowers[28]. Leaves of *Etlingera elatior*, mixed with other aromatic herbs in water, are used by post-partum women for bathing to remove body odour[29]. The mature fruits of *Etlingera elatior* are edible but sour, and are reputed to have antihypertensive, antimicrobial, antioxidant as well as antitumor promoting activities activity[30]. *Curcuma zedoaria* has been used as a stimulant, carminative, expectorant, demulcent, diuretic and rubifacient[31], analgesic[32], hepatoprotective[33], antiinflamatory[34], antiallergic, antimetastatic[35] and antioxidant[36] activities. *Curcuma zedoaria* has well-known effect of zedoary as a stomachic, it has been recently studied for its anti tumor, hepatoprotective[37], anti inflammatory [38] and analgesic[39] effects. *Curcuma kwangsiensis* exhibits antiinflammatory, antitumor, antiallergic and other properties through its main active components, curcuminoids and volatile oil[40,41]. *Curcuma aeruginosa* rhizomes are antiflatulent, antidysentery and antiulcer[12]. *Boesenbergia rotunda* is believed to possess anti-inflammatory effects, provides relief from bacterial dysentery, stomachache, anti-flatulence and helps to promote appetite. The tubers are widely used as local application for suppression of tumors, swellings and wounds[14]. Furthermore, rhizomes of *Boesenbergia rotunda* contain a potential antipyretic, analgesic, antimutagenic, antiinflammatory and antioxidant enzymes[42-45]. In the most recent study, panduratin was shown to have antibacterial and antiviral activity[46,47]. Furthermore, 4-hydroxypanduratin A and panduratin A, isolated from the rhizomes of *Boesenbergia rotunda* were found to show high inhibitory activity towards dengue-2 virus protease at 120 ppm[48]. *Costus speciosus* rhizome serves as a source of antihelmintic compounds and an alternative source of diosgenin[49]. It is also used locally for treatment of diabetes and jaundice. The root extract acts as an astringent, aphrodisiac, purgative and is useful in catarrhal fever, coughs, skin diseases and snake bites[50-52]. The rhizomes can be used as an alternative source of diosgenin[49]. *Cornukaempferia aurantiflora* has been used by local people in Northeastern Thailand to treat infected hemorrhoids and laryngitis[19]. *Zingiber montanum* rhizome is used for postnatal treatment, swelling, rheumatism. In Thailand they are applied for joint pain, intestinal disorders and numb feet[53]. They were reported to have antifungal, anti-inflammatory, analgesic and antioxidant activity[54]. In Malaysia, rhizomes of *Zingiber zerumbet* are consumed to increase appetite, for the treatment of ulcers, stomachaches, diarrhea, asthma, rheumatism and as antiinflammatory[55]. The rhizome macerated in alcohol is regarded as a novel food factor for mitigating experimental ulcerative colitis[56]. However, in South East Asia, *Zingiber zerumbet* is traditionally used for the treatment of fever, constipation and to relieve pain[18]. It possesses antipyretic and analgesic properties[57]. It also possesses antiinflammatory[57] and chemo-preventive activities[58].

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Micropropagation- a tool for regeneration of plants

Development of the plant tissue culture is historically linked to the discovery of the cell and subsequent propounding of the cell theory. The concept of 'Totipotency' which is an inherent part of the cell theory of Schleiden[59] and Schwann[60] is the basis for plant tissue culture. *In vitro* technique dates back to 1902, when Haberlandt predicted the totipotency of plant cells[61]. Totipotency is the ability of a plant cell to develop into a complete plant. The formulation of nutrient media like Murashige and Skoog[62] medium, which is the most commonly used medium for culturing of a large number of horticultural plants. According to Murashige[63] there are three possible methods available for micropropagation.

- Enhanced release of axillary buds
- Production of adventitious shoots through organogenesis.
- Somatic embryogenesis.

Callus mediated organogenesis and somatic embryogenesis are not recommended for clonal propagation since there is a possibility of producing aberrant. In shoot tips and axillary bud cultures, genetic fidelity is maintained to a large extent. *In vitro* somatic embryogenesis is limited to a few species but still, acts as the most rapid method of plant regeneration[64]. Currently, *In vitro* clonal propagation strategies have been developed for a number of economically important plant species[63-68].

Importance of in vitro regeneration in Zingibers

In Zingiberaceae plant breeding is seriously handicapped by poor flowering and seed set. So, most crop improvement programs are confined to the conventional methods like evaluation and selection of naturally occurring variants. *In vitro* culture offers a method for producing variations and exploring the resultant variations for crop improvement. *In vitro* culture techniques provide an alternative means of plant propagation and a tool for crop improvement[69]. Moreover overexploitation of Zingiberous crops are making them endangered. *In vitro* regenerated plants are superior to conventionally propagated plants in respect of productivity and disease resistance. As rhizome is used as propagating stock, they become a common source of pathogen. Production of pathogen free plantlets through tissue culture can overcome the problem of transmission of pathogen through rhizome[70].

Establishment of culture and regeneration

The objective is to successfully place an explant into aseptic culture by avoiding contamination and then to provide an *in vitro* environment that promotes stable shoot production. The important aspects of this are explant disinfection, explant selection and

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culture medium[71]. The success of establishment of explant in the culture media is dependent on explant selection, sterilization of explant, culture conditions, and composition of the culture media.

The use of tissue culture as a tool for plant propagation could be particularly relevant for vegetatively propagated crop plants that resist conventional asexual propagation[72] or when methods of mass propagation of single plant is required. The different explants such as axillary bud, shoot tips, meristem tips, root tips are commonly used. Various explants like emerging rhizome buds, rhizome pieces, axillary bud, shoot tip, leaves are used in culture of Zingibers.

The most common explant used for regeneration of Zingibers is the rhizome sprout or the emerging shoot buds. Plant parts like emerging buds of *Alpinia galanga*[7], axillary buds of *Aframomum corrorima*[8], *Etlingera elatior*[11], *Amomum krervanh*[8], rhizome buds of *Alpinia officinarum*[9], *Mantisia wengeri*[10], *Curcuma aeruginosa*[12], *Curcuma kwangsiensis*[76], *Curcuma zedoaria*[17], *Zingiber montanum*[54], *Costus speciosus*[74] thin rhizome sections of *Costus speciosus*[73], sprouting rhizome buds of *Boesenbergia rotunda*[14], *Alpinia purpurata*[15], *Curcuma zedoaria* and *Zingiber zerumbet*[18,75] were used as explants. Leaf explants were used in the regeneration of *Cornukaempferia aurantiflora*[19].

In the process of sterilization of living materials, the sterilant should not hamper the biological activity, but only bacterial or fungal contaminants should be eliminated. The commonly used sterilants are bleach, ethanol, sodium hypochlorite, mercuric chloride. The type of sterilant used, concentration and time depends on the nature of explant and species[77].

Various disinfecting chemicals were used in the culture of Zingibers (Table 1). In all the cases the explants were thoroughly washed under running tap water and were trimmed. The buds were soaked in commercial bleach solution for few minutes. Followed by sterilization in Mercuric chloride (0.1% - 0.2%) for 2-10 minutes. Sterilization without mercuric chloride was undertaken in sterilization of the explants of *Etlingera elatior*[11] and *Aframomum corrorima*[8]. The sterilant and time required for the regeneration of the members of Zingiberaceae depend on the genera and species.

Successful growth and differentiation of excised plant tissues and organs are possible if they are supplied with nutrients required by it. The artificially prepared nutrient medium is called culture medium. The culture media is a mixture of several components like inorganic salts, vitamins, amino acids, sugars, growth regulators, agar or gelrite. The minerals present in the plant tissue culture medium are used by the plant cell as building blocks for the synthesis of organic molecules or as catalysts. The ions of different salts play an important role in

Table: 1-Various disinfecting chemicals used in the culture of Zingibers

Plant species	Sterilant used
Alpinia galanga[7]	5% Tween- 20 solution for 15 min followed by 0.2% Mercuric chloride for 5-7 min
Alpinia officinarum[9]	5 % (V/V) Tween followed 0.2 % (W/V) Mercuric chloride
Alpinia purpurata[15]	2% Bavistin for 5 minutes, 5% Tween – 20 solutions for 15 min, 70% Ethyl alcohol for 30. Finally 0.1% Mercuric chloride solution for 5–7 min
Aframomum corrorima[8]	70% Ethanol for 1 minute; followed by 20 and 10% Hyter (6% v/v Sodium hypochlorite) mixed with 2 ml/l Tween-80 for 10 and 5 minutes, respectively
Boesenbergia rotunda[14]	20% (v/v) commercial Sodium hypochlorite (Clorox) for 15 min followed 0.1% (w/v) aqueous solution of Mercuric chloride ($HgCl_2$) for 5 min
Curcuma aeruginosa[12]	0.2 % w/v Mercuric chloride solution and a few drops of Tween-20 for 8 min
Curcuma speciosus[73]	Tween- 20 for 15 min, followed by 70% ethanol (5 min) and 0.1% Mercuric chloride (2 min)
Curcuma speciosus[74]	0.1% Mercuric chloride (10 mins.)
Curcuma zedoaria[17]	70% Alcohol for 10 s and 0.1% Mercuric chloride for 10 min
Curcuma zedoaria[18]	Detergent for several times followed by mercuric chloride solution (100mg/l) for 5 min. Finally by 20% Clorox with few drops of Tween- 20 for 10 min again by 10% Clorox with few drops of Tween- 20 for 10
Curcuma kwangsiensis[76]	70% Alcohol for 10 s and 0.1% Mercuric chloride for 10 min
Cornukaempferia auranti-flora[19]	Washed with 70% (v/v) Ethyl alcohol for 30 seconds, sterilized with 0.9% Sodium hypochlorite containing 2 drops of Tween- 20 for 15 seconds
Etlingera elatior[11]	60% Clorox added with 6 to 7 drops of Tween- 20 for 30 min. followed by 20% Clorox added with 6 to 7 drops of Tween- 20 for another 15 min
Mantisia wengeri[10]	Tween-20 followed by 0.2% cetramide for 10 mins followed by surface sterilization using 0.1% Mercuric chloride for 5 mins
Zingiber zerumbet[18]	Detergent for several times followed by Mercuric chloride solution (100mg/l) for 5 min. finally by 20% Clorox with few drops of Tween- 20 for 10 min again by 10% Clorox with few drops of Tween- 20 for 10
Zingiber zerumbet[75]	Washing the explants first with 60% Clorox and addition of 6 to 7 drops of Tween- 20 for 30 min followed by washing in 20% Clorox with the addition of 6 to 7 drops of Tween- 20 for another 15 min
Zingiber montanum[54]	75% (w/v) Ethanol for one minute. Without rinsing, they were agitated in 20, 30 or 40 % (w/v) Clorox (5.25 % w/v Sodium hypochlorite) added with 0.1ml/L Tween 20 and four drops of 25% HCl for 20 minutes

transportation or osmotic regulation and in maintaining the electrochemical potential of the plant.

The nutrient requirements for the growth of different plants are not the same. Even, it differs for the different organs of a same plant. Therefore, a single medium is not suitable for optimum growth of all plant tissues. To overcome this, different nutrient solutions were proposed by different authors from time to time. Consequently the most suitable medium for a particular tissue must be determined by trial and error.

Murashige and Skoog's medium[62] was used extensively in the regeneration of the members of Zingiberacee with a few exceptions. In cultures of *Amomum krervanh* and *Aframomum corrorima* four different media including MS, half-macro nutrient MS, MS with half-nitrogen {i.e. MS medium with half of its original nitrate (NO_3^-) and ammonium (NH_4^+) ions}, as well as Schenk and Hildebrandt (SH)[78] medium was used[8]. B_5 basal medium[79] was used in the regeneration of *Costus speciosus*[73] and *Zingiber montanum*[54].

The sugar is supplied in the form of sucrose. Sucrose was added as the source of carbon at a concentration of 3% (w/v) in almost all the experiments. In the culture of *Mantisia wengeri*[10] 3% and 10% Sucrose were used in the medium for induction of callus and shoots regeneration respectively. 3 % - 7 % sucrose and 5% sucrose was used from breading dormancy of the axillary buds and subculture respectively in the culture of *Costus speciosus*[74].

The range of acidity or alkalinity is an important factor that determines the quality of regenerated plantlets from a tissue culture media. The optimum pH for regeneration varies with the plant that is used as an explant. In the cultures of the members of Zingiberaceae pH of 5.8 were generally maintained in most of the cultures. pH of 5.7 were maintained in the cultures of *Boesenbergia rotunda*[14], *Aframomum corrorima*[8], *Amomum krervan*[8].

A medium may be solid or semisolid or liquid. When agar-agar or gelrite is dissolved in the liquid nutrient medium, it makes a solid medium. Agar is obtained from algae like Gelladium or Gracilaria, whereas gelrite is a naturally-derived gelling polymer that can be used in a variety of applications as solidification agent instead of agar.

The media was solidified with agar 0.8% (w/v) in all the cultures with a few exceptions. 0.75 % agar was used in the cultures of *Curcuma zedoaria* and *Zingiber zerumbet*[18]. In cultures of *Alpinia officinarum*, *Cornukaempferia aurantiflora*[19], *Aframomum corrorima* and *Amomum krervanh*[8] media were gelled with 0.7% agaragar. Lower concentrations of agar like 0.55% in *Curcuma kwangsiensis*[76] and 0.2% in *Boesenbergia rotunda*[14] were used to solidify their cultures. In *Zingiber montanum* 0.25%[54] gelrite was used for solidification

of the culture. 4.5 g/l Gelrite was used as a gelling agent in *Etlingera elatior*[11] and *Zingiber zerumbet*[75].

Autoclaving is the most efficient technique for sterilization of media. In all the experiments the media were autoclaved for 20 minutes at 121°C (1.06 kg/cm^2) with a few exceptions. Culture media for regeneration of *Costus* was autoclaved for 15 min[73] and cultures of *Curcuma zedoaria* and *Zingiber zumbret for* 13 min[18].

Many researchers prefer to call plant hormones as plant growth substances or plant growth regulators. Plant hormones added to plant tissue culture media are taken up and increase the level within the tissue. Most of the increase is however, transient because plant hormones are rapidly inactivated after uptake. Usually only very small amounts of the applied hormones remain in the free form. It has been seen that, for auxins, equilibrium exists between the free and conjugated form, of which only less than 1% being present in the freeform. The effect of hormones not only depends on the rate of uptake from the medium, or on the stability in the medium and in the tissue, but also on the sensitivity of the target tissue.

The main plant growth regulators used in tissue culture are auxins (indole-3-acetic acid, indole-3-buturic acid, 1-napthaleneacetic acid, 2, 4-dichlorophenoxyacetic acid, picloram etc); cytokinins (zeatin, 6-benzylamino purine, kinetin, thidiazuron etc); gibberellins ($GA_{1,}$

Table2: A list of the plant species and the plant growth regulator used for its regeneration

Plant species	Plant growth regulator	Organic additives
Aframomum corrorima[8]	TDZ, BA and Kn	Coconut water
Alpinia galanga[7]	Kn, BAP and NAA	-
Alpinia officinarum[9]	Kn, BAP and NAA	-
Mantisia wengeri[10]	BA, Kn and NAA	-
Etlingera elatior[11]	BAP and IAA	-
Costus speciosus[73]	TRIA (Triacontanol)	-
Costus speciosus[74]	BAP and NAA	-
Boesenbergia rotunda[14]	NAA, IAA, 2, 4-D, BAP and Dicamba	-
Alpinia purpurata[15]	BAP and Kn	-
Curcuma kwangsiensis[76]	BAP, NAA and TDZ	-
Curcuma zedoaria[17]	BAP and IBA	-
Curcuma zedoaria[19]	BAP and NAA	-
Zingiber zumbret[18]	BAP and IBA	-
Zingiber zumbret[75]	BAP and IBA	-
Amomum krervanh[8]	TDZ, BA and Kn	Coconut water
Cornukaempferia aurantiflora[19]	2, 4-D and BAP	-
Zingiber montanum[54]	BAP,Kn, 2-isopentyl adenine riboside and TDZ	-

GA3, GA_4, GA_7 etc); abscisic acid; ethylene etc. A list of the plant species and the plant growth regulator used for its regeneration are provided in table (Table 2). In the regeneration of Zingibers plant growth regulators like TDZ, BA, Kn, NAA, IAA, TRIA, 2, 4 -D, Dicamba etc were extensively used. Organic additives like coconut water were used as a supplement in some media for the regeneration of the Zingibers.

Incubation condition is very important for micropropagation. High temperature is likely to lead to dissociation of the culture media and tissue damage while in very low temperature tissue growth is slow. Moreover some tissue grows in dark while other prefers light conditions. The amount of light also has substantial effect on the regeneration. The incubation conditions required by Zingibers are shown in table 3.

Callus tissue is an unorganized and undifferentiated proliferated mass of cells produced from isolated plant cells, tissues or organs when grown aseptically on artificial nutrient

Table3: Incubation conditions required by Zingibers

Plant species	Temperature	Light
Aframomum corrorima[8]	25 ± 2°C	16 hour photoperiod with 28 mmol/m^2s photosynthetic photon flux density
Alpinia galanga[7]	23 ± 2°C	16/8 h photoperiod of 2000 Lux light intensity.
Alpinia officinarum[9]	25 ± 2°C	16/8 h photoperiod of 2000 Lux light intensity.
Mantisia wengeri[10]	25±2 °C	12 h photoperiod of 54 moles m^{-2} s^{-1}light intensity
Etlingera elatior[11]	25 ± 2°C	16 h photoperiod at a photosynthetic flux of 150 µmol m^{-2} s^{-1}
Costus speciosus[73]	25 ± 2°C	16 hrs
Costus speciosus [74]	25 ± 2°C	16 h (day/night) photoperiod with 3000 lux intensity
Boesenbergia rotunda[14]	25 ± 2°C	Dark for callus induction under a photoperiod of 16 h for shoot induction
Alpinia purpurata[15]	25 ± 2°C	16/8 h photoperiod provided by cool white fluorescent tubes (60 µ mol m-2 s-1)
Curcuma kwangsiensis[76]	27 ± 1°C	16 h photoperiod providing 80 lmol m^{-2} s^{-1} fluorescent light.
Curcuma zedoaria[17]	25 ± 2°C	16/8 hours photoperiod under a 31 ¼mol m^{-2} s^{-1}
Curcuma zedoaria[18]	25 ± 2°C	16/8 hours photoperiod under 35µmol/m^2/s
Zingiber zumbret [19]	25 ± 2°C	16/8 hours photoperiod under 35µmol/m^2/s
Zingiber zumbret[75]	25 ± 2°C	16 h photoperiod at a photosynthetic flux of 150 µmol m^{-2} s^{-1}
Amomum krervanh[8]	25 ± 2°C	16-hour photoperiod under cool-white fluorescent light (28 m mol m^{-2} s^{-1})
Cornukaempferia auranti-flora[19]	25 ± 2°C	White, fluorescent light (2,000 lux) with a 16 h photoperiod
Zingiber montanum[54]	25 ± 2°C	White fluorescent light (3000 lux), at 16 h photoperiod

medium in glass vials under controlled experimental conditions. Formation of callus tissue is the outcome of cell expansion and cell division of the cells of the explants.

Initiation of callus culture is done with juvenile parts like leaf, stem segment, roots etc containing meristematic tissue. Such tissue has a pre-existing growth momentum. On implantation, the merstematic tissue absorbs the exogeneously supplied nutrients and growth regulators; divide asynchronously to form the unorganized mass of tissue. During the initial growth phase the cells enlarge or swell to rupture. This indicates the response of tissue to the medium for callus formation. Some endogeneous growth substances oozes out through the injured tissue through the cut end and stimulates the cell division along with the penetration of the exogeneously supplied hormone and nutrients. The unorganized callus tissue gradually increases in size and ultimately the whole part of the explants starts to divide.

In the study of plantlets from rhizomatous callus tissues in Murashige and Skoog medium of *Mantisia wengeri,* Bhowmick and his co researchers described the development of high efficiency regeneration protocol for obtaining large-scale diverse incorporated with various concentration of growth regulators N_6-benzyladenine (BA), α-Naphthalene acetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2,4-D) and sucrose at elevated concentrations[10]. A protocol for high frequency shoot organogenesis and plant establishment from shoot base derived callus was developed for *Boesenbergia rotunda*. The cultures produced callus in MS medium supplemented with various concentrations of dicamba and 2, 4-D. But, embryogenic callus only emerged on 2, 4-D augmented MS medium. In addition, the success of callus induction was achieved from meristem derived callus producing 100% of embryogenic callus[14]. *In Curcuma kwangsiensis* a protocol for callus-mediated shoot regeneration was achieved by using shoot base sections. The frequency of callus formation reached 91% for explants cultured on MS medium containing 1.4 µM TDZ, 4.4 µM BA and 2.3 µM 2, 4-D[76]. In cultures of *Curcuma zedoaria* concentrations of 2.4-D did not show any positive response for callus induction. Treatment of diverse tissues of *Curcuma zedoaria* with different NAA concentrations produced variable callus induction responses. Callogenesis occurred on the shoot apex, leaf sheath base, root apex and root medium region explants. Occurrence of relevant callogenesis was inversely proportional to NAA concentration. Best results were observed when explants from roots (root apexes and median portion of roots) were maintained at NAA concentrations of 1.0 mg/L. Complete absence of light was a requirement for best callus induction[17]. In young leaf mediated callus culture of *Cornukaempferia aurantiflora,* initiation of callus growth was observed on Murashige and Skoog medium supplemented with 3% sucrose and various concentrations of

2, 4-dichlorophenoxyacetic acid. The highest number of callus forming, percentage of callus formation and average weight of callus were obtained from young leaves cultured on the medium supplemented with 2.0 mg/l 2, 4-D[19].

The role of growth regulators and their concentration will have to be carefully chosen for obtaining desired responses in tissue culture. The most important development in the tissue culture of the plant were made with the discovery of growth regulators, auxins, gibberellins, cytokinins and abscisic and other organic compounds.

In *Alpinia galanga* the best shoot multiplication and growth was observed at 3.0 mg/l Kn, where a maximum of 8 shoots could be observed per flask. During subculture, all the plants were divided and cultured into same multiplication medium. They responded in the same manner as the other culture, shoot showing similar shoot multiplication and roots. This multiplication rate did not decrease in the successive subcultures on the same medium even after more than one year. Thus, by repeating this procedure for 4–5 successive culture cycles, an average of 1000 plantlets can be produced per flask and complete plantlets could be produced within 45–50 days[7]. Optimum shoot multiplication of *Alpinia officinarum*[9] in MS medium supplemented with 3% (W/V) sucrose, 3.0 mg/L Kn and 1.0 mg/L NAA. In *Alpinia purpurata*, Murashige and Skoog medium supplemented with BA (3.0 mg/l) and Kn (2.0 mg/l) exhibited regeneration rate up to 6.4±0.32 shoots/explants using rhizome bud explants[15]. In cultures of *Aframomum corrorima* the highest number of shoots and leaves were obtained from MS, while both HMS and HNMS media followed by SH gave the highest root number. Korarima explants cultured on MS medium produced significantly longer shoots, while those on SH were the shortest. However, the shortest roots were produced on the MS medium followed by SH medium. Addition of CW (5%) increased the number of shoots and leaves, mean lengths of shoots, and both fresh and dry weights of the plantlets. The best result was obtained when the culture media was supplemented with 2 mg/l imazalil in combination with 0.5 mg/l thidiazuron[8]. Initiation of shoot buds in *Costus speciosus* was observed when rhizome thin sections of rhizome were cultured on B_5 basal medium supplemented with 5 mg per litre TRIA. Shoots with two to three leaves produced roots when cultured on B_5 basal medium supplemented with 2 mg per litre TRI[73]. The best response for shoot multiplication in *Costus speciosus*[74] was on 1 µM NAA, 50gl-1 sucrose and 7 µM BAP. In *Mantisia wengeri*[10] the bud forming capacity and shoot regeneration capacity to a maximum of 33.5±3.7 was recorded from the organogenic callus tissues of within 8 - 10 weeks in MS medium supplemented with a combination of 10µM of BA and 40µM NAA at 10% sucrose. The axillary bud explants of *Etingera elatior* were cultured on Murashige and Skoog (MS) basal medium, supplemented with 22.2 µM BAP to the MS

medium produced the highest mean number of shoots (3.67) per explants as compared to other concentrations. The best shoots length (4.20 cm) was obtained from the medium containing 26.6 μM of BAP[11]. Multiple shoot induction was achieved from the surface of the callus of *Boesenbergia rotunda* after transferring onto MS medium supplemented with BAP or kinetin (shoot induction medium). The highest frequency of callus producing shoots (49.8%) was achieved on MS medium containing 2.0 mg/L BAP[14]. *In* Curcuma kwangsiensis 8.2 shoots per callus was achieved on MS medium supplemented with 1.4 μM TDZ, 17.8 μM BA and 2.7 μM NAA[76]. Explants of *Curcuma zedoaria* showed high rate of shoot development in the presence of high BAP concentrations and absence of NAA[17]. In *Curcuma zedoaria* and *Zingiber zerumbet*, MS solid media supplemented with 0.5 mg/L BA and 0.5 mg/L IBA showed less number of shoots in MS liquid media supplemented with the same hormones in the same concentrations[18]. In *Zingiber zerumbet* MS medium supplemented with a combination of 5.0 mg/l BAP and 2.0 mg/l IAA or 3.0 mg/l BAP and 0.5 mg/l IAA produced the highest mean number of shoots (5.6) per explant as compared to other concentrations. The best shoots length (9.44 cm) was obtained on the medium containing 1.0 mg/l of BAP and 2.0 mg/l IAA. Thus, combined effects of BAP and IAA significantly improved the shoot growth and proliferation[75]. *In Zingiber montanum*[54] thidiazuron (TDZ) at 0.5 mg/L was found to induce the highest shoot multiplication with a mean of 8.1 shoots per explant. Sectioned buds produced a mean of 4.6 shoots from each explant. As far the culture phase, liquid medium was found to be superior to solid medium. The highest rate of multiplication in *Amomum krervanh* was obtained from MS medium. 5%

Table4: Hardening materials and survival rate of regenerated Zingibers

Plant species	Potting mixture	Survival rate
Aframomum corrorima[8]	Peat moss and river sand (1:1)	93 %
Alpinia galanga[7]	Sterilized potting soil	80 %
Alpinia officinarum[9]	Vermiculite and soil (1:1)	93 %
Mantisia wengeri[10]	Soil and compost (1:1)	90 %
Etlingera elatior[11]	Sterilized peat moss soil	75 %
Costus speciosus[73]	Sterilized Soil	100 %
Costus speciosus[74]	Sterilized Soil	95 %
Boesenbergia rotunda[14]	Soil and sand (1:1w/w)	100 %
Alpinia purpurata[15]	Vermiculite and soil (1:1)	100 %
Curcuma kwangsiensis[76]	Chinese peat: coconut chaff: perlite in the ratio 1: 1: 1	100 %
Curcuma zedoaria[17]	Soil with Mycorrhiza	More
	Soil without Mycorrhiza	Less
Curcuma zedoaria[18]	Organic soil and sand (1:1)	100 %
Zingiber zumbret[18]	Organic soil and sand (1:1)	100 %
Zingiber zumbret[75]	Sterilized peat moss and soil	80 %
Amomum krervanh[8]	Peat moss and river sand (1:1)	90 %
Zingiber montanum[54]	Mixture of 1:1:1 soil, sand and peat	85%

coconut water (CW) was found to produce the best for shoot growth and development, i.e. shoot number, as well as fresh and dry weights. The best media for shoot growth and development was the one with 2 mg/l and 0.5 mg/l TDZ[8]. In young leaf mediated callus culture of *Cornukaempferia aurantiflora s*hoots were successfully regenerated on the medium with 2,4-D and BA[19].These techniques greatly facilitate the in vitro conservation of rare and endangered plants.

In cultures of the members of Zingiberaceae additional plant growth regulator may or may not be required for *in vitro* rooting. Single shoots of *Curcuma kwangsiensis*[76] and *Amomum krervanh*[8] transferred into MS medium free of plant growth regulator rooted well. spontaneous rooting of shoots in *Curcuma zedoaria*[18] and *Zingiber zerumbet.* Similar observations were made in *Alpinia galanga*[7]. Spontaneous rooting of shoots of *Alpinia purpurata* occurred in Murashige and Skoog medium supplemented with BA (3.0 mg/l) and Kn (2.0 mg/l)[16]. In *Alpinia officinarum* optimum and best rooting was observed in media with 0.5mg/L IBA in comparison to IAA and NAA[15]. In *Aframomum corrorima* both HMS and HNMS media followed by SH gave the highest root number. The explants cultured on MS medium produced significantly longer shoots, while those on SH were the shortest. However, the shortest roots were produced on the MS medium followed by SH medium. Inclusion of CW to the culture medium resulted in reduction of both root number and length[8]. In cultures of *Costus speciosus* better rooting was observed in lower concentrations of TRIA[73]. The maximum average number of roots was found in 3 µM BAP treatment[74]. In *Mantisia wengeri* the number of roots formed from the *in vitro* shoots was significantly higher in ½ MS medium supplemented with IBA as compared to IAA supplemented MS medium. A maximum of 11.6±0.3 healthy roots were regenerated from the *in vitro* shoots within 2 weeks in ½ MS medium supplemented with 0.5µM IBA. The roots formed from the shoots in IAA supplemented ½ MS medium was thin and less in number as compared to roots obtained in IBA supplemented medium[10]. For *in vitro* rooting of shoot in *Etingera elatior,* MS medium supplemented with 11.4 µM IAA produced the highest number of root, whereas 34.2 µM IAA gave the longest roots in the medium[11]. In *Zingiber zerumbet* MS medium supplemented with a combination of 5.0 mg/l BAP and 2 mg/l IAA gave the highest number of roots. However, longest roots per explant were obtained with 1.0 mg/l BAP alone[75]. In *Zingiber montanum*[54] rooting of propagules was conducted on B_5 medium devoid of growth regulators. Root growth and elongation of *Alpinia galanga* could be enhanced by supplementing thiamine alone at 10 mg/l in place of standard MS vitamins[7].

One of the major obstacles in the application of tissue culture methods for plant propagation has been the difficulty in successful transfer of plantlets from the laboratory to the field[80].

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The reasons for such a difficulty appear to be related to the dramatic change in the environmental conditions. The environment of the culture vessel is one of low light intensity, with very high humidity (generally 100%) and poor root growth, while the greenhouse and/or field conditions are typified by very high light intensity, low humidity and microflora[80]. Several workers have developed protocols to overcome some of these constraints. These reasons for such a difficulty appear to be related to the dramatic change in the environmental conditions.

In hardening of regenerated tissue cultured plantlets of the family Zingiberaceae hardening of regenerated plantlets can be achieved very easily. Different hardening materials like peat moss and river sand, sterilized potting soil, vermiculite and soil, soil and compost, soil, sand, soil with mycorrhiza, soil without mycorrhiza, organic soil and sand etc have been used (Table 4). The success rate of hardening depends upon the hardening material and the condition of the regenerated plantlet. High rate of survival of regenerated plantlets have been achieved in field in all the cases.

Conclusion

Zingiberous plants have been the subjects of man's curiosity since long time and every civilization has a history of medicinal plant use. High percentage of the people in the developing countries of the world relies on traditional medicine for their primary health care needs. Zingiberous medicinal plants are of special importance as they are widely cultivated or are found in the wild. The members of Zingiberaceae have poor flowering and seed set so their plant breeding is seriously handicapped for which *in vitro* regeneration in very significant in Zingiberous plants. It has been seen that *in vitro* regenerated plants are superior to conventionally propagated plants in respect of productivity and disease resistance. The rates of productivity of Zingiberous plants are high with very profuse rooting. So, they can be easily hardened and transferred to the field with a good rate of survival. *In vitro* culture of these plants offers a method for producing variations and exploring the resultant variations for crop improvement. Moreover overexploitation of Zingiberous crops are making them endangered, regeneration through tissue culture can save the wild population.

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Chapter 8

Biodegradation of Xenobiotic compounds by microbes-a review

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Abstract

Xenobiotics are chemically synthesized compounds that are not occurring naturally. It includes a long list of compounds involving pesticides, polychlorinated biphenyls (PCBs), dyes and chlorinated solvents. Many Xenobiotic compounds are structurally related to natural compounds, and thus can be slowly degraded by an enzyme, that already exists to degrade these natural compounds. In other cases, these compounds are structurally so different from natural compounds, that their degradation rate in nature is extremely slow, if at all. Nevertheless for many Xenobiotic compounds, microorganisms have been found, that can degrade them. This paper reviews pesticide degradation by diverse group of bacteria and fungi with special reference to the mechanisms that microorganisms employ to degrade Xenobiotic compounds.

Keywords

Xenobiotics, Reductive dechlorination, Aerobic dechlorination, Laccases, Peroxidasess

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Introduction

Chemically synthesized compounds containing structural elements uncommon in nature are referred to as Xenobiotic compounds.Infact substances that are present in abnormally high concentration can also be referred to as Xenobiotics.The term Xenobiotics is derived from the Greek word "Xenos" which means foreign and "bios" means life. Xenobiotic includes a long list of compounds such as polychlorinated biphenyl's (PCB's), dyes and chlorinated solvents, pesticides like chlorophenoxyalkyl carboxylic acid, substituted ureas, triazines, nitrophenols, 2,4,5-trichlorophenoxy acetic acid; complex polyaromatic substance like lignin and a diverse range of compounds like TNT, benzopyrerne and plastics.These compounds are structurally so different from natural compounds that their degradation rate is extremely slow, if at all. However nature also contributes to the formation of compounds that are very much identical or analogous to "Xenobiotic pollutants". Some examples are petroleum hydrocarbons produced through natural processes like coaltar pits,some volatile terpenes produced by plants and a large variety of halogenated compounds produced as metabolites by many plants, algae ,fungi, bacteria and other organisms[1] .Presently, the natural formation of vinyl chloride was also reported.

Nowadays, cholesterol biodegradation has become an issue relative to our environment and also to human health indirectly. Excessive use of synthetic steroid compounds like sexual hormones with strong metabolic activities have negative impact to our ecosytem.

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Various sources of xenobiotic compounds

The primary sources of Xenobiotic compounds include various industrial activities such as polychlorinated biphenyls (PCB) from transformers, azo dyes associated with textile industries and increased use of chlorinated solvents in dry cleaning and engine cleaning. Petrochemical refineries also contribute to the sources of Xenobiotic pollutants.Other sources of Xenobiotic compounds include human activities such as increase use of chemical pesticides in agricultural fields and extensive use of chemically synthesized steroid compounds.

Extensive use of explosives like TNT, 2, 4, 5-Trichlorophenoxy Acetic Acid etc. also contributes to various Xenobiotic pollutants. An overview of Xenobiotic compounds and their sources are shown in table 1.

Xenobiotic Compounds – "A threat to environment and public health"

The main environmental threat associated with most Xenobiotic compounds is the toxicity threat they pose to public health. In general, the introduction of Xenobiotic compounds in the environment can affect many organisms in the ecosystem, especially through

Table 1: Various sources of xenobiotic pollutants

Name of the pollutants	Industrial sources
2,4,5-T	Pesticides
Substituted ureas	Pesticides
TNT	Explosives
BTEX	Fossilfuels,solvents,industrial feedstocks
Cholesterol	Synthetic steroid compounds like sexualhormones
Alkanes/Alkenes	Fossil fuels,plastics,industrial feedstocks

TNT – Trinitro toluene; BTEX - benzene, toluene, ethylbenzene and xylene; 2,4,5-T – 2,4,5 – Trichloro phenoxy acetic acid

biomagnification of hydrophobic pollutants upwards in the food chain. Excessive use of chemical pesticides such as chlorophenoxy alkyl carboxylic acid; substituted ureas, nitrophenols etc also contribute to toxicity and pose threat to public health.

A new concern is the recognition that some xenobiotic compounds (phenols, biphenylic compounds, phthalates, etc) have hormonal activities, the so called "endocrine disruptors"[2,3]. This is particularly shocking since hormones have an impact at extremely low concentration.

Current research suggests that, excessive use of pesticides in agricultural fields can lead to disease like cancer. In general, such environmental concerns can persists, if these compounds remain undegraded for a long period of time.

Biodegradation of xenobiotic compounds

Biodegradation is the complete breakdown of the complex and toxic contaminants to non-toxic, simple elements by the action of microbes. Hence, these contaminants act as the microbial food substrate. Biodegradation, in general, can be considered as a series of steps of biological degradation (or pathway) that ultimately result in the oxidation of the compound which most often results in the generation of energy. In other words Xenobiotic pollutants can serve as the electron acceptor of primary metabolism, instead of electron donor.

Xenobiotics in the environment can be degraded by microbes since microbes have the capacity to degrade all naturally occurring compounds as proposed by Alexandra in 1965.Not all Xenobiotic compounds can be degraded by microbes. The compounds that can resist biodegradation and persist in the environment for a long period of time are called recalcitrant compounds. However significant advances have been made in respect to isolation of bacterial cultures, different biochemical mechanisms, and laboratory and field

scale applications for Xenobiotic removal.

Types of biodegradation

Types of biodegradation include two mechanisms, namely the aerobic biodegradation and the anaerobic biodegradation.

Aerobic biodegradation of Xenobiotic compounds requires the presence of oxygen and is quite an expensive process requiring high energy costs and sludge productions when bioreacters are involved. This mechanism is basically employed by the bacterium *Burkholderia cepacia G4* to degrade 2,4,5-Trichlorophenoxy acetic acid and also by several species of *Rhodococcus* .strain RHA1. The mechanism also degrades several petroleum hydrocarbons.

In contrast to aerobic biodegradation, anaerobic biodegradation mechanism is of great environmental significance in relative to its novel pathway. In particular, hydrocarbons and halogenated compounds have long been doubted to be degradable in absence of oxygen, but the isolation of anaerobic hydrocarbon degrading bacteria and reductively dehalogenating bacteria such as *Dehalococcoides* provide an ultimate proof for these processes in nature. Many novel biochemical pathways were also discovered but progress in molecular level of these bacteria were

rather slow since genetic systems are not applicable for most of them. Recent advances are being made in the field of molecular biology as well as in genetics in order to get some improved strain for degrading xenobiotic pollutants.

Compared to the aerobic strategies of biodegradation, anaerobic biodegradation has two important disadvantages.

Firstly a thermodynamic disadvantage in which the alternative electron acceptors have lower standard reduction potential ($E^{0,}$) values as compared to that of oxygen giving rise to lower Gibbs Free Energy change (dG^0) when coupled to the oxidation of any given substrate.

The second disadvantage for anaerobic biodegradation of Xenobiotic compounds is the inert character of the compounds due to a general lack of oxygenated functional groups. The presence of oxygenated functional groups allows organic compounds to enter a common metabolic pathway of degradation. Aerobic organisms overcome the problem with the help of enzyme oxygenase.

Aerobic biodegradation

Aerobic biodegradation commonly known as aerobic dechlorination is basically employed

by the bacterium *Burkholderia cepacia* G4 to degrade 2, 4, 5-T (2, 4, 5-Trichlorophenoxy Acetic Acid) a commonly used pesticide in agricultural field. This organism utilizes 2, 4, 5-T as a substrate for its metabolism to generate energy. The final product of the degradation is succinate and acetate which are further catabolized in the citric acid cycle. An overview of the entire pathway is shown in the Figure 1**.**

Anaerobic biodegradation

Under anaerobic condition, three main types of biodegradation can be distinguished in which the xenobiotic acts as an electron donor for primary metabolism, electron acceptor for primary metabolism or the Xenobiotic is cometabolically reduced.Some examples of xenobiotics acting as an electron donor for primary metabolism include phenols, phthalates [4] and hydrocarbons including BTEX. Even some chlorinated compounds such as simple chlorophenols)[5,6], chloromethane or dichloromethane[7] fall in this category.

Examples of xenobiotic compounds acting terminal electron acceptor (TEA) include processes like halorespiration (reductive dechlorination as terminal electron accepting

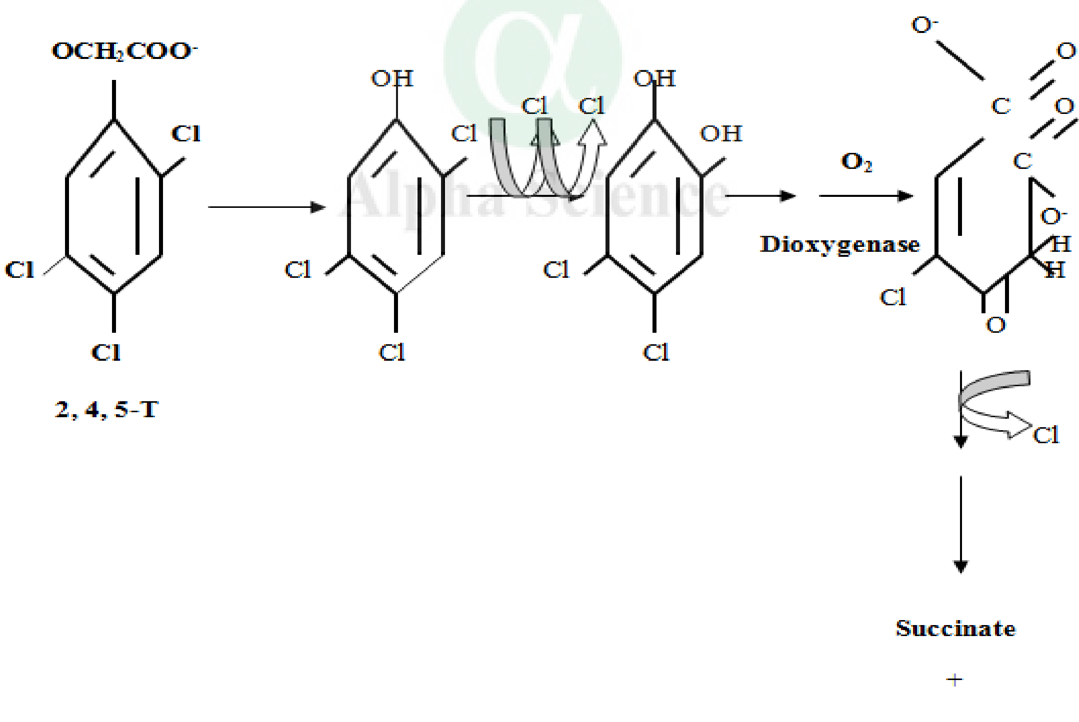

Figure 1: Aerobic biodegradation

process) in which 3-chlorobenzoate is reduced to benzoate[8].

Anaerobic Biodegradation pathways

Several pathways of xenobiotic degradation can be recognized. Among them, the most common is the reductive dechlorination pathway.Benzoyl Co A pathway also fall in this category.

Reductive dechlorination

In reductive dechlorination significant degradation of chlorinated pesticides occur in anoxic environment. In general, the anoxic biodegradation is linked to reductive dechlorination of the molecule. It is one of the novel pathways followed by the bacterium *Desulfomonile* to reduce 3-chlorobenzoate to benzoate and chloride ions as indicated in the reaction below:

$C_7H_4O_2Cl^- + 2H \rightarrow C_7H_5O_2^- + HCl$

Electrons for this reaction come from acetate, formate or hydrogen and the reductive reaction is linked to the establishment of proton motive force to drive ATP synthesis. Thus we can say that the reductive dechlorination of 3-chlorobenzoate is a type of anaerobic respiration.

Direct evidence for reductive dechlorination has also been obtained in case of dichloroethylene, trichloroethylene, tertrachloroethylene, chloroform, dichloromethane and certain brominated and chlorinated compounds.

The bacterium *Dehalococcoides* is unique as it removes all the chlorine from polychlorinated ethylene leaving only the ethylene gas as the final product. The biochemical pathway for this degradation is shown in Figure2.

Benzoyl CoA pathway

It is one of the central pathways of most aromatic compounds. The key enzyme of the pathway is benzoate CoA ligase which attaches the coenzyme to the carboxy group and thus activates the benzoate for metabolism. The initial steps of the pathway are shown in Figure 3. More recently the pathway has been elucidated from the syntrophic fermentative bacterium *Syntrophus gentianae* that grows in coculture with the methanogen, *Methanospirillum hungatei* and thus is more representative of an organism occurring in a methanogenic community.

Biodegradation of petroleum hydrocarbons

Petroleum is a complex mixture of aliphatic, alicyclic and aromatic hydrocarbons and a small portion of non-hydrocarbon compounds such as phenols, napthelic acid, thiols etc.The

Figure 2 The successive reductive dechlorination of perchloroethylene (PCE) to ethene
TCE- Trichloroetene, cis-DCE – cis- Dichloroethene, VC – Vinyl Chloride.

Figure 3: Benzoyl-CoA pathway of the syntrophic fermentative bacterium *Syntrophus gentianae*

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mode of biodegradation varies with the type and size of hydrocarbon molecules. n-alkanes of intermediate chain length (C10 – C20) are degraded most rapidly. Short chain alkanes are toxic to most microorganisms but they generally evaporate from oil slicks rapidly. Aromatic compounds are degraded very slowly. Alicyclic compounds, serving as a sole source of carbon for most microorganisms are degraded via co-metabolism by two or more cooperating microbial strains with complimentary metabolic capabilities.

Fungal biodegradation

Fungi plays an important role in biodegradation of many xenobiotic pollutants such as TNT, Polycyclic aromatic hydrocarbons (PAH's) like benzopyrene and plastics such as poly

ethylene. The white rot fungus *Phanerochaete chrysosporium* has been proposed to degrade many complex polluted compounds like TNT, ployaromatic hydrocarbons.etc. This fungus produces enzymes like laccases and peroxidases that are normally involved in the degradation of lignin which is a complex polyaromatic substance. These enzymes also have the ability to degrade a diverse range of compounds like TNT, benzopyrene and polyethylene.

Fungus like *Aspergillus* and moulds also plays an important role in degrading many refractory compounds like fat, chitin, oils and keratin. Maximum decomposition occur when there is sufficient nitrogen, phosphorous and other essential inorganic nutrients.

Recent advances in the field of xenobiotic biodegradation

Anaerobic biodegradation of xenobiotic compounds has been a subject of interest since the last two decades. Consiquently, our current understanding in the dissipation of xenobiotic compounds in anaerobic environment has considerably improved. Many anaerobic bioreacters and remediation system have been developed to effectively clean up the contaminated media.

However with the increasing application of genomics in the field of environmental microbiology a new and promising perspective is now at hand to obtain molecular insights into these new metabolic properties. During the last few years, several complete genomic sequences have been determined for bacteria capable of degrading several organic pollutants. The ~4.7 Mb genome of the facultative denitrifying *Aromatoleum aromaticum* strain EbN1 was the first to be determined for an anaerobic hydrocarbon degrader (using toluene or ethylbenzene as substrates).

Genomic studies of anaerobic hydrocarbon degrading bacteria are recently completed for iron reducing species like *Geobacter metallireducens* (accession nr. NC_007517) and the perchlorate-reducing bacteria *Dechloromonas aromatica* (accession nr. NC_007298), but not yet published in formal publications. Complete genomes were also determined for bacteria capable of anaerobic degration of halogenated hydrocarbons by halorespiration.These bacteria includes *Dehalococcoides ethenogenes* strain 195 , *Dehalococcoides* sp strain CBDB1 and *Desulfitobacterium hafniense.*

Recently, it has become apparent that some organisms including *Desulfitobacterium chlororespirans* capable of utilizing chlorophenols can also use certain brominated compounds such as herbicide bromoxynil and its major metabolite as electron acceptor for growth.

Recent advances has also been made in the field of cholesterol biodegradation. Since these

compounds are common carbon sources for many microorganisms, their aerobic and anaerobic degradation has been extensively studied.

Discussion

The removal of wide range of pollutants and wastes from the environment is an absolute requirement to develop a healthy society with low environmental impact. Biological processes play a major role in the removal of these contaminants and they take advantage of the catabolic versatility of microorganisms to degrade or covert such polluted compounds. This paper specifically reviews the current mechanisms that microorganisms employ to degrade selected group of xenobiotic compounds like chlorinated hydrocarbons, TNT, 2, 4, 5-trichlorophenoxy acetic acid, benzopyrene and polyethylene. Degradation of petroleum hydrocarbons is also a part of this review paper. The paper also summarizes the recent advances made in the field of xenobiotic biodegradation.

Previous research has been dedicated quite a lot to phenols, pesticides like 2, 4, 5-T, subtituted ureas, hydrocarbons, explosives and chlorinated solvents. New challenges in research should be to investigate the biodegradation of xenobiotic compounds from other chemical families such as organometalloids, organofluorine compounds and organosulfide compounds such as methyl mercaptan and dimethyl sulfide. Methylated arsenicals are used on a large scale as pesticides in cotton and roxarsone (3-nitro-4-hydroxyphenyl-arsonic acid) is used extensively in chicken feed [9]. A large variety of oragnofluorine compounds (especially fluorinated hydrocarbons) are being used in the semiconductor industry yet little is known about their biodegradability in the environment. The petrochemical refinery industry has many types of organosulfide compounds, such as methyl mercaptan and dimethyl sulfide are direct substrates of ceratin methanogens. Thus there is a large unexplored potential for anaerobic processes to treat organosulfide compounds.

Lastly there is also a great need to design mechanisms for degrading various industrial compounds like surfactants in detergents, dyes from aromatic amines with hydroxy and carboxy groups[10].

Several excellent reviews have been published on anaerobic biodegradation of xenobiotics, both in general and focused on specific compounds including petroleum hydrocarbons[11], explosives[12], chlorinated compounds, and pesticides.

Conclusion

Till now, biodegradation of limited number of compounds have been studied. Extensive studies should be carried out in the field xenobiotic biodegradation in addition with genetics

and molecular biology so as to design some new mechanisms of degradation in order to make our environment a healthy place to live in.

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Chapter 9

Advancement of bamboo taxonomy in the era of molecular biology: a review

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Abstract

Extensive loss of the valuable plant species in the past centuries has triggered the conservation of plant resources. However successful conservation depends upon the appropriate identification and characterisation of the plant materials. Bamboo with about 1575 species harbouring both woody and herbaceous bamboo included in the sub family Bambusoideae of Poaceae is of more concern in this regard because of its ever increasing demand. Traditionally the taxonomy of bamboo has been dependant on morphological characters like other plants, but because of infrequent flowering behaviour and extensive polyplodization of the genome, the taxonomy of bamboo is in a state of flux. To overcome these difficulties molecular techniques has been employed since these techniques answer many new evolutionary and taxonomic questions which were not possible with the traditional method based on phenotypic characters. This paper presents an overview of different molecular techniques like Restriction fragment length polymorphisms (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified fragment length polymorphism (AFLP), Sequence-Characterized Amplified Regions (SCARs), Inter-simple sequence repeat (ISSR), Expressed Sequence Tag - Simple Sequence Repeat (EST-SSR), used to access the genetic diversity of the bamboo species and also the precise identification of the bamboo genotypes.

Keywords

Bamboo, Molecular techniques, Microsatellite markers, RAPD, RFLP, Taxonomy

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Introduction

Bamboo with altogether 1575 species including both woody and herbaceous bamboos belongs to the subfamily Bambusoideae of the true grass family Poaceae. Bamboo is popularly known as "Green Gold of the forest" because of its varied applications. Bamboo is naturally distributed round the globe. Major species of bamboo is found in Asia Pacific and South America but much less in Africa[1]. Asia remains the richest continent having two-third of the acerage. Bamboos are mainly classified into three major categories: the pleotrophic woody bamboo; the neotrophic woody bamboos and north temperate wood bamboos. The great diversity of species makes bamboo adaptable to many environments and they are highly palatable to man, domestic animals, and wildlife. India is well endowed with bamboo resources, and is the second largest in the world after China[2]. In India bamboo is encountered in the moist and deciduous forests of all the states except Jammu & Kashmir[3]. Bamboo occupies nearly 11. 4 million hectares of land which represents 16.7 percent of total forest cover in India. But despite this huge acerage under bamboo plantation (both natural and planted), India contributes only 4% to the global market.

Bamboo has emerged as a potential crop having over 1500 diversified uses around the globe ranging from medicine to nutrition and from toys to aircrafts. Bamboo is known as world's best engineering material due to its high tensile strength compared to teak wood and mild steel[4]. They can be grown for processing into beautiful flooring, high quality paper and soft bamboo clothing. They are also strong contenders for large-scale biomass crop cultivation, for the production of renewable carbon-neutral energy and biofuels. Its biological characteristics make it a perfect tool for reducing carbon dioxide levels in atmosphere. It generates more O2 than equivalent strands of trees, protects against UV rays and is an atmospheric and soil purifier[3]. On the other hand, bamboo identification is usually far from easy. It is often unkind even to the expert, and the literature usually looks indigestible and frightening. The growing requirements of different species vary enormously. The intrinsic characteristics of different species that give them their individual aesthetic appeal, or make them suitable for any of the thousands of uses to which they can be put will obviously also vary. Therefore it is important to have a reliable and consistent system of names, so that the best species can be selected for growing in the right environment for a purpose to which it is well suited. Bamboo finds its importance in therapeutic world (chawanprash) because of high antioxidant properties and nutrient values (high flavin and phenolic compounds), low in fat, high in edible fibres and rich in mineral. Despite of its multiple uses the research efforts spent on bamboo seems to be negligible compared to other agricultural crops. The very basic problem faced in this field is the proper identification of bamboo i.e. bamboo

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taxonomy at the species level.

This paper aims at presenting an overview of the recently developed molecular markers in proper identification of the bamboo and systematic grouping.

A. Traditional system of bamboo taxonomy

Traditionally, like all other plant species bamboo also involves morphological features like rhizomes, bud, leaf, branching patterns, inflorescence, flowers and fruits for any documentation or taxonomic treatment. Bamboos are also gifted with some exceptional morphological features like the culm sheath and well developed branching complements that are generally absent in the grasses and thus can play a role in proper identification of the bamboo and systematic grouping. But it is not true since many of these characters are not uniformly applicable to all the species and thus there is absolute necessity to have detailed descriptors which can be followed to identify the species[5] that can be utilized by different group of people dealing with bamboo. Apart from this there is an urgent requirement to refine and better understand the taxonomic techniques and classify the taxonomic diversity in bamboos. As per conventional method the morphological characters were used for taxonomic identification, especially the flowers. This has been problematic in case of bamboo since the number of characters is limited and there is the scarcity of flowering material because of the intriguing flowering behaviour of the bamboos. Flowering in bamboo remains one of the great mysteries in botany as flower of bamboo is unusual and the period may vary between 15-120 years[6]. Incongruity stemmed out among taxonomists due to different interpretations of morphological features and the terminology used for different parts of the plant materials. Thus it the very first step required is to refine the characters that are used currently and also try to find some unique new characters that can prove to be helpful in the long run. Identification and classification of bamboo using the anatomical features didn't prove to be successful as at first hoped. The credit for revealing the importance of branch and buds characteristics in bamboo taxonomy goes to Usui[7]. Following this McClure[8,9] studied the morphology of the rhizome, branching patterns, culm sheath and the inflorescence. Apart from this Soderstrom and Ellis[10] also considered the anatomical characters of leaf for bamboo classification at the subfamilies and tribes level however they failed to apply so at the generic level[11-13] revealed that branching pattern of inflorescence can also be applied in bamboo taxonomy. Two new characters (prophyll keel and branch replication) were detected by Stapleton[14] while studying the Himalayan bamboo and he inferred that these characters can aid in identification of bamboo at the generic level. Considering these morphological features bamboo has been classified variously by different authors.

B. Bamboo taxonomy using molecular markers

The taxonomy of bamboo is in a state of flux and molecular studies are required to help resolve systematic issues. With the advent of molecular biology, the taxonomy of different plants has been revolutionized including bamboo. The use of molecular markers has been increasing at an exponential state in all the fields of biology. The application of molecular marker in classifying bamboo where the basic biology is so little understood can prove to be a landmark. Though the use of molecular markers is innumerable, in bamboo these can be employed for dual function, firstly for precise identification of bamboo genotypes and secondly assessment of genetic variation within species irrespective of the geographic location or other factors responsible to phenotypic variability. Extensive progress has been achieved in bamboo by the implementation of molecular markers. The present study aimed at reviewing the different molecular tools that have been applied to date.

1) DNA fingerprinting method

In recent years, a number of assays have been proposed to detect DNA polymorphism, which has become increasingly precise. The methods employ the use of restriction enzymes or polymerase chain reaction (PCR) or combination of both.

i) *Restriction fragment length polymorphisms (RFLP)*

Nuclear restriction fragment length polymorphisms (RFLP) is based on the differences in the restriction enzymes recognition site between genome sequences. Friar and Kochert[15,16] were the first to use restriction fragment length polymorphisms for bamboo identification of 61 accessions and 20 species of *Phyllostachys*. The study supported the earlier observations of the presence of two distinct sections (*Phyllostachys* and *Heteroclada*) in *Phyllostachys* species pool. However, they disagreed to place *Phyllostachys nigra* under the section Heteroclada and thus contradicted a previous study[17]. The regular use of RFLP in plant genotyping as well as bamboo has been limited mainly due to the requirements of large amount of DNA along with the use of radioactive isotopes.

ii) *Random Amplified Polymorphic DNA (RAPD)*

The big boom of molecular markers came with PCR-technology i.e. Random Amplified Polymorphic DNA (RAPD) developed by Williams and his co-workers[18], where a single and short arbitrary primer is used. Since its discovery this technique has been successfully employed in the evaluation of genetic relationships in bamboos and other plant species. In bamboo, RAPD analysis has been successfully employed to study the population genetic structure of *Yushnia niitakayamensis*[19] and also to study the genetic relationships within *Phyllostachys*[20-22]. Nayak and his co-workers[23] had used this technique to study the genetic

variations among 12 species of tropical bamboo. Using the RAPD based neighbour-joining tree, Sun and his co-workers[24] segregated a thorny core *Bambusa* cluster from a cluster of *Dendrocalamus* species with more capitate inflorescences. Bhattacharya and his co-workers[25] developed a RAPD fingerprint profile for a single bamboo species, *Bambusa tulda*. Das and his team[26] used two independent parameters viz. 32 key morphological descriptors and 120 polymorphic loci in the genomic DNA to assess the phylogenetic relationships between 15 tropical bamboo species. Genetic diversity and relationship among nine species of bamboo belonging to four genera was studied by Ramanayake and his co-workers[27] using RAPD analysis. To access the genetic similarity among the 20 different accessions of *Melocanna baccifera*, Lalhruaitluanga and Prasad[28] used 40 arbitrary RAPD primers. Zhang and his co-researchers[29] performed the RAPD of the chloroplast DNA of 22 bamboo species to assess the polymorphism, similarities and relationships among them.

iii) *Amplified fragment length polymorphism (AFLP)*

Developed by Vos and his co-workers[30], Amplified fragment length polymorphism (AFLP) is a method described as a combination of restriction digestion and PCR amplification. The use of AFLP for identification as well as determining genetic and relationships among bamboo species was first attempted by Loh and his co-workers[31]. They conducted AFLP analysis on 15 species belonging to four genera using eight primer combinations. Unique banding pattern were observed in 13 out of the 15 species experimented. AFLP markers were also used to study the phylogenetic relationships among *Phyllostachys*[32] and clonal structure in *Sasa senanensis*[32]. Marulanda and his co-workers[34] reported distinct genetic differentiations among the American wood bamboos employing this technique. Recently in 2011, Ghosh and his co-workers[35], conducted AFLP analysis on 12 bamboo species belonging to five different genera using six pairs of primer combinations to study the genetic various among them. From the phylogenetic tree it was revealed that all the five species under the genera *Bambusa* were included in one cluster, while the four species under the genera *Dendrocalamus* formed a discrete cluster. However both these clusters had the same origin, while the genus *Melocanna*, *Chimonobambusa*, *Schizostachyum* segregated out as independent clusters.

vi) *Sequence-Characterized Amplified Regions (SCARs)*

Paran and Michelmore[36] developed the Sequence-Characterized Amplified Regions (SCARs) markers which is nothing but the conversion of RAPD markers to overcome the reproducibility problems encountered in the RAPD technique. Das and his co-workers[37] are the sole authority till date to develop the SCARs for bamboo species. They were successful in developing two species-specific SCAR markers, ‘Balco836’ for *Bambusa balcooa* and

'Tuldo609' for *B. tulda*.

v) *Inter-simple sequence repeat (ISSR)*

Inter-simple sequence repeat (ISSR) is a molecular marker which has been used for identification of genetic diversity of many plants including the bamboos. The use of ISSR markers is however limited in case of bamboos. Lin and his co-workers[38] used the ISSR markers to study the genetic diversity of different cultivars of *Phyllostachys pubescens*. Using ISSR markers Lin and his co-workers[39] succeeded in identifying the bamboo hybrids (formed by crossbreeding) from the parents. Twenty five ISSR markers were used by Mukherjee and his co-workers[40] to investigate the genetic diversity among 22 taxa of bamboos of which 12 resulted in reproducible and scorable bands. Lin and his co-workers[41] also used the ISSR markers to study the genetic diversity of different cultivars of *Phyllostachys violascens*.

vi) *Expressed Sequence Tag - Simple Sequence Repeat (EST-SSR)*

With the advent of time ESTs have become valuable and first-hand source of *in silico* mining of simple sequence repeats (SSR) markers providing insight into the organisms genetic diversity. Twenty-five EST-SSR markers derived from maize, wheat, sorghum, and rice were used by Barkley and his co-workers[42] to assess the genetic diversity 92 bamboo accessions classified under 11 genera and 44 species. Polymorphic EST–SSR markers obtained from major cereal crops have also been experimented by Sharma and his colleagues[43] to assess phylogenetic and genetic diversity of twenty five different species of Bambusoideae. Twelve EST-SSR markers were used by Mukherjee and his co-workers[40] to investigate the genetic diversity among 22 taxa of bamboos of which 4 resulted in reproducible and scorable bands. Dong and his co-workers[44] report the use of Bambusa expressed sequence tags (ESTs) to develop and validate additional microsatellite markers, determine their cross-species transferability and use them to identify bamboo interspecies hybrids. Markers BOM01 and BOM02 transferred successfully to most of the caespitose bamboo species showed rich polymorphism, and are therefore potentially valuable as species-specific alleles for the identification of caespitose bamboo interspecies hybrids.

vii) *Transposons*

Transposable elements are mobile genetic elements broadly classified into two classes (Retrotransposons or Class I and DNA Transposons or Class II) based on their mechanism of transpositions[45]. Transoposons occupy considerable proportions of many eukaryotic genomes[46]. In 1995, Huttley and his co-workers reported the presence of Ac-like transposable element in *Bambusa multiplex* while Gielis[47] also found the presence of Ac-like transposable

element in *Bambusa vulgaris, Sasa veitchii and Phyllostachys edulis*. Applying PCR based Keukeleire and his co-workers[48] detected hAT group-related sequences in Bambusa vulgaris (hATbrna1). Zhou and his co-researchers[49,50] performed molecular phylogenetic analysis of 82 *mariner*-like elements (MLE) transposase gene fragments in 44 bamboo species and PIF-like (P instability factor) elements in the Bambusoideae family. Zhong and his co-workers[51] initiated the comprehensive characterization and analysis of *Pong*-like superfamily of transposases in 6 subtribes including 44 species in 38 genera under Bambusoideae subfamily. Two transposable elements Ty1-*copia* and Ty3-*gypsy* are reported in *Phyllostachys pubescens*[52].

2) *DNA Sequence based method*

Several studies have shown the use of DNA sequence based methods for phylogenetic study of grasses and bamboos. The chloroplast genome has been used to assess the phylogenetics of the grasses since the birth of plant molecular systematic. In 1994 Nadot and his co-workers[53] used the chloroplast gene rps4 to study the phylogentics of 28 poaceae species including bamboo. They succeeded in resolving the position of Bambusoids in relationships with other groups and also how closely the rice and bamboo are associated. On the basis of the rbcL gene, Barker and his team[54] revealed relationships between monophyletic bamboos and Pooideae. Clark and his group[55] sequenced the chloroplast gene ndhF to address the phylogenetic relationships among the 47 grass sequences including two outgroup sequences. Their study resolved the Streptochaeteae and Anomochloeae (tribes of the neotropical herbaceous bamboos) as the most basal clade within the family. The trnL-F has been attempted by several researchers[56,57]. In 2005, Qiang and his co-workers[58] performed the preliminary analysis of the genera Arundinaria in comparison with other closely related genera like Pleoblastus, Pseudosasa, Bashania, Clavinodum and Oligostachyum to screen the phylogenetic relationships among them using trnL-F region of the cpDNA. The trnL-F based sequencing method has also been attempted by Yang and his co-workers[59] to establish a phylogenetic of major gropus of Paleotrophical Woody Bamboos; matK (e.g. Liang and Hilu[60]; Hilu *et al.*[61]). The atpB-rbcL and rps16 regions have not previously been used to study bamboo phylogenetics. However, rps16 has proven useful for plant molecular systematics both for dicots (Caryophyllaceae[62]) and for monocots (Palmae[63] and Marantaceae[64]). Combined analyses of plastid DNA regions are often useful for improving phylogenetic resolution and support[65,66]. Sungkaew and his coworkers[67] performed the combined analysis of five different plastid DNA regions viz. trnL intron, trnL-F intergenic spacer, atpB-rbcL intergenic spacer, rsp16 intron and matK to access the phylogentic relationships among 60 taxa including all the subtribes of Bambuseae and related non-

bambusoid grasses. Their study resolved the non-monophyly of the woody Bamboos. They further emphasized that the classification of Bambuseae needs to be revised to have a clear picture of the different genera of bamboos.

Conclusion

In contrast to the vast majority of studies done to date on bamboo taxonomy and systematics, investigations on genetic diversity at the population level are in its infancy. This paper presents precisely how the molecular marker helps in sorting out the problems related to genotype identification in general and bamboo taxonomy in particular. This review provides a clear picture of the application of various molecular techniques in the population studies especially in bamboo. Though the progress in this field is encouraging, yet these methods should not be considered appropriate for phylogenetic studies above the species level. These markers are undoubtedly useful tools to address the population genetics but for phylogeny reconstruction and taxonomy these might be problematic and misleading, so they must be used with caution. Molecular genetics is a fast-moving field and new techniques are likely to be developed in the near future which will have their own strengths and limitations. Thus it is necessary that these concerns motivate bamboo researchers to a wise and well considered implementation of molecular markers as tools for complementing other techniques.

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Chapter 10

Ethnobotanically important plants of Malda and Uttar Dinajpur - a review

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Abstract

At the beginning of human civilization, knowledge of useful plants has been transmitted from generation to generation through visual memory and it has been an asset to every early civilization. In India such tradition can be traced back to 5000 B.C. The indigenous ethnic communities practice their own traditional treatment systems in their daily life to cure from different ailments. The ongoing growing recognition of local medicinal plants is due to less side effect and low cost. The medicinal properties of plants could be based on the antioxidant, antimicrobial and antipyretic effects of the phytochemicals present in plants. The Medical science has proved that the medicinal plants contain large amounts of antioxidants such as polyphenols, vitamin C, vitamin E, selenium, β-carotene etc., which is responsible for curing several diseases. So, medicinal plants would be the best source to obtain a variety of herbal drugs. Therefore, medicinal plants should be reviewed in every aspect for better understanding of their properties, safety and parallel efficacy. The present review deals with medicinal plants found in both two districts, based on the intensive survey as well as from various literatures. Such investigation will definitely contribute for better understanding of the plants and subsequently conservation and commercial strategy may be adopted as a whole.

Keywords

Ethnobotany, Malda, Uttar Dinajpur, Medicinal plants, Phytochemicals, Antioxidant

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Introduction

The history of medicine is as old as the history of the planet. The human body is not merely a biological entity; the possession of a soul gives rise to psychological, spiritual and psychosomatic values. Man being a sum total of biological and spiritual existence is liable to disease pertaining to both spheres of life. Needless to say, his desire for a quick recovery has sent him on the quest for Medicine.

Greece, Rome, China and India are the pioneering contributors of ancient medicines. The great physician Hippocrates, known as the "Father of Medicine", developed the '*Hippocratic Oath*' which is still using today in Unani system of medicine of Geek. Later, this system was further developed by the wisdom of many Greek scholars and spreaded in different parts of the world, such as, Egypt, Syria, Iraq, Persia, India, China and Middle East and Far Eastern countries[1].

The ancient Roman medicine included a number of specializations such as internal medicine, ophthalmology and urology. The Romans were great believers in a healthy mind equaling a healthy body and favored the prevention of diseases over the cures of them. Many Greek medical ideas were adopted by the Romans and Greek medicine had a huge influence on Roman medicine[2].

However, ancient China medical treatments were very different than those in the West, and there is much less reliance on more conventional methods such as prescription medications. Massage, acupuncture and manipulation of body part were more commonly used forms of treatment, and so natural is really the way to go in Chinese medicine. The Chinese believed that the patient's body should be viewed as an organic whole rather than as individual parts. This is why it is the preferred choice of a large number of people around the world[3].

The beginning of medical science in India started at the age of Vedic literature which is familiar with '*osadhi*' i.e. plants with medicinal properties. As time progressed, the domain of osadhi expanded at the expense of 'bhesaj' and osadhi itself transcended plants. The discipline of Ayurveda was born as an affiliate of the *Atharvaveda*[4]. Ayurveda can be defined as an ancient Indian health care system, comprising both practice and theory and devoted to a systematized quest for a long, healthy, vigorous and happy life comprising two distinct traditions: botanical and the metallurgical[5].

'*Charaka Samhita'* is the first treatise of Ayurveda in India, written by Charaka. About the same time Sushruta, an eminent surgeon of Vedic era, also wrote '*Susruta-samhita*' with more emphasis on surgery in the 4th and the 5th century[6].

Besides Ayurveda, the Unani and Siddha system of medicine was also popular in India. The

knowledge of Unani medicine came to India through Arabian and Persian and got enriched with the medicine of the sub continent and became a part of Indian systems of medicine. This system achieved great efficacy in curing diseases. Actually, the Unani and Ayurvedic medicines are contemporary systems of 400 BC, one took birth in Greek and the other one in India. Their philosophies are different but ideas can be correlated[7]. The Siddha system of medicine is one of the oldest traditional treatment system generated from Dravidian culture and flourished in the period of Indus Valley. Believed to be more than 10,000 years old, the Siddha system of medicine is considered to be one of the most antiquated traditional medical systems where use of metals, minerals and chemical products were predominant. The Siddha literature was also written in Tamil and is mainly practiced in Tamil speaking regions of the country and abroad. However, Ayurveda was prevalent in the north, Siddha was flourishing in the southern part of India[8].

Recent Perspective

The plant-based traditional medicine system, play a very essential role in human health care. Chandel *et.al,*[9] reported that nearly about 70% of Indian population depends on herbal medicines for various ailments. The information about medicinal properties of plants came down traditionally generation after generation since immemorial. The medicinal plants not only provide access and affordable medicine to poor people, but also a source of foreign exchange for developing countries. Most of herbs, shrubs and plant parts are used to prevent several human diseases. Almost all the developing countries have greater value of traditional healing treatments in these days[10,11]. According to Sahai[12], there are two levels of medicine system in India, the first one is classical well documented systems of Ayurveda, Siddha, Unani and the other one is folk medicine of system which is informal and exists in communities and passed orally from generation to generation and this folk system has survived for thousands of years because of their common use with cultural believes.

WHO[13] reported that more than 80% of the world's population relies on traditional herbal medicine for their primary health care. In almost every Asian country, there is a vast indigenous knowledge of the use of medicinal plants. However, the biodiversity of medicinal and aromatic plants is yet to be studied thoroughly in many countries. A few countries including China, India and Sri Lanka have formulated legislation to conserve their natural resources of medicinal and aromatic plants whereas some countries have ceased the practice of wild collection[14].

Area of interest

Having rich in biodiversity and with a handful of several ethnic communities (Santal,

Rajbansi, Polia, Oraon, Mundas, Malpaharias etc.), Malda and Uttar Dinajpur districts have great potential to be explored. Therefore, the present review deals with the medicinal properties of common medicinal plants which are commonly practiced in these two districts (Fig. 1) of West Bengal in India. The District of Uttar Dinajpur lies between latitude 25°11' N to 26°49' N and longitude 87°49' E to 90°00' E occupying an area of 3142 Sq. Km enclosed by Bangladesh on the East, Bihar on the west, Darjeeling & Jalpaiguri District on the north and Malda District on the south[15] whereas Malda District is located between the latitude and longitude figures of 24°40’20”N to 25°32’08”N and 88°28’10”E to 87^{0}45’50”E respectively and surrounded by Bangladesh and South Dinajpur in the east, Santal Parganas

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Figure 1: Map of Malda and Uttar dinajpur districts.

of Jharkhand state in the west, Uttar Dinajpur in the north and Murshidabad in the south. The total geographical area of this district is 3455.66 Sq Km[16].

***Culture with flora and fauna in the studied area*:**

Uttar Dinajpur is basically an agro and horticulture based city. Bengali is the main language but few portion of district consist of Urdu, Bihari and Hindi speaking people at Islampur Sub-Division showing mixture of culture. On the bank of Kulik River, the 'Raiganj Bird Sanctuary' is a major attraction of this district, spreading over an area of 35 acre and inhabited by a wide array of bird species including migratory birds such as night herons, open bill stroks, egrets, little cormorant etc. from South Asian countries and coastal regions.

Malda district shows a different type of colorful and enjoyable culture. Folk dances like, Gambhira, Jatra, Charak dance, Santhal dance, Lathi dance are most popular in this district performed by various types of ethnic communities. Besides, there are so many places of interest, such as Historical place like Gour and Jagjibanpur, Adina Deer Park, Ramkeli mela, Pandua Masjid etc. The soil of the district is particularly suited to the growth of mulberry and mango, for the production of both of which Malda has become famous, though the district is mainly agriculture based.

Literature on ethnobotany:

Previous literature on ethnobotany proved a number of immense works had been done in India. The importance of medicinal plants of India was described by Chopra *et al.*[17]. The ethnobotanical uses of plants by tribal of Madhya Pradesh were studied by Jain[18] whereas Rai *et al*.[19] described the ethnobotanical use of some areas Sikkim and Darjeeling Himalaya. S.G. Joshi[20] wrote a handbook on medicinal plants, comprising a huge data on the use of plants of India. H. Lalramnghinghlova[21] studied the ethnobotanical use of plants of Mizoram where as medicinal plants of Mysore were studied by Kshirsagar and Singh[22]. The folk usage of medicinal plants of Rajasthan was studied by Katewa *et al.*[23] and Sharma and Kumar[24] whereas Kuru[25] did the same kind of work for Tamil Nadu.

The eastern part of India particularly West Bengal has a wide array of natural vegetation which starts from the mountain of the Himalayan hills of Darjeeling in north to the unique vegetation of the coastal areas of Sundarbans in south. The Gangetic plain deals with the lush evergreen natural vegetation. During the last few decades, several works had been done with the ethnobotanical information among the different districts of West Bengal. An extensive ethnobotacinal survey of Bankura district, W.B., had been done by various workers[26-28]. De[29] reported the use of plants from Purulia District of W.B. whereas Chaudhuri *et al.*,[30-32] reported the same kind of work for 24- parganas, Midnapore and

Jalpaiguri Districts of W.B. Further survey was carried out by Molla and Roy[33] in Jalpaiguri district. Bandyopadhyay and Mukherjee[34,35] reported the floristic diversity and ethno-botanical survey of Koch Bihar District. A few workers[36-40] reported on the floristic composition and ethnomedicinal use of plants for Uttar and Dakshin Dinajpur districts.

A few floristic work had been done by Sur *et al*.[37,38] and Acharya and Das[41] for Malda district. Later, Chowdhury and Das[42] reported wet-land vegetation and some ethnomedicinal uses of plants of Malda district. Therefore, Malda and Uttar Dinajpur districts have a great potentiality to be explored.

Ethnomedicinal use

According to Schultes[43], ethnobotany is "the study of the relationship which exists between people of primitive societies and their plant environment". Ethnobotany is a new field of research and if this field is investigated thoroughly and systematically, it will yield results of great value to the ethnologists, archaeologists, anthropologists, plant-geographers and pharmacologists etc. It has been realized all over the world that much valuable knowledge about the uses of plants including medicinal uses is still endemic among many tribal or rural human societies. The traditional system of medicine not only provides cure for a large number of general and chronic diseases but it also strengthens the inner body strength. According to a survey of World Health Organization[13] and Siddiqui[44], the practitioners of traditional system of medicine treat about 80% of patients in India, 85% in Burma and 90% in Bangladesh.

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According to the workers[45-47], the Indian medicinal plants have been successfully used in management of various ailments like bronchial asthma, chronic fever, cold and cough, malaria, dysentery, convulsions, diabetes, diarrhea, arthritis, emetic syndrome, skin diseases, insect bite etc. and in treatment of gastric, hepatic, cardiovascular and immunological disorders. Therefore, now-a-days there is a trend to switch over to the old traditional healing system of medicine which is highly effective and has no adverse side effect. The present review also deals with some common medicinal plants having different values in Malda and Uttar Dinajpur Districts.

Indigenous knowledge

Indigenous knowledge is derived from interaction between people and their environment and is a characteristic feature of all cultures. Rural India, inhabited by a number of ethnic groups with their diverse cultural practices, heavily depends on traditional system of medicine as a part of their lifestyle. It can be said that the same plant, used by different ethnic communities of different region, may treat same or different ailments. But, each

ethnic community has their own traditional system of methods and uses plant species either singly or with other remedies to treat diseases. De[48] described the close association of human society with the plant life, since time immemorial and also described that, from the ancient time plants have been a primary source of medicine and have influenced culture, thought and economic activity human beings through the ages[49].

Herbarium resources

Herbarium sheets and field notes have also proved to be a good source of ethnobotanical data. These sheets have been used for various purposes including plant species identification by various workers in their study. The most outstanding example of this type of research is findings of 5,178 useful notes of drugs and food value from about 2.5 million plant specimens in Harvard University Herbarium by Dr. Altschul[50].

Medicinal plants in curing diseases

No plants could be treated as weed as because of every plant has its own story. The literature showed the ethnomedicinal use of plants along with the mode of preparation of medicine and doses. The Indian folk medicinal knowledge and ethnobotanical uses of plants had been described by Jain[51,52]. Singh and Pandey[53,54] reported the medicinal uses of plants by ethnic communities of Rajasthan, India.

Sini, *et al*.[55] reported that *Cassia occidentalis* and *Clitoria ternatea*, which are commonly found beside the roadside or railway tracks or at garden, used for general infection and root juice in headache and swelling respectively, while *Datura metel* is used in asthma, depression, motion sickness, analgesic and hallucinations. They also worked on the uses of several other medicinal plants. Mitra and Mukherjee[56] reported more than 70 plants having ethnobotanical value of West Dinajpur district, West Bengal.

Joshi[57] reported that *Acorus calamus*, (known as 'bach' in the study areas) normally found in the wet-lands, used to treat dysentery and cough while *Cuscuta reflexa* commonly called 'swarnalata' used to treat jaundice, cough and stomach disorders. Ghimire and Bastakoti[58] reported on the ethnobotanical uses of several medicinal plants. They reported that *Achyranthes aspera* cures fever, swelling and rheumatism while *Artemisia vulgaris* is used for scabies, gastric, headache.

Sesbania grandiflora, (known as 'bakphul' in those two districts) used in dysentry, fevers, headaches, rheumatism, small pox and stomatitis, anaemia, bronchitis, tumors and biliousness[59]. Goyal *et al*.[60] reported that *Asparagus racemosus*, (known as 'satamul' in those two districts) is used to cure dyspepsia, diarrhoea, inflammations, hyperdipsia, infertility and leucorrhoea whereas *Azadirachta indica* have been reported to be a good

anthelmintic and also used for cough and cold, stomach worms, remittent fever, eczema, diabetes, leprosy and several skin infections[61].

The Ayurvedic Pharmacopoeia of India[62] reported that *Gymnema sylvestre* is used as antidiabetic, antiviral, diuretic, antiallergic, hypoglycemic and hypolipidemic.

Medicinal Properties

The study of various workers[17,20,63-69] proved that different aromatic and medicinal plants are used to cure various ailments. According to Chattopadhyay and Khan[70], a large number of traditional preparations are effective over modern allopathic drugs. For example, herpes virus infections, which cannot be completely cured by the available anti-herpes drugs like nucleoside analogs but curable only by traditional preparations as mentioned by Chattopadhyay and Khan[70]. Recently, interest has been raised in many countries on the commercial extraction of medicine from plants that contribute to cure for major diseases like cancer, AIDS etc.

The medicinal plants contain large amounts of antioxidants, such as polyphenols, vitamin C, vitamin E, selenium, β-carotene, lycopene, lutein, and other carotenoids, anthocyanin, coumarin lignans, catechins and isocatechins, which play important roles in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides[71]. Therefore, there is an increasing interest in searching antioxidants from natural origin to scavenge free radicals to prevent human body from oxidative stress produced by ROS (reactive oxygen species) and RNS (reactive nitrogen species). The free radicals can lead to oxidative damage to biomolecules and may cause disorders such as cancer, diabetes, inflammatory disease, asthma, cardiovascular diseases, neurodegenerative diseases and premature aging[72]. Plant secondary metabolites such as flavonoids and terpenoids also play an important role in defense against free radicals[73]. Data from various studies indicate that medicinal plants contain a wide variety of natural antioxidants, such as phenolics, flavonoids, and tannins, which possess more potent antioxidant activity than common dietary plants.

Middleton *et al.*[74] reported that the compounds responsible for antioxidant activity can be isolated and used for prevention and treatment of free radical-related disorders whereas Aqil *et al.*[75], reported that various herbs and spices exhibit antioxidant activity, including *Ocimum sanctum*, *Piper cubeba* Linn., *Allium sativum* Linn., *Terminalia bellerica*, *Camellia sinensis* Linn., *Zingiber officinale* Roscoe etc. and also reported this antioxidant activity is due to the flavones, isoflavones and flavonoids.

Gautam[76] reported that, several plant sources, like curcumin, resveratrol, baicalein,

boswellic acid, betulinic acid, ursolic acid, and oleanolic acid which are plant in origin, are the possible drugs for the future, against inflammatory diseases. Amara, *et al.*[77] showed various edible plant extracts have promising anti-tumorigenic activity. Various anti-cancerous agents like vinblastine, vincristine, nevelbine, etoposide, teniposide, taxol, taxotere, topotecan, and irinotecan, derived from plant sources, currently being used or undergoing clinical trials[78].

Therefore, the interest has refocused on traditional medicine because of the high cost of modern drugs, time and expenditure which is necessary to bring a drug to market after appropriate clinical trials. Besides these, there are various types of serious side-effects of modern drugs due to their carcinogenicity. As a result, scientists are now taking an active interest in traditional medicinal preparations of indigenous peoples, which are mostly plant based, having naturally occurring antioxidants and simultaneously trying to replace synthetic antioxidants.

This review summarizes the ethnomedicinal uses of some common medicinal plants which are found in Malda and Uttar Dinajpur districts and their antioxidant activities. This review is comprehensive enough to be useful to find as well as assemble the medicinal properties, main chemical constituents and antioxidant activity of those plants.

Study of the antioxidant activity

Antioxidants are the substances that when present in body at low concentration compared with that of an oxidizable substrate markedly delay or prevent the oxidation of that substrate. They have also been of interest to biochemist and health professionals because they may help the body protect itself against the oxidative damage caused by reactive oxygen species (ROS) and diseases like cancer, aging etc. It can interfere with the oxidation process by reacting with free radicals, chelating, catalytic metals, and also by acting as oxygen scavengers[79].

The present review deals with a list of medicinal plants, found in Malda and Uttar Dinajpur districts, which are being used as a source of medicine since long, based on information collected from various literatures dealing with herbs having antioxidant properties.

Several methods have been used by the different workers to measure the antioxidant activity of medicinal plants. From the previous literature, the most widely used methods are the DPPH methods, super oxide radical scavenging method, scavenging of hydrogen peroxide, hydroxyl radical scavenging assay and ABTS etc. For example, Sini *et al.*[55] determined free radical scavenging assay by DPPH method and found 84.23±0.004 % and 82.87±0.246% inhibition (absorbance at 515 nm) in case of *Cassia occidentalis* and *Clitoria ternatea*

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respectively in respect of Ascorbic acid standard solution 96.540 ± 0.652 %. They also determined free radical scavenging assay by DPPH method and presence of other chemical content in plant extracts.

Rachh *et al.*[80] showed maximum antioxidant activity of *Gymnema sylvestre* (Retz.) was 57.10% at 100 µg/ml conc. among different concentration and IC_{50} value is 85.28 µg/ml. (absorbance at 517 nm) while Fazal *et al.*[81] showed the alkaloids and ash value of *Gymnema* are 1.33% and 7.90 % respectively whereas the antioxidant activity of *Curcuma longa* was determined by Sathisha *et al.*[82] with the help of DPPH method at IC_{50} value of 0.32±0.12 mg/mL while *Trigonella foenum-graecum* shows 0.81±0.21 mg/mL at IC_{50} more antioxidant activity (absorbance at 517 nm).

Phytoconstituents in plants

The active constituents in plants are the chemicals that have a medicinal effect on the body. Medicinal plants have been used for centuries as food, fooder, fertilizer and sources of chemicals for the pharmaceutical, food and chemical industries. These chemicals have been divided into several groups such as- alkaloids, flavonoids, phenols, saponins, coumarins, anthocyanins, essential oils etc. In this review, a few active constituents with structure of medicinal plants have been shown in Table 1.

Conclusion

The aim of the present review was to develop a comprehensive and accurate list of some common plants, growing in Malda and Uttar Dinajpur districts that have identified as folk medical uses, and have antioxidant properties. The medicinal plants have occupied a distinct place in the human life right from the earlier era to till date and provide the information of the usage and management of various illnesses is due to their phytochemical constituents, which are naturally occurring in plants. There are so many evidences that indigenous antioxidants may be useful in preventing of oxidative stress in body and there is an increasing interest of natural antioxidants, found in medicinal plants, spices and in herbs. This knowledge of use of therapeutic plants has started to decline and become obsolete due to lack interest by younger generations as well as rapid socioeconomic, cultural changes, overgrazing, natural calamity and deforestation results lose of important medicinal plants from their habitats. Therefore, medicinal plants should be reviewed to better understand their properties, safety and efficacy.

Hence, the present review will play a significant role not only in documentation of this worthy knowledge of these districts but also for shorting out the plant species for conservation. So, proper documentation of this cultural heritage is urgently needed for the

Table- 1. Active constituents of medicinal plants

Plant Species	Active constituents (with structure)	References
Cassia occi-dentalis L.	Emodin	83
Clitoria ter-natea L.	Pterocarpin	84
Acacia nilotica L.	Gallic acid; Protocatechuic acid	85
Pergularia daemia (Forssk.) Chiov.	ß- sitosterol; Stigmesterol	86
Datura metel L.	Atropin	87

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Plant Species	Active constituents (with structure)	References
Plumbago zeylanica L.	Plumbagin	88
Achyranthes aspera L.	Oleanic acid	89
Asparagus racemosus Willd.	Aspargine	90
Azadirachta indica A. Juss	Nimbidin; Azadirachtin	91
Mentha arvensis L.	Menthol; Menthone	92
Ocimum sanctum L.	Luteolin; Ocimarin	93

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Plant Species	Active constituents (with structure)	References
Curcuma longa L.	Curcumin	94
Justicia adha-toda L.	Vasicine Adhatodine	95

forthcoming future as well as it can generate new ideas in the field of modern drug development, which can be very useful in medical biology. So, further pharmacological and biochemical investigations are required to screen out some more effective user friendly potential medicine for human welfare. It is hoped that this review will serve as an encouragement to all the researchers, involved in this field.

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Chapter 11

Antibiotic sensitivity, heavy metal tolerance and carbohydrate fermentation profile of coliforms isolated from lakes of Darjeeling

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Abstract

Twenty coliform (Enterobacteriaceae) strains isolated from three different lakes of Darjeeling Hills were assessed for antibiotic sensitivity, heavy metal tolerance and carbohydrate fermentation pattern. All the isolates were identified as *Escherichia coli* by comparing their properties referred in Bergey's Manual of Systematic Bacteriology. All the isolates were resistant to ampicillin and sensitive to six of the eight antibiotics tested. Three of the isolates were also resistant to Co-trimoxazole at tested concentration (25 µg/ml). Considerable tolerances to heavy metals (Hg^{++}, Cd^{++} and Cu^{++}) were also observed, MIC values indicative of metal tolerance were 54.3 µg/ml for Hg^{++}, 250 µg/ml for Cd^{++} and 1700 µg/ml for Cu^{++}. Carbohydrate fermentation pattern showed 90% homology to that of *E. coli*. The results indicate that lake waters of Darjeeling hills are subjected to heavy metal stress and microbiologically unsafe for use.

Keywords

Antibiotic, heavy metal, carbohydrate fermentation, coliforms, lake water, Darjeeling

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Introduction

Fresh water is indispensable for the existence of life on earth. Scarce availability of fresh water on the surface of the earth is becoming polluted by various activities. Water pollution can range from purely natural to several man-made sources. The effects of water pollution can broadly be classified into physico-chemical, biological, toxic and pathogenic[1].

Presence of chemicals like heavy metals, biocides, cyanide etc. make water toxic for aquatic life as well as human health. They (especially non-biodegradable) accumulate in body of organisms and biomagnify along the tropic levels causing long term effects[1].

Heavy metals are available in the water in small quantities and are further added due to soil erosion and leaching of minerals. Insecticides and heavy metals, known to cause widespread environmental contamination, are the object of study on account of their toxicity and ubiquity, moreover they are known to remain stable in the aquatic environment[2,3]. However, in the recent past, freshwater pollution by heavy metals has become a hazard due to discharge of industrial effluents[4]. The classical cadmium poisoning is associated with urea protein .

Besides, few wastes like sewage also contain several pathogenic and non pathogenic microorganisms, protozoa and viruses. Consumption of polluted water imposes serious threat to the health, economy and the environment[1].

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Antimicrobial resistance has become an important problem worldwide[5]. Bacterial resistance to antimicrobial agents has been emerging and rapidly disseminating among many nosocomial and community acquired pathogens[6]. Sensitivity patterns and treatment must be guided by laboratory investigations[7]. The development of antibiotic resistance in *E. coli* has important clinical implications. The development of resistance to older agents such as ampicillin and trimethoprim-sulfamethoxazole, as well as the emerging problem of fluoroquinolone resistance, may substantially limit our antibiotic choices[8,9]. Studied antibiotic resistance profiles and relatedness of enteric bacterial pathogens isolated from HIV/AIDS patients with and without diarrhoea and their household drinking water in rural communities in Limpopo Province, South Africa and found that about 50% of *E. coli* isolated from the various study cohorts showed multiple antibiotic resistance to penicillin, amoxicillin, ampicillin, erythromycin, tetracycline, doxycycline and cotri-moxazole, whereas less than 10% resistance was consistently reported for ofloxacin, gentamicin, meropenem cefotaxime, cefuroxime and imipenem.

Keeping in view, the aforesaid facts, this study was taken up with the objectives to determine the antibiotic susceptibility pattern, heavy metal tolerance range and carbohydrate

fermentation homology of the twenty *E. coli* isolates collected from the three lakes of Darjeeling.

Materials and methods

Bacterial isolates

In total 20 *E. coli* isolates were collected from three lakes *viz*. Mirik lake, Jorepokhari lake and Nakhapani lake of Darjeeling following standard methods[10]. They were identified by morphological, Gram Staining and biochemical characters based on Bergey's Manual of Determinative Bacteriology (1994).

Antimicrobial sensitivity pattern

Antibiotic sensitivity pattern of isolated *E. coli* strains to eight different antibiotics was determined by the Kirby-Bauer disc diffusion method[11] using Octodisc (HIMedia).The antibiotic discs and their concentrations per disc included: chloramphenicol (C 30µg), ampicillin (A 10µg), tetracycline (T 30µg), gentamycin (G 10µg), kanamycin (K 30µg), Co-Trimoxazole (Co 25µg), amikacin (Ak 30µg) and streptomycin (S 25µg). Inhibition zones were measured using HiMedia HiAntibiotic ZoneScale™-C.

Heavy metal tolerance pattern

All isolates were also tested to determine the minimal inhibitory concentrations (MICs) of three metals *viz*. Hg^{2+}, Cd^{2+} and Cu^{2+}[12]. The experimental tubes were prepared by supplementing Muller-Hinton medium with metal salts for cationic concentrations of 2.7, 13.57, 27.15, 54.3 mg/ml for Hg^{2+}; 12.5, 25, 50, 100, 200, 400, 800 mg/ml for Cd^{2+}; and 100, 200, 400, 800, 1600, 3200, 6400 mg/ml for Cu^{2+}. The compounds used were $CuSO_4$, $CdCl_2$, and $HgCl_2$. One milliliter of the test organism suspension (1×10^6 CFU/ml) was added to each tube. The tubes were incubated for 18 h at 37^0C and visual turbidity is noted at 540nm in a spectrophotometer (AIMIL, INDIA). Minimal Inhibitory Concentration (MIC) of heavy metals was determined by tube dilution method.

Carbohydrate fermentation pattern

Carbohydrate fermentation pattern was determined by taking 15 sugars. The sugars included: adonitol, dulcitol, L-rhamnose, D-raffinose, meso-inositol, D-mannose, starch, maltose, D-xylose, D -sorbitol, sucrose, dextrose, D-trehalose, Lactose, D-galactose, following the procedure described in HiMedia Manual (1998).

Results and discussion

Results presented showed the isolates showed multiple resistance to ampicillin, tetracycline and co-trmoxazole. Similar result observed by Obi *et al.*[9]. All the isolates were resistant to

Table 1A: Antibiotic sensitivity pattern of twenty selected isolates of *E. coli*

	E. coli* isolates **Diameter of inhibition zone (mm)**									
Antibiotics Discs	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Chloramphenicol (C 30µg)	18	18	19	18	23	20	20	20	20	18
Ampicillin (A 10µg)	0	0	0	0	0	0	0	0	0	0
Tetracycline (T 30µg)	10	25	11	12	27	17	15	17	20	20
Gentamycin (G 10µg)	18	26	24	20	24	14	13	15	13	20
Kanamycin (K 30µg)	17	26	23	20	25	15	20	12	15	18
Co-Trimoxazole (Co 25µg)	0	26	0	0	0	12	0	20	13	20
Amikacin (Ak 30µg)	20	26	26	24	27	16	18	20	15	20
Streptomycin (S 25µg)	15	20	19	16	22	20	20	20	20	20

Table 1B: Antibiotic sensitivity pattern of twenty selected isolates of *E. coli*

	E. coli* isolates **Diameter of inhibition zone (mm)**									
***Antibiotic Discs**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Chloramphenicol (C 30µg)	21	21	21	21	21	20	18	20	18	18
Ampicillin (A 10µg)	0	0	0	0	0	0	0	0	0	0
Tetracycline (T 30µg)	19	21	20	19	10	0	0	0	0	20
Gentamycin (G 10µg)	20	19	21	19	18	20	20	20	20	20
Kanamycin (K 30µg)	19	20	21	18	19	20	20	20	20	20
Co-Trimoxazole (Co 25µg)	25	25	25	23	0	18	0	20	8	20
Amikacin (Ak 30µg)	22	21	22	22	22	20	20	20	20	20
Streptomycin (S 25µg)	20	22	20	21	22	10	15	15	13	15

*1= ECL1, 2=ECL2, 3=ECL3, 4= ECL4, 5=ECL5, 6=ECL6, 7=ECL7, 8=ECL8, 9=ECL10, 10=ECL10, 11=ECL11, 12=ECL12, 13=ECL13, 14=ECL14, 15=ECL15, 16=ECL16, 17=ECL17, 18=ECL18, 19=ECL19, ECL20
*C=Chloramphenicol, A=Ampicillin, T=Tetracycline, G=Gentamicin, K=Kanamycin, Co=Cotrimoxazole, Ak=Amikacin, S=Streptomycin

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Table 2: Carbohydrate fermentation pattern of *E. coli* isolates

Sugars tested	*E. coli* isolates*																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
D-Galactose	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	B	B	B	B	B
D-Sorbitol	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
Adonitol	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
D-Xylose	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
Maltose	B	B	B	B	B	B	G	B	B	B	B	B	B	B	B	B	G	B	B	B
D-Trehalose	B	B	B	A	B	B	B	B	B	B	B	B	B	A	B	B	B	B	B	B
Dextrose	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	G	B	B	B	B
Lactose	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
Starch	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Meso-Inositol	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Sucrose	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
D-Mannose	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	B	B
D-Raffinose	--	--	A	A	B	A	B	B	B	B	--	A	A	A	A	B	B	B	B	B
Dulcitol	A	B	A	A	--	B	B	B	B	B	B	A	A	B	--	B	B	B	B	B
Rhamnosee	B	B	G	G	G	B	B	B	B	B	A	A	A	--	A	A	--	A	A	B

A = Only Acid produced; G = Only Gas produced; B= Both Acid and Gas produced; -- = Acid and Gas both not produced. *1= ECL1, 2=ECL2, 3=ECL3, 4= ECL4, 5=ECL5, 6=ECL6, 7=ECL7, 8=ECL8, 9=ECL10, 10=ECL10, 11=ECL11, 12=ECL12, 13=ECL13, 14=ECL14, 15=ECL15, 16=ECL16, 17=ECL17, 18=ECL18, 19=ECL19, ECL20

ampicillin and sensitive to six of the eight antibiotics tested. Nine isolates were resistant to Co-trimoxazole at tested concentration of 25 µg /ml. The highest rate of resistance was observed against ampicillin, same result are observed[13,14]. This may be due to the indiscriminate use of inexpensive antibiotics in our country, India[14]. The resistance to ampicillin may be due to production of beta-lactamase enzymes and the most common mechanism of for resistance to cotrimoxazole is acquisition of plasmid mediated, variant diaminopyrimidine folate reductase enzymes.

Antibiotic susceptibility profile of microorganisms vary from country to country, province to province, town to town, and hospital to hospital in the same town as well as between private and public healthcare facilities in the same area[15]. In addition, different pathologies may alter antibiotic sensitivity patterns, consequently, periodic evaluation of antibiotic susceptibility is recommended to guide management of patients requiring antibiotic treatment[9]. It is speculated that the widespread use of antibiotics may create pressure

that encourages the selection of multi-drug resistance among bacteria[16-18].

All the isolates exhibited multiple tolerance levels to heavy metals tested. MIC values indicative of metal tolerance were 54.3 µg/ ml for Hg^{++}, 250 µg/ ml for Cd^{++} and 1700 µg/ ml for Cu^{++}. Positive correlations between tolerance to high levels of Cu^{2+}, Pb^{2+}, Zn^{2+} and multi-resistant bacteria isolated from drinking water have previously been reported[19]. Ghosh *et al.*[20] have also reported on transferable plasmids encoding resistance to various heavy metals and antibiotics of *Salmonella abortus equi*.

Among the 15 sugars tested adonitol, meso-inositol, and starch were not fermented by all the isolates which is commonly observed in *E. coli.* (table 2). Acid and gas from glucose, fructose, galactose, lactose, maltose, arabinose, xylose, rhamnose and mannitol; sucrose, raffinose, salicin, esculin, dulcitol and glycerol may or may not be fermented; inulin, pectin and adonitol rarely fermented; dextrin, starch, glycogen and inositol not fermented (Bergey's manual, 1994). Gonzatez *et al.*[21] observed two fermentation patterns in enterotoxigenic *Escherichia coli* strains of serotype O153:H45 isolated in Spain, as rhamnose fermenters (four strains) and rhamnose non-fermenters (five strains) and the ability to ferment rhamnose was the only differential characteristic found among 49 carbohydrate fermentation tests used to establish fermentation patterns[21].

Conclusion

From the study it can be concluded that though several antibiotics are active against the isolates, periodic monitoring of antibiotic sensitivity is imperative to detect any changing patterns. The multiple resistance of isolates to some antibiotic classes are of great public health concern and calls for caution in the indiscriminate use of antibiotics on humans. The study of the isolates also indicate that the water of the three lakes are subjected to heavy metal stress and are microbiologically unsafe for domestic use and drinking.

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Chapter 12

Screening of antioxidant activity of lichens *Everniastrum* sp. and *Cladonia* sp. from Darjeeling hills

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Abstract

Oxidative stress can lead to the production of free radicals which may cause many degenerative diseases. These free radicals can be eliminated with the help of antioxidants which may be of a natural origin. The aim of this study was to examine the antioxidant activity of two common lichens namely *Everniastrum* sp. and *Cladonia* sp. from Darjeeling hills. The antioxidant assay of different concentration of ethanolic and methanolic extracts of lichens was determined by means of four techniques viz., DPPH radical scavenging activity, total antioxidant activity, reducing power ability and determination of phenolic content. The DPPH radical scavenging activity ranged from 34 to 51%, for reducing power measured values of absorbance varied from 0.442 to 0.863. In addition, contents of total phenolic compounds and total antioxidants were appreciable. Tested lichen species were found to possess effective antioxidant activities and can be used as good natural sources of antioxidants

Keywords

Antioxidant, DPPH, phenolics, *Everniastrum*, *Cladonia*, Darjeeling

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Introduction

Free radicals which have one or more unpaired electrons are produced in normal or pathological cell metabolism. Reactive oxygen species (ROS) react easily with free radicals to become radicals themselves. ROS are various forms of activated oxygen, which include free radicals such as superoxide anion radicals (O^-) and hydroxyl radicals (OH^-), as well as non-free radical species (H_2O_2) and the singlet oxygen ($^1O_2$)[1-4]. Also, excessive generation of ROS, induced by various stimuli and which exceed the antioxidant capacity of the organism, leads to a variety of pathophysiological processes such as inflammation, diabetes, genotoxicity, and cancer[5-7]. Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents, and pesticides[3,8-10]. The most effective way to eliminate free radicals which cause the oxidative stress is with the help of antioxidants.

Antioxidants, both exogenous and endogenous, whether synthetic or natural, can be effective in preventing free radical formation by scavenging and suppressing such disorders[11, 12-14]. Currently, there is a growing interest toward natural antioxidants of herbal resources[10-12]. Epidemiological and *in vitro* studies on medicinal plants and vegetables strongly supported this idea that plant constituents with antioxidant activity are capable of exerting protective effects against oxidative stress in biological systems[13-16].

Lichens are the symbiotic organisms including a fungal partner and an algal partner and are known to have therapeutic effects on various diseases in folk medicine of many countries. Recently, much attention has been paid to lichens as resources of natural antioxidants. Scientist already investigated the antioxidant activity of some species of lichens, such as *Bryoria fuscescens, Cetraria islandica, Dermatocarpon intestiniformis, Parmelia saxatilis, Peltigera rufescens, Platismatia glauca, Ramalina pollinaria, R. polymorph, Umbilicaria nylanderiana, Usnea ghattenis, and U. longissima* and some of them have very good antioxidant activity [17-21].

India is a rich centre of lichen diversity, contributing nearly 15% of the 13,500 species of lichens so far recorded worldwide[18]. The Darjeeling Hills also known as the ‘Queen of Hills’ is rich in flora and fauna. Lichens occur in abundance in Darjeeling Hills of West Bengal, India since it is less polluted. Different types of lichens are recorded in and around Darjeeling hills. However little study has been attempted to evaluate *in vitro* antioxidant activity of *Everniastrum* sp. and *Cladonia* sp. Hence this work was set out in order to screen the antioxidant activity of two lichen species namely *Everniastrum* sp. and *Cladonia* sp. of Darjeeling hills.

Materials and methods

Collection and identification of lichen materials

The collection of lichens have been made from different areas of Darjeeling district, namely Mirik with altitude of 1495m, Sukia pokhari (3638m) and Darjeeling town (2280m). Darjeeling is situated at coordinates 27˚13' N to 26˚27' N, and 88˚53 " E to 87˚58' E. It has an area of 3,149 sq km. Its annual mean maximum temperature is 14.9˚C.and annual mean minimum temperature is 8.9˚C.Average annual rainfall is 3092mm.

Mirik is located at coordinates 26°9' N 88°17'E. It has an average elevation of 1,495 m (4,905 ft). The highest point is Boker Monastery at about 1,768 m (5,801 ft). Mirik Lake at about 1,494 m (4,902 ft) is the lowest point.

The lichen specimens was collected from the wild and characterised with the help of their morphology, anatomy, colour reaction, thin layer chromatography, etc. and identified with the help of guidelines described in the communication of British Lichen Society, the Key to Macrolichens[22] and the Key to Microlichens[23,24]. Specimen samples is preserved in the herbarium of Darjeeling Government College, Darjeeling.

Extract preparation

Lichen specimen were air dried at room temperature to constant weights. The dried plant materials were ground separately to powder. One hundred gram of each plant material were shaken separately in methanol and ethanol for 48 hours in shaker. Each extract was filtered with Whatman No. 1 filter paper. Each filtrate was concentrated to dryness in open air.

Chemicals used

1,1-Diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid alumunium nitrate, potassium acetate, quercetin, Folin-Ciocalteus's phenol reagent, sodium carbonate, tannic acid, potassium ferricyanide, phosphate buffer, trichloroacetic acid, ferric chloride, butylated hydroxytoluene (BHT), sodium phosphate, ammonium molybdate α-tocopherol were obtained from Himedia laboratories, Mumbai, India. All the chemicals used including the solvents, were of analytical grade.

Determination of total phenolics

Using modified Folin-Ciocalteu method[25] total phenol contents in the extracts were determined. An aliquot of the extract was mixed with 5 ml Folin-Ciocalteu reagent (previously diluted with water at 1:10 v/v) and 4 ml (75 g/l) of sodium carbonate. The tubes were vortexed for 15 sec and allowed to stand for 30 min at 40ºC for color development. Absorbance was then measured at 765nm using the UV-1700 PharmaSpec UV-VS

Spectrophotometer, Shimadzu, Japan. Samples of extracts were evaluated at a final concentration of 0.1 mg/ml. Total phenolic content were expressed as mg/g tannic acid equivalent using the following equation based on the calibration curve: y = 0.0.008x, R^2= 0.928, where x was the absorbance and y was the tannic acid equivalent (mg/g).

DPPH radical scavenging assay

The effect of the extracts on DPPH radical was estimated using the method of Liyana-Pathiranan and Shahidi[26]. A solution of 0.135 mM DPPH in methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of extract in methanol containing 0.02–0.1 mg of the extract. The reaction mixture left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid was used as reference. The ability to scavenge DPPH radical was calculated by the following equation: DPPH radical scavenging activity (%) = [($Abs_{control}$ – Abs_{sample})]/ ($Abs_{control}$) x 100 where $Abs_{control}$ is the absorbance of DPPH radical + methanol or ethanol; Abs_{sample} is the absorbance of DPPH radical + sample extract /standard.

Evaluation of total antioxidant capacity

The total antioxidant capacity of lichen extracts, and its different fractions was evaluated by the method of Prieto *et al.*[24]. An aliquot of 0.1 ml of sample (100 µg) solution was combined with 1 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95^0 C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank in UV-1700 PharmaSpec UV-VS Spectrophotometer, Shimadzu, Japan. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. For samples of unknown composition, antioxidant capacity was expressed as equivalents of α-tocopherol (mg/g).

Determination of Ferric reducing antioxidant power (FRAP)

Reducing power of both extracts of the lichen specimens were measured by method of Oyaizu's. According to this method the reduction of Fe^{3+} to Fe^{2+} was determined by measuring absorbance of the Perl's Prussian blue complex. This method is based on the reduction of (Fe^{3+}) ferricyanide in stoichiometric excess relative to the antioxidants. For this purpose, different concentrations (25,50,100 and 200 mg/ml) of lichen extracts in ethanol and methanol, and standard antioxidants (BHT) was added to the each tube, volumes were adjusted with distilled water to 0.75 ml, separately. Then, they were mixed with 1 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 1 ml (1%) of potassium ferricyanide [$K_3Fe(CN)_6$].

The mixtures were incubated at 50°C for 20 min. After 20 min of incubation, the reaction mixtures were acidified with 1 ml of trichloroacetic acid (10%). Finally, 0.25 ml of $FeCl_3$ (0.1%) was added to this solution. Distilled water was used as blank and for control. Absorbance of these mixture were measured at 700 nm using a UV spectrophotometer[27]. Decreased absorbance indicates ferric reducing power capability of sample[27].

Results and discussion

Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS[28]. The synthetic antioxidants like BHA, BHT, gallic acid esters etc., have been suspected to cause or prompt negative health effects. Strong restrictions have been placed on their application[29,30]. In recent years much attention has been devoted to natural antioxidant and their association with health benefits[31]. The activity of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging[32]. Numerous antioxidant methods and modifications have been proposed to evaluate antioxidant activity and to explain how antioxidants function. Of these, total antioxidant activity, reducing power, DPPH assay, metal chelating, ROS quenching assays are commonly used for evaluation of antioxidant activities of extracts [33,34].

DPPH radical scavenging assay

The change in absorbance produced by reduced DPPH was used to evaluate the ability of test compounds to act as free radical scavengers. It was observed (fig.1) that methanolic extracts of *Everniastrum* sp. and *Cladonia* sp. showed potent radical scavenging activity with IC_{50} value of 4.7 µg/ml and 4.4 µg/ml respectively. Removal of free radical increased by 34 to 51 % in accordance with the increase of the concentrations of the extract from 25µg/ml to 200µg/ml, compared to the negative control and moreover, the scavenging ability of the extract was as moderately less as that of ascorbic acid at all the concentrations tested. Significant correlation was found between the free radical scavenging activity and the concentration of lichen extract or the compounds used as positive controls. The DPPH assay is a widely used method to evaluate antioxidant activities in a relatively short time compared to other methods[35]. The reduction capability on the DPPH radical is determined by the decrease in its absorbance at 517 nm. The decrease in absorbance of DPPH radical caused by antioxidants is due to the reaction between antioxidant molecules and radical, which results in the scavenging of the radical by hydrogen donation. This is visualized as a discoloration from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate

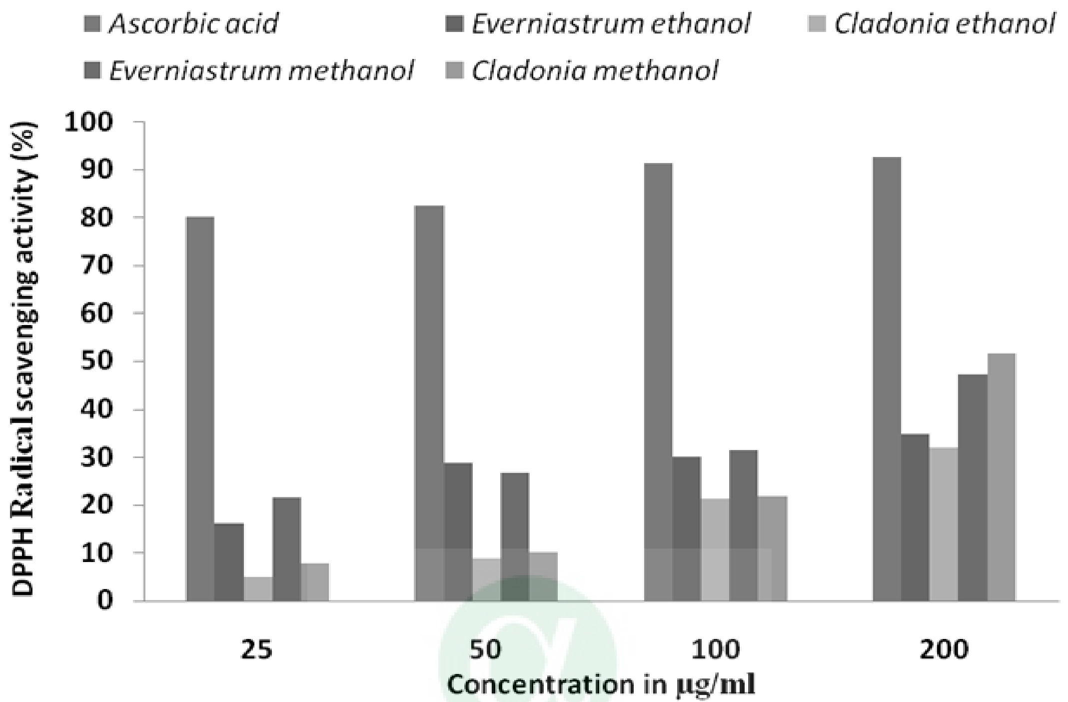

Fig.1: DPPH radical scavenging of the ethanol and methanol extracts of the lichen *Everniastrum* sp. *and Cladonia* sp.

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antioxidant activity[36]. In this study, a significant decrease in the concentration of DPPH radical due to the scavenging ability of the extract was observed. Though the DPPH radical scavenging abilities of the extract was less than that of standard, the study showed that the extract has proton-donating ability and could serve as a natural antioxidant.

Ferric reducing antioxidant power

Reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Antioxidant compounds are able to donate electrons to reactive radicals, reducing them into more stable and unreactive species[20,21]. The reducing power of the extracts was compared with standard BHA and it increased with increasing concentration of the extracts (fig. 2). The ethanolic extracts of *Everniastrum* sp. showed high reducing ability with absorbance of 0.610 at concentration of 200µg/ml and absorbance of *Cladonia* sp. was higher as 0.863 at 200µg/ml Antioxidant compounds cause the reduction of ferric (Fe^{3+}) form to the ferrous (Fe^{2+}) form because of their reductive capabilities. Prussian blue coloured complex formed by adding $FeCl_3$ to the ferrous (Fe^{2+})

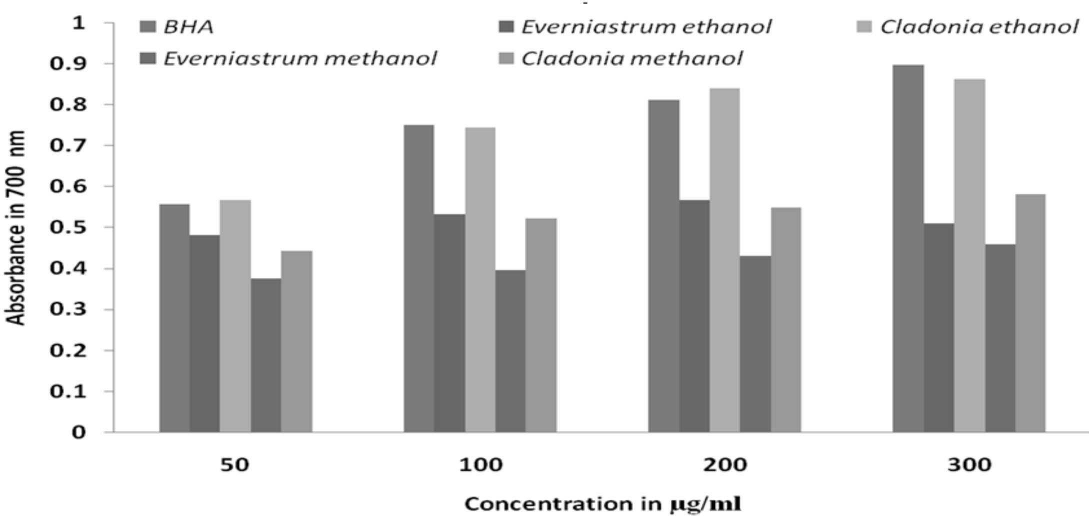

Fig 2: Reducing power of the ethanol and methanol extracts of lichen *Everniastrum and Cladonia* sp.

form. Therefore, reduction can be determined by measuring the formation of Perl's Prussian blue at 700 nm[22,23]. In this assay, yellow colour of the test solution changes to green or blue colour depending on the reducing power of antioxidant samples. A higher absorbance indicates a higher ferric reducing power

Total phenolic content

Results obtained in the present study revealed that the level of phenolic compounds in the methanol extracts of the *Cladonia* sp. and *Everniastrum* sp. were considerable (fig. 3). Polyphenolic compounds are known to have antioxidant activity and it is likely that the activity of the extracts is due to these compounds[31,32]. This activity is believed to be mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides[33]. In fact, many medicinal plants contain large amounts of antioxidants such as polyphenols. Many of these phytochemicals possess significant antioxidant capacities that are associated with lower occurrence and lower mortality rates of several human diseases[34-36]. Phenols are very important constituents because of their scavenging ability due to their hydroxyl groups[37-39].

Total antioxidant activity

The phosphomolybdenum method has been used to investigate the total antioxidant capacity of the extracts. This method is quantitative, since the total antioxidant capacity is expressed as α- tocopherol equivalents. The ethanolic and methanolic extracts of *Everniastrum*

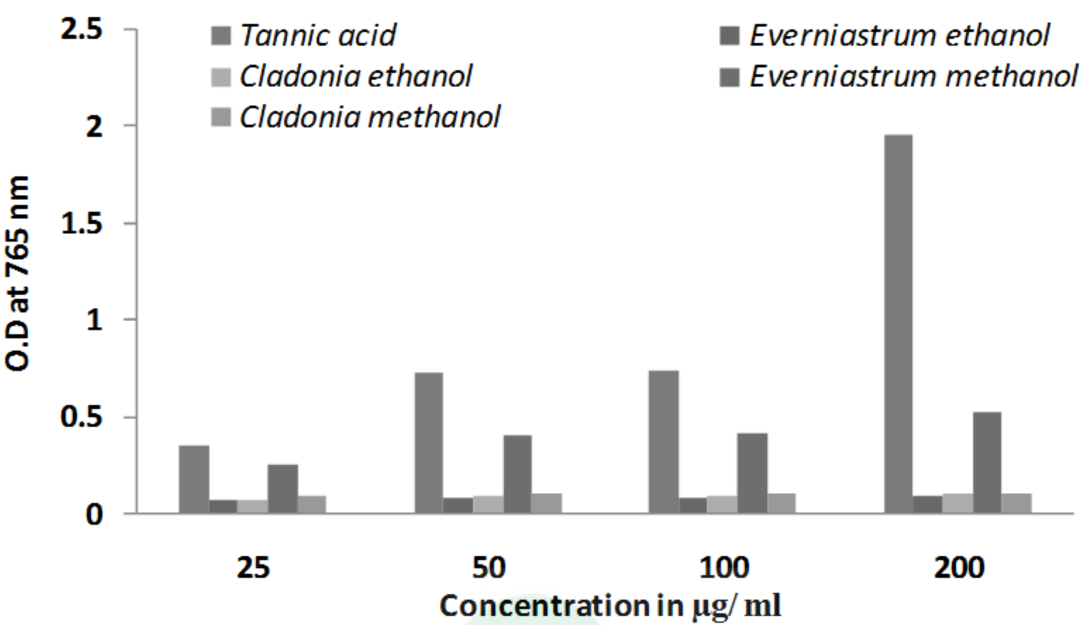

Fig 3: Total phenolic content of lichen extracts

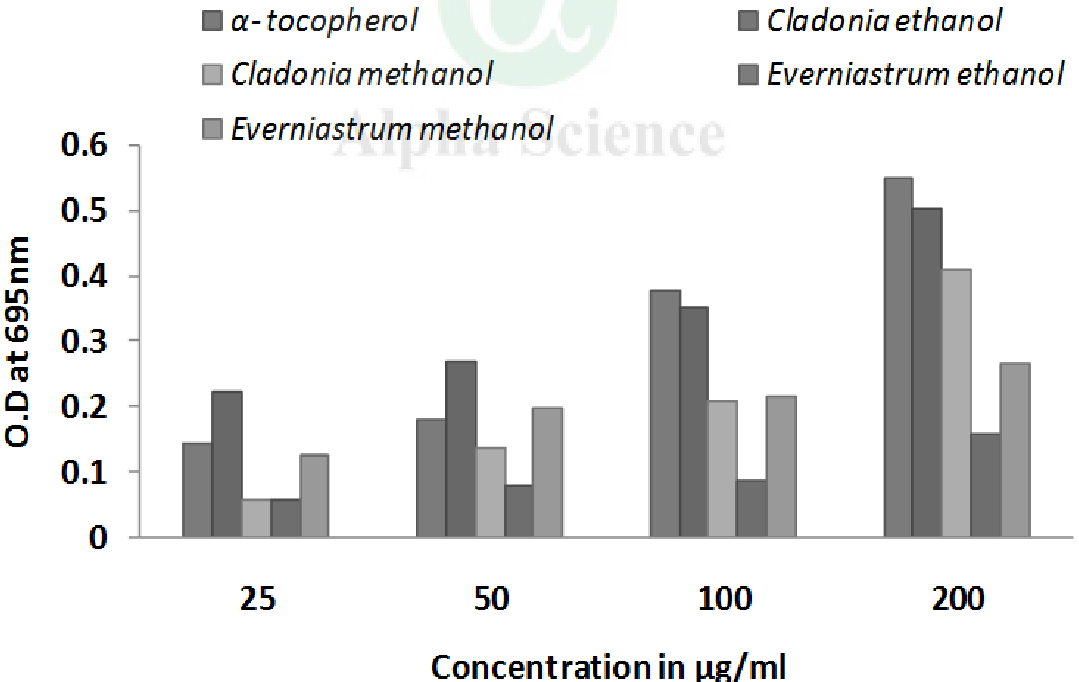

Fig 4: Total antioxidant activity of lichen extracts

contained 0.00831 and 0.00853μg vitamin E equivalent /mg and that of *Cladoina* is 0.008452 and 0.00826μg equivalent /mg respectively.

Conclusion

The investigation for bioactive compounds from natural resources to improve pharmaceutical, cosmetic and agriculture applications is extremely advancing till date. The lichen compounds are also simultaneously investigated. There is an increase in demand for drugs of natural origin for healing several diseases. Peroxidation (auto-oxidation) of lipids exposed to oxygen is responsible not only for deterioration of foods (rancidity) but also damage to tissue *in vivo*, where it may be a cause of cancer, inflammatory disease, ageing etc.

On the basis of the results, it can be concluded that tested lichen extracts show a strong antioxidant activity *in vitro*. The intensity of antioxidant activity depended on the tested lichen species and the solvent that used for extraction. Different antioxidant activity of different solvents depend on their different capabilities to extract bioactive substances[40]. Ethanolic and methanolic extracts of *Everniastrum* sp. and *Cladonia* sp. possessed moderate potent antioxidant activity and DPPH radical scavenging activity. Presence of an appreciable amount of phenol could suggest for the use of these extracts as natural source of antioxidants. Further the bioactive substances from these samples are under investigation.

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Chapter 13

Removal of cadmium from aqueous solutions with *Sargassum* sp.

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Abstract

The release of large quantities of heavy metals into the natural environment has resulted in a number of environmental problems. Cadmium is widely used and extremely toxic at relatively low dosages. The main anthropogenic pathway through which cadmium (II) enters the environment is via wastes from industrial process. As heavy metals cannot be destroyed in the natural environment, technologies can remove and recover the heavy metals from the wastewater. At present, a number of technologies, such as chemical precipitation, evaporation, electroplating, adsorption and ion exchange processes, are used for the treatment of heavy metal- containing wastewater streams. However, these technologies are expensive and not effective. Biosorption of heavy metals is an emerging technology for the treatment of industrial wastewater. In this study, biomass of *Sargassum* sp. was used for the removal of cadmium from aqueous solutions. The influence of pH, temperature, biomass size and agitation were evaluated. The result indicates that the maximum uptake were observed in pH5, temperature 30°C, agitation 150 and biomass size 0.5mg/g. The maximum metal uptakes were 27.24mg/g, 27.20mg/g, 31.30mg/g and 22.22 mg/g respectively. Freundlich model gave a better fit to the experimental data in comparison with the other isotherm models. The kinetic model fitted best with the pseudo-first order.

Keywords

Sargassum, cadmium, heavy metals, biosoption

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Introduction

India's environment is becoming fragile and environmental pollution is one of the undesirable side effects of industrialization, urbanization, population growth and unconscious attitude towards the environment[1]. At present, environmental protection is the main need of the society. In general, sewage effluents from industries and municipal origin contain appreciable amounts of plants nutrients and variable amount of metallic cations like Zn, Cu, Fe, Mn, Pb, Ni, Cd, etc[2]. Long-term irrigation with such effluents increases organic carbon content and heavy metals accumulation in soils and the chances of their entrance in food chain, and this ultimately causes significantly health concern. Thus, it becomes necessary to study the composition of sewage waters and heavy metals accumulation, with the help of advance techniques.

Heavy metal ions present in waste waters are released by various industries such as mining, electroplating, electronic equipment, battery- manufacturing processes, etc.[3]. Biosorption is an emerging and attractive technology which involves the sorption of dissolved substances by the biomaterial. It is a potential technique used for the removal of toxic heavy metals from the solutions and the recovery of precious metals[4]. The major advantages of biosorption over conventional-treatment methods include low cost, high efficiency of metal removal from the dilute solutions, minimization of chemical and/or biological sludge, no additional nutrient requirement, regeneration of biosorbents and the possibility of metal recovery.

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Biomass of brown marine macro algae is a biological resource that is available in large quantities and can form a good base for the development of biosorbent material[5]. Metal ion uptake by biomass is believed to occur through interactions with the cell walls. This is due to the presence of various functional groups such as carboxyl, amino, sulphate and hydroxyl groups, which can act as binding agents, include ionic interactions and complex formation between metal cations and ligands on the surface of the seaweeds[6]. The objective of the present work was to evaluate the influence of different parameters such as pH, temperature, biomass size and agitation on the metal adsorption. The work also considered the isotherm and kinetic studies on the adsorption of cadmium using the biomass *Sargassum* sp.

Materials and methods

Biomass preparation

The biomass used in the present study was the brown seaweed *Sargassum* sp., collected from the rocky seashores near Uvari, Tirunelveli District, Tamilnadu, India. After harvesting from sea, the samples were washed in distilled water to remove particulate

materials and salts from the surface. They were then dried in an oven at 60°c for 24hrs and then used as biosorbent for further studies[7].

Metal solution

Analytical grade $CdCl_2$ was dissolved in distilled water in order to obtain a solution containing chromium. This solution was used for metal biosorption experiments. Concentrations of standard and process solutions were evaluated by Atomic Absorption Spectrometry.

Batch mode adsorption studies

Batch mode adsorption studies for metal compounds were carried out to investigate the effect of different parameters such as Agitation, pH, Temperature and Biomass size. Solution containing adsorbate and adsorbent was taken in 250ml capacity beakers and agitated at 150 rpm in a mechanical shaker at predetermined time intervals. The adsorbate was decanted and separated from the adsorbent using whatman No.1 filter paper. Metal free and biosorbent free blanks were used as control. The residual ion concentration in the solution was analyzed using Atomic Absorption Spectrometry.

Adsorption isotherm models

Langmuir isotherm

The Langmuir isotherm is used to obtain the maximum adsorption capacity produced from complete monolayer coverage of adsorbent surface. The Langmuir's equation is given by Eq (1).

$$\theta = \frac{Q_e}{Q_m} = \frac{bC_e}{1 + bC_e} \quad \text{..........(1)}$$

Freundlich isotherm

For adsorption from solution, the Freundlich isotherm is expressed as given by Eq (2).

Sl. No	Parameters	Optimized parameters	Metal uptake (mg/g)
1	pH	5	27.24
2	Temp (ºC)	30	27.20
3	Rpm	150	31.30
4	Size (mg)	0.5	22.22

Table 1: Maximum metal uptake in different parameters

Pseudo First Order		Pseudo second Order		
k^2	R^2	k^2	h	R^2
0.1150	0.982	0.0010	7.29	0.862

Table 2- pseudo first – order and second- order kinetic models for cadmium

$$q_e = K_f C_e n_F \quad \text{...(2)}$$

Redlich – Peterson isotherm

Redlich – Peterson Isotherm can be described by Eq (3).

$$q_e = \frac{AC_e}{1 + BC_e g} \quad \text{...(3)}$$

Tempkin isotherm

The nonlinear form of Tempkin equation is given by Eq (4).

Table 3: Isotherm model parameters for the metal Cadmium

Langmuir model				**Freundlich model**			
pH	**q_{max}(mg/g)**	**b(L/mg)**	**R^2**	**pH**	**Kf (L/g)**	**nF**	**R^2**
2	**26.31579**	0.002676	0.973	2	0.180717	1.410437	0.989
3	37.03704	0.00284	0.925	3	1.516062	0.709	0.951
4	31.25	0.001758	0.941	4	0.089743	1.410437	0.975
5	32.25806	0.008566	0.961	5	1.229541	0.709	0.982
6	16.94915	0.003561	0.925	6	16.96448	1.410437	0.917
7	12.19512	0.003494	0.851	7	0.212814	0.709	0.94
8	27.02703	0.004401	0.908	8	1.632352	1.410437	0.959
Temp	**q_{max}(mg/g)**	**b(L/mg)**	**R^2**	**Temp**	**K_F (L/g)**	**n_F**	**R^2**
20	27.02703	0.003445	0.92	20	0.234963	1.472754	0.949
30	**38.46154**	0.004175	0.895	30	0.369828	1.422475	0.949
40	**22.22222**	0.003158	0.979	40	0.199526	1.477105	0.98
50	**32.25806**	0.001641	0.824	50	0.105925	1.261034	0.971
Size	**q_{max}(mg/g)**	**b(L/mg)**	**R^2**	**Size**	**K_F (L/g)**	**n_F**	**R^2**
0.1	19.23077	0.003428	0.955	0.1	0.130617	1.349528	0.956
0.2	37.03704	0.00134	0.905	0.2	0.076033	1.172333	0.983
0.3	31.25	0.001763	0.8	0.3	0.406443	1.893939	1
0.4	18.86792	0.002484	0.885	0.4	0.152055	1.488095	0.988
0.5	27.77778	0.004063	0.979	0.5	0.533335	1.47929	0.969
0.6	28.57143	0.003012	0.898	0.6	0.271644	1.508296	0.987
0.7	13.88889	0.003907	0.853	0.7	0.406443	1.893939	1
0.8	20.40816	0.002982	0.819	0.8	0.25704	1.639344	0.982
0.9	20.40816	0.002007	0.806	0.9	0.757007	1.193317	0.923
Rpm	**q_{max}(mg/g)**	**b(L/mg)**	**R^2**	**Rpm**	**K_F (L/g)**	**n_F**	**R^2**
50	28.57143	0.001754	0.906	50	0.08356	1.219512	0.982
100	38.46154	0.002317	0.842	100	0.331131	1.47929	0.972
150	12.98701	0.008333	0.969	150	0.398107	1.876173	0.923

Table 3 continued to next page

Table 3 continued from previous page

Tempkin model				**Redlich-Peterson**			
Ph	**AT**	**BT**	R^2	**pH**	**A**	**B**	R^2
2	0.294834	9.596	0.9	2	0.326	62.86566	0.896
3	0.30666	13.79	0.922	**3**	0.11	262.1718	0.468
4	3.56E-06	0.962	0.9	4	0.321	4.486173	0.898
5	0.754617	8.464	0.932	5	0.414	73.25892	0.847
6	0.38805	6.613	0.903	6	0.431	36.56165	0.953
7	0.406405	9.85	0.891	7	0.415	64.26403	0.821
8	0.401921	9.887	0.823	8	0.312	118.7476	0..833
Temp	A_T	B_T	R^2	**Temp**	**A**	**B**	R^2
20	0.321438	10.3	0.902	20	0.322	68.03348	0.907
30	0.327486	15.82	0.921	**30**	0.27	47.27587	0.843
40	0.304765	8.416	8.416	40	0.098	395.0451	0.311
50	0.280419	9.548	0.82	50	0.165	241.2901	0.386
Size	A_T	B_T	R^2	**Size**	**A**	**B**	R^2
0.1	0.321913	7.522	0.912	0.1	0.351	34.74376	0.904
0.2	0.286058	9.62	0.85	0.2	0.28	120.061	0.827
0.3	0.286654	9.612	0.875	0.3	0.334	45.69551	0.93
0.4	0.353104	6.123	0.879	0.4	0.272	248.6385	0.873
0.5	0.33928	10.75	0.942	**0.5**	0.35	16.92852	0.914
0.6	0.337075	9.72	0.843	0.6	0.245	411.5786	0.755
0.7	0.35731	4.974	0.956	0.7	0.35	107.1254	0.946
0.8	0.423443	6.32	0.865	0.8	0.345	237.2229	0.651
0.9	0.294834	9.596	0.9	0.9	0.094	859.1985	0.626
Rpm	A_T	B_T	R^2	**Rpm**	**A**	**B**	R^2
50	0.308742	8.274	0.861	50	0.458	5.606911	0.947
100	0.406023	10.4	0.873	100	0.418	47.56038	0.748
150	0.48336	4.89	0.844	150	0.541	1.197217	0.978
200	0.430593	6.391	0.918	200	0.262	94.63241	0.772

$$q_e = \frac{R_T}{b_T}\ln\left(A_T C_e\right) \qquad (4)$$

ADSORPTION KINETICS

Pseudo First– Order Kinetics

The non linear form of pseudo first order equation is given by Eq (5).

$$\frac{dq_t}{d_t} = k_{ad}(q_e - q_t) \qquad (5)$$

Pseudo Second- Order Kinetics

Applicability of the second order kinetics has to be tested with the rate equation given by Eq (6).

$$\frac{dq_t}{d_t} = k_2(q_e - q_t)2 \qquad \ldots\ldots(6)$$

Result and discussion

Batch method

Marine algae contain high content of ionizable groups (carboxyl from mannuronic and guluronic acids) on the cell wall polysaccharides, which suggests that the biosorption process could be affected by changes in the solution pH. The effect of biomass on the removal of cadmium was studied in the size range of 0.1-1 mg. The uptake of cadmium was evaluated by varying the temperature from 20-50°c and agitation from 50 to 200. Table 1 showed that the uptake of cadmium was higher at pH5, biomass size 0.5mm, temperature 30°C and the agitation was 150rpm. The maximum uptake was 27.24 mg/g, 22.22 mg/g, 27.20mg/g and 31.30 mg/g respectively.

Adsorption isotherm and kinetic study

In the present study, four equilibrium models were analyzed to investigate the suitable adsorption isotherm. Table 3 shows the model constants along with correlation coefficients obtain from four isotherm models. By comparing all the R^2 value it was observed that the R^2 values were found to be higher in Freundlich isotherm. In order to understand the kinetics of metal absorption using *Sargassum* sp. as an adsorbent, pseudo first–order and second-order kinetic models were tested with the experimental data. The values were calculated and listed in Table 2. The maximum correlation coefficient was obtained in pseudo first-order kinetics. On the basis of correlation coefficients values, it can be concluded that pseudo first-order model may be suitable for the present systems.

Conclusion

Biosorption of heavy metals is one of the promising technologies involved in the removal of heavy metals from waste waters. *Sargassum sp*. was selected for studying biosorption due to its originality and to assess the possibility of utilizing a waste biomass for heavy metal removal. Batch experiments provided fundamental information regarding optimum pH, temperature, agitation, biomass size and maximum metal uptake. The adsorption isotherms could be well fitted by the Freundlich equation. The kinetics was best described with pseudo second order model.

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Chapter 14

Medicinal plants used by Garos of Terai and Duars of West Bengal

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Abstract

Duars and terai region of West Bengal is one of the most tribal populated regions of the state. The important tribes are Rava, Garo, Meich, Oraons, Munda etc. These peoples are closely associated with the plant resources of these area. This association forms the basis of their rich traditional ethnomedicobotanical knowledge. The objective of the present study was to explore this knowledge which is well protected from the rest of the world. Many plant species were recorded which are used to treat stomach disorder, amoebiasis, urinary disorder, toothache, skin disease etc. The scientific name, family, local name, plant parts or material used and medicinal uses of each species were also recorded. Emphasis should be given to conserve these plants as well as this traditional knowledge.

Keywords

Ethnomedicobotany, Garo, Duars and terai region of West Bengal

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Introduction

The Duars region is situated in the eastern part of Jalpaiguri district of West Bengal. This district is one of the most tribal populated districts of West Bengal. It has a rich tribal heritage and culture. Ethnomedicobotanical exploration of this region is still far beyond satisfaction. The rapid urbanization, change in traditional values as well as socio-economic conditions remarkably hamper this knowledge. The major tribal communities are Meich, Rava, Munda, Santhal, Paik, Toto, Garo, Oraon etc. However, Garo tribe is very poorly represented in Duars region and most of them are still living in the rural areas and conserve their traditional lifestyle[1]. Many of them are good traditional healer and almost every house use their traditional way of treatment through various ethnomedicines.

The ethnomedicine is a new field which draws attention of many botanists. This system of medicine is not only patient-friendly but also low-priced. Various system of medicine finds their roots in ethnomedicine[2]. However, this knowledge is passed down verbally from generation to generation and preserved from publicity[3]. A few good works have been done in the field of ethnomedicine among tribes of duars and terai region of West Bengal[3,4], but the ethnomedicinal knowledge of Garo tribe is largely unexplored in this region. Thus the aim of the present work is to explore the traditional ethnomedicobotanical knowledge of the Garo community.

Materials and Methods

Present study has been undertaken among the Garo tribes residing at duars and terai region of West Bengal. The study area comprises an area which lies between 26°16’N to 27°N latitude and 88°25'E to 89°53'E longitude. This area also includes three subdivisions and fourteen blocks. The Garo population is very infrequent in this region. Relevant ethnobotanical information was collected from Garo traditional healers and elderly persons.

Plant specimens were collected, identified and preserved for herbarium by standard herbarium technique[5-7]. The correct botanical name, local name, family, synonym(s) and medicinal uses were recorded on the basis of available literature[2,8-11].

Results and Discussion

Altogether sixteen (16) species were recorded from the present study. Detail description of the medicinal plant found in the present study is given in the table 1. Most of the medicinal plants have common uses and follow the same concept of the treatment of the other region of the district. Use of leaf decoction or raw leaf juice of *Justicia adatoda* for the treatment of chronic bronchitis, cough and cold or use of raw leaf of *Centella asiatica* in Diarrhoea and

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Table 1: List of medicinal plants uses by Garo tribes

Sl no	Botanical name of the plant with family	Garo name	Disorders	Plant parts used	Mode of use
1	*Centella asiatica* (L.) Urbn	Mishinachhil	Diarrhea and dysentery.	Leaf	Cooked or eaten fresh paste
2	*Costus speciosus* (Koen. Ex. Retz.) Smith (Costaceae)	Madagonglek	Snake-bite	Rhizome	Fresh
3	*Oroxylum indicum Vent.*(Bignoniaceae)	Khiringbibal	Stomach pain and chest pain	Fruit, seed and bark.	Fresh or dried material is soaked in water and made into paste
4	*Asparagus racemosus* Willdenow. (Liliaceae)	Mimang Thamachi	Stomach disorder	Root	Raw
5	*Bombax ceiba* L. (Bombacaceae)	Balchhu	Amoebiosis	Root	Raw
6	*Cassia fistula* L. (Fabaceae)	Hamak Mithi	Antihelminthic	Stem bark	Raw
7	*Clerodendrum viscosum* Vent (Verbenaceae)	Samakhi	Skin diasease	Leaf	Raw leaf paste
8	*Justicia adhatoda* L. (Acanthaceae)	Allok gufok	Chronic bronchitis, cough and cold	Leaf	Leaf decoction and fresh juice
9	*Phyllanthus emblica* L. (Euphorbiaceae)	Ambri	Constipation	Unripe fruit	Eaten fresh
10	*Curcuma caesia* Roxb. (Zingiberaceae)	Dikki Gissim	Bone fracture	Rhizome	Paste
11	*Physalis minima* L. (Solanaceae)	Chitaku	Edema	Root	Tied on the hand
12	*Murraya koenigii* (L.) Spreng (Rutaceae)	Samsuang	Diarrhoea, dysentery and vomiting	Leaf	After cooking
13	*Artocarpus heterophylus* Lam. (Moraceae)	Thibrong	Food source	Seed	Cooked
14	*Cajanus cajan* (L.) Millsp. (Fabaceae)	Jehu	Jaundice	Leaf	decoction
15	*Solanum indicum* L. (Solanaceae)	Khujka	Stop itching	Leaf and fruit	Leaves and fruits mixed with sugar externally applied
16	*Alstonia scholaris* (L.) R.Br (Apocynaceae)	Loksomfang	Prevents acidity and child constipation and increase the lactation in cattle	Latex and Bark	Raw

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dysentery follow this way of treatment. However it is note worthy that *Physalis minima* root is used to treat edema. Similarly rhizome of *Costus speciosus* is used to treat snake bite and leaf paste of *Clerodendrum viscosum* is used to treat skin disease. These findings indicate the uniqueness of ethnomedicinal knowledge of this tribe and will help in ethnomedicobotanical understanding of this region.

With rapid spread of urbanization, modern medicine and health care system, this traditional ethnomedicinal knowledge is restricted within some remote places. This knowledge is sinking not only due to loss of biodiversity but also due to loss of cultural diversity. However this indigenous knowledge is a powerful discovery engine for not only newer and safer but also an affordable medicine[12]. It is also interesting to note here that these natural plant products are more successful against multidrug resistant infectious disease[13]. This information collected from the Garo tribe may play an important role in scientific research; new drug discovery may forms the basis of primary health care system of Garo community. This knowledge would be an excellent addition to the current knowledge of folk medicine and this community may open a new horizon in the field of ethnomedicobotany.

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Chapter 15

Effect of bioinoculants on growth and yield attributes in cotton

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Abstract

Cotton, the ‘white gold’ is major fibre crop grows throughout the world including western and north-western part of India. Compared to global yield, low yield of Indian cotton is attributed to infection of various insect pests. The present study therefore aimed at evaluating the effect of bioinoculants on growth and yield in cotton. Results revealed that all the bioinoculant treatments (seed treatment, soil drenching and foliar application) significantly enhanced the growth parameters as well as seed cotton yield in Cv. PH-316. However, seed treatment and soil application of the *Azospirillum lipoferum* + *Pseudomonas striata* (PSB) + *Methylobacterium* spp. (PPFM) recorded significantly high root and shoot growth. This treatment also recorded significantly high boll growth in terms of number and weight.. Thus, it is inferred that the application of bioinoculants found promising in enhancing plant growth, yield in cotton.

Keywords

Gossypium spp., *Azospirillum lipoferum, Pseudomonas striata, Methylobacterium* spp., *Bacillus circulans*, bioinoculants, growth parameters, seed cotton yield

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Introduction

Cotton (*Gossypium* spp.) known as "White Gold", is one of the major fibre crops grown globally especially in the tropical and subtropical regions. In India, it is grown on large scale in the states of Maharashtra, Gujarat, Andhra Pradesh, Madhya Pradesh, Rajasthan, Tamil Nadu and Karnataka which accounts more than 95% area of the country. Compared to world productivity of cotton, it is quite low in India. Of the several reasons for low cotton productivity in India; diseases, insect pests, insufficient nutrients (macro & micro) supply are the major constraints. Therefore, present study was undertaken to evaluate the efficacy of bioinoculants in enhancing the growth and yield contributing parameters in cotton Cv. PH -316, which is popularly grown in the state of Maharashtra.

Materials and Methods

The field experiment was conducted during Monsoon, 2010 at Cotton Research Scheme, M.K.V., Parbhani. At onset of the monsoon, the popularly grown cotton Cv. PH-316 was sown in the plots (4.5 m X 3.6 m) at 60cm (plant -plant) X 45 cm (row-row) spacing and after germination one seedling / hill was maintained. The crop was grown and maintained applying recommended packages of practices.

Bioinoculants *viz.*, *Methylobacterium* spp. (pink puigmented facultative methylotrophs = PPFM), *Bacillus circulans* (Silicate Solubilizing bacterium = SSB), *Pseudomonas striata* (Phosphate solubilizing bacterium = PSB) and *Azospirillum lipoferum* (associative, non-symbiotic N_2 fixer) were obtained from the Biocontrol Laboratory, Department of Plant Pathology and BNF Scheme (AICRP), Department of Soil Science and Agril. Chemistry, M.K.V., Parbhani.

The experiment was designed with RBD and a total of eight treatments replicated thrice were imposed. The details of the treatments imposed were:

T_1: Untreated Control

T_2: *Azospirillum lipoferum* (ST +SD)

T_3: *Pseudomonas striata* (ST +SD)

T_4: *Methylobacterium* spp. (ST +PS)

T_5: *Bacillus circulans* (ST +SD)

T_6: *A. lipoferum* + *P. striata* + *Methylobacterium* spp

T_7: *A. lipoferum* + *P. striata* + *Methylobacterium* spp + *B. circulans*

T_8: Recommended dose of fertilizers (RDF).

All the test bioinoculants were applied as seed treatment (ST) @ 25g/kg seed before sowing and soil drenching (SD) @ 5 ml (10^7 CFU /ml.) per hill at 30 DAS. *Methylobacterium* spp. (PPFM) was applied as seed treatment @ 25g /kg seed and phyllosphere spray (PS) @ 5 ml (10^7 CFU /ml) / lit of water at 45, 90 and 135 DAS.

For recording observations, five plants / treatment / replications were selected randomly and tagged. Observations on growth parameters *viz*., plant height, shoot and root length, shoot and root fresh weight, shoot and root dry weight were recorded at appropriate growth stages (45, 90 and 135 DAS) following standard procedures. Observations on number of bolls, boll weight and seed cotton yield were recorded picking – wise. The data obtained on growth and yield parameters was averaged and analyzed statistically following standard procedure[1].

Results and Discussion

Growth parameters

Results (Table 1) revealed that all the treatments significantly influenced the growth parameters in cotton Cv. PH-316. Length, fresh weight and dry weight of both roots and shoots were found to be increased significantly with all the treatments over untreated control.

Root characters

Results (Table 1) indicated that all the treatments significantly enhanced the average root length, its fresh and dry weight over untreated control. However, significantly highest root length (38.67 cm), its fresh weight (26.72 g) and dry weight (10.65 g) were recorded with the treatment T_6 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp). The second and third best treatments found were, treatment T_7 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp + *B. circulans*) and T_4 (*Methylobacterium* spp) which recorded root length, respectively of 36.91 and 32.27 cm, its fresh weight, respectively of 23.63 and 23.26 g both of which were on at par and its dry weight of 10.28 and 10.22 g, respectively. This was followed by T_5 (length: 31.50cm, fresh weight: 23.19 g and root dry weight: 9.35 g), T_2 - *A. lipoferum* (length: 29.43cm, fresh weight :22.36 g and dry weight : 8.91 g), T_8 - RDF (length: 27.30cm, fresh weight :22.27 g and dry weight : 8.72 g) and T_3 - *P. striata*. Comparatively minimum root length (24.14 cm) its fresh weight (19.22 g) and dry weight (8.53 g) were recorded with untreated control (T_1).

Shoot characters

Results (Table 1) revealed that all the treatments exerted similar effects on shoot characters (length, fresh and dry weight) as that of root characters. All the treatments were found to influence and enhance the shoot characters in cotton cv. PH-316 over untreated control.

However, significantly highest shoot length (109.70 cm), its fresh weight (513.90 g) and dry weight (151.30 g) were recorded with the treatment T_6 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp). The second and third best treatments found were, treatment T_7 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp + *B. circulans*) and T_4 (*Methylobacterium* spp) which recorded shoot length of 107.26 and 106.26 cm, shoot fresh weight of 465.50 and 467.67 g and shoot dry weight of 141.84 and 137.93 g, respectively. Both of these treatments were on par in respects to shoot length and shoot fresh weight. This was followed by the treatments T_5 (*B. circulans*) which recorded 103.74 cm, 442.84 g and 134.83 g shoot length, fresh weight and dry weight, respectively; treatment T_2 - *A. lipoferum* (shoot length :102.83 cm, fresh weight : 429 g, dry weight :132 g), T_8- RDF (shoot length : 102.18 cm, fresh weight: 424.33 g, dry weight :129 g) and T_3- *P. striata* (shoot length : 100.37 cm, fresh weight: 420.83g, dry weight : 121.90 g). Comparatively minimum length (91.67cm), fresh weight (362.33g) and shoot dry weight (112.37g) were recorded in untreated control (T_1).

Thus, treatment T_6 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp), followed by T_7 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp + *B. circulans*) and T_4 (*Methylobacterium* spp) were found most effective which significantly enhanced the growth characteristics *viz,* root / shoot length, root /shoot fresh weight and root / shoot dry weight in cotton cv. PH-316.

Table 1: Effect of bioinoculants on growth parameters in cotton Cv. PH-316

Treatments	**Av. Growth Parameters ***					
	Root (length in cm, wt. in g)			Shoot (length in cm, wt. in g)		
	Length	Fresh Wt.	Dry Wt.	Length	Fresh Wt.	Dry Wt.
T1	24.14	19.22	08.53	91.67	362.33	112.37
T2	29.43	22.36	08.91	102.83	429.17	132.78
T3	26.55	21.91	08.62	100.37	420.83	121.90
T4	32.27	23.26	10.22	106.26	464.67	137.93
T5	31.50	23.19	09.35	103.74	442.84	134.83
T6	38.67	26.72	10.65	109.70	513.90	151.30
T7	36.91	23.63	10.28	107.26	465.50	141.84
T8	27.30	22.27	08.72	102.18	424.33	129.47
SE±	**0.57**	**0.22**	**0.24**	**0.81**	**7.35**	**0.51**
CD (at 5%)	**1.73**	**0.68**	**0.72**	**2.46**	**22.30**	**1.55**

T1: Control; T2: *Azospirillum lipoferum* **;** T3: *Pseudomonas striata* **;** T4:*Methylobacterium* spp. **;** T5: *Bacillus circulans;* T6: *A. lipoferum* + *P. striata* + *Methylobacterium* spp; T7: *A. lipoferum* + *P. striata* + *Methylobacterium* spp + *B. circulans;* T8: RDF

* = Mean of three replications

Similar beneficial effects of bioinoculants alone and co-inoculantion of *Azospirillum, Pseudomonas, Bacillus* and PPFM - *Methylobacterium* spp in many cereals, pulses and vegetables were reported earlier by several workers . Associative N_2 fixer *Azospirillum lipoferum* and *Azospirillum brasilensi* and phosphobacteria application (alone or co-inoculation) was reported to improve growth characteristics, biomass, dry matter etc. in the crops like sorghum, pearl millet. Rice, cotton, sunflower and safflower[2,3,4]. Co-inoculation of bioinoculants *viz*., *Azospirillum, Bacillus, Pseudomonas, Methylobacterium* etc. in various crop plants were reported to improve biometric parameters earlier by several workers[5,6,7].

Yield and yield contributing parameters

Results (Table 2) revealed that, all the treatments significantly improved the yield and yield contributing traits in cotton Cv. PH-316 over untreated control. However, treatment T_6 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp) was found most effective and recorded significantly highest average number of bolls (28.67 / plant), average boll weight (17.47 g), average seed cotton yield (1349.70 kg/ ha) and highest increase (41.36%) in seed cotton yield over untreated control (T_1).

The second and third best treatment found were, T_7 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp + *B. circulans*) and T_8 (RDF) which recorded average boll number of 26.00 and 24.66 / plant, average boll weight of 16.76 and 16.60 g, average seed cotton yield of 1280 and 1261 kg / ha and increase in seed cotton yield by 34.06 and 32.11 per cent, respectively over untreated control (T_1). This was followed by treatments T_4 (av. bolls / plant: 23.66, boll weight : 16.06 g, seed cotton yield : 1249 kg /ha and 30.87 % increase in seed yield), T_2 (av. bolls/ plant : 23.33, boll weight : 13.53g, seed cotton yield : 1153 kg /ha and 20.76 % increase in yield) and T_5 (av. bolls / plant: 21.33,boll weight : 16.12g, seed cotton yield : 1057.80 kg / ha and 10.78% increase in seed cotton yield) as against untreated control (T_1). Treatment T_3 (*P. striata*) was found comparatively least effective and recorded minimum number of bolls (21.00 / plant) boll weight (12.04 g), seed cotton yield (1045.00 kg/ ha) and 09.44 percent increase in seed cotton yield over untreated control. (T_1).

Thus, all the treatments significantly enhanced the yield and yield contributing parameters in cotton Cv. PH-316 over untreated control. However, treatment T_6 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp) followed by T_7 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp + *B. circulans*),T_8 (RDF) and T_4 (*Methylobacterium* spp.) were found to be the best treatments in improving the boll number, boll weight and seed cotton yield in cotton cv. PH-316.

Table 2 : Effect of bioinoculants on seed cotton yield and yield traits in cotton Cv . PH-316

Treatments	Avg. Bolls/ Plant *	Avg. Boll weight (g)*	Avg. Yield (Kg/ha)*	Yield Increase. (%) over control
T1	17.33	12.32	954.90	- -
T2	23.33	13.53	1153.20	17.18
T3	21.00	12.04	1045.00	09.44
T4	23.66	16.06	1249.70	30.87
T5	21.33	16.12	1057.80	10.78
T6	28.67	17.47	1349.70	41.36
T7	26.00	16.76	1280.10	34.06
T8	24.66	16.60	1261.50	32.11
SE±	**1.36**	**1.34**	**19.08**	**--**
CD (at 5%)	**4.12**	**4.08**	**57.80**	**--**

T1: Control; T2: *Azospirillum lipoferum* **;** T3: *Pseudomonas striata* **;** T4:*Methylobacterium* spp. **;** T5: *Bacillus circulans;* T6: *A. lipoferum* + *P. striata* + *Methylobacterium* spp; T7: *A. lipoferum* + *P. striata* + *Methylobacterium* spp + *B. circulans;* T8: RDF
* = Mean of three replications

Results of the present study obtained on beneficial role of the test bioinoculants application and their contribution to the test enhancement of growth parameters along with seed cotton yield and yield parameters may be attributed to the accelerated nutrient supply, phosphate solubilization and availability, augmentation of the beneficial microflora both in rhizosphere as well as phyllosphere of the cotton crop. Results of the present study obtained on enhancement of seed cotton yield and yied parameters are in agreement with those reported earlier by several workers[3,6,7,8,9,10]. Though, all the treatments in the present study were found to improve growth parameters as well as see cotton yield and yield attributes in cotton Cv. PH- 316; but the treatments in the order of the merit *viz.,* T_6 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp), T_7 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp + *B. circulans*), T_8 (RDF) and T_4 (*Methylobacterium* spp.) which were found most promising in present study could further be exploited on large scale in integrated nutrient management of cotton crop.

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Chapter 16

Efficacy of bioinoculants against major diseases and sucking pest complex in cotton cv. PH-316

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Abstract

In present study, the efficacy of bioinoculants *viz*., *Azospirillum lipoferum* (N_2 fixer), *Pseudomonas striata* (PSB), *Methylobacterium* spp. (PPFM) and *Bcillus circulans* (SSB) against major diseases and sucking pest complex in cotton Cv. PH-316 was evaluated. Results revealed that all the treatments significantly reduced the incidence of major diseases as well as sucking pest complex in cotton Cv. PH-316. However, significantly least incidence of angular leaf spot (7.96%), root rot (5.24%), grey mildew (10.25%) and reddening (20.93%) were recorded with the treatment T_6 (*Azospirillum lipoferum* + *Pseudomonas striata* + *Methylobacterium* spp.). The same treatment also recorded significantly least populations of the sucking pests *viz*., aphids, jassids, thrips and white flies. The second best treatment found was (*A. lipoferum* + *P. striata* + *Methylobacterium* spp. + *Bacillus circulans*. All the treatments recorded significant increase in seed cotton yield and were found cost effective. Thus, for economical and eco-friendly management of major diseases and sucking pest complex in cotton the bioinoculants found effective in present study could be incorporated in integrated insect pest and disease management of cotton and exploited commercially.

Keywords

Gossypium spp., *Azospirillum lipoferum, Pseudomonas striata, Methylobacterium* spp., *Bacillus circulans***,** disease, sucking pest complex

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Introduction

Cotton (*Gossypium* spp.), popularly known as "White Gold", is one of the important cash crops playing key role in socio-economic status of the world. India is the second largest cotton growing country after USA in the world. The major states in India contributing to the cotton economy are: Maharashtra, Andhra Pradesh, Madhya Pradesh, Gujarat, Tamil Nadu, Karnataka, Punjab, Hariyana and Rajasthan. During 2009-10 area, production and productivity of cotton in India were 103.10 lakh ha, 295 lakh bales and 486 kg lint/ha, respectively. As compared to world's cotton production and productivity, India's cotton production and productivity are too low. Of the various factors responsible for low production and productivity of cotton in India, diseases and insect pests are the major one.

Application of bioinoculants *viz*., *Azospirillum lipoferum / A. brasilence* (associative non-symbiotic N_2 fixer), *Pseudomonas striata* (PSB), *Methalobacterium* spp. (pink pigmented facultative methylotrophic bacterium) and *Bacillus circulans* (silicate solubilizing bacterium) have been reported to augment availability of nutrients, phosphate solubilization, plant growth enhancement and yield improvement in cotton and many other non-leguminous crop hosts[1,2,3,4]. These bioinoculants have also been reported to impart tolerance / resistance against sucking pest complex and major diseases in cotton[5,6].

Therefore, present field studies were undertaken during monsoon, 2010-11, to evaluate the efficacy of bioinoculants *viz*., *Azospirillum lipoferum*, *Pseudomonas striata* (PSB), *Methylobacterium* spp. (PPFM) and *Bacillus circulans* (PSB) against major diseases and sucking pest complex in cotton Cv. PH-316.

Materials and Methods

The field experiment was conducted at Cotton Research Scheme, MKV, Parbhani. A total of eight treatments replicated thrice with RBD were undertaken.

The details of the treatments imposed were:

T_1: Untreated Control

T_2: *Azospirillum lipoferum* (ST +SD)

T_3: *Pseudomonas striata* (ST +SD)

T_4: *Methylobacterium* spp. (ST +PS)

T_5: *Bacillus circulans* (ST +SD)

T_6: *A. lipoferum* + *P. striata* + *Methylobacterium* spp

T_7: *A. lipoferum* + *P. striata* + *Methylobacterium* spp + *B. circulans*

T_8: Recommended dose of fertilizers (RDF).

For recording observations on major diseases and sucking pest complex, five plants / treatment / replication were selected randomly and tagged. Three leaves one each from bottom, middle and top on each observation plant were selected for recording the observations.

Observations on incidence of major diseases *viz*., angular leaf spot (*Xanthomonas axonopodis* pv. *malvacearum*), gray mildew (*Ramularia auriola*), root rot (*Rhizoctonia bataticola*) and disorder reddening were recorded applying standard disease rating scale[7].

Observations on the population of sucking pests *viz*., ahids, thrips, jassid and white flies were recorded by counting visually the number of pest individual. For major diseases, observations were recorded at 45, 90 and 135 DAS and that of sucking pests were recorded at forth-night interval and continued till 50 per cent bolls opening was completed in cotton Cv. PH-316. The data obtained on disease incidence and sucking pests population were averaged and analyzed statistically following standard procedure[8].

Results and Discussion

Incidence of Diseases

Results (Table 1) revealed that all the treatments significantly influenced incidence of the disease *viz*., angular leaf spot, gray mildew and root rot and disorder reddening in cotton cv. PH-316 as compared to untreated control. However, treatment T_6 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp) was found to be most effective and recorded significantly least incidence of the diseases *viz*., angular leaf spot (7.96%), gray mildew (10.25%), root rot (5.24%) and disorder reddening (20.93%).The second and third best treatments found were : T_7 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp + *B. circulans*) and T_4 (*Methylobacterium* spp.) which recorded the incidence of angular leaf spot (11.98 and 14.76 %, respectively), gray mildew (13.44 and 14.72%, respectively) and root rot (10.14 and 10.34 %, respectively) and disorder reddening (24.93 and 27.10 %, respectively). This was followed by the treatments T_2 (*A. lipoferum*), T_5 (*B. circulans*), T_3 (*P. striata*) and T_8 (RDF). The untreated control recorded comparatively maximum incidence of angular leaf spot (35.90 %), gray mildew (30.52%) and root rot (19.39%) and disorder reddening (53.70%).

All the treatments significantly reduced incidence of the diseases recorded over untreated control (T_1) in cotton Cv. PH-316. However, significantly highest reduction of 77.83, 66.42, 72.98 and 61.02 per cent, respectively of angular leaf spot, gray mildew, root rot and

Table 1: Effect of bioinoculants on incidence of major diseases in cotton Cv. PH-316

Treatments	Disease Incidence * (%)				Reduction (%) over control (T_1)			
	ALS	GM	RRR	RD	ALS	GM	RRR	RD
T1	35.90 (36.80)	30.52 (33.52)	19.39 (26.10)	53.70 (47.11)	--	--	--	--
T2	17.86 (24.99)	14.80 (22.60)	10.54 (18.59)	30.53 (33.53)	50.25	51.51	45.64	43.15
T3	26.90 (31.23)	21.80 (27.82)	14.33 (22.71)	41.98 (40.37)	25.07	28.57	26.09	21.82
T4	14.76 (22.59)	14.72 (22.55)	10.34 (18.75)	27.10 (31.36)	58.89	51.77	46.67	49.53
T5	19.36 (26.10)	19.56 (26.23)	12.30 (20.51)	34.13 (35.34)	46.07	21.69	36.56	36.44
T6	07.96 (16.31)	10.25 (18.65)	5.24 (13.23)	20.93 (27.22)	77.83	66.42	72.98	61.02
T7	11.98 (20.23)	13.44 (21.49)	10.14 (18.56)	24.93 (29.95)	66.63	55.96	47.71	53.57
T8	29.36 (32.80)	23.34 (28.87)	16.97 (24.31)	48.50 (44.13)	18.22	23.53	16.61	09.68
SE±	**0.54**	**0.69**	**0.67**	**0.46**	--	--	--	--
CD (at 5%)	**1.64**	**2.11**	**2.04**	**1.41**	--	--	--	--

T1: Control; T2: *Azospirillum lipoferum* **;** T3: *Pseudomonas striata* **;** T4:*Methylobacterium* spp. **;** T5: *Bacillus circulans;* T6: *A. lipoferum* + *P. striata* + *Methylobacterium* spp; T7: *A. lipoferum* + *P. striata* + *Methylobacterium* spp + *B. circulans;* T8: RDF
ALS = Angular Leaf Spot (*Xanthomonas axonopodis* pv. *malvacearum*) ; RRR = Rhizoctonia Root Rot (*Rhizoctonia bataticola*); GM = Gray Mildew (*Ramularia auriola*); RD = Reddening (Physiological disorder);
*=Mean of three replications (Figure in parentheses are angular transformed value)

reddening were recorded with the treatment T_6 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp). The second and third best reduction in disease incidence were recorded by the treatments, T_7 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp + *B. circulans*) and T_4 (*Methylobacterium* spp.) which recorded reduction in angular leaf spot (66.63 and 58.89 %,respectively), gray mildew (55.96 and 51.77%, respectively), root rot (47.71 and 46.67 %, respectively) and reddening (53.57 and 49.53%, respectively), respectively. This was followed by the treatment T_2 (*A. lipoferum*) which recorded 50.25, 51.51, 45.64 and 43.15 per cent reduction respectively in angular leaf spot, grey mildew, root rot and reddening. Treatments T_8 (RDF) and T_3 (*P. striata*) were found comparatively least effective in reducing incidence of these diseases.

Though, all the treatments were found effective in reducing the diseases *viz.*, angular leaf spot, gray mildew, root rot and disorder reddening; but the treatments in the order of merit *viz.*, T_6, T_7, T_4 and T_5 were found most effective. Thus the bioinoculants tested and found

Table 2: Effect of bioinoculants on population of the sucking pests in cotton cv. PH-316

Treatments	Average population*/plant				Reduction (%) over control (T_1)			
	Aphids	**Thrips**	**Jassid**	**White flies**	**Aphids**	**Thrips**	**Jassid**	**White flies**
T1	65.00	28.00	6.47	14.66				
T2	38.00	10.66	3.31	10.67	41.54	61.93	48.84	27.22
T3	43.34	18.33	4.17	12.67	33.32	34.54	35.55	13.57
T4	34.00	10.00	3.23	08.67	47.69	64.29	50.07	40.86
T5	38.67	16.66	3.54	11.66	40.51	40.50	45.28	20.46
T6	26.00	6.33	2.47	05.34	60.00	77.39	61.82	63.57
T7	30.67	9.67	2.54	06.00	52.82	65.46	60.74	59.07
T8	52.67	20.00	5.38	12.67	18.97	28.57	16.84	13.57
SE±	**1.25**	**1.57**	**0.32**	**1.52**				
CD (at 5%)	**3.79**	**4.76**	**0.99**	**4.63**				

T1: Control; T2: *Azospirillum lipoferum* **;** T3: *Pseudomonas striata* **;** T4:*Methylobacterium* spp. **;** T5: *Bacillus circulans;* T6: *A. lipoferum + P. striata + Methylobacterium* spp; T7: *A. lipoferum + P. striata + Methylobacterium* spp + *B. circulans;* T8: RDF
*=Mean of three replications

effective in the present study could be exploited commercially on large scale for integrated management of cotton diseases.

Insect pest population

Results (Table 2) revealed that, all the treatments had influenced the population of sucking pests as that of diseases in cotton Cv. PH-316. The trend of effectiveness of all the treatments was found to be similar as that was found for diseases. Amongst the treatments, treatment T_6 (*A. lipoferum + P. striata + Methylobacterium* spp) was found most effective and recorded significantly least population of sucking pests *viz*., aphids (26.00 / plant), thrips (6.33/ plant), jassids (2.47 / plant) and white flies (5.34 / plant). The second and third best treatments found were T_7 (*A.. lipoferum + P. striata + Methylobacterium* spp + *B. circulans*) and T_4 (*Methylobacterium* spp.) which recorded aphids (30.67 and 34.00 / plant, respectively), thriphs (9.67 and 10.00 / plant, respectively), jassids (2.54 and 3.23 / plant, respectively) and white flies (6.00 and 8.67 / plant, respectively), respectively. This was followed by the treatments T_2 (*A. lipoferum*) and T_5 (*Bacillus circulans*) both of which were on par and T_3 (*P. striata*). Treatment T_8 (RDF) was found comparatively least effective and recorded 52.67, 20.00, 5.38 and 12.67, respectively aphids, thriphs, jassids and whiteflies / plant.

Results (Table 2) indicated that all the treatments significantly reduced the population of the sucking pests recorded over untreated control (T_1). However, significantly highest reduction

Table 3: Seed cotton yield and cost : benefit ratio (CBR) as influenced by bioinoculants in cotton Cv. PH-316

Treatments	Yield (Kg / ha)	Yield increase (%) over Control (T_1)	CBR
T1: Untreated Control	954.90	-	1:2.07
T2: *Azospirillum lipoferum*	1153.20	20.76	1:2.23
T3: *Pseudomonas striata*	1045.00	9.44	1:2.06
T4:*Methylobacterium* spp.	1249.70	30.87	1:2.34
T5: *Bacillus circulans*	1057.80	10.78	1:2.14
T6: *A. lipoferum* + *P. striata* + *Methylobacterium* spp	1349.70	41.36	1:2.58
T7: *A. lipoferum* + *P. striata* + *Methylobacterium* spp + *B. circulans*	1280.10	34.06	1:2.43
T8: RDF	1261.50	32.11	1:2.41
SE±	**1.91**	**--**	**--**
CD (at 5%)	**5.78**	**--**	**--**

in the population of sucking pests *viz*., aphids (60.00%), thrips (77.39%), jassids (61.82%) and whiteflies (63.57%) was recorded with the treatment T_6 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp). The second and third best treatments found in respect of reduction in the population of sucking pests were T_7 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp + *B. circulans*) and T_4 (*Methylobacterium* spp.) which recorded second and third best reduction in the population of sucking pests *viz*., aphids (52.82 and 47.69 %, respectively), thrips (65.46 and 64.29 %, respectively), jassids (60.74 and 50.07 %, respectively) and white flies (59.07 and 40.86, respectively). This was followed by the treatments T_2 (*A. lipoferum*) which recorded 41.54, 61.93, 48.84 and 27.22 per cent reduction in the population, respectively of aphids, thrips, jassids and whiteflies; T_3 (*P. striata*) which recorded 33.32, 34.54, 35.55 and 13.57 per cent reduction in the population, respectively of aphids, thrips, jassids and whiteflies. The treatment T_8 (RDF) was found comparatively least effective in reducing the population of sucking pests and recorded 18.97, 28.57,16.84 and 13.57 per cent reduction, respectively of aphids, thrips, jassids and whiteflies over untreated control (T_1).

Thus, all the treatments except T_8 (RDF) were found effective in reducing the population of sucking pest over untreated control in cotton Cv. PH-316. The treatments in the order of merit *viz.,* T_6, T_7, T_4 and T_2 were found most promising against sucking pests of cotton in the present study, could further be incorporated in the integrated pest management of cotton.

Results (Table 3) revealed that all the treatments significantly influenced the seed cotton

yield in cotton Cv. PH-316 were found cost effective over untreated control (T_1). However, significantly highest seed cotton yield (1349.70 kg/ha) and highest increase (41.36%) in yield were recorded with the treatment T_6 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp.) which also recorded maximum CBR (1 : 2.58). The second and third best treatments found were T_7 (*A. lipoferum* + *P. striata* + *Methylobacterium* spp + *B. circulans*) and T_8 (RDF) which recorded seed cotton yield, respectively of 1280.10 and 1261.50 kg/ha, 34.06 and 32.11 per cent increase in yield, respectively and CBR respectively of 1:2.43 and 1: 2.41. This was followed by the treatments T_4 (*Methylobacterium* spp.) and T_2 (*A. lipoferum*) which recorded seed cotton yield, respectively of 1249.70 and 1153.20 kg / ha, 30.87 and 20.76 per cent increasing yield and CBR, respectively of 1: 2.34 and 1 : 2.23.

The beneficial effects of boinoculants tested in the present study against the diseases and sucking pests might be due to tolerance or resistance imparted in cotton, augmentation of the beneficial microflora in the rhizosphere as well as phyllosphere of the cotton crop, excretion of the plant growth promoting hormones and auxines, accelerated solubilization and availability of the nutrients like nitrogen and phosphorus, enhanced methanol and silicate oxidation (PPFM and SSB) etc.

Results of the present study obtained on beneficial effects of the bioinoculants tested on reduction in disease incidence, sucking pests populations, increase in seed cotton yield and cost effectiveness of the treatments recorded in cotton Cv. PH-316 are in consonance with these reported earlier by several workers[9,10,11,12].

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Chapter 17

Economically important underutilized fruit plants of Darjeeling hills

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Abstract

The wild edible plants form an important constituent of traditional diets. Darjeeling one of the renowned hill stations in the world is not only blessed with the natural sites but is also rich in flora and fauna. There are number of wild (underutilized) edible plants having good nutritive value as well as medicinal properties in this region. The present work deals with the commonly known edible yet underutilized fruits of Darjeeling Himalayas like Kusum (*Baccaurea sapida*, Mull), lupsi (*Spondias axillaris* ,Roxb) Muslerhi/maldhero (*Elaeagnus latifolia* ,Linn) lapche kawlo/phumsi (*Machilus edulis*, King) Mael (*Eriolobus indica),* pummelo (*Citrus grandis*), Fig (*Ficus roxburghii)* etc. These fruits are commonly found and are taken directly (raw fruit) as well as used for pickles. However, due to population growth and increase in demands of various products, unplanned use of these plants increasing day by day. Therefore, planned cultivation and sustainable use is required for prevention of their extension in future.

Keywords

Darjeeling hills, fruits, natural resources, biodiversity

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Introduction

The Himalaya offers an array of forest types with diversity in forest produce such as medicine, vegetables, nuts and wild edible fruits from time immemorial. There is no clear-cut demarcation that a particular species of fruit tree or shrub should be available in a particular area. Darjeeling one of the renowned hill station in the world is not only blessed with the natural sites but is also rich in flora and fauna, there are number of wild (underutilized) edible plants having good nutritive value as well as medicinal properties. Most of them have a fairly wide adaptability. During the course of human civilization nearly 3000 plant species have been used as food but only about 150 species have been cultivated and less that 10 plant species are meeting over 90% of the world food demand. Many such food resources and valuable plants are still to be explored.

Wild fruits were an important source of food for humankind even before the dawn of civilization. These wild fruits have played a very vital role in supplementing the diet of the people. Wild fruits have been important sources of indispensable nutrients. The fact that most wild fruits have high nutritional value, especially in protein, carbohydrate, vitamin C and some essential minerals. The increase in urbanization and gradual exploration of forest and waste land has led to the threat of the extinction of wild species. Few peoples in rural areas still use them extensively as a supplement to their basic food requirement. Some are preserved for use during periods of scarcity. They are sometimes sold in the urban market.

The scientific and systematic knowledge of wild edible fruit will increase the nutritional status of local inhabitants and this will help in horticultural and agricultural field. Nutritional value of fruits cannot be judged without analysis. The commonly known edible yet underutilized fruits of Darjeeling Himalayas like Kusum-(*Baccaurea sapida*, Mull), lupsi-(*Spondias axillaris*, Roxb) Muslerhi/maldhero-(*Elaeagnus latifolia*, Linn) lapche kawlo/phumsi-(*Machilus edulis*, King) Mael-(*Eriolobus indica),* pummelo (*Citrus grandis*), Fig (*Ficus roxburghii)*, are commonly found and taken as raw fruit or used for pickles.

Ripe fruits are mostly eaten raw whereas unripe fruits are used variedly as vegetable, mixed with curry, eaten with salt or cooked. Some of the economically important underutilized fruit plants of Darjeeling hills are as follows:

1) *Baccaurea sapida* Muell. Arg

Description

The generic name is derived from Latin 'Baccaurea' referring to the golden yellow colour of the fruit. It is a small to medium sized, dioecious tree. The haploid chromosome number of latka is reported to be 13[1]. It is a semi-evergreen tree which grows up to 10 m in height. The

Table: Description and utilization of these important lesser known fruits has been presented below

Botanical name	Common name	Family	Flower-ing time	Harvesting time
Baccaurea sapida	Burmese grape	Euphorbeaceae	Mar-Apr	July-Aug
Elaeagnus latifolia	Bastard oleaster	Elaeagnaceae	Dec-Jan	April-May
Elaeocarpus sikkimensis	Indian olive	Elaeaocarpaceae	Apr-May	Nov-Dec.
Eriolobus indica	Assam apple/Indian crab apple	Rosaceae	Mar-Apr	Oct-Nov
Machilus endulis	Wild avocado	Lauraceae	May-Jun	Dec–Jan
Spondias axillaris	Indian hog plum	Anacardiaceae	Jun-July	Oct-Nov

yellowish fruits are edible when ripe, and are available during May–July. The local name of the fruit is Latka. It grows in evergreen forests on a wide range of soils. Latka fruits are roundish to oval in shape, with cauliflory bearing habit. Latka is traditionally propagated by seed, but the dioecious nature of the plant is one of the major limiting factors of seed propagation. Attempts had been made to propagate latka plant by means of mature stem cutting[2]. Fruits can be kept fresh for 4–5 days, or boiled and mixed with salt after which it can be kept well in a closed jar.

Uses

The flesh or aril around the seed coat can be eaten, and tastes delicious. The rind of the fruits is occasionally used for making chutney. Commercially, squash-making has increased the value of the fruits. *B. sapida* can be a good source of vitamin C[4]. Fresh peel yields pectin useful in preparation of jellies, jams etc. Leaves and barks are used in dyeing. Leaves and flowers are also edible. The fruit is harvested and used locally, eaten as a fruit, stewed or made into wine. In Bangladesh, it is cultivated chiefly for production of valuable dye called 'annatto' from seeds. A seed contains 4.8-6% annatto dye. It is used to dye silk, cotton and other textile materials[2]. The Bark, roots and woods are dried and ground before boiling in water. They are used medicinally to treat skin diseases. The bark, roots and wood are harvested for medicinal uses[3].

2) *Elaeagnus latifolia* Linn.

Description

The species is found in subtropical and temperate Himalaya from Kumaon through Sikkim, Darjeeling, Bhutan and Khasi hills in Meghalaya. *E. latifolia* is a liana (woody climber) and is mostly found/ grown in semi wild condition in the back yard garden of many houses of

the region. The plant requires well-drained soil and can be grown in nutritionally poor acid soil. It can be grown in dry condition and can tolerate drought. The flowers are hermaphrodite and are pollinated by bees. The fruit is oblong in shape with dark pink color at the time of ripening. It flowers during December-January and the light pink coloured fruits are harvested during March-April in 3-4 picking. The fruits are perishable and can be stored only for 3-5 days at room temperature[5].

Uses

Fruits are eaten raw or used in pickles and making chatnies, jam. jelly and refreshing drinks. Leaves are used as fodder. The fruit contains a single large seed that has very short viability.

3) *Elaeocarpus sikkimensis*

Description

Trees are usually 25 m tall. Branchlets striate, pilose. Petiole 2–4.5 cm, prominently swollen at upper end; leaf blade broadly oblong, usually tapered to base, thinly leathery, glabrous, apex acuminate. Raceme short, 4cm; peduncle yellow-brown. Flowers bisexual; buds oblong, 5–7 mm, Sepals 5, Petals 5, Stamens 30–32.

Uses

The fruits are eaten raw, or processed into pickles, chutneys etc.

4) *Eriolobus indica* Schn.

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Description

This is a tree of lower temperate zone. *E. indica* is mainly distributed in the eastern Himalaya, particularly in eastern Nepal, Sikkim, Darjeeling and Bhutan. This tree attains a height of up to 9–12 m. Fruits are round shaped and pale green in colour when ripe. The fruit contains about 71.73% carbohydrate, 0.35% fat and 1.75% protein[3].

Uses

The fruit extract is made into a semi-solid gel locally known as 'chuk', which is considered to be a good medicine for stomach disorders. Nearly 15 kg of fruit is required to make 1 kg of chuk which can be stored for a longer duration. They are eaten either fresh or processed into pickle[3].

5) *Machilus edulis* King.

Description

The species is an evergreen tree of about 15–20 m height, with a straight bole and spreading branches It is found growing eastward from Nepal to Sikkim, Bhutan, Arunachal Pradesh

and the whole northeastern region. Fruits are commonly sold in the market and are available during November–March. They are medium size trees, 15-30 m tall at maturity. The leaves are simple, lanceolate to broad lanceolate, varying with species from 5-30 cm long and 2-12 cm broad, and arranged spirally or alternately on the stems. The flowers are in short panicles, with six small greenish-yellow perianth. The fruit is an oval or pear-shaped drupe, with a fleshy outer covering surrounding the single seed. After the fruits are picked they are stored in warm, dark, non-airy enclosures for curing. This increases the flavour before bringing the fruits to the market. The outer fleshy pulp, which comes out attached with the skin, is scooped out and eaten. The fruits are highly nutritious, with high fat and carbohydrate content[3].

Uses

The fruit is used for table purpose and also as fodder for animals.

6) *Spondias axillaris* Roxb. (synonym: *Choerospondias axillaris* (Roxb.) Burtt and Hill)

Description

S. axillaris is known as Lupsi in the hills. It is a light-demanding, medium-sized, deciduous tree, which grows up to 25 m tall and has a diameter up to 50 cm. The bark is thin, dark brown or dark grey; the leaves are spirally arranged. The leaflets are arranged in three to five opposite pairs. Leaflet blades are thin, ovate to oblong-ovate with an acuminate apex and an obliquely acute base. The margin is mostly entire, less often serrate. The inflorescence is axillary and the fruit is a fleshy, ovoid drupe, usually one per in frutescence. The mesocarp is pulpy. The wood is soft, light and used for interior finishes, drawers, crates, carvings, turnery, plywood and pulp. The leaves are used as fodder and are consumed by people after boiling. The fruit can be eaten fresh or made into a variety of sweetmeats and chutney.

Uses

The fruits of this species are nutritious and highly valued as a source of traditional medicine. The fruits (ripe or unripe) are traditionally eaten by local peoples as raw and in the form of different product like pickle, chutney, etc. The processed product has a considerably long shelf-life (up to 5 years)[3]. The ripened fruits (pulp) are eaten raw. Pickle and processed candies of this fruit are popular among the local people. Fruits are pickled or processed into a variety of sweet. Sour fruit products are locally called “Mada” or “Titaura”. It is a rich source of vitamin C. It is also believed to aid in digestion and is often consumed after a protein rich meal[6].

Conclusion

These species of economically important underutilized fruit plants of Darjeeling hills are nutritionally rich, even more nutritious than some of the commercial fruits. Therefore, consuming wild edible plants can play a major role in meeting dietary requirement of the tribal population in remote areas. Use of wild edible plants can substantiate vitamin, protein and fat contents in the human diet. The value addition can increase the cash return from wild edible plants 2–3 folds by processing the products into pickle, jam, jelly and squash. These fruit trees besides yielding edible fruits, is also considered as a good fodder and is therefore lopped heavily. Collection of fruits is also done by lopping the branches of big trees; this decreases the yield during the next season. The density of some species is so low in natural stands that managing these species for sustainable harvest does not appear to be a feasible option. Therefore proper cultivation is required for sustainable utilization of these plants species can replace staple or commercial fruits, thereby contributing handsomely to the economy of the subsistence farmers in the mountains.

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Chapter 18

Exploring the riddles of intestinal probiogenomics

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Abstract

An intestinal population of beneficial commensal microorganisms helps maintain the steady state of human health, and some of these bacteria have been found to significantly reduce the risk of gut-associated disease and to alleviate disease symptoms. The world of intestinal probiotics is a fascinating one with lots of information at its repertoire. There has been a lot of advancements in the research area pertaining to the pathogenic microbes, a concern to human health. However, the globe of probiotics, their adaptive strategies to maintain a close concord with human niche, subtle evolutionary tricks and of course, most inevitably the mechanisms of immune modulation bestowing beneficial health effects on the human host, still remains elusive. This review certainly peeps into the advancements achieved, so far, and also discusses future scopes and challenges that need to be accomplished to gain a better insight into the riddles of probiotic bacterial community.

Keywords

Bioinformatics, Bifidobacterium , probiotics, immune-modulation, comparitive genomics

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Introduction

History of probiotic research in pre-genomic era

During the last decades, it has been quite prominent that the human body lives in close concord with a complex ecosystem that is composed of more than 100 million different bacterial species that dwell in the oral cavity, upper respiratory tract, gastro-intestinal tract (GIT), vagina, and skin. This collection of microbial population is commonly known as the microbiota and is acquired soon after birth and persists throughout life. These pools of microbes play an important role in the physiology and sustainability of their host, including the digestion and assimilation of nutrients, protection against pathogen colonization. Human microbiota is also known to prohibit endogenous and exogenous pathogen infectivity, stimulate modulation of host immune responses, trigger intestinal angiogenesis and other functions leading to the amelioration of host immune responses[1].

The sequencing of human genome[2,3] has greatly helped in delving into the potential role of genetic factors in health and disease. Intestinal 'microbiome', known to play a crucial role in maintaining human health and well being, has always been a fascinating field of research. The intestinal 'microbiome' may contain more than 100 times the number of genes in the human genome[5] and provides many functions that human system has developed by itself. The indigenous intestinal microbiota provides a barrier against pathogenic bacteria and other harmful food components[4,5,6]. It has also been shown to have a direct impact on the morphology of the gut[7], and many intestinal diseases can be linked to disturbances in the intestinal microbial flora[8].

However, the principles and subtle intricacies underlying the complex mechanisms exhibited by intestinal microbiota remain obscure and thus a major challenge to be accomplished. One major complexity lies in correlating the health status of the host with the presence or absence of certain bacterial species, keeping in attention that the microbiota varies extensively among individuals. Thus the study of microbe-microbe and microbe-host interactions has a plethora of information at its gamut- a challenging yet interesting task.

The complex domain of research on intestinal microbial flora also encompasses the globe of probiotic bacteria that can contribute substantially to gain better knowledge of beneficial microbe host interactions. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benifit on the host[9]. Although the term probiotic cannot merely be used for beneficial set of intestinal microflora, members of intestinal pool are often sources from which probiotics are isolated based on recommended properties such as specific health benefits, survival and persistence in the host, proven safety, and stability[10].

The era of ''omics'' we are living in at this time, promises to make crucial contributions as it enables the analysis and comparison of whole genomes, transcriptomes, metabolomes, proteomes and secretomes[11]. This all will lead to the identification and characterization of probiotic properties, distinguishing real probiotics from the false-positive ones leading to a

scope of proper probiotic formulations, an essential parameter, in administering proper probiotic usage.

The novel idea of modifying the gut flora by replacing the harmful microbes with the beneficial ones was first proposed by Russian scientist and Nobel laureate Élie Metchnikoff in the beginning of the 20th century. According to Metchnikoff, toxic substances like phenols, indols and ammonia produced by proteolytic bacteria such as clostridia, a part of normal gut microflora, were detrimental factors in "intestinal auto-intoxication", leading to physical changes with old age.

Elie Metchnikoff also suggested that the long, healthy life of Bulgarian peasants resulted from their consumption of fermented milk products. He believed that when consumed, the fermenting Bacillus (Lactobacillus) positively influenced the microflora of the colon, decreasing toxic microbial activities[12,13].

Henry Tissier of Pasteur Institute first isolated Bifidobacteria from a breast-fed infant. The isolated bacterium named *Bacillus bifidus* communis[14] was later renamed to the genus *Bifidobacterium*. It was observed by Tissier that bifidobacteria are dominant in the gut flora of breast-fed babies and had clinical benefits in treating diarrhea in infants. The claimed effect was a result bifidobacterial community displacing proteolytic bacteria causing the disease.

It was quite articulate that bacteria originating from the gut were more likely to produce the desired effect in the gut, and in 1935 certain strains of *Lactobacillus acidophilus* were found to be very active when incorporated in the human digestive tract[15].

The term "probiotics" was first cioned in 1953 by Werner Kollath[16] in 2003. In 1989, Roy Fuller suggested a definition of probiotics that has been widely used: "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance"[17].

Types

Lactic acid bacteria (LAB) and bifidobacteria are the most common types of microbes used as probiotics; but certain yeasts and bacilli may also be used. Probiotics are commonly consumed as part of fermented foods with specially added active live cultures; such as in yogurt, soy yogurt, or as dietary supplements.

The most common strains belong to the two genera *Lactobacillus* and *Bifidobacterium*, but other microorganisms including *Enterococcus, Streptococcus, Escherichia* and *Saccharomyces* species have also been used.

Lactobacilli and *Bifidobacteria* are considered the golden stars among probiotic bacteria. Probiotics are subject to several stressful conditions during industrial processing as well as in nature, where a fast stress-response is essential for survival[18].

This particular review mainly deals with the research advancements being made in the field of probiotic research on *Lactobacilli* and *Bifidobacteria*.

Post Genomic Era

The revelation of the human genome sequence has increased our understanding of the genetic deviations that lead to or predispose to gastrointestinal disease as well as to diseases associated with the gut, such as food allergies. In 1995, the first genome of a free-living organism, the bacterium *Haemophilus influenzae*, was sequenced[19]. Since then, over 300 bacterial genome sequences, mainly of pathogenic microorganisms, have been completed. The first genome of a mammalian lactic-acid bacterium, that of *Lactococcus lactis*, a microorganism of great industrial interest, was completed in 2001[20]. More recently, the genomes of numerous other lactic-acid bacteria[21], bifidobacteria[22] and other intestinal microorganisms[23,24,25] have been sequenced.

These great breakthroughs have demonstrated that evolution has adapted both microbes and humans to their current state of cohabitation, or even symbiosis, which is beneficial to both parties and facilitates a healthy and relatively stable but adaptable gut environment.

Information from the Genomes

Lactic-acid bacteria and bifidobacteria can act as biomarkers of gut health by giving early warning of aberrations that represent a risk of specific gut diseases. Many members of the genera Lactobacillus and Bifidobacterium that are potential probiotics have been completely sequenced with the advancements in genome sequencing technologies. The prudency of these micro-organisms is the litheness and flexibility that they exhibit in acclimatization with the changes in the host environments.

This flexibility is emphasized in the completed genomes of intestinal and probiotic microorganisms. The complete genome sequence of the probiotic *Lactobacillus acidophilus* NCFM has already been reported by Altermann *et al.,*[26]. The genome is relatively small and the bacterium appears to be unable to synthesize several amino acids, vitamins and cofactors. It also encodes a number of permeases, glycolases and peptidases for rapid uptake and utilization of sugars and amino acids from the human intestine, especially the upper gastrointestinal tract. The authors also report a number of cell-surface proteins, such as mucus- and fibronectin binding proteins, that enable this strain to adhere to the intestinal epithelium and to exchange signals with the intestinal immune system. Flexibility is guaranteed by a number of regulatory systems, including several transcriptional regulators. The genome of another probiotic, *Lactobacillus johnsonii*[27], also lacks some genes involved in the synthesis of amino acids, purine nucleotides and numerous cofactors, but contains numerous peptidases, amino-acid permeases and other transporters, reflecting a strong reliance and dependence on the host.

The presence of bile-salt hydrolases and transporters in these bacteria clearly points to the adaptive strategies that these bacteria are accoutered with to inhabit in the acidic and bile-rich environments of the upper gastrointestinal tract[27].In this regard, bile-salt hydrolases have been found in most of the sequenced genomes of bifidobacteria and lactic-acid bacteria[21], and these enzymes can have a significant impact on bacterial survival. Another

lactic-acid bacterium, *Lactobacillus plantarum* WCFS1, has been reported to contain a large number of genes related to carbohydrate transport and utilization, and has genes for the production of exopolysaccharides and antimicrobial agents[22], indicating a good adaptation to a variety of environments, including the human small intestine[5]. In general, flexibility and adaptability are reflected by a large number of regulatory and transport functions.

Microorganisms that inhabit the human colon, such as *Bifidobacterium thetaiotaomicron* and *Bifidobacterium longum*[29], have a great number of genes devoted to oligosaccharide transport and metabolism, indicating adaptation to life in the large intestine and differentiating them from, for example, *L.johnsonii*[27]. Genomic research has also provided initial information on the relationship between components of the diet and intestinal microorganisms. The genome of *B. longum*[29] suggests the ability to scan for nutrient availability in the lower gastrointestinal tract in human infants. This strain is adapted to utilizing the oligosaccharides in human milk along with intestinal mucins that are available in the colon of breast-fed infants. On the other hand, the genome of *L. acidophilus* has a gene cluster related to the metabolism of fructo-oligosaccharides, carbohydrates that are commonly used as prebiotics[30].

Mechanisms of probiotic action

Probiotics exhibit a broad range of functions that have been well established to be beneficiary for their host. The various mechanisms and strategies that have been well associated with health promoting activities are discussed below:

(i) Probiotics might be able to modulate the host's defense including the innate as well as the acquired immune system. They are also reported in the improvement of epithelial barrier functions[31].

(ii) Probiotics are reported to have direct influence on pathogen inhibition and restoration of proper microbial equilibrium in human gut, most essential for maintaining gut homeostasis. This principle is used for the prevention and therapy of infections and restoration of the microbial equilibrium in the gut[31].

(iii) Finally, probiotic effects may be based on actions affecting microbial products like toxins, host products e.g., bile salts and food ingredients. Such actions may result in inactivation of toxins and detoxification of harmful bacterial and food components in the gut.

Immune modulatory effects

Probiotics can influence the immune system by products like metabolites, cell wall components and DNA. Obviously, immune modulatory effects might be even achieved with dead probiotic bacteria or just probiotics-derived components like peptidoglycan fragments or DNA. Probiotic products are recognized by host cells sensitive for these because they are e.g. equipped with recognition receptors. The main target cells in that context are therefore gut epithelial and gut-associated immune cells. The interaction of probiotics with host

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(epithelial) cells by adhesion itself might already trigger a signaling cascade leading to immune modulation. Alternatively, release of soluble factors can trigger signaling cascades in immune cells or in epithelial cells which subsequently affect immune cells. The direct adhesion of probiotics to host epithelial cells has been demonstrated in many in vitro experiments. However, probiotic and commensal bacterial adherence to gut epithelial cells in vivo has not been demonstrated. Rather, such bacteria adhere just to and invade the outer mucus layer but do not reach the epithelial cells themselves[32]. Another direct contact between probiotics and host cells in the gut occurs by internalization of bacteria by DCs which are localized below the epithelial cells (non M-cells)[33].

Teichoic acid, a component of the Gram-positive cell wall, of *L. plantarum* is involved in the anti-inflammatory activity of this probiotic. A mutant with enhanced anti-inflammatory capacity incorporated much less D-ala in its teichoic acids than the wild- type strain and dramatically reduced secretion of pro-inflammatory cytokines by peripheral blood mononuclear cell sand monocytes resulting in significant increase in IL10 production. The effects observed were clearly TLR-2 dependent. This mutant was also more protective in a murine colitis model than its wild- type counterpart[34.] This study clearly provides an understanding of the probable involvement of TLR-2 in the probiotic action of *L. plantarum* highlighting the importance of TLRs for probiotic actions.

However, probiotics are also able to protect the integrity of the mucosal gut barrier against the destructive action of enteropathogenic *Escherichia coli* in a TLR-independent way. They achieve this by changing protein kinase C signaling resulting in an amplification of expression and redistribution of zonula occludens protein 2 (ZO-2) in T84 cells[35]. This makes perfect sense because ZO-2 is an important factor for the preservation of tight-junction function in the gut epithelium. Induction by the probiotic *E. coli* strain Nissle 1917 (EcN) of ZO-2 as well as ZO-1expression in vivo was demonstrated in the mouse model. EcN was even able to protect mice with dextran sodium sulphate-induced colitis by reducing the loss of body weight and colon shortening as a consequence of increasing intestinal barrier function[36]. Anti-inflammatory effect of EcN on human gut epithelial cells (HCT15) was demonstrated for live bacteria but without direct contact. Rather a secreted factor mediated this effect by suppressing the TNFα-induced IL-8 transactivation by a mechanism independent of NF-kβ inhibition[37].

Probiotics are also known to alter cytokine production by modulation of cellular signal transduction mechanism. They can either block degradation of the inhibitor Ikβ by inhibiting the ubiquitination of this inhibitor or influence RelA localization via the receptor ʏ-dependent signal cascade which finally is activated through a peroxisome proliferator[24].

According to Yan *et al.,* two soluble proteins from *Lactobacillus rhamnosus* GG promote intestinal epithelial cell survival and growth.by inhibiting TNF-a-mediated apoptosis by activation of the anti-apoptic factor Akt and the protein kinase B. They are also known to inactivate the pro-apoptotic p38 nitrogen activaying protein kinase signaling pathway in epithelial cells[38].

The flagella of EcN have been identified as an inducing agent for human β-defensin. This particular modulatory effect has been instrumental in keeping away commensals and pathogens from intestinal epithelium[39].

DNA of certain probiotic strains are reported to show systematic anti-inflammatory effect in contrast to pathogenic strains in presence of Toll-like receptor 9(TLR9)[40].

Even some regulatory T (Treg) cells are induced by some probiotics. This might explain how subcutaneously applied probiotics exert an anti-inflammatory effect[41].

All these effects of probiotics are sufficient enough to bolster the epithelial barrier and keep away the commensals and pathogens. Thus the probiotics adjust the host immune machinery to combat the threats posed by the intestinal pool of pathogenic microbes.

Direct effects on other microorganisms

Antimicrobial substances produced by probiotics

Bacteriocins: It has been prominent from in vitro studies that pathogen replication can be inhibited by low molecular weight substances. The most common among this group is lactic acid. Hydrogen peroxide is known for similar actions. Low-molecular-weight bacteriocins (LMWB) and high-molecular-weight bacteriocins (class III) are produced by lactobacilli. LMWB can be grouped into three classes: (class I) lantibiotics, post-translationally modified peptides harboring unusual amino acids such as lanthionin, (class II) heat stable non-lantibiotics, (class IV) cyclic antimicrobial peptides[42]. The broad-spectrum classII bacteriocin Abp118 produced by *Lactobacillus salivarius* strain UCC118 is able to protect mice against infection with the invasive foodborne pathogen *Listeria monocytogenes* [43].

The production of the antibiotic reuterin (3-hydroxypropionaldehyde) by *Lactobacillus reuteri* strain ATCC55730 has been reported. Reuterin is a broad- spectrum antibiotic active not only against Gram-positive and Gram-negative bacteria but also against yeast, fungi, protozoa and viruses [44]. Microcines, peptides with narrow range of activity, are also produced by many probiotics.

Deconjugated bile acids: Probiotic bacteria are able to produce so-called deconjugated bile acids which are derivatives of bile salts. Deconjugated bile acids show a stronger antimicrobial activity compared to the bile salts synthesized by the host organism. However it is yet to be explored that how the probiotic strains themselves protect themselves from these “self-made” metabolites.

Competition for limiting resources

Iron is an important limited substance in human host. Almost all bacteria require iron as an essential element. However *Lactobacilli* do not need in their natural habitat [45]. This might be a crucial advantage for competition and survival of *Lactobacilli* in human niche. *L. acidophilus* and *L. delbrueckii* are able to bind ferric hydroxide at their cell surface, rendering it unavailable to pathogenic micro-organisms [46]. EcN is also able to compete very effectively for the limited resource of iron because it encodes at least seven different iron

uptake systems[47, 48].

Anti-adhesive effects

Probiotic bacteria have the ability to bind to epithelial cells, as seen in cell culture assays, thereby blocking adherence of pathogens. It is extrapolated from this mode of action of probiotics that they are potent enough in keeping away the pathogens from binding the host receptor factors. The anti-adhesive property might be a result of competition between probiotic and pathogenic strains for the same receptor in host system. It was reported indeed induction of MUC3 mucin in HT20-MTX cells when co-cultured with *L. plantarum* 299v or *L. rhamnosus* GG. MUC3 mucin inhibited subsequently the adhesion of entero-pathogenic *E.coli* strainE2348/69. Even adhesion of pathogenic Salmonella, Clostridium and *E. coli* strains to pig intestinal mucus could be reduced in the presence of probiotic *Bifidobacterium lactis* Bb12and/or *L. rhamnosus* LGG[49]. However the ability to inhibit pathogens from binding to human intestinal epithelial cells also depends on the specific strain of the probiotic and the pathogen concerned. Furthermore, some commercial probiotic strains are also reported to even increase the adhesion of *E. coli*, *L. monocytogenes* and *Salmonella typhimurium* to human mucus[50]. The most prominent adhesions of probiotics are surface proteins of Lactobacilli, e.g. Mub-Mucus binding protein of *L. reuteri* 1063[51].

Anti-invasive effects

Invasion of the epithelial cells is a major criterion for pathogenicity among gut pathogens. Probiotics also display some anti-invasive effects. The standard assay for quantification of invasiveness is the gentamicin protection assay[52] that supports the evidence. In this cell culture assay gentamicin kills the extracellular bacteria. The number of intracellular bacteria is enumerated after lysis of the epithelial cells. Anti- microbial substances produced by probiotics result in reduced numbers of intracellular bacteria although these substances do not directly inhibit invasion but just kill the pathogens. Some probiotics are able to specifically interfere with bacterial host cell invasion. EcN inhibits invasion of various gut epithelial cell lines by *S. typhimurium*, *Yersinia enterocolitica*, *Shigella flexneri*, *Legionella pneumophila* and *L. monocytogenes*. The extent of inhibition is dependent on the number of EcN bacteria administered and only observed with live EcN strains. A direct contact is not necessary in this case. Some secretion factors are the tools that play their part in such an anti -invasive mechanism[53]. Secreted factors of some probiotic lactobacilli and *Bifidobacterium bifidum* strain Bb12 are also interfering with invasion of host epithelial cells by *S. typhimurium*[49,54]. For *Lactobacillus kefir* strains the S-layer protein is known to play such a role[55.]

Antitoxin effects

Toxins are the most detrimental group of virulence factors. However, certain probiotics have the tremendous ability to protect the host against toxins. This protection can result from inhibition of toxin expression in pathogens. *Bifidobacterium breve* Yakult and *Bifidobacterium pseudocatenulatum* DSM20439 are known to inhibit shiga toxin expression

in *E. coli* (STEC) O157:H7. High concentration of acetic acid produced by strain Yakult was responsible for inhibition of Shiga toxin expression[56].

Probiotics and other clinical implications

The majority of clinical studies on probiotics have been performed with regard to the prevention or treatment of disturbances in the normal gastrointestinal microflora. However, increased awareness of the importance of the indigenous microflora has resulted in the assessment of potential benefits of probiotics in new areas, such as the prevention of recurrences of acute and secretory otitis media, and streptococcal pharyngotonsillitis in children. Sprays containing a-haemolytic streptococci isolated from healthy children have been tested in placebo-controlled studies. α-Haemolytic streptococci had previously been tested for their ability to inhibit growth of pathogens. Spray application following treatment with antimicrobial agents was found to reduce the incidence of recurrence and thereby the need for further treatment with antimicrobial agents[51,57]. The microflora of the nasopharynx and the nasal cavity has been shown to differ between sinusitis-prone children and children not prone to sinusitis[58].

The probiotic concept has been regarded as attractive also in the prevention of dental caries. Dairy microorganisms have been tested *in vitro* for their ability to become a part of the supragingival dental biofilm and for their ability to compete with cariogenic microorganisms, and promising results have been obtained for some strains. In addition, milk fermented with *Lactobacillus* GG was recently shown to reduce the adherence of *Streptococcus mutans* to saliva-coated hydroxyapatite beads[59].

The use of probiotics to maintain health must be considered promising, although much remains to be elucidated. In addition, the pharmacokinetic and the pharmacodynamic properties, the safety and the risks of acquisition of resistance to antimicrobial agents should be taken into prime consideration.

Exploring the puzzles of probiotic globe– a bioinformatics way

The achievement of complete genome sequences of numerous species combined with the tremendous advancement in computation that occurred in the last few decades allow the use of new holistic approaches in the study of genome structure, organization and evolution, as well as in the field of gene prediction and functional classification. The comprehensive analysis of entire genomes has the potential to provide a profound understanding of genetics, biochemistry, and pathogenesis of microorganisms.

While initial bacterial genomics efforts were largely dedicated to pathogenic bacteria, probiotic microbes have gained a lot recently (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi/).

This wealth of information has and will continue to change our perspective of the functionality of these bacterial genera. *Bifidobacterium* are characterized by a high degree of conservation and synteny across the entire genomes[3,60], with limited phylogenetic

diversity and with only a few breakpoint regions of genomic plasticity identified by genome comparison of species with distinct eco-phenotypes.

The *Lactobacillus* genomes reflect the phylogenetic, phenotypic, and ecological diversity of this genus. Analogously, a recent whole-genome and single-marker comparative phylogenetic study was proposed to reclassify the lactobacilli and the sequencing of some key taxonomic lactobacilli to provide the molecular depth required for the proposed reclassification [61].

Genome sequence mining and comparative genomics of bifidobacteria and lactobacilli have revealed their differential focus of activity in the gastrointestinal tract.

Metagenomic perspectives

Valuable studies performed over the past few decades enumerated and detected lactobacilli and bifidobacteria in intestinal samples following, for example, consumption of pre- or probiotics, temporal variation, and disease, initially using culturing and later using 16S rRNA-based molecular technologies, especially fluorescent *in situ* hybridization (FISH) and the fingerprinting technique PCR-denaturing gradient gel electrophoresis, and more recently quantitative real-time PCR (qPCR)[62].

High-throughput sequencing of DNA and pyrosequencing based methods from an ecosystem or microarray analysis may supersede these techniques for certain purposes once affordable by general laboratories. *Lactobacilli* and *Bifidobacteria* are considered culturable, but molecular studies indicated that there are still novel, not yet cultured, members within the human gut[52,63].

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Bifodibacterial genomics

Bifidobacterium genomics has underlined their relative broad autotrophy for amino acids, nucleotides, vitamins, and cofactors and has confirmed their broad capacity for degradation and utilization of complex carbohydrates[3]. The carbohydrate sequestering capacity, especially, is thought to create a considerable competitive advantage in the intestinal tract. Genome annotation revealed that a relatively large proportion (>10%) of the bifidobacterial genome is dedicated to carbohydrate uptake and metabolism, with many, predominantly intracellular, glycosyl hydrolases required for the initial degradation of complex carbohydrates such as arabinogalactans, arabinoxylans, starch, and related polysaccharides[6,64,29,65]. Associated with these glycosyl hydrolases, transport systems for the internalization of structurally diverse carbohydrates were identified that include docking sites for carbohydrate binding to the bacterial cell wall[66], which presumably prevents loss to nearby competitors. Different bifidobacteria are considered to harbor differential capacities for complex carbohydrate utilization, thereby specifying their ecological specificity. This is illustrated by an operon dedicated to the breakdown of starch, amylopectin that is exclusively present in the genomes of *B. breve* strains[67] and might explain their relative abundance in the infant microbiota[68].In addition, the determination of the complete genome of the oral *B. dentium* (2.7 Mb) revealed

the capacity to metabolize a broad range of simple sugars in addition to complex carbohydrates, which is in agreement with the nutrient availability in the oral habitat[69].

Genomics of lactobacillus

Contrary to the bifidobacteria, the *Lactobacillus* genomes revealed a considerable degree of auxotrophy for amino acids and/or other cellular building blocks. Lactobacilli compensate for these auxotrophies by a large array of transport functions. Comparisons of the genomes between typical intestinal lactobacilli and plant or milk isolates exemplified functional groups that represent their niche adaptation. Although the typical milk-adapted *L. bulgaricus* and *L. helveticus* genomes[68,70] contain many pseudogenes related to the utilization of various carbohydrates and are largely adapted to grow on lactose, lactobacilli associated with the intestinal niche in general encode a large capacity for sugar internalization and utilization[3,68], as exemplified by the numerous sugar-uptake systems encoded by *L. plantarum*[22]. In addition, specific intestinal adaptation is also apparent through the enrichment of (mucus-binding) cell surface proteins and enzyme complexes that are predicted to be involved in carbohydrate degradation[71,72,73].

Phylogenomics of probiotics

Whole-genome, 16S rRNA and single-marker based phylogenetic approaches have been instrumental in the classification of Firmicutes. Qualitative and quantitative gene analysis of these groups resulted in three findings: there is a relatively small number of group-specific proteins, the majority of which are poorly characterized; major groupings are functionally better distinguishable by absent genes rather than gained/retained genes; and, finally, a gene cluster possibly involved in purine metabolism is uniquely present in four lactobacilli associated with meat. Phylogenetic ambiguities were effectively visualized with cluster networks.

Future scopes and conclusion

Genome mining and comparative genomics will shed light on the evolutionary mechanisms underlying speciation within the genus and the specific adaptation to respective ecological niches. These efforts have already made an important contribution to the identification of candidate probiotic effector molecules that may underlie the health benefits associated with the consumption of specific probiotic strains[69,74]. Codon and amino acid usage properties of the diverse groups of *Bifidobacterium* and *Lcatobacillus* still need to be explored with a global perspective. Though the codon usage features have already been discussed with respect to seventy sequenced genes in different *Lactobacillus* species way back in 1994[75], this particular field still demands a better insight. Investigating and comparing the codon bias in these probiotic genomes certainly promises to provide a lot of information that may prove to be a key to solve the mystery of proper probiotic strategy that these microbes display.

The availability of probiotic genomes will be very important for predicting the capabilities of the various probiotic microorganisms[76], and will also allow the development of genetic

tools to analyze the functionality of these strains as probiotics[77]. This will also provide information about their mechanisms of action, facilitating the development or selection of a new generation of probiotics. Such data will also enable us to know which factors influence the performance of probiotics, thus allowing a rational approach to strain improvement.

The comparative genomics of probiotic and symbiotic microorganisms and pathogens will provide valuable information on the features of these different lifestyles. This will, in turn, shed light on the detailed functional properties of probiotics and their safety, as well as their evolutionary relationships.

In conclusion, genetic studies on the current generation of probiotic microorganisms will increase our understanding of their biological mechanisms and provide an important step toward understanding human biology in its most complete sense.

Comparative genomic analysis aided by synteny based approaches still need to be performed to gain a better understanding of the various gene insertion, duplication, incorporation and deletion events that may have associations in the adaptive and acclimatizing nature of the probiotic strains.

Exploring the metabolic pathways using bioinformatic and metabolomic approaches can certainly aid to screen and filter the genes and proteins of the probiotic microbes that play the strings and music of symbiosis with human host.

Another domain that deserves an immediate scrutiny is the co-evolution of probiotic genomes with human gut. Probing deep into the secrets of evolution of the various probiotic genes, secretomes and genobiotic gene clusters that play crucial roles in the well being of the host will surely be the key to the riddles and tricks that these probiotic genomes possess in their repertoire.

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Chapter 19

Insights into the nitrogenase protein - an *in silico* approach

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Abstract

Biological nitrogen fixation is an important source of fixed nitrogen for the biosphere. Biological nitrogen fixation is accomplished by prokaryotes through the catalytic action of complex enzyme system known as nitrogenase and it is the only known family of metalloenzyme which accomplishes this process. Nitrogenase is a two protein component system that catalyzes the reduction of dinitrogen to ammonia coupled to the hydrolysis of ATP. This protein is highly conserved through evolution and shares structural, mechanistic as well as evolutionary relationships many other proteins. With recent developments in the field of proteomics, a large amount of biological data is now available in the public domain. This data include amino-acid sequences of nitrogenase proteins from a wide range of microbes. However, very little is known about the 3D structure and role of all these proteins. The obstacles to studying nitrogenase are multiple and varied: they include the extreme oxygen labile condition of the proteins; the size and complexity of the iron-sulfur containing metallocenters and difficulties in obtaining crystals of nitrogen bound to nitrogenase. Theoretical modelling of the protein will be able to peer inside the nitrogenase functionality and achieve a detailed mechanistic description This review mainly focuses on the application of bioinformatics tools in sequence conservation analysis and structural studies of this protein and the use of structure-based phylogenetic approaches to garner information about the structural and functional divergence of the nitrogenase protein.

Keywords

Nitrogenase, 3D structure, metalloprotein, modeling, phylogenetic analysis

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Introduction:

Nitrogen is the essential component of organic molecules such as DNA, RNA and proteins, the building blocks of life. Growth of all organisms depends on the availability of nitrogen. Atmospheric nitrogen represents the primary source of nitrogen on earth. It comprises approximately 78% of the total atmosphere. Despite its abundance in the air we breathe, most of this nitrogen is unavailable for use by organisms. This is because the strong covalent triple bond between the N atoms in atomspheric N_2 molecules makes it relatively inert. In fact, in order for plants and animals to be able to use nitrogen, N2 gas must first be converted to more a chemically available form such as ammonium (NH4+), nitrate (NO3-), or organic nitrogen (e.g. urea - $CO(NH_2)_2$). The process representing the transformation of nitrogen in nature is called the nitrogen cycle. Nitrogen cycle processes include assimilation, ammonification, nitrification, denitrification, anaerobic ammonium oxidation, and nitrogen fixation. One of the most important parts of the nitrogen cycle is nitrogen fixation. A relatively small amount of atmospheric nitrogen is fixed by lightning. The enormous energy of lightning breaks nitrogen molecules and enables their atoms to combine with oxygen in the air forming nitrogen oxides. These dissolve in rain, forming nitrates that are carried to the earth. Some ammonia also is produced industrially by the Haber-Bosch process, using an iron-based catalyst, very high pressures and fairly high temperature. But the major conversion of N_2 into ammonia, is achieved by microorganisms in the process called biological nitrogen fixation.

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Biological nitrogen fixation

Biological nitrogen fixation is the reduction of atmospheric N_2 gas to biologically available ammonium, mediated by prokaryotic organisms in symbiotic relationships, associative relationships, and under free-living conditions[1]. Some eubacteria and a few archebacteria can also fix nitrogen - but no eukaryotic cells have this unique ability. The amount of biologically fixed nitrogen produced is estimated to be 2 X 10^{13} g/year[2]. In contrast, lightning discharge—the primary abiotic source of fixed nitrogen—accounts for 1012 to 1013 g/year. Thus the relevance of biological nitrogen fixations in nature cannot be underestimated. The discovery of nitrogen fixation was attributed to the German scientists Hellriegel and Wilfarth, who in 1886 reported that legumes bearing root nodules could use gaseous (molecular) nitrogen. Shortly afterwards, in 1888, Beijerinck, a Dutch microbiologist, succeeded in isolating a bacterial strain from root nodules. The fixed nitrogen that is provided by biological nitrogen fixation is less prone to leaching and volatilization and therefore the biological process contributes an important and sustainable input into agriculture. Biological nitrogen fixation also has an important role in the marine

nitrogen cycle and can influence the capacity of the ocean biota to sequester atmospheric CO2[3].

The nitrogen-fixing organisms

Although nitrogen fixation is not found in eukaryotes,it is widely distributed among the Bacteria and the Archaea, revealing considerable biodiversity among diazotrophic organisms. The ability to fix nitrogen is found in most bacterial phylogenetic groups, including green sulphur bacteria, Firmibacteria, actinomycetes, cyanobacteria and all subdivisions of the Proteobacteria. In Archaea, nitrogen fixation is mainly restricted to methanogens. The ability to fix nitrogen is compatible with a wide range of physiologies including: aerobic (e.g. *Azotobacter*), facultatively anaerobic (e.g. *Klebsiella*) or anaerobic (e.g. *Clostridium*) heterotrophs; anoxygenic (for example, *Rhodobacter*) or oxygenic (for example, *Anabaena*) phototrophs; and chemolithotrophs (for example, *Leptospirillum ferrooxidans*). Diazotrophs are found in a wide variety of habitats including free-living in soils and water, associative symbioses with grasses, actinorhizal associations with woody plants, cyanobacterial symbioses with various plants. Some microorganisms also enter into symbiotic association with a number of leguminous plants. Symbioses with higher plants offer an ecological niche for the particular microbe to fix nitrogen[4]. The rhizobia groups of bacteria consisting of genera like *Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium*. Different species of *Rhizobium* generally forms association with peas, clover, beans while *Bradyrhizobium* and *Azorhizobium* forms associations with soyabeans and *Sesbania* respectively[5]. It has been reported that most of the legume symbionts are host specific. Symbiotic association is not limited to the legumes but to a number of non-legumes. The most significant amongst them are the actinorhizal plants-*Frankia* association. This association involves a number of woody dicot plants like *Casuarina, Hippophae, Alnus, Myrica*, etc. belonging to different families[6]. Some cereals like rice, wheat, maize, millets, grasses etc. have associative relations with microorganisms accomplishing the process of nitrogen fixation. Prominent among these microbes are *Azospirillium, Acetobacter and Azoarcus*[7]. It has been established[8] that among cyanobacteria both heterocystous and non heterocystous forms like *Anabaena, Nostoc, Trichodesmium, Lyngbya, Plectonema* etc. fix nitrogen[5]. These occur in wide ranging habitats and ecological niches. Besides, the *Anabaena-Azolla* association[9], *Nostoc–Gunnera* association[4] can fix a substantial amount of nitrogen. Isolation of microorganisms from surface-sterilized roots led to the discovery of nitrogen fixing endophytes[10,11]. Cycads in association with cyanobacterial species can also fix nitrogen[12]. Methanogenic Archaea gave a wider angle to the field of biological nitrogen fixation. Zinder and Daniels were the first to discover

nitrogen fixation in different methanogenic Archaea[13,14].

In the rhizobia and *Frankia* the root nodules are the sites for nitrogen fixation. Compared to legume symbiotic microorganisms the first isolation of *Frankia* a non legume symbiont was achieved from nodules of *Comptonia peregrine* in 1978[15]. *Frankia* has two distinctive developmental structures vesicles and spores crucial for its survival[16]. Vesicles are the sites for nitrogen fixation, while spores contained in are the reproductive structures in *Frankia*. In symbiotic nitrogen fixation, the establishment of the bacterium inside the host root and nodule development is a complex process and involves the processes like recognition and infection of the host root, differentiation of the root nodules, proliferation of the bacteria and transformation of the bacteroids in nodules. In cyanobacteria a light and thick walled cell structure called heterocyst is the point where nitrogen fixation takes place. However, in members like *Lyngbya, Plectonema* etc. where heterocyst is absent nitrogen fixation occur in internally organized cells[5].

Genetics and genomics of nitrogen fixation

Symbiotic and free living nitrogen fixers have a set of genes which are responsible for effective nodulation and nitrogen fixation. These are the *nod*, *nol*, *noe*, *nif*, *fix* and some hydrogenase genes. Various techniques like mutations, deletion mapping, cloning vectors etc. have facilitated the identification of genes associated with nitrogen fixation. The work on the genetics of nitrogen fixation was first started in *Klebsiella oxytoca* M5a1 and first ever detailed organization of *nif* genes were reported in this organism[17]. A number of studies[18-21] have established that core *nif* genes like *nif*H, *nif*D, *nif*K, *nif*Y, *nif*B, *nif*Q, *nif*E, *nif*N, *nif*X, *nif*U, *nif*S, *nif*V, *nif*W, *nif*Z are essential for nitrogen fixation. On the basis of mutational studies the natures of different *nif* gene products were determined. Studies confirmed that *nif* HDK encodes nitrogenase while *nif*LA had regulatory functions. In *Azotobacter vinelandii*, the genes coding for the Mo-dependent nitrogenase components (*nif*HDK) and their regulatory and assembly systems are located in two discrete regions[22]. The established functions of the essential and other accessory *nif* genes in *Azotobacter* are described in table 1.

In cyanobacteria especially *Anabaena* sp. 7120 it was established that the organization of *nif*HDK differed from that of *Klebsiella pneumoniae*. Rao[8] reported that it was Johnston and his co-workers who discovered the presence of nodulation genes in a plasmid of *Rhizobium leguminosarum* and mutation of those genes rendered them useless. Later on studies[23,24] ascertained that *nod*, *nol* and *noe* genes produce nodulation signals. The interplay of different *nod* genes, triggering of the creation of root nodule, signaling cascades and

development of nodule meristem were reported by a number of researchers[25-27]. Like the rhizobia, *Azosprillium* includes a megaplasmid and sequences similar to *nod* genes[28]. *Frankia* on the other hand houses a number of *nif* genes but researchers failed to spot *nod* genes in *Frankia*[29]. However, groundwork on *Frankia* genomes exposed some putative nod-like genes which did not emerge in organized clusters and failed to detect the *nod*A gene[30].

Recent advances in genome sequencing have opened exciting new perspectives in the field of genomics by providing the complete gene inventory of rhizobial microsymbionts. The genome of *Mesorhizobium loti* was the first symbiotic bacterium to be sequenced and it was followed by *Sinorhizobium meliloti*[31]. The completion of the genomes of *Rhizobium leguminosarum bv viciae*[32], *Rhizobium etli*[33], *Bradyrhizobium* strains, *Frankia* strains[34] and sequences for a number of free-living diazotrophs spanning different habitat and ecological niches bolstered nitrogen fixation. The studies on the genomes exposed new evidences pertaining to evolution and structure, interactions between plants and microbes and diversity amongst the diazotrophs. Comparisons between these rhizobial sequences revealed that genes and regions involved in symbiosis and nitrogen fixation display an unexpected diversity. Transcriptome analysis is likely to reveal several regulatory networks and will give new insights into the infection process and symbiosis.

In the post genomic era, application of bioinformatics tools in comparative genomics has led to the belief that every genome has its own story. Particularly the genetic code and its usage preferences is one of the most interesting aspects of biological science. In the early period, majority of work on codon usage patterns focused upon *E. coli*[35]. Gradually the bioinformatics analysis of codon usage was applied upon mammalian, bacterial, bacteriophage, viral and mitochondrial genes[36] were the pioneers in developing the Codon Adaptation Index (CAI) to assess the similarity amid the synonymous codon usage of a gene to that of the reference set. Besides CAI, several indices such as GC content, GC3 content , effective number of codons (Nc)[37], relative synonymous codon usage (RSCU)[38], Codon Bias Index (CBI), Fop (frequency of optimal codons)[39] are very significant in studies concerning codon usage patterns.

Very preliminary work on codon usage of nitrogen fixing diazotrophs was initiated by Mathur and Tuli[40]. Ramseier and Gottfert [41] reported differences in codon usage and GC content in *Bradyrhizobium* genes. Moderate codon bias was attributed to translational selection in nitrogen fixing genes of *Bradyrhizobium japonicum* USDA 110[42]. The analysis of synonymous codon usage patterns of three *Frankia* genomes (strains CcI3, ACN14a and EAN1pec) revealed that codon usage was highly biased, but variations were noticed among the three strains[43]. Using codon adaptation index (CAI) highly expressed genes in *Frankia*

were predicted. Synonymous codon usage analysis in *Azotobacter vinelandii* divulged considerable amount of heterogeneity[44]. Highly expressed genes were rich in GC codons. About 503 potentially highly expressed genes were identified and most of them were linked to metabolic functions of which 10 were associated with the core nitrogen fixing

Table 1: *nif* genes and their products in *Azotobacter vinelandii*

Gene	Protein Name	Function in nitrogen fixation
nifH	Fe protein, nitrogenase reductase	Provides electrons to nitrogenase and ATP-derived energy for catalysis
nifD	α-chain of nitrogenase or MoFe protein	Catalyzes reduction of nitrogen to ammonia
nifK	ß-chain of nitrogenase or MoFe protein	
nifT		Unknown
nifY		Putative intermediate carrier of FeMo-cofactor
nifE		FeMo-cofactor assembly protein
nifN		
nifX		Putative intermediate carrier of FeMo-cofactor
FeSII	Shethna protein	Provides respiratory protection
iscANif	$IscA^{Nif}$	Involved in Fe–S cluster assembly protein
nifU	Fe–S cluster assembly scaffold protein	Assembles Fe-S clusters for nitrogen fixing proteins
nifS	Cysteine desulfurase	Catalyzes transfer of sulfur from cysteine to NifU for Fe–S cluster formation
nifV	Homocitrate synthase	Required in biosynthesis of homocitrate moiety of FeMo-cofactor
cysE1	Serine O-acetyl transferase	Involved in biosynthesis of cysteine, which is used as a sulfur source of Fe–S cluster biosynthesis
nifW		Involved for biogenesis of functional MoFe protein
nifZ		Involved in biogenesis of functional MoFe protein
nifM	Peptidyl/prolyl cis/ transisomerase	Required for biogenesis of functional NifH
clpX	Clp protease	Subunit for ATP-dependent protease. Function in nitrogen fixation is unknown.
nifF	Flavodoxin	Involved in transfer of electrons to nitrogenase
nifL		Negative transcription regulatory element
nifA		Positive transcription regulatory element
nifB		Involved in biosynthesis of NifB-cofactor, a FeMo-cofactor precursor.
fdx	Ferredoxin	
nifO		Thioredoxin-like protein. Function in nitrogen fixation is unknown.
nifQ		Molybdenum chaperone
rhdNif	Putative rhodanese	Function in nitrogen fixation is unkown
grx5Nif	Glutaredoxin	Function in nitrogen fixation is unkown

mechanism .Other than codon usage, molecular evolution of genes is another aspect which needs to be investigated. A more reliable index of genetic drift over evolutionary time is the ratio of Ka (nonsynonymous substitutions per site) to Ks (synonymous substitutions per site) for a large set of genes, based on comparisons of related species. The Ka/Ks ratio, which is almost always less than one, is widely used as an indicator of the extent of purifying selection acting to conserve coding sequences. This parameter has been widely applied in the analysis of adaptive molecular evolution, and is regarded as a general method of measuring the rate of sequence evolution in biology. These parameters have been used to assess the molecular evolution of in plant haemoglobin genes[45]; secretory protein genes in *Streptomyces* and yeast[46] and in various disease causing genes. Among diazotrophs, Crossman *et al.* [47] measured the rates of synonymous (Ks) and non-synonymous substitutions (Ka) in orthologous genes of *R. etli* and *R. Leguminosarum.* Other than the whole genome, molecular evolution of the genes responsible for symbiotic association and nodulation such as nodule specific genes[48] and recently *SymRK*[49] have been specifically analyzed. But still a lot of symbiotic genes from wide range of diazotrophs have still to be analyzed to gather a complete scenario of their evolutionary rate in terms of their sequence features.

Nitrogenase system and its functioning

A family of enzymes known as nitrogenase (EC 1.18.6.1) catalyze biological nitrogen fixation[50,51]. The overall stoichiometry of dinitrogen reduction under optimal conditions is as follows:

$N_2 + 8e^- + 8H^+ + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$

The enzyme mechanism requires reduction of the Fe protein by electron donors such as ferredoxin and flavodoxin, transfer of single electrons from the Fe protein to the MoFe protein (which is dependent on MgATP hydrolysis) and finally internal electron transfer in the MoFe protein by the P cluster to the FeMo cofactor substrate-binding site. Each electron -transfer step requires an obligatory cycle of association of the Fe and MoFe proteins to form a complex, after which the two components dissociate. Nitrogenase is a relatively slow enzyme with a turnover time of ~5 s–1, and dissociation of the complex is the rate-limiting step. Complex formation has a crucial role in the enzyme mechanism as it is required for the coupling of ATP hydrolysis to electron transfer.These are complex metalloenzymes with conserved structural and mechanistic features. Nitrogenase contains two components, which are named based on their constituent metals. The nitrogenase cofactors may contain vanadium, V; molybdenum, Mo; or iron, Fe. There are three types of nitrogenase systems

available based on their cofactors; the molybdenum- nitrogenase, the vanadium-nitrogenase, and the iron only system. *K. pneumoniae* has a Mo-nitrogenase system. It consists of two oxygen sensitive metalloproteins, an iron (Fe) protein and a molybdenum-iron (MoFe) protein[52]. The vanadium-nitrogenase system has two components. It has a Fe protein which is the same as other nitogenase systems and the second component is a vanadium-iron (VFe) containing protein which is different than two other systems. This type of nitrogenase has been detected in *A. vinelandii* and *A. chroococcum*[53]. Vanadium-nitrogenase has a very similar amino acid sequence to the molybdenum-nitrogenase.The third type of nitrogenase, iron only, contains an iron (Fe) protein and another protein, which is very similar to MoFe protein and VFe protein, while it has only Fe as its cofactor. This type of protein has also been detected in *A. vinelandii* nitrogenase[53].Nitrogenase has some unique characteristics, which distinguish it from some other enzymes. It is highly sensitive to oxygen, and is degraded by the reaction of oxygen within the iron component. The high sensitivity of the enzyme to oxygen means the nitrogen-fixing bacteria need some protection mechanism to prevent the degradation. Further more, the turnover time of the enzyme is relatively slow, therefore, the organism needs to synthesize large quantities of nitrogenase[20]. Nitrogenase also requires considerable amounts of energy for catalyzing nitrogen fixation.

The smaller component of nitrogenase is the Fe protein, which acts as a redox-active agent and transfers electrons to the MoFe protein for reduction of substrates from available electron donor in the system. Although this transfer of electrons is the main function of the Fe protein, it has some other functions. The Fe protein is needed for initial biosynthesis of the MoFe cofactor. Following the biosynthesis of MoFe cofactor, the insertion of the preformed MoFe cofactor into the MoFe protein requires the Fe protein[54].The Fe protein is ATP-dependant and hydrolyses MgATP using the acquired energy to transfer electrons to the MoFe protein. It has two identical subunits. The Fe protein of nitrogenase contains one iron sulfur cluster [4Fe-4S], which bridges the two subunits. The oxidation state of the Fe protein can vary through the iron sulfur cluster. The Fe protein has one MgATP binding site in each subunit that binds to two MgATP molecules. Binding of MgATP to the Fe protein induces conformational changes, which facilitate the electron transfer from the Fe protein to the MoFe protein.

The larger component of nitrogenase is the MoFe protein, which is a $\alpha 2\beta 2$ tetramer, containing two $\alpha\beta$ dimer subunits. Each dimer contains one MoFe cofactor and one P-cluster, [8Fe-7S]. The MoFe cofactor is located in the active site of the protein where the reduction of substrates occurs. The main role of the P-cluster is electron transfer by accepting an electron from the Fe protein and donating it to the MoFe cofactor. The $\alpha\beta$

dimeric units communicate and contact each other through their "subunits"[54]. The P cluster bridges between each α and β subunit while the MoFe cofactor is placed on the α subunits.

Nif H, D K: structural and divergence studies

With recent developments in the field of proteomics, a large amount of biological data is now available in the public domain. This data include amino-acid sequences of nitrogenase proteins from a wide range of microbes. However, very little is known about the structure and role of all these proteins. Two technologies, X-ray and NMR, are by far the two most common means used to determine protein structure experimentally. In 1992, Kim and Rees[55] provided a detailed cystallographic structure of molybdenum-iron protein of the *Azotobacter vinelandii* nitrogenase. The crystal structure of nitrogenase molybdenum iron protein has also been described from *Clostridium pasteurianum* by Kim and his colleagues[56]. The X-ray crystal structure of *Klebsiella pneumoniae* nitrogenase component 1 (Kp1) has also been determined and refined to a resolution of 1.6 Å by Mayer *et al.* [57]. Georgiadis *et al.*[58], obtained the 2.9 Å crystal structure of the NifH protein from *Azotobacter vinelandii*. It revealed that each of the subunits of the NifH dimer consisted of mixed α-helix/β-sheet polypeptide fold, with a consensus topology of an eight-stranded β-sheet flanked by nine α-helices. A β-sheet core in each monomer was formed of one short antiparallel and seven parallel β-strands. The binding sites critical to NifH function were determined by loops at the carboxy-terminal ends of several β-strands. The Nucleotide Binding Sites (NBSs) were situated in the loops following β1 and β2 and the cysteine ligands (C 97 and 132) for the [4Fe-4S] cluster were in the loops following β5 and β6 [58,59]. Both cluster ligands are located near the amino terminal ends of α-helices that are directed toward the cluster. As indicated by spectroscopic studies, a prominent feature of the Fe protein is the exposure of the [4Fe-4S] cluster to the solvent[60], which is also probably one of the reasons that it is an oxygen sensitive protein. In addition to the [4Fe-4S] cluster, there are numerous Van der Waals and polar interactions in the interface beneath the cluster that help stabilize the dimer structure. NifH displays structural similarity to other nucleotide-binding proteins including signal transduction molecules such as G-proteins and ras and energy transduction systems such as myosin[61]. It is known to be similar to the nucleotide binding proteins based on the presence of the following structural features: (i) mainly parallel β-sheets flanked by α-helices (ii) a phosphate-binding loop (P-loop) or Walker A motif, containing the G-X- X- X- X-G-KS/T consensus sequence and (iii) two switch regions, Switch I and Switch II that interact with the γ-phosphate group of the bound nucleoside triphosphate. Analysis of the position of a bound ADP molecule in the X-ray structure of the Fe protein from *Azotobacter vinelandii*[58] and the properties of other site-

specifically altered Fe proteins, has established the position of the nucleotide binding sites, one on each subunit,19 Å away from the [4Fe-4S] cluster. The nucleotide dependent switch regions are responsible for communication between the sites responsible for nucleotide binding and hydrolysis and the [4Fe-4S] cluster of the Fe protein and the docking interface that interacts with the MoFe protein upon macromolecular complex formation. In *A. vinelandii* NifH, residues 38 to 43 and 125 to 132 form the Switch I and Switch II respectively. The switch regions undergo conformational changes upon hydrolysis to the nucleoside diphosphate with consequent loss of the interaction with the terminal phosphate group. Substitutions in the nucleotide dependent switch regions of the NifH of *Azotobacter vinelandii* showed that the altered NifH proteins formed a trapped complex subsequent to a single electron transfer event[62]. The studies suggested that whereas in the structure of the native enzyme the analogous interaction between the side chains of D39 and 125 was precluded due to electrostatic repulsion, the D39N substitution allowed the formation of a hydrogen bond between the Switch I D39 and the Switch II D125; this demonstrated that the electrostatic repulsion between D39 and D125 was important for dissociation of the Fe protein: MoFe protein complex during catalysis[62]. Thus, the switch regions play a critical role in transmitting information concerning the nucleotide state to other effector molecules that bind to these regions. Investigations of the dimer interface of the NifH protein have shown that in the NifH dimer, residues with >30Å of buried surface area include K 41, E 92,P 93, V 95, A 98, D 129, V 130, V 131, C 132, M 156, Y 159, K 166 and K 170. These residues, along with other residues adjacent in the sequence, mediate the subunit-subunit interactions through a series of primarily polar (hydrogen bond and salt-bridge) interactions[63]. The C-terminus of NifH has been speculated to provide an additional degree of intersubunit interaction in NifH as residues from this region wrap around the body of the opposing subunit and enhance the overall stabilization of the NifH dimer. Also, in the C-terminus region, cross-subunit salt bridges have been known to form between K 224-E 277 and K 233- E 287[63]. An integral part of the nitrogenase mechanism is MgATP binding to the Fe protein and hydrolysis by the Fe protein-MoFe protein complex. Through the studies of Jang *et al*.[64], it was confirmed that the protein interactions with the Mg2+ were essential for the transduction of the nucleotide hydrolysis event. Analysis of the MgADP bound crystallized Fe protein revealed that S16 binds the Mg2+ and α, β and δ phosphates of nucleotides and appears to form a hydrogen bond with D125 of the switch II region[64]. Yet another region found to be critical for the MgATP induced conformational change was the highly conserved span of the Fe protein around A157 located at the subunit interface. This region is part of the α 5helix extending from residue 151 to 176 at the subunit interface and shows substantial difference when the free Fe protein structure is compared with the

structure of the Fe protein in the MgATP bound complex. Moreover, the mutation of A 157 to Ser resulted in a protein that could still bind MgATP normally but was unable to undergo the MgATP-induced conformational change[65]. Further insight into MgATP binding and hydrolysis was obtained by studies based on residues K 15 and D 125. It was suggested that the breaking of a salt bridge between these two residues by MgATP binding was responsible for triggering a conformational change. The substrate reduction mechanism of the nitrogenase enzyme involves the key step of MoFe protein docking on the Fe protein. Recent studies dealing with the biochemical and structural characterization of the cross-linked complex of nitrogenase have shown the specificity of the residues involved in the transient complex formation between the Fe and MoFe proteins. It was found that only E112 from one of the two NifH subunits and K400 of the NifK subunit were cross-linked, although these residues were surrounded by numerous other charged residues that could potentially participate in this process[66]. Most recently, when the Fe and MoFe proteins were crystallized under the conditions of (i) no nucleotide (ii) MgADP and (iii) MgAMPPCP (an MgATP analog), the Fe protein molecules were found to be capable of occupying different interaction sites on the MoFe protein[67]; three separate docking areas were thereby identified on the surface of the MoFe protein[67]. Other studies that substituted Ala in place of F 125 of the α and β subunits of the MoFe protein, separately and in combination, showed that the doubly substituted MoFe protein was unable to form a tight complex with the MgADP-$AlF4^{-}$ treated NifH or when using the altered L127 ΔNifH, thereby suggesting that the F 125 residues were involved in an early event(s) that occurred upon component protein docking and could be involved in eliciting MgATP hydrolysis[68].

However, tertiary structures of large number of nitrogenase proteins from different diazotrophs particularly those of symbiotic ones has not been yet resolved. The exact mechanism of working of these proteins is also relatively unknown due to the difficulty in obtaining crystals of nitrogen bound to nitrogenase. This is because the resting state of MoFe protein does not bind nitrogen. Moreover, in the recent years quite a number of discrepancies have also crept out regarding the protein structures resolved by X-ray crystallography leading to retraction of papers. In this regard, a viable alternative approach is to predict 3D structure of proteins based on homology modeling technique and validate it properly. Homology modelling is a reliable technique that can consistently predict the 3D structure of a protein with precision akin to one obtained at low-resolution by experimental means[69]. This technique depends upon the alignment of a protein sequence of unknown structure (target) with that of a homologue of known structure (template). This technique is particularly quite important in organisms with slow growth rate which poses difficulties in

purification of subsequent proteins. Homology modeling approaches have been used for structural analysis of nitrogenase iron protein from *Trichodesmium* sp., a marine filamentous nitrogen-fixing cyanobacteria[70]. Standard homology modeling approaches have been also been used to generate reliable models of the nitrogenase Fe protein from thermophilic *Methanobacter thermoautotrophicus* based on structure of the *Azotobacter vinelandii* nitrogenase Fe protein[71]. In recent times, the structure of the *Frankia* NifH protein was determined using homology modelling technique[72]. Metal binding sites and functionally important regions of the protein were analyzed. These models based on homology are quite useful in providing conformational properties and structure-function relationship of these proteins. But the model generated by this technique requires proper validation and assessment.

Availability of 3D structures has lead to the structural divergence studies of theses proteins. The studies revealed that various proteins have structural and mechanistic similarities as well as evolutionary relationships with the NifH protein, notable among them being: light independent protochlorophyllide (Pchlide) reductase (ChlL/FrxC or bChL), arsenite pump ATPase (ArsA), 2-hydroxyglutaryl dehydratase Component A (CompA) involved in glutamate degradation and MinD that functions in spatial regulation of cell division[73-76]. Although involved in diverse biological processes, these proteins have been found to bear considerable structural resemblance to the NifH protein. Mechanistically, they do play a role similar to NifH in their respective complexes, based upon the presence of ATP binding motifs in each of them[66]. Whereas ChlL, ArsA and MinD belong to the same superfamily of proteins as NifH, known as the P-loop containing nucleoside triphosphate hydrolases,the Component A of 2-hydroxyglutaryl dehydratase (CompA) is a member of the Actin-like ATPase domain superfamily, as designated by the SCOP database[77]. An analogy between NifH and ChlL, ArsA, CompA and MinD lies in the organization of these proteins as members of their respective two component systems. In the nitrogenase complex, NifH functions as the obligate electron donor to its specific partner, the MoFe protein. This intermolecular electron transfer process requires ATP hydrolysis. In a similar manner, for the dehydratase system, CompA is the site of ATP hydrolysis and is the electron donor to the sec component, CompD, ArsA exists in a complex with the ArsB protein forming an arsenite-antimonite [As(III)/Sb(III)]- translocating ATPase. As a result of the ATP hydrolysis in ArsA, a more compact conformation of the enzyme is generated, allowing the vectorial movement of the arsenite or antimonite ions into ArsB; It has been speculated that the MinDMinE membrane complex is analogous to the NifHMoFe complex and results in ATP hydrolysis, as aconsequence of which, MinD and MinE are released from the

membrane; Comparison of the molecular architecture between nitrogenase and the light independent Pchlide reductase has shown that the NifH counterpart in the light independent Pchlide reductase is ChlL. ChlL is suggested to be involved in ATPdependent transfer of electrons from a reductant, such as ferredoxin, to the ChlB-ChlN complex via the Fe:S center[73]. Thus, in a manner similar to NifH, the proteins ArsA, ChlL, CompA and MinD also couple hydrolysis of nucleoside triphosphates to redox reactions from a metallic cluster.The protein sequence comparison of NifH, ArsA, ChlL, CompA and MinD gives an overview of their similar regions. However, it is difficult to detect protein relationships and the presence of different domains by direct comparisons of sequences belonging to different protein families and it is more reasonable to determine the presence or absence of domains and their family relationships by comparison of their three dimensional structures . How proteins with similar protein folding patterns perform varied functions is a prevalent question and it has been suggested that popular folding patterns have thermodynamic advantages enabling them to be stabilized by random sequences and these few advantageous folds can probably tolerate various primary structures and therefore perform different functions. The structural comparison of each of the four proteins with NifH creates a general model that indicates their common ancestry at some point.

Structure-based phylogeny as a diagnostic for functional characterization

In the post genomic era, the accessibility of the genes, proteins and completely sequenced genomes have made it feasible to comprehend the impact of complex genetic events like paralogy, lateral gene transfer, gene duplication events in the evolution of nitrogen fixation. It has now become possible to understand clearly the forces influencing the evolution of nitrogenase at the molecular level. Raymond *et al.*[78] reported that nitrogenase evolved in multiple lineages and there are evidences of loss, duplications and horizontal and vertical transfers for the nitrogenase genes and operons during the course of evolution. Most, if not all, phylogenetic analyses in diazotrophs use as query the amino acid sequence of the well-studied Mo-dependent nitrogenase reductase also known as iron protein coded by *nif*H. Genes coding for NifH or proteins similar to NifH have been found in all known diazotrophs with sequenced genomes. The strict requirement of NifH in biological nitrogen fixation and its universal presence in diazotrophs has resulted in this protein serving as a sequence tag or bar code for the identification of nitrogen fixers. Such analysis has proved successful in the identification of species able to express not only Mo-dependent but also vanadium- and iron-only nitrogenases. A sometimes *nif*D gene sequence is also used for phylogenetic analysis. However, these analyses are not straight forward due to the existence of paralogous genes in these genomes. However,there are relatively few *nif*D and K

sequences available, so the use of these genes at this time is limited in scope for phylogenetic analysis or phylogenetic comparisons. These genes, when developed as phylogenetic markers, promise to provide more resolution among closely related strains and better differentiate different *nif* gene family members such as the V nitrogenases. Henson *et al*.,[79] re-examined the phylogeny of nitrogen fixation by analyzing only the molybdenum containing *nif* D gene from a cyanobacteria, proteobacteria as well as gram-positive bacteria. They compared the *nif*D phylogeny with that of the 16S rRNA phylogeny. The rationale behind this was that in cases where *nif* and rRNA topologies matched, vertical descent could be inferred otherwise lateral gene transfer could be the reason. They applied the parsimony technique, maximum likelihood technique and neighbour joining technique to analyze the evolution of the *nif* D genes. Henson *et al*.,[79] inferred that their studies on *nif* D phylogenies are harmonious with that of the 16S rRNA based phylogenies and supported the hypothesis of vertical descent. Their results also matched with the *nif*H phylogenies with respect to vertical descent as described by Zehr *et al.* [80]. Henson *et al*., [79] reported that parsimony analysis of the amino acid sequences and maximum likelihood studies concerning the nucleotide sequences of *nif*D genes support monophyletic origins of cyanobacterial and actinobacterial strains strengthening their hypothesis on vertical descent. Conversely, distance matrix analysis of nucleotide sequences for *nif*D genes bolstered the concept of horizontal gene transfer. They also argued that the parsimony and maximum likelihood methodologies are probably more superior compared to other methodologies based on sequence alignments for resolving the evolutionary relationships between organisms. Kechris *et al*.,[82], however strongly lent support for horizontal gene transfer events.

However, phylogenetic techniques based on sequence alignment and structures are inadequate in studying evolution since, sequence similarity becomes erratic at identity levels below 25%[83]. Some workers [84,85] have also pointed out fallacies in sequence alignment based methods. To escape from the twilight zone of protein sequence alignments, structure based phylogeny can provide a suitable alternative. It is well known that the 3-D structures and structural features of homologous proteins are conserved better than their amino acid sequences[86,87]. It has been demonstrated several times that the homologous proteins could diverge beyond recognition at the level of their amino acid sequences but maintain similar structure and function. In several cases of low sequence similarity proteins retain the fold as well as retain the broad biochemical features and/or functional properties, suggesting an evolutionary connection[77,88]. Hence, it is more appropriate to use similarities in 3-D structure of proteins rather than the amino acid sequence similarities in modelling evolution

of distantly related proteins. Though construction of phylogenetic trees using 3-D structures has been applied for a variety of protein families like Short-chain Alcohol Dehydrogenases[89] and metallo-β-lactamases[90]. More recently 3D structure-based phylogenetic approach have been utilized for functional characterization of proteins with cupin folds[91]. In fact it was revealed that structure-based clustering of members of cupin superfamily reflects a function-based clustering. Thus Structure-based phylogeny of cupins can influence identification of functions of proteins of yet unknown function with cupin fold. Similar approaches can utilize to assess the phylogenetic relationships of nitrogenase proteins which shares low sequence similarity but high structural similarities with many proteins with diverse biological functions.

Conclusion

As we enter into the post genomics era, the bioinformatics tools have emerged as important means in research of biological nitrogen fixation. As newer and newer diazotrophs are discovered and sequenced novel insights are being gained. Over the years, detail the structural framework of nitrogenase has emerged, our understanding of the of the molecular details by which this system is assembled and its functioning system is still quite hazy.. The nitrogenase enzyme system has many unknown facets which can be explored. Even though the sequence evolution has been a matter of much debate but the functional evolution of this protein still poses many unsolved mysteries. With the advent of *in silico* protein modelling techniques and new algorithms for measuring structural divergence, the problems associated with nitrogenase system can be tackled in a better way and new glimpses can be gained.

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Chapter 20

Role of cloud computing in bioinformatics research for handling the huge biological data

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Abstract

Bioinformatics, the branch of science applying the information, computer and computational science in the biological world is considered a parasite on the computer and its different fields as host on research aspects. The data flooded by the next and third generation sequencing techniques like pyrosequencing, nanopore sequencing and non optical Ion chip techniques piled up petabytes of data per day and put a question mark on analysis of such a huge data in a smooth and convenient way. The IT field accepting the challenge replied with an alternative of working in virtual environment with no real resources. It considered all the resources virtual and started using this service in virtual mode and termed this as Cloud computing. As Bioinformatics too has been hit by the same storm it is the best time to adopt this technology in the biological world to complement the research works in a smooth and convenient way. Here in the present manuscript an overview of implemented cloud in biology with respect to the time and cost is presented showing the prerequisites for adoption of cloud computing in research in a convenient way.

Keywords

DNA cloud, Cloud computing, Sequence analysis, Bioinformatics, Biological data

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Introduction

Earlier the term cloud was used as the metaphor for internet representing telephone and computer network. But today it has become a buzz word changing the scenario and even its definition. Forrester defined cloud as a pool of abstracted, highly scalable, and managed compute infrastructure capable of hosting end-customer applications and billed by consumption[1]. In a broader sense, it is an elastic execution environment of resources involving multiple stakeholders and providing a metered service at multiple granularities for a specified level of quality i.e. service[2]. Now-a-days cloud comprises of application used to simulate information from raw data, the database storing all the information, physical storage system and servers.

Types of cloud

Based on the availability of the cloud and its components to the clients using this service they are of following 3 types [3]

- ***Public clouds***: Cloud owned and operated by third parties and available publicly with some constraint of security and data variance.
- ***Private clouds***: Enterprise maintaining cloud for its personal issues and the services are accessed only with enterprise permission.
- ***Hybrid clouds***: Mixture of Public and Private cloud and the some parts of services are publicly available whereas some are private and can be accessed only by organization and its permission.

Cloud computing is a service rather technology which is provided by some vendors on 'pay as you go' manner. The concept of cloud computing was introduced by John McCarthy in 1961 and according to him this kind of computation may someday be organized as public utility (Speech given to MIT centennial) Today it has a giant meaning and aspect.

Models of cloud computing

The services provided under cloud computing can be grouped into following three categories:

- ***Software as a Service (SaaS)***: As name suggests, software (the complete application) is served on demand to the clients. Multiple users are serviced using the single instance of software without investing in servers, licenses. Only provider has to pay and as a single instance's running cost is much lower than on multiple servers.
- ***Platform as a Service (PaaS)***: Here a working platform is provided as a service by encapsulating the required software and the working environment to the provider and this

platform can be used by clients. For example, Many platforms are predefined such as Restricted J2EE (Java 2 Enterprise Edition), RUBY, LAMP (Linux, Apache, MySql, PHP), APPERENDA to fulfill the manageable and scalability requirement.

- ***Infrastructure as a Service (Iaas)***: This service provides computing capabilities and basic storage over the network. Here networking equipments, data center space, servers are provided as standardized service. Most common are Amazon, Joyent, GoGrid, Skytap. Skytap promises to give 1 million virtual machines.

Service Providers

There are many IT companies providing the cloud computing services in India. We have summarized the name of the company with specialty, their official and web address in Table 1.

Table 1: List of vendors providing cloud computing services in India with their address and their specification

Sl No	Company	Address	Cloud Offering	Web Link
1	Cyanpse India	Dheerajm Sagar, Malad Mumbai, Maharashtra	Cyn.in	http://www.cynapse.com/
2	Infosys	Infosys Technologies Ltd. Electronics City, Bangalore, Karnataka	Cloud based Solution for Auto Sector	http://www.infosys.com
3	Netmagic solutions	Netmagic Solutions Pvt. Ltd., Nirlon Knowledge Park, Goregaon (East) Mumbai, Maharashtra	Cloud Net Cloud Serve, Private Cloud	http://www.netmagicsolutions.com/
4	OrangeEscape	Orange Escape Tech Ltd., TIDEL Park, Taramani, Chennai	Orange Escape Cloud	http://www.orangescape.com/
5	Qualispace	Jyoti Bldg, Gokhale Road, Thane, Maharashtra	Flexi Cloud Server	http://www.qualispace.in
6	Reliance Data Center	Reliance Industries Limited, Nirman Point, Mumbai, Maharashtra	Reliance cloud computing services	http://www.relianceidc.com/
7	Spinco Biotech	Devi Darshan, S.P Mukherjee Road, Kolkata, West Bengal	Biobase	http://www.spincobiotech.com/
8	TCS	Nirmal Building, Nariman Point, Mumbai, Maharashtra	ITaas	http://www.tcs.com/
9	Wipro Technologies	Doddakannelli, Sarjapur Road, Bangalore, Karnataka	Wipro -w-saas	http://www.wipro.com/
10	Wolf Frame works	WOLF Frameworks. Level Prestige Omega No.104, Whitefield Bangalore, Karnataka	Wolf Paas	http://www.wolfframeworks.com/
11	Zenith	Zenith Infotech Ltd. M.G. Road, Bangalore, Karnataka	PROUD	http://www.zenithinfotech.com/

Applications in Biology

Comparative genomics

Reciprocal Shortest Distance (RSD) algorithm is used for comparative genomics studies in bioinformatics which further increases with the increase in genome size to be analyzed. RSD uses three bioinformatics application BLAST, ClustalW and Codeml for comparative studies. The sequence is searched, aligned and using maximum parsimony shortest distance is calculated and based on best score sequence is annotated. Then using phylogenetic analysis sequence having shortest distance is retained and it is checked by reciprocal blast against genome containing the query sequence. The shortest distance is calculated from hits for original sequence. This is iterated several times for complete studies. This is an extensive computation demanding procedure and consumed a lot of time and was going impractical with the tremendously increasing data.

This algorithm implemented in the cloud and calculated orthologs with the analysis of whole genome data[4,5]. The code and the executables were installed on the master node and using Amazon's Elastic Map Reduce (EMR) algorithm, the input sequences were distributed (mapped) into chunks and process was distributed over slave nodes and the results from the slave nodes were reduced to a single file. More than 300,000 cloud processes on 100 high capacity nodes simultaneously the computation was over in just 70 hours with a cost of $6302 which is much more reliable.

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Metagenomics

To date, the iterated version of the genomic analysis is known as metagenomics. The sequence is searched for the whole genome data to find the best match and helps in recognition of the organism and physiological function. Metagenomics is an approach through which we may get the preliminary information of groups, class, genus or species from any environmental samples like marine, hot spring, intestinal cavity etc and where we don't need to grow the samples in the laboratory. In traditional approach, the data is chunked into smaller fragments and the chunks are searched for its homologues and identical fragments to identify the organism. The search is performed by the bioinformatics tool BLAST. Meta Genome Rapid Annotation using Subsystem Technology (MGRAST) based on this algorithm is a tool used for the metagenomic studies. This BLAST computation part is heavy computation demanding and needs heavy system resources[6]. With the advancement in sequencing techniques the data to be analysed has increased and thus computational time too rapidly increased. In a study of merely 10Kbp of metagenomic data for analysis where the computation required by blast was also computed in Cloud services,

dedicated servers as well as local environment, it was observed that the cloud computing services was not cheaper than the local and dedicated environment with respect to time and cost[7].So it can be concluded that to use cloud computing services in a beneficial way we must select the instances and wisely with a clear concept.

Genome informatics and biomedical information

Till 1980s trivial approach for genome informatics was going and working smoothly. But after the introduction of the Next Generation sequencing techniques the data flood forced the operating procedure to think for an alternative. In the trivial approach users accessed data or information directly from the databases or the organization maintaining the websites downloaded them and maintained local copies in their own resources and then analyzed in their own environment. All the transfer took place with the help of file transfer protocol or hyper text transfer protocol. But it was going hectic due to network conflicts in maintaining the demands with the exponential increase in the data by new sequencing techniques.

Cloud computing came as a ray of hope for the researchers and database organizers. This approach just condensed the resources, data and all tools in the cloud and users can access those data in the virtual mode and can work in cloud itself with that data without downloading and maintaining a local copy in personal system[8,9]. This service was charged on pay as you use and was beneficial for both database organizers and users with respect to the data storage and tools installation.

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Neurosciences

The core heart of any research work is the experimental data produced and using them to interpret the message conveyed. This is also true with the neurological research. Advancement in the technology is producing the data with a great rate and promise to increase with exponential rate in future. Unfortunately, the neurological data are not shared due to the data format produced by tools are in their own informal metadata format thus retarding the growth in neurological research growth rate. To solve out this challenge CARMEN was developed. CARMEN is an e-science cloud platform designed to share, analyse and integrate data over web. So an architecture implementing cloud was designed to manage the metadata formatting and manage the whole dataset. The implementation of cloud provided the privilege of metadata management without any extra effort of installation for metadata formatting and management of services to users[10]. This rapidly increased the sharing and increased the research rate.

RNA analysis

Myrna is a pipeline for calculating differential gene expression and complementary to RNA

sequence analysis existing tool ERANGE and Cufflinks. It can be used with Hadoop, MapReduce or on singleton computer under cloud infrastructure. To calculate differential gene expression of 1.1 billion RNA-seq reads it took almost 4 hours 20 minutes with 1 master and 10 worker nodes, 2 hours 32 minutes with 1 master and 20 worker nodes and 1 hour 28 minutes with 1 master and 40 worker nodes. The performance in the cloud increased with the increase in the nodes implementing parallel computing. However some load misbalancing for nodes and slow data transfer in the cloud affected the speed of the performance. However it is concluded that a large data (1.1 billion sequences read) were analyzed within less than 2 hours with a cost of $66 using Amazon Elastic Compute Cloud which is much more beneficial[11].

Genome analysis and SNP detection

SNP detection and validation is much more laborious and time consuming in wet lab. Even bioinformatics approach a single conventional computer required weeks of time to analyze a deep coverage human re-sequencing project and annotate the whole genome[12]. The cloud application (Hadoop, implementing MapReduce) whereas solved same analysis problem in less than 3 hours without compromising the accuracy rate[13,14].

The implemented cloud computing used an efficient whole genome genotyping tool, Crossbow, combining two software tools[15], the sequence aligner 'Bowtie' and the SNP caller 'SOAPsnp'. This combination analyzed large sets of DNA sequences rapidly maintaining a minimum accuracy rate of 98.9% with simulated datasets of individual chromosomes and better than 99.8% concordance with the Illumina 1 M BeadChip assay of a sequenced individual. This also did not require any extensive software engineering for parallel computation. The input file system was distributed over several nodes in cloud environment (MapReduce) where Bowtie was called for alignment and then SOAPsnp for SNP detection on every allotted slave server node.

Conclusion

Cloud computing is necessarily a blind man's stick for the bioinformatics research. The cloud implementation in the bioinformatics research can mainly be studied into following two main categories (Figure 1A and Figure 1B). However, the case study suggests that the shifting to cloud is not always beneficial especially in the case of small research group where they fluctuate with the use in the cloud environment. To harness the cloud in the beneficial and best possible way one need to completely rely and use it in an uninterrupted way. To achieve this one need to first optimize the commands in a proper channel in order to avoid termination and recreation of the cloud instances. This could be achieved by coding

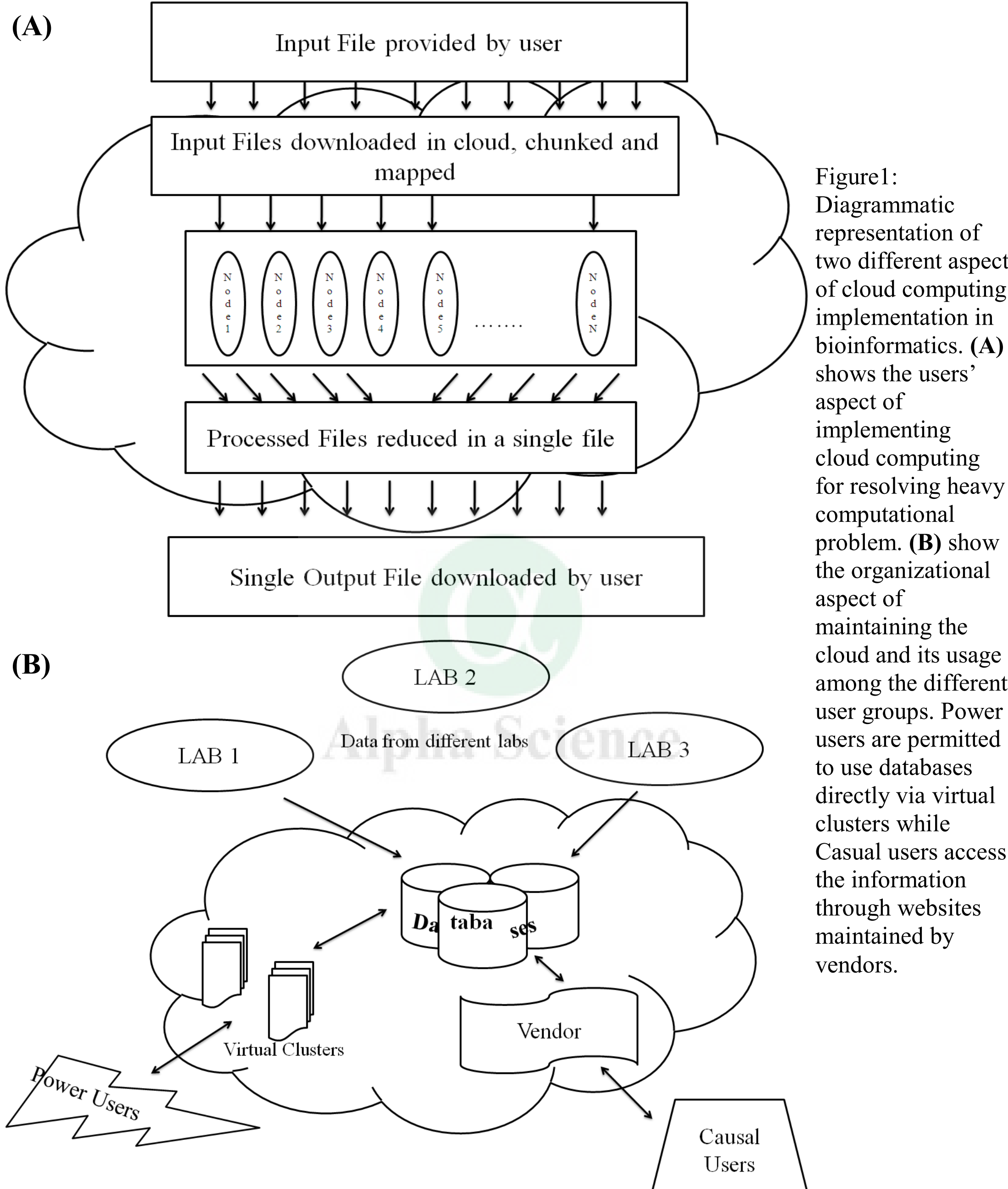

Figure1: Diagrammatic representation of two different aspect of cloud computing implementation in bioinformatics. **(A)** shows the users' aspect of implementing cloud computing for resolving heavy computational problem. **(B)** show the organizational aspect of maintaining the cloud and its usage among the different user groups. Power users are permitted to use databases directly via virtual clusters while Casual users access the information through websites maintained by vendors.

the executables call according to a workflow in a single program so that the cloud instances can be used uninterruptedly. However data security and the backup recovery still is the key

question in the adoption of the cloud to the research works and its needs to be worked upon to ensure the users to shift to cloud. Furthermore, debugging in the case of an error during execution becomes a challenge because reduce program of MapReduce reduces the results in single file and cannot be studied about the slave node used. As it is implementing a parallel computing the speed may vary and can give worse results due to load unbalancing and slow data transfer on the slave nodes. So a perfect and technical hand is required to handle the cloud infrastructure for beneficial, economic and reliable results.

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Chapter 21

Tryptophan biosynthetic pathway genes and their organization

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Abstract

Since, the whole genome contains all of the information for producing the phenotype of that organism; it is an efficient tool for comparison of two organisms or in between two species of same organism also. Presence of similar genes or conservation of the families of protein is the example of this kind of genomic information. Conservation of gene order is important as it can provide information for both about the function and interaction of the proteins, which are coded by those genes, and about the evolution of the genomes and the organisms. The objective of this work was to study the genes and their organization in the Tryptophan biosynthetic pathway from a variety of organism, by doing a comparative analysis of the nucleotide and amino acid sequences available for the completely-sequenced microbial genomes. This work is grouped as follows-i) Gene-Enzyme organization and their variation in different organisms of the pathway. ii) Identification of gene fusion. iii) Graphical representation of variation in gene organization of the pathway in different organism and their groups. The results show a variety of organization of the genes coding for the five enzymes in the tryptophan biosynthetic pathway in genomes of the different micro-organisms, even though the metabolic reactions are fully conserved in all organisms.

Keywords

Anthranilate Synthase, Anthranilate Phosphoribosyl Transferase, Phosphoribosyl Anthranilate Isomerase, Indole -3-Glycerol Phosphate Synthase, Tryptophan Synthase, Enzyme Code

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Introduction

The synthesis of the three aromatic amino acids, Tryptophan, Phenylalanine, and Tyrosine, in microorganisms takes place in a common metabolic pathway. Chorismate is the first branch point metabolite of this pathway, with one branch leading to Tryptophan and the other to Phenylalanine and Tyrosine. The branched pathway leading to Tryptophan synthesis is highly conserved in a diverse group of bacteria, eukaryotes, fungi and plants. This pathway of synthesizing Tryptophan is a 5 steps reaction process with intermediate substrates. These reaction steps are catalyzed by five enzymes, Anthranilate Synthase (AS: EC 4.1.3.27), Anthranilate Phosphoribosyl Transferase (PRAT: EC 2.4.2.18), Phosphoribosyl Anthranilate Isomerase (PRAI: EC 5.3.1.24), Indole-3-Glycerol Phosphate Synthase (IGPS: EC 4.1.1.48) and Tryptophan Synthase (TS: EC 4.2.1.20). The involvement of these enzymes in this biochemical pathway of Trp synthesis is shown in Figure 1 [1,2,3,4,5].

In the present study, a comparative analysis of the genes encoding these five enzymes among different organisms was carried out. The objective was to find out whether the genes exists as clustered, fused, scattered, rearranged, grouped or in disordered condition.

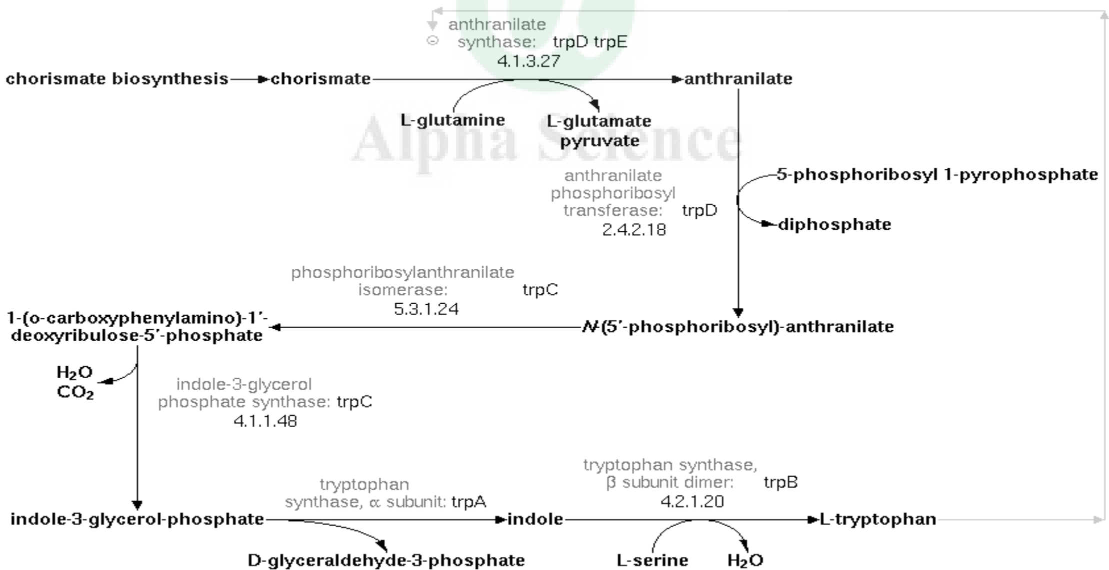

Figure 1: The involvement of the enzymes, biochemical reactions and regulation for tryptophan bio-synthesis

Materials and methods

Collection of Data

The microorganisms chosen for the present analysis were obtained from KEGG database[6]. Fourteen organisms were chosen from the Proteobacteria group, three from Bacillales, two from each of Lactobacillales, Chlamydia, Radioresistant bacteria, Hyperthermophilic bacteria and Archaea (Crenarchaeota), one from each of the others like Clostridia, Spirochete, Cyanobacteria and Green sulfur bacteria. These were represented in table 1, along with the enzymes, their corresponding genes and respective number in the Trp Biosynthetic Pathway[7]. Genes existing in fused condition are colored in table1. The Table shows that the number of genes varies from 5 to 7.

Geneplot

GenePlot[8] is a pairwise comparison tool that allows the user to visualise similarities among bacterial genomes. To construct a GenePlot, genes are numbered sequentially along the genomic sequences of two organisms and the two corresponding sets of predicted proteins are compared using BLAST. The GenePlot link from an organism's genome record[9] can be used to obtain a GenePlot against the organism with which it shares the highest number of reciprocal best hits. Comparisons between other organisms can be made using pull-down menus. Comparisons between other organisms can be made using pull-down menus.

For nearly identical genomes, the GenePlot consists of a diagonal line running from the lower left to the upper right-hand corners. Closely related genomes that may have undergone rearrangements, shows segments displaced from, or running perpendicular to, the main diagonal. The basis of the choice of the organisms chosen for Geneplot has also been shown in table 1.

Results and Discussion

Representation of the structural genes of the trp operon

In *E. coli* (eco) - The five genes (trpEDCBA) involved in the Trp biosynthetic pathway is being represented in Figure 2. The three rows of the genes correspond to three reading frames. The lengths of these proteins, their corresponding genes and the nucleotide positions of the genes are given in table 2. It has been observed that, trpA and trpB genes have a single nucleotide overlap; 11 nucleotide gaps exists between trpB and trpC; 3 nucleotide gap exists between trpC and trpD and finally, trpD and trpE genes have a single nucleotide overlap.

In *Bacillus halodurans* (bha) - The positions of the five genes (trpEGDFCBA) are

Table 1: List of organisms, their classifications, no. of Trp genes, their status and the basis of the choice of the organisms. In the above Table, the organisms corresponding to the shaded 'Sl. No.' are selected for the comparative analysis. The selection was based on different issues nes are in cluster, scatter, in cluster the genes are ordered or not, fused genes category

Sl. No.	Classification	Organisms Name	Anthranilate synthase Component I (trpE)	Component II (trpG)	Anthranilate phosphoribosyl transferase (trpD)	Anthranilate isomerase (trpF)	Indole-3-glycerol phosphate synthase (trpC)	Tryptophan synthase beta (trpB)	alpha (trpA)	Total no. of Genes	Basis of Choice for GenePlot
1.	γ-Proteobacteria	*Escherichia coli K-12 MG1655 (eco)*	b1264	b1263		b1262		b1261	b1260	5	Five genes are in side by side (clustered).
2.		*Salmonella typhi CT18 (sty)*								5	
3.		*Yersinia pestis (ype)*								6	trpC&F are fused
4.		*Buchnera aphidicola (buc)*								6	
5.		*Haemophilus influenzae (hin)*								6	
6.		*Xylella fastidiosa (xfa)*								7	Seven genes. But genes are organized in two groups
7.		*Vibrio cholerae (vch)*								6	
8.		*Pseudomonas aeruginosa (pae)*								7	
9.	β-Proteobacteria	*Neisseria meningitidis MC58 (nme)*								7	7 genes present, trpA&B are not in side by side, scatter (except G&D)
10.		*Ralstonia solanacearum GMI1000 (rso)*								7	
11.	δ/ε-Proteobacteria	*Helicobacter pylori 26695 (hpy)*								6	trpF&C are in fused condition
12.		*Campylobacter jejuni (cje)*								6	
13.	α-Proteobacteria	*Brucella melitensis (bme)*								6	trpE & G are fused
14.		*Caulobacter crescentus (ccr)*								7	
15.	Bacillales	*Bacillus haloduranss (bha)*								7	pabA (trpG) functioning in Folate biosynthesis
16.		*Staphylococcus aureus N315 (sau)*								7	Not in order
17.		*Staphylococcus epidermidis (sep)*								7	
18.	Lactobacillales	*Streptococcus pneumoniae TIGR4 (spn)*								7	
19.		*Streptococcus mutans (smu)*								7	Same as sau but other group
20.	Clostridia	*Clostridium acetobutylicum (cac)*								7	
21.	Actinobacteria	*Mycobacterium tuberculosis (mtu)*								7	
22.		*Corynebacterium glutamicum (cgl)*								6	
23.		*Corynebacterium efficiens (cef)*								6	
24.		*Streptomyces coelicolor (sco)*								7	
25.	Chlamydia	*Chlamydia trachomatis (ctr)*								7	
26.		*Chlamydia muridarum (cmu)*								7	
27.	Spirochete	*Leptospira interrogans (lil)*								7	
28.	Cyanobacteria	*Synechocystis sp. PCC6803 (syn)*								7	Scattered
29.	Green sulfur bacteri	*Chlorobium tepidum (cte)*								7	
30.	Radioresistant	*Thermus thermophilus (tth)*								7	
31.	bacteria	*Deinococcus radiodurans (dra)*								7	
32.	Hyperthermophilic	*Aquifex aeolicus: (aae)*								7	Scattered
33.	bacteria	*Thermotoga maritima (tma)*								6	
34.		*Methanococcus jannaschii (mja)*								7	
35.		*Methanosarcina acetivorans (mac)*								7	
36.	Euryarchaeota	*Methanosarcina mazei (mma)*								7	
37.	(Archaea)	*Methanobacterium thermoautotrophicum (mth)*								7	
38.		*Methanopyrus kandleri (mka)*								7	
39.		*Archaeoglobus fulgidus (afu)*								7	trpC&D are in fused condition
40.		*Thermoplasma acidophilum (tac)*								7	
41.		*Thermoplasma volcanium (tvo)*								7	
42.		*Pyrococcus furiosus (pfu)*								7	Not in order
43.	Archaea	*Sulfolobus solfataricus (sso)*								7	Not in order
44.	(Crenarchaeota)	*Pyrobaculum aerophilum (pai)*								7	

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Table 2: Trp genes in *E. coli*, corresponding Locus Tag number, protein length, start and end Nucleotide positions and gene length in genome

Organism Name	Protein Name	Locus Tag No.	Protein Length	Nucleotide Positions	Gene Length
	trpE	b1264	520	1319408-1320970	1563
	trpD	b1263	531	1317813-1319408	1596
eco	trpC	b1262	452	1316451-1317809	1359
	trpB	b1261	397	1315246-1316439	1194
	trpA	b1260	268	1314440-1315246	807

Table 3: Trp genes in *Bacillus halodurans*, corresponding Locus Tag number protein length, start and end-nucleotide positions, and gene length in genome

Organism Name	Protein Name	Locus Tag No.	Protein Length	Nucleotide Position	Gene Length
	trpE	BH1659	502	1733136-1734644	1509
	trpG/pabA	BH0091	198	111031-111627	597
	trpD	BH1660	342	1734637-1735665	1029
bha	trpF	BH1662	218	1736415-1737071	657
	trpC	BH1661	257	1735658-1736431	774
	trpB	BH1663	399	1737049-1738248	1200
	trpA	BH1664	265	1738241-1739038	798

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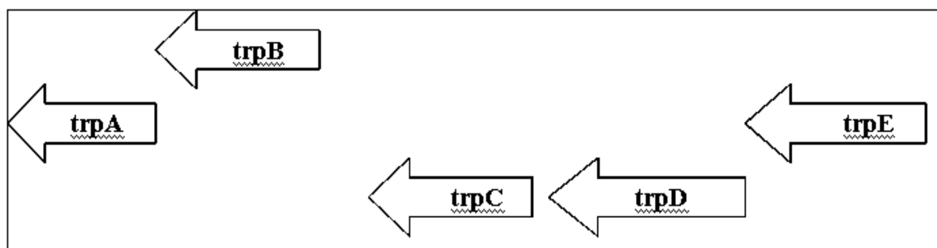

Figure 2: Organization of the Trp biosynthetic pathway genes in the genome of *E.coli*

represented in Figure 3. The rows of the genes correspond to three reading frames. The trpG gene is also known as pabA. The lengths of the five proteins, their corresponding genes and nucleotide positions of these genes are given in table 3. It has been observed that many

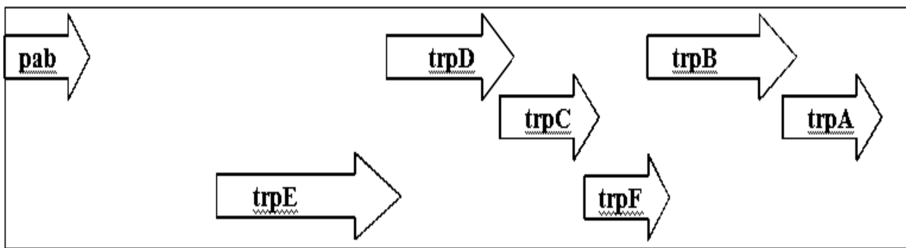

Figure 3: Organization of Trp biosynthetic pathway genes in the genome of *Bacillus halodurans*

nucleotide gaps exists between pabA/trpG and trpE; 6 nucleotides overlap between trpE and trpD; 6 nucleotides overlap between trpD and trpC; 15 nucleotides overlap between trpC and trpF; 22 nucleotides overlap between trpF and trpB and finally, 6 nucleotides overlap between trpB and trpA.

It should be noted that the genes in these two organisms are arranged in opposite directions (Figure 2 & 3), thereby indicating that trp genes in eco and bha are on different strands (eco genes are in upper strand and that of bha are in bottom strand).

Visualization of the Organization of trp genes in genomes of different organisms using GenePlot

The visualization of the trp genes organization can be represented via six different cases:

Firstly, if *E. coli* is chosen for comparison, a diagonal line is obtained (as expected), implying that for each protein in any one organism the other has the same protein and at the same position in genome also. Therefore in all other cases, *E. coli* K12 has been selected as a reference, to study the trp gene organization in different organism and the species.

The five genes of the tryptophan operon are positioned side by side in *E. coli* K12 genome i.e., the genes are organized in a cluster and gene order is conserved. (See Figure 2 also).

Secondly, it's found that the trp gene organization in γ-proteobacteria (eco, sty, ype, hin, vch, buc) and δ-proteobacteria (hpy) are approximately the same, i.e. - all of them have trpCF gene fusion. Inspite, a trpGD gene fusion has also been found in eco and sty. The GenePlot between *E. coli* k12 and Yersinia pestis CO92 is being represented by Figure 5. Many genes are organized differently when compared to Figure 4, and so the plot shows lot of scatter. Further an existence of 6 genes is observed rather than 5 and the gene order is conserved. There is also a presence of a trpCF gene fusion.

Thirdly, Actinobacteria (cgl/cef) like the previous group (γ/δ-proteobacteria) also shows the

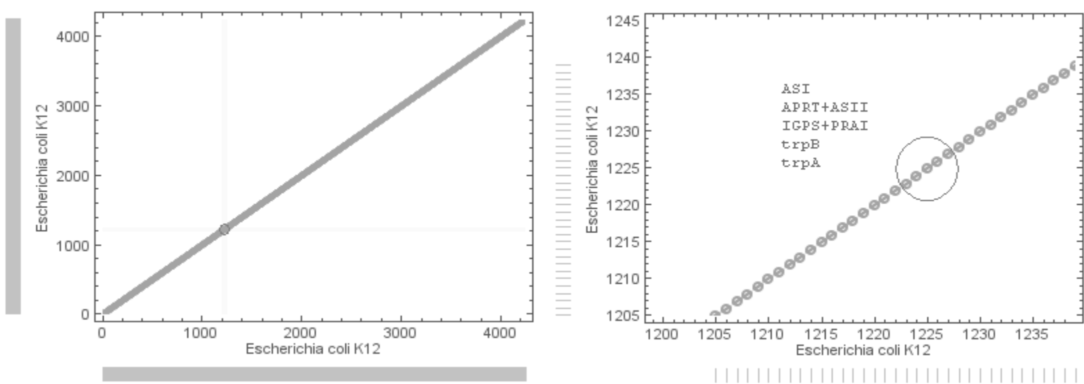

Figure 4: Genome comparison between the same strain of *E.coli* (K12).

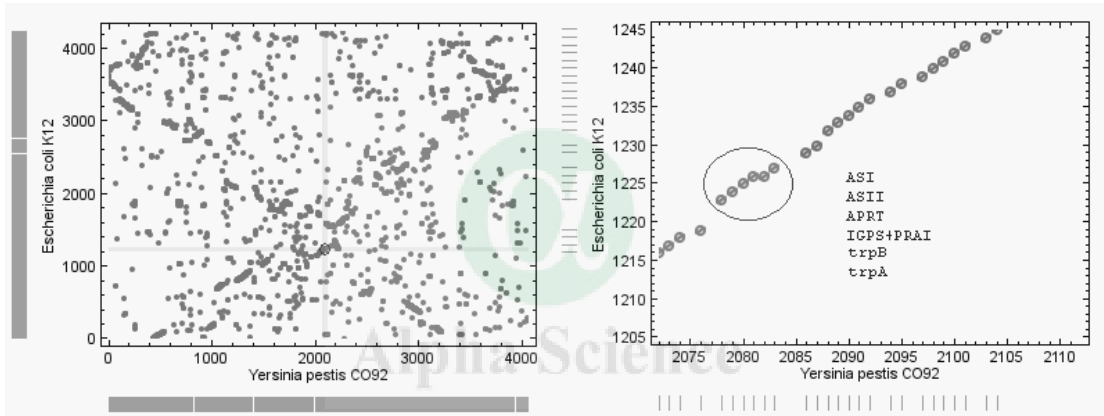

Figure 5: Genome comparison between *E.coli* k12 and *Yersinia pestis* CO92

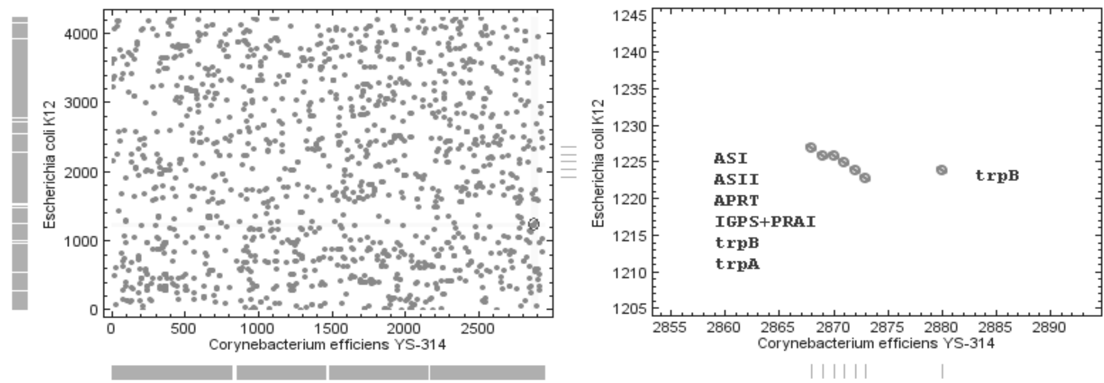

Figure 6: Genome comparison between *E.coli* k12 and *Corynebacterium efficiens* YS-314

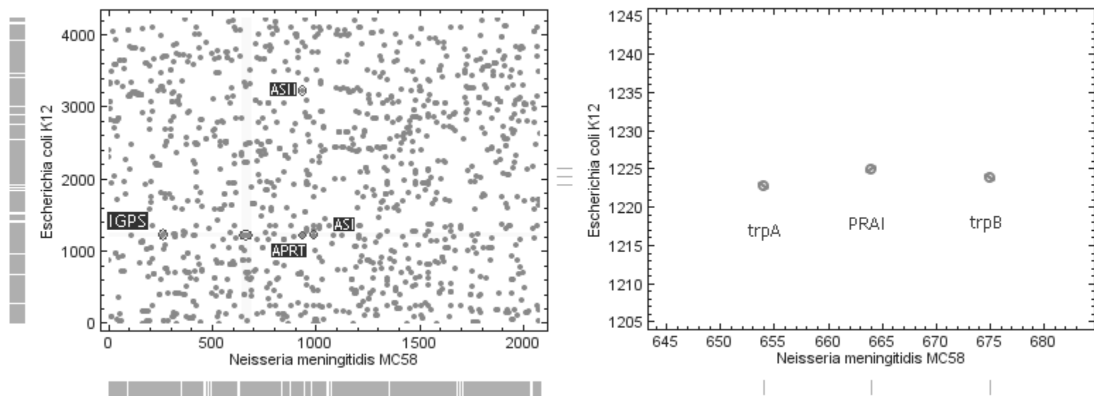

Figure 7: Genome comparison between *E.coli* k12 and *Neisseria meningitides* MC58

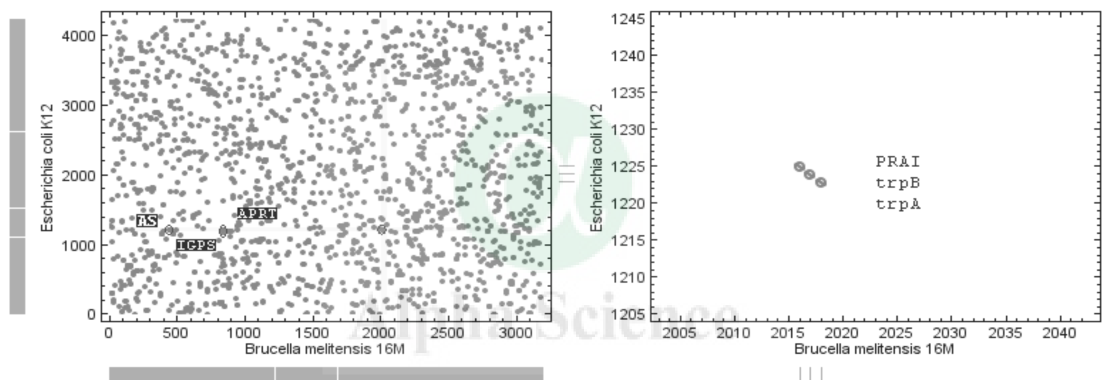

Figure 8: Genome comparison between *E.coli* k12 and *Brucella melitensis* 16M

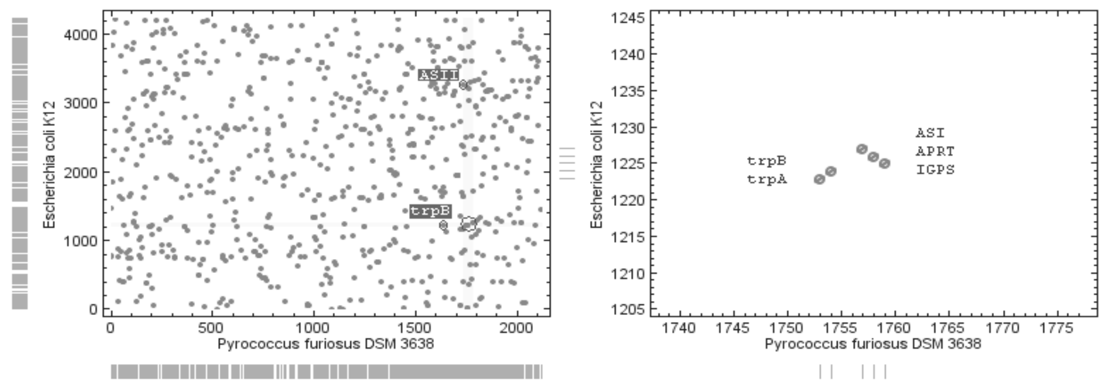

Figure 9: Genome comparison between *E.coli* k12 and *Pyrococcus furiosus* DSM 3638

trpCF gene fusion. Figure 6 shows the GenePlot between *E. coli* k12 and Corynebacterium efficiens YS-314. In cef another gene coding the trpB subunit is observed. Also the trp pathway genes in cef are inverted (similar to Figure 3) when compared to eco.

Further, in γ-proteobacteria (xfa, pae) and β- (nme, rso), the trp genes are not in a cluster and rather exist in different operons i.e., split operon (scattered genes). Figure 7 shows the GenePlot between *E. coli* k12 and Neisseria meningitides MC58. In nme trpA and trpB are not contiguous, separated by the gene for PRAI. The ASII subunit of nme matches with para -aminobenzoate synthase component II (pabA), which is involved in folic acid biosynthesis.

It is also observed that only in bme (α-protobacteria) ASI and ASII subunits are coded by a single gene. Figure 8 shows the GenePlot between *E. coli* k12 and Brucella melitensis 16M. There are five genes for the pathway, and a single gene codes for both the subunits of Anthranilate Synthase (ASI and ASII) protein. There is inversion in gene order here.

And finally, it is found that in euryarchaeota, 7 trp genes are present in a cluster. Figure 9 shows the GenePlot between *E. coli* k12 and Pyrococcus furiosus DSM 3638. Analysis showed, genes in pfu are not in order in comparison to *E. coli*. Further few genes (for ASI, APRT and IGPS) are in reverse orientation.

Conclusion

Pair-wise genome comparisons of protein homolog, analysis of the genes and their corresponding proteins involved in the Tryptophan biosynthetic pathway, in different organism/species (proteobacteria, archaea, bacillus etc) has been done to observe whether they are clustered, fused, scattered, ordered, rearranged, duplicated in the genome, etc. It has also been found that there are 7 genes coding for the domains of the 5 enzymes catalyzing the pathway. They are ASI (trpE), ASII (trpG), APRT (trpD), PRAI (trpF), IGPS (trpC), TS-α (trpA) and TS-β (trpB).

Different organisms/species show different organization pattern of these genes. In most of the organisms trpA and trpB are encoded by two individual genes which are positioned side by side. In most of the organism AS is encoded by two genes, except in *Brucella melitensis* (bme), which is encoded by a single gene i.e.- a gene fusion between trp E and trpG. Moreover it is found that for a particular protein there might be greater than one gene in the genome i.e.- gene duplication (Example: trpB in cef). Further it's seen the five enzymes in *E. coli* are coded by five genes while in organisms like Yersinia pestis and Neisseria meningitides MC58, there exist six and seven genes respectively. Lastly, in some cases the trp genes exists in cluster i.e. - in a single operon (eco, ype etc) and in some organisms (bme, nme etc) they are positioned in different places of the genome i.e., not in a single

group (split operon).

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Chapter 22

Analysis of hypothetical proteins in *Trichomonas vaginalis* proteome

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Abstract

Trichomonas vaginalis is a unicellular protozoan described first by Donne in 1836. It causes trichomoniasis, number one nonviral and second most sexually transmitted disease (STD). The world health organization (WHO) has estimated 250 million infections in the world each year. Trichomoniasis is associated with high morbidity, bad pregnancy outcomes and enhanced risk for HIV & cervical cancer. Its genome draft was published in 2007 which reveals many unusual genomic and biochemical features like, exceptionally large genome size of 160 Mb nearly ten times larger than predicted earlier, large number of hypothetical proteins, the presence of hydrogenosome instead of mitochondria, gene duplication, lateral gene transfer mechanism, presence of miRNAs, drug resistance etc. Large numbers of hypothetical proteins (HPs) have been identified. Therefore, it is important to carry out analysis of hypothetical proteins. This group is of utmost importance to complete genomic and proteomic information. There will be new structures with so far unknown conformations and new domains and motifs will be arising. New HPs may be serving as markers and pharmacological targets. Analysis of such proteins will enhance our understanding in designing and development of novel methods for diagnosis and treatment of *T. vaginalis* infection.

Keywords

T. vaginalis, hypothetical proteins, Ankyrin repeat

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Introduction

Trichomonas vaginalis is a unicellular protozoan described first by Donne in 1836[1]. Infection with *T. vaginalis* causes trichomoniasis, number one nonviral and second most sexually transmitted disease (STD) and for women with 250 million infections in the world each year this is associated with high rate of morbidity[2]. The disease is characterized by vulvovaginitis, cervicitis, and urethritis. This may be associated dysuria, dyspareunia, and abdominal pain. Cervical and Prostate cancer is a major gynecological cancer in developing countries like India. *T. vaginalis* is transmitted mostly by sexual contact. It is a cause of urethritis and prostate cancer in men and both urethritis and vaginitis in women. Adverse consequences to women with trichomoniasis include enhanced risk for human immunodeficiency virus transmission which plays an important role in HIV transmission dynamics other complications resulting from infection are cervical cancer and bad pregnancy outcomes[3-4]. The genome sequence of the *Trichomonas vaginalis*, a sexually transmitted human pathogen has been published. But its genome presents many unusual genomic and biochemical features like, exceptionally large genome size and the presence of hydrogenosome[5]. At present around 50,539 total proteins are present in UNIPROTKB database out of which 39,083 protein sequences are available as putative uncharacterized proteins which are hypothetical proteins in the *Trichomonas vaginalis* proteome. These are proteins predicted from nucleic acid sequences only and protein sequences with unknown function with no experimental evidence about their *in vivo* expression. This opens the wide opportunity to explore the uncharacterized hypothetical proteome of the *Trichomonas vaginalis*[6]. Therefore the need of the hour is to carry out the annotation and characterization of rest of the *T. vaginalis* proteome by using bioinformatics approaches so that we can understand the various biological mechanisms involved in genome evolution, genome expansion, pathogenesis, drug resistance, metabolic pathways and related activities, etc. The putative uncharacterized proteins can be annotated with the sequence, signature, motif, pattern, protein-protein interaction, subcellular localization, signal peptide identifications, physicochemical properties and structural information with the help of different bioinformatics approaches[7]. This will help in data mining of the uncharacterized proteome of the *T. vaginalis*. Having more annotated information and characterization of the proteome will help in gaining insight into the various metabolic, gene regulatory mechanism and the functional aspects of this unique organism. Therefore, the present work involves the extensive use of bioinformatics tools for a complete annotation of the hypothetical protein.

A2EBP0_TRIVA in *T. vaginalis* as this hypothetical protein was predicted as essential protein. Bioinformatics approach includes BLAST searches to determine identities/similarities

or homologies to known proteins, the protein sequence is examined for the presence of functional domains using databases PROSITE, PRINTS, InterPro, ProDom, Pfam and SMART, subjected to searches for motifs and protein secondary structure prediction[8].

Materials and methods

The hypothetical proteins data set was retrieved from UNIPROTKB database (www.expasy.org). Proteins were categorized according to their amino acid length. Database of Essential Genes (http://www.essentialgene.org/) was searched with BLAST program[9] (http://blast.ncbi.nlm.nih.gov/Blast) to filter essential genes. As a case study, A2EBP0_TRIVA found to be essential protein was selected for *in silico* analysis and characterization. The physicochemical properties of the selected protein were calculated by ProtParam (web.expasy.org/protparam), motif prediction by motif search (www.genome.jp/tools/motif/), secondary structures prediction by GOR IV method[10], predicting Pfam families (http://pfam.sanger.ac.uk/), conserved domain identification by (NCBI CD search tool). The subcellular localization was predicted by Psort II (http://psort.hgc.jp/cgi-bin/runpsort.pl).

Results and discussion

At present around 50,539 total proteins are present in UNIPROTKB database out of which 39,083 protein sequences are available as putative uncharacterized proteins which are hypothetical proteins in the *Trichomonas vaginalis* proteome. Protein A2EBP0_TRIVA was identified as one of the essential proteinsusing database of essential gene. The various

Table 1. Physicochemical properties of A2EBP0_TRIVA

Physicochemical properties	**A2EBP0_TRIVA**
Number of amino acids	412
Molecular weight	48016.8
Theoretical pI	6.20
negatively charged	51
positively charged	46
Extinction coefficients	44990
Estimated half-life	30 hours
Instability index	39.53
Aliphatic index	85.70
Grand average of hydropathicity	-0.224

physiochemical properties were calculated Table 1. Hypothetical proteins sequences were classified according to amino acid length. Maximum number of protein sequences were of length between 100 to 500 amino acids (29852), 4383 proteins sequences were having length less than 100 and therefore this group of proteins sequences can be excluded from further analysis as they are insignificant from functional point of view Figure 1 (number of hypothetical protein vs range of amino acid length). The protein A2EBP0_TRIVA is a stable protein as its instability index is less than 40. The amino acid composition involves major portion Ile and Leu (9.0%) Phe (8.5%), Lys (8.0 %), Ser (7.5%), Asn (7.0%) and Glu while Tpr was minimum (0.2%) Table 2.

The secondary structure of A2EBP0_TRIVA predicted by GOR4 contains Alpha helix 33.74% (least frequently occurred), Extended Strand 16.02% and Random coil 50.24% (most frequently occurred). The cytoplasmic subcellular localization was predicted by PROSA II as its possibility was very high 65.2%.

Two motifs of different length were identified in protein A2EBP0_TRIVA and both of them represent the ankyrin repeat region Table 3.

By using different tools for protein A2EBP0_TRIVA, the Ankyrin repeats family and An-

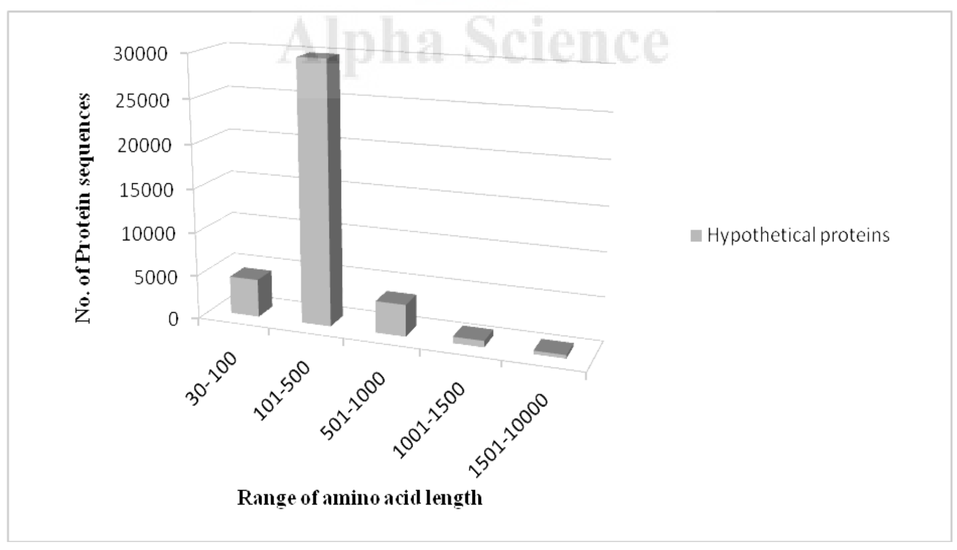

Figure 1. Hypothetical protein sequences in *T. vaginalis* proteome

Table 2. Amino acid composition of A2EBP0_TRIVA

S. No	Amino Acid	Amino Acid(%)
1	Ile	9.0%
2	Leu	9.0%
3	Phe	8.5%
4	Lys	8.0%
5	Ser	7.5%
6	Glu	7.0%
7	Asn	7.0%
8	Tyr	6.3%
9	Asp	5.3%
10	Ala	5.1%
11	Gly	4.4%
12	Arg	3.2%
13	Thr	3.2%
14	Cys	2.9%
15	Gln	2.9%
16	Pro	2.4%
17	His	2.9%
18	Met	1.5%
19	Trp	0.2%
20	Val	3.6%

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Table 3. Motifs predicted for A2EBP0_TRIVA

S.No	Motif found	Motif Position	Description
1	SVLEYFISNGCNINAKENKFKRTALHIAVTTGNKE-LIELLLYKGANVHSKDSYGEKSLSYAVGLHYNKIVETLIKFGADYTSKDIIGAALFAAARSNNYDVAEFLILNGANVNI	247..400	Ankyrin repeat region
2	FKRTALHIAVTTGNKELIELLLYKGANVHSKDS	306..338	Ankyrin repeat region

Table 4. Domain and family predicted for A2EBP0_TRIVA

S.No.	Protein	Pfam	NCBI Conserved Domain	ProDom
1.	A2EBP0_TRIVA	Ankyrin repeats	Ankyrin repeats	Ankyrin repeats

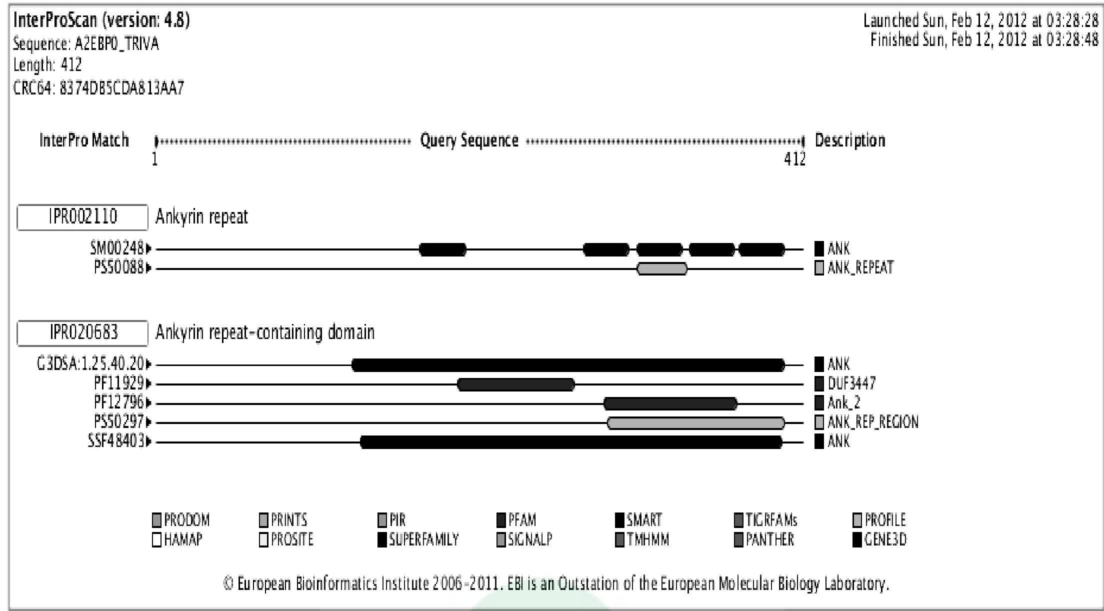

Figure 2. Domain predicted by InterProScan in A2EBP0_TRIVA

kyrin domain was predicted Figure2 and Table 4.

Ankyrin (ANK) repeats are about 33 amino acids long and occur in at least three consecutive copies. They are involved in protein-protein interactions. The core of the repeat seems to be a helix-loop-helix structure. The ankyrin repeat is one of the most common protein-protein interaction motifs in nature[11]. ANK repeat occurred in three consecutive copies along the entire length of the potein in an overlapping fashion (Figure 2). One feature of the internal repeats is a predicted central hydrophobic alpha helix, which is likely to interact with other repeats. Ankyrin repeats occur in a large number of functionally diverse proteins mainly from eukaryotes. The repeat has been found in proteins of diverse function such as transcriptional initiators, cell-cycle regulators, cytoskeletal, ion transporters and signal transducers[12]. Therefore, identification of Ankyrin domain and Ankyrin motif in A2EBP0_TRIVA indicate active role of this protein in *T. vaginalis.* Moreover since A2EBP0_TRIVA was also identified as essential protein for *T. vaginalis* it can also be treated as a pharmacologically significant protein target[13-14]. Similar analysis can also be carried out with other hypothetical proteins to complete genomic and proteomic information. This will not only offer new motifs, new structures and new domains but also new functions. Analysis of such proteins will help in the identification of novel drug target or for

the vaccine designing.

Conclusion

Prediction of hypothetical protein A2EBP0_TRIVA as an essential proteins having significant Ankyrin (ANK) repeats reflects the applications of bioinformatics approaches in the characterization and annotation of hypothetical proteins. *In silico* analysis and function prediction of hypothetical proteins would be of benefit to genomics and proteomics enabling the discovery of so far unknown or even predicted genes. The characterized essential and non essential hypothetical proteins may be serving as markers and pharmacological targets for the further research. Also the experimental characterization of these proteins is expected to reveal new, crucial aspects of *T. vaginalis* and could also lead to better functional prediction for medically relevant hypothetical proteins.

Acknowledgement

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Chapter 23

Studies of host-pathogen interaction in economically important plants using computational systems biology approach

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Abstract

Infections from pathogens are a major bottleneck in crop yields and animal stocks and causes great damage to the worldwide economy. The complex mechanisms by which pathogens evade the host defense system and re-program the host physiology to support colonization and reproduction is unclear. Therefore, understanding host-pathogen interactions is important both socially and a scientifically. Recent advances have given us exquisite detailed views of host-pathogen interactions; novel computational and analytical approaches have come up which are necessary to appreciate the vast amount of data being generated. Genome sequencing on pathogens and related microbes was a pioneering step. Plant epidemiologists should be more proactive in exploring potential applications of their concepts and procedures in rapidly expanding disciplines such as statistical genetics or bioinformatics. This review is about the novel computational, theoretical and computation-driven experimental approaches for studying host-pathogen interactions, and to illuminate points of synergy between different approaches.

Keywords

Pathogen, plant, expression, system, profiling

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Introduction

A major cause of human disease and reduction of crop yield and animal stocks are infections caused by pathogens and thus cause immense economical damage worldwide. In the ever changing environmental conditions, significance of infectious diseases is expected to grow since the pathogens can gain easy access to new hosts and ecological niches. From a systems perspective, the complex mechanisms by which a host is evaded by a pathogen and causes different changes by reprogramming the host physiology is very incompletely understood. Thus, understanding of host-pathogen interaction is important both socially and scientifically.

Recently many rapid advances have occurred on certain aspects of plant pathogenic bacteria but despite that many economically important pathosystems are still unexplored. The latent infection, end-stage disease, survival of the host and interactions among multiple microbes in a plant are very little known and understood. The systems biology research eras have successfully introduced tools of disease physiology, single gene molecular genetics and genomics for the study of molecular factors enabling microbial pathogens to cause plant diseases. A unified model of interactions of biotrophic and hemibiotrophic pathogens emerged from this work which revealed that successful pathogens mainly defeat two levels of plant defense by translocating cytoplasmic effectors that suppress the first defense (surface arrayed against microbial signatures) while evading the second defense (internally arrayed against effectors). As predicted from this model and confirmed by sequence pattern discovery of large number of cytoplasmic effectors in the genomes of many pathogens, the co-evolution of (hemi) biotrophic pathogens and their hosts has generated pathosystems featuring extreme complexity. These findings have shown the need of a fourth research era of systems biology in which virulence factors are studied as pathosystem components.

Methods

Host-pathogen interactions are dynamic and environmental stresses like humidity, temperature, drought stress have significant effects but they are largely unknown. Rhizospheres and leaf surfaces support complex communities of microbes that are mostly uncultured and undescribed as has been shown by Metagenomics[1] and these affect the plant pathogen but they are generally ignored in experiments. Now by using extensive genomic and gene expression data and computational tools, these complex traits can also be modeled. An advancing field is Microarray-based profiling of pathogen gene under various conditions. Another way to measure the specific conditions that are experienced by a pathogen in plant is by using Biosensors. Some other promising methods are heavy isotope labeling, semi-robotic sample processing and ordered gene knockout collections[2].

mRNA profiling

Gene expression analysis by mRNA profiling is the most prominent and powerful tool in functional genomics. According to the experiment type that is the number of genes that are investigated in a single experiment, the gene expression analysis is divided into two categories: first category includes the analysis of a single gene at a time, whereas in the second category a global view of the genome and its transcript file is provided. Gene expression analysis on a single gene level are labor-intensive, time consuming and less informative compared to high-throughput screening methods. Single gene based expression analysis includes the techniques quantitative real time PCR (qRT-PCR) which are most widely used.

Global gene expression analysis

Quantitative and qualitative identification of differentially regulated genes and transcript profiles of a genome is very important. Transcript pattern analysis gives useful and valuable information for assessing the roles of novel sequences in an organism since comparison of expression pattern sequences of known genes with unknown genes might indicate the functional genomics of the unknown genes[3].

Transcript profile, pattern determination and identification of differentially related genes in plants can be done by various techniques such as serial analysis of gene expression (SAGE), DNA microarrays, differential display, cDNA fragment sizing combined amplified fragment length polymorphism, expressed sequence tag sequencing, differential screening of cDNA libraries and massively parallel signature sequencing. These techniques are basically divided into two classes; direct or indirect analysis. In direct analysis nucleotide sequencing and fragment sizing are involved whereas in indirect analysis, nucleic acid hybridization of mRNA or cDNA fragments is employed[4].

Microarray-based profiling

A wide range of plant defenses are generated due to interaction of plant-pathogen. In a recent development, microarray-based expression profiling method along with genomic and/ or ESTs data that is available for some plant species are used for characterization of plant-pathogenesis related responses. Though the expression profiling studies completed till date are small in number yet a number of genes have been identified. Similarities and differences between different defense signaling pathways have been revealed in initial expression profiling work. Whole genome arrays are now becoming available and many undefined interactions can be studied. Microarray-based profiling in association with other genomic tools are progressively used for the better understanding of plant-pathogen interactions and

the defense signaling pathways.

In microarray technique, hybridization occurs between probe and target. Probe are the specific genetic fragments that are fixed on a solid surface like a glass slide, which makes the array itself, and target is the labeled RNA or DNA strand in solution. DNA microarrays are a useful tool for the identification of the potential defense related genes. The technology is evolving constantly but the two basic types that are widely used are: cDNA microarrays and oligonucleotides-based arrays, these are most widely used[5]. Due to their relative simplicity, high throughput and comprehensive sampling capacity the DNA-microarray technique is suitable for monitoring of gene expression changes in the plant during plant-pathogen interaction. The main advantage of DNA microarray is that responses of thousands of genes can be studied simultaneously on a single treatment. Using expression profiling the differentially present mRNA species can be identified and hypothesized.

In cDNA microarrays, PCR amplified cDNA fragments are spotted on glass slides using arrayers, as probes. The designing of probe is very crucial aspect of DNA microarrays and they are designed by taking the sequence information from genomic or EST sequence databases and the probes are designed to complement the 3' end of expressed sequences. 25 bp oligonucleotides each constitute the probe pairs in a GeneChip, in which each pair has a perfect-match (PM) and mismatch (MM) probe. The MM and PM have identical sequences except that at the central base which functions as an internal control[6].

For oligonucleotides and cDNA arrays there are separate techniques for the construction of arrays and placement of probes on solid surface at specific locations. Light-directed synthesis is employed in GeneChips for the construction of high density arrays, this includes two techniques: photolithography and solid-phase DNA synthesis. The physical size of the array and the available lithographic resolution are the only factors which can limit the amount of nucleic acid information encoded on the array. Spotters or arrayers are used in cDNA microarrays for transferring the probe solution and placing them on specific regions of substrate.

The two types of microarrays also have different sample preparation and labeling techniques. In GeneChip, double stranded cDNA synthesis is done from isolated RNA which is then converted to cRNA via *in vitro* transcription and the cRNA is labeled with biotin during transcription. Whereas in cDNA microarrays, during the synthesis of cDNA from RNA, the cDNA are labeled with fluorescent dyes, Cy3 and Cy5[7].

Recently, a large number of genes have been identified as potent plant-defense related genes by carrying out only a small number of DNA microarray experiments. For example,

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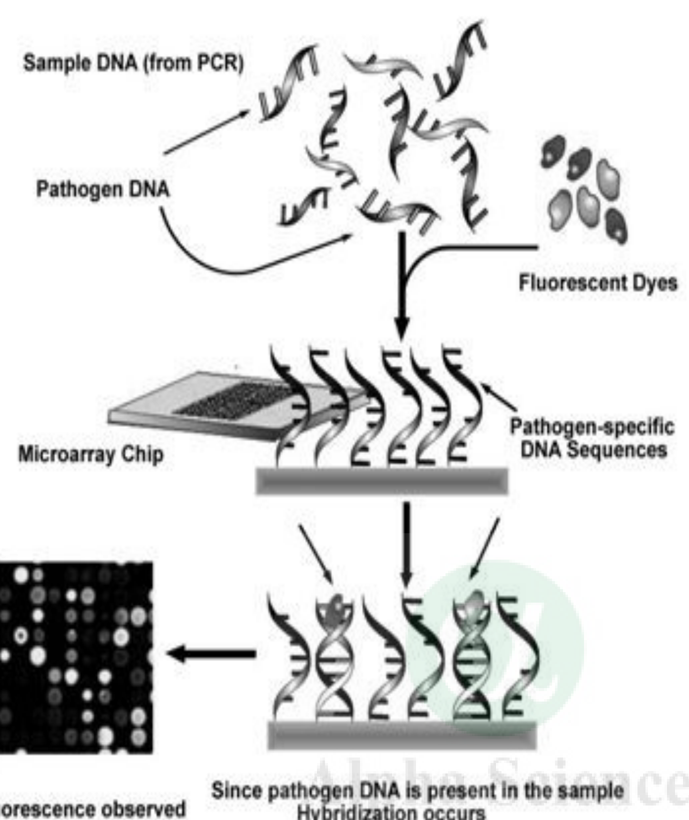

Figure 1 Simple representation of a microarray experiment

Baldwin *et al*[8], showed that the mRNA expression in maize altered 6 hour after various treatments with a fungal pathogen *Cochliobolus carbonum*, he used a maize DNA microarray representing 1,500 genes and identified 117 genes that consistently showed this alteration.

A particularly well developed experimental system is *Arabidopsis*. In an experimental study gene expression changes were studied under 14 different SAR-inducing or repressing conditions, 413 ESTs were identified that were associated with SAR. Also, expression analyses of crop plants such as rice, soyabean, wheat, potato, barley and tomato have been reported.

In the area of plant-pathogen interactions, expression profiling has provided better understanding of the various mechanisms that are in relation with plant-pathogen interactions such as host/non-host resistance, basal defense and specific gene resistance,

biotrophy/nectrotrophy and many others. Hence, genomic technologies, mainly expression profiling has facilitated the identification and interconnection of the unique features that are involved in the interactions of host and pathogen and provided us a system wide approach.

Conclusion

For the better understanding of host-pathogen interactions it is very important that the pathogenic microorganism is accurately identified and detected, this is a demanding requirement for pest management strategies and in clinical diagnostics. Traditionally, culture-based morphological approaches were the main tools for characterization but it is very time consuming. To increase the efficiency, multiplex expression profiling or microarray based profiling enabled the detection of several pathogens simultaneously. Though, this approach is an improvement over the conventional PCR-based assays but still there are serious limitations like targeting a conserved genome region will limit the analysis of similar but taxonomically defined pathogens. Recently, the microarray analysis combined with universal amplification is suggested as the best and unbiased approach for pathogen detection, but the sensitivity at present for diagnostic purposes is not sufficient and research needs to be done in future for increasing the sensitivity. Padlock probe's (PLPs), which are long oligonucleotides of approximately 100 bases whose ends are complimentary to adjacent target sequences, are used in combination with microarray-based profiling and it has provided a means for combining universal amplification and pathogen-specific molecular recognition.

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Not only in plants but in other organisms as well microarray based analysis is likely to be widely used to study different biological processes and provides a system wide approach of the biological processes. With the complete genomic sequence availability of *Arabidopsis* it is possible that an expression profile containing all ORF's of this plant is built and the effect of different pathogens can be studied taking the abiotic stresses into consideration as well.

Expression analysis on crop plants for different pathogens like bacteria, fungi, viruses, nematode, oomycetes and in different abiotic conditions like water stress, high salinity, high temperature etc. have been carried out and the improvement of the sensitivity of these expression based analysis is the current future need in systems biology.

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Chapter 24

Symptomatic approach for disease treatment

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Abstract

Health information recording and clinical data repositories help to produce immediate access to patient diagnoses, allergies, and lab test results that enable better and time-efficient medical decisions. The Machine Learning (ML) field has gained its momentum in almost any domain of research and just recently has become a reliable tool in the medical domain. The empirical domain of automatic learning is used in tasks such as medical decision support, medical imaging, protein-protein interaction, extraction of medical knowledge, and for overall patient management care. ML is envisioned as a tool by which computer-based systems can be integrated in the healthcare field in order to get a better, more efficient medical care. This paper describes a ML-based methodology for building an application that is capable of identifying and disseminating healthcare information. It extracts sentences from published medical papers that mention diseases and treatments, and identifies semantic relations that exist between diseases and treatments. Our evaluation results for these tasks show that the proposed methodology obtains reliable outcomes that could be integrated in an application to be used in the medical care domain. The potential value of this paper stands in the ML settings that we propose and in the fact that we outperform previous results on the same data set. This can also be implemented using the CLOUD concepts for time efficiency.

Keywords

Diseases, health, healthcare, machine learning

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Introduction

Now a day's people care deeply about their health and want to be more securely prevented from them. The medicine that is practiced today is an *Evidence Based Medicine* (EBM) – in this medical expertise is not only based on years of practice and also on latest discoveries. Google Health and Microsoft Health Vault make people to know more details about their health care and management. This health care system is working on Internet. *Electronic Health Records* (EHR) are becoming the standard in health care domain. The benefits of having EHR system are;

Health information recording and clinical data repositories– Immediate access to patient's diagnosis and lab results that enable accurate and time efficient medical decisions.

Medication management– Rapid access to information regarding drug reaction, immunization etc.

Decision support – Provide ability to capture and use quality medicine.

This EHR needs better, fast and more reliable access to information. In medical domain the richest and most used source of information is medicine, a database which contains extensive life science published articles. The maintenance and updating process are difficult. Our paper focuses on two tasks:

Automatically identifying sentence : From Medline

Automatically identifying semantic relation: Relation between Disease and Treatment

Focuses on 3 semantic relation **:** Cure, Prevent and Side Effect.

This framework identifies and disseminates health care information which is fast and reliable, the people wants, depend on their workflow and habits.

Our objective for this work is to show what Natural Language Processing (NLP) and Machine Learning (ML) techniques– what representation of information and what classification algorithms are suitable to use for identifying and classifying relevant medical information in short text. In our research, we focus on disease and information and their relation that exists between these two entities. The patients are mainly focused on the beneficial treatment & medicine and they don't care about the side effects. So in order to get awareness the information by health care providers is up-to-date. In order to provide better and efficient tool we use both NLP and ML approach, similar to an RSS feed, capable to identify and disseminate textual information related to disease and treatment. This study is aim that designing and examining various techniques in combination with various learning methods to identify and extract biomedical relations from literature.

To provide fast reliable result, it is better to identify and eliminate first the sentence that don't contain relevant information and then classify the rest of the sentence by relations of interest, instead of doing everything in one step by classifying sentence into one of the relations of Internet.

Related work

The most relevant related work is the work done by Rosario and Hearst[1]. The authors of this paper are the ones who created and distributed the data set used in our research. The data set consists of sentences from Medline5 abstracts annotated with disease and treatment entities and with eight semantic relations between diseases and treatments. The main focus of their work is on entity recognition for diseases and treatments. The authors use Hidden Markov Models and maximum entropy models to perform both the task of entity recognition and the relation discrimination.

There are three major approaches used in extracting Relations between entities: co-occurrences analysis, rule-based approaches, and statistical methods. The co-occurrences methods are mostly based only on lexical knowledge and words in context, and even though they tend to obtain good levels of recall, their precision is low. Good representative examples of work on Medline abstracts include Jenssen *et al.*[2] and Stapley and Benoit[3].

In biomedical literature, rule-based approaches have been widely used for solving relation extraction tasks. The main sources of information used by this technique are either syntactic: part-of-speech (POS) and syntactic structures; or semantic information in the form of fixed patterns that contain words that trigger a certain relation. One of the drawbacks of using methods based on rules is that they tend to require more human-expert effort than data driven methods (though human effort is needed in data driven methods too, to label the data). The best rule-based systems are the ones that use rules constructed manually or semiautomatically extracted automatically and refined manually. A positive aspect of rule-based systems is the fact that they obtain good precision results, while the recall levels tend to be low.

Syntactic rule-based relation extraction systems are complex systems based on additional tools used to assign POS tags or to extract syntactic parse trees. It is known that in the biomedical literature such tools are not yet at the state-of-the-art level as they are for general English texts, and therefore their performance on sentences is not always the best[4]. Representative works on syntactic rule-based approaches for

relation extraction in Medline abstracts and full-text articles are presented by Thomas *et al.*[5], Yakushiji *et al.*[6], and Leroy *et al.*[7]. Even though the syntactic information is the result of tools that are not 100 percent accurate, success stories with these types of systems have been encountered in the biomedical domain. The winner of the BioCreative II.57 task was a syntactic rule-based system, OpenDMAP described in Hunter *et al.*[8]. A good comparison of different syntactic parsers and their contribution to extracting protein-protein interactions can be found in Miyao *et al.*[9].

Various learning algorithms have been used for the statistical learning approach with kernel methods being the popular ones applied to Medline abstracts.

The semantic rule-based approaches suffer from the fact that the lexicon changes from domain to domain, and new rules need to be created each time. Certain rules are created for biological corpora, medical corpora, pharmaceutical corpora, etc. Systems based on semantic rules applied to full-text articles are described by Friedman *et al.*[10], on sentences by Pustejovsky *et al.*[11], and on abstracts by Rindflesch *et al.*[12.] Some researchers combined syntactic and semantic rules from Medline abstracts in order to obtain better systems with the flexibility of the syntactic information and the good precision of the semantic rules, e.g., Gaizauskas *et al.*[13] and Novichkova *et al.*[14].

Statistical methods tend to be used to solve various NLP tasks when annotated corpora are available. Rules are automatically extracted by the learning algorithm when using statistical approaches to solve various tasks. In general, statistical techniques can perform well even with little training data. For extracting relations, the rules are used to determine if a textual input contains a relation or not. Taking a statistical approach to solve the relation extraction problem from abstracts, the most used representation technique is bag-of-words. It uses the words in context to create a feature vector[15,16]. Other researchers combined the bag-of-words features, extracted from sentences, with other sources of information like POS[17,18] used two sources of information: sentences in which the relation appears and the local context of the entities, and showed that simple representa-tion techniques bring good results.

The proposed approach

Tasks and Data Sets

The two tasks that are undertaken in this paper provide the basis for the design of an information technology frame-work that is capable to identify and disseminate

healthcare information. The first task identifies and extracts informative sentences on diseases and treatments topics, while the second one performs a finer grained classification of these sentences according to the semantic relations that exists between diseases and treatments.

The first task (task 1 or sentence selection) identifies sentences from Medline published abstracts that talk about diseases and treatments. The task is similar to a scan of sentences contained in the abstract of an article in order to present to the user-only sentences that are identified as containing relevant information (disease treatment information).

The second task (task 2 or relation identification) has a deeper semantic dimension and it is focused on identifying disease-treatment relations in the sentences already selected as being informative (e.g., task 1 is applied first). We focus on three relations: Cure, Prevent, and Side Effect, a subset of the eight relations that the corpus is annotated with.

We decided to focus on these three relations because these are most represented in the corpus while for the other five, very few examples are available. Table 1 presents the original data set, the one used by Rosario and Hearst[1], that we also use in our research. The numbers in parentheses represent the training and test set size. For example, for Cure relation, out of 810 sentences present in the data set, 648 are used for training and 162 for testing.

The approach used to solve the two proposed tasks is based on NLP and ML techniques. In a standard supervised ML setting, a training set and a test set are required. The training set is used to train the ML algorithm and the test set to test its performance. The objectives are to build models that can later be deployed on other test sets with high performance.

For the first task, the data sets are annotated with the following information: a label indicating that the sentence is informative, i.e. containing disease-treatment information, or a label indicating that the sentence is not informative. For the second task, the sentences have annotation information that states if the relation that exists in a sentence between the disease and treatment is Cure, Prevent, or Side Effect. These are the relations that are more represented in the original data set and also needed for our future research. We would like to focus on a few relations of interest and try to identify what predictive model and representation technique bring the best results. The task of identifying the three semantic relations is addressed in two ways:

Table 1: Data Set Description, Taken from Rosario and Hearst ('04)

Relationship	Definition and example
Cure 810 (648, 162)	TREAT cures DIS *intravenous immune globulin for recurrent spontaneous abortion*
Only DIS 616 (492, 124)	TREAT not mentioned *Social ties and susceptibility to common cold*
Only TREAT 166 (132, 34)	DIS not mentioned *Flucticasome propionate is safe in recommended doses*
Prevent 63 (50, 13)	TREAT prevents the DIS *Statins for prevention of stroke*
Vague 36 (28, 8)	Very unclear relationship *Phenylbutazone and leukemia*
Side effect 29 (24, 5)	DIS is a result of TREAT *Melignant mesodermal mixed tumor of the uterus following irradiation*
No Cure 4 (3, 1)	TREAT doesnot cure DIS *Evidence for double resistance to permethrin and malathion in head lice*
Total relevant: 1724 (1377. 347)	
Irrelevant 1771 (1416, 335)	TREAT and DIS not present *Patients were followed up for 6 months*
Total: 3495 (2793, 702)	

Setting1. Three models are built. Each model is focused on one relation and can distinguish sentences that contain the relation from sentences that do not. This setting is similar to a two-class classification task in which instances are labeled either with the relation in question (Positive label) or with nonrelevant information (Negative label);

Setting2. One model is built, to distinguish the three relations in a three-class classification task where each sentence is labeled with one of the semantic relations.

The pipeline is similar to a hierarchy of tasks in which the results of one task is given as input to the other. We believe that this can be a solution for identifying and disseminating relevant information tailored to a specific semantic relation because the second task is trying a finer grained classification of the sentences that already contain information about the relations of interest. This framework is appropriate for consumers that tend to be more interested in an end result that is more specific, e.g., relevant information only for the class Cure, rather than identifying sentences that have the potential to be informative for a wider variety of disease-treatment semantic relations.

Classification algorithms and data representations

In ML, as a field of empirical studies, the acquired expertise and knowledge from previous research guide the way of solving new tasks. The models should be reliable at identifying informative sentences and discriminating disease-treatment semantic relations. The research experiments need to be guided such that high performance is obtained. The experimental settings are directed such that they are adapted to the domain of study (medical knowledge) and to the type of data we deal with (short texts or sentences), allowing for the methods to bring improved performance.

Evaluation and results

This section discusses the evaluation measures and presents the results of the two tasks using the methodology described above.

Evaluation Measures

The most common used evaluation measures in the ML settings are: accuracy, precision, recall, and F-measure. All these measures are computed form a confusion matrix[19] that contains information about the actual classes, the true classes and the classes predicted by the classifier.

The test set on which the models are evaluated contain the true classes and the evaluation tries to identify how many of the true classes were predicted by the model classifier. In the ML settings, special attention needs to be directed to the evaluation measures that are used. For data sets that are highly imbalanced (one class is overrepresented in comparison with another), standard evaluation measures like accuracy are not suitable. Because our data sets are imbalanced, we chose to report in addition to accuracy, the macro averaged F-measure. We decided to report macro and not micro averaged F-measure because the macro measure is not influenced by the majority class. The macro measure better focuses on the performance the classified has on the minority classes. The formulas for the evaluation measures are: Accuracy ¼ the total number of correctly classified instances; Recall ¼ the ratio of correctly classified positive instances to the total number of positives. This evaluation measure is known to the medical research community as sensitivity. P recision ¼ the ratio of correctly classified positive instances to the total number of classified as positive. F -measure ¼ the harmonic mean between precision and recall.

Results for the Task of Identifying Informative Sentences (Task 1)

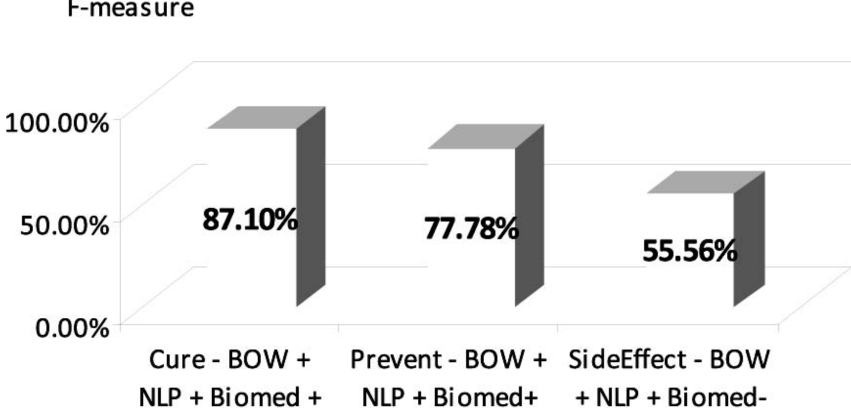

Fig.1: Results for Setting 1

This section presents the results for the first task, the one of identifying whether sentences are informative, i.e., containing information about diseases and treatments, or not. The ML settings are created for a two-class classification task and the representations are the ones mentioned in the previous section, while the baseline on which we need to improve is given by the results of a classifier that always predicts the majority class.

Fig. 1 presents the results obtained when using as representation features verb-phrases identified by the Genia tagger. When using this representation, the results are close to baseline. The reason why this happens for all algorithms that we use is the fact that the texts are short and the selected features are not well represented in an instance. We have a data sparseness problem: it is the case when a lot of features have value 0 for a particular instance.

Fig. 2 presents the results obtained using as representation features noun-phrases selected by the Genia tagger. Compared to previous results, we can observe a slight improvement in both accuracy and F-measure. The best results are obtained by the CNB classifier. We believe that the slight improvement is due to a reduction of the sparseness problem: noun-phrases are more frequently present in short texts than verb-phrases.

Fig. 3 presents the best results obtained so far. An increase of almost 5 percentage

points, for both accuracy and F-measure is obtained when using as representation features biomedical entities extracted by the Genia tagger and CNB as classifier. An increase in results for the other classifiers can be also observed.

Results for the Task of Identifying Semantic Relations (Task 2)

The focus for the second task is to automatically identify which sentences contain information for the three semantic relations: Cure, Prevent, and Side Effect. The reported results are based on similar settings to the ones used for the previous task.

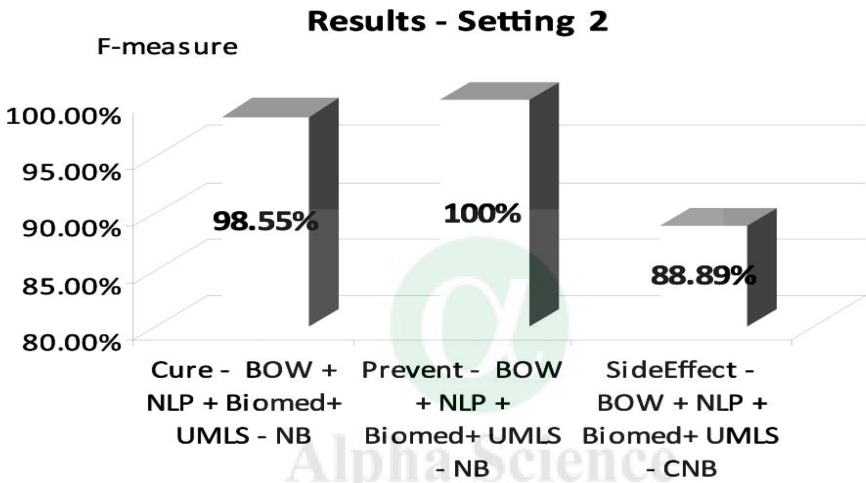

Fig.2: Results for the second task, Setting 2

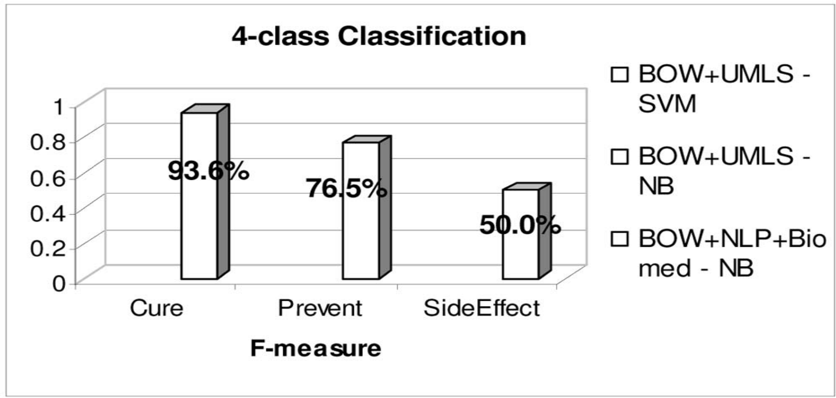

Fig.3: F-measure results for four-class classification

Since imbalanced data sets are used for this task, the evaluation measure that we are going to report is the F-measure. Due to space issues, we are going to present the best results obtained for all settings. The best results are chosen from all the representation techniques and all classification algorithms that we also used for the first task. The labels on the x-axis stand for the name of the semantic relation, the representation technique, and the classification algorithm used.

In Fig. 1, we present the results when using Setting 1,described in Section 3.1, as the setup for the experiment. On the x-axis, we present for each relation the best F-measure result, the representation technique, and the classifier that obtained the result. For example, for the Cure relation, the combination of BOW features, noun-phrases and verb-phrases, biomedical and UMLS concepts, with SVM as a classifier, obtained the 87.10 percent result for F-measure. SVM and NB with rich feature representations are the setups that obtained the best results. Fig. 2 presents the best results that we obtain for the second task, a level of almost 100 percent F-measure for the Cure relation, 100 percent F-measure for Prevent relation, and 75 percent F-measure for Side Effect. For this setting, we train a model for all three relations in the same time, and we distinguish sentences between these three relations.

For this setting, the NB classifier with combinations of various representation features is the one that obtains the best results for all relations. The improvement over the other settings can be due to the fact that the combination of classifier and features has a good predicting value for a model trained on the three relations. Each of the relations can be well-defined and predicted when using the model that we propose in Setting 2. The fact that we achieve close to perfection prediction suggests that the choice of classifier and representation technique are key factors for a supervised classification task, even for semantically charged tasks like ours.

The good performance results that we obtain with the second setting also suggest that a prior triage of sentences, informative versus noninformative can be crucial for a finer grained classification of relations between entities. Setting 1 uses all the sentences, including those that do not contain information about the three relations of interests, while in Setting 2, we used as training data only sentences that we knew a priori to contain one of the three relations. This observation for the results of the second setting also validates our choice of proposing the first task, identify which sentences are informative and which not. For good performance level in the relation classification task, we need to weed out noninformative sentences.

Results for the Pipeline—Task 1 Followed by Task 2

In this section, we present the evaluation results for the pipeline of the two tasks. When looking at the results that we obtain for the second task, the best setting was the one in which we classify sentences already known to be informative (Setting 2). This observation let us believe that a pipeline of the two tasks is a viable solution for our goal.

To show that a pipeline of results is better as a solution for identifying semantic relations in informative sentences, we need to compare its results to the results of a model that classifies sentences into four-classes directly: the three semantic relations Cure, Prevent, Side Effect and the class for sentences that are uninformative.

The results for the pipeline of tasks are obtained by multiplying the evaluation measures acquired by the first task with the evaluation measure for the second task for each semantic relation. To be consistent, we report the F- measure results. For the first task, the best F-measure result of 90.72 percent is obtained by the CNB classifier using a combination of all types of features (Fig. 1). For the second task, the best F-measure results are obtained by the NB classifier using a combination of all types of features for all three semantic relations (Fig. 2).

Discussion

This section discusses the results we obtained for the two tasks in this study. For the first task, the one of identifying informative sentences, the results show that probabilistic models based on Naïve Bayes formula, obtain good results. The fact that the SVM classifier performs well shows that the current discoveries are in line with the literature. These two classifiers have always been shown to perform well on text classification tasks. Even though the independence of features is violated when using Naïve Bayes classifiers, they still perform very well. The AdaBoost classifier was outperformed by the other classifiers, which is little surprising taking into account the fact that it is designed to focus on hard-to-learn concepts. In our previous experience, it was shown to perform well on medical domain texts with imbalanced classes[7]. One reason why the AdaBoost classifier did not perform well might be that fact that in previous experiments we used the entire abstract as source of information while in this current study we use sentences.

Conclusions and future work

The conclusions of our study suggest that domain-specific knowledge improves the results. Probabilistic models are stable and reliable for tasks performed on short texts in the medical domain. The representation techniques influence the results of

the ML algorithms, but more informative representations are the ones that consistently obtain the best results.

The first task that we tackle in this paper is a task that has applications in information retrieval, information extraction, and text summarization. We identify potential improvements in results when more information is brought in the representation technique for the task of classifying short medical texts. We show that the simple BOW approach, well known to give reliable results on text classification tasks, can be significantly outperformed when adding more complex and structured information from various ontologies.

The second task that we address can be viewed as a task that could benefit from solving the first task first. In this study, we have focused on three semantic relations between diseases and treatments. Our work shows that the best results are obtained when the classifier is not overwhelmed by sentences that are not related to the task. Also, to perform a triage of the sentences (task 1) for a relation classification task is an important step. In Setting 1, we included the sentences that did not contain any of the three relations in question and the results were lower than the one when we used models trained only on sentences containing the three relations of interest. These discoveries validate the fact that it is crucial to have the first step to weed out uninformative sentences, before looking deeper into classifying them. Similar findings and conclusions can be made for the representation and classification techniques for task 2.

The above observations support the pipeline of tasks that we propose in this work. The improvement in results of 14 and 18 percentage points that we obtain for two of the classes in question shows that a framework in which tasks 1 and 2 are used in pipeline is superior to when the two tasks are solved in one step by a four-way classification.

CLOUD

In our approach we are creating an azure i.e., private cloud which contains complete details regarding the disease, treatment and side effects. As it contains multiple databases it is very easier to get an accurate result than the search in Internet. These items which contains complete details regarding the disease, treatment and side effects. As it contains multiple databases it is very easier to get an accurate result than the search in Internet are highly valuable to both the normal humans and more effective for the doctors to prescribe best and common medicines and treatment. In this approach, the normal persons also get details

regarding their disease with their symptoms and find out the corresponding treatment and side effects.

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Chapter 25

Object oriented programming for different simulation and emulation

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Abstract

Teaching Biomedical Instrumentation as a subject has been a tough task, especially for the undergraduate courses. But now, since the computer technology is in its advent, and also along with the help of object oriented programming in educational laboratories, it seems the task is getting easier. We are coming even closer for the imitation of these systems. This paper would describe the program in biomedical instrumentation wherever object oriented programming is brought to use. The course explains, widens and emulates the indispensable biomedical instrumentation systems.

The use of computers to carry out the circuit simulation is especially frequent in both the universities and industries. The processor simulations attaches toward to the inside of a data file from the keyboard into a computer and having it in the working out of the results on the predefined structures. These simulations have been established as extremely useful for saving of both time and money, but it has certain restrictions as well. Circuit emulation is a straightforward difference on the subject matter of simulation where the computer system is made to operate in real time in receipt of data continually. These emulation systems are sometimes referred as the virtual instruments so as to keep away from misunderstanding between the words simulation and emulation.

Keywords

Biomedical, emulation, simulation, object oriented programming

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Introduction

The simulation through a computer system typically involves ingoing data file via the keyboard to the computer and calculating fallouts on sets of predefined structures. These simulations have established extremely of use for economy in terms of together time and money.

This paper here presents how virtual instruments could be formed by means of object-oriented programming and applied to Biomedical Instrumentation systems. A subject course using these notions is introduced at the Indian Institute of Technology, Delhi.

The call for electronic instrumentation in the medical field has been getting higher at a swift rate of knots. All sorts of laboratories along with hospitals as well as the physician's offices are getting reliant in the lead to the rapid high quality information from electronic instruments. This data may perhaps be indispensable unprocessed data or may be deduced outcomes. Large amounts of the data desired for elucidations are the measurements of fundamental electronic constraints like voltage, current, frequency, as well as resistance. Measurement of these parameters is fit in the capacity of the undergraduate engineering students.

The biomedical instrumentation curriculum at the Indian Institute of Technology, Delhi is prepared up of three elements: The Instrumentation, Simulation, and Control (ISC) Laboratory (the most current), The Physiology Minor (a wide minor at the university), and the Biomedical Instrumentation course (which this paper is about).

The ISC Laboratory has twelve Macintosh-11 based stations outfitted all (as the least) with a National Instruments (NI) MIO-16 data acquisition (DAQ) card, an interface box, and software packages. The DAQ card makes available eight differential analog inputs, 2 analog outputs, 3 programmable timers and 8 digital inputs or outputs. The Biomedical Instrumentation subject is structured into four sections: (1) Living systems: the structure and function of the major body systems; (2) Transducers: the patient instrument interface; (3) Signal processing: LabVIEW and Instrument emulation; (The use of LabVIEW and ISC Lab is a major change), (4) The user: the instrument-user interface.

Living systems: this part has three chief segments: (a) a general idea of most important body systems in terms of structure, role, and computable structures (e.g. Muscular system - muscle cell structure - relationship between skeletal and nervous systems - movement reflex), (b) an outline of current measurement , practices and mechanisms (e.g. electrical and mechanical - ectromyograph and reflexometer), (c) A supplementary thorough conversation on the Cardiovascular (blood pressure = heart rate x cardiac output),

Respiratory (Volume *8* BTPS =[volume collected *8* T "C] x [273+37/273+T] x [PB - PH~O/ P B - 47]), along with the Sensory systems (eyes ,ears ,optical, sound, pressure characteristics).

Transducers: the patient - instrument interface is separated into three fundamental divisions: (a) a summary of main transducer kinds as connected to the above measurable factors like strain gauges and electrodes. (b) Patient-transducer interface (mechanical, electrical, and chemical). (c) Safety and device conventions.

Signal processing: With the initiation of software such as LabVIEW by the National Instruments (NI) it has happen to be probable to plan and emulate real instruments from input ends to output display with a PC form computer. This division of the course is separated in three chief subdivisions:

(a) An impression of essential medical signal processing method and necessities such as response times, units, and noise reduction , (b) An overview of LabVIEW along with virtual instruments (VI's) , (c) VI improvement and its uses.

The user: the instrument-user interface, the concluding section of the course, has three main divisions. (a) A synopsis of user interface necessities (e.g. user operating requirements, maintenance necessities, and operating atmosphere). (b) Use of VI's to build up enhanced user interfaces (c) Self-test and calibration needs.

Object-oriented programming

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Since there are novel open architectures for structuring instruments and processors with prevailing software tools, a question arises as of how resourceful could you be in this new virtual instrumentation age? Since the computer and virtual instrument revolutions are shifting technologies rapidly, a common user can't keep away from being completely flooded when he puts jointly together a new system. The response is the technology of object-oriented software that put a user requires to make virtual instruments and hardware in an integrated, user friendly atmosphere. We are observing an uprising which will see virtual instruments convert to a mainstream technique. Many environments have by now already accepted the industry-standard for creating VI's. Upcoming adaptations of several computer operating systems feature object-oriented architecture and file system in which modular applications all commune by means of object linking and any embedding language like COBOL.

The primary software employed in the ISC Laboratory is LabVIEW

LabVIEW uses graphical programming and VI's which perform at speeds similar to compiled C programs. A virtual instrument is basically a software unit packaged graphically

to enclose the look and sense of a physical instrument. A front panel acts as an interactive interface for input and output terminals. The block diagram decides the functions of the VI. By linking functional blocks an analyst could illustrate the diagram thus programming in a graphical language.

Since LabVIEW is conceptually very easy, one can focus on the basic substance of the test performed saving precious time on hectic toil and physical data sorting. The object-oriented programming of LabVIEW is easy to use and potent in process.

Figure 1 demonstrates a pH and temperature data acquisition and control system. The pH is observed by a pH transmitter (pHT) and the temperature is measured with a platinum RTD temperature transmitter (TT). The standard 4-20 mA outputs of the transmitters are converted into 1-5V signals, which are fed back to the analog input channels AI-1 and AI-2. A current shunt is placed in series with heater element to sample the heater current value.

Virtual instrument technology gives power to its designers to design the VI revolution, and object-oriented as much as necessary. The computer system is the key to making this technology user friendly.

Object-oriented programming though, is not everybody's C++. Researchers consent that the key to application-specific software is its frameworks. Such frameworks will allow users to work in a familiar environment customized to go with the tactics that they already use in their line of work to a certain extent than requiring wide-ranging special-purpose training. The features of a software structure are: Code reprocessing is utilized by a dependable, application - specific development methodology. Modular workings present a fine example

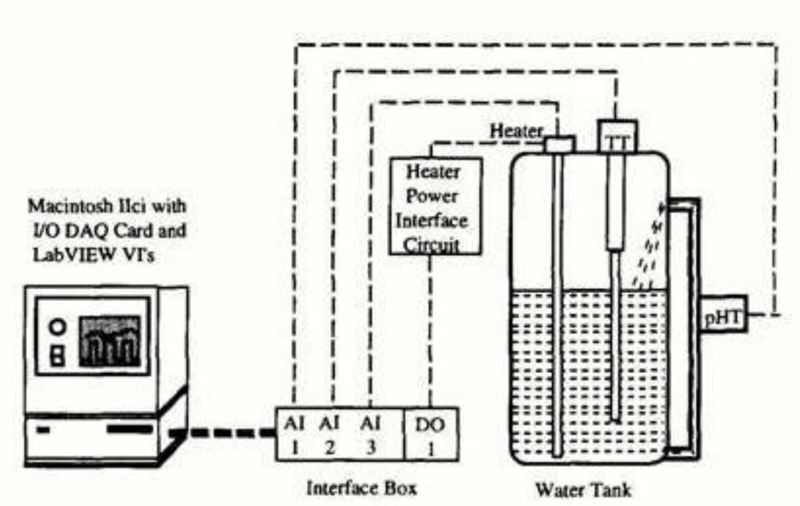

Figure 1. pH and Temperature Data Acquisition System

that demonstrates the plan of software frameworks in the form of a spreadsheet. A current shunt is placed in series with the heater element to sample the heater current. The voltage across this shunt is applied through an instrumentation amplifier to AI-3. The three sampled signals are influenced in software and shown on the "Front Panel" of the VI. To organize the heater from the computer an outer opto-isolated power interface circuit (Figure 2) is desired.

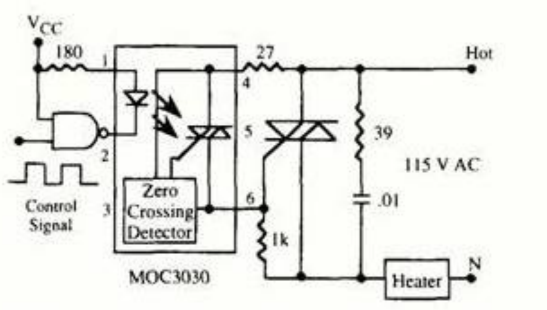

Figure 2: The heater power interface circuit

The circuit comprises of a MOC3030 triac-output optoisolator along a zero-crossing detector driving an NTE5635 triac, which is a 400-V, 10-A instrument. The input of the optocoupler is pulled by a NAND gate to offer a TTL level direct input for association to the computer. Designing a VI comprises of assembling the user-interface "hardware" on the front panel window and then wiring the opposite blocks in the diagram window.

LabVIEW takes in a well-thought-out group of user-interface hardware comprising of each plausible piece for building an instrument control panel (such as switches, knobs, indicators, and graph tools). The front panel consists of two strip charts, an x-y graph, two bar-graph indicators along with digital displays, a digital control to fine-tune the duty cycle of the heater current, and three push-button switches. Each set on the front panel has a pre-built counterpart (terminal) appearing in the diagram window. For instance, a switch has a true/ false icon with a logical output. The block diagram illustrates the entire computation along with its data and control flow of the VI through a graphical wiring diagram. Each VI is recognized by a symbol having a connector with terminals for input and output. A complete system may comprise of a main VI and a number of sub-VIS.

LabVIEW employs a data flow programming loom. The user can get through a number of programming objects for arithmetic, trigonometric, comparison, array, file I/O, and signal-analysis tasks. Five structures (For and While Loops, Case, Sequence, and Equation Node) are also additionally offered to manage looping and execution sequencing.

The For and While loops characteristically highlight auto-indexing for the purpose of data incoming and outgoing of the loop. Therefore, no further indexing is essential for the temperature and pH arrays built by the For loop and passed as the x and y arrays, respectively, to the x-y graph icon. There comes a spool tool which is used to "wire" the objects. A wire's pattern and color typically depends on the type of data it is carrying. A

scalar integer corresponds to a thin blue line, while arrays are represented by wider lines. The thickness of the line is proportional to the dimensions of the array.

The exercising of object-oriented programming software, such as LabVIEW, to emulate systems mainly in the laboratory permits the student to be extra imaginative in the design of an instrument. LabVIEW lets the student design and get familiar with the process of real systems at least expense. Quite a lot of examples of student work follow, the first set of examples will deal with temperature measurement and the second, with heart rate measurement. Each example shown consists of a project done with and without the use of LabVIEW.

Temperature Measurement and Control

A. Without LabVIEW The project was to design an instrument that can compute body temperature of an infant following subsequent typical medical standard. The project consisted of an AD590 temperature sensor and an instrumentation amplifier designed with built-in compensation for the sensor. A printed circuit board (PCB) was designed and made for the amplifier compensation unit. The output device was a digital voltmeter.

It took 4 students 5 weeks to research, acquire parts, build, and test the instrument.

B. With LabVIEW The project was to design an instrument to measure and control the heat of a standard carbon resistor.

The project consisted of an LM35, a temperature sensor from National Semiconductor, a Macintosh IIci computer running LabVIEW with an inbuilt NI DAQ board, an electric fan, and a 1/2 watt carbon resistor.

The LM35 detected the temperature of the resistor. The sensor output was attained by the interface unit and sent to the LabVIEW instrument emulation that changed the reading from the sensor to an accurate value, and further displayed the current and average temperature. A high temperature limit that restricted the fan used to keep the resistor's temperature at a set value. The project was finished in 5 weeks by 2 students. The project described earlier (figures 1-2) which was more complicated was completed in 7 weeks by 2 students.

Heart Rate Monitor

A. Without LabVIEW the project was intended to design an instrument to determine the heart rate of infant following typical medical conditions. The project comprised of a pair of ECG electrodes joined to an Instrumentation amplifier, and a single stage 0.05 to l00 Hz band-pass filter. The input stage of the Instrumentation amplifier consisted of a dual Bifet Op- Amp integrated circuit (IC) and the last stage and bandpass filter consisted of another

dual Op-Amp IC. The output of the amplifier was displayed on an oscilloscope (Note the ECG waveform was displayed just the heart rate. The students did not add rate signal processing). This project was conducted over a period of 5 weeks by 2 students. The circuit diagram is shown below in figure 3.

B. With LabVIEW the project was made to design an instrument to compute heart rate following typical medical specifications. The project consisted of an IR emitter-detector pair (Figure 4) and a Macintosh IIci computer running LabVIEW with an NI interface. The emitter-detector pair sensed the change in blood flow in the finger. This signal was acquired by the interface and made available to LabVIEW. Using LabVIEW a virtual instrument was created that sensed the incoming signal, determined when a true peak had occurred, counted the number of peaks in a 15 second period, multiplied by 4 to get beats per minute, and displayed the rate. Also there was a display of the actual waveform from the sensor. This project was completed by one student over a 10 week period. Shown below are the sensor

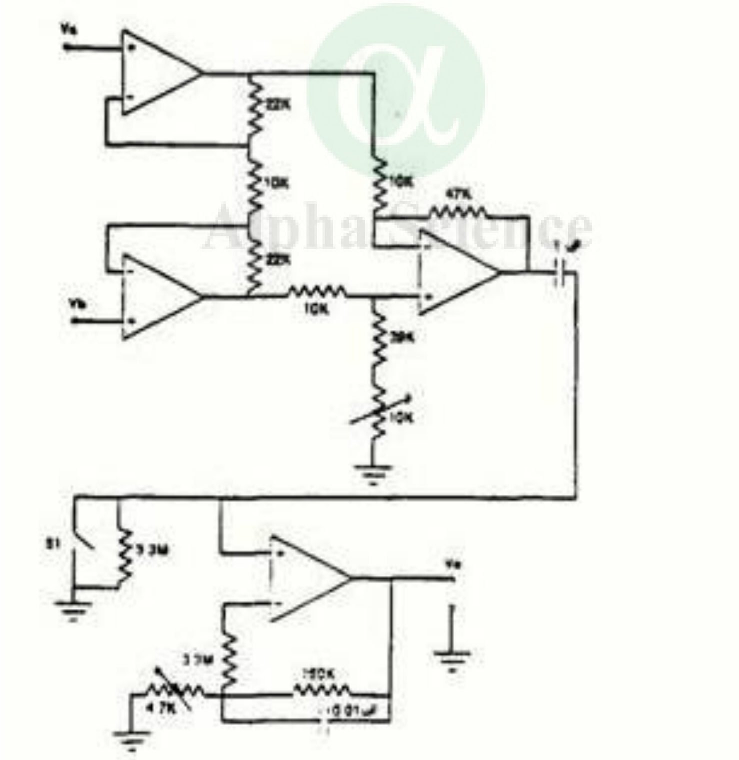

Figure 3: Heart rate circuit diagram

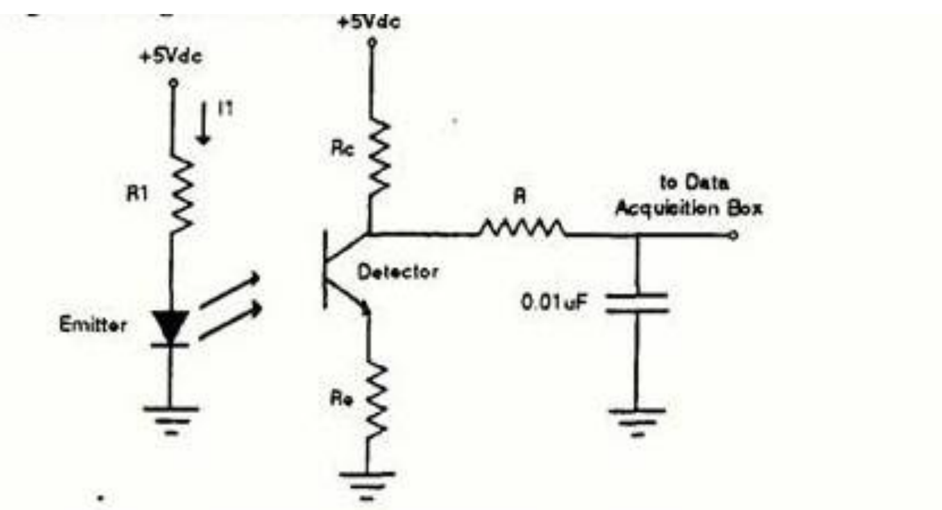

Figure 4: Heart rate sensor circuit diagram

and LabVIEW diagram (figure 4).

As is evident, without LabVIEW one is restricted to very simple systems with minimal signal processing, on the other hand with LabVIEW we are able to design instruments with complicated signal processing and output capabilities.

Conclusions

- We have started the process of developing a greater insight and more creativity in the design process. It is hoped that students will have better feeling for the interaction of all parts of a biomedical system as well as interaction with other systems both living and not.
- The ability to deliver effective engineering education can be greatly enhanced by laboratories equipped with computer hardware and software, virtual-reality simulators, and physical systems
- Even sophisticated software cannot completely cover replacing physical systems in undergraduate biomedical instrumentation education. Comparing software education models and virtual instruments to physical hardware can demonstrate important topics such as the effects of noise and nonlinearities.
- Used appropriately, modeling techniques can be very helpful in meeting the challenge of balancing the difference between software and hardware.

Object oriented programming-Rajhans.

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Chapter 26

Isolation and characterization of erythromycin resistant gram negative bacteria

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Abstract

In past decade, antibiotics found critical in fight against infectious disease caused by microbes. Increasing use, misuse of existing antibiotics in human, veterinary medicine and agriculture; may be reason of it. An alarming increase in penicillin resistant bacteria is already proved. Even multi-drug resistance has also evolved. This was the reason of choosing Erythromycin as work of interest. The brief methodologies used, were- isolation of gram negative bacteria of 2 water samples in Mac Conkey agar media; slant culture of isolated gram negative bacteria; isolation of Erythromycin resistant gram negative bacteria and its pure culture; serial dilution, plating and bacterial colony count of both samples; Plasmid DNA isolation of both samples and observation by agarose gel electrophoresis; Transformation of plasmid in host; plating of cells containing only competent host cells, on Erythromycin containing Luria agar plates to observe transformation. Both samples were transformed in all plates, containing erythromycin. But, no growth was observed in plate containing only competent host cell. This indicated some 'Erythromycin resistant bacteria' contamination to the region, from where water samples were collected. Also can say that antibiotic resistance property of these gram negative bacteria were present in its plasmid DNA.

Keywords

Gram negative bacteria, Morphological characterization, Plasmid isolation, Transformation

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Introduction

Antibiotics plays major role in fighting against infectious diseases caused by microbes. Antimicrobial chemotherapy is the leading cause for dramatic rise of average life expectancy now a days. These disease-causing microbes that become resistant to antibiotics are an increasing public health issue. Wound infection, gonorrhea, tuberculosis, pneumonia, septicemia are a few of diseases that become hard to treat with antibiotics. The problem is that bacteria and other infectious microbes are remarkably resilient and have developed several ways to resist antimicrobial drugs. Also, increasing use and misuse of antibiotics in human and veterinary medicine is an alarming cause of drug resistance. So, unless antibiotic resistance problems are detected as they emerge and actions are taken immediately to avoid it, society can face with previously treatable diseases would become again untreatable, as the days before antibiotics developed. We become exposed to antibiotic resistant bacteria from soil and water of nearby river, ponds, lands, uncooked foods, green vegetables, contaminated foods etc. The use of such kind of water for drinking, cooking etc. is dangerous for health. Moreover, the biomedical waste of hospital which ultimately goes to the pond, river etc will also be harmful and responsible for various types of diseases in human beings. Krutkiewicz *et al.*[1] carried their work on "Resistance to antimicrobial agents of *Campylobacter spp.* strains isolated from animals in Poland". They found that 91.7% were resistant to antimicrobial drugs- amoxicillin, ciprofloxacin, erythromycin, tetracycline and trimethoprim. X Li *et al.*[2] carried out "Emerging macrolide resistance in *Mycoplasma pneumoniae* in children: detection and characterization of resistant isolates". They identified macrolide-resistant *M. pneumoniae* infection in 2 seriously ill hospitalized children with community-acquired pneumonia by MIC testing. Macrolide-resistant *M. pneumoniae* from both children were found to carry an A2063G transition in 23S rRNA gene.

Erythromycin, an antibiotic- Erythromycin the macrolide antibiotic, displays bacteriocidal activity, particularly at higher concentrations by binding to 50s subunit of bacterial 70s rRNA complex and subsequently inhibiting protein synthesis and replication process. It also interferes in aminoacyl translocation, preventing the transfer of tRNA bound at A site of rRNA complex to P site of rRNA complex. Without this translocation, A site remain occupied and thus addition of incoming tRNA and its attached amino acid to nascent polypeptide chain is inhibited. This interferes with production of functionally useful proteins -the basis of antimicrobial action. Erythromycin is mainly used to prevent acne, rosacea, Erythrasma, *Pitvriasis lichenoides*, Infections such as impetigo or boils, Mycoplasma infection, Syphilis, Gonorrheae etc. It is particularly useful in individuals allergic to penicillin. It is active against many gram positive organisms[3,4,5] (*Staphylococcus aureus*,

Streptococcus pyogenes, corynebacteria and clostridia) and few gram negative organisms (*Neisseria gonorrhoeae*).

Materials and Methods

Collection of water sample

Sample 1 –Ganga Water, Armenian Ghat, Kolkata and Sample 2 –Shyamsayar, Burdwan

Materials required

MAc Conkey agar media, Luria agar, Luria broth, Nutrient broth, Nutrient agar, Erythromycin, Distilled water, Colony counter, BOD incubator, UV transilluminator, Centrifuge.

Isolation of gram negative bacteria

1.5g MAc Conkey agar powder was taken and total volume was brought to 30ml by distilled water and mix thoroughly. It was gently heated and brings to boiling (Melting Process). Then it was autoclaved at 15 psi pressure at 121ºC for 15 minute. Plating was done in 2 plates. After 1 hour, streaking was done from both 2 samples. Plates were incubated overnight at 37ºC. As MAc Conkey agar media is a selection media for Gram Negative bacteria, so next day growth of gram negative bacteria was observed on both plates.

Preparation of slant culture

15ml nutrient agar was prepared. It was then melted to distribute equally in 4 test tubes. Then the test tubes were autoclaved and set in slant. Bacteria were streaked in the slants from previously formed MAc Conkey Agar plates. These were then incubated overnight at 37ºc. Next day, bacterial growth was observed.

Isolation of erythromycin resistant gram negative bacteria and their pure culture

Stock solution of Erythromycin (10ml) prepared with sterile distilled water, having concentration 20mg/5ml. Both Bacterial samples were inoculated in nutrient broths. Erythromycin of concentration 10µg/ml (12.5µl) was added to it. This was kept overnight in incubator at 37ºC to observe further growth. Next day, 200µl old culture (resistant to 10µg/ml) added to fresh 10ml nutrient broth. Then, Erythromycin of concentration 30µg/ml (37.5µl) added to it. It was kept overnight in incubator at 37ºC to observe further growth. The same procedure was followed for Erythromycin of strength 50µg/ml (65µl), 70µg/ml (87.5µl), 90µg/ml (112.5µl) and 100µg/ml (125µl).

Plating and bacterial colony count

Serial dilution was done for bacteria which showed growth on 50µg/ml concentration (10^{-1},

10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}). 100ml bacterial culture from 10^{-5}, 10^{-6}, 10^{-7} concentration of both 2 samples was plated on 6 plates prepared before. The plates were incubated overnight at 37ºC. Next day, growth was observed on plates and bacterial colonies were counted and same process was followed for concentration of 70µg/ml, 90µg/ml and 100µg/ml.

Plasmid DNA isolation

1.5ml culture was taken from Erythromycin resistant Bacterial culture (both samples) of highest concentration 100µg/ml, centrifuged for 5min at 10,000 rpm for 3 times. Then the pellet was suspended in 100µl Sol-I, kept at 37ºC for 5min. 200µl Sol-II was added, mixed well by inverting and kept at room temperature for 5 min. 150µl ice cold Sol-III was added mixed well and kept in ice for 5 min. This was then centrifuged at 10,000 rpm for 15 min and supernatant was taken in a fresh test tube. Equal volume of Phenol: Chloroform: isoamyl alcohol (25:24:1) was added and vortexed. It was then centrifuged at 10,000rpm for 2 min at room temperature. The upper aqueous layer was taken in a fresh eppendorf tube and equal volume of 70% ethanol was added. Freezed for 15 min. at 4ºC and again centrifuged at 10,000 rpm 10 min. Supernatant was discarded and pellet was taken and washed in 70% ethanol. It was again centrifuged at 10,000rpm for 5min. Ethanol was evaporated and pellet was resuspended in 50µl tris buffer.

Gel running

Agarose was melted and then cooled. After cooling, 10µl EtBr (Ethidium Bromide) mixed to agarose and a few times later gel formed. Gel set in gel apparatus, containing TBE running buffer- 500ml. Now Sample-1 and Sample-2 taken on eppendorf tip of 10µl each and then 10µl bromophenol blue added to both samples. EtBr + Sample 1/2 placed in gel wells through micropipette in Y-X-Y-X consequence. Electric field applied to gel apparatus and samples run from negative to positive. When bands migrated to last of gel plates, gel was then taken out from TBE buffer and set under UV illumination chamber. Plasmids were observed under UV light.

Transformation of Plasmid

100ml Luria Broth, 60ml Luria Agar, 4 Petri plates prepared. Transformation Host (Escherichia coli) + 0.1ml LB mixed well in a sterile eppendorf. This was streaked on 4 LA plates and kept in incubator at 37ºC for 24 hrs. Transformation host *E. coli*, grows on LA plates were inoculated in 90ml LB and was kept in incubator for 3 hrs. for further growth. Flask was then ice cooled for 20mins. Sterile eppendorf taken and 1.5ml of ice cold LB culture added. This was centrifuged at 6000rpm for 8mins, repeated thrice and each time

1.5ml supernatant discarded. Pellet then resuspended in ice cold 500µl solution-A and kept in ice for 20min. It was then again centrifuged at 3000 rpm 15mins and supernatant discarded. The pellet was resuspended in 300 µl of ice-cold solution-A. Plasmid containing vials (Sample-1 and Sample-2) + 300µl ice cold competent cells mixed properly and kept in ice 20min. Cells then heat shocked by placing vials in 42°C serological water bath for 2mins, followed by quick chilling in ice 5mins. Then 0.5ml sterile LB added to vials and kept in incubator for an hour at 37°C.

Plating of cells

Samples- 1 and 2 were taken from vials, containing competent host cells (*Escherichia coli*) and plated on Erythromycin containing LA plates. Only competent host cell was plated on an LA plate, containing Erythromycin. All plate kept in incubator at 37ºC 24 hrs to observe transformation. [Erythromycin stock Solution (for 60ml LA): $V_1S_1 = V_2S_2$; V_1 (Concentration of Erythromycin) = 1.5ml]

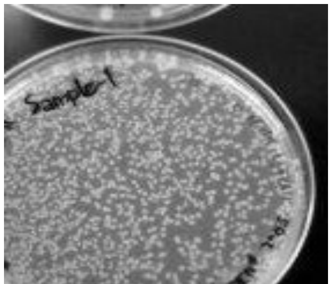
Results and discussion

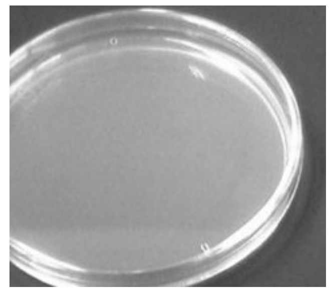
Both Sample-1 and Sample-2 were transformed in LA plate, containing Antibiotic- Erythromycin, as observed the bacterial colonies after a day of incubation in all 4 plates.

But, no growth was observed in the plate containing only competent host cells (Fig 1). Gram negative bacteria were isolated from both sample-1 and sample-2 by selecting them on MAc Conkey agar medium. Then slant culture was prepared from the mother plates.

Both the culture shows growth up to 100µg/ml erythromycin concentration (table 1), from which 50µg/ml, 70µg/ml, 90µg/ml, 100µg/ml bacterial culture were plated and bacterial colony count was done (table 2).

Gel running was done for the highest concentration of antibiotic- erythromycin resistance,





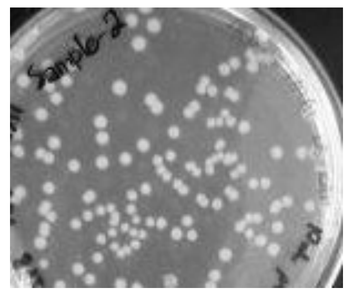


Figure 1: [left] Transformed plate of Sample-1 (Ganga Water, Armenian Ghat, Kolkata), [Middle] Control of only competent host cells, [Right] Transformed plate of Sample-2 (Shyamsayar water, Burdwan)

Table 1: Growth of erythromycin resistant bacteria

Concentration	Sample – 1 (Growth Observed)	Sample – 2 (Growth Observed)
10µg/ml	+	+
30µg/ml	+	+
50µg/ml	+	+
70µg/ml	+	+
90µg/ml	+	+
100µg/ml	+	+

Table 2: Bacterial colony count

Sample-1 (ganga water- armenian Ghat, kolkata)				
Dilution	50µg/ml	70µg/ml	90µg/ml	100µg/ml
10^{-5}	TNTC	TNTC	TNTC	TNTC
10^{-6}	,,	,,	,,	,,
10^{-7}	,,	321×10^{-6} c.f.u	263×10^{-7} c.f.u	152×10^{-8} c.f.u
Sample-2 (shyamsayar water- near burdwan medical college, burdwan)				
Dilution	50µg/ml	70µg/ml	90µg/ml	100µg/ml
10^{-5}	TNTC	TNTC	TNTC	TNTC
10^{-6}	,,	,,	,,	298
10^{-7}	,,	184×10^{-6} c.f.u	172×10^{-7} c.f.u	150×10^{-8} c.f.u

Note- TNTC= Too Numerous To Count
C.F.U (Colony forming Unit) = No. of colony × Dilution factor × Volume of crude sample

Table 3: Plating of cells to observe transformation of plasmid

Plasmid (Antibiotic- Erythromycin resistant)	Result
Sample-2: 1st lane	+
Sample-1: 2nd lane	+
Sample-2: 3rd lane	+
Sample-1: 4th lane	+

that is- 100µg/ml. Bands of plasmid DNA were detected there (table 3).

For the confirmation of presence of plasmid, transformation experiment was carried out. Both the samples-1 and 2 were transformed in LA plates.

From the present work it was evident that the water samples under study, contained Erythromycin resistant bacteria. This indicates some sort of 'Erythromycin resistant bacteria' contamination to the region from where the water samples were collected. Both the

water samples were collected from the areas, where common people consume these contaminated waters for household works and consuming this water for a long time can lead to severe health effects of erythromycin resistance.

So, it is concluded that the characteristics of Erythromycin resistance was present in PLASMIDS of Gram Negative bacteria of both 2 water samples :

Sample-1 (Ganga Water- Armenian Ghat, Kolkata) and Sample-2 (Shyamsayar water, Burdwan)

Hence, we can also say that the enzymes responsible for antibiotic resistance property of these gram negative bacteria may be present in their plasmid DNA.

Acknowledgement

We are thankful to our institute for providing the opportunity to undergo this work. We are also thankful to Mr. Prasenjit Bhattacharya of Subhasree Biotech, Kolkata for giving us valuable suggestions during this work. Thanks are also due to Dr. Mandira Mukherjee, HOD, Dept. of Biochemistry and medical biotechnology of Calcutta School of Tropical Medicine for her continuous encouragement.

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Chapter 27

Study of antimicrobial activity from different citrus peels

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Abstract

Three citrus fruits viz. lemon, orange and musambi have been studied for their possible antimicrobial property. Crude extracts of peel were isolated by hydro-distillation and tested on *E. coli*, *S. aureus* and *Pseudomonas* sp through Agar diffusion method. Lemon and orange extracts were found to have most antimicrobial effect, while musambi peel extracts recorded mild inhibition. Lemon peel extracts showed highest antimicrobial property on *Pseudomonas* (32mm) and *S. aureus* (31mm) while orange peel extracts recorded significantly higher inhibition zone (36mm) in *Pseudomonas sp*.

Keywords

Antimicrobial property, citrus peel extracts, agar diffusion

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Introduction

Citrus is a genus of flowering plants in the family Rutaceae, originating in tropical and subtropical southeast regions of the world. The most well known examples are the orange, the lemon, the grapefruit, the lime and musambi. The peels are a potential source of essential oils in oil sacs of flavedo enriched with sesquiterpene, oxygenated derivatives and aromatic hydrocarbons[1]. The composition of the terpenic mix varies depending on the examined citrus species to which it owns. The mix of each species is in different proportion, made of: limonene, α-pinene, β- pinene, myrcene, linalool and terpinene[2,3,4]. Chemically, these oils are distinct from edible oils, because they are not esters of glycerides. The present communication aims to report inhibitory activity of extracted oils from citrus peels on three common microorganisms.

Materials and Methods

Plant materials

Three common citrus fruits, namely Orange *(Citrus sinensis),* Lemon (*Citrus limonia)* and Musambi (*Citrus limetta*) at mature stage were collected from local market, Badu, Madhyamgram, Kolkata.

Extraction of essential oil and antimicrobial activity

Five hundred gram (50g) of citrus peel from three citrus fruits was subjected to hydro-distillation for 4-hours to obtain crude essential oil. The essential oil was extracted using petroleum ether (80°C b.p) in a separating funnel, concentrated in a hot plate removing any petroleum ether residue and stored in sealed vials at 4°C.

Cultures of three bacterial species *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas sp* were procured from Department of Microbiology of the Institute. The strains were initially revived overnight on LB (*Himedia)* medium. Each of the three strains were grown on Luria Broth (*Himedia*) overnight at 37°C. One millilitre of culture was transferred to 9 ml of broth medium and incubated at 37°C for another 15h; cell concentration was then adjusted to obtain final concentration of 10^6cfu/ml using Luria Broth[5].

The antimicrobial activity of essential oil was evaluated using Agar diffusion method. 100µl of bacterial culture was spread on nutrient agar medium with sterile glass spreader and allowed to diffuse for 10 minutes. Three compact wells were made with cork borer and marked accordingly. 50µl of crude oil extract was added to respective wells each treatment was replicated thrice. Sterile distilled water was used as negative control, while antibiotic streptomycin at a concentration of 50µg/ml was used as positive control. All the plates were

Table 1: Antimicrobial effect of peel extracts on three different microorganism

Treatments	Volume (µl)	Zone of Inhibition (mm)		
		S. aureus	*E.coli*	*Pseudomonas sp.*
Sterile water (Negative control)	50	0	0	0
Streptomycin (Positive control)	50	19.0^{c}	21.0^{b}	14.0^{c}
Citrus limonia	50	31.0^{ab}	26.0^{bc}	32.0^{ab}
Citrus sinensis	50	22.0^{c}	25.0^{c}	36.0^{a}
Citrus limetta	50	16.0^{c}	12.0^{c}	11.0^{d}

Values with different letters show significant difference as ($p \leq 0.05$) as determined by Duncan's Multiple Range Test.

incubated for 24 h at 37°C and size of inhibition zone diameters surrounding was measured.

Results and Discussion

Lemon and orange showed significant antimicrobial property against all three selected microorganisms. Both lemon and orange peel extracts showed good inhibitory activity on *Pseudomonas sp.* (32mm and 36mm zone) (Table 1). Musambi extracts was found to be less effective against all the three tested microorganisms in contrast to other two citrus species recording a maximum of 16mm inhibition zone against *S. aureus.* There was no inhibition zone formation in negative control.

Among the various peel extracts tested, *Citrus sinensis* was most effective (36.0mm) against *Pseudomonas sp*. while extracts from peels of *Citrus limetta* showed lowest (11.0mm) antimicrobial effect. It has been reported previously that the monoterpene composition of the essential oil is accountable for the antibacterial activity. These compounds could be responsible for lethal action on microbial cell through destruction of the cellular integrity of microbial cells accompanied with loss of chemiosmotic control followed by inhibiting the respiration process[6,7]. The antibacterial activity of the essential oil may also be correlated to a synergistic effect of all the chemical components present in the oil[5,8].

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Chapter 28

Importance of polymer in the field of drugs

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Abstract

Polymers are used in all the fields like industries, for the manufacturing of television, fridge, washing machine etc and even we can see in the field of film industries. In the same way now a days we are using polymers in the field of drugs or we can say that we are using for the drug delivery. The pharmaceutical applications of polymers range from their use as binders in tablets to viscosity and flow controlling agents in liquids, suspensions and emulsions. Polymers can be used as film coatings to disguise the unpleasant taste of a drug, to enhance drug stability and to modify drug release characteristics. Basically we want to focus on the use controlled drug delivery applications. Examples of pharmaceutical polymers and the principles of controlled drug delivery are outlined and applications of polymers for controlled drug delivery are described. The field of controlled drug delivery is vast therefore the aim is to provide an overview of the applications of pharmaceutical polymers. Although polymers are used extensively as pharmaceutical packaging, this is concerned with the use of polymers in the formulation of dosage forms. Basically this review is for those who are interested in the pharmaceutical use of polymers, whether as a researcher or as a manufacturer of medical devices.

Keywords

Drugs, Pharmaceuticals, Polymers, human health

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Introduction

A polymer (Greek; poly = many, mer = unit) may be defined as follows. Polymer is a large molecule of very high molecular mass formed by the repeated combination of a very large number of one or more types of small molecules called monomers.

Polymers are the giant molecules of very high molecular masses. They may have molecular masses as high as 50,000 or even more. Such molecules occur is nature and can be synthesized in the laboratory as well. Cellulose, starch, proteins, rubber resins etc. are some common examples of naturally occurring polymers. These polymers find a variety of applications in our day to day life. Besides the natural polymers, we have a wide range of synthetic polymers which have become a part of our daily life. Synthetic fibers e.g., nylons, terylene etc. are used for making clothes, ropes, nets etc. Plastics and synthetic resins e.g. polyethylene, Teflon, Styron, PVC etc. find a variety of applications in our life. Artificial rubbers are also synthetic polymers. In fact, polymers find a wide range of uses starting from common household utensils, automobiles, clothes, furniture, medicines, space crafts and biomedical and surgical operations.

Polymers may be both of inorganic and organic in nature. Among the inorganic polymers, metaphosphoric acid $(HPO_3)_n$, silicates, silicones etc. are important. However, the organic polymers, particularly the synthetic ones, are more important and are the chief products of modern chemical industry today. In the forthcoming sections, we shall study some important aspects related to organic polymers, particularly the synthetic ones.

Advances in polymer science have led to the development of several novel drug-delivery systems. A proper consideration of surface and bulk properties can aid in the designing of polymers for various drug-delivery applications. Biodegradable polymers find widespread use in drug delivery as they can be degraded to non-toxic monomers inside the body. Novel supramolecular structures based on polyethylene oxide copolymers and dendrimers are being intensively researched for delivery of genes and macromolecules.

Different organs, tissues and cellular compartments may have large differences in pH, which makes the pH a suitable stimulus. Chemists in the US20 have developed a three-component polymer that can respond to temperature, pH and the presence of a reducing agent. For That we have to prepared different polymers for different purpose.

Polymer structure affects the diffusion mechanism and release behavior of various drugs. Zero-order release can be achieved under certain conditions of polymer preparation and for specific geometric shapes. Physical, physicochemical, diffusive and toxicological tests for biomaterials used in controlled release applications.

Use of polymers in pharmaceutical and biological field

Tablets are by far the most popular dosage form when administering drugs to patients, and a large proportion of the tablets produced around the world are film coated. With the current awareness of health, safety and environmental problems, film coating is a process that is routinely employed in the preparation of pharmaceutical solid dosage forms.

Tablet coating applications

The application of coating of tablets, which is an additional step in the manufacturing process, increases the cost of the product. Therefore, the decision to coat a tablet is usually based on one or more of the following objectives:

1. To mask the taste, odor, color of the drug.
2. To provide physical and chemical protection for the drug.
3. To control the release of the drug from the tablet.
4. To protect the drug from the gastric environment of the stomach with an acid resistant enteric coating.
5. To incorporate another drug or formula adjuvant in the coating to avoid chemical incompatibilities or to provide sequential drug release.
6. To improve the pharmaceutical elegance by use of special colors and contrasting.

There are three primary components involved in tablet coating:

i. Tablet properties

ii. Coating process

Coating equipment

Parameters of the coating process

Facility and ancillary equipment

Automation in coating process

iii. Coating compositions

Water-Soluble Synthetic Polymers

- Poly (acrylic acid) cosmetic, pharmaceuticals, immobilization of cationic drugs, base for Carbopol polymers.
- Poly (ethylene oxide) Coagulant, flocculent, very high molecular-weight up to a few

millions, swelling agent.

- Poly (ethylene glycol) Mw <10,000; liquid (Mw <1000) and wax (Mw >1000), plasticizer, base for suppositories.
- Poly (vinyl pyrrolidone) Used to make betadine (iodine complex of PVP) with
- less toxicity than iodine, plasma replacement, tablet granulation
- Poly (vinyl alcohol) Water-soluble and in the form of tablet binder even for the tablet coating.
- Polyacrylamide Gel electrophoresis to separate proteins based on their molecular weights, coagulant, absorbent.
- Poly (isopropyl acrylamide) and poly (cyclopropyl methacrylamide).

Thermogelling acrylamide derivatives, its balance of hydrogen bonding and hydrophobic association changes with temperature.

Cellulose-based polymers

- Ethyl cellulose Insoluble but dispersible in water, aqueous coating system for sustained release applications.
- Carboxymethyl cellulose Super disintegrant, emulsion stabilizer.
- Hydroxyethyl and hydroxypropyl celluloses.
- Soluble in water and in alcohol, tablet coating.
- Hydroxypropyl methyl cellulose Binder for tablet matrix and tablet coating, gelatin alternative as capsule material.

Cellulose acetate phthalate enteric coating.

Hydrocolloids

- Alginic acid Oral and topical pharmaceutical products; thickening and suspending agent in a variety of pastes, creams, and gels, as well as a stabilizing
- Agent for oil-in-water emulsions; binder and disintegrant.
- Carrageenan Modified release and viscosifier.
- Chitosan Cosmetics and controlled drug delivery applications, mucoadhesive
- Dosage forms, rapid release dosage forms.

Alpha Science

- Hyaluronic acid reduction of scar tissue especially for cosmetics.

Pectinic acid Drug delivery.

Water-insoluble biodegradable polymers

(Lactide-co-glycolide) polymers Microparticle–nanoparticle for protein delivery.

Starch-based polymers

Sodium starch glycolate super disintegrant for tablets and capsules in oral delivery.

Plastics and rubbers

- Polyurethane (Transdermal patch backing soft, comfortable, moderate moisture transmission), blood pump, artificial heart, and vascular grafts, foam in biomedical and industrial products.
- Silicones Pacifier, therapeutic devices, implants, medical grade adhesive for trans dermal delivery.
- Polycarbonate Case for biomedical and pharmaceutical products.
- Polychloroprene Septum for injection, plungers for syringes, and valve components.
- Polyisobutylene Pressure sensitive adhesives for trans dermal delivery.
- Polycyanoacrylate Biodegradable tissue adhesives in surgery, a drug carrier in nano- and microparticles.
- Poly (vinyl acetate) Binder for chewing gum.
- Polystyrene Petri dishes and containers for cell culture.
- Polypropylene Tight packaging.
- Poly (vinyl chloride) Blood bag.
- Poly (methyl meth acrylate) Hard contact lenses.
- Poly (hydroxyethyl methacrylate) Soft contact lenses.
- Vinyl acetate and methyl acrylate copolymer.
- High cohesive strength pressure–sensitive adhesive for transdermal patches.

Ethylene vinyl acetate and polyethylene, terephthalate.

Biodegradable polymers find widespread use in drug delivery as they can be degraded to non-toxic monomers inside the body. Novel supramolecular structures based on

Alpha Science

polyethylene oxide copolymers and dendrimers are being intensively researched for delivery of genes and macromolecules.

The bio-safety and biocompatibility are the important characteristics needed for the use of polymers in the field of pharmaceutical formulation and in novel drug delivery.

It will not be an exaggeration to say that the age in which we are living today is the age of polymers because we cannot think of smooth running of life without polymers.

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